pension in 5% gum arabic 3 days after adjuvant injection. The surface temperature of the paw was measured by a contact-type thermometer (Thermomex TH-10, Natsume Seisakusho, Tokyo, Japan) as reported previously.[7]

Carrageenin-Induced Paw Edema. Test compounds as a suspension in 5% gum arabic were orally administered to groups of five male Sprague–Dawley rats (Shizuoka Laboratory Animal Center). Paw edema was induced by subplantar injection of 0.05 mL of 1% λ-carrageenin in 0.9% saline into the right hind paw 1 h after the drug administration. At 3 h after carrageenin injection, the increase in hind paw volume was determined by a water-displacement method.[7,8]

Flection Pain Test. Among the rats used in the local hyperthermia test described above, the rats exhibiting squeaking responses to gentle flection of the affected joints were selected 15–20 days after adjuvant injection.[9] Test compounds as a suspension in 5% gum arabic were orally administered to groups of 5–14 rats, and a series of five flection stimuli were applied at 1, 3, 5, 7, 9, and 11 h after administration. The squeaking responses were counted on each occasion.

Adjuvant-Induced Arthritis. Male Fisher strain rats (F_{344}, 6 week old, Charles River) were given 0.05 mL of a 1% suspension of adjuvant (*M. butyricum*) in liquid paraffin by injection into the subplantar tissue of the right hind paw. Test compounds in 5% gum arabic were orally administered daily from the day of adjuvant injection to day 21. At 24 h after the last administration, the increase in volume of the adjuvant-injected paw was measured by a water-displacement method.

PGE₂ and LTB₄ Generation. Rat synovial cells (2×10^5 cells/well) were cultured for 15 h with a factor derived from rat polymorphonuclear leucocytes which stimulated PGE_2 generation as well as collagenase generation.[10] Test compounds in dimethyl sulfoxide and the factor were simultaneously added; the final concentration of dimethyl sulfoxide was 0.1%. PGE_2 in the culture medium was determined by using a radioimmunoassay kit (NEN). Human neutrophils (2×10^5 cells) were incubated with compounds at 37 °C for 10 min, and A23187 at a final concentration of 1 µg/mL was added to the cells.[11] After 10 min, the cells were pelleted by centrifugation, and LTB_4 in the supernatant was quantitated by using a radioimmunoassay kit (Amersham).

Gastric Ulceration. Male Fisher strain rats (7 week old) were fasted for 24 h. Test compounds as a suspension in 5% gum arabic were administered orally to groups of 6–12 rats. After 6 h, the rats were killed, the stomachs were removed, and gastric lesions on the mucosa were determined by using a stereoscopic microscope. "Ulcer" was defined as at least one lesion that was 0.5 mm or more in length. All lesions of more than 0.1 mm in length were summed to obtain the ulcer index.

Plasma Levels of Compounds. Test compounds were orally administered as a suspension in 5% gum arabic to Sprague–Dawley rats weighing 300–350 g. Blood samples were obtained by cardiac puncture, and heparinized plasma was stored at –20 °C until analysis. A mixture of 0.2 mL of plasma, 1 mL of water, and 0.2 mL of methanol containing compound 4 as an internal standard was extracted twice with 4 mL of diethyl ether. The ether phase was evaporated to dryness, the residue was dissolved in 200 µL of acetonitrile, and the 20–50 µL of the solution was injected into the HPLC instrument. HPLC analysis was performed on a Zorbax ODS column (8-µm particles, 4.6 mm × 25 cm) with 30% acetonitrile in water as an elution solvent. The flow rate was 1 mL/min, and the absorbance of the effluent was monitored at 310 nm.

(10) Hashida, R.; Kobayashi, S.; Shirota, H.; Yoshimatsu, K.; Ohsawa, S.; Hori, H.; Hattori, S.; Nagai, Y. *Prostaglandins* 1984, *27*, 697.

(11) Katayama, S.; Tsunoda, H.; Sakuma, Y.; Kai, H.; Katayama, K. *Int. Arch. Allergy Appl. Immunol.* 1987, *83*, 284.

2,4-Diamino-5-benzylpyrimidines as Antibacterial Agents. 8. The 3,4,5-Triethyl Isostere of Trimethoprim. A Study of Specificity[1,2]

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3,4,5-Triethylacetophenone was synthesized in 60% yield by a Friedel–Crafts reaction from 4-ethylacetophenone and converted to 2,4-diamino-5-(3,4,5-triethylbenzyl)pyrimidine (2), a trimethoprim (1) isostere, by standard techniques. This compound is more lipophilic than 1 by three log units (log *P*, octanol/water). Compound 2 was approximately equipotent with 1 in inhibiting *Escherichia coli* dihydrofolate reductase (DHFR), 2-fold more potent against *P. berghei* and *N. gonorrhoeae* DHFR, and 10 and 25 times better an inhibitor of rat and chicken liver DHFR, respectively. Although the 3,4-dimethoxy analogue 19 was 10-fold less inhibitory to *E. coli* DHFR than 1, it was 3–4 times *more* potent on the vertebrate isozymes, whereas the diethyl congener 10 followed 19 in its *E. coli* DHFR binding but was less active on rat and chicken DHFR. Therefore, a significant portion of the selectivity of 1 for bacterial, as opposed to vertebrate, DHFR, involves the methoxy functions. An analysis of the X-ray data on 1 and 2 complexed with chicken DHFR, coupled with kinetic data, led to the conclusion that the differences in binding energies of the methoxy and ethyl compounds probably involve desolvation factors, as well as direct energies of interaction with protein atoms. Thus, one cannot invoke lipophilicity or shape alone in explaining the relationship in properties of 1 and 2.

The 3,4,5-trimethoxy groups of trimethoprim (TMP, 1) were found at an early date to be very important to its antibacterial activity, as well as to its selectivity for bacterial dihydrofolate reductase (DHFR) over the vertebrate enzymes.[3,4] In this paper we compare the biological

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(1) Roth, B.; Aig, E.; Lane, K.; Ferone, R.; Bushby, S. R. M. *Abstracts of Papers*, 164th National Meeting of the American Chemical Society, New York; American Chemical Society: Washington, DC, 1972; MEDI 23.

(2) Roth, B. U.S. Patent 3 772 289, 1973.

properties of **1** with those of its 3,4,5-triethyl counterpart, **2**. The latter compound was expected to have essentially the same shape as **1**, qualified by differing tendencies for the meta substituents to lie in-plane and slightly different bond angles and lengths. However, the lipophilicity, as well as field and resonance characteristics, would be quite different.

1 R = OCH₃ (TRIMETHOPRIM)

2 R = CH₂CH₃
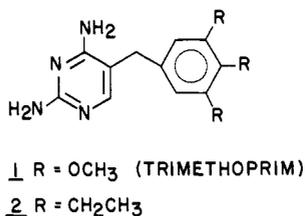
We also compare the activities of related dialkyl analogues with those of the 3,4-dimethoxybenzyl derivative, diaveridine (**19**). The high activity for bacterial DHFR created by the additional 5-methoxy group of TMP compared to that of the 3,4-dimethoxy derivative has been found to involve a cooperative effect resulting from the presence of the coenzyme, as shown by kinetic studies of binary and ternary complexes.[5,6] Recently this has been explained at the molecular level, since *Escherichia coli* DHFR has been crystallized not only with TMP in binary complex[7,8] but now as a ternary complex including the coenzyme.[9] A movement of residues 15–20 in the presence of NADPH results in a closer fit of the complex, such that one *m*-methoxy group that was formerly surrounded by water is now in contact with the Met-20 side chain of the enzyme, as well as the nicotinamide ring of the cofactor.

Both **1** and **2** have been crystallized in ternary complex with chicken liver DHFR and NADPH, which makes it possible to compare the various interatomic interactions and to further develop explanations for relative activities and selectivities with these compounds.[8,10,11]

Chemistry

3,4,5-Triethylacetophenone (**4**), a key intermediate in the synthesis of **2**, has been described by McNulty and Pearson.[12] They obtained **4** in 9% yield, along with the 3-, 3,5-, and 3,4-ethyl-substituted ketones by following the directions of Baddiley for the Friedel–Crafts alkylation of acetophenone.[13]

We chose 4-ethylacetophenone as our starting material to increase the chances of obtaining the 3,4,5-triethyl de-

Scheme I
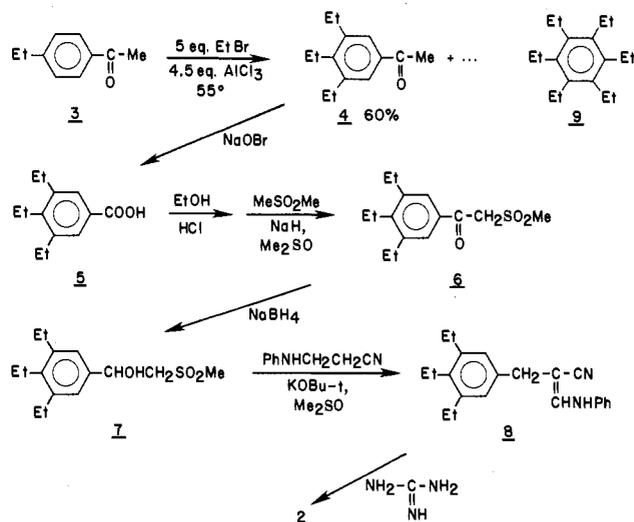
rivative in reasonable yield. Many conditions involving variations in proportions of ethyl bromide, AlCl₃, time, and temperature were tried. Scheme I describes our best reaction, which produced **4** in 60% yield along with other products, which were separated by fractional distillation. The structure of **4** was proven by derivatization[12] and by NMR spectroscopy, which showed two identical ortho protons, both in the case of **4** and in the products that followed. The methyl protons of the *m*-ethyl groups were not equivalent to that in the para position.

In a number of trial experiments using less ethyl bromide, hexaethylbenzene (**9**) was surprisingly obtained.[14]

Conversion of **4** to **5** by hypobromite oxidation[15] was followed by standard reactions leading to benzylpyrimidines, which have been previously described.[16–18] Compound **2** had a pK_a of 7.12 (20 °C), which is identical with that of **1**.[19] The NMR spectrum again showed nonequivalent signals for the *m*- and *p*-methyl moieties. The log *P*, measured in octanol/0.1 N HCl, was 1.33.[20] Trimethoprim in the same medium gave a value of −1.60; as the free base in 0.01 N NaOH, log *P* of **1** was 0.89.[21] Thus, **2** is, as expected, more lipophilic than **1** by nearly 3 log units by the above criteria. TMP, conversely, is more hydrophilic than its parent unsubstituted benzylpyrimidine by 0.7 log unit.[21]

The 3-isopropyl-4-ethyl analogue (**18**) was prepared by Friedel–Crafts alkylation of **3** with 2-bromopropane. The major fraction collected was **17**, which was converted to **18** as in Scheme I. None of the hoped for 3,5-diisopropyl-4-ethylbenzyl derivative was detected. Other (alkylbenzyl)pyrimidines described here (**10, 13, 15, 16**) were prepared similarly from readily available starting materials.

Biological Activity

Table I describes the inhibitory potency expressed as I_{50} values for the various (alkylbenzyl)pyrimidines, com-

(3) Roth, B.; Falco, E. A.; Hitchings, G. H.; Bushby, S. R. M. *J. Med. Pharm. Chem.* **1962**, *5*, 1103.

(4) Hitchings, G. H.; Burchall, J. J.; Ferone, R. *Proc. Int. Pharmacol. Meet., 3rd* **1966**, *5*, 3.

(5) Birdsall, B.; Burgen, A. S. V.; Roberts, G. C. K. *Biochemistry* **1980**, *19*, 3723.

(6) Baccanari, D. P.; Daluge, S.; King, R. W. *Biochemistry* **1982**, *21*, 5068.

(7) Baker, D. J.; Beddell, C. R.; Champness, J. N.; Goodford, P. J.; Norrington, F. E. A.; Smith, D. R.; Stammers, D. K. *FEBS Lett.* **1981**, *126*, 49.

(8) Matthews, D. A.; Bolin, J. T.; Burridge, J. M.; Filman, D. J.; Volz, K. W.; Kaufman, B. T.; Beddell, C. R.; Champness, J. N.; Stammers, D. K.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 381.

(9) Champness, J. N.; Stammers, D. K.; Beddell, C. R. *FEBS Lett.* **1986**, *199*, 61.

(10) Volz, K. W.; Matthews, D. A.; Alden, R. A.; Freer, S. T.; Hansch, C.; Kaufman, B. T.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 2528.

(11) Matthews, D. A.; Bolin, J. T.; Burridge, J. M.; Filman, D. J.; Volz, K. W.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 392.

(12) McNulty, P. J.; Pearson, D. E. *J. Am. Chem. Soc.* **1959**, *81*, 612.

(13) Baddiley, G. *J. Chem. Soc.* **1949**, S229.

(14) Galle, K. *Ber. Dtsch. Chem. Ges.* **1883**, *16*, 1744.

(15) Taylor, E. P.; Watts, G. E. *J. Chem. Soc.* **1952**, 1123.

(16) Cresswell, R. M.; Mentha, J. W. U.S. Patent 3 878 252, 1975.

(17) Cresswell, R. M.; Mentha, J. W.; Seaman, R. L. U.S. Patent 3 697 512, 1972.

(18) Roth, B.; Aig, E.; Lane, K.; Rauckman, B. S. *J. Med. Chem.* **1980**, *23*, 535.

(19) Roth, B.; Strelitz, J. A. *J. Org. Chem.* **1969**, *34*, 821.

(20) We are indebted to Dr. Edna Oppenheimer for this study.

(21) Roth, B.; Strelitz, J. Z.; Rauckman, B. S. *J. Med. Chem.* **1980**, *23*, 379.

Table I. Inhibitory Activities of 2,4-Diamino-5-benzylpyrimidines against Dihydrofolate Reductase[a]

| | benzene substituents | | | inhibitory activity (I_{50}) vs. DHFR × 10^8, M[b] | | | | |
no.	3	4	5	*E. coli*	rat liver	chicken liver	*P. berghei*	*N. gonorrhoeae*
1[c]	OMe	OMe	OMe	0.5–0.7	26000–37000	73000–78000	12	45
2	Et	Et	Et	1.0	3800	3300	7.3	23
19[d]	OMe	OMe		5–10	7800; 9000	30000; 31000	21	53
10	Et	Et		9; 9.6	5900	12000; 10000	6.8	111
18	i-Pr	Et		28	2900			70
20[d]		OMe		50; 38	17000			
16		Et		65	>4000			
15		i-Pr		110	21000			
13		t-Bu		40	23000			

[a] Assays for *E. coli*, rat, and chicken liver DHFR were carried out as described in ref 21. Homogeneous enzyme from chicken liver was a gift from J. Freisheim, University of Cincinnati. Assays for *P. berghei* DHFR were carried out as described by Ferone, R.; Burchall, J. J.; Hitchings, G. H. *Mol. Pharmacol.* 1969, 5, 49; *N. gonorrhoeae* DHFR assays were carried out as described in ref 25. In all cases the compounds were preincubated with enzyme and buffer at 37 °C, followed by the addition of NADPH and then FH_2 to initiate the reaction. [b] With assays carried out over a considerable period of time using different enzyme batches the normal variability is about ±50%. The variability over a short period is closer to ±10%, however. Ranges are shown in some cases for repeat assays. [c] Reference 3. [d] Falco, E. A.; DuBreuil, S.; Hitchings, G. H. *J. Am. Chem. Soc.* 1951, 73, 3758.

Table II. In Vitro Antibacterial Activity of 2,4-Diamino-5-benzylpyrimidines[a]

| | | | | minimum inhibitory concentration, µg/mL[a] | | | | | | |
| | benzene substituents | | | *Staphylococ-cus aureus* CN491 | *Klebsiella pneumon-iae* CN3632 | *Salmonella typhosa* CN512 | *Escheric-hia coli* CN314 | *Shigella flexneri* CN6007 | *Enterobac-ter aerogenes* 2200/86 | *Proteus vulgaris* CN329 |
no.	3	4	5							
1	OMe	OMe	OMe	0.3	1.0	0.03	0.1	0.1	0.3	1.0
2	Et	Et	Et	0.1	10	1	1	3	10	30
19	OMe	OMe		5	5	0.1	0.5	0.5	0.5	5
10	Et	Et		3	30	3	3	10	10	30
18	i-Pr	Et		3	100	10	30	30	100	>100
16		Et		30	30	10	10	10	10	30
15		i-Pr		8		16	16	32		125
13		t-Bu		10	100	10	10	30	30	100

[a] Assays were carried out as described by Bushby, S. R. M.; Hitchings, G. H. *Br. J. Pharmacol. Chemother.* 1968, 33, 72.

pared with their methoxybenzyl counterparts, against DHFR from various sources.[22-24] The activity of the target compound 2 is observed to be very close to that of 1 against bacterial DHFR. Thus, the trimethoxy and ethyl groups behave similarly in their interactions with the *E. coli* enzyme. This strongly suggests that the common feature, which is the shape of these substituents, is the most important factor for good binding in this case, rather than lipophilicity, polarity, or electronic character.[1] The same pattern holds with the disubstituted pair, 10 and 19. The more bulky derivative 18 is less active than 10, as is to be expected by comparison with data on 3,5-dialkyl-4-substituted analogues.[25] Trends are difficult to see with the less active 4-monosubstituted derivatives, the substituents of which may have little contact with the enzyme.

(22) Although the inhibitory activity of TMP as measured by its I_{50} value is approximately 50 000 times greater with *E. coli* DHFR than it is against vertebrate enzymes, the true differential binding is considerably less, since I_{50} values are not kinetic constants; however, they can be related to the true kinetic constant (K_i) as shown in the equation below, where K_m is the Michaelis constant for the competing substrate dihydrofolate, and [S] is the substrate concentration.[23] Since K_m values for

$$I_{50} = K_i(1 + [S]/K_m)$$

vertebrate DHFRs are about 10-fold lower than for *E. coli* DHFR,[24] the true selectivity of TMP for *E. coli* DHFR is closer to 3000.[6] That for the triethyl analogue (2) is only about 300, on the other hand (Table I).

(23) Cheng, Y. C.; Prusoff, W. H. *Biochem. Pharmacol.* 1973, 22, 3099.

(24) Cha, S.; Kim, S. Y. R.; Kornstein, S. G.; Kantoff, P. W.; Kim, K. Y.; Naguib, F. N. M. *Biochem. Pharmacol.* 1981, 30, 1507.

(25) Roth, B.; Rauckman, B. S.; Ferone, R.; Baccanari, D. P.; Champness, J. N.; Hyde, R. M. *J. Med. Chem.* 1987, 30, 348.

A different pattern is seen with the vertebrate enzymes in comparing 2 with 1. Here the more lipophilic compound is about 10 times more inhibitory than is 1 with rat liver DHFR and roughly 25 times more potent with the chicken enzyme. The selectivity of 1 for bacterial DHFR is then about 13–40 times greater than that of 2, in comparisons with rat and chicken isozymes. The dimethoxy and diethyl derivatives (19 and 10) on the other hand differ only 3-fold in their inhibition of chicken DHFR and give essentially the same results within experimental error against rat DHFR despite a difference in log P of over 2 units. (The measured log P of 19 in octanol/0.01 N NaOH is 0.99; that of 10 is calculated to be about 3.6, based on a value of 1.60 for the unsubstituted derivative.[20]) The more lipophilic analogue 18 is about twice as active as 10 with the rat enzyme. No differences of note are seen in comparing 20 with the monosubstituted alkyl derivatives.

A few comparisons are of interest with the *Plasmodium berghei* and *Neisseria gonorrhoeae* DHFR data. Compound 2 is about twice as inhibitory as 1 against both enzymes. However, where 19 is slightly less active than 1 against these DHFRs, 10 equals 2 in its inhibition of *P. berghei* enzyme and is 3 times as inhibitory as 19. On the other hand, 10 has significantly less potency than the other analogues for *N. gonorrhoea* DHFR. Thus the third ethyl group is important for *N. gonorrhoeal* DHFR binding but not for *P. berghei*. It would be of interest to determine binary K_D's to see whether the third ethyl group might be involved in cofactor binding, as with 1 in the *E. coli* complex.[6]

Table II illustrates in vitro antibacterial activities of the (alkylbenzyl)pyrimidines described here, compared to that of TMP. Compound 2 shows surprisingly good potency against representative Gram-positive and Gram-negative organisms, despite its very high lipophilicity, which often
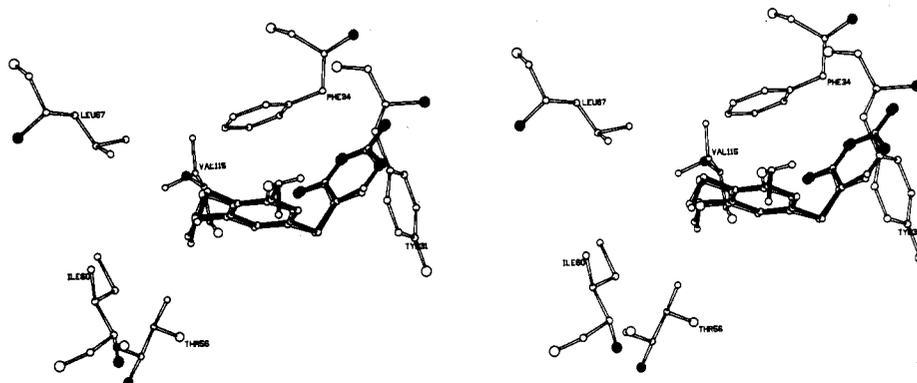
Figure 1. Stereo view of **2** (solid bonds) superimposed on **1** (open bonds) in chicken DHFR, showing neighboring residues in the hydrophobic cleft. Carbon is shown as small open circles, oxygen as larger open circles, nitrogen as large black circles. (For a more complete view of **1** in chicken DHFR, see ref 8, p 388.)

prevents such compounds from reaching the site of action in the cell.[26] It is only one to three dilutions less active than **1** against the various organisms shown. This suggests that shape, as well as optimum lipophilicity, is a factor involved in cell penetration. (See paper 5 of this series for a comparison with more bulky analogues with lower log *P* values than **2** which have low in vitro antibacterial affinity.[26]) Compound **10** is similarly less potent than **19** (although the numbers are not strictly comparable due to a different dilution schedule). Compound **18**, which had lower enzyme inhibition than **10**, is also less active in vitro, as is the case with the monosubstituted analogues.

Discussion

Matthews and co-workers have suggested an explanation for the high selectivity of TMP (**1**) for bacterial DHFR, based on an analysis of the X-ray data in its complexes with chicken liver and *E. coli* isozymes.[11] The stereochemistry of TMP in the avian enzyme is such that a potential hydrogen bond between the 4-amino group and the backbone carbonyl of Val-115 is lost which is present with the corresponding backbone function in *E. coli* DHFR, and this could be the major factor responsible for the much stronger binding to the latter enzyme.

Our finding that **2** was considerably less selective than **1** for bacterial DHFR, although approximately equipotent against that enzyme, suggested that one needs to consider the interaction energies of all the atoms of the ligand in such a complex when trying to interpret reasons for selectivity. Undoubtedly the loss of a hydrogen bond is an important factor that contributes to binding, but it cannot account for the differences observed. In order to make an appropriate comparison between the two compounds, we raised the question as to whether **2** was bound in the same manner as **1** to chicken DHFR. Dr. David Matthews agreed to conduct the appropriate crystallographic experiment, which he has now published separately.[11] Upon refinement to 2.2 Å, he found slight geometrical differences between the orientation of the two benzyl moieties, although the pyrimidine rings were positioned almost identically in the complex. The benzene rings were tilted about 1 Å relative to each other, and the torsion angles differed for the ethyl functions compared to the methoxy groups. This is illustrated in Figure 1, which shows a stereo view of **2** (solid lines) superimposed on **1** in chicken DHFR. From this view the *m*-ethyl group at the front of the binding cavity is seen to be in quite a different confor-

mation from the corresponding methoxy group of TMP. Matthews states this to be the case for the inner meta substituent, which is less obvious from this view.[11]

It is not to be expected that the methoxy and ethyl groups would have the same conformations. In the first place, the sp³ carbon and ether oxygen bond angles would be different. Secondly, the conformational preferences of methoxy- and ethylbenzenes are different, since the latter lacks the resonance capability of the former. In the absence of steric hindrance, methoxy groups attached to aromatic rings prefer planar conformations, presumably because the lone-pair orbitals of the oxygen atoms can then delocalize electrons into the aromatic system.[27] 1,2-Dimethoxybenzene has recently been shown by ¹³C NMR measurements in chloroform to have a planar conformation,[28] although other solution measurements indicate nonplanarity,[29] whereas addition of a third methoxy group at the 3-position forces rotation of the 2-methoxy substituent to a perpendicular conformation. This has been demonstrated by photoelectron spectroscopy,[29] NMR spectroscopy,[28,29] crystal-structure analysis,[30-33] and theoretical calculations,[29] all of which show that the preferred conformation for the 1- and 3-methoxy groups is planar with the ring, as opposed to the perpendicular conformation for the middle methoxy group. We can be confident, therefore, that in *vic*-trimethoxybenzenes a nonplanar conformation for one or both of the *m*-methoxy substituents only occurs when some external source of energy can be coupled in such way as to overcome the torsional barriers restricting out-of-plane rotations.

The situation is different with ethylbenzenes. The in-plane conformation is destabilized by an unfavorable steric interaction between the methyl group and one of the ortho hydrogens.[34,35] The crystal structure of ethylbenzene is

(26) 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; see also ref 32 in that publication.

(27) Radom, L.; Hehre, W. J.; Pople, J. A.; Carlson, G. L.; Fateley, W. G. *J. Chem. Soc., Chem. Commun.* **1972**, 308.

(28) Makriyannis, A.; Fesik, S. *J. Am. Chem. Soc.* **1982**, *104*, 6462.

(29) Anderson, G. M., III; Kollman, P. A.; Domelsmith, L. N.; Houk, K. N. *J. Am. Chem. Soc.* **1979**, *101*, 2344 and references therein.

(30) Bryan, R. F., personal communication, 1969. Phillips, T.; Bryan, R. F. *Acta Crystallogr., Sect. A* **1969**, *A25*, S200.

(31) Haltiwanger, R. C. M.Sc. Thesis, 1971, University of Virginia, Charlottesville, VA.

(32) Koetzle, T. F.; Williams, G. L. B. *J. Am. Chem. Soc.* **1976**, *98*, 1074.

(33) Koetzle, T. F.; Williams, G. J. B. *Acta Crystallogr., Sect. B* **1978**, *B34*, 323.

(34) Hehre, W. J.; Radom, L.; Pople, J. A. *J. Am. Chem. Soc.* **1972**, *94*, 1496.

(35) Bruckwedde, F. G.; Moskow, M.; Scott, R. B. *J. Chem. Phys.* **1945**, *13*, 547.

Table III. Solvent Accessible Surface Areas[a] ($Å^2$), for Ethyl Groups of 2 in Ternary Complex with Chicken Liver DHFR and NADPH,[b] Compared to the Corresponding Accessibility for Trimethoprim Methoxy Groups in a Similar Chicken Liver DHFR Complex[c]

substit atoms	solvent accessible surface area, $Å^2$		percent accessible	
	2 alone[d]	chicken ternary complex	2	1[c]
3-ethyl				
CH_2	24.2	0.0	0	0[e]
CH_3	76.2	2.7	3.5	0
4-ethyl				
CH_2	26.4	0.0	0	11[e]
CH_3	57.7	15.5	26.8	24
5-ethyl				
CH_2	32.0	0.72	2.2	0[e]
CH_3	63.4	36.5	57.5	42

[a] Lee, B.; Richards, F. M. *J. Mol. Biol.* 1971, *55*, 379. [b] The coordinates used are those of Matthews et al., ref 11, for which we are grateful. [c] Reference 25. [d] The coordinates for 2 in the ternary complex were used as a model, in the absence of any crystal coordinates for a vicinally substituted triethylbenzene. [e] Corresponding oxygen atoms.

unknown; however, a number of polysubstituted ethylbenzenes[36-40] and related molecules[34] show out-of-plane conformations in the solid state. In solution, then, it is assumed that the tendency would be for the outer ethyl groups of 2 to exist in nonplanar conformations. We have stated that the NMR spectrum of 2 shows that one of the three ethyl groups has a different chemical shift from the other two; one would conclude that it is the middle substituent that is different from the other two, and that this very likely tilts in the opposite direction from the outside functions.

What is actually observed when 1 is bound to chicken DHFR is that the protein induces very minor changes in all three methoxy torsion angles, which become 6°, 109°, and 177°, respectively.[8] This then has a negligible effect on the conformational potential energy of bound TMP relative to that of the free molecule, so it is difficult to invoke enzyme-induced strain energy as a reason for the lower activity of 1 compared to 2 in chicken DHFR.

One possible contribution to the binding difference between 1 and 2 is that desolvation energy is required to strip the methoxy groups of water as 1 combines with the vertebrate DHFR. According to an extension of this hypothesis, the similar inhibitory activities of 1 and 2 against *E. coli* DHFR could be explained if little or no desolvation of the benzyl substituents is required when these two inhibitors bind to *E. coli* DHFR. In the previous paper of this series[25] we have described the relative exposures of the methoxy groups of TMP in ternary complexes with *E. coli* and chicken DHFR, and have shown that the oxygen atoms of the substituents are considerably more accessible in the bacterial complex than with that from chicken. One methoxy group is totally buried in the latter case. We have now measured the corresponding exposed areas for the ethyl groups of 2 in chicken liver DHFR. The results of

the comparisons of 1 and 2 in the avian enzyme are shown in Table III. It will be observed that there are only minor differences in the percentage exposure to solvent with the two compounds. That vicinally substituted di- and trimethoxybenzenes are actually solvated in the presence of water may be inferred from their partition coefficients in octanol/water, which show the compounds to be much more soluble in the aqueous phase with increased substitution.[29,41] These results suggest that it is appropriate to invoke desolvation energy in explaining the lower activity of 1, compared to 2, against chicken DHFR.

In the introduction we mentioned the cooperative effect in binding TMP to *E. coli* DHFR occasioned by the cofactor. Upon deletion of one *m*-methoxy group to form 19, it will be seen from Table I that the compound is about 10-fold less inhibitory to *E. coli* DHFR than is 1, thus further establishing the important role of the *m*-methoxy groups to binding. Kuyper has calculated minimum-energy conformations for 19 bound to *E. coli* DHFR plus NADPH, and has found two minima, one of which has torsional angles like that of TMP in this complex.[42]

An entirely different situation exists with the vertebrate DHFRs. Here very little cooperativity takes place in binding the coenzyme to a TMP complex,[6] and this ligand is in an entirely different conformation from that found in bacterial DHFR, such that there is no near contact between the benzyl substituents of TMP and the coenzyme. However, all three methoxy groups have contact with the enzyme.[8,25] Note that upon removal of one methoxy group to form 19, this analogue is now 3–4 times *more* active on the chicken and rat DHFRs, respectively, than is TMP. In other words, the third methoxy group now has a deleterious effect on binding. The selectivity of TMP for bacterial DHFR is *then 30–40-fold greater than that of diaveridine* (19) out of a total 3000-fold selectivity, considered on a kinetic basis.[22] Thus a very large portion of the total selectivity is created by adding a third methoxy group! Note, however, that the other two methoxy groups are essential for this result to obtain.

Now let us consider the triethyl–diethyl comparison. Although we have no X-ray information on how 2 binds to *E. coli* DHFR, we are probably safe in assuming that it binds like 1. Once more, the disubstituted analogue 10 binds about 1/10 more weakly. It is the methyl groups of TMP that have closer contact with the protein than the oxygen atoms,[25] and this is very likely the case with 19, as well as with the methyl atoms of the ethyl groups. Turning to the vertebrate DHFRs, the diethyl compound in contrast to its dimethoxy counterpart is now 1.5–3 times *less* inhibitory on rat and chicken DHFRs, respectively, than is the trisubstituted derivative 2, which is in the direction one would expect if *m*-ethyl groups are performing a useful function, although the effect is rather small. It is also of interest to compare 19 and 10 directly, which have very similar activities on rat DHFR, with 10 being 3-fold more potent on the avian enzyme. This is in contrast to the large differences between the trisubstituted derivatives. In one more comparison, 2 is only 3–6-fold more selective than is 10 for bacterial DHFR, unlike TMP and its relative.

Why should the disubstituted pair bear so few apparent differences relative to each other in comparison to the trisubstituted analogues? We would like to suggest that it is with the inner meta substituent in the trisubstituted

(36) Kennard, O.; Giacovazzo, C.; Horn, A. S.; Mongiorgi, R.; di Sanseverino, L. R. *J. Chem. Soc., Perkin Trans. 2* 1974, 1160.
(37) Fornies-Marquina, J.; Courseille, C.; Busett, B. *Cryst. Struct. Commun.* 1972, *1*, 261.
(38) Andrianov, V. G.; Korapetyan, H. A.; Struchkou, T. *Cryst. Struct. Commun.* 1978, 7, 553.
(39) Folting, K.; Huffman, J. C.; Merritt, L. L., Jr. *Cryst. Struct. Commun.* 1975, *4*, 705.
(40) Braun, R. L.; Lingafelter, E. C. *Acta Crystallogr.* 1967, *22*, 787.

(41) Leo, A.; Hansch, C.; Elkins, D. *Chem. Rev.* 1971, *71*, 525.
(42) Kuyper, L. F. *Abstracts of Papers*, 189th National Meeting of the American Chemical Society, April 1985, Miami; American Chemical Society: Washington, DC, 1985; MEDI 88.

derivatives bound to chicken DHFR wherein the greatest differences lie. This substituent is completely buried in a hydrophobic environment in the protein, in contrast to the other two functions (Table III). This suggests that the dimethoxy compound might prefer to avoid that hydrophobic pocket, whereas it would satisfy the diethyl compound quite well. In other words, **10** and **19** could easily undergo a 180° flip in their conformations. This situation is rendered more complex by the fact that Tyr-31 in the chicken DHFR is capable of undergoing large conformational changes in the presence of a ligand, which it does with both **1** and **2**, but not with a closely related 4-isopropenyl analogue.[11] Having undergone this movement, the Tyr-31 phenolic group then hydrogen bonds via a water molecule to the backbone carbonyl of Trp-24. This might possibly happen, for example, with the complex involving **10**, but not **19**, which would complicate the energetics. The fact that the data with chicken DHFR differ from those with rat suggests that this tyrosine may well be involved in these interactions. Although the rat liver DHFR has not been sequenced, S180 mouse and L1210 mouse as well as bovine, porcine, and human liver DHFRs have been sequenced, and all of these mammalian DHFRs except for the porcine isozyme contain Phe, rather than Tyr, at this position and are otherwise highly homologous in the region of the active site.[43]

In summary then, a comparison between the binding properties of TMP and its triethyl analogue, as well as the disubstituted congeners, makes clear the fact that the methoxy groups of TMP are partially responsible for its selectivity for bacterial, as opposed to vertebrate, DHFR. The shapes of the two molecules have been found to very similar when combined with chicken DHFR. However, analysis of X-ray crystallographic data of **1** and **2** suggests that desolvation energy may be an important source of the differences in the total binding potential. To obtain a total picture of the interaction energies in the presence of water molecules will require some very sophisticated molecular dynamics calculations, however.

Compounds **1** and **2** are dramatically different from each other with regard to lipophilicity, a factor that has been shown by QSAR studies to play an important role in binding, at least to the vertebrate DHFRs.[44] It is clear, however, that the phenomenon is actually much more complex. Whether or not the shapes of the two molecules are identical in complex with the bacterial enzyme is not as yet known. The fact that the activities are almost identical suggests that shape plays a key role. QSAR analysis of simpler analogues suggested a major role for MR,[44,45] but it probably does not represent the whole story, as we have shown in a recent analysis of related trisubstituted derivatives.[25]

Experimental Section

All melting points were determined with calibrated thermometers, on either a Hoover or a Thiele tube melting point apparatus. Where analyses are indicated only by the symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values. The analyses were carried out by Dr. Stuart Hurlbert and his staff or by Atlantic Microlabs, Inc., Atlanta, GA. Nuclear Magnetic Resonance (NMR) spectra were recorded on Varian A-60, XL-100, or T-60 spectrophotometers;

chemical shifts are reported in parts per million (δ) from internal tetramethylsilane. Ultraviolet spectra were obtained with a Cary 118 spectrophotometer.

3,4,5-Triethylacetophenone (4).[12] Aluminum chloride (300 g, 2.25 mol) was charged into a flask containing 114 mL (1.48 mol) of cold EtBr, and the contents were then heated with stirring to 50 °C. A solution of 75 g (0.5 mol) of 4-ethylacetophenone (**3**) in 73 mL (0.95 mol) of EtBr was then added dropwise over 135 min. The mixture was then maintained at 55 °C for 20 h, at which time all evolution of HCl and HBr had ceased. The contents were cooled, poured over ice, and extracted well with Et$_2$O. The ether layer was washed with water, NaOH and again with water and dried over CaCl$_2$, the solvent removed, and the residue vacuum distilled. The fraction boiling between 80 and 118° (1.5 mmHg) was analyzed by VPC and shown to contain 61 g (60%) of **4**. It then was fractionally distilled by using a column packed with glass helices equipped with partial take off (approximately 10 theoretical plates); the fraction boiling at 105–119 °C (0.30 mmHg) was found to contain 98% of **4** by VPC analysis. This was redistilled and the fraction boiling at 120–121 °C (2 mm) collected. Anal. (C$_{14}$H$_{20}$O) C, H. NMR: (CCl$_4$) δ 1.14 (t, 3, 4-CH$_2$CH_3, J = 7.5 Hz), 1.26 (t, 6, 3,5-(CH$_2$CH_3)$_2$), 2.45 (s, 3, COCH_3), 2.71 (q, 6, CH_2CH$_3$, J = 7.5 Hz), 7.53 (s, 2 aromatic H).

In earlier experiments, in which the ratio of 4-ethylacetophenone/AlCl$_3$/EtBr was 1:1.5:6, a white solid was collected upon distillation; mp 126–127 °C (EtOH). The NMR spectrum showed no aromatic protons and only Et peaks [(CDCl$_3$) δ 1.18 (t, 3, J = 7.5 Hz), 2.63 (q, 2, J = 7.5 Hz)]. It was concluded that the product was **hexaethylbenzene (9)**, which is reported to melt at 126 °C.[14] Anal. (C$_{18}$H$_{30}$) C, H.

3,4,5-Triethylbenzoic Acid (5).[12] Compound **4** was oxidized by the method of Taylor and Watts[15] to give **5** (77%); mp 141–143 °C (lit.[12] mp 142–143 °C). Anal. (C$_{13}$H$_{18}$O$_2$) C, H.

ω-(Methylsulfonyl)-3,4,5-triethylacetophenone (6). Compound **5** was converted to its methyl ester in methanolic HCl (92%); bp 149–151 °C (10 mmHg). This was used directly in the next reaction. A solution of 3.8 g (0.041 mol) of Me$_2$SO$_2$ in 18 mL of Me$_2$SO was heated to 65 °C under N$_2$, and 1.7 g of NaH (61% in mineral oil, 0.04 mol) was added. The mixture was heated for 105 min and cooled to 45 °C, and then 4.6 g (0.02 mol) of methyl 3,4,5-triethylbenzoate was added dropwise with vigorous stirring over a 25-min period. The resultant mixture was then heated at 60 °C for 90 min, cooled, poured on ice, and acidified with HCl. The mixture was extracted with ether, and the ether extract washed with water and NaHCO$_3$, after which the solvent was evaporated and the residue dissolved in 1 N NaOH. The solution was acidified with hydrochloric acid and cooled, and the precipitate was isolated; weight of **6**, 5.5 g (98%); mp 125–126 °C (EtOH). Anal. (C$_{15}$H$_{22}$O$_3$S) C, H.

Methyl 2-(3,4,5-Triethylphenyl)-2-hydroxyethyl Sulfone (7). A mixture of 4.5 g (0.016 mol) of **6**, 3 mL of EtOH, and 10 mL of water was cooled to 15 °C and a cold solution of 0.2 g (0.006 mol) of NaBH$_4$ in 4 mL of water added dropwise. The mixture was stirred at room temperature for 1.5 h, after which it was heated until a clear solution formed. Water was added until the mixture was cloudy, after which it was cooled and filtered; weight of precipitate, 3.7 g (82%) of **7**; mp 101–103 °C (25% EtOH). Anal. (C$_{15}$H$_{24}$O$_3$S) C, H. NMR: (CDCl$_3$) δ 1.13 (tr, 3, CH$_2$CH_3, J = 7.7 Hz), 1.23 (tr, 6, (CH$_2$CH_3)$_2$, J = 7.6 Hz), 2.67 (q, 6, (CH_2CH$_3$)$_3$, J = 7.6 Hz), 3.05 (s, 3, SO$_2$Me), 3.22–3.66 (m, 2, CH_2SO$_2$), 5.22 and 5.34 (2 tr, 1, CHOH, J = 2.6 Hz), 7.03 (s, 2, Ar); OH not seen.

α-(3,4,5-Triethylbenzyl)-β-anilinoacrylonitrile (8). A mixture of 2.83 g (0.01 mol) of **7**, 1.65 g (0.011 mol) of β-anilinopropionitrile, and 4 mL of dry Me$_2$SO was heated to 40 °C, followed by the addition of 1.13 g (0.01 mol) of KO-t-Bu in 8.3 mL of t-BuOH in small portions. The mixture was heated at 48 °C for 90 min, cooled, and poured over ice. The resultant oil was stirred in ice water until it crystallized; weight, 3.7 g; mp 154–156 °C (EtOH). A small amount was purified by column chromatography on silica gel and eluted with hexane/CH$_2$Cl$_2$ (1:1). Anal. (C$_{22}$H$_{26}$N$_2$·0.25H$_2$O) C, H, N.

2,4-Diamino-5-(3,4,5-triethylbenzyl)pyrimidine (2). To a solution of 2.2 g (0.04 mol) of NaOMe in 40 mL of absolute EtOH was added 2.9 g (0.03 mol) of guanidine hydrochloride. The resultant salt was filtered off, and 3.2 g (0.02 mol) of **8** was added to the ethanolic solution. This mixture[12] was heated under reflux

(43) Champness, J. N.; Kuyper, L. F.; Beddell, C. R. In *Molecular Graphics and Drug Design*; Burgen, A. S. V., Roberts, G. C. K., Tute, M. S., Ed.; Elsevier: Amsterdam, 1986.

(44) Blaney, J. M.; Dietrich, S. W.; Reynolds, M. A.; Hansch, C. *J. Med. Chem.* **1979**, *22*, 614.

(45) Dietrich, S. W.; Blaney, J. M.; Reynolds, M. A.; Jow, P. Y. C.; Hansch, C. *J. Med. Chem.* **1980**, *23*, 1205.

for 25 h, followed by the addition of decolorizing charcoal, clarification, cooling, and isolation of the resultant precipitate. Additional product was obtained by the addition of water to the filtrate; total weight, 1.1 g (39%); mp 175–176 °C (recrystallized twice, Me$_2$CO). Anal. (C$_{17}$H$_{24}$N$_4$) C, H, N. UV: (neutral species, 0.01 N NaOH) λ_{max} 208 nm (ϵ 35 500), 287.5 (7100); (cation, 0.1 N HCl), 202 (57 000), 271 (5400). pK_a = 7.12 ($\pm$0.03) (20 °C) by the method of Roth and Strelitz.[19] NMR: (Me$_2$SO-d_6) δ 1.06 (t, 3, CH$_2$CH_3, J = 7.5 Hz), 1.14 (t, 6, CH$_2$CH_3, J = 7.5 Hz), 2.56 (q, 6, CH_2CH$_3$, J = 7.5 Hz), 3.51 (s, 2, benzylic CH$_2$), 5.65 (s, 2, NH$_2$), 6.01 (br s, 2, NH$_2$), 6.85 (s, 2, 2,6-benzene H), 7.48 (s, 1, pyrimidine 6-H).

2,4-Diamino-5-(3,4-diethylbenzyl)pyrimidine Hydrochloride (10). 3,4-Diethylbenzaldehyde was treated with β-anilinopropionitrile by the method described for 8 (compound 7 behaves like an nascent aldehyde). The crude product was treated directly with guanidine as described by 2, which yielded 10, isolated as the hydrochloride, mp 285–287 °C (EtOH). Anal. (C$_{15}$H$_{20}$N$_4$·HCl) C, H, N. UV: (neutral species, 0.01 N NaOH) sh 238 nm (ϵ 11 600), λ_{max} 286 (7300); (cation 0.01 N HCl) λ_{max} 271 (5250).

ω-(Methylsulfonyl)-4-*tert*-butylacetophenone (11). 4-*tert*-Butylbenzoic acid was converted to its methyl ester in methanolic HCl; bp 130 °C (10 mm). This product was treated with Me$_2$SO$_2$ according to the procedure for 6 to produce 11, mp 88–89 °C (EtOH) (81%). Anal. (C$_{13}$H$_{18}$O$_3$S) C, H.

α-(4-*tert*-Butylbenzyl)-β-anilinopropionitrile (12). Compound 11 was reduced with NaBH$_4$ as for 7 and the crude alcohol treated directly with β-anilinopropionitrile as for 8, which produced 12, mp 179–181 °C (EtOH) (41%). Anal. (C$_{20}$H$_{23}$N$_2$) C, H, N.

2,4-Diamino-5-(4-*tert*-butylbenzyl)pyrimidine (13). With 12 as the starting material, the procedure for 2 was followed, to produce 13, mp 187.5–188.5 °C (50% EtOH) (46.5%). Anal. (C$_{15}$H$_{20}$N$_4$) C, H, N. UV: neutral species, 0.01 N NaOH λ_{max} 287 nm (ϵ 7400); (cation, 0.01 N HCl) 204 (33 000), 219 (29 000), 271 (5200).

α-(4-Isopropylbenzyl)-β-anilinoacrylonitrile (14). A mixture of 14.8 g (0.1 mol) of 4-isopropylbenzaldehyde, 16.5 g (0.11 mol) of β-anilinopropionitrile, and 40 mL of dry Me$_2$SO was heated with stirring to 40 °C. A solution of 11.3 g (0.1 mol) of KO-*t*-Bu in 83 mL of *t*-BuOH was added and the solution then maintained at 45 °C for 3.5 h. The solvent was removed in vacuo and the residue slurried in ice water until the product became crystalline, followed by filtration and washing with very cold EtOH and hexane; weight, 29.4 g; mp 154–156 °C (EtOH) (50% of pure 14). Anal. (C$_{19}$H$_{21}$N$_2$) C, H, N.

2,4-Diamino-5-(4-isopropylbenzyl)pyrimidine (15). The above product (14) was treated by the method used for 2 to yield 15, mp 161–162.5 °C (EtOH) (41%). Anal. (C$_{14}$H$_{18}$N$_4$) C, H, N. UV: (neutral species, 0.01 N NaOH) λ_{max} 287 nm (ϵ 7250); (cation, 0.01 N HCl) 205 (32 600), 219 (28 990), 271 (5320).

2,4-Diamino-5-(4-ethylbenzyl)pyrimidine (16). This substance was made by the procedure described for 2 with *p*-ethylbenzoic acid as the starting material. The intermediates were partially purified and their identities checked by NMR and IR. Compound 16 was obtained in 47% yield from its immediate precursor; mp 163–165 °C (EtOH). Anal. (C$_{13}$H$_{16}$N$_4$) C, H, N.

3-Isopropyl-4-ethylacetophenone (17). To a well-stirred mixture of 62 g (0.5 mol) of 2-bromopropane and 53.2 g (0.4 mol) of AlCl$_3$ at 16 °C was added dropwise over a 3-h period a solution of 30 g (0.2 mol) 4-ethylacetophenone in 62 g of 2-bromopropane. The temperature slowly rose to 32 °C and gas was steadily evolved. The mixture was then maintained at 45 °C for 18 h, after which it was cooled and poured over ice. The very thick mass was broken up by adding ether, followed by further ether extraction. The ether layer was washed with dilute HCl, water, NaOH, and water, dried, and distilled. The fraction boiling at 72–120 °C (1.5 mmHg) was collected; 26.3 g. This was then fractionated on a spinning band column. The fraction boiling at 72 °C (0.28 mm), 11.5 g, was found to be pure by VPC and identified as 17 by NMR spectroscopy. NMR: (CDCl$_3$) δ 1.20 (t, 3, CH$_2$CH_3, J = 7 Hz), 1.25 (d, 6, CH(CH_3)$_2$, J = 7 Hz), 2.55 (s, 3, COCH_3), 2.73 (q, 2, CH_2CH$_3$, J = ~7 Hz), 3.16 (q, 1, CH(CH$_3$)$_2$, J = ~7 Hz), 7.22 (d, 1, Ar 5 H, J = 8 Hz), 7.71 (dd, 1, Ar 6 H, J = 8, J' = 2 Hz), 7.92 (d, 1, Ar 2 H, J' = 2 Hz).

No pure diisopropyl derivative was isolated from this reaction.

2,4-Diamino-5-(3-isopropyl-4-ethylbenzyl)pyrimidine (18). Compound 17 was oxidized to the corresponding acid with NaOBr by the method of Taylor and Watts,[15] esterified, and then carried through the procedures described for 2 without full characterization of the intermediates. The product, 18, was isolated as its hydrochloride salt, mp 270–272 °C (EtOH) (31% from its immediate precursor). Anal. (C$_{16}$H$_{22}$N$_4$·HCl) C, H, N.

UV (neutral species, 0.01 N NaOH) λ_{max} 287 nm (ϵ 7300), sh 241 (10 070); (cation 0.01 N HCl) 274 (5680).

Registry No. 1, 738-70-5; 2, 36821-85-9; 3, 937-30-4; 4, 107771-18-6; 5, 74660-03-0; 5 methyl ester, 74660-04-1; 6, 36821-82-6; 7, 36821-83-7; 8, 36821-84-8; 9, 604-88-6; 10, 110224-64-1; 11, 82652-18-4; 12, 110224-65-2; 13, 110224-66-3; 14, 110224-67-4; 15, 110224-68-5; 16, 110224-69-6; 17, 110224-70-9; 18, 110224-71-0; 19, 5355-16-8; DHFR, 9002-03-3; EtBr, 74-96-4; 2,4-diamino-5-(4-methoxybenzyl)pyrimidine, 20285-70-5; 2-bromopropane, 75-26-3; methyl 4-*tert*-butylbenzoate, 26537-19-9; β-anilinopropionitrile, 1075-76-9; guanidine hydrochloride, 50-01-1; 3,4-diethylbenzaldehyde, 110224-72-1; 4-isopropylbenzaldehyde, 122-03-2; *p*-ethylbenzoic acid, 619-64-7; 2,4-diamino-5-(3-isopropyl-4-ethylbenzyl)pyrimidine, 110224-73-2; 2,4-diamino-5-(3,4-diethylbenzyl)pyrimidine, 110224-74-3.

(46) (a) Meyer, E. J. *CAMCRYST User's Manual: The PROPHET Interface to the Cambridge Crystallographic Data Base*; Bolt Beranek and Newman: Cambridge, MA, 1983. (b) Raub, W. F. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1974**, *33*, 2390.