Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 38 (2003) 719–728
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Original article

Synthesis of quaternised 2-aminopyrimido[4,5-*d*]pyrimidin-4(3*H*)-ones and their biological activity with dihydrofolate reductase

Markus G. Gebauer [1], Carolyn McKinlay [2], Jill E. Gready *

Computational Proteomics and Therapy Design Group, John Curtin School of Medical Research, Australian National University, PO Box 334, Canberra ACT 2601, Australia

Received 13 February 2003; received in revised form 27 May 2003; accepted 28 May 2003

Abstract

In a program to design and develop mechanism-based compounds active as substrates and inhibitors of dihydrofolate reductase (DHFR), we report the synthesis and physical properties of the 6-methyl- (**7**), 8-methyl- (**8a**), and 8-ethyl- (**8b**) derivatives of the parent 2-aminopyrimido[4,5-*d*]pyrimidin-4-(3*H*)-one (**6**). These compounds are the first members of a class of heterocycles related to 8-alkylpterins (N8-alkyl-2-aminopteridin-4(8*H*)-ones) (**2a**–**2c**), which have been shown to be novel substrates for DHFR. Three methods were developed for the synthesis of target compounds **7**, **8a** and **8b**; however, the optimum yields (1–8%) could not be improved because the products decomposed by ring opening (e.g. to 2,4-diamino-5-methyliminomethylpyrimidin-6(1*H*)-one (**9**)) under the reaction conditions. The marked π-electron deficiency of compounds **7**, **8a** and **8b** is the likely cause for the susceptibility of the quaternised pyrimidine ring in the related cations **10**, **15a** and **15b**, respectively, to add nucleophiles, thus promoting the opening of the pyrimidopyrimidine ring system. ^{1}H-NMR spectroscopic studies of compounds **7**, **8a** and **8b** revealed a fast and reversible covalent hydration of the associated cations across the C7–N8 bond for the N6-methyl derivative **7** and across the N6–C7 bond for the N8-methyl derivative **8a**. UV spectroscopic studies of methyl derivatives **7** and **8a** as well as the parent heterocycle **6** showed that protonation of the latter occurred at N1, while methylation with iodomethane proceeded at N6 and N8. The basicities of the *N*-methyl derivatives **7** and **8a** (pK_a ca. 5.5) are similar to those of 8-alkylpterins **2**; this is an essential element of the design to promote binding to DHFR in their protonated form. Enzyme kinetics of **7**, **8a** and **8b** with chicken DHFR confirmed our predictions that they are substrates, with apparent K_m values of 3.8, 0.08, and 0.65 mM, and apparent V_{max} values of 0.47, 2.27, and 0.30 nmol L^{-1} min^{-1} (for enzyme concentration 0.122 μM), respectively. The parent compound **6** was not a substrate.
© 2003 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Antifolate; Dihydrofolate reductase; Pterin; Alkyl-pyrimido[4,5-*d*]pyrimidine; Substrates

1. Introduction

Previously we had shown that whereas pterin **1**, with a basic pK value of 2.31, exhibits negligible enzyme activity towards dihydrofolate reductase (DHFR), its N8-alkyl derivatives **2** (**a**–**c**), which are more basic (pK ca. 5.3–5.6), are enzymically active with K_m values in the micromolar range [1,2]. 8-Methylpterin **2a** binds to chicken liver DHFR with a thermodynamic dissociation constant K_d of 127 μM compared with that of pterin **1** which is negligible [2]. Binding of 8-methylpterin **2a** is assisted by the formation of the N3-protonated cation **3** in the enzyme-bound form [3], which can interact at the active site with a conserved carboxylic acid residue (Glu in mammalian DHFRs) [4]. In contrast, pterin **1** with a considerably lower basic pK [5] would have a negligible concentration of protonated form at the active site under the same pH conditions. The usefulness of *N*-alkylation to increase the basicity of pterin derivatives and to promote binding to DHFR has been further demonstrated in N5-deazapterin **4** in which the N8-alkyl derivatives were found to be inhibitors of this enzyme [6]. N5-Deaza-N8-methylpterin (**5**) is the simplest re-

* Corresponding author.
E-mail address: jill.gready@anu.edu.au (J.E. Gready).
[1] Current address: Pharmacy Department, Repatriation General Hospital, Daws Road, Daw Park, SA 5041, Australia.
[2] Current address: BIOTRON, Innovations Building #124, Australian National University, Canberra, ACT 0200, Australia.

0223-5234/03/$ - see front matter © 2003 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.
doi:10.1016/S0223-5234(03)00140-5

presentative in this series with a K_d of 58 μM whereas the parent N5-deazapterin (**4**) does not bind appreciably [6].

We wish to explore further the usefulness of *N*-alkylation as a design principle with a new series of heterocycles. 2-Aminopyrimido[4,5-*d*]pyrimidin-4-one (**6**) is isomeric with pterin **1**, and we predicted that alkylation at N6 or N8 would yield compounds with similar basicities and enzymic properties to N8-alkylpterins. Here, we report the syntheses, physical and enzymic properties of N6-methyl- (**7**), N8-methyl- (**8a**), and N8-ethyl- (**8b**) 2-aminopyrimido[4,5-*d*]pyrimidin-4-ones, and confirm our prediction (Fig. 1).

2. Chemistry

2.1. Synthetic procedures

We have prepared the 6-methyl, 8-methyl and 8-ethyl derivatives **7**, **8a** and **8b** of 2-aminopyrimido[4,5-*d*]pyrimidin-4(3*H*)-one (**6**) by three different approaches which are illustrated in Scheme 1. For each synthetic method, conditions were optimised by HPLC reaction course studies. Yields of isolated products **7**, **8a** and **8b** were in the range 1–8%.

In the first method (Method A, Scheme 1), we initially prepared the pyrimidopyrimidine **6** in 74% yield according to a modification of the literature procedure [7] and treated **6** with iodomethane in methanol in a sealed tube at 110 °C. Reaction course studies indicated that the amount of 8-methyl isomer **8a** was maximised after 1 h when the mixture cleared, indicating complete consumption of undissolved starting compound **6**. Longer reaction times resulted in the degradation of products **7** and **8a**, e.g. to 2,4-diamino-5-methyliminomethylpyrimidin-6(1*H*)-one (**9**). Purification by preparative HPLC on strong cation exchange and reverse-phase resins yielded the desired methyl derivatives **7** and **8a** in ca. 1 and 5% yield, respectively. Transannular methylation at N6 and N8 of **7** and **8a** was consistent with the high basic pK_a values measured for **7** and **8a** (see below), and was confirmed by nuclear Overhauser effect (NOE) difference spectroscopy of each isomer (see below). Furthermore, the position of 6-methylation in compound **7** was confirmed by synthesis from the 5-methylimino pyrimidinone (**9**). In an attempt to improve on the yields of these reactions, we developed alternative

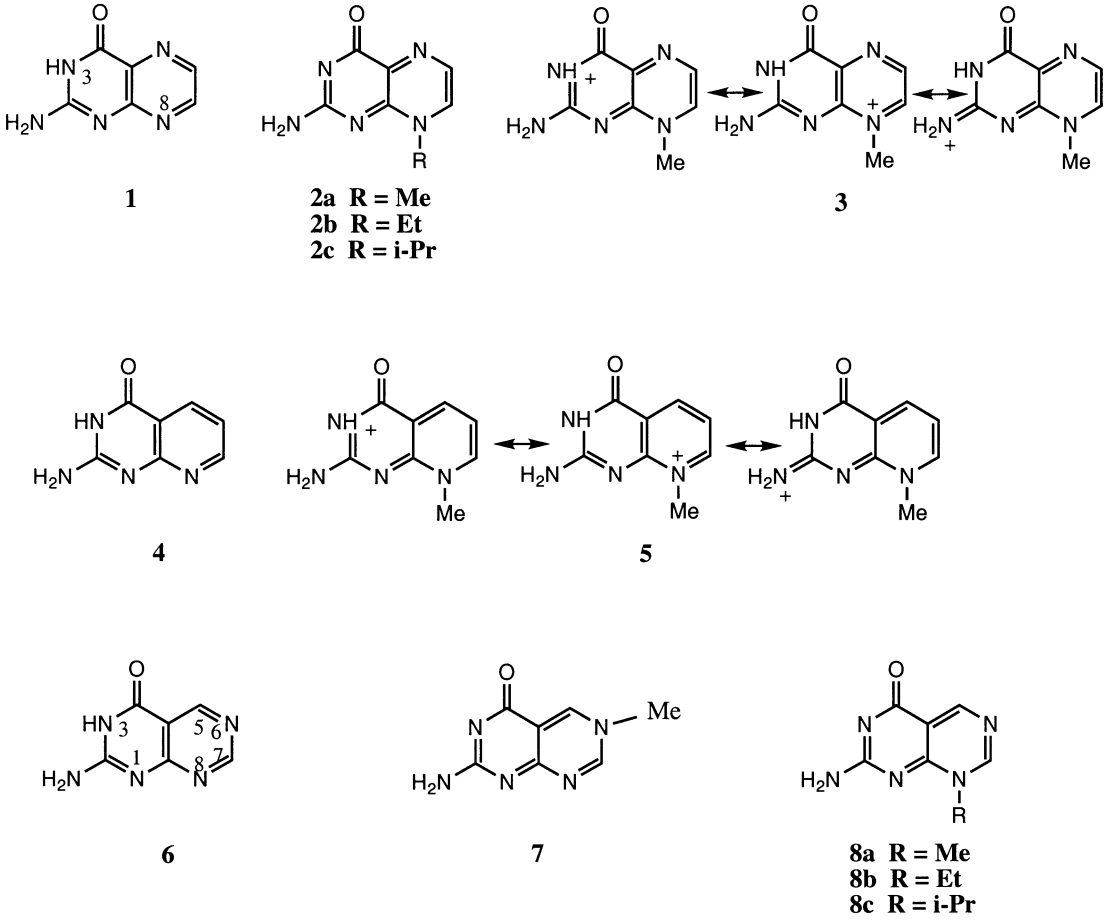
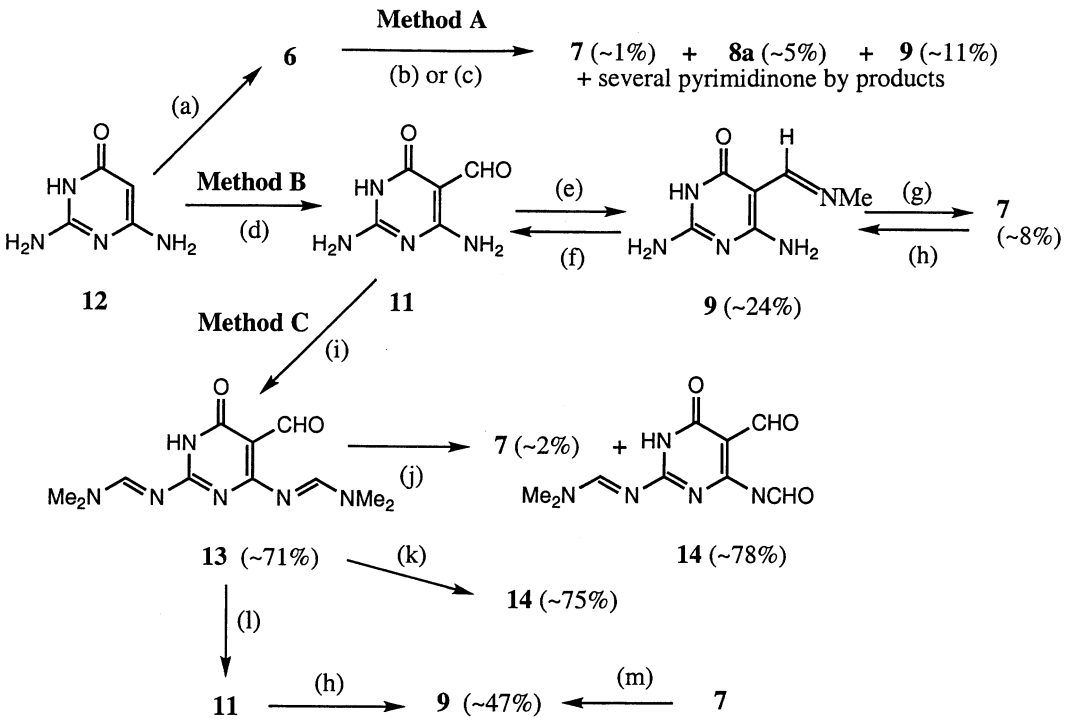


Fig. 1.



Scheme 1. (a) H_2NCHO, (b) MeI–MeOH, (c) MeI–DMSO, (d) $POCl_3$–DMF, (e) $MeNH_2$–MeOH, (f) H_2O-pH > 10, (g) HCOOAc, (h) $AcO^-MeNH_4^+$, DMSO, (i) $MeNHCH(OMe)_2$–DMF, (j) H_2O-pH 5, (k) $MeNH_2$–H_2O-pH 5, (l) $MeNH_2$–DMSO, and (m) $MeNH_2$–DMSO r.t. Yields are given as (%).

conditions by using dimethyl sulphoxide (DMSO) as a solvent and lowering the reaction temperature to 50 °C. Although these conditions appear milder, the yields of products **7** and **8a** were not increased. Before optimisation of the reaction time, the methylation of compound **6** in methanol was conducted for 12 h and pyrimidinimine **9** was isolated in 11% yield as a yellow precipitate. To confirm the structure of compound **9**, it was synthesised in 43% yield by condensation of 2,4-diamino-5-formylpyrimidin-6(1*H*)-one (**11**) with methylamine in ethanol (Scheme 1). The product from the latter synthesis had identical spectral properties to the precipitate formed in the methylation reaction confirming the identity of pyrimidinimine **9**. Compound **9** was converted back into the formylpyrimidine **11** in aqueous solution at pH > 10 or at elevated temperatures at pH < 1. These experiments revealed a low tendency of the imine portion of pyrimidine **9** to hydrolyse, which is consistent with the pronounced hydrolytic stability of a vinylogous amide.

In an attempt to extend the scope of Method A, pyrimidopyrimidine **6** was treated with iodoethane in DMSO to furnish the 8-ethyl derivative **8b** in 2% yield. Further extension of this method to prepare the isopropyl derivative **8c** (as N8-isopropylpterin (**2c**) was a very good substrate for DHFR [2]) by treatment of compound **6** with 2-iodopropane in DMSO at 90 °C resulted in the consumption of compound **6**, but no product was isolated from the very complex reaction mixture.

In a second approach (Method B, Scheme 1), pyrimidinimine **9** was prepared from 2,6-diaminopyrimidin-4(3*H*)-one (**12**) in two synthetic steps in an overall yield of 24%. In an attempt to reverse the ring-opening reaction observed for product **7** during the methylation of compound **6**, we treated compound **9** with acetic formic anhydride in acetic acid at 110 °C. HPLC reaction course studies revealed the regiospecific formation of the 6-methyl derivative **7**, which was isolated in 8% yield.

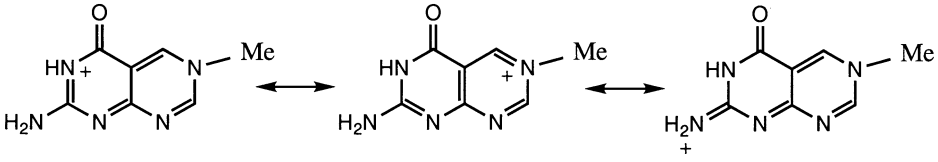
In the third method (Method C, Scheme 1), the key step was the ring closure of an activated precursor 2,4-bis(dimethylaminomethylenamino)-5-formylpyrimidin-6(1*H*)-one (**13**) by treatment with methylamine. Precursor **13** was readily prepared in 71% yield from the 5-formylpyrimidine (**11**) and dimethylformamide dimethyl acetal. In several reactions, precursor **13** was treated with methylamine in aqueous solution buffered at several pH values. For each reaction, a precipitate was observed after 5 min which was identified as 2-dimethylaminomethylenamino-4-formamido-5-formylpyrimidin-6(1*H*)-one (**14**) and accounted for approximately 75% of the starting material. Presumably the formylpyrimidine **14** was formed by a rapid hydrolysis of the more reactive amidate group in position 6 of the starting pyrimidine **13**. We, therefore, treated compound **13** in the absence of methylamine under otherwise identical

reaction conditions (pH 5) and isolated formyl derivative **14** in 78% yield. HPLC analysis of the filtered reaction mixtures containing methylamine indicated various degrees of complexity and a time- and pH-dependent profile. Product **7** was detected as a minor product in all cases and was isolated from the reaction conducted at pH 5 by the usual chromatographic procedure in 2% yield. In an attempt to avoid the formation of pyrimidine **14** by the hydrolysis of starting pyrimidine **13**, the latter was treated with methylamine in anhydrous DMSO. Under these basic conditions, only the deamidated 2,4-diamino-5-formylpyrimidin-6(1*H*)-one (**11**) was detected by HPLC analysis after several minutes. Treatment of precursor **11** with methylammonium acetate in DMSO under non-basic and anhydrous reaction conditions resulted in the rapid formation of pyrimidinimine **9** as the major product. Compound **9** may have been formed via the 6-methyl derivative **7**, which decomposed under the reaction conditions. Indeed, treatment of purified compound **7** with methylammonium acetate under identical conditions resulted in the rapid formation of pyrimidinimine **9** at ambient temperature. Methylation of compound **6** in basic media would have certainly provided the N1 or N3 methylated derivatives of **6**; these would have been much weaker bases (see below) and we did not want them for the enzymic tests (Fig. 2).

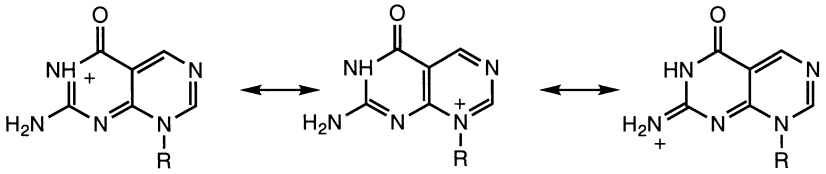
2.2. Physicochemical properties

2.2.1. Ionisation

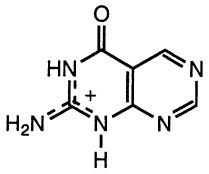
2-Aminopyrimido[4,5-*d*]pyrimidin-4(3*H*)-one (**6**) has a basic pK_a of 2.8 (Table 1) and is slightly more basic than pterin **1**. As for the pterin cation, protonated **6** would be stabilised (in comparison with the neutral molecule) by delocalisation of the positive charge between three vicinal nitrogen atoms in a "guanidinium" resonance as in structure **16**. Similarly protonation of N6 or N8 would produce cations in which the positive charge is delocalised between three nitrogen atoms viz. N6, N3 and $2NH_2$, and N8, N3 and $2NH_2$, respectively. Similarities in the UV spectra (see below) between compounds **6** and **1** and their cations suggest N1 protonation as in **16**, but it is possible that some protonation may occur at N6 and N8 as well. Theoretical calculations may shed light on this issue [8]. Charge delocalisation occurs in the cations **10** and **15a** of the N6- and N8-methylated derivatives **7** and **8a**, and accounts to a large extent for their higher basicities, with pK_a values of 5.5 and 5.4, respectively (Table 1). We had predicted that these values should be similar to those of N8-methylpterin (**2a**). These are, however, equilibrium (microscopic) pK_a values as they include the protonation of anhydrous species as well as hydrated species (see below and Refs. [9–11]). The considerably higher pK_a values of compounds **7** and **8a** (i.e. by ca. 2.6



10



15a R = Me
15b R = Et



16

Fig. 2.

Table 1
Ionisation and ultraviolet spectra of 2-aminopyrimido[4,5-*d*]pyrimidin-4-ones in H_2O at 25 °C

Compound	pK_a [a]	pH	λ_{max}, nm (ε, M^{-1} cm−1) [b]	Species [c]
Parent **6**	2.8 (basic) [d]	1.0	291 (10,000)	+
		4.0	271 (14,420)	O
	7.4 (acidic) [d]	10.0	248 (21,000); 272 (6630); 312 (6100); 340 (2330)	—
N6-Methyl derivative **7**	5.5 (basic) [e,f]	2.0	235 (9050); 303 (10,269)	+ [g]
		7.5	206 (16,820); 264 (12,000); 312 (6500)	O
N8-Methyl derivative **8a**	5.4 (basic) [e,f]	2.0	234 (11,100); 277 (9100); 318 (8980)	+ [g]
		7.5	206 (21,000); 257 (15,600); 335 (6820)	O
N8-Ethyl derivative **8b**	ca. 5.5 (basic) [h]	2.0	236 (7000); 277 (6500); 319 (5470)	+ [g]
		7.5	206 (17,400); 259 (10,120); 337 (4340)	O
Pterin **1** [i]	2.31 (basic)	0.0	242 (8700); 315 (7600)	+
		5.3	270 (11,200); 340 (5800)	O
	7.92 (acidic)	13.0	252 (20,400); 359 (6800)	—

[a] Errors ±0.1.
[b] Errors ±10%.
[c] (+) Cation, (O) neutral species, (—) anion.
[d] Determined by UV spectroscopy.
[e] Determined by ^{1}H-NMR.
[f] Equilibrium pK_a with hydrated species.
[g] Mixture of anhydrous and hydrated cations (see text).
[h] Estimated value.
[i] For comparison from Ref. [12, p. 290].

pK units) exclude any possibility of methylation of compound **6** at N1 or N3, which would have altered the pK_a values by only a small amount comparable with those of 1-methylpterin (pK_a 2.8) and 3-methylpterin (pK_a 2.2) [12].

2.2.2. *H-NMR spectra*

The ^{1}H-NMR spectra of the neutral species in D_2O-pD 7 show the expected signals for the respective compounds **6**, **7**, **8a** and **8b** (Table 2). The cations of the alkylated compounds **7**, **8a** and **8b** in D_2O/DCl-pD 3, on the other hand, revealed two sets of signals. One set for the anhydrous species, e.g. for cation **15a**, and one set for the hydrated species, e.g. for hydrated cation **17** (Fig. 3). Covalent hydration of these *N*-alkyl compounds has occurred across the 7–8 bond. This is evidenced by the upfield shifts of all signals on hydration, but particularly of H7 by almost 3 ppm as a result of disruption of the aromatic resonance of the pyrimidine ring bearing the *N*-alkyl group. This is characteristic of π-deficient nitrogen heterocycles when hydration across a carbon–nitrogen double bond is stabilised by resonance between canonical protonated structures. Charge delocalisation is more readily achieved for C7–N8 hydrated cations than the alternative C5–N6 hydrated cations. Clearly the equilibrium between these species is sufficiently slow on the NMR time scale to show two sets of signals. Measurements of the relative

Table 2
^{1}H-NMR spectra of 2-aminopyrimido[4,5-*d*]pyrimidin-4-ones [δ from Me_4Si (in DMSO-d_6) or from sodium 4-trimethylsilylbutanoate (in D_2O)]

Compound	H7	H5	CH_2	CH_3	Solvent
Parent **6**	8.97	8.99	—	—	DMSO-d_6
N6-Methyl derivative **7**	8.85	8.82	—	4.06	D_2O-pD 7
Anhydrous cation	9.23	9.04	—	4.14	D_2O/DCl-pD 3
Hydrated cation	6.31	8.47	—	3.55	D_2O/DCl-pD 3
N8-Methyl derivative **8a**	8.97	8.97	—	3.93	D_2O-pD 7
Anhydrous cation	9.30	9.37	—	3.87	DMSO-d_6
Anhydrous cation	9.20	9.24	—	4.01	D_2O/DCl-pD 3
Hydrated cation	6.39	8.46	—	3.32	D_2O/DCl-pD 3
N8-Ethyl derivative **8b**	8.83	8.75	4.26 (q, $J = 6$ Hz)	4.06 (t, $J = 6$ Hz)	D_2O-pD 7
Anhydrous cation	9.23	9.24	4.54 (q, $J = 7$ Hz)	1.51 (t, $J = 7$ Hz)	D_2O/DCl-pD 3
Hydrated cation	6.43	8.23	3.83 (q, $J = 7$ Hz)	1.30 (t, $J = 7$ Hz)	D_2O/DCl-pD 3

signal integrals of hydrated and anhydrous cations of the N6-methyl- (**7**), N8-methyl- (**8a**) and N8-ethyl- (**8b**) pyrimidopyrimidines showed ca. 35, 55 and 20% hydration in aqueous solution at pD 3, respectively. The changes in the chemical shifts of the signals of compounds **7** and **8a** with change of pD were used to determine their pK_a values (Table 1). On the other hand, if methylation had occurred at N1 or N3 of compound **6**, covalent hydration of the derived cations is not expected. Moreover, NOE analyses of the N6 and N8 methyl derivatives **7** and **8a** in D_2O/DCl-pD 3 confirmed methylation of compound **6** and also showed magnetisation transfer between the corresponding sets of signals of the hydrated and anhydrous cations. Thus, in the 6-methyl cation **10**, irradiation of the signal for H7 caused the signal size of the methyl group to increase by ca. 2.3% and irradiation of the signal for H5 caused a ca. 1.8% increase in the signal for the methyl. Irradiation of the signal from the methyl group caused ca. 8% enhancements in the signals for H5 and H7. In the 8-methyl compound **15a**, irradiation of the signal for H7 caused a 10% enhancement of the signal for the methyl group and irradiation of the signal for the methyl caused a ca. 8% enhancement for the signal of H7 with little effect on the signal from H5. Such energy transfers are consistent with the structures stated and inconsistent with alkylation on N1 or N3.

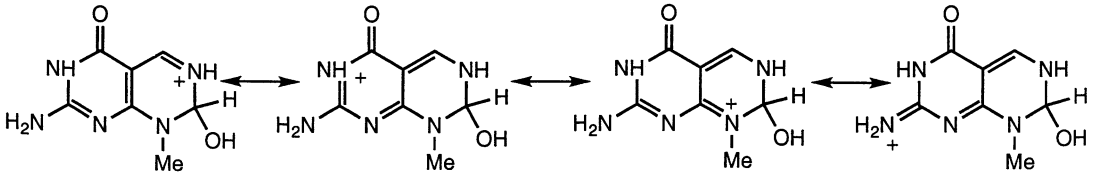
2.2.3. Ultraviolet spectra

The spectra of the pyrimidopyrimidines **6**, **7**, **8a** and **8b** are in Table 1. The spectra of the neutral species and cation of parent compound **6** show the characteristic red shift of the long wavelength band of anhydrous species of π-deficient nitrogen heterocycles [12]. The long wavelength bands in the spectra of the *N*-alkyl derivatives **7**, **8a** and **8b** at pH 2.0 are at shorter wavelength (blue shift by ca. 9–17 nm) than at pH 7.5. This is partly because at pH 2 the solutions consist of hydrated and anhydrous cations. The long wavelength bands of hydrated cations are normally at shorter wavelengths than those of the corresponding neutral species as a result of the disruption of the aromatic system [13].

3. Biological activity

The three *N*-alkyl 2-aminopyrimido[4,5-*d*]pyrimidin-4-ones **7**, **8a** and **8b** are reduced by NADPH in the presence of chicken DHFR and followed Michaelis–Menten kinetics. The kinetic parameters of the enzyme for these three substrates are listed in Table 3. The K_m, V_{max} and V/K are apparent values because they were obtained using one concentration of NADPH (60 μM); we do not know if this is a saturating concentration, and it is assumed that 1 mol of NADPH is consumed per mol of substrate. However, 60 μM is known to be saturating for substrate activity of the 8-substituted pterin series [2]. Also, the pH of the reaction (5.8) is close to the pK_a of the substrates, i.e. the cuvettes contain equal amounts of neutral species and cations, and if the cations are partly hydrated (covalent) then the rates of the equilibria between hydrated and anhydrous cations may contribute to the rates of the enzymic reactions. However, as the rates of these equilibria are in the seconds range (in the NMR time scale, see above), then their contributions to the enzymic rates would be within the errors of the kinetic measurements. The reaction extinction coefficients at 340 nm were determined for each substrate by reducing known concentrations with excess of $NaBH_4$ until reduction was complete and noting the changes in ε values at 340 nm. These were added to the ε value of NADPH (6200 M^{-1} cm^{-1}). These values were used for the enzymic rate measurements and are given in Table 3.

Comparison of the V/K values is the best way of assessing the relative activities of substrates as they combine the two measurable parameters, and because they are the apparent first-order rate constants for the combination of substrate with the enzyme at very low concentrations of the substrate [14,15]. From the V/K values, the N8-methyl derivative **8a** is the better substrate by about two orders and one order of magnitude than compounds **7** and **8b**, respectively, with the former 6-methyl derivative being the poorest substrate. This is possibly because the methyl group at N8 binds to a flexible pocket in DHFR as is the case for N8-alkylpterins **2a**–**2c** [2,16]. Such a contribution to the binding affinity may not be effective when the alkyl



17

Fig. 3.

Table 3
Kinetic parameters of chicken DHFR for 2-amino-pyrimidino[4,5-*d*]pyrimidin-4-ones with NADPH at 60 μM (enzyme concentration at 0.122 μM) at pH 5.8 [a] and 25 °C

Compound	Apparent K_m (mM)	Apparent V_{max} (nmol L^{-1} min^{-1})	Apparent V/K (min^{-1})	Reaction extinction coefficient [b] (M^{-1} cm^{-1})
N6-Methyl derivative **7**	3.76±0.47	0.47±0.04	$0.12\pm0.005\times10^{-6}$	7415
N8-Methyl derivative **8a**	0.082±0.020	2.27±0.4	$28.1\pm3.4\times10^{-6}$	5100
N8-Ethyl derivative **8b**	0.65±0.38	0.30±0.12	$0.46\pm0.11\times10^{-6}$	7852
N8-Methylpterin **2a** (for comparison) [c]	0.025	39.5×10^{3}	1.58	–

[a] Multi-acetate buffer: AcOH (100 mM), Tris (50 mM), ethanolamine (50 mM) and NaCl (200 mM).
[b] At 340 nm.
[c] From Ref. [2]; value for V_{max} converted from turnover of 5.4 s^{-1}.

group is located at N6 (see the high K_m value for compound **7**, Table 3).

When these parameters are compared with those of N8-methylpterin **2a**, it is found that the latter is a very superior substrate in comparison with compounds **7**, **8a** and **8b**. It should be noted that **2a**, in absolute terms, is a very good substrate for DHFR with apparent V_{max} comparable with that for dihydrofolate as substrate [1]. However, if the K_m values are taken as rough estimates for binding, then the value for the N8-methyl derivative **8a** compares favourably with that of N8-methylpterin **2a**. In contrast, the parent substance **6** is not a substrate of chicken DHFR when measured using the same conditions as for its N6- and N8-alkyl derivatives.

4. Conclusions

Enzymic and chemical tests of the new series of N6- and N8-alkylated derivatives of 2-aminopyrimido[4,5-*d*]-pyrimidin-4-one (**6**) have supported our design principle that such compounds should have enhanced basicity allowing them to be substrates of DHFR at neutral pH, whereas the parent compound **6** is not. This result extends our previous work for the isomeric pterin **1** (2-aminopteridin-4(3*H*)-one) nucleus, the heterocyclic fragment of the natural folate substrates, for which N8-alkylated derivatives were also shown to be DHFR substrates at neutral pH whereas the parent pterin is not. Although several strategies were used to synthesise the first members of the class, the target 6-methyl- (**7**), 8-methyl- (**8a**) and 8-ethyl- (**8b**) derivatives of compound **6**, the optimised yields (1–8%) were still low due to product decomposition from ring opening. The marked π-electron deficiency of these compounds is the likely cause for the quaternised pyrimidine ring of their cations to add nucleophiles, and, thus, promote opening of the ring system. The π-electron deficiency underpins other properties of the compounds we have characterised, including covalent hydration of the cations and the UV–Vis spectral properties.

5. Experimental

5.1. Materials and methods

Reagents were purchased from commercial suppliers and used without further purification. All solvents were analytical reagent grade. The following compounds were prepared according to the published procedures: acetic formic anhydride [17], 2,4-diamino-5-formylpyrimidin-6(1*H*)-one (**11**) [18], and 2,6-diamino-pyrimidin-4(3*H*)-one (**12**) [19]. Instruments used in compound identification: UV spectra (Cary 1 Bio UV/VIS spectrophotometer), NMR spectroscopy (Varian 500 MHz); ESMS spectroscopy (Fisons VG quattro II triple quadrupole); EIMS (Fisons VG AutoSpec). Analytical HPLC was performed using an Applied Biosystem Kratos system, which consisted of a UV single-channel detector, a Rheodyne manual injector and a Spectra Physics integrator/recorder. Samples were chromatographed on a 4.6 mm × 250 mm ZORBAX SB-C8 column using an isocratic solvent system consisting of 70% of a 5-mM sodium acetate/acetic acid buffer (pH 5) and 30% of ethanol at a flow rate of 0.8 mL min^{-1}. The eluent was detected at λ 310 nm. Preparative HPLC involved two consecutive steps and was performed using an integrated Beckman system. Samples were chromatographed on a 10 mm × 250 mm ACTIVON Gold column packed with a strong cation exchange resin using an isocratic solvent system consisting of 30% of a 0.1-M sodium formate/formic acid buffer (pH 4) and 70% of ethanol at a flow rate of 8 mL min^{-1}. The eluent was detected at λ 310 nm. Fractions were analysed by UV spectroscopy and analytical HPLC and those containing product were pooled and lyophilised. Residues were resuspended in water and the pH values of samples were adjusted to 7. Samples were chromatographed on a 22 mm × 250 mm ZORBAX SB-C8 column using an isocratic solvent system consisting of 5% ethanol in water at a flow rate of 8 mL min^{-1}. The eluent was detected at λ 310 nm. After each run, the column was washed with a 1:1 mixture of HCl (pH 2) and ethanol for 5 min and then

reconditioned with the running solvent for 10 min. Fractions containing products were analysed by UV spectroscopy and analytical HPLC, pooled and lyophilised.

The samples used for spectroscopic work and enzyme kinetics contained variable amounts of water and in some cases traces of buffer, and did not give consistent elemental microanalytical results for anhydrous compounds. Compounds **7**, **8a** and **8b** were, however, uncontaminated with other organic materials as shown by single peaks in HPLC analyses using more than one column, gave only the desired peaks in the NMR spectra, gave correct accurate molecular weights on mass spectrometric analyses, and gave UV spectra in various buffers with ε values consistent with these structures. If there were any impurities in these three samples, which were not revealed in the above analyses, they could not have amounted to more than 5% and should not invalidate any conclusions made in this work.

5.2. Synthesis

5.2.1. 6-Methyl-2-aminopyrimido[4,5-d]pyrimidin-4(6H)-one (7)

Method A. A mixture of 2-aminopyrimido[4,5-*d*]pyrimidin-4(3*H*)-one (**6**) (394 mg, 2.42 mmol), iodomethane (7.83 g, 43.3 mmol), and methanol (14 mL) was stirred in a sealed glass tube at 110 °C for 1 h. The tube was allowed to reach ambient temperature and the solvent was removed by rotary evaporation. The residue was purified by HPLC. Compound **7** was isolated as a white solid (3 mg, 1%).

Method B. A mixture of 2,6-diamino-5-methyliminomethylpyrimidin-4(3*H*)-one (**9**) (300 mg, 1.80 mmol), acetic formic anhydride (2.27 g, 25.8 mmol), and glacial acetic acid (7 mL) was stirred in a sealed glass tube at 110 °C for 3 h. The tube was allowed to reach ambient temperature and the contents lyophilised. The residue was purified by HPLC. Compound **7** was obtained as a light yellow solid (25 mg, 8%).

Method C. A 33% (w/v) solution of methylamine in ethanol (1.43 g, 15.2 mmol) was added to water (20 mL) and the pH adjusted to 5 by the addition of acetic acid. 2,4-Bis(dimethylaminomethylenamino)-5-formylpyrimidin-6(1*H*)-one (**13**) (792 mg, 3.0 mmol) was added and the resultant mixture stirred. After 1 h, the mixture was filtered and the filtrate lyophilised. The residue was purified by HPLC to yield **7** as a white solid (10 mg, 2%). HR-EIMS *m/z*: 177.0650 [M^+] (Calc. for $C_7H_7N_5O$: 177.0651). ESMS *m/z*: 196 [$(M+H_3O)^+$], 178 [$(M+H)^+$], 177 [M^+].

5.2.2. 8-Methyl-2-aminopyrimido[4,5-d]pyrimidin-4(8H)-one (8a)

We use the same procedure as for compound **7** (Method A). Compound **8a** was also isolated by the HPLC procedure as a white solid (22 mg, 5%). HR-EIMS *m/z*: 177.0650 [M^+] (Calc. for $C_7H_7N_5O$: 177.0651).

5.2.3. 8-Ethyl-2-aminopyrimido[4,5-d]pyrimidin-4(8H)-one (8b)

A mixture of 2-aminopyrimido[4,5-*d*]pyrimidin-4(3*H*)-one (**6**) (351 mg, 2.15 mmol), iodoethane (8.12 g, 52.1 mmol), and DMSO (30 mL) was stirred in a sealed glass tube at 70 °C for 2 h. The tube was allowed to reach ambient temperature and its contents were purified by weak cation exchange chromatography on a CM-Sephadex resin followed by preparative HPLC. Compound **8b** was obtained as a white solid (7 mg, 2%). HR-EIMS *m/z*: 191.0807 [M^+] (Calc. for $C_8H_9N_5O$: 191.0808).

5.2.4. 2-Aminopyrimido[4,5-d]pyrimidin-4(3H)-one (6)

This is a modification of a literature method [7] on a 200-fold increased scale, and was most satisfactory. A mixture of 2,6-diaminopyrimidin-4(3*H*)-one (**12**) (25.0 g, 0.198 mol) in formamide (250 mL) was mechanically stirred under argon at 150 °C for 16 h. The resultant thick paste was cooled to ambient temperature. Water (700 mL) was added and the mixture stirred and refluxed for 10 min. The hot mixture was filtered and the solid was thoroughly washed with boiling water (250 mL). It was suspended in boiling water (250 mL) and a solution of 20% aqueous KOH added until the solid dissolved. Charcoal (5.0 g) was added to the mixture and it was filtered through a pad of celite and the pad washed with boiling water (100 mL). The combined filtrates were heated to boiling and added rapidly to a boiling mixture of acetic acid (60 mL) and water (250 mL) with stirring. The hot mixture was filtered and the resulting off-white solid dried at 100 °C for 2 days. It was further dried over phosphorus pentoxide under vacuum to yield compound **6** as an off-white solid (24.0 g, 74%, lit yield was 64%), m.p. >360 °C (decomp.) ($CHN\cdot 0.58H_2O$). HR-EIMS *m/z*: 163.0495 [M^+] (Calc. for $C_6H_5N_5O$: 163.0494).

5.2.5. 2,6-Diamino-5-methyliminomethylpyrimidin-4(3H)-one (9) sulphate

5.2.5.1. From 2,4-diamino-5-formylpyrimidin-6(1H)-one (11).

A mixture of compound **11** (5.00 g, 32.4 mmol) and a 33% (w/v) solution of methylamine in ethanol (15.3 g, 0.162 mol) was heated in a Parr bomb at 110 °C for 7 h. After the bomb reached ambient temperature, the contents were filtered and the solid suspended in a

mixture of sulphuric acid (8.0 g, 82 mmol) and water (500 mL). The acidic mixture was boiled, decolourised with charcoal and filtered through a pad of celite. After standing for 1 day, the precipitate was filtered off, washed with water and dried at 130 °C under vacuum. Product **9** was obtained as a light yellow solid (2.32 g, 43%), m.p. >360 °C (decomp.) (CHNS for dihydrate hemisulphate). ^{1}H-NMR (D_2O, acetic acid-d_4) δ: 8.48 (s, 1H, CH), 3.36 (s, 3H, CH_3) ppm. UV λ_{max} (H_2O) nm: 231, 309. HR-EIMS *m/z*: 167.0807 [M^+] (Calc. for $C_6H_9N_5O$: 167.0806).

5.2.5.2. From 6-methyl-2-aminopyrimido[4,5-d]-pyrimidin-4(6H)-one (7). A mixture of compound **7** (6 mg, 34 μmol) and methylammonium acetate (9 mg, 102 μmol) was stirred in DMSO (0.4 mL) at ambient temperature under argon. After 30 min, the reaction mixture was filtered and analysed by HPLC. A single peak was observed that had a UV spectrum consistent with compound **9**.

*5.2.5.3. From 2,4-bis(dimethylaminomethylenamino)-5-formylpyrimidin-6(1H)one (**13**).* A mixture of compound **13** (661 mg, 2.5 mmol) and methylammonium acetate (455 mg, 5.0 mmol) was stirred in DMSO (20 mL) at ambient temperature under argon. After 1 h, the reaction mixture was analysed by HPLC and a single peak was observed that had a UV spectrum consistent with compound **9**.

*5.2.6. 2,4-Bis(dimethylaminomethylenamino)-5-formylpyrimidin-6(1H)-one (**13**)*

A mixture of 2,4-diamino-5-pyrimidin-6(1*H*)-one (**12**) (20.2 g, 0.132 mol), *N*,*N*-dimethylformamide dimethyl acetal (47.0 g, 0.394 mol), and dimethylformamide (50 mL) was stirred at 50 °C under argon for 14 h. The mixture was filtered and the solid washed with dimethylformamide (2 × 10 mL) and ether (2 × 30 mL). After drying at ambient temperature under vacuum for 5 h, a yellow solid was obtained (24.6 g, 71%), m.p. 202–208 °C (decomp.) (CHN). ^{1}H-NMR (DMSO-d_6) δ: 11.05 (bs, 1H, NH), 10.30 (s, 1H, CHO), 8.86 (s, 1H, CHN), 8.77 (s, 1H, CHN), 3.22 (s, 3H, CH_3), 3.20 (s, 3H, CH_3), 3.07 (s, 3H, CH_3), 3.06 (s, 3H, CH_3) ppm. UV λ_{max} (DMSO) nm: 321. HR-EIMS *m/z*: 264.1337 [M^+] (Calc. for $C_{11}H_{16}N_6O_2$: 264.1335).

*5.2.7. 2-Dimethylaminomethylenamino-4-formamido-5-formylpyrimidin-6(1H)-one (**14**)*

2,4-Bis-(dimethylaminomethylenamino)-5-formylpyrimidin-6(1*H*)-one (**13**) (1.00 g, 3.78 mmol) was added to a 0.5-M solution of acetic acid–sodium acetate (pH 5, 5 mL). The mixture was stirred for 15 min, filtered and the solid was washed with water (10 mL). After drying at ambient temperature under vacuum for 24 h, a white solid was obtained (0.70 g, 78%). ^{1}H-NMR (acetic acid-d_4, DMSO-d_6) δ: 9.90 (s, 1H, CHO), 9.66 (s, 1H, CHO), 8.93 (s, 1H, CHN), 3.28 (s, 3H, CH_3), 3.16 (s, 3H, CH_3) ppm. UV λ_{max} (HCl, pH 2) nm: 228, 316. EIMS *m/z*: 237 [M^+], 209 [$(M-CO)^+$], 181 [$(M-2CO)^+$]. HR-EIMS *m/z*: 237.0861 [M^+] (Calc. for $C_9H_{11}N_5O_3$: 237.0862).

5.3. Enzyme kinetics

Chicken liver DHFR was purchased from Sigma and NADPH was from Boehringer. A double beam Cary 100 spectrophotometer set at 340 nm and cuvettes (1 cm) thermostated at 25 °C were used to measure the kinetics. The changes in optical density with time were recorded on a computer. The concentration of chicken DHFR was determined by titration with methotrexate [20] and diluted as required. Stock substrate was made in 0.1 M chloroacetic acid in water (pH 2). Stock 0.1 M multi-acetate buffer consisted of acetic acid (100 mM), Tris (50 mM), ethanolamine (50 mM), and NaCl (200 mM) in water. The blank cuvette contained stock buffer (500 μL), substrate aliquot (100–200 μL), NADPH (6 mM, 10 μL), made up to 1 mL with water. The reaction cuvette contained stock buffer (500 μL), substrate aliquot as in blank (100–200 μL), DHFR (15 μL to a final concentration of 0.122 μM) and water (less volume of NADPH to make to 1 mL). The reaction was started by addition of NADPH (6 mM, 10 μL). The reaction was recorded over 5 min and calculated from 1 to 5 min. The absorbance change per min was converted to nmol L^{-1} min^{-1} for various substrate concentrations. The K_m, V_{max} and V/K were computed using Cleland's program [21] and the results are in Table 3. The low solubility of substrate **8b** meant that rates at higher concentrations could not be determined and the parameters were calculated from concentrations below the K_m; hence, the relatively larger error.

Acknowledgements

We are indebted to Dr W.L.F. Armarego for advice and for assistance with the manuscript, to Dr. T.J. Delia for making the spectral data of compound **6** available, and to Dr. D.J. Brown and Prof. W. Pfleiderer for most helpful discussions. We thank Dr. M. Casarotto for assistance with NMR spectroscopy, and the Research School of Chemistry for the mass spectroscopy service.

References

[1] V. Thibault, M.J. Koen, J.E. Gready, Biochemistry 28 (1989) 6024–6049.
[2] M.T.J. Ivery, J.E. Gready, Biochemistry 34 (1995) 3724–3733.
[3] S.-S. Jeong, J.E. Gready, Eur. J. Biochem. 221 (1994) 1055–1062.

[4] J. Kraut, D.A. Matthews, in: F.A. Jurnak, A. McPherson (Eds.), Biological Macromolecules and Assemblies, Vol. 3, Wiley, New York, 1987, pp. 3–71.
[5] W. Pfleiderer, J.W. Bunting, D.D. Perrin, G. Nubel, Chem. Ber. 101 (1968) 1072–1088.
[6] M.T.G. Ivery, J.E. Gready, J. Med. Chem. 37 (1994) 4211–4221.
[7] T.J. Delia, J. Niedzinski, J. Heterocyc. Chem. 24 (1987) 1421–1423.
[8] P. Wormell, J.E. Gready, Chem. Phys. 179 (1994) 55–69.
[9] A. Albert, Ionization constants, in: A.R. Katritzky (Ed.), Physical Methods in Heterocyclic Chemistry, vol. III, Academic Press, New York, 1971, pp. 1–26.
[10] A. Albert, W.L.F. Armarego, Adv. Heterocyc. Chem. 4 (1965) 1–42.
[11] D.D. Perrin, Adv. Heterocyc. Chem. 4 (1965) 42–73.
[12] D.J. Brown, Fused Pyrimidines—Pteridines, Wiley/Interscience, New York, 1988, pp. 453, 455.
[13] W.L.F. Armarego, Ultraviolet spectra of heterocycles, in: A.R. Katritzky (Ed.), Physical Methods in Heterocyclic Chemistry, vol. III, Academic Press, New York, 1971, pp. 67–222.
[14] M. Dixon, E.C. Webb, Enzymes, Academic Press, New York, 1979, p. 64.
[15] A. Fersht, Enzyme Structure and Mechanism, Freeman, Reading, 1985, p. 84.
[16] A.-D. Gorse, J.E. Gready, Prot. Eng. 10 (1997) 23–30.
[17] M. Fieser, L. Fieser, Reagents Org. Synth. 2 (1969) 10–12.
[18] T.J. Delia, R. Otteman, Heterocycles 20 (1983) 1805–1809.
[19] H. Bredereck, F. Effenberger, R. Sauter, Chem. Ber. 95 (1962) 2049–2056.
[20] J.W. Williams, J.F. Morrison, R.G. Duggleby, Biochemistry 18 (1979) 3449–3457.
[21] W.W. Cleland, Meth. Enzymol. 63 (1979) 103–138.