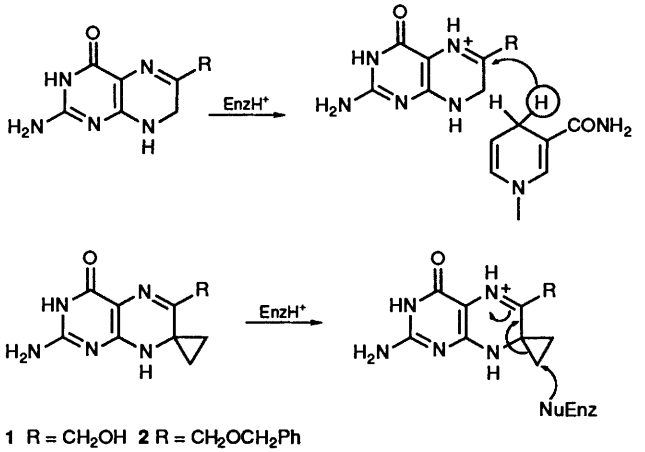
Latent Inhibitors. Part 8. Synthesis and Evaluation of some Mechanism-based Inhibitors of Dihydrofolate Reductase

John McGill, Lilias Rees, Colin J. Suckling* and Hamish C. S. Wood
Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow, G1 1XL, UK

The synthesis of two new pteridine-7-spirocyclopropanes that are time-dependent irreversible inhibitors of dihydrofolate reductase is described. Several related compounds including a cyclopropyl substituted quinoxaline and 2-aminopteridine-4(3*H*),6(5*H*)-dione were also prepared but these compounds were not found to be time-dependent inhibitors. The inhibitors were assessed using several dihydrofolate reductases, *Escherichia coli* RT/39, two mutants of the *E. coli* enzyme, *Lactobacillus casei*, and chicken liver. Activity was found against all enzymes tested but most strongly against *E. coli* wild type enzyme.

At its simplest, the mechanism of action of dihydrofolate reductase (DHFR) can be viewed as the transfer of a proton to N-5 of dihydrofolate (DHF) thereby enhancing the electrophilicity of C-6 to accept hydride from NADPH. Asp-27 (*E. coli* numbering) has been shown to play a significant role in catalysis.[1,2] We applied this concept to the design of the first mechanism-based inhibitor of DHFR,[3] a pteridine-7-spirocyclopropane **1** (Scheme 1). **1** was shown to be a time-dependent

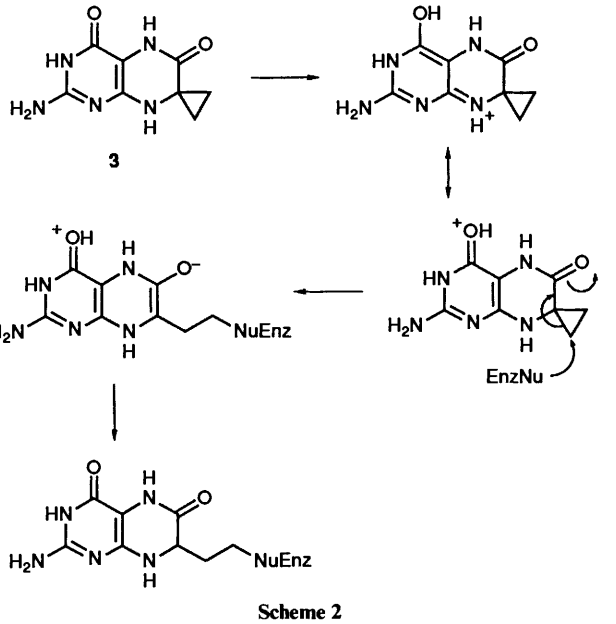


1 R = CH_2OH **2** R = CH_2OCH_2Ph

Scheme 1

irreversible inhibitor of DHFR. Its precise mechanism of action, in particular the nature of the presumed enzymic nucleophile, was not indicated by the kinetic experiments. Unlike most DHFR inhibitors, **1** lacks the usual binding determinants, namely a hydrophobic substituent or a 4-aminoglutamate residue, that would make it straightforward to deduce the likely binding mode to DHFR on the basis of known crystal structures.[4,5] To investigate this problem more closely, we have synthesised a pteridine-7-spirocyclopropane **2** bearing a hydrophobic benzyl substituent at C-6.

Recent discussions of the mechanism of action of DHFR have suggested that reduction of substrate takes place *via* initial protonation of 4-O through a hydrogen bonded relay.[2,6] These ideas led to the design of a new type of DHFR inhibitor illustrated by the 6-oxopteridine-7-spirocyclopropane **3**. The rationale for its mechanism of action is shown in Scheme 2. Protonation of 4-O leads to a delocalised cation in which the cyclopropane function becomes flanked by two electron withdrawing groups. In such a situation, it would be expected that

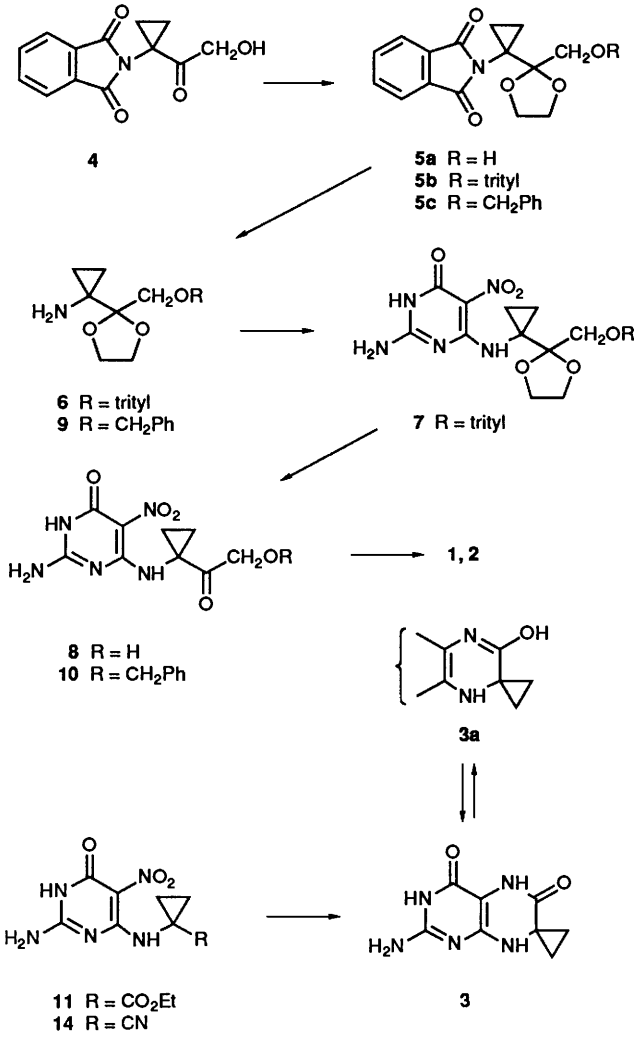


Scheme 2

the cyclopropane ring would be strongly activated towards nucleophilic attack and should lead to inhibition at the enzyme's active site.

Results and Discussion

Synthesis of Inhibitors.—The synthesis of the pteridine-7-spirocyclopropanes followed established routes.[3] A major problem in the synthesis of the inhibitor **1** was the isolation of 1-amino-1-hydroxyacetylcyclopropane, the alicyclic building block required. In order to compare the properties of the new inhibitors with **1**, it was necessary to prepare further samples of **1**. To overcome the isolation problem, we prepared a hydrophobic protected derivative. Thus 1-hydroxyacetyl-1-phthalimidocyclopropane **4** (Scheme 3) was converted into a hydroxy ketal **5a** which was derivatised with trityl chloride to give **5b**. Cleavage of the phthalimido group was accomplished with 4 equiv. of hydrazine hydrate in refluxing 2-methylpropanol and the amino derivative **6** easily isolated as a viscous oil. Treatment of this compound with 2-amino-6-chloro-5-nitropyrimidin-4(3*H*)-one led to the fully protected precursor **7** which on acid hydrolysis afforded the immediate pteridine precursor **8** previously described.[3] Reductive cyclisation of **8** to



Scheme 3

the pteridine **1** was performed using sodium dithionite in hot water and we found that short reaction times (< 2 min) led to greatly improved yields. The short reaction time minimised the extent of decomposition of the pteridine **1** presumably *via* cyclopropane ring opening as discussed previously.[3] Pteridine **2** was prepared similarly from **5a** *via* alkylation with benzyl bromide to give **5c**, cleavage of the phthalimido group affording **9**, coupling with the chloronitropyrimidine and hydrolysis affording **10** and finally cyclisation. Pteridine **3** was prepared similarly by coupling the chloronitropyrimidine with ethyl 1-aminocyclopropane-1-carboxylate to give **11** and subsequent cyclisation in the usual way.

In order to assist developing an understanding of the mechanism of action of these spirocyclopropane inhibitors, we also prepared some potential inhibitors in which the cyclopropane ring was a simple substituent to the heterocyclic ring and not spiro connected. Condensation of ethyl cyclopropylglyoxylate with respectively 2,5,6-triaminopyrimidin-4(3*H*)-one and *o*-phenylenediamine afforded the 2-aminopteridine-4(3*H*), 6(5*H*)-dione derivative **12a**, accompanied by 10% of its isomer **12b**, and **13**. For investigation as an enzyme inhibitor, the mixture of isomers **12** was not separated. In an attempt to extend the range of pteridines for study, the cyano analogue **14** of the ester **11** was prepared. However, it failed to cyclise to the corresponding pteridine under the usual conditions. No products were isolable. Presumably, this result reflects the relatively high basicity and hence lability to cyclopropane ring opening of the required aminopteridine.

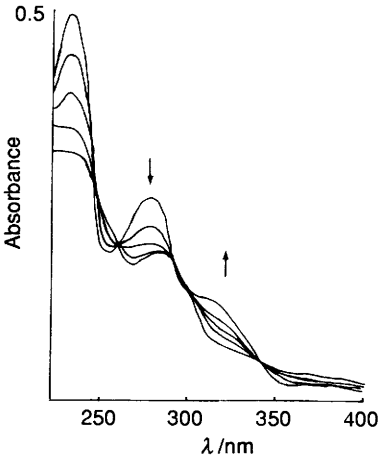
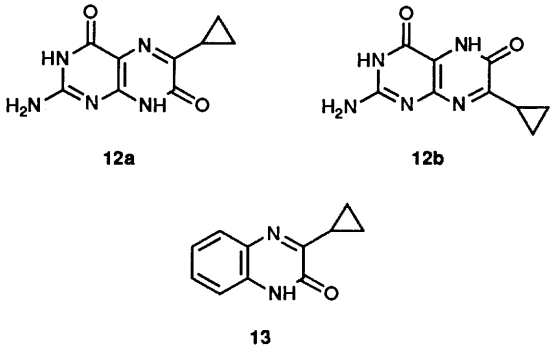


Fig. 1 Time course of the tautomerisation of **3** in aqueous buffer under the conditions used for enzyme assay. Traces were measured at 30 min, intervals.



Chemical Properties of Inhibitors.—Since the proposed inhibition mechanisms involved protonation of the inhibitors followed by nucleophilic ring opening, it was important to establish that the new inhibitors were stable to the assay conditions used. As mentioned above, **1** was shown to be labile in aqueous solutions containing strong nucleophiles (sulfites). Each of the three pteridine inhibitors was, therefore, incubated in the pH 5 tris–succinate buffer used for the assay (see below) and any reactions monitored by HPLC. It was found that all three underwent some changes when left for periods of several hours. The benzyl-substituted inhibitor **2** was only poorly soluble in this buffer and satisfactory solutions could only be obtained using 20% dimethyl sulfoxide as co-solvent. Under these conditions, all of the inhibitors showed better stability and no decomposition was evident within 2–3 h. Dimethyl sulfoxide was therefore used as a co-solvent for all the enzyme assays.

In the cases of inhibitors **1** and **2**, the products of incubation in buffer were complex suggesting that both were unstable under the solution conditions. Inhibitor **3**, in contrast, exhibited a clean change (Fig. 1) on incubation in buffer at pH 7 indicated by the growth of a second peak in the HPLC trace and distinct isosbestic points in the UV spectrum at 247, 259, 295, 306 and 339 nm. These changes are accompanied by an increase in absorbance at 310 nm and a decrease in absorbance at 280 and 233 nm. It is probable that inhibitor **3** is not undergoing decomposition but an isomerisation or possibly hydration. Reversible nucleophilic addition is a reaction that has been described in the case of 7,7-dialkyl-7,8-dihydropteridines[7] but such a reaction would not be expected to be so facile with the 6-oxo substituent present in **3**. Further, the increase in absorbance at longer wavelength is not consistent with the

Table 1 Biological activity of pteridines with respect to dihydrofolate reductases[a]

Compound	*E. coli* wild type	*E. coli* Thr → Val	*E. coli* Tyr → Gly	*L. Casei*	Chicken liver
1	k_i 1.4 × 10^{-3} s^{-1} $t_{1/2}$ 99 min [**1**] = 0.33 mmol dm^{-3} [E] = 0.92 μmol dm^{-3}	t.d.	n.t.	n.t.	n.t.
2	k_i 0.89 × 10^{-3} $t_{1/2}$ 15 min [**2**] = 0.66 mmol dm^{-3} [E] = 0.92 μmol dm^{-3}	t.d.	t.d.	t.d. 35 0.40 0.11	t.d. 76 0.27 1.0
3	k_i 0.70 × 10^{-3} $t_{1/2}$ 30 min [**3**] = 0.43 mmol dm^{-3} [E] = 0.94 mmol dm^{-3}	t.d.	n.i.	n.i.	t.d. 98 1.29 1.0

[a] t.d. [4] time-dependent inhibitor; n.i. = no inhibition; n.t. = not tested.

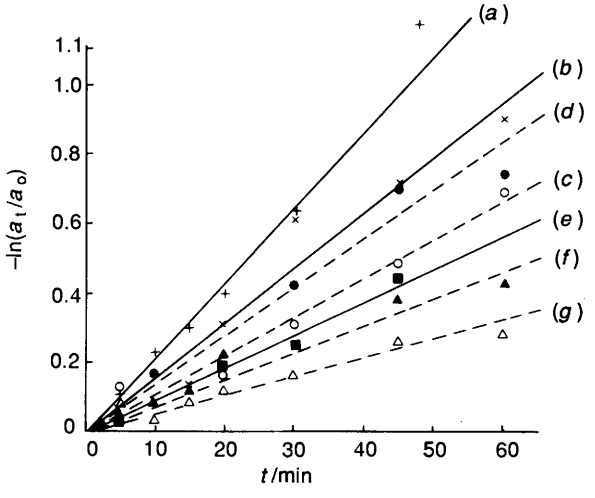


Fig. 2 Comparison of reaction of inhibitors **2** and **3** with DHFRs: first order plots. (*a*) *E. coli* wild type **2** 0.19 mmol dm^{-3} + — + (*b*) *E. coli* wild type **3** 0.27 mmol dm^{-3} × — × (*c*) *E. coli* Thr → Leu **2** 0.23 mmol dm^{-3} ○- - - -○ (*d*) *E. coli* Thr → Leu **3** 0.19 mmol dm^{-3} ●- - - -● (*e*) *L. casei* 0.29 mmol dm^{-3} **2** ■———■ (*f*) chicken liver **2** 0.23 mmol dm^{-3} ▲- - - -▲ (*g*) chicken liver **3** 0.83 mmol dm^{-3} △- - - -△.

formation of what would essentially be a pyrimidine chromophore if hydration were occurring. An alternative explanation is that the changes in the UV spectrum are due to a slow tautomerism from the oxo form, in which compounds such as **3** are usually formulated, to the hydroxy imine form **3a** resulting in the formation of an equilibrium mixture of the two tautomers. Interestingly, this form contains an unprotonated N-5, as in 6-alkylpteridines, to which a proton might be transferred at the enzyme's active site during activation of the inhibitor. This interpretation is supported by the UV spectra of blocked alkyl 7,8-dihydropteridines which typically show a significant absorption above 300 nm giving the compounds a distinct yellow–orange colour.[8]

Enzyme Inhibition Studies.—The enzymes used for evaluating the inhibitors were from *E. coli* RT/39, *L. casei* (provided by courtesy of Prof. G. C. K. Roberts, University of Leicester), chicken liver, and two mutant *E. coli* enzymes Thr-113 → Val-113 and Tyr-100 → Gly-100 (provided by courtesy of Prof. S. J. Benkovic, Pennsylvania State University).[9] Assays were carried out using established procedures[3] by following the decrease in absorbance in NADPH as DHF is reduced in samples removed at intervals from incubations of DHFR solutions with the inhibitors. The well known insolubility of 2-aminopteridin-4(3*H*)-ones made it necessary to dissolve the inhibitors in dimethyl sulfoxide which was then diluted with the assay buffer. Even with this expedient, the range of concentrations of inhibitors within which significant inhibition could be measured was limited by solubility and the relatively low affinity of the inhibitors (see below). Both inhibition and control experiments were performed in pH 5.0 0.1 mol dm^{-3} tris–succinate buffer containing 20% dimethyl sulfoxide. These conditions are below the pH optimum for the enzyme from *L. casei* (pH 7.35) but at this pH, no inhibitory activity was found for any of the compounds tested. To investigate irreversibility, samples of inhibited enzyme were dialysed extensively against cold buffer solution; gel filtration was found to be impractical because DHFR was itself unstable to that technique. The data obtained for the inhibitors with the five enzymes are presented in Table 1. Insufficient samples of enzyme were available for a complete kinetic analysis in all cases. However, it was possible to diagnose the characteristic behaviour of each combination.

None of the compounds studied is a potent inhibitor of DHFR either with respect to affinity or reaction rate. The point of interest is the comparison of reactions of different enzymes with different inhibitors. The most extensive comparison can be made between inhibitors **2** and **3**; **1** was only studied with wild type *E. coli* enzyme and one of the mutants and in those cases, it behaved similarly to its benzyl ether **2**. Typical first order plots for the different enzymes studied are shown in Fig. 2. For wild type *E. coli* enzyme, there was no significant difference observed between **2** and **3**. Both were irreversible inhibitors with similar kinetic constants (K_i 0.30 and 0.55 mmol dm^{-3} and k_i 9.8 × 10^{-4} and 7.0 × 10^{-4} s^{-1}, respectively). Further, all three inhibitors were time dependent inhibitors of the mutant enzyme in which Thr-113 was replaced by valine. Thr-113 is one of a group of amino acids that is believed to bind to the 2-amino-4-oxo substituents of the pyrimidine ring of substrates;[6,9] as such, it would not have been expected to be a competent nucleophile for attack at the cyclopropane ring because of its distance. The behaviour of the inhibitors is consistent with the expectation. The most significant distinction between **2** and **3**, however, is the observation that **3** was not an inhibitor of those enzymes in which a tyrosine was absent from the active site region. Neither the Tyr-100 Gly mutant of *E. coli* nor the *L. casei* enzyme contains this residue. It therefore appears that **2** and **3** are differently bound at the active site of these DHFRs. The result also argues against Tyr-100 as a potential nucleophile in the inhibition of DHFR by **2** and, by extension, **1** as was previously suggested.[3] On the other hand, this residue could be responsible for irreversible inhibition by **3**. From the limited data available the kinetic parameters for inhibition of the *L. casei* enzyme by **2** were estimated to be K_i 1.9 mmol dm^{-3} and k_i 10^{-3} s^{-1}.

The chicken liver enzyme also contains a tyrosine residue at the homologous position to that in *E. coli*. Both **2** and **3** were found to be time dependent inhibitors of the chicken liver enzyme although they appeared to be less reactive than with the wild type *E. coli* enzyme (Fig. 2). For **2**, the kinetic parameters were estimated to be K_i 0.42 mmol dm^{-3} and k_i 4×10^{-4} s^{-1}.

The above results have been discussed in the context of specific ring opening of the cyclopropane ring of the three inhibitors activated by DHFR. Several lines of evidence show that this is an acceptable context. Firstly, 2-amino-7,8-dihydro-6-hydroxymethyl-7,7-pteridin-4(3*H*)-one, the dimethyl analogue of the spirocyclic cyclopropane **1**, is not an inhibitor of DHFR; it acts earlier in the pathway leading to dihydrofolate.[8] Secondly, the cyclopropane must be incorporated into a pteridine; the 2,4-dinitrophenylhydrazone of cyclopropyl methyl ketone and a representative example of dihydroorotate dehydrogenase inhibitors, a phenyl substituted spirocyclopropanebarbiturate[10] were not inhibitors. Thirdly, the position of the cyclopropane ring with respect to the pteridine ring is important. Thus a 6-cyclopropyl-substituted pterin **12a** was not found to be an inhibitor of DHFR. Finally, the pK_as of **1–3** would not be expected to be higher than that of the normal substrate, dihydrofolic acid which has a pK_a of 3.84.[11] Since the compounds tested have been shown to be stable under the conditions of assay, the proton transfer that activates them to enzyme inhibition is most likely to occur at the active site of DHFR. In the case of **1** and **2** transfer to N-5, ultimately, would lead to activation. In the case of **3** the mechanistic design considered protonation at 4-O; however protonation at 6-O through further extended hydrogen bonding would also lead to activation and cannot be ruled out.

In the context of potential medicinal or agrichemical applications of this type of DHFR inhibitor, the results reported here establish that both mammalian and bacterial enzymes are susceptible to inhibition and that clear cut species selectivity exists.

Experimental

NMR spectra were recorded on Perkin-Elmer R32 (90 MHz) or Bruker WH-250 (250 MHz) spectrometers using tetramethylsilane as internal standard. HPLC eluting solvent was as follows: 50 mmol dm^{-3} pH 7.0 tris-HCl–acetonitrile 70:30. HPLC analyses were run on Whatman ODS reversed phase columns (10 μm) in 25 cm stainless steel columns at a flow rate of 60 cm^3 h^{-1}.

1-*Phthalimido*-1-*trityloxyacetylcyclopropane Ethylene Ketal* **5b**.—A solution of 1-hydroxyacetyl-1-phthalimidocyclopropane ethylene ketal[3] (2.33 g, 8.03 mmol) and trityl chloride (2.24 g, 8.05 mmol) in dry pyridine (20 cm^3) was heated at 60–65 °C for 3.5 h. The solution was allowed to cool overnight during which time a white solid precipitated. The solid was filtered off and dried under reduced pressure. Concentration of the pyridine solution to one quarter of its volume caused the precipitation of a further crop of product. The combined products were recrystallised from carbon tetrachloride to yield the *title compound* **5b** (3.2 g 83%) as colourless crystals, m.p. 205–207 °C (Found: C, 77.0; H, 5.3; N, 2.4. $C_{34}H_{29}NO_5$ requires C, 76.8; H, 5.5; N, 2.6%); δ_H(90 MHz; $CDCl_3$) 7.8–7.1 (19 H, m, aromatic), 4.20 (4 H, s, OCH_2CH_2O), 3.33 (2 H, s, CH_2O), 1.2 (2 H, m, cyclopropane) and 0.91 (2 H, m, cyclopropane).

1-*Benzyloxyacetyl*-1-*phthalimidocyclopropane Ethylene Ketal* **5c**.—To a suspension of sodium hydride [80% dispersion, prewashed with tetrahydrofuran (THF); 0.5 g] in dry THF (5 cm^3) a solution of 1-hydroxyacetyl-1-phthalimidocyclopropane ethylene ketal (3.98 g) and benzyl bromide (1.65 cm^3) in dry THF (60 cm^3) was added over 1 h. The solution was heated under reflux for 1 h and then poured into ice–water (100 cm^3). The THF was removed under reduced pressure and then the aqueous solution was extracted twice with chloroform. The chloroform extracts were combined and washed with brine, dried ($MgSO_4$) and evaporated to dryness to give a gum which crystallised with time. Recrystallisation from light petroleum (b.p. 60–80 °C) afforded the required *title compound* **5c** (3.8 g, 70%), m.p. 103–106 °C (Found: C, 69.0; H, 4.9; N, 3.2. $C_{22}H_{21}NO_5$ requires C, 69.6; H, 5.6; N, 3.7%); δ_H(90 MHz; $CDCl_3$) 7.75 (4 H, m, phthalimido-H) 7.25 (5 H, s, C_6H_5), 4.58 (2 H, s, CH_2O), 4.13 (4 H, m, OCH_2CH_2O), 3.75 (2 H, s, OCH_2Ph), 1.29 (2 H, m, cyclopropane) and 1.00 (2 H, m, cyclopropane).

2-*Amino*-6-[1-(2-*hydroxy*-1-*oxoethyl*)*cyclopropylamino*]-5-*nitropyrimidin*-4(3H)-*one* **8**.—1-Amino-1-trityloxyacetylcyclopropane ethylene ketal **6** was prepared from the corresponding phthalimido derivative **5b** by heating a solution of **5b** (20.7 g) in 2-methylpropanol (1 dm^3) with hydrazine hydrate (8.6 cm^3) under reflux overnight. Removal of the solvent under reduced pressure left an oily residue which was extracted with chloroform. The chloroform extracts were filtered, the filtrate washed twice with brine, dried ($MgSO_4$) and then evaporated under reduced pressure to yield the product as a gum (15.5 g); δ_H(90 MHz; $CDCl_3$) 7.48–7.21 (15 H, m, C_6H_5), 3.96 (4 H, s, OCH_2CH_2O), 3.24 (2 H, s, OCH_2), 1.74 (2 H, s, NH_2), 0.79 (2 H, m, cyclopropane) and 0.52 (2 H, m, cyclopropane).

The free-amino compound **6** (15 g) was coupled with 2-amino-6-chloro-5-nitropyrimidin-4(3*H*)-one (7.1 g) in ethanol (dry distilled from Mg; 685 cm^3) in the presence of triethylamine (7.5 cm^3) by heating under reflux for 48 h. The solution was filtered hot and the residue was washed with diethyl ether and dried to give the required product **7** (12.6 g).

The trityl group and the acetal were cleaved by heating a suspension of the foregoing pyrimidine **7** (12 g) in water (1 dm^3) and adding hydrochloric acid (2 mol dm^{-3}; 147 cm^3). Heating was continued for 1 h. The solution was filtered hot and then cooled in an ice bath. The pH was adjusted to 7 with aqueous ammonia and then left overnight during which time the title compound **8** crystallised (6.8 g). The product was spectroscopically identical with material prepared previously without use of the trityl protecting group.[3]

1-*Amino*-1-*benzyloxyacetylcyclopropane Ethylene Ketal* **9**.—The above phthalimido derivative **5c** (2.2 g) was dissolved in 2-methylpropanol (125 cm^3) and heated under reflux in the presence of hydrazine hydrate (1.0 cm^3) overnight. After cooling, the solution was filtered and the filtrate evaporated to dryness under reduced pressure. The residue was extracted twice with chloroform and then the combined chloroform extracts were washed twice with brine. The chloroform solution was dried ($MgSO_4$) and evaporated to dryness to give the required *title compound* **9** as a pale yellow oil (1.26 g), b.p. 170 °C (at 0.1 Torr) (Found: C, 66.7; H, 7.6; N, 5.1. $C_{14}H_{19}NO_3$ requires C, 67.4; H, 7.7; N, 5.6%); δ_H(90 MHz; $CDCl_3$) 7.32 (5 H, s, C_6H_5), 4.61 (2 H, s, OCH_2), 3.98 (2 H, s, OCH_2CH_2O), 3.76 (2 H, s, CH_2Ph), 2.11 (2 H, br s, NH_2), 0.71 (2 H, t, cyclopropane) and 0.52 (2 H, t, cyclopropane).

2-*Amino*-6-[1-(2-*benzyloxy*-1-*oxoethyl*)*cyclopropylamino*]-5-*nitropyrimidin*-4(3H)-*one* **10**.—The above amine **9** (1.6 g) was coupled with 2-amino-6-chloro-5-nitropyrimidin-4(3*H*)-one (1.23 g) in ethanol (60 cm^3) in the presence of triethylamine as described for **7** above. The coupled product (1.1 g) was dissolved

in ethanol (600 cm^3) and hydrochloric acid (2 mol dm^{-3}; 30 cm^3) added. The solution was heated under reflux for 1 h, cooled in ice–water, and the pH adjusted to 7 with aqueous ammonia. The *title compound* **10** (0.62 g) was obtained as a pale yellow solid, and recrystallised from aqueous ethanol, m.p. 257–260 °C (decomp.) (Found: C, 53.4; H, 4.1; N, 17.2. $C_{18}H_{21}N_5O_5 \cdot H_2O$ requires C, 53.3; H, 5.7; N, 17.3%); δ_H[250 MHz; $(CD_3)_2SO$] 10.75 (1 H, br s, NH pyrimidine), 9.80 (1 H, s, NH-cyclopropane), 7.32 (5 H, s, C_6H_5), 4.50 and 4.44 (each 2 H, s, OCH_2) 3.30 (2 H, s, NH_2), 1.51 (2 H, m, cyclopropane) and 1.26 (2 H, m, cyclopropane).

2-*Amino*-6-*benzyloxymethylpteridine*-7-*spirocyclopropan*-4(3H)-*one* **2**.—The foregoing nitropyrimidine **10** (350 mg) was added to hot sodium phosphate buffer solution (pH 8; 0.1 mol dm^{-3}; 75 cm^3) at 85 °C immediately followed by sodium dithionite (1.5 g). The mixture was stirred at 85–90 °C for 1–2 min, and then quickly chilled in ice–water. The *title compound* **2** was precipitated as an orange solid (230 mg), m.p. >300 °C (Found: C, 58.9; H, 5.4; N, 21.9. $C_{16}H_{17}N_5O_2 \cdot H_2O$ requires C, 58.4; H, 5.8; N, 21.3%); δ_H[$(CD_3)_2SO$] 10.0 (1 H, s, NHCO), 7.31 (5 H, narrow m, Ph), 6.85 (1 H, s, NH), 6.39 (2 H, br s, NH_2), 4.42 (2 H, s, OCH_2), 3.88 (2 H, s, OCH_2), 1.06 (2 H, m, cyclopropane) and 0.74 (2 H, m, cyclopropane).

2-*Amino*-6-[1-*ethoxycarbonylcyclopropylamino*]-5-*nitropyrimidin*-4(3H)-*one* **11**.—2-Amino-6-chloro-5-nitropyrimidin-4(3-*H*)-one (1.48 g, 7.74 mmol), ethyl 1-aminocyclopropane-1-carboxylate (1 g, 7.74 mmol) and triethylamine (0.57 cm^3, 7.74 mmol) were dissolved in dry ethanol (70 cm^3) and the solution heated under reflux for 48 h. The resulting precipitate was filtered off, washed with water, ethanol and diethyl ether and then dried over phosphorus pentoxide to give the required *title compound* **11** (1.56 g, 71%), m.p. >300 °C (Found: C, 42.1; H, 4.4; N, 24.5. $C_{10}H_{13}N_5O_5$ requires C, 42.4; H, 4.6; N, 24.7%); δ_H[90 MHz; $(CD_3)_2SO$] 11.5 (1 H, s, NH), 9.6 (1 H, s, NH), 7.2 (2 H, s, NH_2), 4.05 (2 H, q, OCH_2), 1.45 (2 H, m, cyclopropane), 1.3 (2 H, m, cyclopropane) and 1.11 (3 H, t, CH_3); HPLC: R_t 8.8 min.

2-*Amino*-7,8-*dihydropteridine*-7-*spirocyclopropane*-4(3H),6(5-H)-*dione* **3**.—The foregoing pyrimidine **11** (1 g, 3.53 mmol) was dissolved in sodium phosphate buffer solution (pH 8.4; 0.1 mol dm^{-3}; 500 cm^3) and the solution heated to 90 °C. Sodium dithionite (6.15 g, 37.3 mmol) was added rapidly and the temperature of the solution maintained at 95 °C for 3 min. The solution was cooled and evaporated to one third of its volume under reduced pressure to give an orange precipitate. This was filtered off, washed with water, ethanol and diethyl ether, and dried over phosphorus pentoxide to afford the required *title compound* **3** (0.61 g, 83%), m.p. >300 °C (Found: C, 44.3; H, 4.2; N, 33.9. $C_8H_9N_5O_2 \cdot H_2O$ requires C, 44.5; H, 4.5; N, 33.1%; M^+, 207.0762. $C_8H_9N_5O_2$ requires *M*, 207.0756); δ_H[250 MHz; $(CD_3)_2SO$] 10.45 (1 H, s, NH), 8.9 (1 H, s, NH), 6.9 (1 H, s, NH), 6.2 (2 H, s, NH_2), 1.15 (2 H, m, cyclopropane) and 0.8 (2 H, m, cyclopropane); HPLC R_t 4.8 and 5.7 min (see Discussion).

1-*Aminocyclopropane*-1-*carbonitrile Hydrochloride*.—1-Diphenylmethyleneaminocyclopropane-1-carbonitrile[12] (5 g, 20.23 mmol) was dissolved in methanolic hydrogen chloride (0.12 mol dm^{-3}; 250 cm^3) and the solution stirred for 14 h. The solution was evaporated under reduced pressure and the residue recrystallised from methanol–diethyl ether to give the *title compound* as a white solid (1.6 g, 66%), m.p. 223 °C (decomp.) (Found: C, 39.9; H, 5.9; N, 23.7. $C_4H_7ClN_2$ requires C, 40.5; H, 5.95; N, 23.6%); ν_{max}/cm^{-1} 2150; δ_H[90 MHz; $(CD_3)_2SO$] 9.2 (3 H, br s, NH_3^+) and 1.6 (4 H, s, cyclopropane).

2-*Amino*-6-(1-*cyanocyclopropylamino*)-5-*nitropyrimidin*-4(3-H)-*one* **14**.—This compound was prepared in the same manner as the corresponding ester **11** from the foregoing 1-aminocyclopropane-1-carbonitrile hydrochloride in 72% yield (Found: C, 40.7; H, 3.2; N, 35.5%. $C_8H_8N_6O_3$ requires C, 40.7; H, 3.4; N, 35.6%); ν_{max}/cm^{-1} 2255; δ_H[250 MHz; $(CD_3)_2SO$] 10.91 (1 H, s, NH), 9.54 (1 H, s, NH), 8.12 (1 H, br s, NH_2), 6.74 (1 H, br s, NH_2), 1.53 (2 H, m) and 1.40 (2 H, m, cyclopropane).

2-*Amino*-6-*cyclopropylpteridine*-4(3H),7(8H)-*dione* **12a** *and* 2-*Amino*-7-*cyclopropylpteridine*-4(3H),6(5H)-*dione* **12b**.—2,5,6-Triaminopyrimidin-4(3*H*)-one (0.8 g, 3.34 mmol) and methyl cyclopropylglyoxylate were dissolved in water (150 cm^3) and the solution heated under reflux for 48 h. The resulting precipitate was filtered off and washed with water, ethanol and diethyl ether. The residue was dried under reduced pressure over phosphorus pentoxide to give a mixture of the required title compound **12a** together with its isomer **12b** (0.41 g, 56%) (Found: C, 48.1; H, 3.9; N, 31.3. $C_9H_9N_5O_2 \cdot 0.5H_2O$ requires C, 47.4; H, 4.4; N, 30.7%); λ_{max}/nm 222, 253 and 343 (*cf.* isoxanthopterin 221, 253 and 339);[13] δ_H(250 MHz; 1 mol dm^{-3} NaOD) 2.0 (1 H, quintet, CH) and 0.6 (4 H, m, cyclopropane).

3-*Cyclopropylquinoxalin*-2(1H)-*one* **13**.—To a solution of potassium cyclopropylglyoxylate (0.5 g, 3.25 mmol) in water (5 cm^3) a solution of *o*-phenylenediamine dihydrochloride (0.88 g, 5 mmol) in water (5 cm^3) was added. The mixture was left at room temperature for 1 h after which the precipitate was filtered off and the residue recrystallised from ethanol in the presence of activated charcoal to give the *title compound* **13** (0.39 g, 65%), m.p. 246 °C (Found: C, 70.7; H, 5.1; N, 14.9. $C_{11}H_{10}N_2O$ requires C, 71.0; H, 5.4; N, 15.0%); δ_H[90 MHz; $(CD_3)_2SO$] 7.2–7.8 (4 H, m), 2.71 (1 H, m, cyclopropane CH) and 1.05, 1.12 (4 H, cyclopropane CH_2).

Enzyme Assays.—Assays of dihydrofolate reductase inhibition were carried out as described previously[3] using the concentrations of enzymes given in Table 1.

Time-dependent Assays.—These were carried out in 0.1 mol dm^{-3} pH 5.0 succinic acid–tris solution from which samples were removed at appropriate times. Concentrations of the pteridines were in the range 0.18–0.09 mmol dm^{-3}, of DHFRs 0.9–1.1 μmol dm^{-3} and of NADPH 0.06–0.07 mmol dm^{-3}.

Reactions were continued for up to 1 h at 30 °C. Samples of reaction mixture (2.70 cm^3) were removed and a solution of 7,8-dihydrofolic acid (0.3 cm^3) added to initiate reaction. Initial rates were measured. The dihydrofolic acid solution was prepared immediately before use from a suspension of the acid (3.79×10^{-3} mol dm^{-3}; 1.32 cm^3) in 0.005 mol dm^{-3} HCl and 2-mercaptoethanol (100 mm^3) made up to 10 cm^3 with 50 mmol dm^{-3} degassed aqueous potassium dihydrogen phosphate (50 mmol dm^{-3}; pH 7.0).

Irreversibility Tests.—These were carried out using the following reaction mixtures. DHFR [0.25 cm^3 of a solution of enzyme (1.5 mg) in distilled water (5 cm^3)] and pteridine **1** [2 cm^3 of a solution of the compound (7.0 mg) dissolved in DMSO (50 cm^3) made up to 10 cm^3 with pH 5.0 0.1 mol dm^{-3} succinate–tris buffer]. The control experiment contained 2 cm^3 of DMSO in place of inhibitor solution. Two successive dialyses against succinate–tris buffer at room temperature were carried out.

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