Methotrexate Analogues. 34. Replacement of the Glutamate Moiety in Methotrexate and Aminopterin by Long-Chain 2-Aminoalkanedioic Acids[1]

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Eight previously unreported methotrexate (MTX) and aminopterin (AMT) analogues with the L-glutamate moiety replaced by DL-2-aminoalkanedioic acids containing up to 10 CH_2 groups were synthesized from 4-amino-4-deoxy-N^{10}-methylpteroic or 4-amino-4-deoxy-N^{10}-formylpteroic acid. All the compounds were potent inhibitors of purified L1210 mouse leukemia dihydrofolate reductase (DHFR), with IC_{50}'s of 0.023–0.034 μM for the MTX analogues and 0.054–0.067 μM for the AMT analogues. The compounds were not substrates for, but were inhibitors of, partially purified mouse liver folylpolyglutamate synthetase (FPGS). Activity was correlated with the number of CH_2 groups in the side chain. The IC_{50}'s for inhibition of cell growth in culture by the chain-extended MTX analogues were 0.016–0.64 μM against CEM human leukemic lymphoblasts and 0.0012–0.026 μM against L1210 mouse leukemia cells. However, the optimal chain length for growth-inhibitory activity was species-dependent. Our results suggested that CEM cells were inhibited most actively by the analogue with nine CH_2 groups, while L1210 cells were most sensitive to the analogue with six CH_2 groups. Among the AMT analogues, on the other hand, the most active compound against L1210 cells was the one with nine CH_2 groups, which had an IC_{50} of 0.000 65 μM as compared with 0.0046 μM for MTX and 0.002 μM for AMT. A high degree of cross-resistance was observed between MTX and the chain-extended compounds in two MTX-resistant cells lines, CEM/MTX and L1210/R81. All the MTX analogues were active against L1210 leukemia in mice on a qd × 9 schedule, with optimal increases in lifespan (ILS) of 75–140%. Notwithstanding their high in vitro activity, the AMT analogues were more toxic and less therapeutically effective than MTX analogues of the same chain length even though neither series of compounds possessed FPGS substrate activity. These MTX and AMT analogues are an unusual group of compounds in that they retain the dicarboxylic acid structure of classical antifolates yet are more lipophilic than the parent compounds because they have more CH_2 groups and are almost equivalent in vivo to MTX on the same schedule even though they do not form polyglutamates.

Analogues of the antitumor drug methotrexate (MTX, 1) with the L-glutamic acid side chain replaced by other L-2-aminoalkanedioic acids were reported previously from this[2] and other laboratories.[3,4] While the L-aspartate analogue of MTX was less potent than MTX both as a dihydrofolate reductase (DHFR) inhibitor and as an antileukemic agent in cell culture and in vivo,[2] increasing the length of the side chain from two to as many as five CH_2 groups resulted in retention of enzyme-inhibitory and cell-growth-inhibitory activity.[3,4] The chain-extended analogues also possessed in vivo antitumor activity similar to that of MTX against L1210 leukemia in mice, although 5–10-fold higher doses had to be given to elicit the same therapeutic effect.[2] The larger dose of chain-extended analogues required in vivo as opposed to in vitro probably reflects the fact that these compounds either are not substrates or are poor inhibitors of the enzyme folylpolyglutamate synthetase (FPGS)[5] and hence, unlike MTX, cannot be converted to noneffluxing polyglutamate derivatives. It has been suggested[2] that, in the therapy of tumors resistant to MTX by virtue of a polyglutamylation defect,[6-9] dose-limiting toxicity might be mitigated through the use of analogues that cannot form polyglutamates in either the host tissues or tumor.

In our previous study comparing the cell-growth-inhibitory activities of MTX analogues with extended side chains,[2] we observed a 2- and 3-fold increase in potency relative to MTX when the total number of CH_2 groups was four and five, respectively. Consequently it was of interest to examine whether potency could be further enhanced by increasing the number of CH_2 groups beyond five. Since the enantiomerically pure L-2-aminoalkanedioic acids were not readily available, we chose to prepare the desired analogues first in the DL form, with the idea that L enantiomers could be made later depending on the activity of the racemates. This paper describes the synthesis of the chain-extended MTX analogues **2–6** (cf. Table I), in which the number of CH_2 groups ranges from six to 10. Also synthesized were the N^{10}-unsubstituted compounds **7–9** (cf. Table I), which are, to our knowledge, the first examples of aminopterin (AMT, **10**) analogues with a lengthened side chain. Compounds **2–9** were tested as inhibitors of purified DHFR from L1210 murine leukemia cells, as inhibitors of the growth of MTX-sensitive and MTX-resistant L1210 leukemia cells in culture, and as inhibitors of the growth MTX-sensitive L1210 leukemia in mice. In addition, substrate and/or inhibitor activity was assayed in vitro with partially purified FPGS from mouse liver.[5,10]

Chemistry

DL-2-Aminoalkanedioic acids were synthesized from monomethyl esters of long-chain alkanedioic acids from

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(1) For paper 33 in this series, see: Rosowsky, A.; Bader, H.; Cucchi, C. A.; Moran, R. G.; Freisheim, J. H. *J. Med. Chem.*, preceding paper in this issue.
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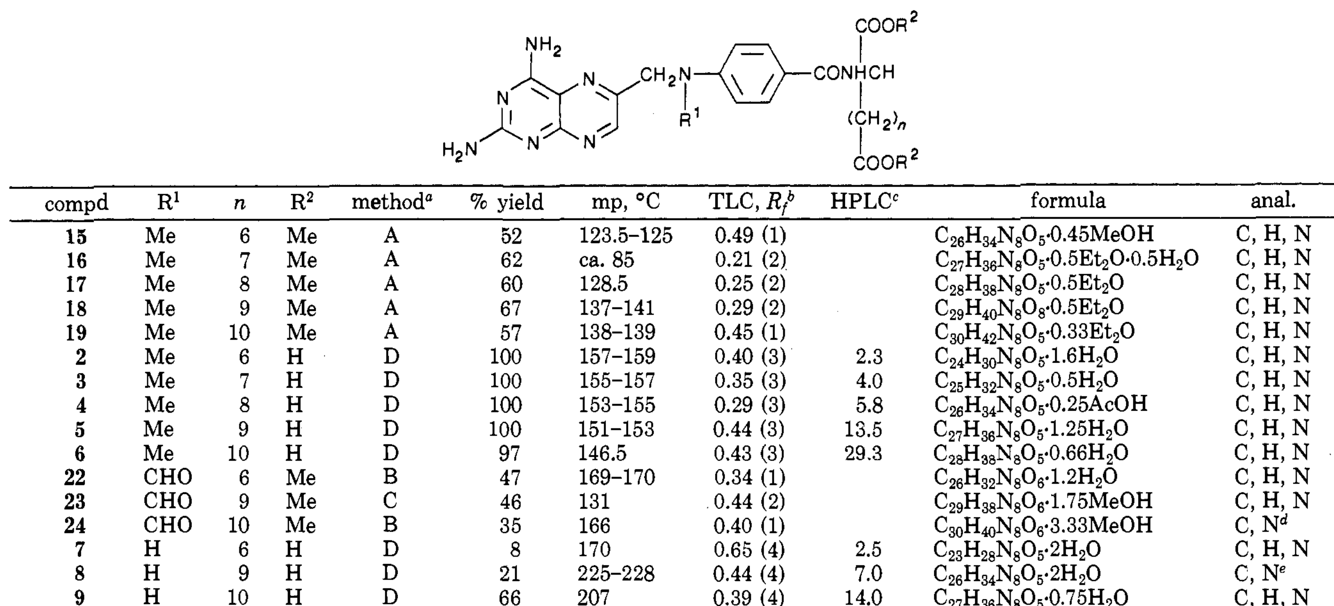
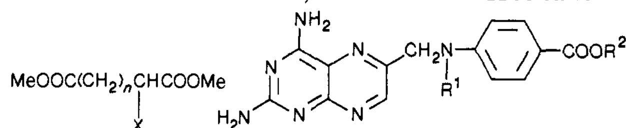
Table I. Chain-Extended MTX and AMT Analogues



compd	R[1]	*n*	R[2]	method[a]	% yield	mp, °C	TLC, R_f[b]	HPLC[c]	formula	anal.
15	Me	6	Me	A	52	123.5-125	0.49 (1)		$C_{26}H_{34}N_8O_5$·0.45MeOH	C, H, N
16	Me	7	Me	A	62	ca. 85	0.21 (2)		$C_{27}H_{36}N_8O_5$·0.5Et_2O·0.5H_2O	C, H, N
17	Me	8	Me	A	60	128.5	0.25 (2)		$C_{28}H_{38}N_8O_5$·0.5Et_2O	C, H, N
18	Me	9	Me	A	67	137-141	0.29 (2)		$C_{29}H_{40}N_8O_5$·0.5Et_2O	C, H, N
19	Me	10	Me	A	57	138-139	0.45 (1)		$C_{30}H_{42}N_8O_5$·0.33Et_2O	C, H, N
2	Me	6	H	D	100	157-159	0.40 (3)	2.3	$C_{24}H_{30}N_8O_5$·1.6H_2O	C, H, N
3	Me	7	H	D	100	155-157	0.35 (3)	4.0	$C_{25}H_{32}N_8O_5$·0.5H_2O	C, H, N
4	Me	8	H	D	100	153-155	0.29 (3)	5.8	$C_{26}H_{34}N_8O_5$·0.25AcOH	C, H, N
5	Me	9	H	D	100	151-153	0.44 (3)	13.5	$C_{27}H_{36}N_8O_5$·1.25H_2O	C, H, N
6	Me	10	H	D	97	146.5	0.43 (3)	29.3	$C_{28}H_{38}N_8O_5$·0.66H_2O	C, H, N
22	CHO	6	Me	B	47	169-170	0.34 (1)		$C_{26}H_{32}N_8O_6$·1.2H_2O	C, H, N
23	CHO	9	Me	C	46	131	0.44 (2)		$C_{29}H_{38}N_8O_6$·1.75MeOH	C, H, N
24	CHO	10	Me	B	35	166	0.40 (1)		$C_{30}H_{40}N_8O_6$·3.33MeOH	C, N[d]
7	H	6	H	D	8	170	0.65 (4)	2.5	$C_{23}H_{28}N_8O_5$·2H_2O	C, H, N
8	H	9	H	D	21	225-228	0.44 (4)	7.0	$C_{26}H_{34}N_8O_5$·2H_2O	C, N[e]
9	H	10	H	D	66	207	0.39 (4)	14.0	$C_{27}H_{36}N_8O_5$·0.75H_2O	C, H, N

[a] Methods: A = DEPC coupling; B = mixed carboxylic-carbonic anhydride coupling; C = nitrophenyl ester displacement; D = alkaline hydrolysis. [b] TLC systems: 1 = silica gel, 2:5:5 MeOH-MeCN-$CHCl_3$; 2 = silica gel, 1:5:5 MeOH-MeCN-$CHCl_3$; 3 = silica gel, 1:5:15 AcOH-MeOH-$CHCl_3$; 4 = silica gel, 1:4:5 NH_4OH-MeOH-$CHCl_3$. [c] HPLC was carried out on a Waters C_{18} μBondapak column, which was eluted with 0.1 M NH_4OAc, pH 6.0, containing 20% MeCN. MTX (1) and analogues of MTX with n = 3-5 could not be distinguished from compound **2** in this system. However, when the MeCN was decreased to 10%, the following retention times were obtained for the shorter-chain analogues: n = 2 (MTX), 7.2 min; n = 3, 9.8 min; n = 4, 15.0 min; n = 5, 33.0 min. [d] H: calcd, 7.51; found, 8.02. [e] H: calcd, 6.66; found, 5.92.

nonanedioic (azelaic) to tridecanedioic. Monoesters, obtained from the corresponding diesters[11a,b] by half-hydrolysis,[12a-c] were treated with $SOCl_2$ in the presence of a catalytic amount of DMF, and the resultant acid chlorides were subjected to α-bromination and MeOH addition according to the method of Schwenk and Papa.[13] The resultant bromo diesters **11a-e**[11a,14a,b] were treated with NaN_3 in refluxing absolute EtOH to form the azides **12a-e**, which were catalytically reduced in the presence of HCl to obtain the amines **13a-e**,[11a,c] isolated as HCl salts.



11a-e: X = Br
12a-e: X = N_3
13a-e: X = NH_2
a, n = 6; **b**, n = 7; **c**, n = 8; **d**, n = 9; **e**, n = 10

14: R^1 = Me, R^2 = H
20: R^1 = CHO, R^2 = H
21: R^1 = CHO, R^2 = $C_6H_4NO_2$-*p*

Condensation of the amino diesters **13a-e** with 4-amino-4-deoxy-N^{10}-methylpteroic acid (mAPA, **14**) was accomplished with the aid of the coupling reagent diethyl phosphorocyanidate (DEPC),[15] which we have used previously to obtain a wide variety of side chain modified MTX analogues.[1,2,16-21] The coupling reaction (method A, Table I) was conducted in DMF at room temperature, with the amino diesters being added in 2-fold excess.

In agreement with our earlier experience,[18] a side product that eluted slightly ahead of the desired compound on silica gel was formed in small amount during DEPC-mediated coupling of each of the amino diesters. The amount of this side product that formed appeared to be time-dependent. Thus, in the condensation of **14** with **13e**, which was allowed to proceed at room temperature for 76 h, the yield of byproduct (based on **14**) was 33%, whereas in the other coupling reactions, which were run for 24 h, this yield was generally <10%. Removal of the byproduct was more easily accomplished at the diester stage than at the diacid stage. Consequently, cleavage of the diesters was carried out only after rigorous purification. When purified diesters were hydrolyzed, typically with 1 N NaOH in MeOH at room temperature for 24 h, the diacids that precipitated at pH 4.0-4.5 upon addition of AcOH required no further purification. Yields of the diacids **2-6** from **15-19**, respectively, were essentially quantitative.

It has been reported recently that peptides can be formed directly from α-azido esters and N-blocked amino acids in toluene at 60-70 °C in the presence of triphenylphosphine.[22-24] We investigated the coupling of

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Table II. Dihydrofolate Reductase Inhibition and Cell-Growth Inhibition by Chain-Extended MTX and AMT Analogues

compd	n	N^{10}	DHFR:[a] IC_{50}, μM	cell growth: IC_{50},[b] μM			
				CEM	CEM/MTX	L1210	L1210/R81
2	6	Me	0.023	0.15	5.8	0.0012	68
3	7	Me	0.032	0.062	1.0	0.0042	73
4	8	Me	0.029	0.056	2.2	0.0031	78
5	9	Me	0.034	0.016	10	0.0071	58
6	10	Me	0.026	0.64	10	0.026	56
7	6	H	0.54	*d*	*d*	0.020	>218
8	9	H	0.081	*d*	*d*	0.00065	110
9	10	H	0.067	*d*	*d*	0.0011	215
MTX (1)	2	Me	0.025	0.032	6.6	0.0046	197
AMT (10)[c]	2	H	0.025	0.001	0.32	0.002	84

[a] See ref 23. The assay mixture contained NADPH (75 μM), DHFR (0.052 μM), and dihydrofolate (50 μM) in 0.05 M Tris-Cl buffer, pH 7.5, at 22 °C, and the reaction was initiated by addition of dihydrofolate after 2 min of preincubation of the other components. The enzyme was obtained from L1210/R71 cells and purified by affinity chromatography on MTX-Sepharose as previously described.[24] [b] See ref 17 and 21; cells were treated for 48 h. Data for the L1210 and L1210/R81 cells are the means of three separate assays. Data for the CEM and CEM/MTX cells are from a single experiment in triplicate. [c] Data taken from ref 18. [d] Not determined.

α-azido diesters directly to **14** but found that when the reaction was attempted at 80 °C in *N*-methylpyrrolidinone (toluene being an unsuitable solvent for **14**), only starting material was recovered.

The AMT analogues **7–9** were obtained from 4-amino-4-deoxy-N^{10}-formylpteroic acid (fAPA, **20**) via a mixed carboxylic-carbonic anhydride (method B, Table I) or via the *p*-nitrophenyl ester **21** (method C, Table I).[17-21] The previously described modified mixed anhydride procedure (repetitive cycles of activation and coupling)[17] was followed. Purification of the diesters was laborious, in that silica gel column chromatography had to be performed several times to completely remove poorly separated minor contaminants. Yields of the rigorously purified N^{10}-formyl diesters **22–24** were 35–50% (based on **20**) and appeared to be comparable by the two methods. However, it is important to note that reactants are present in approximately equimolar proportions in method B, whereas in method C an excess of amino diester is used. Thus, the former route is advantageous because it conserves the amino diester.

Alkaline hydrolysis of **22–24** to diacids **7–9** was more complicated than hydrolysis of the corresponding N^{10}-methyl derivatives, being accompanied by extensive formation of fluorescent side products. As a result, when diesters **22** and **23** were left to stand in base at room temperature (**22**, 92 h; **23**, 48 h), the yield of the deblocked diacids **7** and **8** was <25%. In an attempt to determine the minimal time required to just remove the ester and N^{10}-formyl groups, the reaction was followed by TLC. Ester cleavage occurred cleanly in a few hours, but cleavage of the formyl group was slow, and it was mostly at this stage that the side products were formed. In a control experiment in which pure **8** was left to stand in 0.25 N NaOH at 25 °C, HPLC revealed that only about 30% of the compound remained intact in the solution after 68 h. It thus appeared that side products formed mainly because hydrolysis of the formyl group at 25 °C was too slow. As a possible means to overcome this problem, we examined the effect of raising the temperature to increase the hydrolysis rate. Care was also taken to use deoxygenated water and exclude light in order to minimize oxidative and/or photocatalytic cleavage of the C^9–N^{10} bond, which is rapid in alkaline solutions of AMT. When **24** was kept in degassed alkaline solution at 36 °C for 26 h, a 66% yield of the diacid **9** was obtained. Though the yield was still not quantitative (in contrast to the hydrolysis of the N^{10}-methyl diesters), it was nonetheless a significant improvement over the room temperature hydrolysis. In retrospect, however, we believe that a synthetic strategy employing a more labile N^{10}-protecting group than formyl would have the best chance of improving the overall yield of the AMT analogues.

Because of a need to establish a basis for comparing the lipophilicities of the diacids **2–6** with those of previously reported analogues containing a shorter side chain,[4] HPLC retention times on C_{18}-bonded silica gel were determined. As shown Table I, there was a progressive increase in affinity for C_{18} silica gel as the number of CH_2 groups increased, in accord with our expectation that longer chain compounds would be more lipophilic. It can also be seen that the MTX analogues **5** and **6** eluted more slowly than the corresponding AMT analogues **8** and **9**, reflecting the contribution of the N^{10}-methyl substituent to lipophilicity.

Biological Activity

Compounds **2–9** were assayed spectrophotometrically as inhibitors of dihydrofolate reductase from L1210/R81 murine leukemia cells as described earlier.[23] As shown in Table II, the IC_{50} values ranged from 0.023 to 0.034 μM for the N^{10}-methyl analogues **2–6** and from 0.054 to 0.081 μM for the N^{10}-unsubstituted analogues **7–9**. (While we cannot state with certainty that L enantiomers would be more active than the racemic mixtures for which data are given in this paper, it is known that the antifolate activity of the clinically used L enantiomer of MTX is substantially lower than that of the D enantiomer or the DL mixture.[25] The D enantiomer of MTX is not a substrate for FPGS.[5]) The IC_{50} for both MTX and AMT in these experiments was found to be 0.025 μM. Comparison of IC_{50} values for pairs of compounds with the same side chain but differing in N^{10} substitution (e.g., **2** and **7**) revealed a consistent 2–3-fold difference, suggesting that, at least in this series of α,ω-dicarboxylic acid analogues, N^{10}-methyl substitution favored DHFR binding. The same trend was observed previously with the L-cysteic and L-homocysteic acid analogues of MTX and AMT[17] but not with other pairs, such as the γ-*tert*-butyl esters[18] and DL-2-amino-4-phosphonobutyric acid analogues.[21] It thus appears that the contribution of the N^{10}-substituent to binding to the DHFR active sites has to be viewed in the context of the entire ligand, including the side chain.

Assays of cell-growth inhibition were carried out with the MTX analogues **2–6** against human leukemic lymphoblasts (CEM and CEM/MTX) and with all the ana-

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logues against mouse leukemia cells (L1210 and L1210/R81). The MTX-resistant L1210/R81 cell line has been shown previously to have a profound MTX transport defect, along with a 35-fold increase in DHFR content.[26] The CEM/MTX line has normal DHFR activity but is defective in MTX transport and polyglutamylation.[9,27] As shown in Table II, the IC_{50} of the compounds **2–6** against CEM cells ranged from 0.016 to 0.64 μM and followed a pattern that appeared to be related to the $(CH_2)_n$ group, with potency increasing over the range $n = 6$ to $n = 9$ and decreasing sharply at $n = 10$. The most potent member of the series (**5**, $n = 9$) was twice as potent as MTX.

In assays against L1210 cells (Table II), the IC_{50}'s of the MTX analogues **2–6** ranged from 0.0012 to 0.026 μM, as compared with 0.0046 μM for MTX. However, while **6** ($n = 10$) proved again to be substantially less active than the other members of the series, an increase in activity from $n = 6$ to $n = 9$ was not observed. This contrasted with the CEM cells, for which the data suggested the optimal number of CH_2 groups to be smaller. The L enantiomeric analogues of MTX with $n = 3–5$ were found earlier to have IC_{50}'s of 0.030 ($n = 3$), 0.010 ($n = 4$), and 0.0063 ($n = 5$) μM, respectively. From these results it appears that the optimal value of n for activity against cultured L1210 cells is six. It is unknown at present whether this species specificity reflects differences in structure between murine and human DHFR or differences in the transport kinetics for these compounds between CEM and L1210 cells.

Comparison of the activities of the chain-extended AMT analogues **7–9** with those of the corresponding MTX analogues **2**, **5**, and **6** against L1210 cells led to the unexpected finding that $n = 9$ was superior to N = 6 when N^{10} was unsubstituted. While it is impossible, in the absence of the analogues with $n = 7$ and $n = 8$, to conclude that $n = 9$ is optimal in the AMT series, our results suggest that deletion of the N^{10}-methyl increases the number of side-chain CH_2 groups required for optimal cell growth inhibition, as it does for DHFR inhibition. The most potent inhibitor of L1210 cell growth was **8**, with an IC_{50} of 0.00065 μM as compared with 0.002 μM for AMT. It thus appears that the analogue with $n = 9$ is more growth inhibitory than AMT even though it is not a substrate for FPGS (see below).

Results with the MTX-resistant L1210/R81 cells showed the MTX analogues **2–6** to be 3–4-fold more potent than MTX. Overall, however, the chain-extended compounds, including those most active against the parental cells, showed essentially complete cross-resistance with MTX in this highly resistant line. Since less resistant cells might respond more favorably to the chain-extended analogues, we also tested **2–6** against CEM/MTX cells. The sensitivity of these cells to lipophilic antifolates was previously shown to be similar to, or greater than, that of the parental CEM cells.[4] All the compounds were at least 30-fold less active against the resistant cells than the parent cells. Thus, increasing the lipophilicity of the side chain by addition of up to eight extra CH_2 groups was not a promising approach to overcoming resistance in either highly or moderately MTX resistant cells.

Since it was conceivable that analogues of MTX and AMT containing chain-lengthened amino acids, such as **3** and **5**, might substitute for the natural di- or triglutamates as FPGS ligands, **2–6** and **8** were tested as substrates, as well as inhibitors, of this enzyme. Partially purified FPGS from mouse liver[10] was used in these assays as described earlier,[5] with 500 μM folic acid as the substrate. The extent of labeled glutamic acid incorporation was determined in the presence of 500 μM of each test compound; in some instances the $K_{i,s}$ (estimated K_i; see footnote *b*, Table III) as well as percent inhibition was obtained. None of the compounds had detectable activity as substrates for the enzyme (data not shown). In fact, the chain-lengthened analogues were moderately active FPGS inhibitors (Table III). As expected from our previous results in other series,[5,17,21,24] N^{10}-unsubstituted analogues were more potent inhibitors than were the corresponding N^{10}-methyl derivatives. The most potent inhibitor was **8**, for which a $K_{i,s}$ of 24 ± 6 μM was obtained. Inhibition by the more potent compounds (**8** and **9**) was competitive with AMT as the variable substrate. However, for **2** and **7** a mixed or more complicated pattern of inhibition was observed when AMT was the variable substrate. The only inhibitors thus far found with affinities greater than that of **8** are N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-ornithine (K_i = 20 μM, mouse;[9] 3–4 μM, rat and human[28]), tetrahydrofolic acid γ-monoamide (K_i = 10 μM, hog[29]), N^{α}-(4-amino-4-deoxypteroyl)-DL-2-amino-4-phosphonobutyric acid (K_i = 8.4 μM, mouse;[24] 1.9 μM, human[30]), N^{α}-pteroyl-L-ornithine (K_i = 6 μM, hog[29]) and its reduced derivatives (K_i = 0.2 μM, hog[29]), and N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine (K_i = 0.1–0.3 μM, mouse[19] and human[30]). What makes **8** unique among the diverse compounds in this group is that it is *the only one with a carboxyl group on the end of the side chain.*

Compounds **2–7** and **9** were tested for in vivo antitumor activity against L1210 leukemia in mice. The MTX analogues **2–6** were administered daily for 9 days at doses of 20–80 mg/kg per injection, while **7** and **9** were given on the same schedule at 6.25–50 mg/kg with the expectation, based on previous experience in this laboratory,[17] that the AMT analogues would have a lower tolerated dose. Results of these experiments are presented in Table IV.

Compounds **2–5** ($n = 6–9$) had an optimal therapeutic dose of 40–60 mg/kg, with maximal increases in lifespan

Table III. Inhibition of Folylpolyglutamate Synthetase by Chain-Extended Analogues of MTX and AMT

compd	no. of CH_2's	N^{10}	% inhibn[a]	$K_{i,s}$,[b] μM
mAPA-L-Adi[c]	3	Me	26	
mAPA-L-Pim[c]	4	Me	28	
mAPA-L-Sub[c]	5	Me	33	
2	6	Me	47	320 ± 100[d]
3	7	Me	43	
4	8	Me	49	
5	9	Me	53	
6	10	Me	59	
7	6	H	50	170 ± 4[d]
8	9	H	90	24 ± 6[e]
9	10	H	74	62 ± 7[e]

[a] Assays were performed against partially purified FPGS from mouse liver as described in ref 5. [b] $K_{i,s}$ represents a K_i estimated from a replot of the slopes of lines from double-reciprocal plots of $1/V$ versus $1/S$ where V = reaction velocity and S = substrate concentration. [c] Data taken from ref 5. [d] The kinetics of inhibition was more complex than competitive or mixed with respect to the folate substrate. [e] The kinetics of inhibition was strictly competitive with respect to the folate substrate.

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Table IV. Inhibition of L1210 Leukemia in Mice by MTX and AMT Analogues with a DL-2-Aminoalkanedioic Acid Side Chain

compd	N^{10}	*n*	dose,[a] mg/kg × 9	no. of mice	mean 7-day wt change, %	survival,[b] days		ILS, %
						range	mean ± SD	
2	Me	6	20	1 × 5	+4	14–16	14.8 ± 1.1	+68
			40	2 × 5	–1	15–22[c]	17.0 ± 2.8	+93
			60	1 × 5	+4	17–19	18.0 ± 0.7	+105
			80	1 × 5	–12[d]	9–16	12.6 ± 3.0	+43
3	Me	7	20	1 × 5	+7	13–14	13.6 ± 0.5	+55
			40	3 × 5	+6	14–16[e]	14.6 ± 0.6	+66
			60	2 × 5	+6	12–19	15.3 ± 2.2	+74
			80	2 × 5	+3	12–18[f]	15.2 ± 1.9	+73
4	Me	8	20	1 × 5	+5	15–16	15.2 ± 0.4	+73
			40	3 × 5	+2	13–23	16.9 ± 2.8	+92
			60	2 × 5	+4	10–18	15.0 ± 3.2	+70
			80	2 × 5	–5	9–23	15.2 ± 5.2	+73
5	Me	9	20	1 × 5	+6	16–19	17.4 ± 1.1	+98
			40	2 × 5	+3	14–24	17.2 ± 3.0	+95
			60	2 × 5	+5	15–22	17.6 ± 2.7	+100
			80	1 × 5	+2	12–18	15.2 ± 2.3	+73
6	Me	10	20	1 × 5	+3	19–22	20.4 ± 1.1	+137
			40	2 × 5	+3	12–25	19.0 ± 3.7	+116
			60	2 × 5	+2	9–24	17.3 ± 6.4	+97
			80	1 × 5	+4	9–11	10.0 ± 0.7	+14
7	H	6	6.25	1 × 5	+9	10–13	12.0 ± 3.8	+36
			12.5	1 × 5	+9	10–15	14.2 ± 1.3	+61
			25	1 × 5	+5	13–16	11.8 ± 2.0	+34
			50	1 × 5	+7	8–16	10.6 ± 1.3	+20
9	H	10	6.25	1 × 5	+10	12–13	12.8 ± 0.4	+45
			12.5	1 × 5	+10	11–16	13.8 ± 1.9	+57
			25	1 × 5	–3	6–16	9.6 ± 3.8	+11
			50	1 × 5	+4	8–15	10.4 ± 3.4	+18
MTX	Me	2	4	4 × 5	+8	14–23	17.2 ± 2.6	+95

[a] Protocols for the testing of compounds against L1210 leukemia in $B6D2F_1J$ mice (Jackson Labs, Bar Harbor, ME) were similar to those used at the National Cancer Institute.[34] Compounds were dissolved in sterile saline at pH 7.5–8.5, and solutions were prepared daily and discarded after each use. Solutions were injected intraperitoneally every day for 9 days (qd × 9) to groups of five animals per experiment. [b] Control mice (five groups totaling 54 mice) showed a mean 7-day weight change of +14% and a mean survival of 8.8 ± 1.6 days. ILS = increase in lifespan. [c] Three mice survived >30 days and were excluded from the range. [d] A weight loss > 10% is considered toxic. [e] One mouse survived >30 days and was excluded from the range. [f] The tumor failed to grow in one of the 10 inoculated mice.

(ILS) ranging from 74% (**3**) to 105% (**2**), while **6** ($n = 10$) gave an ILS of 137% at 20 mg/kg. These doses are comparable to the ones typically used in the treatment of mice on the qd × 9 schedule with lipid-soluble antifolates, e.g., piritrexim (BW301U)[31] and trimetrexate (TMQ),[32,33] both of which lack a glutamate side chain and hence cannot form polyglutamates.

The AMT analogues **7** and **9** were more toxic in vivo than the corresponding MTX analogues **2** and **6**. Optimal doses for **7** and **9** were found to be 12.5 mg/kg (qd × 9), but at larger doses (25 and 50 mg/kg) there were early deaths suggesting host toxicity. Maximum lifespan increases with these compounds proved 2-fold lower than those of the MTX analogues. From this we conclude that the introduction of extra CH_2 groups in the AMT side chain is less successful in vivo than the same modification in MTX.

In summary, we have shown that MTX analogues in which the L-glutamate moiety is replaced by longer DL-2-aminoalkanedioic acids are potent DHFR inhibitors and can also inhibit FPGS. Murine and human tumor cells growth in vitro as well as murine tumor growth in vivo were strongly inhibited by several of the compounds. Activity was observed in vivo despite the fact that these analogues are not FPGS substrates and thus cannot form polyglutamates. The most active compound against L1210 leukemia in mice was the one with the highest number of CH_2 groups ($n = 10$). The high potency of the AMT analogues in vivo in comparison with the MTX analogues suggests that polyglutamylation is not the only mechanism responsible for the toxicity of AMT relative to MTX. Although AMT analogues with extra CH_2 groups in the side chain probably do not warrant further study, additional work on chain-extended MTX analogues, especially those with the L configuration, would be of interest.

Experimental Section

Melting points were determined in a Fischer-Johns hot-stage apparatus and are corrected. IR spectra were obtained on a Perkin-Elmer Model 781 double-beam spectrophotometer. Analytical thin-layer chromatography (TLC) was on Whatman MK6F and Baker 250F silica gel plates) or Eastman 13254 cellulose sheets containing a fluorescent indicator. Preparative TLC was on Analtech 1000 μm GF silica gel plates. Spots were visualized uner 254-nm illumination. Conventional column chromatography was on Baker 3405 silica gel (60–200 mesh) or Whatman DE52 [(diethylamino)ethyl]cellulose (DEAE-cellulose), flash chromatography on Baker 7024-1 silica gel (40 μm average particle diameter), and low-pressure liquid chromatography (LPLC) on a Merck LiChroprep RP-18 prepacked C_{18}-bonded silica gel column (40–63 μm particle diameter) eluted with the aid of Milton Roy Mini-Pump. Microanalyses were by Galbraith Laboratories, Knoxville, TN, and Multi Chemical Laboratories, Lowell MA, and were within ±0.4% of theory unless otherwise indicated. Final traces of the known organic solvents used in the preparation of analytical sample of the hydrophobic diesters **15–19**, **23**, and **24** were very difficult to remove. The presence of these solvents (e.g., MeOH, Et_2O) was not rigorously established in every

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instance by physical means but was reflected in the microanalytical data. Moreover, in two representative examples, 300-Mz proton NMR spectra taken in $CDCl_3$ solution revealed the presence of MeOH in **15** as a singlet at δ 3.65 and Et_2O in **17** as a quartet at δ 3.45 (OCH_2CH_3). 4-Amino-4-deoxy-N^{10}-methylpteroic acid (**14**), 4-amino-4-deoxy-N^{10}-formylpteroic acid (**20**), and *p*-nitrophenyl 4-amino-4-deoxy-N^{10}-formylpteroate (**21**) were obtained as previously described.[18] Molar quantities of **14** and **20** were calculated by assuming a sesquihydrate formula, though the amount of water in these compounds may vary from batch to batch.[18] Literature methods were likewise used to prepare monomethyl esters of undecanedioic,[12a] dodecanedioic,[12b] and tridecanedioic[12c] acids. Monomethyl esters of nonadedioic acid (azelaic), decanedioic (sebacic) acid, and other chemicals were from Aldrich, Milwaukee, WI, and Fisher, Boston, MA.

Bromoalkanedioic Acid Diesters. Procedure 1. 2-Bromotridecanedioic Acid Dimethyl Ester (11e). A solution of tridecanedioic acid monomethyl ester (10.5 g, 0.0406 mol) in $SOCl_2$ (12 mL, 0.163 mol) containing 3 drops of DMF was heated under reflux for 3 h. A crystal of I_2 was added, and the refluxing mixture was irradiated with a flood lamp during dropwise addition of Br_2 (2.18 mL, 0.0426 mol) over a period of 4 h. The lamp was turned off, and, after another 20 h, excess $SOCl_2$ was evaporated under reduced pressure. The residue was poured into MeOH (70 mL), and the mixture was left at room temperature for 3.5 h. After removal of the solvent, the residue was taken up in Et_2O, and the solution was washed consecutively with H_2O (50 mL), saturated $NaHCO_3$ 50 mL), and H_2O (25 mL), dried ($MgSO_4$), and evaporated.

Distillation of the residue gave **11e** as an oil: bp 154 °C (0.1 Torr); n_D^{20} 1.4655 [lit.[14a] bp 145–152 °C (0.03 Torr)]; yield 12.1 g (85%). The bromo diester was used directly in the next step.

Other bromo diesters had the following properties. **11a**: 90% yield; bp 132 °C (0.5 Torr); n^{20}_D 1.4685 [lit.[11a,14a] bp 180 °C (12 Torr)]. **11b**: 87% yield; bp 126–128 °C (0.05 Torr); n^{20}_D 1.4707 [lit.[11a] bp 140–148 °C (1.5 Torr), n^{20}_D 1.461–1.470]. **11c**: 85% yield; bp 121 °C (0.02 Torr); n^{20}_D 1.4701 [lit.[11a] bp 167–177 °C (1.5 Torr), n^{20}_D 1.4602–1.4713]. **11d**: 90% yield; bp 162 °C (0.3 Torr); n^{20}_D 1.4681–1.4700 [lit.[11a] bp 175–192 °C (1.5 Torr), n^{20}_D 1.4587–1.4722].

2-Aminoalkanedioic Acid Diesters. Procedure 2. 2-Aminotridecanedioic Acid Dimethyl Ester Hydrochloride (13e·HCl). A solution of **11e** (7.03 g, 0.02 mol) in absolute EtOH (15 mL) containing NaN_3 (1.5 g, 0.023 mol) was stirred under reflux for 15 h and then poured into H_2O (100 mL). The product was extracted with three portions of Et_2O (200 mL total), the extracts were dried ($MgSO_4$), and the solvent was evaporated in vacuo at 35 °C (bath temperature) to obtain the azido diester **12e** as a clear yellowish oil: IR (thin film) ν 2100 (N_3), 1735 (ester C=O) cm^{-1}. The oil was dissolved in a mixture of absolute EtOH (40 mL) and 12 N HCl (4 mL) and was catalytically reduced in a Parr hydrogenation apparatus for 17 h in the presence of 10% Pd–C (0.75 g). The catalyst was filtered off, the solvent was evaporated, and the residue was thoroughly dried by azeotropic distillation (3 times) with toluene. The final residue was extracted three times with warm petroleum ether (bp 30–56 °C), and the combined extracts were evaporated to constant weight under reduced pressure to obtain **13e·HCl** as an oil, which crystallized on standing: yield 5.2 g (80%); mp 60 °C; IR (thin film) ν 3350 (NH_3^+), 2600 (NH_3^+), 1740 (ester C=O).

Catalyst poisoning occasionally prevented hydrogenation of the azido diesters. We found that this could be easily remedied by preliminary treatment of the azido diester with a small amount of Davison sponge nickel in boiling EtOH.

Condensation Reactions with 4-Amino-4-deoxy-N^{10}-methylpteroic Acid (14). Procedure 3. 2-[N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)amino]tridecanedioic Acid Dimethyl Ester (19). Acid **14**·$1.5H_2O$ (1.76 g, 0.005 mol) was added in portions to a stirred solution of DEPC (2.45 g, 0.015 mol) and Et_3N (1.52 g, 0.015 mol) in dry DMF (150 mL). A clear solution was obtained within 10 min. After 4 h, **13e·HCl** (3.24 g, 0.01 mol) and Et_3N (2.03 g, 0.02 mol) in DMF (15 mL) were added, and solution was left to stand at room temperature for 76 h. The solvent was removed under reduced pressure, the residue was taken up in $CHCl_3$ (275 mL), and the solution was washed with 0.15 M NH_4OH (275 mL). The organic layer was evaporated, the residue was redissolved in 10 mL of 2:5:5 MeOH–MeCN–$CHCl_3$, and the solution was applied onto a column of silica gel (400 g), which was eluted with the same solvent mixture. A yellow forerun was obtained as a gum (1 g, 33% of theory) with R_f 0.71 (silica gel, 2:5:5 MeOH–MeCN–$CHCl_3$). The UV spectrum of this fraction [λ_{max} (EtOH) 280, 295 infl, 402 nm] was consistent with a 2- or 4-formamidino derivative of the desired structure. Further elution and pooling of appropriate fractions, followed by evaporation to a small volume, stirring into Et_2O (50 mL), filtration, and drying in vacuo over P_2O_5 at 40 °C, gave a bright yellow solid (1.23 g, 57%): IR (KBr) 3400 (NH_2), 1735 (ester C=O), 1610 (amide C=O) cm^{-1}. Other compounds obtained by this procedure are listed in Table I.

Condensation Reactions with 4-Amino-4-deoxy-N^{10}-formylpteroic Acid (20). Procedure 4. 2-[N-(4-Amino-4-deoxy-N^{10}-formylpteroyl)amino]nonanedioic Acid Dimethyl Ester (22). Isobutyl chloroformate (0.26 mL, 2 mmol) was added at room temperature to a stirred solution of **20**·$1.5H_2O$ (0.732 g, 2 mmol) and Et_3N (0.81 g, 8 mmol) in dry DMF (35 mL). After 20 min, **13a·HCl** (0.536 g, 2 mmol) was added, followed after 15 min by a second portion of isobutyl chloroformate (0.13 mL, 1 mmol). After another 15 min, a second portion of **13a·HCl** (0.268 g, 1 mmol) was added, followed 10 min later by isobutyl chloroformate (0.066 mL, 0.5 mmol) and 15 min later by a third portion of **13a·HCl** (0.134 g, 0.5 mmol). The last cycle of addition of isobutyl chloroformate (0.5 mmol) and **13a·HCl** (0.5 mmol) was repeated one more time, thus bringing the total of these reactants to 4.0 mmol. The progress of coupling was monitored by TLC (silica gel, 2:5:5 MeOH–MeCN–$CHCl_3$) to confirm the formation of product (R_f 0.34, blue fluorescent). After removal of DMF under reduced pressure, the residue was taken up in 2:5:5 MeOH–MeCN–$CHCl_3$ (25 mL) and applied onto a silica gel column (60 g, 29 × 3.0 cm), which was eluted with 1:5:5 MeOH–MeCN–$CHCl_3$. Fractions containing the desired product (TLC) were pooled, evaporated, and rechromatographed on the same column with 1:10:10 MeOH–MeCN–$CHCl_3$ as the eluent. TLC-homogeneous fractions were pooled and rechromatographed on a third column (80 g, 39 × 3.0 cm), which was eluted successively with 98:2 and 95:5 $CHCl_3$–MeOH. The solid remaining after evaporation of pooled product-containing fractions was dissolved in a small volume of MeOH, reprecipitated with Et_2O, collected, and dried in vacuo (60 °C, P_2O_5) to obtain a yellow powder (0.359 g, 47%): IR (KBr) 3340 (NH_2), 1740 (ester C=O), 1645, 1660, 1670, 1610 (amide C=O) cm^{-1}.

2-[N-(4-Amino-4-deoxy-N^{10}-formylpteroyl)amino]tridecanedioic Acid Dimethyl Ester (24). The same method of coupling was followed as in the synthesis of **22**, but a change in purification procedure was used because several minor contaminants made the product tenaciously resistant to purification. The crude material after removal of the DMF was applied onto a silica gel column (80 g, 37 × 3.0 cm), which was eluted successively with 98:2 and 95:5 $CHCl_3$–MeOH. Evaporation of pooled fractions containing mainly the desired product yielded 1.08 g. Other less pure fractions whose TLC showed the presence of product were applied onto another silica gel column (35 g, 26 × 2.0 cm), which was eluted with 1:10:10 MeOH–MeCN–$CHCl_3$ to obtain another 0.23 g. The still slightly impure solids (1.31 g total) were rechromatographed twice more, first on silica gel (80 g) with 98:2 and 95:5 $CHCl_3$–MeOH as the eluent and finally on silica gel (80 g) with 1:20:20 MeOH–MeCN–$CHCl_3$ as the eluent. Pooled fractions, which were now rigorously pure by TLC, were evaporated, and the solid was triturated with Et_2O, filtered, and dried (40 °C, P_2O_5) to obtain a yellow powder (0.50 g, 35%).

Procedure 5. 2-[N-(4-Amino-4-deoxy-N^{10}-formylpteroyl)amino]dodecanedioic Acid Dimethyl Ester (23). A mixture of the nitrophenyl ester **21** (0.691 g, 1.5 mmol), **13d·HCl** (0.510 g, 1.65 mmol), and Et_3N (0.182 g, 1.8 mmol) in dry *N*-methylpyrrolidinone (25 mL) was kept at 55 °C for 114 h. A clear solution formed within 30 min. The reaction was monitored by TLC (silica gel, 1:5:5 MeOH–MeCN–$CHCl_3$) for the appearance of product (R_f 0.44). After solvent evaporation under reduced pressure, the residue was taken up in 1:10:10 MeOH–MeCN–$CHCl_3$ and chromatographed on silica gel (80 g) by successive elution with 98:2, 96.5:3.5, and 95:5 $CHCl_3$–MeOH. Fractions that were TLC-homogeneous were pooled and evaporated, and the solid was triturated with Et_2O, collected, and dried in vacuo (25 °C,

P_2O_5) to obtain a yellow powder (0.452 g, 46%): IR (KBr) 3340 (NH_2), 1740 (ester C=O), 1670, 1660, 1645 (amide C=O) cm^{-1}.

Hydrolysis of 2-[N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)amino]alkanedioic Acid Diesters. Procedure 6. 2-[N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)amino]tridecanedioic Acid (6). A solution of diester **19** (1.18 g, 1.9 mmol) in a mixture of MeOH (25 mL) and 1 N NaOH (6 mL) was kept at room temperature for 24 h. After rotary evaporation of most of the MeOH and dilution with H_2O, the pH was adjusted to 4.5 with 10% AcOH. The solid was filtered and dried, first with the aid of a lyophilizer and then under vacuum at 100 °C over P_2O_5, to obtain a yellow powder (1.06 g, 97%): IR (KBr) 3395 (NH_2), 1710 (acid C=O), 1640, 1610 (amide C=O) cm^{-1}.

Hydrolysis of 2-[N-(4-Amino-4-deoxy-N^{10}-formylpteroyl)alkanedioic Acid Diesters. Procedures 7. 2-[N-(4-Amino-4-deoxypteroyl)amino]decanedioic Acid (8). A solution of **23** (0.427 g, 0.656 mmol) in a mixture of MeOH (13 mL) and 0.5 N NaOH (12 mL) was stirred at 25 °C for 48 h. The progress of the reaction was monitored by TLC (cellulose, pH 7.4 phosphate buffer), which showed a blue UV-fluorescent spot at R_f 0.78 for the N^{10}-formyl monoacid and diacid and a dark UV-absorbing spot at R_f 0.35 for the fully deblocked product. The solution was acidified to pH 4 with 10% AcOH, and the precipitated solid was filtered, redissolved in 3% NH_4HCO_3 (10 mL), and applied onto a DEAE-cellulose column (17 × 2.0 cm), which was eluted first with H_2O and then successively with 1% and 3% NH_4CO_3. Fractions whose TLC (silica gel, 5:4:1 $CHCl_3$–MeOH–NH_4OH) showed a UV-absorbing spot with R_f 0.44 were pooled and freeze-dried to give a yellow powder (0.13 g). This material was redissolved in a 1:3 mixture of EtOH and 0.05 M NH_4HCO_3 and subjected to gradient LPLC on C_{18}-bonded silica gel with 0.05 M NH_4HCO_3, pH 8.8, in reservoir A and a 1:3 mixture of EtOH and 0.05 M NH_4HCO_3 in reservoir B. Fractions containing pure product (elution time 175 min) were evaporated, and the residue was dried, first in a lyophilizer and then in vacuo over P_2O_5 at 100 °C, to obtain a yellow powder (0.079 g, 21%): IR (KBr) 3390 (NH_2), 1640 (acid C=O), 1610 (amide C=O) cm^{-1}.

Procedure 8. 2-[N-(4-Amino-4-deoxypteroyl)amino]tridecanedioic Acid (9). A solution of **24** (0.488 g, 0.682 mmol) in a nitrogen-purged mixture of MeOH (45 mL) and 0.25 N NaOH (25 mL) was sonicated at 36 °C (bath temperature) for 20.5 h and kept at 36 °C for an additional 5.5 h. The progress of the reaction was monitored by TLC as in the preceding experiment. The crude product obtained after acidification to pH 4 with 10% AcOH was redissolved in 3% NH_4HCO_3 (10 mL) and chromatographed on a DEAE-cellulose (22 × 2.0 cm) column, which was successively eluted with H_2O, 1% NH_4HCO_3, and 3% NH_4HCO_3. The partly purified product (0.35 g) was redissolved in 35:65 EtOH-0.05 M NH_4HCO_3 and subjected to gradient LPLC on C18-bonded silica gel with 0.05 M NH_4HCO_3, pH 8.8, in reservoir A and 35:65 EtOH–0.05 M NH_4HCO_3 in reservoir B. The pure product (elution time 137 min) weighed 0.255 g (66%): IR (KBr) 3340 (NH_2), 1640–1620 (broad, acid C=O), 1610 (amide C=O).

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Registry No. **2**, 113976-23-1; **3**, 113976-24-2; **4**, 113976-25-3; **5**, 113976-26-4; **6**, 113976-27-5; **7**, 113976-28-6; **8**, 113976-29-7; **9**, 113976-30-0; **11a**, 108208-10-2; **11b**, 113976-39-9; **11c**, 113976-40-2; **11u**, 113976-41-3; **11e**, 113976-42-4; **12**, 113976-43-5; **13a**·HCl, 5722-16-7; **13b**·HCl, 5686-32-8; **13c**·HCl, 5686-31-7; **13d**·HCl, 5686-30-6; **13e**·HCl, 113976-44-6; **14**, 19741-14-1; **15**, 113976-31-1; **16**, 113976-32-2; **17**, 113976-33-3; **18**, 113976-34-4; **19**, 113976-35-5; **20**, 89043-75-4; **21**, 11976-36-6; **22**, 113976-37-7; **23**, 113976-38-8; **24**, 95485-01-1; DHFR, 9002-03-3; FPGS, 63363-84-8; H-Glu-OH, 56-86-0; $HOOC(CH_2)_{11}COOMe$, 3927-59-1.

Only One Pharmacophore Is Required for the κ Opioid Antagonist Selectivity of Norbinaltorphimine[1]

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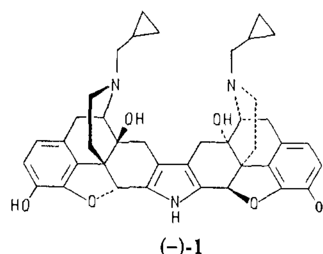
We have investigated whether one or two pharmacophores are required for the κ opioid receptor selectivity of the bivalent opioid antagonist norbinaltorphimine, (−)-1 (nor-BNI), by the synthesis and testing of its meso isomer 2. In smooth muscle preparations 2 was more potent than 1 and about half as selective as a κ antagonist. Since 2 contains only one antagonist pharmacophore but yet retains substantial κ selectivity, it is concluded that κ selectivity is not dependent on the presence of two (−)-naltrexone-derived pharmacophores of 1. It is suggested that the κ selectivity of (−)-1 and 2 is derived from the portions of the second halves of these molecules in that they mimic key "address" components of dynorphin at κ opioid receptors.

We recently have reported on the structure–activity relationship of a series of bimorphinans with potent and selective κ opioid antagonist activity.[2,3] Since these ligands have the highest κ selectivity reported, they are of interest as pharmacologic tools in opioid research. One of the compounds in this series, norbinaltorphimine, (−)-1 (nor-BNI), is presently employed for this purpose.

A key question in the structure–activity studies[3] of nor-BNI and its congeners pertained to whether or not two pharmacophores are required for κ antagonist selectivity. One possibility was that selectivity is due to the simultaneous interaction of both pharmacophores of nor-BNI with two proximal opioid receptors. A second possibility was that a unique recognition site proximal to the binding site of one of the pharmacophores is responsible for the κ selectivity. While prior structure–activity relationship studies[2] tended to implicate the latter, the former could



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