Synthesis and Characterization of a Fluorescent Analogue of Methotrexate

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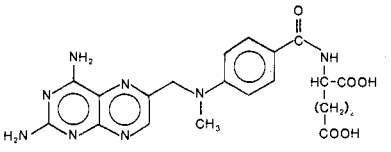
The lysine analogue (4) of methotrexate (1) was treated with 5-(dimethylamino)naphthalene-1-sulfonyl chloride to give the corresponding dansyl analogue 5. The product isolated by preparative TLC was further purified on a preparative HPLC column. Analogue 5 exhibited fluorescence properties characteristic of the dansyl moiety and was a potent inhibitor of dihydrofolate reductase purified from *Lactobacillus casei*, chicken liver, and a human lymphoblastoid cell line.

In the past few years the structural details of the binding interaction of dihydrofolate reductase (EC 1.5.1.3) with the potent anticancer drug methotrexate (MTX, 1) has been elucidated.[1,2]



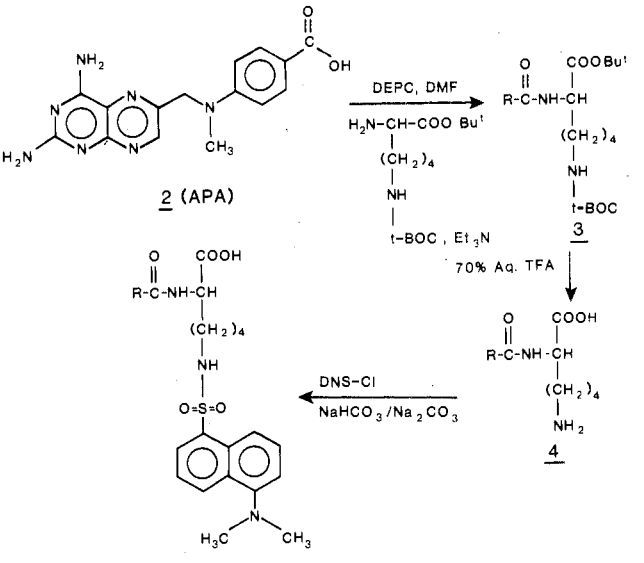
1 (Methotrexate)

Several derivatives[3-9] and analogues[10,11] of MTX have been synthesized with the intention of improving its transport into, and its activity against, MTX-resistant tumor cells. In 1975 the synthesis and properties of a fluorescein conjugate of MTX were reported.[12] The interaction of this derivative with two cell lines and its use as an intracellular marker for the target enzyme dihydrofolate reductase has been investigated by two groups.[13,14] The synthesis of a MTX analogue with a terminal fluorescein moiety was recently reported.[15,16]

We had earlier reported the synthesis and initial characterization of the ornithine and lysine analogues of MTX starting from 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA, 2).[17] The results indicate that lengthening of the carbon chain, as well as replacement of the γ-carboxylate of MTX by an amino moiety, does not significantly lower the affinity of these analogues for dihydrofolate reductase. As a continuation of our work, we report the synthesis of a fluorescent analogue of MTX derived from N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine (4) and 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride, DNS-Cl) and its interaction with the enzyme dihydrofolate reductase.

Our earlier report for the synthesis of the lysine analogue (4) involved condensation of APA (2) with N^{ϵ}-carbobenzoxy-L-lysine *tert*-butyl ester by the mixed anhydride technique, followed by removal of the protecting groups. The use of diethyl phosphorocyanidate (DEPC) to couple amino acids was first reported in 1976.[18] Recently, Rosowsky et al.[8] employed this reagent to bring about the condensation of APA (2) with C-protected amino acids. In the present work, N^{ϵ}-(*tert*-butyloxycarbonyl)-L-lysine *tert*-butyl ester was coupled to DEPC-activated APA (2) (Scheme I). The product (3) was isolated in good yield and was converted to the lysine analogue (4) following the removal of protecting groups with 70% aqueous trifluoroacetic acid. This new synthesis of 4 has several advantages over our previously reported synthesis.[17] First, the DEPC coupling, in our hands, has been found to be more reproducible than either the mixed anhydride or

Scheme I



dicyclohexylcarbodiimide technique, the yield is higher, and its purification is simpler. The use of N^{ϵ}-(*tert*-but-

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Table I. Inhibition of Dihydrofolate Reductases by MTX Analogues

enzyme source	I/E_{100}[a] MTX	4	5
L. casei[b]	1.0	1.0	1.9
WIL2[c]	1.0	1.0	2.9
chicken liver[d]	1.0	1.4	3.5

[a] Molar ratio of inhibitor to enzyme for 100% inhibition. Enzyme was incubated with increasing concentrations of inhibitor and assayed for residual enzyme activity as described under Experimental Section. [b] Enzyme concentration 7.2×10^{-8} M. [c] Enzyme concentration 7.5×10^{-8} M. [d] Enzyme concentration 18.4×10^{-8} M.

yloxycarbonyl)-L-lysine *tert*-butyl ester in place of N^{ϵ}-carbobenzoxy-L-lysine *tert*-butyl ester has produced better yields of 4 under milder conditions. Overall yields of 4 from APA (2) have averaged around 45%. Condensation of the lysine analogue (4) with DNS-Cl under alkaline conditions resulted in the product N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-[5-(dimethylamino)-1-naphthalenesulfonyl]-L-lysine (5, Scheme I).

The structural formula of the fluorescent analogue (5) is shown in Scheme I. TLC and HPLC methods were used to establish the purity of the compound. TLC studies on Whatman KC18F plates indicated the presence of minor impurities, in addition to a major fluorescent spot with an R_f value of 0.64 (precursor R_f 0.72). Hence, the compound was further purified by HPLC on a C-18 column. Compound 5 elutes with a retention time of 68 min. Precursor 4 has a retention time of 31 min.

The chemical identity of the fluorescent analogue (5) was examined by mass spectrometry. The results indicated the presence of a molecular ion with a mass of 725.23843 (calcd for M^+ + potassium 725.23145). The ultraviolet spectrum of 5 in 0.1 N KOH showed absorption peaks at 252, 304, and 370 nm, characteristic of a pteridine moiety. A similar absorption was observed for the precursor 4. Compound 5 in 0.1 M potassium phosphate, pH 7.0, exhibited an emission maximum at 560 nm when excited at 338 nm. An emission maximum of 540–580 nm has been reported for dansyl amino acids when excited at 335 nm in aqueous media.[19]

Biological Activity. Table I shows the results of the interaction of compounds 4 and 5 with dihydrofolate reductase from three different sources. The results are presented as I/E_{100} and indicate the molar excess of a particular analogue required for complete enzyme inhibition. The values for 100% inhibition were obtained by extrapolation of the inhibition profiles, which were linear up to 85 to 90% inhibition. Such I/E_{100} values provide a direct comparison of the relative potencies of MTX analogues to the parent compound without a correction for enzyme concentration. The compounds 4 and 5 were effective inhibitors of the enzyme as compared to MTX. The results also indicate that the fluorescent analogue (5) and its precursor (4) were slightly more effective against the *L. casei* enzyme than for the avian enzyme. The attachment of the bulky dansyl group to the ϵ-amino group of 4 does not significantly alter its ability to inhibit dihydrofolate reductase. The interaction of the dansyl moiety with hydrophobic regions of proteins leads to an enhancement of the fluorescence of the dansyl group. Preliminary results with the chicken liver dihydrofolate reductase have shown that the binding of fluorescent analogue 5 to the enzyme leads to a 4-fold enhancement of the fluorescence of the dansyl group. Experiments are in progress to elucidate in detail the mode of binding of the fluorescent analogue (5) to the dihydrofolate reductase.

Experimental Section

Melting points were taken on a Fisher-Johns apparatus and are uncorrected. Infrared spectra were obtained on a Perkin-Elmer 735 spectrometer either in a KCl solution cell or as KBr pellets. Analytical and preparative TLC studies were done on either Analtech silica gel plates or Whatman KC18F plates.

Reversed-phase analytical and preparative HPLC of compounds 4 and 5 were carried out on a Waters Associates HPLC system using a Waters μBondapak C-18 column and 1-propanol gradients. Prior to HPLC analysis, 4 was purified by ion-exchange chromatography on a CM-Sephadex C-25 column (1.5 × 20 cm; 0.05 M sodium acetate, pH 4.5). Compound 4 was eluted from the column employing a linear gradient of 0.05 M sodium acetate (pH 4.5) to 0.2 M sodium acetate (pH 7.8). The acid hydrolysis of 4 was carried out at 110 °C in evacuated tubes with 6 N HCl. The hydrolyzed sample was analyzed on a Durram D-500 amino acid analyzer.

The visible and ultraviolet absorption spectra were recorded with a Cary 219 spectrophotometer, and the sample compartment was maintained at room temperature (22 °C). The fluorescence emission spectrum of 5 was obtained with a Perkin-Elmer MPF44A spectrofluorometer operated in the ratio mode, with excitation and emission monochromators both being set at 6 nm.

Dihydrofolate reductases from *Lactobacillus casei*, chicken liver, and human lymphoblastoid cell line WIL2, purified by affinity chromatography, were used in the inhibition studies. Dihydrofolate reductase was assayed as previously described.[17] Inhibition studies were carried out by preincubating the enzyme and the inhibitor in the assay buffer for 2 min prior to the addition of NADPH and dihydrofolic acid (FAH_2). The residual activity was determined by measuring the rate of change of absorbance at 340 nm in a Cary 219 spectrophotometer at 22 °C.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(*tert*-butyloxycarbonyl)-L-lysine *tert*-Butyl Ester (3). Triethylamine (0.2 mL, 1.43 mmol) was added to a stirred mixture, at 0 °C, of APA (2; 201 mg, 0.168 mmol) and N^{ϵ}-(*tert*-butyloxycarbonyl)-L-lysine *tert*-butyl ester hydrochloride (308 mg, 0.909 mmol) in dry DMF (60 mL). This was followed by the immediate addition of a solution of diethyl phosphorocyanidate (239 mg, 1.82 mmol) in dry DMF (1 mL). An additional volume of dry DMF (10 mL) was added to increase dissolution. The reaction vessel was flushed with argon, and the mixture was stirred at 0 °C for 2.5 h. At the end of this time, the temperature was raised to 23 °C, and stirring was continued for 18 h. After solvent removal by vacuum distillation, the residue was partitioned between $CHCl_3$ and 10% NH_4OH saturated with NaCl. The organic layer was washed with saturated brine, dried with Na_2SO_4, and evaporated. The residue was redissolved in $CHCl_3$ (1.5 mL) and applied to a silica gel column (2.5 × 30 cm), which was eluted with 9:1 $CHCl_3$–MeOH (500 mL). The analytically pure fractions (as ascertained by silica gel TLC in 9:1 $CHCl_3$–MeOH, R_f 0.36) were combined, evaporated to dryness, and triturated with Et_2O. The resultant yellow solid was filtered, and the residue was washed with Et_2O and dried in vacuo to give 194.5 mg (56.1%) of 3: mp 131–134 °C; IR ($CHCl_3$) 3515, 3410, 1710, 1640, 1605 cm^{-1}; UV (CH_2Cl_2) λ_{max} 257, 303, 370 nm (ε 24 300, 14 700, 7000). Anal. ($C_{30}H_{43}N_9O_5 \cdot 0.5H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine (4). A solution of aqueous TFA (7:3 TFA–H_2O, 29.1 mL) was added to a mixture of 3 (146 mg, 0.239 mmol) in anisole (0.18 mL, 1.66 mmol). After a few minutes of vigorous agitation, the solution was allowed to stand at room temperature for 4.5 h. The solution was then dried by rotary evaporation. The viscous orange residue was dissolved in a minimum of 4:1 EtOH/NH_4OH (concd) and

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chromatographed on a preparative TLC plate [silica gel, 1500 μm, 20 × 20 cm, 3:1:1 *i*-PrOH–MeOH–NH_4OH (concd)]. The main yellow band was eluted with 4:1 EtOH–NH_4OH (concd), and the elutions were rotary evaporated to near dryness. Coevaporation of remaining solvent with 1:1 benzene–EtOH afforded 81.9 mg (75.3%) of **4** as a yellow powder, which was essentially homogeneous on silica gel TLC [3:1:1 *i*-PrOH–MeOH–NH_4OH (concd), R_f 0.28; ninhydrin positive]: UV (0.1 N KOH) λ_{max} 258, 302, 370 nm (ε 25 100, 24 800, 8000); UV (0.1 M potassium phosphate, pH 7.0) λ_{max} 258, 302, 370 nm (ε25 600, 25 100, 8200); UV (30% CH_3COOH, pH 1.8) λ_{max} 304 nm (ε 27 100).

The product was further purified by chromatography on a CM-Sephadex C-25 column. The major peak from the gradient elution was isolated and repurified by HPLC on a preparative C-18 column. Compound **4** emerged from the HPLC column with a retention time of 31 min. Amino acid analysis of the HPLC-purified **4** indicated the presence of lysine (recovery >85%) following hydrolysis in 6 N HCl (110 °C, 20 h).

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-[5-(dimethylamino)-1-naphthalenesulfonyl]-L-lysine (5). A solution of dansyl chloride (144 mg, 0.53 mmol) in 7 mL of acetone was added to a solution of **4** (40 mg, 0.09 mmol) in a 0.5 M $Na_2CO_3/NaHCO_3$ buffer (6 mL, pH 11.5). The homogeneous reaction was allowed to stand for 1.5 h (pH 10.24), and the solvents were removed by rotary evaporation. Triturating with benzene removed excess dansyl chloride. The residue was taken up in DMF and purified by preparative reversed-phase TLC (Whatman KC18F, with preadsorbent area, 3:1 methanol–2% aqueous acetic acid). A sharp yellow band at R_f 0.64 (ninhydrin negative; precursor R_f 0.72) was isolated, and the product was separated from the stationary phase with methanol. Removal of the solvent gave 13 mg (22%) of **5**: mp 190–198 °C (glassy melt); UV (0.1 N KOH) λ_{max} 252, 304, 370 nm (ε 15 300, 13 200, 3600); UV (0.1 M potassium phosphate, pH 7.0) λ_{max} 252, 302, 370 nm (ε 17 000, 12 100, 3800); UV (30% CH_3COOH, pH 1.8) λ_{max} 296 nm (ε 15 300). Fluorescence: λ excitation 338 nm, λ emission 560 nm in 0.1 M potassium phosphate, pH 7.0. Mass spectrum[20] calcd for $C_{33}H_{38}N_{10}O_5S$ plus potassium, 725.23145; found, 725.23843.

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Registry No. **2**, 19741-14-1; **3**, 83399-36-4; **4**, 80407-56-3; **5**, 83416-29-9; N^{ϵ}-(*tert*-butyloxycarbonyl)-L-lysine *tert*-butyl ester hydrochloride, 13288-57-8; dansyl chloride, 605-65-2; dihydrofolate reductase, 9002-03-3.

(20) 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). We thank Dr. Catherine E. Costello for performing the analysis.

2-(Hydroxyphenyl)indoles: A New Class of Mammary Tumor Inhibiting Compounds

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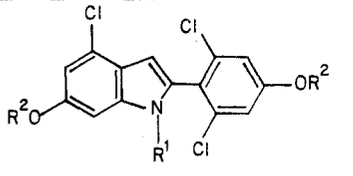
1-Alkyl-4-chloro-2-(2,6-dichloro-4-hydroxyphenyl)-6-hydroxyindoles (**4**, alkyl = CH_3, C_2H_5, C_3H_7) were synthesized by thermolysis of the corresponding *N,N'*-dialkyl-1,2-diphenylethylenediamines and subsequent ether cleavage. They showed an affinity for the estrogen receptor (1% of 17β-estradiol) and inhibited the growth of the 9,10-dimethyl-1,2-benz[*a*]anthracene (DMBA) induced mammary carcinoma of the Sprague-Dawley (SD) rat. The best result was obtained by the ethyl compound (**4b**), which reduced the original tumor area by 50% after 4 weeks administration of 6 × 18 (mg/kg)/week. Since **4a** and **4b** show uterotrophic activity and cytostatic effects against hormone-independent cells, a dual mode of action has to be considered for the tumor inhibition.

In previous papers we have reported attempts to develop antiestrogens for the treatment of hormone-dependent mammary carcinoma by replacing the methylene groups in the synthetic estrogen hexestrol by isosteric imino groups. The resulting 1,2-diarylethylenediamines showed either a too weak antitumor activity[1,2] or proved to be true estrogens.[3] Starting from these ethylenediamines, we searched for another appropriate structure with an affinity for the estrogen receptor and a growth-inhibiting effect on the mammary carcinoma.

From previous studies,[4] we knew that *o*-chloro-substituted diphenylethylenediamines can be converted into 2-phenylindoles. Thermolysis of *meso*- or (±)-*N,N'*-dialkyl-1,2-bis(2,6-dichloro-4-methoxyphenyl)ethylenediamines (**1**)[3] at 215 °C afforded 1-alkyl-4-chloro-2-(2,6-dichloro-4-methoxyphenyl)-6-methoxyindoles (**3**). The free phenolic compounds **4a–c** were obtained by ether cleavage with BBr_3 (Scheme I).

Table I. 1-Alkyl-4-chloro-2-(2,6-dichloro-4-hydroxyphenyl)-6-hydroxyindoles (**4**) and Dimethyl Ether Derivatives (**3**)



compd	R^1	R^2	mp, °C	formula	RBA,[a] %
3a	CH_3	CH_3	145–146	$C_{17}H_{14}Cl_3NO_2$	
3b	C_2H_5	CH_3	157–158	$C_{18}H_{16}Cl_3NO_2$	
3c	C_3H_7	CH_3	134–136	$C_{19}H_{18}Cl_3NO_2$	
4a	CH_3	H	215–216	$C_{15}H_{10}Cl_3NO_2$	1.3
4b	C_2H_5	H	126–129	$C_{16}H_{12}Cl_3NO_2$	1.9
4c	C_3H_7	H	70–72	$C_{17}H_{14}Cl_3NO_2$	1.0

[a] Relative binding affinities for the calf uterine estrogen receptor = ratio of molar concentrations of 17β-estradiol (E2) and inhibitor required to decrease the amount of bound [^{3}H]E2 by 50% × 100.

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