(1:8): mp 296–298 °C dec; IR 3453, 3237, 3073, 1599, 1529, 1487, 1375, 1327, 1167, 1093 cm^{-1}; NMR δ 11.35 (s, 2 H, exchangeable with D_2O), 11.08 (s, 2 H, exchangeable with D_2O), 7.79 (m, 10 H), 7.67 (s, 2 H), 7.50 (s, 2 H), 7.11 (s, 2 H); (-)-FABMS *m/e* (relative intensity) 982 ($[M - Na]^-$, 9), 962 (38), 961 (30), 960 (42), 556 (100). Anal. ($C_{32}H_{20}N_2O_{18}S_6Na_4 \cdot H_2O$) C, H, N.

3,3'-[4,4'-Biphenyldiylbis(sulfonylamino)]bis(1,5-naphthalenedisulfonic acid) (8). 3-Amino-1,5-naphthalenedisulfonic acid (7, 1 g, 3.3 mmol) and 4,4'-biphenyldisulfonyl chloride (1 g, 2.8 mmol) were heated to 120 °C, after which time dry pyridine (10 mL) was added and the mixture was heated under reflux for 5 h. Pyridine was evaporated and the remaining solid was washed with chloroform (5 × 10 mL), and the residue was dissolved in methanol (ca. 10–15 mL) and filtered. The filtrate was triturated with ether (50 mL) to produce a solid which was filtered. The solid was dissolved in 0.1 N NaOH (10 mL), warmed on a water bath for 1 h, and evaporated to dryness to yield a solid. The product was washed with absolute ethanol (5 × 10 mL) to produce a yellow powder isolated as the tetrasodium salt (0.91 g, 56%). An analytical sample was prepared by recrystallization from methanol–chloroform: mp 338–340 °C dec; IR 3445, 1624, 1597, 1333, 1309, 1196, 1163, 1093, 1041 cm^{-1}; NMR δ 10.56 (s, 2 H, exchangeable with D_2O), 8.83 (s, 2 H), 8.73 (d, 2 H, J = 8.7 Hz), 8.00 (d. 4 H, J = 7.8 Hz), 7.92 (d, 4 H, J = 7.8 Hz), 7.90 (s, 2 H), 7.83 (d, 2 H, J = 8.4 Hz), 7.31 (t, 2 H, J = 7.8 Hz); (-)-FABMS *m/e* (relative intensity) 949 ($[M - Na - H]^-$, 43), 905 (100). Anal. ($C_{32}H_{20}N_2O_{16}S_6Na_4 \cdot H_2O$) C, H, N.

Antiviral Assay Procedures. Activity of the compounds against the replication of HIV-1 (HTLV-III_B strain) and HIV-2 (ROD strain) was based on the inhibition of virus-induced cytopathogenicity in MT-4 cells. MT-4 cells were infected with HIV at a multiplicity of infection of 0.02 and incubated in the presence of various concentrations of test compounds. After a 4-day incubation, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) method, as previously described.[76] The giant cell formation assay was carried out according to established protocol.[77] MOLT-4 cells were cultured with an equal number of HUT-78/HTLV-III_B for 24 h, and the number of giant cells was determined microscopically. The HIV RT assay was carried out as follows. HIV-1 RT was obtained from disrupted virions which had been partially purified and concentrated. The assay was performed at 37 °C for 30 min with 50 μL of a reaction mixture containing 50 mM Tris-HCl (pH 8.4), 2 mM dithiothreitol, 100 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 μCi of [*methyl*-^{3}H]dTTP (30 Ci/mmol), 0.01 unit of poly(rA)·oligo(dT), test compound, and enzyme. The reaction was terminated with 200 μL of trichloroacetic acid (5%, v/v). The precipitated materials were collected on glass-fiber filters and analyzed for their radioactivity in a liquid-scintillation counter. Variation in the experiments was 10% at maximum.

Acknowledgment. This work was supported in part by Grant No. 030, Campus Research Board, University of Illinois at Chicago. P.M. is the recipient of a Scholar Award (No. 700065-5-RF) from the American Foundation for AIDS Research.

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Potential Antitumor Agents. 61. Structure–Activity Relationships for in Vivo Colon 38 Activity among Disubstituted 9-Oxo-9*H*-xanthene-4-acetic Acids

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Analogues of 9-oxo-9*H*-xanthene-4-acetic acid (XAA) bearing small, lipophilic 5-substituents are among the most dose-potent compounds yet reported with the capability of causing hemorrhagic necrosis of implanted colon 38 tumors in mice. To further extend structure–activity relationships among this class of compound, a series of XAA derivatives bearing two small lipophilic groups at various positions have been prepared and evaluated. The 5,6-disubstituted compounds in particular show consistently high levels of both dose potency and activity, suggesting this is the optimal configuration among substituted 9-oxo-9*H*-xanthene-4-acetic acids. The 5,6-dimethyl and 5-methyl-6-methoxy are the most effective analogues, showing in vivo colon 38 activity comparable to that of FAA at 10–15-fold lower doses and superior activity to FAA at the respective optimal doses, and the former has been selected for detailed evaluation.

The drug flavone-8-acetic acid (1; FAA, NSC 347512) has come under intense scrutiny as a potential antitumor agent, since it shows remarkable activity against advanced experimental colon tumors in mice,[1-3] high activity as a biological response modifier,[4,5] good ability to induce cytokines,[6] and has dramatic effects on shutting down blood flow in solid tumors.[7,8] However, the drug has low dose potency and has not shown activity in man, despite extensive clinical trials.[9]

We have previously reported[10] on the synthesis and evaluation of a related class of compounds, the 9-oxo-9*H*-xanthene-4-acetic acids, which have a similar biological profile. The parent compound (2; XAA) is as active as FAA against colon 38 tumors in mice, while being somewhat more dose-potent.[10] Studies[10,11] of structure–activity relationships among monosubstituted analogues of XAA

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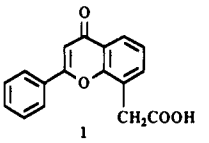
Table I. Physicochemical and Biological Properties of Disubstituted 9-Oxo-9*H*-xanthene-4-acetic Acids



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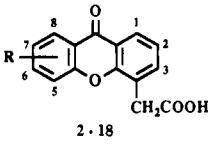

2-18

no.	R	mp, °C	formula	analyses	method[a]	Rm[b]	colon 38 OD[c]	colon 38 activity[d]
1	(FAA)		ref 1			0.06	330	++
2	H (XAA)		ref 10			0.64	220	++
3	5-Me		ref 10			0.62	30	++
4	5-Cl		ref 10			0.60	150	++
5	1,5-Me_2	243–244	$C_{17}H_{14}O_4$	C, H	A	0.75	65	++
6	2,5-Me_2	247–248	$C_{17}H_{14}O_4$	C, H	A	0.80	220	++
7	3,5-Me_2	242–243	$C_{17}H_{14}O_4$	C, H	A	0.84	45	++
8	5,6-Me_2	259–261	$C_{17}H_{14}O_4$	C, H	B	0.49	30	++
9	5-Me,6-F	133–134	$C_{16}H_{11}FO_4$	C, H, F	B	0.69	45	++
10	5-Me,6-Cl	251–252	$C_{16}H_{11}ClO_4$	C, H, Cl	B	0.82	30	++
11	5-Me,6-Br	258–260	$C_{16}H_{11}BrO_4$	C, H, Br	B	0.83	45	++
12	5-Me,6-OMe	260–261	$C_{17}H_{14}O_5$	C, H	D	0.61	30	++
13	5-Me,6-NMe_2	233–235	$C_{18}H_{17}NO_4$	C, H, N	D	0.63	45	–
14	5,6-Cl_2	240–242	$C_{15}H_8Cl_2O_4$	C, H, Cl	B	0.79	100	+
15	5-Cl,6-Me	226–227	$C_{16}H_{11}ClO_4$	C, H, Cl	B	0.68	100	++
16	5,6-$(CH_2)_3$	233–234	$C_{18}H_{14}O_4$	C, H	B	0.69	45	++
17	5,6-benzo	282–284	$C_{19}H_{12}O_4$	C, H	C	0.54	65	++
18	6,7-benzo	275–278	$C_{19}H_{12}O_4$	C, H	B	0.53	330	+

[a] A–D = methods of synthesis as shown in Scheme I and detailed in the text. [b] Rm = relative measure of drug lipophilicity, determined by liquid–liquid chromatography as detailed in ref 24, with 4′-(9-acridinylamino)methanesulfonanilide (AMSA) as internal standard. [c] OD = the *minimum* dose of drug in milligrams/kilogram, administered intraperitoneally as the sodium salt in water in a single dose, to achieve the level of activity cited. [d] Animals bearing subcutaneous colon 38 tumors (4–8 mm in diameter) were treated with drug at the optimal dose (determined by carrying out a full dose profile at 1.5-fold dose intervals). Tumors were removed after 24 h, fixed in formalin, stained in hematoxylin/eosin (ref 14), and examined histopathologically as detailed in the text. ++ = >90% necrosis across examined sections, + = 50–90% necrosis.

showed that small lipophilic substituents in the 5-position enhance dose potency, with the 5-methyl derivative (**3**) being about 8-fold more potent than FAA. A comparative study[12] of FAA and a series of monosubstituted derivatives of XAA showed there was an excellent correlation between the ability of the drugs to stimulate NK activity in vivo and their ability to induce tumor necrosis. This suggests not only a common pathway for these two activities but also a similar underlying mechanism of action (albeit still poorly understood) for both FAA and the XAA family of compounds. A recent report[13] has suggested that both FAA and XAA derivatives act by the stimulation of activated macrophages to produce the cytotoxin nitric oxide.

Given the clear structure–activity relationships found to date for XAA analogues and the potential utility of compounds with FAA-like activity but considerably greater dose potency, we have continued our development of the XAA class of compounds and in this paper report the synthesis and evaluation of a series of disubstituted (largely 5,6-disubstituted) derivatives of XAA.

Chemistry

Compounds **5–7**, containing one substituent in each ring, were synthesized by coupling the sodium salts of 2-iodo-3-methylbenzoic acid (**47**) and the appropriate methyl-substituted 2-hydroxyphenylacetic acid (e.g. **46**) as recorded previously,[10] with the phase-transfer catalyst tris-[2-(2-methoxyethoxy)ethyl]amine (TDA-1),[14] to give the diacids (e.g. **34**) in good yield, followed by acid-catalyzed cyclodehydration (method A of Scheme I). The substituted 2-hydroxyphenylacetic acids for this route were prepared from the corresponding benzoic acids by a published method.[10] The majority of the compounds (all the 5,6-disubstituted derivatives[15] and the 6,7-benzo analogue **18**) were prepared by essentially the same route, coupling the salts of 2-hydroxyphenylacetic acid (**51**) with the requisite disubstituted 2-iodobenzoic acids (e.g. **26**), which were synthesized from the corresponding anilines by the isatin route (method B of Scheme I). However, the difficulty of obtaining 1-iodonaphthalene-2-carboxylic acid meant that the 5,6-benzo analogue (**17**) was prepared by elaboration of the corresponding methylxanthenone (**53**) (method C of Scheme I),[9] which was prepared straightforwardly from 1-naphthol.

Finally, compounds **12** and **13** were prepared by nucleophilic displacement of chlorine from 6-chloro-5-methyl-9-oxo-9*H*-xanthene-4-acetic acid (**10**) with methoxide and dimethylamine, respectively (method D).

Results and Discussion

Table I gives results for the in vivo evaluation of the compounds against the subcutaneously implanted C38 colon tumor in mice, with the short-term histology assay[16] to measure antitumor effect. Compounds were given as a single intraperitoneal injection of the sodium salt in water, over a range of doses increasing at 1.5-fold intervals up to the maximum tolerated dose, with at least two (and usually three) independent determinations at each dose

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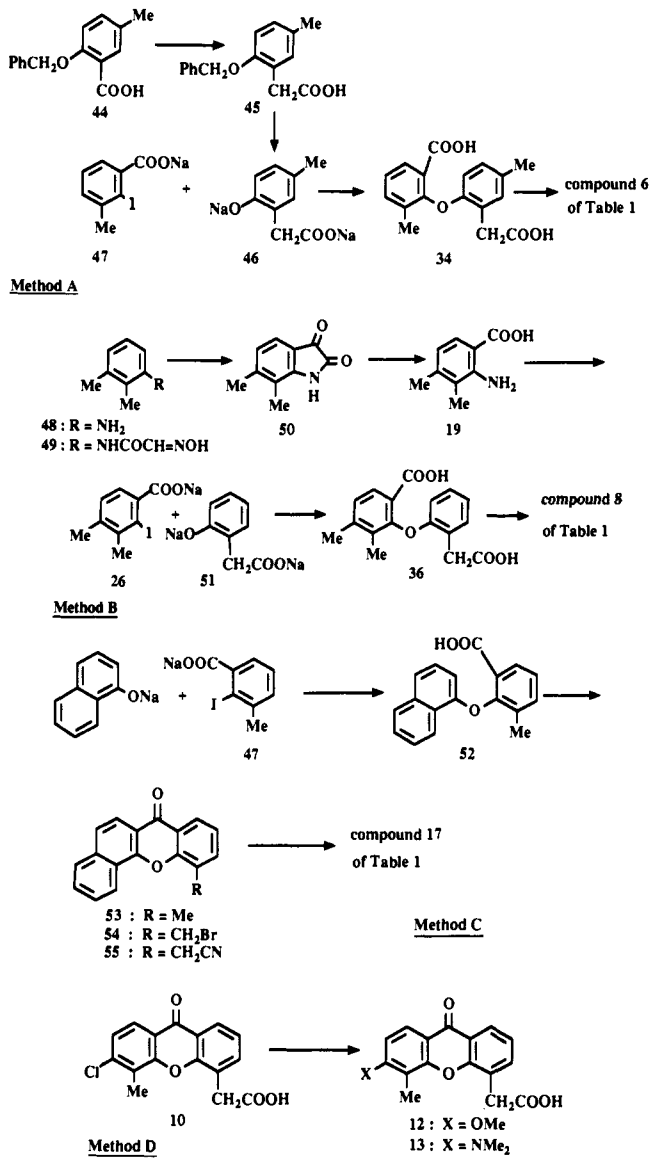
Scheme I



level, using groups of five mice per experiment. All the disubstituted XAA analogues tested except the linear 6,7-benzo compound (**18**) proved more dose-potent (lower maximum tolerated dose) than either FAA (**1**) or the parent (**2**), in agreement with earlier-derived[10,11] structure–activity relationships which suggested the need for small, lipophilic substituents.

Since the earlier work[10,11] had shown 5-methyl compound **3** to be the most dose-potent, compounds **5–8** explored the SAR for 5,*x*-dimethyl derivatives. Both the 1,5- and 2,5-dimethyl compounds (**5** and **6**) were highly active, with the 1,5 derivative also showing good dose potency. 3,5-Dimethyl compound **7** was also highly active and more dose-potent, but the 5,6-dimethyl and 5-methyl-6-methoxy compounds **8** and **12** showed the best overall effects. 5,6-Dimethyl compound **8** was highly active (100% tumour necrosis) at doses of 65 and 45 mg/kg and still showed ++ levels of activity (>90% tumor necrosis) at 30 mg/kg, making it approximately 10–15-fold more dose-potent than FAA.

Compounds **9–13** were a set of 5-methyl derivatives carrying an additional lipophilic 6-substituent of varying electronic and steric properties. In agreement with earlier conclusions drawn from results on monosubstituted compounds,[11] small lipophilic 6-substituents (F, Cl, OMe) were

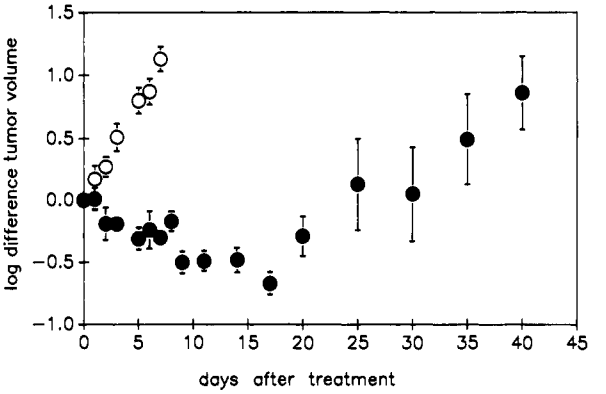


Figure 1. Plot of the logarithm of the difference in tumor volume against the number of days after subcutaneous implantation of colon 38 tumors in BDF_1 mice. Open symbols (○) represent a group of five untreated controls, and filled symbols (●) represent the treated group (15 animals). The error bars represent the standard errors. The large increase in standard error after about day 20 is because the data points from then on represent only the three mice in which the tumor was not eradicated by this time.

well-accepted. However, the 6-NMe_2 substituent was not tolerated, with compound **13** being inactive. This substituent is still quite lipophilic, but it is considerably larger. As expected, all the compounds were more lipophilic than the parent. A comparison of the isomeric dimethyl compounds shows that the 5,6-configuration is the least lipophilic.

Since we previously showed that a 5-Cl group was quite well-tolerated,[10] compounds **14** and **15** explored the effects of 5-Cl,6-X-disubstitution, but neither compound provided the combined degree of high activity and potency shown by the 5-Me,6-X compounds. Compounds **16** and **17** investigated the effects of tying the two methyl groups of **8** into either a polymethylene (**16**) or an aromatic (**17**) ring. While both compounds were highly active, they showed slightly lower potency than **8**, while linear-fused aromatic compound **18** was much less effective.

5,6-Dimethyl compound **8** was evaluated in a growth-delay assay (Figure 1). A single dose of 30 mg/kg resulted in 12/15 (80%) long-term survivors, with the remaining three animals showing an average tumor-growth delay of 20 days.

Conclusions

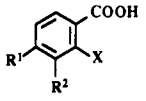
The set of disubstituted 9-oxo-9*H*-xanthene-4-acetic acids studied here show consistently high activity, with 11/14 scoring a ++ activity level. This may be contrasted with results (using the same assay system) for the set of monosubstituted analogues we published previously,[10] where only 5/33 compounds showed ++ levels of activity. In addition, the set of 5,6-disubstituted compounds (**8–12**) show consistently high levels of dose potency, suggesting that this is the optimal configuration among substituted 9-oxo-9*H*-xanthene-4-acetic acids. The pharmacokinetic and metabolic profiles of 5,6-dimethyl derivative **8** are now being evaluated.[17]

Experimental Section

Where analyses are indicated by the symbols of the elements, resulted were within ±0.4% of the theoretical values; analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were determined on an Electrothermal apparatus using the supplied, stem-corrected thermometer and are as read. All compounds had NMR spectra

(17) Kestell, P.; McKeage, M. J., unpublished results, this laboratory.

Table II. 3,4-Disubstituted 2-Amino- and 2-Iodobenzoic Acids



no.	R^1	R^2	X	mp, °C	formula	anal. or lit mp, °C
19	Me	Me	NH_2	183–185		184–186[a]
20	Me	Cl	NH_2	195	$C_8H_8ClNO_2$	C,H,N,Cl
21	Cl	Cl	NH_2	236–238		238[b]
22	Cl	Me	NH_2	210–212		211–212[c]
23	F	Me	NH_2	195–206 d	$C_8H_8FNO_2$	C,H,N
24	Br	Me	NH_2	221–223	$C_8H_8BrNO_2$	C,H,N
25	$-(CH_2)_3-$		NH_2	221–222 d	$C_{10}H_{11}NO_2$	C,H,N
26	Me	Me	I	137–138	$C_9H_9IO_2$	C,H,I
27	Me	Cl	I	176–177	$C_8H_6ClIO_2$	C,H,I
28	Cl	Cl	I	176	$C_7H_2Cl_2IO_2$	C,H,I
29	Cl	Me	I	164	$C_8H_6ClIO_2$	C,H,I
30	F	Me	I	185–186.5	$C_8H_6FIO_2$	C,H,I
31	Br	Me	I	160	$C_8H_6BrIO_2$	C,H,I
32	$-(CH_2)_3-$		I	158–160	$C_{10}H_9IO_2$	C,H

[a] Reference 19. [b] Reference 25. [c] Reference 26.

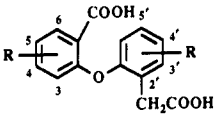
(measured on a Bruker WP-60, Me_4Si) consistent with their assigned structure. Quoted NMR data were acquired on a Bruker AM-400 spectrometer.

Preparation of 2,5-Dimethyl-9-oxo-9*H*-xanthene-4-acetic Acid (6), Example of Method A of Scheme I. 2-[2-(Phenylmethoxy)-5-methylphenyl]acetic Acid (45). 2-(Phenylmethoxy)-5-methylbenzoic acid (44;[18] 31 g, 0.13 mol) was heated under reflux for 30 min in excess $SOCl_2$ containing 2 drops of DMF, followed by removal of the volatiles under reduced pressure. The residual crude acid chloride was then added slowly to a stirred solution of $NaBH_4$ (10 g) in diglyme (200 mL) at 10 °C. After 10 min the excess $NaBH_4$ was carefully neutralized with water, and most of the solvent was removed under reduced pressure. The residue was extracted with EtOAc, and the organic layer was washed with 2 N HCl and 2 N aqueous $NaHCO_3$, dried and evaporated to give crude 2-(phenylmethoxy)-5-methylbenzenemethanol. This was dissolved in benzene and treated with PBr_3 (6 mL, 65 mmol) at 20 °C. After 15 min the solution was basified with aqueous NaOH, and the organic layer was separated and worked up to give crude 2-(bromomethyl)-4-methyl-1-(phenylmethoxy)benzene as an oil.

This was dissolved in CH_2Cl_2 and reacted with NaCN (12.5 g, 0.26 mmol) in water in a two-phase system, in the presence of the phase-transfer catalyst tetrabutylammonium bromide (4 g, 12 mmol). The mixture was rapidly stirred for 15 h at 20 °C, and the organic layer was then separated, washed twice with water, dried, and evaporated to give the crude acetonitrile. This was hydrolyzed by heating under reflux in 2 N KOH in 65% aqueous EtOH for 6 h. The EtOH was then removed by distillation, and the aqueous layer was extracted twice with benzene to remove neutral impurities. Acidification of the aqueous layer with 2 N HCl then gave [2-(phenylmethoxy)-5-methylphenyl]acetic acid (45; 23.6 g, 72% overall yield based on the starting benzoic acid 44): mp (aqueous MeOH) 78 °C; ^1H NMR ($CDCl_3$) δ 11.30 (s, 1 H, COOH), 7.45–6.55 (m, 8 H, aromatic protons), 4.99 (s, 2 H, OCH_2), 3.64 (s, 2 H, CH_2CO), and 2.24 (s, 3 H, CH_3). Anal. ($C_{16}H_{16}O_3$) C, H, N.

2-[2-(Carboxymethyl)-4-methylphenoxy]-6-methylbenzoic Acid (34). Catalytic hydrogenation of 45 in MeOH over Pd/C gave (2-hydroxy-5-methylphenyl)acetic acid (46).[19] A mixture of the disodium salt of this compound (4.2 g, 20 mmol), the sodium salt of 2-iodo-3-methylbenzoic acid (47; 5.7 g, 20 mmol), CuCl (0.2 g, 2 mmol), and tris[2-(2-methoxyethoxy)ethyl]amine (0.6 g, 2 mmol) in dry 1,4-dioxane (200 mL) was heated under reflux for 2 h. Excess solvent was removed under reduced pressure, and the residue was diluted with water, filtered, and acidified to give

Table III. Substituted 2-[(2-Carboxymethyl)phenoxy]benzoic Acids



no.	R	mp °C	formula	anal.
33	3,5′-Me_2	189–190	$C_{17}H_{16}O_3$	C, H
34	3,4′-Me_2	172	$C_{17}H_{16}O_5$	C, H
35	3,3′-Me_2	232–233	$C_{17}H_{16}O_5$	C, H
36	3,4-Me_2	240–242	$C_{17}H_{16}O_5$	C, H
37	3-Cl,4-Me	228–230	$C_{16}H_{13}ClO_5$	C, H
38	3,4-Cl_2	212–214	$C_{15}H_{10}Cl_2O_5$	C, H, Cl
39	3-Me,4-Cl	241–242	$C_{16}H_{13}ClO_5$	C, H, Cl
40	3-Me,4-F	200–201	$C_{16}H_{13}FO_5$	C, H
41	3-Me,4-Br	250–252	$C_{16}H_{13}BrO_5$	C, H
42	3,4-$(CH_2)_3$	208–209	$C_{18}H_{16}O_5$	C, H
43	4,5-benzo	197–200	$C_{19}H_{14}O_5$	C, H

2-[2-(carboxymethyl)-4-methylphenoxy]-6-methylbenzoic acid (34; 4.52 g, 75% yield): mp (PhH) 172 °C; ^1H NMR (CD_3SOCD_3) δ 7.83–6.66 (m, 6 H, aromatic protons), 3.74 (s, 2 H, CH_2CO), and 2.24 (s, 6 H, CH_3, CH_3). Anal. in Table III.

2,5-Dimethyl-9-oxo-9*H*-xanthene-4-acetic Acid. Compound 6 of Table I. Cyclodehydration of 34 in 90% H_2SO_4 using published procedures[10] gave 2,5-dimethyl-9-oxo-9*H*-xanthene-4-acetic acid (6), mp (EtOH) 247–248 °C. Anal. in Table I.

Preparation of 5,6-Dimethyl-9-oxo-9*H*-xanthene-4-acetic Acid (8). Example of Method B of Scheme I. 2,3-Dimethyl-α-isonitrosoacetanilide (49). Chloral hydrate (90 g, 0.54 mol) and anhydrous Na_2SO_4 (453 g, 3.2 mol) were added to water (1.5 L) with rapid stirring. A slurry of hydroxylamine sulfate (410 g, 2.5 mol) and 2,3-dimethylaniline (48; 58.1 g, 0.48 mol) in water (500 mL) containing concentrated HCl (50 mL) was then added, and the stirred mixture was heated slowly to 45 °C over 90 min, to 52 °C over 45 min, and finally to 75 °C for 60 min. After cooling, the precipitate was collected, washed with water followed by petroleum ether, and dried to give the crude isonitroso compound 49 (80.2 g, 87%), mp 130–132 °C (lit.[20] mp 131–132 °C).

6,7-Dimethylisatin (50). Crude, powdered, isonitroso compound (60.0 g, 0.31 mol) was added in portions to stirred CH_3SO_3H (240 mL) at 70–80 °C. The cooled mixture was poured onto ice and diluted with water, and the precipitate was collected and dissolved in excess 1 N aqueous NaOH. Neutralization with AcOH precipitated impurities which were removed by filtration, and acidification (HCl) of the clarified solution gave isatin 50 as an orange-red solid (35.8 g, 65%), mp 252–253 °C (lit.[20] mp 230–232 °C).

3,4-Dimethylanthranilic Acid (19). A solution of 6,7-dimethylisatin (37.8 g, 0.22 mol) in water (400 mL) containing KOH (14.0 g) and KCl (35 g) was vigorously stirred and treated dropwise at 8–10 °C with 27% H_2O_2 (ca. 45 g) over 45 min. The reaction mixture was then stirred at 20 °C until TLC analysis indicated complete reaction. Addition of AcOH precipitated the crude anthranilic acid 19, which was washed with water and dried (23.2 g, 65%), mp 183–185 °C (lit.[20] mp 184–186 °C).

3,4-Dimethyl-2-iodobenzoic Acid (26). A suspension of powdered 3,4-dimethylanthranilic acid (19; 21.0 g, 0.13 mol) in water (280 mL) containing concentrated H_2SO_4 (43 mL) was boiled until homogeneous and then cooled and diazotized with $NaNO_2$ (9.2 g, 0.13 mol) in water (20 mL) at 10 °C. The resulting solution was filtered and added to a solution of KI (65 g) in water (300 mL), and the mixture was then heated to 100 °C with occasional swirling. After cooling, the precipitate was collected, dissolved in Na_2CO_3, filtered, and reacidified. The resulting precipitate was collected, dried, and crystallized from benzene–petroleum ether to give pure 3,4-dimethyl-2-iodobenzoic acid (26; 24.1 g, 69%), mp 139–140 °C. Anal. ($C_9H_9IO_2$) in Table II.

2-[(2-Carboxymethyl)phenoxy]-3,4-dimethylbenzoic Acid (36). A mixture of the sodium salt of 3,4-dimethyl-2-iodobenzoic

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acid (26; 25.0 g, 84 mmol), the disodium salt of 2-hydroxyphenylacetic acid 51; 19.8 g, 114 mmol, tris[2-(2-methoxyethoxy)ethyl]amine (TDA-1; 4.9 g), and CuCl (2.9 g) in DMSO (50 mL) was stirred at 95 °C for 12 h. The bulk of the solvent was removed under reduced pressure (water pump, 130 °C), and the residue was dissolved in water, saturated with NaCl, and acidified. The resulting solid was collected, dissolved in hot Na_2CO_3, clarified by charcoal treatment, and acidified with AcOH to give the crude diacid. This was collected, dried, and dissolved in the minimum volume of boiling MeOH, diluted with a large excess of EtOAc, and boiled down until incipient crystallization. After thorough cooling, the product (36) was collected (15.8 g, 63%). A sample recrystallized from EtOAc, mp 240–242 °C. Anal. ($C_{17}H_{16}O_5$) in Table III.

5,6-Dimethyl-9-oxo-9*H*-xanthene-4-acetic Acid (Compound 8 of Table I). A mixture of the above diacid (36; 15.0 g), concentrated H_2SO_4 (72 mL), and water (8 mL) was heated at 80 °C for 10 min and then cooled and poured onto ice. The precipitate was collected, washed well with water, and dried. It was then suspended in MeOH (250 mL) and stirred under reflux until finely divided, cooled, collected, washed with MeOH, and dried to give pure acid 8 (9.88 g, 70%). A sample crystallized from MeOH, mp 259–260 °C. Anal. in Table I.

The acid was dissolved in water containing $NaHCO_3$ (3.10 g) and filtered, and the filtrate was evaporated to dryness. The residue was crystallized from MeOH/EtOAc to provide the pure sodium salt as white, water-soluble prisms (9.44 g, 62% from the diacid), mp 316–318 °C.

Similar treatment of the other anthranilic acids listed in Table II gave the corresponding 9-oxoxanthene-4-acetic acids via the diacids of Table III. Many of the anthranilic acids were known compounds, and the following new ones were prepared.

3-Chloro-4-methylanthranilic acid (20) was obtained from 2-chloro-3-methylaniline via 2-chloro-3-methyl-α-isonitrosoacetanilide [Mp (aqueous MeOH) 151–152 °C. Anal. ($C_9H_9ClN_2O_2$) C, H, N.] and 7-chloro-6-methylisatin [Mp (MeOH) 217–218 °C. Anal. ($C_9H_6ClNO_2$) C, H, N.].

4-Fluoro-3-methylanthranilic Acid (23) was obtained from 3-fluoro-2-methylaniline via 3-fluoro-2-methyl-α-isonitrosoacetanilide [Mp (aqueous MeOH) 127–128 °C. Anal. ($C_9H_9FN_2O_2$) C, H, N.] and 6-fluoro-7-methylisatin [Mp (MeOH) 215–216 °C (lit.[21] mp 204–206 °C).].

4-Bromo-3-methylanthranilic acid (24) was obtained from 3-bromo-2-methylaniline via 3-bromo-2-methyl-α-isonitrosoacetanilide [Mp (aqueous MeOH) 152–153 °C. Anal. ($C_9H_9BrN_2O_2$) C, H, N.] and 6-bromo-7-methylisatin [Mp (MeOH) 267–269 °C. Anal. ($C_9H_6BrNO_2$) C, H, N.].

4-Aminoindan-5-carboxylic acid (25) was obtained from 4-aminoindan[22] via 4-(α-isonitrosoacetamino)indan [Mp (aqueous MeOH) 180–181 °C. Anal. ($C_{11}H_{12}N_2O_2$) C, H, N.] and 2,3-dioxo-1,2,3,6,7,8-hexahydrocyclopent[*g*]indole [Mp (MeOH) 283–284 °C. Anal. ($C_{11}H_9NO_2$) C, H, N.].

Preparation of 7-Oxo-7*H*-benzo[*c*]xanthene-11-acetic Acid (17) by Method C of Scheme I. A mixture of equimolar amounts of sodium 1-naphthoate and the sodium salt of 2-iodo-3-methylbenzoic acid (47) were coupled with TDA-1 by using published methods[10,11] to give 3-methyl-2-(1-naphthyloxy)benzoic acid (52), which was cyclodehydrated to 11-methyl-7*H*-benzo[*c*]xanthen-7-one (53), mp (MeOH) 230–231 °C. Anal. ($C_{18}H_{12}O_2$) C, H. This was elaborated to 7-oxo-7*H*-benzo[*c*]xanthen-11-acetic acid (17) via 11-(bromomethyl)-7*H*-benzo[*c*]xanthen-7-one (54) [Mp (MeOH/PhH) 254–255 °C. Anal. ($C_{18}H_{11}BrO_2$) C, H, Br.] and 7-oxo-7*H*-benzo[*c*]xanthene-11-acetonitrile (55) [Mp (AcOH) 221.5–222 °C. Anal. ($C_{19}H_{11}NO_2$) C, H, N.] using published methods.[10,11]

Preparation of Compound 13 of Table I. Example of Method D of Scheme I. A solution of 6-chloro-5-methyl-9-oxo-9*H*-xanthene-4-acetic acid (10; 3.5 g, 11.6 mmol) in 40% aqueous dimethylamine (100 mL) was heated at 100 °C in a sealed pressure vessel for 2 weeks. Excess dimethylamine was removed under reduced pressure, and the solution was diluted with water and filtered. Acidification of the filtrate with AcOH gave a crude product which was converted to the methyl ester (MeOH/3% H_2SO_4/reflux). This was purified on SiO_2, elution with CH_2Cl_2, giving methyl 6-(dimethylamino)-5-methyl-9-oxo-9*H*-xanthene-4-acetate (1.8 g, 48%): mp (MeOH) 113–114 °C; ^1H NMR ($CDCl_3$) δ 8.04 (dd, *J* = 7.75 and 1.6 Hz, 1 H, H1), 7.91 (d, *J* = 8.8 Hz, 1 H, H8), 7.73 (dd, *J* = 7.3 and 1.6 Hz, 1 H, H3), 7.36 (t, *J* = 7.7 Hz, 1 H, H2), 7.07 (d, *J* = 8.8 Hz, 1 H, H7), 4.01 (s, 2 H, CH_2), 3.68 (s, 3 H, OMe), 2.84 (s, 6 H, NMe_2), 2.30 (s, 3 H, Me). Anal. ($C_{19}H_{19}NO_4$) C, H, N.

Hydrolysis of this compound with 2 N NaOH, followed by acidification with AcOH in aqueous MeOH, gave 6-(dimethylamino)-5-methyl-9-oxo-9*H*-xanthene-4-acetic acid (13), mp (EtOH) 233–235 °C. Anal. in Table I.

Similar treatment of 10 with methanolic sodium methoxide (heating under reflux in the absence of light[23] for 10 weeks) gave an 86% yield of 6-methoxy-5-methyl-9-oxo-9*H*-xanthene-4-acetic acid (12) directly. The compound was recrystallized from EtOH, mp 260–261 °C. Anal. in Table I.

Biological Testing Colon 38 fragments were implanted subcutaneously in BDF_1 mice and allowed to grow to a diameter of 4–8 mm when drug was given as a single intraperitoneal dose of the sodium salt in water. Because of the photosensitivity of solutions of these compounds,[23] precautions were taken to exclude light during all operations. Each compound was tested at a range of doses escalating by 1.5-fold up to a maximum dose of 750 mg/kg or at the maximum tolerated dose if this was lower. The maximum tolerated dose was defined as the highest dose in the above protocol which did not cause death in 24 h. After 24 h the tumor was surgically removed and fixed in formalin. Sections were stained and examined histologically for evidence of necrosis. Flavoneacetic acid (1) was used as a standard and when given at a dose of 330 mg/kg caused necrosis across 90–100% of the tumor section (scored as ++). Compounds showing lesser but still extensive necrosis (50–90%) were scored at +. the assay provides a stringent criterion of activity, since compounds showing less than 50% necrosis were scored as negative (–).

The growth-delay experiment with 8 used a control group of five and a treatment group of 15 $B6D2F_1$ mice with sc tumors (5–10 mm diameter). The mice were randomized with respect to tumor size into treatment and control groups, and the treatment group was injected intraperitoneally with a single dose (30 mg/kg) of 8 as a solution of the sodium salt in water. Tumors were measured thereafter three times weekly with digital callipers. For mice with palpable tumors, tumor volumes were calculated as $0.52a^2b$, where *a* and *b* are the minor and major axes of the tumor. Means and standard errors were calculated on the basis of the logarithms of tumor volumes and the results expressed as a fraction of the initial mean tumor volume (typically 0.3 cm^3).

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Registry No. 1, 87626-55-9; 2, 35614-21-2; 3, 117570-47-5; 4, 117570-49-7; 5, 129833-18-7; 6, 129833-19-8; 7, 129833-20-1; 8, 117570-53-3; 9, 129833-21-2; 10, 129833-22-3; 11, 129833-23-4; 12, 129095-11-0; 13, 129833-24-5; 14, 129833-25-6; 15, 129833-26-7; 16, 129833-27-8; 17, 117570-54-4; 18, 117570-79-3; 19, 50419-58-4; 20, 27696-37-3; 21, 20776-62-9; 22, 98968-68-4; 23, 129833-28-9; 24, 129833-29-0; 25, 129833-30-3; 26, 129833-31-4; 27, 129833-32-5; 28, 129833-33-6; 29, 129833-34-7; 30, 129833-35-8; 31, 129833-36-9; 32, 129833-37-0; 33, 129833-38-1; 34, 129833-39-2; 35, 129833-40-5; 36, 117570-93-1; 37, 129833-41-6; 38, 129833-42-7; 39, 129833-43-8; 40, 129833-44-9; 41, 129833-45-0; 42, 129833-46-1; 43, 129833-47-2; 44, 67127-92-8; 45, 129833-48-3; 46, 41873-66-9; 47, 108078-14-4;

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Folate Analogues. 34. Synthesis and Antitumor Activity of Non-Polyglutamylatable Inhibitors of Dihydrofolate Reductase[1]

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Five analogues of methotrexate (MTX), 10-deazaaminopterin (10-DAM), and 10-ethyl-10-deazaaminopterin (10-EDAM) in which the glutamate moiety was replaced by either a γ-methyleneglutamate or β-hydroxyglutamate were synthesized and evaluated for their antifolate activity. These analogous are 4-amino-4-deoxy-N^{10}-methylpteroyl-β-hydroxyglutamic acid (1), 4-amino-4-deoxy-10-deazapteroyl-β-hydroxyglutamic acid (2), 4-amino-4-deoxy-N^{10}-methylpteroyl-γ-methyleneglutamic acid (3, MMTX), 4-amino-4-deoxy-10-deazapteroyl-γ-methyleneglutamic acid (4, MDAM), and 4-amino-4-deoxy-10-ethyl-10-deazapteroyl-γ-methyleneglutamic acid (5, MEDAM). None of these compounds were metabolized to the respective polyglutamate derivative as judged by their inability to serve as substrates for CCRF-CEM human leukemia cell folylpolyglutamate synthetase (FPGS) in vitro. All compounds inhibited recombinant human-dihydrofolate reductase (DHFR) at nearly equivalent magnitude as MTX. Growth-inhibition studies with H35 hepatoma, Manca human lymphoma, and CCRF-CEM human leukemia cells established greater cytotoxic effects with compounds 3–5 than with compounds 1 and 2. γ-Methyleneglutamate derivatives 3–5 were transported to H35 hepatoma cells better than MTX or β-hydroxyglutamate derivatives 1 and 2. Compound 3 was 2.5 times better than MTX in competing with folinic acid transport in H35 hepatoma cells. Compound 1 did not have a significant inhibitory effect on folinic acid transport even at 50 μM under identical conditions. The IC_{50} for compound 1 against H35-hepatoma cell growth was 8.5-fold higher than MTX. Compounds with the γ-methyleneglutamate moiety (3–5) exhibited almost equal or lower IC_{50} values than MTX against the growth of CCRF-CEM human leukemia cells. These studies show that on continuous exposure, the non-polyglutamylatable inhibitors DHFR (3–5) can exhibit superior antifolate activity compared to the polyglutamylatable methotrexate, presumably due to their enhanced transport to these cell lines. Compounds 3–5 appear to be excellent models to study the role of polyglutamylation of antifolates in antitumor activity and host toxicity.

The isolation and identification of the poly-γ-glutamyl metabolites of the well-known anticancer drug methotrexate (MTX) from human red blood cells were first reported in 1973.[2] With use of ^{14}C-labeled methotrexate, the formation of these metabolites in rodent tissues was subsequently confirmed.[3,4] Chemical synthesis of the poly-γ-glutamyl metabolites of methotrexate was accomplished by the solid-phase procedure, and these synthetic standards were utilized for the detailed study of their biochemical pharmacology[5,6] and role in methotrexate cytotoxicity.[5,6] Significant results of the investigations with synthetic MTX-polyglutamates that have therapeutic implications include (a) MTX polyglutamates inhibit dihydrofolate reductase (DHFR) and the IC_{50} values for DHFR inhibition decrease progressively as the number of glutamate residues is increased,[7] (b) the formation of MTX-polyglutamates in mammalian tissues is dose and time dependent,[8] (c) MTX-polyglutamates with longer chain lengths are retained longer within the cell,[8] (d) thymidylate synthase (TS) is inhibited more effectively by MTX-polyglutamates than the parent compound,[9] and (e) MTX-polyglutamates effectively inhibit aminoimidazole carboxamide ribonucleotide formyltransferase (AICARFTase).[10] Like MTX, the antitumor agents 10-deazaaminopterin and 10-ethyl-10-deazaaminopterin are also metabolized to their respective poly-γ-glutamyl derivatives in animal tissues.[11-13] Addition of γ-glutamate residues to 10-deazaaminopterin, 10-ethyl-10-deazaaminopterin,

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