stirred mixt of **13** (1.0 g, 0.0039 mole) in liquid NH_3 (60 ml) and Et_2O (25 ml). After 2 hr, EtOH (5 ml) was added and the NH_3 removed. The residual mixt was cooled (ice) and acidified with dil HCl. The aqueous layer was separated and extracted with Et_2O. The combined Et_2O soln was washed (saturated aqueous NaCl), dried (Na_2SO_4), and concentrated. The residual solid was recrystallized from Skellysolve B to give **14** (0.65 g), mp 117–119°. Two recrystallizations gave analytical material: mp 120–122°; nmr ($CDCl_3$) δ 6.16 (m, 1 H, $CH{=}CHCH_2$), 5.92 (m, 1 H, $CH{=}CHCH_2$), 4.37 (m, 1 H, $>CHCO_2H$), and 3.40 ppm (m, 2 H, $CH{=}CHCH_2$).

6-Cyclohexyl-3,4-dihydro-1-naphthoic Acid (15). A soln of **14** (2.5 g) in 2 *N* NaOH (125 ml) was heated under reflux for 2.5 hr. The warm soln was treated with Norit, filtered, cooled, and acidified with 5 *N* H_2SO_4. The ppt was dried and recrystallized from Skellysolve B to give **15** (1.21 g), mp 171–178°. Recrystallizations from MeOH (Norit) followed by Skellysolve B gave **15** as colorless crystals: mp 182–183°; nmr ($CDCl_3$) δ 7.80 (d, 1 H, ArH), 7.30 (t, 1 H, vinylic), 7.04 (m, 2 H, ArH), 2.55 (broad m, 5 H, allylic and benzylic), and 1.55 ppm [broad m, 10 H, $(CH_2)_5$].

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References

(1) P. F. Juby, W. R. Goodwin, T. W. Hudyma, and R. A. Partyka, *J. Med. Chem.*, **15**, 1297 (1972).
(2) P. F. Juby, R. A. Partyka, and T. W. Hudyma, U. S. Patent 3,565,904 (1971).
(3) Ciba S. A., Belgium Patent 740,314 (1970).
(4) C. A. Winter, E. A. Risley, and G. W. Nuss, *Proc. Soc. Exp. Biol. Med.*, **111**, 544 (1962).
(5) J. L. Marshall and T. K. Folsom, *J. Org. Chem.*, **36**, 2011 (1971).
(6) (a) T. Mitsui and A. Tamura, *Nippon Nogei Kagaku Kaishi*, **25**, 17 (1951); *Chem. Abstr.*, **47**, 9302*b* (1953); (b) M. Inaba and T. Mitsui, *Bull. Agr. Chem. Soc. Jap.*, **20**, 42 (1956); (c) R. A. Heacock, R. L. Wain, and F. Wightman, *Ann. Appl. Biol.*, **46**, 352 (1958); (d) T. Fujita, S. Imai, K. Koshimizu, T. Mitsui, and J. Kato, *Nature (London)*, **184**, 1415 (1959); (e) K. Kawazu, T. Fujita, T. Mitsui, J. Kato, and M. Katsumi, *ibid.*, **187**, 694 (1960); (f) T. Fujita, K. Koshimizu, S. Imai, T. Mitsui, and J. Kato, *Agr. Biol. Chem.*, **25**, 710 (1961); (g) T. Fujita, K. Kawazu, T. Mitsui, M. Katsumi, and J. Kato, *ibid.*, **30**, 1280 (1966); (h) T. Fujita, K. Kawazu, T. Mitsui, and M. Katsumi, *Phytochemistry*, **6**, 889 (1967).
(7) (a) K. Kawazu, T. Fujita, and T. Mitsui, *J. Amer. Chem. Soc.*, **81**, 932 (1959); (b) A. Fredga, *Chem. Ber.*, **89**, 322 (1956).
(8) E. C. Horning and A. F. Finelli, "Organic Syntheses," Collect. Vol. IV, Wiley, New York, N. Y., 1963, p 461.
(9) E. C. Horning and A. F. Finelli, ref 8, p 776.
(10) E. Testa, A. Bonatti, G. Pagani, and E. Gatti, *Justus Liebigs Ann. Chem.*, **647**, 92 (1961).
(11) H. A. Lloyd and E. C. Horning, *J. Amer. Chem. Soc.*, **76**, 3651 (1954).
(12) A. Bayer, *Justus Liebigs Ann. Chem.*, **266**, 169 (1891).

Folic Acid Analogs. Modifications in the Benzene-Ring Region. 2. Thiazole Analogs†

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Two thiazole analogs of folic acid, in which the benzene ring is replaced by a thiazole ring, were synthesized as part of a continuing program to design and obtain folic acid analogs with a potentially altered ability to function as one-carbon transfer agents. The reductive condensation of 2-acetamido-6-formylpteridin-4(3*H*)-one (**3**) with diethyl *N*-[(2-amino-4-thiazolyl)carbonyl]glutamate (**2**) followed by hydrolysis of the blocking groups afforded thiazole analog **6**. Thiazole analog **10** was obtained similarly from **3** and diethyl *N*-[(2-amino-5-thiazolyl)carbonyl]glutamate (**8**). Compounds **2**, **6**, **8**, and **10** were not active against leukemia L1210 in mice in tests on a single-dose schedule and were not cytotoxic to HEp-2 cells in culture. Analogs **6** and **10** displayed modest inhibition of *Streptococcus faecalis* ATCC 8043 but were noninhibitory toward pigeon liver dihydrofolate reductase.

Tetrahydrofolate derivatives serve as agents in the transfer of one-carbon units in biological systems.[1] The manner in which certain structural alterations in the folic acid molecule may alter its capacity ultimately to function as a one-carbon transfer agent has been described.[2] For example, the potential of reduced folic acid type molecules for forming one-carbon transfer agents may possibly be decreased by decreasing the electron availability at position 10 (N^{10}). In the two folic acid analogs herein reported, in which the benzene ring is replaced by the thiazole ring, the electron availability at N^{10} may be expected to be diminished. Additionally, because of its attachment to a five-membered instead of a six-membered ring, the geometric and spatial relationship of the carbonylglutamate moiety to the remainder of the molecule has been altered. Either or both of these structural changes may affect folic acid metabolism at stages other than, or in addition to, the reduction by dihydrofolate reductase. These folic acid analogs were synthesized as part of a continuing program whose goal is to obtain potentially useful antineoplastic agents.

Chemistry. The method of synthesis of the folic acid analogs **6** and **10** is based on the method of Sletzinger, *et al.*,[3] in which folic acid had been obtained *via* the reductive condensation of **3** with *p*-aminobenzoylglutamic acid in the presence of *p*-toluenethiol. The syntheses of 2′-azafolic acid and 3′-azafolic acid have been accomplished similarly.[2] Intermediate **2**, obtained by the coupling of **1**[4] with diethyl glutamate, was condensed reductively with **3**, and the crude N^2-deacetylated product **4** was obtained. Acetylation of **4** yielded **5**, and purification was accomplished at this stage. Complete and selective hydrolysis in 0.1 *N* NaOH of the glutamate ester groups and the N^2- and N^{10}-acetamide functions of **5** afforded the desired thiazole analog **6**. Analog **6** was obtained more conveniently from **4** by direct hydrolysis of the ester functions.

Similarly, reductive condensation of **3** with **8**, obtained by the coupling of **7b** with diethyl glutamate, afforded **9**. Hydrolysis of the ester functions of **9** yielded thiazole analog **10** (Scheme I).

Hydrated samples of **6** and **10** having satisfactory elemental analyses and uv and pmr spectral properties were obtained by DEAE-cellulose column chromatography, but tlc analysis of the analogs revealed the presence of a blue-fluorescent impurity in **6**. Its identity was established as 2-amino-6-formylpteridin-4(3*H*)-one (**11**) by tlc compari-

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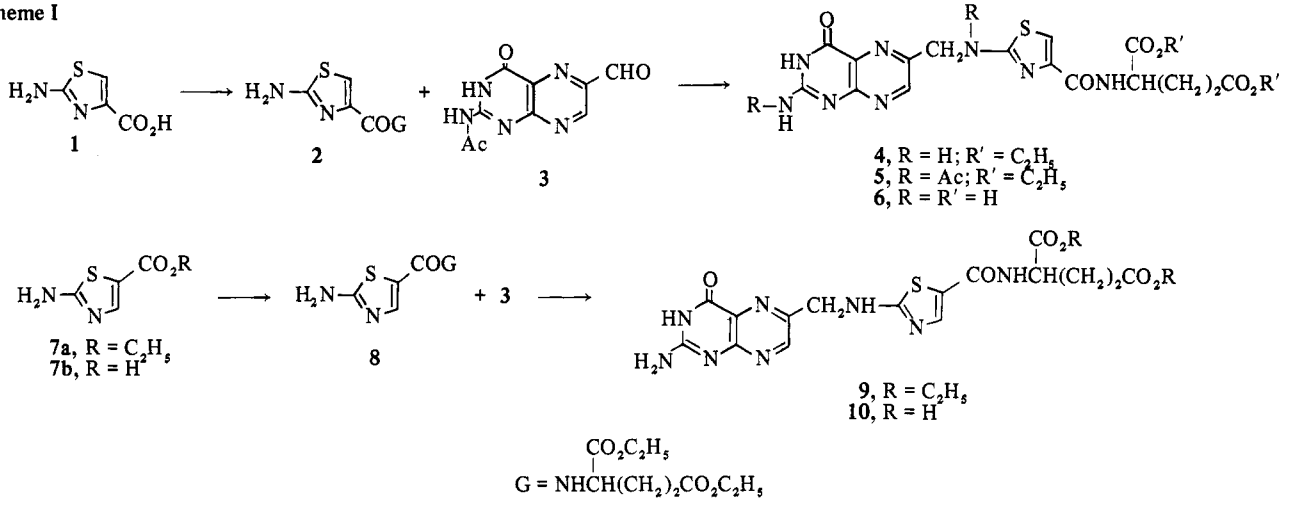
Scheme I



sons with an authentic sample in several chromatographic systems. Impurity **11** was presumed to have arisen by air oxidation of **6** during chromatography.[2,5] The level of occurrence of **11** in **6** was shown by comparative tlc to be less than 1%. This impurity **(11)** was not detected on tlc analysis of analog **10**.

Biological Evaluation. Compounds **2, 6, 8,** and **10** were administered on a single dose schedule to mice within 24 hr of implantation ip with 10^5 L1210 cells. Compounds **2** and **8**, at a dose of 400 mg/kg, produced no evidence of toxicity and no increase in life-span. Analogs **6** and **10** were toxic at all doses above 75 and 100 mg/kg, respectively; at these specified doses, no significant increase ($T/C \geq 25\%$) in life-span was observed.

Compounds **2, 6, 8,** and **10** exhibited no cytotoxicity when tested at concentrations of 1, 10, and 100 mcg/ml *vs.* HEp-2 cells in culture.

Compounds **6** and **10** were found to produce 50% inhibition of pigeon liver dihydrofolate reductase at concentrations of 1.9 and 1.5×10^{-4} *M*, respectively; methotrexate was 50% inhibitory at 1.4×10^{-8} *M*.

Analog **6** produced 50% inhibition of *Streptococcus faecalis* ATCC 8043 at a concentration of 6.0×10^{-6} *M*. In a simultaneous test, methotrexate was 1700 times more effective ($I_{50} = 3.5 \times 10^{-9}$ *M*) than **6**, and tetrahydrohomofolate was 40 times more effective ($I_{50} = 1.5 \times 10^{-7}$ *M*). Analog **10** produced 50% inhibition of *Strep. faecalis* at a concentration of 2.5×10^{-6} *M*; methotrexate and tetrahydrohomofolate were, respectively, 700 and 17 times more effective than **10**. Although the inhibition of *Strep. faecalis* by **6** and **10** is relatively modest, it is in sharp contrast to the growth-supporting activity (toward *S. faecalis*) previously observed[2] for 2'- and 3'-azafolic acids. The lack of inhibition of pigeon liver dihydrofolate reductase suggests that **6** and **10** may be exerting their modest inhibitory effect on *Strep. faecalis* by inhibiting at sites other than dihydrofolate reductase.

Analog **6** was tested[6] *vs. Plasmodium berghei* KBG 173 malaria in mice and was found inactive at doses of 20, 40, 80, 160, 320, and 640 mg/kg.

Experimental Section

Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of theoretical values. Mp's were determined with a Kofler Heizbank (gradiently heated bar) apparatus. Pmr data were determined with a Varian A-60A spectrometer and are given in ppm downfield from Me_4Si. Uv spectra were determined with a Cary Model 17 recording spectrophotometer, and wavelengths are given in nm. Linde Type 4A molecular sieves were used to dry solvents designated anhydrous, and in certain reaction procedures where indicated. Na_2SO_4 was used to dry soln in organic solvents, and solvent evaporations were done *in vacuo*. The DEAE-cellulose used was Mannex (Mann Research Laboratories) of the capacity noted. Before chromatographic use, the DEAE-cellulose was converted to the phosphate form by washing with a large excess of 0.5 *M* potassium phosphate buffer (pH 7.0); this was followed by washes with H_2O, and with H_2O containing mercaptoethanol. Soln used in elution were maintained at pH 7.0 by the use of 0.005 *M* potassium phosphate buffer and were 0.2 *M* in mercaptoethanol. Linear NaCl gradient elutions utilized 0.6 *M* NaCl in the reservoir and buffer soln in the mixing bottle. Isolations and washings by centrifugation were done at 1800–2000 rpm; washing by centrifugation of **6** and **10** was done with dil HCl: 3–4 drops of 1 *N* HCl per 50 ml of H_2O. Analytical samples reported as hydrated were handled in a dry atmosphere.

Tlc was run on Analtech, Inc., cellulose-coated plates (Cell-A), Bakerflex cellulose sheets (Cell-B), Bakerflex DEAE-cellulose sheets (Cell-DEAE), and Brinkmann's silica gel H (SGH). SGH was also used for column chromatography. Solvent systems used were: A, BuOH–AcOH–H_2O (5:2:3); B, BuOH–AcOH–H_2O (4:1:5, upper layer); C, 5% aqueous Na_2HPO_4; D, 5% aqueous NH_4OH–3% aqueous NH_4Cl; E, 0.5 *M* NaCl, 0.2 *M* in mercaptoethanol, in 0.005 *M* potassium phosphate buffer at pH 7.0; F, $CHCl_3$–MeOH (98:2); G, $CHCl_3$–MeOH (97:3).

Diethyl *N*-[(2-Amino-4-thiazolyl)carbonyl]glutamate (2). 2-Aminothiazole-4-carboxylic acid·HBr[4] (**1**, 9.0 g, 0.04 mole) was dissolved in 1200 ml of pyridine and allowed to react with 8.25 g (0.04 mole) of DCI and 9.6 g (0.04 mole) of diethyl glutamate·HCl. After 4 days, the mixture was chilled and the ppt was removed by filtration and washed with $CHCl_3$. The filtrate was evaporated, and the residue was triturated with $CHCl_3$. The $CHCl_3$ filtrate was applied to a chromatography column (SGH) which was eluted with solvent F. Impure product-containing fractions were rechromatographed. Combination of the tlc-homogeneous (SGH, solvent G) fractions afforded **2**: 9.78 g, 74% yield, mp 115°; pmr (CF_3CO_2D) δ 7.82 (s, 1 H, C_5-H); the remainder of the pmr spectrum was as expected. *Anal.* ($C_{13}H_{19}N_3O_5S$) C, H, N.

2-Aminothiazole-5-carboxylic Acid (7b). Ethyl 2-aminothiazole-5-carboxylate‡ (**7a**, 17.2 g, 0.1 mole) was dissolved in 500 ml of CH_3OH, and 500 ml of aqueous 2 *N* KOH was added. After stirring at room temp for 3 hr, the reaction was acidified to pH 4.2 and refrigerated overnight. The crystalline solid was isolated by filtration and washed with cold CH_3OH–H_2O before drying (P_2O_5) *in vacuo* at 78°; 14.3 g, 99% yield; pmr (CF_3CO_2D) δ 8.03 (s, 1 H, C_4-H). *Anal.* ($C_4H_4N_2O_2S$) C, H, N.

Diethyl *N*-[(2-Amino-5-thiazolyl)carbonyl]glutamate (8). Compd **7b** (12.95 g, 0.09 mole) was dissolved in 1250 ml of pyridine and allowed to react with 18.55 g (0.09 mole) of DCI and 21.6 g (0.09 mole) of diethyl glutamate·HCl for 2 days. The product was isolated as described for **2**: 15.9 g, 53% yield; mp 106–108°; pmr (CF_3CO_2D) δ 8.06 (s, 1 H, C_4-H); the remainder of the pmr spectrum was as expected. *Anal.* ($C_{13}H_{19}N_3O_5S$) C, H, N.

‡The ethyl chloromalonaldehydate, used for the preparation[7] of **7a**, was not a liquid as reported,[8] but crystallized after distillation: bp (15 mm) 86–88° (lit.[8] bp (14 mm) 60–104°).

N-[(2-{[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino}-4-thiazolyl)carbonyl]glutamic Acid (6). A soln of *p*-toluenethiol (43.5 g, 0.35 mole) in 1700 ml of anhydrous methoxyethanol was heated to vigorous reflux in a flask fitted with a N_2 inlet and a reflux condenser atop a condensate trap filled with molecular sieves; the reaction was protected from atm moisture and light. The flask was flushed with N_2, and **2** (18.1 g, 0.055 mole) and **3** (11.65 g, 0.05 mole) were added. After 12-hr reflux under N_2, the cooled reaction mixture was poured into 4 l. of Et_2O, and the resulting solid was isolated by filtration and washed with Et_2O. Et_2O trituration of the total filtrate residue, obtained by evaporation, yielded more solid (14.72 g total, 58% crude yield).

The combined solids were hydrolyzed at room temp in 3500 ml of 0.1 *N* NaOH in a N_2 atm. After 17 hr, the soln was filtered and acidified to pH 6.5, the solid was isolated by centrifugation, and the supernatant was retained. This solid was twice redissolved (dil NH_4OH) and reprecipitated at pH 6.5 before discarding. The three supernatant soln of pH 6.5 were combined and acidified to pH 3.5, and the solid thus obtained was isolated and washed with dil HCl by centrifugation; after freeze-drying, the solid weighed 2.1 g.

This material was dissolved in a minimum amount of dil NH_4OH, and the soln was diluted to 2000 ml and made 0.2 *M* in mercaptoethanol. This soln was applied to a DEAE-cellulose column (standard capacity, phosphate form, 1800-ml column volume). The column was washed with 2000 ml of 0.2 *M* mercaptoethanol and then eluted with 2000 ml of 0.2 *M* NaCl. The column was next eluted with 0.35 *M* NaCl; the fractions were monitored by uv absorption. The desired fractions were combined, acidified to pH 3.5 with HCl, and refrigerated. The solid was isolated and washed with dil HCl by centrifugation. It was redissolved in 1500 ml of dil NaOH containing 15 ml of mercaptoethanol, and the soln was filtered and acidified (HCl) to pH 3.5 and refrigerated. The yellow ppt was isolated and washed four times with dil HCl by centrifugation before drying (P_2O_5) *in vacuo*; 1.417 g, 6% from **3**. The product was pulverized before final drying (P_2O_5) for 24 hr at 0.5 mm: uv_{max} (0.1 *N* HCl) 250 (22,400), 322 (8700); uv_{max} (pH 7) 234 (24,300), 275 (20,300), 348 (7020); uv_{max} (0.1 *N* NaOH) 255 (32,600), 365 (8350); pmr (CF_3CO_2D) δ 5.20 (s, 2 H, NCH_2), 7.92 (s, 1 H, C_5'-H), 9.00 (s, 1 H, C_7-H); the remainder of the pmr spectrum was as expected; analysis of **6** by tlc on Cell-B (solvent A) produced a pattern having a uv-absorbing spot followed by a separate uv-absorbing tail; two-dimensional tlc indicated this pattern was a chromatographic artifact. On Cell-DEAE (solvent E), **6** was found to contain a major uv-absorbing component and a minor blue-fluorescent impurity; side-by-side tlc comparisons with standards at several concentrations indicated this impurity to be **11** whose amount in these samples was less than 1% (Cell-DEAE, solvent E). *Anal.* ($C_{16}H_{16}N_8O_8S \cdot 1.25H_2O$) C, H, N.

N-[(2-{[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino}-5-thiazolyl)carbonyl]glutamic Acid (10). The reductive condensation of **8** (5.92 g, 18 mmoles) with **3** (3.82 g, 16.4 mmoles) in anhydrous methoxyethanol (700 ml) in the presence of *p*-toluenethiol (14.2 g, 0.115 moles) was conducted as described for **2** with **3**. The cooled reaction mixture was poured into 1500 ml of Et_2O, and two portions of solid were obtained (5.75 g total).

The combined solids were hydrolyzed in 3500 ml of deaerated 0.1 *N* NaOH in a N_2 atm. The ppt obtained by acidification of the filtered hydrolysis soln was freed of solids insoluble at pH 6.5 as before. The supernatant soln of pH 6.5 was acidified to pH 3.5, and the solid thus obtained was isolated and washed as before.

The crude product was dissolved in dil NH_4OH (2000 ml containing 15 ml of mercaptoethanol; soln pH 6.5–7), and the soln was applied to a DEAE-cellulose column (27 g, standard capacity, basic form) which was next washed with H_2O containing mercaptoethanol. The column was eluted with 1.0 *M* NaCl until the eluate yielded no ppt on acidification (HCl) to pH 3.5. The ppt obtained by acidification of the total eluate to pH 3.5 was isolated and washed with dil HCl by centrifugation; drying (P_2O_5) *in vacuo* yielded 927 mg of a yellow powder.

The yellow material was dissolved in dil NH_4OH (2000 ml containing 300 ml of mercaptoethanol; soln pH 6.5), and this soln was applied to a DEAE-cellulose column (31 g, standard capacity, phosphate form); the column was washed with H_2O containing mercaptoethanol before elution with a linear NaCl gradient (3000 ml total vol). The desired fractions were treated as before, and the yellow solid thus obtained weighed 377 mg; 5% yield from **3**. The product was pulverized before final drying (P_2O_5) for 24 hr at 0.5 mm; uv_{max} (0.1 *N* HCl) 250 (15,200), 281 (20,600), 320 (9650); uv_{max} (pH 7) 279 (18,800), 302 (20,800), 342 (7920); uv_{max} (0.1 *N* NaOH) 255 (24,300), 303 (20,400), 364 (9220); pmr (CF_3CO_2D) δ 5.15 (s, 2 H, NCH_2), 8.27 (s, 1 H, C_4'-H), 9.05 (s, 1 H, C_7-H); the remainder of the pmr spectrum was as expected. Analysis of **10** by tlc on Cell-B (solvents A, B, C, D) and Cell-DEAE (solvent E) indicated it to be homogeneous. *Anal.* ($C_{16}H_{16}N_8O_6S \cdot 1.5H_2O$) C, H, N.

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References

(1) M. Friedkin, *Annu. Rev. Biochem.*, **32**, 185 (1963); F. M. Huennekens in "Biological Oxidations," T. P. Singer, Ed., Wiley, New York, N. Y., 1968, pp 439–513.
(2) E. C. Roberts and Y. F. Shealy, *J. Med. Chem.*, **14**, 125 (1971).
(3) M. Sletzinger, D. Reinhold, J. Frier, M. Beachem, and M. Tishler, *J. Amer. Chem. Soc.*, **77**, 6365 (1955).
(4) J. M. Sprague, R. M. Lincoln, and C. Ziegler, *ibid.*, **68**, 268 (1946).
(5) J. M. Whiteley, J. Drais, J. Kirchner, and F. M. Huennekens, *Arch. Biochem. Biophys.*, **126**, 955 (1968).
(6) T. S. Osdene, P. B. Russell, and L. Rane, *J. Med. Chem.*, **10**, 431 (1967).
(7) O. Dann, *Chem. Ber.*, **76**, 424 (1943).
(8) A. Dornow, F. Boberg, and L. Schürer, *Arch. Pharm. (Weinheim)*, **286**, 494 (1953).