13 in absolute 2-propanol (30 mL) (distilled over CaO) was placed in standard apparatus under N_2. While the suspension was rapidly stirred, 1.9 mL (4.26 g, 0.03 mol) of methyl iodide was added slowly. After all the methyl iodide was added, the suspension cleared and a transparent yellowish solution resulted which was then boiled at about 85 °C by means of oil bath (3 h). After the first 30 min, the solution became cloudy and a white solid appeared and accumulated up to 3 h. When the mixture cooled, the solid was filtered (0.59 g, 63%, mp 220–222 °C) and recrystallized (water–2-propanol). Yellow needles of salt **13a** were obtained [0.486 g (52%), mp 222.5–224 °C]. Anal. ($C_{11}H_{12}N_3I$) C, H, N.

Attempted Preparation of 6-Hexadecyl-4,5-dihydro-1*H*-pyrazolo[3,4-*f*]quinolinium Bromide. A solution of 0.513 g (0.003 mol) of pyrazole 13 dissolved in absolute ethanol (25 mL) was boiled with 3.05 g (0.01 mol) of cetyl bromide with constant stirring under N_2. The solution was then poured into an excess (ca. 150 mL) of absolute ether, and a cloudy mixture formed. Filtration gave a white precipitate, 1.1 g (70%), mp 200 °C; this was recrystallized three times (absolute methanol–ether) and gave white crystals, 0.5 g (32%), mp 262–265 °C. NMR spectral data and the elemental analysis support the structure of the hydrobromide **13b**. Anal. ($C_{10}H_{10}N_3Br$) C, H, N. A variety of other conditions produced the same result.

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References and Notes

(1) For previous studies in this area from our laboratory, see (a) M. M. Hashem, K. D. Berlin, R. W. Chesnut, and N. N. Durham, *J. Med. Chem.*, **19**, 229 (1976); (b) E. A. Mawdsley, K. D. Berlin, R. W. Chesnut, and N. N. Durham, *ibid.*, **19**, 239 (1976); (c) M. M. Hashem, K. D. Berlin, R. W. Chesnut, and N. N. Durham, *J. Carbohydr., Nucleosides, Nucleotides*, **2**, 357 (1975); (d) N. N. Durham, R. W. Chesnut, D. F. Haslem, and K. D. Berlin, *Ann. Okla. Acad. Sci.*, 77 (1974), and references cited therein; (e) M. M. Hashem, Ph.D. Dissertation, Oklahoma State University, May 1975.

(2) (a) H. D. Brown, Ed., "Chemistry of the Cell Interface—Part A", Academic Press, New York, N.Y., 1971, Chapter I (by J. T. Van Briggen) and Chapter IV (by H. T. Tien and L. K. James, Jr.); (b) A. Korolkova, "Essentials of Molecular Pharmacology—Background for Drug Design", Wiley-Interscience, New York, N.Y., 1970; (c) C. Silipo and C. Hansch, *J. Med. Chem.*, **19**, 62 (1976); (d) N. Bodor, E. Shek, and T. Higuchi, *ibid.*, **19**, 102 (1976); (e) E. J. Ariens in "Drug Design", Vol. I, E. J. Ariens, Ed., Academic Press, New York, N.Y., 1971, Chapter 6; (f) E. J. Lien, C. Hansch, and S. M. Anderson, *J. Med. Chem.*, **11**, 430 (1968).

(3) S. N. Ananchenko, V. Ye Limanov, V. N. Leonov, V. N. Rzheznikov, and I. V. Torgov, *Tetrahedron*, **18**, 1355 (1962).

(4) J. G. Morgan, K. D. Berlin, N. N. Durham, and R. W. Chesnut, *J. Heterocycl. Chem.*, **8**, 61 (1971).

(5) (a) N. D. Weiner and G. Zographi, *J. Pharm. Sci.*, **54**, 436 (1965); (b) N. D. Weiner, H. C. Parreira, and G. Zographi, *ibid.*, **55**, 187 (1966).

(6) G. F. Duffin, *Adv. Heterocycl. Chem.*, 1 (1964).

(7) C. Rufer, H. J. Kessler, and E. Schroder, *J. Med. Chem.*, 18, 253 (1975).

Thymidylate Synthetase Inhibitors. Synthesis of N-Substituted 5-Aminomethyl-2'-deoxyuridine 5'-Phosphates

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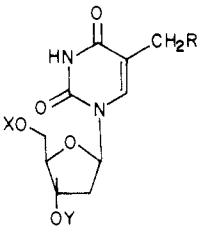
A series of substituted 5-aminomethyl-2'-deoxyuridines was synthesized as analogues of 5-thymidylyltetrahydrofolic acid, a proposed intermediate in the thymidylate synthetase catalyzed reaction. 1-(3,5-Di-*O*-*p*-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-chloromethyluracil (**3**) was treated with the appropriate amine to give the ester protected 5-aminomethyl nucleoside. Removal of the ester groups was accomplished with anhydrous potassium carbonate in methanol to afford the free β-nucleoside. In this way 5-(2-dimethylaminoethylaminomethyl)-2'-deoxyuridine (**5a**), 5-dimethylaminomethyl-2'-deoxyuridine (**5b**), 5-*N*-methylpiperazinylmethyl-2'-deoxyuridine (**5c**), and 5-pyrrolidinylmethyl-2'-deoxyuridine (**5d**) were prepared. Compounds **5a,b,d** were converted to the respective 5'-phosphates **6a,b,d**. All three compounds were substrate competitive inhibitors of thymidylate synthetase purified from *Escherichia coli*, calf thymus, and Ehrlich ascites tumor cells. The most active compound was **6a** with K_I's of 6, 3.1, and 14 μM observed for the respective enzymes.

As part of a program to design selective cancer cell thymidylate synthetase inhibitors, a series of substituted 5-aminomethyl-2'-deoxyuridines with variations in the amine substituent was synthesized in order to elucidate structural criteria that provided strong binding of a 5-substituted 2'-deoxyuridine to the enzyme. Further modifications on the derived structure could then be made to probe for differences between cancer cell and normal cell thymidylate synthetase.[1] Ultimately, it is hoped that a study of this kind will lead to the synthesis of an agent that will selectively and irreversibly inhibit tumor cell thymidylate synthetase. Only one example of isozyme selectivity has been demonstrated for thymidylate synthetase.[2]

The rationale for the synthesis of the 5-aminomethyl-2'-deoxyuridine series of compounds is based on the postulated formation of 5'-thymidylyltetrahydrofolic acid (**1**) as an intermediate in the conversion of deoxyuridine 5'-phosphate to thymidine 5'-phosphate.[3] According to Friedkin's proposed mechanism, **1** undergoes a rearrangement via a 1,3-hydride shift to give the observed products.[3,4]

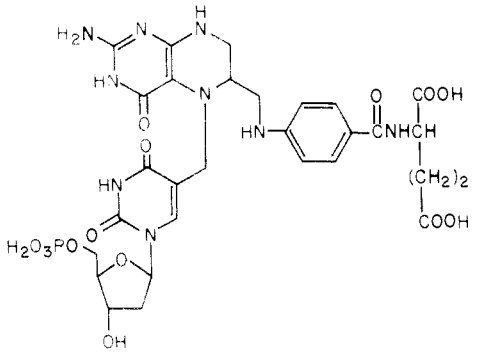
Compounds **6a** and **6c** are analogues of 5-thymidylyltetrahydrofolic acid (**1**). As such, they include binding sites of both the normal substrate, dUMP, and N_5 and N_8 of the cofactor, tetrahydrofolate, and should, therefore, have a strong binding affinity for the enzyme and act as potent inhibitors of it. Compounds **6b** and **6d**, containing only

Table I. Ultraviolet, Chromatographic, and Electrophoretic Characteristics of N-Substituted 5'-Aminomethyl-2'-deoxyuridines and Their Monophosphate Derivatives



Compd	R	X	Y	UV absorption, λ_{max} (ϵ) [λ_{min} (ϵ)] 0.1 M HCl	H_2O	0.1 M NaOH	Chromatography[a]	Electrophoresis[b]
5a	$-NHCH_2CH_2N(CH_3)_2$	-H	-H	268 (9500) [231 (3300)	266 (7800) 236 (4400)	268 (7300) 245 (6000)]	2.7	-0.82
6a	$-NHCH_2CH_2N(CH_3)_2$	$-PO_3H_2$	-H				1.4	0.27
5b	$-N(CH_3)_2$	-H	-H	266 (9000) [230 (2700)	266 (7300) 235 (3200)	267 (6300) 245 (4300)]	2.0	0.57
6b	$-N(CH_3)_2$	$-PO_3H_2$	-H				1.4	1.2
5c	$-c-N(CH_2CH_2)_2N-CH_3$	-H	-H	268 (9400) [232 (2500)	266 (8700) 236 (3100)	267 (8500) 247 (6500)]	7[c]	0.2
6c[d]	$-c-N(CH_2CH_2)_2N-CH_3$	-H	$-PO_3H_2$				2.0[c]	0.69
5d	$-c-NC_4H_8$	-H	-H	267 (10500) [233 (3300)	266 (9500) 237 (4600)	268 (9000) 246 (7000)]	2.2	-0.4
6d	$-c-NC_4H_8$	$-PO_3H_2$	-H				1.1	0.5

[a] Mobility on Whatman No. 1 paper is reported relative to that of thymidine 5'-phosphate using 2-propanol-ammonia-water (7:1:2). [b] Mobility on Whatman 3 Chroma paper is reported relative to uridine 2'(3')-phosphate using 0.05 M phosphate buffer, pH 7.5. Negative numbers indicate migration to the cathode. [c] Mobility for this material is relative to that of uridine 5'-phosphate. [d] This compound is resistant to the action of 5'-nucleotidase.



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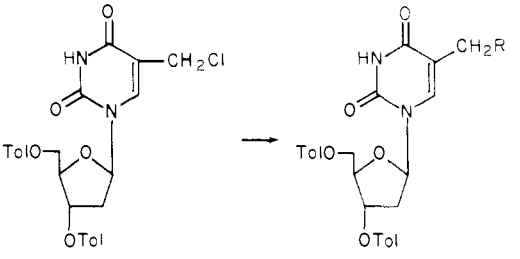
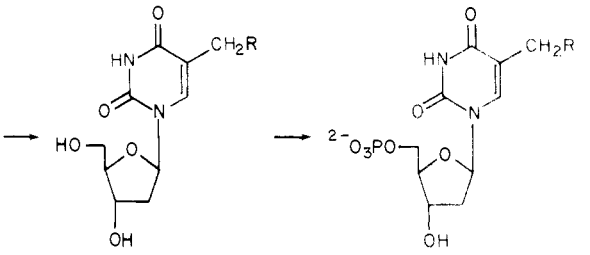


3 4



5 6

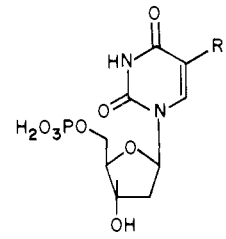
a, R = $-NHCH_2CH_2N(CH_3)_2$
b, R = $-N(CH_3)_2$
c, R = $-c-N(CH_2CH_2)_2N-CH_3$
d, R = $-c-NC_4H_8$

the N_5 analogue of the cofactor, were prepared to evaluate the binding advantage offered by the second amine function found in **6a** or **6c**. The syntheses of **6a**, **6b**, and **6d** are described as is the isolation of a phosphate of **5c** which is proposed to be the 3'-phosphate.

The syntheses of substituted 5-aminomethyl-2'-deoxyuridines were generally the same for all amine substituents on the 5-methyl group within each particular class of compounds (e.g., the protected nucleosides **4**, the free nucleosides **5**, or the 5'-monophosphates **6**). Thus, for the synthesis of the protected nucleosides **4**, the protected 5-chloromethyl nucleoside[4,5] **3** with the appropriate amine in a dioxane solution gave the ester protected nucleosides **4** which could be rapidly purified on a silica gel column to give the product in good yield (67–86%). Removal of the ester groups was accomplished by stirring **4** in dry methanol in the presence of a 2–3-equiv excess of potassium carbonate to give the free nucleoside **5**. The work-up of **5** involved the addition of concentrated hydrochloric acid at the end of the reaction period to precipitate most of the potassium salt. The white precipitate was filtered and the filtrate evaporated under vacuum to a yellow-brown oil. Except for **5d**, addition of ethanol to this oil induced crystallization of the nucleoside, which was then filtered and immediately recrystallized from boiling ethanol. The need for immediate recrystallization was because of the extreme hygroscopic properties of the impure nucleoside, which was attributed to the presence of potassium salt impurities. After one recrystallization, nonhygroscopic, pure nucleoside was obtained.

For the free nucleoside **5d**, the addition of ethanol to the brown oil left after evaporation of the filtrate did not lead to the desired crystallization of the nucleoside from the oil. However, pure **5d** could be obtained from the oil using a methanol–ethyl acetate solvent system to give a

Table II. Inhibition of Thymidylate Synthetase Purified from Three Sources[a]



Compd	R	K_m (no inhibn), K_i values in μM (binding free energy, kcal/mol)		
		E. coli	Calf thymus	Ehrlich ascites
dUMP	-H	7.6 (-7.2)	8 (-7.2)	6.5 (-7.3)
6a	$-CH_2NCH_2CH_2N(CH_3)_2$	6 (-7.4)	3.1 (-7.8)	14 (-6.8)
6b	$-CH_2N(CH_3)_2$	90 (-5.7)	110 (-5.6)	73 (-5.8)
6d	$-CH_2$-c-NC_4H_8	56 (-6)	43 (-6.2)[b]	27 (-6.4)

[a] Experimental conditions have been described (ref 8). [b] This is the only observed noncompetitive inhibition.

good yield (78%) of the nonhygroscopic crystalline product.

For the determination of K_i values it was necessary to convert the nucleosides into the corresponding 5'-monophosphates. This has been achieved chemically for the two nucleosides **6a** and **6b** using the procedure of Yoshikawa et al.[6] We obtained low yields (10% or less) of the 5'-monophosphates and attributed this to the lack of solubility of the nucleosides in the trimethyl phosphate solvent. However, since enough desired material was obtained for in vitro enzyme testing, no further attempt has been made to improve the yields. Compounds **6a** and **6b** were purified by paper chromatography and characterized by their electrophoretic properties and hydrolysis by a 5'-nucleotidase enzyme (Table I).

The procedure of Sowa and Ouchi[7] was used for the synthesis of **6d**; in contrast to our results using the trimethyl phosphate procedure,[6] complete solution of **5d** was observed during reaction. When the phosphorylation of **5c** was attempted, an analytically pure nucleotide product was obtained that was presumed to be the 5'-phosphate. However, the product was not hydrolyzed by a nonspecific 5'-nucleotidase and it was completely inactive as an enzyme inhibitor. We are suggesting that the product of this reaction is the 3'-phosphate.

Biological Results. The purification of the enzyme from the three sources and the assay for inhibition have been described.[8] Analysis for inhibition constants was done by a double reciprocal plot of the velocity vs. the substrate concentration using five different substrate concentrations and at least two inhibitor concentrations. All three compounds, **6a,b,d**, were competitive with substrate against the three enzymes with the exception of **6d** which showed uncompetitive inhibition when tested against the calf thymus preparation (Table II).

These compounds should be competitive with the substrate and not with the cofactor, tetrahydrofolic acid. This was verified using **6a**, the dimethylaminoethylaminomethyl derivative, since it was the most potent substrate competitive inhibitor. A double reciprocal plot of velocity vs. tetrahydrofolic acid concentration at two concentrations of inhibitor gave lines parallel with the K_m line (no inhibitor) using the *Escherichia coli* enzyme. This suggests uncompetitive inhibition classically seen in sequential enzyme mechanisms.[9] In preliminary studies using ascites tumor enzyme, compound **6a** shows the same pattern; the inhibition observed is not competitive with the cofactor.

The 5-dimethylaminomethyl nucleotide **6b** was a weak inhibitor with K_i's greater than the K_m for the substrate. This loss of affinity was unexpected in view of the results observed with the pyrrolidinomethyl compound **6d** which was twice as active as **6b**.

The most potent compound was the substituted ethylenediamine derivative **6a**. A K_i of 3 μM was observed against the calf thymus enzyme which was also the most sensitive enzyme using this inhibitor. In previous studies, we have observed that the isozyme specificity showed the ascites tumor enzyme to be the most sensitive of the three enzymes.[8] Thus, the fourfold difference seen in the activity of **6a** is unusual in that the thymus enzyme is more sensitive than the ascites enzyme.

That compound **6a** is at least ten times more effective than **6b** or **6d** against the thymus and *E. coli* enzymes indicates an additional binding site contributing approximately 1.5 kcal/mol to complex formation. The finding that **6a** binds as strongly as the substrate confirms earlier studies which showed that the enzyme can tolerate reasonably large substituents in the 5 position of the substrate (compare the 5-benzyloxymethyl nucleotide with a binding energy of -7.1 kcal/mol).[8]

The monoamines **6b** and **6d** are not particularly effective. However, the diamino compound **6a**, a closer analogue of **1**, is a good inhibitor. At physiological pH the amine would be protonated. Electrostatic binding to a nucleophile in this region of the enzyme would support earlier conclusions reached with the active-site-directed irreversible inhibitor, 5-iodoacetamidomethyl-2'-deoxyuridine 5'-phosphate[2] which is proposed to alkylate a nucleophile on the enyzme at a site in a region four atoms removed from C-5 of the pyrimidine ring.

Experimental Section

All melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra are measured with a Beckmann IR-33, UV spectra with a Cary 14 recording spectrophotometer, and NMR spectra with a Varian Model EM-360 or T-60. Microanalyses (±0.4% of theory) were obtained from a Hewlett-Packard 185B at the Department of Medicinal Chemistry, University of Kansas. Silica gel chromatography was done using 1-butanol–acetic acid–water (5:2:3); paper chromatography was done on Whatman No. 1 or 3MM (preparative) paper using 2-propanol–ammonia–water (7:1:2). Electrophoresis was done on Whatman Chroma 3 paper using 0.05 M potassium phosphate buffer, pH 7.5. Compounds **6c** and **6d** were purified on a 2.6 × 35 cm DE52 (Whatman) DEAE cellulose resolved with a gradient of 0.001–0.4 M ammonium formate buffer, pH 4.4. NMR assignments are given only for the 5-substituent.

1-(3,5-Di-*O*-*p*-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-(2-dimethylaminoethylaminomethyl)uracil (4a). To a solution of unsymmetrical dimethylethylenediamine (1.2 g, 13.6 mmol) in 10 mL of dry dioxane was slowly added a solution of 1-(3,5-

di-O-p-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-chloromethyluracil[4,5] (3, 2.0 g, 3.9 mmol) in 25 mL of warm dioxane. The reaction mixture was stirred overnight at room temperature. The solvent was then evaporated and the resulting oil dissolved in 100 mL of chloroform. This solution was shaken with 100 mL of a saturated sodium bicarbonate solution and 50 mL of a saturated sodium chloride solution, and the organic layer was collected. The aqueous layer was washed twice more with 100-mL portions of chloroform. The organic layers were pooled and dried over sodium sulfate. TLC (ethyl acetate–methanol, 50:50) on silica showed one major low R_f product and several minor high R_f products. Separation was effected on a silica gel column using the same eluent as for TLC. Fractions containing the low R_f product were combined and evaporated to give 1.46 g of **4a** (67%): mp 101–102 °C; UV (EtOH) λ_{max} 243 nm (ϵ 27650), λ_{min} 217 (9350); NMR ($CDCl_3$) δ 7.48 (s, 1, H-6), 3.28 (s, 2, $-CH_2NH$), 2.20 (s, $-NCH_3$). Anal. ($C_{30}H_{36}N_4O_7$) C, H, N.

1-(3,5-Di-O-p-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-dimethylaminomethyluracil (4b). Dimethylamine was bubbled into 40 mL of dioxane at 0–10 °C for 15 min. To this solution was then added dropwise a solution of **3** (2.0 g, 3.9 mmol) in 50 mL of dioxane. After the addition was complete, dimethylamine was bubbled into the reaction mixture for 10 min. Stirring was continued for another 45 min. TLC (2% methanol in chloroform) on silica showed one spot, R_f 0.2. Work-up was the same as that for **4a** except that the sodium chloride solution was not necessary. Purification was done with a silica gel column using 2.5% methanol in chloroform as eluent. Fractions containing the appropriate R_f product were combined and evaporated to give 1.64 g of **4b** (81%): mp 165.5–166.5 °C; UV (EtOH) λ_{max} 242 nm (ϵ 29450), λ_{min} 216 (10500); NMR ($CDCl_3$) δ 7.60 (s, 1, H-6), 2.20 (s, $-NCH_3$). Anal. ($C_{28}H_{31}N_3O_7$), C, H, N.

1-(3,5-Di-O-p-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-N-methylpiperazinylmethyluracil (4c). To a solution of freshly distilled N-methylpiperazine (2.0 g, 19.9 mmol) in 10 mL of dry dioxane was added a warm solution of **3** (2.0 g, 3.9 mmol) in 50 mL of dioxane. The reaction mixture was then stirred at room temperature for 15 h. During this time, a deep yellow color developed. TLC on silica using a 3% methanol in chloroform solvent system showed one UV-quenching spot that barely moved off the origin. Work-up was again the same as that for **4a**. Purification was achieved on a silica gel column using 10% methanol in ethyl acetate as the eluent. Combination and evaporation of the fractions containing the low R_f product yielded 1.81 g of **4c** (81%): mp 80–82 °C; UV (EtOH) λ_{max} 242 nm (ϵ 35300), λ_{min} 217 (17700). Anal. ($C_{31}H_{36}N_4O_7 \cdot 0.5H_2O$).

1-(3,5-Di-O-p-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-pyrrolidinylmethyluracil (4d). To freshly distilled pyrrolidine (1.5 g, 21 mmol) in 10 mL of dioxane was added, dropwise, a warm solution of **3** (2.0 g, 3.9 mmol) in 50 mL of dioxane. The resulting clear solution was stirred at room temperature for 3 h. TLC (3% methanol in chloroform) showed one UV-quenching spot with a very low R_f. Work-up was the same as described for **4b**. A silica gel column using 10% methanol in ethyl acetate as eluent was utilized for purification. **4d** (1.82 g) was obtained (86%): mp 167–168 °C; UV ($CHCl_3$) λ 249 nm (ϵ 34500); NMR ($CDCl_3$) δ 7.60 (s, 1, H-6), 3.23 (s, 2, CH_2N), 1.63 (m, 4, $-CH_2CH_2CH_2CH_2$). Anal. ($C_{30}H_{33}N_3O_7$) C, H, N.

5-(2-Dimethylaminoethylaminomethyl)-2′-deoxyuridine Dihydrochloride (5a). A suspension of **4a** (1 g, 1.8 mmol) and 0.5 g of potassium carbonate (3.6 mmol) was stirred in 50 mL of dry methanol at room temperature for 2 h. About 15 mL of concentrated hydrochloric acid was then added and the resulting white precipitate filtered. The filtrate was evaporated to an oil. The addition of 35 mL of ethanol to this oil induced copious white crystal formation. These crystals were then filtered and immediately recrystallized from hot ethanol. The yield of the nonhygroscopic product (**5a**) was 0.55 g (77%), mp 182–183 °C. Anal. ($C_{14}H_{26}N_4O_5Cl_2$) C, H, N.

5-Dimethylaminomethyl-2′-deoxyuridine Hydrochloride (5b). Utilizing the same procedure as described for **5a**, 1.33 g (2.6 mmol) of **4b** and 0.6 g (4.3 mmol) of anhydrous potassium carbonate were stirred for 2 h in 50 mL of methanol. After work-up, immediate recrystallization from hot ethanol yielded 0.49 g of **5b** (60%), mp 197–198 °C. Anal. ($C_{12}H_{20}N_3O_5Cl$) C, H, N.

5-N-Methylpiperazinylmethyl-2′-deoxyuridine Dihydrochloride (5c). A mixture of **4c** (1 g, 1.7 mmol) and potassium carbonate (0.5 g, 3.6 mmol) in 20 mL of methanol was stirred for 2 h at room temperature. The work-up was exactly the same as that used for **5a**. Recrystallization from hot ethanol gave 0.56 g of **5c** (78%), mp 178–179 °C. Anal. ($C_{15}H_{26}N_4O_5Cl_2$) C, H, N.

5-Pyrrolidinylmethyl-2′-deoxyuridine Hydrochloride (5d). To 1.92 g of **4d** (3.51 mmol) in 50 mL of dry methanol was added 1.0 g (7.24 mmol) of potassium carbonate and the resulting mixture stirred at room temperature for 2 h. Concentrated hydrochloric acid (12 mL) was then added and the white precipitate that formed was filtered. The filtrate was then evaporated to a brown oil. An ethyl acetate–methanol solvent mixture was added to the oil and the resulting solution heated to cloud point and then left to stand at room temperature overnight. Pure **5d** (0.95 g, 78%) was obtained as nonhygroscopic, off-white crystals, mp 183.5–184.5 °C. Anal. ($C_{14}H_{22}N_3O_5Cl \cdot 0.5H_2O$) C, H, N.

5-(2-Dimethylaminoethylaminomethyl)-2′-deoxyuridine 5′-Monophosphate (6a). A mixture of trimethyl phosphate (2.3 mL), phosphorus oxychloride (0.31 g, 2 mmol), water (0.01 mL, 0.5 mmol), and 0.102 g of **5a** (0.03 mmol) was stirred at 0 °C overnight. The product (**6a**) was obtained by preparative paper chromatography (Whatman 3MM paper) with 2-propanol–ammonium hydroxide–water (7:1:2) as solvent. After development, a narrow band with intense UV quenching (R_f 0.48) was cut out and eluted with a 2% ammonium hydroxide solution. This solution was then lyophilized to yield 0.024 g of a white solid. By UV analysis, utilizing the molar extinction coefficients of **5a**, it was determined that **6a** was obtained in 2.2% yield: UV (H_2O) λ_{max} 266 nm, λ_{min} 236 nm; UV (1 M HCl) λ_{max} 268 nm, λ_{min} 231 nm; UV (1 M NaOH) λ_{max} 266 nm, λ_{min} 254 nm.

5-Dimethylaminomethyl-2′-deoxyuridine 5′-Monophosphate (6b). Using the same procedure as indicated for **6a**, trimethyl phosphate (2.3 mL), phosphorus oxychloride (0.31 g, 2 mmol), water (0.01 mL, 0.5 mmol), and **5b** (0.17 g, 0.5 mmol) were stirred at 0 °C overnight. The product **6b** was obtained by preparative paper chromatography (same solvent as for **6a**) to yield 0.071 g of a green powder. By UV analysis, it was determined that **6b** was obtained in 10% yield: UV (molar extinction coefficients estimated to be same as those for **5b**) (H_2O) λ_{max} 267 nm, λ_{min} 238 nm; UV (1 M HCl) λ_{max} 266 nm, λ_{min} 236 nm; UV (1 M NaOH) λ_{max} 267 nm, λ_{min} 255 nm.

5-N-Methylpiperazinylmethyl-2′-deoxyuridine 3′-Phosphate (6c). Using the method of Sowa and Ouchi[7] 124 mg of nucleoside **5c** (0.3 mmol) was added to a cold (0 °C) solution of acetonitrile (300 μL), pyridine (115 μL), water (15 μL), and phosphorus oxychloride (120 μL) prepared cold in the above sequence. The thick suspension was occasionally shaken while maintaining the temperature at 0 °C. Complete solution had occurred after 6 h; 4 mL of cold water was added and the mixture was allowed to stand overnight at 0 °C. The entire mixture after neutralization was diluted fivefold and resolved on a DEAE cellulose column. A 70% yield of a monophosphate of **5c** was calculated from ultraviolet analysis of the resolved mixture. The sample was analyzed after evaporation and drying at 60 °C in a vacuum for 12 h. This product was resistant to the action of a 5′-nucleotidase. Anal. ($C_{15}H_{24}N_4PO_8 \cdot H_2O$) C, H, N.

Enzyme Assays. The procedures used for determining the substrate competitive inhibition have been reported.[8]

The assay of *E. coli* thymidylate synthetase using varying amounts of cofactor with saturating substrate levels contained in each 0.1 mL of 0.02 M formaldehyde, 0.02 M $MgCl_2$, 0.05 M Tris–HCl, pH 7.4, 0.05 M mercaptoethanol, 25 μM [5-^{3}H]-2′-deoxyuridine 5′-phosphate (400 μCi/μmol), inhibitor, and tetrahydrofolic acid (5.7–22.8 μM). Assays were incubated for 7 min and samples treated as in previous studies.

The assay for ascites tumor thymidylate synthetase was run in a total volume of 0.1 mL and contained 0.0033 M formaldehyde, 0.033 M mercaptoethanol, 0.1 M potassium phosphate, pH 6.7, 25 μM [5-^{3}H]-2′-deoxyuridine 5′-phosphate (400 μCi/μmol), inhibitor, and tetrahydrofolic acid (2.9–11.6 μM).

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References and Notes

(1) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors", Wiley, New York, N.Y., 1967.
(2) R. L. Barfknecht, R. A. Huet-Rose, A. Kampf, and M. P. Mertes, *J. Am. Chem. Soc.*, **98**, 5041 (1976).
(3) M. Friedkin, *Adv. Enzymol.*, **38**, 235 (1973).
(4) R. S. Wilson and M. P. Mertes, *Biochemistry*, **12**, 2879 (1973).
(5) R. Brossmer and E. Rohm, *Hoppe-Seyler's Z. Physiol. Chem.*, **348**, 1431 (1967).
(6) M. Yoshikawa, T. Kato, and T. Takenishi, *Bull. Chem. Soc. Jpn.*, **42**, 3505 (1969).
(7) T. Sowa and S. Ouchi, *Bull. Chem. Soc. Jpn.*, **48**, 2084 (1975).
(8) A. Kampf, R. L. Barfknecht, P. J. Shaffer, S. Osaki, and M. P. Mertes, *J. Med. Chem.*, **19**, 903 (1976).
(9) I. H. Segel, "Enzyme Kinetics", Wiley, New York, N.Y., 1975, p 136.

N-(2,4,5-Trihydroxyphenethyl)normetazocine, a Potential Irreversible Inhibitor of the Narcotic Receptor

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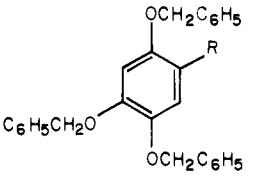
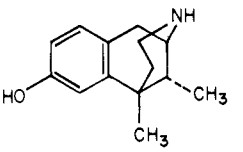
The reaction of *N*-(2,4,5-tribenzyloxyphenyl)ethyl methanesulfonate, prepared in a seven-step sequence, with normetazocine followed by hydrogenolysis of the benzyloxy-protecting groups, gave *N*-(2,4,5-trihydroxyphenethyl)normetazocine. This compound was prepared to study the effect of a narcotic analgesic containing a functional group which could be activated in situ to a moiety potentially capable of reacting irreversibly with the narcotic receptor. This 6-hydroxydopamine derivative of normetazocine did not prove to be a useful affinity label. Its low toxicity could indicate the necessity for the formation of an aminochrome system for the expression of toxicity by 6-hydroxydopamine.

Much effort is currently being expended in search of a useful affinity label for the opiate receptor.[2,3] The ideal reagent for this purpose would interact specifically with the opiate receptor with high affinity so that it could be employed at very low concentrations. A reactive group is needed in the reagent, capable of forming a covalent bond with one or more types of functional groups on the receptor. It is probably also important, to ensure the greatest specificity, that the reactive group be neither too readily reactive nor too weakly so. Alternatively, groups which can be activated after binding to receptors can be employed. One such approach has been the use of photoaffinity probes.[2] We chose to make use of a functional group which may require oxidation by molecular oxygen prior to reaction. The 2,4,5-trihydroxyphenethylamine moiety may be such a group and its potential usefulness is explored in this communication.

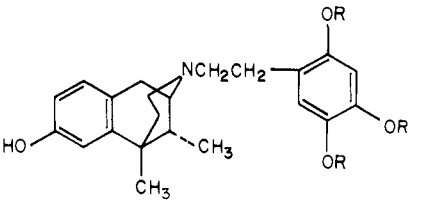
2,4,5-Trihydroxyphenethylamine (6-hydroxydopamine) is known to effect the release of norepinephrine and cause long-term reduction of cardiac uptake of norepinephrine and selective destruction of peripheral noradrenergic nerve terminals.[4] The central administration of 6-hydroxydopamine results in a profound depletion of both dopamine and norepinephrine as a consequence of the selective destruction of catecholamine-containing nerve terminals.[4] A possible mechanism for the cytotoxic action of 6-hydroxydopamine results from the ability of the initial oxidation product, the *p*-quinone of 6-hydroxydopamine, to bind covalently to sulfhydryl groups on macromolecules leading to the polymerization of proteins at the plasma membrane and within the neuronal terminal.[5-7] We hoped to deliver a 6-hydroxydopamine-like molecule to the vicinity of the narcotic receptor in the brain by combining it with a compound from which potent analgesics have been prepared, namely, 5,9α-dimethyl-2′-hydroxy-6,7-benzomorphan (normetazocine, **6**).[8] Thus, *N*-(2,4,5-trihydroxyphenethyl)normetazocine (**8**) was synthesized and the biological activity of the hydroiodide salt of **8** was explored.



1, R = CH_2Br
2, R = CH_2CN
3, R = CH_2COOH
4, R = CH_2CH_2OH
5, R = $CH_2CH_2OSO_2CH_3$



6



7, R = $CH_2C_6H_5$
8, R = H

N-Phenethyl-substituted benzomorphans and morphinans, whether or not the aromatic ring has a para substituent, are very potent analgesics and have high affinity for the narcotic receptor.[9] Thus, *N*-phenethylnormetazocine is about ten times more potent than morphine in vivo (in mice, hot-plate assay, subcutaneous injection). *N*-*p*-Hydroxyphenethylnormetazocine has been