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Notes

Synthesis of Aza Homologues of Folic Acid

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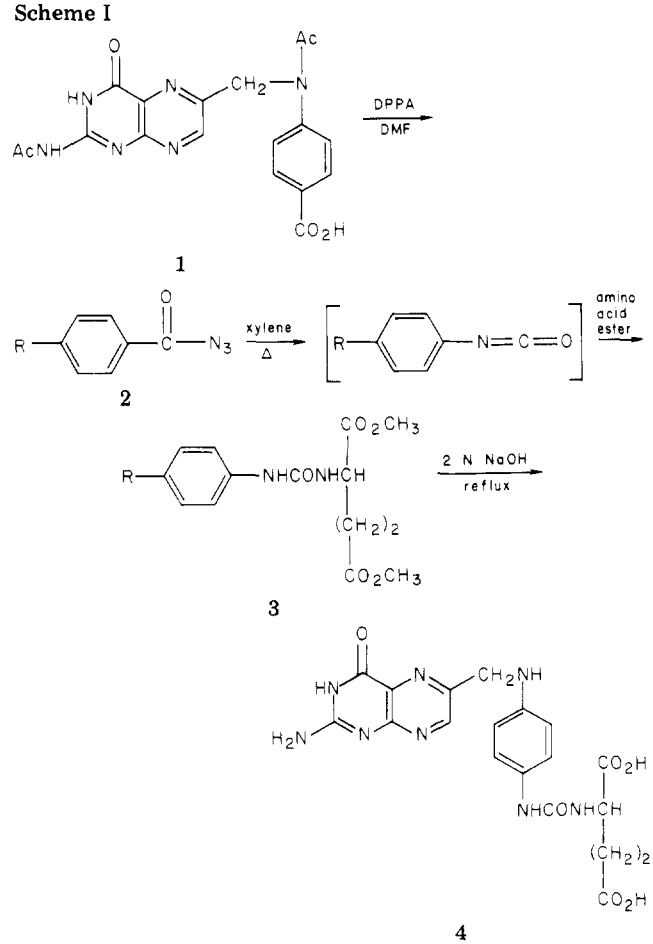
Folic acid analogues containing an additional nitrogen atom between the phenyl ring and the carbonyl group of the side chain were synthesized. None of the compounds showed significant inhibitory activity against human lymphoblastic leukemia cells (CCRF-CEM) in culture or against *Lactobacillus casei* (ATCC 7469) growth. Against L1210 leukemia in mice, the aza homologue of folic acid, **4**, and the aspartic acid analogue, **14**, showed no increase in life span over control animals. These compounds were more toxic in vivo than the corresponding methotrexate analogues. Compound **4** supported the growth of *Streptococcus faecium* (ATCC 8043), and its tetrahydro derivative supported the growth of *Pediococcus cerevisiae* (ATCC 8081). These results strongly suggest that **4** can substitute for folate derivatives as cofactors for serine transhydroxymethylase, thymidylate synthetase, and dihydrofolate reductase.

The development of methotrexate (MTX) analogues with potentially superior clinical properties and whose sole mode of action is inhibition of dihydrofolate reductase (DHFR) is generally held to have little promise.[1,2] However, the design of folate analogues which act as substrates for DHFR,[3] producing "spurious" coenzymes which inhibit other enzymes in the folate cycle, remains an attractive rational strategy[4,5] since its initial proposal.[6] The potent inhibition of thymidylate synthetase and the antitumor activity of several reduced folate, MTX, and quinazoline analogues, in particular homofolic acid derivatives, support this strategy. Accordingly, we have synthesized new aza homologues of folic acid in which an additional nitrogen atom separates the phenyl ring and the carbonyl group.

Chemistry. Folic acid was hydrolyzed with the enzyme carboxypeptidase G to pteroic acid in quantitative yield.[7] Attempts to convert pteroic acid to its pure carbonyl azide with diphenylphosphoryl azide (DPPA) were unsuccessful due to the extremely low solubility of pteroic acid and the carbonyl azide in organic solvents. To improve its solubility, pteroic acid was diacetylated[8] to **1**, which reacted with DPPA in DMF to give **2** in high yield (Scheme I). Photolytic Curtius rearrangement of **2** in the presence of excess dimethyl L-glutamate to yield **3** was impractical because the product was greatly contaminated with the dimethyl folate derivative formed by direct peptide coupling. A similar result precluded the *N*-formylamino protection during the Curtius rearrangement of *p*-aminobenzoyl azide (see below). It is evident that N^{10}-acylation retards resonance deactivation of the carbonyl azide toward nucleophilic attack.[9] Thermolysis of a suspension of **2** in refluxing xylene gave the crude isocyanate which reacted with dimethyl L-glutamate in DMF to give pure **3** in 40% yield. Complete hydrolysis of **3** to the deacylated diacid required refluxing aqueous alkaline conditions and provided racemic **4** in only 10% yield after DEAE-cellulose chromatography. The low overall yield prompted a different approach.

Scheme I



p-Aminophenyl isocyanate, formed by photolysis of *p*-aminobenzoyl azide,[10] reacted in situ with dimethyl

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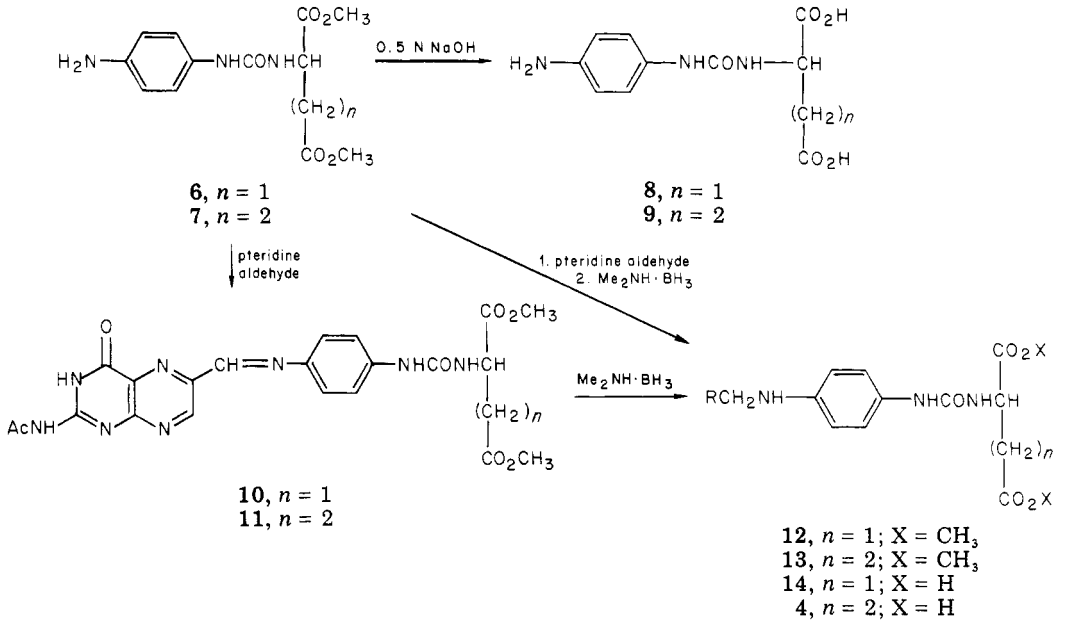
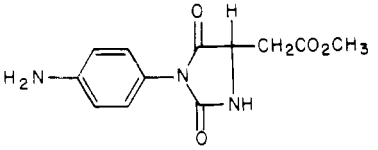
Scheme II



L-aspartate and dimethyl L-glutamate to produce in moderate yields the optically active diesters **6** and **7**, respectively (Scheme II). The 270-MHz NMR spectrum indicated that **7** contained no (<0.2%) dimethyl *p*-aminobenzoylglutamate that formed via direct coupling prior to Curtius rearrangement. Mild alkaline hydrolysis of **6** gave racemic diacid **8**, and similar hydrolysis of **7** gave diacid **9**, which retained partial optical activity. It is probable that hydrolysis of the aspartate analogue **6** proceeds via a hydantoin intermediate, which is rapidly



racemized under the alkaline conditions,[9] while the glutamate **7** is hydrolyzed by a competition of hydrolysis of hydantoin intermediate vs. direct ester hydrolysis.

The diesters **6** and **7** reacted rapidly with 2-acetamido-3,4-dihydro-4-oxopteridine-6-carbaldehyde[11] to give anils **10** and **11**, respectively, which were isolated as a mixture of syn and anti isomers, consistent with previous reports.[11,12] It was more convenient to reduce the anils in situ with a slight excess of dimethylamine–borane to yield the optically active diesters **12** and **13**. Mild alkaline hydrolysis of diester **12** gave racemic diacid **14**, and hydrolysis of **13** gave the aza homologue of folic acid, **4**, almost completely racemized.

Biological Results. Compounds **4** and **10–14** were inactive as inhibitors of human lymphoblastic leukemia cells (CCRF-CEM) in culture[13] at the highest dose tested (10 μg/mL). In comparison, MTX showed 50% inhibition at 0.02 μg/mL. As inhibitors of *L. casei* (ATCC 7469) growth,[14] compound **14** showed 50% inhibition at 1.6 μg/mL, while the others were inactive at 2.0 μg/mL (ID_{50} for MTX = 0.01 mμg/mL). Both **4** and **14** showed minimal inhibition of *L. casei* thymidylate synthetase at $ID_{50} = 1.8 \times 10^{-4}$ M, approximately one-half that of MTX.

Compound **4** supported the growth of *S. faecium* (ATCC 8043) to 2% that of folic acid. The tetrahydro derivative of **4**, purified by DEAE-cellulose chromatography, had a UV spectrum similar to *dl*,L-tetrahydrofolate and supported the growth of *P. cerevisiae* (ATCC 8081) at 0.5% that of *dl*,L-formyltetrahydrofolate (leucovorin, CF). These results provide strong evidence that compound **4** can substitute for folic acid and be converted to derivatives which can substitute for folate derivatives as cofactors for enzymes of thymidylate formation, namely, serine transhydroxymethylase, thymidylate synthetase, and dihydrofolate reductase.

The diacids **4** and **14** were also evaluated for antitumor activity in vivo against L1210 leukemia in mice. Male BDF/1 mice were inoculated ip with 10^5 L1210 leukemia cells. The compounds, dissolved in 0.1 M phosphate buffer (pH 8), were injected ip (20–100 (mg/kg)/injection) 24 h after tumor implantation (day 1) and on days 4 and 7 (qd 1, 4, and 7). Both compounds were found to be toxic at dose levels of 60 mg/kg. At the nontoxic doses, these compounds produced no increase in life span over control animals. It is interesting to note that these compounds were three to seven times more toxic than the corresponding methotrexate analogues.[9]

Experimental Section[9]

4-[[(2-Acetamido-3,4-dihydro-4-oxo-6-pteridinyl)methyl]acetamido]benzoyl Azide (2). To a solution of **1** (15.85 g, 40 mmol) and Et_3N (8.07 g, 80 mmol) in DMF (100 mL) was added diphenylphosphoryl azide (16.5 g, 60 mmol). After stirring for 20 h, the mixture was diluted with H_2O (200 mL) and cooled, and the solid was filtered and washed with H_2O, EtOH, and Et_2O to yield 14.0 g (83%) of almost white solid. For analysis, a sample was chromatographed on silica gel with 10% MeOH–$CHCl_3$: mp 165–170 °C dec; NMR (CF_3CO_2H) δ 2.2 (s, 3 H, N^{10}-CH_3CO), 2.6 (s, 3 H, 2-CH_3CON), 5.35 (s, 2 H, C^9-H_2), 8.0 (A_2B_2 center, J = 10 and 32 Hz, 4 H, arom), 9.22 (s, 1 H, C^7-H). Anal. ($C_{18}H_{15}N_9O_4$) C, H, N.

Dimethyl *N*-[4-[[(2-Acetamido-3,4-dihydro-4-oxo-6-pteridinyl)methyl]acetamido]phenylcarbamoyl]-L-glutamate (3). A suspension of **2** (2.11 g, 5.0 mmol) in xylene (50 mL; bp 138–140 °C) was heated at reflux for 25 min. The mixture was cooled and filtered to give the crude isocyanate. The IR spectrum (KCl) showed isocyanate absorption at 2350 (sh) and 2300 cm^{-1} and no azide absorption.

To a solution of dimethyl L-glutamate hydrochloride (3.16 g, 15 mmol) in DMF (40 mL) was added Et_3N (2.02 g, 20 mmol). After stirring the solution for 5 min, the Et_3N·HCl was filtered off. To the filtrate was added the crude isocyanate, and the yellow solution was stirred for 2 h. The solution was evaporated to dryness with a mechanical pump, and the residue was dissolved in CH_2Cl_2 (100 mL) and filtered to remove some polymeric

material. The filtrate was washed with 0.1 N HCl, dried (Na_2SO_4), and evaporated to leave a pale yellow syrup. This was dissolved in warm CH_3CN (10 mL) and EtOAc (30 mL) was added. Cooling overnight and filtering gave 1.15 g (40%) of white solid: mp 157–165 °C; $[\alpha]^{56}_D$ −4.36 (*c* 2.98, MeOH); NMR (CF_3CO_2H) δ 2.2 (s, 3 H, N^{10}-CH_3CO), 2.3–2.9 (m, 4 H, $-CH_2CH_2-$), 2.56 (s, 3 H, 2-CH_3CON), 4.8 (m, 1 H, α-CH), 5.45 (s, 2 H, C^9-H_2), 7.68 (s, 4 H, arom), 9.4 (s, 1 H, C^7-H). Anal. ($C_{25}H_{28}N_8O_8$) C, H, N.

***N*-[4-[[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]phenylcarbamoyl]-DL-glutamic Acid (4).** A mixture of **3** (2.27 g, 40 mmol) and 2 N NaOH (50 mL) was refluxed under N_2 for 3 h. The solution was diluted with H_2O (50 mL) and acidified with concentrated HCl to pH 3.5. The gelatinous precipitate was centrifuged, washed twice with dilute HCl (pH 3.0), and lyophilized to 1.5 g of tan solid. The solid was stirred with 0.005 M phosphate buffer (1 L, pH 7.0) containing 0.1 M 2-mercaptoethanol and then filtered. The filtrate was placed on a column of Whatman DE-52 cellulose, and the product was gradient eluted with 0.1–0.4 M NaCl in phosphate buffer at pH 7. The desired fractions were pooled, adjusted to pH 3 with concentrated HCl, and centrifuged. The gelatinous residue was washed with dilute HCl (pH 3) and lyophilized to yield 178 mg (9.8%) of rust-colored product: mp 310–320 °C dec; UV (0.1 N HCl) λ_{max} 243 nm (ε 24 030), 280 (sh, 4090), 319 (6830), 360 (sh); UV (0.1 N NaOH) λ_{max} 255 nm (ε 32 540), 366 (9030); NMR (CF_3CO_2D) δ 1.9–2.8 (m, 4 H, $-CH_2CH_2-$), 4.6 (m, 1 H, α-CH), 5.0 (s, 2 H, C^9-H_2), 7.35 (s, 4 H, arom), 8.65 (s, 1 H, C^7-H). Anal. ($C_{19}H_{20}N_8O_6 \cdot 0.5H_2O \cdot 0.5HCl$) C, H, N, Cl. This product was identical (Co-TLC, IR, UV, and NMR) with that obtained from diester **13**.

4-Aminobenzoyl Azide (5). *p*-Aminobenzoic acid and diphenylphosphoryl azide afforded **5** in 71% yield. This compound has been previously described.[10]

Dimethyl *N*-(4-Aminophenylcarbamoyl)-L-glutamate (7) and the L-Aspartate Analogue 6. Dimethyl L-glutamate (0.15 mol) and compound **5** (6.4 g, 0.0395 mol) were photolyzed for 7 h in CH_2Cl_2 (300 mL). The solution was evaporated and the residue was recrystallized twice from EtOAc to yield 3.7 g (0.012 mol, 30%) of **7**: mp 155–155.5 °C; $[\alpha]^{25}_D$ −23.6 (*c* 2.48, 1 N HCl); UV (EtOH) λ_{max} 253 nm (ε 18 160), 298 (1880); NMR (CF_3CO_2D) δ 2.1–2.9 (m, 4 H, $-CH_2CH_2-$), 3.85 (s, 3 H, γ-carbomethoxy), 3.93 (s, 3 H, α-carbomethoxy), 4.8 (m, 1 H, α-CH), 7.55 (s, 4 H, arom). Anal. ($C_{14}H_{19}N_3O_5$) C, H, N.

Similarly, compound **6** was obtained from **5** and dimethyl L-aspartate in 41% yield: mp 160–161 °C; $[\alpha]^{25}_D$ −6.8 (*c* 4.5, 1 N HCl); UV (EtOH) λ_{max} 253 nm (ε 20 960), 300 (2090); NMR (CF_3CO_2D) δ 3.25 (d, *J* = 5 Hz, 2 H, β-CH_2), 3.9 (s, 3 H, γ-carbomethoxy), 4.0 (s, 3 H, α-carbomethoxy), 5.15 (t, *J* = 5 Hz, 1 H, α-CH), 7.65 (s, 4 H, arom). Anal. ($C_{13}H_{17}N_3O_5$) C, H, N.

***N*-(4-Aminophenylcarbamoyl)glutamic Acid (9) and the Aspartate Analogue 8.** A mixture of **7** (996 mg, 3.54 mmol) and 0.5 N NaOH (20 mL) was stirred at room temperature for 3 h. The solution was acidified to pH 3.5 with concentrated HCl, and the precipitate was filtered and dried to yield 670 mg (2.38 mmol, 67%) of **9**: mp 195 °C dec; $[\alpha]^{25}_D$ −7.5 (*c* 2.0, 1 N HCl); UV (0.1 N NaOH) λ_{max} 247 nm (ε 15 290), 290 (sh, 1590); NMR (CF_3CO_2D) δ 2.0–2.8 (m, 4 H, $-CH_2CH_2-$), 4.6 (m, 1 H, α-CH), 7.35 (s, 4 H, arom). Anal. ($C_{12}H_{15}N_3O_5 \cdot 0.5H_2O$) C, H, N.

Similarly, diester **6** was hydrolyzed to racemic **8** in 84% yield: mp 196 °C dec; UV (0.1 N NaOH) λ_{max} 247 nm (ε 15 800), 290 (sh, 1585); NMR (CF_3CO_2D) δ 3.2 (d, *J* = 5 Hz, 2 H, β-CH_2), 4.95 (t, *J* = 5 Hz, 1 H, α-CH), 7.35 (s, 4 H, arom). Anal. ($C_{11}H_{13}N_3O_5 \cdot 0.33H_2O$) C, H, N.

2-Acetamido-3,4-dihydro-4-oxopteridine-6-carbaldehyde. Folic acid was cleaved with bromine and HBr by the method of Thijssen[15] to give 2-amino-3,4-dihydro-4-oxopteridine-6-carbaldehyde. The yield was raised to almost quantitative by K_2CO_3 neutralization of the acidic aqueous solution. The 2-amino group was acetylated with acetic anhydride by the method of Bieri and Viscontini.[11]

Dimethyl *N*-[4-[[(2-Acetamido-3,4-dihydro-4-oxo-6-pteridinyl)methyl]imino]phenylcarbamoyl]-L-aspartate (10) and the L-Glutamate Analogue 11. A solution of 2-acetamido-3,4-dihydro-4-oxopteridine-6-carbaldehyde (466 mg, 2.0 mmol) and diester **6** (650 mg, 2.20 mmol) in acetic acid (10 mL) was heated briefly to 75 °C and then stirred for 30 min while cooling to room temperature. The solution was diluted with Et_2O (50 mL), and the yellow solid was filtered, washed with Et_2O, and dried to yield 795 mg (1.56 mmol, 78%) of **10**: mp 239 °C dec; NMR (CF_3CO_2D) δ 2.55 (s, 2 H, 2-CH_3CON), 3.2 (d, *J* = 5 Hz, 2 H, β-CH_2), 3.9 (s, 3 H, β-carbomethoxy), 4.0 (s, 3 H, α-carbomethoxy), 5.1 (t, *J* = 5 Hz, 1 H, α-CH), 7.55 and 7.9 (s and A_2B_2 center, *J* = 10 and 20 Hz, arom, syn and anti isomers), 9.8 (s and m, 2 H, C^9-H and C^7-H). Anal. ($C_{22}H_{22}N_8O_7$) C, H, N.

Similarly, compound **11** was prepared from the pteridine aldehyde and diester **7** in 66% yield: mp 237 °C dec; NMR (CF_3CO_2D) δ 2.0–2.9 (m, 4 H, $-CH_2CH_2-$), 2.55 (s, 3 H, 2-CH_3CON), 3.9 (s, 3 H, γ-carbomethoxy), 3.98 (s, 3 H, α-carbomethoxy), 4.75 (m, 1 H, α-CH), 7.6 and 7.95 (s and A_2B_2 center, *J* = 10 and 20 Hz, arom, syn and anti isomers), 9.85 (s, 1 H, C^7-H). Anal. ($C_{23}H_{24}N_8O_7$) C, H, N.

Dimethyl *N*-[4-[[(2-Acetamido-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]phenylcarbamoyl]-L-glutamate (13) and the L-Aspartate Analogue 12. A mixture of 2-acetamido-3,4-dihydro-4-oxopteridine-6-carbaldehyde (1.478 g, 6.30 mmol) and diester **7** (1.961 g, 6.34 mmol) in acetic acid (25 mL) was stirred at room temperature for 1 h, followed by the addition of dimethylamine–borane (0.408 g, 6.93 mmol). After 30 min, Et_2O (100 mL) was added and the mixture was then cooled and filtered to yield 2.99 g (5.68 mmol, 89.6%) of **13**: mp 198 °C dec; $[\alpha]^{25}_D$ −16.5 (*c* 0.665, 1 N HCl); NMR (CF_3CO_2D) δ 2.0–2.6 (m, 4 H, $-CH_2CH_2-$), 2.4 (s, 3 H, 2-CH_3CON), 3.7 (s, 3 H, γ-carbomethoxy), 3.80 (s, 3 H, α-carbomethoxy), 4.45 (m, 1 H, α-CH), 5.0 (br s, 2 H, C^9-H_2), 7.4 (s, 4 H, arom), 8.8 (s, 1 H, C^7-H). Anal. ($C_{23}H_{26}N_8O_7$) C, H, N.

Similarly, compound **12** was prepared from the pteridine aldehyde and **6** in 89.5% yield: mp 213 °C dec; $[\alpha]^{25}_D$ −23.6 (*c* 0.55, 1 N HCl); NMR (CF_3CO_2D) δ 2.2 (s, 3 H, 2-CH_3CON), 3.10 (d, *J* = 5 Hz, 2 H, β-CH_2), 3.7 (s, 3 H, β-carbomethoxy), 3.8 (s, 3 H, α-carbomethoxy), 4.85 (t, *J* = 5 Hz, 1 H, α-CH), 5.10 (br s, 2 H, C^9-H_2), 7.35 (s, 4 H, arom), 8.8 (s, 1 H, C^7-H); NMR (Me_2SO-d_6) δ 2.2 (s, 3 H, 2-CH_3CONH), 2.8 (m, 2 H, β-CH_2), 3.7 (d, 6 H, α- and β-carbomethoxy), 4.6 (m, 3 H, α-CH and C^9-H_2), 6.9 (A_2B_2 center, *J* = 7 and 31 Hz, 4 H, arom), 8.9 (s, 1 H, C^7-H). Anal. ($C_{22}H_{24}N_8O_7$) C, H, N.

***N*-[4-[[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]phenylcarbamoyl]-DL-aspartic Acid (14) and -DL-glutamic Acid (4).** A solution of **12** (1.025 g, 2.0 mmol) and 0.1 N NaOH (90 mL) in MeOH (60 mL) was stirred at room temperature under N_2 for 5 h. The pH was then adjusted to 3.5 with concentrated HCl, and the gelatinous precipitate was centrifuged, washed with H_2O (pH 3), resuspended in H_2O (pH 3), and lyophilized to leave 787 mg (1.79 mmol, 89.5%) of **14** as a rust-colored solid: mp 340–355 °C dec; UV (0.1 N HCl) λ_{max} 243 nm (ε 24 030), 319 (6830), 280 (sh, 4210), 360 (sh, 585); UV (0.1 N NaOH) λ_{max} 255 nm (ε 32 540), 366 (9030); NMR (CF_3CO_2D) δ 3.2 (d, 2 H, β-CH_2), 5.1 (m, 3 H, α-CH and C^9-H_2), 7.45 (s, 4 H, arom), 8.9 (s, 1 H, C^7-H). Anal. ($C_{18}H_{18}N_8O_6 \cdot 2H_2O \cdot 0.6HCl$) C, N, Cl; H: calcd, 4.55; found, 3.91.

The diester **13** was hydrolyzed in a similar manner to yield **4** in 60% yield: $[\alpha]^{25}_D$ +0.9 ± 0.2 (*c* 0.65, 0.1 N NaOH). This product was identical with that obtained by hydrolysis of **3**.

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Dipeptides of *O*-Methyl-L-threonine as Potential Antimalarials

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L-Leucyl-*O*-methyl-L-threonine, *O*-methyl-L-threonyl-L-leucine, and *O*-methyl-L-threonyl-*O*-methyl-L-threonine were prepared and compared with *O*-methyl-L-threonine and L-leucine for antimalarial activity against *Plasmodium berghei* in mice. *O*-Methyl-L-threonine significantly prolonged survival time at doses of 160, 320, and 640 mg/kg. *O*-Methyl-L-threonyl-*O*-methyl-L-threonine was less active, significantly prolonging survival time only at 640 mg/kg. L-Leucine, as well as the other two dipeptides, exhibited no activity in this test.

Since protozoans were known to have only limited capability for de novo synthesis of amino acids from simple sources, it is believed that the proteins of intraerythrocytic malarial plasmodia are made primarily from preformed amino acids of the host.[2] Existing evidence indicates that the free amino acid pools of red cells are too small to be significant and that most serum amino acids are not taken up by the plasmodium to form protein.[3,4] Serum proteins are also not utilized, and, therefore, the chief source of parasite protein must of necessity be derived from the hemoglobin of the red cells of the host.[2]

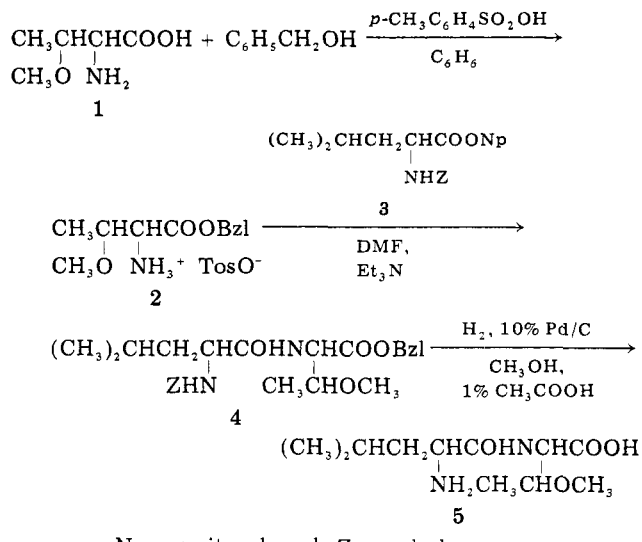
Electron microscopic studies of *Plasmodium lophurae* and *P. berghei* indicated that the parasites feed by phagocytosis of hemoglobin.[5,6] This was further demonstrated by growing *P. lophurae* in duck red cells containing radioactive hemoglobin. Radioactivity was recovered in the proteins of the parasite which were uncontaminated by host cell hemoglobin or parasite pigment.[7] Studies on the nutritional requirements of malarial parasites revealed that L-isoleucine was essential for the growth of the erythrocytic forms of *P. knowlesi*.[8,9]

It was shown that *O*-methyl-L-threonine [L-Thr(Me)], a known isoleucine antagonist,[10] strongly inhibited in vitro growth of *P. knowlesi*. Reversal was achieved with L-isoleucine, and growth repression was attributed to inhibition of protein synthesis.[11,12]

L-Isoleucine and L-leucine are structurally related, and a number of mutually antagonistic effects with these amino acids have been observed. A high L-leucine diet fed to rats results in marked reductions in L-isoleucine in the plasma. Loading human infants with L-leucine caused a marked[13] depression in L-isoleucine concentration in the plasma.[14]

A rational approach toward the control of the *Plasmodia*, causing malaria, may be made by interfering with the nutrition of the parasite. Since it was found that *P. knowlesi* requires L-isoleucine and hemoglobin is known to be a poor source of this amino acid, L-isoleucine must be obtained from the plasma of the host. The malaria-parasitized erythrocyte takes up 150 times as much L-isoleucine as the normal red cell.[15] The parasite thus appears to be vulnerable at several stages of its nutrition: proteolysis of the host's proteins, synthesis of its own proteins, and amino acid transport.

Scheme I. L-Leucyl-*O*-methyl-L-threonine [L-Leu-L-Thr(Me)] (5)



Np = *p*-nitrophenyl; Z = carbobenzoxy

Pertinent facts concerning the role of peptides in biological systems were summarized by Meister.[16] Amino acids in peptide linkage are protected from destructive reactions that are specific for the corresponding free amino acids. Thus, gradual hydrolysis of a peptide can be a more efficient source of an amino acid than an equivalent quantity of the free amino acid. Certain peptides are transported across membranes more effectively than their constituent amino acids. When an amino acid antagonist exerts its effect on transport, peptides transported by another permeation mechanism would be expected to relieve the inhibition due to the antagonism, provided the

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