Structural Studies on Bioactive Compounds. 28.[1] Selective Activity of Triazenyl-Substituted Pyrimethamine Derivatives against *Pneumocystis carinii* Dihydrofolate Reductase

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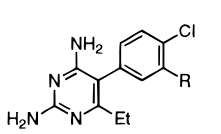
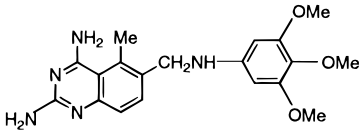
Triazenyl-substituted pyrimethamine derivatives **10a**−**s** have been prepared by coupling diazotized 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethyl pyrimidine (**1c**) with a series of secondary amines in aqueous sodium carbonate solution. The triazenes which are stable and poorly soluble as free bases form more soluble, but unstable, salts with alkanesulfonic acids. The lead dimethyltriazene 2,4-diamino-5-[4-chloro-3-(3,3-dimethyltriazen-1-yl)phenyl]-6-ethylpyrimidine (**4a**) forms a crystalline ethanesulfonic acid salt (solvated with 2-propanol), which is protonated at the pyrimidine N-1 position as determined by X-ray crystallography. The ability of these new triazenes to inhibit *Pneumocystis carinii* dihydrofolate reductase *in vitro* has been compared to that of triazene **4a**. The most potent and selective compound, 2,4-diamino-5-[3-[3-[2-(acetyloxy)ethyl]-3-benzyltriazen-1-yl]-4-chlorophenyl]-6-ethylpyrimidine (**14a**), has an IC_{50} value of 0.17 μM against the microbial enzyme and potentially useful selectivity (rat liver IC_{50}/*P. carinii* IC_{50} = 114).

Introduction

The emergence of the HIV pandemic has created a population of immune-supppressed patients who are vulnerable to opportunistic microorganisms. Infections by *Pneumocystis carinii* elicit a pneumonia (PCP) which is the leading cause of mortality in AIDS sufferers in the USA.[2,3] *P. carinii*, like most microorganisms, synthesizes folates from PABA[4] and generates reduced folates by employing a dihydrofolate reductase enzyme (DHFR) which is structurally distinct from the mammalian variant.[5,6] Combinations of sulfamethoxazole and trimethoprim are considered standard chemotherapy for PCP, but their use is associated with unpredictable, and occasionally severe, side effects.[7] Although the search for effective treatments for PCP has broadened to include a range of diverse structures of different biochemical mechanisms,[8] there is still scope for the development of selective inhibitors of *P. carinii* DHFR. Most recently, new series of lipophilic 2,4-diamino-5-deaza-[9] and 2,4-diamino-8-deazapteridines[10] have been shown to be superior to the benchmark trimetrexate **2** as inhibitors of *P. carinii* and *Toxoplasma gondii* DHFR.

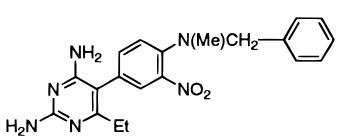
In the past, we have utilized the diaminopyrimidine pyrimethamine **1a** as a starting material to prepare the corresponding nitro (**1b**) and amino analog (**1c**) in order to develop synthetic routes to lipophilic inhibitors of mammalian DHFR with potential antitumor activity. Thus the *m*-azidopyrimethamine **1d**,[11] used clinically as the ethanesulfonic acid salt (MZPES),[12] had a shorter biological half-life in humans than etoprim **1e** but was withdrawn from clinical trial because it elicited unpredictable neurotoxicity.[13] An alternative lipophilic pyrimethamine derivative, methylbenzoprim **3**, is one of the most potent known inhibitors of mammalian (rat liver) DHFR (K_i 0.009 nM),[14] possibly because it is competitive with NADPH as well as dihydrofolate.[15] Among a large series of related structures submitted to the NCI Developmental Therapeutics Program for antitumor evaluation, including the above, the dimethyltriazenyl-substituted pyrimethamine **4a** was subsequently compared with pyrimethamine as an inhibitor of *P. carinii* and *T. gondii* DHFR. The useful selectivity (rat liver IC_{50}/*P. carinii* IC_{50} = 6.75[16] and rat liver IC_{50}/*T. gondii* IC_{50} = 61[17]) and confirmation that the triazene-inhibited *P. carinii* growth in culture with an IC_{50}



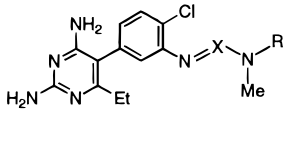


1a: R = H (pyrimethamine)
1b: R = NO_2
1c: R = NH_2
1d: R = N_3
1e: R = Cl
1f: R = N_2^+ BF_4^-

2 (trimetrexate)



3 (methylbenzoprim)



4a: X = N, R = Me
4b: X = CH, R = Me
4c: X = N, R = H

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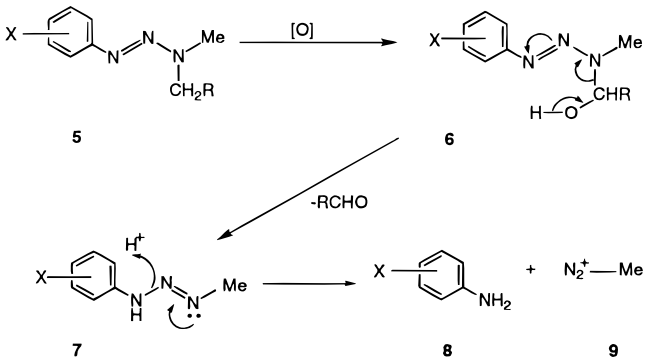
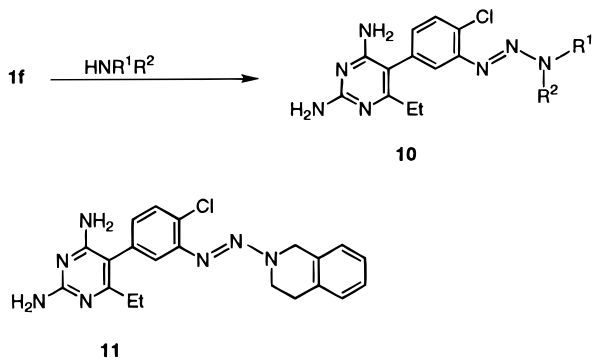
Scheme 1



Scheme 2



	R^1	R^2
a:	Me	Et
b:	Me	Pr
c:	Me	CH_2Ph
d:	Me	$(CH_2)_2NEt_2$
e:	Et	Et
f:	^{t}But	^{t}But
g:	—— $(CH_2)_4$ ——	
h:	—— $(CH_2)_5$ ——	
i:	— $(CH_2)_2NMe(CH_2)_2$ —	
j:	— $(CH_2)_2CHOH(CH_2)_2$ —	
k:	— $(CH_2)_2O(CH_2)_2$ —	

	R^1	R^2
l:	Me	$(CH_2)_2OH$
m:	Et	$(CH_2)_2OH$
n:	Pr	$(CH_2)_2OH$
o:	^{t}But	$(CH_2)_2OH$
p:	CH_2Ph	$(CH_2)_2OH$
q:	$(CH_2)_2OH$	$(CH_2)_2OH$
r:	$(CH_2)_2OMe$	$(CH_2)_2OMe$
s:	Me	CH_2 — 

value of 5.5 μM[18] marked out compound **4a** as a suitable lead compound on which to base a synthesis effort for further structure−activity evaluation. Interestingly, the related pyrimethamine **4b**, with a dimethylformamidine side chain (which is presumably protonated at physiological pH), is less potent and selective againstthe *P. carinii* enzyme (rat liver IC_{50}/*P. carinii* $IC_{50} = 0.33$).[16]

The literature on 1,1-dialkyl-3-aryltriazenes related in structure to the dimethyltriazene **4a** alerted us to the concern that methyl- and ethyltriazenes might be mutagenic.[19] Thus, in those 1-alkyl-1-methyl-3-aryltriazenes of general structure **5**, where the alkyl group has a free H atom on the α-carbon, metabolic oxidation can generate unstable (hydroxyalkyl)triazenes **6**,[20] which readily lose a carbonyl fragment (formaldehyde from a hydroxymethyltriazene)[21] to liberate a mutagenic monomethyltriazene **7** (Scheme 1). In the case of the dimethyltriazene **4a**, metabolic demethylation would afford the known monomethyltriazene **4c**.[11] Although monomethyltriazenes are relatively stable above pH 7.4,[22] generally in acid media they undergo proteolytic fragmentation to generate an arylamine **8** (amine **1c** from the monomethyltriazene **4c**)[11] and the fugitive methyldiazonium species **9** which methylates O^6- and N^7-positions of guanine residues of DNA.[23] Monoethyltriazenes can similarly ethylate DNA and the aforementioned chemistry is, largely, independent of the nature of aryl substituent X.[20] Although in the present study our main target was limited to identify inhibitors of *P. carinii* DHFR with enhanced potency and selectivity relative to the lead dimethyltriazene **4a**, our ultimate objective is to select modified dialkyltriazenes which could not be metabolized to mutagenic methylating or ethylating agents. Furthermore, by incorporating bulky substituents containing polar oxygen substituents at the terminal triazene N-atom, we hoped to block protonation at this site, thereby enhancing acid stability and solubility. In fact, in all the examples studied, there is provision of an alternative site for protonation at N-1 on the diaminopyrimidinyl residue.[24]

Results and Discussion

Synthetic Chemistry. The starting material for the synthesis of the required triazenes **10** was the diazonium tetrafluoroborate **1f**, which was prepared from the corresponding amine **1c** by diazotization in aqueous tetrafluoroboric acid. We have shown previously that this diazonium salt, isolated as a hydrotetrafluoroborate (hemihydrate) salt,[11] can be stored safely in bulk quantity (25 g) for periods >10 years at 25 °C. The water-soluble salt was coupled in aqueous sodium carbonate solution at 0 °C with a range of dialkylamines to yield the triazenylpyrimidines **10a**−**s** in yields of 75−85% (Scheme 2). Interaction of the diazonium salt **1f** with tetrahydroisoquinoline gave the triazene **11**, which can be considered as a modified *N*-benzyl-*N*-ethyltriazene. The triazene **10p** formed (70%) by coupling diazonium tetrafluoroborate **1f** with *N*-benzylethanolamine **12** furnished an *O*-acetyl derivative **14a** (35%) when treated with acetyl chloride in pyridine at 25 °C with a catalytic amount of DMAP: this acetate and the corresponding propionate **14b** were formed more efficiently by coupling the diazonium chloride derived from the arylamine **1c** and the hydrochloride salts of *N*-benzylethanolamine esters **13a,b** in aqueous sodium carbonate in 75 and 80% yields, respectively (Scheme 3).

All of the aforementioned triazenes could be crystallized unchanged from aqueous polar solvents (acetone, methanol, or ethanol); they were characterized as triazenes by their production of red azo-dye colorations when heated with 2-naphthol in acetic acid. Examination of the ^{1}H NMR spectra of "pure" (single-spot TLC) samples of some of the triazenes revealed the presence of solvents which were tenaciously held even on prolonged vacuum drying. Solvation is a characteristic feature of other diaminopyrimidines of the pyrimethamine class.[11,14]

Physical Properties of Triazenes. The solubility of the triazene derived from diethanolamine **10q** in water at 25 °C was disappointingly low (<0.1 mg/mL) although solubility was enhanced (to 3.85 mg/mL) at pH 4 in citrate buffer in which the triazene is protonated. However qualitative stability experiments monitored by

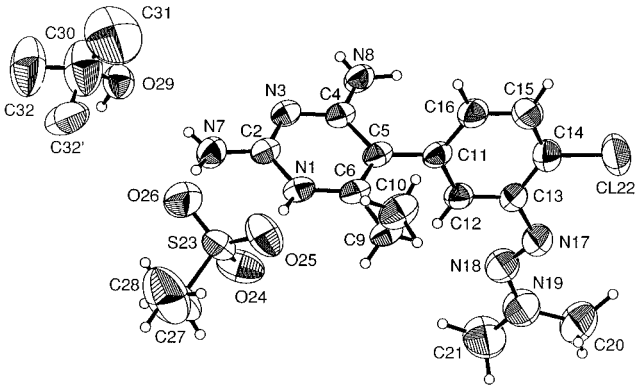


Figure 1. ORTEP drawing of 2,4-diamino-5-[4-chloro-3-(3,3-dimethyltriazen-1-yl)phenyl]-6-ethylpyrimidine (**4a**) as the ethanesulfonic acid salt (2-propanol solvate) and the crystallographic numbering scheme.

HPLC showed that ethanol solutions of three triazenes **10p**, **10q**, and **14a** were rapidly degraded by 1 M HCl, 1 M NaOH, and aqueous H_2O_2 solutions. A detailed evaluation of the stability of **10q** at pH 4 showed that the compound was essentially undegraded when maintained at −20 °C for 1 h, but suffered >25% decomposition at 4 °C over 1 h. Although the degradation products have not been identified, it is probable that heterolytic fission of the triazene linkage generates an unstable diazonium species which then suffers reduction by the ethanol.

These results notwithstanding, methanesulfonic and ethanesulfonic acid salts of some of the triazenes were prepared successfully from the corresponding bases with methanesulfonic or ethanesulfonic acids (1 molar equiv) in 2-propanol and were shown to be monoprotonated pyrimidines (^{1}H NMR) although they gave unreliable C, H, N analyses because of association with nonstoichiometric quantities of solvents. Crystals of the ethanesulfonic acid salt of the lead compound **4a** suitable for an X-ray analysis were grown from 2-propanol to confirm the site of protonation and to facilitate future molecular modeling studies and interpretation of the biological data.

X-ray Crystallography. The contents of the asymmetric unit, comprising one molecule of **4a** protonated at N-1 of the heterocycle, one ethanesulfonate counter ion, and a disordered 2-propanol solvent molecule, are shown with their crystallographic numbering scheme in an ORTEP[25] drawing in Figure 1. The cation of **4a** consists of three moieties, each of which is planar within rms deviation of 0.01 Å; the pyrimidine ring, the benzene ring, and the dimethyltriazene chain. The first two intersect at an angle of 70.7(1)°, and the last two at 24.8(4)°. A structural feature common to simple 1-aryl-3,3-dimethyltriazenes[26] is the large C12−C13−N17 bond angle [124.1(3)°], which alleviates interference between the triazene and aryl groups at the expense in the present structure of a close contact [2.925(5) Å] between N17 and Cl22 which is not relieved by any similar expansion of the C13−C14−Cl22 bond angle [119.7(3)°]. The terminal triazene N atom exhibits sp^2 character, and N17−N18 at 1.271(5) Å shows more double-bond character than N18−N19 at 1.322(5) Å.

As is typical for antifolate drugs,[27] the protonated pyrimidine ring is heavily hydrogen bonded, donating hydrogen bonds to counterions at the 1 and 2 positions, forming a centrosymmetric dimer at 3 and 4, and directing one H atom from each amino group toward the 2-propanol O29 atom. No triazene N atom is involved in hydrogen bonding.

Biological Results. Results of the *in vitro* assays of triazenyl-substituted pyrimethamine derivatives against *P. carinii* and rat liver DHFR are recorded in Table 1. Included in this table are previously published results from one of us[16] which allows for an analysis of the influence of substitution at the 3′-position of the pyrimethamine nucleus. Dealing with the reference compounds first, pyrimethamine **1a** and its nitro (**1b**) and azido analog (**1d**) are relatively weak inhibitors of *P. carinii* DHFR and more selectively inhibit the rat liver enzyme, in particular the nitropyrimethamine **1b**. The reactive diazonium tetrafluoroborate **1f,** which, presumably, can react covalently with nucleophilic components of the target enzyme, is the most potent of the four simple pyrimethamine congeners with an IC_{50} of 0.19 μM against *P. carinii* enzyme but is still selectively active against the mammalian enzyme. Trimetrexate **2** and methylbenzoprim **3** (the structure of which was incorrectly assigned in earlier reports)[16,17] are 1000-fold more potent than pyrimethamine against the mammalian enzyme: they differ in that trimetrexate discriminates little between the *P. carinii* and mammalian enzymes whereas methylbenzoprim **3** is an exquisitely selective inhibitor of the rat liver variant. Indeed, this compound is one of the most active known nonclassical inhibitors of mammalian DHFR and the nitro group (cf. **1b**) is an essential requirement for high potency in the series.[14,15]

The lead dimethyltriazene **4a** is approximately equi-

Scheme 3

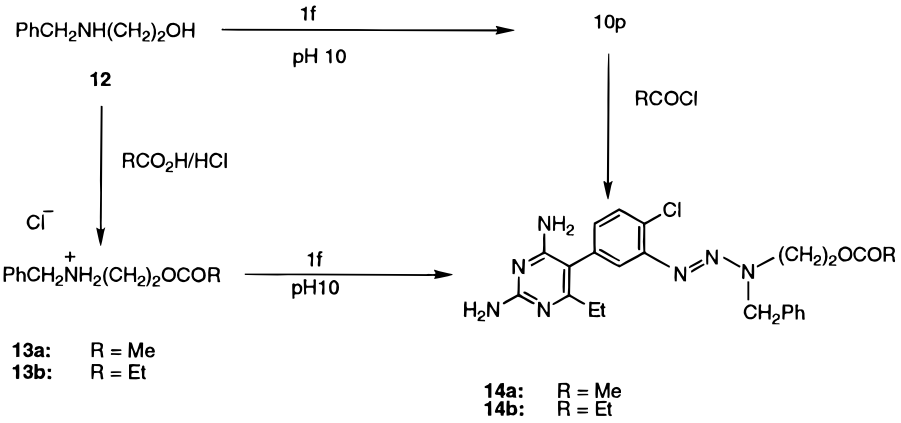


Table 1. Inhibition Data for Triazenyl-Substituted 2,4-Diaminopyrimidines and Reference Compounds against *P. carinii* and Rat Liver DHFR

compound	IC_{50} (μM) vs DHFR[a]		selectivity index[b] (rl/pc)
	P. carinii	rat liver	
1a (pyrimethamine)	3.65	2.3	0.63[c]
1b	0.85	0.015	0.02[c]
1d	1.33	0.33	0.25[c]
1f	0.19	0.032	0.17[c]
2 (trimetrexate)	0.042	0.003	0.071[c]
3 (methylbenzoprim)	1.6	0.0032	0.002[c]
4a	2.8	18.9	6.8[c]
4a ($EtSO_3H$ salt)	6.0	7.8	1.3
4b	10.6	3.5	0.33[c]
4c	4.6	2.3	0.5
10a	14.1	202	14
10a ($MeSO_3H$ salt)	6.6	0.57	0.09
10b	10.2	>4[d]	
10b ($MeSO_3H$ salt)	7.6	0.89	0.12
10c	2.8	1.8	0.64
10d	18.2	17.3.	1
10e	29.3	17.7	0.6
10f	>11[e]	>11	
10g	>11[e]	>11	
10h	40.4	66.8	1.7
10i	8.5	16.2	1.9
10j	3.3	7.2	2.2
10k	1.7	26.3	15
10k ($MeSO_3H$ salt)	4.7	1.1	0.23
10l	3.5	27.9	8
10m	11.5	27	2.3
10n	2.5	0.44	0.2
10o	4.9	10.3	2.1
10p	0.26	7	27
10q	0.44	5	11
10r	0.91	26.1	29
10s	0.57	0.5	0.9
11	5.0	1.7	0.34
14a	0.17	19.4	114

[a] See ref 16 for method. [b] IC_{50} (μM) for rat liver DHFR/IC_{50} (μM) for *P. carinii* DHFR; values of >1 denote compounds selective toward the enzyme from *P. carinii*. [c] Results from ref 16. [d] No inhibition of rat liver DHFR at 3.2 μM. [e] No inhibition of *P. carinii* DHFR at 11 μM.

active with pyrimethamine **1a** as an inhibitor of the *P. carinii* enzyme but a weaker inhibitor of the mammalian enzyme (Table 1). This results in a 10-fold enhancement in selectivity for **4a** relative to pyrimethamine **1a**. This selectivity is lost in the dimethylformamidine **4b** and the monomethyltriazene **4c**. Overall, with a few exceptions (see later), the new triazenes are less potent than pyrimethamine against the *P. carinii* enzyme: the observed increase in selectivity (rl/pc) is due to reduced potency against the rat liver enzyme. Thus, the ethylmethyltriazene **10a** is a 4-fold less potent inhibitor of the *P. carinii* enzyme than the corresponding dimethyltriazene **4a** but its significantly weaker activity against rat liver DHFR gives **10a** an improved selectivity index (rat liver IC_{50}/*P. carinii* IC_{50} = 14 compared to 6.5 for the dimethyltriazene). In a consistent pattern the other *N*,*N*-dialkyltriazenes **10b**−**f** displayed only moderate potency against the *P. carinii* enzyme (IC_{50} > 10 μM) with a tendency to weaker activity as the size of the hydrophobic attachment increased. When both alkyl groups are constrained within a heteroalicyclic ring as in **10g**−**k** the role of an extra nitrogen or oxygen atom in increasing potency toward *P. carinii* DHFR in the triazenes is clearly seen in a comparison between the activities of the piperidino- (**10h**) and morpholinotriazene (**10k**). The morpholino compound is >20-fold more potent against the *P. carinii* enzyme and significantly more selective (rat liver IC_{50}/*P. carinii* IC_{50} = 15), with the highest favorable index thus far observed in this series.

Table 2. Activity of Triazenyl-Substituted 2,4-Diaminopyrimidines against *P. carinii* Microorganisms Extracted from Immunocompromised Rats

compound	IC_{50} (μg/mL)[a]	compound	IC_{50} (μg/mL)[a]
10k	49.7	**10r**	30.5
10p	11.1	**14a**	43.9
10q	46.6	pentamidine isethionate	2.5

[a] Concentration required to inhibit uptake of [^{3}H]-4-aminobenzoic acid over 24 h.

The acyclic triazenes **10l**−**s,** with one or more oxygen functionalities attached to the triazene moiety, displayed a significant divergence of potency and selectivity. Within the homologous alkyltriazenes bearing (2-hydroxyethyl) substituents **10l**−**o** no significant structure−activity trends were discernable and the compounds had only low selectivity against the *P. carinii* DHFR. However, replacement of an alkyl group for benzyl in the benzyl(hydroxyethyl)triazene **10p** gave an agent with potency (IC_{50} = 0.26 μM) and selectivity against the *P. carinii* enzyme (rat liver IC_{50}/*P. carinii* IC_{50} = 27). This compares with the low potency and selectivity of the "cyclic" benzyltriazene **11** derived from tetrahydroisoquinoline (rat liver IC_{50}/*P. carinii* IC_{50} = 0.34). The acetoxy derivative **14a** of the benzyltriazene **10p** is the most potent compound in the series (IC_{50} = 0.17 μM) with a selectivity index (114) which is one of the highest thus far recorded for all DHFR inhibitors.[8] Incorporation of two oxygen atoms in different arms of the triazene side chain **10q,r** also gave compounds with relatively high potency against the *P. carinii* DHFR (IC_{50} < 1 μM) and, in the case of the bis(2-methoxyethyl)triazene **10r**, a favorable selectivity index of 29. This selectivity was lost when both oxygen atoms were in the same alkyl group **10s**.

The activities of the alkanesulfonic acid salts of certain of the triazenes were generally similar to those of the free bases toward *P. carinii* DHFR, but in all cases the IC_{50} values of the salts against rat liver DHFR were markedly reduced (Table 1). The chemical instability of the triazenes at pH 4 has been noted previously. Possibly, variations in the experimental conditions may allow for increased breakdown of the salts in the rat liver DHFR assays. The product of such (heterolytic) degradation would be the diazonium species **1f** which is a known potent inhibitor of rat liver DHFR (IC_{50} = 0.032 μM).[16]

Certain of the significant triazenes identified above were also tested for their ability to inhibit the viability of *P. carinii* organisms extracted from lungs of immunosuppressed (dexamethasone) rats. Microorganism viability over 24 h was measured by monitoring uptake of [^{3}H]-4-aminobenzoic acid. The most active compound was the triazene **10p**, although this compound was less potent than the reference compound pentamidine isethionate (Table 2).

Some of the more active triazenes against *P. carinii* DHFR were also tested against the enzyme from *T. gondii*. Results of these *in vitro* assays, together with those of related reference compounds, are summarized in Table 3. Pyrimethamine is inhibitory to the protozoal enzyme with an IC_{50} of 0.3 μM and reasonable selectivity (rat liver IC_{50}/*T. gondii* IC_{50} = 5.9): the dimethyl-

Table 3. Comparative Inhibition Data for Triazenyl-Substituted 2,4-Diaminopyrimidines and Reference Compounds against *T. gondii* and Rat Liver DHFR

compound	IC_{50} (μM) v DHFR[a]		selectivity index[b]
	T. gondii	rat liver	(rl/tg)
1a (pyrimethamine)	0.39	2.3	5.9[c]
1b	0.013	0.015	1.1[c]
1d	0.69	0.33	0.5[c]
1f	0.023	0.032	1.4[c]
2 (trimetrexate)	0.01	0.003	0.3[c]
3 (methylbenzoprim)	0.091	0.0032	0.04[c]
4a	0.31	18.9	61[c]
4b	1.6	3.5	2.2[c]
10k	0.19	26.3	138
10r	8.8	26.1	3
14a	0.69	19.4	28

[a] See ref 17 for method. [b] IC_{50}(μM) for rat liver DHFR/IC_{50} (μM) for *T. gondii* DHFR; values of >1 denote compounds selective toward the enzyme from *T. gondii*. [c] Results from ref 17.

triazene is equipotent against the parasitic enzyme but 10-fold more selective (rat liver IC_{50}/*T. gondii* IC_{50} = 61). The morpholinotriazene **10k** is the most potent (IC_{50} = 0.19 μM) of the limited number of new triazenes evaluated in this work and displays potentially useful selectivity (rat liver IC_{50}/*T. gondii* IC_{50} = 138). The benzyl(acetoxyethyl)triazene **14a**, the most effective compound (combination of potency and selectivity) against *P. carinii* DHFR, also exhibited reasonable potency and selectivity toward the *T. gondii* enzyme.

Conclusion

We have shown previously by ^{19}F and ^{1}H NMR studies that 2,4-diamino-5-(4-fluoro-3-nitrophenyl)-6-ethylpyrimidine (**1b**; Cl = F)[28] and methylbenzoprim **3** [29] bind to *Lactobacillus casei* DHFR in two different conformations which are unequally populated and not interconvertible in the bound state. These conformations are generated by restricted rotation about the pivotal pyrimidine−phenyl bond and position the critical nitro group in two different environments within the active site of the enzyme. The X-ray structure of the dimethyltriazenylpyrimidine **4a** (Figure 1), which also has an asymmetrically-substituted 5-aryl group, suggests that similar steric factors will determine the binding potency and specificity of the sterically demanding triazenyl moieties to *P. carinii* DHFR and the rat liver enzyme.

Basing a synthetic effort on the lead dimethyltriazene **4a**, we have achieved the following:

(i) An efficient high-yielding synthesis (three steps from pyrimethamine **1a**) of a series of triazenyl pyrimidines with varying substituents at the triazene terminus.

(ii) Replacement of the potentially mutagenic dimethyltriazene fragment with other groups which do not have the potential for metabolic conversion to potential DNA-alkylating monoalkyltriazenes.

(iii) Identification of derivative **14a** which is 16-fold more potent than **4a** as an inhibitor of *P. carinii* DHFR (IC_{50} 0.17 μM) and 16-fold more selective (rat liver IC_{50}/*P. carinii* IC_{50} = 114). Intriguingly, compound **14a** shares with methylbenzoprim **3** the presence of an *N*-benzyl grouping but the differing selectivity for DHFR from different sources—*P. carinii* DHFR for **14a** and rat liver enzyme for **3**—is especially notable. A trimethoprim analog epiroprim (Ro11-8958) has been reported to have a selectivity (human DHFR IC_{50}/*P. carinii* IC_{50}) of 200.[30] However, it has been shown by one of us[8] that, when this agent was retested in the standard assay employed in the current work, a selectivity of 44 was measured for human recombinant DHFR and 28 for rat liver DHFR. The selectivity of epiroprim can be explained by its very low potency toward mammalian DHFR.

Experimental Section

Mass spectra were recorded on an AEI MS-902 or a VG Micromass 7070E spectrometer. IR spectra were determined in KBr on a Mattson 2020 GALAXY series FT-IR spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AC250 or ARX250 spectrometers in DMSO-d_6 solutions TLC systems for routine monitoring of reaction mixtures and confirming the homogeneity of analytical samples employed Kieselgel 60F_{254} (0.25 mm) with either $CHCl_3$ or $CHCl_3$−2% ethanol as developing solvents.

General Procedure for the Synthesis of Triazenyl-substituted 2,4-Diamino-5-(4-chlorophenyl)-6-ethylpyrimidines. To a stirred solution of the diazonium tetrafluoroborate **1f**[11] in water at 0 °C was added the appropriate secondary amine (1 molar equiv) and sufficient solid sodium carbonate to adjust the mixture to pH 10. The suspension was stirred at 0 °C for 2 h, and the solid triazene was collected and washed with copious amounts of water. The products were isolated in yields of 80−95%. The triazenes could be purified by crystallization from methanol or aqueous acetone, or by flash chromatographic fractionation on Sorbsil silica gel C 60-H (40−60 mm), using an ethyl acetate−hexane solvent mixture. Alternatively, an aqueous solution of the diazonium chloride hydrochloride salt (prepared *in situ* by diazotization of amine **1c** in 3 M hydrochloric acid at 0 °C)[11] was coupled with the appropriate amines at pH 10. The following compounds were prepared.

2,4-Diamino-5-[4-chloro-3-(3,3-dimethyltriazen-1-yl)-phenyl]-6-ethylpyrimidine (4a): mp 233−235 °C (efferv) [lit.[11] mp 233−236 °C (efferv)]; ^{1}H NMR δ 1.00 (t, 3 H), 2.18 (q, 2 H), 3.25 (s, 3 H), 3.56 (s, 3 H), 5.85 (br, 2 H), 6.02 (br, 2 H), 7.00 (dd, 1 H), 7.29 (d, 1 H), 7.55 (d, 1 H).

2,4-Diamino-5-[4-chloro-3-(3-ethyl-3-methyltriazen-1-yl)phenyl]-6-ethylpyrimidine (10a): mp 235−236 °C (efferv); ^{1}H NMR δ 0.95 (t, 3 H), 1.21 (t, 3 H), 2.09 (q, 2 H), 3.17 and 3.45 (2 x s, 3 H), 3.81 (q, 2 H), 5.62 (br, 2 H), 5.88 (br, 2 H), 6.90 (dd, 1 H), 7.15 (d, 1 H), 7.44 (d, 1 H). Anal. ($C_{15}H_{20}ClN_7$) C, H, N.

2,4-Diamino-5-[4-chloro-3-(3-methyl-3-propyltriazen-1-yl)phenyl]-6-ethylpyrimidine (10b): mp 189−191 °C (efferv); ^{1}H NMR δ 0.83 (t, 3 H), 0,95 (t, 3 H), 1.63 (q, 2 H), 2.08 (q, 2 H), 3.18 (s, 3 H), 3.73 (t, 2 H), 5.62 (br, 2 H), 5.87 (br, 2 H), 6.90 (dd, 1 H), 7.15 (d, 1 H), 7.44 (d, 1 H). Anal. ($C_{16}H_{22}ClN_7$) C, H, N.

2,4-Diamino-5-[3-(3-benzyl-3-methyltriazen-1-yl)-4-chlorophenyl]-6-ethylpyrimidine (10c): mp 196−197 °C (efferv); ^{1}H NMR δ 1.06 (t, 3 H), 2.26 (q, 2 H), 3.15 and 3.55 (2 s, 3 H), 5.03 (s, 2 H), 7.32 (m, 10 H), 8.1 (br, 1 H), 12.0 (br, 1 H). Anal. ($C_{20}H_{22}ClN_7$) C, H, N.

2,4-Diamino-5-[4-chloro-3-[3-[2-(diethylamino)ethyl]-3-methyltriazen-1-yl]phenyl]-6-ethylpyrimidine (10d): mp 157−159 °C (efferv); ^{1}H NMR δ 0.93 (3 t, 9 H), 2.11 (q, 2 H), 2.44 (q, 4 H), 2.65 (m, 2 H), 3.51 (s, 3 H), 3.85 (m, 2H), 5.62 (br, 2 H), 5.90 (br, 2 H), 6.92 (dd, 1 H), 7.19 (d, 1 H), 7.46 (d, 1 H); ^{13}C NMR δ 12.04, 13.39, 27.65, 35.38, 46.54, 51.19, 53.90, 105.79, 120.61, 126.91, 128.22, 130.46, 135.63, 147.16, 162.11, 162.28, 166.54. Anal. ($C_{19}H_{29}ClN_8$) C, H, N.

2,4-Diamino-5-[4-chloro-3-(3,3-diethyltriazen-1-yl)phenyl]-6-ethylpyrimidine (10e): mp 225−226 °C (efferv); ^{1}H NMR δ 0.96 (t, 3 H), 1.21 (t, 6 H), 2.08 (q, 2 H), 3.75 (q, 4 H), 5.63 (br, 2 H), 5.89 (br, 2 H), 6.90 (dd, 1 H), 7.14 (d, 1 H), 7.44 (d, 1 H). Anal. ($C_{16}H_{22}ClN_7$) C, H, N.

2,4-Diamino-5-[4-chloro-3-(3,3-diisobutyltriazen-1-yl)-phenyl]-6-ethylpyrimidine (10f): mp 185−189 °C (efferv); ^{1}H NMR δ 0.83 (m, 12 H), 0.92 (t, 3 H), 2.05 (q, 2 H), 2.10 (m,

2H), 3.51 (d, 4 H), 5.72 (br, 2 H), 5.95 (br, 2 H), 6.86 (dd, 1 H), 7.10 (d, 1 H), 7.41 (d, 1 H). Anal. ($C_{20}H_{30}ClN_7$) C, H, N.

2,4-Diamino-5-[4-chloro-3-(pyrrolidin-1-ylazo)phenyl]-6-ethylpyrimidine (10g): mp 247−251 °C (efferv); ^{1}H NMR δ 0.94 (t, 3 H), 1.96 (m, 4 H), 2.09 (q, 2 H), 3.58 (s, 2 H), 3.88 (s, 2 H), 5.62 (br, 2 H), 5.89 (br, 2 H), 6.89 (dd, 1 H), 7.13 (d, 1 H), 7.44 (d, 1 H). Anal. ($C_{16}H_{20}ClN_7$) C, H, N.

2,4-Diamino-5-[4-chloro-3-(piperidin-1-ylazo)phenyl]-6-ethylpyrimidine (10h): mp 250−252 °C (efferv); ^{1}H NMR δ 1.00 (t, 3 H), 1.63 (m, 6 H), 2.11 (q, 2 H), 3.79 (m, 4 H), 5.63 (br, 2 H), 5.88 (br, 2 H), 6.93 (dd, 1 H), 7.18 (d, 1 H), 7.45 (d, 1 H). Anal. ($C_{17}H_{22}ClN_7$) C, H, N.

2,4-Diamino-5-[4-chloro-3-(4-methylpiperazin-1-ylazo)-phenyl]-6-ethylpyrimidine (10i): mp 215−216 °C (efferv); ^{1}H NMR δ 0.96 (t, 3 H), 2.10 (q, 2 H), 2.25 (s, 3 H), 3.80 (m, 8 H), 5.63 (br, 2 H), 5.89 (br, 2 H), 6.97 (dd, 1 H), 7.20 (d, 1 H), 7.49 (d, 1 H); ^{13}C NMR δ 13.36, 27.68, 45.72, 105.64, 120.48, 127.40, 129.20, 130.60, 135.79, 146.47, 162.10, 162.32, 166.57. Anal. ($C_{17}H_{23}ClN_8$) C, H, N.

2,4-Diamino-5-[4-chloro-3-[(4-hydroxypiperidin-1-yl)-azo]phenyl]-6-ethylpyrimidine (10j): mp 224−225 °C (efferv); ^{1}H NMR δ 0.98 (t, 3 H), 1.50 (m, 2 H), 1.82 (m, 2 H), 2.10 (q, 2 H), 3.40−4.10 (m, 5 H), 4.90 (m, 1H), 5.65 (br, 2 H), 5.89 (br, 2 H), 6.91 (dd, 1 H), 7.19 (d, 1 H), 7.46 (d, 1 H); ^{13}C NMR δ 13.39, 27.69, 48.81, 65.20, 105.73, 120.45, 127.22, 128.87, 130.56, 135.73, 146.76, 162.15, 162.30, 166.58. Anal. ($C_{17}H_{22}ClN_7O$) C, H, N.

2,4-Diamino-5-[4-chloro-3-(morpholin-4-ylazo)phenyl]-6-ethylpyrimidine (10k): mp 255−257 °C (efferv); ^{1}H NMR δ 0.95 (t, 3 H), 2.09 (q, 2 H), 3.78 (m, 8 H), 5.61 (br, 2 H), 5.88 (br, 2 H), 6.98 (dd, 1 H), 7.20 (d, 1 H), 7.49 (d, 1 H). Anal. ($C_{16}H_{20}ClN_7O$) C, H, N.

2,4-Diamino-5-[4-chloro-3-[3-(2-hydroxyethyl)-3-methyltriazen-1-yl]phenyl]-6-ethylpyrimidine (10l): mp 217−218 °C (efferv); ^{1}H NMR δ 0.97 (t, 3 H), 2.11 (q, 2 H), 3.37 (s, 3 H), 3.66 (d, 2 H), 3.85 (m, 2 H), 4.85 (br, 1 H), 5.66 (br, 2 H), 5.91 (br, 2 H), 6.92 (dd, 1 H), 7.19 (d, 1 H), 7.47 (d, 1 H); ^{13}C NMR δ 13.41, 27.70, 36.62, 58.26, 59.60, 105.81, 120.72, 126.95, 128.32, 130.47, 135.65, 147.16, 162.13, 162.29, 166.59. Anal. ($C_{15}H_{20}ClN_7O$) C, H, N.

2,4-Diamino-5-[4-chloro-3-[3-ethyl-3-(2-hydroxyethyl)-triazen-1-yl]phenyl]-6-ethylpyrimidine (10m): mp 214−215 °C (efferv); ^{1}H NMR δ 0.97 (t, 3 H), 1.23 (t, 3 H), 2.11 (q, 2 H), 3.8 (m, 6 H), 4.86 (br, 1 H), 5.64 (br, 2 H), 5.91 (br, 2 H), 6.92 (dd, 1 H), 7.16 (d, 1 H), 7.47 (d, 1 H); ^{13}C NMR δ 13.4, 27.7, 42.1, 50.7, 57.1, 60.0, 105.8, 120.5, 127.0, 128.3, 130.4, 135.7, 147.0, 162.1, 162.3, 166.5. Anal. ($C_{16}H_{22}ClN_7O$) C, H, N.

2,4-Diamino-5-[4-chloro-3-[3-(2-hydroxyethyl)-3-propyltriazen-1-yl]phenyl]-6-ethylpyrimidine (10n): mp 186−187 °C (efferv); ^{1}H NMR δ 0.88 (m, 3 H), 0.98 (t, 3 H), 1.70 (m, 2 H), 2.12 (q, 2 H), 3.8 (m, 6 H), 4.85 (br, 1 H), 5.70 (br, 2 H), 5.93 (br, 2 H), 6.93 (dd, 1 H), 7.17 (d, 1 H), 7.47 (d, 1 H); ^{13}C NMR δ 11.20, 11.80, 13.42, 18.38, 21.83, 27.70, 49.57, 50.39, 56.59, 57.23, 59.80, 105.81, 120.45, 127.04, 128.34, 130.44, 135.66, 147.10, 162.15, 162.29, 166.60. Anal. ($C_{17}H_{24}ClN_7O$) C, H, N.

2,4-Diamino-5-[3-[3-*tert*-butyl-3-(2-hydroxyethyl)triazen-1-yl]-4-chlorophenyl]-6-ethylpyrimidine (10o): mp 224−226 °C (efferv); ^{1}H NMR δ 0.98 (t, 3 H), 1.41 (s, 9 H), 2.10 (q, 2 H), 3.66 (m, 2 H), 3.81 (m, 2 H), 4.78 (br, 1 H), 5.60 (br, 2 H), 5.89 (br, 2 H), 6.91 (dd, 1 H), 7.12 (d, 1 H), 7.46 (d, 1 H); ^{13}C NMR δ 13.52, 27.67, 28.02, 47.22, 57.11, 60.82, 105.85, 120.68, 127.00, 128.26, 130.44, 135.67, 147.50, 162.13, 162.29, 166.59. Anal. ($C_{18}H_{26}ClN_7O$) C, H, N.

2,4-Diamino-5-[3-[3-benzyl-3-(2-hydroxyethyl)triazen-1-yl]-4-chlorophenyl]-6-ethylpyrimidine (10p): mp 112−114 °C (efferv); ^{1}H NMR δ 0.98 (t, 3 H), 2.12 (q, 2 H), 3.71−4.10 (m, 4 H), 4.91 (br, 1 H), 5.05 (br, 2 H), 5.68 (br, 2 H), 5.91 (br, 2 H), 6.96 (dd, 1 H), 7.4 (m, 7 H). Anal. ($C_{21}H_{24}ClN_7O$) C, H, N.

2,4-Diamino-5-[4-chloro-3-[3,3-bis(2-hydroxyethyl)triazen-1-yl]phenyl]-6-ethylpyrimidine (10q): mp 219−220 °C (efferv); ^{1}H NMR δ 0.98 (t, 3 H), 2.12 (q, 2 H), 3.71 (m, 4H), 3.88 (m, 4H), 4.89 (br, 2 H), 5.74 (br, 2 H), 5.94 (br, 2 H), 6.94 (dd, 1 H), 7.19 (d, 1 H), 7.49 (d, 1 H). Anal. ($C_{16}H_{22}ClN_7O_2$) C, H, N.

2,4-Diamino-5-[4-chloro-3-[3,3-bis(2-methoxyethyl)triazen-1-yl]phenyl]-6-ethylpyrimidine (10r): mp 130−132 °C (efferv); ^{1}H NMR δ 0.96 (t, 3 H), 2.09 (q, 2 H), 3.26 (d, 6 H), 3.62 (d, 4H), 3.95 (m, 4 H), 5.63 (br, 2 H), 5.89 (br, 2 H), 6.94 (dd, 1 H), 7.16 (d, 1 H), 7.47 (d, 1 H). Anal. ($C_{18}H_{26}ClN_7O_2$) C, H, N.

2,4-Diamino-5-[4-chloro-3-[3-(1,3-dioxol-2-ylmethyl)-3-methyltriazen-1-yl]phenyl]-6-ethylpyrimidine (10s): mp 213−214 °C (efferv); ^{1}H NMR δ 0.97 (t, 3 H), 2.11 (q, 2 H), 3.24 (s, 3 H), 3.9 (m, 6 H), 5.08 (m, 1 H), 5.68 (br, 2 H), 5.91 (br, 2 H), 6.96 (dd, 1 H), 7.18 (d, 1 H), 7.48 (d, 1 H); ^{13}C NMR δ 13.38, 27.69, 36.59, 57.55, 64.66, 101.93, 105.71, 120.85, 127.16, 128.78, 130.50, 135.73, 146.87, 162.12, 162.30, 166.60. Anal. ($C_{17}H_{29}ClN_7O_2$) C, H, N.

2,4-Diamino-5-[4-chloro-3-(1,2,3,4-tetrahydroisoquinolin-2-ylazo)phenyl]-6-ethylpyrimidine (11): mp 170−172 °C (efferv); ^{1}H NMR δ 0.98 (t, 3 H), 2.17 (q, 2 H), 3.01 (s, 2 H), 4.08 (s, 2 H), 4.90 (s, 2 H), 6.98 (dd, 1 H), 7.20 (m, 9 H), 7.52 (d, 1 H). Anal. ($C_{21}H_{22}ClN_7$) C, H, N.

2,4-Diamino-5-[3-[3-[2-(acetyloxy)ethyl]-3-benzyltriazen-1-yl]-4-chlorophenyl]-6-ethylpyrimidine (14a). A solution of *N*-benzylethanolamine (2.0 g) in acetic acid (30 mL) was saturated with dry hydrogen chloride gas at 20 °C for 1 h. The mixture was maintained at 20 °C (24 h) and solvent removed by vacuum evaporation to yield 2-(*N*-benzylamino)-ethyl acetate hydrochloride **13a** (98%). The salt was coupled with the diazonium chloride solution prepared from the arylamine **1c** in the presence of excess aqueous sodium carbonate to furnish triazene **14a** (75%): mp 166−168 °C (efferv); IR (KBr) 3453, 3326 (NH_2), 1723 (C=O) cm^{-1}; ^{1}H NMR δ 0.98 (t, 3 H), 1.90 (s, 3 H), 2.10 (q, 2 H), 3.9−4.3 (m, 4 H), 5.04 (br, 2 H), 5.80 (br, 2 H), 5.98 (br, 2 H), 6.96 (dd, 1 H), 7.31−7.47 (m, 7 H); ^{13}C NMR δ 14.1, 21.4, 28.3, 51.1, 53.7, 59.4, 60.1, 62.6, 106.4, 121.4, 128.0, 128.1, 129.0, 129.2, 129.6, 129.8, 131.3, 136.4, 137.0, 137.6, 147.2, 162.9, 167.1, 171.0. Anal. ($C_{23}H_{26}ClN_7O_2$) C, H, N.

The same (acetoxyethyl)benzyltriazene (35%) was prepared by treating the (hydroxyethyl)benzyltriazene **10p** with acetyl chloride (1 molar equiv) and catalytic DMAP in pyridine at 25 °C.

2,4-Diamino-5-[4-chloro-3-[3-[2-(propionyloxy)ethyl]-3-benzyltriazen-1-yl]phenyl]-6-ethylpyrimidine (14b). Similarly prepared, from 2-(*N*-benzylamino)ethyl propionate hydrochloride **13b** and the diazonium salt prepared from arylamine **1c**, the [(propionyloxy)ethyl]benzyltriazene (80%) had mp 184−186 °C (efferv): IR (KBr) 3338, 3137 (NH_2), 1730 (C=O) cm^{-1}; ^{1}H NMR δ 0.92 (t, 3 H), 1.04 (t, 3 H), 2.18 (q, 2 H), 2.20 (q, 2 H), 3.94−4.32 (m, 4 H), 5.00 (br, 2 H), 6.46 (br, 2 H), 6.70 (br, 2 H), 7.01 (dd, 1 H), 7.34−7.52 (m, 7 H). Anal. ($C_{24}H_{28}ClN_7O_2$) C, H, N.

General Procedure for the Synthesis of Alkanesulfonic Acid Salts of Triazenyl-Substituted 2,4-Diamino-5-(4-chlorophenyl)-6-ethylpyrimidines. The triazene (0.5 g) in a minimum of 2-propanol at 50 °C was treated with the alkanesulfonic acid (1.1 molar equiv) and immediately cooled to 5 °C. The crystalline salts were collected and washed with a minimum of ice-cold 2-propanol. The following salts were prepared.

Ethanesulfonic acid salt of 2,4-diamino-5-[4-chloro-3-(3,3-dimethyltriazen-1-yl)phenyl]-6-ethylpyrimidine (4a), which crystallized as a 2-propanol solvate, mp 160 °C (dec) (see Crystallography Section).

Methanesulfonic acid salt of 2,4-diamino-5-[4-chloro-3-(3-ethyl-3-methyltriazen-1-yl)phenyl]-6-ethylpyrimidine (10a): mp 155 °C (dec); ^{1}H NMR δ 0.98 (t, 3 H), 1.10 (t, 3 H), 2.15 (q, 2 H), 2.37 (s, 3 H), 2.85 (q, 2 H), 3.14 (s, 3 H), 3.77 (q, 2 H), 6.95 (dd, 1 H), 7.21 (m, 1 H), 7.50 (m, 1 H), 7.6 (br s, 2 H), 8.1 (br s, 1 H), 8.3 (br s, 1 H), 12.25 (br s, 1 H).

Methanesulfonic acid salt of 2,4-diamino-5-[4-chloro-3-(3-methyl-3-propyltriazen-1-yl)phenyl]-6-ethylpyrimidine (10b): mp 150 °C (dec); ^{1}H NMR δ 0.94 (t, 3 H), 1.08 (t, 3 H), 1.67 (q, 2 H), 2.24 (q, 2 H), 2.49 (s, 3 H), 2.88 (q, 2 H),

3.25 (s, 3 H), 3.81 (q, 2 H), 7.05 (dd, 1 H), 7.29 (m, 1 H), 7.60 (m, 1 H), 7.7 (br s, 2 H), 8.2 (br s, 1 H), 8.4 (br s, 1 H), 12.4 (br s, 1 H).

Methanesulfonic acid salt of 2,4-diamino-5-[4-chloro-3-(morpholin-4-ylazo)phenyl]-6-ethylpyrimidine (10k): mp 205 °C (efferv); ^{1}H NMR δ 0.97 (t, 3 H), 2.19 (q, 2 H), 2.34 (s, 3 H), 3.06 (m, 2 H), 3.73 (m, 6 H), 7.03 (dd, 1 H), 7.25 (d, 1 H), 7.53 (m, 1 H), 7.55 (br s, 2 H), 8.1 (br s, 1 H), 8.7 (br s, 1 H), 12.0 (br s, 1 H).

Crystallography. The ethanesulfonate salt of **4a** crystallized from 2-propanol at 5 °C as rosettes, from which an irregular brown specimen crystal 0.7 × 0.5 × 0.3 mm was obtained. Crystal data: unit cell dimensions $a = 8.438(1)$ Å, $b = 9.933(1)$ Å, $c = 16.969(2)$ Å, $a = 99.86(1)°$, $b = 92.92(1)°$, $g = 113.92(2)°$, $V = 1269.4(2)$ Å^3, $C_{14}H_{19}ClN_7^+$ $C_2H_5O_3S^-$·C_3H_8O, FW = 490.03, $Z = 2$, $D_c = 1.282$ g cm^{-3}, triclinic, P$\bar{1}$, Mo Kα radiation ($\lambda = 0.7107$ Å), 4420 reflections measured by $\omega-2\theta$ scans, 2906 observed [$I > 2\sigma(I)$].

Structure Determination and Refinement. The structure was solved by direct methods with MULTAN [31] and refined by the full-matrix least-squares technique with SHELXL.[32] Two alternative positions were found for one of the methyl groups of the 2-propanol solvent molecule. Positions and anisotropic displacement parameters were refined for all non-hydrogen atoms. Hydrogen atoms were located in difference electron density maps and freely refined with isotropic temperature factors, except for the C20 and C21 methyl groups, which were constructed and refined as rotating rigid groups, the C27−C28 ethyl group, for which a riding model was used, and the H atoms attached to 2-propanol C atoms, which were omitted entirely. The weighting scheme $w = 1/[\sigma^2(F_o^2) + (0.1193P)^2 + 0.7850P]$ was applied, where $P = (F_o^2 + 2F_c^2)/3$. At termination no shift exceeded 0.008 esd, discrepancy indices were $R = 0.061$ for observed reflections, $wR(F^2) = 0.217$ for all reflections. No feature on a difference electron density map exceeded +0.48 or −0.38 e Å^{-3}. Final non-hydrogen fractional coordinates, bond distances, and bond angles have been deposited as supplementary material.

***In Vitro* Evaluation of Triazenyl-Substituted Pyrimethamine Derivatives.** *P. carinii* microorganisms were recovered from the lungs of immunosuppressed rats by rupturing the host cells, washing away the soluble mammalian DHFR, and harvesting intact organisms in association with the mammalian plasma membranes. *P. carinii* DHFR was isolated from the 100000*g* supernatant from sonicated organisms and the inhibitory activities of triazenes and reference compounds were measured according to an established method.[16]

T. gondii RH was obtained in high yield from culture in RPMI medium on a line of Chinese hamster ovary cells lacking DHFR activity (ATCC 3952 dhfr$^-$; American Type Culture Collection). DHFR preparations were obtained from harvested organisms and the inhibitory activities of triazenes, and reference compounds were measured according to an established method.[17]

For assessment of the activitity of triazenes against intact microorganisms *in vitro*, *P. carinii* was extracted from the lungs of rats immunosuppressed for 6−10 weeks with dexamethasone.[33] Serial dilutions of a 10 mg/mL stock solution of each compound in DMSO were made to give a final concentration range of 0.2−100 μg/mL. The *P. carinii* and compound were incubated together in Glasgow Modification of Eagle's Medium (GMEM) with [^{3}H]-4-aminobenzoic acid (pABA) at 37 °C for 24 h in alveolar air (5% CO_2, 95% air). Uptake of [^{3}H]-pABA into the folates of *P. carinii* was utilized as a selective indicator of the *in vitro* viability of the organism. The inhibitory activity of the compounds was expressed as the concentration required to inhibit the uptake of pABA by 50% compared with uptake by untreated control cultures (IC_{50}).

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