Nonclassical Antimetabolites. XXVI. Inhibitors of Thymidine Kinase. II.[1,2] Inhibition by Functionalized 1-Alkyluracils

B. R. BAKER AND THOMAS J. SCHWAN

Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, New York 14214

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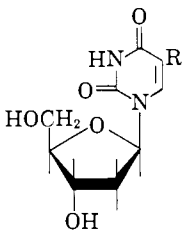
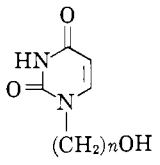
Nineteen 1-alkyluracils, most of which had a functional group on the alkyl radical were investigated as possible inhibitors of thymidine kinase. Only two compounds were found to inhibit the enzyme at 25–50 times the level required for 2′-deoxyuridine to give 50% inhibition. They are 1-(4′-hydroxybutyl)uracil and 1-(*p*-carboxamidobenzyl)uracil (XVIII). Other simple 1-(ω-hydroxyalkyl)uracils (IV) or 1-(3,5-dihydroxypentyl)uracil (VI) failed to show inhibition at high concentrations. No hydrophobic bonding could be detected with 1-butyl-, 1-benzyl-, or 1-phenylpropyluracil.

In the previous paper of this series, the mode of binding of pyrimidine nucleosides to the thymidine kinase from *Escherichia coli* B was studied.[2] Evidence was presented that the 3′- and 5′-hydroxyl groups of thymidine (I) contributed to the enzyme binding; that the furanose oxygen of I contributed to binding was less certain. In this paper is described the search for 1-alkyluracils bearing functional groups on the alkyl group which might bind to thymidine kinase at least 5–10% as well as thymidine (I) or 2′-deoxyuridine (II). Such a molecule could then serve as a prototype structure which could be further modified in order to design active-site-directed irreversible inhibitors[3] for this enzyme.



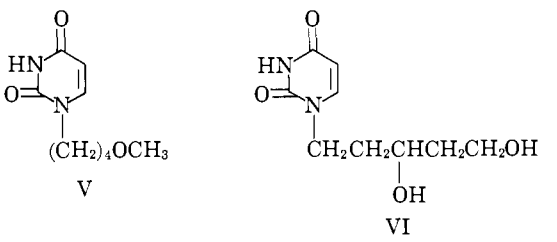
I, R = CH_3
II, R = H
III, R = F



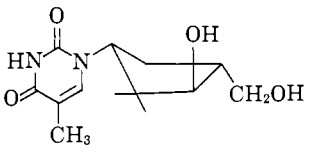
IV, n = 2–5

Enzyme Results.—A series of ω-hydroxyalkyluracils (IV) were synthesized to see if one hydroxyl group would impart sufficient binding to the 1-alkyluracils to be useful; only one of these compounds, 1-(4′-hydroxybutyl)uracil (IV, n = 4), showed measurable inhibition at 5 mM, which is 25 times the concentration at which 2′-deoxyuridine shows 50% inhibition.[2] The possible mode of binding of this compound will be discussed later. Similarly, 1-(4′-methoxybutyl)uracil (V) showed no inhibition at 5 mM.

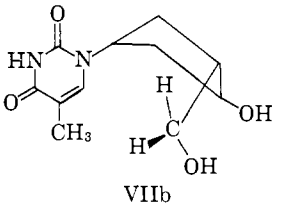
In order to determine whether two hydroxyl groups would give better binding, VI was synthesized. It has hydroxyl groups positioned corresponding to the 3′- and 5′-hydroxyl groups of 2′-deoxyuridine (II). Unfortunately, VI also showed no inhibition at 5 mM, even though the cyclopentane analog VII of thymidine at 2 mM showed 44% inhibition of thymidine kinase.



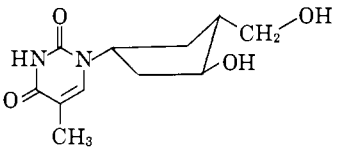
V VI



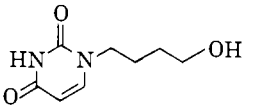
VIIa



VIIb



VIIc



IVa (n = 4)

The pyrimidine ring of I–III and VII must have some given conformation when complexed to the enzyme; this conformation is arbitrarily depicted as in VII. The semienvelope of the cyclopentane can place either the hydroxymethyl or the pyrimidine ring in an equatorial conformation. The second of the two groups will change little in proton interaction at the base of the envelope when the conformation of the first group is shifted from equatorial (VIIa) to axial (VIIb). Since the uracil ring is planar, there is little difference in proton interaction of the 6-H of the uracil and the cyclopentane protons, between the two conformations, VIIa and VIIb. However, there is slightly less proton interaction of the hydroxymethyl group of VIIc compared to VIIa or VIIb; therefore conformation VIIc is the most stable one. The cyclopentane ring of VIIc still has considerable interaction among the ring protons that is less than an eclipsed butyl group, but more than a skewed butyl group. Thus, for VI to assume the conformation of VIIa–VIIc, 2–4 kcal./mole of energy would probably be required from the energy released as the inhibitor complexes to the enzyme,[4] since about

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(2) For the paper XXV of this series which contains the biological rationale for this problem see B. R. Baker, T. J. Schwan, and D. V. Santi, *J. Med. Chem.*, **9**, 66 (1966).

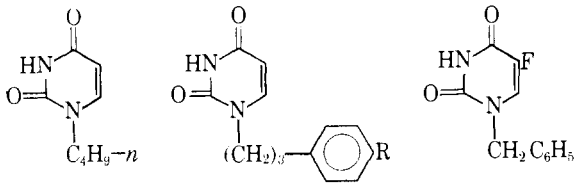
(3) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).

(4) B. R. Baker, B.-T. Ho, and D. V. Santi, *ibid.*, **54**, 1415 (1965).

0.8 kcal. is needed for a skewed and 5.3 kcal. for an eclipsed conformation. A 50–1000-fold loss in binding will occur unless the enzyme-inhibitor complex can change in conformation, so that less energy is required. Thus, the inability of VI to bind as well as VIIc is most probably due to the energetically unfavorable conformation that VI must assume for enzyme binding.

It is now possible to try to rationalize the binding of 1-(4′-hydroxybutyl)uracil (IV, n = 4) which showed 38% inhibition at a concentration of 5 mM. The conformation IVa, which is essentially staggered except for a C-1′-C-2′ proton interaction, should require less than 0.5 kcal./mole to overcome this slightly unfavorable conformation. The hydroxyl group is then almost exactly juxtaposed with the 5′-hydroxyl of VIIc or the similarly juxtaposed 5′-hydroxyl of thymidine. The 5′-hydroxyl of thymidine was previously shown to contribute to binding since, in its absence, a 30-fold loss in binding occurred.[2] The absence of the 3′-hydroxyl on thymidine caused an 80-fold reduction in binding; in contrast, the cyclopentane analog (VII) of thymidine was 120-fold less effective than thymidine.[2] It can be estimated that the 1-(4′-hydroxybutyl)uracil (IV, n = 4) was complexed one-fiftieth as well as 2′-deoxyuridine, which showed 50% inhibition at 0.18 mM. Thus the 4-hydroxybutyl group on uracil would appear to bind equally to what could be expected for 2′,3′-dideoxyuridine. Stated another way, since thymidine complexes to thymidine kinase 9 times better than 2′-deoxyuridine, 1-(4′-hydroxybutyl)thymine might be expected to give 50% inhibition at about 1 mM. Studies on the mode of binding of the 4-hydroxybutyl group are continuing.

Since utilizable hydrophobic bonding of 5-alkylpyrimidines to dihydrofolic reductase[4] and 9-alkyladenines of adenosine deaminase[5] has been observed, the possibility of such hydrophobic bonding with thymidine kinase was investigated. 1-(n-Butyl)uracil (VIII) showed no inhibition of thymidine kinase at 5 mM. At their maximum solubility, IX[6] (1.5 mM), X (1.5 mM), and XI (0.75 mM) showed no inhibition; similarly, XII was ineffective at 3 mM.

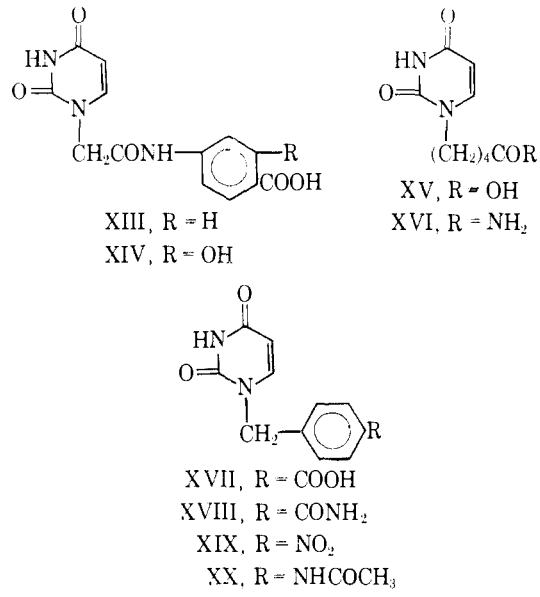


VIII X, R = H IX

XI, R = $COOC_2H_5$

XII, R = COOH

A number of 1-alkyluracils bearing functional groups on the alkyl groups were available from a previous study[7] on the inhibition of thymidylate synthetase. Of these compounds (XIII–XVIII) only XVII showed inhibition of thymidine kinase at a concentration of 3 mM; XVIII showed 39% inhibition, thus being about 24-fold less effective than 2′-deoxyuridine (II).[2] The related amide XX, which would be more suitable for construction of active-site-directed irreversible inhibitors,[3] was then synthesized *via* XIX. Neither XIX



XIII, R = H
XIV, R = OH

XV, R = OH
XVI, R = NH_2

XVII, R = COOH
XVIII, R = $CONH_2$
XIX, R = NO_2
XX, R = $NHCOCH_3$

nor XX, at their maximum solubility of 0.75 mM, showed any inhibition of thymidine kinase. Studies on the mode of binding of XVIII, as well as its possible utility in construction of active-site-directed irreversible inhibitors, are continuing.

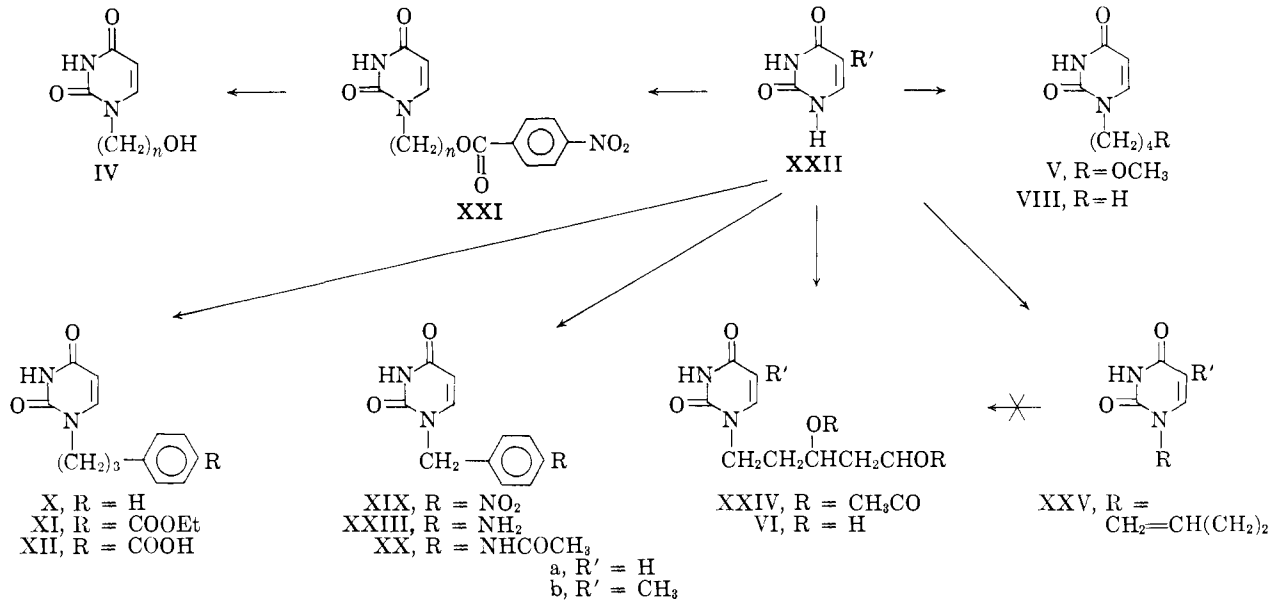
Molecular models show that the NH group of both XVIII and XX approach closely the position of the 5′-hydroxyl of 2′-deoxyuridine. If the binding of XVIII is due to the amide NH simulating the 5′-hydroxyl of 2′-deoxyuridine, then it is possible that there is no bulk tolerance for the methyl group of the acetamido moiety of XX.

Chemistry.—A route to 1-alkyluracils by alkylation of excess uracil in dimethyl sulfoxide in the presence of potassium carbonate has been reported from this laboratory.[7] This route was further modified for preparation of 1-(5-hydroxypentyl)uracil (IV, n = 5) *via* XXI (n = 5) (see Scheme I) by alkylation of excess uracil in the presence of sodium iodide with 5-chloropentyl p-nitrobenzoate.[8] The analogs of XXI with n = 2–4 were prepared similarly except that with n = 3, 3-bromopropyl p-nitrobenzoate was employed. The p-nitrobenzoyl group of the XXI analogs was removed with methanolic butylamine. Since all of the 1-(ω-hydroxyalkyl)uracils (IV) were extremely soluble in water, the by-products from the p-nitrobenzoyl moiety were readily removed by washing an aqueous solution of IV with chloroform.

Other compounds prepared by direct alkylation of uracil with the appropriate alkyl halides were V, VIII, X, XI, XIX, and XXVa. All of these compounds were 1-substituted uracils as shown by the lack of a bathochromic shift in their ultraviolet spectra in basic solution.[9] All but V were obtained as crystalline products; V was obtained as an oil that was purified by preparative thin layer chromatography. Saponification of XI afforded 1-(p-carboxyphenylpropyl)uracil (XII). Catalytic reduction of XIX to 1-(p-aminobenzyl)uracil (XXIII) in the presence of platinum oxide catalyst was rapid, thus avoiding concomitant reduction of the uracil 5,6 double bond. Acetylation

(5) H. J. Schaeffer, and D. Vogel, *J. Med. Chem.*, **8**, 507 (1965).
(6) B. R. Baker and G. D. F. Jackson, *J. Pharm. Sci.*, in press.
(7) B. R. Baker and G. B. Cbheda, *ibid.*, **54**, 25 (1965).
(8) B. R. Baker, G. D. F. Jackson, and G. B. Cbheda, *ibid.*, in press.
(9) D. Shugar and J. J. Fox, *Biochim. Biophys. Acta*, **9**, 199 (1952).

SCHEME I



of an aqueous suspension of XXIII with acetyl chloride in acetone in the presence of potassium carbonate proceeded satisfactorily to 1-(*p*-acetamidobenzyl)uracil (XX).

A number of routes to 1-(3,5-dihydroxypentyl)uracil (VIa) or thymine (VIb) can be envisioned. Such a 1,3-diol type of compound is probably best prepared by the Prins reaction.[10] The alternative routes depend upon what stage the Prins reaction is performed on a 1-substituted 3-butene. Since Murdock and Angier[11] used a Prins reaction on 1-(3-cyclopenten-1-yl)-thymine to prepare the cyclopentane analog VII of thymidine, this route was investigated first.

1-(3-Butenyl)uracil (XXVa), prepared by alkylation of excess uracil with 4-bromo-1-butene, was subjected to reaction with paraformaldehyde in glacial acetic acid in the presence of sulfuric acid; that substitution on the 5-position of the uracil moiety occurred was shown by the 5-mμ bathochromic shift in the ultraviolet spectral peak normally observed with uracils.[9] Although 1-substituted uracils can be hydroxymethylated with formaldehyde in aqueous solution, one could not necessarily assume that the Prins conditions would lead to 5-acetoxymethylation of the uracil moiety.

In order to circumvent reaction at the 5-position, thymine (XXIIb) was employed at the start of the sequence. Alkylation of thymine with 4-bromo-1-butene under the conditions used for uracil gave 1-(3′-butenyl)thymine (XXVb) in 54% yield; that this was a 1-substituted thymine was shown by its ultraviolet spectrum being independent of pH.[9] When XXVb was subjected to the Prins reaction, a multiplicity of products was formed; thin layer chromatography showed five spots in addition to starting material.

3-Buten-1-ol was converted to 4-acetoxytetrahydropyran by the Prins reaction.[12] The 4-acetoxytetrahydropyran was converted to 3,5-diacetoxypentyl chloride[13a] with acetyl chloride and zinc chloride by the general method used by Cloke and Pilgrim.[13b]

Alkylation of excess uracil with 3,5-diacetoxypentyl chloride gave the crude product (XXIVa) as an oil that could not be crystallized. The crude diacetate (XXIVa) could be purified by preparative thin layer chromatography which gave pure XXIVa in 65% yield. Deacetylation of the crude diacetate XXIVa with butylamine in methanol gave the desired VIa as an oil that was also purified by preparative thin layer chromatography to give an over-all yield of 19% (based on uracil) of analytically pure, but oily, 1-(3,5-dihydroxypentyl)uracil (VIa); VIa was further characterized as its crystalline bis-*p*-nitrobenzoate.

Experimental Section

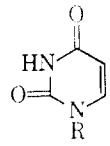
Melting points were taken in capillary tubes on a Mel-Temp block, and those below 230° are corrected. Infrared spectra were determined in Nujol mull with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined with a Perkin-Elmer 202 spectrophotometer; spectra at pH 1 and 13 were determined in 10% ethanol and pH 7 spectra in 95% ethanol. Thin layer chromatography (t.l.c.) was run on Brinkmann silica gel GF_{254} with benzene–methanol (3:1), and spots were located by visual examination under ultraviolet light. Inhibition of the thymidine kinase from *E. coli* B was determined[14] with 0.1 m*M* 5-fluoro-2′-deoxyuridine (III) as substrate as described in the accompanying paper.[2]

1-(4-O-*p*-Nitrobenzoyl-4-hydroxybutyl)uracil (XXI, n = 4).—A mixture of 4.2 g. (16 mmoles) of 4-chlorobutyl *p*-nitrobenzoate,[15] 5.4 g. (48 mmoles) of uracil, 6.6 g. (48 mmoles) of anhydrous K_2CO_3, 2.4 g. (16 mmoles) of sodium iodide, and 100 ml. of dimethyl sulfoxide was magnetically stirred in an oil bath at 90° for 3 hr.[8] The cooled mixture was poured into 100 g. of iced water, acidified to pH 2 with 5% aqueous hydrochloric acid, then extracted with five 100-ml. portions of $CHCl_3$. The combined, dried extracts were spin evaporated *in vacuo*, and the

(10) E. Arundale and L. A. Mikeska, *Chem. Rev.*, **51**, 505 (1952).
(11) K. C. Murdock and R. B. Angier, *J. Am. Chem. Soc.*, **84**, 3758 (1962).
(12) S. Olsen and G. Aksnes, *Acta Chem. Scand.*, **4**, 993 (1950).
(13) (a) R. Paul and S. Tchelitcheff, *Bull. soc. chim. France*, 550 (1951); (b) J. B. Cloke and F. J. Pilgrim, *J. Am. Chem. Soc.*, **61**, 2667 (1939).
(14) The technical assistance of Gail Westley with these assays is acknowledged.
(15) 4-Chlorobutyl,[16] 3-bromopropyl,[17] and 2-chloroethyl *p*-nitrobenzoates were prepared by fusion of the corresponding alcohol with *p*-nitrobenzoyl chloride at 100° for 3 hr. as described for the 2-chloroethyl ester by T. Kaku, Y. Kase, and T. Sakuma, *J. Pharm. Soc. Japan*, **74**, 732 (1954); *Chem. Abstr.*, **49**, 9557 (1955).
(16) L. M. Smorgonskii and Y. L. Goldfarb, *J. Gen. Chem. USSR*, **10**, 1113 (1940); *Chem. Abstr.*, **35**, 4011 (1941).
(17) O. A. Barnes and R. Adams, *J. Am. Chem. Soc.*, **49**, 1307 (1927).

TABLE I

PHYSICAL CONSTANTS OF



Compd.	R[a]	Method	Yield, %	M.p., °C.	Calcd., % C	Calcd., % H	Calcd., % N	Found, % C	Found, % H	Found, % N
IV	CH_2CH_2OH	B	27[b,c]	141–143[d]	46.1	5.16	17.9	45.9	4.99	18.2
IVb	$(CH_2)_3OH$	B	28[b,c]	122–124	49.4	5.92	16.5	49.2	6.02	16.2
IVc	$(CH_2)_4OH$	B	26[b,c]	124–126	52.2	6.57	15.2	52.0	6.74	15.5
IVd	$(CH_2)_5OH$	B[e]	28[b,c]	78–80	54.5	7.12	14.1	54.8	6.95	14.3
V	$(CH_2)_4OCH_3$	A[f]	49	Glass[g]	54.5	7.12	14.1	54.8	7.24	14.0
VIa	$CH_2CH_2CHOHCH_2CH_2OH$	B	19[h]	Glass	50.5	6.59	13.1	50.6	6.70	12.9
VIII	C_4H_9-*n*	A	38[i]	100–103[c,j]	57.1	7.19	16.7	57.0	7.01	16.5
X	$(CH_2)_3C_6H_5$	A	74	115–117[c]	67.8	6.13	12.2	68.1	6.34	12.3
XI	$(CH_2)_3C_6H_5COOC_2H_5$-*p*	A[k]	60	163–166[c]	63.6	6.00	9.27	63.7	6.09	9.30
XIX	$CH_2C_6H_4NO_2$-*p*	A[l]	41	236–240[m,n]	53.5	3.67	17.0	53.3	3.61	16.7
XXIa	$CH_2CH_2O_2CC_6H_4NO_2$-*p*	A[o]	16	243–246	51.1	3.63	13.8	50.9	3.74	13.5
XXIb	$(CH_2)_3O_2CC_6H_4NO_2$-*p*	A	32	184–186[c]	52.7	4.10	13.2	52.9	4.29	12.9
XXIc	$(CH_2)_4O_2CC_6H_4NO_2$-*p*	A	47	172–174[c]	54.1	4.54	12.6	53.8	4.72	12.4
XXIVa	$(CH_2)_2CHOAc(CH_2)_2OAc$[p]	A	65[q]	Oil	52.4	6.08	9.39	52.2	6.17	9.54
XXVa	$CH_2CH_2CH{=}CH_2$	A	37	93–95[r]	57.8	6.07	16.9	57.7	6.09	16.8
XXVb	5-CH_3-1-$CH_2CH_2CH{=}CH_2$[s]	A	25	133–135[c]	60.0	6.71	15.6	60.0	6.66	15.8
XII	$(CH_2)_3C_6H_4COOH$-*p*	C[t]	78	207–210[u]	61.3	5.14	10.2	61.5	5.03	10.2
XXIII	$CH_2C_6H_4NH_2$-*p*	E[v]	44[b,w]	218–220	60.8	5.10	19.3	60.7	5.24	19.2
XX	$CH_2C_6H_4NHCOCH_3$-*p*	D[x]	54	308–310	60.2	5.05	16.2	60.7	5.24	19.2

[a] All compounds had ultraviolet and infrared spectra in agreement with their assigned structure and were uniform on t.l.c. [b] Yield of analytically pure material; no attempt was made to work-up the mother liquors. [c] Recrystallized from ethyl acetate. [d] M. Pyrstas and J. Gut [*Collection Czech. Chem. Commun.*, **27**, 1054 (1962)] have recorded m.p. 136–137° for this compound prepared from uracil with ethylene carbonate. [e] For the intermediate *p*-nitrobenzoate, see ref. 8. [f] The intermediate 4-methoxybutyl bromide was prepared according to P. Karrer and H. Schmid, *Helv. Chim. Acta*, **27,** 124 (1944). [g] Purified by preparative t.l.c. [h] Over-all yield of pure material from uracil after preparative t.l.c. as described for XXIVa. This gave a bis-*p*-nitrobenzoate with *p*-nitrobenzoyl chloride in pyridine that formed yellow crystals from toluene; m.p. 152–154°. *Anal.* Calcd. for $C_{23}H_{20}N_4O_{10}$: C, 53.9; H, 3.93; N, 10.9. Found: C, 54.3; H, 4.00, N, 10.7. [i] The crude product remaining after evaporation was extracted with hot petroleum ether (b.p. 70–110°); the residue from this extract was recrystallized from ethyl acetate. [j] A m.p. 101–103° has recently been recorded by C. C. Cheng and L. R. Lewis, *J. Heterocyclic Chem.*, **1,** 260 (1964), for this compound prepared by a different route. [k] The intermediate ethyl *p*-(3-bromopropyl)benzoate was obtained by esterification of the corresponding benzoic acid prepared according to F. F. Blicke and W. M. Lilienfeld, *J. Am. Chem. Soc.*, **65,** 2281 (1943). [l] Ethyl acetate was used in place of $CHCl_3$ for extraction. [m] Recrystallized from absolute ethanol. [n] The product separated from the aqueous dimethyl sulfoxide and was recrystallized from acetone. [o] See ref. 12 for starting pentyl chloride. [p] See experiment for purification procedure. [q] Recrystallized from toluene. [r] 1-(3-Butene-1-yl)thymine. [s] By saponification of XI with 0.1 *N* aqueous NaOH at 100° for 3 hr. [t] Recrystallized from water. [u] By catalytic hydrogenation of XIX in 2-methoxyethanol in the presence of platinum oxide. [v] By acetylation of XXIII with acetyl chloride in aqueous acetone in the presence of potassium carbonate.

residual dimethyl sulfoxide was removed in high vacuum. The residue was extracted with three 100-ml. portions of boiling ethyl acetate. The combined extracts were clarified by filtration, concentrated to 25 ml. *in vacuo*, and stored at −20°. The product was collected on a filter and washed with cold ethyl acetate; yield 2.5 g. (47%), m.p. 151–163° (suitable for the next step). Recrystallization from ethyl acetate gave nearly white crystals: m.p. 172–174°; λ_{max}^{EtOH} 265 mμ; λ_{max} 3.65–3.75 (acidic H), 5.82 (ester C=O), 5.92, 6.13, 6.25 (uracil), 6.56, 7.45 (NO_2), 7.75 (ester C–O–C), 12.02 μ (*p*-C_6H_4). For analytical data see Table I. Other compounds prepared in this manner are listed in Table I under method A.

1-(4-Hydroxybutyl)uracil (IV, *n* = 4).—A solution of 0.8 g. (2.4 mmoles) of XXI (*n* = 4) and 3.0 g. of butylamine in 15 ml. of methanol was refluxed for 24 hr., and spin evaporated *in vacuo*. The residue was warmed with 200 ml. of water, then cooled and washed with four 50-ml. portions of $CHCl_3$ to remove *p*-nitrobenzoylated by-products. The aqueous solution was spin evaporated *in vacuo*. The residue was dissolved in 50 ml. of absolute ethanol, 25 ml. of toluene was added, and the mixture was spin evaporated *in vacuo* to remove water. Three recrystallizations from ethyl acetate gave 0.11 g. (26%) of analytical sample as white crystals: m.p. 124–126°; λ_{max} (pH 7, 13) 267 mμ; λ_{max} 2.90 (OH), 5.97, 6.15 (uracil), 9.55 μ (COH) (no *p*-nitrobenzoate absorption near 5.82, 6.56, or 7.75 μ). See Table I for analytical data. Other compounds prepared by this method are listed in Table I under method B.

1-(3,5-Di-O-acetyl-3,5-dihydroxypentyl)uracil (XXIVa).—Reaction of 2.1 g. (15 mmoles) of uracil with 1.1 g. (5 mmoles) of 1-chloro-3,5-diacetoxypentane[12,13] as described for the preparation of XXI gave, on evaporation of the $CHCl_3$, 1.2 g. (81%) of crude residual XXIVa. A concentrated solution of 147 mg. of crude product in ethanol was streaked across a 200 × 200 mm. plate coated with 20 g. of silica gel GF. The plate was developed with benzene–methanol (3:1). The zone of lower R_f was scraped from the plate and eluted with two 25-ml. portions of boiling acetone. Evaporation of the acetone *in vacuo* gave 118 mg. (65%) of analytical sample as a viscous oil; λ_{max} (pH 1, 7, 13) 268 mμ; λ_{max} (film) 5.75–6.05 (broad ester and uracil bands), 8.1–8.2 μ (ester C–O–C). See Table I for analytical data.