# Folate Analogues. 21. Synthesis and Antifolate and Antitumor Activities of $N^{10}$-(Cyanomethyl)-5,8-dideazafolic Acid[1]

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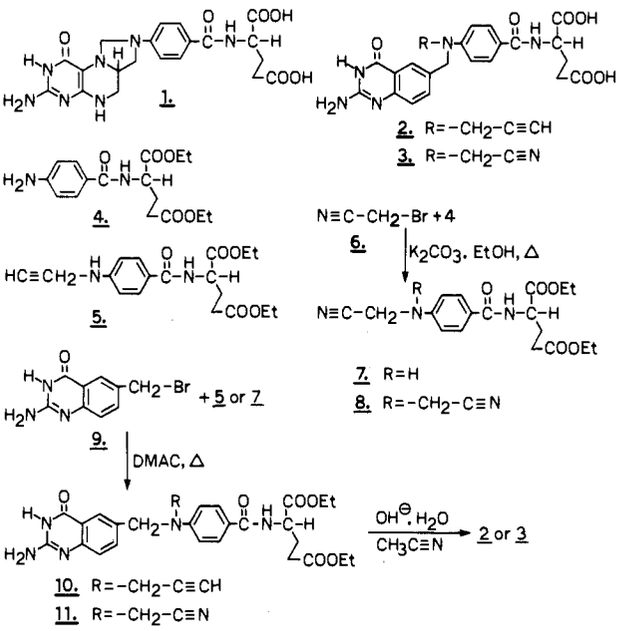
A close analogue of the antileukemic agent 5,8-dideaza-$N^{10}$ propargylfolic acid (**2**) was synthesized by replacing the propargyl moiety of **2** with a cyanomethyl group. This compound, $N^{10}$-(cyanomethyl)-5,8-dideazafolic acid (**3**), was evaluated for its antifolate and antitumor activities in several biological test systems. Alkylation of diethyl *N*-(4-aminobenzoyl)-L-glutamate with bromoacetonitrile gave diethyl *N*-[4-[(cyanomethyl)amino]benzoyl]-L-glutamate (**7**). Reaction of **7** with 2 amino-6-(bromomethyl)-4-hydroxyquinazoline (**9**) in dimethylacetamide gave the corresponding diethyl ester **11**, which was hydrolyzed to the target compound **3**. The known antileukemic agent **2** was also synthesized for comparative studies by employing a modified procedure, which resulted in a better yield of this product. Both compounds **2** and **3** were evaluated for their antifolate activities by using two folate-requiring microorganisms, *Streptococcus faecium* and *Lactobacillus casei*. They were further evaluated as inhibitors of thymidylate synthase and dihydrofolate reductase derived from the above organisms, as well as for their antitumor activity by using selected tumor cells in culture. Compound **2** was found to be as equally potent as methotrexate (MTX) against *S. faecium*, and it was an excellent inhibitor of *L. casei* thymidylate synthase. The cyanomethyl analogue **3** was less active than **2** in all the test systems, except the inhibition of dihydrofolate reductase.

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) to thymidylate (dTMP) in which $N^5$,$N^{10}$-methylenetetrahydrofolate (**1**) participates as a cofactor. This is the terminal step of the de novo synthesis of thymidine nucleotides required exclusively for DNA biosynthesis. Because of this unique feature of the enzyme, it continues to be a prime target for cancer chemotherapy.[2] Theoretically, two types of inhibitors of thymidylate synthase can be developed. Those, belonging to the substrate class, that are analogues of dUMP are well known and are currently being used as anticancer drugs against various forms of human cancers.[2,3] However, powerful inhibitors of TS, belonging to the coenzyme class, are relatively rare,[4] except for the recently described 5,8-dideaza-$N^{10}$-propargylfolic acid (**2**), which was shown to be an excellent inhibitor of the L1210 enzyme.[5] The actual mechanism by which **2** inhibits TS is not clear. Presumably, this inhibition, which is competitive with respect to $N^5$,$N^{10}$-methylenetetrahydrofolate, is due to the fact that **2** is a transition-state analogue of the coenzyme and that the propargyl substitution at the $N^{10}$-position increases the affinity of this drug toward the catalytic site of the enzyme.[5] Although **2** was considerably less active than methotrexate in vitro against the growth of L1210 tumor cells in culture,[5] at higher doses it was shown to be an excellent antitumor agent in mice bearing L1210 leukemia. However, in preclinical toxicology studies with mice, precipitation of the drug in the nephron and subsequent renal failure was observed.[6] Therefore, it was of interest to synthesize and evaluate analogues of **1** and **2** with a view to develop better anticancer drugs, which are TS inhibitors belonging to the coenzyme class. In this report, we describe the chemical synthesis and preliminary biological evaluation of $N^{10}$-(cyanomethyl)-5,8-dideazafolic acid (**3**), which is a close analogue of the antileukemic agent **2**.

Diethyl *p*-aminobenzoyl-L-glutamate (**4**) was commercially available. The propargyl derivative **5** was prepared by reacting **4** with propargyl bromide and chromatographic purification of the reaction products. Reaction of **4** with bromoacetonitrile (**6**) in alcoholic $K_2CO_3$ resulted in the formation of two products, **7** and **8**. The relative ratios of these products varied with the reaction conditions. Under a precise set of conditions, the yield of the desired product **7** could be improved to a maximum of 60%, which was accompanied by the formation of 30% of the dialkylation product **8**. These two products were separated by column chromatography (Scheme I).

Scheme I




The preparation of the required 6-(bromomethyl)-quinazoline (**9**), which was used as a common intermediate for the synthesis of both **2** and **3**, was carried out according to published procedures.[7] Reaction of **9** with either **5** or

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(1) For the previous paper in this series, see Nair, M. G.; Otis, E. B.; Kisliuk, R. L.; Gaumont, Y. *J. Med. Chem.* **1983**, *26*, 135.
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Table I. Antimicrobial Activities of $N^{10}$-Propargyl-5,8-dideazafolic Acid (2), $N^{10}$-(Cyanomethyl)-5,8-dideazafolic Acid (3), and Methotrexate (MTX)

| | ng/mL for 50% inhibition | | | |
|---|---|---|---|---|
| compd | *S. faecium* (ATCC 8043) | *S. faecium* MTX resistant | *L. casei* (ATCC 7469) | *L. casei* MTX resistant |
| 2 | 0.12 | 18.0 | 0.29 | 8 000 |
| 3 | 1.00 | 180.0 | 0.70 | >10 000 |
| MTX | 0.12 | 2900.0 | 0.017 | >500 000 |

Table II. Enzyme Inhibitory Properties of $N^{10}$-Propargyl-5,8-dideazafolic Acid (2), $N^{10}$-(Cyanomethyl)-5,8-dideazafolic Acid (3), and Methotrexate (MTX)

| | concentration for 50% inhibition, M | | | |
|---|---|---|---|---|
| | dihydrofolate reductase | | thymidylate synthase | |
| compd | *S. faecium* | *L. casei* | *S. faecium* | *L. casei* |
| 2 | $3 \times 10^{-7}$ | $2.2 \times 10^{-5}$ | $1 \times 10^{-8}$ | $1.3 \times 10^{-8}$ |
| 3 | $4.6 \times 10^{-7}$ | $2.6 \times 10^{-6}$ | $1 \times 10^{-6}$ | $6.4 \times 10^{-7}$ |
| MTX | $1.2 \times 10^{-9}$ | $8.0 \times 10^{-9}$ | $1.5 \times 10^{-4}$ | $6.0 \times 10^{-5}$ |

7, at elevated temperatures in dimethylacetamide and subsequent workup gave the corresponding diethyl esters 10 and 11 in 50% yield. Saponification of these diethyl esters with NaOH in aqueous acetonitrile, followed by acidification of the hydrolysate, gave the target compounds 2 and 3 in good yield. The UV spectra of 2 were identical with those reported by Jones and co-workers.[5] The NMR spectra of both 2 and 3 in TFA were very similar, and the observed resonances were in complete agreement with those expected for structures 2 and 3. The UV spectrum of 3 in 0.1 N NaOH was also very similar to that of 2.

The antifolate activities of compounds 2 and 3 were investigated with the use of two folate-requiring microorganisms. They were the methotrexate-sensitive and -resistant strains of *Lactobacillus casei* (ATCC 7649) and *Streptococcus faecium* (ATCC 8043). It can be seen from Table I that $N^{10}$-propargyl-5,8-dideazafolate (2) exhibited strong antifolate activity against both these organisms. For *S. faecium* it was as potent an inhibitor as methotrexate. The cyanomethyl analogue 3 was one-half as potent as 2 against *L. casei* and approximately 10 times less active as methotrexate or 2, against *S. faecium*. Compound 2 also shows significant activity against the methotrexate-resistant strain of *S. faecium*.

Next, the inhibitory potencies of 3 toward dihydrofolate reductase (EC 1.5.1.3) and thymidylate synthase derived from both *S. faecium* and *L. casei* were investigated, and these data were compared with those of 2 and methotrexate. These results are summarized in Table II. The propargyl derivative 2 was a very potent inhibitor of TS from both *S. faecium* and *L. casei*. With dihydrofolate reductase, it is 100 times more inhibitory to the *S. faecium* enzyme than to the *L. casei* enzyme. Compound 3 was 100 times more effective an inhibitor of TS derived from both species than methotrexate, although it was less effective than 2 in all of the systems tested above.

Finally, the antitumor activities of [illegible]ainst the growth of selected tumor cells in culture were evaluated. These results were compared with the antitumor activities of both 2 and methotrexate under identical conditions and are shown in Table III. Compounds 2 and 3 were much less toxic to these cell lines than methotrexate, a result which was expected on the basis of their sluggish inhibition of dihydrofolate reductase. However, if one compares the inhibitory potency of 3 with the known antileukemic agent 2 against the most responsive L1210 tumor, it can be seen from Table III that the former is 5 times less active than 2. It has also been revealed that 3 is much less effective as an inhibitor of both S180 and Ehrlich tumor cells in culture than the propargyl analogue.

Table III. Antitumor Activities of $N^{10}$-Propargyl-5,8-dideazafolic Acid (2), $N^{10}$-(Cyanomethyl)-5,8-dideazafolic Acid (3), and Methotrexate (MTX)[a]

| | growth inhibn ($IC_{50}$),[b] μM | | |
|---|---|---|---|
| compd | L1210 | S180 | Ehrlich |
| 2 | 1.17 | 1.73 | 1.82 |
| 3 | 5.52 | 12.3 | 15.2 |
| MTX | 0.00084 | 0.0054 | 0.0126 |

[a] For methods, see Sirotnak, F. M. *Pharmacol. Ther.* **1980**, *8*, 71. [b] $n = 3 \pm$ SE (>20%).

It is apparent from these results that the replacement of the propargyl group with a cyanomethyl group at the $N^{10}$-position of the 5,8-dideazafolate framework resulted in a decrease in biological activity. Since the cyanomethyl group is comparatively more polar than the propargyl group, it appears that the introduction of polar substituents at this position decreases the binding affinity of such analogues to the catalytic site of TS. However, such polarity differences did not appear to affect the binding of these analogues to dihydrofolate reductase. Further modifications of the $N^{10}$-position of the 5,8-dideazafolate framework with less polar functionalities, such as cyclopropyl, cyclopropylmethyl, and methylpropyne, would appear to result in the development of better inhibitors of thymidylate synthase of the coenzyme class.

## Experimental Section

Melting points were determined on a Fisher Model 355 digital melting point analyzer. NMR spectra were run in $CDCl_3$ or $CF_3COOH$ on a 90-MHz Perkin-Elmer R-32 spectrometer with $Me_4Si$ as internal lock signal. Field strengths of the various proton resonances are expressed in parts per million, and coupling constants are in hertz. Peak multiplicity is depicted as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broadened singlet or unresolved multiplet; c, complex set of signals, the center of which is given. UV spectra were determined on a Beckman Model 25 spectrophotometer. Ion-exchange chromatography was carried out on DEAE-cellulose in the chloride form with 2.5 × 20 cm packing unless otherwise specified. A linear NaCl gradient of 1 L each of 0–0.5 M NaCl in 0.005 M phosphate buffer pH 7 was used to elute the column. Elemental analyses were by Galbraith Laboratories, Inc., Knoxville TN. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements or functions were within ±0.4% of the theoretical values.

**Preparation of Diethyl *N*-[4-[(Cyanomethyl)amino]benzoyl]-L-glutamate (7).** In a 100-mL round-bottomed flask fitted with a reflux condenser were added 3.22 g (10 mmol) of diethyl *p*-aminobenzoyl-L-glutamate and 1.38 g (10 mmol) of powdered potassium carbonate. After 30 mL of reagent alcohol was added to this mixture, it was stirred at 70 °C with the gradual addition of 1.2 g (10 mmol) of bromoacetonitrile during a period of 5 min and was vigorously stirred for an additional 3 h at this temperature. TLC examination at this point (silica gel GF, 3% MeOH/$CHCl_3$) revealed the formation of two products, both more polar than the starting material, in a roughly 3:1 ratio. Careful control of the reaction temperature is critical to the formation of the desired monoalkylation product. Further reaction, under these conditions, resulted in the formation of more of the dialkylation product. The solvent was removed by rotary evaporation

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at 50 °C, and the residue was extracted in 100 mL of ethyl acetate; the ethyl acetate layer was washed three times with 30-mL portions of distilled water, dried over anhydrous sodium sulfate, and evaporated to obtain the crude product, which was chromatographed over 150 g of silica gel CC7. [The column was made in hexane, the sample was applied in $CH_2Cl_2$ (10 mL), and the column was eluted with 3:1 methylene chloride and hexane.] The desired product **7** was completely separated from the dialkylation product **8** by this procedure. However, all fractions containing **7** were contaminated with the starting material. Therefore, these fractions were combined and rechromatographed on a Florisil column, with methylene chloride as the eluting solvent. The pure monoalkylation product **7** was obtained in ~40% yield by this procedure. It was recrystallized from minimum methylene chloride and hexane: mp 135 °C; NMR ($CCDl_3$) δ 7.8 and 6.8 (2 d, 4 H, aromatic) 4.25 (t, 1 H, α proton of glutamate), 4.27 (s, 2 H, cyanomethyl) 4.25 (c, 4 H, ethoxy), 2.5–2.3 (c, 4 H, glutamate), 1.3 (2 t, 6 H, ethoxys). The NMR spectrum of the dialkylation product **8** was very similar to that of **7**, except that the two-proton singlet at δ 4.27 was replaced with a four-proton singlet. Anal. ($C_{18}H_{23}N_3O_5$) C, H, N, O.

**Preparation of $N^{10}$-(Cyanomethyl)-5,8-dideazafolic Acid (3).** The preparation of 2-amino-6-(bromomethyl)-4-hydroxyquinazoline (**9**) was accomplished by procedures described in the literature.[7] The alkylation was carried out by stirring 254 mg (1 mmol) of **9** and 360 mg (1 mmol) of **7** in a 20-mL test tube with 4 mL of dimethylacetamide at 110 °C for 18 h under nitrogen. The contents of the test tube were poured into 50 g of crushed ice and triturated. The creamy white solid thus obtained was filtered and dried. This compound **11** showed only a single spot on the TLC plate (silica gel GF, 5% MeOH/$CHCl_3$), and no trace of either of the starting materials was detected: yield 285 mg (57%).

Hydrolysis of **11** was accomplished by stirring the entire amount of **11** in a mixture of 200 mL of 0.1 N NaOH and 35 mL of acetonitrile for 16 h at 25 °C. The solution was concentrated to ~150 mL by rotary evaporation at 30 °C under vacuum, and the turbid solution thus obtained was filtered. The clear filtrate was acidified to pH 3.5 with glacial HOAc and chilled overnight, whereupon cream-colored crystals of **3** were formed. These crystals were filtered, and they were washed with distilled water and dried: yield 200 mg (79%). The compound eluted as a single UV-absorbing peak from a DEAE chloride column: mp 223–225 °C; NMR (TFA) δ 7.8, 7.5, and 7.1 (c,7 H, aromatic), 4.7 (br, 3 H, cyanomethyl and α proton of glutamic acid), 4.4 (s, 2 H, benzylic), 2.4–2.0 (c, 4 H, glutamate); UV (0.1 N NaOH) max 300, 275 nm. Anal. ($C_{23}H_{22}N_6O_6 \cdot H_2O$) C, H, N, O.

**Preparation of $N^{10}$-Propargyl-5,8-dideazafolic Acid (2).** The propargyl derivative **5** was prepared according to the procedure of Jones.[5] Reaction of **5** with **9** was carried out as described for the cyanomethyl analogue but at a lower temperature of 80 °C. The reaction product **10** was hydrolyzed with 0.1 N NaOH in acetonitrile and worked up as described above. Overall yield from 1-mmol scale reaction was 240 mg (49%): mp 240 °C; UV (0.1 N NaOH) max 301, 276 nm; NMR (TFA) δ 7.9, 7.6, 7.4, and 7.1 (c, 7 H, aromatic) 4.8 and 4.3 (2 s, 4 H, benzylic, propargyl), 4.6 (t, proton of glutamate), 2.4–2.0 (c, 4 H, glutamate).

**Biological Evaluation.** The antimicrobial activities of these compounds were evaluated by procedures that have been published previously.[8,9] All assays with thymidylate synthase were performed according to the procedure of Friedkin.[10] Methods involving dihydrofolate reductase and detailed procedures regarding the evaluation of the growth-inhibitory potency of analogues against various tumor cell lines have been published elsewhere.[11,12]

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