CRABP was purified through the DEAE-cellulose step at pH 8.3 and incubated with 3×10^{-6} M [^{3}H] *all-trans*-retinoic acid alone (control) or in the presence of retinoids. The competition for binding was determined by Sephadex G-25 gel filtration on 2-mL columns as described.[60] Binding measurements were performed in triplicate.

Retinoid Binding to Chick Embryo CRABP. CRABP from 12- to 13-day-old chick skins was used.[61] Affi-Gel Blue column chromatography was used to remove albumin, which also binds retinoids. Portions of the protein eluates (1 mg of protein/0.4 mL) were incubated with saturable amounts of [^{3}H]-*all-trans*-retinoic acid in the presence or absence of 1-, 5-, 10-, and 25-fold molar excess of unlabeled retinoid. Free retinoids were removed by adsorption on dextran-coated charcoal, the solution was filtered (0.65-μm membrane), and the amount of radioactivity bound was determined. The specific binding of [^{3}H]-*all-trans*-retinoic acid to CRABP was calculated as the difference between the totally bound radioactivity at a particular concentration of 1 and the total nonspecifically bound radioactivity after competition with a 25-fold molar excess of unlabeled 1. ID_{50} values were calculated from the semilog plots of the molar concentration of the retinoid against the percent inhibition of labeled retinoic acid by the retinoid. Binding measurements were performed in triplicate.

Acknowledgment. This research was supported by USPHS Grants CA30512 (M.I.D.), CA32428 (M.I.D.), DK36870 (J.L.N.), and CA42092 (J.L.N.).

Registry No. **2**, 71441-28-6; **3**, 119999-05-2; (*Z*)-**3**, 119999-31-4; **4**, 119999-06-3; (±)-**5**, 119999-07-4; **6**, 107430-51-3; **7**, 86471-16-1; **8**, 119999-08-5; **10**, 6683-48-3; **11**, 119435-90-4; **11** (dibromide), 119999-29-0; **12**, 119436-52-1; **13**, 1443-80-7; **14**, 119999-10-9; (*Z*)-**14**, 119999-30-3; **15**, 119999-11-0; (*Z*)-**15**, 119999-32-5; **16**, 92654-79-0; **16** (acid), 103031-30-7; **17**, 119999-12-1; (±)-**18**, 119999-13-2; (±)-**19**, 119999-14-3; (±)-**20**, 119999-15-4; (±)-**21**, 119999-16-5; (±)-**22**, 119999-17-6; **23**, 119999-18-7; (*Z*)-**23**, 119999-34-7; **24**, 119999-19-8; (±)-**26**, 120022-39-1; (±)-*cis*-**26**, 119999-33-6; **27**, 580-13-2; **28**, 5798-75-4; **29**, 119999-21-2; **30**, 13275-18-8; **31**, 107430-52-4; **32**, 116233-16-0; **33**, 116233-17-1; **34**, 638-51-7; **35**, 119999-22-3; **36**, 86471-14-9; **37**, 119999-23-4; **38**, 108-86-1; **40**, 34598-49-7; **41**, 119999-25-6; **42**, 119999-26-7; **43**, 3294-60-8; **44**, 119999-27-8; **45**, 27452-17-1; **46**, 119999-28-9; 4-$OHC_6H_4CO_2Et$, 6287-86-1; 4-$H_2NN{=}CHC_6H_4CO_2Et$, 119999-20-1; (±)-5-bromo-1-methyl-1-indanol, 119999-24-5.

Supplementary Material Available: Complete biological data for the tracheal organ culture, transglutaminase inhibition, cross-linked envelope formation, ornithine decarboxylase inhibition, F9 plasminogen activator release assays, and rat testis and chick embryo CRABP binding studies (5 pages). Ordering information is given on any current masthead page.

(60) Jetten, A. M.; Rearick, J. I.; Smits, H. L. *Biochem. Soc. Trans.* **1986**, *14*, 930–933.
(61) (a) Sani, B. P.; Hill, D. L. *Cancer Res.* **1976**, *36*, 409–413. (b) Sani, B. P.; Dawson, M. I.; Hobbs, P. D.; Chan, R. L. C.; Schiff, L. J. *Cancer Res.* **1984**, *44*, 190–195. (c) Sani, B. P.; Titus, B. C.; Banerjee, C. K. *Biochem. J.* **1978**, *171*, 711–717.

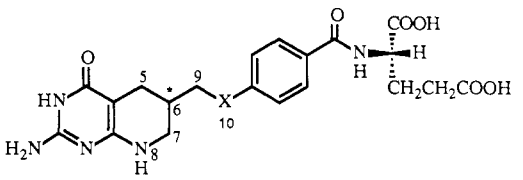
Synthesis and Antitumor Activity of 5-Deaza-5,6,7,8-tetrahydrofolic Acid and Its N^{10}-Substituted Analogues

Edward C. Taylor,*[†] James M. Hamby,[†] Chuan Shih,[‡] Gerald B. Grindey,[‡] Sharon M. Rinzel,[‡] G. Peter Beardsley,[§] and Richard G. Moran[∥]

Department of Chemistry, Princeton University, Princeton, New Jersey 08544, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana 46285, Department of Pediatrics, Yale University, New Haven, Connecticut 06510, and Departments of Pediatrics, Biochemistry, and Pharmacy, University of Southern California, Los Angeles, California 90027.
Received October 11, 1988

Syntheses of 5-deaza-5,6,7,8-tetrahydrofolic acid (**7a**) and its 10-formyl (**7b**), 10-acetyl (**7c**), and 10-methyl (**7d**) derivatives are described. These compounds, prepared as analogues of 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF), the lead compound of a new class of folate antimetabolites, exhibit potent growth inhibition against leukemic cells in culture as well as substantial antitumor activity against transplantable murine solid tumors in vivo.

Recently we reported the synthesis and biological activity of 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (**1**) (DDATHF), the lead compound of a new class of folate



1, X = CH_2

7a, X = NH

antimetabolites possessing unique biochemical properties and potent antitumor activity in experimental animals.[1-5] DDATHF has a novel mode of action as compared to conventional antifolates such as methotrexate [which inhibits dihydrofolate reductase (DHFR)] or 10-propargyl-5,8-dideazafolic acid (CB3717) [which inhibits thymidylate synthase (TS)].[6] DDATHF inhibits purine biosynthesis in cultured mouse (L1210) and human (CCRF-CEM)

[†] Princeton University.
[‡] Lilly Corporate Center.
[§] Yale University.
[∥] University of Southern California.

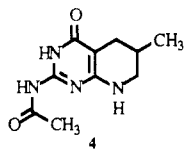
(1) Taylor, E. C.; Harrington, P. J.; Fletcher, S. R.; Beardsley, G. P.; Moran, R. G. *J. Med. Chem.* **1985**, *28*, 914.
(2) Moran, R. G.; Beardsley, G. P.; Taylor, E. C. *Proc. Am. Assoc. Cancer Res.* **1985**, *26*, 231.
(3) Taylor, E. C.; Wong, G. S. K.; Fletcher, S. R.; Harrington, P. J.; Beardsley, G. P.; Shih, C. *Chemistry and Biology of Pteridines*; Cooper B. A., Whitehead, V. M., Eds.; de Gruyter: Berlin, 1986; p 61.
(4) Beardsley, G. P.; Taylor, E. C.; Grindey, G. B.; Moran, R. G. *Chemistry and Biology of Pteridines*; Cooper, B. A., Whitehead, V. M., Eds.; de Gruyter: Berlin, 1986; p 953.
(5) Beardsley, G. P.; Taylor, E. C.; Shih, C.; Poore, G. A.; Grindey, G. B.; Moran, R. G. *Proc. Am. Assoc. Cancer Res.* **1986**, *27*, 259.
(6) (a) Jones, T. R.; Calvert, A. H.; Jackman, A. L.; Brown, S. J.; Jones, M.; Harrap, K. R. *Eur. J. Cancer* **1981**, *17*, 11. (b) Calvert, A. H.; Alison, D. L.; Harland, S. J.; Robinson, B. A.; Jackman, A. L.; Jones, T. R.; Newell, D. R.; Siddik, Z. H.; Wiltshaw, E.; McElwain, T. J.; Smith, I. E.; Harrap, K. R. *J. Clin. Oncol.* **1986**, *4*, 1245.

0022-2623/89/1832-1517$01.50/0

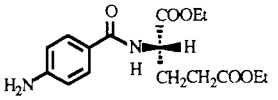
lymphoblastic leukemic cells apparently by inhibiting the folate cofactor-requiring enzyme glycinamide ribonucleotide transformylase (GAR TFase) in the purine de novo biosynthetic pathway.[7,8] As part of our continuing efforts to understand the structural features that determine activity for this unique class of antitumor agents, we describe herein the preparation and biological properties of 5-deaza-5,6,7,8-tetrahydrofolic acid (**7a**) (5-DATHF), a close structural analogue of DDATHF with substitution of NH for CH_2 at the 10-position of DDATHF, and its 10-formyl (**7b**), 10-acetyl (**7c**), and 10-methyl (**7d**) derivatives. We find that 5-DATHF is at least as active as DDATHF by several criteria, suggesting that it is the substitution of C for N at position 5 of DDATHF, rather than that at position 10, which is responsible for its remarkable activity as an inhibitor of de novo purine synthesis.

Chemistry

Our initial synthesis of **7a**, summarized in Scheme I, involved reductive amination of 2-acetyl-6-formyl-5-deazapterin (**2a**) with diethyl *N*-(4-aminobenzoyl)glutamate (**6**) to give intermediate **3a**, followed by catalytic reduction of the pyridine ring of **3a** to give **5a** and final alkaline hydrolysis of the acetyl and ester protecting groups. This approach, however, was not completely satisfactory because (1) the previously described multistep preparation of **2a**[9] was tedious and not suitable for scaleup and (2) catalytic hydrogenation of **3a** led primarily to the benzylic hydrogenolysis products **4** and **6** and gave only a small amount (3–5%) of the desired product **5a**.



4



6

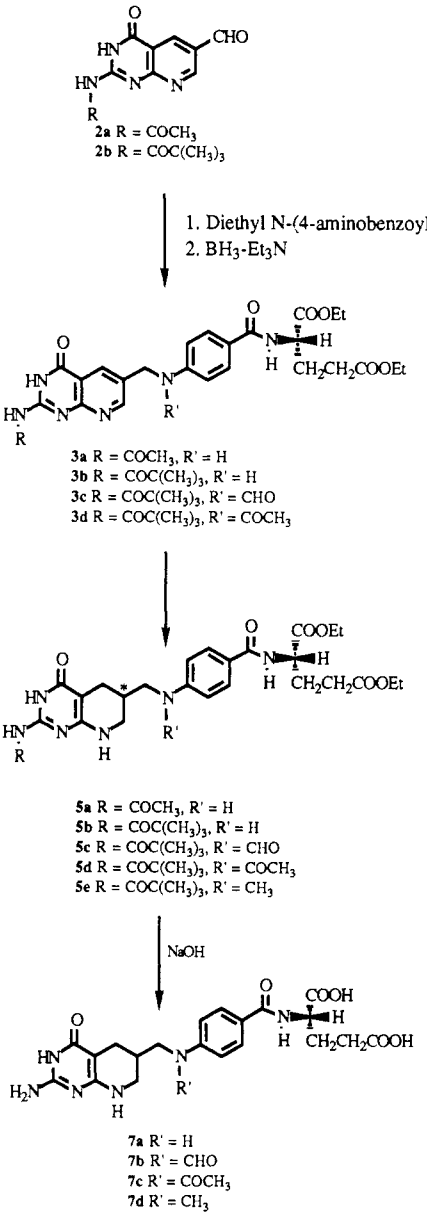
Two major improvements have been made in this general strategy leading to **7a**. First, we have developed a simple and expedient synthesis of 2-pivaloyl-6-formyl-5-deazapterin (**2b**) by ozonolysis of the corresponding 6-styryl derivative **2c**, which in turn was prepared by palladium-catalyzed coupling of styrene with 2-pivaloyl-6-bromo-5-deazapterin[10] (Scheme II). Second, we have found that benzylic hydrogenolysis can be completely prevented by acylation of the 10-nitrogen atom of **3**.

Thus, **2b** was condensed with diethyl *N*-(4-aminobenzoyl)-L-glutamate (**6**) at room temperature in glacial acetic acid, and the resulting imine was reduced with the borane–triethylamine complex to give **3b** in 64% yield. As with the corresponding 2-acetyl derivative **3a**, attempts to reduce the pyridine ring of **3b** under catalytic hydrogenation conditions were unsuccessful. This was due either to facile hydrogenolysis of the benzylic carbon–nitrogen bond (with Pd/C or PtO_2) or to formation of a complex and inseparable mixture of products (with Rh/C). However, treatment of **3b** with a mixture of acetic anhydride and formic acid at 25 °C for 1.5 h gave the 10-formyl derivative **3c** in quantitative yield, which smoothly underwent catalytic reduction of the pyridine ring (PtO_2 in glacial acetic acid) without concurrent benzylic cleavage to produce intermediate **5c** as a 1:1 mixture of two diastereomers (*d*L + *l*L). Target compound **7a** (5-DATHF) was then readily prepared from **5c** in 61% yield, also as a mixture of two diastereomers, upon hydrolysis with 1 N NaOH at 25 °C for 3 days.

Acetylation of **3b** to give **3d** was also accomplished in quantitative yield with acetyl chloride in methylene chloride at 25 °C (acetic anhydride gave only unchanged starting material). Again, catalytic reduction of **3d** proceeded smoothly under the above conditions to give the tetrahydro derivative **5d** (86% yield). In this case, however, treatment of **5d** with 1 N NaOH at 25 °C for 3 days gave an inseparable mixture of **7a** and its 10-acetyl derivative **7c**. The use of 0.1 N NaOH at 25 °C for 5 days, however, smoothly yielded the 10-acetyl derivative of 5-DATHF (**7c**). Alternatively, **7c** could be prepared in rather low yield (23%) by direct acetylation of **7a** with acetyl chloride in dimethylacetamide.

The natural cofactor for GAR TFase is 10-formyltetrahydrofolic acid, and it was therefore of considerable interest to examine the biochemical properties of 10-formyl-5-deaza-5,6,7,8-tetrahydrofolic acid (**7b**). An attempt to prepare this compound from **5c** by selective hy-

Scheme I



(7) Moran, R. G.; Taylor, E. C.; Shih, C.; Beardsley, G. P. *J. Biol. Chem.* Manuscript in preparation.
(8) Beardsley, G. P.; Moroson, B. A.; Taylor, E. C.; Moran, R. G. *J. Biol. Chem.* **1989**, *264*, 328.
(9) Taylor, E. C.; Fletcher, S. R.; George, T. J.; Harrington, P. J.; Palmer, D. C.; Tseng, C. P. *J. Org. Chem.* **1983**, *48*, 4852.
(10) Taylor, E. C.; Yoon, C. M. *Synth. Commun.* **1988**, *18*, 1187.

Scheme II

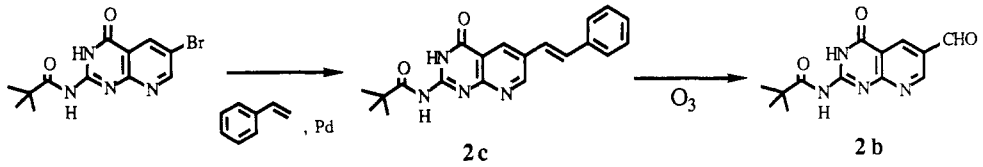


Table I. Effect of 5-Deazatetrahydrofolic Acid and Its 10-Substituted Analogues on Growth of CCRF-CEM Leukemic Cells in Culture

compd	IC_{50}, μM	compd	IC_{50}, μM
7a	0.010	DDATHF-A	0.005
7b	0.050	DDATHF-B	0.008
7c	0.600	**7a**-A	0.006
7d	0.040	**7a**-B	0.006
DDATHF	0.020		

drolysis of the pivaloyl and ester protecting groups with 0.1–0.5 N NaOH failed, since the extraordinary insolubility of the resulting mixtures of **7a** and **7b** made their separation impossible. Although conversion of **5c** to **7b** could be accomplished with methanolic NaOH, these conditions caused some racemization of the L-glutamate moiety. The desired 10-formyl derivative **7b** was finally obtained in moderate yield (47%) by selective formylation of **7a** with acetic anhydride–formic acid. All three of the 10-formyl derivatives **3c**, **5c**, and **7b** exhibited hindered rotation about the N–CHO bond; coalescence of the signals due to the individual rotomers was observed above 70 °C.

Introduction of a methyl group at N-10 prior to reduction of the pyridine ring would not prevent the benzylic hydrogenolysis process, and thus the 10-methyl derivative **7d** was prepared by a slightly different strategy. Selective hydrolysis of the 10-formyl group of **5c** with 3% H_2SO_4 in absolute ethanol gave **5b** in 80% yield; no racemization of the L-glutamate was observed. The 10-methyl group was then introduced by using a modified method of Borch[11] by treatment of **5b** with aqueous formaldehyde in acetonitrile followed by sodium cyanoborohydride reduction to give **5e**. Final hydrolysis of **5e** with 1 N NaOH gave **7d** in almost quantitative yield.

All of the 5-DATHF derivatives **7a–d** were prepared as a mixture of two diastereomers (*d*L + *l*L) which defied separation by conventional means. Satisfactory separations and analyses were achieved, however, by the use of (1) an Astec chiral Cyclobond I reverse-phase HPLC column (with β-cyclodextrin bonded to silica as the chiral stationary phase) and elution with a solvent system of 0.1% aqueous triethylamine–acetic acid buffered at pH 7.0 or (2) a C-18 reverse-phase HPLC column and elution with a solvent system of acetonitrile (25%) in aqueous triethylamine–acetic acid buffered at pH 7 (75%) and containing 0.01 M β-cyclodextrin as the chiral mobile phase.

Biological Results and Discussion

Compounds **7a–d** were evaluated against human lymphoblastic leukemic cells (CCRF-CEM) in vitro (Table I). The IC_{50} values of these analogues indicate that 5-deaza-5,6,7,8-tetrahydrofolic acid (**7a**, mixture of two diastereomers) exhibits the most potent growth inhibition in this series. The in vitro potency of **7a** is superior to that of DDATHF; it is 4–60-fold more potent than analogues having substituents at N-10. Introduction of one-carbon substituents at N-10 (the 10-methyl derivative **7d** and the 10-formyl derivative **7b**) results in only minor decreases

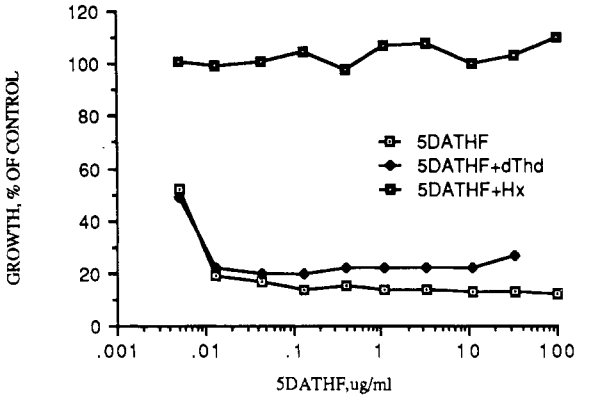


Figure 1. Reversal of 5-DATHF cytotoxicity by hypoxanthine (100 μM) and thymidine (5 μM) in CCRF-CEM cells (72 h).

Table II. Comparison of the Activities of 5-DATHF and DDATHF for Folylpolyglutamate Synthetase[a]

compd	N^b	K_m(apparent)	$V_{max}{}^c$	k'^c
folic acid	49	140 ± 47	1.0	1.0
aminopterin	13	21 ± 4.8	1.59 ± 0.01	10.1 ± 4.2
5-DATHF (**7a**)	2	1.06 ± 0.08	1.30 ± 0.01	170 ± 2
DDATHF	5	7.3 ± 1.1	1.26 ± 0.07	32 ± 7.3

[a] Folylpolyglutamate synthetase was partially purified from mouse liver, and enzyme reactions were performed as previously described.[12] Because of the extremely rapid reaction times observed with 5-DATHF, low levels of enzyme activity were used for these experiments, and the reaction volume used was scaled up to 1.0 mL to minimize substrate consumption. In each experiment, folic acid or aminopterin was used as an internal control. [b] The number of experiments used for each estimate. [c] Relative to folic acid.

in activity, but activity declines sharply (60-fold less potent) when a bulkier acetyl group is introduced into this position. The growth inhibition caused by **7a** in CCRF-CEM cells is prevented by the addition of hypoxanthine (100 μM) but not by the addition of thymidine (5 μM) (Figure 1). This pattern of reversal is also observed for DDATHF.[2,8]

The two diastereomers of 5-DATHF were separated and found to be equally active in inhibiting the growth of CCRF-CEM leukemic cells in culture (Table I). The two diastereomers of DDATHF had been found previously to be equipotent.[7]

Compound **7a** was also shown to be an excellent substrate for mouse liver folate polyglutamate synthetase (FPGS)[7] (Table II). 5-DATHF has an even lower K_m than DDATHF (K_m = 1.1 ± 0.08 μM compared to K_m = 7.3 ± 1.1 μM for DDATHF) and an equal V_{max}. The first-order rate constant (k', which is an excellent descriptor of the relative rate of reaction of substrates at low substrate concentrations) for this reaction differs by a factor of 5 between DDATHF and 5-DATHF.

Compounds **7a** and **7b** were tested in vivo in C3H mice with transplantable murine solid tumors (C3H mammary adenocarcinoma and 6C3HED lymphosarcoma); results are shown in Tables III and IV. Compound **7a** was toxic at

(11) Borch, R. F.; Hassid, A. I. *J. Org. Chem.* **1972**, *37*, 1673.

(12) Moran, R. G.; Colman, P. D.; Rosowsky, A.; Forsch, R.; Chan, K. K. *Mol. Pharmacol.* **1985**, *27*, 156.

Table III. In Vivo Antitumor Activity[a] of Compounds **7a** and **7b**

	7a			7b		
	dose,[b] mg/kg	% inhibn	tox/ total	dose,[b] mg/kg	% inhibn	tox/ total
6C3HED lympho-sarcoma[c]	100	100	9/10	100	100	7/10
	50	100	6/10	50	100	5/10
	25	100	3/10	25	100	1/10
	12.5	100	1/10	12.5	95	1/10
	6.0	77	0/10			
C3H mammary adeno-carcinoma[d]	50	tox	10/10	50	tox	10/10
	25	tox	10/10	25	98	6/10
	12.5	tox	10/10	12.5	86	0/10
	6.25	96	3/10			

[a] For details, see Experimental Section. [b] Compounds were suspended in 2.5% Emulphor in 0.9% saline and administered ip. [c] Dose administered daily × 8. [d] Dose administered daily × 10.

Table IV. In Vivo Antitumor Activity of Compound **7a** in Every Other Day Schedule

6C3HED lymphosarcoma			C3H mammary adenocarcinoma		
dose,[a] mg/kg	% inhibn	tox/total	dose,[a] mg/kg	% inhibn	tox/total
200	98	3/10			
100	93	0/9	100	94	0/10
50	74	0/10	50	81	0/10
25	51	0/10	25	64	0/10
12.5	34	0/10	12.5	61	0/10
			6.25	50	0/10

[a] Compound **7a** was suspended in 2.5% Emulphor in 0.9% saline and administered ip on days 1, 3, 5, 7, and 9.

25 mg/kg given daily × 8 to animals bearing the 6C3HED lymphosarcoma, and at 6.25 mg/kg when given daily × 10 to animals bearing the C3H mammary adenocarcinoma, and compound **7b** was toxic at 50 mg/kg (6C3HED lymphosarcoma) and 25 mg/kg (C3H mammary adenocarcinoma) when administered ip on these schedules (Table III). However, when compound **7a** was given on an every other day schedule, considerably less toxicity and good antitumor activity were observed in both models (Table IV).

In conclusion, the similar growth inhibitory activity of 5-DATHF compared to that of DDATHF in cell culture, the similar reversal patterns of hypoxanthine and by thymidine on growth inhibition in vitro, the superior substrate activity of 5-DATHF for mouse liver FPGS, and their comparable in vivo antitumor activity against murine solid tumors all suggest that substitution of nitrogen for carbon at position 10 of DDATHF is not detrimental either to the overall biochemical properties or to the antitumor activity of DDATHF. Structure–activity studies based upon modification of other parts of the DDATHF molecule are currently in progress.

Experimental Section

General Methods. Melting points are uncorrected and were determined in open capillary tubes by using a Thomas-Hoover apparatus for temperatures below 250 °C and a Meltemp apparatus for temperatures above 250 °C. Room temperature ^{1}H NMR data were recorded with a General Electric QE 300-MHz instrument with chemical shifts reported in ppm in DMSO-d_6 with DMSO (δ 2.49) as the internal standard. Variable-temperature ^{1}H NMR data were obtained with a JEOL GSX 270-MHz instrument. Mass spectral data were obtained by Dr. Dorothy Little on a Kratos MS50TC spectrometer. Elemental analyses were performed by Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN. Radial chromatography was carried out by using a Chromatotron Model 7924 apparatus. The radial chromatographic plates were made with Kieselgel 60 PF_{254} with gypsum added as a binder.

HPLC Procedures. Separation of diastereomeric mixtures of the target compounds was carried out by reverse-phase HPLC using a Perkin-Elmer series 2 liquid chromatograph equipped with a LC-75 spectrophotometric detector. Separation of the diastereomers of **7a** and **7d** was carried out on an Astec C18 reverse-phase column (250 mm × 4 mm i.d.) with a solvent flow rate of 1 mL/min. The mobile phase was prepared as follows: Triethylamine (10 mL) was diluted to 1 L with HPLC-grade water, and the pH of this solution was adjusted to 7 by the dropwise addition of acetic acid using a pH meter. HPLC-grade acetonitrile (250 mL) was then diluted to 1 L with the above buffer solution and β-cyclodextrin (11.35 g, 0.01 M) dissolved in this mixture. This solution was filtered, degassed, and used as the eluting solvent for compounds **7a** and **7d**.

The HPLC separation of compounds **7b** and **7c** was performed by using an Astec Cyclobond I column with a flow rate of 1 mL/min. The solvent system utilized in these analyses was an aqueous triethylamine–acetic acid buffer prepared as described above, but with omission of acetonitrile and β-cyclodextrin.

Diastereomer A in the data reported below for compounds **7a–d** refers to the first diastereomer to elute from the column under the specified conditions, and diastereomer B refers to the second diastereomer to elute from the column. The absolute configurations of these diastereomers have not yet been determined.

Cell Culture Study. CCRF-CEM cells, a human leukemic cell line,[13] were grown as previously described.[14] Dose–response curves were generated for various compounds to determine the concentration required for 50% inhibition of growth (IC_{50}). Cluster plates were prepared in duplicate with the compound at various concentrations. Test compounds were made initially in Me_2SO at a concentration of 4 mg/mL and further diluted with solvent to the desired concentration. Cells in Roswell Park Memorial Institute 1640 media supplemented with 10% dialyzed fetal bovine serum, 16 mM HEPES, and 8 mM MOPS buffers were added to the well at a final concentration of 4.8×10^4 cells/well in a total volume of 2.0 mL. After 72 h of incubation (95% air, 5% CO_2), cell numbers were determined on a ZBI Coulter counter. Cell number for indicated controls at the end of incubation is usually (4–6) × 10^5 cells/well.

In Vivo Antitumor Activity. For the various solid tumors, 1–2-mm^2 tumor fragments were implanted sc by trocar in the axillary region of syngenic mice (C3H). Treatment was initiated 24 h after tumor implantation. One day after the final dose, the inhibition of tumor growth was determined by comparing the tumor volume of the treated group to that of controls. Tumor volume (V) was calculated by measuring the tumor width (W) and length (L) and using the equation $V = LW^2/2$. For all studies, the compounds were suspended in 2.5% Emulphor in 0.9% saline and administered ip.

Diethyl *N*-[*N*-[[2-(Pivaloylamino)-4-hydroxypyrido[2,3-*d*]pyrimidin-6-yl]methyl]-4-aminobenzoyl]glutamate (3b). A mixture of **2b** (3.0 g, 10.94 mmol) and diethyl *N*-(4-aminobenzoyl)-L-glutamate (3.53 g, 10.94 mmol) in glacial acetic acid (60 mL) was stirred at 25 °C for 14 h. To this solution was added borane–triethylamine complex (0.42 g, 0.54 mL, 3.6 mmol), and the mixture was stirred at 25 °C for 2 h. The solvent was removed under reduced pressure, and the residue was dissolved in methylene chloride (150 mL) and extracted with a saturated aqueous solution of sodium bicarbonate (2 × 75 mL). After back-extraction of the aqueous layers with methylene chloride (75 mL), the combined organic layers were dried over anhydrous magnesium sulfate, and the solvent was removed under reduced pressure. The residue was taken up in ethyl acetate (35 mL) and stored at 0 °C for 18 h. The white solid was collected by filtration to give 4.76 g (61% yield) of **3b**: mp 150–153 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.10–1.16 (m, 6 H), 1.23 (s, 9 H), 1.88–2.10 (m, 2 H), 2.37 (t, 2 H, J = 5.87 Hz), 6.60–6.63 (AA'BB', 2 H), 6.96 (t, 1 H, J = 8.68 Hz), 7.60–7.63 (AA'BB', 2 H), 8.23 (d, 1 H, J = 7.45 Hz), 8.37 (m, 1 H), 8.85 (m, 1 H); mass spectrum, m/z (relative intensity) 580 (15, M^+), 399 (27), 398 (27), 379 (30), 378 (100), 350 (40), 348 (41),

(13) Foley, G. E.; Lazarus, H.; Forbes, S.; Uzman, B. G.; Boone, B. A.; McCarthy, R. E. *Cancer* **1965**, *18*, 522.
(14) Grindey, G. B.; Wang, M. C.; Kinahan, J. *J. Mol. Pharmacol.* **1979**, *16*, 601.

331 (21). Anal. ($C_{29}H_{36}N_6O_7$) C, H, N.

Diethyl *N*-[*N*-[[2-(Pivaloylamino)-4-hydroxypyrido[2,3-*d*]pyrimidin-6-yl]methyl]-N^{10}-formyl-4-aminobenzoyl]-glutamate (3c). To a stirred solution of **3b** (2.59 g, 4.5 mmol) in 98% formic acid (25 mL) at 25 °C was added acetic anhydride (0.92 g, 0.85 mL, 9.0 mmol). The reaction mixture was stirred at 25 °C for 1.5 h and the solvent removed under reduced pressure. The residue was taken up in methylene chloride (150 mL) and extracted twice with a saturated aqueous solution of sodium bicarbonate (75 mL). The aqueous extracts were back-extracted with methylene chloride (75 mL), and the combined organic layers were dried over anhydrous magnesium sulfate. After the suspension was filtered, the filtrate was evaporated under reduced pressure to give a quantitative yield of **3c**. This product was used in the next step without further purification: ^{1}H NMR (Me_2SO-d_6) δ 1.09–1.16 (m, 6 H), 1.21 (s, 9 H), 1.90–2.10 (m, 2 H), 2.39 (t, 2 H, J = 7.37 Hz), 3.96–4.09 (m, 4 H), 4.31–4.43 (m, 1 H), 5.22 (s, 2 H), 7.50–7.53 (AA'BB', 2 H), 7.83–7.86 (AA'BB', 2 H), 8.21 (m, 1 H), 8.71 (d, 1 H, J = 5.87 Hz), 8.72 (m, 1 H), 8.80 (s, 1 H).

Diethyl *N*-[*N*-[[2-(Pivaloylamino)-4-hydroxypyrido[2,3-*d*]pyrimidin-6-yl]methyl]-N^{10}-acetyl-4-aminobenzoyl]-glutamate (3d). To a solution of **3b** (4.0 g, 6.9 mmol) in methylene chloride (50 mL) at 0 °C was added sodium acetate (1.24 g, 15.2 mmol) followed by acetyl chloride (0.6 g, 0.54 mL, 7.6 mmol). The reaction mixture was stirred at 0 °C for 15 min and then at 25 °C for 1 h. Methylene chloride was added to the reaction mixture, and the organic layer was extracted with water (75 mL), a saturated solution of sodium bicarbonate (75 mL), and again with water (75 mL). The aqueous layers were back-extracted with methylene chloride (75 mL), and the combined organic layers were dried over anhydrous magnesium sulfate. After filtering, the filtrate was evaporated under reduced pressure to give 4.3 g (100%) of **3d**. This product was used in the next step without further purification: ^{1}H NMR (Me_2SO-d_6) δ 1.10–1.17 (m, 6 H), 1.22 (s, 9 H), 1.88 (s, 3 H), 1.89–2.13 (m, 2 H), 2.39 (t, 2 H, J = 7.42 Hz), 3.97–4.08 (m, 4 H), 4.33–4.42 (m, 1 H), 5.13 (s, 2 H), 7.34–7.36 (AA'BB', 2 H), 7.83–7.86 (AA'BB', 2 H), 8.19 (d, 1 H, J = 2.09 Hz), 8.65 (d, 1 H, J = 2.09 Hz), 8.75 (d, 1 H, J = 7.39 Hz).

Diethyl *N*-[*N*-[[2-(Pivaloylamino)-4-hydroxy-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl]methyl]-N^{10}-formyl-4-aminobenzoyl]glutamate (5c). To a solution of **3c** (2.98 g, 4.9 mmol) in glacial acetic acid (60 mL) was added platinum oxide catalyst (447 mg), and the suspension was hydrogenated (50 psi) in a Parr apparatus for 3 h at 25 °C. The reaction mixture was filtered through Celite and the filtrate evaporated under reduced pressure. The residue was dissolved in methylene chloride (150 mL) and extracted with an aqueous saturated solution of sodium bicarbonate (2 × 75 mL). The aqueous layers were back-extracted with methylene chloride (75 mL) and the combined organic layers dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure to give 2.48 g (83%) of **5c**. Recrystallization from ethyl acetate afforded pure **5c**: mp 152–153 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.12–1.17 (m, 6 H), 1.17 (s, 9 H), 1.90–2.18 (m, 4 H), 2.43 (t, 2 H, J = 7.40 Hz), 2.80–2.94 (m, 1 H), 3.12–3.20 (m, 1 H), 3.89 (d, 2 H, J = 5.59 Hz), 4.00–4.11 (m, 4 H) 4.60–4.73 (m, 1 H), 6.40 (m, 1 H), 7.52–7.55 (AA'BB', 2 H), 7.92–7.94 (AA'BB', 2 H), 8.62 (s, 1 H), 8.75 (d, 1 H, J = 7.33 Hz); mass spectrum, m/z (relative intensity) 567 (4), 382 (4), 249 (100), 165 (10), 84 (6). Anal. ($C_{30}H_{40}N_6O_8$) C, H, N.

Diethyl *N*-[*N*-[[2-(Pivaloylamino)-4-hydroxy-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl]methyl]-N^{10}-acetyl-4-aminobenzoyl]glutamate (5d). To a solution of **3d** (4.29 g, 6.9 mmol) in glacial acetic acid (80 mL) was added platinum oxide catalyst (668 mg), and the suspension was hydrogenated (50 psi) in a Parr apparatus at 25 °C for 3 h. The reaction mixture was filtered through Celite and the filtrate evaporated under reduced pressure. The residue was dissolved in methylene chloride (200 mL) and extracted with an aqueous saturated solution of sodium bicarbonate (2 × 75 mL). The aqueous layers were back-extracted with methylene chloride (100 mL) and the combined organic layers dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure and the residue chromatographed (radial chromatography), eluting with 5% methanol in methylene chloride to give 3.7 g (86%) of **5d** (mp 119–123 °C). Recrystallization from ethyl acetate afforded the analytical sample: mp 120–123 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.09–1.19 (m, 6 H), 1.16 (s, 9 H), 1.82 (s, 3 H), 1.90–2.18 (m, 4 H), 2.42 (t, 2 H, J = 7.40 Hz), 2.80–2.94 (m, 1 H), 3.15–3.20 (m, 1 H), 3.68 (d, 2 H, J = 6.63 Hz), 3.98–4.12 (m, 4 H) 4.38–4.45 (m, 1 H), 6.38 (m, 1 H), 7.44–7.46 (AA'BB', 2 H), 7.90–7.93 (AA'BB', 2 H), 8.78 (d, 1 H, J = 7.36 Hz); mass spectrum, m/z (relative intensity) 626 (4, M^+) 581 (6), 277 (12), 261 (10), 250 (30), 249 (100), 165 (14). Anal. ($C_{31}H_{42}N_6O_8$) C, H, N.

***N*-[*N*-[(2-Amino-4-hydroxy-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)methyl]-4-aminobenzoyl]glutamic Acid (7a).** A solution of **5c** (1.0 g, 1.63 mmol) in 1 N NaOH (15 mL) was stirred at 25 °C for 72 h. Charcoal was added to the reaction mixture, which was then filtered through Celite. The filtrate was acidified to pH 4 with 0.5 N HCl, and the white solid was collected after 1 h at 0 °C to give 0.73 g (61%) of **7a**; mp, slowly decomposes above 198 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.85–2.02 (m, 6 H), 2.43 (t, 2 H, J = 7.40 Hz), 2.81–2.88 (m, 1 H), 3.23–3.32 (m, 2 H), 4.2–4.4 (m, 1 H), 5.92 (s, 2 H), 6.29 (s, 1 H), 6.34 (t, 1 H, J = 5.28 Hz), 6.55–6.57 (AA'BB', 2 H), 7.61–7.64 (AA'BB', 2 H), 8.08 (d, 1 H, J = 7.64 Hz), 9.70 (br s, 1 H); HPLC (retention time) diastereomer A, 6.28 min, and diastereomer B, 8.48 min. Anal. ($C_{20}H_{24}N_6O_6$·0.75H_2O) C, H, N.

***N*-[*N*-[(2-Amino-4-hydroxy-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)methyl]-N^{10}-formyl-4-aminobenzoyl]-glutamic Acid (7b).** To a solution of **7a** (0.30 g, 0.67 mmol) in 97% formic acid (5 mL) was added acetic anhydride (0.075 g, 0.07 mL, 15.75 mmol), and the reaction mixture was stirred at 25 °C for 3 h. The solvent was removed under reduced pressure and the residue dissolved in 2.0 N NaOH. Charcoal was added, and the suspension was filtered through Celite. The filtrate was acidified to pH 4 with 0.5 N HCl and stored at 0 °C for 2 h. The white solid was collected by filtration and dried over P_2O_5 to give 0.150 g (47%) of **7b**: mp, slowly decomposes above 178 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.88–2.06 (m, 5 H), 2.25–2.32 (m, 3 H), 2.69–2.80 (m, 1 H), 3.03–3.07 (m, 1 H), 3.84 (d, 2 H, J = 5.75 Hz), 4.25–4.40 (s, 1 H), 5.95 (s, 2 H), 6.19 (s, 1 H), 7.50–7.55 (AA'BB', 2 H), 7.89–7.92 (AA'BB', 2 H), 8.55 (d, 1 H, J = 7.48 Hz), 8.59 (s, 1 H), 9.73 (br s, 1 H); HPLC (system B, retention time) diastereomer A, 12.67 min, and diastereomer B, 13.34 min. Anal. ($C_{21}H_{24}N_6O_7$) C, H, N.

***N*-[*N*-[(2-Amino-4-hydroxy-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)methyl]-N^{10}-acetyl-4-aminobenzoyl]glutamic Acid (7c). Method A.** A solution of **5d** (1.89 g, 3.0 mmol) in 0.1 N NaOH (145 mL) was stirred at 25 °C for 120 h. Charcoal was added and the mixture filtered through Celite. The filtrate was acidified to pH 4 with 0.5 N HCl and stored at 0 °C. After 3 h the white solid was collected, washed with water, and dried over P_2O_5 to give 0.8 g (54%) of **7b**. The analytical sample was recrystallized from ethanol: mp, slowly decomposes above 195 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.60–2.18 (m, 8 H), 2.29–2.36 (m, 3 H), 2.74–2.80 (m, 1 H), 3.10–3.12 (m, 1 H), 3.36–3.39 (m, 2 H), 4.33–4.39 (m, 1 H), 5.90 (s, 2 H), 6.18 (s, 1 H), 7.40–7.43 (AA'BB', 2 H), 7.89–7.92 (AA'BB', 2 H), 8.65 (d, 1 H, J = 7.54 Hz), 9.74 (br s, 1 H); HPLC (retention time) diastereomer A, 13.94 min, and diastereomer B, 14.59 min. Anal. ($C_{22}H_{26}N_6O_7$·H_2O) C, H, N.

Method B. To a solution of **7a** (0.2 g, 0.45 mmol) in dimethylacetamide (2.5 mL) was added acetyl chloride (0.071 g, 0.9 mmol). The reaction mixture was stirred at 25 °C for 3 h and diluted with ether (20 mL). The solvent was decanted off, leaving a gummy residue in the flask which was taken up in water (10 mL). The pH of the solution was adjusted to 9 by adding 1 N NaOH dropwise. Charcoal was added to the solution and the mixture filtered through Celite. The filtrate was acidified to pH 4 with 0.5 N HCl, and the white solid that had separated was collected after 1 h at 0 °C by filtration to give 0.05 g (23%) of **7c**. Compound **7c** prepared by this method was identical in all respects with the above sample from method A.

Diethyl *N*-[*N*-[[2-(Pivaloylamino)-4-hydroxy-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl]methyl]-4-aminobenzoyl]glutamate (5b). To a 3% solution of H_2SO_4 in absolute ethanol (40 mL) was added **3c** (2.0 g, 3.3 mmol), and the mixture was stirred at 25 °C for 16 h. The reaction mixture was cooled to 0 °C and poured into a cold aqueous saturated solution of sodium bicarbonate (300 mL). The mixture was extracted with

methylene chloride (3 × 100 mL), and the combined organic layers were dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure and the residue chromatographed (radial chromatography), eluting with 5% methanol in methylene chloride. The fractions containing the product were combined and evaporated under reduced pressure. The residue was triturated with ether and the insoluble product collected by filtration to give 1.62 g (85%) of **5b**: mp 133–134 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.04–1.16 (m, 6 H), 1.18 (s, 9 H), 1.94–2.06 (m, 4 H), 2.38 (t, 2 H, J = 7.37 Hz), 2.90–3.00 (m, 1 H), 3.00–3.10 (m, 1 H), 6.41 (m, 1 H), 6.47 (s, 1 H), 6.57–6.59 (AA′BB′, 2 H), 7.62–7.65 (AA′BB′, 2 H), 8.21 (d, 1 H, J = 7.40 Hz); mass spectrum, m/z (relative intensity) 381 (14), 260 (17), 249 (29), 248 (100), 247 (23), 164 (17), 131 (19), 119 (19). Anal. ($C_{29}H_{40}N_6O_7$) C, H, N.

Diethyl *N*-[*N*-[[2-(Pivaloylamino)-4-hydroxy-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl]methyl]-*N*^{10}-methyl-4-aminobenzoyl]glutamate (5e). To a stirred solution of **5b** (2.52 g, 4.31 mmol) and 37% formaldehyde (2 mL) in acetonitrile (20 mL) at 25 °C was added sodium cyanoborohydride (0.81 g, 12.93 mmol). Glacial acetic acid (0.5 mL) was added to the reaction mixture dropwise over a period of 10 min. After stirring for 2 h, additional glacial acetic acid (0.5 mL) was added, and the mixture was stirred for 18 h, poured into water (300 mL), and extracted with methylene chloride (3 × 100 mL). The combined organic layers were dried over anhydrous magnesium sulfate and the solution evaporated under reduced pressure. The residue was chromatographed (radial chromatography), eluting with 2% methanol in methylene chloride, to give 1.3 g of **5e** (50%). Recrystallization from ethyl acetate afforded pure **5e**: mp 181–183 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.11–1.18 (m, 6 H), 1.17 (s, 9 H), 1.92–2.02 (m, 4 H), 2.39 (t, 2 H, J = 7.43 Hz), 2.97 (s, 3 H), 2.93–3.02 (m, 1 H), 3.15–3.23 (m, 1 H), 3.65 (d, 2 H, J = 6.71 Hz), 3.98–4.10 (m, 4 H), 4.32–4.42 (m, 1 H), 6.41 (m, 1 H), 6.45 (s, 1 H), 6.70–6.73 (AA′BB′, 2 H), 7.69–7.72 (AA′BB′, 2 H), 8.28 (d, 1 H, J = 7.40 Hz); mass spectrum, m/z (relative intensity) 598 (4, M^+), 396 (33), 349 (41), 262 (42), 261 (40), 249 (100), 248 (45), 247 (26), 148 (20). Anal. ($C_{30}H_{42}N_6O_7$) C, H, N.

***N*-[*N*-[(2-Amino-4-hydroxy-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)methyl]-*N*^{10}-methyl-4-aminobenzoyl]glutamic Acid (7d).** A solution of **5e** (0.212 g, 0.35 mmol) in 1 N NaOH was stirred at 25 °C for 72 h and the reaction mixture acidified to pH 6 with 0.5 N HCl. The white solid was collected by filtration and washed with water. The product was allowed to air-dry on the filter and was then triturated with ether to remove residual pivalic acid to give 0.160 g (99%) of **7d** after drying overnight in a vacuum desiccator over P_2O_5: mp, gradually decomposes >180 °C; HPLC (retention time) diastereomer A, 6.46 min, and diastereomer B, 8.73 min; ^{1}H NMR (Me_2SO-d_6) δ 1.80–2.15 (m, 6 H), 2.31 (t, 2 H, J = 7.4 Hz), 2.37–2.39 (m, 1 H), 2.84–2.91 (m, 2 H), 2.96 (s, 1 H), 3.09–3.13 (m, 1 H), 3.3 (d, 2 H, J = 6 Hz), 4.30–4.37 (m, 1 H), 6.07 (s, 2 H), 6.35 (s, 1 H), 6.67–6.70 (AA′BB′, 2 H), 7.69–7.72 (AA′BB′, 2 H), 8.17 (d, 1 H, 7.4 Hz), 9.97 (br s, 1 H). Anal. ($C_{21}H_{26}N_6O_6$·0.75H_2O) C, H, N.

Acknowledgment. We gratefully acknowledge financial support of this work by the National Institutes of Health, National Cancer Institute (CA42367), and by Eli Lilly and Co., Indianapolis, IN 46285. R.G.M. is a Scholar of the Leukemia Society of America.

Registry No. (6*R*)-**1**, 106400-81-1; (6*S*)-**1**, 106400-18-4; **2a**, 87373-56-6; **2b**, 120040-44-0; **2c**, 120040-43-9; **3a**, 115499-15-5; **3b**, 120145-37-1; **3c**, 115499-18-8; **3d**, 115499-19-9; **4**, 120145-38-2; (6*R*)-**5a**, 120203-03-4; (6*S*)-**5a**, 120203-04-5; (6*R*)-**5b**, 120145-39-3; (6*S*)-**5b**, 120145-40-6; (6*R*)-**5c**, 120203-05-6; (6*S*)-**5c**, 120203-06-7; (6*R*)-**5d**, 120203-07-8; (6*S*)-**5d**, 120203-08-9; (6*R*)-**5e**, 120145-41-7; (6*S*)-**5e**, 120145-42-8; **6**, 13726-52-8; (6*R*)-**7a**, 115587-73-0; (6*S*)-**7a**, 115587-72-9; (6*R*)-**7b**, 120203-09-0; (6*S*)-**7b**, 120203-10-3; (6*R*)-**7c**, 120145-43-9; (6*S*)-**7c**, 120145-44-0; (6*R*)-**7d**, 120145-45-1; (6*S*)-**7d**, 120145-46-2.

Binary Antidotes for Organophosphate Poisoning: Aprophen Analogues That Are both Antimuscarinics and Carbamates

Haim Leader,† Ruthann M. Smejkal, Charlotte S. Payne, Felipe N. Padilla, B. P. Doctor, Richard K. Gordon, and Peter K. Chiang*

Department of Applied Biochemistry, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100.
Received September 6, 1988

Prophylaxis against organophosphate poisoning can be achieved by pretreatment with physostigmine or pyridostigmine, which are carbamates, and aprophen, which is an anticholinergic agent. Thus, a series of aprophen analogues was synthesized with carbamyl substitutions on the phenyl rings (carbaphens). The rationale behind this design is that such compounds might exhibit most of the therapeutic characteristics of aprophen, as well as the ability to protect prophylactically by chemically masking cholinesterase enzymes. Compounds **4** (dimethylhydroxycarbaphen), **15** (dimethylcarbaphen), and **16** (monomethylcarbaphen) were found to inactivate human butyrylcholinesterase in a time-dependent manner with potencies similar to those of physostigmine or pyridostigmine, and the latter two exhibited almost the same antimuscarinic profile as aprophen. In contrast to the potent inactivation of butyrylcholinesterase by these compounds, marginal inactivation of acetylcholinesterase activity was observed, and only at much higher drug concentrations. The noncarbamylated analogues had no effect on the activity of either cholinesterase. The carbaphen compounds are hence prototype drugs that can interact with either muscarinic receptors or butyrylcholinesterase. Furthermore, these compounds are prodrugs, since after carbamylation of the cholinesterase, the leaving group **14** (hydroxyaprophen) is a potent antimuscarinic itself.

Aprophen [(*N*,*N*-diethylamino)ethyl 2,2-diphenylpropionate] is a potent anticholinergic and antispasmodic agent possessing a wide number of distinct pharmacological actions, including both antimuscarinic and noncompetitive nicotinic antagonist activities.[1-9] The potent antimuscarinic and antinicotinic effects of aprophen make it a potential drug of choice in the therapy of poisoning by organophosphate/anticholinesterase agents.[3,10,11]

* To whom correspondence should be addressed.
† On leave from the Israel Institute for Biological Research, Ness-Ziona, Israel.

(1) Mashkovsky, M. D.; Liberman, S. S. *Farmakol. Toksikol.* **1957**, *20*, 354.
(2) Volkova, Z. V. *Farmakol. Toksikol.* **1959**, *22*, 348.
(3) Witkin, J. M.; Gordon, R. K.; Chiang, P. K. *J. Pharmacol. Exp. Ther.* **1987**, *242*, 796.
(4) Carroll, F. I.; Abraham, P.; Parham, K.; Griffith, R. C.; Ahmad, A.; Richard, M. M.; Padilla, F. N.; Witkin, J. M.; Chiang, P. K. *J. Med. Chem.* **1987**, *30*, 805.