Latent Inhibitors. Part 5.[1]† Latent Inhibition of Dihydrofolate Reductase by a Pteridine-spiro-cyclopropane

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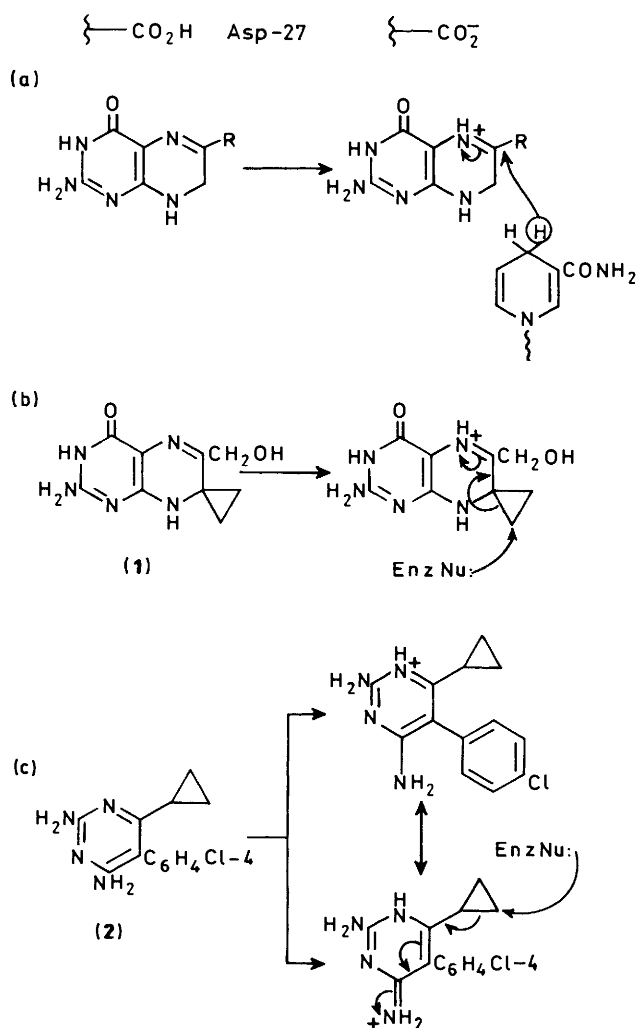
The design of cyclopropane-containing enzyme activated inhibitors of dihydrofolate reductase is presented. The synthesis of two examples, 2-amino-7,8-dihydro-6-hydroxymethylpteridine-7-spirocyclopropan-4(3*H*)-one and 2,4-diamino-5-(4-chlorophenyl)-6-cyclopropylpyrimidine, is described. Kinetic studies with dihydrofolate reductase from *E. coli* are presented to show that the former is a time-dependent inhibitor of the enzyme whereas the latter is a typical competitive inhibitor. The results are interpreted with regard to the active site structure of dihydrofolate reductase.

Dihydrofolate reductase (DHFR) is the target for many effective drugs for the treatment of bacterial and protozoal infections and also cancer.[3] Typical drugs are derivatives of 2,4-diaminopyrimidines or -pteridines and the binding of such compounds to the enzyme has been studied in detail both by n.m.r. spectroscopy[4] and by *X*-ray crystallography.[5] There have, however, been no reports of pteridines with the natural 2-amino-4-oxo functions having significant inhibitory properties. Such compounds might be expected to lack the toxicity of some members of the 2,4-diamino series.

In our previous work we developed versatile syntheses of 7,7-dialkyl-7,8-dihydropteridines.[6] We have also shown that cyclopropane-containing compounds can inhibit alcohol and lactate dehydrogenases[7] and carboxypeptidase A[8] by a mechanism involving the enhancement of positive charge at the carbon atom adjacent to the cyclopropane ring. The importance of an aspartic acid residue (Asp-27 in the *E. coli* enzyme) in the binding of 2,4-diamino-pteridine and -pyrimidine inhibitors has been demonstrated by n.m.r. spectroscopy[4] and *X*-ray crystallography.[5] Site specific mutagenesis experiments in which the aspartate has been replaced by asparagine have further shown that proton transfer from aspartate is important for catalysis[9] although direct transfer of a proton to N-5 of dihydrofolate may not occur (Scheme 1a). We reasoned that such a proton transfer would also be suitable for activating a suitably substituted cyclopropane-containing pteridine such as 2-amino-7,8-dihydro-6-hydroxymethylpteridine-7-spirocyclopropan-4(3*H*)-one (**1**) (Scheme 1b). Similarly, protonation of a suitably substituted pyrimidine such as 2,4-diamino-5-(4-chlorophenyl)-6-cyclopropylpyrimidine (**2**), could lead to an activated cyclopropane ring best shown by the canonical forms in Scheme 1c. The synthesis of the two compounds was therefore undertaken.

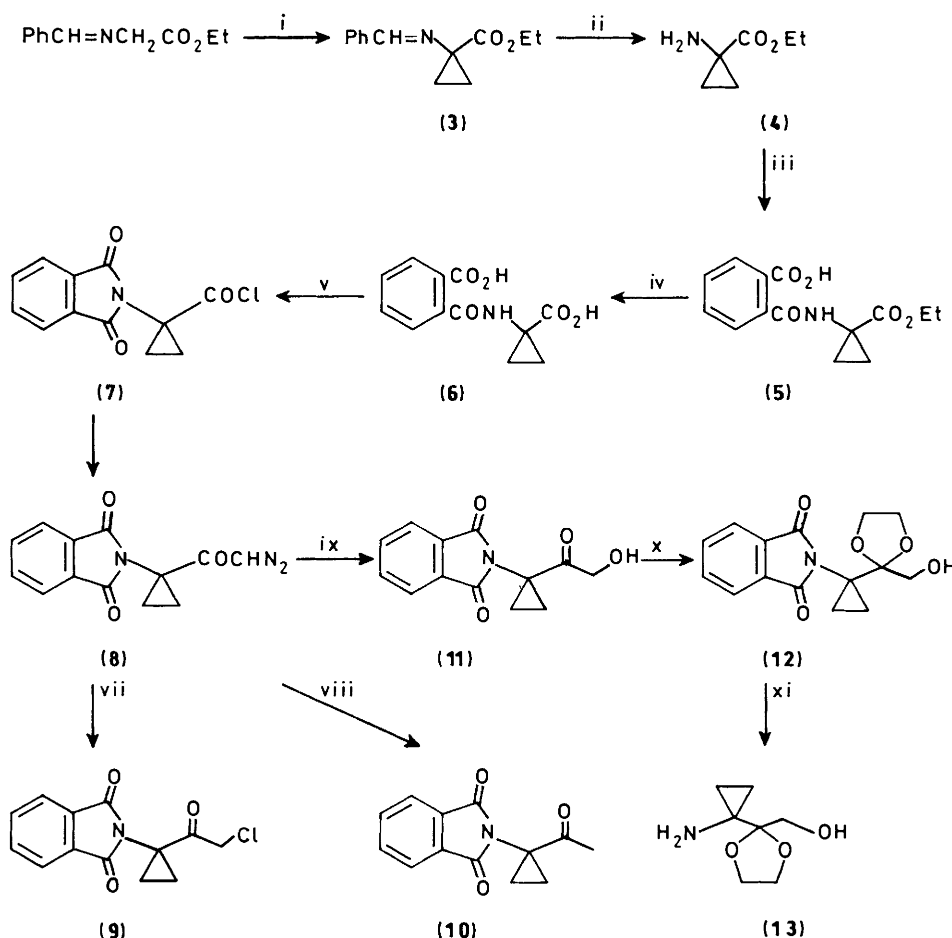


Scheme 1. Mechanistic hypothesis for inhibitor design

Results and Discussion

Synthetic Studies.—The synthesis of the pteridine-spiro-cyclopropane (**1**) was modelled upon our established routes to 7,7-dialkyl-7,8-dihydropteridines (Scheme 2).[6] Alkylation of ethyl benzylideneglycinate with 1,2-dibromoethane using a procedure modified from that of Schöllkopf[10] afforded ethyl 1-benzylideneaminocyclopropane-1-carboxylate (**3**). The labile benzylidene protecting group was then replaced by the stable phthalimido group through a series of hydrolysis and acylation reactions [(**3**) ⟶ (**7**)], the final ring closure of the phthalimide being effected with concomitant formation of the acid chloride

† This paper is also considered as part 10 in the series Specific Inhibitors in Vitamin Biosynthesis.[2]



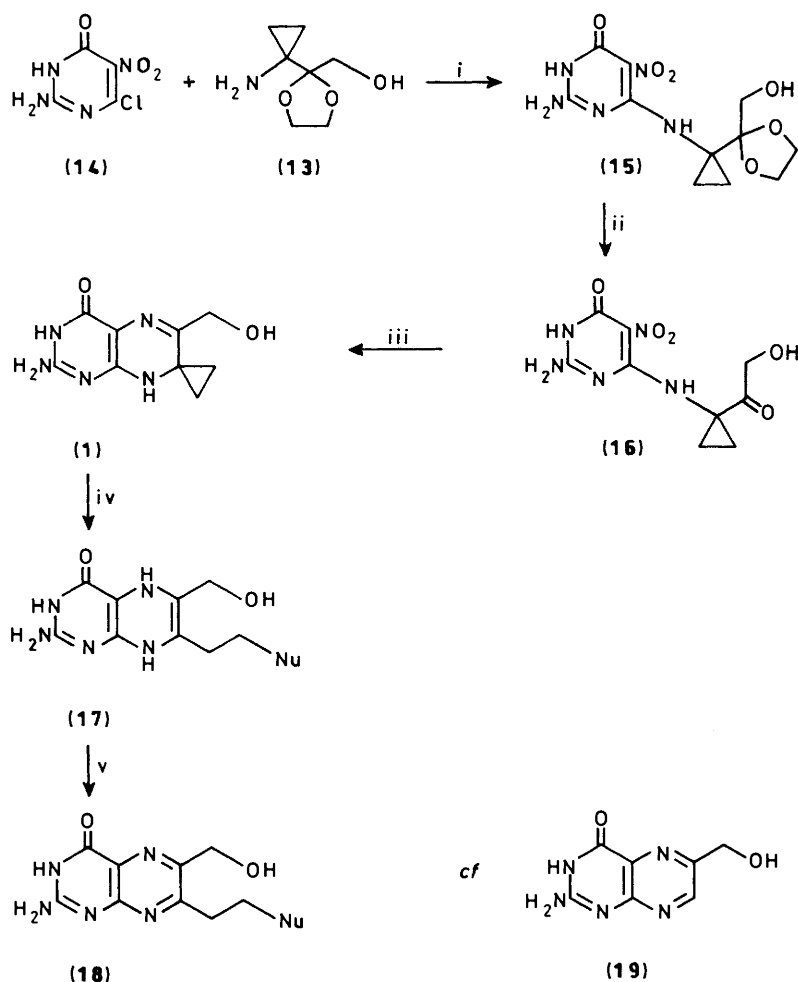
Scheme 2. Synthesis of the protected amino ketone (**13**). *Reagents:* i, $Br(CH_2)_2Br$, NaH; ii, 1M HCl; iii, phthalic anhydride; iv, 1M NaOH; v, $SOCl_2$; vi, CH_2N_2; vii, 1M HCl; viii, 55% aq. HI; ix, 0.25 M H_2SO_4; x, $HO(CH_2)_2OH$, 4-$MeC_6H_4SO_3H$; xi, $MeNH_2$ then aq. KOH

(**7**) using a large excess of thionyl chloride. The cyclopropane group appeared to be fully stable to this series of reactions. Treatment of (**7**) with diazomethane led to the diazo ketone (**8**). This intermediate was chosen for its potential to be transformed into a variety of α-substituted ketones which would lead to a number of 6-substituted pteridines. The diazo ketone (**8**) was converted into the chloromethyl ketone (**9**) with 1M hydrochloric acid and into the methyl ketone (**10**) with 55% hydriodic acid. Because of difficulties experienced in the removal of the phthalimido protecting group explained below, these compounds were not used further in this work although they remain available for further studies. The intermediate taken further in this work was the hydroxymethyl ketone (**11**) which was obtained from (**8**) on treatment with dilute sulphuric acid.

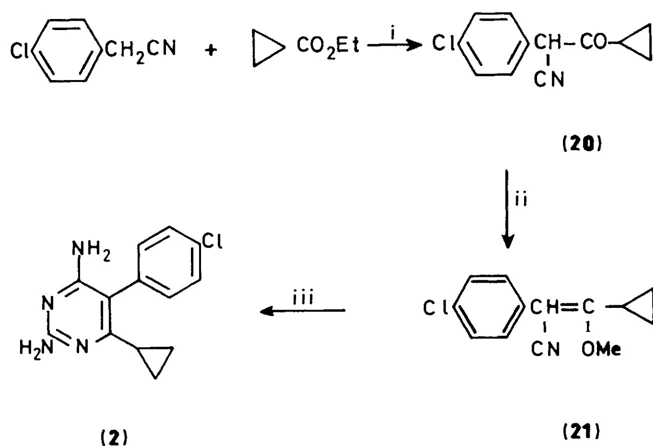
The hydroxymethyl ketone (**11**) contains all the necessary functionalities for the preparation of (**1**) and, in principle, of analogues in which the hydroxymethyl group is derivatised. We attempted to cleave the phthalimide protecting group using dilute acid; although it was labile enough to be cleaved, we were unable to isolate the required amino ketone hydrochloride. The phthalimide was also cleaved by methylamine and hydrazine as shown by the isolation of the appropriate phthalamides but condensation with the ketone not surprisingly occurred also and no useful products were isolated. To minimise potential side reactions, the ketone was protected as its ethylenedioxy ketal (**12**) and the phthalimide removed from this compound. After much experimentation, a two-stage process was adopted in which the imide was half opened with methylamine and the resulting amide cleaved with aqueous potassium hydroxide to give (**13**). The isolation of (**13**) was also troublesome; it is extremely water soluble and a pure sample was only separated using ion exchange chromatography. We have recently found that the analogous methyl ketone is similarly tricky to handle.

The elaboration of the amino ketone (**13**) into the pteridine (**1**) was not achieved without difficulty (Scheme 3). Coupling with the nitrochloropyrimidine (**14**) was effected in the presence of triethylamine giving (**15**) which was converted into the corresponding ketone (**16**) with aqueous hydrochloric acid under carefully controlled conditions. In principle, all that remained was to reduce the nitro group of (**16**) and to allow cyclisation to occur *in situ* leading to (**1**). The reductive cyclisation, however, proved very difficult to accomplish. All purely aqueous conditions failed to produce any pteridine-7-spirocyclopropane and evidence was obtained which suggested that opening of the cyclopropyl ring had rapidly followed cyclisation [(**1**) ⟶ (**17**) ⟶ (**18**)]. The u.v. spectrum of the product closely resembled that of the 7-unsubstituted hydroxylmethylpteridine (**19**)[11] and, like (**19**), the product had a blue fluorescence. We formulate the product as (**18**) although it was not isolated and characterised. Finally we found that the required pteridine (**1**) could be obtained if the reductive cyclisation was carried out using sodium dithionite in aqueous dimethylformamide (DMF) although in very low yield.

Fortunately, the synthesis of the pyrimidine (**2**) was straightforward following established procedures[12] (Scheme 4). 4-Chlorophenylacetonitrile was acylated with ethyl cyclopropanecarboxylate in the presence of sodium ethoxide and the resulting ketone (**20**) converted into its imino ether (**21**) with



Scheme 3. Synthesis of cyclopropylpteridine (**1**). *Reagents:* i, Et_3N; ii, aq. HCl; iii, $Na_2S_2O_4$, aq. DMF; iv, aq. $Na_2S_2O_4$; v, air



Scheme 4. Synthesis of cyclopropylpyrimidine (**2**). *Reagents:* i, NaOEt; ii, CH_2N_2; iii, guanidine, NaOEt

diazomethene. Cyclisation with guanidine led to the required pyrimidine (**2**).

Enzyme Inhibition Studies.—The target compounds (**1**) and (**2**) were tested as inhibitors of DHFR from *E. coli* kindly supplied by Wellcome Research using the decrease in the u.v. absorption of the coenzyme NADPH as a measure of the progress of the reaction. The pyrimidine (**2**) proved to be a good competitive inhibitor of DHFR as shown by the Dixon plot (Figure 1) with a

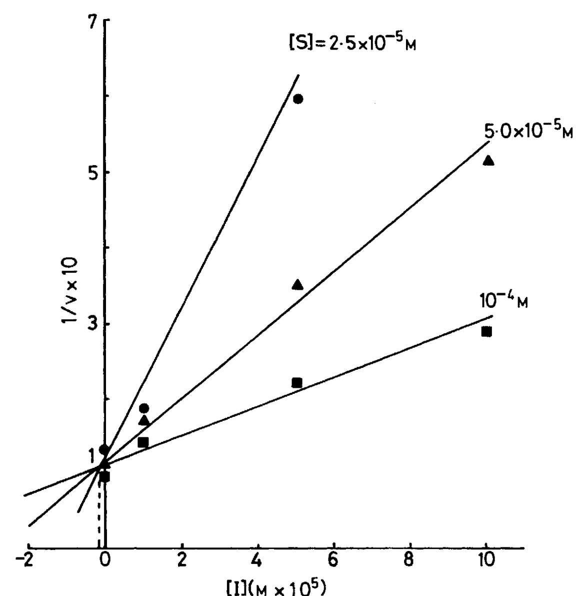


Figure. Dixon plot for the competitive inhibition of DHFR by cyclopropylpyrimidine (**2**)

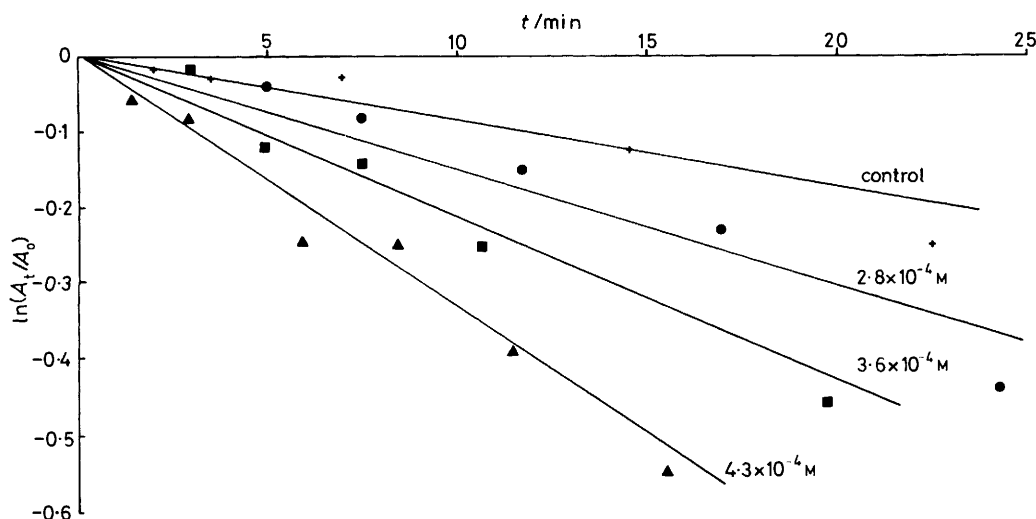


Figure 2. First order plots for the time-dependent inhibition of DHFR by cyclopropylpteridine (**1**)

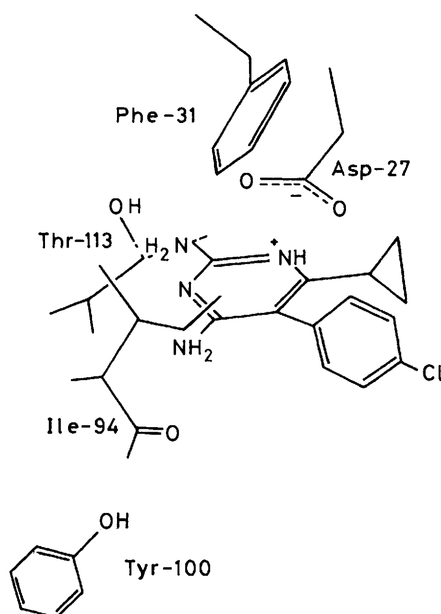


Figure 3. Schematic representation of binding of cyclopropylpyrimidine (**2**) to DHFR showing the proton donor (Asp-27) and amino acids around the binding site

K_i of $\sim 10^{-6}$M. There were, however, no indications of time dependent or irreversible inhibition. On the other hand, the pteridine (**1**) inhibited DHFR in a time dependent manner showing first order kinetics for the loss of enzymic activity (Figure 2). These data led to a rate constant of 1.4×10^{-3} s^{-1} at 25 °C. Unfortunately, it was not possible to determine any further kinetic parameters for the interaction of (**1**) with DHFR partly because of the complexity of the association kinetics [13] and partly because of shortage of material. The inhibition reaction was shown to be irreversible with respect to dialysis by the following experiment. A sample of enzyme and inhibitor dissolved in aqueous 0.1M succinate/Tris buffer pH 5.0 containing dimethyl sulphoxide (DMSO) was prepared and inhibition allowed to proceed for 3.5 h after which time 43% of the initial activity remained. The sample was dialysed overnight against the same buffer without DMSO and the enzyme activity assayed; 36% of the initial activity remained. A control sample without inhibitor showed no loss of activity when treated under the same conditions. The enzyme was not stable to gel filtration. To our knowledge, the pteridine (**1**) is the first compound with the natural 2-amino-4-oxo substitution pattern to be shown to be an inhibitor of DHFR and is also the first mechanism-based inhibitor of the enzyme.[14] We are currently investigating improved methods of synthesis of (**1**) and analogues.

The design of both inhibitors followed a similar mechanistic premise and yet only (**1**) showed any characteristics of time-dependence. This difference required some explanation. In order to evaluate the results and to provide a basis for the design of further experiments and inhibitors, we investigated the binding of both compounds to DHFR by means of molecular graphics using the INTERCHEM suite of programs developed at Strathclyde.[15] The crystal structure of a binary complex of DHFR from *E. coli* with the inhibitor methotrexate has been described [5] and we used these co-ordinates to build a model of the active site. The diaminopteridine methotrexate and the pyrimidine inhibitor (**2**) would be expected to bind to DHFR so that N-1 of both compounds is protonated by Asp-27 as indicated by the crystal structure, pH dependence and n.m.r. studies.[4,5,16] To assess the interaction of (**2**) with DHFR, we replaced methotrexate with (**2**) so that the pyrimidine fragments of both compounds were identically bound (Figure 3). This mode of binding locates the 4-chlorophenyl group and the cyclopropane ring in a well-characterised hydrophobic pocket of the enzyme [17] such that there are no identifiable nucleophiles within range of the cyclopropane ring. In other cases of enzyme inhibition by cyclopropane-containing compounds, we found that a nucleophile has usually been available about 2.5 Å away from an activated apex of the cyclopropane ring in a suitable position for bonding.[18] Assuming that the pyrimidine (**2**) binds to DHFR analogously to all the diamino-pyrimidines and -pteridines so far studied, it seems likely that it acts only as a competitive inhibitor because of the absence of suitable nucleophiles within bonding distance of the cyclopropane ring.

In contrast to the probability of the orientation of binding of (**2**), the binding of the pteridine (**1**) is ambiguous. The ambiguity arises because the pteridine inhibitor (**1**) lacks the binding determinants of the *p*-aminobenzoyl glutamate side chain present in methotrexate and the substrate, and also the hydrophobic substituent present in the pyrimidine inhibitor (**2**). A mechanistically satisfactory binding mode for (**1**) must, however, satisfy the requirements that *N*-5 be protonated and that there be a nucleophile within range of the cyclopropane ring. The closest potential nucleophiles are threonine-113 and

tyrosine-100.[19] The possibility that these or other groups act as nucleophiles is being investigated using mutant enzymes in collaboration with Professor S. J. Benkovic.

Experimental

^{1}H N.m.r. spectra were recorded on Perkin-Elmer R12 (90 MHz) or Bruker WH-250 (250 MHz) spectrometers using tetramethylsilane as an internal standard. I.r. spectra were determined using Perkin-Elmer 397 or 257 spectrometers and u.v. spectra using Pye-Unicam SP 8000 and SP 800A spectrophotometers. H.p.l.c. was carried out on Merck CGC reversed phase columns (LiChrosorb RP-18; 5 μm) using acetonitrile–50 mM Tris HCl, pH 7.0 (5:95) as the mobile phase and a flow rate of 38 ml h^{-1}. The detector used was a Cecil Instruments CE 2012 variable wavelength spectrophotometer operating at 340 nm. T.l.c. was carried out on Polygram Silica G/UV_{254} sheets (type 1), Whatman $MKC_{18}F$ reversed phase plates (type 2) and Fluka OPTi-UPC_{12} reversed phase plates (type 3) using as solvents; (A) ethyl acetate–light petroleum (b.p. 60—80 °C) (5:1) and (B) acetonitrile–water (55:45).

Ethyl Benzylideneaminoacetate.[20]—Ethyl glycinate hydrochloride (100.1 g, 0.72 mol) and anhydrous sodium sulphate (61 g) were added to a solution of anhydrous dichloromethane (1.5 l), freshly distilled benzaldehyde (76.7 g, 0.72 mol), and redistilled triethylamine (200 ml, 1.45 mol) and the resulting suspension was stirred at room temperature for 86 h. The suspension was filtered and the solvent removed. The resulting oil was extracted with ether and water, the ethereal layer being washed with brine and then dried (Na_2SO_4). Concentration gave the imine as a green oil (114.18 g, 82%); $\nu_{max.}$(CCl_4) 2 840 (CH=N), 1 740 (CO_2Et), and 1 645 cm^{-1} (C=N); δ_H (90 MHz; CCl_4) 8.18 (1 H, s, CH=N), 7.70 (2 H, s, ArH), 7.35 (3 H, m, ArH), 4.25 (2 H, s, NCH_2), 4.15 (2 H, q, *J* 7 Hz, CO_2CH_2) and 1.25 (3 H, t, *J* 7 Hz, $CO_2CH_2CH_3$) (Found: C, 68.0; H, 6.9; N, 7.2. Calc. for $C_{11}H_{13}NO_2$: C, 69.1; H, 6.85; N, 7.3%).

Upon distillation of a portion, decomposition occurred. A small sample of pure amine was obtained as a pale green oil, b.p. 98 °C/0.075 mmHg (lit.,[21] decomp. >140 °C/1 atm); n_D^{20} 1.532—1.539. The distilled and undistilled products had identical spectral data.

Ethyl 1-Benzylidineaminocyclopropane-1-carboxylate (**3**).—By modification of the method of Schöllkopf.[10] 60% Sodium hydride dispersion in mineral oil (49.5 g, 1.24 mol) was weighed into a 5 l three-necked flask, purified by washing/decanting with anhydrous ether (× 5) and then covered with anhydrous ether (200 ml) under oxygen-free dry nitrogen. A solution of ethyl 2-benzylideneaminoacetate (105.19 g, 0.55 mol), distilled 1,2-dibromoethane (47.5 ml, 0.55 mol), fresh DMSO (550 ml), and sodium dried ether (1 500 ml) was added dropwise, with vigorous stirring, at ice–salt temperature. The mixture was allowed to equilibrate to room temperature half-way through the addition. At the end of gas evolution, the mixture was refluxed for 30 min with vigorous stirring and then cooled to room temperature. The orange-brown suspension was quenched in ice-cold water (1.4 l) and the organic layer retained. The aqueous layer was washed with ether (3 × 700 ml) and then the combined organic portions were washed with water (2 × 275 ml), dried (Na_2SO_4), and concentrated to give a green oil (86.38 g, 72%). A portion (6.44 g) was distilled using a Kugelrohr electric oven, to give the *title compound* (**3**) as a pale green oil (1.94 g), b.p. 96—98 °C/0.05 mmHg, n_D^{20} 1.546; $\nu_{max.}$(CCl_4) 2 868 (CH=N), 1 720 (CO_2Et), and 1 640 cm^{-1} (C=N); δ_H(90 MHz; CCl_4) 8.53 (1 H, s, CH=N), 7.65 (2 H, m, ArH), 7.3 (3 H, m, ArH), 4.15 (2 H, q, *J* 7 Hz, CO_2CH_2), 1.60 (2 H, m, cyclopropane H), 1.26 (3 H, overlapping t, *J* 7 Hz, $CO_2CH_2CH_3$), and 1.25 (2 H, overlapping m, cyclopropane H) [Found: C, 71.2; H, 6.7; N, 6.2%; *m/e*, 217.1108 (M^+). $C_{13}H_{15}NO_2$ requires C, 71.9; H, 7.0; N, 6.5; *M*, 217.1103].

During distillation decomposition occurred, a thick, dark red residue being left in the distillation flask. I.r. and ^{1}H n.m.r. spectra of the distilled and undistilled oils were identical and the imine (**3**) was subsequently used without further purification.

Ethyl 1-Aminocyclopropane-1-carboxylate (**4**).—The benzylidine derivative (**3**) (44.50 g; 0.20 mol) was stirred in the presence of 1M hydrochloric acid (1 l) for 1 h at room temperature[22] and extracted with dichloromethane (5 × 200 ml). The aqueous phase was basified to pH 11 with solid sodium carbonate and then continuously extracted with dichloromethane for 116 h. The organic extract was dried (Na_2SO_4) and then concentrated under reduced pressure at room temperature, to give a light brown oil (14.68 g). Distillation gave the pure amine as a clear oil (11.19 g, 43%), b.p. 53 °C/6.5 mmHg, n_D^{20} 1.4450; $\nu_{max.}$ (CCl_4) 3 380 (NH_2), 3 100 (cyclopropane), and 1 720 cm^{-1} (CO_2Et); δ_H[90 MHz; $(CD_3)_2SO$] 4.05 (2 H, q, *J* 7 Hz, CO_2CH_2), 2.33 (2 H, br NH_2), 1.19 (3 H, overlapping t, *J* 7 Hz, $CO_2CH_2CH_3$), 1.10 (2 H, overlapping m, cyclopropane H) and 0.85 (2 H, m, cyclopropane H).

Ethyl 1-(2-Carboxybenzamido)cyclopropane-1-carboxylate (**5**).—Phthalic anhydride (12.74 g, 86 mmol) was added to a solution of ethyl 1-aminocyclopropane-1-carboxylate (**4**) (11.11 g, 86 mmol) in sodium-dried ether (750 ml). The resulting clear solution was stirred at room temperature for 48 h after which the resulting white precipitate was filtered off (Buchner), washed with anhydrous ether, and sucked dry to give the *amide* as a white amorphous solid (22.32 g, 94%), m.p. 151—152 °C; $\nu_{max.}$(KCl) 3 300 (NH), 3 200—2 300 (CO_2H), 1 718sh (CO_2H), 1 724 (CO_2Et), and 1 640 (CONH) and 1 590 cm^{-1} (ArC=C); δ_H[90 MHz; $(CD_3)_2SO$] 8.8 (1 H, s, NHCO), 7.9—7.3 (4 H, m, ArH), 4.1 (2 H, q *J* 7 Hz, CO_2CH_2), 1.21 (3 H, overlapping t, *J* 7 Hz, $CO_2CH_2CH_2$), and 1.6—1.0 (4 H, overlapping m, cyclopropane H); t.l.c. (type 1, solvent A), R_f 0.36.

Further purification was achieved by recrystallisation from chloroform (Found: C, 60.8; H, 5.5; N, 5.0. $C_{14}H_{15}NO_5$ requires C, 60.6; H, 5.5; N, 5.0%).

1-(2-Carboxybenzamido)cyclopropane-1-carboxylic Acid (**6**).—The ester (**5**) (48.19 g; 0.174 mol) was dissolved in 1M sodium hydroxide solution (3 mol equiv.) prepared from sodium hydroxide (20.88 g, 0.522 mol) and water (522 ml). The resulting solution was warmed gently on a steam-bath for 1 h, cooled to room temperature, and then acidified to pH 1 with 1M hydrochloric acid (590 ml). A white precipitate formed which was filtered off (Buchner), washed with water, and then freeze dried to give white crystals of the *diacid* (40.15 g, 93%), m.p. 226—228 °C; $\nu_{max.}$(KCl) 3 300 (NH), 3 200—2 200 (CO_2H), 1 700 (CO_2H), 1 630 (CONH), and 1 590 cm^{-1} (ArC=C); δ_H[90 MHz; $(CD_3)_2SO$] 8.71 (1 H, s, CONH), 7.9—7.3 (4 H, m, ArH), 1.38 (2 H, m, cyclopropane H), and 1.18 (2 H, m, cyclopropane H); t.l.c. (type 1, solvent A), R_f 0.22.

Further purification was achieved by recrystallisation from ethanol–water (Found: C, 57.4; H, 4.5; N, 5.5. $C_{12}H_{11}NO_5$ requires C, 57.8; H, 4.5; N, 5.6%).

1-Phthalimidocyclopropane-1-carbonyl Chloride (**7**).—The diacid (**6**) (39.63 g, 0.159 mol) was refluxed in thionyl chloride (62 ml, 0.86 mol) previously distilled from triphenyl phosphite for 3 h, until all traces of the diacid (**6**) had disappeared.[23] The reaction was followed by t.l.c. (type 1, solvent A): diacid (**6**), R_f 0.22; acid chloride (**7**) R_f 0.69. The unchanged thionyl chloride was removed *in vacuo* to leave a crystalline residue which was washed with anhydrous ether, the latter being evaporated off.

Washing and evaporation were repeated twice after which the residue was recrystallised from chloroform–light petroleum (b.p. 60—80 °C) to give colourless crystals of *acid chloride* (31.14 g, 79%), m.p. 147—148 °C; $\nu_{max.}$(CCl_4) 1 770 (phthalimido C=O and COCl) and 1 730 cm^{-1} (phthalimido C=O) δ_H[90 MHz; $CDCl_3$] 7.82 (4 H, m, ArH), 2.11 (2 H, m, cyclopropane H) and 1.75 (2 H, m, cyclopropane H) (Found: C, 57.1; H, 3.15; N, 5.4; Cl, 14.6. $C_{12}H_8ClNO_3$ requires C, 57.7; H, 3.2; N, 5.6; Cl, 14.2%).

1-*Diazoacetyl*-1-*phthalimidocyclopropane* (**8**).—An alcohol-free ethereal solution of diazomethane[24] was prepared, observing the precautions advised by De Boer,[25] by adding Diazald (51.42 g, 0.24 mol) dissolved in ether (380 ml) dropwise to a flask containing a solution of potassium hydroxide (15.46 g), water (27 ml), diethylene glycol (90 ml), and ether (150 ml), the mixture being warmed on a water-bath at 45—55 °C. The diazomethane solution formed *in situ* was collected by distillation, dried over potassium hydroxide pellets and kept cold in an ice–salt bath. Triethylamine (10.60 ml, 76 mmol) was added[26] followed by the dropwise addition of the acid chloride (**7**) (19 g, 76 mmol) in anhydrous dichloromethane (500 ml); the mixture was then stirred overnight, in the dark, in an ice–salt bath that was allowed to equilibrate to room temperature. The mixture was evaporated to dryness under reduced pressure with an aqueous acetic acid trap to destroy excess of diazomethane. The resulting yellow-green solid was recrystallised from ethyl acetate (with the filtrate being cooled very slowly without agitation) to give the *diazo ketone* as bright green crystals (13.13 g, 68%), m.p. 184—185 °C; $\nu_{max.}$(KCl) 3 085 (cyclopropane), 2 110 (CHN_2), 1 770 and 1 710 (phthalimido C=O), and 1 604 cm^{-1}($COCHN_2$); δ_H(90 MHz; $CDCl_3$) 7.84 (4 H, m, ArH), 5.30 (1 H, s, CNH_2), 1.88 (2 H, m, cyclopropane H), and 1.47 (2H, m, cyclopropane H) (Found: C, 61.5; H, 3.2; N, 16.7. $C_{13}H_9N_3O_3$ requires C, 61.2; H, 3.55; N, 16.5%); t.l.c. (type 2, solvent A), R_F 0.59.

1-*Hydroxyacetyl*-1-*phthalimidocyclopropane* (**11**).—The diazo ketone (**8**) (12.90 g, 50.5 mmol) was added to a solution of 0.25M sulphuric acid (210 ml) in dioxane (130 ml) and the resulting suspension was warmed on a steam-bath, with mechanical stirring for 45 min, until all effervescence had ceased.[27] The solution was then cooled in an ice-bath, basified with solid sodium hydrogen carbonate, and extracted with chloroform (4 × 100 ml). The organic extracts were then washed successively with aqueous sodium hydrogen carbonate (2 × 100 ml), water (2 × 100 ml), and brine (2 × 100 ml), dried (Na_2SO_4), and concentrated to give the *α-hydroxy ketone* as a white powder (11.28 g, 91%), m.p. 163—166 °C; $\nu_{max.}$(KCl) 3 470 (OH), 3 100 (cyclopropane), 1 770 and 1 712 (phthalimido C=O), and 1 700 cm^{-1} ($COCH_2OH$); δ_H(90 MHz; $CDCl_3$) 7.87 (4 H, m, ArH), 4.33 (2 H, d, *J* 5 Hz), CH_2OH), 3.02 (1 H, t, *J* 5 Hz, OH), 1.95 (2 H, m, cyclopropane H), and 1.60 (2 H, m, cyclopropane H); T.l.c. (type 1, solvent A), R_f 0.51.

Further purification was achieved by recrystallisation from ethanol (Found: C, 63.85; H, 4.3; N, 5.6. $C_{13}H_{11}NO_4$, requires C, 63.7; H, 4.5; N, 5.7%).

1-*Chloroacetyl*-1-*phthalimidocyclopropane* (**9**).—The diazo ketone (**8**) (6.61 g, 26 mmol) was refluxed vigorously with stirring, in a solution of 1M hydrochloric acid (225 ml) and dioxane (65 ml) for 22 h.[28] The resulting suspension was cooled, basified with solid sodium hydrogen carbonate, and extracted with chloroform. The organic phase was washed with aqueous sodium hydrogen carbonate, water, and brine, dried (Na_2SO_4), and concentrated to give the α-chloro ketone as a white solid (3.20 g, 47%). Further purification was achieved by recrystallisation from ethyl acetate–light petroleum (b.p. 60—80 °C) to give a sample, m.p. 190—192 °C; $\nu_{max.}$(KCl) 1 771 and 1 720 (phthalimido C=O), 1 708 ($COCH_2Cl$), and 721 cm^{-1} (Ar C–H); δ_H(90 MHz; $CDCl_3$) 7.87 (4 H, m, ArH), 4.27 (2 H, s, CH_2Cl), and 2.20—1.45 (4 H, m, cyclopropane H) (Found: C, 59.65; H, 3.9; Cl, 13.5; N, 5.5. $C_{13}H_{10}ClNO_3$ requires 59.2; H, 3.8; Cl, 13.5; N, 5.3%).

1-*Acetyl*-1-*phthalimidocyclopropane* (**10**).—Then diazo ketone (**8**) (0.25 g, 1 mmol) was dissolved in a solution of chloroform (10 ml) and 55% aqueous hydrogen iodide (1.50 ml) and the resulting dark brown solution was stirred at room temperature for 4 h.[29] The organic phase was separated off, washed with water, dilute aqueous[29] sodium thiosulphate (until free of iodine), water again, and then dried (Na_2SO_4). Upon concentration the methyl ketone was obtained as a thick oil which solidified, with time, to a white waxy solid (0.15 g, 65%), m.p. 153—155 °C $\nu_{max.}$(KCl) 3 100 (cyclopropane), 1 770 and 1 720 (phthalimido C=O), and 1 710 cm^{-1} ($COCH_3$); δ_H(90 MHz; $CDCl_3$) 7.85 (4 H, m, ArH), 2.11 (3 H, s, $COCH_3$) and 2.10—1.37 (4 H, m, cyclopropane H).

1-*Hydroxyacetyl*-1-*phthalimidocyclopropane Diethylene Ketal* (**12**).—The α-hydroxy ketone (**11**) (26.09 g, 104.4 mmol), ethylene glycol (29 ml, 0.522 mol), benzene (500 ml), and toluene-*p*-sulphonic acid monohydrate (100 mg) were refluxed together for 20 h using a Dean and Stark apparatus to remove water.[30] It was on occasions necessary to add a further 5 equiv., [with respect to ketone (**11**)] of ethylene glycol and to reflux for a further 20 h. The reaction was followed by t.l.c. (type 1, solvent A): ketone (**11**), R_f 0.54; ketal (**12**), R_f 0.38. On completion of the reaction the mixture was concentrated and the residue extracted with chloroform. The organic phase was washed with aqueous sodium hydrogen carbonate, water, and then brine, dried (Na_2SO_4), and concentrated to give a quantitative yield of the *ketal* as a white powder; $\nu_{max.}$(KCl) 3 460 (OH), 3 095 (cyclopropane), 1 770 and 1 700 (phthalimido C=O), and 1 040 cm^{-1} (ketal C–O); δ_H(90 MHz $CDCl_3$) 7.78 (4 H, m, ArH), 4.50—3.85 (4 H, m, OCH_2CH_2O), 3.63 (2 H, d, *J* 7 Hz), CH_2OH), 3.12 (1 H, t, *J* 7 Hz, OH), 1.33 (2 H, m, cyclopropane H), and 1.00 (2 H, m, cyclopropane H); t.l.c. (type 1, solvent A), R_f 0.43.

Further purification was achieved by recrystallisation from ethanol, giving a sample, m.p. 119—112 °C; (Found: C, 62.2; H, 5.2; N, 4.7. $C_{15}H_{15}NO_5$ requires C, 62.3; H, 5.2; N, 4.8%).

1-*Hydroxyacetyl*-1-[2-(N-*methylcarboxamido*)*benzamido*)]-*cyclopropane Diethylene Ketal*.—The ketal (**12**) (30.65 g, 95.7 mmol) was dissolved in a solution of 33% ethanolic methylamine (50 ml) and ethanol (200 ml) and stirred at room temperature.[31] A thick suspension formed immediately and this was left at room temperature overnight. The solvent and excess of methylamine were removed *in vacuo* and the solid recrystallised from ethanol to give the *diamide* as a white amorphous powder (25.59 g, 84%), m.p. 208 °C; $\nu_{max.}$(KCl) 3 420 (OH), 3 250 (CONH), 1 630 (CONH), 1 545 (NH) and 1 035 cm^{-1} (ketal CO); δ_H[90 MHz; $(CD_3)_2SO$)] 8.57 (1 H, s, CONH), 8.08 (1 H, br, CON$H$$CH_3$), 7.42 (4 H, s, ArH), 4.79 ([1 H, t, (exchanges with D_2O), *J* 7 Hz, CH_2OH], 3.90 (4 H, s, OCH_2CH_2O), 3.50 (2 H, d, *J* 7 Hz, CH_2OH), 2.71 (3 H, d, *J* 5 Hz, $NHCH_3$) and 1.10—0.50 (4 H, m, cyclopropane H) (Found: C, 59.6; H, 6.2; N, 8.4. $C_{16}H_{20}N_2O_5$ requires C, 60.0; H, 6.3; N, 8.7%); t.l.c. (type 3, solvent B), R_f 0.59.

1-*Amino*-1-*hydroxyacetylcyclopropane Diethylene Ketal* (**13**).—The above diamide (23.19 g, 72.4 mmol) was dissolved in a solution of potassium hydroxide (12.18 g, 217 mmol) in methanol (250 ml) and refluxed on a steam-bath for 1 h.[32] The reaction mixture was concentrated and the residue dissolved in the minimum quantity of water. The product was isolated by passing the aqueous solution through an Amberlite CG-120

column (100 g, free acid form), washing the column with water (2 l), eluting with concentrated aqueous ammonia (*d* 0.88) and then freeze-drying the coloured fractions. Pure *amine* was obtained as a white waxy solid (2.68 g, 23%) which was purified further by recrystallisation from chloroform–petroleum (b.p. 60—80 °C) to give a sample, m.p. 87—88 °C; $\nu_{max.}$(KCl) 3 700—2 300 (OH, NH_2), 1 625 (NH), and 1 040 cm^{-1} (ketal CO); δ_H[250 MHz; $(CD_3)_2SO$] 3.88 (4 H, m, OCH_2CH_2O), 0.56 (2 H, m, cyclopropane H), and 0.31 (2 H, m, cyclopropane H) (Found: C, 52.9; H, 8.3; N, 8.8. $C_7H_{13}NO_3$ requires C, 52.8; H, 8.2; N, 8.8%).

The column water washings were left for 2 weeks, after which time white needles had formed. These were filtered off, washed with water, dried, and identified as the α-hydroxy ketone (**11**) (4.50 g, 25%), m.p. 165—167 °C.

2-*Amino*-6-[1-(1-*ethylenedioxy*-2-*hydroxyethyl*)*cyclopropylamino*]-5-*nitropyrimidin*-4(3H)-*one* (**15**).—The amine (**13**) (2.62 g, 16.5 mmol), 2-amino-6-chloro-5-nitro-pyrimidin-4(3*H*)-one (**14**)[6,33] (3.10 g; 16.5 mmol) and triethylamine (2.30 ml, 16.5 mmol) were refluxed in anhydrous ethanol (310 ml) for 22 h. The resulting precipitate was cooled to room temperature, filtered off, washed with ethanol and then ether, and dried *in vacuo* over phosphorous pentaoxide. The *pyrimidine* was obtained as a beige powder (4.43 g, 86%), m.p. 245 °C (decomp.); $\nu_{max.}$(KCl)3 700—3 020 (NH_2, NH, OH, NHCO), 1 685 (NHCO), 1 655 (ArC=N) 1 570 (ArC=C), and 1 525 cm^{-1} (NO_2). δ_H[250 MHz; $(CD_3)_2SO$] 9.80 (1 H, s, CONH), 4.72 (1 H, t, *J* 6.8 Hz, CH_2OH), 4.06—3.88 (4 H, m, OCH_2CH_2O), 3.45 (2 H, d, *J* 6.8 Hz, CH_2OH), 1.24 (1 H, br, NH), 1.15 (2 H, s, NH_2), and 1.02—0.85 (4 H, m, cyclopropane H); $\lambda_{max.}$(2M HCl) 221, 328 nm; (2M NaOH) 232, 347 nm (Found: C, 42.3; H, 4.9; N, 22.2. $C_{11}H_{15}N_5O_6$ requires C, 42.2; H, 4.8; N, 22.4%); t.l.c. (type 3, solvent B), R_f 0.62; h.p.l.c., R_t 8.1 min. If required, recrystallisation can be achieved from water.

2-*Amino*-6-[1-(2-*hydroxy*-1-*oxoethyl*)*cyclopropylamino*]-5-*nitropyrimidin*-4(3H)-*one* (**16**).—A suspension of the nitro ketal (**15**) (300 mg, 1 mmol) in water (25 ml) was stirred and brought to the boil. 2M Hydrochloric acid (3.50 ml, 7 mmol) was added and the green solution was filtered, cooled to room temperature, and taken to pH 7 with aqueous ammonia (*d* 0.88). The white precipitate was filtered off, washed with water, ethanol, and ether, and then dried *in vacuo* over phosphorus pentaoxide. The *pyrimidine* was obtained as a slightly off-white powder (180 mg, 68%), m.p. 220 °C (decomp.); $\nu_{max.}$(KCl) 3 460 (OH), 3 280 and 3 215 (NH, NH_2), 1 715 ($COCH_2OH$), 1 670 (NHCO), 1 630 (ArC=N), and 1 560 cm^{-1} (ArC=C); δ_H[250 MHz; $(CD_3)_2SO$] 9.69 (1 H, s, NHCO), 4.78 (1 H, t, *J* 5.6 Hz, CH_2OH), 4.37 (2 H, d, *J* 5.6 Hz, CH_2OH), 1.46 (2 H, m, cyclopropane H), 1.23 (3 H, m, cyclopropane 2-H + NH), and 1.13 (2 H, s, NH_2); $\lambda_{max.}$(2M HCl) 215, 240 infl, and 330 nm; (2M NaOH) 230 and 346 nm; t.l.c. (type 3, solvent B) R_f 0.75; h.p.l.c., R_t 35.3 min. Further purification was achieved by recrystallisation from water (Found: C, 39.3; H, 4.1; N, 26.2. $C_9H_{11}N_5O_5$ requires C, 40.2; H, 4.1; N, 26.0%).

2-*Amino*-7,8-*dihydro*-6-*hydroxymethylpteridin*-7-*spiro*-*cyclopropane*-4(3H)-*one* (**1**).—The nitro ketone (**16**) (100 mg, 0.37 mmol) was dissolved in hot DMF (5 ml) (steam bath) and the green solution stirred at 50 °C. 85% Sodium dithionite (0.42 g, 2.10 mmol) was added, followed by the dropwise addition of distilled water (5 ml) (after addition of 2 ml a turquoise colour was visible). After being stirred for a further 10 min the mixture was diluted to 50 ml with water and cooled to 0 °C. The pH was adjusted to pH 4 with glacial acetic acid and then the faint, pale-yellow suspension was filtered off. The solid was washed with water (× 2), each time being recovered by centrifugation, and then dried *in vacuo* over phosphorus pentaoxide. The *pteridine* (**1**) was obtained as a pale yellow powder (5 mg, 6%), m.p. 295 °C (decomp.); $\nu_{max.}$(KCl) 3 330 (OH), 3 255 and 3 130 (NH, NH_2), 1 655 (CONH), 1 634 (ArC=N), and 1 595 and 1 560 cm^{-1}; $\lambda_{max.}$(1M NaOH) 248, 275 infl and 347 nm; (2M HCl) 220, 246, 265, 275 infl, and 374 nm; δ_H[250 MHz; $(CD_3)_2SO$] 9.88 (1 H, s, NHCO), 6.79 (1 H, s, NH), 4.67 (1 H, t, *J* 5.3 Hz, CH_2OH), 3.78 (2 H, d, *J* 5.3 Hz, CH_2OH), 1.22 (2 H, s, NH_2), 1.08 (2 H, m, cyclopropane H), and 0.72 (2 H, m, cyclopropane H) [Found: *m*/*z* 221.0918 (M^+). $C_9H_{11}N_5O_5$ requires *M*, 221.0913] (Found: C, 46.7; H, 4.8; N, 30.1. $C_9H_{11}N_5O_2 \cdot 0.5H_2O$ requires C, 46.9; H, 5.3; N, 30.4%).

1-(4-*Chlorophenyl*)-1-*cyclopropylcarbonylacetonitrile* (**20**).—Sodium (0.99 g, 43.2 mmol) was dissolved in anhydrous ethanol (22 ml) and a mixture of 4-chlorophenylacetonitrile (6.55 g, 43.2 mmol), ethyl cyclopropanecarboxylate (4.93 g, 43.2 mmol), and anhydrous ethanol (7 ml) was added.[12] The solution was refluxed gently, with stirring, for 5 h after which the solvent was removed *in vacuo*. The residue was dissolved in water (150 ml), extracted with ether, and then the aqueous solution acidified with 1M sulphuric acid. The separated oil was extracted with ether and the ethereal solution washed with water, dried (Na_2SO_4), and concentrated. The resulting yellow semi-solid (3.67 g) was recrystallised from ether–light petroleum (b.p. 40—60 °C) to give the *cyano ketone* (**20**) as a white waxy solid (1.97 g, 21%), m.p. 45—46 °C; $\nu_{max.}$(Nujol) 2 242 and 2 200 (CN) and 1 700 cm^{-1} (C=O); δ_H(90 MHz; $CDCl_3$), 7.38 (4 H, s, ArH), 4.87 (1 H, s, PhCH), 2.25—1.95 (1 H, m, cyclopropane 1-H) and 1.30—0.88 (4 H, m, cyclopropane H) (Found: C, 65.0; H, 4.5; N, 6.3; Cl, 16.5. $C_{12}H_{10}ClNO$ requires C, 65.6; H, 4.6; N, 6.4; Cl, 16.1%); T.l.c. (type 1, solvent A), R_f 0.67.

The initial ethereal extractions were washed with water, dried (Na_2SO_4), and concentrated. The resulting red oil (2.9 g, 44%) was identified as 4-chlorophenylacetonitrile by i.r. spectroscopy.

2,4-*Diamino*-5-(4-*chlorophenyl*)-6-*cyclopropylpyrimidine* (**2**).—A dry, alcohol-free, ethereal solution of diazomethane (50 ml) was prepared from Diazald (3.16 g, 14.8 mmol) by the method previously described for the preparation of (**8**) and kept in an ice-salt bath. 1-4-Chlorophenyl-1-cyclopropylcarbonylacetonitrile (**20**) (1.62 g, 7.4 mmol) in anhydrous ether (25 ml) was added dropwise, with stirring, and the mixture stirred for 3 days at room temperature. The mixture was evaporated to dryness under reduced pressure with an aqueous acetic acid trap to destroy excess of diazomethane. The enol ether (**21**) was obtained as a brown oil (1.66 g, 95%), $\nu_{max.}$(liquid film) 2 200 cm^{-1} (conjugated CN). This was used without further purification in the following reaction.

Guanidine hydrochloride (0.67 g, 7 mmol) was added to a solution of sodium (0.16 g, 7 mmol) and anhydrous ethanol (10 ml) and the mixture was refluxed on a steam-bath for 2 min; it was then filtered. To the filtrate was immediately added a solution of the enol ether (**21**) (1.66 g 7 mmol) in anhydrous ethanol (5 ml) and the solution refluxed on a steam-bath for 6 h. The solvent was removed and concentrated aqueous sodium hydroxide solution added to the residue. The insoluble material was filtered off, washed with water, dissolved in glacial acetic acid, and the solution filtered. The filtrate was basified with aqueous sodium hydroxide and the precipitate filtered off, washed with water, ethanol, and then ether, to give the *pyrimidine* (**2**) as a white powder (0.66 g, 35%). Recrystallisation from ethanol gave an analytically pure sample (0.37 g, 19%), m.p. 213—215 °C; $\nu_{max.}$(KCl) 3 470 and 3 300 (NH_2), 1 615 (Ar C=N), and 1 550 cm^{-1} (ArC=C); δ_H(90 MHz; $CDCl_3$), 7.26 (2 H, m, ArH), 7.41 (2 H, m, ArH), 4.62 (2 H, s, NH_2), 4.42 (2 H, s, NH_2), 1.51 (1 H, m, cyclopropane 1-H), 1.04 (2 H, m,

cyclopropane H), and 0.74 (2 H, m, cyclopropane H) (Found: C, 60.0; H, 5.05; Cl, 13.9; N, 21.6. $C_{13}H_{13}ClN_4$ requires C, 59.9; H, 5.0; Cl, 13.6; N, 21.5%).

Enzyme Inhibition Studies.—Dihydrofolate reductase (DHFR: *E. coli* RT/39 Form II) was assayed following Wilmanns[34] using the decrease of absorbance of NADPH at 340 nm with time as a measure of the rate of reaction.

Inhibitor constants. K_i Values were determined using the Dixon plot[35] with concentrations of inhibitor in the range 10^{-5}—10^{-4}M and substrate concentrations of 5×10^{-5}, 2.5×10^{-5}, and 10^{-4}M.

Time dependent assays. These were carried out using the following reaction mixtures from which samples were removed at appropriate times:

Reaction buffer:	0.1M pH 5.0 succinic acid/Tris.
Pteridine (**1**):	2.84×10^{-4}, 3.55×10^{-4}, 4.26×10^{-4}M
DHFR:	5.75×10^{-7} g ml^{-1}
NADPH:	6.58×10^{-5}M

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

Irreversibility tests. These were carried out using the following reaction mixtures. DHFR: 0.25 ml of a solution of 1.5 mg enzyme in 5.0 ml distilled water; pteridine (**1**): 2 ml of 7.0 mg dissolved in 5 ml DMSO up to 10 ml with pH 5.0 0.1M succinate/Tris buffer.

The control experiment contained 2 ml of DMSO in place of inhibitor solution. Two successive dialyses against succinate/Tris buffer at room temperature were carried out.

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References

1 Part 4, I. G. Buntain, C. J. Suckling, and H. C. S. Wood, *J. Chem. Soc., Perkin Trans. 1*, 1988, 3175.
2 Part 9, S. S. Al-Hassan, R. Cameron, S. H. Nicholson, D. H. Robinson, C. J. Suckling, and H. C. S. Wood, *J. Chem. Soc., Perkin Trans. 1*, 1985, 2145.
3 'Inhibition of Folate Metabolism in Chemotherapy,' ed. G. H. Hitchings, Springer Verlag, Berlin, 1953.
4 G. C. K. Roberts in 'Chemistry and Biology of Pteridines,' ed. J. A. Blair, de Gruyter, Berlin, 1983, p. 197.
5 D. A. Matthews, R. A. Alden, S. T. Freer, N. Xuong, and J. Kraut, *J. Biol. Chem.*, 1979 **254**, 4144; J. T. Bolin, D. J. Filman, D. A. Matthews, R. C. Hamlin, and J. Kraut, *ibid.*, 1982, **257**, 13650; D. J. Filman, J. T. Bolin, D. A. Matthews, and J. Kraut, *ibid.*, 1982, **257**, 13663.
6 S. S. Al Hassan, R. Cameron, A. W. C. Curran, W. J. S. Lyall, S. H. Nicholson, D. H. Robinson, A. Stuart, C. J. Suckling, I. Stirling, and H. C. S. Wood, *J. Chem. Soc., Perkin Trans. 1*, 1985, 1645; R. Cameron, S. H. Nicholson, D. H. Robinson, C. J. Suckling, and H. C. S. Wood, *ibid.*, 1985, 2133.
7 I. MacInnes, D. C. Nonhebel, S. T. Orszulik, C. J. Suckling, and R. Wrigglesworth, *J. Chem. Soc., Perkin Trans. 1*, 1983, 2771.
8 S. Ner, C. J. Suckling, A. Bell, and R. Wrigglesworth, *J. Chem. Soc., Chem. Commun.*, 1987, 480.
9 J. E. Villafranca, E. E. Howell, D. H. Voet, M. S. Strobel, R. C. Ogden, J. N. Abelson, and J. Kraut, *Science*, 1983, **222**, 782; E. E. Howell, J. E. Villafranca, M. S. Warren, S. J. Oatley, and J. Kraut, *ibid.*, 1986, **231**, 1123.
10 U. Schöllkopf, R. Harms, and D. Hoppe, *Justus Liebigs Ann Chem.*, 1973, 611; U. Schöllkopf, D. Hoppe, and R. Jentsch, *Chem. Ber.*, 1975, **108**, 1580.
11 J. R. Sweeney, Ph.D. Thesis, University of Strathclyde, 1975.
12 P. B. Russell and G. H. Hitchings, *J. Am. Chem. Soc.*, 1951, **73**, 3763.
13 R. Eisenthal and A. Cornish-Bowden, *Biochem. J.*, 1974, **139**, 715.
14 J. Haddow, C. J. Suckling, and H. C. S. Wood, *J. Chem. Soc., Chem. Commun.*, 1987, 478; U.K. Pat. Appl. 8619103/1986.
15 P. Bladon and R. Breckenridge, INTERCHEM, Interprobe Chemical Services Ltd., University of Strathclyde.
16 S. R. Stone and J. F. Morrison, *Biochemistry*, 1984, **23**, 2753.
17 V. Cody, *J. Mol. Graphics*, 1986, **4**, 69; C. D. Selassie, S.-X. Fang, R. L. Li, C. Hansch, T. Klein, R. Langridge, and B. T. Kaufman, *J. Med. Chem.*, 1986, **29**, 621.
18 R. J. Breckenridge and C. J. Suckling, *Tetrahedron*, 1986, **42**, 5665.
19 P. A. Charlton, D. W. Young, B. Birdsall, J. Feeney, and G. C. K. Roberts, *J. Chem. Soc., Chem. Comm.*, 1979, 922; J. C. Fontecilla-Camps, C. E. Bugg, C. Temple, J. D. Rose, J. A. Montgomery, and R. L. Kisluik, *J. Am. Chem. Soc.*, 1979, **101**, 6114.
20 G. Stork, A. Y. W. Leong, and A. M. Touzin, *J. Org. Chem.*, 1976, **41**, 3491; L. Velluz, G. Amiard, and R. Heymes, *Bull. Soc. Chim. Fr.*, 1954, 1012.
21 O. Gerngross and A. Olcay, *Chem. Ber.*, 1963, **96**, 2550.
22 P. Bey and J. P. Vevert, *Tetrahedron Lett.*, 1977, 1455.
23 E. Testa, L. Fontanella, G. F. Cristiani, and L. Mariani, *Helv. Chim. Acta*, 1959, **42**, 2370.
24 A. Stuart and H. C. S. Wood, B. P. 1 303 171, 1 303 173, 1973.
25 Th. J. de Boer and H. J. Backer in *Org. Synth.*, Coll. Vol. IV, 1963, p. 250.
26 M. S. Newman and P. Beal, *J. Am. Chem. Soc.*, 1949, **71**, 1506.
27 F. Kipnis, H. Soloway, and J. Ornfelt, *J. Am. Chem. Soc.*, 1948, **70**, 142.
28 J. S. Pizey, 'Synthetic Reagents,' Ellis Horwood, Chichester, 1974, vol. 2, pp. 65—142.
29 M. L. Wolfram and R. B. Bennett, *J. Org. Chem.*, 1965, **30**, 458.
30 E. J. Salmi, *Chem. Ber.*, 1938, **71**, 1803; S. W. Smith and M. S. Newman, *J. Am. Chem. Soc.*, 1968, **90**, 1249.
31 S. Wolfe and S. K. Hasan, *Can. J. Chem.*, 1970, **48**, 3572.
32 F. F. Blicke and M. U. Tsao, *J. Am. Chem. Soc.*, 1946, **68**, 905.
33 W. Pfleiderer and H. Walter, *Justus Liebigs Ann. Chem.*, 1964, **677**, 113.
34 W. Wilmanns in 'Methods of Enzymatic Analysis,' ed. H. U. Bergmeyer, Verlag Chemie-Academic Press, New York, 1974, 2nd edn., vol. 2, p. 666.
35 M. Dixon, *Biochem. J.*, 1953, **55**, 170.

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