at least 45 min. The residual activity was tested twice with 1.5 × 10^{-7} M 1 about 45 min apart. Reference tissues were treated exactly the same way; however, the irradiation was omitted.

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Notes

Lysine and Ornithine Analogues of Methotrexate as Inhibitors of Dihydrofolate Reductase[1]

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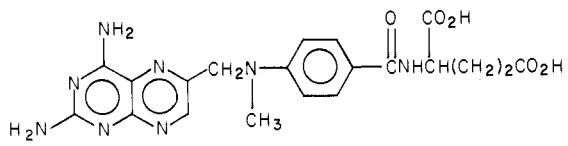
The ornithine (**6a**) and lysine (**6b**) analogues of methotrexate (1) have been synthesized via condensation of 4-amino-4-deoxy-N^{10}-methylpteroic acid (**2**) with N^{δ}-carbobenzoxy-L-ornithine *tert*-butyl ester (**3a**) and N^{ϵ}-carbobenzoxy-L-lysine *tert*-butyl ester (**3b**), respectively. Removal of the protecting groups gave **6a** and **6b**. Compounds **6a** and **6b** and their precursor Cbz acids (**5a** and **5b**) show significant inhibition of dihydrofolate reductase.

Methotrexate (MTX, **1**) has been employed in the

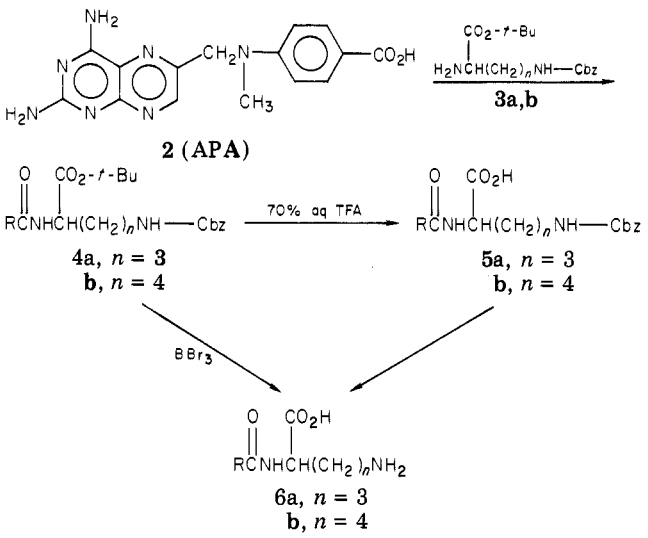


1 (methotrexate)

chemotherapeutic treatment of leukemias, lymphomas, psoriasis, and other clinical disorders.[2] The major intracellular receptor for MTX appears to be dihydrofolate reductase (DHFR). Inhibition of the enzyme by MTX depletes the tetrahydrofolate pool, resulting in a decreased synthesis of thymidylate and, in turn, an inhibition of DNA synthesis. One of the major clinical problems associated with MTX therapy is the development of resistance to the drug.[3,4] Furthermore, MTX in vivo forms polyglutamates via the γ-carboxylate, resulting in prolonged intracellular retention of the drug and additional toxicity.[5] These clinical findings have prompted the synthesis of a variety of analogues of MTX, with a significant portion of the work directed toward modification of the glutamate moiety in MTX. We wish to report the synthesis of the ornithine (**6a**) and lysine (**6b**) analogues of methotrexate and preliminary studies of the ability of these compounds and their precursors to inhibit dihydrofolate reductase.

Chemistry. Carboxypeptidase G_1 cleavage of methotrexate afforded the starting compound for the syntheses, 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA, **2**).[6] We, like others,[7] encountered difficulty in getting APA to dissolve in organic solvents to any appreciable extent. The acid has shown the greatest solubility in *N*,*N*-dimethylformamide (DMF) but only to the extent of about 1 g/100 mL at room temperature.[8] We have found, however, that APA is approximately three times as soluble in *N*-methyl-2-pyrrolidinone as it is in DMF, and on this basis the former solvent was chosen for the following reactions.

Scheme I



Treatment of APA (**2**) with N^{δ}-carbobenzoxy-L-ornithine *tert*-butyl ester (**3a**)[9] and N^{ϵ}-carbobenzoxy-L-lysine *tert*-

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(1) Presented in part at the 28th IUPAC Congress, Vancouver, British Columbia, Canada, Aug 16–21, 1981.
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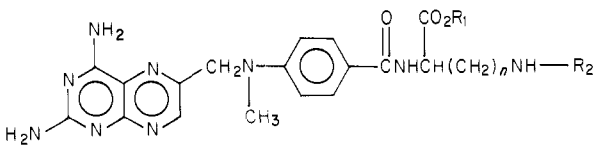
(8) Dr. Andre Rosowsky has recently reported that the solubility of APA in DMF is greatly improved if the acid is isolated by precipitation from aqueous solution using acetic acid rather than hydrochloric acid. We thank Dr. Rosowsky for this personal communication.

0022-2623/82/1825-0475$01.25/0

Table I.



compd	R_1	R_2	n	I_{50},[a] $\times 10^8$ M
1 (MTX)				9
4a	*t*-Bu	Cbz	3	310
4b	*t*-Bu	Cbz	4	95
5a	H	Cbz	3	33
5b	H	Cbz	4	38
6a	H	H	3	25
6b	H	H	4	13

[a] The enzyme concentration was 18.4×10^{-8} M.

butyl ester (**3b**) using both the mixed anhydride (isobutyl chloroformate, *N*-methylmorpholine) and dicyclohexylcarbodiimide (DCC, 1-hydroxybenzotriazole) methods, with *N*-methyl-2-pyrrolidinone as the solvent, afforded the coupled products **4a** and **4b**, respectively (Scheme I). The yields were slightly higher with the DCC method; however, purification of the products from the mixed anhydride method proved easier, and this became the method of choice for the synthesis of **4**. When either method was used, significant amounts of unreacted starting material were recovered, and the yields based on consumed starting material were variable and never greater than 60%.

Selective deprotection of the carboxylate in **4** was achieved with 70% aqueous trifluoroacetic acid (TFA) for 6 h at room temperature,[10] giving the Cbz acids **5a** and **5b** in good yields. These acids were normally carried on to the next step without purification. They were identified by the absence of a *tert*-butyl resonance in their NMR spectra, solubility in dilute base, and lesser mobility than their precursors on silica gel TLC. No evidence (TLC) of loss of the Cbz protecting group was observed under these conditions. Removal of the Cbz group in **5** proved more difficult. In order to avoid possible lactamization of the resulting amino acid, mild catalytic hydrogenolysis of **5** was first investigated. Reaction of Cbz acids **5** with 10% palladium on charcoal in glacial acetic acid using either hydrogen (normal hydrogenolysis) or 1,4-cyclohexadiene (transfer hydrogenolysis[11]) gave in each case a mixture of products (TLC), several of which stained with ninhydrin. On the other hand, **5** reacted smoothly, if sluggishly (7 days, 25 °C), with neat TFA or with boron tris(trifluoroacetate)(BTFA) in TFA (9 h, 25 °C),[12] giving the ornithine (**6a**) and lysine (**6b**) analogues of methotrexate. Alternately, **6a** and **6b** were prepared in good yield directly from Cbz esters **4a** and **4b**, respectively, by simultaneous removal of both protecting groups with 1 M BBr_3 in methylene chloride.[13]

Biology. The inhibition of chicken liver DHFR by compounds **3–6** was measured spectrophotometrically by following the decrease in absorbance at 340 nm caused by the oxidation of the coenzyme NADPH and the reduction of the substrate dihydrofolic acid by DHFR. The results are shown in Table I. Relative to MTX, both the ornithine (**6a**) and lysine (**6b**) analogues showed considerable affinity for DHFR, with the lysine analogue being nearly as potent an inhibitor of DHFR as MTX itself.

Substitution of the α-carboxyl group of the ornithine derivative as the *tert*-butyl ester (**4a**) decreased the enzyme inhibitory potency ca. 10-fold as compared with the N^{δ}-Cbz derivative (**5a**). The corresponding disubstituted lysyl derivative (**4b**) was a better inhibitor than **4a**, and the N^{ϵ}-Cbz derivative (**5b**) was a 2.5-fold better inhibitor. The presence or absence of a Cbz group on N^{δ} of the ornithine analogue did not significantly affect enzyme inhibition (**5a** vs. **6a**). Removal of the N^{ϵ}-Cbz moiety of the lysine analogue, however, increased inhibitory potency ca. 3-fold (**5b** vs. **6b**). These results agree with the findings of others[14-16] that the γ-carboxylate in MTX can be replaced by groups with considerable electronic and steric variety without significantly reducing the affinity of the molecule for DHFR.

Experimental Section

All reactions were run under an atmosphere of argon. Melting points were taken on a Fisher-Johns apparatus and are uncorrected. Infrared spectra were obtained on a Perkin-Elmer 735 spectrophotometer either on the neat liquid held between sodium chloride plates or as KBr pellets. The ^1H NMR spectra were recorded on a Varian EM 360 spectrometer; chemical shifts are reported in parts per million downfield from a tetramethylsilane internal standard (δ scale). The ^1H NMR data for the assignable resonances are presented in the form: value of signal (peak multiplicity, coupling constant (if any), integrated number of protons).

All analytical and preparative TLC studies were done on Analtech silica gel plates. The data are presented as follows: TLC R_f value (solvent system). The following solvent systems were used: A, 10% methanol in chloroform; B, ethanol–concentrated ammonia, 4:1; C, 2-propanol–water–concentrated ammonia, 8:1:1; D, 2-propanol–methanol–concentrated ammonia, 3:1:1. All compounds which were evaluated for biological activity were tested for homogeneity by TLC and HPLC. The HPLC analyses were run on a Waters 720 system using a 25-cm RP-8 column (Brownlee Labs). Microanalyses were performed by Galbraith Laboratories, Knoxville, TN. Analytical results for the elements indicated were within ±0.4% of the theoretical values.

Standard Enzyme Assay. Dihydrofolate reductase was assayed spectrophotometrically as previously described.[17] Initial rates were derived from the change in absorbance continuously recorded with a Cary Model 219 spectrophotometric system. The assay mixture consists of 0.1 mM dihydrofolate, 0.14 mM NADPH, and 0.05 M potassium phosphate buffer (pH 7.0) in a total volume of 1.0 mL. The standard unit of enzyme activity was calculated from the change in absorbancy at 340 nm using a $\Delta\epsilon$ for the reaction of 12 300 at 22 °C.[18]

Inhibition studies were carried out by perincubating the enzyme and the inhibitor in the assay buffer for 2 min at 20 °C, and residual enzyme activity was determined after the addition of dihydrofolate and NADPH. The remaining activity was expressed as percentage of activity compared to the activity of the enzyme obtained in the absence of inhibitor.

***N*$^{\delta}$-Carbobenzoxy-L-ornithine *tert*-Butyl Ester (3a).** This compound was prepared by a slight modification of the procedure

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of Widmer and Keller-Schierlein.[9] To 162 mL of *tert*-butyl acetate, N^{δ}-carbobenzoxy-L-ornithine (3.00 g, 11.3 mmol) and 70% perchloric acid (1.1 mL) were added. The mixture was stirred until it was homogeneous (ca. 2 h) and allowed to stand for 1 week. The solution was then chilled in an ice bath and extracted five times with ice-cold 0.5 N HCl as rapidly as possible. The combined aqueous layers were immediately made alkaline with ice-cold 1 N NaOH and extracted three times with ether. The ether layers were dried with sodium sulfate and then concentrated to yield 3.22 g (81%) of a colorless viscous oil, which gave one spot on TLC: R_f 0.77 (C), 0.57 (A); NMR (CCl_4) δ 1.40 (s, 9, *t*-Bu), 4.97 (s, 2, $PhCH_2$), 7.23 (s, 5, Ph); IR (neat) 3350 (NH_2), 1735 (ester C=O), 1710 (shoulder, carbamate C=O) cm^{-1}.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{δ}-carbobenzoxy-L-ornithine *tert*-Butyl Ester (4a). To 7 mL of 1-methyl-2-pyrrolidinone was added APA (**2**; 200 mg, 0.638 mmol). The mixture was heated to 80 °C, and the resulting solution was cooled to 0 °C in an ice bath. To the cooled solution were added 64.5 mg (0.638 mmol) of *N*-methylmorpholine and 87 mg (0.638 mmol) of isobutyl chloroformate. The solution was stirred at 0 °C for 20 min and then allowed to warm to room temperature. Then **3a** (400 mg, 1.24 mmol) was added, and the flask was heated at 60 °C for 1 h. The solvent was removed by vacuum distillation, and the residue was partitioned between 0.3 N NH_4OH and $CHCl_3$. The aqueous phase was extracted with $CHCl_3$ until the $CHCl_3$ layer was colorless (four or five extractions). The $CHCl_3$ extractions were dried with sodium sulfate and concentrated. Acidification of the aqueous layer to pH 3 precipitated unreacted starting material. The yellow product was purified on preparative TLC, R_f 0.43 (A), to yield 105 mg (27%; 54% based on consumed starting material): mp 103–112 °C; IR (KBr) 3630–3010 (NH, CH), 1735 (shoulder 1710, C=O) cm^{-1}. Anal. ($C_{32}H_{39}N_9O_5$) C, H, N.

In one experiment, the intermediate isobutyl mixed anhydride was isolated and recrystallized from absolute EtOH: mp 168–175 °C (lit.[7] mp 170–180 °C); TLC R_f 0.70 (A); ^1H NMR ($CDCl_3$/Me_2SO-d_6, 1:1) δ 1.0 [d, J = 6.5 Hz, $C(CH_3)_2$], 1.93 (m, CH), 3.3 (s, NCH_3), 4.05 (d, J = 6.5 Hz, OCH_2), 4.85 (br s, C-9 H_2), 6.87 and 7.85 (AA'BB' pattern, aromatic), 8.62 (s, C_7 H).

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-carbobenzoxy-L-lysine *tert*-Butyl Ester (4b). To 7 mL of 1-methyl-2-pyrrolidinone was added compound **2** (200 mg, 0.638 mmol). The mixture was heated to 80 °C, and the resulting solution was cooled to 0 °C in an ice bath. To the cooled solution were added 129 mg (1.28 mmol) of *N*-methylmorpholine and 87 mg (0.638 mmol) of isobutyl chloroformate. The solution was stirred at 0 °C for 20 min and then allowed to warm to room temperature. Then N^{ϵ}-carbobenzoxy-L-lysine *tert*-butyl ester hydrochloride (**3b**; 462.4 mg, 1.24 mmol) was added, and the flask was heated at 60 °C for 1 h. The yellow product was isolated following the procedure for **4a**: yield 110 mg (31%, 71% based on consumed starting material); TLC R_f 0.67 (C); 0.46 (A); mp 101–112 °C. Anal. ($C_{33}H_{41}N_9O_5 \cdot 1.5H_2O$)C, H, N.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{δ}-carbobenzoxy-L-ornithine (5a). A solution of 20 mL of 70% aqueous trifluoroacetic acid and **3a** (50 mg, 0.08 mmol) was allowed to stand at room temperature for 6 h. The solution was concentrated on a rotary evaporator, redissolved in EtOH–benzene (1:1), and concentrated again to yield 39 mg (86%) of yellow **5a**, which was purified by preparative TLC: R_f 0.74 (B); mp 148–160 °C.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-carbobenzoxy-L-lysine (5b). A solution of 20 mL of 70% trifluoroacetic acid and **4b** (61 mg, 0.095 mmol) was allowed to stand at room temperature for 6 h. The yellow product (**5b**) was isolated as described above for **5a**. Purification by preparative TLC, R_f 0.69 (D), gave 37 mg (67%) of **5b**, mp 178–187 °C.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-ornithine (6a). BTFA Method.[12] A solution of **5a** (100 mg, 0.170 mmol) and of 1 M boron tris(trifluoroacetate)(BTFA) in TFA (1.6 mL) was allowed to stand at 0 °C for 7 h, MeOH was added, and the reaction mixture was evaporated to dryness. Purification was accomplished using preparative TLC, R_f 0.28 (B), to yield 49 mg (64%) of yellow crystals of **6a**: mp 185–200 °C dec; medium-resolution mass spectrum,[19] m/e 421 [$(M - H_2O)^+$], 323, 308, 247, 175.

TFA Method. A solution of **5a** (62 mg, 0.108 mmol) and 8 mL of 100% TFA was allowed to stand at room temperature for 7 days. After the TFA was removed, the residue was redissolved in EtOH and purified as described above to yield 11 mg (23.4%) of **6a**.

BBr_3 Method.[13] A solution of **4a** (45 mg, 0.071 mmol) in 4 mL of CH_2Cl_2 was chilled to −10 °C, and 1 M BBr_3 in CH_2Cl_2 (0.36 mL) was introduced dropwise with stirring. The resulting mixture was stirred for 1 h at −10 °C and then for 2 h at 25 °C, and the reaction was terminated by the addition of cold water. The yellow aqueous layer was separated and lyophilized. Pure **6a** was isolated from the residue by preparative TLC as described above: yield 17 mg (55%). Anal. ($C_{20}H_{25}N_9O_3 \cdot 0.5CF_3CO_2H \cdot 3.5H_2O$) C, N; H: calcd, 5.85; found, 6.30.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine (6b). BTFA Method.[12,20] The Cbz acid **5b** (92 mg, 0.15 mmol) was dissolved in 3.2 mL of TFA, and then 0.86 mL of 1 M BTFA in TFA was added. The solution was allowed to stand at 5 °C for 9 h. The reaction was quenched with MeOH and evaporated to dryness. The residue was purified via preparative TLC, R_f 0.33 (B) or 0.35 (D), to yield 31 mg (44%) of yellow **6b**: mp 180–190 °C dec.

TFA Method. A solution of **5b** (187 mg, 0.318 mmol) in 32 mL of 100% TFA was allowed to stand at room temperature for 7 days. After the TFA was removed, the residue was redissolved in MeOH and purified as above: yield 60 mg (42%).

BBr_3 Method.[13] A solution of Cbz ester **4b** (98 mg, 0.152 mmol) in 4 mL of CH_2Cl_2 was cooled to −10 °C, and a solution of BBr_3 in CH_2Cl_2 (0.76 mL of 1 M; 5 equiv) was added dropwise with stirring. The resulting mixture was stirred at −10 °C for 1 h, then at 25 °C for 2 h, and quenched with cold water. The yellow aqueous layer was separated, washed once with CH_2Cl_2, and lyophilized. The crude product was purified as above: yield 28 mg (41%). Anal. ($C_{21}H_{27}N_9O_3 \cdot 0.5HBr \cdot 2.5H_2O$) C, N; H: calcd, 6.08; found, 6.65.

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(19) This spectrum was run on a Finnigan MAT 731 field desorption instrument which was funded by Grant RR-00317 from the Division of Research Resources, National Institutes of Health, to the Massachusetts Institute of Technology (K. Biemann, principal investigator).

(20) During the preparation of this paper, we became aware of an alternate preparation of **6b**: A. Rosowsky, J. E. Wright, C. Ginty, and J. Uren, in "Abstracts of Papers", 182nd National Meeting of the American Chemical Society, New York, Aug 23–28, 1981, American Chemical Society, Washington, DC, 1981, Abstr MEDI 87.