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

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Synthetic procedures are described for the preparation of a variety of pyrimidine acyclonucleosides, 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, 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 = 2.7 μM. The same compound was a reasonably good inhibitor of the reverse, synthetic, reaction, with K, 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 IC50 ~ 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), [2,14C]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-tetramethyleneuracil in 100 ml acetonitrile was added 11.6 g (20 mmol) 1,2-epoxypropane and 0.1 ml SnCl₄ (92:8, v/v), S2 (butanol:water, 86:14, v/v), S3 (chloroform:ethanol, 7:3, v/v), S4 (butanol:acetic acid: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-Benzoxoxyethoxy)methyl chloride. A sample of 2-benzoxoxyethoxy methane (b.p. 128 °C/8 mm Hg, nD₂₅ 1.5033) obtained by esterification of 2-methoxyethanol-1 with benzoyl chloride, was dissolved in double the volume of CCl₄ 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 Na₂CO₃, then with water, and dried over anhydrous MgSO₄. Rectification under reduced pressure led to the desired compound, b.p. 124–132 °C/2 mm Hg, nD₂₅ 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—CH₂—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-benzoxoxyethoxy)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-2-0-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'-propoxy)-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-chloropropene and 0.1 ml SnCl₂ 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 Na₂CO₃ and water. The organic phase was dried over MgSO₄ and brought to small volume, from which it crystallized overnight to yield 0.92 g (35%), m.p. 174–175 °C (R₁ 0.42 with S1 and 0.79 with S2). UV (pH 7) λmax 271 nm (εmax 10.5 × 10⁵). NMR (CDCl₃), 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) $\lambda_{\text{max}}$ 273 (t$_{\text{max}}$ 8.4 x 10$^3$). NMR (CDCl$_3$), $\delta$ 3.82 ppm (d, 2, H-1'), 4.20 ppm (m, 1, H-2'), 1.39 ppm (d, 3, H-3').

$I$-(3'-hydroxypropyl)-5,6-tetramethyleneuracil (5). To 1.4 g (4.5 mmol) of the silylated tetramethyleneuracil was added 13.2 g (34.3 mmol) of 1-bromo-3-benzyloxypropane, and the mixture heated for 5 h at 100 °C. The resulting precipitate was washed with water, taken up in 20 ml CH$_3$OH containing 3 mmol CH$_3$ONa and heated to boiling. The cooled solution was deposited on a 1 x 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 0.96 g (27%), m.p. 188–189 °C ($R_f$ 0.35 with S1 and 0.61 with S2). UV (pH 7) $\lambda_{\text{max}}$ 275 nm (t$_{\text{max}}$ 10.0 x 10$^3$). NMR (D$_2$O), $\delta$ 3.67 (m, 2, H-1'), 1.65 ppm (m, 2, H-2'), 3.94 ppm (m, 2, H-3').

$I$-(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) $\lambda_{\text{max}}$ 274 nm (t$_{\text{max}}$ 9.8 x 10$^3$). NMR (CDCl$_3$), $\delta$ 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)-I-(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-isopropylidenglycerine 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 x 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 (solute 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$_3$COOH. Solvent was removed and the residue crystallized from 95% ethanol to yield 60 mg (10%) of (S)-3, m.p. 178–180 °C, [d$_{25}$] $-$75 °C (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 x 30 sec with 1 min breaks, using an MSE 100 watt ultrasonic disintegrator. The sonicate was centrifuged for 15 min at 20,000 x g at 6 °C, and the resulting supernatant again centrifuged for 1 h at 100,000 x 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 Maxtrel 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, $\Delta$ε$_{280}$ = 2.1 x 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 $\Delta E_{275} = 2.42 \times 10^3$.

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-fr(u(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-benzyloxyethoxy)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 benzyolated derivatives of 1f–1h. All the foregoing were then deblocked, as indicated in Table I, either with NH$_3$/CH$_3$OH or (when this proceeded slowly) CH$_3$ONa/CH$_3$OH, 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).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Blocked product</th>
<th>Yield</th>
<th>m.p.</th>
<th>UV (pH 7)</th>
<th>NMR in CDCl$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conden­sation with</td>
<td>[%]</td>
<td>[°C]</td>
<td>$\lambda_{max}$ (nm)</td>
<td>$\epsilon_{max} \times 10^3$</td>
</tr>
<tr>
<td>1a</td>
<td>A</td>
<td>70</td>
<td>86–87</td>
<td>72</td>
<td>138–140$^a$</td>
</tr>
<tr>
<td>1b</td>
<td>A</td>
<td>85</td>
<td>117–119</td>
<td>68</td>
<td>143–144b</td>
</tr>
<tr>
<td>1c</td>
<td>B</td>
<td>36</td>
<td>121–122</td>
<td>54</td>
<td>145–147</td>
</tr>
<tr>
<td>1d</td>
<td>B</td>
<td>47</td>
<td>80–83</td>
<td>53</td>
<td>140–142</td>
</tr>
<tr>
<td>1e</td>
<td>B</td>
<td>55</td>
<td>68–69</td>
<td>74</td>
<td>120</td>
</tr>
<tr>
<td>1f</td>
<td>B</td>
<td>68</td>
<td>93–97</td>
<td>99</td>
<td>127–128</td>
</tr>
<tr>
<td>1g</td>
<td>B</td>
<td>85</td>
<td>144–149</td>
<td>80</td>
<td>170–173</td>
</tr>
<tr>
<td>1h</td>
<td>B</td>
<td>84</td>
<td>159–160</td>
<td>72</td>
<td>186–188</td>
</tr>
</tbody>
</table>

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$_2$O.
Table II. Preparative and analytical data for 1-(1',3'-dihydroxy-2'-propoxy)methyl uracils (Series 2 analogues, for structure see Table III).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Blocked product Yield [%]</th>
<th>m.p. [°C]</th>
<th>Acyclonucleoside following deblock Yield [%]</th>
<th>m.p. [°C]</th>
<th>$R_f$ with solvent</th>
<th>UV (pH 7) $\lambda_{max}$ (nm)</th>
<th>$\epsilon_{max} \times 10^3$</th>
<th>NMR in D$_2$O H1' H4' H3'H5'</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>70</td>
<td>oil</td>
<td>31</td>
<td>115$^a$</td>
<td>0.34</td>
<td>0.71</td>
<td>260</td>
<td>9.6</td>
</tr>
<tr>
<td>2b</td>
<td>93</td>
<td>93–96</td>
<td>64</td>
<td>159–163b</td>
<td>0.49</td>
<td>0.76</td>
<td>265</td>
<td>10.9</td>
</tr>
<tr>
<td>2c</td>
<td>78</td>
<td>oil</td>
<td>37</td>
<td>96–98</td>
<td>0.59</td>
<td>0.81</td>
<td>264</td>
<td>9.1</td>
</tr>
<tr>
<td>2d</td>
<td>80</td>
<td>oil</td>
<td>58</td>
<td>105–107</td>
<td>0.66</td>
<td>0.84</td>
<td>264</td>
<td>9.9</td>
</tr>
<tr>
<td>2e</td>
<td>85</td>
<td>69–71</td>
<td>47</td>
<td>148–150</td>
<td>0.63</td>
<td>0.81</td>
<td>268</td>
<td>11.1</td>
</tr>
<tr>
<td>2f</td>
<td>95</td>
<td>45–50</td>
<td>33</td>
<td>oil</td>
<td>0.72</td>
<td>0.90</td>
<td>266</td>
<td>9.8</td>
</tr>
</tbody>
</table>

$^a$ 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-chloropropionate and 1,2-epoxypropionate, 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 $^1$H NMR spectral data.

Acyclonucleoside analogues as inhibitors of phosphorylase

The extent to which the various synthetic acyclonucleosides inhibited phosphorylase 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 phosphorylase, 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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$R_1$</th>
<th>$R^2$</th>
<th>$K_i$ [μM]</th>
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<tbody>
<tr>
<td>1a</td>
<td>−H</td>
<td>−H</td>
<td>35</td>
</tr>
<tr>
<td>1b</td>
<td>−CH$_3$</td>
<td>−H</td>
<td>77</td>
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<tr>
<td>1c</td>
<td>−CH$_3$</td>
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<td>53</td>
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<tr>
<td>1d</td>
<td>−C$_2$H$_5$</td>
<td>−H</td>
<td>15</td>
</tr>
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<td>1e</td>
<td>−C$_2$H$_5$</td>
<td>−H</td>
<td>14</td>
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<tr>
<td>1f</td>
<td>−CH(CH$_3$)$_2$</td>
<td>−H</td>
<td>58</td>
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<tr>
<td>1g</td>
<td>−(CH$_2$)$_3$</td>
<td>−</td>
<td>100</td>
</tr>
<tr>
<td>1h</td>
<td>−(CH$_2$)$_3$</td>
<td>−</td>
<td>27</td>
</tr>
<tr>
<td>2a</td>
<td>−H</td>
<td>−H</td>
<td>23</td>
</tr>
<tr>
<td>2b</td>
<td>−CH$_3$</td>
<td>−H</td>
<td>21</td>
</tr>
<tr>
<td>2d</td>
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<td>5</td>
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<td>2f</td>
<td>−CH(CH$_3$)$_2$</td>
<td>−H</td>
<td>7</td>
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<tr>
<td>2h</td>
<td>−(CH$_2$)$_3$</td>
<td>−</td>
<td>2.7</td>
</tr>
<tr>
<td>2i</td>
<td>−CH$_2$C$_6$H$_5$</td>
<td>−H</td>
<td>0.75</td>
</tr>
</tbody>
</table>

(RS)-3 $\text{CH}_2\text{C}(\text{OH})\text{CH}_2\text{OH}$  26

(S)-3 $\text{CH}_2\text{C}(\text{OH})\text{CH}_2\text{OH}$ inactive

(RS)-4 $\text{CH}_2\text{C}(\text{OH})\text{CH}_2\text{Cl}$  26

5 $\text{CH}_2\text{C}(\text{OH})\text{CH}_2\text{OH}$  40

(RS)-6 $\text{CH}_2\text{C}(\text{OH})\text{CH}_2\text{Cl}$  43

7 $\text{CH}_2\text{C}(\text{OH})\text{CH}_2\text{Cl}$  300

8 $\text{CH}_2\text{C}(\text{OH})\text{CH}_2\text{Cl}$  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 [1].
**Series 1 analogues.** Amongst these (see Table III), the most effective, albeit moderate, inhibitors are 1d ($K_s = 15 \mu M$) and 1e ($K_s = 14 \mu M$). 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 $K_s$). 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 $K_s$ from 77 $\mu M$ to 53 $\mu M$). This is clearly relevant to the previous finding [25] that 6-methyluridine and 5,6-dimethyluridine, both of which are in the fixed $\psi\mu$ 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 $K_s$ (23 $\mu M$) is only minimally affected as compared to 1a ($K_s = 35 \mu M$). But with 5-methyluracil as the aglycone, the $K_s$ is reduced from 77 $\mu M$ for 1b to 21 $\mu M$ for 2b. A similar 3-fold reduction is observed when the aglycone is 5-ethyluracil ($K_s$ of 1d is 15 $\mu M$, and of 2d 5 $\mu M$).

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

Compound 2i, with 5-benzyluracil as the aglycone, turned out to be the most effective inhibitor ($K_s = 0.7 \mu M$), 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.

**Anallogues 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 $K_s$ value one-half that found for the racemate of 3 (26 $\mu M$, Table III), i.e. 13 $\mu M$. 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 $K_s$ values of 19 $\mu M$ for competition with uracil and 15 $\mu M$ 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 $K_s$ values of 15 $\mu M$ and 3 $\mu M$, respectively. With the bacterial enzyme, our values are 35 $\mu M$ 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 and 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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