Folate Analogues Altered in the C^9–N^{10} Bridge Region. 18. Synthesis and Antitumor Evaluation of 11-Oxahomoaminopterin and Related Compounds[1]

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The chemical synthesis of 11-oxahomoaminopterin (1) has been carried out using procedures which were also found to be applicable to the synthesis of 11-oxahomofolic acid (2). Reaction of 1-bromo-4-[*p*-(carbomethoxy)phenoxy]-2-butanone (10) with sodium azide gave 1-azido-4-[*p*-(carbomethoxy)phenoxy]-2-butanone (11). Protection of the carbonyl group of 11 as the ethylene ketal and subsequent base hydrolysis of the product gave 1-azido-4-(*p*-carboxyphenoxy)-2-butanone ketal (13). The glutamate conjugate 14 was prepared from 13 by the isobutyl chloroformate method and was hydrogenated to diethyl *N*-[(α-amino-2-oxo-4-butanoyl)-*p*-anisoyl]-L-glutamate ketal (15). Reaction of 15 with 6-chloro-2,4-diamino-5-nitropyrimidine (16) and 2-amino-6-chloro-4-hydroxy-5-nitropyrimidine (17) and deprotection of the corresponding products gave the intermediates 18 and 19, which were elaborated to 1 and 2 using a series of steps involving deprotection, dithionite reduction, cyclization, oxidation, and hydrolysis. Although 11-oxahomoaminopterin showed antifolate activity against two folate-requiring microorganisms and inhibited *Lactobacillus casei* DHFR, it was inactive against L-1210 leukemia in mice at a maximum dose of 48 mg/kg. Compound 1 was also tested for its ability to be transported via the methotrexate transport system using the L-1210 and Ehrlich tumor cell lines, and these results are compared with those of related analogues. The growth inhibitory activity of 1 in the L-1210 cell lines in culture was found to be 15 times weaker than that of methotrexate.

Antagonists of the vitamin folic acid have been successfully used in the chemotherapy of neoplastic and infectious diseases.[2-4] Although methotrexate (MTX) was synthesized as early as 1949, it continues to be the most versatile and widely used folate antagonist for the chemotherapy of various forms of cancers.[2,3] Trimethoprim[4] is a nonclassical analogue of the pyrimidine portion of folic acid, which is a very effective inhibitor of bacterial folate reductases but a poor inhibitor of the mammalian enzymes;[5] it is therefore used as a safe and effective antibacterial drug.[4,6] In spite of a vigorous research effort involving structural modification of the vitamin in several laboratories, no compound having a better chemotherapeutic index than MTX has emerged during the past 3 decades.

However, quite recently there has been a series of observations indicating that subtle changes in the C^9–N^{10} bridge region of classical aminofols have a profound influence on their transport to mammalian cell lines.[7-10] These changes, however, do not appear to affect their enzyme inhibitory activities.[1] Two classical analogues, 10-deazaaminopterin[11] and 10-oxaaminopterin[12] are examples. These two compounds, although having undergone structural changes in the bridge region, were shown to be equally potent as methotrexate with regard to their inhibition of *Lactobacillus casei* dihydrofolate reductase (DHFR) and bacterial growth and yet were remarkably different in their in vivo antitumor activities. The differences in antitumor activities between methotrexate and 10-deazaaminopterin were attributed to the ability of the latter compound to be transported and accumulated more efficiently in the tumor compared to MTX.[9,13,14] In HeLa cell cultures, 10-oxaaminopterin was found to be capable of competing with folate transport, indicating its potential ability to be transported via the folic acid transport system.[7]

For these reasons, our laboratories are engaged in a program to investigate bridge-altered analogues of aminopterin and MTX in an attempt to identify generalized criteria and structural features which could be useful for the proper design of potential analogues capable of exhibiting the desired biological properties, such as selective transport and accumulation in tumor cells. In this report we describe the chemical synthesis and preliminary antitumor evaluation of 11-oxahomoaminopterin (1) and related compounds.

Quite recently we described the chemical synthesis and antifolate activities of 11-oxahomofolate (2) and its reduced

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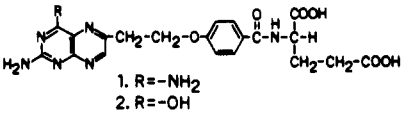
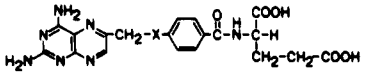
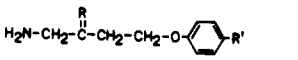


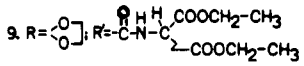
1. R=-NH_2
2. R=-OH

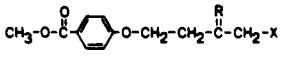


3. X=-NH
4. X=-NH-CH_3
5. X=-CH_2-
6. X=-O-
7. X=-S-
7b. X=-CH_2-S-

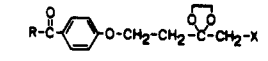


8. R==N-OH; R'=-C(=O)-OCH_3

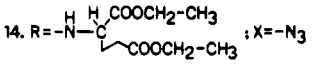




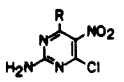
10. X=Br; R==O
11. X=-N_3; R==O
12. X=-N_3; R=



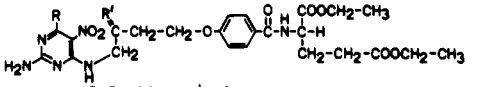
13. R=-OH; X=-N_3



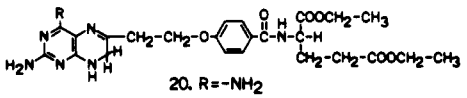
14. ; X=-N_3



16. R=NH_2
17. R=-OH



18. R=-NH_2; R'==O
19. R=-OH; R'==O



20. R=-NH_2
21. R=-OH

derivatives which exhibited some very interesting biological properties in *L. casei*.[15] Our current plans to investigate the activities of these compounds using mammalian cell lines in vitro and tumors in vivo necessitated the resynthesis of this compound. Therefore, the synthetic chemistry which was employed for the construction of 1 was also applied for the synthesis of its 4-hydroxy analogue 2. These results are also included as part of this report.

Chemistry. The strategy of elaborating 8 to 4-amino-4-deoxy-11-oxahomopteroic acid by modifications on the Boon–Leigh procedure and its subsequent conversion to 1 by glutamate conjugation was considered an unattractive approach for the following reasons. First, by analogy with the synthesis of 11-oxahomofolic acid, the 11-oxahomopteroate framework was expected to be unstable to the conditions of solid-phase coupling procedures.[15,16] Secondly, in our experience, the solution phase coupling of various pteroates with diethyl glutamate under a prescribed set of conditions (where protection of the pteroate moiety was found to be unnecessary) was not always quantitative. Therefore, contamination of the coupled product with the pteroate was possible after base hydrolysis. Separation of the pteroate from the glutamate conjugate involves repeated and tedious ion-exchange chromatography. An alternate approach for introducing the glutamate moiety to the side chain, such as in 9, prior to the closure of the pyrazine ring of the pteridine system, would effectively circumvent the problems associated with pteroate coupling procedures and with ion-exchange chromatography.[1] Therefore, we decided to explore this latter chemical route to the synthesis of 11-oxahomoaminopterin (1) and its 4-hydroxy analogue (2).

At the outset, the construction of side chain 9, which can be used as a common intermediate for the syntheses of both 1 and 2, was examined. Our previous attempts to convert the known bromo ketone 10 to the keto azide 11 in protic solvents using sodium azide resulted in a retro-Michael reaction with the formation of methyl *p*-hydroxybenzoate.[15,17] However, when this reaction was carried out using strictly anhydrous conditions and nonaqueous workup, the required azide 11 was obtained in good yield. The carbonyl function of 11 was protected as the ethylene ketal with *p*-toluenesulfonic acid as a catalyst in refluxing benzene. This noncrystalline product 12 was purified by column chromatography over silica gel and hydrolyzed with aqueous NaOH in acetonitrile. The crystalline acid which was obtained after workup exhibited NMR and IR spectra consistent with structure 13. The carboxyl group of 13 was activated as the mixed anhydride with the use of isobutyl chloroformate at 25 °C in DMF and was coupled with diethyl L-glutamate hydrochloride as described under Experimental Section. The desired diethyl ester was separated from the nonreacted 13 by aqueous bicarbonate extraction and freed of a few minor impurities by column chromatography. The diethyl ester 14 thus obtained in acceptable yield was subjected to catalytic hydrogenation whereby a quantitative yield of the desired side chain 9 was obtained.

Both 6-chloro-2,4-diamino-5-nitropyridine (16) and 2-amino-6-chloro-4-hydroxy-5-nitropyrimidine (17) were prepared according to published procedures.[18,19] Reaction of 9 with each of these pyrimidines (16 and 17) in methanol gave the corresponding pyrimidine intermediates 18a and 19a, respectively. The ketal protective groups of these intermediates were removed efficiently with the use of a 1:1 mixture of trifluoroacetic acid and 0.1 N HCl at 50 °C,[15,16] and the corresponding deprotected products, 18 and 19, were subjected to dithionite reductions. These reductions were carried out in aqueous DMF as previously described.[1,20] A cyclization procedure[15] involving treatment of each of the reduction products with 0.1 N NaOH for 1.5 h was necessary to obtain the dihydropteridine structures 20 and 21. However, it was also observed that after 1.5 h, excess NaOH in combination with acetonitrile[22] could be used to hydrolyze the ester functionalities of 20 or 21 without their isolation. The 7,8-dihydro derivatives of 1 and 2 thus obtained were oxidized to 1 and 2 in methanolic NaOH with the use of 5% potassium permanganate. In fact, the whole series of reactions starting from the dithionite reduction products to compounds 1 and 2 could be carried out in one reaction vessel with remarkable simplicity. Acidification of the filtrate obtained from permanganate oxidation of each of the dihydrofolate analogues gave crude 1 and 2, which were collected by filtration. These compounds were freed from minor impurities by chromatography over DEAE-cellulose using a linear sodium chloride gradient as previously described from our laboratories.[1] Compound 2 was compared with an authentic sample[15] and was found to be identical in all respects. An alternative procedure using a combination of Me_2SO and DMF was also employed for the oxidation of crude 20 to diethyl-11-oxahomoaminopterin, which was

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Table I. Antifolate Activities of Bridge-Altered Analogues of Aminopterin

compd	antitumor act.[a] (L-1210 leukemia) % ILS	antitumor act.[a] (L-1210 leukemia) dose, (mg/kg)/day	I_{50} for [^{3}H]MTX influx,[b] M (HeLa cells)	*L. casei* concn for 50% inhibn of growth, ng/mL	*S. faecium* concn for 50% inhibn of growth, ng/mL	DHFR (*L. casei*) concn for 50% inhibn, M
methotrexate (4)				0.01 (11)[c]	0.15 (11)[c]	3.3×10^{-9} (11)[c]
10-oxaaminopterin (6)	125 (125)	0.38	0.74×10^{-6}	0.05 (12)	0.5 (12)	4×10^{-9} (12)
	143 (130)	0.75				
	168 (154)	1.50				
	179 (190)	3.0				
10-thioaminopterin (7)	101 (124)	6.0 (0.37)	2.7×10^{-6}	0.01 (21)	0.4 (21)	4×10^{-9} (21)
	110 (144)	12.0 (0.75)				
	117 (153)	24.0 (1.5)				
	130 (133)	48.0 (3.0)				
11-oxahomoaminopterin (1)	87 (137)	0.38	21×10^{-6}	1.0	5.0	2×10^{-7}
	90 (175)	0.75				
	87 (175)	1.50				
	87 (128)	3.0				
11-thiohomoaminopterin (7b)	96 (121)	0.38	25×10^{-6}	0.1 (1)	2.0 (1)	4×10^{-7}
	100 (116)	0.75				
	104 (133)	1.5				
	95 (144)	3.0				
10-deazaaminopterin (5)	127 (115)[d]	6.0		0.02 (11)	0.2 (11)	4×10^{-9} (11)
	168 (136)	9.0				
	172 (150)	12.0				
isoaminopterin	(117)	0.5	9.0×10^{-6}	0.04	1.98	1.5×10^{-8} (23)
	94 (138)	1.0				
	110 (164)	2.0				
	132	4.0				

[a] These numbers in parentheses are for MTX, which was used as a positive control. [b] MTX present at 0.8×10^{-6} M. [c] Figures in parentheses denote references to studies where the data were obtained. [d] These values are for MTX, taken from ref 25.

converted to 1 by base hydrolysis in acetonitrile.

Biological Evaluation and Discussion

Three analogues of aminopterin in which alterations were made in the C^9–N^{10} bridge region either by isosteric replacement of the 10-amino group by heteroatoms such as sulfur[21,22] and oxygen,[12] as in the case of 10-oxa- and 10-thioaminopterin (6 and 7), or by reversal of substituents, as in the case of isoaminopterin,[23] have been previously investigated for their antifolate activities in our laboratories using several test systems. These included enzyme inhibition studies on DHFR and thymidylate synthase derived from *L. casei*, human leukemic DHFR, microbiological assays using *L. casei* and *Strepcococcus faecium*, and evaluation of antifolate activities[7,24] and transport characteristics in HeLa cells. These compounds were also investigated for their activities against L-1210 leukemia in mice at the National Cancer Institute. These results are summarized in Table I along with the I_{50} of each analogue for MTX transport in HeLa cells. 10-Oxaaminopterin is more efficiently transported than is 10-thioaminopterin. Reversal of substituents in bridge region, as in isoaminopterin, resulted not only in a decrease in its ability to compete with MTX transport but also resulted in less effective dihydrofolate reductase inhibition.

By examining the data concerning transport, microbiological activity, and enzyme inhibition of these compounds, one could conclude that 10-oxaaminopterin and MTX should have comparable antitumor activities, whereas 10-thioaminopterin should be less active. Examination of the in vivo antitumor activities shown in Table I substantiates this point of view.

Recently there has been considerable interest in unraveling structure–activity relationships which exist among transport, enzyme inhibition, and structural changes in the C^9–N^{10} bridge region of various folate analogues.[7-9,24] Since our laboratories have been engaged in a study concerning development of folate analogues altered in the C^9–N^{10} bridge region, it was of interest to examine the biological consequence of extending the bridge region of 10-oxafolic acid and 10-thiofolic acid by one methylene unit. We have recently described the synthesis and preliminary biological evaluation of one such compound, 11-thiohomoaminopterin (7b).[1]

With the use of two folate-requiring microorganisms, *L. casei* (ATCC 7469) and *S. faecium* (ATCC 8043), 11-oxahomoaminopterin was investigated for its antifolate activity. A comparison of the antimicrobial activities of 1 with those of methotrexate (4) and closely related analogues 5–7 and 7b, are summarized in Table I. It can be seen from these results that the antifolate activities of the "bridge-elongated" analogues 1 and 7b are considerably lower than those of analogues in which the length of the bridge has been preserved. This trend was very obvious in both the organisms. In Table I we have also summarized the I_{50} values of 1 with regard to inhibition of *L. casei* DHFR. None of these compounds inhibited *L. casei* thymidylate synthase significantly at 5×10^{-5} M. Introduction of one more methylene unit in the bridge region of 10-oxaaminopterin resulted in a dramatic decrease in DHFR inhibitory potency, which was also reflected in its antifolate activity in the microorganisms. The same results were seen with the sulfur analogues, where the I_{50} of the homo analogue fell to 4×10^{-7} M from 4.5×10^{-8} M for 10-thioaminopterin (7). Since replacement of the amino group at the 10 position of aminopterin with either a

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Table II. Transport and Growth Inhibition of the Homofolate Analogues in L-1210 and Ehrlich Cells

compd	IC_{50}, M (L-1210 cells)[a]	influx (L-1210 cells) (K_i), M	Ehrlich cells[b]
aminopterin	0.61×10^{-9}	1.2×10^{-6}	2.05×10^{-6}
11-oxahomoaminopterin (1)	40.4×10^{-9}	23.7×10^{-6}	12.3×10^{-6}
11-thiohomoaminopterin	125×10^{-9}	22.8×10^{-6}	12.9×10^{-6}
methotrexate	2.7×10^{-9}	3.65×10^{-6}	10.1×10^{-6}

[a] Reference 30. [b] SE = greater than ±19%; n = 3–4 (ref 26).

methylamino, oxygen, sulfur, or methylene group did not significantly alter the enzyme inhibitory potency of these compounds (Table I), the present results with the homo analogues indicate that changes in the length of the bridge regions of related analogues are susceptible to enzyme binding in spite of the fact that all bonds connecting the pteridine and benzene rings are capable of free rotation.

Although 11-oxahomoaminopterin and 11-thiohomoaminopterin were found to be inferior in their biological potencies in the bacterial system compared to MTX and their parent compounds **6** and **7**, we were interested in examining the effect of these structural modifications with regard to their activities in mammalian cell lines and in selected tumor systems in vivo and in vitro.

First, the abilities of **1** and **7b** to inhibit the uptake of [^{3}H]MTX in HeLa cells were examined under identical experimental conditions previously employed for compounds **6** and **7**. The I_{50} values of these 11-substituted analogues (**1** and **7b**) were more than one order of magnitude higher than those for the 10-substituted analogues (Table I). As an extension of this, the homo analogues were also investigated for their influx into L-1210 and Ehrlich ascites cells in culture,[26] and the results were compared with those of MTX and aminopterin (Table II). These results (K_i values) revealed that in L-1210 cells the homo analogues are not transported as efficiently as MTX, which was also the case with HeLa cells. Therefore, the inactivity of **1** and **7b** against L-1210 leukemia can be explained in terms of decreased transport and DHFR inhibition. It is of interest that tumor specificity with regard to transport is observed in Ehrlich cells where the influx of the homo analogues is approximately equal to that of MTX, whereas in L-1210 cells the homo analogues are less active.

These results with the five bridge-altered analogues of aminopterin revealed that the in vivo antitumor activity of such analogues can be correlated with data concerning their inhibition of *L. casei* DHFR, growth of microorganisms, and the transport influx into tumor cells. Although the transport data of these compounds are favorable in Ehrlich ascites cells, they may not be superior to MTX in their activities in these cell lines because of their decreased enzyme inhibition.

Finally, **1** and **7b** were examined for their activity against the growth of L-1210 leukemia cell lines in culture and, in confirmation of expectations, were found to be much less active than methotrexate or aminopterin.

These results, with regard to antimicrobial activity, dihydrofolate reductase inhibition, carrier mediated influx, and in vivo and in vitro antitumor activities of the bridge-altered folate analogues, indicate the following: (a) structural alterations in the bridge region of analogues of aminopterin, in which the bridge length is not altered, do not significantly affect DHFR inhibition but do result in changes in transport as with 10-deaza, 10-oxa, and 10-thio analogues; (b) elongation of the bridge region of these analogues results in decreased enzyme inhibition and transport and, as a consequence, lower antitumor activities.

Experimental Section

Melting points were determined on a Fisher Model 355 digital melting point analyzer. NMR spectra were run in $CDCl_3$ or CF_3COOH on a 90-MHz Perkin-Elmer R-32 spectrometer with Me_4Si as internal lock signal. Field strength of the various proton resonances are expressed in δ (parts per million) and coupling constants in hertz. Peak multiplicity is depicted as s, singlet; d, doublet; t, triplet, q, quartet, br, broadened singlet or unresolved multiplet, the center of which is given. UV spectra were determined on a Beckman Model 25 spectrophotometer. Ion-exchange chromatography was carried out on DEAE-cellulose in the chloride form with 2.5 × 20 cm packing unless otherwise specified. A linear NaCl gradient of 1 L each of 0–0.5 M NaCl in 0.005 M phosphate buffer, pH 7, was used to elute the column. Elemental analyses were by Galbraith Laboratories, Inc., Knoxville, TN. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements or functions were within ±0.4% of the theoretical values.

1-Azido-4-[*p*-(carbomethoxy)phenoxy]-2-butanone (11). To 200 mL of dry acetone in an Erlenmeyer flask were added 3 g (46.15 mmol) of sodium azide and 0.3 g (1.8 mmol) of finely pulverized potassium iodide. To this solution was added 3 g (10 mmol) of bromo ketone **10** dissolved in 75 mL of dry acetone during a period of 5 min, and the formation of the azide was monitored by TLC. When all the starting bromo ketone was consumed (~2 h), the reaction mixture was filtered and the filtrate evaporated to dryness. The syrup-like product thus obtained was dissolved in benzene (75 mL), and upon adding hexane, needle-shaped crystals of **11** formed. After chilling, the solvents were removed by decantation, and the crystals were collected: yield 2.16 g (82%); mp 66–68 °C. Anal. ($C_{12}H_{13}N_3O_4$) C, H, N.

Protection of 11. Preparation of 1-Azido-4-[*p*-(carbomethoxy)phenoxy]-2-butanone Ethylene Ketal (12). To 15 mL of ethylene glycol in a 500-mL round-bottomed flask were added 6 g (22.8 mmol) of **11** and 800 mg (4.21 mmol) of *p*-toluenesulfonic acid monohydrate. The mixture was heated to ~100 °C and 2 mL of benzene was added. After the mixture was stirred for 5 min, gentle vacuum was applied to remove traces of benzene, which facilitated the dissolution of both reactants in ethylene glycol. At this point, 250 mL of dry benzene was added to the mixture and it was refluxed for 6 h with use of a Dean–Stark apparatus for the continuous removal of water. Toward the end of the reflux period most of the benzene was removed through the Dean–Stark apparatus and the remainder by rotary evaporation at reduced pressure. After cooling to 25 °C, the contents of the flask were treated with 6 g (60 mmol) of solid $KHCO_3$ and stirred vigorously while adding 500 mL of a saturated solution of $KHCO_3$ in water. The crude ketal was extracted from this aqueous solution with 200 mL of ethyl acetate. The ethyl acetate layer was separated, washed three times with distilled water, dried with anhydrous Na_2SO_4 and evaporated to obtain crude **12**: yield 5.75 g (82%); about 98% pure by TLC. The material was freed from very minor impurities by filtration through an activated alumina column (50 g) with the aid of benzene. Evaporation of the benzene eluate gave pure **12**: NMR ($CDCl_3$) δ 8.05 and 6.95 (2 d, 4 H, aromatic), 4.22 (t, 2 H, ethylene), 4.13 (br s, 4 H, ethylene ketal), 3.92 (s, 3 H, methoxy), 3.39 (s, 2 H, azidomethyl), 2.29 (t, 2 H, ethylene). This compound could not be crystallized.

Hydrolysis of 12. Preparation of 1-Azido-4-(*p*-carboxyphenoxy)-2-butanone Ethylene Ketal (13). The hydrolysis of **12** to **13** was carried out by stirring 5.75 g (18.73 mmol) of the ester **12** with 100 mL of 0.5 N NaOH and 50 mL of acetonitrile at room temperature for 18 h. The acetonitrile was removed by evaporation under reduced pressure and the clear aqueous solution of the sodium salt of **13** thus obtained (~50 mL) was chilled and acidified to pH 4.0 with glacial acetic acid. White crystals of **13**

(26) Sirotnak, F. M. *Pharmacol. Ther.* **1980**, *8*, 71.

thus formed and were filtered, washed, and dried: yield 5.13 g (93.5%); mp 111 °C; NMR ($CDCl_3$) δ 8.12 and 7.0 (2 d, 4 H, aromatic), 4.25 (t, 2 H, ethylene), 4.12 (br s, 4 H, ketal), 3.4 (s, 2 H, azidomethyl), 2.3 (t, 2 H, ethylene). Anal. ($C_{13}H_{15}N_3O_5$) C, H, N, O.

Preparation of Diethyl *N*-[*p*-(1-Azido-2-oxo-4-oxabutanoyl)benzoyl]-L-glutamate Ketal (14). Under strictly anhydrous conditions, and taking the necessary precautions to avoid moisture, a solution of 2.93 g (10 mmol) of **13** in 90 mL of DMF was treated with 1.41 mL (12.5 mmol) of freshly distilled *N*-methylmorpholine, followed by 1.31 mL (10 mmol) of isobutyl chloroformate (freshly distilled and stabilized with $CaCO_3$), and stirred for 20 min. To this reaction mixture was added 2.26 mL (20 mmol) of an additional amount of *N*-methylmorpholine and the mixture was then stirred at room temperature for 18 h with 4.9 g (20 mmol) of diethyl L-glutamate hydrochloride. Dimethylformamide was evaporated off under vacuum at 65 °C and the residue was treated with 200 g of crushed ice. After the ice had melted, the crude product was extracted in 200 mL of ethyl acetate and the aqueous layer was discarded. The ethyl acetate layer was then extracted three times with 75-mL portions of saturated sodium bicarbonate. The bicarbonate layers were combined and acidified to pH 4.0 with glacial HOAc, whereupon the unreacted **13** precipitated and was collected by filtration. The ethyl acetate layer containing crude **14** was washed three times with 100-mL portions of distilled water, dried with Na_2SO_4, and evaporated. The noncrystalline viscous product, which showed only a single spot on TLC, was dissolved in 50 mL of benzene and passed through a column made of 40 g of silica gel CC_7 in benzene. The column was washed with 200 mL of benzene, and evaporation of the combined benzene eluates gave 3.2 g of **14** as a colorless glassy product (67%): NMR ($CDCl_3$) δ 7.7 and 6.85 (2 d, 4 H, aromatic), 4.15 (t, 1 H, α proton of glutamate), 3.95 (br s, 4 H, ethylene ketal), 3.9–4.2 (c, 6 H, ethoxymethylenes and anisoylmethylene), 3.25 (s, 2 H, azidomethyl), 1.8–2.5 (c, 4 H, glutamate), 2.12 (t, 2 H, methylene), 1.1–1.3 (c, 6 H, methyl groups).

Hydrogenation of the Azide 14. Preparation of Diethyl *N*-[*p*-(1-Amino-2-oxo-4-oxabutanoyl)benzoyl]-L-glutamate Ketal (9). The diethyl glutamate conjugate **14** [3.2 g (6.7 mmol)], which was obtained from the previous experiment, was dissolved in 150 mL of methanol and hydrogenated at 25 psi for 18 h using 500 mg of 5% palladium on carbon as a catalyst. Examination of this hydrogenation mixture at this stage by TLC revealed that the hydrogenation was complete. The methanolic solution of **9** was separated from the catalyst by filtration and was used for the next step.

Preparation of 1-[*N*-(2,4-Diamino-5-nitropyrimidin-6-yl)amino]-4-[diethyl *N*-*p*-anisoyl-L-glutamate]-2-butanone (18). A solution of 1.35 g (8.6 mmol) of 6-chloro-2,4-diamino-5-nitropyrimidine (**16**) in 200 mL of methanol was reacted under reflux with 4.38 g (9.7 mmol) of **9** for 1 h. To this refluxing reaction mixture was added 1 mL of *N*-methylmorpholine, and the reaction continued for an additional 2 h. On evaporation and addition of 100 g of crushed ice to the residue, a yellow semisolid was obtained, which was separated by decantation: yield 4.6 g (88%); UV (0.1 N NaOH) λ_{max} 337 nm 250. The NMR spectrum of this compound (TFA) retained all the resonances of **9**, confirming the structure of this product. Due to the noncrystalline nature of this material, it was used for the next step without further characterization. The ketal protective group was removed by dissolving the product in 50 mL of TFA and slowly adding 50 mL of 1 N HCl during a period of 20 min at 55 °C. The reaction mixture was evaporated to dryness at reduced pressure, and upon addition of 100 g of crushed ice, a yellow solid was obtained, which was separated by filtration and dried: yield 4.04 g (94.8%); mp 145 °C; UV (0.1 N NaOH) λ_{max} 335 nm, 287. Anal. ($C_{24}H_{31}N_7O_9$) C, H, N, O.

Preparation of 1-[*N*-(2-Amino-4-hydroxy-5-nitropyrimidin-6-yl)amino]-4-(diethyl *N*-*p*-anisoyl-L-glutamate)-2-butanone (19). A methanolic solution (100 mL) of the reaction product **9**, which was obtained by hydrogenation of 3.19 g (6.67 mmol) of **14**, was refluxed with a solution of 0.952 g (5 mmol) of **17** in 100 mL of methanol for 1.5 h. After this period, 0.59 mL of *N*-methylmorpholine was added to the reaction mixture, the refluxing was continued for an additional period of 3 h, and the mixture was then evaporated to dryness. Upon adding 250 g of crushed ice, a yellow noncrystalline product was obtained, which was deprotected by dissolving in 20 mL of trifluoracetic acid and treating with an equal volume of 0.1 N HCl at 55 °C for 20 min. The clear solution of **19** thus obtained was evaporated to dryness at reduced pressure; after 200 g of crushed ice was added and trituration, a light yellow solid was obtained, which was collected by filtration, washed with water, and dried: yield 1.02 g (34%); mp 183 °C; NMR (TFA) δ 7.6 and 6.8 (2 d, 4 H, aromatic), 4.55 (br s, 2 H, aminomethyl), 3.95–4.35 (c, 6 H, ethoxymethylenes and anisoylmethylene), 3.0 (2 H, ethylene bridge), 2.08–2.7 (c, 4 H, glutamate), 1.25 (2 t, 6 H, overlapping methyl groups); UV (0.1 N NaOH) λ_{max} 287 nm, 340. Anal. ($C_{24}H_{30}N_6O_{10}$) C, H, N, O.

Preparation of 4-Amino-4-deoxy-11-oxahomoaminopterin (1) and 11-Oxahomofolic Acid (2). (a) Dithionite Reductions of 18 and 19. The reduction of the 5-nitro group of each of these compounds to the corresponding amino group was carried out by similar procedures as follows. A water bath was kept ready and maintained at 55 °C. A solution of 2.5 g (~4.1 mmol) of either **18** or **19** in 50 mL of DMF was prepared by first heating and then cooling to ~55 °C; to this solution was carefully added 20 g of sodium dithionite before placing in the water bath and stirring. With the aid of a transfer pipet, 50 mL of distilled water was added to this mixture over a period of 20 min. At one stage, close to the end of the addition, the reaction mixture became homogeneous in both cases and the color lightened. When the addition was complete, the mixture was poured over 760 g of crushed ice and stirred vigorously. After 30 min a white precipitate formed, which was collected by filtration. The yield of the reduction product ranged between 40 and 50%. These reduction products (~2 mmol) were suspended in 100 mL of 0.1 N NaOH and stirred vigorously for 1.5 h to induce cyclization to **20** and **21**. These cyclized products were collected by filtration after adjusting the pH of the cyclization medium to 7.2. UV of **21** (0.1 N NaOH) λ_{max} 325 nm, 280 (s), 240; UV of **20** (0.1 N NaOH) λ_{max} 325 nm, 290, 245.

(b) 11-Oxahomofolic Acid (2). The conversion of **19** to **2** was accomplished without the isolation of **21** according to the following procedure. To 1.25 g (~2 mmol) of the reduction product in a 500-mL Erlenmeyer flask was added 100 mL of 0.1 N NaOH and the mixture was stirred vigorously for 1.5 h. An additional 10 mL of 1.0 N NaOH was added to the solution, and the reaction mixture was chilled for 18 h in a refrigerator. Stirring was continued for another 4 h at room temperature to ensure ester hydrolysis. At this point, UV examination of the hydrolysate revealed that no significant oxidation of the dihydropteridine to the pteridine had taken place. Therefore, the compound was oxidized by adding 12 mL of alcohol, followed by the dropwise addition of 6 mL of 5% $KMnO_4$ over a 5-min interval. The mixture was stirred for another 20 min at room temperature, filtered, and acidified to pH 4.0 with glacial HOAc. The yellow precipitate of **2** thus obtained was collected by filtration, washed, and freed from minor impurities by gradient elution with NaCl on a DEAE column as described previously: yield 600 mg (66%). Compound **2** was found to be identical with an authentic sample of **2** in all respects, including its biological activities.

(c) 11-Oxahomoaminopterin (1). The product, diethyl-7,8-dihydro-11-oxahomoaminopterin (**20**), was dried overnight in vacuo (800 mg, 1.56 mmol) and suspended in 40 mL of dry DMF. The mixture was stirred vigorously with the addition of 1 mL of Me_2SO. The rate of oxidation was monitored by UV spectroscopy by examining the appearance of a λ_{max} at 360 nm and the disappearance of the λ_{max} at 325 nm. Since the oxidation was proceeding at a slow rate, an additional 4 mL of Me_2SO was added to this mixture during the first several hours and a last 1 mL after 23 h. Stirring continued under aerobic conditions for an additional 24 h (total 47 h). From UV criteria, the oxidation was judged to be complete: UV (0.1 N NaOH) λ_{max} 367 nm, 256. Evaporation of DMF under vacuum and dilution of the residue with 500 mL of ice-cold water gave a bright yellow product, the NMR spectrum of which showed resonances expected for the structure, viz., diethyl-11-oxahomoaminopterin. This compound was hydrolyzed to **1** using 0.1 N NaOH and acetonitrile[22] and purified by ion-exchange chromatography over DEAE-cellulose in the chloride form, using a sodium chloride gradient at pH 7.0 as described

previously:[1] yield 550 mg (77.5% based on **20**): UV (0.1 N NaOH) λ_{max} 367 nm (ϵ 5416), 259 (29 204); UV (0.1 N HCl) λ_{max} 335 nm (ϵ 7677), 242 (22 000); NMR (TFA) δ 8.96 (s, 1 H, pteridine), 7.85 and 7.05 (2 d, 4 H, aromatic), 4.6 and 3.6 (t, t, 4 H, ethylene bridge); 4.01 (t, 1 H, α proton of glutamic acid), 2.05–2.85 (c, 4 H, glutamic acid). Compound **1** was also prepared using the procedure which was employed for the conversion of **21** to **2**. Anal. ($C_{20}H_{19}N_7O_6 \cdot H_2O$) C, H, N, O.

Methods Used for Biological Evaluation. Microbiological assays using the different strains of *Lactobacillus casei* and *Streptococcus faecium* were carried out as described previously.[1,27] Thymidylate synthase assays were performed according to the procedure of Friedkin.[28] Methods involving dihydrofolate reductase were also described previously.[29] The antitumor data on all compounds were collected under the auspices of the National Cancer Institute using the standard protocol (Instruction 14) for evaluating methotrexate analogues in the L-1210 lymphoid leukemia test system in mice (treatment schedule QD 1D × 09). Detailed procedures regarding the transport of some of these analogues in L-1210 and Ehrlich cells in culture, as well as procedures used to evaluate the growth inhibition potency of the analogues in the L-1210 cells, have been published recently from one of these laboratories.[26,30]

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Antimicrobial 3-Methyleneflavanones

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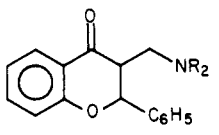
The antimicrobial activity previously attributed to flavanone Mannich bases was found to be due to their breakdown products, 3-methyleneflavanones. Among the latter compounds, highest potency was observed when the flavanone phenyl ring contained bromine or chlorine substituents. 3-Methylene-2-phenylflavanone (**8**) was synthesized and shown to be equal to hexachlorophene in tests against representative Gram-positive microorganisms.

In recent years two reports have appeared attributing in vitro antifungal and antibacterial activity to the Mannich bases (I) of flavanones.[1,2] However, although the



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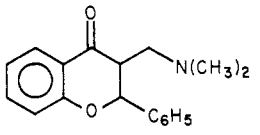

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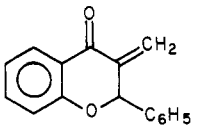

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synthesis of several dozen analogues of I were reported, no clear structure–activity pattern was apparent. For example, bromo and methyl substituents on the benzo ring often affected activity in the same way; however, if they were attached to the phenyl ring, their effects were opposed. Analogues of I having cycloalkyl substituents on nitrogen displayed high activity, while aliphatic substituents of similar lipophilicity were inactive. Nevertheless, because of the potential usefulness of lipophilic analogues of I as topical antifungal agents, we reinvestigated the chemistry and biological activity of flavanone Mannich bases and their derivatives.

We first examined 3-[(dimethylamino)methyl]flavanone (**1**) which had been reported to be both active and inactive. Although this compound could be prepared as the HCl salt by the reaction of flavanone with formaldehyde and dimethylamine, its purification was difficult. Furthermore, when converted to the free base, **1** decomposed extensively on standing, even in nonpolar solvents. When a carefully purified sample of the HCl salt of **1** was neutralized with aqueous $NaHCO_3$ solution, extracted into ether, dried, and evaporated, 3-methyleneflavanone (**2**) was isolated in good yield. In our hands, the HCl salt of **1** displayed potent antimicrobial activity, and, when tested against 14 microorganisms, **2** possessed the same potency and spectrum of antimicrobial activity as purified **1**.

The Mannich bases appear to act as prodrugs for their unsaturated derivatives. Their instability probably explains the inconsistent structure–activity pattern observed earlier. Different substituents on nitrogen and inconsistencies of handling may have led to varying degrees of conversion of the bases to the corresponding 3-methyleneflavanones. Support for this view was obtained by synthesizing 3-[(dimethylamino)methyl]-3-methylflavanone (**3**). This compound, which cannot eliminate dimethylamine, was inactive when tested against 22 fungi and bacteria.

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