according to the Wilcoxon nonparametric W test, the agent was considered active at the given dose. The value ∞ means that more than 50% of treated animals in the group had been cured.

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Notes

Syntheses of α- and γ-Substituted Amides, Peptides, and Esters of Methotrexate and Their Evaluation as Inhibitors of Folate Metabolism[1]

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N-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamic acid α-benzyl ester (**2**) and γ-benzyl ester (**6**) served as key intermediates in syntheses of precursors to amides and peptides of methotrexate (MTX) involving both the α- and γ-carboxyl groupings of the glutamate moiety. Coupling of **2** and **6** at the open carboxyl grouping with amino compounds was effected by the mixed anhydride method (using isobutyl chloroformate); carboxyl groupings of amino acids coupled with **2** and **6** were protected as benzyl esters. *N*-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamic acid γ-methyl ester (**5**), a precursor to MTX γ-methyl ester, was prepared from L-glutamic acid γ-methyl ester and 4-[[(benzyloxy)carbonyl]methylamino]benzoyl chloride (**1**) in a manner similar to that used to prepare **2** and **6**. The precursor to MTX α-methyl ester was prepared from γ-benzyl ester **6** by treatment with MeI in DMF containing $(i\text{-Pr})_2$NEt. Benzyl and (benzyloxy)carbonyl protective groupings were removed by hydrogenolysis, and the deprotected side-chain precursors were converted to α- and γ-substituted amides, peptides, and esters of MTX by alkylation with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**12**). Biochemical–pharmacological studies on the prepared compounds aided in establishing that the α-carboxyl grouping of the glutamate moiety contributes to the binding of MTX to dihydrofolate reductase while the γ-carboxyl does not. Other studies on the peptide MTX-γ-Glu (**13h**) are concerned with the contribution toward antifolate activity of this metabolite of MTX. The compounds prepared were also evaluated and compared with MTX with respect to cytotoxicity toward H.Ep.-2 cells and effect on L1210 murine leukemia.

Methotrexate (MTX) has been in clinical use for more than 30 years.[2,3] It remains a mainstay in the treatment of acute leukemia[4] and choriocarcinoma[5] and has also proved beneficial in treatment of osteogenic sarcoma[6,7] and carcinoma of the head and neck.[8] The usefulness of MTX in the treatment of human leukemia is limited by the ability of the malignant cells to develop resistance to the drug.[8,9] Some tumors are naturally resistant while others acquire resistance after a period of response. Two factors known to be connected with resistance are (1) increased intracellular levels of dihydrofolate reductase (DHFR), the enzyme whose inhibition is the main cause of the arrest of cell proliferation by MTX, and (2) loss of the active-transport system by which MTX enters cells.[10-13] Certain murine tumors deficient in this transport system are resistant to MTX but sensitive to other inhibitors of DHFR which enter cells by passive diffusion.[11,14] Acquired resistance in some leukemia cell lines has been shown to result from loss of the active-transport mechanism.[12,15-17] These observations have prompted searches for synthetic analogues of MTX which might have favorably altered transport characteristics and still have the capacity to bind to DHFR.

The tight binding of MTX to DHFR is readily demonstrated in vitro, and, until recently, the concept that MTX acted without metabolic activation was accepted.[18]

(1) Most of the synthetic work described in this paper was briefly summarized earlier [J. R. Piper and J. A. Montgomery, "Chemistry and Biology of Pteridines", Proceedings of the International Symposum on the Chemistry and Biology of Pteridines, 6th, La Jolla, CA, 1978. See *Dev. Biochem.*, 4, 261 (1979)].
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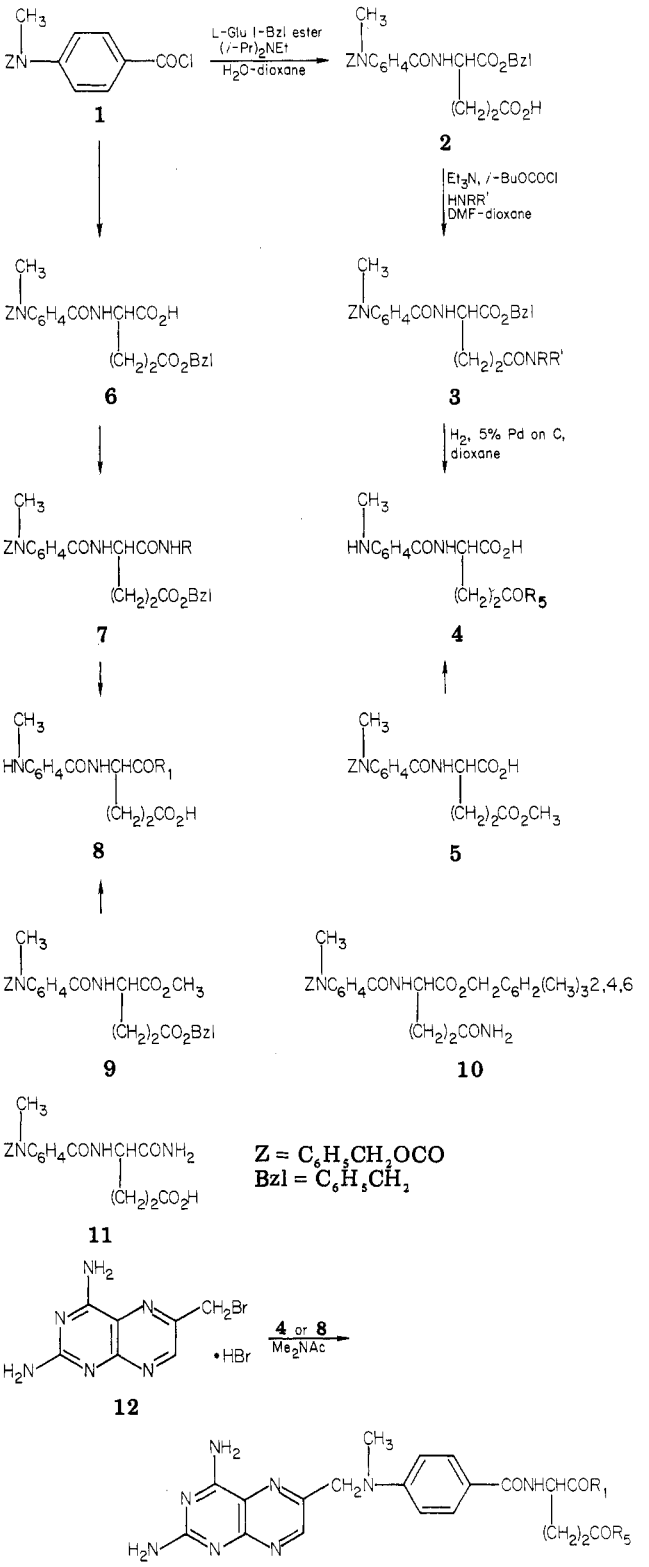
Burgeoning interest and active investigations in this area have resulted in rapid accumulation of new information which suggests that γ-glutamyl peptides formed intracellularly following MTX administration appear to contribute significantly to the pharmacological activity of MTX.[19-22] The finding that *N*-[*N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-γ-glutamyl]-L-glutamic acid (**13h**, Scheme I), a significant metabolite of MTX in a number of mammalian tissues,[19] is equivalent to MTX as an inhibitor of DHFR from L1210 leukemia cells[20] led us to synthesize **13h** and a number of other γ- and also α-amides and peptides of MTX for biological studies.

The synthesis consisted of preparing *N*-[4-(methylamino)benzoyl]-L-γ- and -α-glutamyl precursors of structural types **4** and **8** by unequivocal routes and then introducing the (2,4-diamino-6-pteridinyl)methyl grouping by alkylation with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**12**).[23] All but two of the protected side-chain precursors (types **3** and **7**) were prepared by acylation of the appropriate amino compounds with the α- and γ-benzyl esters **2** and **6** using the mixed-anhydride method. Carboxyl groups of amino acids used in this coupling procedure were protected by benzyl groups. The protected precursor **10** was prepared from **1** and L-glutamine 2,4,6-trimethylbenzyl ester,[24] and **11** was prepared by ammonolysis of **2**. The protective groupings of all precursors except **10** were removed by hydrogenolysis. Deprotection of **10** was effected by HBr. The resulting **4** and **8** types were then treated with **12** to give the amide and peptide types **13** and **14**. The previously reported unambiguous synthesis of **13h** was by the Merrifield method.[19,20] The solution methods described in this paper offer facile adaptability to large-scale syntheses.

Rosowsky and Yu described the use of monoethyl esters of MTX in coupling reactions to prepare compounds related to some described in this paper.[25] The ester approach offers possible advantages over the present method. For example, if the amide bears a grouping incompatible with reducing conditions, intermediates of types **3** and **7**, which are best deprotected by hydrogenolysis, would not be suitable. In this connection we developed straightforward approaches that allow ready access to monoalkyl esters of MTX. Commercial L-glutamic acid γ-methyl ester was coupled with **1** to give **5**, the precursor of MTX γ-methyl ester (**15**). L-Glutamic acid α-methyl ester of suitable purity was not readily available, but treatment of the γ-benzyl ester **6** with methyl iodide in DMF containing *N*,*N*-diisopropylethylamine gave the α-methyl γ-benzyl ester **9**, which served as a precursor to MTX α-methyl ester (**16**). Interest in MTX esters was also heightened recently by the finding by Rosowsky et al. that dibutyl and γ-butyl esters of MTX are active against certain tumor cell lines that do not effectively transport MTX itself.[29,27]

Scheme I



13a-h, 14a-d (Table I)
15, R_1 = OH, R_5 = OCH_3
16, R_1 = OCH_3, R_5 = OH

Biological Data. The inhibitory effects of **13a-h** and **14a-d** on L1210 cell DHFR and the transport properties of these compounds with respect to L1210 cells have been examined; data from these studies are included in Table

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Table I. Biological Test Results

compd	R_1	R_5	cytotoxicity to H.Ep.-2 cells[a] ED_{50}, μM	MTX ratio	act. vs. leukemia L1210[b] optimal dose, mg/kg, qd1-9	% ILS	L1210 cell DHFR inhibn: K_i,[c] nM	L1210 cell membrane transport[c] influx K_m, μM	influx K_i, μM	efflux k, min^{-1}
MTX	OH	OH	0.0024	1.0	1.3	48	0.0043	3.3	3.5	0.23
13a	OH	NH_2	0.028	12.0	180.0	58	0.0027	9.4	8.4	0.15
13b	OH	$NHCH_3$	0.027	11.0	100.0	58	0.0027	27.6		0.21
13c	OH	$N(CH_3)_2$	0.059	25.0	50.0	44	0.0039	48.4		0.26
13d	OH	$NH(CH_2)_4CH_3$	0.055	23.0	200.0	55	0.0035	16.9	16.8	0.26
13e	OH	$NHCH_2C_6H_5$	0.21	88.0	100.0	33	0.0036	3.6	3.8	0.19
13f	OH	Gly	0.0059	2.5	5.0	74	0.0029	3.9		2.1
13g	OH	Asp	0.33	140.0	5.0	65	0.0028	>300.0		
13h	OH	Glu	0.0052	2.2	2.5	65	0.0037	49.3		0.22
14a	NH_2	OH	0.61	250.0	25.0	34	460.0			
14b	Gly	OH	0.59	370.0	50.0	41	208.2		66.6	
14c	Asp	OH	0.52	217.0	25.0	47	208.4		146.2	
14d	Glu	OH	0.18	75.0	25.0	67	170.1		61.0	

[a] Procedure of L. L. Bennett, Jr., M. H. Vali, P. W. Allan, and S. C. Shaddix, *Biochem. Pharmacol.*, **22**, 1221 (1973). [b] Protocol described by R. I. Geran, N. H. Greenberg, M. M. Macdonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep., Part 3*, 3(2) (1972). [c] See ref 28-30.

I. The γ-substituted compounds **13a–h** inhibit the enzyme as effectively as MTX, but α-substituted compounds **14a–d** are markedly less inhibitory. Thus, it is apparent that a free α-carboxyl group is required for effective binding but a γ-carboxyl is not. Both the α- and γ-carboxyl groupings are involved in transport as evidenced by reduced influx of derivatives substituted at either of these positions. These results were reported earlier[28,29] and were also discussed in a recent review.[30]

These compounds were also included in a study by Cheng et al. on the effects of folate and MTX analogues on human thymidylate synthetase.[31] It was concluded that a free α-carboxyl on the glutamyl moiety or a free carboxyl group in that vicinity is essential for binding. This conclusion was based in part on the observation that α-amide **14a** showed a markedly lower inhibitory effect than MTX, but the α-glutamate derivative **14d** gave the same level of inhibition as MTX. It was further concluded that a free γ-carboxyl is not required for binding. The γ-amide gave the same inhibitory effect as MTX, and, more important, the γ-aspartate (**13g**) and the γ-glutamate (**13h**) showed significantly greater inhibitory potency. This finding is clearly an important observation in light of the in vivo metabolism of MTX to **13h** and higher poly(γ-glutamates).

Results from in vivo tests against murine leukemia L1210 are listed in Table I. Each of these compounds produced increases in life span comparable to that given by MTX. None proved significantly more effective. Optimal doses extended over a wide range, and each of these compounds is subject to conversion in vivo to MTX.

Cloning suppression test results summarized in Table I are in general accord with DHFR inhibition capacity. The γ-aspartate **13g** is by far the least suppressive of the γ-subsituted group. This result is probably due to poor transport in light of the earlier finding that **13g** is transported into L1210 cells much less effectively than any of the other compounds evaluated.[32]

Experimental Section

High-pressure liquid chromatographic assays were made with a Waters Associates ALC-242 liquid chromatograph equipped with a UV detector (254 nm) and an M-6000 pump using a 30 × 0.29 cm C_{18} μBondapak column. Methods used were like those described earlier.[23] ^1H NMR spectra (determined in Me_2SO-d_6 with a Varian XL-100-15 spectrometer), UV spectra (determined in 0.1 N HCl, pH 7 buffer, and 0.1 N NaOH with a Cary 17 spectrophotometer), and mass spectra (determined on a Varian MAT 311A mass spectrometer equipped with a combination electron impact/field ionization/field desorption ion source[33]) obtained for all of the compounds listed in Tables II and III were consistent with assigned structures as was the ^{13}C NMR spectrum obtained for **15**. Analytical results indicated by element symbols were within ±0.4% of the theoretical values. Spectral determinations and some of the elemental analyses were performed in the Molecular Spectroscopy Section of the Southern Research Institute under the direction of Dr. W. C. Coburn, Jr. Elemental Analyses were also performed by Galbraith Laboratories, Knoxville, TN. Unless other conditions are specified, evaporations were performed with a rotary evaporator and a water aspirator, and products were dried in vacuo (<1 mm) at room temperature over P_2O_5.

4-[[(Benzyloxy)carbonyl]methylamino]benzoyl chloride (1) was prepared in two steps as follows. (A) A solution of 4-(methylamino)benzoic acid (45.0 g, 0.298 mol) in dioxane (270 mL) and 2 N NaOH (153 mL) was stirred at 0–5 °C while separate solutions of 2 N NaOH (225 mL) and benzyl chloroformate (61 g, 0.36 mol) in dioxane (180 mL) were added at approximately the same rate during 0.5 h. The cloudy mixture was stirred for 0.5 h longer at 0–5 °C before the cooling bath was removed. The mixture was then treated with H_2O (675 mL), and stirring at 25 °C was continued 0.5 h longer. The mixture, now nearly clear, was clarified (Norit, Celite mat) and treated with glacial AcOH (90 mL). White solid separated and, after being kept overnight in a refrigerator, was collected, washed with H_2O followed by cold MeCN, and dried. This material (84 g) was recrystallized from MeCN (1 L required) to give pure 4-[[(benzyloxy)carbonyl]-methylamino]benzoic acid, mp 153 °C (Kofler Heizbank) [lit.[34] mp 149.5–151.5 °C (from AcOH)], in 86% yield (73 g). (B) A

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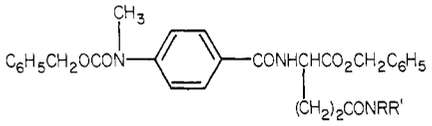
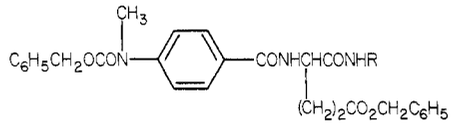
Table II



3a-g

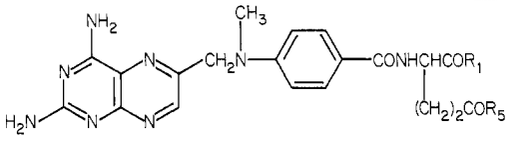


7a-c

compd	R	R^1	yield, %	mp, °C	mol formula[a]
3a	H	CH_3	78	124-125	$C_{29}H_{31}N_3O_6$
3b	CH_3	CH_3	80	oil[b]	$C_{30}H_{33}N_3O_6$
3c	H	$(CH_2)_4CH_3$	70	119-120	$C_{33}H_{39}N_3O_6$
3d	H	$CH_2C_6H_5$	82	142-143	$C_{35}H_{35}N_3O_6$
3e	H	$CH_2CO_2CH_2C_6H_5$	70	128-130	$C_{37}H_{37}N_3O_8$
3f	H	$CHCO_2CH_2C_6H_5$ | $CH_2CO_2CH_2C_6H_5$	89	119-122	$C_{46}H_{45}N_3O_{10}$
3g	H	$CHCO_2CH_2C_6H_5$ | $(CH_2)_2CO_2CH_2C_6H_5$	78	87-89	$C_{47}H_{47}N_3O_{10}$
7a	$CH_2CO_2CH_2C_6H_5$		61	114-118	$C_{37}H_{37}N_3O_8$
7b	$CHCO_2CH_2C_6H_5$ | $CH_2CO_2CH_2C_6H_5$		41	102-104	$C_{46}H_{45}N_3O_{10}$
7c	$CHCO_2CH_2C_6H_5$ | $(CH_2)_2CO_2CH_2C_6H_5$		49	117-120	$C_{47}H_{47}N_3O_{10}$

[a] Anal. C, H, N. [b] Did not crystallize, but the ^1H NMR spectrum was as expected for pure **3b**.

Table III



compd	R_1	R_5	yield, %	mol formula[a]
13a	OH	NH_2	75	$C_{20}H_{23}N_9O_4 \cdot 2.6H_2O$
13b	OH	$NHCH_3$	78	$C_{21}H_{25}N_9O_4 \cdot 3H_2O$
13c	OH	$N(CH_3)_2$	34	$C_{22}H_{27}N_9O_4 \cdot 2.6H_2O$
13d	OH	$NH(CH_2)_4CH_3$	83	$C_{25}H_{33}N_9O_4 \cdot 2.2H_2O$
13e	OH	$NHCH_2C_6H_5$	71	$C_{27}H_{29}N_9O_4 \cdot 1.5H_2O$
13f	OH	Gly	72	$C_{22}H_{25}N_9O_6 \cdot 1.9H_2O$
13g	OH	Asp	39	$C_{24}H_{27}N_9O_8 \cdot 2H_2O$
13h	OH	Glu	56	$C_{25}H_{29}N_9O_8 \cdot 2H_2O$
14a	NH_2	OH	96	$C_{20}H_{23}N_9O_4 \cdot 4H_2O$
14b	Gly	OH	82	$C_{22}H_{25}N_9O_6 \cdot 1.5H_2O$
14c	Asp	OH	86	$C_{24}H_{27}N_9O_8 \cdot 2.5H_2O$
14d	Glu	OH	77	$C_{25}H_{29}N_9O_8 \cdot 2H_2O$
15	OH	OCH_3	84	$C_{21}H_{24}N_8O_5 \cdot 3H_2O$
16	OCH_3	OH	89	$C_{21}H_{24}N_8O_5 \cdot 0.36HBr \cdot H_2O$

[a] Anal. C, H, N for all compounds listed; also Br for **16**. [b] Precursor **10** was deblocked by a reported procedure[24] to give **4a**·HBr, which was treated in Me_2NAc with an equimolar amount of Et_3N prior to the addition of **12**.

solution of the N-protected carboxylic acid just described (32.0 g, 0.112 mol) in dry C_6H_6 (400 mL) containing freshly distilled $SOCl_2$ (50 mL) was refluxed for 2 h, cooled, and evaporated under reduced pressure. The white solid residue was repeatedly (four times) dissolved in C_6H_6 (100-mL portions), which was removed by evaporation under reduced pressure. The product was then removed from the flask, pulverized under ligroin (boiling range 30–60 °C), collected by filtration under N_2, and dried in vacuo (25–30 °C over NaOH pellets and paraffin chips) to give **1**, mp 70–72 °C, in 99% yield (33.8 g) and of purity suitable for use in the preparations described below.

***N*-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamic Acid α-Benzyl Ester (2).** A solution of **1** (5.10 g, 16.8 mmol) in dioxane (50 mL) was added dropwise during 1 h to a cold (0–5 °C), stirred solution of L-glutamic acid α-benzyl ester[35] (4.20 g, 17.7 mmol) in dioxane–H_2O (140 mL, 4:3, v/v) containing $(i\text{-Pr})_2NEt$ (5.4 g, 42 mmol). The resulting solution was left for 16 h in a refrigerator, then kept for 2 h at 25 °C, and evaporated under reduced pressure until most of the dioxane had been removed. The aqueous solution was then diluted with H_2O to (150 mL) and extracted with two portions of Et_2O (50 mL each), which were discarded. The aqueous phase was freed of remaining Et_2O by a brief evaporation period under reduced pressure and was then treated with 12 N HCl (5 mL) in portions with shaking. The white gummy precipitate that formed was extracted into $CHCl_3$ (200 mL), and the $CHCl_3$ solution was washed twice with H_2O (50-mL portions), dried (Na_2SO_4), filtered (Celite mat), and evaporated under reduced pressure to give a viscous oil. The oil was freed of residual $CHCl_3$ by dissolving it in MeCN and evaporating the solution under reduced pressure. The $CHCl_3$-free residue was dissolved in MeCN (25 mL), and the solution was added dropwise to rapidly stirred, cold H_2O (250 mL). The product first separated as an oil but soon solidfied while stirring with ice-bath cooling was continued for about 2 h. The white solid was triturated with H_2O, collected, and dried to give pure **2** in 89% yield (7.60 g), mp 110–112 °C. Anal. ($C_{28}H_{28}N_2O_7$) C, H, N.

(35) H. Sachs and E. Brand, *J. Am. Chem. Soc.*, **75**, 4610 (1958).

***N*-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamic acid γ-benzyl ester (6)** was prepared from 1 (10.2 g, 33.6 mmol) and L-glutamic acid γ-benzyl ester (8.00 g, 33.7 mmol) in dioxane–H_2O (280 mL, 4:3, v/v) containing $(i\text{-Pr})_2NEt$ (10.0 g, 77.0 mmol) as described above for **2**. Pure **6**, mp 101–102 °C, was obtained in 68% yield (11.6 g). Anal. ($C_{28}H_{28}N_2O_7$) C, H, N.

Benzyl Esters of N^2-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-*N*-substituted-L-glutamines (3a–d). The mixed anhydride from **2** and *i*-BuOCOCl was prepared as described below under the preparation of **3g** and was treated at −10 °C with a solution of the appropriate amine (1.1 mol equiv with respect to **2**) in DMF. Subsequent treatment and workup was essentially like that described for **3g**. Additional data are listed in Table II.

***N*-[*N*-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-γ-glutamyl]-L-glutamic Acid Tribenzyl Ester (3g).** A solution of **2** (2.52 g, 5.00 mmol) and Et_3N (550 mg, 5.45 mmol) in DMF (30 mL) was treated at −10 °C (crushed ice–MeOH bath) with stirring during 10 min with a solution of freshly distilled *i*-BuOCOCl (0.70 g, 5.1 mmol) in dry dioxane (10 mL). Stirring at −10 °C was continued for 1 h before solid L-glutamic acid dibenzyl ester hydrochloride (1.82 g, 5.00 mmol) was added in one portion followed by a solution of Et_3N (550 mg) in DMF (10 mL). The cooling bath was removed, and the mixture was stirred at 25 °C for 2 h. Solvents were then removed by evaporation in vacuo (<1 mm, bath at 25–30 °C), and the residue was stirred with H_2O (100 mL). The mixture containing gummy white precipitate was kept in a refrigerator overnight. The supernatant was removed by decantation, and the now semisolid residue was stirred with H_2O (100 mL). The precipitate soon solidified and was triturated sequentially with H_2O, 3% $NaHCO_3$, and 0.1 N HCl, with filtration following each treatment. The solid was finally washed with H_2O, dried, and recrystallized from EtOH. Additional data are given in Table II.

The analogous **L-aspartic acid derivative 3f** and the **glycine derivative 3e** were prepared from L-aspartic acid dibenzyl ester hydrochloride and glycine benzyl ester *p*-toluenesulfonate in the manner described for **3g**. Results are listed in Table II.

***N*-[*N*-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-α-glutamyl]-L-glutamic Acid Tribenzyl Ester (7c).** This compound and analogues **7a** and **7b** were prepared by coupling **6** with the appropriate benzyl esters using the procedure described above for preparation of **3e–g** from **2**. Pure products were obtained by repeated recrystallizations from EtOH, and results are listed in Table II.

***N*-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamic Acid γ-Methyl Ester (5).** Treatment of L-glutamic acid γ-methyl ester with **1** as described for the preparation of **2** afforded **5**. The gummy precipitate that formed on acidification of the reaction solution solidified when the mixture was chilled. Successive recrystallizations from MeOH–H_2O and MeOH alone gave pure **5**, mp 115 °C after sintering at about 55 °C and changing crystalline form, in 52% yield (2.23 g from a 10-mmol run). Anal. ($C_{22}H_{24}N_2O_7$) C, H, N.

***N*-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamic Acid α-Methyl γ-Benzyl Ester (9).** A solution of **6** (5.05 g, 10.0 mmol), CH_3I (1.56 g, 11.0 mmol), $(i\text{-Pr})_2NEt$ (1.42 g, 11.0 mmol), and DMF (15 mL) was kept in a stoppered flask at 25 °C for 4 days. Evaporation in vacuo (<1 mm, bath to 35 °C) gave a viscous oil, which was dissolved in C_6H_6 (75 mL). The C_6H_6 solution was washed with H_2O, 2 N NH_4OH, and again with H_2O, then dried (Na_2SO_4), and evaporated. The oily residue crystallized readily from C_6H_6–ligroin (bp 30–60 °C) to give pure **9**, mp 66–69 °C, in 86% yield (4.28 g). Anal. ($C_{29}H_{30}N_2O_7$) C, H.

N^2-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamine 2,4,6-Trimethylbenzyl Ester (10). A mixture of **1** (13.5 g, 44.4 mmol) and L-glutamine 2,4,6-trimethylbenzyl ester hydrochloride[24] (14.0 g, 44.5 mmol) was stirred at 25 °C with CH_2Cl_2 (500 mL). When a smooth suspension had formed, a solution of $(i\text{-Pr})_2NEt$ (14.0 g, 0.108 mol) in CH_2Cl_2 (50 mL) was added dropwise during 20 min. The solution that formed was left at 25 °C for 64 h and then evaporated. The residue was triturated with H_2O, and the white solid was collected and then recrystallized from EtOH (1.8 L required) to give pure **10**, mp 196–198 °C, in 83% yield (20.2 g). Anal. ($C_{31}H_{35}N_3O_6$) C, H, N.

N^2-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]isoglutamine (11). A solution of **2** (2.50 g, 4.97 mmol) in 28% NH_4OH solution (50 mL) was kept in a stoppered flask for 1 week. Evaporation gave a viscous oil which was dissolved in H_2O (50 mL), and the solution was treated dropwise with 12 N HCl to pH 3. A gummy precipitate formed, and the mixture was kept overnight in a refrigerator. A small amount of solid material separated and was collected on a filter when the supernatant was decanted from the gummy precipitate. The gum was then dissolved in EtOH from which it crystallized when the solution was seeded with the small amount of solid collected earlier. The yield of pure **11**, mp 147–149 °C, was 62% (1.28 g). Anal. ($C_{21}H_{23}N_3O_6$) C, H, N.

Deblocking of the Side-Chain Precursors. The protective groupings of all precursors except **10** were removed by hydrogenolysis. Deprotection of **10** was effected by HBr.[24] The starting amount of pure blocked precursor determined the amount of bromomethyl compound **12** used in the final step. A typical hydrogenolysis operation is represented by the deblocking of **3f**, precursor of **13g**. A solution of **3f** (3.30 g, 4.13 mmol) in dioxane (60 mL) containing 5% Pd on C (0.60 g) was stirred under H_2 at ambient conditions of the laboratory for 20 h. During this time, the mixture absorbed a volume of H_2 (458 mL) close to that calculated (with vapor-pressure corrections) for the expected conversion. The catalyst was removed by filtration (Celite mat), and the filtrate was evaporated (final conditions <1 mm, bath at 30 °C) to give the deblocked side-chain precursor of **13g** as a deliquescent solidified foam weighing about 10% above the quantitative yield. The ^1H NMR spectrum of this material revealed the presence of dioxane but was as expected otherwise. Three compounds (**3a**, **6b**, and **11**) were treated differently from the example described because of low solubility of the deblocked products in dioxane. Partial separation of the products occurred during hydrogenolysis of **3a** and **6b**. In these examples, the precipitated material was collected along with the catalyst, and the gray solid mixture was stirred with MeOH. The catalyst was filtered off as before, and the methanolic filtrate was combined with the filtered dioxane solution for evaporation. Compound **11** was dissolved in dioxane–MeOH (3:1, v/v 13 mL/mmol) for the hydrogenolysis, and the product remained in solution. The ^1H NMR spectrum of the latter three deblocked products revealed the prescence of both dioxane and MeOH but otherwise were as expected.

***N*-[4-(Methylamino)benzoyl]-L-glutamic acid γ-methyl ester (4, $R_5 = OCH_3$)** solidified after evaporation of the dioxane. Trituration with Et_2O gave pure **4** ($R_5 = OCH_3$), mp 122–125 °C, in 93% yield (1.28 g from 2.00 g of **5**). Anal. ($C_{14}H_{18}N_2O_5$) C, H, N. The isomeric **α-methyl ester 8 ($R_1 = OCH_3$)** was obtained as a viscous oil whose mass spectrum showed the expected M^+ of m/e 294. This material was used directly for conversion to **16**.

Alkylation of the Side-Chain Precursors with 12. The procedure for the preparation of compounds listed in Table III is typified by that for **14b**. Precursor **8** (R_1 = Gly, from 4.60 mmol of **7a**) was dissolved in Me_2NAc (20 mL), and **12** (3.84 mmol) was added. The mixture was stirred at 25 °C for 5 days. The yellow-orange solution that formed was combined with cold H_2O (150 mL), and the pH of the solution was raised from 2.2 to 3.8 by the dropwise addition with stirring of 1 N NaOH. The product separated readily, and, after the mixture had been kept at 0–5 °C for 5 h, the yellow–orange solid was collected by filtration and washed with cold H_2O. The dried product, which weighed 1.63 g immediately after removal from a desiccator, underwent a weight increase on exposure to ambient conditions of the laboratory to 1.70 g. This sample did not require further purification. Spectral data (^1H NMR, UV, and mass) were as expected, and assay by HPLC established the sample to be of high purity (~99% with respect to UV-absorbing material) and free of MTX.

The **14** series and **13f** did not receive or require further purification, but the remainder of the **13** types were redissolved by treatment of stirred suspensions in H_2O with sufficient 1 N NaOH to produce pH 7–8, and the solutions were treated with Norit and filtered (Celite and powdered cellulose mat). The solutions were then treated dropwise with stirring with 1 N HCl to pH 3.6–3.8 to give reprecipitated products, which gave satisfactory spectral

data and HPLC assay results (>98% pure and free of MTX). Samples first dried in vacuo at 25–30 °C (over P_2O_5 and NaOH pellets) were allowed to attain constant weight by exposure to ambient conditions of the laboratory (relative humidity typically 50%) before analysis.

Methotrexate γ-methyl ester (15) was isolated as described for the 14 types. The α-methyl ester **16** was also isolated similarly, but it precipitated at a lower pH than was intended. Just after the reaction solution containing **16** was combined with cold H_2O, a yellow solid began separating, before the planned base treatment to raise the pH from 2.2 to 4.0 was started. The treatment with 1 N NaOH with ice-bath cooling was done after much solid had already separated, and then the mixture at pH 4.0 was stirred at 0–5 °C for 0.5 h. After centrifugation and decantation, the solid was collected by filtration from a small volume of H_2O. The dried and equilibrated product turned out to be a partial hydrobromide of **16** as listed in Table III.

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Adriamycin Analogues. Preparation and Biological Evaluation of Some *N*-Perfluoroacyl Analogues of Daunorubicin, Adriamycin, and *N*-(Trifluoroacetyl)adriamycin 14-Valerate and Their 9,10-Anhydro Derivatives[1]

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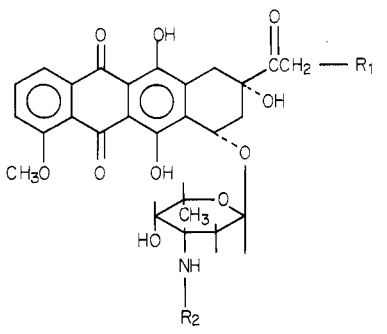
The experimental and clinical antitumor activity, as well as the low toxicity, of *N*-(trifluoroacetyl)adriamycin 14-valerate (AD 32), a non-DNA binding anthracycline analogue, has led us to prepare and evaluate several *N*-perfluoroacyl analogues of daunorubicin, adriamycin, and *N*-(trifluoroacetyl)adriamycin 14-valerate. Target compounds were prepared by reaction of the appropriate perfluoroacyl anhydride with daunorubicin in chloroform–ether, with adriamycin in cold pyridine, and with adriamycin 14-valerate in ethyl acetate. In connection with this work, it was found that reaction of perfluoroacyl anhydrides with N-acylated or N-unsubstituted anthracyclines in pyridine at room temperature afforded with ease and in good yield the corresponding 9,10-anhydro-N-acylated derivatives. A number of products showed good to highly significant antitumor activity in vivo against the murine P388 leukemia system. However, the lack of in vivo antitumor activity of the pentafluoropropionyl and heptafluorobutyryl analogues of *N*-(trifluoroacetyl)adriamycin 14-valerate is noteworthy. The results continue to show that non-DNA binding anthracycline analogues can exhibit in vivo antitumor activity. Loss of the anthracycline 9-carbinol function by dehydration leads to reduction of biological activity as compared to the parent compound.

The anthracycline antibiotic adriamycin (**2**) is widely used in the clinical management of leukemias and various solid tumors.[2-5] The value of this agent is compromised, however, by its toxic side effects, two of which (acute myelosuppression and total accumulated dose-dependent cardiac toxicity) are serious enough to be dose limiting. In addition, inadvertant paravenous extravasation can result in severe local tissue ulceration and necrosis, and gastrointestinal toxicity, manifested as nausea and vomiting, is almost universal.



1 (daunorubicin), $R_1 = R_2 = H$
2 (adriamycin), $R_1 = OH$; $R_2 = H$
3 (AD 32), $R_1 = OCO(CH_2)_3CH_3$; $R_2 = COCF_3$

N-(Trifluoroacetyl)adriamycin 14-valerate (AD 32; **3**)[6,7] is an adriamycin analogue conceived and developed in these laboratories and currently undergoing phase II clinical evaluation. In animal model systems, **3** is therapeutically superior, sometimes dramatically so, to adriamycin.[6,8,9] In addition, in animals **3** produces less toxicity in general and significantly less cardiotoxicity in particular compared to adriamycin.[8,10,11] Clinical antitumor activity and low toxicity have been documented for **3** in connection with phase I/II trials.[12,13] No patient has had evidence

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