Table V. Chronic Administration (Test 2)

Compd 2 doses, mg/kg/day po	% decrease of euglobulin lysis time after day				
	15	30	60	30 after cessation of treatment	30 after starting again
100	33	51	54	0	52
p values	$0.05 \leq p \leq 0.02$	$0.02 \leq p \leq 0.01$	$p \leq 0.001$	0	$0.02 \leq p \leq 0.01$

greater the activity of the compound, the narrower the active concentration range. However, this test could only be performed on water-soluble compounds. Like other authors, we have chosen *o*-thymotic acid (OTA) as reference compound.[9]

Test 2. Measurement of Systemic Fibrinolytic Activity by Determination of Plasma Euglobulin Lysis Time (ELT) after Oral Administration to Rat.[10] This method consisted of precipitating by means of a flow of carbon dioxide the euglobulin fraction of diluted plasma. The resultant precipitate was removed by centrifugation, redissolved in a buffer solution, and then coagulated by thrombin. The euglobulin clot was incubated at 37° and the time of complete lysis determined.

Surprisingly, OTA was found inefficient as an *in vivo* reference compound and, consequently, the studied compounds could not be compared to this known standard. When compounds of high activity were detected using this test, we have attempted to study their possible effects on thromboembolic diseases by means of the following techniques.

Test 3. Decrease or Inhibition of Rabbit Blood Platelet Adhesiveness on Glass Bead Column.[11] The per cent adhesiveness was calculated by determining the number of platelets before and after passage through the column. The platelet count was carried out in triplicate by means of an electronic particle counter.

Test 4. Decrease or Inhibition of the Platelet Aggregating Effect of ADP, Thrombin, and Epinephrine on Rat and Rabbit Plasma Measured by Means of a Photometric Technique.[12] The compounds were incubated in platelet-rich plasma for 5 min before addition of the aggregating agent. The results were expressed as the percentage inhibition of aggregation induced by the compound as measured by determination of the planimetric relationship of the surfaces obtained with the aggregating agent alone on the one hand, and the compound and aggregating agent on the other, at the different concentrations tested.

Test 5. Decrease of Fibrin Monomer Level in the Rabbit after Intravenous Perfusion.[13] In addition, the acute toxicity (LD_{50} in mice) of active compounds was assessed by the Probit method[14] up to 2000 mg/kg po.

Results and Discussion

Only two compounds were sufficiently soluble under our experimental conditions (aqueous buffered solution at pH 7) to be tested against standard hanging clots (test 1). Compound **2** was active from 0.02 to 0.007 mol/l. while OTA was active at 0.02–0.008 mol/l. On the other hand, compound **8** although soluble was inactive.

Five compounds (**2**, **11–13**, and **15**) out of the 14 tested possessed marked activity in test 2. The toxicities of active compounds and the results of test 2 are listed in Table I.

Tests 3, 4, and 5 were performed on the most active compound **2** which induced a marked decrease in platelet stickiness (*cf.* Table II), a pronounced inhibition of platelet aggregation (*cf.* Table III) induced by ADP, thrombin, or epinephrine in rabbit, rat, and human plasma, and a decrease of fibrin monomer levels (*cf.* Table IV) after iv injection. Contrary to nicotinic acid,[15] this compound was able to decrease the ELT in the rat after daily administration over a period of 2 months (*cf.* Table V). No tachyphylatic phenomena were apparent.

Although only 14 derivatives have been tested, some features of a structure-activity relationship were apparent from the *in vivo* results (test 2). Most of the compounds containing an asymmetrical anion (**2**, **11**, **12**, and **15**) were active. This fact supports a recent hypothesis concerning the presence of such an anion in a series of benzoic acid derivatives found to be active *in vitro*.† Furthermore, most of the nonanionic compounds (**1**, **4–6**, **8–10**) were inactive.

The reason why the hydroxamic acid derivative **3** and the morpholid **7** (both with $R' = H$; $R'' = CH_3$) were inactive while the corresponding compounds **12** and **13** (both with $R' = H$; $R'' = C_2H_5$) were active is not clear. This could be due to the higher lipid solubility of **12** and **13**.

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†K. N. von Kaulla, personal communication.

Synthesis of 5-Trifluoromethyl-2′-deoxyuridine 5′-Phosphate and 5-Trifluoromethyl-2′-deoxyuridine 5′-Triphosphate†

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This laboratory has been interested in the biochemistry of 5-trifluoromethyl-2′-deoxyuridine (F_3dThd‡), a fluorinated analog of thymidine with clinical antitumor and antiviral activity.[1] The nucleotide, F_3dThd-5′-P, is a powerful inhibitor[2,3] of thymidylate synthetase, the enzyme responsible for metabolic conversion of deoxyuridylic acid to thymidylic acid. F_3dThd also is incorporated into DNA,[4] presumably after conversion *in vivo* to the 5′-triphosphate (F_3dThd-5′-PPP). To facilitate our studies on the mech-

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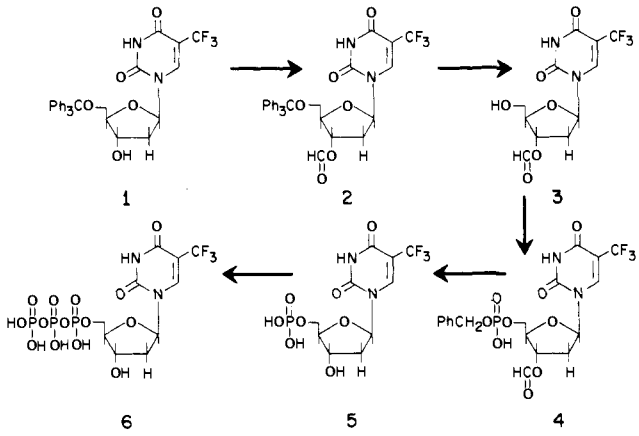
‡Approved IUPAC abbreviation.

anisms of these processes, it became necessary to develop chemical syntheses of F_3dThd-5'-P and F_3dThd-5'-PPP. Previously, the only source of the nucleotide had been a laborious small-scale enzymatic phosphorylation of the nucleoside, F_3dThd.[2]

Chemistry. The base lability of the trifluoromethyl group of F_3dThd[5] precluded the use of any phosphorylating agents and/or blocking groups that require basic conditions at some stage in their application. The synthetic route shown in Scheme I requires only mildly acidic conditions and reliably gives a moderate yield of F_3dThd-5'-P. 5'-Trityl-F_3dThd[6] (**1**) was treated with formic-acetic anhydride[7] to give the 3'-formyl derivative **2**. Although acid labile, the formyl group was not hydrolyzed under the mild acidic conditions employed to cleave the trityl moiety.[6] The salient feature of this synthesis is the treatment of 3'-formyl-F_3dThd (**3**) with benzyl phosphate[8] and dicyclohexylcarbodiimide to achieve phosphorylation of the 5'-hydroxyl group (**4**). Other phosphorylating agents such as phosphorus oxychloride in triethyl phosphate[9] and dibenzyl phosphochloridate[10] proved to be unsatisfactory. The formyl group was lost during the subsequent hydrogenation of **4** to remove the benzyl group, resulting in a product (**5**) indistinguishable in all respects from enzymatically prepared F_3dThd-5'-P.

Scheme I



The triphosphate **6** was obtained by activation of **5** with 1,1'-carbonyldiimidazole, followed by treatment with inorganic pyrophosphate.[11] Purification was achieved by preparative paper chromatography, since **6** adhered so tightly to ion-exchange resins that it could not be readily eluted. The structure of the product was verified by uv spectroscopy and phosphorus analyses. As further proof, it was hydrolyzed for 7 min in refluxing 1 *N* HCl, a procedure which cleaved the β and γ phosphates to give the 5'-monophosphate.[12]

To obtain the corresponding radioactive nucleotides, the identical sequence of reactions was carried out on [6-^{3}H]-F_3dThd, which was prepared by catalytic exchange of the 6-hydrogen with tritium. Compounds **5** and **6** with specific activities of 60 mCi/mmol were obtained.

The F_3dThd-5'-P (**5**) prepared by the method depicted in Scheme I inhibited thymidylate synthetase from Ehrlich ascites carcinoma cells[13] and *Lactobacillus casei*,[14] with dissociation constants (K_i values) of 5×10^{-8} and 10^{-9} *M*, respectively. The former value is in agreement with that obtained previously using enzymatically synthesized **5**.[2]

F_3dThd-5'-PPP (**6**) did not inhibit thymidylate synthetase at concentrations up to 10^{-5} *M*. However, the aforementioned 7-min hydrolysate of **6** gave a substance that inhibited the enzyme with a K_i value similar to that of the monophosphate. The triphosphate **6** was found to be competitive with thymidine triphosphate in several mammalian DNA polymerase systems and was also incorporated by them into DNA (H. Tone and C. Heidelberger, unpublished data).

Experimental Section§

1-(2-Deoxy-3-formyl-5-triphenylmethyl-β-D-ribofuranosyl)-5-trifluoromethyluracil (2). 5'-Trityl-F_3dThd[6] (**1**) (536 mg, 1.0 mmol) was dissolved in 5 ml of dry pyridine and the mixture cooled in an ice bath to 5°. Then 2 ml of 98% formic acid followed by 1.6 ml of acetic anhydride were added. This addition was repeated after 4 hr. The reaction was allowed to warm to room temperature overnight. The solvents were removed *in vacuo*, and the residue was taken up in 50 ml of benzene. The benzene solution was washed with two 50-ml portions of water and then dried over magnesium sulfate. Crystallization of the product was effected by slow addition of cyclohexane to the benzene solution and placing the mixture in the cold. There resulted 510 mg (90%) of the product as a white solid. There was no definite melting point, but the solid began to soften at 80° with bubbling which ceased at 95°. The uv spectrum showed λ_{max}^{MeOH} 261 nm (ϵ 9500). *Anal.* ($C_{30}H_{25}F_3N_2O_6 \cdot 0.5H_2O$) C, H, N.

5-Trifluoromethyl-2'-deoxyuridine 5'-Phosphate (5). 3'-Formyl-5'-trityl-F_3dThd (**2**) (1.03 g, 1.8 mmol) was treated with 5 ml of ice-cold 98% formic acid. The mixture was stirred for ~2 min to bring all particles in contact with the acid, and then the acid was evaporated under high vacuum at room temperature. Traces of the formic acid were removed by distillation with two 5-ml portions of dioxane. Water (10 ml) was then added to the residue and the precipitate removed by filtration. The water was evaporated *in vacuo* and the residue dried by a threefold evaporation of 5-ml portions of pyridine. Silica gel tlc showed one spot at R_f 0.5 ($CHCl_3$–MeOH, 9:1), compared to F_3dThd which had an R_f of 0.25 in this system. Without further purification, the 3'-formyl-F_3dThd (**3**) was dissolved in 20 ml of dry pyridine along with 4 mmol of benzyl phosphate (pyridinium salt) and 2 g of dicyclohexylcarbodiimide. The reaction was allowed to stand at room temperature for 48 hr, when the dicyclohexylurea was removed by filtration and the filtrate was evaporated. The residue was extracted with two 10-ml portions of ether and then dissolved in 100 ml of 40% aqueous MeOH. To this solution was added several drops of concentrated HCl and 100 mg of 10% Pd/C. Hydrogenation was carried out in a Parr shaker at 2.8×10^3 g/cm^2 for 12 hr. After removal of the catalyst by filtration, the volume of the solution was reduced to 5 ml and the solution filtered. The solution was placed on a 1.5 × 20 cm Dowex-1 (formate) column, which was then eluted with a gradient of 4 *M* formic acid (200 ml) in the mixing chamber and 4 *M* formic acid–1.5 *M* ammonium formate (200 ml) in the reservoir. The elution was monitored by silica gel tlc using the solvent system *n*-PrOH–MeOH–formic acid (6:4:0.8) in which the monophosphate has an R_f of 0.5. The product was obtained between elution volumes of 135–225 ml. These fractions were combined and passed through a Dowex-50 (H^+) ion-exchange resin for desalting. The solvent was evaporated to dryness under vacuum, and the residue was dissolved in 5 ml of MeOH. NH_4OH was added dropwise until the solution was slightly basic to pH paper. Addition of 10 vol of acetone gave a precipitate that was collected by centrifugation and then washed with acetone. After drying over P_2O_5 *in vacuo*, 200 mg (26%) of the ammonium salt of **5** was obtained as a white, amorphous solid, $\lambda_{max}^{H_2O}$ 262 nm (ϵ 9300).

The product was chromatographically homogeneous and identical with an authentic sample of **5** obtained by enzymatic phosphorylation of F_3dThd.[2] R_f values by paper chromatography were 0.60 (*n*-BuOH–AcOH–H_2O, 5:2:3), 0.85 (4 *M* formic acid–0.1 *M* ammonium formate), and 0.60 on DEAE-cellulose paper (4 *M* formic acid–0.1 *M* ammonium formate).

5-Trifluoromethyl-2'-deoxyuridine 5'-Triphosphate (6). A slight

§ Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Uv spectra were recorded on a Beckman DB-G spectrophotometer. Elemental analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich., and were within 0.4% of the theoretical values. Analytical tlc was performed on Eastman Chromatogram silica gel sheets with fluorescent indicator. Paper chromatography was carried out on Whatman No. 40 paper and Whatman DE-81 DEAE-cellulose paper.

modification of the method of Hoard and Ott[11] was used to prepare the triphosphate. The tripentylammonium salt of **5** (0.1 mmol) was dissolved in 3 ml of dry DMF and to this solution was added 160 mg (1.0 mmol) of 1,1'-carbonyldiimidazole. The mixture was allowed to stand overnight and then treated simultaneously with MeOH (73 μl, 1.8 mmol) and tripentylammonium pyrophosphate (1.0 mmol). This mixture was stirred vigorously at room temperature for 24 hr. The precipitated imidazolium pyrophosphate was removed by filtration and washed with several portions of DMF. An equal volume of MeOH was added to the filtrate, and after 30 min the solution was evaporated to dryness at reduced pressure. The residue was dissolved in 0.5 ml of H_2O and the solution streaked onto two sheets (46 × 57 cm) of Whatman No. 40 paper. Ascending chromatography was carried out with *n*-BuOH–AcOH–H_2O, 6:2:2. The major band at R_f 0.33 was cut out and the material eluted from the paper with H_2O. By uv, the yield of **6** was 20%, $\lambda_{max}^{H_2O}$ 262 nm. *Anal.* Calcd base–phosphorus ratio, 1:3; found, 1:2.9.

The product was homogeneous by polyethyleneimine–cellulose tlc (R_f 0.3, 0.8 *M* LiCl) and paper chromatography (R_f 0.35, *n*-BuOH–AcOH–H_2O, 5:2:3) and had an R_f of 0.0 on DEAE-cellulose paper (4 *M* formic acid, 0.1 *M* ammonium formate) which agrees with a previously published value.[15] Hydrolysis in 1 *N* HCl for 7 min regenerated F_3dThd-5'-P.

[6-^{3}H]-5-Trifluoromethyl-2'-deoxyuridine 5'-Phosphate and 5'-Triphosphate. The tritiation of F_3dThd was carried out for this laboratory by the Amersham-Searle Co. F_3dThd (300 mg, 1.02 mmol) was dissolved in 70% aqueous AcOH containing [^{3}H]H_2O and heated for 5 hr at 80° in the presence of prereduced PtO_2. This procedure resulted in the incorporation of 60 mCi/mmol of non-exchangeable tritium into F_3dThd. Synthetic procedures identical with those described afforded [6-^{3}H]F_3dThd-5'-P and [6-^{3}H]F_3dThd-5'-PPP.

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Synthesis and Carcinogenicity of Compounds Related to 6-Hydroxymethylbenzo[*a*]pyrene†

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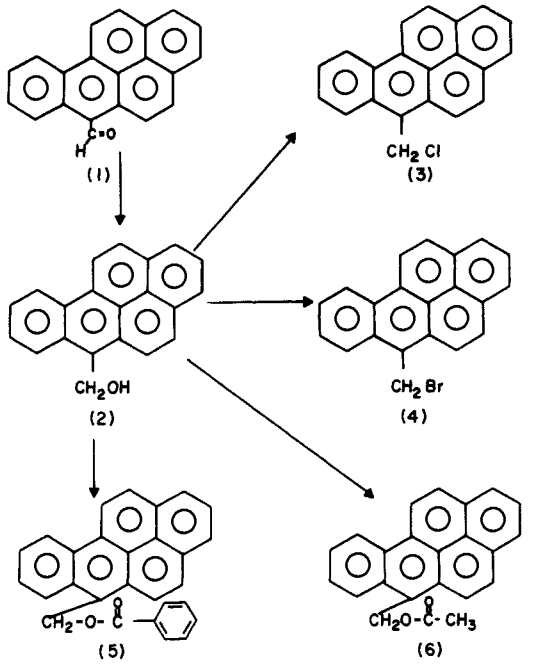
In earlier studies from this laboratory, it was postulated that 7-hydroxymethyl-12-methylbenz[*a*]anthracene, a metabolite of 7,12-dimethylbenz[*a*]anthracene, functions as a proximate carcinogen.[1,2] Evidence in support of this hypothesis was provided by observations of the carcinogenicity of 7-iodomethyl, 7-bromomethyl, 7-chloromethyl, 7-benzoyloxymethyl, 7-acetoxymethyl, 7-methoxymethyl, and 7-formyl derivatives of 12-methylbenz[*a*]anthracene. In accord with this hypothesis, each of the compounds which would be expected to be converted to 7-hydroxymethyl-12-methylbenz[*a*]anthracene was, in fact, shown to be converted, in part, to this compound. Thus, according to this hypothesis, the first step in carcinogenesis by 7,12-dimethylbenz[*a*]anthracene is hydroxylation of the 7-methyl group to form 7-hydroxymethyl-12-methylbenz[*a*]-anthracene. The second is the formation of a reactive ester bearing a good leaving group which would generate a highly reactive carbonium ion. The carbonium ion would be expected to react with critical cellular nucleophiles to initiate the chain of cellular events which result in cancer.

The observation that 7-hydroxymethyl-12-methylbenz[*a*]-anthracene and related compounds are carcinogenic suggested that a similar series of derivatives of benzo[*a*]pyrene might also be carcinogenic. The synthesis of 6-hydroxymethylbenzo[*a*]pyrene and related compounds was therefore undertaken to determine whether this is, in fact, the case.

The synthesis of 6-hydroxymethylbenzo[*a*]pyrene (**2**) was accomplished as illustrated in Scheme I by reduction

Scheme I



of 6-formylbenzo[*a*]pyrene (**1**) with $NaBH_4$. The aldehyde required for this synthesis was prepared by treatment of benzo[*a*]pyrene with *N*-methylformanilide in the presence of phosphorus oxychloride as described by Fieser and Hershberg.[3] 6-Hydroxymethylbenzo[*a*]pyrene was then converted to the chloromethyl (**3**), bromomethyl (**4**), benzoyloxymethyl (**5**), and acetoxymethyl (**6**) compounds as described in the Experimental Section.

The carcinogenicity of the compounds tested is presented in Table I. Each of the compounds tested is clearly a potent carcinogen as indicated by the high tumor incidence and short latent period. Benzo[*a*]pyrene and its formyl derivative are well-established carcinogens.[4] The carcinogenicity of 6-hydroxymethylbenzo[*a*]pyrene was recently reported from this laboratory but this is the first report of the other compounds in this series.[5]

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