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Received June 25, 1979

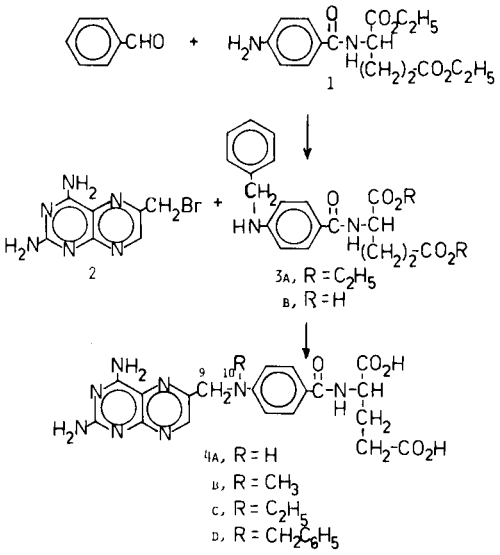
Substitution of a benzyl group for the methyl at N-10 of Methotrexate caused only a slight decrease in the inhibitory properties of the folate analog against L1210 leukemia in cell culture.

J. Heterocyclic Chem., **16**, 1645 (1979).

Studies on the transport of aminopterin (**4a**) (Scheme 1), 10-methylaminopterin (**4b**) (Methotrexate), and 10-ethylaminopterin (**4c**) by isolated murine intestinal epithelial cells (2) and L1210 tumor cells (2-4) indicated that alkyl substitution at position 10 reduced the affinity of the transport system in either cell type for the folate analog molecule. Whereas either alkyl substitution resulted in an approximate 3-fold reduction in the affinity of the carrier system in tumor cells, as compared to aminopterin, the affinity of the carrier in isolated intestinal epithelial cells was considerably more sensitive to these structural alterations. It was decreased 12-fold for 10-methyl aminopterin and 17-fold for 10-ethylaminopterin, relative to that for aminopterin. Efflux of either folate analog from the two cell types was not appreciably affected by changes in the substituent at the 10 position. The greater reduction in the affinity of the carrier in normal cells than in tumor cells for the 10-alkyl derivatives results in a greater persistence of drug in tumor cells than in normal tissue *in situ* (2,5-7) and provides a basis for the observed selective antitumor action of the 10-substituted folate analogs.

Since substitution of larger alkyl groups at the 10 position of the aminopterin molecule could conceivably increase the selectivity for tumor cells, the synthesis of 10-benzylaminopterin (**4d**) (Scheme 1) was undertaken. Previous syntheses of 10-substituted aminopterins have introduced the alkyl group at that position by alkylation of an *N*-sulfonyl derivative of ethyl *p*-aminobenzoate (8), then ester hydrolysis and introduction of the diethyl L-glutamyl moiety or by alkylamination of diethyl *N*-(*p*-iodobenzoyl)glutamate (9). A more direct approach that appeared to have general applicability to the synthesis of a variety of 10-substituted derivatives of **4a** was the direct reductive alkylation of diethyl *N*-(*p*-aminobenzoyl)-L-glutamate (**1**) with benzaldehyde in the presence of sodium cyanoborohydride (10). This procedure afforded the benzylated amine (**3a**) in one step in good yield (70%). Ester hydrolysis of **3a** to **3b** was accomplished in acetone/aqueous sodium hydroxide. The reaction of **3b** with 6-bromomethylpteridine (11,12) in DMAC afforded 10-benzylaminopterin (**4d**) in excellent yield (90%) and high purity, as determined by nmr, tlc and elemental analyses.

SCHEME 1



Biological Evaluation.

The potency of 10-benzylaminopterin as a cytotoxic agent was assessed in parallel with that of **4b** (13) by determining the concentrations of each that inhibited the growth of L1210 murine leukemia cells in culture by 50% (ID_{50}) during a 72 hour exposure by procedures described (14). The results from three separate experiments demonstrated that 10-benzylaminopterin (ID_{50} = 5.2 $\pm 2.0 \times 10^{-8}$ *M*) was 5-6 fold less potent than methotrexate (ID_{50} = $0.9 \pm 0.4 \times 10^{-8}$ *M*) against the L1210 leukemia. Those studies indicate that substantial alterations in the structure of **4a** can be introduced at the 10 position with retention of significant antitumor activity. Further studies to assess the effect of the highly lipophilic moiety at N-10 of **4d** on the inhibition of dihydrofolate reductase and on its transport by tumor and normal cells are planned.

EXPERIMENTAL

Nmr spectra were determined in the solvents indicated using TMS as an internal standard with a JEOL PFT-100 NMR Spectrometer, uv spectra were determined with a Cary 15 Recording Spectrophotometer, and melting points were determined with a

0022-152X/79/081645-02$02.25

Mel-Temp Apparatus and were uncorrected. Microanalyses were performed by Spang Microanalytical Laboratory, Eagle Harbor, Michigan.

Diethyl *N*-(*p*-Benzylaminobenzoyl)-L-glutamate (**3a**).

Diethyl *N*-(*p*-aminobenzoyl)-L-glutamate (**1**) (2.0 g., 4.9 mmoles) and benzaldehyde (0.6 ml., 5.4 mmoles) were dissolved in 45 ml. of ethanol and 0.5 ml. of saturated ethanolic hydrogen chloride, then sodium cyanoborohydride (155 mg., 2.54 mmoles) was added and the solution was allowed to stir at 25°. Progress of the reaction was monitored by tlc (silica gel, dichloromethane:methanol). After 6 days the mixture was filtered and the solvents were removed from the filtrate under vacuum. The residue was dissolved in ethyl acetate and the solution was extracted with water, then the ethyl acetate was removed under vacuum and the residue was recrystallized from ether-petroleum ether, yield 1.7 g. (70%), m.p. 117-118°; uv λ max (ε) (ethanol): 299 nm (23,900); nmr (deuteriochloroform): δ 7.67 (d, 2, J = 8.9, arom C_2H_2), 7.33-7.26 (m, 5, C_6H_5), 6.67 (d, 2, J = 8.9, arom C_2H_2), 4.38 (s, 2, CH_2), 4.33-3.99 (m, 4, CH_2's), 1.36-1.14 (m, 6, CH_3's).

An analytical sample was recrystallized from ether-petroleum ether and dried over phosphorus pentoxide (70°/2 hours).

Anal. Calcd. for $C_{23}H_{28}N_2O_5$: C, 66.97; H, 6.84; N, 6.79. Found: C, 66.95; H, 6.84; N, 6.77.

N-(*p*-Benzylaminobenzoyl)-L-glutamic Acid (**3b**).

A sample of the ester (**3a**) (1.2 g., 2.38 mmoles) was dissolved in 50 ml. of acetone and 4 equivalents of sodium hydroxide (383 mg. in 12 ml. of water) were added. After reacting for 18 hours the solvents were removed under reduced pressure, the residue was dissolved in water, the solution was acidified and extracted with ethyl acetate. The ethyl acetate extracts were combined and the solvent was removed under vacuum to afford an oil which solidified upon the addition of water. The precipitate was collected, washed with water and dried, yield 840 mg. (78%), m.p. 149-154°; uv λ max (ε) (ethanol): 291 nm (21,200); nmr (DMSO-d_6): δ 12.30 (s, 1, exch, O*H*), 12.22 (s, 1, exch, O*H*), 8.10 (d, 1, exch, J = 6.7, N*H*), 7.63 (d, 2, J = 8.9, arom, C_2H_2), 7.37 (m, 5, C_6H_5), 7.25 (t, 1, exch, N*H*), 6.58 (d, 2, J = 8.5, arom, C_2H_2), 4.33 (d, 2, J = 5.5, CH_2), 2.28 (d) and 1.98 (m, glu, C*H*'s).

An analytical sample recrystallized from ether-petroleum ether was dried over phosphorus pentoxide (70°/2 hours).

Anal. Calcd. for $C_{19}H_{20}N_2O_5 \cdot \frac{1}{2}H_2O$: C, 62.45; H, 5.79; N, 7.66. Found: C, 61.92; H, 5.57; N, 7.63.

N-[4-[(2,4-Diamino-6-pteridinyl)methyl]benzylamino]benzoyl-L-glutamic Acid (10-Benzylaminopterin) (**4d**).

N-(*p*-Benzylaminobenzoyl)-L-glutamic acid (**3b**) (840 mg., 1.8 mmoles) and 6-bromomethylpteridine (11,12) (**2**) (700 mg., 2.0 mmoles) dissolved in 6 ml. of DMAC were allowed to react at 50-60° for 2 days, then the solution was added dropwise with stirring to 200 ml. of water. The *p*H of the solution was then adjusted to 8 with ammonium hydroxide and the solution was treated with charcoal and filtered. The filtrate was acidified with acetic acid and chilled and the precipitate collected, yield 850 mg. (90%); m.p. dec. > 174°; uv λ max (ε) (pH 1): 243 nm (21,600), 307 (29,400), 345 sh (12,500); (*p*H 7): 258 nm (27,900), 304 (29,600), 370 (9600); (*p*H 13): 259 nm (27,300), 305 (29,300), 370 (9600); nmr (DMSO-d_6): δ 8.63 (s, 1, C_7H), 7.66 (d, 2, J = 8.6, arom, C_2H_2), 7.29 (s, 5, C_6H_5), 6.83 (d, 2, J = 9.2, arom, C_2H_2), 6.64 (s, 2, exch, NH_2), 4.92 (s, 2, CH_2), 4.36 (s, 2, CH_2), 2.31-1.95 (m, glu, C*H*'s); **4d** migrated as a single component (R_f = 0.66) on silica gel tlc developed in acetonitrile-water-28% ammonium hydroxide (7:2:1) at a lower R_f than those of **2** (R_f = 0.77) and **3b** (R_f 0.71).

The sample was equilibrated with water for 3 days before analysis.

Anal. Calcd. for $C_{26}H_{22}N_8O_5$: C, 53.79; H, 4.86; N, 19.30. Found: C, 53.83; H, 4.82; N, 19.12.

Acknowledgement.

We thank Dr. George Bosworth Brown for his interest and support, Dr. John Montgomery of the Southern Research Institute for helpful discussions, Dr. Charles R. Watson and Mr. Michael T. Ricciardi for assistance in preparing **2**, and Mr. Marvin J. Olsen for determining the nmr spectra.

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(1) This investigation was supported in part by Grants CA-08748 and CA-18856, awarded by the National Cancer Institute, DHEW, and an Ann Tennis Kurz Memorial Grant CH-15 from the American Cancer Society.

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(13) The sample of **4b** was kindly supplied by Dr. Harry B. Wood, Jr., Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland.

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