Thymidylate Synthetase. Model Studies of Inhibition by 5-Trifluoromethyl-2′-deoxyuridylic Acid*

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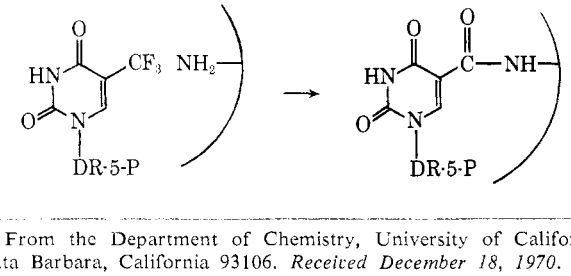
ABSTRACT: The mechanisms of hydrolytic reactions of 5-trifluoromethyluracil and its N-alkylated derivatives have been examined to provide insight into the mechanism of irreversible inhibition of thymidylate synthetase by 5-trifluoromethyl-2′-deoxyuridylic acid. All reactions appear to proceed by formation of a highly reactive intermediate having an exocyclic difluoromethylene group at the 5 position which subsequently reacts with water or hydroxide ion in a series of rapid steps to give corresponding 5-carboxyuracils. For those derivatives which possess an ionizable proton at the 1 position, the predominant mechanism involves ionization to the conjugate base and assistance by the 1 anion in the expulsion of fluoride ion. Further assistance is provided by ionization of the 3-NH in the case of 5-trifluoromethyluracil to give the dianionic species. When ionization at the 1 position is precluded by the presence of an alkyl substituent, acylation reactions proceed by rate determining attack of hydroxide ion at the 6 position of the neutral or negatively charged (3-anion) heterocycle to provide the reactive intermediate. In order to obtain suitable intramolecular models, and to verify the primary site of reaction of 1-substituted derivatives, a series of 1-(ω-aminoalkyl)trifluoromethyluracils were prepared and their hydrolyses examined. Neighboring group participation was apparent where attack of the amino group on the 6 position of the heterocycle results in the formation of five-, six-, and seven-membered rings; in the case of 1-(3-aminopropyl)-5-trifluoromethyluracil apparent first-order constants were more than 10^4 times greater than simple 1-alkyl derivatives not possessing a neighboring nucleophile. An analogous mechanism for the acylation of thymidylate synthetase by 5-trifluoromethyl-2′-deoxyuridine is proposed in which a nucleophilic group of the enzyme active-site participates in the activation of the trifluoromethyl group.

5-Trifluoromethyl-2′-deoxyuridylic acid (F_3TDRP[1]) is a potent reversible inhibitor of thymidylate synthetase, the enzyme which catalyzes the reductive methylation of 2′-deoxyuridine 5′-monophosphate to thymidine 5′-monophosphate using 5,10-methylenetetrahydrofolic acid as cofactor. Reyes and Heidelberger (1965) have also observed that upon preincubation F_3TDRP causes irreversible inhibition of this enzyme. Based on the observation that F_3T acylates amines in aqueous media to give uracil-5-carboxamides (Heidelberger *et al.*, 1964), it has been suggested that the irreversible inactivation of thymidylate synthetase may result from a similar acylation of an amino group at or near the active site of the enzyme (Reyes and Heidelberger, 1965).



The question that immediately arises is why the trifluoromethyl group at the 5 position of uracil derivatives should be at all susceptible to these reactions. The carbon–fluorine bond is quite strong (Pauling, 1960) and an outstanding characteristic of trifluoromethyl groups is their unusual resistance toward chemical degradation. As relevant examples, it is noted that benzotrifluorides, derivatives of 6-trifluoromethyluracils (Giner-Sorolla and Bendich, 1958), derivatives of 2-trifluoromethyl-4-oxopyrimidines (Barone, 1963), and 5-trifluoromethyl-6-azauracil (Dipple and Heidelberger, 1966) are quite stable toward hydrolytic reactions. In contrast, F_3T is rapidly converted into 5-carboxyuracil (CU) (Heidelberger *et al.*, 1964) in basic media and, although somewhat slower, nucleosides of F_3T are converted into the corresponding nucleosides of CU (Shen *et al.*, 1965; Khwaja and Heidelberger, 1969; Ryan *et al.*, 1966). *In vivo*, the metabolism of F_3T and F_3TDR provides CU and not the normal products expected from pyrimidines (Heidelberger *et al.*, 1965). With the exception of derivatives of 5-trifluoromethyluracil, we have been able to locate few reports of trifluoromethyl groups which demonstrate high reactivity toward nucleophiles. For example, the *o*- and *p*-trifluoromethylphenols are rapidly converted into the corresponding *o*- and *p*-hydroxybenzoic acids in basic media (Jones, 1947); in contrast, the isomeric *m*-trifluoromethylphenol is completely stable toward hot, aqueous sodium hydroxide. Although qualitative, these results suggest that a secondary driving force, perhaps assistance by the *o*- and *p*-phenolate anion in the above case, is necessary for acylation reactions of trifluoromethyl groups, and direct displacement reactions (S_{N2} type) are probably not operative. Nestler and Garrett (1968) have examined the kinetics of solvolysis of 5-trifluoromethyl-2′-deoxyuridine and suggested that the mechanism involves direct displacement of fluoride by hydroxide to give 5-hydroxydifluoromethyl-2′-deoxy-

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[1] Abbreviations used are: F_3T, 5-trifluoromethyluracil; 1(3)-MF_3T, 1(3)-methyl-5-trifluoromethyluracil; BF_3T, 1-*n*-butyl-5-trifluoromethyluracil; DMF_3T, 1,3-dimethyl-5-trifluoromethyluracil; F_3TR, 5-trifluoromethyluridine; IpF_3TR, 2′,3′-*O*-isopropylidene-5-trifluoromethyluridine; UDR, 2′-deoxyuridine; F_3TDR, 5-trifluoromethyl-2′-deoxyuridine; F_3TDRP, 5-trifluoromethyl-2′-deoxyuridylic acid; CU, 5-carboxyuracil; 1(3)-MCU, 1(3)-methyl-5-carboxyuracil; DMCU, 1,3-dimethyl-5-carboxyuracil; NF_3T, 1-(ω-aminoalkyl)-5-trifluoromethyluracil; NCU, 1-(ω-aminoalkyl)-5-carboxyuracil.

uridine as an intermediate; in view of the aforementioned stability of the trifluoromethyl group toward this type of reaction, this proposal appears subject to question.

It is apparent that if acylation reactions of 5-trifluoromethyluracils must proceed by anomalous reaction mechanisms, such pathways would also be likely in the proposed acylation of thymidylate synthetase by F_3TDRP. In this report we describe the kinetics and mechanisms of hydrolytic reactions of F_3T and derivatives, and their relevance to the enzymic counterpart and catalytic mechanism.

Materials and Methods

General. Melting points were determined on a Mel-Temp block and are corrected below 230°. Ultraviolet spectra were obtained with Cary Models 14 or 15 recording spectrophotometers with 1-cm path-length cells. Rate measurements were made on a Beckman DU monochromator combined with a Gilford Model 2000 multiple-sample absorbance recorder. Infrared spectra were recorded on a Perkin-Elmer Model 337 spectrophotometer. A Radiometer Model 26 pH meter was used for pH measurements using Radiometer GK 2021 B or Metrohm EA 125 U combined glass and reference electrodes. Potentiometric pK_a determinations were made by manual titration using a Radiometer buret syringe; spectrophotometric pK_a determinations were made using the titration apparatus described by Bruice and Maley (1970). Thin-layer chromatography was run on silica gel GF_{254} (Merck) plates and Brinkmann MN-Polygram-Cel 300/uv cellulose plates. Paper chromatography was performed on Whatman No. 1 strips using an ascending technique. Silica gel solvent systems used were ethyl acetate (A), chloroform–ethanol (3:1) (B), and ethyl acetate–petroleum ether (bp 65–110°) (4:1) (C); for cellulose tlc, ethyl acetate–formic acid–water (7:2:1) (D) and 1-butanol–acetic acid–water (2:1:1) (E) were used; for paper chromatography, systems D and E were utilized as well as *tert*-butyl alcohol–methyl ethyl ketone–water–ammonium hydroxide (4:3:2:1) (F). Compounds were detected visually under ultraviolet light at 254 nm. F_3T was obtained from PCR, Inc., Gainesville, Fla. F_3TR and F_3TDR were gifts from Dr. Charles Heidelberger and Cancer Chemotherapy National Service Center, respectively. IpF_3TR was prepared by the method of Fromageot *et al.* (1967). All other materials were reagent grade. Microanalysis were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Kinetic Measurements. All solutions for kinetic measurements were prepared from doubly distilled water which had been boiled and purged with nitrogen before use. Ionic strength was maintained at 1.0 M with sodium perchlorate. Unless otherwise specified, buffer catalysis was not observed and buffer concentration was normally maintained at 0.05 M. External buffers used were phosphate (pH 5.7–8.0), borate (pH 7.6–9.2), and carbonate (pH 9.2–10.5); sodium hydroxide was used for pH 10.5–14.0.

For kinetic measurements of derivatives having half-lives of less than 10 hr, 0.10 ml of a 1–2 mM solution of the F_3T derivative was added to solutions which were preequilibrated at the desired temperature (30.0 or 50.0 ± 0.1°) in 10-mm quartz cells in the thermostated cell compartment of the spectrophotometer; reactions were continuously recorded for at least 8 half-lives at appropriate wavelengths. For longer runs, 10 ml of a 0.5 mM solution in the appropriate buffer was incubated at 50 ± 0.1°, withdrawn periodically, and cooled, and the optical density at the appropriate wavelength determined. The reactions were monitored at wavelengths where the difference in absorbance of the starting material and uracil-5-carboxylic acids were sufficiently different: for F_3T, 290 nm was used between pH 7.5 and 14, and 250 nm below pH 7.5; for 3-MF_3T, 300 nm was used for pH 8 to 11.4 and 275 nm below pH 8; for all 1-substituted derivatives of F_3T, reactions were monitored at 280 nm. The observed first-order rate constants were obtained from the equation $\ln (OD_t - OD_\infty) = \ln (OD_0 - OD_\infty) - k_{obsd}t$, where OD_0, OD_t, and OD_∞ are the measured absorbances at times 0, t, and after completion of the reaction.

Product Analysis. Identification of N-substituted derivatives of CU was most readily accomplished by ultraviolet spectral analysis. Generally, conversion of F_3T derivatives into the corresponding CU is accompanied by a bathochromic shift of 10–12 nm at pH 13. CU and 3-MCU showed maxima at *ca.* 286 nm at pH 13 with higher extinction coefficients than F_3T and 3-MF_3T; all 1-substituted CU derivatives showed maxima at 272–275 nm. Authentic samples of CU, 3-MCU, 1-MCU, and DMCU were prepared for direct ultraviolet spectral and chromatographic comparisons to hydrolytic products. Although authentic samples of the 1-(ω-aminoalkyl)-CU derivatives were not available, their identity could be verified by sodium bisulfite catalyzed decarboxylation (Isono and Suzuki, 1970) to the known 1-(ω-aminoalkyl)-uracils (Santi and Brewer, 1970; D. V. Santi and C. F. Brewer, unpublished results).

1-Methyl-5-trifluoromethyluracil (1-MF_3T). A mixture of 900 mg (5 mmoles) of F_3T, 740 mg (5 mmoles) of anhydrous potassium carbonate, and 710 mg (5 mmoles) of methyl iodide in 15 ml of dry dimethylformamide was stirred at ambient temperature for 2 hr. After removal of solvent *in vacuo* the residue was extracted with ethyl acetate, clarified with charcoal, and concentrated to *ca.* 5 ml. Addition of petroleum ether (bp 65–110°) gave 600 mg (61%) of white crystals, mp 244–248°; the product showed one spot on thin-layer chromatography with systems A and B, and was identical with a sample prepared by an alternative route (Sakai *et al.*, 1968). The spectrophotometrically determined pK_a at 50° and μ = 1.0 was found to be 8.20.

1-Butyl-5-trifluoromethyluracil (BF_3T) was synthesized as described for 1-MF_3T using *n*-butyl bromide and a reaction time of 9 hr; yield 46%, mp 174–175°. *Anal.* Calcd for $C_9H_{11}F_3N_2O_2$: C, 45.76; H, 4.70; N, 11.85. Found: C, 45.79; H, 4.64; N, 11.86.

1,3-Dimethyl-5-trifluoromethyluracil (DMF_3T). A mixture of 225 mg (1.25 mmoles) of F_3T, 345 mg (2.5 mmoles) of anhydrous potassium carbonate, and 5 ml of methyl iodide in 15 ml of anhydrous dimethylformamide was stirred at room temperature for 3 hr at which time thin-layer chromatography indicated loss of all starting material. The mixture was evaporated *in vacuo* to a yellow oil which was dissolved in chloroform, clarified with charcoal, and taken to dryness under reduced pressure. The residual clear oil crystallized upon standing to give 220 mg (85%) of a white solid, mp 101–102°; thin-layer chromatography with systems B and C showed one spot. A 100-mg portion was recrystallized from ethyl acetate–petroleum ether (bp 65–110°) to give the analytical sample, 65 mg, mp 101–101.5°; λ_{max} (H_2O) 262 (ε 6630) (pH 1), 262 (ε 6300) (pH 7), 262 nm (ε 6970) (pH 13). *Anal.* Calcd for $C_7H_7F_3N_2O_2$: C, 40.39; H, 3.39; N, 13.46. Found: C, 40.63; H, 3.24; N, 13.45.

3-Methyl-5-trifluoromethyluracil (3-MF_3T). This was prepared by the method described for F_3T (Mertes *et al.*, 1966). A mixture of 0.50 g (2.9 mmoles) of 3-methyl-5-carboxyuracil

(Whitehead, 1952), 0.3 ml of H_2O, and 23 g of SF_4 were heated at 100° with shaking for 16 hr. The reaction vessel was cooled; the gases were vented into a 20% NaOH solution. The residue was dissolved in 5 ml of ethyl acetate, filtered free of insolubles, and eluted from a silica gel column (2.5 × 25 cm) with ethyl acetate. Concentration of the ultraviolet-absorbing fraction (*ca.* 50–100 ml) gave 125 mg (25%) of a white solid. Recrystallization from ethyl acetate–petroleum ether (bp 30–60°) gave 60 mg (12%) of flat white needles, mp 185–190°; thin-layer chromatography in system B showed one spot; λ_{max} (H_2O) 256 (ε 5200) (pH 1), 256 (ε 5000) (pH 7), 278 nm (ε 7700) (pH 13). The pK_a at 30° (μ = 1.0) was 7.60 by potentiometric titration. *Anal.* Calcd for $C_6H_5F_3N_2O_2$: C, 37.13; H, 2.60; N, 14.43. Found: C, 37.31; H, 2.64; N, 14.60.

1-(ω-Bromoalkyl)-5-trifluoromethyluracils. A mixture of 360 mg (2 mmoles) of F_3T, 1.5 ml of hexamethyldisilazane, and a drop of trimethylchlorosilane was heated to dissolution (approximately 0.5 hr at 150°). The resulting clear solution was treated with 5 ml of the appropriate α,ω-dibromoalkane for 6–10 days at 50° protected from moisture. After the addition of 1 ml of water, the mixture was evaporated *in vacuo* and the residue partitioned between 100 ml of chloroform and 50 ml of water. The chloroform fraction was dried ($MgSO_4$), clarified with charcoal, and evaporated under reduced pressure. The residue was crystallized from ethyl acetate–petroleum ether (65–110°) to give white crystals; thin-layer chromatography in system B showed one spot.

Ethyl derivative: yield 38% (6 days), mp 178–180°; λ_{max} (H_2O) 262 (ε 9800) (pH 1), 262 (ε 9430) (pH 7), 260 nm (ε 6410) (pH 13). *Anal.* Calcd for $C_7H_6BrF_3N_2O_2$: C, 29.29; H, 2.11; N, 9.76. Found: C, 29.55; H, 1.92; N, 9.69.

Propyl derivative: yield 82% (10 days), mp 143–144°; λ_{max} (H_2O) 264 (ε 9940) (pH 1), 264 (ε 8700) (pH 7), 262 nm (ε 5960) (pH 13). *Anal.* Calcd for $C_8H_8BrF_3N_2O_2$: C, 31.92; H, 2.68; N, 9.30. Found: C, 31.85; H, 2.65; N, 9.30.

Butyl derivative: yield 85% (8 days), mp 172–173°; λ_{max} (H_2O) 264 (ε 9750) (pH 7), 262 nm (ε 6970) (pH 13). *Anal.* Calcd for $C_9H_{10}BrF_3N_2O_2$: C, 34.25; H, 3.20; N, 8.89. Found: C, 34.50; H, 3.18; N, 9.03.

Pentyl derivative: yield 60% (8 days), mp 153–154°; λ_{max} (H_2O) 265 (ε 9840) (pH 1), 265 (ε 10,970) (pH 7), 262 nm (ε 7030) (pH 13). *Anal.* Calcd for $C_{10}H_{12}BrF_3N_2O_2$: C, 36.49; H, 3.68; N, 8.51. Found: C, 36.69; H, 3.63; N, 8.43.

1-(ω-Azidoalkyl)-5-trifluoromethyluracils. A mixture of 0.5 mmole of the appropriate bromoalkyl derivative and 150 mg of sodium azide in 2 ml of anhydrous dimethylformamide was heated 18–20 hr on a steam bath. After evaporation *in vacuo*, the residue was treated with water and the mixture extracted with chloroform. The chloroform extract was dried ($MgSO_4$) and concentrated to an oil which, with the exception of the azidopentyl compound, gave a semisolid on standing. Yields in all cases were approximately 80%. Thin-layer chromatography in system A showed one spot different from starting material; ν_{max} (KBr) 2110 cm^{-1} (N_3); ultraviolet spectra were consistent with 1-substituted uracils (Shugar and Fox, 1952). These compounds were sufficiently pure for subsequent transformations.

1-(ω-Aminoalkyl)-5-trifluoromethyluracils, Hydrochloride Salts. A solution of 0.5 mmole of the appropriate 1-(ω-azidoalkyl)-F_3T in 10 ml of absolute ethanol containing approximately 3 mmoles of anhydrous HCl was hydrogenated at atmospheric pressure in the presence of 5 mg of platinum oxide for 15–20 hr. The catalyst was filtered and the solvent removed under reduced pressure. The residue was recrystallized from ethanol–ether to give a white solid, one spot on cellulose thin-layer chromatography in systems D and E.

Ethyl derivative: yield 35%, mp 290–295 dec; λ_{max} (H_2O) 261 (ε 9320) (pH 1), 261 (ε 8720) (pH 7), 262 nm (ε 6540) (pH 13). The pK_a of the heterocycle was found to be 7.69 by spectrophotometric titration at 50° (μ = 1.0). *Anal.* Calcd for $C_7H_9ClF_3N_3O_2$: C, 32.38; H, 3.49; N, 16.18. Found: C, 32.64; H, 3.66; N, 15.99.

Propyl derivative: yield 48%, mp 237–240°; λ_{max} (H_2O) 263 (ε 10,000) (pH 1), 263 (ε 9890) (pH 7), 262 nm (ε 8050) (pH 13). *Anal.* Calcd for $C_8H_{11}ClF_3N_3O_2$: C, 35.11; H, 4.05; N, 15.36. Found: C, 34.99; H, 4.19; N, 15.16.

Butyl derivative: yield 66%, mp 211–213°; λ_{max} (H_2O) 264 (ε 10,100) (pH 1), 264 (ε 9640) (pH 7), 262 nm (ε 7230) (pH 13). The pK_a of the heterocycle was 7.76 as determined spectrophotometrically at 50° (μ = 1.0). *Anal.* Calcd for $C_9H_{13}ClF_3N_3O_2$: C, 37.58; H, 4.56; N, 14.53. Found: C, 37.74; H, 4.62; N, 14.45.

Pentyl derivative: yield 58%, mp 237–239°; λ_{max} (H_2O) 265 (ε 9690) (pH 1), 265 (ε 9850) (pH 7), 262 nm (ε 7390) (pH 13). The pK_a by spectrophotometric titration was found to be 8.10 at 50° (μ = 1.0). *Anal.* Calcd for $C_{10}H_{15}ClF_3N_2O_2$: C, 39.81; H, 5.01; N, 13.93. Found: C, 39.73; H, 5.02; N, 13.87.

1,3-Dimethyl-5-carboxyuracil (DMCU). DMF_3T (60 mg, 0.29 mmole) was added to 10 ml of 0.5 N NaOH and the mixture was stirred to dissolution at room temperature (*ca.* 5 min). The solution was acidified to pH 1 with concentrated HCl and cooled to give 35 mg (65%) of white crystals, mp 188–189° dec; cellulose thin-layer chromatography with system E showed one spot; λ_{max} (H_2O) 283 (ε 9400) (pH 1), 278 nm (ε 8000) (pH 13). *Anal.* Calcd for $C_7H_8N_2O_4$: C, 45.66; H, 4.38; N, 15.21. Found: C, 45.50; H, 4.21; N, 15.09.

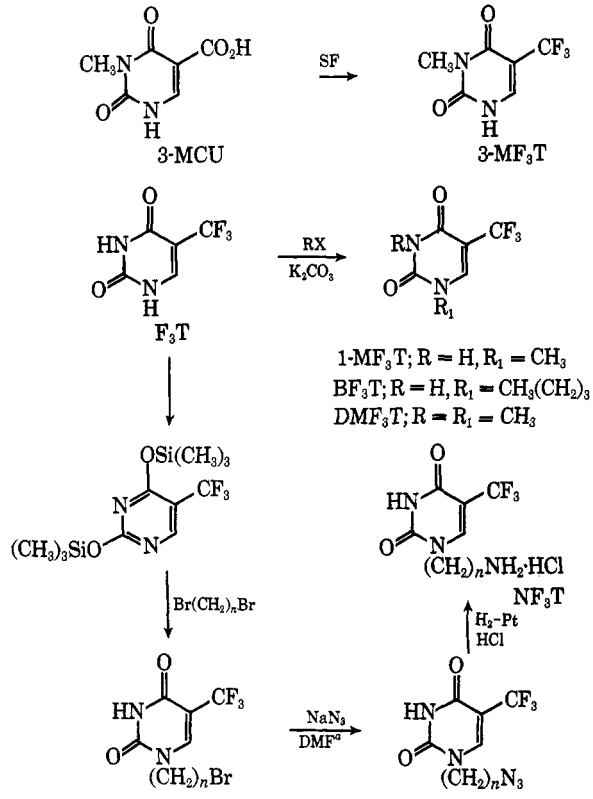
Isolation of 1-Methyl-5-carboxyuracil (1-MCU). 1-MF_3T (100 mg, 0.52 mmole) was dissolved in 5 ml of 2 N KOH and heated at 70° for 1.5 hr. The solution was adjusted to pH 1 with concentrated HCl and evaporated *in vacuo*. The residue was recrystallized from water–ethanol to give 50 mg (56%) of white crystals: mp 259–261° dec; λ_{max} (H_2O) 279 (ε 11,600) (pH 1), 276 (ε 11,400) (pH 7), 271 nm (ε 9200) (pH 13). As characteristic of 5-carboxyuracils (Isono and Suzuki, 1970), treatment of this compound with a 2- to 3-fold excess of sodium bisulfite in aqueous solution at 37° for 5 hr gave a quantitative yield of 1-methyluracil and provided verification of the structure assignment.

Results

Synthesis of N-Substituted F_3T Derivatives. The most direct routes to N-substituted derivatives of F_3T involve either fluorination of the appropriate N-substituted 5-carboxyuracil or alkylation of F_3T. The successful routes used for the syntheses of the desired derivatives of F_3T are depicted in Scheme I. 1-MF_3T and 1-BF_3T could be prepared in good yield by direct alkylation of F_3T in dimethylformamide and 1 equiv of anhydrous K_2CO_3. Treatment of F_3T with 2 equiv of K_2CO_3 and an excess of methyl iodide afforded DMF_3T in excellent yield. 3-MF_3T was prepared by fluorination of 3-methyl-5-carboxyuracil with sulfur tetrafluoride, as described for the preparation of F_3T (Mertes *et al.*, 1966).

Several synthetic routes were attempted for the synthesis of the 1-(ω-aminoalkyl)-5-trifluoromethyluracils. Direct alkylation of F_3T with 3-phthalimidopropyl bromide in dimethylformamide–K_2CO_3 gave 1-(3-phthalimidopropyl)-F_3T, but attempted removal of the blocking group with hydrazine gave an intractable mixture of products. Similarly, direct alkylation of F_3T with α,ω-dibromoalkanes did not appear promising.

SCHEME I[a]



[a] DMF = dimethylformamide.

A number of 1-substituted pyrimidine-2,4-diones, including 1-MF_3T, have recently been prepared (Sakai *et al.*, 1968; Browne, 1968) by treatment of the bis(*O*-trimethylsilyl) derivative of the unsubstituted pyrimidines with excess alkyl halide. When the bis(*O*-trimethylsilyl) derivative of F_3T was treated with an excess of the appropriate α,ω-dibromoalkane at 50° for 6–10 days, the 1-(ω-bromoalkyl)-5-trifluoromethyluracils were obtained in good yield. Treatment with sodium azide in dimethylformamide at 100° gave the corresponding 1-(ω-azidoalkyl)-F_3T derivatives which could be catalytically reduced in ethanolic HCl to give the desired 1-(ω-aminoalkyl)-5-trifluoromethyluracils (NF_3T) as stable hydrochloride salts.

Hydrolysis of F_3T and N-Methylated Derivatives. The hydrolysis of DMF_3T to DMCU in the pH range of 9–13 (Figure 1) is characterized by a bathochromic shift of λ_{max} from 262 to 278 nm and follows the expression

$$k_{obsd} = k_{OH}[OH^-] \qquad (1)$$

where k_{OH} = 10.6 M^{-1} min^{-1} at 30° and 83.4 M^{-1} min^{-1} at 50°. A subsequent slower shift in the maximum to 284 nm was noted which was second order in hydroxide (k_{OH^2} = 1.6×10^{-2} M^{-2} min^{-1}) followed by loss of ultraviolet absorbance. These changes were sufficiently slow that they did not interfere with the reaction of concern. On the basis of the known instability of 1,3-dimethyluracils in aqueous base (Santi and Brewer, 1970; Shugar and Fox, 1952), these reactions are believed to involve complex cleavage of the heterocycle of DMCU; they are not observed with any of the derivatives discussed below.

The pH–log k_{obsd} profile for the hydrolysis of 1-MF_3T at 50° is given in Figure 1. The dependence of k_{obsd} on an ionizable group (pK_{app} = 8.2) suggests that at $a_H \ll K_a$ the hydrolysis proceeds by reaction of hydroxide with the neutral pyrimidine (1-MF_3T); the specific hydroxide region at high pH represents hydroxide reaction with the 3-anion (1-MF_3T^-). The rate data may be described by the equation

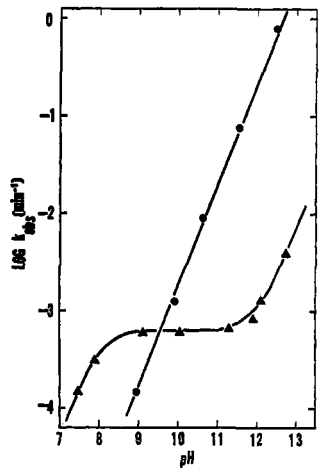


FIGURE 1: The pH–log k_{obsd} profiles for the hydrolysis of DMF_3T (●) at 30° and 1-MF_3T (▲) at 50°. Points are experimental and the lines are calculated from eq 1 and 2.

$$k_{obsd} = k_{OH}[OH^-]\frac{a_H}{K_a + a_H} + k_{OH}'[OH^-]\frac{K_a}{K_a + a_H} \qquad (2)$$

where k_{OH} (79.5 M^{-1} min^{-1}) and k'_{OH} (6.6×10^{-2} M^{-1} min^{-1}) are the specific rate constants associated with hydroxide attack on 1-MF_3T and 1-MF_3T^-, respectively, K_a (6.3×10^{-9}) the acid dissociation constant of 1-MF_3T, and a_H the hydrogen ion activity as measured by the glass electrode.

The conversion of 3-MF_3T into 3-MCU was examined as a function of pH at 30° (Figure 2). The apparent first-order rate constants at pH values above 5.5 may be expressed by the equation

$$k_{obsd} = k_1\frac{K_a}{K_a + a_H} \qquad (3)$$

where k_1 is the specific rate constant for the reaction of the monoanion (3-MF_3T^-) with water and K_a the acid dissociation constant of 3-MF_3T^-. Unlike 1-MF_3T, no significant change in rate is observed at high pH ($a_H \ll K_a$) and k_{obsd} becomes equal to k_1 (3.14×10^{-3} min^{-1}). The apparent pK_a of 3-MF_3T

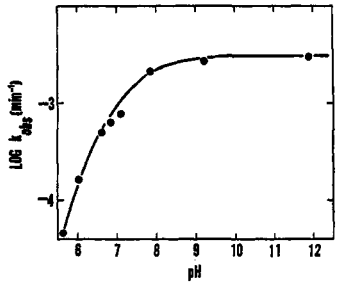


FIGURE 2: The pH–log k_{obsd} profile for the hydrolysis of 3-MF_3T at 30°. Points are experimental and the curve is calculated from eq 3.

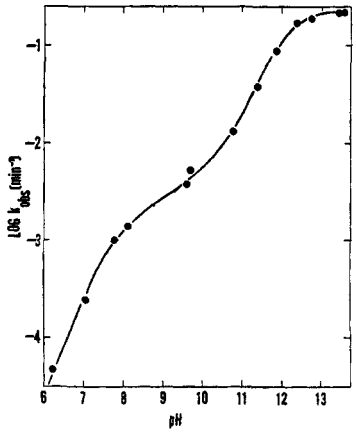


FIGURE 3: The pH–log k_{obsd} profile for the hydrolysis of F_3T at 30°. Points are experimental and the curve is calculated from eq 5.

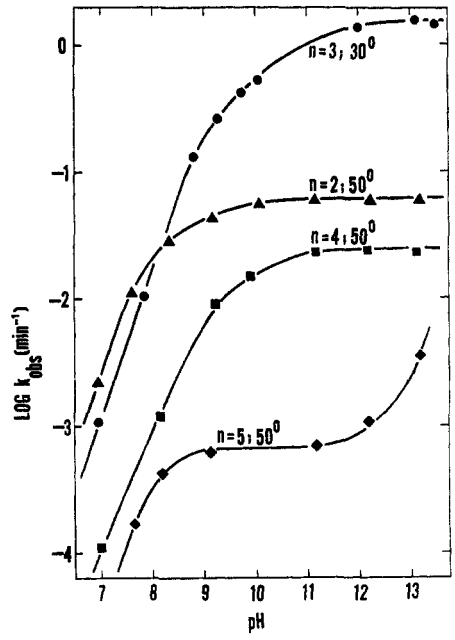


FIGURE 4: The pH–log k_{obsd} profiles for the hydrolysis of the labeled 1-(*n*-aminoalkyl) F_3T's at the given temperatures. Points are experimental and the curves are calculated from eq 7 using the constants given in Table II.

obtained is 7.60, in good agreement with the measured value of 7.59.

Rates for the conversion of F_3T into uracil-5-carboxylic acid between pH 11.4 and 13.3 have been reported (Nestler and Garrett, 1968) but the data are insufficient to establish the reactive ionic species. The complete pH–log k_{obsd} profile for the hydrolysis of F_3T at 30° is given in Figure 3 and indicates the involvement of at least two acidic protons with apparent pK_a values of 8.0 and 12.0. F_3T possesses two ionizable protons which may give rise to four molecular species in solution. An empirical expression in accord with Figure 3 which describes the possible involvement of the various ionic species is given in eq 4, where k_{OH} is the second-order rate constant associated

$$k_{obsd} = k_{OH}[OH^-]f_{F_3T} + k_1 f_{F_3T\text{-}1^-} + k_1' f_{F_3T\text{-}3^-} + k_2 f_{F_3T^{2-}} \quad (4)$$

with the reaction of hydroxide ion with the mole fraction of un-ionized F_3T (f_{F_3T}), k_1 and k_1' are the specific rate constants for spontaneous reaction of the mole fractions of the N-1 ($f_{F_3T\text{-}1^-}$) and N-3 ($f_{F_3T\text{-}3^-}$) anions, respectively, and k_2 the rate constant for the reaction of the mole fraction of the dianion ($f_{F_3T^{2-}}$). The first three terms of eq 4 are kinetically equivalent and are included for descriptive purposes. As will be shown later (see Discussion) the reaction of hydroxide with F_3T and the spontaneous reaction of F_3T-3$^-$ are sufficiently slow as not to contribute to observed hydrolysis, and the first and third terms of eq 4 need not be considered. The first kinetic dissociation constant (pK_a = 8.0) does not agree with the measured pK_a for F_3T of 7.59; however, it will be shown that the unreactive F_3T-3$^-$ accounts for approximately 15% of the monoanions present in solution and the resulting nonproductive equilibrium is responsible for this discrepancy. Making these assumptions for the present, eq 4 may be expanded through material balance, a_H, and the dissociation constants of each acidic species. The resultant expression is given in eq 5 and can be fitted to the profile in Figure 3 to

$$k_{obsd} = \left(k_1 + \frac{k_2 K_2}{a_H}\right) \frac{K_1 a_H}{K_1 K_2 + (K_1 + K_3) a_H + a_H^2} \quad (5)$$

give the values $k_1 = 1.25 \times 10^{-3}$ min^{-1}, $k_2 = 0.221$ min^{-1}, and $K_1 = 2.75 \times 10^{-8}$ for dissociation of the N-1 proton, $K_2 = 1 \times 10^{-12}$ for the composite dissociation constants of F_3T-1$^-$ and F_3T-3$^-$ to give the dianion F_3T^{2-}, and $K_3 = 5 \times 10^{-9}$ for the dissociation of the N-3 proton of F_3T to give the unreactive F_3T-3$^-$. Reactions performed in the presence of 0.5 M fluoride, bromide, iodide, or nitrate ions at pH 12.5 showed no depression in rate.

Hydrolysis of 1-(ω-Aminoalkyl)-F_3T. In Figure 4, the pH–log k_{obsd} profiles are given for the hydrolysis of a series of 1-(ω-aminoalkyl)-5-trifluoromethyluracils (NF_3T) to the corresponding carboxylic acids (NCU) at 30 or 50°. Unlike the simple 1-alkyl derivatives described previously, there are four possible reactive ionic species which are shown in Scheme IV. Hydrolytic rates of the pentyl derivative (NF_3T, n = 5) at 50° exhibit the same pH–rate dependence as previously described for 1-MF_3T. The reaction may be described by eq 2 where k_{OH} and k_{OH}' (79.5 and 3.1 × 10^{-2} M^{-1} min^{-1}) are the specific rate constants associated with hydroxide attack on the species having an un-ionized heterocycle ($^+HNF_3T$, NF_3T) and the species in which the 3-NH of the pyrimidine is dissociated ($^+HNF_3T^-$, NF_3T^-). The fact that the constants obtained (k_{OH} = 79.5 M^{-1} min^{-1}, k_{OH}' = 3.1 × 10^{-2} M^{-1} min^{-1}, and K_a = 6.3 × 10^{-9}) from a fit of the hydrolytic data to eq 2 are identical with those obtained for 1-MF_3T indicates that the state of ionization of the primary amine group has no effect on the dissociation (K_a) or reactivity (k_{OH}, k_{OH}') of the heterocycle. With the exception of the aminopentyl derivative (NF_3T, n = 5), all NF_3T derivatives display a rate saturation effect above apparent pK_a values of 8.4–9.8 and there is no indication of hydroxide ion attack on the 3-monoanions (NF_3T^-, n = 2–4). In addition, rate enhancements of up to 10^4 are observed as compared to 1-MF_3T and NF_3T, n = 5, which are a function of the length of the alkyl chain separating the heterocycle from amine (Table I). An empirical expression which is in accord with the profiles given in Figure 4 and describes the possible involvement of the various ionic species is given in eq 6, where f represents the fraction of the sub-

TABLE I: Comparison of the Relative Rates of Hydrolysis of 1-Substituted 5-Trifluoromethyluracils at 50°.[a]

1-Substituent	k_{rel}	$t_{1/2}$ (min)
CH_3	1.0	1000
$CH_3(CH_2)_3$-	0.573	1741
$H_2N(CH_2)_2$-	82.7	12.0
$H_2N(CH_2)_3$-	10,700[b]	0.084[b]
$H_2N(CH_2)_4$-	33.2	30.0
$H_2N(CH_2)_5$-	1.0	1004

[a] Calculated from k_{obsd} at rate saturation of the reaction of the neutral heterocycle with hydroxide or kinetic equivalent (pH 11.0). [b] Estimated by comparison with rates of hydrolysis of other NF_3T's at 30°.

$$k_{obsd} = k_{OH}[OH^-]f^+_{HNF_3T} + k_{OH}'[OH^-]f_{NF_3T} + k_1 f_{NF_3T} + k_2 f_{NF_3T^-} \quad (6)$$

scripted species present in solution, k_{OH} and k_{OH}' are specific rate constants associated with reactions of hydroxide with the species having a neutral pyrimidine ($^+HNF_3T$, NF_3T), and k_1 and k_2 are specific rate constants for the spontaneous hydrolysis of the species having an unprotonated primary amino group (NF_3T, NF_3T^-). The first and third terms as well as the second and fourth terms of eq 6 are kinetically indistinguishable but are given for descriptive purposes since they represent feasible reaction pathways. Since the microscopic dissociation constants described in Scheme IV cannot be directly ascertained, it is necessary to make a number of simplifying reasonable assumptions before expansion of eq 6. First, it is assumed that for each derivative the state of ionization of either the heterocycle or the primary amino group will not greatly affect the pK_a of the other (*i.e.*, $K_1 \simeq K_4$ and $K_2 \simeq K_3$); although this may be subject to argument in the case of the ethyl derivative (NF_3T, $n = 2$) the effect would likely be small enough to make the error negligible (see Discussion), and separation of the ionizable groups of longer alkyl chains would almost certainly eliminate such inductive effects. Second, it is assumed that the rate of hydroxide attack on the heterocycle will not be greatly affected by the state of ionization of the amine and is similar in magnitude to that observed for 1-MF_3T and the aminopentyl derivative (NF_3T, $n = 5$). From this, it follows that the first two terms of eq 6, which are associated with bimolecular attack of hydroxide on the neutral heterocycle, are negligible and may be omitted from consideration. The resulting expression involving unimolecular hydrolysis of NF_3T and NF_3T^- is given in eq 7, where K_1 and K_2 are

$$k_{obsd} = \left(\frac{k_1 a_H}{K_1} + k_2\right) \frac{K_1 K_2}{K_1 K_2 + (K_1 + K_2)a_H + a_H^2} \quad (7)$$

the dissociation constants for the 3-NH of the heterocycle and the ammonium ion, respectively; k_1 and k_2 are specific rate constants for the unimolecular reactions of the neutral and ionized species of the heterocycle, respectively. Values for the above constants obtained by best fits of eq 7 to the hydrolytic data are given in Table II.

F_3TDR and IpF_3TR. Ultraviolet spectral scans of 5-trifluoromethyl-2′-deoxyuridine (F_3TDR) in 0.126 M NaOH at 30° taken at various times are given in Figure 5A. The conditions

TABLE II: Rate and Dissociation Constants for the Hydrolyses of NF_3T.

n	Temp (°C)	k_1 (min^{-1})	k_2 (min^{-1})	pK_1	pK_2
2	50	1.0	0.06	7.67	9.28
3	30	7.7	1.06	8.46	10.21
4	50	0.07	0.02	7.76	9.60

used are identical with those reported by Nestler and Garrett (1968), but the rapid increase in absorbancy in the 290–310-nm range described by these workers was not observed. In Figure 5B, similar scans are shown in which a small amount of F_3T, a possible hydrolytic product of F_3TDR, has been added. Spectral changes identical with those reported are obtained and the difference spectrum (inset) is identical with 5-CU. In Table III are listed apparent first-order rate constants for the hydrolyses of IpF_3TR and F_3TDR.

Discussion

An interesting property of 5-trifluoromethyluracils is the abnormal reactivity of the CF_3 group toward nucleophilic reagents. In a study of the hydrolysis of F_3TDR at 29.5°, Nestler and Garrett (1968) observed a rapid increase in absorbancy in the 290- to 310-nm region prior to formation of CUDR. This change was interpreted as evidence for the accumulation of 5-hydroxydifluoromethyl-UDR as a kinetic intermediate which was converted into CUDR in a slower step. The proposed mechanism is not consistent with the known stability of the trifluoromethyl group toward direct nucleophilic displacement and the data described in this report. Furthermore, in none of the conversions of F_3T derivatives described here were kinetic intermediates detectable by spectral scans of progressing reactions. We have obtained evidence which demonstrates that the initial rapid increase in absorbance observed in the hydrolysis of F_3TDR does not reflect the formation of an intermediate, but is a result of contamination by F_3T, probably resulting from hydrolysis of F_3TDR upon storage in slightly acidic media. From the reported data it is noted that the rate constant for the initial increase in absorbancy assigned to 5-hydroxydifluoromethyl-UDR was similar to that obtained for F_3T. In addition, construction of difference spectra before and after formation of this product gave a spectrum which was identical with 5-carboxyuracil. When

TABLE III: Rate Constants for the Hydrolysis of IpF_3TR and F_3TDR at 50°.

pH	k_{obsd} (min^{-1}) × 10^2		k_{IpF_3TR}/k_{F_3TDR}
	IpF_3TR	F_3TDR[a]	
10.4	0.47	0.54	0.87
11.2	0.84	0.56	1.51
12.1	2.3	0.70	3.29
13.1	10.1	2.87	4.65

[a] Values for k_{obsd} are calculated from the data of Nestler and Garrett (1968).

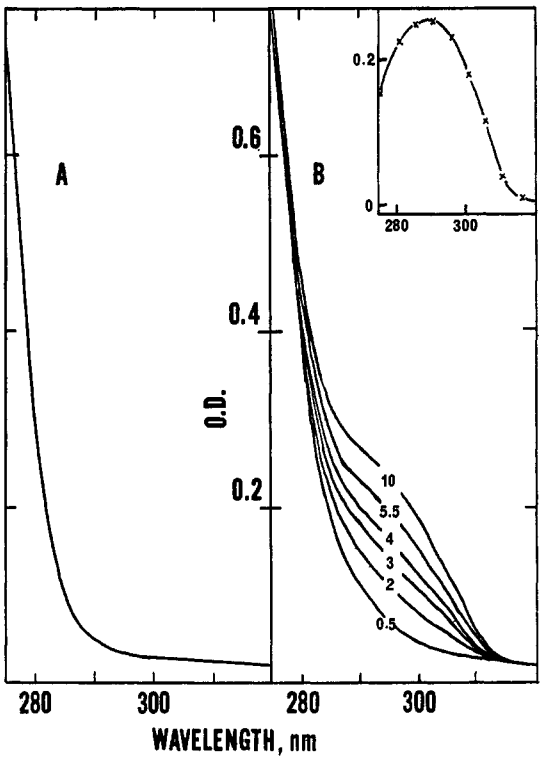


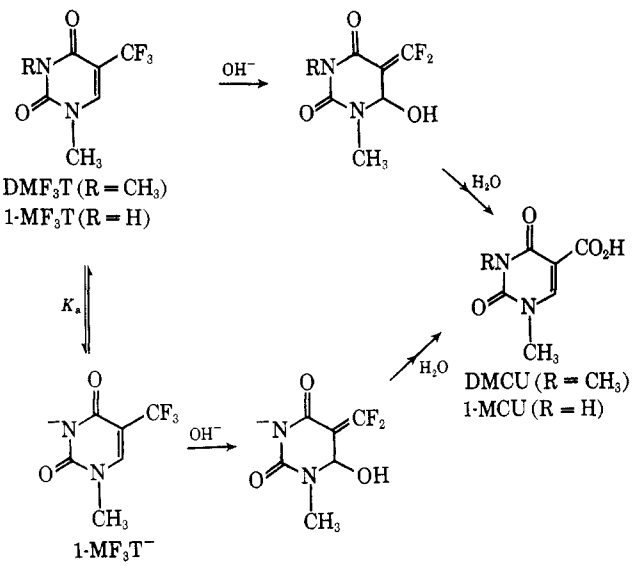
FIGURE 5: (A) Spectrum of 3.4×10^{-4} M F_3TDR in 0.126 N NaOH at 30°. Scans were taken up to 10 min. (B) Same solution with F_3T added to 1.85×10^{-5} M. Scans are labeled as to the number of minutes after the addition of F_3T. The inset shows the difference spectrum of the product formed in part B.

a chromatographically pure sample of F_3TDR was treated under identical conditions as those reported, we observed no indication of a kinetic intermediate. However, by introduction of a small amount of F_3T, spectral changes identical with those assigned to 5-hydroxydifluoromethyl-UDR were obtained (Figure 5).

Hydrolytic Reactions of F_3T and Its Derivatives. The hydrolysis of DMF_3T to the corresponding carboxylic acid between pH 9.0 and 13 is first order in hydroxide ion and serves as a model for reactions of the un-ionized heterocycle. The chemical unlikelihood of direct displacement of fluoride ion by hydroxide suggests that the primary site of reaction is not the trifluoromethyl group, but rather the pyrimidine ring; as will be discussed later, direct evidence for this is provided by the 1-(ω-aminoalkyl)-5-trifluoromethyluracils. Reactions of the 6 position of pyrimidine-2,4-diones with nucleophilic reagents are well documented, and in many cases have been implicated in assisting reactions at the 5 position of the heterocycle (Santi and Brewer, 1970; Garrett and Yakatan, 1968). It is reasonable to expect that the high electronegativity of the trifluoromethyl group will result in an even greater polarization of the 5,6-double bond and susceptibility of the 6 position toward nucleophilic attack. The most likely mechanism for the hydrolysis of DMF_3T (Scheme II, R = CH_3) involves hydroxide attack at the 6-carbon of the heterocycle with concomitant expulsion of fluoride ion to give a reactive exocyclic difluoromethylene intermediate which rapidly reacts with solvent.

At $a_H \gg K_a$, the conversion of $1\text{-}MF_3T$ proceeds by hydroxide ion attack on the neutral species or the kinetically equivalent spontaneous reaction of the N-3 monoanion with water. If the former pathway is the case, the bimolecular rate

SCHEME II

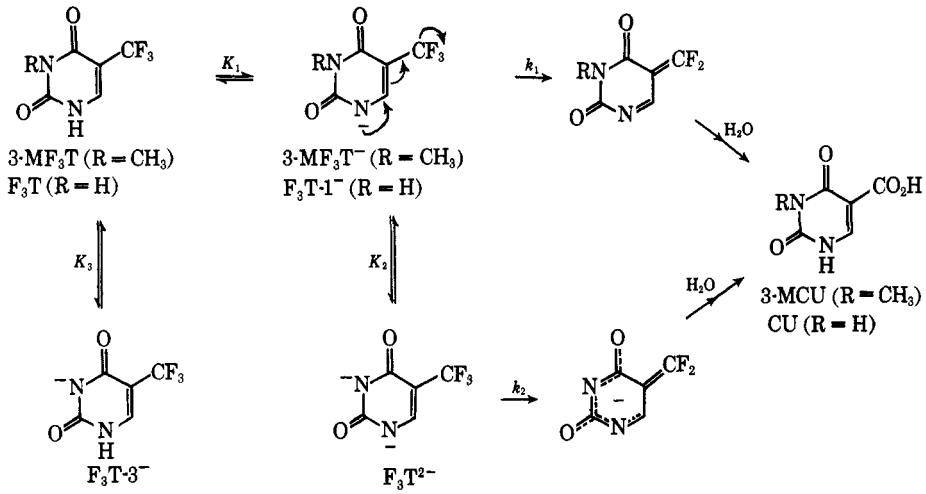


constant is expected to be of similar magnitude as that observed for DMF_3T. The calculated second-order rate constant for the reaction of $1\text{-}MF_3T$ (eq 2) with hydroxide ion at 50° is 79.5 M^{-1} min^{-1}, and compares very favorably with the value of 83.4 M^{-1} min^{-1} obtained for hydrolysis of DMF_3T at 50°. In the region $a_H \ll K_a$, the data are in accord with either hydroxide ion reaction with the anionic species or second-order hydroxide ion reaction with the neutral species. The latter possibility may be excluded on the basis that DMF_3T, a model for the neutral species of $1\text{-}MF_3T$, does not exhibit the reaction at high pH; analogous intramolecular reactions are in support of this conclusion (*vide supra*) and demonstrate the initial site of reaction to be the 6 position of the heterocycle. A mechanism which is consistent with the hydrolytic data and the above considerations is given in Scheme II (R = H). It is of interest to note that hydroxide attack on the neutral species (k_{OH} = 79.5 M^{-1} min^{-1}) is some 1300 times faster than on the monoanion ($k_{OH} = 6.6 \times 10^{-2}$ M^{-1} min^{-1}). Although no attempt was made to differentiate kinetically equivalent mechanisms in the hydrolysis of F_3TDR (Nestler and Garrett, 1968), the reaction exhibits a similar pH dependence as that described for $1\text{-}MF_3T$ and probably proceeds by an identical mechanism.

A similar rationalization may be used to choose between the two kinetically equivalent schemes which are in accord with the hydrolytic data for $3\text{-}MF_3T$. If the reaction involves hydroxide attack on the neutral heterocycle, the reaction rates are expected to be of the same magnitude as observed for DMF_3T and $1\text{-}MF_3T$. With this assumption, eq 2 was used to calculate a bimolecular rate constant of 5.36×10^3 M^{-1} min^{-1}, which is some 500 times greater than would be expected. This suggests that the reaction proceeds by an alternate pathway (Scheme III, R = CH_3) in which the monoanion ($3\text{-}MF_3T^-$) provides anchimeric assistance for the formation of the reactive difluoromethylene intermediate leading to 3-MCU.

Interpretation of the hydrolytic data for F_3T is complicated by the existence of four possible reactive spcies and numerous kinetically equivalent pathways (Scheme III, R = H); in addition, the microscopic equilibrium constants relating the concentrations of each species cannot be ascertained by direct measurement. However, by utilizing the methylated deriva-

SCHEME III



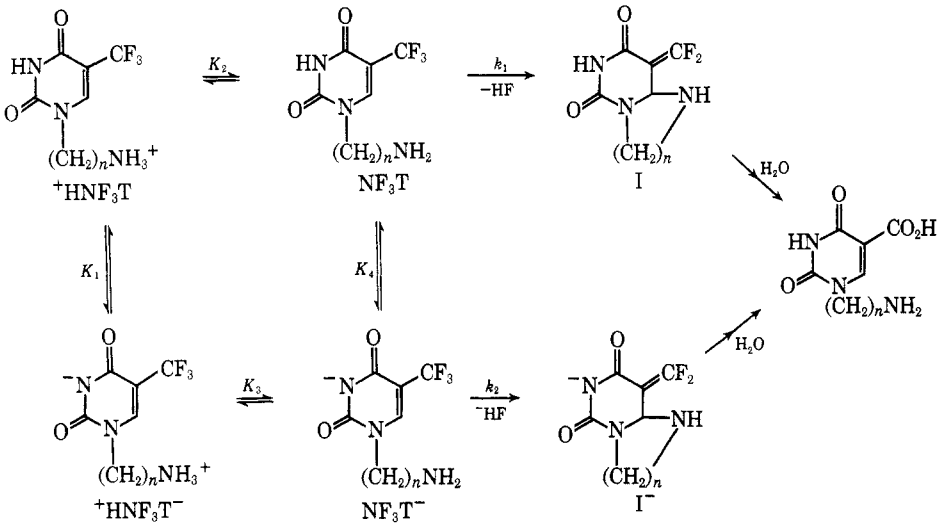
tives of F_3T described above, conclusions may be reached which permit assignment of the pertinent routes of reaction. The 1- and 3-NH of 2,4-pyrimidinediones often have similar dissociation constants so that significant amounts of both monoanions exist in solution (Nakanishi *et al.*, 1961; Shapiro and Kang, 1969; Santi and Brewer, 1970). Taking the dissociation constants of 1-MF_3T and 3-MF_3T as approximations of K_1 and K_3, it may be calculated that the ratio of F_3T-1$^-$ and F_3T-3$^-$ present in solution at any pH is 5.5 to 1. Using these values and the equation (Tucker and Irvin, 1951) $K_{F_3T} = K_{3-MF_3T} + K_{1-MF_3T}$, a calculated p$K_a$ of 7.53 is obtained for the first ionization of F_3T which is in excellent agreement with the measured value of 7.6 and provides verification for the assumption that substitution of a methyl group for hydrogen has only minor electronic effects on the heterocycle.

At pH values below the first apparent dissociation constant, the concentration of the dianion F_3T^{2-} is negligible and the possible pathways which are in accord with the kinetic data are (a) reaction of the neutral species with hydroxide or (b) reaction of either, or both, of the monoanions (F_3T-1$^-$, F_3T-3$^-$) with water. Using the hydrolysis of DMF_3T and 1-MF_3T as models, the bimolecular rate constant (k_{OH}) for the reaction of the neutral species is expected to be about 80 M^{-1} min^{-1}. With this value, calculated first-order rate constants are found to be 250-fold lower than that actually observed; on this basis it may be concluded that reaction of the neutral species with hydroxide is not a significant pathway in the overall reaction. Similarly, the reaction of F_3T-3$^-$ with water may be eliminated at the outset since 1-MF_3T^- undergoes no analogous reaction. Thus, it would appear that the most likely pathway in this pH range is the reaction of F_3T-1$^-$ with water, to give a reactive difluoromethylene intermediate as described for 3-F_3T-1$^-$ (Scheme III). The first kinetic pK_a derived from Figure 3 is 0.4 unit higher than the measured value for the first dissociation of F_3T. From the above arguments it is apparent that this is likely a result of the low reactivity of F_3T-3$^-$ and its presence in a nonproductive equilibrium. If this is indeed the case, compensation for this equilibrium should correct the discrepancy of the apparent pK_a value and the specific rate constants of 3-MF_3T^- and F_3T-1$^-$ would be expected to be similar. Using the values for K_1 and K_3 described above, a good fit of eq 5 to the hydrolytic data is obtained and the specific rate constant (k_1) for the hydrolysis of F_3T-1$^-$ is close to that of 3-MF_3T^-. It is noted that, in analogy to the hydrolysis of 6-trichloromethylpurine (Cohen and Dinar, 1965), Dipple and Heidelberger (1966) have suggested that basic hydrolysis of F_3T proceeds *via* the 1-anion.

At higher pH ($a_H \ll K_1$), the hydrolytic data are in accord with hydroxide ion reaction with either of the two monoanions or spontaneous elimination of fluoride from the dianion (F_3T^{2-}). Reactions of hydroxide ion with F_3T-1$^-$ need not be considered since a similar reaction is not observed with the methyl analog, 3-MF_3T^-. Although the reaction of hydroxide ion with F_3T-3$^-$ is analogous to the observed reaction with 1-MF_3T^-, the bimolecular rate constant for hydrolysis of the latter is 250-fold less than the calculated value for F_3T-3$^-$. On this basis, the most probable pathway for hydrolysis of F_3T at high pH is *via* spontaneous reaction of the dianion, F_3T^{2-} (Scheme III). The higher reactivity of F_3T^{2-} (k = 0.22 min^{-1}) as compared to F_3T-1$^-$ ($k = 1.3 \times 10^{-3}$ min^{-1}) may be rationalized in terms of the higher electron density available for assistance which would reside at the 1 position as a result of ionization of the 3-NH.

The aforementioned studies strongly suggest that acylation reactions of 1-substituted 5-trifluoromethyluracils proceed by rate-determining attack of a nucleophile at the 6 position of the heterocycle to give reactive 5-difluoromethylene intermediates. In order to verify this mechanism we sought to design suitable intramolecular models in which a sterically disposed nucleophile on the 1-substituent could participate in the reaction only by nucleophilic addition to the 6 position of the heterocycle to form cyclic intermediates. In previous studies (Santi and Brewer, 1968), we have demonstrated that base-catalyzed exchange of the 5 proton of certain uracil furanosides proceeds by intramolecular nucelophilic attack of the 5′-oxyanion at the 6 position of the heterocycle to give 5′,6-*O*-cyclonucleoside intermediates. Reaction rates were shown to be a function of the structure of the sugar moiety, and 5-H exchange of 2′,3′-*O*-isopropylideneuridine proceeded some 66-times faster than 2′-deoxyuridine. With this precedent, it was hoped that a similar rate enhancement would be observed with IpF_3TR as compared to F_3TDR. The apparent first-order rate constants at pH $\simeq$ 10.5 were quite similar (Table III), but at higher pH values where more of the 5′-hydroxyl is ionized [p$K_a \simeq$ 13.9 at 30° (D. V. Santi and C. F. Brewer, unpublished results)] a 5.5-fold rate difference was observed. Although limitations of the amount of sample available did not permit complete studies, the data may be

SCHEME IV



taken as marginal evidence for participation of the 5′-oxyanion in the hydrolysis of IpF_3TR. The small effect is attributable to hydroxide ion competition at the high pH necessary to obtain significant ionization of the 5′-hydroxyl group; furthermore, the heterocycle is largely present as the 3-anion which, on the basis of the results obtained with 1-MF_3T, is anticipated to be approximately 1000-fold less reactive toward negatively charged nucleophiles than the neutral species.

With the 1-(ω-aminoalkyl)F_3T derivatives, many of these difficulties are circumvented since the participating group is sufficiently nucleophilic under conditions where the heterocycle is present largely as the neutral species and where bimolecular reaction with hydroxide is minimal. The pH–log k_{obsd} profile of 1-(5-aminopentyl)F_3T is virtually superimposable on that of 1-MF_3T and implies identical solvolytic mechanisms. This is not surprising since intramolecular nucleophilic attack of the amino group at the 6 position of the heterocycle would require the unfavorable formation of an eight-membered ring. In contrast, neighboring group participation is evident in the solvolysis of NF_3T, $n = 3$, which, at pH > pK_{app}, is hydrolyzed some 10,000 times faster than is 1-MF_3T; bimolecular reaction with hydroxide ion is not significant throughout the entire pH range. The aminoethyl and aminobutyl derivatives of F_3T behave similarly, albeit with lower apparent rate enhancements. The relative rate enhancements observed are a function of the ease of formation of the five-, six- and seven-membered cyclic intermediates (*i.e.*, six-membered ring > five > seven). The observation that the aminopropyl derivative hydrolyzes some 134 times faster than the aminoethyl-F_3T is at first somewhat surprising since formation of five- and six-membered rings are usually comparably facile. However, in this case considerable restrictions are imposed by the coplanarity of the C-6, N-1, and adjacent methylene carbons of the alkyl chain. Inspection of molecular models shows that in entering the transition state, the necessary overlapping of the unshared electrons of the nitrogen of NF_3T, $n = 2$, with the p orbital at C-6 may only occur with considerable strain and resulting increase in the energy of activation.

The most likely mechanism for the hydrolysis of NF_3T, $n = 2,3,4$, which is in accord with the kinetic data is shown in Scheme IV. The rate-determining step probably involves attack of the free ω-amino group at the 6 position of the neutral or anionic heterocycle with concomitant expulsion of fluoride ion to produce the highly reactive difluoromethylene intermediates, I and I^-. Alternatively, the difluoromethylene intermediates may exist in steady-state concentrations in a preequilibrium prior to the rate-determining step; available data do not permit these two possibilities to be distinguished.

Although the exact pathways leading from the difluoromethylene intermediates proposed for all of the foregoing reactions are not known, they probably involve a sequence of rapid addition–elimination reactions, perhaps with the intermediacy of 5-hydroxydifluoromethylpyrimidines and acyl fluorides as suggested for F_3TDR (Nestler and Garrett, 1968). Alternatively, enolate intermediates having negative charge delocalized over the 5-carbon and 4-oxygen atoms may be involved, in which case the participating nucleophile at the 6 position of the heterocycle need not be eliminated until the hydrolysis of the CF_3 group is complete.

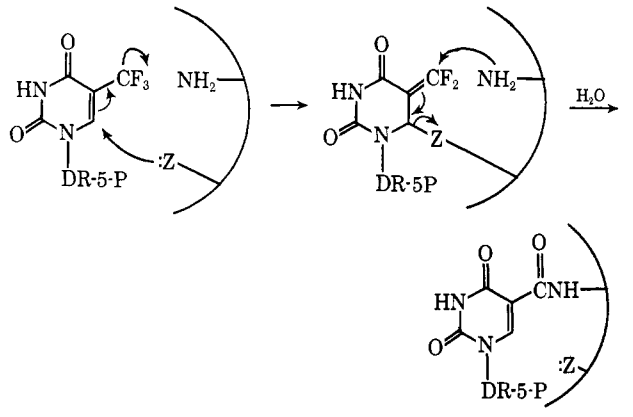
From the foregoing discussion of bimolecular reactions of 1-substituted derivatives of F_3T, it is apparent that the neutral heterocycle is inherently more susceptible to nucleophilic attack than the anionic species. It is interesting that intramolecular nucleophilic attack of an amino group proceeds with similar facility on both neutral and anionic heterocycles, whereas the negatively charged heterocycle of 1-substituted F_3T derivatives is some 10^3-fold less reactive to attack by hydroxide ion; likewise, the ionized heterocycle does not appear to be any more susceptible to intramolecular attack of the 5′-oxyanion of IpF_3TR. Using arguments similar to those of Holmquist and Bruice (1969), these observations are explicable in terms of electrostatic effects on the transition state of reactions involving a negatively charged heterocycle. With the nitrogen nucleophiles, the transition state should involve significant bond formation, and the developing positive charge on the nucleophilic nitrogen atom could provide stabilization by electrostatic interaction with the delocalized negative charge of the heterocycle. On the other hand, with highly basic nucleophiles such as hydroxide and oxyanions, the transition state will be reached before much bond formation has occurred and interactions with the heterocycle will be much less than in the above case; in fact, electrostatic destabilization of the transition state might result from interactions of the negatively charged heterocycle and the anionic nucleophiles.

Relationship between Model Reactions and Inhibition of

Thymidylate Synthetase by F_3TDRP. The salient feature of the above studies is that the trifluoromethyl group of F_3T derivatives may only behave as an acylating agent when a secondary driving force is furnished by reactions which occur at other parts of the heterocycle. If the 1 position is unsubstituted, the necessary assistance may be provided by the N-1 anion, whereas with 1-substituted derivatives of F_3T, it is necessary that a nucleophile is added to the 6 position of the heterocycle; in this manner, the normally inert trifluoromethyl group is converted into a highly reactive exocyclic difluoromethylene intermediate which rapidly reacts with nucleophilic reagents. It is probably relevant that chemical reactions of 1-substituted uracils and thyminyl derivatives (Santi and Brewer, 1968, 1970; Santi and Pogolotti, 1968, 1971) which may serve as possible models of the thymidylate synthetase reaction proceed by pathways analogous to those described for the hydrolysis of F_3T derivatives.

In accord with previous proposals (Santi and Brewer, 1968) for the possible involvement of nucleophilic catalysis in the enzymic reaction, the studies described herein lead us to propose a related minimal mechanism for the irreversible inactivation of thymidylate synthetase by F_3TDRP. In the pathway depicted in Scheme V, it is suggested that juxtaposed

SCHEME V



within the active site, a nucleophilic group of the enzyme (:Z) adds to the 6 position of F_3TDRP, promoting the expulsion of fluoride ion and the formation of a reactive exocyclic difluoromethylene intermediate similar to those encountered in our model studies. The reactive intermediate would then be trapped by a proximate nucleophilic group of the enzyme (perhaps an ϵ-amino group of lysine) to give, after a number of steps, the acylated enzyme. Alternatively, one can envisage an enzyme-catalyzed addition of hydroxide to the 6 position of F_3TDRP to form a similar reactive intermediate. In this case, the general base involved in the initial addition reaction could, after loss of a proton, be the nucleophile which is acylated by F_3TDRP.

References

Barone, J. A. (1963), *J. Med. Chem. 6*, 39.

Browne, D. T. (1968), *in* Synthetic Procedures in Nucleic Acid Chemistry, Vol. 1, Zorbach, W. W., and Tipson, R. S., Ed., New York, N. Y., Interscience, pp 96–98.

Bruice, T. C., and Maley, J. R. (1970), *Anal. Biochem. 34*, 275.

Cohen, S., and Dinar, N. (1965), *J. Amer. Chem. Soc. 87*, 3195.

Dipple, A., and Heidelberger, C. (1966), *J. Med. Chem. 9*, 715.

Fromageot, H. P. M., Griffin, B. E., Reese, C. B., and Sulston, J. E. (1967), *Tetrahedron 23*, 2315.

Garrett, E. R., and Yakatan, G. J. (1968), *J. Pharm. Sci. 57*, 1478.

Giner-Sorolla, A., and Bendich, A. (1958), *J. Amer. Chem. Soc. 80*, 5744.

Heidelberger, C., Boohar, J., and Kampschroer, D. (1965), *Cancer Res. 25*, 377.

Heidelberger, C., Parsons, D. G., and Remy, D. C. (1964), *J. Med. Chem. 7*, 1.

Holmquist, B., and Bruice, T. C. (1969), *J. Amer. Chem. Soc. 91*, 2985.

Isono, K., and Suzuki, S. (1970), *Tetrahedron Lett.*, 425.

Jones, R. J. (1947), *J. Amer. Chem. Soc. 69*, 2347.

Khwaja, T. A., and Heidelberger, C. (1969), *J. Med. Chem. 12*, 543.

Mertes, M. P., Saheb, S. E., and Miller, D. (1966), *J. Med. Chem. 9*, 876.

Nakanishi, K., Suzuki, N., and Yamazaki, F. (1961), *Bull. Chem. Soc. Jap. 34*, 53.

Nestler, H. J., and Garrett, E. R. (1968), *J. Pharm. Sci. 53*, 1117.

Pauling, L. (1960), The Nature of the Chemical Bond, Ithaca, N. Y., Cornell University Press, p 85.

Reyes, P., and Heidelberger, C. (1965), *J. Mol. Pharmacol. 1*, 14.

Ryan, K. J., Acton, E. M., and Goodman, L. (1966), *J. Org. Chem. 31*, 1181.

Sakai, T. T., Pogolotti, A. L., Jr., and Santi, D. V. (1968), *J. Heterocycl. Chem. 5*, 849.

Santi, D. V., and Brewer, C. F. (1968), *J. Amer. Chem. Soc. 90*, 6236.

Santi, D. V., and Brewer, C. F. (1970), *J. Heterocycl. Chem. 7*, 903.

Santi, D. V., and Pogolotti, A. L., Jr. (1968), *Tetrahedron Lett.*, 6159.

Santi, D. V., and Pogolotti, A. L., Jr. (1971), *J. Heterocycl. Chem. 8*, 265.

Shapiro, R., and Kang, S. (1969), *Biochemistry 8*, 1806.

Shen, T. Y., Ruyle, W. V., and Lewis, H. M. (1965), *J. Org. Chem. 30*, 835.

Shugar, D., and Fox, J. J. (1952), *Biochim. Biophys. Acta 9*, 199.

Tucker, G. F., Jr., and Irvin, J. L. (1951), *J. Amer. Chem. Soc. 73*, 1923.

Whitehead, C. W. (1952), *J. Amer. Chem. Soc. 74*, 4267.