Acyclonucleoside Analogues Consisting of 5- and 5,6-Substituted Uracils and Different Acylic Chains: Inhibitory Properties *vs* Purified *E. coli* Uridine Phosphorylase

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Pyrimidine Acyclonucleosides, Analogues, Uridine Phosphorylase, Inhibitors, Kinetics

Synthetic procedures are described for the preparation of a variety of pyrimidine acyclonucleoside analogues, in which the aglycones are 5- and 5,6-substituted uracils, and the ribose moiety is replaced by different acyclic chains. These were examined as potential inhibitors of purified *E. coli* uridine phosphorylase. None of the compounds was a substrate for uridine phosphorylase, or either a substrate or inhibitor of *E. coli* thymidine phosphorylase. Kinetic measurements were employed to determine inhibition constants, K_i, for inhibition of uridine phosphorylase. One of the more effective of these was 1-(1′,3′-dihydroxy-2′-propoxy)methyl-5,6-tetramethyleneuracil, with $K_i = 2.7$ μM. The same compound was a reasonably good inhibitor of the reverse, synthetic, reaction, with K_i values of 19 μM *vs* uracil as the variable substrate, and 15 μM *vs* α-D-ribose-1-phosphate as the variable substrate. For one of the analogues, which was a racemate, 1-(2′,3′-dihydroxypropyl)-5,6-tetramethyleneuracil, it was shown that only one of the enantiomers (*R*) was an inhibitor, the (*S*) enantiomer being totally inactive. For several of the analogues, the corresponding isomeric N(3)-acyclonucleosides were inactive as inhibitors. The results for several of the good inhibitors were compared with those of other observers for inhibition of uridine phosphorylase from mammalian sources. Preliminary measurements with several of our analogues demonstrated that some of them were indeed one to two orders of magnitude more effective against the enzyme from mammalian sources.

Introduction

The potential importance of inhibitors of uridine and thymidine phosphorylases was recognized many years ago by Baker *et al.* [1], who found, *e.g.* that 5-benzyluracil is a relatively good inhibitor of uridine phosphorylase from Walker 256 carcinoma, with a $I_{50} \sim 5$ μM. In more recent studies it has been found that acyclonucleosides of uracil and thymine are effective inhibitors of the enzyme from Sarcoma 180 cells [2–5]. Parallel investigations also point to purine acyclonucleosides as good inhibitors of purine nucleoside phosphorylase [6].

We have profited from the findings of Vita and Magni [7] to isolate from *E. coli* a highly homogeneous uridine phosphorylase with a specific activity, 250 μmol/min/mg protein, higher than any hitherto reported. We now describe some structure-activity relationships amongst several types of acyclonucleosides with various uracil analogues as the heterocyclic bases, as inhibitors of this purified enzyme, and compare some of the results with those reported with the use of Sarcoma 180 cell extracts as the source of enzyme [2, 4].

Apart from the utility of such inhibitors for further elucidation of the role of pyrimidine nucleoside phosphorylases in both bacterial and mammalian systems, they are equally potentially useful agents for modulating the activities of antitumour compounds such as 5-fluorouracil and 5-fluoro-2′-deoxyuridine [5, 8]. A preliminary report of some of our findings has appeared [9].

Materials and Methods

Uridine was obtained from BDH (Poole, UK), uracil and thymine from Reanal (Budapest, Hungary), [2-^{14}C]uracil (60 mCi/mmol) from Amersham (Buckinghamshire, UK), α-D-ribose-1-phosphate from Sigma (St. Louis, MO., USA), Matrex Gel Green A from Amicon (Lexington, MA., USA). Uracil analogues were synthesized as described else-

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where for 5,6-dimethyluracil [10], 5-ethyluracil [11], 5-propyl and 5-isopropyl uracils [12], 5-benzyluracil [13] and 5,6-trimethyleneuracil and 5,6-tetramethylene uracil [14]. All other reagents were analytical grade. The foregoing compounds were checked for chromatographic homogeneity with at least two solvent systems.

Melting points (uncorr.) were measured on a Boetius microscope hot stage. Elementary analyses (C, H, N) were performed by Dr. J. Binkowski of the Polish Academy of Sciences (Łódź). Mass spectra were obtained with the aid of an LKB 9000 spectrometer. NMR spectra were recorded with a Bruker 90 instrument, and chemical shifts are relative to internal TMS (tetramethylsilane).

Ultraviolet absorption spectra were run on a Hungarian Spectromom 195 instrument. TLC made use of Merck (Darmstadt, GFR) GF-254 silica gel plates with four solvent systems: S1 (chloroform:ethanol, 92:8, v/v), S2 (butanol:water, 86:14, v/v), S3 (chloroform:ethanol, 7:3, v/v), S4 (butanol:acetone:water, 4:5:1, v/v).

Chemical syntheses

(see Table III, below, for structures)

(2-Acetoxyethoxy)methyl chloride was prepared essentially as described by Arbuzov and Ukhvatova [15].

(2-Benzoyloxyethoxy)methyl chloride. A sample of 2-benzoyloxyethoxy methane (b.p. 128 °C/8 mm Hg, n_D^{20} 1.5033) obtained by esterification of 2-methoxyethanol-1 with benzoyl chloride, was dissolved in double the volume of CCl_4 and chlorinated with an equimolar amount of chlorine at 0 °C, with exposure of the reaction mixture to a daylight lamp. The solution was washed several times with a saturated solution of Na_2CO_3, then with water, and dried over anhydrous $MgSO_4$. Rectification under reduced pressure led to the desired compound, b.p. 124–132 °C/2 mm Hg, n_D^{20} 1.525–1.529, identical with the same compound prepared by an alternative route [16]. The product exhibited a characteristic ^1H signal for the two protons of the fragment $-O-CH_2-Cl$ at 5.45 ppm.

1-(2'-Hydroxyethoxy)methyl uracils (**1a–1h**). The *bis*-(trimethylsilyl) derivative of each uracil analogue was obtained by treatment with trimethylchlorosilane and triethylamine [17] and purified by distillation under reduced pressure. Condensation of 10 mmol of a silylated uracil analogue with 20 mmol (2-acetoxyethoxy)methyl, or (2-benzoyloxyethoxy)-methyl, chloride was conducted at room temperature without solvent. When the reaction (monitored by TLC with solvent S1) was judged complete (16 h), the mixture was taken up in 50 ml 95% ethanol, filtered through Celite, brought to dryness and crystallized from ethanol-ether (4:1). The chromatographically homogeneous product was deblocked with methanolic ammonia or a stoichiometric amount of sodium methoxide in methanol, the mixture passed through Dowex 50W (H^+), brought to dryness and crystallized from 95% ethanol (see Table I, below, for details for each analogue).

1-(1',3'-Dihydroxy-2'-propoxy)methyl uracils (**2a**, **2b**, **2d**, **2e**, **2f**, **2h**, **2i**). Condensation of 30 mmol of a silylated uracil congener with 1,3-di-O-benzyl-2-O-chloromethyl glycerol (12.9 g, 40 mmol), prepared as described elsewhere [18] was performed at room temperature without added solvent. When the reaction (monitored by TLC) was complete, the mixture was brought to dryness, the residue taken up in 50 ml 95% ethanol and precipitated with ether. The product was deblocked by hydrogenation over 1 g PdO (85% Pd) per 10 mmol in aqueous ethanol at atmospheric pressure. The catalyst was removed by filtration, the filtrate deposited on a 3×15 cm column of silica gel, and eluted with solvent S3. The pooled fractions of product were brought to dryness and the residue crystallized from ethanol-ether (4:1). See Table II, below, for data.

1-(3'-chloro-2'-hydroxypropyl)-5,6-tetramethyleneuracil (**4**). To 2 g (9.7 mmol) of 2,4-*bis*-(trimethylsilyl)-5,6-tetramethyleneuracil in 50 ml acetonitrile was added 4.65 g (50 mmol) 1,2-epoxy-3-chloropropane and 0.1 ml $SnCl_4$ and the mixture stored for 1 day at room temperature. It was then brought to dryness and the residue taken up in dichloroethane and washed with saturated aqueous Na_2CO_3 and water. The organic phase was dried over $MgSO_4$ and brought to small volume, from which it crystallized overnight to yield 0.92 g (35%), m.p. 174–175 °C (R_f 0.42 with S1 and 0.79 with S2). UV (pH 7) λ_{max} 271 nm (ε_{max} 10.5×10^3). NMR ($CDCl_3$), δ 3.63 ppm (d, 2, H-1'), 4.15 ppm (m, 1, H-2'), 4.04 ppm (d, 2, H-3').

1-(2'-hydroxypropyl)-5,6-tetramethyleneuracil (**6**). To 5 g (16 mmol) of the silylated tetramethyleneuracil in 100 ml acetonitrile was added 11.6 g (20 mmol) 1,2-epoxypropane and 0.1 ml

$SnCl_4$. Subsequent steps were as for **4**, above, to yield 0.96 g (27%), m.p. 188–189 °C (R_f 0.35 with S1 and 0.68 with S2). UV (pH 7) λ_{max} 273 (ε_{max} 8.4×10^3). NMR ($CDCl_3$), δ 3,82 ppm (d, 2, H-1′), 4.20 ppm (m, 1, H-2′), 1.39 ppm (d, 3, H-3′).

1-(3′-hydroxypropyl)-5,6-tetramethyleneuracil (**5**). To 1.4 g (4.5 mmol) of the silylated tetramethylene uracil was added 13.2 g (34.3 mmol) of 1-bromo-3-benzoyloxypropane, and the mixture heated for 5 h at 100 °C. The resulting precipitate was washed with water, taken up in 20 ml CH_3OH containing 3 mmol CH_3ONa and heated to boiling. The cooled solution was deposited on a 1 × 15 cm column of Amberlite IR-120 (H^+) and the product eluted with 50 ml of 50% aqueous methanol. The eluate was concentrated to small volume, leading to crystallization to give 0.25 g (25%), m.p. 152 °C, chromatographically homogeneous (R_f 0.34 with S1 and 0.61 with S2). UV (pH 7) λ_{max} 275 nm (ε_{max} 10.0×10^3). NMR (D_2O), δ 3.67 (m, 2, H-1′), 1.65 ppm (m, 2, H-2′), 3.94 ppm (m, 2, H-3′).

1-(2-tetrahydrofuranyl)-5,6-tetramethyleneuracil (**8**). To 10.4 g (30 mmol) of the silylated tetramethyleneuracil in 20 ml anhydrous benzene was added 3.4 g (30 mmol) 2-chlorotetrahydrofuran, and the mixture stored overnight. Solvent was removed under reduced pressure, the residue taken up in 100 ml water and extracted with $CHCl_3$. The organic phase was dried over $MgSO_4$ and reduced in volume to yield 1.2 g (17%), m.p. 205–206 °C (R_f 0.65 with S1). UV (pH 7) λ_{max} 274 nm (ε_{max} 9.8×10^3). NMR ($CDCl_3$), δ 6.0 ppm (m, 1, H-1′), 2.56 ppm (m, 4, H-2′ and H-3′), 4.3 ppm (q, 2, H-4′).

(S)-1-(2′,3′-dihydroxypropyl)-5,6-tetramethyleneuracil ((*S*)-**3**). To the sodium salt of tetramethyleneuracil, from 0.83 g (5 mmol) tetramethyleneuracil in 30 ml methanol containing 0.23 g (10 mmol) Na, was added 1.43 g (5 mmol) of 3-O-tosyl-1,2-O-isopropylideneglycerine in 10 ml dimethylformamide. The mixture was heated for 10 h at 100 °C, solvent removed under reduced pressure, and the residue taken up in benzene and deposited on a 1 × 25 cm column of silica gel. Elution with benzene-ethyl acetate (8:2, v/v) at a flow-rate of 0.5 ml/min, with collection of 10 ml fractions, gave three peaks with R_f values (solvent S1) of 0.53 (fractions 200–205, substituted on N-1), 0.59 (fractions 173–180, substituted on N-3), 0.74 (fractions 120-135, substituted on N-1, N-3). The fractions with R_f 0.53 were pooled, brought to small volume and heated to boiling in 5 ml 80% CH_3COOH. Solvent was removed and the residue crystallized from 95% ethanol to yield 60 mg (10%) of (*S*)-**3**, m.p. 178–180 °C, $[\alpha_{20}^D]$ −75 ° (*c* = 0.75, water), chromatographically homogeneous with R_f values and UV spectra like the racemate of **3**.

Enzyme purification
(uridine phosphorylase, EC 2.4.2.3)

Cells of *E. coli* B grown, harvested and suspended as described by Vita and Magni [7], were disrupted by sonication, 6 × 30 sec with 1 min breaks, using an MSE 100 watt ultrasonic disintegrator. The sonicate was centrifuged for 15 min at 20,000 × *g* at 6 °C, and the resulting supernatant again centrifuged for 1 h at 100,000 × *g*. The final supernatant was dialyzed overnight *vs* 20 mM Tris-HCl buffer pH 7.5 containing 5 mM 2-mercaptoethanol, and then subjected to purification by chromatography on a column of Matrex Gel Green A according to Vita and Magni [7].

Protein determinations were performed by the method of Bradford [19], with bovine serum albumin as standard.

As elsewhere reported [9], the uridine phosphorylase was homogeneous on polyacrylamide gel electrophoresis, with a specific activity of 250 μmol/min/mg protein at pH 7.5 and 37 °C for phosphorolysis of uridine.

The column effluent, following binding of uridine phosphorylase, was employed as a source of thymidine phosphorylase. It was totally devoid of uridine phosphorylase activity (unpublished).

Enzyme assays

Phosphorolysis assays were performed spectrophotometrically with uridine as substrate. Phosphorolysis was followed by continuous recording of the decrease in absorption at 280 nm, using a Cary 118 recording instrument fitted with a temperature-controlled cuvette compartment maintained at 37 °C. For conversion of uridine to uracil, $\Delta\varepsilon_{280} = 2.1 \times 10^3$. The incubation mixture, 2,5 ml in a 10 mm cuvette, contained 100 mM phosphate buffer pH 7.5, 80–240 μM uridine and inhibitors at concentrations given in figures. The reaction was initiated by addition of enzyme (0.4 μg/ml). Initial reaction rates were employed for determination of *v*. Apparent K_m and apparent K_i were evaluated from double reciprocal plots of $1/v$ *vs* $1/S$.

Phosphorolysis of thymidine by thymidine phosphorylase was followed spectrophotometrically as for uridine phosphorylase, but at 275 nm, with $\Delta\varepsilon_{275} = 2.42 \times 10^3$.

Reverse reaction (synthesis of uridine). The incubation medium, 50 µl of 50 mM Tris-HCl buffer pH 7.5, contained (a) 2.5 mM ribose-1-phosphate and [2-^{14}C]uracil (0.25–1 mM, 0.25 µCi/mM), or (b) 2.5 mM [2-^{14}C]uracil (0.25 µCi) and ribose-1-phosphate (0.1–0.6 mM). The reaction was initiated at 37 °C by addition of enzyme, and terminated after 5 min by insertion of the tube in boiling water for 2 min. The sample was cooled in ice and centrifuged to collect moisture condensed on the walls. A 5-µl aliquot was deposited on a thin sheet of F-254 cellulose, followed by 1 µl each of 10 mM solutions of uracil and uridine as standards, and the sheet was developed with water-saturated *n*-butanol. The spots of uridine ($R_f = 0.17$) and uracil ($R_f = 0.33$) were located with a dark UV-lamp, cut out, transferred to a vial containing toluene scintillation fluid, and radioactivity counted in a Beckman LS-9000 instrument.

Results and Discussion

Synthetic procedures

The general procedure adopted for the synthesis of the acyclonucleoside analogues (for details see above) was based on a Hilbert-Johnson type condensation of a 1,3-*bis*(trimethylsilyl) derivative of the desired uracil analogue with the appropriately blocked reactive acyclic synthon without added solvent, at room temperature. This led, as expected, to N(1)-acyclonucleosides, which exhibited the characteristic decrease in UV absorption on transfer from neutral to alkaline medium.

For the series **1** compounds (**1a–1h**, see Table I), (2-acetoxyethoxy)methyl chloride, prepared from acetyl chloride and 1,3-dioxalane [15] was initially employed in the condensation reaction to obtain **1a–1e**. However, the resulting acetylated products of **1d** and **1e** were obtained as oils, which proved difficult to purify. Attention was then directed to the use of (2-benzoyloxyethoxy)methyl chloride, previously reported, but without experimental details [16]. This was now synthesized by a different route (see Chemical syntheses, above), and its use in the condensation reaction led to isolation in crystalline form of the benzoylated derivatives of **1c–1h**. All the foregoing were then deblocked, as indicated in Table I, either with NH_3/CH_3OH or (when this proceeded slowly) CH_3ONa/CH_3OH, to give **1a–1h** in crystalline form from 95% ethanol.

The synthesis of the compounds of series **2** (Table II) was based on the use of 1,3-di-O-benzyl-2-O-chloromethyl glycerol as the acyclic synthon, essentially as described by Martin *et al.* [17] for other analogues. This series was further extended to include 5-benzyluracil as the aglycone (**2i** in Table II), previously reported a strong inhibitor of the enzyme from mammalian sources [4].

Table I. Preparative and analytical data for 1-(2′-hydroxyethoxy)methyl uracils (Series **1** analogues, for structure see Table III).

Compound	Blocked product			Acyclonucleoside following deblocking						NMR in $CDCl_3$	
	Condensation with	Yield [%]	m.p. [°C]	Yield [%]	m.p. [°C]	R_f with solvent S1	S2	UV (pH 7) λ_{max} (nm)	$\varepsilon_{max} \times 10^3$	H1′	H4′H5′
1a	A	70	86– 87	72	138–140[a]	0.08	0.22	260	8.5	515s	3.50s
1b	A	85	117–119	68	143–144[b]	0.17	0.33	265	9.4	5.65s[c]	4.12s[c]
1c	B	36	121–122	54	145–147	0.22	0.36	268	11.3	5.33s	3.67s
1d	B	47	80– 83	53	140–142	0.24	0.42	265	9.6	5.20s	3.73s
1e	B	55	68– 69	74	120	0.28	0.50	268	10.0	5.22s	3.73s
1f	B	68	93– 97	99	127–128	0.29	0.48	265	9.3	5.23s	3.75s
1g	B	85	144–149	80	170–173	0.23	0.31	269	10.6	5.27s	3.75s
1h	B	84	159–160	72	186–188	0.28	0.43	270	10.9	5.35s	3.74s

A = (2-acetoxyethoxy)methyl chloride.
B = (2-benzyloxyethoxy)methyl chloride.
[a] Robins and Hatfield [26] report 147–148 °C (from methanol) and Abrams *et al.* [16] 140 °C (from ethanol).
[b] Robins and Hatfield [26] report 150–152 °C (from methanol) and Abrams *et al.* [16] 139 °C (from ethanol).
[c] In D_2O.

Table II. Preparative and analytical data for 1-(1′,3′-dihydroxy-2′-propoxy)methyl uracils (Series **2** analogues, for structure see Table III).

Compound	Blocked product		Acyclonucleoside following deblocking						NMR in D_2O		
	Yield	m.p.	Yield	m.p.	R_f with solvent		UV (pH 7) λ_{max}				
	[%]	[°C]	[%]	[°C]	S3	S4	(nm)	$\varepsilon_{max} \times 10^3$	H1′	H4′	H3′H5′
2a	70	oil	31	115[a]	0.34	0.71	260	9.6	5.73s	4.20m	4.05m
2b	93	93–96	64	159–163[b]	0.49	0.76	265	10.9	5.60s	4.17m	4.03m
2d	95	oil	42	118–120	0.59	0.81	265	9.1	5.56s	4.04m	3.89m
2e	78	oil	37	96– 98	0.65	0.83	264	9.3	5.46s	4.01m	3.84m
2f	80	oil	58	105–107	0.66	0.84	264	9.9	5.71s	4.18m	4.06m
2h	85	69–71	47	148–150	0.63	0.81	268	11.1	5.73s	4.16m	3.93m
2i	95	45–50	33	oil	0.72	0.90	266	9.8	5.37s	4.05m	3.73m

[a] Martin *et al.* [27] report 117–118 °C.
[b] Martin *et al.* [27] report 156–157 °C.

Preliminary measurements of inhibitory properties *vs E. coli* uridine phosphorylase of compounds in the series **1** and **2** directed our attention to those containing 5,6-tetramethyleneuracil as the aglycone (**1h** and **2h**), in particular the fact that **2h** was a 10-fold more effective inhibitor than **1h** (see Table III). This suggested the possible utility of attachment of other acyclic analogues of the pentose ring to the N(1) of this aglycon, *e.g.* compound **3** may be considered as containing the "lower" fragment of the ribose ring; whereas compounds **5** and **6**, each with a hydroxyl group, are models of the parent 2′- and 3′-deoxyribosides. Furthermore the acyclic chains of **4** and **7** mimic structurally the corresponding chloro- and anhydro-sugars. Finally, compound **8**, in which the ribose ring is replaced by tetrahydrofuran, should indicate to what extent the sugar hydroxyls are necessary for effective inhibition.

Compounds **4** and **6** were prepared with the use of 1,2-epoxy-3-chloropropane and 1,2-epoxypropane, respectively; **5** with the use of 1-bromo-3-benzoyloxypropane, and **8** with 2-chlorotetrahydrofuran [20]. The syntheses of **7**, and its product of oxidation, **3**, have been previously described [21, 22].

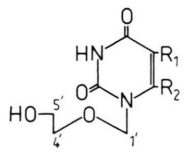
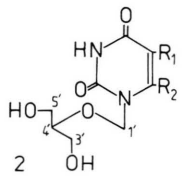
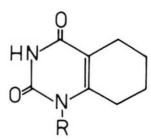
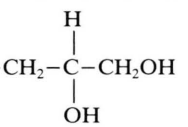
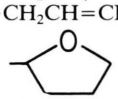
Compounds **3**, **4**, **6** and **8** are racemates. Since **3** was found to be a moderate inhibitor, attempts were made to prepare the *R* and *S* enantiomers, but without success for the former. (*S*)-**3** was prepared with the use of 3-tosyl-1,2-isopropylidene glycerine [23], which was condensed with the sodium salt of 5,6-tetramethyleneuracil. The products were found to include, in addition to the desired 1-substituted derivative, of appreciable proportions of the 3-substituted and 1,3-disubstituted analogues. The mixture was readily fractionated on a silica gel column (see Chemical syntheses, above). Only the 1-substituted product was examined as a potential inhibitor, since we had found, earlier in this study, that the N(3) isomers of **3**, **5** and **7**, prepared by an independent route, were very poor inhibitors of uridine phosphorylase, with K_i values in excess of 200 μM.

All the synthesized compounds, most of which are new, were chromatographically homogeneous in the two solvent systems employed (Table I, II). Their UV absorption spectra exhibited the typical decrease in extinction of about 22% for 1-substituted uracil analogues on transfer from pH 7 to pH 12. All of them gave satisfactory elementary analyses for C, H, N, and the mass spectra exhibited a major peak at M^+ (with the exception of **2a**), and significant peaks B and B + 1, characteristic for 1-substituted uracils [24]. The entire fragmentation patterns (available on request) were consistent with the postulated structures, further supported by the ^1H NMR spectral data.

Acyclonucleoside analogues as inhibitors of phosphorolysis

The extent to which the various synthetic acyclonucleosides inhibited phosphorolysis of uridine is shown in Table III, which gives the inhibition constants, K_i. None of them was a substrate of the enzyme, or a substrate or inhibitor of *E. coli* thymidine phosphorylase. With uridine phosphorylase, all acyclonucleosides were competitive inhibitors of uridine phosphorolysis, as illustrated by the appropriate data for one of the more effective ones, **2h** (Fig. 1).

Table III. Inhibition constants, K_i, for inhibition of *E. coli* uridine phosphorylase by acyclonucleoside analogues.

Inhibitor		R_1		R^2	K_i [μM]
	**1a**	−H		−H	35
	1b	$-CH_3$		−H	77
	1c	$-CH_3$		$-CH_3$	53
	1d	$-C_2H_5$		−H	15
	1e	$-C_3H_7$		−H	14
	1f	$-CH(CH_3)_2$		−H	58
	1g		$-(CH_2)_3-$		100
1	**1h**		$-(CH_2)_4-$		27
	**2a**	−H		−H	23
	2b	$-CH_3$		−H	21
	2d	$-C_2H_5$		−H	5
	2e	$-C_3H_7$		−H	10
	2f	$-CH(CH_3)_2$		−H	7
	2h		$-(CH_2)_4-$		2.7
2	**2i**	$-CH_2C_6H_5$		−H	0.75
		R			
	(*RS*)-**3**	$-CH_2CH(OH)CH_2OH$			26
	(*S*)-**3**				inactive
	(*RS*)-**4**	$-CH_2CH(OH)CH_2Cl$			26
	5	$-CH_2CH_2CH_2OH$			40
3 - 8	(*RS*)-**6**	$-CH_2CH(OH)CH_3$			43
	7	$-CH_2CH=CH_2$			~ 300
	8				~1000

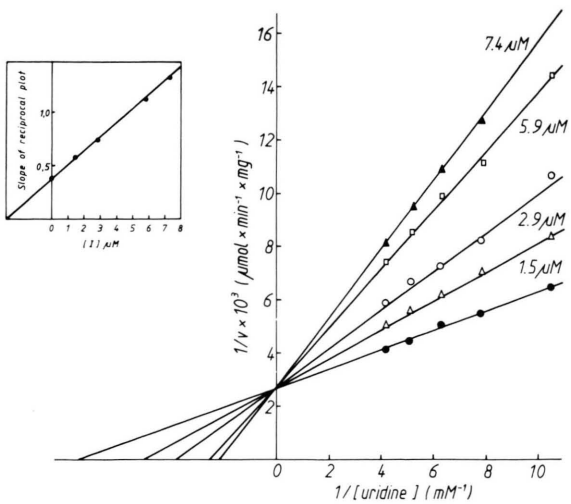


Fig. 1. Lineweaver-Burk double reciprocal plots for inhibition of uridine phosphorylase by compound **2h**. Inhibitor concentrations varied from 0 to 7.4 μM, as indicated beside each plot. Phosphorolysis was followed spectrophotometrically by procedure A (see Materials and Methods), with 0.4 μg/ml enzyme. Insert: plot of slopes of reciprocal plots *vs* inhibitor concentration [I].

*Series **1** analogues.* Amongst these (see Table III), the most effective, albeit moderate, inhibitors are **1d** ($K_i = 15$ μM) and **1e** ($K_i = 14$ μM). Note, however, that a change of the 5-substituent of the uracil moiety from propyl (**1e**) to the isomeric isopropyl (**1f**) leads to a marked reduction in affinity for the enzyme (4-fold increase in K_i). By contrast, a change in the aglycon from 5-methyluracil (**1b**) to 5,6-dimethyluracil (**1c**) actually slightly enhances affinity for the enzyme (decrease in K_i from 77 μM to 53 μM). This is clearly relevant to the previous finding [25] that 6-methyluridine and 5,6-dimethyluridine, both of which are in the fixed *syn* conformation about the glycosidic bond, are reasonably good substrates of the enzyme.

Quite striking is the fact that **1h** is a 4-fold more effective inhibitor than **1g**. The corresponding nucleoside analogues, 5,6-tetramethyleneuridine and 5,6-trimethyleneuridine, have been shown to be substrates for the bacterial enzyme, with similar V_{max} values; but the K_m for the former (corresponding to **1h**) is 4-fold higher than for the latter (corresponding to **1g**) [25], hence consistent with **1h** being 4-fold more effective an inhibitor than **1g**.

*Series **2** analogues.* The results for this series demonstrate the rather striking effects of branching of the acyclic chain. With uracil as the aglycone (**2a**), the K_i (23 μM) is only minimally affected as compared to **1a** ($K_i = 35$ μM). But with 5-methyluracil as the aglycone, the K_i is reduced from 77 μM for **1b** to 21 μM for **2b**. A similar 3-fold reduction is observed when the aglycone is 5-ethyluracil (K_i of **1d** is 15 μM, and of **2d** 5 μM).

With 5-propyluracil as the aglycone, the K_i values of **1e** and **2e** are similar. Hence all the more striking is the fact that, with 5-isopropyluracil as the aglycone, the K_i of **1f** (58 μM) is reduced almost 9-fold in **2f** (7 μM). This effect is even more pronounced with 5-tetramethyleneuracil as the aglycone, the K_i for **1h** being reduced from 27 μM to 2.7 μM for **2h**.

Compound **2i**, with 5-benzyluracil as the aglycone, turned out to be the most effective inhibitor ($K_i = 0.7$ μM), in agreement with the finding of Niedzwicki *et al.* [3] and Lin and Liu [4] that the highly hydrophobic 5-benzyl group leads to a marked enhancement of inhibition with the enzyme from mammalian sources.

*Analogues **3**–**7**.* As mentioned above, these compounds, all with 5,6-tetramethyleneuracil as the aglycone, but with acyclic chains differing from those in series **1** and **2**, were prepared following the observation that **2h** is 10-fold more effective an inhibitor than **1h**. None of them proved as effective as **2h**.

It should, however, be noted that **3**, **4**, **6** are racemates. In the case of **3**, it proved possible to synthesize the *S* enantiomer, and this proved totally inactive as an inhibitor. Hence the *R* enantiomer would be expected to exhibit a K_i value one-half that found for the racemate of **3** (26 μM, Table III), *i.e.* 13 μM. Presumably similar results would prevail for the other racemates. This observation underlines the high specificity of interaction of these acyclic chains with the enzyme.

Inhibition of reverse, synthetic, reaction

Since the reaction catalyzed by uridine phosphorylase is a reversible one, it was of obvious interest to examine the extent to which the foregoing inhibitors of phosphorolysis affect the synthesis of uridine from ribose-1-phosphate and uracil. Compound **2h** was selected for such experiments, and effectively inhibited the synthesis of uridine.

Lineweaver-Burke plots of initial activities for synthesis of uridine with various initial concentrations of uracil (and constant concentration of ribose-1-phosphate), and varying initial concentrations of ribose-1-phosphate (and constant concentration of uracil), are shown in Fig. 2. The resulting K_m for ribose-1-phosphate in the presence of 2.5 mM uracil was 0.52 mM; for uracil in the presence of 2.5 mM ribose-1-phosphate, the K_m was 0.72 mM.

From Fig. 2 it will be seen that inhibition by **2h** is competitive in character (as for phosphorolysis) with respect to both uracil and ribose-1-phosphate. A replot of the slope of each reciprocal plot *vs* inhibitor concentration, [I], led to K_i values of 19 μM for competition with uracil and 15 μM as a competitive inhibitor of ribose-1-phosphate.

Concluding remarks

Several of the acyclonucleoside analogues we have examined were also prepared, by different routes, by Niedzwicki *et al.* [2] and Lin and Liu [4], and examined as inhibitors of the enzyme in cytosol extracts of mammalian cells, presumably free of thymidine phosphorylase.

For compounds **1a** and **1b**, Niedzwicki *et al.* [2] report K_i values of 15 μM and 3 μM, respectively. With the bacterial enzyme, our values are 35 μM for

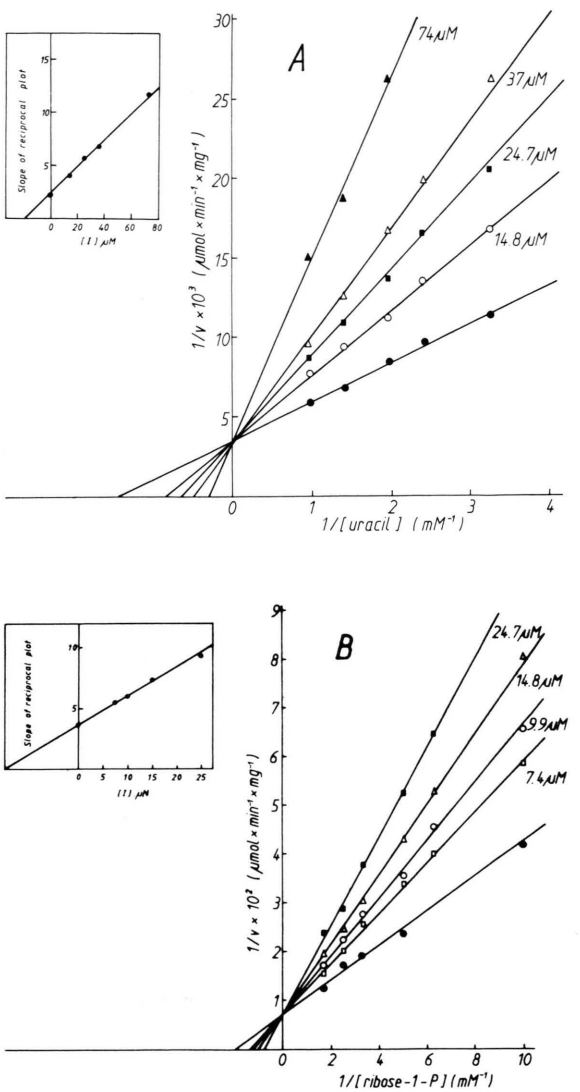


Fig. 2. Double reciprocal plots for inhibition, by compound **2h**, of uridine phosphorylase-catalyzed synthesis of uridine from uracil and α-D-ribose-1-phosphate. The reaction was followed radiochemically (see Materials and Methods), with an enzyme concentration of 0.4 μg/ml. Inhibitor concentrations are indicated beside each plot: (A) with uracil as the variable substrate, and constant concentration of ribose-1-phosphate (2.5 mM). $K_m = 0.71$ mM, $v = 333$ μmol/min/mg enzyme. Insert: plot of slopes of reciprocal plots *vs* inhibitor concentration. (B) with ribose-1-phosphate as variable substrate and constant uracil concentration (2.5 mM). $K_m = 0.52$ mM, $v = 143$ μmol/min/mg enzyme. Insert: plot of slopes of reciprocal plots *vs* inhibitor concentration.

1a und 77 μM for **1b** (Table III). Two points here call for comment. First, with the mammalian enzyme, the thymine analogue **1b** is 5-fold more effective as inhibitor than the uracil analogue **1a**; with our bacterial enzyme, the reverse holds, **1a** being twice as effective as **1b**. The second is the remarkably low value of K_i for **1b** with the mammalian enzyme (3 μM) as compared to the bacterial enzyme (77 μM).

Furthermore, Lin and Liu [4] also synthesized **2i** by a different route and, using a Sarcoma 180 cell extract as source of uridine phosphorylase, report a K_i of 0.1 μM as compared to our value of 0.7 μM for the bacterial enzyme.

It must be concluded from the foregoing that, as in the case of purine nucleoside phosphorylases, where differences in effectiveness of various inhibitors exist not only between the mammalian and bacterial enzymes, but also between the mammalian enzymes from different sources, also holds for pyrimidine nucleoside phosphorylases. We have consequently undertaken a study of the inhibitory properties of our acyclonucleoside analogues, using a partially purified enzyme from mouse intestinal mucosa, and some preliminary results confirm the foregoing. For example, the K_i values for **1a** and **1b** are approximately 5.2 μM and 2.2 μM, hence they are not only better inhibitors with this enzyme system than that employed by Niedzwicki *et al.* [2], but the thymine analogue (**1b**) is more effective than the uracil analogue (**1a**). Similar exploratory results indicate that several of the compounds in the series **2** are more effective inhibitors in the mammalian enzyme system, and these studies are being continued, in part because of their potential utility in chemotherapy. It is hoped to extend these investigations with the use of some highly purified enzyme from mammalian sources.

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