# Synthesis of a Pyrimidine Analog of Tetrahydrohomofolic Acid[1]

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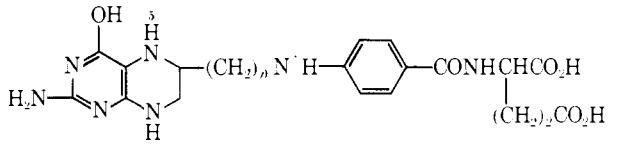
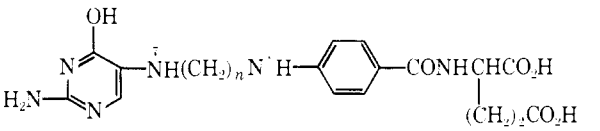
The synthesis of N-[*p*-(N-{3-[N-(2-amino-4-hydroxy-5-pyrimidinyl)amino]propyl}amino)benzoyl]-L-glutamic acid (IV), an analog of tetrahydrohomofolic acid, is described. Reductive alkylation was used to join together the properly protected isocytosine, malonaldehyde, and *p*-aminobenzoic acid fragments to give a product which, in the form of the triformamidobenzoic acid XII, was coupled with diethyl L-glutamate using N,N′-carbonyldiimidazole. Hydrolysis in concentrated HCl afforded IV. Qualitatively, IV resembles tetrahydrohomofolic acid in its inhibition of thymidylate synthetase, but not of dihydrofolate reductase.

Tetrahydrofolic acid ($fH_4$, I) serves as a one-carbon transfer agent in many biological systems. Five-membered cyclic compounds involving the $N^5$ and $N^{10}$ atoms of $fH_4$ and the one-carbon fragment are intermediates in the one-carbon transfer at both the formyl and hydroxymethyl oxidation level.[2]

Recently, homofolic acid has been synthesized.[3] The tetrahydro derivative (II) of homofolic acid was found



I, $n = 1$; $N^* = N^{10}$
II, $n = 2$; $N^* = N^{10}$

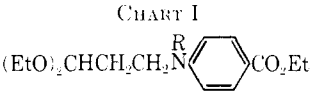


III, $n = 2$; $N^* = N^{10}$
IV, $n = 3$; $N^* = N^{10}$

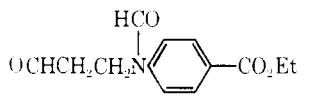
to be a quite specific inhibitor of the enzyme thymidylate synthetase.[3b] This inhibition may be a result of inserting the additional carbon atom between $N^5$ and $N^*$, thereby altering the geometry of the one-carbon transfer intermediates. Compound III, a pyrimidine analog of $fH_4$ was also synthesized recently and found to have some biological activity.[4] Chemical experiments demonstrated that the $N^7$- or $N^{10}$-formyl derivative of III can form a five-membered cyclic methenyl compound (XIV). As a continuation of our folic acid antagonist studies, it was interesting to prepare IV, which is related to III as tetrahydrohomofolic acid (II) is related to $fH_4$ (I). This paper reports the synthesis of N-[*p*-(N-{3-[N-(2-amino-4-hydroxy-5-pyrimidinyl)-amino]propyl}amino)benzoyl]-L-glutamic acid (IV) and some of its properties.

The general scheme of synthesis of IV resembles that of III[4] in that mild reductive alkylation conditions were used: first, to combine the malonaldehyde and *p*-aminobenzoic acid fragments, and then to join that product to 2-acetamido-5-amino-4-hydroxypyrimidine[4] to give the homopteroic acid analog VIII. In the case of III it had been possible to employ a *p*-aminobenzoyl-L-glutamic acid fragment. When rigorously purified 3,3-diethoxypropionaldehyde[5] was used in the first step of the synthesis to give V, it was easy to obtain pure formamide (VI), convert it to the aldehyde (VII),[6] and then to VIII without purification of the intermediates (see Chart I).

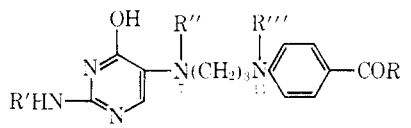
CHART I



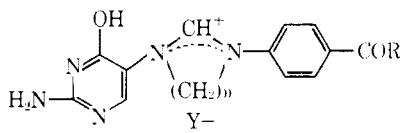
V, R = H
VI, R = CHO



VII



VIII, R = OEt; R′ = Ac; R″ = H; R‴ = CHO
IX, R = OH; R′ = H; R″ = H; R‴ = H
X, R = OEt; R′ = Ac; R″ = CHO; R‴ = CHO
XI, R = OH; R′ = H; R″ = CHO; R‴ = H
XII, R = OH; R′ = CHO; R″ = CHO; R‴ = CHO
XIII, R = Q; R′ = CHO; R″ = CHO; R‴ = CHO



XIV, $n = 2$; R = S
XV, $n = 3$; R = S
XVI, $n = 3$; R = OH

Q = NHCH(CO$_2$Et)(CH$_2$)$_2$CO$_2$Et
S = NHCH(CO$_2$H)(CH$_2$)$_2$CO$_2$H

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(2) For a recent review of folic acid metabolism see M. Friedkin in *Ann. Rev. Biochem.*, **32**, 185 (1963).

(3) (a) J. I. DeGraw, J. P. Marsh, Jr., E. M. Acton, O. P. Crews, Jr., C. W. Mosher, A. N. Fujiwara, and L. Goodman, *J. Org. Chem.*, **30**, 3404 (1965); (b) L. Goodman, *et al.*, *J. Am. Chem. Soc.*, **86**, 308 (1964).

(4) G. L. Tong, W. W. Lee, and L. Goodman, *ibid.*, **86**, 5664 (1964).

(5) L. A. Yanovskaya and V. F. Kucherov, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, 667 (1962); *Chem. Abstr.*, **57**, 16378 (1962).

(6) The formamidopropionaldehyde VII and its glutamate homolog (diethyl N-{*p*-[N-(2-formylethyl)formamido]benozyl}-L-glutamate; not fully characterized) were not as labile as the corresponding formamidoacetaldehyde (see footnote 7 in ref 4).

The diamido ester VIII was hydrolyzed by heating at 65–70° in 12 *N* HCl to afford the amino acid IX as the trihydrochloride, analytically pure without recrystallization. Hydrolysis of VIII with 0.5 *N* NaOH also afforded IX, but with some $N^7$-formyl acid XI as by-product. The migration of the formyl group from $N^{11}$ of VIII to $N^7$ of XI during treatment with base was similar to that observed for the corresponding formyl derivatives of tetrahydrofolic acid[7] and III.[4] When VIII was first converted to the triamido ester X and then hydrolyzed with 0.5 *N* NaOH, the $N^7$-formyl acid XI was the principal product. Either the acid IX or the mixture of IX and XI were satisfactory for the next step in which all the amino groups were formylated by treatment with formic acetic anhydride in pyridine to give XII.

The best yields in coupling the triformamido acid XII to diethyl L-glutamate were obtained when XII was heated at 45–50° for 1 hr with 1 equiv of N,N′-carbonyldiimidazole[8] in N,N-dimethylformamide followed by reaction with 1.2 equiv of diethyl glutamate at room temperature for 20 hr. The reaction of the acid XII with N,N′-carbonyldiimidazole was evidently slow because the use of either shorter reaction time or lower reaction temperature diminished the yield of XIII. Other coupling methods using the acid chloride, or the mixed anhydride as described by Erlanger and colleagues,[9] gave very poor yields of XIII. Hydrolysis of the amido ester XIII in concentrated HCl at 37° for 1.5 hr[10] gave the desired acid IV in reasonable yield.

No evidence for the formation of the cyclic methenyl derivative XV was observed in the 12 *N* acid hydrolysis of XIII to IV, contrary to the case of the lower homolog III.[4] Another set of experiments also showed that IV, unlike III, did not readily form a stable cyclic methenyl derivative. Each of the compounds, III, IV, and IX, was reformylated in formic acid at 100° and the ultraviolet spectrum of each product in 0.1 *N* HCl was examined at intervals. The spectrum of the product from III indicated that the cyclic methenyl derivative XIV had formed immediately. On the other hand, the spectra of the products from IV and IX indicated the absence of cyclic methenyl derivatives (XV and XVI) and supported the presence of normal N-formyl groups. The spectra of all three products (from III, IV, and IX) did not change much over an interval of 1 day. These results indicated that the formation of a six-membered methenyl ring is not as favorable as formation of a five-membered one. However, a cyclic intermediate may have been involved in the transfer of the $N^{11}$-formyl group from VIII to the $N^7$ position in XI during base hydrolysis.

The biological activity of IV has been determined in several microbiological and enzyme systems.[11] The results are given in Tables I and II, together with previous data for II[3b] and III[4] for comparison. Thus, IV was a poorer inhibitor than II and III against *Streptococcus faecalis*, *Lactobacillus casei*, and *Pediococcus cerevisiae*. Compounds III and IV were about equal in their ability to inhibit thymidylate synthetase; however, only III was an inhibitor of dihydrofolate reductase. Qualitatively, IV resembled tetrahydrohomofolate (II) in its inhibition of thymidylate synthetase, but not of dihydrofolate reductase.[3b]

TABLE I

INHIBITION OF MICROBIAL GROWTH[a]

| Compd | 0.5 max inhib concn, mμg/ml: S. faecalis[b] | L. casei[b] | P. cerevisiae[c] |
|---|---|---|---|
| II[d] | 0.7 | 6 | >1,000 |
| III | 0.5 | 20 | 1,500 |
| IV | 4.5 | 40 | >10,000 |

[a] Assay procedures are those used in ref 3b. [b] Folate, 1.0 mμg/ml. [c] Calcium *dl*-L-5-formyltetrahydrofolate, 1 mμg/ml. [d] Data from ref 3b.

TABLE II

INHIBITION OF ENZYME PREPARATIONS[a]

| Compd | 0.5 max inhib concn, *M*: Dihydrofolate reductase[b] | Thymidylate synthetase[c] |
|---|---|---|
| II[d] | ... | $2 \times 10^{-6}$ |
| III | $3.6 \times 10^{-5}$ | $3 \times 10^{-5}$ |
| IV | No inhib at $10^{-3}$ | $\sim 2 \times 10^{-5}$ |

[a] For the assay procedures, see ref 3b. [b] From mouse leukemia cells; dihydrofolate, $3 \times 10^{-5}$ *M*. [c] From *E. coli*; *dl*-L-tetrahydrofolate, *ca.* $3 \times 10^{-4}$ *M*. [d] From ref 3b.

## Experimental Section[12]

**3,3-Diethoxypropionaldehyde** has been prepared from 1,1-diethoxy-3-butene by ozonolysis[13] and from 1,1-diethoxy-3,4-dihydroxybutane by oxidation with lead tetracetate[5] according to the procedure described for the preparation of 2,2-diethoxyacetaldehyde.[14] We tried several modifications of the oxidation of 1,1-diethoxy-3,4-dihydroxybutane, including the use of periodate; all of these gave product containing some 3-ethoxyacrolein which was detected by its absorption at $\lambda_{max}^{EtOH}$ 242.5 mμ (ε 13,900).[5] The purest 1,1-diethoxypropionaldehyde was obtained by a slight modification of the literature method.[14] After the reaction, the mixture was filtered, and the filtrate was concentrated at 35° (20 mm) to one-fourth volume, diluted with 5 vol of ether, filtered, neutralized with $K_2CO_3$ solution, and distilled to afford a 58% yield of 1,1-diethoxypropionaldehyde, bp 74–75° (19–20 mm), containing only 1% 3-ethoxyacrolein.

**Ethyl *p*-(3,3-Diethoxypropylamino)benzoate (V).**—Reductive alkylation of 0.495 g (3.0 mmoles) of ethyl *p*-aminobenzoate and 0.46 g (3.15 mmoles) of 3,3-diethoxypropionaldehyde by the literature method for the preparation of ethyl *p*-(2,2-diethoxyethylamino)benzoate[4,15] afforded a crystalline residue after work-up. Recrystallization from 7 ml of hot methanol and 4 ml of water afforded 0.38 g (42%) of V as yellow crystals, mp 65–67°, and a second crop, mp 56–66° (total, 57%), suitable for use in the next step. Two recrystallizations from aqueous methanol

(7) J. C. Rabinowitz, *Enzymes*, **2**, 185 (1960).

(8) R. Paul and G. W. Anderson, *J. Am. Chem. Soc.*, **82**, 4596 (1960).

(9) B. F. Erlanger, W. V. Curran, and N. Kokowsky, *ibid.*, **81**, 3051 (1959).

(10) R. B. Merrifield and D. W. Woolley, *ibid.*, **78**, 4646 (1956).

(11) We express our appreciation to Drs. R. L. Kisliuk, and M. L. Friedkin, Department of Pharmacology, Tufts University School of Medicine, who obtained the microbiological and enzymatic data metioned here.

(12) Melting points were determined with the Fisher-Johns apparatus and were corrected. Paper chromatography was done by the descending technique on Whatman No. 1 paper, except solvent A, which was done on Schleicher and Schuell No. 2496 acetylated paper. The spots were detected by visual examination under ultraviolet light. Adenine was used as a standard; the spots were located relative to $R_{Ad}$ 1.00. The solvent systems were: (A) benzene–water–methanol (2:1:6), (B) water, (C) 1-butanol–acetic acid–water (5:2:3), (D) benzene–water–methanol (2:1:6), (E) 5% aqueous disodium hydrogen phosphate pH 8.9, (F) 1-butanol–water (saturated). Anhydrous $MgSO_4$ was used as the drying agent.

(13) S. Hattori, *Yuki Gosei Kagaku Kyokai Shi*, **19**, 453 (1961); *Chem. Abstr.*, **55**, 20926 (1961).

(14) H. O. L. Fischer and E. Baer, *Helv. Chim. Acta*, **18**, 514 (1935).

(15) We performed similar reductive alkylation experiments with dimethyl *p*-aminobenzoylglutamate, using 3,3-diethoxypropionaldehyde from early batches, before we appreciated the adverse effect of the impurities in 3,3-diethoxypropionaldehyde. Thus we obtained the glutamate analogs of V, VI, and VII. The last compound exhibited an ultraviolet spectrum with unexpected by-product absorption at 310 mμ. Since the glutamate analogs were all syrups it was necessary to turn to the benzoate series where V was a crystalline solid that could be purified.

afforded the analytical sample of V: mp 68–68.5°; $\lambda_{max}^{Nujol}$ ($\mu$) 2.95 (NH), 5.92 and 7.79 (ester), 8.9 and 9.45 (COC); $\lambda_{max}^{EtOH}$ 225 m$\mu$ ($\epsilon$ 8050), 307 (27,000); it moved as a single spot in solvent A with $R_{Ad}$ 0.74.

*Anal.* Calcd for $C_{16}H_{23}NO_4$: C, 65.1; H, 8.54; N, 4.74. Found: C, 65.0; H, 8.52; N, 4.82.

**Ethyl *p*-[N-(3,3-Diethoxypropyl)formamido]benzoate (VI).**—A solution of 9.14 g (31 mmoles) of V in dry pyridine was treated with a solution of formic acid anhydride according to the literature procedure for the preparation of dimethyl N-[*p*-[N-(2,2-diethoxyethyl)formamido]benzoyl]-L-glutamate[3] to afford, after work-up, a residue of 10.1 g (100%) of VI as an orange-yellow oil; $\lambda_{max}^{film}$ ($\mu$) 5.80, 5.91 (ester and amide); $\lambda_{max}^{EtOH}$ 266 m$\mu$ ($\epsilon$ 16,200); it moved as a single spot in solvents A and B with $R_{Ad}$ 0.88 and 1.77, respectively. A portion of the oil was further dried at 56° (0.1 mm) for 4 hr for analysis.

*Anal.* Calcd for $C_{17}H_{25}NO_5$: C, 63.1; H, 7.79; N, 4.33. Found: C, 63.2; H, 7.75; N, 4.43.

**Ethyl-*p*-[N(2-Formylethyl)formamido]benzoate (VII).**—A solution of 9.65 g (29.9 mmoles) of VI in 30 ml of 90% formic acid was allowed to stand for 30 min at room temperature, then poured into 150 ml of ice water and extracted with two 300-ml portions of benzene. The benzene solution was washed once with 100 ml of saturated $NaHCO_3$ solution, twice with 200-ml portions of $H_2O$, dried, treated with Norit, filtered, and evaporated *in vacuo* to afford 7.44 g (100%) of VII as a yellow oil; $\lambda_{max}^{film}$ ($\mu$) 3.68 (CHO), 5.78–5.93 (C=O of ester, aldehyde, and amide); $\lambda_{max}^{EtOH}$ 266 m$\mu$ ($\epsilon$ 15,400); it moved as a single spot in solvents A and B with $R_{Ad}$ 1.02 and 1.27, respectively. A sample of oil was dried at 56° (0.1 mm) for 4 hr and analyzed.

*Anal.* Calcd for $C_{13}H_{15}NO_4$: C, 62.6; H, 6.07; N, 5.62. Found: C, 62.9; H, 6.16; N, 5.60.

Allowing the hydrolysis to proceed for 1 hr did not affect the quality of the product. Reaction of VII with 5,5-dimethyl-1,3-cyclohexanedione gave a 90% yield of the dimethon, mp 140.5–141.5°.

*Anal.* Calcd for $C_{29}H_{37}NO_7$: C, 68.1; H, 7.29; N, 2.74. Found: C, 67.8; H, 7.42; N, 2.58.

The 2,4-dinitrophenylhydrazone of VII was also prepared. It had mp 100–102°, resolidified at 107–145°, remelted at 145–146°.

*Anal.* Calcd for $C_{19}H_{19}N_5O_7$: C, 53.1; H, 4.46; N, 16.3. Found: C, 52.6; H, 4.40; N, 16.2.

**Ethyl *p*-(N-{3-[N-(2-Acetamido-4-hydroxy-5-pyrimidinyl)-amino]propyl}formamido)benzoate (VIII).**—The reductive alkylation of 0.69 g (4.1 mmoles) of 2-acetamido-5-amino-4-hydroxypyrimidine[4] and 1.081 g (4.3 mmoles) of VII in N,N-dimethylformamide with 0.346 g of 5% Pd–C was completed after 15 hr at room temperature and gave, after work-up using the same procedure as for V, a gum. This was recrystallized from 25 ml of hot ethyl acetate to afford 1.18 g (71%) of VIII as a yellow powder, mp 157–158.5°. Similar material from an earlier run was recrystallized from acetone to give the analytical sample of VIII: mp 160.5–161°; $\lambda_{max}^{Nujol}$ ($\mu$) 3.2 (NH), 5.84, 5.95, 6.10 (ester and amides); $\lambda_{max}^{EtOH}$ 271 m$\mu$ ($\epsilon$ 22,600), 310 shoulder (~9900); it moved as a single spot in solvents C and D with $R_{Ad}$ 1.42 and 1.62, respectively.

*Anal.* Calcd for $C_{19}H_{23}N_5O_5$: C, 56.9; H, 5.78; N, 17.5. Found: C, 57.3, 57.0; H, 5.87; 5.71; N, 17.4.

***p*-(N-{3-[N-(2-Amino-4-hydroxy-5-pyrimidinyl)amino]propyl}-amino)benzoic Acid (IX). A. Acid Hydrolysis.**—A solution of 2.05 g (5.1 mmoles) of the diamido ester VIII in 100 ml of HCl (12 *N*) was stirred and heated at 65–70° under nitrogen for 17 hr. The partially crystalline mixture was kept at 0° for 5 hr and then the precipitate was collected, washed with cold 12 *N* HCl and dried to afford 1.69 g (80%) of IX·3HCl as a white crystalline powder: mp 234–236° (yellowing at 226°); $\lambda_{max}^{Nujol}$ ($\mu$) 3.04, 3.22, 3.26 (OH, NH), 3.7–4.15 ($NH^+$, COOH), 5.81, 5.93 (COOH and pyrimidine ring); $\lambda_{max}^{pH\ 1}$ 226 m$\mu$ ($\epsilon$ 15,000), 268 (10,500), 302 (9200); $\lambda_{max}^{pH\ 13}$ 285 m$\mu$ ($\epsilon$ 21,400); it moved as a single spot in solvents E, C, and D with $R_{Ad}$ 1.4 (blue fluorescene), 1.24, and 1.19 (fluorescent ring around spot), respectively. The analytical sample was dried over $P_2O_5$ for 5 hr at 56° and <1 mm.

*Anal.* Calcd for $C_{14}H_{17}N_5O_3 \cdot 3HCl$: C, 40.7; H, 4.88; Cl, 25.8; N, 17.0. Found: C, 40.9; H, 5.08; Cl, 25.8; N, 16.8.

**B. Base Hydrolysis.**—A solution of 5.25 (13.1 mmoles) of VIII in 131 ml of 0.5 *N* NaOH was heated at 60–65° under nitrogen for 15 hr treated with charcoal, filtered, and diluted with 115 ml of water. The filtrate was cooled in an ice bath and acidified to pH 4.5 with 1 *N* HCl. After 3 hr, the precipitate was collected, washed successively with water and acetone, and dried *in vacuo* to afford 3.56 g of a mixture of IX (mostly) and XI, mp 231–232.5° dec, as indicated by ultraviolet analysis and paper chromatography in solvent E (which showed two spots: $R_{Ad}$ 1.22 (major spot, IX) and 1.86 (minor spot, XI). For analysis, a portion of the product mixture was dissolved in 0.2 *N* NaOH and reprecipitated at pH 4.5 to give the acid IX as an off-white powder, mp 246–248° (decomposed; browning begins at 200°); $\lambda_{max}^{Nujol}$ ($\mu$) 2.98, 3.02, 3.2 (NH, OH), 5.95 (COOH); it moved as one spot in solvents E, C, and D with $R_{Ad}$ 1.25, 1.37, and 1.05, respectively. The sample was dried at 56° (1 mm) for 15 hr over $P_2O_5$ for analysis.

*Anal.* Calcd for $C_{14}H_{17}N_5O_3 \cdot 0.33H_2O$: C, 54.4; H, 5.76; N, 22.6. Found: C, 54.3; H, 5.82; N, 22.6.

***p*-(N-{3-[N-(2-Amino-4-hydroxy-5-pyrimidinyl)formamido]-propyl}amino)benzoic Acid (XI).**—A solution of 0.40 g (1.0 mmole) of the diamido ester VIII in 8 ml of pyridine was formylated by the procedure used to prepare VI to afford 0.44 g (103%) of a solid yellow foam (that had the infrared, nmr, and ultraviolet [$\lambda_{max}^{EtOH}$ 266 m$\mu$ ($\epsilon$ 18,900) and 305 shoulder (~7600)] spectra compatible with structure X and was homogeneous in solvents C and D with $R_{Ad}$ 1.32 and 1.68, respectively. This foam could not be crystallized.

A solution of 0.35 g (0.82 mmole) of the triamidoester X in 10 ml of 0.5 *N* NaOH was treated by the procedure described for the preparation of IX to afford 0.160 g (59%) of the monoformamido acid XI, mp 206–210° (softening from 157°). Two recrystallizations from methanol–water (5:1) gave the analytical sample of XI: mp 157–176°, resolidified at 177°, remelted at 219–221°; $\lambda_{max}^{Nujol}$ ($\mu$) 3.0, 3.2 (NH), 3.6–4.2 ($CO_2H$), 6.02 (pyrimidine); $\lambda_{max}^{pH\ 1}$ 224 m$\mu$ ($\epsilon$ 18,400), 270 (11,900), 302 shoulder (~7200); $\lambda_{max}^{pH\ 13}$ 281 m$\mu$ ($\epsilon$ 23,300); it moved as one spot in solvents E, C, and D with $R_{Ad}$ 1.9, 1.24, and 1.20, respectively.

*Anal.* Calcd for $C_{15}H_{17}N_5O_4 \cdot 2H_2O$: C, 49.0; H, 5.75; N, 19.1. Found: C, 48.7; H, 5.51; N, 19.4.

***p*-(N-{3-(2-Formamido-4-hydroxy-5-pyrimidinyl)formamido}-propyl}formamido)benzoic Acid (XII).**—A suspension of 3.57 g (11.5 mmoles) of the unblocked acid IX in pyridine was treated with a formic acetic anhydride mixture as described for VI to yield, after work-up and trituration with hot 2-methoxyethanol, 3.94 g (88%) of the triformamido acid XII as a pale yellow crystalline powder: mp 274.5–276° dec; $\lambda_{max}^{Nujol}$ ($\mu$) 2.93 (NH), 5.91 (acid, amides); $\lambda_{max}^{pH\ 1}$ 262 m$\mu$ ($\epsilon$ 16,500); $\lambda_{max}^{pH\ 13}$ 238 m$\mu$ ($\epsilon$ 17,600), 275 shoulder (~10,800); it moved as a single spot in solvents F and D with $R_{Ad}$ 0.14 (blue fluorescence) and 1.13, respectively.

*Anal.* Calcd for $C_{17}H_{17}N_5O_6$: C, 52.7; H, 4.42; N, 18.1. Found: C, 52.6, 52.8; H, 4.96, 4.89; N, 18.0.

**Diethyl N-{*p*-(N-{3-[N-(2-Formamido-4-hydroxy-5-pyrimidinyl)formamido]propyl}formamido)benzoyl}-L-glutamate (XIII).**—The triformamido acid XII (1.59 g, 4.1 mmoles) was dissolved in 200 ml of dry N,N-dimethylformamide by heating on a steam bath. The solution was cooled to room temperature, combined with 0.66 g (4.1 mmoles) of N,N'-carbonyldiimidazole, and heated at 45–50° for 1 hr with protection from moisture. To this solution, cooled to room temperature, was added a freshly prepared solution of 1.00 g (4.9 mmoles) of diethyl L-glutamate in 1 ml of N,N-dimethylformamide, and the resultant solution was stirred for 20 hr at room temperature with protection from moisture. The solution was treated with charcoal, filtered, and evaporated *in vacuo*. The residue was partitioned between 100 ml of $CH_2Cl_2$ and 25 ml of $H_2O$ by gentle stirring. The aqueous phase was further extracted with two 50-ml portions of $CH_2Cl_2$. The combined $CH_2Cl_2$ extracts were dried, filtered, and evaporated *in vacuo* to a syrup. A solution of this syrup in 2 ml of $CH_2Cl_2$ was added dropwise to 250 ml of ether with stirring. The precipitate was triturated, collected, washed with ether, and dried to yield 1.44 g (61%) of the diethyl ester (XIII), mp 86–93°. For analysis, a sample was reprecipitated from ether to furnish a pale yellow powder: mp 92–98°; $\lambda_{max}^{Nujol}$ ($\mu$) 3.01, 3.2 (NH, OH), 5.74, 6.00 (esters, amides); $\lambda_{max}^{EtOH}$ 262 m$\mu$ ($\epsilon$ 18,400), 300 shoulder (~9200). In solvent F, it has $R_{Ad}$ 1.38 with a trace of fluorescence at $R_{Ad}$ 0.69; in solvent D, $R_{Ad}$ 1.53 (major spot) and 1.09 (trace fluorescence). This sample was analyzed after drying for 15 hr over $P_2O_5$ at 56° (1 mm).

*Anal.* Calcd for $C_{26}H_{32}N_6O_9$: C, 54.5; H, 5.63; N, 14.7. Found: C, 54.6; H, 5.83; N, 15.3, 15.1, 15.2.

When the acid XII was allowed to react with 1 equiv of N,N'-carbonyldiimidazole at 45–50° for 30 min in 1-methyl-2-pyrrolidone before the reaction with diethyl glutamate, the yield of

XIII was 37%. When XII and N,N'-carbonyldiimidazole were allowed to react in N,N-dimethylformamide for 1 hr at room temperature, the yield of XIII was 11%. When the acid XII was treated with 1.05 equiv of $SOCl_2$ in N,N-dimethylformamide at room temperature for 15 min and then allowed to react with diethyl glutamate and triethylamine for 20 hr at room temperature, a very small amount of XIII was formed, as indicated by paper chromatography. The mixed anhydride method[9] was also tried. A mixture of XII and 1.1 equiv of triethylamine in N,N-dimethylformamide treated with 1.2 equiv of isobutyl chloroformate at room temperature for 45 min followed by 2.5 equiv of diethyl glutamate hydrochloride and 2.5 equiv of triethylamine at room temperature for 21 hr yielded 8% of XIII.

**N-[*p*-(N-{3-[N-(2-Amino-4-hydroxy-5-pyrimidinyl)amino]-propyl}amino)benzoyl]-L-glutamic Acid (IV).**—A solution of 1.00 g (1.74 mmoles) of the triformamido ester XIII in 25 ml of 12 *N* HCl was hydrolyzed at 37° for 1.5 hr under $N_2$ and evaporated to a syrup *in vacuo* at 25°. The syrup was dissolved in 25 ml of water, treated with charcoal, and filtered. The filtrate was adjusted to pH 2.5 with 1 *N* NaOH and decanted from a small amount of yellow gummy precipitate. The supernatant liquid was adjusted to pH 4 and cooled in ice for 30 min. The precipitate was collected, washed with $H_2O$ and ethanol, and dried to yield 0.45 g (57% as IV·1.33$H_2O$) as a white powder, mp 164–168°, which gradually acquired a pink color during subsequent handling, perhaps because of air oxidation. Two recrystallizations from $H_2O$ afforded IV as a pink powder: mp 165–167.5°; $\lambda_{max}^{Nujol}$ ($\mu$) 3.0 (NH, OH), 5.73, 5.88 (COOH); $\lambda_{max}^{pH\ 1}$ 222 m$\mu$ ($\epsilon$ 14,800), 257–260 plateau (11,000), 295 shoulder (7200); $\lambda_{max}^{pH\ 13}$ 295 m$\mu$ ($\epsilon$ 25,800); $[\alpha]^{22}D$ −9° (*c* 1.0, 1 *N* HCl); it moved as a single spot in solvents E, D, and C with $R_{Ad}$ 1.86 (blue fluorescence), 1.05 (spot with fluorescent ring), and 0.95 (spot with fluorescent ring), respectively.

*Anal.* Calcd for $C_{19}H_{24}N_6O_6 \cdot 1.33H_2O$: C, 50.0; H, 5.89; N, 18.4. Found: C, 50.1, 50.1; H, 5.83, 6.02; N, 18.3, 18.4.

**Comparison of Ease of Cyclic Methenyl Derivative Formation.**—Compounds III, IV, and IX were each dissolved in 97–100% formic acid in the proportion of 0.25 mmole to 5 ml of acid and heated on the steam bath for 1 hr.[16] The solution was evaporated to dryness *in vacuo*. The gummy residue was triturated in $CHCl_3$ or ether and again evaporated to give a foam or powder. The spectrum of the product in 0.1 *N* HCl was measured at intervals. The results are given in Table III, and should be compared with the maximum (313 m$\mu$) for the methenyl compound XIV.[4]

TABLE III
ULTRAVIOLET SPECTRA AT pH 1 AFTER FORMYLATION

| | $\lambda_{max}$, m$\mu$ for product from | | |
|---|---|---|---|
| Time | III | IV | IX |
| 0.25 hr | 317 | 268 | 269 |
| 1 day | 313 | 268 | 270 |

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(16) D. E. Wolf, R. C. Anderson, E. A. Kaczka, S. A. Harris, G. E. Arth, P. L. Southwick, R. Mozingo, and K. Folkers, *J. Am. Chem. Soc.*, **69**, 2753 (1947).

# Derivatives of Fluorene. XXII.[1a,b] Nitrogen Mustards. II[1c]

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In a continuation of earlier work, synthesis of a number of new N-2-fluorenyl mustards and such analogs as bis-2-bromoethylamino- and bis-2-halopropylaminofluorenes with various substituents in the 7 position is reported. Dimethyl sulfoxide is found to be a good medium for di-2-hydroxy ethylation; higher yields were obtained in shorter times than in the usual media. Biological data are presented showing that some of these compounds, particularly with activating groups in the 7 position, inhibit some tumor systems and have relatively low toxicity. Synthesis of several new 7-amido-N-fluoren-2-ylamines is described. Ultraviolet spectral properties in neutral and acidic solutions are recorded.

Since the only N-2-fluorenyl mustard (reported earlier[1c]) that showed any tumor inhibitory effect, and that minimal, was the one with a 7-dimethylamino group,[2] we felt that other electron donor groups, particularly in the 7 position, might confer interesting biological properties on compounds in this series. Table I lists data concerning the new chloro and bromo mustards, the corresponding bis-2-halopropyl compounds, and their precursors.

Table II presents the results of biological testing for some of these compounds as supplied by the Cancer Chemotherapy National Service Center and by the Chester Beatty Research Institute. In particular, three of the compounds gave total inhibition of the Walker rat tumor 256.

(1) (a) Paper XXI in this series by H.-L. Pan and T. L. Fletcher appeared in *J. Med. Chem.*, **8**, 491 (1965). (b) Supported in part by a grant (CA-01744) from the National Cancer Institute, National Institutes of Health, and in part by Research Career Development Award 5-K3-GM- (now CA-) 14,991 (T. L. F.). (c) For part I, see T. L. Fletcher and W. H. Wetzel, *J. Org. Chem.*, **25**, 1348 (1960).

(2) This was obtained and tested only as an impure oil.

An improved method of di-2-hydroxyethylation in dimethyl sulfoxide (DMSO) was used in two cases as described below, and we suggest that such use of DMSO may be valuable with amines having low solubility in the usual media. In this work we found a simpler approach (A in the general chlorination procedure) with high yields, for chlorinating the more stable compounds. Syntheses of the new 7-nitro- and 7-amino-N-2-fluorenylformamides, -propionamides, -urethans, and -N'-*n*-propylureas are reported. All of these 7-amino derivatives (plus the previously reported 7-amino-N-2-fluorenylacetamide), except the formamide, gave analytically pure di-2-hydroxyethylated and di-2-chloroethylated derivatives.

We also have extended the ultraviolet spectral data recorded in our first paper with similar information about the new compounds, including the effect of various concentrations of acid in causing a characteristic increase in complexity of the spectra. The latter effect depends markedly on the nature of the substituents in the 7 position. Examination of the spectral