2,4-Diamino-5-benzylpyrimidines and Analogues as Antibacterial Agents. 9. Lipophilic Trimethoprim Analogues as Antigonococcal Agents[1]

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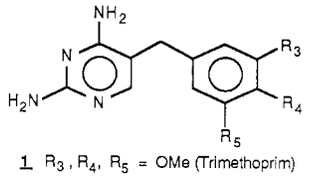
Lipophilic analogues of trimethoprim (1) bearing 3,5-dialkyl-4-hydroxy substituents in the benzene ring are much more active in vitro against *Neisseria gonorrhoeae* than is 1. The 3,5-diisopropyl-4-hydroxy derivative (2) was selected as a candidate for clinical evaluation as an antigonococcal agent, and as part of the preliminary evaluation it was submitted to extended pharmacokinetic and metabolism studies in dogs. Although the compound was not extensively conjugated by metabolic enzymes, one of the methyl groups was metabolized to produce a 3-isopropyl-4-hydroxy-5-(α-carboxyethyl)benzyl derivative (**43**), which was rapidly excreted. Related analogues were likewise extensively metabolized.

In paper 7 of this series,[2] we described the activities of a series of trimethoprim (TMP) (1) analogues bearing alkyl substituents in the 3- and 5-positions of the benzene ring and alkoxy, hydroxy, or amino functions in the 4-position against four dihydrofolate reductase (DHFR) isozymes. Some of these compounds were found to be much more active than trimethoprim against *Neisseria gonorrhoeae* DHFR, particularly derivatives with *n*-propyl or isopropyl substituents at the 3- and 5-positions. This paper is focused largely on the 3,5-dialkyl-4-hydroxybenzyl derivatives as potential antigonococcal agents, with particular emphasis on the 3,5-diisopropyl-4-hydroxybenzyl derivative (**2**).



1. R_3, R_4, R_5 = OMe (Trimethoprim)
2. R_3, R_5 = Pr-i; R_4 = OH

General in vitro antibacterial screening results on the more promising compounds of paper 7 are detailed here, along with data on selected compounds in a more specific in vitro antigonococcal screen. No convenient animal model is available for assessing activity against this organism.[3] Therefore, several of the most promising leads were selected for extended pharmacokinetic and metabolic studies in rats and dogs to assess their potential utility, the results of which are described here, as are some improved synthetic methods.

Chemistry

In paper 6 of this series,[4] we described the acid-catalyzed condensation of 2,6-dialkylphenols with 2,4-diamino-5-(hydroxymethyl)pyrimidine to yield 2,4-diamino-5-(3,5-dialkyl-4-hydroxybenzyl)pyrmidines in a single-step condensation. Although this reaction worked reasonably well with lower alkyl derivatives, it produced only 20% of **2** and only traces of the di-*tert*-butyl analogues under the conditions described. At an earlier date, we had prepared the 3,5-diisopropyl and di-*tert*-butyl-4-methoxy derivatives in overall yields of 16–38% from phenolic Mannich condensations with 2,4-diamino-6-(methylthio)pyrimidine, followed by alkylation of the phenol and dethiation.[5] Although this method was successful, it was lengthy, and the Raney nickel dethiation was somewhat erratic. We never tried to make the phenolic 6-unsubstituted pyrimidines by this procedure. Since we had found that uracil reacted successfully with 2,6-diisopropyl-4-[(dimethylamino)-methyl]phenol,[5] we decided to pursue this approach further, as well as the coupling of 5-(hydroxymethyl)uracil with 2,6-disubstituted phenols.

Scheme I illustrates the coupling of **3** with **4**, compared with the reaction of **5** plus **6**. Both reactions proceeded satisfactorily to give **7**, but the Mannich procedure gave a far superior yield. Chlorination of both **7** and **8** gave good yields of **9** and **10**. Amination proceeded in excellent yield with **10** to produce **2**, but under similar conditions the di-*tert*-butyl analogue was only partially diaminated, for reasons that are not clear. Further attempts were made to produce **11** from **12** and **4**, which finally resulted in achievement of a 10% yield of the desired product, along with 5% of **13**, in which one of the *tert*-butyl groups was lost. No further attempts were made to improve the route to **11**, since **2** proved a considerably more interesting target.

Scheme II illustrates the route used to obtain radiolabeled **2**, which required additional steps, but was highly satisfactory.

Biological Data

A. In Vitro Antibacterial Activities. Table I lists the in vitro antibacterial activities of (3,5-dialkyl-4-hydroxybenzyl)- and (3,5-dialkyl-4-methoxybenzyl)pyrimidines against 10 selected organisms, relative to that of trimethoprim (1). In most cases, serial dilutions were carried out at levels of 0.1, 0.3, 1, 3, etc., and differences of ±1 dilution are not significant. In practically every case the compounds were significantly less active than 1, particularly against Gram-negative organisms. This is very often the case with analogues of 1 that are more lipophilic.[6] Compounds **25** and **27** were nearly equipotent with **1**, and several of the compounds were very active against such Gram-positive organisms as *Staphylococcus aureus* and *Streptococcus faecium* (not listed here). However, none could be considered outstanding for the standard series of organisms tested. The compounds most active against *Escherichia coli* DHFR had been found to be the 3-ethyl-5-*n*-propyl-4-hydroxy and 3,5-di-*n*-propyl-4-hydroxy derivatives **27** and **28**.[2] Compound **27** is the most active of these against whole cells.

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(1) Roth, B.; Baccanari, D. P.; Sigel, C. W.; Hubbell, J. P. Abstracts of the 6th International Symposium on Future Trends in Chemotherapy, Tirrenia (Pisa), May 1984, p 170.
(2) Roth, B.; Rauckman, B. S.; Ferone, R.; Baccanari, D. P.; Champness, J. N.; Hyde, R. M. *J. Med. Chem.* **1987**, *30*, 348.
(3) DiGiacomo, R. F.; Gale, J. L.; Holmes, K. K.; Buchanan, T. M. *Infect. Immun.* **1977**, *15*, 670.
(4) Stuart, A.; Paterson, T.; Roth, B.; Aig, E. *J. Med. Chem.* **1983**, *26*, 667.
(5) Roth, B.; Aig, E.; Lane, K.; Rauckman, B. S. *J. Med. Chem.* **1980**, *23*, 535.
(6) Roth, B.; Aig, E.; Rauckman, B. S.; Strelitz, J. Z.; Phillips, A. P.; Ferone, R.; Bushby, S. R. M.; Sigel, C. W. *J. Med. Chem.* **1981**, *24*, 933.

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Table I. Comparative in Vitro Antibacterial Activity of (3,5-Dialkylbenzyl)pyrimidines and Analogues against Selected Organisms vs. Trimethoprim Standard (MIC Compound/MIC Trimethoprim)[a]

	benzene subst			organism[b]								
no.	3	4	5	*Staph. aureus*	*Vibrio chol.*	*Myco. smeg.*	*Sal. typh.*	*Shig. flex.*	*E. coli*	*Serr. marc.*	*Kleb. pneum.*	*Entero. aerog.*	*Proteus vulgaris*
1	OMe	OMe	OMe	1	1	1	1	1	1	1	1	1	1
2	Pr-*i*	OH	Pr-*i*	10			100		300		300	100	300
11	Bu-*t*	OH	Bu-*t*	30		10	>1000	>1000	>1000	>100	>1000	>1000	>1000
24	Me	OH	Me	10			10		10		3	1	3
25	Me	OH	Et	1		1	3	3	3	1	3	3	1
26	Et	OH	Et	3	1	3	10	10	3	10	10	10	10
27	Et	OH	Pr-*n*	0.3	1	3	3	3	10	0.3	3	10	3
28	Pr-*n*	OH	Pr-*n*	0.3	1	3	10	10	10	0.3	30	30	30
29	Me	OH	Bu-*t*	3		1	100	30	100	3	100	100	100
30	Me	OMe	Me	100			100		1000		300	1000	300
31	Me	OMe	Et	3		3	3	30	30	3	30	30	3
32	Et	OMe	Et	0.3			3	3	3		10	10	30
33	Et	OMe	Pr-*n*	0.3	10	3	30	30	30	1	30	100	30
34	Pr-*n*	OMe	Pr-*n*	1	10	1	100	100	30	1	100	100	>30
35	Pr-*i*	OMe	Pr-*i*	30			3000		3000		>1000	>1000	>1000
36	Me	OMe	Bu-*t*	10	10	10	300	300	100	10	>100	300	>30
37	Bu-*t*	OMe	Bu-*t*	30			3000		10000		>1000	>1000	>1000

[a] MIC = minimal inhibitory concentration, determined as micrograms per milliliter. Numbers greater than one indicate less activity for the test compound than for trimethoprim. Typical MIC values (μg/mL) for 1 with the organisms listed are 0.3–1, 0.3–1, 1–3, 0.03–0.1, 0.1–0.3, 0.1–0.3, 10, 0.03–1.0, 0.1–0.3, and 1–10. [b] *Staphylococcus aureus* CN 491, *Vibrio cholerae* ATCC 14035, *Mycobacterium smegmatis* S3254, *Salmonella typhosa* CN 512, *Shigella flexneri* CN 6007, *Escherichia coli* CN 314, *Serratia marcescens* CN 2398, *Klebsiella pneumoniae* CN 3632, *Enterobacter aerogenes* 2200/186, *Proteus vulgaris* CN 329.

Table II. Comparison of Inhibitory Activities of Selected 3,4,5-Substituted (Alkoxy-, Alkyl-, and Alkenylbenzyl)pyrimidines against *Neisseria gonorrhoeae* DHFR with Their in Vitro Antibacterial Activities (MIC) against *Neisseria gonorrhoeae*

	benzene subst			I_{50}, M, × 10^8 vs *N. gonorrhoeae* DHFR[a]	MIC, μM, vs *N. gonorrhoeae*
compd no.	3	4	5		
2	Pr-*i*	OH	Pr-*i*	2.9	9.2
33	Et	OMe	Pr-*n*	4.4	17.5
27	Et	OH	Pr-*n*	4.9	9
37	Bu-*t*	OMe	Bu-*t*	5.8	13.0
34	Pr-*n*	OMe	Pr-*n*	6.0	20
11	Bu-*t*	OH	Bu-*t*	6.1	35
38[b]	OMe	OMe	Pr-*n*	6.7	54
39[b]	OMe	OMe	CH=CHMe	9.3	110
40[b]	OMe	OMe	$CH_2CH=CH_2$	11	110
41[b]	OMe	OMe	OEt	17	180
31	Me	OMe	Et	31	140
30	Me	OMe	Me	37	200
1	OMe	OMe	OMe	45	240

[a] See ref 2 for methodology. [b] Roth, B.; Tidwell, M. Y.; Ferone, R.; Baccanari, D.; Sigel, C.; Elwell, L., manuscript in preparation.

Table III. Compounds Chosen for Pharmacokinetic Studies and Their Inhibitory Activities against *Neisseria gonorrhoeae* and Rat Liver DHFR, Compared to 1

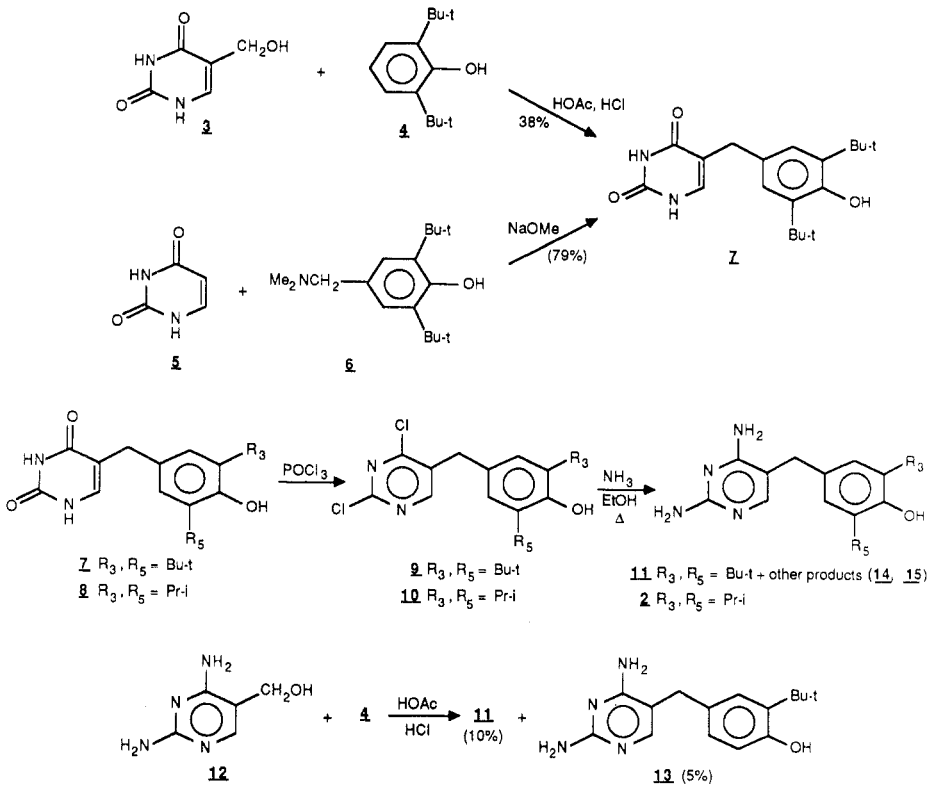
	benzene subst			I_{50}, M, × 10^8 vs DHFR	
compd no.	3	4	5	*N. gonorrhoeae*	*Rat Liver*
2	Pr-*i*	OH	Pr-*i*	2.9	720
11	Bu-*t*	OH	Bu-*t*	6.1	3450
26	Et	OH	Et	12	1600
33	Et	OMe	Pr-*n*	4.4	3000
37	Bu-*t*	OMe	Bu-*t*	5.8	3100
42[a]	Pr-*n*	OH	Allyl	2.0	363
1	OMe	OMe	OMe	45	34000

[a] See footnote b, Table II.

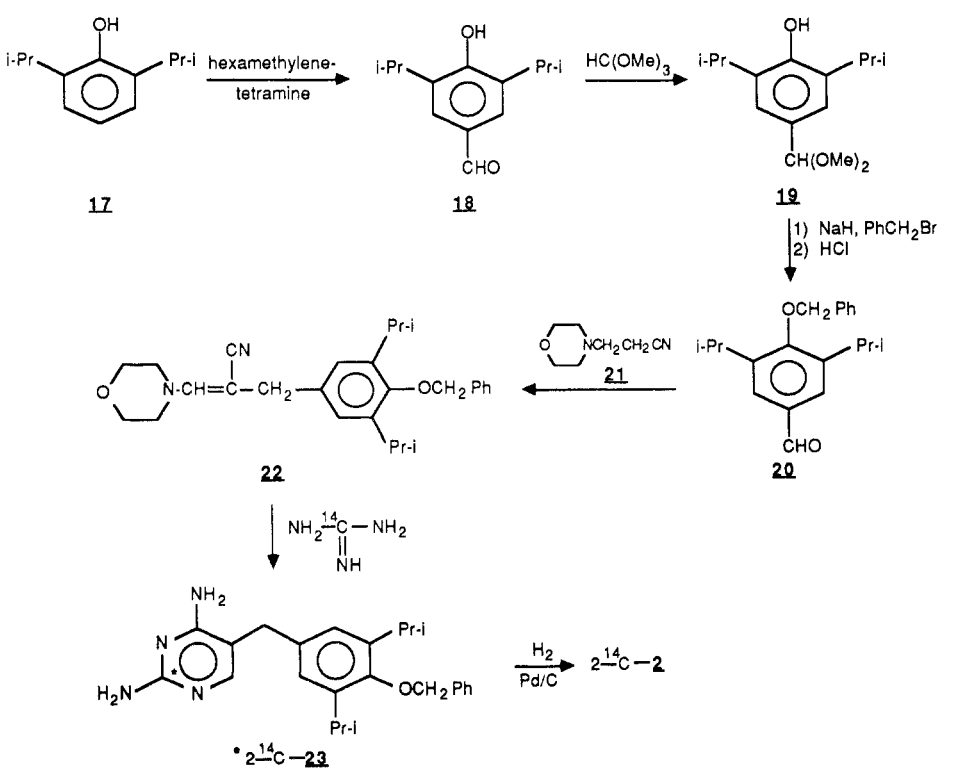
Initial screening for *N. gonorrhoeae* was carried out with clinical isolates. Among a very large series of compounds tested, the only ones showing activity superior to **1** were the lipophilic (3,5-dialkylbenzyl), (3,4-dimethoxy-5-alkylbenzyl, or -alkenylbenzyl), or (3,4-dimethoxy-5-aralkylbenzyl)pyrimidines. The latter series will be discussed in a separate paper. A subset of the most active compounds of Table I, as well as compounds **38–41**, were selected for careful comparison in a separate test against a single strain of *N. gonorrhoeae* (F62). These results are shown in Table II. The MIC data are compared with inhibitory activities against DHFR from the same strain, and it will be observed that the relative activities are very well correlated. The most active compounds are **2** and **27**; **2** is 15 times better as a gonococcal enzyme inhibitor than **1**, with 27-fold greater in vitro antigonococcal activity.

B. Pharmacokinetic Studies in Dogs. The concentrations in blood, urine, and tissues of mice after oral administration were determined for several compounds showing significant antigonococcal activity (S. R. M. Bushby, unpublished results). Six compounds were then chosen for further pharmacokinetic study in the dog, on the basis of all previous screening data. These are shown in Table III. Since the most active compounds were the 4-hydroxy derivatives, consideration was also given to the relative probabilities of glucuronide or sulfate formation

Scheme I



Scheme II



in vivo, which would lead to rapid excretion. Trimethoprim itself is metabolized in part to a 4-hydroxybenzyl derivative, which is rapidly converted to a glucuronide, followed by rapid excretion.[7] Flanking of the 4-hydroxy substituent by branched-chain alkyl substituents might be expected to minimize such enzymatic inactivation, but a comparison of branched and straight-chain analogues was considered essential.

The dog was chosen for the more extended studies, since from previous data with analogues of **1**, the dog kinetic data correlated better with human results than did other laboratory animals.[7]

For oral absorption studies, four beagle dogs (two males and two females) received single doses (5 mg/kg) of each drug in a gelatin capsule. Only very low concentrations of compounds **11**, **37**, and **42** were observed in serum

(7) Sigel, C. W. in *Handbook of Experimental Pharmacology*; Hitchings, G. H., Ed.; Springer-Verlag: Heidelberg, 1983; Vol. 64, pp 164–184.

Table IV. Tissue Concentrations (μg/g) in Male Rats, Ovary Concentrations (μg/g) in Female Rats, and Serum Concentrations (μg/mL) in Male and Female Rats of Compound **2** (Equivalents) at Various Times following Oral Administration of 2-^{14}C-**2** (Average of Three Rats)

time after dose, h	stomach	intestine	liver	kidney	lung	muscle	brain	testes	ovary	serum male	serum female
0.25	386.97 ± 114.44	157.50 ± 61.60	17.42 ± 1.70	2.98 ± 1.59	2.52 ± 1.35	0.44 ± 0.29	0.13 ± 0.05	1.34 ± 0.29	2.06 ± 1.06	0.45 ± 0.21	0.49 ± 0.12
0.50	303.84 ± 103.15	114.48 ± 27.89	29.9 ± 6.35	8.43 ± 1.45	8.31 ± 3.41	1.45 ± 0.87	0.58 ± 0.13	1.07 ± 0.44	4.06 ± 2.18	1.12 ± 0.29	0.90 ± 0.17
0.75	319.16 ± 51.06	112.23 ± 85.55	23.13 ± 2.06	10.29 ± 0.19	11.33 ± 0.38	2.31 ± 0.84	1.01 ± 0.10	1.77 ± 0.34	4.48 ± 0.78[a]	1.13 ± 0.13	1.44 ± 0.30
1.00	46.97 ± 15.90	25.97 ± 11.09	13.51 ± 1.58	7.78 ± 1.79	11.88 ± 2.26	2.36 ± 0.47	0.91 ± 0.20	1.32 ± 0.25	3.33 ± 0.78[a]	0.92 ± 0.08	1.09 ± 0.42
1.50	97.08 ± 70.22	14.91 ± 7.75	13.46 ± 0.45	6.74 ± 0.63	7.17 ± 0.22	1.84 ± 0.23	0.65 ± 0.12	1.52 ± 0.21	4.50 ± 0.83	0.80 ± 0.08	1.09 ± 0.15
2.00	46.08 ± 32.77	11.59 ± 3.83	10.88 ± 0.97	4.08 ± 0.71	4.98 ± 2.45	1.40 ± 0.40	0.43 ± 0.23	1.24 ± 0.43	1.93 ± 0.57	0.49 ± 0.04	0.68 ± 0.17
3.00	8.92 ± 5.71	7.34 ± 1.47	9.56 ± 0.95	4.11 ± 0.15	4.18 ± 0.92	1.38 ± 0.29	0.30 ± 0.03	1.79 ± 0.19	2.06 ± 1.09	0.47 ± 0.08	0.61 ± 0.20
4.00	14.75 ± 10.14	5.57 ± 1.38	8.23 ± 1.27	2.07 ± 0.66	2.30 ± 1.63	0.67 ± 0.43	0.19 ± 0.15	1.01 ± 0.56	1.55 ± 0.13	0.42 ± 0.14	0.53 ± 0.12
6.00	5.22 ± 5.39	4.13 ± 1.50	5.14 ± 1.05	1.55 ± 0.26	0.52 ± 0.11	0.25 ± 0.05	0.05 ± 0.01	0.41 ± 0.07	0.71 ± 0.56	0.27 ± 0.08	0.34 ± 0.18
10.00	2.00 ± 1.54	2.29 ± 1.33	5.67 ± 0.97	1.13 ± 0.51	0.40 ± 0.27	0.13 ± 0.04	0.04 ± 0.02	0.66 ± 0.72	0.23 ± 0.09	0.23 ± 0.09	0.16 ± 0.06
24.00	1.49 ± 2.40	0.85 ± 1.24	2.71 ± 0.94	0.50 ± 0.32	0.10 ± 0.08	0.04 ± 0.01	0.004 ± 0.003	0.15 ± 0.03	0.08 ± 0.06	0.08 ± 0.06	0.14 ± 0.04

[a] Average of two rats.

samples (<0.04 μg/mL), indicating that they were either poorly absorbed or extensively metabolized. Insufficient serum concentration data were obtained to calculate meaningful pharmacokinetic parameters. Compounds **2** and **26** reached significantly greater concentrations in serum; mean peak concentrations occurred at 2 h and were 1.16 and 0.41 μg/mL, respectively; the mean serum $t_{1/2}$ values for **2** and **26** were 2.2 and 1.0 h.

Serum drug concentrations for compounds **2**, **11**, and **33** were measured following iv administration to two male beagle dogs. The mean serum $t_{1/2}$ values were as follows: **2**, 1.1 h; **11**, 1.1 h; **33**, 0.5 h. All of these compounds had considerably shorter half-lives in the dog than **1** (mean half-life = 2.5 h in 12 dogs).[8] The rapid rates of elimination of these compounds were suggestive of extensive metabolism. Analyses of 24-h cumulative urine collections showed that for all of the compounds less than 1% of the dose was present in the urine as intact drug. A major route of metabolism for the 4-hydroxypyrimidines is by direct conjugation with sulfate or glucuronic acid. Upon treatment of the urine with glucuronidase/sulfatase, 19% of the dose of **26**, 7.5% of **2**, and 6% of **11** were recovered as parent compound. As predicted then, the branched-chain analogues produced less conjugation than did the diethyl derivative **26**. This is still a low recovery, however, and is additional evidence for either poor absorption or possibly other significant metabolic pathways.

A difference in the metabolic profile was seen in the comparison of the drug serum concentration data for male and female dogs receiving **26**. The mean peak serum concentrations for female and male dogs were 0.69 and 0.13 μg/mL, respectively, and the AUC values (areas under the concentration/time curves) were 2.57 and 0.92 μg/mL per h, respectively. Both male and female dogs excreted less than 1% of **26** in the urine as free drug and excreted similar quantities of conjugated metabolites (18–21% of the dose). These results suggest that sex-related differences in metabolism may not result from differences in the extent of conjugation, but may be due to other metabolic reactions. Compound **2** did not show an analogous sex-related difference in the pharmacokinetic profile.

The mean absolute bioavailability of **2** was estimated to be 110% by comparison of the AUC values after iv and oral administration. Of the benzylpyrimidines with promising antigonococcal activity, **2** had the most desirable pharmacokinetic profile in dogs. On that basis, it was chosen for more detailed disposition studies.

C. Disposition Studies in Rats. (1) Bioavailability and Elimination from Serum. Compound **2** was rapidly absorbed by male and female rats and reached peak serum concentrations by 0.75 h following oral treatment (Table IV). Mean peak serum concentrations of total radiocarbon (equvalents of **2**) were 1.44 ± 0.30 μg/mL for female rats and 1.13 ± 0.13 μg/mL for male rats. At 2 h after the dose, <20% of the dose remained in the stomach.

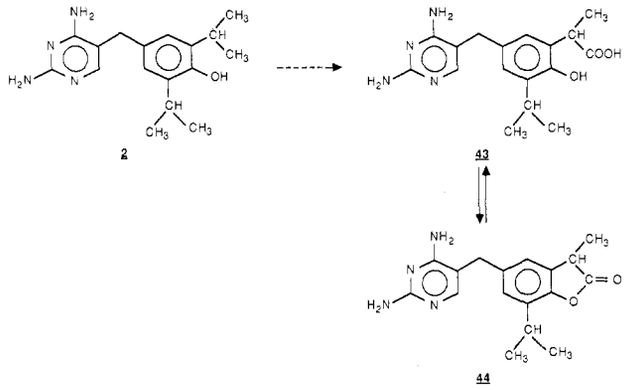
The overall elimination profile of total radiocarbon from serum was at least biphasic; during the first 2 h after the dose, the elimination was rapid with $t_{1/2} \cong 1$ h. After about 6 h, the $t_{1/2}$ was extended to several hours.

(2) Excretion of 2. During the 72 h following oral administration, a mean of 98.3 ± 2.5% and 97.5 ± 4.1% of the dose was recovered in the excreta and cage washings from male and female rats. For males 31.2 ± 4.2% was excreted in urine and 60.8 ± 0.4% in feces, while for females 40.5 ± 8.7% was excreted in the urine and 50.0 ± 2.6% in the feces. An average of 0.91 ± 0.36% and 2.03 ± 1.1% of the dose was excreted in the urine as **2** for male and female rats. Likewise, for both sexes, only ~6.5% of the dose was present in feces as **2**. Most of the drug-related material in excreta was found to be a polar metabolite (see below). These studies are suggestive that most of the dose was absorbed and extensively metabolized. Intact drug and the polar metabolite were eliminated by excretion into both the bile and urine.

(3) Tissue Distribution. Concentrations of total radiocarbon of **2** in liver, kidney, lung, muscle, brain, and gonads for male and female rats were measured and are summarized in Table IV. Peak concentrations in tissues occurred within 1 h, and for all tissues except testes and

(8) Sigel, C. W.; Ling, G. V.; Bushby, S. R. M.; Woolley, J. L.; DeAngelis, R. L.; Eure, S. *Am. J. Vet. Res.* **1981**, *42*, 996.

Scheme III



ovaries, the elimination profiles paralleled the serum profiles closely. Of the nonexcretory organs, lung had the highest tissue/serum ratios. The concentrations in brain tissue were very similar to corresponding serum concentrations.

(4) Metabolite Identification. Serum samples collected during the first 2 h after treatment were analyzed by TLC, before and after extraction. By 0.5 h, about 40% of the radioactivity in serum was a polar compound(s), which did not migrate from the origin of the TLC plate with a $CHCl_3$/*i*-PrOH/NH_4OH (25:20:1) solvent system. With time, the percentage of the polar material increased. As determined with a scanning spectrodensitometer, the distribution of radiocarbon on thin-layer chromatograms of extracts of urine and feces showed similar patterns. Further TLC studies indicated that an *n*-BuOH/AcOH/H_2O (6:1:1) system converted the metabolite into a much less polar compound that migrated with the above $CHCl_3$/*i*-PrOH/NH_4OH or an aqueous propanol solvent system. The polar metabolite did not appear to be a conjugate of **2** since enzymatic hydrolysis did not alter it.

To obtain a larger quantity of the metabolite, urine was collected for 2 days from 10 rats that were dosed (20 mg/kg) with unlabeled **2**. The metabolite was isolated by sequential purification steps by using XAD-2 column chromatography and preparative TLC and HPLC. High resolution MS data was consistent with a $C_{17}H_{20}N_4O_2$ formula, which was indicative of the addition of one oxygen and loss of four hydrogens from **2**. On that basis, structure **44** was proposed; however, that compound would not have the polarity of the unknown metabolite. A more reasonable structure for the polar metabolite would be the hydroxy acid **43**, which might be converted to the lactone **44** on heating or acid treatment (as observed during TLC analyses) (Scheme III). The NMR spectrum of the isolated material provided further evidence that one isopropyl methyl was altered, but it was not possible to distinguish between the hydroxy acid or lactone structure. There is precedent in the literature for the metabolic oxidation of an isopropyl methyl to a carboxyl group. Isopropylbiphenyl is converted to 2-biphenylpropionic acid by the rat.[9]

Discussion

New agents are needed for the treatment of gonorrhea. Although penicillin is still considered the therapy of choice, a large amount must be given in combination with probenecid. In addition, the incidence of antibiotic-resistant gonorrhea, and penicillinase-producing *N. gonorrhoeae* (PPNG) in particular, is rapidly increasing. Between 1984 and 1986, there was a 4-fold increase in PPNG (to 16 000 cases) in the United States. Unfortunately, once antibiotic resistance becomes endemic, eradication is extremely difficult and expensive.[10]

Trimethoprim (**1**) has been found effective in the treatment of gonorrhea when used in combination with a sulfonamide, such as sulfamethoxazole.[11] However, relatively high doses are required, and treatment failures sometimes occur.

The 25-fold greater in vitro antigonococcal activity of **2**, compared to **1**, suggested that it might serve as a therapeutic agent for gonorrhea, without need for a sulfa synergist.

In the absence of an animal model for this disease, early pharmacokinetic and metabolic studies with these compounds were considered essential for their therapeutic evaluation. Experience with **1** suggested that conjugation and excretion might well occur with the straight-chain, but not branched-chain, 3,5-dialkyl-4-hydroxybenzyl derivatives, and this proved to be the case. Despite lower selectivity for bacterial DHFR, the phenolic derivatives were prime targets for study because of their considerably greater activity than their 4-methoxy counterparts. A relatively poor therapeutic index compared to **1** would probably assume less importance for the short-term therapy envisioned here, in contrast with long-term treatment of other diseases.

The actual metabolic fate of **2**, to produce **43** as a major metabolic product, with less than 1% excreted as intact drug in the urine after 24 h, was a surprising and unfortuitous result. Although animal metabolism is often exaggerated over that of man, the dog, in contrast to rodents, has been a reasonably reliable model for man for analogues of **1**. The extensive metabolism of **2** became one of the factors that led to a decision to discontinue development of **2** and other compounds in this series for treatment of gonorrhea.

Experimental Section

A. Chemistry. Melting points were determined with a Thomas-Hoover melting point apparatus and are uncalibrated. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.04% of the theoretical values. Nuclear magnetic resonance (NMR) spectra were recorded on Varian XL-100 and T-60 spectrophotometers; chemical shifts are reported in parts per million (δ) from internal tetramethylsilane. The elemental analyses were performed by Dr. Stuart Hurlbert and his staff, and by Atlantic Microlab, Inc., Atlanta, GA. Synthesis of [2-^{14}C]-**2** was carried out by Dr. John Hill of these laboratories.

2,4-Dichloro-5-(3,5-diisopropyl-4-hydroxybenzyl)pyrimidine (10). Two grams (6.6 mmol) of 5-(3,5-diisopropyl-4-hydroxybenzyl)uracil[12] (**8**) was chlorinated by heating for 1.5 h in $POCl_3$, followed by removal of excess $POCl_3$, addition of the residue to ice water, and neutralization. The resultant precipitate (1.93 g, **10**) was crystallized from 70% EtOH: mp 105–108 °C. Anal. ($C_{17}H_{20}Cl_2N_2O$·0.3H_2O) C, H, N.

2,4-Diamino-5-(3,5-diisopropyl-4-hydroxybenzyl)pyrimidine (2).[4] A mixture of 1.57 g (4.6 mmol) of **10** and 35 mL of saturated NH_3/EtOH was heated in a bomb at 165 °C for 8 h. The solvent was removed, and 150 mL of $CHCl_3$ and 90 mL of 0.5 M $NaHCO_3$ were added. The product was extracted into the $CHCl_3$, and the aqueous layer was extracted four times with 50-mL portions of $CHCl_3$/MeOH (3:1). The combined organic layers

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were dried, and the solvent was removed; 1.2 g (86%) (**2**): mp 244–246 °C (MeOH); NMR (Me_2SO-d_6) δ 1.13 (d, 12, ($CHMe_2$)$_2$), 3.27 (septet, 2, ($CHMe_2$)$_2$), 3.48 (s, 2, CH_2), 5.60 (s, 2, NH_2), 6.00 (s, 2, NH_2), 6.82 (s, 2, Ar), 7.42 (s, 1, pyr-6-H), 7.78 (br, 1, ArOH).

5-(3,5-Di-*tert*-butyl-4-hydroxybenzyl)uracil (7). A. From 5-(Hydroxymethyl)uracil (3). A mixture of 7.1 g (0.05 mol) of **3**,[13] 10.3 g (0.05 mol) of 2,6-di-*tert*-butylphenol (**4**), 200 mL of glacial AcOH, and 7 mL of concentrated HCl was heated under reflux for 6 h, after which the solvent was removed in vacuo. The residual white solid was mixed with 100 mL of water, and the pH was adjusted to neutrality with dilute NaOH. The resultant heavy precipitate was isolated and recrystallized from β-methoxyethanol/H_2O; 6.3 g (38.4%) (**7**): mp 326–329 °C dec; NMR (Me_2SO-d_6) δ 1.35 (s, 18, (CMe_3)$_2$), 3.38 (s, 2, CH_2), 6.18 (br, 1, ArOH), 6.95 (s, 2, Ar), 7.12 (s, 1, pyr-6-H), 10.8 (br, 2, (NH)$_2$). Anal. ($C_{19}H_{26}N_2O_3$·0.25H_2O) C, H, N.

B. From Uracil (5) plus a Phenolic Mannic Base (6). To 65 mL of ethylene glycol were added 5.6 g (0.05 mol) of **5**, 13.2 g (0.05 mol) of 2,6-di-*tert*-butyl-4-[(*N*,*N*-dimethylamino)-methyl]phenol (**6**)[14] and 2.7 g (0.05 mol) of NaOMe. The reaction mixture was heated under nitrogen to 150 °C for 6 h, and the Me_2NH that was released was trapped in portions of dilute sulfuric acid in order to follow the course of the reaction. The mixture was cooled and diluted with 250 mL of H_2O, followed by the addition of acetic acid to adjust the pH to about 4.5. The resultant precipitate was filtered and washed with water and ether; yield, 13.0 g (78.7%) of **7**. The NMR spectrum was identical with that obtained by method A.

2,4-Dichloro-5-(3,5-di-*tert*-butyl-4-hydroxybenzyl)pyrimidine (9). A 3.3 g portion of **7** was heated for 2.5 h in 40 mL of $POCl_3$, followed by removal of the excess chlorinating agent. The residual oil was slurried in 100 mL of ice water and neutralized with Na_2CO_3, which yielded a white precipitate, 2.6 g (70.9% crude, **9**: mp 145.5–146.5 °C (EtOH); NMR ($CDCl_3$) δ 1.42 (s, 18, (CMe_3)$_2$), 3.95 (s, 2, CH_2), 3.95 (s, 2, CH_2), 5.18 (s, 1, ArOH), 7.00 (s, 2, Ar), 8.29 (s, 1, pyr-6-H). Anal. ($C_{19}H_{24}Cl_2N_2O$) C, H, Cl, N.

2,4-Diamino-5-(3,5-di-*tert*-butyl-4-hydroxybenzyl)pyrimidine (11). A. By Amination of 9. A 3.77-g (0.010-mol) portion of **9** in 35 mL of absolute EtOH was mixed with 30 mL of EtOH saturated with NH_3 at 0 °C and heated in a sealed vessel at 165 °C for 8 h. The solvent was removed, and 100 mL of 0.05 M $NaHCO_3$ was added; the pH was adjusted to 8.5. The aqueous mixture was extracted four times with 100 mL of $CHCl_3$, and the $CHCl_3$ was evaporated. The residue was purified on a silica gel column, with $CHCl_3$ elution, followed by $CHCl_3$/MeOH, first 50:1 and then 25:1. There was isolated 1.72 g (24.7%) of **11**: mp 208–210 °C (MeOH); NMR ($CDCl_3$) δ 1.43 (s, 18, (CMe_3)$_2$), 3.63 (s, 2, CH_2), 4.56 (br, 2, NH_2), 5.12 (s, 1, ArOH), 6.96 (s, 2, Ar), 7.73 (s, 1, pyr-6-H). Anal. ($C_{19}H_{26}N_4O$) C, H, N.

Other products isolated from the reaction included **2-(or 4-)-amino-4-(or 2-)-chloro-5-(3,5-di-*tert*-butyl-4-hydroxybenzyl)pyrimidine (14)** and **2-(or 4-)-amino-4-(or 2-)-ethoxy-5-(3,5-di-*tert*-butyl-4-hydroxybenzyl)pyrimidine (15).** These were identified by NMR and MS, giving M^+ ions of 348 and 357, respectively. **14**: NMR ($CDCl_3$) δ 1.44 (s, 18, (CMe_3)$_2$, 3.69 (s, 2, CH_2), 4.85 (br, 1, ArOH), 5.24 (br d, 2, NH_2), 6.88 (s, 2, Ar), 7.91 (s, 1, pyr-6-H). **15**: NMR ($CDCl_3$) δ 1.3 (s, 21, (CMe_3)$_2$), CH_2CH_3), 3.6 (s, 2, CH_2), 4.3 (q, 2, CH_2CH_3), 4.5 (br, 1, ArOH), 4.7 (br, 1, NH), 5.0 (br, 1, NH), 6.90 (s, 1, Ar), 6.95 (s, 1, Ar), 7.76 (s, 1, pyr-6-H). Several similar aminations that were carried out in ethanol saturated with NH_3 at 140 °C for 7–8 h gave poorer yields of **11**. A somewhat higher temperature and a saturated NH_3 solution is recommended. No mono-(*tert*-butylbenzyl)pyrimidine was isolated by this reaction sequence.

B. From 2,4-Diamino-5-(hydroxymethyl)pyrimidine (12). A mixture of **12** (7.0 g, 0.05 mol), **4** (10.3 g, 0.05 mol), 225 mL of glacial AcOH, and 6.7 mL of concentrated hydrochloric acid was heated together at reflux for 11 h. The solvent was removed, and the yellow residual oil was taken up in 0.5 M $NaHCO_3$ solution and $CHCl_3$. The aqueous layer was adjusted to pH 8.5, and several extractions with $CHCl_3$ were combined, dried over $MgSO_4$, clarified, and evaporated to dryness. The residue was purified on a silica gel column with $CHCl_3$/MeOH (30:1) elution initially and then 18:1 and 9:1 elution ratios. Two products were isolated and identified as **11** and **2,4-diamino-5-(3-*tert*-butyl-4-hydroxybenzyl)pyrimidine (13).** The former (**11**) was recrystallized as the free base from MeOH; mp 208.5–210.5 °C (10.3%). The NMR spectrum was identical with that from experiment A.

Compound **13** was crystallized as the hydrochloride salt: mp 274–281 °C (absolute EtOH); 5.4%; NMR (Me_2SO-d_6) δ 1.32 (s, 9, CMe_3), 3.54 (s, 2, CH_2), 6.75 (m, 2, Ar), 7.01 (br, 1, Ar), 7.36 (s, 1, pyr-6-H), 7.54 (br, 2, NH_2), 7.90 (br, 2, NH_2), 9.24 (s, 1, ArOH), 12.10 (br, 1, NH^+); MS, *m/e* 272 (M^+), 257, 215, 123. Anal. ($C_{15}H_{18}N_4O$·HCl) C, H, N.

A repeat preparation on a 0.1-mol scale gave 4.9% and 4.2%, respectively, of **11** and **13**.

2,4-Diamino-5-(3-*tert*-butyl-4-methoxybenzyl)pyrimidine (16). Compound **13** (0.82 g, 3 mmol) was methylated in Me_2SO (10 mL) to which was added 0.2 g (3 mmol) of KOH. After all was in solution, 0.19 mL (3 mmol) of MeI was added, and the mixture was allowed to stand overnight under N_2 at room temperature. The precipitate was then isolated and recrystallized twice as the HCl salt from MeOH: mp 267–270 °C; NMR (Me_2SO-d_6) δ 1.30 (s, 9, CMe_3), 3.59 (s, 2, CH_2), 3.76 (s, 3, OMe), 6.91–7.08 (m, 3, Ar), 7.38 (s, 1, pyr-6-H), 7.47 (br, s, NH_2), 7.93 (br, 2, NH_2), 11.8 (s, 1, NH). Anal. ($C_{16}H_{22}N_4O$·HCl·0.5H_2O) C, H, N.

3,5-Diisopropyl-4-hydroxybenzaldehyde (18).[15] A mixture of 267.5 g (1.5 mol) of 2,6-diisopropylphenol (**17**), 420.6 g (3.0 mol) of hexamethylenetetramine, 1245 mL of glacial AcOH, and 255 mL of water was heated to refluxing for 5 min, at which time a distillation head was introduced, and approximately 100 mL of distillate was collected; the pot temperature was then at 118 °C. The distillation was discontinued, and refluxing was continued for 5.5 h, after which the mixture was partially cooled, and 300 mL of water was added. The mixture was chilled to 0 °C, resulting in the precipitation of a yellow crystalline solid, which was isolated, washed with three 2-L portions of cold water, followed by isolation of the product; yield, 250.7 g (81%). Recrystallization from *i*-PrOH produced a product showing a single spot by TLC (3:2 hexane/Et_2O): mp 101–103 °C. Anal. ($C_{13}H_{18}O_2$) C, H.

2,6-Diisopropyl-4-(dimethoxymethyl)phenol (19). A mixture of 240 g (1.16 mol) of **18**, 787 g (7.42 mol) of methyl orthoformate, 2.46 g (0.046 mol) of NH_4Cl, and 700 mL of MeOH was refluxed for 3.5 h, cooled, and added to 2760 mL of 3 N NH_4OH at 10 °C. A yellow oily suspension was formed, which was extracted with CH_2Cl_2, followed by extraction of the CH_2Cl_2 solution with water and then drying the organic solution over Na_2CO_3. The solvent was removed, leaving a dark oil, 316 g, which was dissolved in approximately 300 mL of hot hexane and allowed to cool slowly. This resulted in the crystallization of **19**: mp 56–58 °C. Anal. ($C_{15}H_{24}O_3$) C, H.

4-(Benzyloxy)-3,5-diisopropylbenzaldehyde (20). To a suspension of 51.1 g (1.06 mol) of 50% NaH in 200 mL of dry DMF was added 243 g (0.96 mol) of **19** dissolved in 350 mL of DMF over a 25-min period, while the flask was cooled in an ice bath. The solution was stirred for 20 min, the ice bath was removed, and then 181 g (1.06 mol) of benzyl bromide in 200 mL of DMF was added dropwise over 1 h. During this time, the temperature rose to 40 °C. The mixture was allowed to stand overnight, followed by the slow addition of 50 mL of 1 N HCl. The solvent was removed in vacuo, and the residue was mixed with 1 L of acetone, followed by filtration from salts. Then, 150 mL of 1 N HCl was added, and the mixture heated under reflux for 30 min. Most of the solvent was removed, and 1 L of CH_2Cl_2 was added, followed by washing this with 1 N HCl and then water and drying over $MgSO_4$. The solvent was removed, leaving a yellow oil (**20**) (275 g), which crystallized after standing for 10 days at 0 °C: mp 48–50 °C (*i*-PrOH/H_2O). Anal. ($C_{20}H_{24}O_2$) C, H.

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3-Anilino-2-[4-(benzyloxy)-3,5-diisopropylbenzyl]acrylonitrile (22). A solution of 156.7 g (1.12 mol) of β-morpholinopropionitrile (**21**) in 315 mL of Me_2SO was heated to 65 °C, followed by the addition of 24.2 g (0.45 mol) of NaOMe in 350 mL of Me_2SO. The mixture was heated to 80 °C for 45 min, followed by the addition of 265 g (0.89 mol) of **20** in 500 mL of Me_2SO over a 20-min period. Heating was continued for 2 h, followed by cooling and addition of 3 L of H_2O plus 6 N HCl to neutrality. The product was extracted into Et_2O, washed with 1 N HCl and water, and dried over $MgSO_4$, and the solvent was evaporated, leaving 338 g of a dark yellow oil, which did not solidify on standing several days. This was then dissolved in 500 mL of hot 95% EtOH, and 112.8 g (0.87 mol) of aniline hydrochloride in 400 mL of EtOH was added with stirring. An exothermic reaction occurred, and a precipitate formed. The solution was refluxed for 1 h, filtered, and washed with EtOH; yield of product, 204 g (61% crude **22**): mp 187–195 °C (EtOH/Me_2CO, 5:1). Anal. ($C_{29}H_{32}N_2O$) C, H, N.

2,4-Diamino-5-[4-(benzyloxy)-3,5-diisopropylbenzyl]pyrimidine (23). Guanidine hydrochloride (90.8 g, 0.95 mol) was slurried in 500 mL of EtOH and added to a solution of 76.7 g (1.42 mol) of NaOMe in 500 mL of EtOH. The mixture was heated to the boil for several minutes, cooled, and filtered directly into a reaction flask containing 201 g (0.47 mol) of **22** in 1 L of EtOH. The mixture was heated under reflux for 18 h, at which time the reaction was complete, as indicated by TLC. A distillation head was added to the apparatus, and 1 L of solvent was removed. The mixture was cooled, and the precipitate was isolated; yield, 164 g (89% crude **23**): mp 210–212 °C (80% EtOH). Anal. ($C_{24}H_{30}N_4O$) C, H, N.

2,4-Diamino-5-[4-(benzyloxy)-3,5-diisopropylbenzyl][2-^{14}C]pyrimidine (2-^{14}C-23). The preparation of **23** was repeated on a 2.535 mmol scale with 2-[^{14}C]guanidine hydrochloride, assayed at 14.2 mCi/mmol (22.76% isotopic abundance). The mixture was kept under argon during the course of the reaction and worked up as before; yield, 909 mg (91.8%) of 2-^{14}C-**23**, which was identical by TLC with **23**.

2,4-Diamino-5-(4-hydroxy-3,5-diisopropylbenzyl)-[2-^{14}C]pyrimidine (2-^{14}C-2). To a suspension of 105 mg of 5% Pd/C in 10 mL of glacial HOAc was added 909 mg of **23**, which was transferred with the aid of 65 mL of HOAc. The mixture was warmed for a few minutes to dissolve the pyrimidine, and the mixture was then hydrogenated at atmospheric pressure, with hydrogen over a calibrated water reservoir. The hydrogenolysis was allowed to proceed over a 22-h period, while the mixture was stirred well. At this time, 1 atm of H_2 had been consumed, and TLC with $CHCl_3$/MeOH/NH_4OH (90:10:1) showed single spot material, corresponding to **2**. The product was filtered through Celite, and after thorough washing, the solvent was removed in vacuo, leaving an off-white crystalline solid. This was washed well with NH_4OH and water and dried under high vacuum; yield, 612 mg (87%). No impurities were detected by scanning a TLC plate with a Vangard VS-940 plate scanner. The specific activity of the product was found to be 16.4 mCi/mmol.

B. In Vitro Antibacterial Assays. Routine minimal inhibitory concentration (MIC) tests were carried out in Wellcotest Sensitivity Agar containing 5% lysed horse blood.[6] For these studies, the compounds were sterilized by dissolving in dimethylformamide. After 30 min, the solutions were diluted into sterile distilled water, and the compounds that precipitated were dissolved as the isethionate salts by slowly adding 1 N isethionic acid. MIC values for selected organisms are shown in Table I. The antibacterial assays against *N. gonorrhoeae* were performed in a separate broth dilution test with strain F62 obtained from Dr. P. F. Sparling.[16] Briefly, stock inhibitor solutions were prepared by dissolving the compound in deionized water. Dilute HCl was added, if necessary, to facilitate dissolution. The solution was then neutralized and filter sterilized by membrane filtration (0.2-μM pore size; Nucleopore Corp.), and the concentration of the compound was determined spectrophotometrically. Tubes containing 1.0-mL volumes of test compounds diluted in GCB broth (Difco) were inoculated with about 3×10^7 cfu and incubated 18 h at 37 °C under an 8% CO_2 atmosphere. Endpoints correspond to approximately 90% inhibition of growth as measured turbidimetrically.

C. Enzyme Assays and I_{50} Determinations. The standard assay for DHFR activity was performed in 0.1 M imidazole chloride buffer, pH 6.4, 12 mM mercaptoethanol, with 60 μM NADPH and 40 μM dihydrofolate. Reaction velocities were monitored by decreases in 340-nm absorbance. The concentration of inhibitor necessary to inhibit DHFR activity by 50% is called the I_{50} value.

D. Pharmacokinetics and Metabolism. For quantitative TLC analysis, samples were applied with an electronically driven "TLC multispotter" (Analytical Instrument Specialties, Libertyville, IL). UV absorbance measurements on TLC plates were made by scanning with a Schoeffel SD 3000 spectrodensitometer (Kratos Schoeffel Instruments, Westwood, NJ). The total reflected emission from the surface of the plate was determined by using a Schoeffel SDC 300 density computer. Peak areas for samples and reference compounds were simultaneously recorded on a Honeywell Electronik 124 recorder and integrated with an Autolab System IV integrator. Samples were analyzed for total radiocarbon by combustion with a Packard Tri-Carb Sample Oxidizer, Model 306. Liquid scintillation counting (LSC) was carried out with a Packard Tri-Carb, Model 3320, liquid scintillation spectrometer. Radioactive zones on TLC plates were scanned with a Berthold Model LB 2760 scanning spectrodensitometer (Shandon Southern, Sewickley, PA). The NMR spectra were measured on a Varian XL-100-15-FT spectrometer.

Disposition Studies in Dogs. For oral absorption studies, four beagle dogs (two males and two females) received single doses (5 mg/kg) of the drug in a gelatin capsule. The dogs were fasted for 18 h before the dose, but were fed a standard ration 5 h after the dose. The dogs were housed in metal metabolism cages that were equipped with stainless steel collection pans to facilitate collection of urine, which was done for the 24-h period before the dose and at 24-h intervals for 3 days after the dose. Blood samples (7 mL) were obtained from each dog by jugular venous puncture before each dose and at 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, and 24.0 h after the dose. Each sample was allowed to clot for 15 min at room temperature and centrifuged at 2500 rpm for 15 min. The serum was removed and stored at −20 °C until assayed.

For iv studies, compounds dissolved in propylene glycol were administered (5 mg/kg) to two male beagle dogs. Blood samples were obtained at 0.17, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, and 8.0 h after the dose. Serum was collected as described above. Urine was collected for 24 h after the dose.

Drug Concentration in Dog Serum and Urine. The concentrations of the benzylpyrimidines in serum and urine were measured by making appropriate modifications to the sensitive and specific quantitative TLC methods developed in this laboratory for **1** and related compounds.[17] The compounds with hydroxy groups on the benzene ring were extracted from serum (1 mL) with two 5-mL portions of *i*-PrOH/CH_2Cl_2 (1:4) after adjustment of the serum to pH 8.0 with 1 N NaOH. Otherwise, the pH was adjusted to 12.5. Chloroform was used to extract the compounds from urine at the appropriate pH. The reproducibility of recovery (N = 5 samples) of the compounds added to serum (0.75–1.0 μg/mL) was **2**, 93.4 ± 4.6; **26**, 102.1 ± 12.3; **33**, 71.3 ± 18.5; **37**, 74.2 ± 1.0; **61**, 98.2 ± 7.7; **11**, 98.4 ± 11.5. For quantitative analysis, silica gel TLC plates (EM brand) were developed in $CHCl_3$/*i*-PrOH/28% NH_4OH (320:80:1) for compounds **2**, **37**, and **61** and in $CHCl_3$/*i*-PrOH/28% NH_4OH (80:20:1) for compounds **11**, **26**, and **33**. Plates were scanned in the absorbance mode with a Schoeffel spectrodensitometer.[17]

Pharmacokinetic parameters were estimated from serum concentrations of the benzylpyrimidines administered iv and for those compounds that had measurable blood levels following an oral dose. The elimination rates were calculated by a least-squares linear regression analysis of the mean serum concentration–time curves. By applying the trapezoidal rule, the areas under the serum concentration–time curves (AUC) were calculated.[18]

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Disposition Studies in Rats. a. Animals. Thirty-six male (group I) and 36 female (group II) Charles River Sprague–Dawley rats (100–150 g) were used. During a 1–2-week acclimation period, the rats were maintained on standard ration (Wayne Lab Blox) and had free access to water. The rats were fasted for 18 h prior to drug treatment. Three rats from both groups I and II were housed in individual glass metabolism cages that separated urine and feces. The remaining rats from each group were divided into 11 subgroups of three animals, and each subgroup was housed in a separate cage. The cages and animals were marked as to the time of sacrifice. The cages were set up so that the rats could not come into contact with excreta.

b. Drug Treatment and Sample Collection. The rats were weighed just prior to treatment and received an appropriate dose (~0.5 mL) orally of 10 mg/kg of 2-^{14}C-**2** dissolved in 0.02 N lactic acid (0.055 mCi/animal). The subgroups (three animals each) were killed at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6, 10, and 24 h after treatment. Rats from each group housed in metabolism cages were killed at 72 h after the dose. Blood (3–5 mL) was obtained from each rat; it was allowed to clot at room temperature for about 30 min and centrifuged to remove serum. Serum was stored frozen (−20 °C) until assay. From each of the rats, the following tissues were obtained: liver, kidney, muscle, brain, lung, stomach, intestines, and testes or ovaries. Cumulative urine and feces were collected at 24-h intervals for the rats housed in metabolism cages.

c. Assay Methods. Samples were analyzed for total radiocarbon by combustion and LSC. Two-dimensional TLC autoradiography was used to define the pattern of urinary metabolites, including the extent of conjugation (analysis of urine before and after treatment with β-glucuronidase and sulfatase). Quantities of intact drug and the major metabolite in blood and excreta were measured by LSC following TLC separation.

Isolation and Identification of a Metabolite of 2. To obtain a sufficient quantity of the **2** metabolite for characterization, 10 rats were dosed on 2 consecutive days with unlabeled **2** (20 mg/kg), and urine was collected for 48 h. To expedite purification of the metabolite, some of the urine from rats that received radiolabeled drug was added to the urine from the cold experiment. The combined urine was applied to an XAD-2 column, which was washed with water and eluted with methanol. The methanol fraction had 91% of the radioactivity. After evaporation of the solvent, the residue was applied to several 0.5-mm, 20 × 20 silica gel TLC plates, which were developed with a 20% aqueous *n*-PrOH solvent system. The major metabolite ($R_f \cong 0.6$) was eluted from the silica gel with MeOH and further purified by HPLC with a μ-Bondapak C_{18} column with an acetonitrile/water (1:4) mobile phase. The solvent was evaporated, and the residue analyzed by MS. The accurate mass of the highest ion observed was determined by peak matching at 10 000 resolution, 10% valley definition, and found to be 312.1581 (calcd for $C_{17}H_{20}N_4O_2$ 312.1576). The NMR spectrum indicated that one isopropyl methyl was altered: NMR (Me_2SO-d_6) δ 1.11 (m, 6), 1.25 (d, 3), 3.1–3.6 (m, 2), 3.42 (s, 2), 5.59 (s, 2), 5.92 (s, 2), 6.57 (d, 1), 6.75 (d, 1), 7.40 (s, 1). These spectral data were consistent with a **2,4-diamino-5-[4-hydroxy-3-isopropyl-5-(α-carboxyethyl)benzyl]pyrimidine** structure (**43**) for the metabolite.

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Registry No. **2**, 42310-33-8; 2-^{14}C-**2**, 110798-52-2; **3**, 4433-40-3; **4**, 128-39-2; **5**, 66-22-8; **6**, 88-27-7; **7**, 73943-43-8; **8**, 84876-25-5; **9**, 73943-44-9; **10**, 110798-40-8; **11**, 73554-80-0; **12**, 42310-45-2; **13**·HCl, 110798-41-9; **14** (isomer 1), 110798-42-0; **14** (isomer 2), 110798-48-6; **15** (isomer 1), 110798-43-1; **15** (isomer 2), 110798-49-7; **16**, 110798-44-2; **16**·HCl, 110798-50-0; **17**, 2078-54-8; **18**, 10537-86-7; **19**, 110798-45-3; **20**, 108402-07-9; **21**, 4542-47-6; **22**, 110798-46-4; **23**, 110798-47-5; 2-^{14}C-**23**, 110798-51-1; **24**, 42310-36-1; **25**, 105639-88-1; **26**, 36821-97-3; **27**, 73554-79-7; **28**, 73554-78-6; **29**, 105639-89-2; **30**, 73576-30-4; **31**, 105639-85-8; **32**, 36821-88-2; **33**, 105639-86-9; **34**, 36821-94-0; **35**, 36821-90-6; **36**, 105639-87-0; **37**, 39667-06-6; **38**, 110798-54-4; **39**, 110798-55-5; **40**, 110798-56-6; **41**, 110798-57-7; **62**, 110798-53-3; 2-[^{14}C]guanidine hydrochloride, 73549-39-0; benzyl bromide, 100-39-0; guanidine hydrochloride, 50-01-1.

Evaluation of the Importance of Hydrophobic Interactions in Drug Binding to Dihydrofolate Reductase

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The interaction of dihydrofolate reductase (DHFR) from *Escherichia coli* with drugs such as methotrexate (MTX) and 2,4-diamino-6,7-dimethylpteridine (DAM) has been studied by means of site-directed mutagenesis, fluorescence spectroscopy, and steady-state as well as transient kinetics. A strictly conserved residue at the dihydrofolate binding site of DHFR, phenylalanine-31, has been replaced with tyrosine or valine to ascertain the importance for binding of this hydrophobic amino acid, which interacts with both the pteridine ring and the *p*-aminobenzoyl moiety. The first mutation (Phe-31 → Tyr) has a minimal effect on the binding of the classical inhibitor, DAM. On the other hand, the second mutation (Phe-31 → Val) has increased the dissociation constant of DAM from the DHFR·NADPH·DAM ternary complex over 150-fold (>3 kcal/mol). The dissociation constant of DAM from the (Val31-DHFR)·DAM binary complex was too large to be measured fluorometrically. More importantly, these mutations have decreased the overall tight binding of MTX, from 100- to 140-fold (corresponding to a loss of binding energy of 2.2–2.4 kcal/mol) for the Tyr-31 and Val-31 mutants, respectively. These results indicate that hydrophobic interactions between MTX and DHFR are at least as important as formation of the MTX·DHFR salt bridge in the tight binding of MTX.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3; DHFR) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F). The enzyme is necessary for maintaining intracellular pools of H_4F and its derivatives which are essential cofactors in the many important biosynthetic reactions which require the transfer of one-carbon units. An anti-folate drug, methotrexate (MTX), differs from the natural folates in only two positions: a methyl group replaces the N-10 hydrogen and an amino group replaces the C-4 keto function of the pteridine ring. These slight molecular changes produce a potent inhibition of DHFR and ultimately inhibit the synthesis of purines, pyrimidines, and DNA due to the loss of nucleotide production. Because of this enzyme's biological and pharmacological importance, it has been the subject of extensive studies, both structural and kinetic, over the past three decades.[1-3]

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