Structural Studies on Bio-active Compounds. Part 6.[1] Determination of the Sites of Protonation on Three 2,4-Diaminopyrimidines of Pharmaceutical Importance by Proton-coupled ^{13}C and ^{1}H Nuclear Magnetic Resonance Spectroscopy

Michael D. Threadgill,* Roger J. Griffin, Malcolm F. G. Stevens, and Sang K. Wong
Pharmaceutical Sciences Institute, Department of Pharmaceutical Sciences, Aston University, Aston Triangle, Birmingham B4 7ET

The protonation of three 2,4-diaminopyrimidines of interest as antitumour agents has been studied by proton-coupled ^{13}C n.m.r. and by ^{1}H n.m.r. spectroscopy in solution in dimethyl sulphoxide. Assignment of ^{13}C resonances was achieved through chemical-shift considerations and through ^{13}C–^{1}H coupling patterns, supported in one case by a DEPT experiment and by heteronuclear decoupling by selective irradiation in the proton spectrum. In each case, first protonation occurs at pyrimidine N-1 with the second, much weaker, basic site of 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine being at the aniline amino group. The observations are consistent with current hypotheses of structural requirements for inhibitory activity against dihydrofolate reductase.

The 2,4-diaminopyrimidines, and particularly 2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidine (pyrimethamine) (**1**), are important in the control of infectious disease through their potent inhibition of dihydrofolate reductase (DHFR).[2] Inhibition of the corresponding mammalian enzyme with the folate analogue methotrexate has also proved a successful strategy in the chemotherapy of neoplastic disease.[3] Recently, 2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine (MZP) (**2**) has entered clinical trial as an antitumour agent as its monoethanesulphonate salt (MZPES). Protonation at the ring nitrogen N-1 at physiological pH (pH 7.4) has been suggested to be of considerable importance in the inhibitory binding of 2,4-diaminopyrimidines to the active site of DHFR,[4,5] although the site of protonation of the pyrimidines in solution has hitherto been inferred from solid-state crystallographic techniques.[6] It was therefore of interest to determine the sites of protonation of 2,4-diaminopyrimidines in solution through study of the ^{13}C n.m.r. spectra of these compounds in protonated and unprotonated forms.

The pyrimidines studied were two compounds of therapeutic importance (**1**) and (**2**) and 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine (**3**) which is a metabolite, photoproduct, and thermal degradation product[7] of compound (**2**). Chemical-shift data from ^{13}C n.m.r. experiments are potentially more useful than ^{1}H data for this study owing to the lack of protons bonded directly to pyrimidine ring carbons. Thus, ^{1}H n.m.r. spectroscopy can only report protonation of the pyrimidine ring at long range in these compounds. Confident assignment of the ^{13}C resonances is essential; hence proton-coupled ^{13}C spectra were obtained and, in one case, selective heteronuclear decoupling was employed. Dimethyl sulphoxide (DMSO) was used as spectroscopic solvent in most experiments, owing to the very poor solubility of the free bases of compounds (**1**)—(**3**) in water.

The proton-coupled ^{13}C n.m.r. spectral data of (**1**), the simplest of the three pyrimidines studied, is listed in Table 1 (numbering scheme shown in Figure 1). Assignment of the resonances corresponding to C-1″ and C-2″, the carbon atoms of the ethyl group attached to C-6 of the pyrimidine, was carried out in a straightforward manner from chemical-shift considerations with C-1″ resonating at δ 27.32 and C-2″ at δ 13.76. Confirmation was achieved by consideration of carbon–proton coupling. The signal from C-1″ is found to be a triplet of quartets with a one bond C–H coupling constant (^{1}J 126.7 Hz) typical for an sp^3-hybridised carbon atom; the two-bond coupling constant corresponding to coupling of the methyl protons with C-1″ (^{2}J 4.5 Hz) is also consistent with reported values for related systems.[8] The ^{1}J and ^{2}J values for the quartet of triplets from the methyl carbon (C-2″) are very similar to those of C-1″.

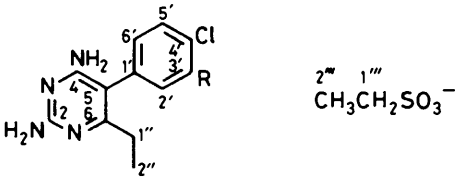

Figure 1. Numbering scheme for carbon atoms in pyrimidines (**1**; R = H), (**2**; R = N_3), and (**3**; R = NH_2) and in ethanesulphonate anion

Assignment of the ^{13}C resonances from the benzene ring of (**1**) was carried out in the opposite order, *i.e.* consideration of multiplicity allowed the four resonances between δ 110 and δ 145 (the usual aromatic range) to be separated into groups of tertiary carbons (with coupling > 150 Hz) and quaternary carbon (all C–H coupling < 10 Hz). Careful comparison of chemical shifts with accepted typical values[8] then enabled individual assignment of ^{13}C resonances as in Table 1. The one-bond C–H coupling constants (^{1}J 161.0 and 165.4 Hz) correspond with the usual values for sp^2-hybridised carbon atoms. Longer range couplings (^{2}J 4.7 Hz and ^{3}J 7.5, 7 Hz) are also of typical magnitude for an aromatic ring, although $^{2}J_{C\text{-}2',3'\text{-}H}$ 7.1 Hz is unusually large[8] (Figure 2). Little has been published on the ^{13}C n.m.r. spectra of 2,4-diaminopyrimidines and, as no protons are bonded directly to the carbon atoms of the pyrimidine ring in compound (**1**), use was made of the longer range C–H couplings observed. Clearly, the three carbon atoms (C-2, -4, and -6) adjacent to ring nitrogens (some bearing amino groups) should experience considerable deshielding and the resonances of all three appear in the range δ_C 162—167. C-6 Is identifiable as giving rise to the resonance at δ_C 166.56, the signal at lowest field in the spectrum of pyrimethamine (**1**), which is a quartet (^{3}J 5 Hz) owing to three-bond C–H coupling through to the exocyclic C-2″ methyl protons. C-2 and C-4 are far from coupling protons and appear as singlets at δ_C 162.06 and 162.19, although differentiation between these two is not possible in this

Table 1. Proton-coupled ^{13}C n.m.r. spectral data of pyrimethamine (**1**) in $(CD_3)_2SO$

	Free base			Salt (1 × $EtSO_3H$)			
Carbon	δ_C	Mult.	J (Hz)[a]	δ_C	Mult.	J (Hz)[a]	$\Delta\delta$[b]
2	162.06/	s		154.88	s		−7.18/
	162.19[c]	s					−7.31
4	162.06/	s		164.20	s		−2.14/
	162.19[c]	s					+2.01
5	105.32	m	*d*	107.26	m	*d*	+1.94
6	166.56	q	5	154.58	m	*d*	−11.98
1′	135.11	t	7.5	133.70	tt	10.8, 3	−1.41
2′/6′	132.64	dd	161.0, 7.1	132.63	dd	162.5, 7.0	−0.01
3′/5′	128.97	dd	165.4, 4.7	129.56	dd	166.3, 4.4	+0.59
4′	131.87	t	7	130.22	t	8.1	−1.65
1″	27.52	tq	126.7, 4.5	23.66	tq	131.0, 4	−3.86
2″	13.76	qt	126.9, 4.5	12.71	qt	128.8, 4.1	−1.05
1‴				45.37	tq	132.9, 4.6	
2‴				9.79	qt	127.4, 4.1	

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] δ (1 × $EtSO_3H$) − δ (free base). [c] Assignment uncertain between the resonances indicated. [d] J < 10 Hz but poorly resolved.

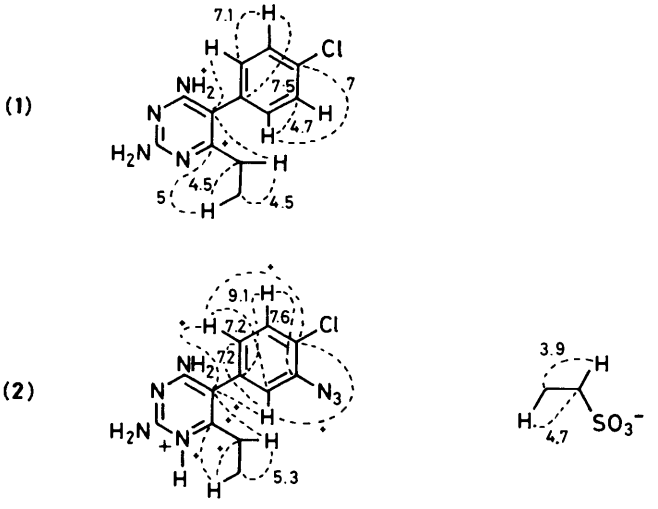

Figure 2. Illustrative ^{13}C–^1H long-range coupling systems observed for the free base of (**1**) and the monoprotonated form of MZP (**2**). Numbers are coupling constants in Hz, * represents poorly resolved coupling with 4 Hz < J < 8 Hz. ^1J Couplings are omitted for clarity

spectrum. The C-5 atom, which is not adjacent to nitrogen, gives a resonance at δ_C 105.32 as a narrow multiplet owing to 3-bond C–H coupling with the protons bonded to C-1″, -2′, and -6′.

When the spectrum of the monoethanesulphonate salt of compound (**1**) was examined, the same methods of assignment were used (Table 1). In this spectrum, however, not only has the coupling from C-1′ to 3′, 5′-H been increased to ^3J 10.8 Hz but the usually very small two-bond aromatic coupling between this carbon and 2′-, 6′-H has been resolved (^2J 3 Hz). The effects of monoprotonation of pyrimethamine (**1**) on the chemical shifts of pyrimidine ring carbons are profound, with a lesser effect on the ethyl group and, as expected owing to the local lack of basic centres, absent in the carbocyclic ring. In the pyrimidine moiety, C-2 and C-6 experience large upfield shifts (*ca.* 7 and *ca.* 12 p.p.m. respectively). These shifts are similar in sign and magnitude to the changes on protonation in the chemical shifts of the *ipso* carbon atoms of anilines, as recently reported by Faure *et al.*;[9] there is also good agreement with the $\Delta\delta$ on protonation observed by Smal *et al.*[10] using specific ^{13}C-labelled trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine]. Thus, it can be inferred that N-1 of the pyrimidine has been protonated, since C-2 and C-6 are both directly bonded to this nitrogen. That protonation has occurred exclusively at N-1 and not at N-3 is shown by the observation that the signal for C-4 does not experience this upfield shift but rather moves slightly downfield.

The ^{13}C n.m.r. spectrum of the free base of the other therapeutic pyrimidine in the study (**2**) is similar to that of pyrimethamine (**1**), with the only exceptions arising in the benzene ring as a result of the additional (azido) substituent at C-3′ (Table 2). Again, no differentiation is possible between the singlet resonances at δ_C 162.01 and 162.30 for C-2 and C-4 in the free base; similarly, the doublets with ^3J coupling at δ_C 136.86 and 136.92 are assigned to C-1′ and C-3′, not necessarily respectively. The contribution of the azido group to the ^{13}C chemical shifts in benzene rings has hitherto not been reported for many compounds. In this case, comparing spectra of compounds (**1**) and (**2**), the effects are approximately +7 p.p.m. (*ipso* carbon; C-3′), −5 p.p.m. (*ortho* carbons; C-2′ and C-4′), +2 p.p.m. (*meta* carbons; C-1′ and C-5′), and −10 p.p.m. (*para* carbon; C-6′). These general values reflect well the nature of the azido group as a pseudohalogen similar to bromine in electronic character and steric factors but are at some quantitative variance with one report[11] for the azido group. When protonated by one equivalent of ethanesulphonic acid, the carbon chemical shifts in the spectrum of MZP (**2**) (Table 2) change very much as do those of pyrimethamine (**1**), again indicating exclusive protonation at N-1. Interestingly, C-1′ experiences a greater upfield shift (some 5 p.p.m.) upon protonation in compound (**2**) than in the case with (**1**) (*ca.* 1.4 p.p.m.).

The analysis and implications of the ^{13}C n.m.r. spectra of the amino analogue (**3**) are more complex, since a basic site attached to the benzene ring can be identified in addition to those on the 2,4-diaminopyrimidine moiety. It is not absolutely clear, *a priori*, whether N-1 or the 3′-amino group would be the site of first protonation, although comparison of the measured pK_a of the conjugate acid of pyrimethamine (**1**) (reported as 7.30[1] and 7.34[5]) with that reported for 2-chloroanilinium ion (2.65),[12] as model compounds for the constituent parts of the amine (**3**), would tend to predict initial protonation at N-1. No prediction is available to select 3′-NH_2 or N-3 (or, indeed, 2-NH_2 or 4-NH_2) as the site of the second protonation.

The spectroscopic data for the free base of compound (**3**) with

Table 2. Proton-coupled ^{13}C n.m.r. spectral data of MZP (**2**) in $(CD_3)_2SO$

	Free base			Salt (1 × $EtSO_3H$)			
Carbon	δ_C	Mult.	J (Hz)[a]	δ_C	Mult.	J (Hz)[a]	$\Delta\delta$[b]
2	162.01/	s		154.88	s		−7.13/
	162.30[c]	s					−7.42
4	162.01/	s		164.05	s		+2.04
	162.30[c]	s					+1.75
5	104.80	m	*d*	106.67	m	*d*	+1.87
6	166.70	m	*d*	154.7[e]	m	*d*	−12.0
1′	136.86/	d	8.0	131.77	d	9.1	−5.09/
	136.92[c]	d	6				−5.15
2′	128.74	dd	164.2, 7.1	128.41	dd	165.3, 7.2	−0.33
3′	136.86/	d	8.0	137.60	d	7.6	+0.74/
	136.92[c]	d	6.0				+0.68
4′	122.22	m	*d*	124.0[e]	m	*d*	−1.8
5′	130.86	d	166.6	131.31	d	167.7	+0.45
6′	122.75	dd	161.2, 7.4	123.01	dd	162.9, 7.2	+0.26
1″	25.27	tq	127.2, 4.1	23.70	tq	133.1, *d*	−3.87
2″	13.17	qt	126.8, 4.8	12.62	qt	128.4, 5.3	−0.55
1‴				45.31	tq	133.3, 4.7	
2‴				9.70	qt	127.4, 3.9	

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] δ (1 × $EtSO_3H$) − δ (free base). [c] Assignment uncertain between the resonances indicated. [d] $J < 10$ Hz but poorly resolved. [e] Estimated graphically.

Table 3. Proton-coupled ^{13}C n.m.r. spectral data of compound (**3**) in $(CD_3)_2SO$

	Free base			Salt (1 × $EtSO_3H$)			
Carbon	δ_C	Mult.	J (Hz)[a]	δ_C	Mult.	J (Hz)[a]	$\Delta\delta_{1-0}$[b]
2	161.96/	s		154.92	s		−7.04/
	162.17[c]	s					−7.25
4	161.96/	s		164.14	s		+2.18/
	162.17[c]	s					+1.97
5	106.29	m	*d*	107.96	m		+1.67
6	166.42	q	4.6	154.40	m	*d*	−12.02
1′	135.59	d	6.8	130.62	d	9.0[e]	−4.97
2′	117.28	dm	167.1, *d*	116.92	dm	157.0,[f] *d*	−0.31
3′	145.04	d	8.6	145.42	d	6.0[f]	+0.38
4′	116.65	m	*d*	117.75	m	*d*	+1.10
5′	129.57	d	162.8	130.03	d	163.4[e]	+0.46
6′	119.19	dd	170.3, 7.7	118.55	dd	160.3,[f] 7.2	−0.64
1″	27.60	tq	127.5, 3.9	23.69	tq	131.1, 4	−3.91
2″	13.53	qt	127.1, 5.0	12.88	qt	128.7, 4	−0.65
1‴				45.35	tq	132.4, 4.6	
2‴				9.72	qt	128.3, 4	

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] δ (1 × $EtSO_3H$) − δ (free base). [c] Assignment uncertain between the resonances indicated. [d] $J < 10$ Hz but poorly resolved. [e] Decoupled by irradiation of proton resonance centred at δ_H 7.273 (5′-H). [f] J reduced by *ca.* 10 Hz by irradiation as in (*e*).

one equivalent of ethanesulphonic acid are given in Table 3. In the case of the spectrum of the free base, initial assignments were confirmed by a ^1H broadband-decoupled DEPT experiment; C-2′, -5′, -6′, and -2″ appearing as positive peaks, C-1″ as a negative peak, and the remaining (quaternary) carbons giving no signal. Supporting evidence for assignment of the spectrum of the monoethanesulphonate came from selective heteronuclear decoupling. Irradiation at the centre of the 5′-H signal (δ_H 7.273) in the proton spectrum removed the two three-bond couplings of this proton (^3J 9.9 Hz to C-1′ and ^3J 6.0 Hz to C-3′) and, surprisingly in view of the low decoupler power involved, removed the ^1J 163.4 Hz coupling to C-5′. Accidental irradiation of the two other aromatic protons was negligible, resulting only in slight reductions in the magnitudes of ^1J for C-2′ and C-6′. Yet again, the C-2 and C-4 resonances occur as two very close singlets (δ_C 161.96 and 162.17) in the free base. The changes in chemical shift, in the presence of one equivalent of acid, for compound (**3**) parallel closely those observed for MZP (**2**) and also correlate well with the $\Delta\delta$ for protonation of pyrimethamine (**1**). Hence, it can be deduced that the initial protonation of the amine (**3**) occurs exclusively at N-1. However, when the experiment was carried out in the presence of two equivalents of ethanesulphonic acid, *i.e.* when the crystalline bis(ethanesulphonate) salt[1] of compound (**3**) is dissolved in DMSO, the resulting ^{13}C n.m.r. spectrum (Table 4) shows only minor to moderate differences from that of the monoethanesulphonate. The maximum deviation in chemical shift of pyrimidine and 6-ethyl carbons betwen the 1 × $EtSO_3H$ and 2 × $EtSO_3H$ spectra is merely 0.23 p.p.m., showing that no change in protonation status has occurred in the pyrimidine

Table 4. Proton-coupled ^{13}C n.m.r. spectral data of compound (**3**) in $(CD_3)_2SO$

Carbon	Free base δ_C	Salt (2 × $EtSO_3H$) δ_C	Mult.	J (Hz)[a]	$\Delta\delta_{2-0}$[b]	$\Delta\delta_{2-1}$[b]
2	161.96/ 162.17[c]	154.82	s		−7.14/ −7.35	−0.10
4	161.96/ 162.17[c]	164.15	s		+2.19/ +1.98	+0.01
5	106.29	107.73	m	*d*	+1.44	−0.23
6	166.42	154.29	m	*d*	−12.13	−0.11
1′	135.59	130.69	d	8.6	−4.90	+0.07
2′	117.28	118.63	dd	160.5, 5	+1.35	+1.66
3′	145.04	143.06	m	*d*	+1.98	−2.36
4′	116.65	119.35	m	*d*	+2.70	+1.60
5′	129.57	130.27	d	164.4	+0.70	+0.24
6′	119.19	118.55	dd	169.7, 5	+1.53	+2.17
1″	27.60	23.60	tq	132.7, *d*	−4.00	−0.09
2″	13.53	12.79	qt	127.0, 5.0	−0.74	−0.09
1‴		45.38	tq	128.8, 4.6		+0.03
2‴		9.54	qt	128.2, 4.2		−0.18

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] $\Delta\delta_{2-0}$: δ (2 × $EtSO_3H$ salt) − δ (free base), $\Delta\delta_{2-1}$: δ (2 × $EtSO_3H$ salt) − δ (1 × $EtSO_3H$ salt). [c] Assignment uncertain between the resonances indicated. [d] J < 10 Hz but poorly resolved.

Table 5. Proton-coupled ^{13}C n.m.r. spectral data of compound (**3**) in CF_3CO_2D compared with that of free base in $(CD_3)_2SO$

Carbon	MZP (**2**) Free base δ_C	CF_3CO_2D δ_C	Mult.	J (Hz)[a]	$\Delta\delta_{TFAD-0}$[b]	$\Delta\delta_{TFAD-1}$[b]
2	161.96/ 162.17[c]	152.12	s		−9.84/ −10.05	−2.80
4	161.96/ 162.17[c]	160.08	s		−1.88/ −2.09	−4.06
5	106.29	107.36	m	*d*	+1.07	−0.60
6	166.42	159.76	m	*d*	−6.66	+5.36
1′	135.59	127.90/ 128.43[c]	m br d	*d* 8.0	−7.69/ −7.16	−2.72/ −2.19
2′	117.28	127.25	dd	164.0, 8.0	+10.67	+10.98
3′	145.04	127.90/ 128.43[c]	m br d	*d* 8.0	−17.14/ −16.61	−17.52/ −16.99
4′	116.65	131.80	m	*d*	+15.15	+14.05
5′	129.57	133.72	d	171.9	+4.15	+3.69
6′	119.19	134.24	dd	165.6, 7.4	+15.05	+15.69
1″	27.60	24.78	tq	132.5, 5.0	−2.82	+1.09
2″	13.53	10.55	qt	130.6, 5.1	−2.98	−2.33

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] $\Delta\delta_{TFAD-0}$: δ(CF_3CO_2D) − δ [free base/$(CD_3)_2SO$], $\Delta\delta_{TFAD-1}$: δ(CF_3CO_2D) − [1 × $EtSO_3H$ salt/$(CD_3)_2SO$]. [c] Assignment uncertain between the resonances indicated. [d] J < 10 Hz but poorly resolved.

ring. Changes in chemical shift of the benzene ring carbons vary from −2.36 p.p.m. for C-3′ to +2.17 p.p.m. for C-6′. Consideration of the signs and relative magnitudes of these $\Delta\delta_{2-1}$, shown in Table 4, and comparison with reported shifts on protonation of aniline in DMSO solution suggest that a small amount of protonation remains at the 3′-NH_2 in solution in an equilibrium which is rapid on the n.m.r. time-scale. When the changes in chemical shift observed here on partial protonation of the 3′-NH_2 (−2.36 p.p.m. for the *ipso* C-3′, +1.66 p.p.m. for the *ortho* carbons, +0.07 and +0.24 p.p.m. for the *meta* carbons, and +2.17 p.p.m. for the *para* carbon) are compared with those reported[9] for full protonation of aniline in the same solvent (*ipso* −16.7 p.p.m., *ortho* +9.4 p.p.m., *meta* +1.0 p.p.m., and *para* +12.3 p.p.m.), it can be inferred that the 3′-amino group is *ca.* 16% protonated. Hence, the 3′-ammonium function which is present in the solid state dissociates to the extent of *ca.* 84% upon dissolution in DMSO.

Since compound (**3**) is largely only monoprotonated in the presence of two equivalents of ethanesulphonic acid, it was necessary to make use of much higher acid concentrations to examine the spectra of multiprotonated species from compound (**3**). The proton-coupled ^{13}C n.m.r. spectral data of amine (**3**) in neat deuteriotrifluoroacetic acid are listed in Table 5. This spectrum, although slightly less well resolved than the others, is still capable of full assignment except that the resonances for C-1′ and C-3′ could not be distinguished between signals at δ_C 127.90 and 128.43. Clearly, the 3′-amino group is fully ionised in this medium and corresponding characteristic chemical-shift differences between the spectrum of the free base of (**3**) in DMSO (and, incidentally, the monoethanesulphonate) and that in CF_3CO_2D solution are observed. The $\Delta\delta_{TFAD-1}$ values for the benzene-ring protons are: *ipso* carbon (C-3′) *ca.* −17 p.p.m., *ortho* carbon (C-2′) +10.98 p.p.m., *ortho* carbon (C-4′) +14.05 p.p.m., *meta* carbon (C-1′) *ca.* −2.5 p.p.m., *meta* carbon (C-5′) +3.69 p.p.m., *para* carbon (C-6′) +15.69 p.p.m. These data are in good agreement with the changes in chemical shift on

Table 6. ^{1}H N.m.r. spectral data of compound (**3**) ethanesulphonate salts in $(CD_3)_2SO$. Only C–H signals are shown

	Salt (1 × $EtSO_3H$)			Salt (2 × $EtSO_3H$)		
Proton	δ_H	Mult.	J (Hz)	δ_H	Mult.	J (Hz)
2′-H	6.65	d	2.0	6.69	d	2.0
5′-H	7.27	d	8.1	7.32	d	8.1
6′-H	6.40	dd	8.1, 2.0	6.47	dd	8.1, 2.0
1″-H_2	2.23	br q	7.6	2.22	br q	7.6
2″-H_3	1.05	br t	7.6	1.04	t	7.6
1‴-H_2	2.57	q	7.4	2.53	q	7.4
2‴-H_3	1.12	t	7.4	1.09	t	7.4

Table 7. ^{1}H N.m.r. spectral data of compound (**3**) in $(CD_3)_2SO$ and in CF_3CO_2D. Only C–H signals are shown

	Free base [$(CD_3)_2SO$]			CF_3CO_2D		
Proton	δ_H	Mult.	J (Hz)	δ_H	Mult.	J (Hz)
2′-H	6.65	d	1.5	7.84	br d	1.5[a]
5′-H	7.25	d	8.0	7.93	d	8.3
6′-H	6.38	dd	8.0, 1.5	7.61	dd	8.3, 1.5
1″-H_2	2.18	q	7.5	2.60	q	7.5
2″-H_3	1.00	t	7.5	1.29	t	7.5

[a] Estimated graphically.

protonation of aniline reported[9] by Faure *et al.* (see above), subject to the effects of change of solvent (predicted to be <3 p.p.m.).[9] Examination of the resonances from the pyrimidine carbons in CF_3CO_2D (Table 5) and comparison with the mono-(N-1)-protonated species shows some additional protonation on this heterocycle or its amino substituents. More detailed examination of chemical-shift differences between amine (**3**) in its monoprotonated form in DMSO and in trifluoroacetic acid (TFA) shows upfield shifts for C-2 and C-4 and a downfield shift for C-6 (Table 5), indicative of protonation at N-3. The magnitudes of the $\Delta\delta$ values involved, when compared with the magnitudes of the effects of protonation at N-1, suggest however that this protonation is incomplete even in this highly acidic medium.

Proton n.m.r. spectra were also recorded for the solutions of amine (**3**) and acids as detailed above for the ^{13}C n.m.r. spectra; the data are recorded in Tables 6 and 7. The resonances for the C–H protons are very similar in the spectra of amine in DMSO in the presence of 0, 1, and 2 equiv. of ethanesulphonic acid, confirming that only slight protonation of the 3′-NH_2 group occurs under these conditions. In neat TFA, full protonation at 3′-NH_2 is reported by downfield shifts of *ca.* 1.1 p.p.m. for 2′-H and 6′-H (*ortho* and *para* respectively to the amino group) and of 0.66 p.p.m. for 5′-H (*meta*).

In conclusion, it can be seen from the above data that the first protonation of all three 2,4-diaminopyrimidines studied takes place on N-1 in DMSO solution, as predicted by *X*-ray crystallographic work.[6] This protonation is complete in the presence of only 1 equiv. of ethanesulphonic acid, indicating a relatively high basicity of N-1 in these molecules, which is consistent with values for the overall values of pK_a of the conjugate acids determined by conventional techniques [7.3 for (**1**),[1,5] 7.2 for (**2**),[1] and 7.5 for (**3**)[13]]. The second protonation of amine (**3**) has been shown to take place extensively only at very high acid strengths and then at 3′-NH_2. This conclusion allows the rationalisation of the observation that amine (**3**) is an inhibitor of DHFR [although weaker than (**1**) or (**2**)][1] according to the classical requirements for protonation at N-1, but at no other sites, at physiological pH for binding to the active site of the enzyme.

Experimental

Spectroscopy.—N.m.r. spectra were obtained at 100.8 MHz (^{13}C) and at 400 MHz (^{1}H) with a Bruker WH400 spectrometer. ^{13}C Spectra were recorded at natural abundance without decoupling from protons except that selective irradiation of the proton resonance at δ_H 7.273 was used in one ^{13}C experiment using the monoethanesulphonate of compound (**3**) in order to confirm assignment of certain ^{13}C resonances. All chemical shifts are referenced to tetramethylsilane as internal standard. Spectra were recorded at 300 ± 2 K at *ca.* 10% w/v concentration.

Materials.—Pyrimethamine (**1**) was a kind gift of The Wellcome Foundation Ltd., Dartford, Kent. The amino analogue (**3**) was prepared by nitration (HNO_3–H_2SO_4) and subsequent reduction ($SnCl_2$) of compound (**1**); diazotisation (aq. HCl–$NaNO_2$) and treatment with sodium azide furnished the free base of compound (**2**) as previously reported.[1] The crystalline monoethanesulphonate salts of compounds (**1**) and (**2**) and bis(ethanesulphonate) salt of compound (**3**) were prepared by treatment of the free bases with aqueous ethanesulphonic acid followed by recrystallisation.[1] The spectroscopic sample for the monoethanesulphonate salt of compound (**3**) was prepared by dissolution of equimolar amounts of compound (**3**) (free base) and ethanesulphonic acid in $(CD_3)_2SO$; correct proportions were checked by integration of the proton n.m.r. spectrum.

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