Potential Anticancer Agents: 5-(*N*-Substituted-aminocarbonyl)- and 5-(*N*-Substituted-aminothiocarbonyl)-5,6,7,8-tetrahydrofolic Acids

Carroll Temple, Jr.,* Robert D. Elliott, and John A. Montgomery

Kettering-Meyer Laboratory, Southern Research Institute, P.O. Box 55305, Birmingham, Alabama 35255-5305.
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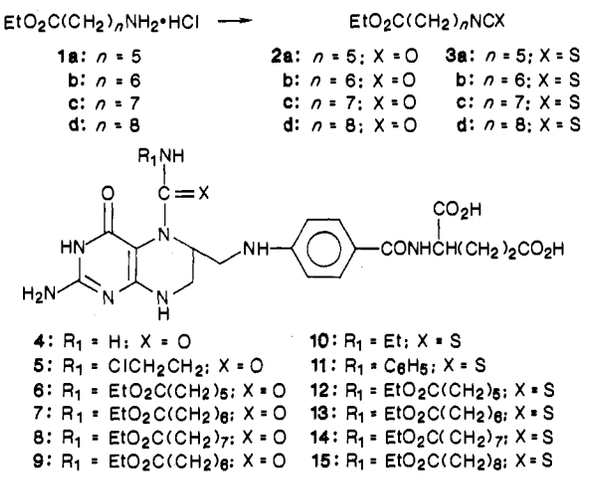
5-[[*N*-[(Ethoxycarbonyl)alkyl]amino]carbonyl] (**6–9**) and the corresponding aminothiocarbonyl (**12–15**) derivatives of 5,6,7,8-tetrahydrofolic acid were prepared as multisubstrate analogues of the substrate–cofactor adduct in the reactions catalyzed by the folate-mediated one-carbon transfer reactions. Evaluation in vitro showed that **7** (alkyl = hexyl) was cytotoxic to H.Ep.-2 cells (ED_{50}, 4 μM) but noncytotoxic to proliferating L1210 cells. No activity was observed for **7** against the P388 leukemia in mice.

Thymidylate synthase (TS), glycinamide ribotide transformylase (GAR Tase), and 5-amino-4-imidazole carboxyamide ribotide transformylase (AICR Tase) are key enzymes in folate metabolism. In the reactions catalyzed by TS and the Tases, a one-carbon moiety is transposed from folate cofactors to substrates that eventually provide the purine and one of the pyrimidine nucleotides of the nucleic acids. Work by Danenberg, Santi, and others indicated that the TS transformation occurs via a covalent ternary complex of enzyme, cofactor, and substrate (Figure 1).[1a] Reactions catalyzed by the Tases might proceed via enzyme complexes that contain the cofactor and substrate as covalent binary adducts (Figure 1).[1b] For the proposed adducts to exist, a minimum requirement is the binding of both the phosphate and THF moieties to the enzyme. The bonds connecting these binding sites are indicated by solid lines in the structures.

In recent work on antifolates containing the 2-amino-4(3*H*)-oxopyrimidine moiety, 5-[(2′-deoxyuridin-5-yl)-methyl]-8-deaza-5,6,7,8-tetrahydrofolic acid 5′-monophosphate was identified as an inhibitor of TS.[2] Substitution at the 5-position of folate is not a requirement for TS activity as demonstrated by 10-propargyl-5,8-dideazafolic acid, a potent inhibitor of TS.[3] In addition, the activity of 5,10-dideaza-5,6,7,8-tetrahydrofolic acid[4,5] against solid tumors was attributed to the inhibition of GAR Tase.[6]

Although 5- and 8-deaza-5,6,7,8-tetrahydrofolic acids are more stable than 5,6,7,8-tetrahydrofolic acid (THF) toward oxidative degradation, our work was directed toward the preparation of derivatives of THF. The chemistry of THF is complicated by its array of functional groups, but work in our laboratory indicated that isocyanates react selectively at N-5 of THF to give stable aminocarbonyl derivatives.[7] Krumdieck and co-workers isolated an aminocarbonyl derivative of THF from the preparation of liver extracts in 6 M urea and postulated correctly that cyanate presence in the urea solution had reacted at the N-5 position.[8] Diastereoisomeric derivatives of THF were prepared by treatment of THF with (*R*)-1-naphthylethyl isocyanate to afford the N-5 derivatives.[9] Our goal was to prepare stable 5-(aminocarbonyl) and 5-(aminothiocarbonyl) derivatives of THF, which might mimic the binary adduct in the one-carbon transformation reactions. In the target compounds (**6–9, 12–15**), the distance between folate and the carboxylate group was varied to account for differences in the length of the backbone chains (Figure 1). It was anticipated that the tetrahydrofolate moiety would allow these compounds to enter cells readily, which might be followed by conversion of the ester group to a carboxylate by esterases.

Scheme I



Chemistry. Previously, we reported on methods for the preparation, isolation, and storage of large amounts of THF.[7,10] Reduction of the pyrazine ring of folic acid in an aqueous medium with excess $NaBH_4$ by modification of the procedure of Blair and Saunders[11] and isolation of the product in the presence of ascorbic acid gave a high yield of THF. If protected from light and oxygen, THF prepared in this manner could be used over a period of 3–4 weeks for the synthesis of derivatives. These samples contained varying amounts of H_2O and boron impurities; thus, molecular weights were calculated from elemental analyses.

The synthesis of the target compounds required the preparation of the isocyanates **2** and isothiocyanates **3** (Scheme I). The amino ester hydrochlorides **1** were

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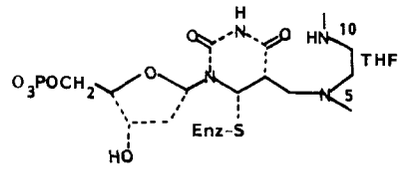
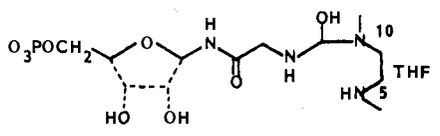
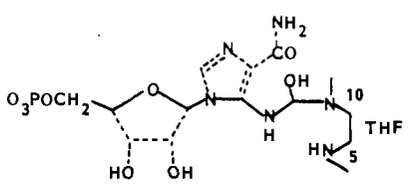
Table I. 5-Carbamyl- and 5-Thiocarbamyl-5,6,7,8-tetrahydrofolic Acids

		reaction				UV absorption spectra λ_{max}, nm ($\epsilon \times 10^{-3}$)			^{1}H NMR, δ			
compd	method	THF, mmol	RNCX, mmol	time, h	yield, %	0.1 N HCl	pH 7	0.1 N NaOH	phenylene peaks		formula	anal.
4	A	1.36	2.04	4	69	289 (23.2)	283 (29.9)	279 (26.3)	6.46 d	7.66 d	$C_{20}H_{24}N_8O_7 \cdot 1.3H_2O$	CHN
5	A	8.42	12.8	4	68	284 (22.3)	283 (26.9)	281 (24.5)	6.46 d	7.68 d	$C_{22}H_{27}ClN_8O_7 \cdot 0.3H_2O$	CHClN
6	A	3.21	4.82	1[a]	62	285 (23.9)	284 (30.7)	280 (27.6)	6.67 d	7.65 d[b]	$C_{28}H_{38}N_8O_9 \cdot 0.8HCl$	CHN
7	A	4.07	5.29	4	21	292 (21.4)	292 (28.5)	288 (26.1)	6.55 d	7.66 d	$C_{29}H_{40}N_8O_9 \cdot 0.5H_2O$	CHN
8	A	3.01	3.91	4	8	284 (21.7)	285 (22.9)	281 (23.4)	6.55 d	7.67 d	$C_{30}H_{42}N_8O_9 \cdot H_2O$	CHN
9	B	3.53	5.30	48	19	285 (22.7)	285 (30.0)	281 (26.7)	6.52[c]	7.62[c]	$C_{31}H_{42}N_8O_9 \cdot Ca \cdot 4.5H_2O$	CHN
10	A	2.73	4.10	2	78	287 (28.1)	288 (32.7)	283 (31.3)	6.58 d	7.69 d	$C_{22}H_{28}N_8O_6S \cdot 2H_2O$	CHN
11	A	8.17	10.6	2	86	288 (31.0)	288 (35.6)	283 (33.1)	6.82 d	7.72 d[b]	$C_{26}H_{26}N_8O_6S \cdot Ca \cdot 0.9EtOH \cdot 0.3H_2O$	CHN·Ca
12	A	3.13	3.75	16	28	288 (26.9)	288 (33.8)	285 (31.7)	6.58 d	7.68 d	$C_{28}H_{38}N_8O_8S \cdot 0.1HCl$	CHN
13	A	2.64	3.16	64	54	287 (27.4)	288 (33.9)	283 (31.4)	6.58 d	7.66 d	$C_{29}H_{40}N_8O_8S \cdot 0.8H_2O$	CHN
14	A	4.65	6.98	3	6	287 (26.2)	287 (33.0)	284 (30.5)	6.57 d	7.63 d	$C_{30}H_{42}N_8O_8S \cdot 1.7H_2O$	CHN
15	B	3.60	5.40	24	60	288 (25.5)	288 (33.4)	285 (30.9)	6.55[c]	7.65[c]	$C_{31}H_{42}N_8O_8S \cdot Ca \cdot 2.7H_2O$	CHN

[a] After 1 h at room temperature, the solution was maintained at 5 °C for 18 h. [b] Spectra determined in D_2O–NaOD. [c] Broad peak.







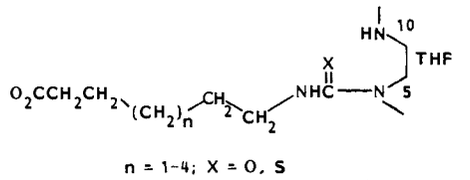


Figure 1. THF, 5,6,7,8-tetrahydrofolic acid.

prepared in high yields from the corresponding acids by treatment of the latter at room temperature with a premixed solution of $SOCl_2$ in EtOH.[12] Reaction of a suspension of the hydrochlorides in refluxing toluene with phosgene, as reported for the preparation of **2a**, afforded the isocyanates **2**.[13] This procedure gave good yields except for **2b** in which the amount (38%) was decreased by the reaction of **2b** with **1b** to give the corresponding urea. Similarly, treatment of solutions of **1** in water with thiophosgene in $CHCl_3$ while continuously extracting the product into $CHCl_3$[14] gave good yields of the isothiocyanates **3**.

For the preparation of model compounds, THF was dissolved in deoxygenated H_2O by the addition of NaOAc. Treatment of solutions of THF with KNCO, 2-chloroethyl isocyanate, ethyl isothiocyanate, or phenyl isothiocyanate gave **4**, **5**, **10**, and **11**, respectively (Table I).[7] Similarly, addition of **2a–c** and **3a–c** to aqueous solutions of THF gave the aminocarbonyl (**6–8**) and aminothiocarbonyl (**12–14**) products, respectively. Because of the water insolubility of **2d** and **3d**, these reactants were added to solutions of THF in *N,N*-dimethylacetamide (DMAC) to give **9** and **15**. As an initial step in the purification, the acids were converted to calcium salts. The latter was precipitated from the aqueous solution by the addition of EtOH. Further purification was effected by fractional precipitation of the products from aqueous solutions of the salts by adjusting the pH in increments with 1 N HCl. Because of the low solubility of **9** and **15** in aqueous media, these compounds were characterized as their calcium salts.

Several methods were used to ascertain the position of substitution on THF. Both THF and its 10-substituted derivatives undergo decomposition readily, whereas, 5-formyl-THF is stable under neutral and alkaline conditions.[1c] Products **4–15** were dissolved in 0.01 N NaOH, and these solutions were used in the determination of the UV absorption spectra in 0.1 N HCl, pH 7 buffer, and 0.1 N NaOH (Table I). Substitution at N-5 of THF was indicated by stable UV maxima in the 280–290-nm region, which is similar to that observed for 5-formyl-THF (λ_{max},

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pH 7, 287 nm).[1c] Previously, another method was developed to differentiate between substitution at N-5 and N-10.[7] The ^{1}H NMR spectra of DMSO-d_6 or D_2O solutions of derivatives of THF substituted at N-5 (e.g., CO) exhibited peaks for the phenylene protons in the range 6.5–7.0 and 7.5–7.9 ppm, which is consistent with the chemical shifts observed for **4–15** (Table I). In contrast, THF derivatives substituted with an unsaturated group (e.g., CO) at N-10 gave ranges of 7.4–7.7 and 7.8–7.9 ppm in which the chemical shift of one pair of phenylene protons was deshielded. In addition, the absence of a peak near that of the 7-CH of folic acid (δ 8.73)[1c] indicated that oxidation had not occurred at the 7,8-positions of **4–15**.

Biological Evaluation. Previously, the model compounds **4**, **5**, **10**, and **11** were reported to be inactive against 10 isolated folate-utilizing enzymes.[7] In addition, these compounds were inactive against L1210 in mice as were **6**, **7**, **9**, and **15** against P388.[21] The long-chain derivatives were evaluated for their effect on the viability of H.Ep.-2 cells in the colony-forming assay.[22] Concentrations inhibiting colony formation by 50% for the aminothiocarbonyl derivatives **12–15** exceeded 25 μM, whereas the aminocarbonyl derivatives **6–9** gave ED_{50} values of 13, 4, 24, and 35 μM, respectively. Neither **6** nor **7** inhibited the proliferation of L1210 cells at concentrations 7-fold greater than that observed for inhibition in the H.Ep.-2 system. The longer time span of the H.Ep.-2 assay suggested that the observed cytotoxicity of **7** could be caused by a delayed effect. However, preincubation of H.Ep.-2 cells with **7** for 4, 24, and 48 h prior to addition of radiolabeled thymidine, uridine, and formate produced no significant inhibition of macromolecular synthesis. Thus, the cytotoxicity of **7** cannot be attributed to inhibition of the folate enzymes and remains unexplained. Currently, the mechanism of action of **7** is being studied in another program in our laboratories.

Experimental Section

Ultraviolet absorption spectra were determined with a Cary Model 17 spectrophotometer on solutions prepared from the samples dissolved in 0.01 N NaOH. The ^{1}H NMR spectra were determined with a Varian XL-100-15 spectrometer (internal Me_4Si) on solutions of the samples dissolved in Me_2SO-d_6 (unless otherwise noted). Mass spectra were taken with a Varian Mat 311A spectrometer operating in either the electron-impact or the field-desorption mode. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

General Procedure for Esterifications. The amino ester hydrochlorides were prepared by modification of the procedure of Brenner and Huber in which the amino acid was added to a mixture of $SOCl_2$ and EtOH at room temperature.[12]

Ethyl 6-aminocaproate hydrochloride (**1a**) from $SOCl_2$ (120 mL, 1.67 mol), EtOH (480 mL), and 6-aminocaproic acid (100 g, 0.762 mol) for 18 h: yield 142 g (95%, EtOAc–Et_2O); mp 110 °C (lit.[15] mp 111 °C).

Ethyl 7-aminoheptanoate hydrochloride (**1b**) from $SOCl_2$ (27.1 mL, 378 mmol), EtOH (110 mL), and 7-aminoheptanoic acid (17.0 g, 117 mmol) for 20 h: yield 23.2 g (95%, EtOAc–Et_2O); mp 121–123 °C (lit.[15] mp 123 °C).

Ethyl 8-aminocaprylate hydrochloride (**1c**) from $SOCl_2$ (20.0 mL, 274 mmol), EtOH (80 mL), and 8-aminocapryloic acid (20.0 g, 126 mmol) for 18 h: yield 26.7 g (95%, EtOH–EtOAc); mp 122–123 °C (lit.[15] mp 121 °C).

Ethyl 9-aminononanoate hydrochloride (**1d**) from crude 9-bromononanoic acid (20.5 g)[16] and concentrated NH_4OH in a stainless steel bomb at 100 °C for 1 h to give crude 9-aminononanoic acid (17.3 g, 79% yield),[17] which was added to a mixture of $SOCl_2$ (20.0 mL, 274 mmol) and EtOH (80 mL) and stirred for 48 h: yield 15.3 g (74%, EtOAc); mp 125 °C (lit.[18] mp 143 °C); MS-EI, m/e 201 $(M)^+$; TLC (silica gel, 5:1 $CHCl_3$–MeOH), R_f 0.32 with trace impurity at R_f 0.71.

General Procedure for Preparation of Isocyanates. Compounds **2a–d** were prepared by the method of Taub and Hino in which a suspension of **1** in refluxing toluene (2 mL/g) was treated with phosgene gas at a fast rate for 20–30 min.[13]

Ethyl 6-isocyanatocaproate (**2a**) from **1a** (19.6 g, 100 mmol): yield 12.8 g (69%); bp 80–83 °C (0.5 mm) [lit.[13] bp 96–98 °C/(2 mm)].

Ethyl 7-isocyanatoheptanoate (**2b**) from **1b** (10.5 g, 50.0 mmol): yield 3.75 g (38%); bp 96 °C/(1.25 mm) [lit.[19] bp 98 °C/(1.5 mm)]. The residue (5 g) from the distillation was identified as the corresponding urea: MS-EI, m/e 372 $(M)^+$.

Ethyl 8-isocyanatocaprylate (**2c**) from **1c** (10.0 g, 44.6 mmol): yield 7.98 g (84%); bp 88–91 °C/(0.25 mm) [lit.[19] bp 111 °C/(1.4 mm)]; MS-EI, m/e 168 (213 – OEt)$^+$.

Ethyl 9-isocyanatononanoate (**2d**) from **1d** (6.13 g, 25.8 mmol): yield 3.55 g (61%); bp 88–92 °C/(0.175 mm); MS-FD, m/e 228 $(M + 1)^+$.

General Procedures for Preparation of Isothiocyanates. Compounds **3a–d** were prepared by the method of Floch and Kovac in which a solution of **1** (5–6 g) in a mixture of H_2O (20 mL) and $CHCl_3$ (10 mL) was treated with a solution of thiophosgene (5% excess) in $CHCl_3$ while neutralizing the generated HCl with 2 N NaOH.[14]

Ethyl 6-isothiocyanatocaproate (**3a**) from **1a** (5.00 g, 25.6 mmol): yield 4.47 g (87%); bp 91–92 °C/(0.15 mm) [lit.[20] bp 92–95 °C/(0.04 mm)].

Ethyl 7-isothiocyanatoheptanoate (**3b**) from **1b** (5.40 g, 25.7 mmol): yield 4.93 g (89%); bp 100–103 °C/(0.25 mm). Anal. ($C_{10}H_{17}NO_2S$) C, H, N.

Ethyl 8-isothiocyanatocaprylate (**3c**) from **1c** (5.20 g, 23.2 mmol): yield 3.43 g (65%); bp 116–119 °C/(0.35 mm). Anal. ($C_{11}H_{19}NO_2S$) C, H, N.

Ethyl 9-isothiocyanatononanoate (**3d**) from **1d** (6.35 g, 26.7 mmol): yield 3.48 g (54%); bp 123–125 °C/(0.4 mm). Anal. ($C_{12}H_{21}NO_2S$) C, H, N.

General Procedures for Isocyanate and Isothiocyanate Reactions. A. 5-[[[6-(Ethoxycarbonyl)hexyl]amino]carbonyl]-5,6,7,8-tetrahydrofolic Acid (**7**). A solution of 5,6,7,8-tetrahydrofolic acid (2.07 g, 4.07 mmol)[7,10] and NaOAc·$3H_2O$ (2.04 g, 15.0 mmol) in deoxygenated H_2O (130 mL) was treated with **2b** (1.05 g, 5.29 mmol), stirred vigorously under N_2 for 4 h, treated with $CaCl_2$ (859 mg, 7.74 mmol), adjusted to pH 7.5 with 10% NaOH, and diluted with EtOH (600 mL). The precipitate of calcium salt was collected by filtration, washed with 5:1 EtOH–H_2O, and dried in vacuo. A stirred solution of the calcium salt in deoxygenated H_2O (130 mL) was treated dropwise with 1 N HCl to give a pH of 4.6. The mixture was filtered, and the filtrate was adjusted to pH 4.0 with 1 N HCl. The product was collected, washed with H_2O at pH 4.6, and dried in vacuo (P_2O_5): yield 548 mg.

The following products were isolated from solutions of the calcium salt in water at the indicated pH: **4** (pH 3), **8** (pH 4), **10** (pH 1.6), **13** (pH4), and **14** (pH 3.5). Compound **12** was isolated at pH 3.7 and then purified by reprecipitation from a DMSO solution by the addition of water. Compound **11** was characterized as the calcium salt. After the reaction mixture was filtered to remove byproducts, both **5** and **6** were isolated directly from the filtrates at pH 3 and pH 2, respectively.

B. 5-[[[8-(Ethoxycarbonyl)octyl]amino]thioxomethyl]-5,6,7,8-tetrahydrofolic Acid (**15**). A solution of 5,6,7,8-tetra-

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hydrofolic acid (1.73 g, 3.60 mmol)[7,10] in deoxygenated DMAC (10 mL) was treated under N_2 with **3d** (1.31 g, 5.40 mmol). After being stirred in a stoppered flask for 24 h, the reaction mixture was diluted with Et_2O (100 mL) and refrigerated. The liquid was decanted, and the gummy residue was stirred with H_2O (90 mL) under N_2 while a pH of 8 was maintained by dropwise addition of a suspension of CaO in H_2O. A homogenizer was used to disperse the lumps. When the pH of the suspension remained constant at pH 8, the calcium salt was collected by filtration, washed with H_2O, and dried in vacuo (P_2O_5): yield 1.68 g.

To isolate **9**, the reaction mixture was diluted with acetone (300 mL) and ether (100 mL). The resulting precipitate was dissolved in DMAC (25 mL), the solution was treated with CaO (156 mg), the mixture was filtered, and the filtrate was diluted with acetone: yield 527 mg.

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Registry No. **1a**, 3633-17-8; **1b**, 29840-65-1; **1c**, 29833-31-6; **1d**, 111823-29-1; **2a**, 5100-36-7; **2b**, 78241-56-2; **2c**, 78241-55-1; **2d**, 78241-57-3; **3a**, 16424-03-6; **3b**, 111823-30-4; **3c**, 111823-31-5; **3d**, 111823-32-6; **4**, 72973-87-6; **5**, 72988-03-5; **6**, 111823-23-5; **7**, 111823-24-6; **8**, 111823-25-7; **9** (Ca salt), 111848-30-7; **9** (free acid), 111823-33-7; **10**, 72973-88-7; **11** (Ca salt), 111848-31-8; **11** (free acid), 72973-89-8; **12**, 111823-26-8; **13**, 111823-27-9; **14**, 111823-28-0; **15** (Ca salt), 111848-32-9; **15** (free acid), 111823-34-8; $Br(CH_2)_8COOH$, 41059-02-3; $H_2N(CH_2)_8COOH$, 1120-12-3; $ClCH_2CH_2NCO$, 1943-83-5; EtNCS, 542-85-8; PhNCS, 103-72-0; 5,6,7,8-tetrahydrofolic acid, 135-16-0.

Additions and Corrections

1988, Volume 31

Vittoria Colotta, Lucia Cecchi,* Guido Filacchioni, Fabrizio Melani, Giovanna Palazzino, Claudia Martini, Gino Giannaccini, and Antonio Lucacchini: Synthesis, Binding Studies, and Structure–Activity Relationships of 1-Aryl- and 2-Aryl[1]benzopyranopyrazol-4-ones, Central Benzodiazepine Receptor Ligands.

Page 1. This manuscript was published as a Communication. It should have been published as a Note.