bial activities of these compounds involving acylation by the mesoionic compound at unknown loci.

1-Methyl-3-isobutylxanthine was the most active of a series of 57 xanthine derivatives studied for their lipolytic potencies in epididymal fat cells.[9] Close correlation between this lipolytic activity and inhibition of cyclic AMP phosphodiesterase (PDE) was observed. Detracting somewhat in this case from this parallel structural relationship is the increase in PDE inhibition produced by alkyl groups in the 8 position of theophylline.[10] Compounds in this report are currently being evaluated as potential inhibitors of c-AMP phosphodiesterase.

Experimental Section

Melting points (uncorrected) were determined on a Mel-Temp melting point apparatus. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn.

n-**Pentyl Isothiocyanate.** Pentylamine (17.4 g, 0.2 mol) was added dropwise with stirring to a mixture of CS_2 (15.2 g, 0.2 mol) and 8% aqueous sodium hydroxide (100 ml). The mixture was stirred at room temperature for 1 hr, heated on a steam bath for 1 hr, and then chilled in an ice bath. Ethyl chloroformate (21.6 g, 0.2 mol) was added dropwise to the chilled mixture which was then stirred for 1 hr at room temperature. The organic layer was separated and distilled to give 18 g (64.7%) of *n*-pentyl isothiocyanate, bp 54–56° (3 mm) (lit.[11] bp 191°).

The Pr, *i*-Pr, Bu, *i*-Bu, hexyl, heptyl, and benzyl isothiocyanates were prepared in the same manner and used without further purification.

4-*n*-**Pentylthiosemicarbazide** (3, **R** = *n*-C_5H_{11}). Hydrazine (3.2 g, 0.1 mol) was *slowly* added to a solution of pentyl isothiocyanate (12.9 g, 0.1 mol) in Et_2O (50 ml) at 0°.‡ The reaction mixture was stirred at room temperature for 1 hr and the product collected by filtration. Recrystallization from PhH–petroleum ether gave 14.8 g (92%) of 3 (R = *n*-C_5H_{11}) as white crystals, mp 46–48°. *Anal.* ($C_6H_{15}N_3S$) C, H, N, S.

4-*n*-**Hexylthiosemicarbazide** (3, **R** = *n*-C_6H_{13}) was prepared by the above method from *n*-hexyl isothiocyanate and obtained in 87% yield following recrystallization from PhH/petroleum ether, mp 50–51°. *Anal.* ($C_7H_{17}N_3S$) C, H, N, S.

4-*n*-**Heptylthiosemicarbazide** (3, **R** = *n*-C_7H_{15}) was prepared by the above method from *n*-heptyl isothiocyanate and obtained in 93% yield following recyrstallization from petroleum ether, mp 49–50°. *Anal.* ($C_8H_{19}N_3S$) C, H, N, S.

2-*n*-**Pentylamino-1,3,4-thiadiazole** (4, **R** = *n*-C_5H_{11}; **R′ = H**). A solution of 3 (R = *n*-C_5H_{11}) (3.2 g, 20 mmol), triethyl orthoformate (3.0 g, 20 mmol), and concentrated HCl (0.1 ml) in 95% EtOH (20 ml) was stirred at room temperature for 1 hr and then refluxed for 1 hr. The solvent was removed *in vacuo* and the residual oil was dissolved in EtOAc (10 ml) and petroleum ether added to give a white cyrstalline precipitate. Recrystallization from EtOAc–petroleum ether gave 3.0 g (88%) of 4 (R = *n*-C_5H_{11}; R′ = H), mp 49–50°. *Anal.* ($C_7H_{13}N_3S$) C, H, N, S.

2-*n*-**Hexylamino-1,3,4-thiadiazole** (4, **R** = *n*-C_6H_{13}; **R′ = H**) was prepared by the above method from 3 (R = *n*-C_6H_{13}) and obtained in 61% yield following recrystallization from PhH–petroleum ether, mp 80–81°. *Anal.* ($C_8H_{15}N_3S$) C, H, N, S.

2-*n*-**Heptylamino-1,3,4-thiadiazole** (4, **R** = *n*-C_7H_{15}, **R′ = H**) was prepared by the above method from 3 (R = *n*-C_7H_{15}) and obtained in 87% yield following recrystallization from PhH–petroleum ether, mp 70–71°. *Anal.* ($C_9H_{17}N_3S$) C, H, N, S.

2-Isopropylamino-1,3,4-thiadiazole (4, **R** = *i*-C_3H_7; **R′ = H**) was prepared by the above method from 3 (R = *i*-C_3H_7) and obtained in 89% yield following recrystallization from THF–petroleum ether, mp 109–110°. *Anal.* ($C_5H_9N_3S$) C, H, N, S.

2-Ethylamino-5-methyl-1,3,4-thiadiazole (4, **R = C_2H_5; R′ = CH_3**). 2-Acetamido-5-methyl-1,3,4-thiadiazole (1.5 g, 10 mmol) was added in small portions to a stirred suspension of $LiAlH_4$ (0.38 g, 10 mmol) in THF (25 ml) at 0°. After refluxing for 2 hr, water was added dropwise until the evolution of gas ceased. The resultant mixture was filtered and the filtrate evaporated *in vacuo*. Recrystallization of the residue from 95% EtOH gave 0.72 g (53%) of 4 (R = C_2H_5; R′ = CH_3), mp 67–68° (lit.[12] mp 67–68°).

2-Benzylamino-5-methyl-1,3,4-thiadiazole (4, **R = $PhCH_2$; R′ = CH_3**) was prepared by the above method from 2-benzamido-5-methyl-1,3,4-thiadiazole and obtained in 56% yield following recrystallization from EtOH, mp 134–136° (lit.[13] mp 138–139°).

Anhydro-6-methyl-8-*n*-pentyl-5-hydroxy-7-oxo-1,3,4-thiadiazolo[3,2-*a*]pyrimidinium Hydroxide (2n). An intimate mixture of 4 (R = *n*-C_5H_{11}; R′ = H) (0.86 g, 5 mmol) and bis(2,4,6-trichlorophenyl) methylmalonate (2.38 g, 5 mmol) was heated on an oil bath (160°) under a slow stream of N_2 until a clear melt was obtained (4 min). The cooled oil was triturated with Et_2O and the resulting precipitate collected by filtration. Recrystallization from MeCN gave 1.1 g (87%) of **2n** as white crystals, mp 190–191°. *Anal.* ($C_{11}H_{15}N_3SO_2$) C, H, N, S.

The mesoionic thiadiazolopyrimidines **2a**–t (Table I) were prepared in an identical manner with that for **2n** employing either methyl, ethyl, benzyl, or the unsubstituted bis(2,4,6-trichlorophenyl) malonate ester.[14]

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‡On one occasion a moderate rate of addition of hydrazine to hexyl isothiocyanate resulted in a violent eruption of the reaction mixture.

Synthesis of the α and β Anomers of 1-(2-Deoxy-D-ribofuranosyl)-4-pyridone†

Warren James Woodford, Barbara Anne Swartz, Conrad J. Pillar, Arieh Kampf, and Mathias P. Mertes*

Department of Medicinal Chemistry, School of Pharmacy, The University of Kansas, Lawrence, Kansas 66045.
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Deaza and deoxy analogs of the naturally occurring nucleosides recently have been popular design factors in the search for new cancer chemotherapeutic agents. While the 1-deaza analogs of uridine and 2′-deoxyuridine[1] were unstable and were not growth inhibitory, the 3-deaza analogs clearly show a broad spectrum of antiviral activity.[2]

Previous studies of the inhibition of thymidylate syn-

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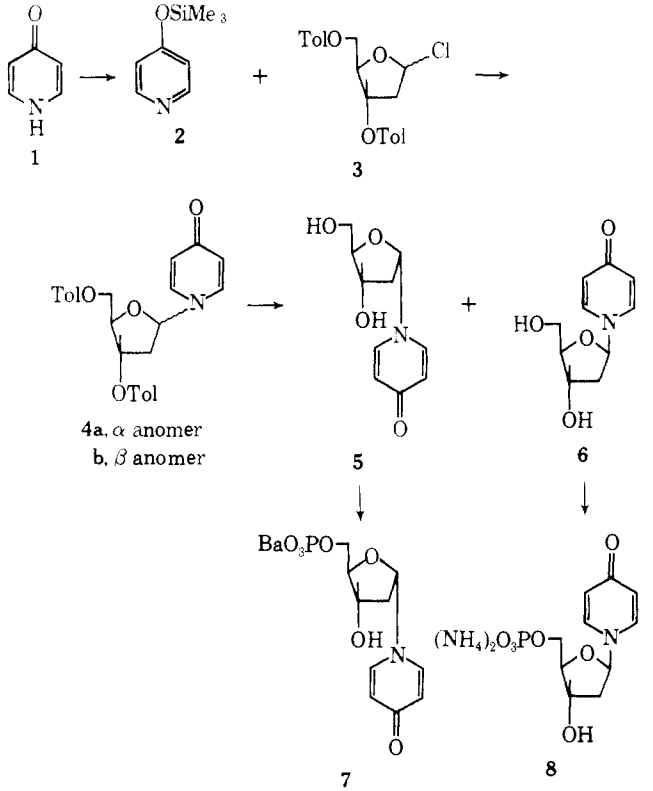
thetase have shown that significant inhibition is found in substrate analogs having a pK_a in the range of 7–9. For example, 5-fluoro-2′-deoxyuridine 5′-phosphate and the 5-trifluoromethyl analog are potent thymidylate synthetase inhibitors. The pK_a's for dissociation of the pyrimidine bases are 8.1 and 7.3, respectively.[3] 5-Trifluoromethyl-6-azauracil with a pK_a of 5.8 would be appreciably ionized at physiological pH;[4] it was inactive when tested as the deoxyribonucleotide against the enzyme. 2,6-Dihydroxypyridine has a pK_a of 4.5; the inactivity of the deoxyribonucleoside in growth inhibition studies[1] is attributed to the fact that the corresponding 2′-deoxyribonucleoside would be completely ionized at physiological pH.

Several years ago we set out to examine analogs with a higher pK_a to assess the effect on enzyme binding. The first compound synthesized in this series was the *N*-2′-deoxyribonucleoside of 2-pyridone which was also reported by several other research groups.[5] This report describes the synthesis and separation of the α and β anomers of the title compound and their 5′-phosphates. The ribosyl analog of 4-pyridone has been reported by Pischel and Wagner.[6]

On treatment with hexamethyldisilazane, 4-hydroxypyridine (**1**) was readily converted into 4-trimethylsiloxypyridine (**2**), which was condensed with 3,5-di-*O*-(*p*-toluyl)-2-deoxy-D-ribofuranosyl chloride (**3**) to afford the protected pyridine derivative **4** as a mixture of anomers. These were separated by silica chromatography to 4a and 4b which were deacylated to the title compounds **5** and **6** (Scheme I).

Scheme I



The uv spectra of **5** and **6** were consistent with an N-substituted 4-pyridone. Relative configurations of **5** and **6** were assigned on the basis of nmr spectra which exhibited a quartet centered at δ 6.03 for **5** and a triplet centered at δ 6.02 for **6**. Other chemical shifts in the nmr were consistent with the 2-pyridone analogs prepared earlier in these laboratories.

The optical rotatory dispersion curve of **5** showed a well-defined negative cotton effect in agreement with the α configuration as assigned from nmr considerations. The β anomer **6** gave a positive cotton curve as expected from the pattern found in the pyrimidine nucleosides.

The 5′-phosphate esters were prepared for the purpose of *in vitro* testing for inhibition of thymidylate synthetase. The α anomer **5** was phosphorylated using triphenylphosphine, dibenzyl phosphate, and diethyl diazodicarboxylate in dry tetrahydrofuran.[7] After palladium-catalyzed hydrogenation the phosphate **7** was isolated as the barium salt. The β anomer **6** was phosphorylated using $POCl_3$ and water in trimethyl phosphate[8] to give the phosphate **8**.

Preliminary biological studies were performed on growth inhibition of *Escherichia coli* B. No inhibition was observed for either **5** or **6** using the turbidometric method of estimating growth: concentrations ranged from 1×10^{-3} to 1×10^{-5} *M*. The α nucleoside **5** (NSC 144744) was inactive when tested against L1210 lymphoid leukemia.

The *in vitro* assay of *E. coli* thymidylate synthetase was run using a modification of the method of Roberts.[9] No inhibition of the enzyme was detected for the α anomer phosphate **7** in an assay concentration of 1×10^{-3} *M*, approximately 100 times greater than the substrate 2′-deoxyuridine 5′-phosphate. The β anomer **8** was a weak inhibitor; reciprocal plotting showed competitive inhibition with a K_i estimated to be 5×10^{-3} *M*.

We conclude from this and previous studies that an acidic ring proton (in the range pK_a = 7–9) corresponding to the N_3H of the substrate is an essential affinity site for enzyme binding.

Experimental Section

Melting points were recorded from a calibrated Thomas-Hoover Unimelt unit or a microscope hot stage. Spectra were recorded on Beckman IR10, Beckman DU, Cary 14, Cary 60, and Varian H-60A spectrometers. Microanalyses were run on an F & M Model 185 C, H, and N analyzer.

1-(3,5-Di-*O*-*p*-toluyl-2-deoxy-D-ribofuranosyl)-4-pyridone (4a,b). Technical grade 4-hydroxypyridine (4.65 g, 0.05 mol) and a catalytic amount of ammonium sulfate were combined with an excess of hexamethyldisilazane (10 ml) and refluxed for 6 hr. After cooling, the reaction mixture was filtered and the excess of silylating reagent was removed *in vacuo*. The resulting oily residue was used directly without further purification.

The silyl derivative of 4-hydroxypyridine (**2**) was dissolved in dry benzene (100 ml). After the addition of 3,5-di-*O*-*p*-toluyl-2-deoxy-D-ribofuranosyl chloride (**3**, 19.50 g, 0.05 mol) and molecular sieves (10 g), the reaction mixture was stirred at 25° overnight. The molecular sieves were removed by filtration and washed well with dry benzene. The filtrate and washings were pooled and evaporated to a yellow oily product which was dissolved in dry ethanol to remove the silyl group. The solvent was evaporated and the anomeric mixture (**5a,b**) was resolved by column chromatography on silica using methanol in chloroform. The α anomer **4a** was recrystallized from ethanol to give 6.7 g (30%): mp 141–144°. *Anal.* ($C_{26}H_{25}NO_6 \cdot 2H_2O$) C, H, N.

The β anomer **4b** crystallized from methanol-chloroform to give 1.75 g (7.9%): mp 180–182°; nmr as expected. *Anal.* ($C_{26}H_{25}NO_6$) C, H, N.

1-(2-Deoxy-α-D-ribofuranosyl)-4-pyridone (5). The α anomer **4a** (11.8 g, 0.026 mm) was dissolved in 100 ml of absolute methanol-benzene (1:1) and 2 ml of approximately 1 *M* sodium methoxide in absolute methanol was added. The mixture was allowed to stand overnight at 25°. Neutralization to pH 7 with Amberlite IRC-50 followed by evaporation of solvent gave a yellow oily residue. Crystallization from methanol-acetone (1:4) gave a total of 1.79 g (33%) of white microcrystals: mp 154–156°; nmr (D_2O) δ 6.03 (q, 1 proton) (the remainder of the assignments were as expected); uv λ_{max} (H_2O) 263 mμ (ϵ 15,050); uv λ_{max} (0.1 *M* HCl) 243 mμ (ϵ 12,900); uv λ_{max} (0.1 *M* KOH) 263 mμ (ϵ 18,500); ORD (c 0.00840, H_2O, 22°) Φ_{233} +5725°, Φ_{250} +1901°, Φ_{254} 0°, Φ_{263} −8244°. *Anal.* ($C_{10}H_{13}NO_4$) C, H, N.

1-(2-Deoxy-β-D-ribofuranosyl)-4-pyridone (6). Treatment of

4b as described in the synthesis of 5 gave a yellow semisolid: nmr (D_2O) δ 6.02 (t, 1 proton) (the remainder of the assignments were as expected); uv λ_{max} (H_2O) 263 mμ (ε 15,800); uv λ_{max} (0.1 *M* HCl) 243 mμ (ε 11,800); uv λ_{max} (0.1 *M* KOH) 263 mμ (ε 18,500); ORD (*c* 0.00810, H_2O, 22°) Φ_{225} −4488°, Φ_{245} 0°, Φ_{250} +3481°, Φ_{262} +847°. *Anal.* ($C_{10}H_{13}NO_4 \cdot 2H_2O$) C, H, N.

1-(*N*-4-Pyridone)-2-α-deoxyribose 5-Phosphate (7). This was prepared according to the procedure of Mitsunobu and coworkers[7] from 211 mg of **5**, 393 mg of triphenylphosphine, and 417 mg of dibenzyl phosphate in 1 ml of dimethoxyethane. After stirring for 5 min at 25°, 261 mg of diethyl azodicarboxylate in 1 ml of dimethoxyethane was added and stirring continued for 6 hr at 50°. After evaporation of the solvent the residue was dissolved in 50 ml of 75% ethanol containing palladium on carbon. Hydrogenolysis was slow and required several additions of fresh catalyst. The solution was filtered and the volume reduced to 10 ml; the product (195 mg, 50%) was isolated as the barium salt. *Anal.* ($C_{10}H_{12}NO_7PBa$) N, P.

1-(*N*-4-Pyridone)-2-β-deoxyribose 5-Phosphate (8). This was prepared using 169 mg of **6**, 377 mg of $POCl_3$, and 16 mg of water in 2.1 ml of trimethyl phosphate according to the procedure of Yoshikawa, *et al.*[8] Preparative chromatography was performed on Whatman 3MM paper using an isopropyl alcohol–concentrated NH_4OH–water (7:1:2) mixture as eluent. The monophosphate band was eluted with water; the resulting solution was lyophilized to yield 103 mg of **8** as the ammonium salt (61% yield).

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A Single-Step Phosphorylation of 5-Fluoro-2′-deoxyuridine to 5-Fluoro-2′-deoxyuridine 5′-Phosphate

Tony M. K. Chiu and R. Bruce Dunlap*

Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208. *Received March 29, 1974*

Thymidylate synthetase, which plays a vital role in the biosynthesis of DNA as a catalyst for the methylene tetrahydrofolate dependent conversion of 2′-deoxyuridine 5′-phosphate to thymidine 5′-phosphate, is strongly inhibited by 5-fluoro-2′-deoxyuridine 5′-phosphate (5-fluoro-dUMP).[1,2] However, the mode of substrate, product, and inhibitor binding to the enzyme is still a controversial matter. Santi and coworkers[3,4] and Heidelberger and collaborators[5,6] have presented evidence supporting the formation of a ternary covalent complex involving 5-fluoro-dUMP, methylene tetrahydrofolate, and thymidylate synthetase. However, the two groups of investigators proposed different covalent structures for the ternary complex. Recently, gel electrophoresis has been employed to detect two types of ternary complexes of thymidylate synthetase, differing in stoichiometry of inhibitor and coenzyme interaction with the enzyme.[7] In order to study the properties of the ternary complexes in detail, it was important to have a readily available source of moderate quantities (50 mg) of both 5-fluoro-dUMP and its labeled analogs.

This report deals with the direct syntheses of 5-fluoro-2′-deoxyuridine 5′-phosphate and [6-^{3}H]-5-fluoro-dUMP by a selective phosphorylation of the deoxynucleoside with phosphorus oxychloride in triethyl phosphate in a one-step procedure.[8,9] Careful control of the stoichiometry of the phosphorylating agent to the deoxynucleoside makes it possible to effect selective phosphorylation at the 5′ position in a reasonable yield. Pure 5-fluoro-dUMP is then readily isolated by preparative tlc, which also permits the recovery and recycling of unreacted starting material.

Experimental Section

5-Fluoro-2′-deoxyuridine and unlabeled 5-fluoro-dUMP were obtained from Terra-Marine Bioresearch Co. [6-^{3}H]-5-Fluoro-2′-deoxyuridine (790 mCi/mmol) was obtained from the Radiochemical Center, Amersham. Thin-layer chromatography was performed on a cellulose plate and developed with the following systems: A, 2-propanol–28% aqueous ammonia (50:50 v/v); B, 2-propanol–saturated ammonium sulfate–0.5 *M* sodium acetate (2:79:19 v/v). Paper chromatograms were developed by the ascending technique on Whatman No. 1 paper with solvent A. The separated materials were detected with uv light or by spraying the chromatogram with the molybdate perchloric acid reagent [60% v/v perchloric acid–1 *N* HCl–4% ammonium molybdate–H_2O (5:10:25:60)]. They were also identified by a comparison of the experimentally determined R_f values with those of authentic samples. Both the product obtained and the starting material used were recovered by eluting the appropriate samples of cellulose from the preparative thin-layer chromatography system with water, followed by evaporation. Evaporations were carried out *in vacuo* with bath temperatures kept below 40°.

[6-^{3}H]-5-Fluoro-2′-deoxyuridine 5′-Phosphate Disodium Salt. Unlabeled 5-fluoro-2′-deoxyuridine (35.7 mg, 0.156 mmol) was added to an aqueous solution of [6-^{3}H]-5-fluoro-2′-deoxyuridine (500 μl, 250 μCi). The resulting solution was evaporated and then azeotropically evaporated three times with ethanol–toluene to dryness. The white powder was added to a cold (0°) solution of triethyl phosphate (0.7 ml) and phosphorus oxychloride (63.0 mg, 0.41 mmol) with stirring for 3 hr. The reaction mixture, containing a trace of water, was then held at −5° for 2 days. After this time period, another portion of phosphorus oxychloride (35.0 mg, 0.23 mmol) was added. The mixture was vigorously agitated with ice (3 g) and ether (5 ml). The ether layer was separated and discarded, and the aqueous layer was further extracted with three portions of ether (10 ml). The aqueous solution was carefully neutralized with 1% NaOH solution and concentrated *in vacuo* to a small volume and then applied to four thin-layer cellulose plates (20 × 20 cm). Bands in the chromatograms were removed and eluted with water and evaporated to give 24.0 mg of product (70% based on the amount of starting material consumed) and 14.5 mg of starting material was recovered. Spectral data (uv and ir) and R_f values from paper chromatograms and thin-layer chromatograms were identical with that of the authentic sample of 5-fluoro-2′-deoxyuridine 5′-phosphate. The synthetically prepared nucleotide inhibited thymidylate synthetase to an extent identical with that of an authentic sample of 5-fluoro-dUMP (Aull, Lyon, and Dunlap, unpublished results).

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