Methotrexate Analogues. 28. Synthesis and Biological Evaluation of New γ-Monoamides of Aminopterin and Methotrexate

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Received December 6, 1985

Lipophilic γ-monoamide derivatives of aminopterin (AMT) were synthesized in high overall yield from 4-amino-4-deoxy-N^{10}-formylpteroic acid and γ-*N*-*tert*-alkyl-, γ-*N*-aralkyl-, or γ-*N*-arylamides of α-benzyl L-glutamate via a modification of the mixed carboxylic–carbonic anhydride coupling method. Coupling was also accomplished with *p*-nitrophenyl 4-amino-4-deoxy-N^{10}-formylpteroate. Compounds obtained in this manner included the γ-*tert*-butylamide, γ-(1-adamantylamide), γ-benzylamide, γ-(3,4-dichlorobenzylamide), γ-(2,6-dichlorobenzylamide), γ-anilide, γ-(3,4-methylenedioxyanilide), and γ-(3,4-dihydroxanilide) derivatives of AMT. Also prepared, from 4-amino-4-deoxy-N^{10}-methylpteroic acid via diethyl phosphorocyanidate coupling, was the γ-(3,4-methylenedioxyanilide) of MTX. The methylenedioxyanilides were cleaved smoothly to dihydroxyanilides with boron tris(trifluoroacetate) in trifluoroacetic acid. All the γ-monoamides were tested as inhibitors of purified dihydrofolate reductase (DHFR) from murine L1210 leukemia cells and as inhibitors of the growth of wild-type L1210 cells and a subline (L1210/R81) with high-level resistance to MTX and AMT based mainly on a defect in drug uptake via active transport. Several compounds were also tested against human leukemic lymphoblasts (CEM cells) and a resistant subline (CEM/MTX) whose resistance is likewise based on uptake. The IC_{50} of the γ-monoamides against DHFR was 1.5- to 5-fold higher than that of the parent acids, but the IC_{50} against cultured cells varied over a much broader range, suggesting that uptake and/or metabolism rather than DHFR binding are principal determinants of in vitro growth inhibitory activity for these compounds. γ-*N*-Aryl and γ-*N*-aralkyl derivatives appeared to be more potent than γ-*N*-*tert*-alkyl derivatives. Where comparison could be made, AMT γ-monoamides were more potent than MTX γ-monoamides. Several of the γ-monoamides showed potency comparable to that of the parent acid against wild-type L1210 and CEM cells; all of them were more potent than MTX against the L1210/R81 subline; and some of the AMT γ-monoamides were also more potent than the parent acid against resistant CEM/MTX cells. As a group, however, the γ-monoamides were considerably more active against the murine cells than against the human cells, suggesting that the former may take up the amides better or may be able to metabolize them more efficiently than the parent acids. All the γ-monoamides were tested in vivo against L1210 leukemia in mice. The γ-*N*-*tert*-alkylamides were inactive, whereas significant activity (>50% increase in survival at optimal doses) was shown by every γ-*N*-aralkylamide and γ-*N*-arylamide in the series. The γ-*N*-arylamides were more toxic than the γ-*N*-aralkylamides. The most therapeutically effective compound was AMT γ-(3,4-dichlorobenzylamide), which produced an increase in lifespan (ILS) of 110% at 70 mg/kg per day × 9 as compared with 122% for AMT itself at 0.5 mg/kg per day × 9. The fact that this compound was the most active in vivo but among the least active in cell culture indicates that activity is not due to the presence of AMT contamination in the test sample and suggests that bioconversion of the amide to the parent acid may be occurring in the host animal independently of whether it also occurs in the tumor.

γ-Monoamides of the well-known antineoplastic drug methotrexate (MTX, 1)[1] have been synthesized in this[2] and other[3,4] laboratories as potential prodrugs of MTX or as active agents in their own right. Modification of the γ-carboxyl in MTX does not greatly affect binding to the target enzyme dihydrofolate reductase (DHFR).[2,3] Thus, even if formation of free MTX did not occur from the γ-monoamides inside the cell, these derivatives might still inhibit tumor cell growth.

An important consequence of γ-substitution in MTX is that the replacement of a negative charge in the glutamate side chain by a hydrophobic group may favor uptake into the cell via a mechanism other than the one for MTX itself.[5] This energy-requiring, carrier-mediated pathway appears to have a preference for the transport of dianions.[6,7] Hydrophobic γ-monoamides of MTX are therefore potentially attractive agents to use against MTX-resistant tumor cells with a defect in MTX transport, such cells having been shown to remain sensitive in culture to other γ-substituted MTX derivatives[8–10] as well as to lipid-soluble nonclassical antifolates.[8,11–16]

A further consequence of γ-substitution in MTX is that γ-polyglutamylation, which is currently viewed as a major contributor to the potency and antitumor selectivity of MTX and other classical antifolates,[17–21] is rendered impossible. In this context, a γ-substituted MTX derivative would normally be expected to be less potent than the parent drug, and possibly less discriminant in its effects

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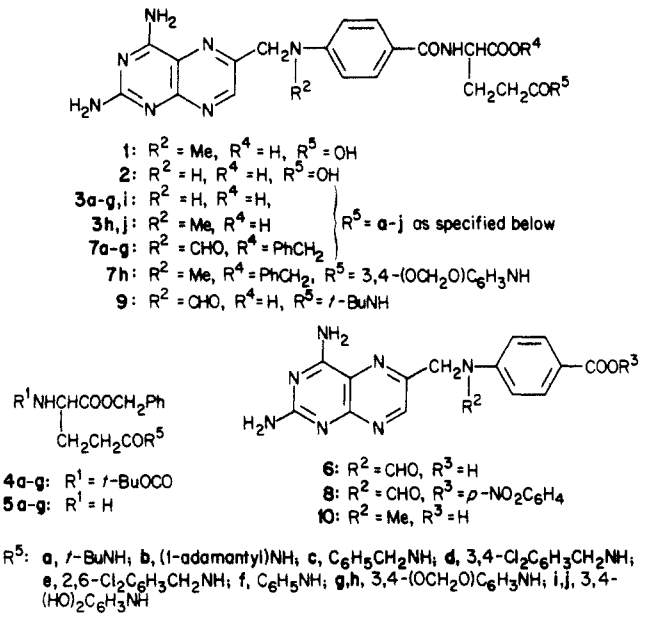

Figure 1. Structures of compounds **3a–j** and the intermediates used in their synthesis.

on tumor vs. host tissues. On the other hand there have been literature reports[9,14,22,23] of MTX-resistant tumor lines with an impaired capacity for polyglutamylation. If this were an operative mechanism of resistance in the in vivo setting, i.e., if the dominant cell population in the tumor of a patient lacked the ability to polyglutamylate MTX, it is conceivable that an MTX derivative that *cannot* form polyglutamates might have an improved therapeutic index in comparison with MTX.[24]

The in vitro and in vivo potency of MTX γ-monoamides as inhibitors of MTX-sensitive tumor cell growth has been too low, for the most part, to allow these compounds to be considered for therapeutic use in the resistance setting. Accordingly, we turned our attention to γ-monoamides of the more potent antifolate aminopterin (AMT, **2**). Rational support for this initiative was provided by recent observations that the γ-*tert*-butyl ester of AMT[10] and the analogues of AMT in which the glutamate side chain is replaced by homocysteic acid[24] or 2-amino-4-phosphonobutyric acid[25,26] are all more potent than the corresponding MTX analogues. At least in enzyme inhibition assays, this trend is also followed by MTX and AMT analogues containing an ornithine side chain.[26,27] Interestingly, none of these compounds is capable of being polyglutamylated, and indeed the homocysteic acid, 2-amino-4-phosphonobutyric acid, and ornithine analogues of AMT are strong competitive inhibitors of folylpolyglutamate synthetase, the enzyme responsible for the cellular conversion of MTX and AMT to polyglutamyl conjugates.[25-27] The present paper describes the synthesis of a series of lipophilic AMT γ-monoamides (**3a–g**), as well as the preparation of the heretofore unreported MTX γ-monoamide **3h**. The methylenedioxy group in **3g** and **3h** was found to be removable with boron tris(trifluoroacetate), giving rise to the interesting γ-(3,4-dihydroxyanilide) derivatives **3i** and **3j**, respectively. Compounds **3a–h** were tested as DHFR inhibitors and as inhibitors of tumor cell growth in culture and in vivo. Compounds **3i** and **3j**, which were synthesized in limited quantity, were tested only for their ability to inhibit DHFR activity and the growth of cultured cells. The structures of **3a–j** and of the intermediates used in their synthesis are presented in Figure 1.

Chemistry. A series of *N*-(*tert*-butyloxycarbonyl)-L-glutamic acid γ-monoamides (**4a–g**) were obtained by condensing α-benzyl-*N*-(*tert*-butyloxycarbonyl)-L-glutamate with the desired amine in the presence of diphenylphosphoryl azide and Et_3N in dry DMF. The reaction proceeded well at room temperature and was generally complete in 24–72 h. With the exception of aniline, which gave only 58% of **4f**, the yields of protected monoamides were in excess of 90%. Deprotection was effected smoothly by heating **4a–g** with 1 molar equiv of *p*-toluenesulfonic acid in refluxing benzene. The resulting crystalline tosylate salts were obtained in >90% yield. In one instance the tosylate was converted to a hydrochloride (**5b**·HCl), but this led to a decreased yield and provided no advantage in terms of the subsequent coupling step. Further reaction of the salts of **5a–g** with 4-amino-4-deoxy-N^{10}-formylpteroic acid (**6**)[10] to obtain **7a–g** was carried out via the modified mixed carboxylic–carbonic anhydride procedure we developed earlier in the synthesis of other AMT analogues.[10,24] In this modification, which affords greatly improved yields in comparison with the standard method, the alkyl chloroformate and amine are added in several decreasing portions so as to permit in situ reactivation of any **6** that is unavoidably formed by wrong-way attack of the mixed anhydride by the amine. We have found that four cycles of activation (5 min) and coupling (15 min) produce satisfactory results, giving yields of 60–70% in most cases. One compound (**7e**) was prepared from *p*-nitrophenyl 4-amino-4-deoxy-N^{10}-formylpteroate (**8**), which had been used earlier in our laboratory in the synthesis of AMT γ-*tert*-butyl ester.[10] The yield of **7e** via the modified mixed anhydride method and the *p*-nitrophenyl ester method was comparable. However, it may be noted that the latter method uses only 1 molar equiv of the amine component, whereas the former uses two. Thus, when the amine component is in limited supply the *p*-nitrophenyl ester method has an obvious advantage.

Simultaneous removal of the N^{10}-formyl and α-benzyl groups in **7a–g** was accomplished in 80–95% yield by

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Table I. Dihydrofolate Reductase Inhibition and Cell Growth Inhibition by γ-Monoamides of Aminopterin and Methotrexate

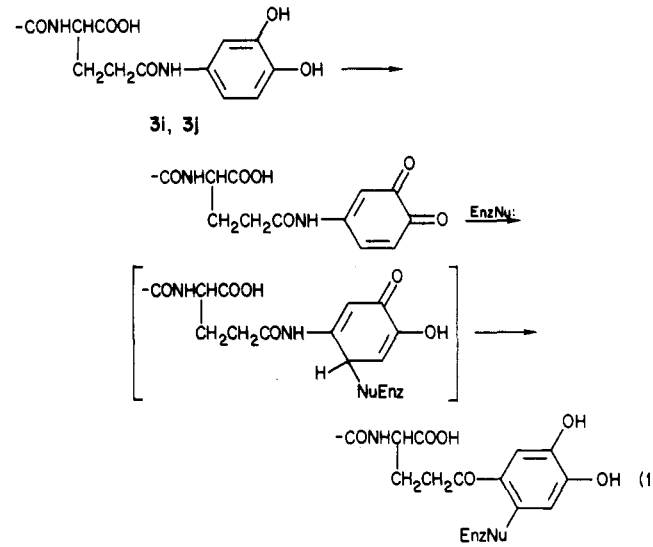
	DHFR[a]		cells IC_{50},[b] μM				
compd	IC_{50}, μM	IC_{50}/[E]	L1210[c]	L1210[d]	L1210/R81[d]	CEM	CEM/MTX
3a	0.044	1.1	0.12	0.032	22[e]	5.8[e]	36
3b	0.10	2.5	0.68	0.51	25	4.5	16
3c	0.087	2.2	0.005	0.0071	17	0.065	6.0
3d	0.13	3.3	0.046	0.034	32	nd	nd
3e	0.080	2.0	0.0037	0.002	61	nd	nd
3f	0.060	1.5	0.0035	0.0036	10	0.18[e]	4.7[e]
3g	0.045	1.1	0.0032	0.00039[f]	28	0.14	1.0
3h	0.042	1.1	nd	0.0029	25	nd	nd
3i	0.057	1.4	0.037	0.006	23	nd	nd
3j	0.031	0.8	0.030	0.0084	38	nd	nd
AMT	0.020	0.5	0.003	0.002	84	0.0010	0.32
MTX	0.020	0.5	0.01–0.03[g]	0.002	220	0.032	6.6

[a] See ref 27; the assay mixture contained NADPH (75 μM), DHFR (0.04 μM), and dihydrofolate (50 μM) in 0.05 M Tris chloride buffer, pH 7.5. The reaction was conducted at 22 °C and was initiated by addition of the dihydrofolate to the other components after 2 min of preincubation. [b] L1210 cells were exposed for 48 h and CEM for 72 h; see ref 31, 32, and 35 for culture conditions; nd = not determined. [c] Experiments at the Dana-Farber Cancer Institute. [d] Experiments at the Medical College of Ohio and the University of Cincinnati Medical Center. [e] Mean of two separate assays. [f] Mean of four separate assays. [g] Range of values from a number of assays over several years.

treatment with 10–20 molar equiv of NaOH in aqueous MeOH. The progress of deblocking could be monitored readily by TLC. The blue fluorescent spot corresponding to starting material was replaced, generally within 30 min, by another slower moving blue fluorescent spot corresponding to cleavage of the ester group. With time, the second fluorescent spot gave way to a UV-absorbing spot representing final product. Complete deprotection typically was complete in 12–24 h, but one instance (**7b**) required 72 h. Use of $Ba(OH)_2$ instead of NaOH was investigated with one compound (**7a**) and found to produce a mixture containing mainly the N^{10}-formyl derivative **9** (65% yield) and a smaller amount of the fully deprotected product **3a** (15% yield). Treatment of **9** with NaOH converted it smoothly to **3a**. Compounds **9** and **3a** could be separated by ion-exchange chromatography on DEAE-cellulose, with 3% NH_4HCO_3 as the eluent.

Synthesis of the MTX γ-monoamide **3h** was achieved in two steps (67% combined yield) by condensation of **5g** with 4-amino-4-deoxy-N^{10}-methylpteroic acid (**10**) in the presence of diethyl phosphorocyanidate and Et_3N, followed by hydrolysis of the ester group in the coupling product (**7h**) with NaOH in aqueous MeOH. Both steps proceeded in 82% yield. This method was used previously in our laboratory to obtain the γ-*n*-butylamide and γ-benzylamide of MTX.[2]

Cleavage of the 3,4-methylenedioxy group in **3g** and **3h** required some preliminary experimentation. Several attempts to effect the reaction with boron trichloride in CH_2Cl_2 gave either unchanged starting material or complex mixtures of products. Likewise unsuccessful were attempts to carry out the reaction in AcOH, to use boron tribromide instead of boron trichloride, or to increase the solubility of the pteridine by presilylation. Satisfactory cleavage conditions were finally found to involve treatment of the methylenedioxy compound with 0.5 M boron tris(trifluoroacetate) in trifluoroacetic acid. After 15 min at 0 °C the reaction mixture was left at room temperature for 2–3 h. Excess reagent was destroyed with MeOH, and the products (**3i, 3j**) were isolated from Et_2O as trifluoroacetate salts. The correct identity of **3i** and **3j** was established by microchemical analysis and on the basis of their NMR spectra, which showed disappearance of the OCH_2O group (cf. Table IV, footnotes e and f). In addition, the presence of a catechol structure was confirmed by a positive ferric chloride test and the fact that alkaline solutions of the compounds darkened on exposure to air. To our knowledge, this is the first reported use of boron tris(trifluoroacetate) to cleave an aromatic methylenedioxy group and form a catechol. Compounds **3i** and **3j** were of special interest because of the possibility that in tumor cells with a high tyrosinase content the catechols would be converted to *o*-quinones.[28,29] The latter might thereupon react with nucleophilic thiol or amino groups in DHFR, and perhaps other enzymes of the folate pathway, to give stable covalent adducts (eq 1).



Biological Activity. Compounds **3a–j** were tested as inhibitors of purified DHFR from L1210/R71 murine leukemia cells as previously described.[30] The enzyme from these MTX-resistant cells has the same kinetic properties as the one from the parental line. IC_{50} values were obtained spectrophotometrically by measurement of the ΔA_{340} that accompanies oxidation of NADPH and the reduction of dihydrofolate. As indicated in Table I, the IC_{50}'s for the AMT γ-monoamides **3a–g** and **3i** ranged from 44 nM (**3a**) to 130 nM (**3d**). As a group, therefore, these compounds were less potent than AMT (IC_{50} = 20 nM), but not greatly so. No clearcut structure–activity correlation emerged with respect to whether *N*-*tert*-alkyl, *N*-aralkyl, or *N*-aryl derivatives are the best inhibitors. There was little or no IC_{50} difference between pairs of identically γ-substituted MTX and AMT derivatives, e.g., **3g**/**3h** and **3i**/**3j**. It thus appears that the ability of the γ-monoamides to bind to DHFR is not influenced much

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by N^{10}-methyl substitution. These results are in qualitative agreement with what we have observed for other modifications of the γ-region in MTX vs. AMT, including replacement of the glutamate moiety by homocysteic acid,[24] ornithine,[26,27] or 2-amino-4-phosphonobutyric acid.[25,26] It should be noted that the IC_{50}'s for DHFR inhibition by catechols **3i** and **3j** were obtained with no special steps to either induce or avoid oxidation to *o*-quinones and that 2-mercaptoethanol was not present in the assay mixture. The spectrophotometric DHFR assay was typically completed within 15 min of dissolving the test compound in the reaction mixture, and we do not consider it likely that this was long enough, in the absence of added oxidant, to convert all the catechol to a quinone. Therefore, we believe the IC_{50} data for **3i** and **3j** are mainly for the binding of intact catechol and do not reflect irreversible inactivation of the enzyme via covalent linkage. Separate studies of time-dependent DHFR inactivation by **3i** and **3j** after prior oxidation to quinones would be of interest.

The AMT and MTX γ-monoamides **3a–j** were also tested as inhibitors of cell growth in culture, using L1210 murine leukemia cells and the MTX-resistant subline L1210/R81, which is characterized by a profound defect in MTX uptake along with some increase in DHFR content.[31] The cells were exposed continuously for 48 h and IC_{50}'s were determined from growth curves. As shown in Table I, there was a much broader range in the IC_{50}'s for cell growth inhibition than in the IC_{50}'s for enzyme inhibition. The least potent growth inhibitor was the γ-(1-adamantylamide) **3b** and the most potent was the γ-(3,4-methylenedioxyanilide) **3g**, whose IC_{50} of 0.000 39 μM (n = 4, range 0.00028–0.00045 μM) was 8-fold lower than that of AMT. The most active and least active compounds differed by 3 orders of magnitude in their effect on cell growth, even though their DHFR-inhibitory activity differed only 2-fold. There appeared to be a trend in the growth-inhibitory potency of the γ-monoamides, in that the N-alkyl and N-aralkyl derivatives were for the most part less active than the N-aryl derivatives, at least in this limited series. The IC_{50}'s of the AMT γ-monoamides **3a–g** and **3i** against the MTX-resistant L1210/R81 cells ranged from 10 μM (**3f**) to 61 μM (**3e**), but most of the IC_{50} values were in the 15–35 μM range. The IC_{50} of AMT itself against these cells was 84 μM as compared with 0.002 μM for the parental line. It thus appears that AMT γ-monoamides are more potent than AMT against MTX- and AMT-resistant L1210 cells. The IC_{50}'s of the MTX γ-monoamides **3h** and **3j** against the L1210/R81 cells were 25 and 38 μM, respectively, whereas the IC_{50} of MTX was 220 μM. These results suggest that γ-monoamides of MTX, like those of AMT, may be more potent than the parent drug against cells that owe their resistance mainly to impaired transport. These findings are qualitatively consistent with what we have recently observed for the γ-*tert*-butyl esters of AMT and MTX.[10]

A question we wished to address with regard to cell growth inhibition by the AMT γ-monoamides was whether these compounds would be more potent than AMT against other MTX-resistant cells with a level of resistance lower than that of the L1210/R81 line. Accordingly, several members of the series were tested against CEM human leukemic lymphoblasts[32] and a subline (CEM/MTX) that is approximately 200-fold MTX-resistant and 300-fold AMT-resistant.[10] Resistance in these cells has been determined to be associated with decreased MTX uptake.[8] As shown in Table I, the IC_{50}'s of the five compounds tested ranged from 0.18 μM (**3f**) to 5.8 μM (**3a**) against CEM cells and from 1.0 μM (**3g**) to 36 μM (**3a**) against CEM/MTX cells. The N-alkyl derivatives once again were the least active. The IC_{50} of AMT itself against CEM/MTX cells was 0.32 μM. In this case, therefore, the γ-monoamides were not more active than the parent drug against resistant cells. γ-*tert*-Butyl AMT likewise has been found not to be more active than AMT against CEM/MTX cells even though it is more active than AMT against L1210/R81 cells. An interesting aspect of the data in Table III is that wild-type CEM cells are much less sensitive than wild-type L1210 cells to growth inhibition by the AMT γ-monoamides even though the two lines are almost equally sensitive to AMT.

In addition to the in vitro studies described above, γ-monoamides **3a–h** were tested in vivo for their ability to prolong survival in mice with L1210 leukemia. The tumor (10^5 cells) was implanted intraperitoneally and drugs were given by the same route in aqueous solution (pH 7.5–8.5) on a qd×9 schedule starting 1 day after tumor implantation.[33] As shown in Table II, increases in lifespan (ILS) of 50% or more were obtained with six of the eight compounds, and with one compound (**3d**) the ILS at the maximum dose tested was comparable to the ILS with

Table II. Effect of Aminopterin and Methotrexate γ-Monoamides on Survival of Mice with L1210 Leukemia

compd		dose, mg/kg per day	7-day wt change, %	survival, days: range	survival, days: median, T/C	% ILS
3a		20	+17[a]	8–11	9/9	0
		40	+7[a]	8–12	10/9	+11
		80	+9[a]	9–12	11/9	+22
3b		20	nd[b]	8–9	8/9	−11
		40	nd	9–10	9/9	0
		80	+17[a]	10–13	10/9	+22
3c		20	+13[a]	12–13	12/9	+33
		40	+3[a]	10–15	14/9	+56
		80	−26[a]	7–11	toxic	toxic
3d		50	+2	14–18	15/10	+50
		60	0	15–18	17/10	+70
		70	−3	15–25	21/10	+110
		80	−16	9–14	toxic	toxic
3e	expt 1	40	+6	11–13	13/9	+44
		80	+3	13–16	14/9	+56
	expt 2	80	+9	12–13	13/9	+44
		100	−3	9–15	15/9	+67
3f	expt 1	10	+17	12–13	12/9	+33
		20	0	9–14	12/9	+33
	expt 2	7.5	+4	11–14	13/10	+30
		10	+6	11–15	15/10	+50
		15	+2	13–16	13/10	+30
3g	expt 1	10	+7	11–13	12/9	+33
		20	+3	13–15	14/9	+56
	expt 2	40	−20	9–14	toxic	toxic
		80	−26	8–10	toxic	toxic
3h	expt 1	20	+11	11–13	11/9	+22
		40	+10	12–14	13/9	+44
		80	+6	13–17	15/9	+67
	expt 2	60	+3	14–17	14/9	+56
		80	−5	15–18	16/9	+78
		100	+1	15–16	16/9	+78
AMT		0.5[c]	−4	13–21	17/9	+89
		1	−5	15–20	20/9	+122

[a] Weight change was determined on day 10. [b] Not determined. [c] Data were pooled from three separate experiments using five mice/experiment.

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AMT at its maximally tolerated qd×9 dose of 0.5 mg/kg per day. There was considerable variation in the tolerated dose among the amides, as illustrated by the fact that **3f** and **3g** became extremely toxic at 40 mg/kg per day, whereas the *N*-alkyl derivatives **3a** and **3b** showed no signs of toxicity, such as weight loss or early deaths, even at 80 mg/kg per day. The *N*-aralkyl derivatives **3c–e** were intermediate in their toxicity, with **3c** and **3d** showing >10% weight loss but no early deaths at 80 mg/kg per day. AMT γ-(3,4-dichlorobenzylamide) (**3d**) was more therapeutically effective than its 2,6-dichloro analogue **3e** even though it was less inhibitory to cell growth in culture and was the least potent member of the series against purified DHFR (Table I). These findings suggest that bioconversion of **3e** to AMT may be occurring in the host animal independently of whether it also occurs in the tumor cells. AMT γ-benzylamide (**3c**) gave a 55% ILS at 40 mg/kg per day and a 33% ILS at 10 mg/kg per day. This compared favorably with MTX γ-benzylamide, which has been reported to give a 33% ILS only at a higher dose of 100 mg/kg per day (qd×9).[3] We also have found that MTX γ-benzylamide is only marginally active against L1210 leukemia in vivo. Thus, for two examples where direct comparison was possible, the γ-(3,4-methylenedioxyanilides) and the γ-benzylamides, we found that removal of the N^{10}-methyl group did produce some increase in potency as had been hoped.

An important issue with regard to the in vivo data obtained with the MTX and AMT γ-monoamides was whether the observed effects could be due to small amounts of the parent acids in the test sample. That this was unlikely was evident from the enzyme and cell growth inhibition data (Table I), which showed that if AMT or MTX were the active species they would have to be present in amounts easily detectable by TLC, i.e., much more than the 0.5–1.0% that would account for the in vivo activity. On this basis there seems little doubt that if the in vivo activity is due to the parent drug, the latter must be generated postinjection. In preliminary experiments with MTX γ-benzylamide (unpublished results) we have observed that plasma of mice injected intraperitoneally with a single 250 mg/kg dose of drug contained no HPLC-detectable MTX over a 2.5-h period. During this time the level of unchanged amide was in the 50–200 μM range. The HPLC assay would have readily detected MTX at a concentration of 0.5 μM. Thus, in vivo hydrolysis of the γ-amide bond in this MTX derivative appears to be insignificant if it takes place at all. In the absence of similar experiments on the AMT derivatives, on the other hand, we cannot at this time rule out the possibility that these compounds do undergo some bioconversion to AMT in vivo.

In summary, a method of synthesis of AMT γ-monoamides has been developed, which proceeds in high overall yield and is amenable to be used with a variety of amines. The AMT γ-monoamides have a strong affinity for DHFR and, depending on the nature of the amide group, exhibit inhibitory activity against murine and human tumor cells in culture and against a murine tumor in vivo. Additionally, they are more potent than either AMT or MTX against at least one mutant cell line in which resistance to these drugs is associated with an uptake defect. The availability of the γ-substituted derivatives provides an opportunity to further explore the potential of lipid-soluble analogues of the classical antifolates in cancer therapy.

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer, and NMR spectra were obtained on a Varian T60A instrument with chemical shifts (δ) reported relative to Me_4Si. TLC analyses were performed on fluorescent Baker Si250F silica gel plates or Eastman 13254 cellulose sheets. Spots were visualized under 254-nm UV illumination or with the aid of ninhydrin. Column chromatography was carried out on Baker 3405 silica gel (60–200 mesh) or Baker 7024-1 Flash silica gel (40 μm). Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA) and are corrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and MCL Laboratories, Lowell, MA. Chemicals were purchased from Aldrich Chemical Co., Milwaukee, WI, Chemical Dynamics Corp., South Plainfield, NJ, Bachem, Torrance, CA, and Pierce Chemical Co., Rockford, IL. Previously described methods were used to prepare 2,6-dichlorobenzylamine,[34] **5c**,[2] **6**,[10] and **8**.[10]

Preparation of *N*-(*tert*-Butyloxycarbonyl)-L-glutamic Acid α-Benzyl Ester γ-Amides via Diphenylphosphoryl Azide Coupling (Method A). *N*-(*tert*-Butyloxycarbonyl)-L-glutamic Acid α-Benzyl Ester γ-*tert*-Butylamide (4a). To a stirred solution of α-benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate (1.7 g, 5 mmol) and *tert*-butylamine (0.48 g, 6 mmol) in dry DMF (35 mL) at 0 °C were added successively diphenylphosphoryl azide (1.5 g, 55 mmol) and Et_3N (1.11 g, 11 mmol). Stirring was continued for 5 h at 0 °C and then 40 h at 25 °C, and the resulting solution was poured onto ice (300 g). Filtration and drying in vacuo over P_2O_5 gave a colorless solid (1.96 g).

***N*-(*tert*-Butyloxycarbonyl)-L-glutamic Acid α-Benzyl Ester γ-(1-Adamantylamide) (4b).** To a stirred solution of α-benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate (2.53 g, 7.5 mmol) and 1-adamantylamine hydrochloride (1.69 g, 9 mmol) in dry DMF (100 mL) at 0 °C was added a solution of diphenylphosphoryl azide (2.27 g, 8.25 mmol) in dry DMF (5 mL), followed by Et_3N (2.57 g, 25.7 mmol). After 5 h at 0 °C and 16 h at 25 °C, the solution was poured into ice–H_2O (600 mL) and the mixture was stirred for 1 h. The gummy precipitate was filtered and redissolved in CH_2Cl_2. The solution was washed successively with 10% AcOH, saturated $NaHCO_3$, and H_2O, then dried ($MgSO_4$), and evaporated to a clear oil (3.43 g), which gradually solidified.

***N*-(*tert*-Butyloxycarbonyl)-L-glutamic Acid α-Benzyl Ester γ-(3,4-Dichlorobenzylamide) (4d).** Diphenylphosphoryl azide (3.03 g, 11 mmol) was added to a stirred solution of α-benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate (3.37 g, 10 mmol), 3,4-dichlorobenzylamine (93% purity, 1.99 g, 10.5 mmol), and Et_3N (2.22 g, 22 mmol) in dry DMF (85 mL) at 5 °C. After 6 h at 0 °C and 65 h at 25 °C, the solution was poured into ice–H_2O (800 mL) containing AcOH (80 mL). The resulting emulsion was extracted with CH_2Cl_2 (300 mL), and the extract was washed with saturated $NaHCO_3$ and rinsed with H_2O. The solvent was evaporated and the residue taken up in toluene (100 mL). The solution was washed twice with H_2O, dried ($MgSO_4$), and evaporated to constant weight, giving an oil (4.95 g) which solidified on standing.

N^{α}-Protected amides **4e–g** were prepared by the same method as amide **4d**; yields and melting points are given in Table III. Infrared and ^1H NMR spectra were consistent with the assigned structures.

Preparation of L-Glutamic Acid γ-Benzyl Ester γ-Amides (Method B). L-Glutamic Acid α-Benzyl Ester γ-*tert*-Butylamide *p*-Toluenesulfonate Salt (5a·TsOH). A solution of **4a** (1.47 g, 3.75 mmol) and *p*-toluenesulfonic acid monohydrate (0.714 g, 3.75 mmol) in benzene (15 mL) was heated under reflux with a Dean–Stark trap for 1 h. The solution was concentrated to a volume of 2 mL, and Et_2O was added to induce crystallization. Filtration and drying at 75 °C over P_2O_5 gave colorless microcrystals (1.63 g).

L-Glutamic Acid α-Benzyl Ester γ-(1-Adamantylamide) Hydrochloride Salt (5b·HCl). A solution of **4b** (3.4 g, 6.9 mmol) and *p*-toluenesulfonic acid monohydrate (1.31 g, 6.9 mmol) in benzene (20 mL) was heated under reflux with a Dean–Stark trap

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Table III. Physical Constants of Glutamic Acid Derivatives

compd	method	% yield	mp, °C	formula[b]
4a	A	98	122–123	$C_{21}H_{32}N_2O_5$ (C, H, N)
4b	A	92	*c*	$C_{27}H_{38}N_2O_5 \cdot 0.1C_{12}H_{10}N_3O_2P$[d] (C, H, N)
4d	A	100	79–80	$C_{24}H_{28}Cl_2N_2O_5$ (C, H, N)
4e	A	94	107–108[e]	$C_{24}H_{28}Cl_2N_2O_5$ (C, H, N)
4f	A	58	106–107[f]	$C_{23}H_{28}N_2O_5$ (C, H, N)
4g	A	95	113–113.5[g]	$C_{24}H_{28}N_2O_7$ (C, H, N)
5a·TsOH	B	94	107–108	$C_{16}H_{24}N_2O_3 \cdot CH_3C_6H_4SO_3H \cdot 0.67H_2O$ (C, H, N)
5b·HCl	B	68	93–94	$C_{22}H_{30}N_2O_3 \cdot 1.1HCl \cdot 0.67H_2O$ (C, H, Cl, N)
5d·TsOH	B	90	161–162	$C_{19}H_{20}Cl_2N_2O_3 \cdot CH_3C_6H_4SO_3H$ (C, H, Cl, N, S)
5e·TsOH	B	98	177–180	$C_{19}H_{20}Cl_2N_2O_3 \cdot CH_3C_6H_4SO_3H$ (C, H, Cl, N, S)
5f·TsOH	B	97	171–173	$C_{18}H_{20}N_2O_3 \cdot CH_3C_6H_4SO_3H \cdot 0.25H_2O$ (C, H, N, S)
5g·TsOH	B	97	165.5–167	$C_{19}H_{20}N_2O_5 \cdot CH_3C_6H_4SO_3H$ (C, H, N, S)

[a] Key: A, diphenylphosphorylazide coupling; B, *tert*-butyloxycarbonyl group cleavage. [b] Correct microchemical analyses within ±0.4% of theoretical values were obtained for elements in parentheses. [c] Oil solidifying partially on standing. [d] The sample contained 0.1 mol of diphenylphosphoryl azide, which could be discerned by TLC and in the IR spectrum (KCl pellet, ν 2200 cm^{-1}). This impurity did not interfere with the next step. [e] Recrystallized from EtOAc–hexane. [f] Recrystallized from Et_2O–hexane. [g] Recrystallized from toluene.

Table IV. Physical Constants of Aminopterin and Methotrexate Derivatives

compd	method[a]	% yield	mp, °C[b]	TLC data, R_f[c]	formula[d]
3a	E	15	190–195	0.66 (1)	$C_{23}H_{29}N_9O_4 \cdot 3.5H_2O$ (C, H, N)
3b	F	78	220		$C_{29}H_{35}N_9O_4 \cdot 2.9H_2O$ (C, H, N)
3c	F	94	170	0.71 (1)	$C_{26}H_{27}N_9O_4 \cdot 2.25H_2O$ (C, H, N)
3d	F	84	180	0.78 (1)	$C_{26}H_{25}Cl_2N_9O_4 \cdot 2H_2O$ (C, H, Cl, N)
3e	F	92	190	0.48 (2)	$C_{26}H_{25}Cl_2N_9O_4 \cdot 1.25H_2O$ (C, H, N)
3f	F	92	190	0.13 (3)	$C_{25}H_{25}N_9O_4 \cdot 1.25H_2O$ (C, H, N)
3g	F	93	200	0.74 (1)	$C_{26}H_{25}N_9O_6 \cdot 2H_2O$ (C, H, N)
3h	G	85	190	0.70 (3)	$C_{27}H_{27}N_9O_6 \cdot CF_3CO_2H \cdot CH_3OH \cdot H_2O$ (C, H, F, N)
3i	F	82	190	0.32 (2)	$C_{25}H_{25}N_9O_6 \cdot 0.75H_2O$ (C, H, N)
3j	G	79	170		$C_{26}H_{27}H_9O_6 \cdot 0.85CF_3CO_2H \cdot 0.75CH_3OH \cdot 2H_2O$ (C, H, F, N)
7a	C	71	ca. 125	0.29 (4)	$C_{31}H_{35}N_9O_5 \cdot 0.5CH_3C_6H_4SO_3H \cdot 2H_2O$ (C, H, N)
7b	C	67	147–150		$C_{37}H_{41}N_9O_5 \cdot 1.4H_2O$ (C, H, N)
7c	C	44	ca. 115		$C_{34}H_{33}N_9O_5 \cdot 1.5CH_3OH$ (C, H, N)
7d	C	82	128–132		$C_{34}H_{31}Cl_2N_9O_5 \cdot 0.75H_2O$ (C, H, Cl, N)
7e	C	67	141–143	0.83 (4)	$C_{34}H_{31}Cl_2N_9O_5 \cdot 0.25H_2O$ (C, H, Cl, N)
	D	65			
7f	C	69	209–210		$C_{33}H_{31}N_9O_5 \cdot 0.25H_2O$ (C, H, N)
7g	C	73	146[e]		$C_{34}H_{31}N_9O_7 \cdot H_2O$ (C, H, N)
7h	H	82	144–146	0.53 (5)	$C_{34}H_{33}N_9O_6 \cdot CH_3OH \cdot H_2O$ (C, H, N)
9	E	69	ca. 165	0.78 (1)	$C_{24}H_{29}N_9O_5 \cdot 2CH_3OH \cdot H_2O$ (C, H, N)

[a] Key: C, mixed-anhydride coupling; D, *p*-nitrophenyl ester coupling; E, $Ba(OH)_2$ hydrolysis; F, NaOH hydrolysis; G, boron tris(trifluoroacetate); H, diethyl phosphorocyanidate coupling. [b] Good melting points were not obtained with compounds **3b–j**; values given represent temperatures at which decomposition, sometimes with charring, was observed. [c] Numbers in parentheses correspond to the following TLC systems: 1, silica gel, 5:4:1 $CHCl_3$–MeOH–concentrated NH_4OH; 2, cellulose, pH 7.4 potassium phosphate buffer; 3, cellulose, 1:2 DMF–0.1 N HCl; 4, silica gel, 9:1 $CHCl_3$–MeOH; 5, silica gel, 5:5:2 $CHCl_3$–MeCN–MeOH. [d] Correct microchemical analyses within ±0.4% of theoretical values were obtained for elements in parentheses. [e] The OCH_2O group in this compound was seen in the ^1H NMR spectrum, in CF_3CO_2H solution, as a singlet at τ 5.98 (2 H). [f] Loss of the OCH_2O group in this compound was confirmed by the disappearance of the τ 5.98 singlet in the ^1H NMR spectrum (CF_3CO_2H solution); see footnote e.

for 1 h, cooled, washed successively with H_2O (50 mL) and 1 N NaOH (10 mL), dried ($MgSO_4$), and evaporated to dryness. The residue was taken up in Et_2O and treated with HCl/Et_2O to form a precipitate, which was filtered and dried at 45 °C in vacuo over P_2O_5 to obtain **5b**·HCl as a hygroscopic solid (1.9 g).

Deprotected ester amide salts **5d**·TsOH–**5g**·TsOH were prepared by the same method as salt **5a**·TsOH; yields and melting points are given in Table III.

Preparation of N^{10}-Formylaminopterin α-Benzyl Ester γ-Amides via Mixed-Anhydride Coupling (Method C). N^{10}-Formylaminopterin α-Benzyl Ester γ-*tert*-Butylamide (7a). *i*-BuOCOCl (0.28 mL, 2.15 mmol) was added at room temperature to a stirred solution of **6** (0.797 g, 2.15 mmol, containing 1.75 molar equiv of H_2O by elemental analysis) in dry DMF (35 mL) to which Et_3N (0.87 g, 8.6 mmol) had been added. After 15 min, **5a**·TsOH (1.02 g, 2.15 mmol) was added, followed after 5 min by a second portion of *i*-BuOCOCl (0.14 mL, 1.08 mmol). After another 15 min, a second portion of **5a**·TsOH (0.512 g, 1.08 mmol) was added, followed again after 5 min by *i*-BuOCOCl (0.07 mL, 0.537 mmol). After another 15 min, a third portion of **5a**·TsOH (0.256 g, 0.537 mmol) was added, followed 5 min later by *i*-BuOCOCl (0.07 mL) and 15 min later by a final portion of **5a**·TsOH (0.256 g). The reaction was monitored by TLC (silica gel, 9:1 $CHCl_3$–MeOH) to confirm the formation of product (R_f 0.29, blue fluorescent spot). After removal of the DMF under reduced pressure, the residue was taken up in 95:5 $CHCl_3$–MeOH (10 mL) and applied onto a silica gel column (40 g), which was eluted with the same solvent mixture. Fractions containing the product were pooled and evaporated, and the residue was triturated with Et_2O to obtain **7a** as a hemi-*p*-toluenesulfonate; yield 1.13 g.

Aminopterin derivatives **7b–g** were prepared by the same method as derivative **7a**; yields and melting points are given in Table IV.

N^{10}-Formylaminopterin α-Benzyl Ester γ-(2,6-Dichlorobenzylamide) (7e) via Nitrophenyl Ester Coupling (Method D). A mixture of 8^{10} (0.552 g, 1.2 mmol), **5e**·TsOH (0.681 g, 1.2 mmol), and Et_3N (0.132 g, 1.3 mmol) in dry DMF (50 mL) was kept at 55 °C for 72 h. A clear solution formed within 40 min. The reaction was monitored by TLC (silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH), which showed the gradual replacement of 8 (R_f 0.85) by **7e** (R_f 0.91). After evaporation of the DMF, the residue was taken up in 95:5 $CHCl_3$–MeOH (14 mL) and adsorbed onto a silica gel column (70 g), which was eluted with the same solvent mixture. Fractions containing **7e** were pooled and evaporated, and the residue was redissolved in a minimum volume of 95:5 $CHCl_3$–MeOH. The solution was added in a stream to rapidly stirred Et_2O, and the precipitate was filtered and dried in vacuo at 100 °C over P_2O_5; yield 0.561 g.

N^{10}-Formylaminopterin γ-Amides via Barium Hydroxide Hydrolysis (Method E). N^{10}-Formylaminopterin γ-*tert*-Butylamide (9) and Aminopterin γ-*tert*-Butylamide (3a).

A solution of **7a** (1.1 g, 1.5 mmol) in a mixture of EtOH (60 mL) and H_2O (50 mL) was stirred with $Ba(OH)_2 \cdot 8H_2O$ (0.553 g, 1.75 mmol) for 20 h at 25 °C. A solution of NH_4HCO_3 (0.152 g, 1.92 mmol) in H_2O (3 mL) was then added and the precipitated $BaCO_3$ was filtered off. The filtrate was partially evaporated, acidified to pH 5.0 with AcOH, and freeze-dried. TLC (cellulose, pH 7.4 phosphate buffer) showed a UV-absorbing spot at R_f 0.21 and a blue fluorescent spot at R_f 0.64. The solid was dissolved in 3% NH_4HCO_3 (15 mL); the pH was adjusted to 9.7 with ammonia; and the solution was applied onto a DEAE-cellulose column (Whatman DE52, HCO_3^- form, 100 g). The column was eluted first with distilled H_2O (150 mL) to remove inorganic salts and then with 3% NH_4HCO_3. The first 90 mL of 3% NH_4HCO_3 eluate (R_f 0.64 spot) were combined and freeze-dried to obtain **9** as a pale-yellow powder (0.654 g). Further elution of the column with 350 mL of 3% NH_4HCO_3 and freeze-drying gave **3a** as a bright yellow powder (0.128 g).

Aminopterin γ-Amides via Sodium Hydroxide Hydrolysis (Method F). Aminopterin γ-(1-Adamantylamide) (3b). A solution of **7b** (1.05 g, 1.46 mmol) in a mixture of MeOH (40 mL) and 0.25 N NaOH (60 mL) was kept at 25 °C for 72 h, then acidified to pH 4.5 with 10% AcOH, refrigerated for 1 h, and filtered to give, after drying in vacuo, a bright yellow powder (0.714 g).

Other aminopterin γ-amides (**3a**, **3c–g**) and the methotrexate γ-amide **3i** were prepared by the same method as the amide **3b**, with minor differences in the percent of MeOH (40–60%) and in the length of reaction (14–28 h); yields and melting points are given in Table IV.

Aminopterin γ-(3,4-Dihydroxyanilide) (3h) (Method G). **3g** (100 mg, 0.168 mmol) was added to 4 mL of 0.5 M boron tris(trifluoroacetate) in TFA at 0 °C. After 15 min, the solution was allowed to warm up to 25 °C, at which temperature it was kept for 2 h. The solvent was removed in vacuo, MeOH added, and the solution reevaporated. Another portion of MeOH was added, and the solution was concentrated to a volume of 3 mL and added dropwise to stirred Et_2O (40 mL). The precipitated solid was filtered and dried at 60 °C in vacuo over P_2O_5 with protection from light; yield 101 mg.

Similar treatment of the methotrexate derivative **3i** afforded **3j** (cf. Table IV).

Methotrexate α-Benzyl Ester γ-(3,4-Methylenedioxyanilide) (7h) (Method H). **10** (2.78 g, 7.9 mmol) was added in small portions to a stirred solution of diethyl phosphorocyanidate (3.87 g, 2.37 mmol) and Et_3N (2.4 g, 23.7 mmol) in dry DMF (250 mL). Complete solution was achieved within 30 min. After 4 h, **5g** (4.17 g, 7.9 mmol) and Et_3N (1.6 g, 1.58 mmol) were added, and stirring was continued for 24 h at 25 °C. The solvent was removed in vacuo, and the residue was suspended in $CHCl_3$ (450 mL). The resulting mixture was washed by stirring with 10% NH_4OH (400 mL). The solid and the organic phase were combined, and the $CHCl_3$ was evaporated. Flash chromatography on silica gel with 5:5:1 $CHCl_3$–MeCN–MeOH as the eluent was performed, and appropriate fractions containing pure **7h** were pooled and evaporated. The residue was taken up in a small volume of MeOH, and the solution was added in a slow stream to stirred Et_2O. The precipitated solid was filtered and dried in vacuo at 100 °C over P_2O_5; yield 4.63 g.

Acknowledgment. This work was supported in part by Grants CA25394 (A.R.), CA19589 (A.R., M.M.W.), and CA41461 (J.H.F.) from the National Cancer Institute, DHHS. The technical assistance of Paul Genereux in obtaining in vivo antitumor data and of Judson Ratliff in obtaining some of the L1210 cell culture data is gratefully acknowledged.

Registry No. **3a**, 102650-87-3; **3b**, 102650-88-4; **3c**, 102650-89-5; **3d**, 102650-90-8; **3e**, 102650-91-9; **3f**, 102650-92-0; **3g**, 102650-93-1; **3h**, 102682-15-5; **3i**, 102650-94-2; **3j**, 102650-95-3; **4a**, 102651-05-8; **4b**, 102651-06-9; **4c**, 29421-22-5; **4d**, 102651-07-0; **4e**, 102651-08-1; **4f**, 102651-09-2; **4g**, 102651-10-5; **5a**·TsOH, 102651-12-7; **5b**·HCl, 102651-13-8; **5d**·TsOH, 102651-15-0; **5e**·TsOH, 102651-17-2; **5f**·TsOH, 102651-19-4; **5g**·TsOH, 102651-21-8; **6**, 89043-75-4; **7a**, 102650-96-4; **7b**, 102650-97-5; **7c**, 102650-98-6; **7d**, 102650-99-7; **7e**, 102651-00-3; **7f**, 102651-01-4; **7g**, 102651-02-5; **7h**, 102651-03-6; **8**, 95485-01-1; **9**, 102651-04-7; **10**, 19741-14-1; DHFR, 9002-03-3; *t*-$BuNH_2$, 75-64-9; $PhCH_2NH_2$, 100-46-9; 3,4-$Cl_2C_6H_3CH_2NH_2$, 102-49-8; 2,6-$Cl_2C_6H_3CH_2NH_2$, 6575-27-5; $PhNH_2$, 62-53-3; 3,4-$(OCH_2O)C_6H_3NH_2$, 14268-66-7; 3,4-$(OH)_2C_6H_3NH_2$, 13047-04-6; L-BOCGluOCH$_2$Ph, 30924-93-7; TsOH, 104-15-4; 1-adamantylamine, 768-94-5; methotrexate, 59-05-2.

Synthesis of Anthraquinonyl Glucosaminosides and Studies on the Influence of Aglycone Hydroxyl Substitution on Superoxide Generation, DNA Binding, and Antimicrobial Properties

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A series of anthraquinonyl glucosaminosides (**10a–e**) were synthesized by Koenigs–Knorr glycosidation of the corresponding aglycones (**11a–e**) with bromo sugar **12** followed by saponification. These glycosides were intended to serve as models to study the role played by the hydroxyl substituents on the aglycone portion of the antitumor anthracycline antibiotics. Superoxide generation as measured in rat heart sarcosomes was found to increase with the addition of successive hydroxyl groups to the anthraquinone nucleus. The 1,8-dihydroxy pattern was determined to generate significantly less superoxide than the 1,4-dihydroxy pattern. Hydroxyl substitution was also observed to stabilize the complex formed between the anthraquinones and DNA and was required for antibacterial activity against a number of Gram-positive organisms.

The anthracycline antibiotics daunomycin (**1**) and adriamycin (**2**) have achieved prominence in recent years due to their demonstrated clinical activity against a wide variety of tumor types.[1,2] Their utility, however, is severely limited by a tendency to produce potentially fatal dose-dependent cardiotoxicity.[2] Although the mechanism of action of these drugs has yet to be determined, there is substantial evidence that, with certain notable exceptions

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