Folate-Synthesizing Enzyme System as Target for Development of Inhibitors and Inhibitor Combinations against *Candida albicans*—Synthesis and Biological Activity of New 2,4-Diaminopyrimidines and 4′-Substituted 4-Aminodiphenyl Sulfones

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The paper describes the design, synthesis, and testing of inhibitors of folate-synthesizing enzymes and of whole cell cultures of *Candida albicans*. The target enzymes used were dihydropteroic acid synthase (SYN) and dihydrofolate reductase (DHFR). Several series of new 2,4-diaminopyrimidines were synthesized and tested as inhibitors of DHFR and compared with their activity against DHFR derived from mycobacteria and *Escherichia coli*. To test for selectivity, also rat DHFR was used. A series of substituted 4-aminodiphenyl sulfones was tested for inhibitory activity against SYN and the I_{50} values compared to those obtained previously against *Plasmodium berghei*- and *E. coli*-derived SYN. Surprisingly, QSAR equations show very similar structural dependencies. To find an explanation for the large difference in the I_{50} values observed for enzyme inhibition (SYN, DHFR) and for inhibition of cell cultures of *Candida*, mutant strains with overexpressed efflux pumps and strains in which such pumps are deleted were included in the study and the MICs compared. Efflux pumps were responsible for the low activity of some of the tested derivatives, others showed no increase in activity after pumps were knocked out. In this case it may be speculated that these derivatives are not able to enter the cells.

Introduction

Infections caused by fungi have increased dramatically during the past decades.[1] The reasons for this are manifold. In principle, one has to differentiate between mycoses of the skin or nails and systemic mycoses. Systemic mycoses mainly appear concomitant with other diseases[2] or are caused by treatment with chemotherapeutics, for instance with cytostatics.[3] At risk are patients after organ transplantation treated with immunosuppressives[4] or those suffering with a weakened immune system, for example patients with AIDS.[5] Endocarditis caused by fungi has been observed with drug abuse.

Despite several interesting targets for the therapy of candidiasis, only four classes of drugs are presently used,[6] namely polyenes (amphotericin B, nystatin) which can be used systemically, 5-fluorocytosine, azoles (miconazole, ketoconazole, etc.), and allylamines (naftifine, terbinafine); the latter, however, show limited efficacy in case of candida infections.[4] Also morpholine derivatives such as amorolfine show activity against *Candida albicans*, and thiocarbamates have been used successfully in the treatment of AIDS patients suffering from secondary infections by *C. albicans*. Most of these drugs act on targets within the ergosterol biosynthesis at different steps (e.g. inhibition of squalene epoxidase) of fungi.[7] Many attempts have been undertaken to reduce the frequency of side effects and the problem of resistance.[1]

The folate-synthesizing enzyme system of fungi could be an alternative target.[8] It is a well-established drug target in microorganisms.[9] Various bacterial infections[9] (e.g. *Pneumocystis carinii*,[10] *Toxoplasma gondii*[11]) and malaria[12−14] can successfully be treated by folate inhibitors. *C. albicans* also possesses the complete folate-synthesizing enzyme system, despite being an eukaryont.[15] Therefore, many attempts have lately been made to develop suitable new inhibitors of the folate-synthesizing enzyme system of various microorganisms, such as *P. carinii* and *T. gondii*,[16−18] Leishmaniasis,[19] *Plasmodium falciparum*,[20] and *C. albicans*.[21] So far, however, none of the new compounds could be introduced into therapy, mainly for two reasons: (i) poor selectivity and (ii) problems in getting into the cytoplasm of fungal cells and/or in gastrointestinal absorption.

In case of bacterial infections, the simultaneous inhibition of the two enzymes dihydropteroate synthase (SYN), and dihydrofolate reductase (DHFR) has been proven to be synergistic and highly effective. Several examples for synergism on combining sulfonamides or

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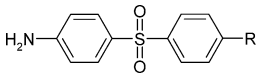
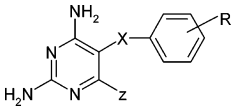
Table 1. Observed and Calculated Inhibitory Activity, I_{50} (μM), of Substituted 4-Aminodiphenyl Sulfones against SYN Derived from *C. albicans* and Observed Activity against SYN from *P. berghei*, *M. lufu* (DDS sensitive/resistant), and *E. coli*



	C. albicans		I_{50} obsd				
R	I_{50} obsd	I_{50} calcd (eq 1)	*M. lufu* sens/res	*P. berghei*	*E. coli*	Δppm2/6	f_i[a]
4-NH_2 (DDS)	1.78	2.88	1.20/1.18	12.41	34.36	−0.108	0
4-$NHCH_3$	2.81	2.99	1.29/2.00	26.70	46.21	−0.105	0
4-NHC_2H_5	3.16	3.29	2.75/1.48		41.71	−0.098	0
4-H	13.26	12.30	12.06/12.0	104.0	116.50	0	0
4-CH_3	10.11	8.55	8.08/5.92	48.00	89.85	−0.027	0
4-OCH_3	9.05	7.37	7.55/9.33		128.2	−0.038	0
4-OH[b]	2.40	2.06	1.50/1.61	32.23	34.92	−0.054	0.788
4-Cl	14.33	13.33	12.99/18.7			0.006	0
4-COOH	2.78	4.30	3.60/3.17		74.24	0.022	1
4-$N(CH_3)_2$	3.83	3.71	1.76/2.04	21.51		−0.089	0
4-$NHCH_2COOH$	1.22	0.89	0.87/0.82	17.77	37.05	−0.095	1
4-$NHCH_2COOCH_3$	1.33		2.24/2.63		101.24	−0.095	0
4-$NHCOCH_3$			7.01/4.05	33.26	75.65	−0.036	0
4-$COOCH_3$			11.75/13.8	147.0	149.29	0.026	0
4-$CONHNH_2$			12.72/9.60	76.51	155.39	0.022	0

[a] $f_i = 1/(1+10^{pK_a-pH})$. [b] 4-OH: $pK_a = 7.83$;[40] tests were performed at pH = 7.75 ($f_i = 0.454$) for *E. coli* and *M. lufu* and at pH = 8.4 ($f_i = 0.788$) for *P. berghei* and *C. albicans*.

Table 2. Code, Structure of 2,4-Diaminopyrimidines and I_{50} Values (μMol/L) against Cell-Free DHFR from Various Sources

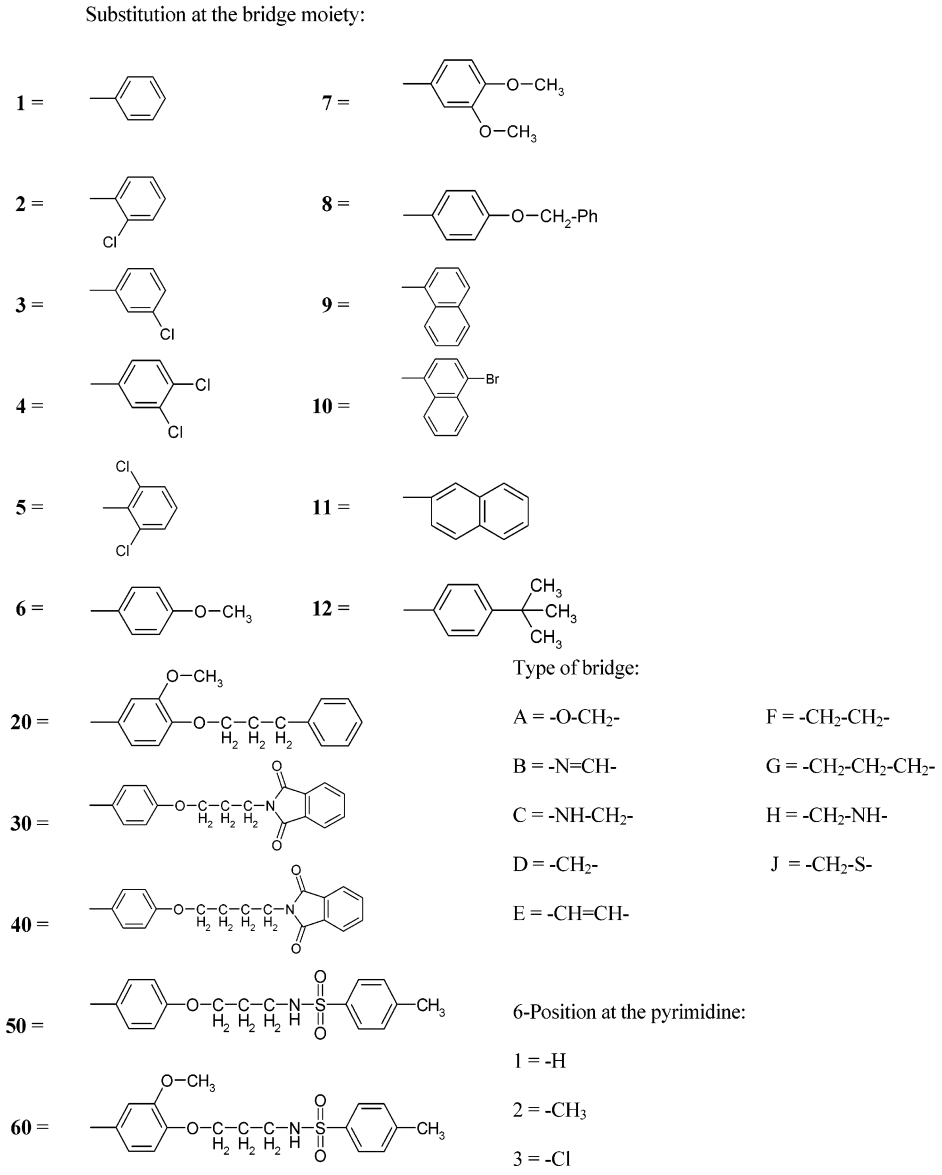


compd	X	Z	R	*C. albicans*	rat	*M. lufu*	*E. coli*
TMP	$-CH_2-$	H	3,4,5-$(OCH_3)_3$	30.30	190.0	0.27	0.0022
D.1.1.	$-CH_2-$	H	H	90.45	115.3	14.77	0.16
D.1.9.	$-CH_2-$	H	2,3-Benzo	53.46	29.4	0.58	0.16
D.1.11.	$-CH_2-$	H	3,4-Benzo	4.77	61.2	0.57	0.16
A.1.1.	$-OCH_2-$	H	H	1.24	10.1	12.3	3.92
A.1.2.	$-OCH_2-$	H	2-Cl	0.73	3.53	3.93	1.48
A.1.4.	$-OCH_2-$	H	3,4-Cl_2	3.72	56.75	1.22	1.43
A.1.5.	$-OCH_2-$	H	2,6-Cl_2	3.10	2.57	5.77	5.61
A.1.6.	$-OCH_2-$	H	4-OCH_3	26.1	99.7	7.32	2.59
A.1.8.	$-OCH_2-$	H	4-OCH_2-C_6H_5	2.81	39.4	2.98	0.43
A.1.9.	$-OCH_2-$	H	2,3-Benzo	0.43	2.63	1.01	0.17
A.1.10.	$-CH_2-$	H	4-Br-2,3-Benzo	0.49	2.82	0.15	0.04
A1.12.	$-OCH_2-$	H	4-$C(CH_3)_3$	23.8	106.0	5.07	0.66
C.1.1	$-NHCH_2-$	H	H	7.94	58.2	105.0	11.29
C.1.2.	$-NHCH_2-$	H	2-Cl	6.63	71.53	8.74	2.69
C.1.3.	$-NHCH_2-$	H	3-Cl	13.9	49.8	35.2	2.81
C.1.4.	$-NHCH_2-$	H	3,4-Cl_2	84.8	>350[a]	3.09	1.84
C.1.6.	$-NHCH_2-$	H	4-OCH_3	52.3	146.2	11.73	3.18
C.1.7.	$-NHCH_2-$	H	3,4-$(OCH_3)_2$	125.8	197.3	4.88	1.12
C.1.9.	$-NHCH_2-$	H	2,3-Benzo	1.39	5.9	2.46	0.33
C.1.11.	$-NHCH_2-$	H	3,4-Benzo	16.1	71.3	1.98	1.29
F.1.1.	$-CH_2CH_2-$	H	H	3.37	39.9	30.7	6.75
Ro12-6099	$-CH_2CH_2-$	H	3,4-$(OCH_3)_2$	35.4	315.3	4.35	1.00
A.2.1.	$-CH_2-$	CH_3	H	1.85	3.36	4.95	2.83
A.2.4.	$-OCH_2-$	CH_3	3,4-Cl_2	1.34	4.58	0.39	0.33
E.1.1.	$-CH=CH-$	H	H	5.72	130.6	45.0	10.52
H.1.1.	$-CH_2NH-$	H	H	59.4	133.1	24.7	1.09
J.1.1.	$-CH_2S-$	H	3,4-Benzo	30.9	110.6	4.42	3.71
F.2.1.	$-CH_2CH_2-$	CH_3	H	4.20	12.8	9.97	6.07
G.2.1.	$-(CH_2)_3-$	CH_3	H	9.90	0.82	0.27	0.62

[a] No inhibitory activity up to the indicated concentration.

sulfones, inhibitors of SYN, with inhibitors of DHFR are documented in the literature. The most prominent and first example is the combination of sulfisoxazole with TMP[22] (Bactrim, Eusaprim) used mainly against infections with Gram-negative bacteria. The combination of dapsone (DDS) or a sulfonamide with pyrimethamine (e.g. Fansidar) has been used in the treatment of malaria,[23] and the combination of DDS with brodimoprim is synergistically active against *Mycobacterium leprae*.[24] Such a combination approach could also improve treatment of candidiasis by increasing selectivity and avoiding severe side effects. Side effect reduction may be

Chart 1. Nomenclature of the Newly Synthesized Substituted 2,4-Diaminopyrimidines



observed because the single doses of the combination partners could be reduced. Therefore, the aim of this study was:

(1) to evaluate the activity of sulfones against SYN derived from *C. albicans*; (2) in case of favorable activity, to optimize the inhibitory activity; and (3) to find new lead structures for a selective inhibition of DHFR derived from *C. albicans*, which could then be used in combination with inhibitors of SYN.

Materials and Methods

Chemistry. The pathway for synthesis of 2,4-diaminopyrimidines, the so-called morpholino−anilino procedure, has been reported by Kompis and co-workers.[25] For the synthesis of the planned derivatives, however, only very few examples are found in the literature. Stogryn[26] described the synthesis of a TMP analogue with a $-NHCH_2-$ bridge and Ponsdorf[27] a TMP analogue with a $-OCH_2-$ bridge. In a review, Kompis et al.[28] mentioned a 5-phenylethyl analogue of TMP [2,4-diamino-5-(3,4,5-trimethoxyphenylethyl)pyrimidine] and 2,4-diamino-5-(3,4-dimethoxyphenylethyl)pyrimidine (Ro12-6099, Table 2), which is not described elsewhere. Weinstock et al.[29] described a 2,4-diaminopyrimidine with a $-NHCH_2-$ bridge between the heterocycle and the phenyl ring. The phenyl ring is substituted only by a carboxyl group. Other references of procedures for the synthesis of the compounds to be synthesized within this project could not be found in the literature.

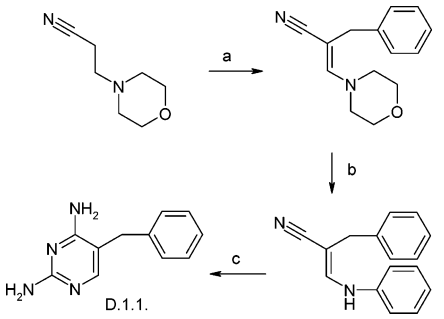
The following nomenclature has been introduced to describe the newly synthesized substituted 2,4-diaminopyrimidines:

The first letter indicates the type of bridge; the first number describes the substituent in 6-position of the pyrimidine and the second the substitution at the bridge moiety (see Chart 1).

The synthetic routes for the 2,4-diaminopyrimidines bearing different bridging groups are exemplified in Schemes 1−4 (for details see the Experimental Section).

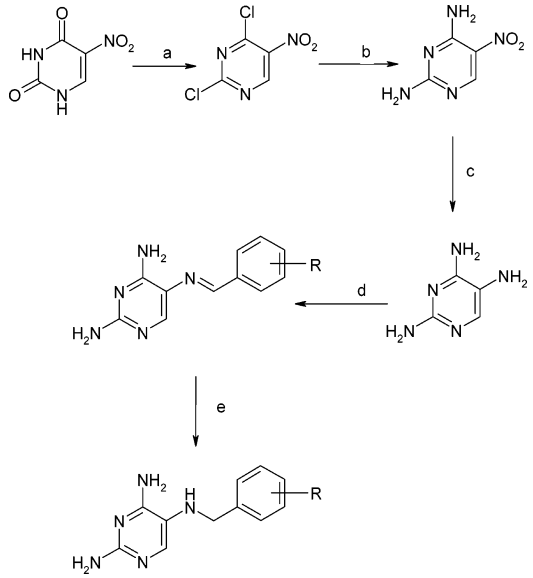
Synthesis of 5-Benzyl-2,4-diaminopyrimidines (Series D). The general procedure (Scheme 1) describes the synthetic route on the example of unsubstituted 5-benzyl-2,4-diaminopyrimidine (**D.1.1.**). The acrylo-

Scheme 1[a]



[a] (a) Ph−CHO; CH_3ONa/DMSO; (b) Ph−NH_2; HCl/EtOH; (c) guanidine hydrochloride; CH_3ONa/EtOH.

Scheme 2[a]



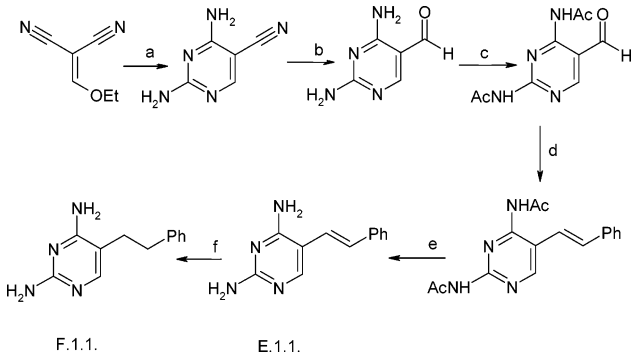
[a] (a) $POCl_3$/Ph-NMe_2; (b) NH_3/EtOH; (c) Zn/HCl/H_2O; (d) R−Ph−CHO/EtOH; (e) $LiBH_4$ or KBH_4/EtOH.

nitrile intermediates are mostly present as a mixture of *E*/*Z*-isomers. *β*-Alkoxy[30] or *β*-aminopropionitriles[25,31] were heated with the substituted aldehydes in the presence of sodium methylate. The resulting α-benzyl-*β*-alkoxy or aminoacrylonitriles were cyclized with guanidine to the corresponding substituted 5-benzyldiaminopyrimidines. Higher yields were obtainable when *β*-morpholinopropionitrile (see Scheme 1) was used with subsequent exchange of the amino group of the morpholine moiety by aniline instead of using *β*-ethoxypropionitrile.[31,32]

Synthesis of 5-Benzylideneamino-2,4-diaminopyrimidines (Series B). The general synthetic pathway is shown in Scheme 2. 5-Nitrouracil was reacted with $POCl_3$. The very unstable intermediate 2,4-dichloro-5-nitropyrimidine reacted under heat and pressure with ethanolic ammonia solution to give the 2,4-diamino-5-nitropyrimidine. This compound was reduced by zinc powder[33] and HCl/H_2O to 2,4,5-triaminopyrimidine, which was then treated with substituted benzaldehydes according to Zhang et al.[33] to give the colored azomethines.

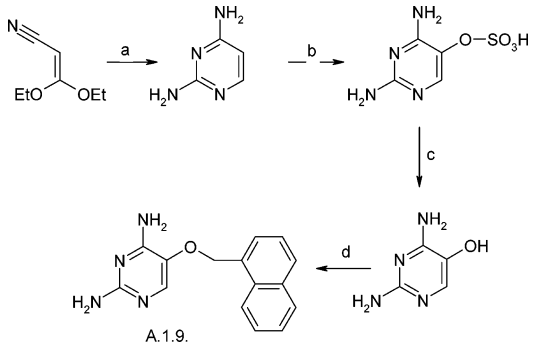
Synthesis of 5-Benzylamino-2,4-diaminopyrimidines (Series C). The azomethines of series B were reduced by alkali borohydrides according to Zhang[33] to

Scheme 3[a]



[a] (a) Guanidine hydrochloride; EtONa/EtOH; (b) H_2/Pd/C; 2 N HCl; 3 h, 60 °C, 4.2 bar; (c) Ac_2O/DMF; (d) $Ph_3P-CH_2Ph^+Cl^-$/DMF; + DBN; (e) KOH/CH_3OH; (f) H_2/Pd/C.

Scheme 4[a]



[a] (a) Guanidine hydrochloride; EtONa/EtOH; (b) ammonium peroxodisulfate; 5 N NaOH; (c) 5 N HCl; (d) 1-chloromethylnaphthalene; EtONa/EtOH.

the corresponding 5-benzylaminopyrimidines (Scheme 2). Lithium borohydride resulted in the highest yield.

Synthesis of 2,4-Diamino-5-styrylpyrimidine (E.1.1.) and 2,4-Diamino-5-phenylethylpyrimidine (F.1.1.). After many unsuccessful attempts, the derivatives could be synthesized following the synthetic route proposed by Kompis[31] (Scheme 3). A solution of guanidine in sodium/ethanol was treated with ethoxymethylenemalononitrile to give 5-cyano-2,4-diaminopyrimidine. The latter was reduced to the aldehyde by a modified reduction method. The reaction mixture was heated and the intermediately formed imine transformed to the aldehyde. The subsequent steps were performed as described by Baker et al.[34] for derivatives with longer chains in the 5-position. The amino groups in the 2- and 4-positions were acetylated to increase the carbonyl reactivity of the aldehyde. This was followed by a Wittig reaction using benzyltriphenylphosphonium chloride. 1,5-Diazabicyclo[4.3.0]non-5-ene (DBN) was used as the base. **E.1.1.** was obtained by removing the acetyl groups in alkaline solution. Reduction with H_2/Pd/C led to derivative **F.1.1**. The synthetic route given in Scheme 3 was used to synthesize also the derivatives **F.2.1.** and **G.2.1**.

Synthesis of 5-Benzyloxy-2,4-diaminopyrimidines (Series A). Many unsuccessful reactions were performed before this class of derivatives could be obtained. This was done by following the procedure of Cavalieri and Bendich.[35] We started with the cyclization of diethoxypropionitrile and guanidine to obtain 2,4-diaminopyrimidine, which was further reacted with am-

monium peroxodisulfate and subsequently with HCl according to Elbs to yield the corresponding phenol[36,37] (Scheme 4). This was then reacted with the appropriate benzyl chloride to the final products (Williamson ether synthesis). For this step many variations have been tested. The best yields were obtained using sodium ethylate in ethanol or potassium carbonate in acetone as reaction medium. Compounds had to be purified by column chromatography according to a method developed in our laboratories (see Experimental Section).

Biology

Determination of Inhibitory Activity against *C. albicans*-Derived DHFR. The isolation and purification of *Candida albicans*-derived DHFR followed the procedures described previously for *E. coli* ATCC 11775, *Mycobacterium lufu* L209, and *P. berghei*.[38,39] The DHFR from rats was commercially available (Sigma D 5519). The inhibition of reduction of dihydrofolate to tetrahydrofolate was followed photometrically. The concentration of inhibitor (I_{50}) leading to 50% reduction in the reaction rate was determined. Results are listed in Table 2.

Inhibition of SYN. The isolation and purification of SYN derived from *C. albicans* was performed in accordance with the general procedure developed for its isolation from *E. coli, M. lufu, M. leprae, and P. berghei*.[38,40] An HPLC-technique was developed to determine quantitatively the inhibition of dihydropteroate synthesis by sulfones.[39]

Results and Discussion

Inhibition of SYN. I_{50}, the concentration leading to 50% decrease in dihydropteroic acid production as compared to the uninhibited control, was determined. The sulfone derivatives used have been described previously as well as their activities against SYN derived from *E. coli*, mycobacteria, and plasmodia.[39,40] Physicochemical data and I_{50} values used in the derivation of regression equations are summarized in Table 1. I_{50} data for *E. coli, M. lufu*, and *P. berghei* have been taken from our previous papers,[39,40] including a larger data set of sulfone derivatives. For better comparison with the new data on inhibition of SYN derived from *C. albicans*, the QSAR (eqs 2−4) was determined from selected identical derivatives.

For the investigated sulfone derivatives, QSAR equations could be derived that may guide their further optimization as inhibitors of *C. albicans* SYN. The QSAR results point to a very similar and conservative enzyme structure of SYN in prokaryotic and eukaryotic cells. For all investigated microorganisms the inhibitory effect depends solely on the electronic nature of the substituents (described by ^{1}H NMR shifts: Δppm2/6) and on the degree of ionization (f_i) in the case of ionizable substituents. Regression equations with high predictive power were obtained.

C. albicans:

$$\log 1/I_{50} = -5.843(\pm 1.851)\Delta\text{ppm2/6} + 0.585(\pm 0.206)f_i - 4.910(\pm 0.146) \quad (1)$$

$$n = 11 \quad r = 0.955 \quad s = 0.122 \quad q = 0.856$$

E. coli:

$$\log 1/I_{50} = -4.157(\pm 1.454)\Delta\text{ppm2/6} + 0.239(\pm 0.191)f_i + 3.933(\pm 0.101) \quad (2)$$

$$n = 12 \quad r = 0.920 \quad s = 0.110 \quad q = 0.824$$

M. lufu (DDS sensitive):

$$\log 1/I_{50} = -7.230(\pm 1.519)\Delta\text{ppm2/6} + 0.535(\pm 0.216)f_i + 4.997(\pm 0.108) \quad (3)$$

$$n = 15 \quad r = 0.957 \quad s = 0.133 \quad q = 0.919$$

P. berghei:

$$\log 1/I_{50} = -6.287(\pm 1.874)\Delta\text{ppm2/6} + 4.119(\pm 0.127) \quad (4)$$

$$n = 10 \quad r = 0.939 \quad s = 0.126 \quad q = 0.892$$

For DDS-resistant *M. lufu*, an almost identical equation (not shown) was obtained as for the DDS-sensitive strain (eq 3). In eqs 1 and 2, the 4-$NHCH_2COOCH_3$ derivative was omitted (as an outlier because of partial hydrolysis of the ester group in the enzyme test). In the regression equations, n is the number of compounds considered, r is the correlation coefficient, s is the standard error of the estimate, and q is the cross-validated correlation coefficient derived from the predictive residual sum of squares (PRESS, leave-one-out method). Regression coefficients are given with their 95% confidence intervals. Δppm2/6 is the ^{1}H NMR chemical shift of the 2/6 protons of the phenyl group bound to the sulfonyl group, and f_i is the fraction of ionized sulfone (i.e. 4-OH, 4-COOH, or 4-$NHCH_2COOH$) at the pH of the enzyme test (pH 8.4 for SYN from *C. albicans*, otherwise pH 7.75). In eq 4 the descriptor f_i was not significant, but in this case only two of 10 derivatives bear an ionizable substituent (Table 1).

As for the larger data set,[40] no influence of lipophilicity (capacity factors log k' from RP-HPLC[40]) could be detected, a fact which presents the possibility to influence transport, absorption functions, and elimination pharmacokinetics without losing binding affinity for the receptor protein.

The similar dependence of the inhibition of SYN on molecular properties of the sulfones for various classes of microorganisms is supported by a principal component analysis (PCA). In a previous publication[40] we had reported PCA results including I_{50} values of cell-free SYN from DDS-sensitive *E. coli, M. lufu*, and *Mycobacterium smegm.* and of cell-free SYN derived from a *M. lufu* strain resistant to sulfones. Furthermore, also I_{25} values for *E. coli* whole cell cultures had been included. In the present study, a PCA analysis with similar results was performed (details not shown) using the I_{50} data of Table 1 together with the I_{25} values of whole cell *E. coli* reported in our previous paper.[40] *P. berghei* data of Table 1 had to be excluded, because the number of identical derivatives was too small, and thus, the data set would become too small. As before,[40] two principal components were derived containing 97% of the information inherent in the five original variables. The first principal component (PC) is loaded by log $1/I_{50}$ data

obtained from four cell-free test systems including the data for SYN derived from sensitive and resistant *M. lufu* (Table 1). This remarkable information showed that the resistance is not due to changes in the target enzyme but rather to changes in the membrane or to an increase in the production of transport proteins of the p-gp type. As before,[40] the second PC is loaded by the log $1/I_{25}$ values obtained from the whole cell cultures of *E. coli* and pointed again to the importance of the bacterial cell wall permeation for the antibacterial activity of these chemotherapeutics. The I_{25} values obtained in whole cell cultures of *E. coli* could be correlated with lipophilicity.[40] The important influence of cell wall permeation on the inhibitory activity of sulfones becomes again obvious when the significant decrease in inhibitory activity against whole cell *C. albicans* cultures is considered.

Effect of Sulfones on Whole Cell *C. albicans*. The inhibitory effect on *C. albicans* ATCC 11651 cultures was determined by a yeast cell generation technique. The generation kinetics was determined by a Coulter counter technique, analogously to previous papers on *E. coli*[41] and mycobacteria.[42] As observed for the effect of SYN inhibitors on *E. coli* and *M. lufu*, a constant lag phase in onset of inhibition appears. The onset is, however, not depending on the inhibitor concentration so that a diffusion controlled effect can be excluded. A disappointingly low inhibitory effect (data not shown) was observed even for the most potent sulfone derivatives, which showed low I_{50} values against extracted SYN. Also the ratio between the two I_{50} values was disappointing (for DDS, 1.70/150 μmol/L).

Development of New Benzylpyrimidines with Inhibitory Activity against *C. albicans*-Derived DHFR. Trimethoprim (TMP, Table 2) is one of the best and most selective inhibitors of bacterial DHFR. It shows, however, low activity against DHFR isolated from *C. albicans* (I_{50} = 30 μmol/L) and no activity against whole cell *C. albicans*. The main aim of this study was to develop new lead structures for inhibitors of *C. albicans*-derived DHFR by studying

(a) the influence of the distance between the pharmacophoric diaminopyrimidine group and the aryl ring [It has been shown that some substituted 2,4-diaminoquinazolines are strong inhibitors of the target DHFR.[21,43] It could therefore be hypothesized that an increase in the distance between the ring systems could be an advantage. Therefore, compounds were synthesized in which the distance between the rings is bridged by a $-CH_2CH_2-$ fragment.],

(b) the influence of changes in bridge atoms [Instead of $-CH_2CH_2-$, other bridges containing different heteroatoms such as $-OCH_2-$, $-NHCH_2-$, or $-CH_2NH-$ were synthesized.];

(c) the influence of the variation in the 6-position of the pyrimidine ring like in pyrimethamine;

and (d) the influence of substitution in 4-position of the benzyl moiety to test if an additional binding site may be recognized as found for other TMP derivatives in the case of *M. lufu*- and *E. coli*-derived DHFR.[44−48]

The newly synthesized compounds were tested against cell-free DHFR derived from *C. albicans* and from other species including DHFR from rats to test for selectivity (Tables 2 and 3).

SAR Results. The following ranking in activity toward DHFR of *C. albicans* as a function of the "bridge elements" is observed:

$$-OCH_2- > -CH_2CH_2- > -CH{=}CH- > -NHCH_2- > -CH_2NH- > -CH_2-$$

The unsubstituted analogue **A.1.1.** with the $-OCH_2-$ bridge is 20 times more potent than TMP. Compared to the unsubstituted 5-benzyl derivative **D.1.1.**, it is even 90 times more potent.

Derivative **F.1.1.** bearing a $-CH_2CH_2-$ bridge shows also increased activity compared to TMP, but the increase is significantly lower (9 times).

Derivative **C.1.1.** possessing a $-NHCH_2-$ bridge is about 4 times more active than TMP and the analogue with the inverse bridge $-CH_2NH-$ (derivative **H.1.1.**) is less active (than TMP).

The increase in bridge length, **G.2.1.**, led to high toxicity (compared to **F.2.1.**): the I_{50} against rat DHFR decreased to 0.82 μmol/L.

Introduction of a methyl group in the 6-position of the pyrimidine ring also led to an increase in toxicity (**A.1.1.**/**A.2.1.**).

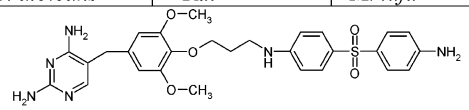
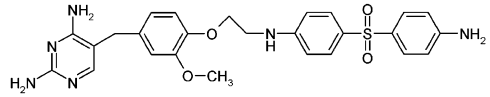
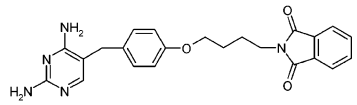
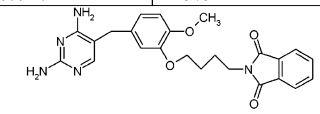
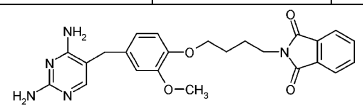
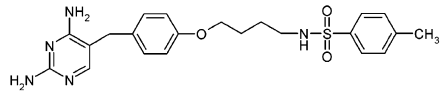
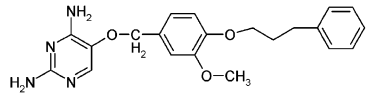
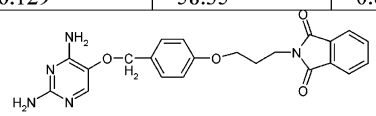
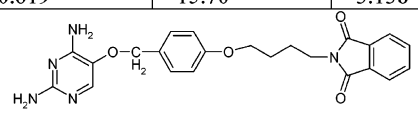
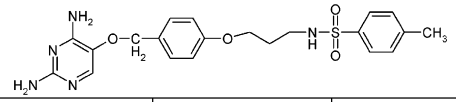
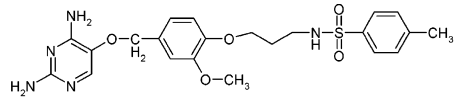
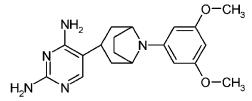
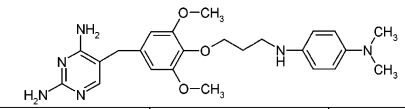
Changes in the substitution pattern of the benzyl ring led to a further increase in activity. Introduction of the 1-naphthyl group reduced the I_{50} toward *C. albicans* DHFR to 0.5 μM. Substitution by methoxy groups led to a decrease in activity. This was in contrast to the positive influence of methoxy groups observed so far on 5-benzyl-2,4-diaminopyrimidines when tested against other species.

The tendency, however, could not be overlooked that an increase in inhibitory activity against *C. albicans*-derived DHFR was always paralleled by an increase in activity against rat DHFR. A similar tendency was also observed by Kuyper et al.[21] for new 7,8-dialkyl-1,3-diaminopyrrolo[3,2-*f*]quinazolines.

Derivatives possessing larger substituents (i.e. spacer sequences with polar end groups) are supposed to reach additional binding sites at the DHFR. This was first shown by Kuyper[44] for 2,4-diamino-5-benzylpyrimidines bearing long chain fatty acids, in which the carboxylate group could reach and interact with Arg 57 of the active center of *E. coli*-derived DHFR as was also found for methotrexate. Similarly, additional binding sites had also been postulated for derivatives substituted by a diaminodiphenylsulfone connected by a spacer of three methylene groups to the ether oxygen in 4-position of the benzyl ring of TMP.[45−48] One of these derivatives, K-130,[45] together with the derivatives HH-133, HH-135, HH-136, HH-154[49] and the newly synthesized **A.1.30.**−**A.1.60.**, is presented in Table 3 together with the biological activities against the different DHFRs.

The most active derivative is **A.1.40.**, which is comparable to the activity of the benzylpyrimidine HH-133. It is 15 times more active compared with the basic structure **A.1.1.** However, note that the activity of **A.1.40.**, if compared to the activity of HH-133 (possessing only a $-CH_2-$ bridge), is not increased and the selectivity remains the same. The derivatives with a sulfonamide group, **A.1.50.**, **A.1.60.**, and HH-154, are more potent than the phenylpropyl-substituted com-

Table 3. Biological Activity (Cell-Free DHFR, I_{50}, μM/L) of 2,4-Diamino-5-benzyl(oxy)pyrimidines with Extended Substituents at the Benzyl Ring

Compound	*C. albicans*	Rat	*M. lufu*	*E. coli*
K-130				
	0.29	24.0	0.040	0.00074
K-220				
	0.34	7.23	0.217	0.009
HH-133				
	0.047	3.644	1.626	0.085
HH-135				
	0.061	1.763	0.116	0.023
HH-136				
	0.031	1.814	0.499	0.031
HH-154				
	0.581	1.724	0.774	0.075
A.1.20.				
	6.129	58.35	0.844	0.20
A.1.30.				
	0.619	15.70	3.138	0.53
A.1.40.				
	0.078	6.070	1.997	0.64
A.1.50.				
	0.435	4.426	1.603	0.54
A.1.60.				
	0.538	4.527	0.976	0.22
Ro 17-3279				
	0.026	0.084	0.00024	---
KC 1308				
	0.97	---	0.204	0.0027

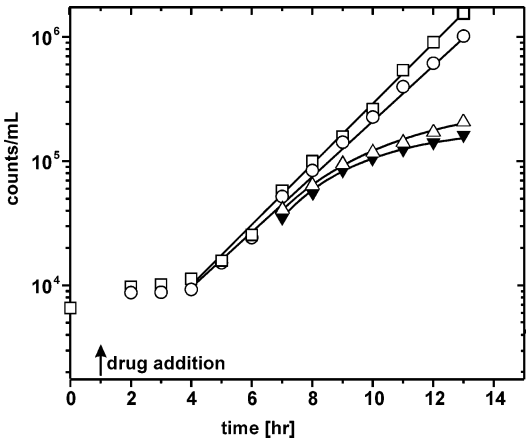


Figure 1. *C. albicans* generation rate in the absence and presence of DDS and KC 1308, alone and in combination: (□) control growth rate: 1.56×10^{-4} sec^{-1}; (○) 100 μM DDS, 1.38×10^{-4}; (△) 50 μM KC 1308, 0.60×10^{-4}; (▼) 100 μM DDS + 50 μM KC 1308, 0.55×10^{-4}.

pound **A.1.20.**, but they are less potent than the derivatives substituted by the phthalimido group and also less selective.

It can be assumed that **A.1.40.** could be a promising new lead structure. It is about 400 times more active against candida DHFR than the standard compound TMP. Its inhibitory activity against rat DHFR is lower in comparison to the other new derivatives possessing a $-OCH_2-$ bridge, i.e. it shows improved selectivity. The question remains, however, is the ability of the new derivatives sufficient to permeate the candida cell wall and to reach the cytoplasm?

Experiments To Affect the Generation Rates of *C. albicans* Cells. Growth kinetic experiments were performed analogously to those reported for *E. coli* and *M. lufu* cultures in the presence and absence of inhibitors.[41,42] The results were disappointing. Despite the low I_{50} values against isolated DHFR of most of the compounds, only **A.1.9.** ($-OCH_2-$ bridge and 1-naphthyl group, $I_{50} = 0.43$ μM) showed a significant inhibitory effect (54%) at 50 μM, whereas the more potent derivative **A.1.40.** ($I_{50} = 0.078$ μM) showed no effect at this concentration.

The effect of a combination of a sulfone (DDS) and a benzylpyrimidine (KC 1308,[47] see Table 3) on the growth rate of *C. albicans* cultures is shown in Figure 1. The combination led to an almost complete stop of growth and shows at least an additive effect. The high concentrations needed for inhibition of growth indicates that the permeability of the *C. albicans* cell wall seems to be the decisive step. This barrier prevents the drugs from reaching the target enzyme in sufficient time and amounts. This argument is supported by data published previously by Chan et al.[50] These authors developed 2,4-diaminoquinazolines as inhibitors of *C. albicans* DHFR. Their most potent derivatives possess I_{50} values of 0.008−0.02 μM, similar to our most potent derivatives HH-133 and **A.1.40.** with an I_{50} of 0.047 and 0.078 μM, respectively, and with similar selectivity indices. But in contrast to our compounds, most of the quinazolines are also highly active against *C. albicans* cell cultures. This observation showed that the inhibition of DHFR of *C. albicans* cultures is possible. The reason for the low activity of our compounds despite their high activity

µg / mL		Cyclosporin A										
		5	4.5	4	3.5	3	2.5	2	1.5	1	0.5	0
Fluconazole	5								+	+	+	+
	4.5								+	+	+	+
	4								+	+	+	+
	3.5								+	+	+	+
	3							+	+	+	+	+
	2.5							+	+	+	+	+
	2						+	+	+	+	+	+
	1.5					+	+	+	+	+	+	2+
	1		+	+	+	+	+	+	+	+	+	2+
	0.5	+	+	+	+	+	+	+	+	+	2+	2+
	0	2+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+

Figure 2. Checkerboard titration of the antifungal activity of fluconazole in combination with cyclosporin A.

against the isolated enzyme is still not fully understood, but possibly it is due to problems in cell wall permeation. The quinazolines possess a lower molecular weight and have a more compact structure. This together with other properties such as lipophilicity and charge might be reasons responsible for the higher activity of 2,4-diaminoquinazolines compared to the 2,4-diamino-5-benzylpyrimidines. The argument of a too high molecular weight for the latter as the only reason for its low activity does, however, not explain the low whole cell activity observed for the low molecular weight 4-aminodiphenyl sulfones.

Effect of Efflux Pumps on Inhibitory Activity. Another reason for inactivity or low activity of many potential drugs in whole cell cultures could be the presence of efflux pumps. The recent discovery of drug-efflux-mediated resistance mechanisms in yeasts opens up a new therapeutic concept. It had been recognized that *C. albicans* expresses multidrug efflux transporter (MET) proteins belonging to two different classes, the ATP-binding-cassette transporters and the major facilitators.[51] METs mediate the efflux of a broad range of compounds, including azoles such as fluconazole. Different MET genes have been identified in *C. albicans*, and the upregulation of some of these genes results in a decrease of the intracellular level of, for example, fluconazole in azole-resistant cells. Thus, possibly also the low activity of our DHFR inhibitors is induced by an efflux system. Recently Sanglard et al.[51] investigated drugs that, by interfering with the MET-mediated active efflux of antifungals, could potentiate the activity of azoles. These authors described that cyclosporin, which had no intrinsic antifungal activity, showed a potent antifungal effect in combination with fluconazole. In a specific test system the combination even seemed to be synergistic. To investigate whether cyclosporin could also potentiate the activity of our diaminopyrimidines, we determined the minimum inhibitory concentrations (MIC values) of potential antifungals toward *C. albicans*. Furthermore, this test was extended to enable the investigation of synergistic, additive, or antagonistic effects of drug combinations by checkerboard titration of combination MICs. In a first test of the combination of cyclosporin A with fluconazole, we could confirm the findings of Sanglard et al. In Figure 2 the downward convex transition from growth (3+, growth like uninhibited control) to nongrowth clearly demonstrates

Table 4. MICs[a] (μg/mL) of 2,4-Diaminopyrimidines against Various Efflux Strains of *C. albicans*

compd	YEM12 WT	YEM13 BENup	YEM14 WT	YEM15 CDR1CDR2up	YEM30 WT	YEM27 quad deletion
A.1.9.	32	>64	>64	>64	>64	32
K-130	>64	>64	>64	>64	>64	>64
K-220	>64	>64	>64	>64	>64	>64
HH-135	>64	>64	>64	>64	>64	>64
HH-136	>64	>64	>64	>64	>64	>64
Ro17-3279	16	>64	64	>64	32	1
fluconazole	1	64	64	64	2	≤0.25

[a] MIC determination was performed in RPMI medium; readings were taken after 48 h.

A.1.9. µg/mL	Cyclosporin A 10	9	8	7	6	5	4	3	2	1	0
100											
90											
80											
70											+
60	+	+	+	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+	+	+	2+
40	+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+
30	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+
20	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+
10	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
0	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+

Figure 3. Checkerboard titration of the antifungal activity of **A.1.9.** in combination with cyclosporin A.

synergism (MIC of single drugs: cyclosporin > 20 μg/mL, fluconazole > 5 μg/mL).

Unfortunately the checkerboard test of the combination of cyclosporin A with the DHFR inhibitor **A.1.9.** showed no potentiating effect (Figure 3). In the tested concentration range growth levels were almost identical with those observed in the right-hand column with no cyclosporin A. This unfavorable finding may be the result of (a) a different efflux system, which is not inhibited by cyclosporin A, or (b) an effective barrier function of the candida cell wall.

To answer this question, a selection of benzylpyrimidines was tested against *C. albicans* mutants possessing either overexpressed efflux pumps or mutants where certain pumps were deleted. At this stage we got access to mutants of *C. albicans* and *Candida glabrata* possessing these properties (from Dr. George H. Miller, Exec. VP, Research and Development, Essential Therapeutics Inc., Mountain View, CA).

The strains used (see Tables 4 and 5) are outlined below.

YEM12 is the wild type of the corresponding YEM13 strain in which the BEN pump is overexpressed. BEN is a member of the major facilitator family of pumps whose only known clinical substrate is fluconazole.

YEM14 is the wild type host for YEM15 in which mutant two coordinately regulated ABC cassette pumps (CDR1, CDR2) are overexpressed. These pumps are capable of effluxing not only fluconazole but also all other clinically used azoles and terbinafine, as well as a large number of experimental compounds.

YEM30 is the wild type *C. albicans* for YEM27 in which four pumps have been deleted, BEN, CDR1, CDR2, and yet another major facilitator pump, FLU, which is known to efflux fluconazole in laboratory strains but is not thought to be a determinant of clinically observed resistance.

Table 5. MICs[a] (μg/mL) of 2,4-Diaminopyrimidines against Various Efflux Strains of *C. glabrata*

compd	YEM75 WT	YEM88 CgCDR1CgCDR2up	YEM216 pCDR1pCDR2pBEN
A.1.9.	16	64	16
K-130	>64	>64	>64
K-220	>64	>64	>64
HH-135	>64	>64	>64
HH-136	>64	>64	>64
Ro17-3279	4	32	4
fluconazole	4	128	2

[a] MIC determination was performed in RPMI medium; readings were taken after 48 h.

Six selected derivatives, namely **A.1.9.**, HH-135, HH-136, K-130, K-220, and Ro17-3279 (see Tables 2 and 3 for structures) were tested against these candida strains and mutants and the results are compared with fluconazole results in Table 4.

For Ro17-3279, showing the lowest I_{50} in vitro (0.025 μmol/L, 0.009 μg/mL), the MIC against the wild types YEM12, YEM14, and YEM30 is 16, 64, and 32 μg/mL, respectively. But when all pumps are knocked out, as in YEM27, the MIC drops to 1 μg/mL. This means about a 32-fold increase in effect, whereas all other compounds (with the exception of **A.1.9.**) show MICs exceeding 64 μg/mL and no effect is seen when the pumps are knocked out, despite their similar activity against the isolated DHFR, e.g. in case of HH-135 and HH-136 (Table 3). This could mean that their inhibitory effect against the isolated enzyme (their I_{50} is about 2−20 times higher than that of Ro17-3279) is not sufficient to influence the growth of *C. albicans* cultures. However, considering the small differences in in vitro activity, this argument does not seem to be very likely. Another factor that may influence activity differently is the different rate of drug influx into candida cells in response to changes in properties and molecular structure. Obviously, the effect is always a balance between uptake and efflux. Efflux may be more constant between similar compounds, since it can be assumed that in a given cell the pumps tend to operate at a "fixed" rate.

The interpretation of results obtained with **A.1.9.** is more problematic. The MIC against the wild-type YEM 12 is 32 μg/mL (120 μmol/L), whereas the MIC of Ro17-3279 is 16 μg/mL (45 μmol/L), i.e. the molar MICs differ by a factor of 3 only. In contrast, the I_{50} values for the cell free system differ by a factor of about 18. It remains unclear whether the uptake of **A.1.9.** is better or whether it is not as good a substrate as Ro17-3279 for one of the four pumps that are deleted in YEM27. Compound **A.1.9.** is a smaller molecule (MW 266) and is more lipophilic (log $k' = 2.14$) than Ro17-3279 (MW 355, log $k' = 1.76$). This could point to a higher influx rate of **A.1.9.**, possibly because of lower molecular

weight. The compound shows about 20 times lower activity in the isolated system but exhibits similar inhibitory activity against whole cell candida as Ro17-3279. This argument would also explain the low whole cell activity of HH-135 and HH-136, which possess similar inhibitory activity against DHFR as Ro17-3279. Both HH derivatives possess higher molecular weight and more polar substituents. Other derivatives in the test set, K-130, K-220, **A.1.40.**, and **A.1.60.**, have also higher MW (MW 450−560) compared to **A.1.9.** and Ro17-3279.

In light of the negative results with **A.1.9**. in the presence of the pump blocker cyclosporin A (Figure 3), it could be possible that fluconazole and **A.1.9.** are transported by different pumps.

In addition to the studies on *C. albicans*, also *C. glabrata* mutants were used to study the effect of pumps on the activity of the folate inhibitors (Table 5).

Strain YEM75 is a wild type and YEM88 represents the glabrata version of overexpressed CDR1CDR2; YEM216 is the glabrata version in which three of the pumps are deleted. The overexpression of the CDR1CDR2 pumps led to an 8-fold increase in MICs of Ro17-3279, but deletion of pump activity did not change the MIC. This seems paradoxical at first glance. It could mean that with overexpression Ro17-3279 can be a substrate of the CDR1CDR2 pumps, but that the basal level of expression present in YEM75 does not affect MICs, because there is no further effect when the pumps are knocked out. Thus, one may conclude that CDR1CDR2 is a potential source of resistance but does not affect basal activity against *C. glabrata*. This may support the tentative explanation why **A.1.9.** acts slightly better against *C. glabrata* if it indeed penetrates better than the other five compounds. It can of course not be excluded that **A.1.9.** is a better inhibitor of the *C. glabrata* DHFR enzyme.

A comparison of the activity of Ro17-3279 against the deletion strains YEM27 (*C. albicans*, MIC = 1 μg/mL) and YEM216 (*C. glabrata*, MIC = 4 μg/mL) indicates that either the uptake of this compound by *C. albicans* is better than by *C. glabrata* or it is less active against the DHFR enzyme of the latter species.

Conclusion

New highly active inhibitors of *C. albicans*-derived SYN and DHFR were synthesized. For the sulfones, inhibitors of SYN, a straightforward QSAR could be derived. Remarkably, from the results of this QSAR it can be concluded that a great similarity seems to exist between the active site of SYN of various microorganisms, including eukariotic *C. albicans*. Therefore, resistance does not seem to be due to changes in the active site. In contrast, a great dissimilarity was observed for the sensitivity of DHFR of prokaryotic microorganisms on one hand and DHFR derived from eukaryotic cells such as *C. albicans* and from rats on the other.

Replacement of the trimethoprim CH_2 group by other bridge atoms as well as introduction of longer spacer groups bearing polar end groups led in most cases to derivatives with increased inhibitory activity. The most active compounds against DHFR (**A.1.40.** and HH-133, -135, -136) possess about 400 times lower I_{50} values as compared to trimethoprim and also interesting selectivity. These derivatives could be candidates for further development of antifungal drugs.

Two problems remain unsolved: (i) the still insufficient selectivity of the new DHFR inhibitors and (ii) their low activity against whole cell cultures of *C. albicans*. It could be shown that the discrepancy between the degree of inhibition of isolated enzymes and of whole cell cultures is at least in part due to the presence of efflux pumps. Other factors as problems in permeability caused by too high molecular weight, by charge, and/or by low lipophilicity cannot be excluded.

Experimental Section

^{1}H NMR Spectra. All synthesized derivatives were characterized by ^{1}H NMR at 360 MHz (Bruker A360 spectrometer) with TMS as internal standard and DMSO-d_6 as solvent. NMR data of newly synthesized compounds are listed in the Supporting Information.

C, H, N Analyses were done by Mikroanalytisches Labor Ilse Beetz, Kronach, Germany.

General Procedure for the Synthesis of Benzyloxy-Substituted 2,4-Diaminopyrimidines (Series A). Some indicated compounds had to be purified by column chromatography according to a method developed in our laboratories: Crude final products were suspended with silica gel in dichloromethane and dried, and the mixture was placed on top of the column [length 10−15 cm, 2 cm diameter; mobile phase, toluene/2-propanol/freshly distilled diethylamine 60/30/10 (by vol)].

5-Benzyloxy-2,4-diaminopyrimidine (A.1.1.). To a solution of sodium ethylate (140 mg Na in 5−20 mL ethanol) was added 400 mg (2 mmol) of 2,4-diamino-5-hydroxypyrimidine·2HCl and the solution warmed to 60 °C for 30 min. Thereafter 253 mg (2 mmol) of benzyl chloride was added and the mixture heated to reflux for 48 h. The solvent was then evaporated. The dry mass was suspended in dichloromethane, 100 mg of silica gel added, and the solvent again evaporated. The mixture was purified by column chromatography as described above. The reaction product was recrystallized two times in ethanol followed by recrystallization in water: yield 150 mg (35%), mp 128 °C. Anal. ($C_{11}H_{12}N_4O$): C, H, N.

5-(2-Chlorobenzyloxy)-2,4-diaminopyrimidine (A.1.2.). Instead of benzyl chloride, 413 mg (2 mmol) of 2-chlorobenzyl bromide was used: yield 400 mg (79%), mp 164 °C (MeOH). Anal. ($C_{11}H_{11}ClN_4O$): C, H, N.

2,4-Diamino-5-(4-methoxybenzyloxy)pyrimidine (A.1.6.). 4-Methoxybenzyl chloride (313 mg, 2 mmol) was used: yield 120 mg (24%), mp 156 °C (EtOH). Anal. ($C_{12}H_{14}N_4O_2$): C, H, N.

2,4-Diamino-5-(1-naphthylmethyloxy)pyrimidine (A.1.9.). 1-Chloromethylnaphthalene (440 mg, 2.5 mmol) was used: yield 400 mg (60%), mp 190 °C (EtOH). Anal. ($C_{15}H_{14}N_4O$): C, H, N.

5-(4-Bromo-1-naphthylmethyloxy)-2,4-diaminopyrimidine (A.1.10.). 4-Bromo-1-bromomethylnaphthalene (602 mg, 2 mmol) was used: yield 200 mg (29%), mp 185 °C (EtOH). Anal. ($C_{15}H_{13}BrN_4O$): C, H, N.

For the following derivatives, 140 mg of Na (6 mmol) and 9 mL of ethylene glycol monomethyl ether were used instead of sodium ethylate, and a small amount of KI was added.

2,4-Diamino-5-(3,4-dichlorobenzyloxy)pyrimidine (A.1.4.). 3,4-Dichlorobenzyl chloride (390 mg, 2 mmol) was used. After 3 days stirring at 20−22 °C, the reaction product was isolated (addition of 10 mL of H_2O) and recrystallized in methanol: yield 250 mg (44%), mp 163 °C. Anal. ($C_{11}H_{10}Cl_2N_4O$): C, H, N.

5-(4-Benzyloxybenzyloxy)-2,4-diaminopyrimidine (A.1.8.). 2,4-Diamino-5-hydroxypyrimidine·2HCl (300 mg, 1.5 mmol) was reacted with 320 mg (1.4 mmol) of 4-benzyloxybenzyl chloride under conditions as for **A.1.4.**: yield 320 mg (54%), mp 142 °C (EtOH). Anal. ($C_{10}H_{18}N_4O_2$): C, H, N.

2,4-Diamino-5-(2,6-dichlorobenzyloxy)pyrimidine (A.1.5.). The reaction conditions for this derivative were slightly changed. 2,4-Diamino-5-hydroxypyrimidine·2HCl (400 mg, 2 mmol) and 482 mg (2 mmol) of 2,6-dichlorobenzyl bromide were mixed with 830 mg (60 mmol) of potassium carbonate in 15 mL of acetone, and a small amount of KI was added. The mixture was refluxed for 72 h. The purification of the mixture was done by column chromatography as described: yield 150 mg (26%), mp 125 °C (EtOH). Anal. ($C_{11}H_{10}Cl_2N_4O$): C, H, N.

5-(4-*tert*-Butylbenzyloxy)-2,4-diaminopyrimidine (A.1.12.). 4-*tert*-Butylbenzyl bromide (456 mg, 2 mmol) was used: yield 200 mg (37%), mp 157 °C (EtOH). Anal. ($C_{15}H_{20}N_4O$): C, H, N.

All following derivatives of series A were also reacted with sodium/ethanol with the addition of a small amount of KI. Some were obtained only in low yield and had to be purified by column chromatography. All were identified by NMR and mostly also by C, H, N analysis.

2,4-Diamino-5-[3-methoxy-4-(3-phenylpropoxy)benzyloxy]pyrimidine (A.1.20). 2,4-Diamino-5-hydroxypyrimidine·2HCl (356 mg, 1.7 mmol) was reacted with 600 mg (1.7 mmol) of 3-methoxy-4-(3-phenylpropoxy)benzyl bromide (Scheme 4). The latter was obtained by reacting 0.055 mol of NaH in DMF with 0.05 mol of 4-hydroxy-3-methoxybenzaldehyde and 0.05 mol of 1-bromo-3-phenylpropane in the presence of a small amount of KI. The benzaldehyde was reduced to the corresponding alcohol with sodium borohydride. The corresponding benzyl bromide was obtained by dissolving 1.5 mmol of the alcohol in dichloromethane. To the solution was added 0.5 mmol of PBr_3 and the mixture stirred for 1 h. Ice water was added and the acid removed by washing. The benzyl bromide was immediately used: yield 200 mg (29%), mp 148 °C (EtOH). Anal. ($C_{21}H_{24}N_4O_3$): C, H, N.

***N*-{3-[4-(2,4-Diaminopyrimidin-5-yloxymethyl)phenoxy]propyl}phthalimide (A.1.30.).** 2,4-Diamino-5-hydroxypyrimidine·2HCl (532 mg, 2.7 mmol) was mixed with 1.0 g (2.7 mmol) of *N*-[3-(4-bromomethylphenoxy)propyl]phthalimide in 35 mL of ethanolate: yield 350 mg (31%), mp 176 °C (EtOH). Anal. ($C_{22}H_{21}N_5O_4$): C, H, N.

The *N*-[3-(4-bromomethylphenoxy)propyl]phthalimide was synthesized by the following reaction steps: 0.021 mol of NaH was suspended in 60 mL of DMF, and 0.019 mol of 4-methylphenol was added under stirring. After the reaction had stopped, the corresponding substituted phthalimide (*N*-(3-bromopropyl)phthalimide) was added together with a small amount of KI and heated to reflux for16 h. The reaction mixture was evaporated to dryness and taken up in dichloromethane/1.5 N NaOH, followed by extraction with dichloromethane twice. *N*-[3-(4-methylphenoxy)propyl]phthalimide (3.4 mmol) was then reacted with 3.7 mmol of NBS (*N*-bromosuccinimide) in 10 mL of CCl_4 under reflux and UV-light and with repeated addition of $Me_2C(CN)-N=N-C(CN)Me_2$: yield 79%, mp 90 °C.

***N*-{4-[4-(2,4-Diaminopyrimidin-5-yloxymethyl)phenoxy]butyl}phthalimide (A.1.40.).** 2,4-diamino-5-hydroxypyrimidine·2HCl (418 mg, 2.1 mmol) was mixed with 800 mg (2.1 mmol) of *N*-[4-(4-bromomethylphenoxy)butyl]phthalimide in 20 mL of ethanolate: yield 200 mg (22%), mp 157 °C (EtOH). Anal. ($C_{23}H_{23}N_5O_4$): C, H, N.

The *N*-[4-(4-bromomethylphenoxy)butyl]phthalimide was obtained following the procedure described for *N*-[3-(4-bromomethylphenoxy)propyl]phthalimide: yield 1.5 g (78%), mp 107 °C.

2,4-Diamino-5-{4-[3-(4-tolylsulfonamido)propoxy]benzyloxy}pyrimidine (A.1.50). 2,4-Diamino-5-hydroxypyrimidine·2HCl (200 mg, 1 mmol) was mixed with 400 mg (1 mmol) of 4-[3-(4-tolylsulfonamido)propoxy]benzyl bromide in 10 mL of ethanolate: yield 100 mg (22%), mp 125 °C (EtOH). Anal. ($C_{21}H_{25}N_5O_4S$): C, H, N.

The 4-[3-(4-tolylsulfonamido)propoxy]benzyl bromide was synthesized by the following steps. First, *N*-(3-bromopropyl)-4-tolylsulfonamide was synthesized using the procedure of Henning[49] with the exception that ethanol is used as solvent instead of water: yield 36%, mp 105 °C. Following the general procedure, the product is reacted with 4-hydroxybenzaldehyde to the ether. The aldehyde is then reduced to the corresponding alcohol and finally transformed to the benzyl bromide by treatment with PBr_3 as described above to obtain the raw product 4-[3-(4-tolylsulfonamido)propoxy]benzyl bromide: yield 67%.

2,4-Diamino-5-{3-methoxy-4-[3-(4-tolylsulfonamido)-propoxy]benzyloxy}pyrimidine (A.1.60.). 2,4-Diamino-5-hydroxypyrimidine·2HCl (200 mg, 1 mmol) was mixed with 450 mg (1 mmol) of 3-methoxy-4-[3-(4-tolylsulfonamido)propoxy]benzyl bromide in 10 mL of ethanolate: yield 90 mg (18%), mp 124 °C (EtOH). Anal. ($C_{22}H_{27}N_5O_5S$): C, H, N.

The 3-methoxy-4-[3-(4-tolylsulfonamido)propoxy]benzyl bromide was synthesized analogously to the procedure given above for the 4-[3-(4-tolylsulfonamido)propoxy]benzyl bromide: yield 64% raw product.

General Procedure for the Synthesis of Benzylideneamino-Substituted 2,4-Diaminopyrimidines (Series B, Preproducts for Series C). 5-Benzylideneamino-2,4-diaminopyrimidine (B.1.1.). Similar to the procedure described by Zhang et al.,[33] equimolar amounts of 2,4,5-triaminopyrimidine (**b.1.3.**) were mixed with the corresponding benzaldehyde and heated in ethanol under reflux temperature for about 12 h. After cooling in a refrigerator, the precipitate is isolated and recrystallized in 70−99% ethanol. The compounds are characterized by ^{1}H NMR and C, H, N analysis. Synthesis of **B.1.1.**: 250 mg of **b.1.3** and 233 mg of benzaldehyde in 4 mL of ethanol: yield 250 mg (59%), mp 168 °C. Anal. ($C_{11}H_{11}N_5$): C, H, N.

General Procedure for the Synthesis of the 5-Benzylamino-2,4-diaminopyrimidines (Series C). Similar to the method used by Zhang,[33] alkali borohydrides were used for reduction of the B-derivatives. However, different alkali borohydrides were used, a larger excess was used, and the time for reaction was prolonged. Also the purification procedure had to be modified. The substituted 5-benzylideneamino-2,4-diaminopyrimidines (series B) (0.76−1.2 mmol) were used with the given amounts of alkali borohydrides and heated under reflux for 3 h. After cooling in a refrigerator, the precipitate was recrystallized in 70−99% ethanol. ^{1}H NMR (Table 8, Supporting Information) and C, H, N analysis were used for characterization.

5-Benzylamino-2,4-diaminopyrimidine (C.1.1.). 5-Benzylidenamino-2,4-diaminopyrimidine (250 mg, 1.2 mmol) dissolved in 8 mL of ethanol was reduced with 250 mg (4.8 mmol) of potassium borohydride: yield 128 mg (51%), mp 146 °C (EtOH). Anal. ($C_{11}H_{13}N_5$): C, H, N.

5-(2-Chlorobenzylamino)-2,4-diaminopyrimidine (C.1.2.). 5-(2-Chlorobenzylideneamino)-2,4-diaminopyrimidine (249 mg, 1.4 mmol) dissolved in 9 mL of ethanol was reduced with 122 mg (5.6 mmol) of lithium borohydride: yield 300 mg (85%), mp 231 °C (EtOH). Anal. ($C_{11}H_{12}ClN_5$): C, H, N.

5-(3-Chlorobenzylamino)-2,4-diaminopyrimidine (C.1.3.). 5-(3-Chlorobenzylideneamino)-2,4-diaminopyrimidine (200 mg, 0.9 mmol) dissolved in 5 mL of ethanol was reduced with 80 mg (3.6 mmol) of lithium borohydride: yield 80 mg; (34%), mp 108 °C (EtOH). Anal. ($C_{11}H_{12}ClN_5$). C, H, N.

2,4-Diamino-5-(3,4-dichlorobenzylamino)pyrimidine (C.1.4.). 2,4-Diamino-5-(3,4-dichlorobenzylideneamino)pyrimidine (271 mg, 0.9 mmol) dissolved in 6 mL of ethanol was reduced with 206 mg (38 mmol) of potassium borohydride: yield 150 mg (55%), mp 186 °C (70% EtOH). Anal. ($C_{11}H_{11}Cl_2N_5$): C, H, N.

2,4-Diamino-5-(4-methoxybenzylamino)pyrimidine (C.1.6.). 2,4-Diamino-5-(4-methoxybenzylideneamino)pyrimidine (300 mg, 1.2 mmol) dissolved in 7 mL of ethanol was reduced with 258 mg (4.8 mmol) of potassium borohydride: yield 200 mg (66%), mp 164 °C (EtOH). Anal. ($C_{12}H_{15}N_5O$): C, H, N.

2,4-Diamino-5-(3,4-dimethoxybenzylamino)pyrimidine (C.1.7.). 2,4-Diamino-5-(3,4-dimethoxybenzylideneamino)pyrimidine (330 mg, 1.2 mmol) dissolved in 8 mL of ethanol was reduced with 550 mg (9.7 mmol) of potassium

borohydride: yield 220 mg (66%), mp 190 °C (EtOH). Anal. ($C_{13}H_{15}N_5O_2$): C, H, N.

2,4-Diamino-5-(1-naphthylmethylamino)pyrimidine (C.1.9.). 2,4-Diamino-5-(1-naphthylideneamino)pyrimidine (200 mg, 0.7 mmol) dissolved in 6 mL of ethanol was reduced with 328 mg (6 mmol) of potassium borohydride: yield 100 mg (50%), mp 175 °C (EtOH). Anal. ($C_{15}H_{15}N_5$): C, H, N.

2,4-Diamino-5-(2-naphthylmethylamino)pyrimidine (C.1.11.). 2,4-Diamino-5-(2-naphthylideneamino)pyrimidine (200 mg, 0.7 mmol) dissolved in 5 mL of ethanol was reduced with 132 mg (6 mmol) of lithium borohydride: yield 150 mg (74%), mp 214 °C (70% EtOH). Anal. ($C_{15}H_{15}N_5$): C, H, N.

Synthesis of 5-(Substituted-benzyl)-2,4-diaminopyrimidines (Series D). The synthesis was performed using the procedure of Kompis et al.[31] and according to the modification of Hachtel.[32] The 2-substituted morpholinoacrylonitriles were prepared in DMSO with solid sodium methylate. Used amounts were between 0.03 and 0.05 mol. In all cases the product was a red oil and was directly used to prepare the 2-substituted anilinoacrylonitriles as described by Hachtel[32] in a 2-propanol/HCl solution with aniline and with heating to reflux. The 2-substituted 3-anilinoacrylonitriles were mixed with solid sodium methanolate and guanidine hydrochloride and heated in ethanol for 36 h under reflux condition.

5-Benzyl-2,4-diaminopyrimidine (D.1.1.): yield 2.1 g (79%), mp 192 °C (EtOH).

2,4-Diamino-5-(1-naphthylmethyl)pyrimidine (D.1.9.): yield 3.2 g (90%), mp 227 °C (EtOH). Anal. ($C_{15}H_{14}N_4$): C, H, N.

2,4-Diamino-5-(2-naphthylmethyl)pyrimidine (D.1.11.): yield 1.3 g (74%), mp 226 °C (EtOH). Anal. ($C_{15}H_{14}N_4$): C, H, N.

Synthesis of 2,4-Diamino-5-styrylpyrimidine (E.1.1.). The synthesis (Scheme 3) was performed in accordance with a synthesis scheme given by Kompis.[52] Procedures for the different steps were taken from the literature and modified if necessary.

5-Cyano-2,4-diaminopyrimidine was prepared according to the method of Huber[53] and reduced to the corresponding 2,4-diamino-5-formylpyrimidine by H_2/Pd/C at 4.2 bar in HCl/water. Following the procedure of Baker,[34] 225 mg of 2,4-diamino-5-formylpyrimidine was heated to 100 °C in 1.8 mL of acetanhydride and 2 mL of DMF under stirring. After cooling to −5 °C the precipitate was recrystallized in DMF/toluene: yield 250 mg (69%) 2,4-diacetylamino-5-formylpyrimidine.

2,4-Diacetylamino-5-styrylpyrimidine. A Wittig reaction was applied as described by Baker[31] for the synthesis of similar compounds. Some modifications were introduced. 2,4-Diacetylamino-5-formylpyrimidine (100 mg) was mixed with 175 mg of benzyltriphenylphosphonium chloride in 1 mL of DMF. Within 10 min, 56 mg of DBN was added and the solution color was changing to dark red. After 10 min, 10 mL of toluene was added and the solution was stirred for 12 h. The solution was evaporated under reduced pressure. The oily residue was dissolved in acetonitrile. The crystals formed were recrystallized in DMF: yield 80 mg (60%).

2,4-Diamino-5-styrylpyrimidine (E.1.1.). 2,4-Diacetylamino-5-styrylpyrimidine (1.4 g) was heated in 50 mL of 3 N methanolic potassium hydroxyde solution (8.4 g of KOH/50 mL of methanol) for 2 h under reflux. The solvent was evaporated under vacuum and the residue taken up in water. The remaining yellow powder was recrystallized in ethanol: yield 850 mg (85%), mp 179 °C (EtOH). Anal. ($C_{12}H_{12}N_4$): C, H, N.

2,4-Diamino-5-(2-phenylethyl)pyrimidine (F.1.1.). E.1.1. (481 mg, 2.3 mmol) and 68 mg of 10% Pd/C were suspended in 40 mL of ethanol and hydrogenated under 1.5 bar for 2.5 h. The catalyst was filtered off and the solvent evaporated. The residue was suspended in water under vigorous stirring and the pH changed with ammonia. The precipitate was recrystallized in water: yield 390 mg (80%), mp 139 °C. Anal. ($C_{12}H_{14}N_4$): C, H, N.

General Procedure for the Synthesis of 5-Phenylalkyl-substituted 6-methyl-2,4-diaminopyrimidines F.2.1. and G.2.1. 2-Amino-4-chloro-6-methyl-5-(2-phenylethyl)pyrimidine and 2-Amino-4-chloro-6-methyl-5-(3-phenylpropyl)pyrimidine. α-Substituted acetylacetic acid esters[54] (Ac−CHR−COOEt, R = $(CH_2)_2$Ph, $(CH_2)_3$Ph) were heated under reflux for 4 h with guanidine carbonate in ethanol to obtain 2-imido-6-methyl-5-(2-phenylethyl)uracil and 2-imido-4-methyl-5-(3-phenylpropyl)uracil: yield ∼27%, mp 226 and 230 °C, respectively.

Following the procedure of Roth et al.,[55] 0.025 mol of the imido product was heated to reflux with 100 mL of $POCl_3$ for 3 h. The main fraction of $POCl_3$ was evaporated under vacuum, and the residue was treated with ice and extracted with dichloromethane. The solution was washed twice with $NaHCO_3$/H_2O and then dried over Na_2SO_4 and filtered. The solvent was evaporated, and 2-amino-4-chloro-6-methyl-5-(2-phenylethyl)-pyrimidine or the corresponding 2-amino-4-chloro-6-methyl-5-(3-phenylpropyl)pyrimidine was obtained as oily products: yield 34% and 78%, respectively. The products were immediately used for synthesis of **F.2.1.** and **G.2.1.**: The corresponding preproducts were treated in an autoclave in ethanolic ammonia for 10 h at 170 °C. The reaction mixture was evaporated to dryness, taken up with 3 N NaOH, and extracted with dichloromethane; the organic phase was dried with Na_2SO_4, filtered, and evaporated. The residue was recrystallized in ethanol.

2,4-Diamino-6-methyl-5-(2-phenylethyl)pyrimidine (F.2.1.). 2-Amino-4-chloro-6-methyl-5-(2-phenylethyl)pyrimidine (1.0 g) was autoclaved in 100 mL of NH_3/EtOH: yield 200 mg (23%), mp 141 °C (EtOH). Anal. ($C_{13}H_{16}N_4$): C, H, N.

2,4-Diamino-6-methyl-5-(3-phenylpropyl)pyrimidine (G.2.1.). 2-Amino-4-chloro-6-methyl-5-(3-phenylpropyl)pyrimidine (2.4 g) was heated in an autoclave in 160 mL of NH_3/EtOH: yield 500 mg (23%), mp 130 °C (EtOH). Anal. ($C_{14}H_{18}N_4$): C, H, N.

5-Anilinomethyl-2,4-diaminopyrimidine (H.1.1.). Following the procedure of Tieckelmann et al.,[56] 500 mg of 2,4-diamino-5-formylpyrimidine were suspended in 20 mL of water and treated with a solution of 100 mg of sodium borohydride as reported. For the following steps the synthetic route of Weinstock[29] was followed but with several modifications. The obtained 2,4-diamino-5-hydroxymethylpyrimidine was heated for 3 h in 30% HBr/acetic acid to reflux. The mixture was evaporated to dryness and, because of instability, the 5-bromomethyl-2,4-diamino-pyrimidine·HBr intermediate (314 mg) was further reacted with 205 mg of freshly distilled aniline in 10 mL of dry DMF; after 6 h DMF was removed by distillation under vacuum. The residue was extracted with 1.5 N NaOH/dichloromethane. The organic phases were combined and dried over Na_2SO_4, and again the solvent was removed. The oily residue was treated with a small amount of diethyl ether and kept at −8 °C. Crystals were recrystallized in ethanol: yield 45 mg (19%), mp 165 °C (EtOH). Anal. ($C_{11}H_{13}N_5$): C, H, N.

2,4-Diamino-5-(2-naphthylthiomethyl)pyrimidine (J.1.11.). NaH (206 mg) was suspended in 20 mL of DMF and 1.058 g of thionaphthol added. After gas development had stopped, 620 mg of 5-bromomethyl-2,4-diaminopyrimidine·HBr (see above) was added. The mixture was stirred at 20−22 °C for 1 week. The solvent was removed by distillation under vacuum. The residue was treated with 3 N NaOH and extracted with dichloromethane. The organic phases were combined, reduced to a volume of about 5 mL, a small amount of silica gel was added, and the mixture evaporated and put on a column for chromatography as described above: yield ca. 15 mg (2.4%), mp 130 °C (EtOH). Anal. ($C_{15}H_{14}N_4S$): C, H, N.

Synthesis of 5-Benzyloxy-6-methyl-2,4-diaminopyrimidines (A.2.1. and A.2.4.). 2,4-Diamino-6-methylpyrimidine 5-Hydrogen Sulfate. The synthesis of 2,4-diamino-6-methylpyrimidine 5-hydrogen sulfate was performed as described by Hull[36] and Hurst.[37] The intermediate was then hydrolyzed in 8 mL of 5 N HCl to the 5-OH derivative. After heating, the reaction mixture was then titrated to pH 6 with 11 N NaOH and NH_4Cl to obtain the 2,4-diamino-5-hydroxy-6-methylpyrimidine hydrochloride.

5-Benzyloxy-2,4-diamino-6-methylpyrimidine (A.2.1.). 2,4-Diamino-5-hydroxy-6-methylpyrimidine·HCl (600 mg) and 468 mg of benzyl chloride were mixed with 986 mg of K_2CO_3 in 30 mL of acetone. After the reaction was finished, the solvent was removed by distillation under vacuum and the residue treated with diethyl ether. Crystals were recrystallized in H_2O/EtOH: yield 580 mg (74%), mp 135 °C (10% EtOH). Anal. ($C_{12}H_{14}N_4O$): C, H, N.

2,4-Diamino-5-(3,4-dichlorobenzyloxy)-6-methylpyrimidine (A.2.4.). 2,4-Diamino-5-hydroxy-6-methylpyrimidine·HCl (400 mg) was dissolved in alcoholate made from 150 mg of sodium and 10 mL of ethylene glycol monomethyl ether, and 430 mg of 3,4-dichlorobenzyl chloride and a small amount of KI were added. The mixture was stirred at 20−22 °C for 48 h, then water was added and the precipitate recrystallized from ethanol: yield 250 mg (38%), mp 181 °C. Anal. ($C_{12}H_{12}Cl_2N_4O$): C, H, N.

Ro12-6099 and Ro17-3279 were a generous gift of Hoffmann-LaRoche, Basel, Switzerland. The derivatives **HH-133, HH-135, HH-136, and HH-154** of Table 3 were synthesized as described by Henning.[49]

Synthesis of Substituted 4-Aminodiphenyl Sulfones. The synthesis has been described elsewhere.[40]

Biology. For the preparation of partially purified enzyme extracts with dihydropteroate synthase (SYN) activity from *E. coli* ATCC 11775, *M. lufu* L209, and *P. berghei*, see Coats et al.,[40] Wiese et al.,[39] and Bartels and Bock.[38] This procedure was also used to obtain partially purified extracts from *Candida albicans* ATCC 11651.

Inhibition Studies of SYN by Sulfones. The rate of folate production with respect to time was determined by periodic sampling of the incubation mixtures and quenching of the reaction by trichloroacetic acid. Amounts of folate produced were determined using folate requiring *Streptococcus faecium*. The incubation temperature was 37 and 31 °C for *E. coli* and *M. lufu*, respectively. For details, see Coats et al.;[40] deviating from this procedure the inhibitory activity against *P. berghei*- and *C. albicans*-derived SYN was determined by HPLC analysis of the produced amount of dihydropteroic acid after oxidation to pteroic acid. For details, see Wiese et al.[39]

Preparation of Partially Purified Enzyme Extracts with Dihydrofolate Reductase (DHFR) Activity. The purification was performed as described in detail[38] for *E. coli* starting with ion exchange chromatography on a DEAE-Sepharose Cl-6B column with a salt gradient, followed by hydrophobic-interaction chromatography on Phenyl-Sepharose Cl-4B and size-exclusion chromatography on Ultrogel AcA 54.

Inhibition of DHFR. The DHFR activity was assayed in 0.1 M Tris buffer, pH 7.24, by monitoring the decrease in absorbance photometrically at 340 nm as a function of time. After incubation of the DHFR with 0.1 mM NADPH and various amounts of inhibitor for 5 min at 25 °C, the reaction was started by adding 0.03 mM dihydrofolate. I_{50} values were calculated by nonlinear regression analysis as the concentration of inhibitor required for 50% decrease in velocity of the enzyme reaction.[45,47]

In Vitro Whole Cell Antifungal Activity Assay. Whole cell antifungal activity was obtained by determination of the minimum inhibitory concentrations (MIC) using the serial dilution method. The MIC was defined as the lowest concentration of test compounds that resulted in a culture with turbidity ≤20% of the value of a control culture. The strains presented in Tables 4 and 5 were grown in RPMI medium, whereas results shown in Figures 2 and 3 (*C. albicans* ATCC 11651) were obtained (at 35 °C, pH 7) in vitamin-free yeast base medium (Difco) supplemented with 1.5 g of asparagine, 3 mg of thiaminiumdichloride, 0.05 mg of biotin, and 0.2 mL of Tween 80 per liter. Here readings were taken after ∼30 h.

Furthermore, the inhibitory effect on *C. albicans* ATCC 11651 whole cell cultures was determined quantitatively by a yeast cell generation technique. The generation kinetics were determined by a Coulter counter technique analogously as described in previous papers for *E. coli*[41] and mycobacteria.[42] Samples were taken over 15 h, and the number of cells counted in a Coulter counter using a capillary equipped with a 38 μm orifice.

Linear Regression Analysis and Principal Component Analysis. Both types of analysis were performed using standard in-house computer programs (K.J.S.).

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Supporting Information Available: ^{1}NMR data (chemical shifts, ppm) and shift assignment of substituted 2,4-diaminopyrimidines (series **A**−**J**). This material is available free of charge via the Internet at http://pubs.acs.org.

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