through 300 transfers (K = 0.28) gave 63 mg of chromatographically homogeneous white powder: R_f(I) 0.55, R_f(II) 0.37, R_f(III) 0.34. An acid hydrolysate had Ala 1.01, Val 1.03, Tyr 1.04, Ile 0.95, His 0.98, Pro 0.99, Phe 0.98, peptide content 76%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Ala 1.13, Val 0.02, Tyr 0.05, aIle 0.01, His 0.07, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −55.6° (c 1, 1 N AcOH).

Nva-Val-Tyr-Ile-His-Pro-Phe (4). This was synthesized as for **2** giving 280 mg of deep yellow powder. Purification by CCD through 200 transfers (K = 0.43) gave 100 mg of chromatographically homogeneous white powder: R_f(I) 0.58, R_f(II) 0.41, R_f(III) 0.38. An acid hydrolysate had Nva 0.97, Val 1.01, Tyr 1.04, Ile 0.97, His 0.99, Pro 0.99, Phe 1.01, peptide content 77%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Nva 0.04, Val 0.02, Tyr 0.05, aIle 0.01, His 0.07, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −46.1° (c 1, 1 N AcOH).

D-Nva-Val-Tyr-Ile-His-Pro-Phe (5). This was synthesized as for **2**, giving 185 mg of yellow powder, and purified as for **4** through 200 transfers (K = 0.42) giving 33 mg of chromatographically homogeneous white powder: R_f(I) 0.60, R_f(II) 0.41, R_f(III) 0.40. An acid hydrolysate had Nva 0.96, Val 1.04, Tyr 1.04, Ile 0.99, His 0.98, Pro 0.99, Phe 1.02, peptide content 72%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Nva 0.96, Val 0.02, Tyr 0.04, aIle 0.01, His 0.05, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −65.3° (c 1, 1 N AcOH).

Val-Tyr-Ile-His-Pro-Phe (6). A 5.0-g (1.0 mmol) portion of **1** was subjected to thiolysis by 6 ml of $HSCH_2CH_2OH$ in 18 ml of DMF; then the peptide was cleaved from the polymer as for **2** giving 600 mg of yellow powder. Purification by CCD through 500 transfers (K = 0.23) gave 240 mg of chromatographically homogeneous off-white powder: R_f(I) 0.55, R_f(II) 0.39, R_f(III) 0.35, R_f(IV) 0.65. An acid hydrolysate had Val 1.00, Tyr 1.04, Ile 0.97, His 0.99, Pro 1.02, Phe 0.98, peptide content 73%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Val 0.02, Tyr 0.04, aIle 0.01, His 0.07, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −31.8° (c 0.4, 1 N AcOH).

Propionyl-Val-Tyr-Ile-His-Pro-Phe (7). To a solution of 25 mg (0.025 mmol) of **6** in 1.7 ml of purified DMF[16] were added 0.011 ml (0.15 mmol) of Et_3N and 10.3 mg (0.053 mmol) of p-nitrophenyl propionate.[17] After 24 hr at 25°, the peptide **7** was precipitated by the addition of 17 ml of anhydrous Et_2O. The precipitate was collected by centrifugation, washed with 17 ml of Et_2O, and lyophilized from 1 N AcOH giving 23 mg of chromatographically homogeneous white powder: R_f(I) 0.64, R_f(II) 0.43, R_f(III) 0.54, R_f(IV) 0.69. An acid hydrolysate had Val 1.02, Tyr 1.00, Ile 0.97, His 0.98, Pro 1.00, Phe 1.00, peptide content 61%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Val 0.02, Tyr 0.03, aIle 0.01, His 0.05, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −48.3° (c 1, 1 N AcOH).

Valeryl-Val-Tyr-Ile-His-Pro-Phe (8). This was prepared in the same manner as **7** using p-nitrophenyl valerate.[18] There was obtained 22 mg of chromatographically homogeneous white powder: R_f(I) 0.68, R_f(II) 0.49, R_f(III) 0.60, R_f(IV) 0.69. An acid hydrolysate had Val 1.04, Tyr 1.00, Ile 0.98, His 0.98, Pro 1.04, Phe 1.03, peptide content 57%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Val 0.02, Tyr 0.03, aIle 0.01, His 0.04, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −42.6° (c 0.8, 4 N AcOH).

Ac-Val-Tyr-Ile-His-Pro-Phe (9). This was prepared in the same manner as **7** using p-nitrophenyl acetate: yield, 24 mg of chromatographically homogeneous white powder; R_f(I) 0.62, R_f(II) 0.38, R_f(III) 0.52, R_f(IV) 0.60. An acid hydrolysate had Val 1.03, Tyr 1.01, Ile 1.01, His 0.99, Pro 0.96, Phe 0.97, peptide content 59%. An L-amino acid oxidase digest of the acid hydrolysate[4] had Val 0.02, Tyr 0.03, aIle 0.01, His 0.04, Pro 1.00, Phe 0.04;‡ $[\alpha]^{23}D$ −54.1° (c 0.5, 1 N AcOH).

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Folic Acid Antagonists. Methotrexate Analogs Containing Spurious Amino Acids. Dichlorohomofolic Acid†

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Received August 30, 1973

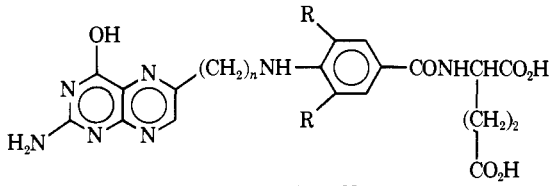
The synthesis and some biological testing results are reported for dichlorohomofolic acid (**2a**) and three methotrexate analogs in which the L-glutamic acid moiety has been replaced by D-glutamic acid, glutaric acid, and L-lysine to give **4b**, **4c**, and **4d**, respectively. Compounds **4b–d** were all like MTX in being strong inhibitors of dihydrofolate reductase, but only **4b** demonstrated activity against leukemia L1210 in the mouse.

In connection with our cancer chemotherapy research, we have long been interested in analogs of folic acid (**1**).[1] We now report the synthesis and some biological evaluations of 3′,5′-dichlorohomofolic acid (**2a**) and some methotrexate (MTX, **4a**)[2] analogs, **4b–d**.

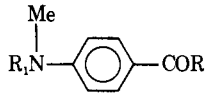
Both MTX (**4a**) and its dichloro derivative are active antitumor agents,[3] with the latter being less toxic. Since homofolic acid (**2b**) in the tetrahydro form[4] has extremely interesting biological properties,[4,5] it seemed worthwhile to prepare the dichloro derivative **2a** for testing.

The MTX analog in which the glutamic acid moiety is replaced by aspartic acid[6] has shown antitumor activity against advanced lymphoid leukemia L1210.[3] This and the fact that natural folate derivatives may have various numbers of glutamate residues[7] suggest that MTX ana-

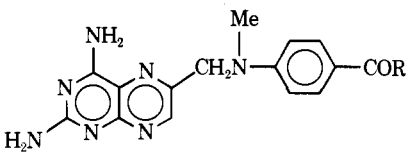
† This work was performed under the auspices of Drug Research and Development (DRD), National Cancer Institute, National Institutes of Health, Public Health Service, Contract No. NO1-CM-33742. The opinions expressed in this paper are those of the authors and not necessarily those of DRD.



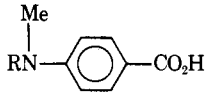
1, n = 1; R = H
2a, n = 2; R = Cl
b, n = 2; R = H



3a–p
(see Table I)



4a, R = L-Glu
b, R = D-Glu
c, R = NHCH(CH_2CO_2H)$_2$ = Gtr
d, R = Lys

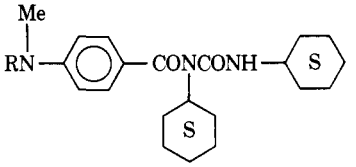


5a, R = Cbz
b, R = CHO
c, R = H

logs accommodating considerable changes in the glutamate moiety may retain their activity. Thus, we prepared the MTX analogs **4b–d** in order to assess this possibility. These analogs represent fairly broad changes in the amino acid moiety—from the L series **4a** to the D series in D-MTX (**4b**); from an α-amino to β-amino acid in **4c**; and from a dicarboxylic acid to a monocarboxylic acid with a free amino group in **4d**.

Homofolic acid[1,4] (**2b**) was readily chlorinated in 6 N hydrochloric acid[8] to give **2a** in good yield. The MTX analogs were all prepared by the same general scheme. The appropriate p-methylaminobenzoyl derivative **3** of the amino acid was prepared and converted by the Waller reaction[9] to the MTX analog. The general procedures are described briefly in the Experimental Section, while the physical properties of intermediates and products are summarized in Table I.

Initially p-(N-carbobenzyloxymethylamino)benzoic acid‡ (**5a**) was used as starting material for both D-MTX (**4b**) and the β-aminoglutarate analog **4c**, but it was later replaced by the more convenient p-(N-methylformamido)-benzoic acid (**5b**).[11] This was smoothly converted to the acid chloride with phosphorus pentachloride in ether. Reaction with diethyl D-glutamate or diethyl β-aminoglutarate[12] proceeded readily to afford **3e** and **3j**, respectively. These were best converted to **3c** and **3i**, respectively, by saponification of the ester and then treatment with hydrochloric acid to remove the formyl group. Complete deblocking with hydrochloric acid in a single step was less satisfactory because of the formation of a persistent impurity. Using dicyclohexylcarbodiimide to couple **5b** or p-methylaminobenzoic acid (**5c**) with either diethyl glutamate or diethyl β-aminoglutarate was unsatisfactory under a variety of conditions. There was facile formation



6a, R = CHO
b, R = H

of the acylureas **6a** and **6b**, respectively; no evidence was found for side reactions involving the amino group of **5c**. However, low yields of the analytically pure products **3d**§ and **3l** could be obtained by preparative thick-layer chromatography.

In the lysine series, the commercially available methyl ester of N^{ϵ}-benzyloxycarbonyl-L-lysine[14] was used to prepare the formamidobenzoyl **3m**, which could be saponified to afford **3n**. However, **3m** was generally hydrolyzed carefully to remove the formyl group without affecting the benzyloxycarbonyl group. The ester **3o** was then saponified to give an excellent yield of **3p** for use in the Waller reaction.

Slight modifications of the literature procedure[2] for the Waller reaction,[9] using N-(p-methylamino)benzoyl-D-glutamic acid (**3c**) with 2,4,5,6-tetraaminopyrimidine and 2,3-dibromopropionaldehyde, and of Noble's[15] purification procedure afforded very pure D-MTX (**4b**) in 6.5% yield. For β-[p-(methylamino)benzamido]glutaric acid (**3i**), the Waller reaction was run at pH 5.6 instead of pH 3.7, and larger excesses of pyrimidine and aldehyde were required to give an 11.3% yield of very pure glutarate analog **4c**. For the lysine analog **4d**, the Waller reaction of **3p** required some methanol in the solvent for solubility. The crude reaction product was similarly purified by Noble's[15] procedure, giving a 7.3% yield of **4e** of analytical purity. The N^{ϵ}-benzyloxycarbonyl group was then removed by brief treatment with hydrogen bromide in trifluoroacetic acid to give a 92% yield of analytically pure **4d** as the trihydrobromide trihydrate. These results represent our experience with a considerable number of runs with the Waller reaction. They show that, with suitable choice of reaction conditions and purification, the Waller reaction can give low but consistent yields of purified product in the 5–10% range.

The final products and a number of intermediates were submitted to the National Cancer Institute (NCI) for biological screening. The data for the products **2a** and **4b–d** are summarized in Table II. The tests against leukemia L1210 in the mouse show **4b**, D-MTX, to be less active and considerably less toxic than MTX itself [LD_{10} = 5 mg/kg/day; qd 1–5 days (ref 16, Figure 3)]. The other final products were inactive against L1210 at doses up to 400 mg/kg/day, as were the intermediates **3a,b,e–k,m,o,p**. For comparison, some data for **2b** and **4a** are included in Table II.

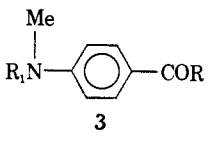
Against dihydrofolate reductase (DHFR), the final products **4b–d** all acted as strong inhibitors of the enzyme from both mouse and human leukemia cells. Dichlorohomofolic acid (**2a**) was a weak inhibitor of DHFR. Whether the reduced tetrahydro form of **2a** is a strong inhibitor of thymidylate synthetase, as is tetrahydrohomofolate,[4] is not presently known. The intermediates **3a,b,e–i,m,o,p** were all weak inhibitors of DHFR. The compounds **2b**, **4b**, and **4d** are being tested further under the auspices of the NCI.

The biological results show that the L-glutamyl moiety of MTX can be modified and still retain some activity

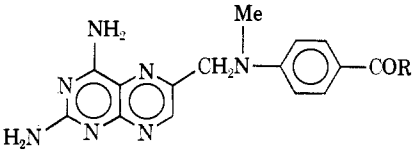
‡ Fu, *et al.*,[10] used p-(carbobenzyloxymethylamino)benzoic acid for an improved synthesis of the L isomer of **3c**.

§ Santi[13] has synthesized the L isomer of **3d** starting from ethyl N-tosyl-p-aminobenzoate.

Table I. Some MTX Analogs and Their Intermediates 3 and 4



3



4

Compd	R_1	R	Yield, %[a]	Mp, °C[a]	Solvent[b]	Chromatography, R_f (solvent)[c]	Formula	Analyses
3a	Z	D-Glu-OEt	(82)	68.0–68.5 (oil)	A	0.91 (PB), 0.95 (PA), 0.52 (TA)	$C_{25}H_{30}N_2O_7$	C, H, N
3b	Z	D-Glu	(100)	64–65 (foam)	B		$C_{21}H_{22}N_2O_7$	C, H, N
3c	H	D-Glu	(100)	(Foam)				
3d	H	D-Glu-OEt	Low	73–80		0.36 (TA)	$C_{17}H_{24}N_2O_5$	C, H, N
3e	Fo	D-Glu-OEt	90	Oil[d]		0.21 (TA)	$C_{18}H_{24}N_2O_6$	C, H, N
3f	Fo	D-Glu	62 (100)	153–153.5	C	0.30 (TM)	$C_{14}H_{16}N_2O_6$	C, H, N
3g	Z	Gtr-OEt	(92)	Oil	*d*	0.32 (TA), 0.93 (PA)	$C_{25}H_{30}N_2O_7$	C, H, N
3h	Z	Gtr	(79)	167.5–168.5 (165.5–166.5)	E	0.20 (TC), 0.79 (PA)	$C_{21}H_{22}N_2O_7$	C, H, N
3i	H	Gtr	80	195.5–196.5	B	0.80 (PH)	$C_{13}H_{16}N_2O_5 \cdot H_2O$	C, H, N
3j	Fo	Gtr-OEt	76	Foam		0.66 (TB)	$C_{18}H_{24}N_2O_6$	C, H, N
3k	Fo	Gtr	55 (78)	181–182	F	0.0 (TD)	$C_{14}H_{16}N_2O_6$	C, H, N
3l	H	Gtr-OEt	Low	Oil		0.25 (TA)	$C_{17}H_{24}N_2O_5$	H, N; C[e]
3m	Fo	Z-Lys-OMe	(93)	87–88	G	0.50 (TE), 0.59 (TB)	$C_{24}H_{29}N_3O_6$	C, H, N
3n	Fo	Z-Lys	44 (87)	118–119.5	G	0 (TE)	$C_{23}H_{27}N_3O_6$	C, H, N
3o	H	Z-Lys-OMe	80	89–90		0.80 (TE)	$C_{23}H_{29}N_3O_5$	C, H, N
3p	H	Z-Lys	90 (97)	134–135[f] (132–133)	H	0.20 (TD)	$C_{22}H_{27}N_3O_5$	C, H, N
4b		D-Glu	6.5 (9)	200–205	J	0.65 (PU), 0.49 (PZ)	$C_{20}H_{22}N_8O_5 \cdot 3H_2O$	C, H, N
4c		Gtr	11.3	283–284 (270–280)	K	0.47 (PU), 0.64 (PZ)	$C_{20}H_{22}N_8O_5 \cdot 0.25H_2O$	C, H, N
4d		Lys	92	Hygroscopic	L	0.55 (PG), 0.48 (PR)	$C_{21}H_{27}N_9O_3 \cdot 3HBr \cdot 3H_2O$	C, H, N
4e		Z-Lys	7.3	158–160	M	0.47 (PU), 0.33 (PZ)	$C_{29}H_{33}N_9O_5 \cdot 1.5H_2O$	C, H, N
2a			(81)	191–194 (190–194)		0.83 (PU), 0.72 (PZ)	$C_{20}H_{19}Cl_2N_7O_6 \cdot {}^2/_3H_2O$	C, H, N, Cl

[a] Yield data and melting point data are for analytical samples, except for the values in parentheses, which are for homogeneous products suitable for the next reaction. [b] Crystallization solvents are: A, Et_2O–petroleum ether (bp 30–60°); B, HCl, pH 3.5; C, *n*-hexane–EtOH; E, EtOAc; F, EtOH; G, toluene; H, EtOAc–Et_2O; J, HCl, pH 4; K, 1 *N* HOAc; L, CF_3COOH–Et_2O; M, HCl, pH 4.7. [c] Solvent systems for tlc are: TA, $CHCl_3$–MeOH (8:2); EtOAc–MeOH mixtures in these respective ratios: TB (9:1); TC (8:2); TD (7:3); TE, EtOAc; TM, MeOH. Solvent systems for paper chromatography are: PA, C_6H_6–H_2O–MeOH (2:1:6); PB, *n*-BuOH–H_2O (saturated); PH, *n*-BuOH–HOAc–H_2O (5:2:3), PG, *i*-PrOH–2 *N* HCl (65:35); PR, 0.1 *N* HCl in 85% aqueous EtOH; PU, 0.5% Na_2CO_3; PZ, 3% NH_4Cl and 5% NH_4OH. [d] Analytical sample was obtained by preparative tlc using solvent TA. $[\alpha]^{21}D$ +4.6° (*c* 0.5, DMF) for **3e**. [e] C: calcd, 60.8; found, 60.1. [f] $[\alpha]^{21}D$ −11.1° (c 1, MeOH).

against L1210 (as with D-MTX) and high activity as inhibitors of DHFR. However, it seems likely that modification of other portions of the MTX molecule (as in dichloro MTX) may be more fruitful in providing analogs with better antitumor activity. Work is under way in these laboratories to examine other changes in the methylaminobenzoyl moiety of MTX.

Experimental Section

Methods. Melting points (uncorrected) were determined on a Fisher-Johns apparatus, and optical rotations were measured at the D line on a Perkin-Elmer 141 polarimeter. Thick-layer preparative chromatography was done on Brinkman silica gel HF of 2-mm thickness; for thin-layer chromatography, the thickness was 0.25 mm. The solvent systems used are given in footnote *c*, Table I. Paper chromatograms were run by the descending technique on Whatman No. 1, except for solvent system PA, which was run on No. 3. Spots were detected mostly by uv light and occasionally by I_2 vapor. Solvents were removed on a spin evaporator under reduced pressure (water aspirator) with a bath temperature of 40–45° (unless specified) and finished on a mechanical pump when required. Anhydrous magnesium sulfate was used as the drying agent.

***N*-[4-[*N*-[(2-Amino-4-hydroxy-6-pteridyl)ethyl]amino]-3,5-dichlorobenzoyl]-β-L-glutamic Acid (Dichlorohomofolic Acid, 2a).** A 1.00-g (2.2 mmol) sample of homofolic acid (**2b**) was chlorinated with 5.28 mmol of Cl_2 in 6 *N* HCl at ice-bath temperature for 30 min by the literature procedure[8] and then evaporated at room temperature. The solid was collected, triturated with 75 ml of H_2O for 36 hr, filtered, and dried (at 56° and 1 mm) to afford 1.00 g (81%) of **2a**. The use of formamide as reaction solvent was unsatisfactory.

Diethyl *N*-[*p*-(*N*-Methylformamido)benzoyl]-D-glutamate (3e). A mixture of 630 mg (3.5 mmol) of *p*-(*N*-methylformamido)-benzoic acid (**5b**)[11] (prepared by Sheehan and Yang's procedure[17]) and 0.90 g (4.3 mmol) of PCl_5 in 30 ml of dry ether was stirred for 2.5 hr at room temperature. The ether solution was washed with 25 ml of ice-cold water and immediately added—without drying—to a cold (ice bath), stirred mixture of 950 mg (3.5 mmol) of diethyl D-glutamate hydrochloride, 2.35 g of $NaHCO_3$, 15 ml of EtOAc, and 15 ml of H_2O. The mixture was continuously stirred for 0.5 hr at ice-bath temperature and for 1 hr at room temperature. The organic layer was separated, washed, dried, and evaporated to afford 1.13 g (90%) of **3e** as a faintly yellow oil, homogeneous and of analytical purity. Similar preparation was used for compounds **3j** and **3m**. Compound **3m** had $[\alpha]^{21}D$ −9.3° (*c* 0.5, DMF).

The condensation of either the amido acid **5b** or the amino acid **5c** with either diethyl D-glutamate or diethyl β-aminoglutarate using dicyclohexylcarbodiimide (DCC) under a variety of conditions with different solvents gave poor yields of the desired products **3d**, **3e**, and **3l**, which were heavily contaminated by acylurea

Table II. Test Results[a]

Compd	L1210 Doses[b] No.	mg/kg	Surv[c]	Wt[d] diff, g $T - C$	% T/C[e]	DHFR M[f]	H[f]
2a	9	400	3/6	−3.5		7×10^{-5}	
	9	300	3/6	−4.5			
	9	150	4/6	−2.0	104		
	1	400	6/6	−1.4	96		
4b	1	400	6/6	−1.6	132	3.35×10^{-9}	3.3×10^{-9}
	1	200	6/6	−1.6	129		
	1	100	6/6	−0.7	125		
4c	9	400	0/6	−0.9		1×10^{-8}	8×10^{-9}
	9	100	5/6	−2.7	118		
	1	400	4/6	−3.2	102		
4d	1	400	6/6	−0.8	96	5×10^{-9}	3×10^{-9}
4a	9	2	6/6	0	154	3.5×10^{-9}	
2b	1	75[g]	8/8	0	104	*h*	

[a] These compounds were screened under the auspices of NCI against: (1) lymphoid leukemia L1210 in the mouse according to the protocols given in *Cancer Chemother. Rep.*, **3** (2), 7 (1972), and (2) as inhibitors of dihydrofolate reductase (DHFR) according to the procedure of J. P. Perkins, B. L. Hillcoat, and J. R. Bertino, *J. Biol. Chem.*, **242**, 4771 (1967). The results are given as molar concentrations of the inhibitor required for 50% inhibition of DHFR activity under the test conditions. Concentrations required are: 10^{-8}–10^{-9} *M* for strong inhibitors; 10^{-6}–10^{-7} *M*, moderate; 10^{-4}–10^{-5} *M*, weak. [b] Administered intraperitoneally, either one dose or nine doses (once per day) beginning on day 1. [c] Number of survivors over total animals started on test as determined on day 5. The number of survivors is a measure of lack of toxicity. [d] Average weight difference between treated (*T*) and control (*C*) animals. [e] The numerical results are ratios (*T*/*C*) of survival times of treated over control mice, expressed as per cent values ≥125% are positive, denoting activity. [f] Dihydrofolate reductase from: M, mouse L1210 cells; H, human leukemia cells. [g] Also inactive at other doses ranging from 150 to 18.7 mg/kg. [h] Compound **2b** at the dihydro stage is as effective a substrate as dihydrofolate.

by-products. Thus, the reaction of 11.1 mmol each of diethyl D-glutamate and **5c** (1.68 g) in 50 ml of pyridine with 3.1 g (15 mmol) of DCC at room temperature for 20 hr afforded 2.73 g of oil containing two major components (R_f 0.36 and 0.64 in solvent TA). Preparative tlc gave the analytical samples of **3d** and **6b**. Compound **3d** had $[\alpha]^{25}D$ −2.0° (*c* 0.5, DMF) and $[\alpha]^{25}D$ +17.8° (*c* 0.5, 1 *N* HCl), as compared with the L isomer with $[\alpha]^{25}D$ −21° (*c* 1, 1 *N* HCl); it was analyzed as an oil that crystallized on standing. Table I lists the other properties. Data for *N*-(*p*-methylaminobenzoyl)-*N*,*N*′-dicyclohexylurea (**6b**) are mp 161–162°; R_f 0.64 in solvent TA. *Anal.* ($C_{21}H_{31}N_3O_2$) C, H, N. Compound **3l** was similarly obtained.

The similar condensation of **5b** and diethyl β-aminoglutarate using DCC gave a crude oil, R_f 0.21 and 0.43 in solvent TA. Crystallization from ether afforded a 31% yield of *N*-[*p*-(*N*-methylformamido)benzoyl]-*N*,*N*′-dicyclohexylurea (**6a**): mp 160–160.5°; R_f 0.43 and 0.71 in solvents TA and TE, respectively. *Anal.* ($C_{22}H_{31}N_3O_3$) C, H. The other product (R_f 0.21 in solvent TA) was not isolated in pure form but had properties (tlc, ir) like that of authentic **3j**, prepared from the acid chloride.

***N*-[*p*-(*N*-Methylformamido)benzoyl]-D-glutamic Acid (3f).** An ice-cold solution of 1.13 g (3.1 mmol) of **3e** in 10 ml of MeOH was treated with 6.2 ml of 1 *N* NaOH solution and then stirred at room temperature for 2 hr. Evaporation of the solution left a residue that was redissolved in water and neutralized with 6.2 ml of 1 *N* HCl. This was extracted with EtOAc (5 × 75 ml) to afford 0.96 g (100%) of homogeneous **3f**, which was recrystallized to afford the analytical sample. Compounds **3k** and **3n** were similarly prepared [compound **3n** had $[\alpha]^{21}D$ −1.4° (*c* 0.5, DMF)]. However, in later runs, the formamido derivatives **3f** and **3k** were not isolated but were used immediately, as illustrated in the next experiment.

β-(*p*-Methylaminobenzamide)glutaric Acid (3i). A cold MeOH solution of 9.11 g (25.0 mmol) of **3j** was treated with 50 mol of NaOH—as was **3f**—to afford, after work-up, a white residue of **3k**. This was heated in 200 ml of concentrated HCl for 4.0 hr at 39 ± 1° and then evaporated to dryness. The residue was redissolved in 100 ml of H_2O, filtered, and adjusted to pH 3.5 with 4 *N* NaOH and refrigerated overnight. The crystalline material was collected, washed with 0.001 *N* HCl, and dried to afford 6.01 g (80% overall from **3j**) of analytically pure **3i**.

Methyl N^{α}-[*p*-(*N*-Methylamino)benzoyl]-N^{ϵ}-carbobenzoxy-L-lysinate (3o). A solution of 120 mg (0.26 mmol) of **3m** in 40 ml of MeOH and 0.25 ml (2.9 mmol) of concentrated HCl was heated 1.5 hr at reflux, diluted with 20 ml of toluene, and evaporated at 25°. The residue was washed in 75 ml of EtOAc ($NaHCO_3$ solution and H_2O), dried, and evaporated to leave 120 mg of **3o** as an oil. This was taken up in MeOH, filtered, and evaporated. The residue on trituration with ether and evaporation left 90 mg (80%) of **3o** as an analytically pure, homogeneous, colorless oil: uv max (pH 7) 296 nm (ε 21,000).

Waller Reaction for the Preparation of MTX Analogs. D-Methotrexate (4b). The procedure of Seeger, *et al.*,[2] was followed for the reaction of 47 mmol of **3c**, with the pH at 3.7 ± 0.2 (monitored by pH meter). The work-up was modified as follows. After refrigeration, the crude precipitated product was collected and washed with 500 ml of cold 10^{-3} *N* HCl. The combined filtrate and washes were set aside as solution 1. The product was mixed with 470 ml of 1 *N* NaOH, diluted to 2 l., and stirred at room temperature for 0.5 hr. The insoluble inorganic barium salts were collected and washed with 500 ml of water. The filtrate and the wash, containing the product, were combined with solution 1, and the resulting basic solution (~pH 11) was charged on a 3.2 × 55 cm column containing Dowex 1 (OAc) and purified by Noble's procedure[15] with these modifications. Elution with 6 l. of 1 *N* NaOAc solution was followed by 0.25 *N* HOAc until all by-products were eluted and product elution began (~2.5 l.; paper chromatography in solvent system PU and PZ). The product was collected in the next 1.6 l. of eluent. The solvent was removed, and the residue was triturated with absolute methanol, collected, and dried, affording 2.11 g of **4b**. A final charcoal treatment with 7.5 g of a charcoal–cellulose mixture by Noble's procedure[15] afforded 1.54 g (6.5%) of **4b**, mp 200–205°. Tlc showed one spot and a very faint trace at the origin but no streaks of fluorescent material, and the R_f was identical with that of highly purified L-MTX: $[\alpha]^{22}D$ −7.2° (*c* 0.4, DMF). Table I lists the other properties of **4b**.

4c. The procedure used for **4b** was followed to produce **4c**, except that the reaction was run at pH 5.6 to prevent the acid **3i** from precipitating out, and a 3.4 molar excess of the pyrimidine and aldehyde was required. During the column purification by Noble's procedure,[15] the product crystallized out at the top of the Dowex column on elution with the 0.25 *N* acetic acid. This portion of the resin was removed and washed with 1 *N* sodium hydroxide and water, and the product was extracted from the resin with 10% acetic acid. Adjustment to pH 3.5 and refrigeration of the solution then gave the product **4c**.

4d. The procedure for **4b** was modified by adding 55 ml of methanol to ensure the solubility of **3p** (4.8 mmol) and by running the Waller reaction at pH 5.7 for 1 hr at 45° after all the reactants had been added. Addition of *N*,*N*-dimethylformamide instead of methanol to the reaction gave poorer results. Some of the highly purified **4e** (50 mg) was dissolved in trifluoroacetic acid (2 ml for 50 mg) and then saturated was gaseous hydrogen bromide. After standing for 20 min, the solution was diluted with 30 ml of ether. The precipitate of **4d**, as the very hygroscopic hydrobromide, was collected, washed with *n*-hexane (25 ml), and dried for 2 hr at 50° and 1 mm and then for 3 days in a desiccator again at 1 mm over phosphorus pentoxide and potassium hydroxide to afford 58 mg (92%) of **4d**·3HBr·3H_2O of analytical purity.

Acknowledgments. We thank Mr. Robert B. Bicknell and his staff for the large-scale preparations; Dr. Peter Lim and his staff for the spectral data; and Dr. David W. Henry for helpful discussions.

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Structure–Activity Relationships of Fenamic Acids†

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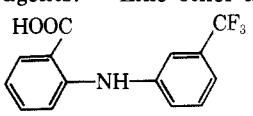
Physicochemical properties such as the dissociation constant in H_2O and partition coefficient with the 1-octanol–H_2O system have been determined for a set of 3′-substituted fenamic acids including flufenamic and mefenamic acids. It has been found that the log K_A value is linearly related with the Hammett σ constants and the Π value of 3′ substituents is practically identical with those derived from substituted phenols. With the use of substituent parameters, σ and Π, the BSA binding affinity and uncoupling activity of the acids have been analyzed. An important role of hydrophobicity is found for each of the activities. The uncoupling activity of fenamic acids is indicated to vary according to common features with other sets of uncouplers such as phenols, benzimidazoles, and methylenebisphenols, hydrophobically as well as electronically, suggesting that they interact quite similarly with the energy conservation mechanism of mitochondria.

Flufenamic acid [*N*-(3′-trifluoromethylphenyl)anthranilic acid] and mefenamic acid [*N*-(2′,3′-dimethylphenyl)anthranilic acid] are known to be potent analgesic and antiinflammatory agents.[1,2] Like other nonsteroidal anti-



flufenamic acid

inflammatory agents, these compounds have uncoupling activity of respiratory chain phosphorylation in isolated mitochondria.[3-7] Recently, we measured the uncoupling activity of a series of 3′-substituted fenamic acids (*N*-phenylanthranilic acids) with the use of rat liver mitochondria. We also determined partition coefficients with *n*-hexane–H_2O, *n*-heptane–H_2O, and chloroform–H_2O systems, and the dissociation constant in acetone–H_2O, and attempted to elucidate their uncoupling activity in terms of these physicochemical properties. An important role of hydrophobic character in the activity of the molecules was found.[8]

In order to obtain more precise information on the biological activity of this class of compounds, the present study was undertaken to analyze the effect of 3′ substituents on uncoupling activity as well as binding affinity with serum albumin in terms of the electronic and hydrophobic substituent parameters, σ and Π, which were evaluated from the dissociation constant in H_2O and the partition coefficient with the 1-octanol–H_2O system, respectively.

Physicochemical Parameters. The 14 fenamic acids used in this study are listed in Table I. Since the potentiometric titration cannot be applied due to their limited solubility, we determined the dissociation constant, K_A, by the "pH-dependent solubility method."[9] pK_A values are listed in Table I. They are considerably different from corresponding values obtained by potentiometric titration in 5–10% aqueous acetone.[8] The 5–10% acetone may not be enough to solubilize the acids during titration. The present values are linearly related to the Hammett σ constant of the 3′ substituent as shown in eq 1. In eq 1, n is the number of acids studied, s is the standard deviation, r is the correlation coefficient, and the values in parentheses are 95% confidence intervals. The pK_A values for some derivatives which were not determined experimentally are calculated from eq 1 by means of σ constants.

$$\log K_A = 0.738\sigma\ (\pm 0.388) - 4.144\ (\pm 0.097) \qquad (1)$$

n	s	r
6	0.076	0.935

The apparent partition coefficient, P', of fenamic acids was measured with the 1-octanol–phosphate buffer system. The partition coefficient, P, for the neutral form was then calculated by eq 2. The P value was also determined directly for some acids by equilibrating the 1-octanol solution with 10^{-2} N HCl. The log P' and log P values are listed in Table I. The coincidence of log P values from

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