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Reduced 2-Methyl-4-oxo, 2-Methylamino-4-oxo, 2-Methylthio-4-oxo and 2-Amino-4-thioxo 6,7-Dimethylpteridines, and 6,8-Dimethylpterin as Substrates for Dihydropteridine Reductase

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Abstract

The syntheses of unsubstituted (4), 6-methyl- (6), *cis*-6,7-dimethyl- (8), *cis*-2,6,7-trimethyl- (10), *cis*-6,7-dimethyl-2-thioxo-(1H)- (12) and *cis*-6,7-dimethyl-2-methylthio- (13) 5,6,7,8-tetrahydropteridin-4(3*H*)-one and the deuterated derivatives (5), (7), (9) and (14), and a *cis* and *trans* mixture of 2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-thione (11) are described. The existence of the transient quinonoid species (15)–(17) had been previously demonstrated by showing that they are substrates for the enzyme dihydropteridine reductase from human brain. We report that the transient quinonoid species *cis*-2,6,7-trimethyl- (18), *cis*-2-methylthio-6,7-dimethyl- (25), *cis*-2-methylamino-6,7-dimethyl- (26), and 2-amino-6,8-dimethyl- (29) 7,8-dihydropteridin-4(6*H*)-one, and 2-amino-6,7-dimethyl-7,8-dihydropteridin-4(6*H*)-thione (28), but not *cis*-6,7-dimethyl-2-thio-7,8-dihydropteridin-4(6*H*)-one (27) are substrates for this enzyme showing that a variety of substituents in position 2 of the pteridine ring can be tolerated, and confirming that the predominant tautomer (1) of the quinonoid species in aqueous solution is also the reactive tautomer at the active site of this enzyme.

Introduction

Several quinonoid dihydropterins are active substrates for the enzyme dihydropteridine reductase and can exit in three possible tautomeric forms (1), (2) and (3) in aqueous solution at near neutral pH.[1] ^{15}N n.m.r. spectral studies of ^{15}N enriched 6,7-dimethylpterins have shown that species (1) are predominant in solution,[2] and tautomer (3) could not be detected in the ^{1}H n.m.r. spectra.[3] However, the predominant tautomer in solution is not necessarily the structure that is enzymically active. In an endeavour to deduce the tautomeric structure of pterin substrates at the active site we have synthesized the 5,6,7,8-tetrahydropteridin-4(3*H*)-ones (4)–(10), and the related thioxo and methylthio compounds (11)–(14) which we now report together with their spectral properties. The substrate activities of the transient quinonoid species (15)–(17), derived by oxidation of the tetrahydropteridinones (4), (6) and (8),

[1] Armarego, W. L. F., Randles, D., and Waring, P., *Med. Res. Rev.*, 1984, **4**, 267.
[2] Benkovic, S. J., Sammons, D., Armarego, W. L. F., Waring, P., and Inners, R., *J. Am. Chem. Soc.*, 1985, **107**, 3706.
[3] Lazarus, R. A., DeBrosse, C. W., and Benkovic, S. J., *J. Am. Chem. Soc.*, 1982, **104**, 6871.

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towards dihydropteridine reductase have been reported,[4] and the activities of the quinonoid pteridinone (18), the thio compounds (25), (27) and (28), and the pterin (26) are briefly described here. The biological activities strongly support the tautomeric structure (1) as the enzymically active species, and show that the enzyme can tolerate quinonoid dihydropteridines with 2-methyl-4-oxo (18), 2-methylamino-4-oxo (26), 2-methylthio-4-oxo (25), 2-amino-4-thioxo (28) and only 4-oxo (15)–(18) substituents in addition to the 2-amino-4-oxo substituents that are present in the natural substrate, quinonoid 7,8-dihydro(6*H*)biopterin and analogues such as (1).[1]

We have indicated in the Experimental section where our syntheses differed from published procedures and have included unpublished spectral data. Only new preparations and unusual features of the syntheses will be discussed here.

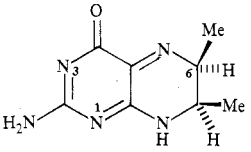
Preparation of Pteridin-4(3*H*)-ones

Pteridin-4(3*H*)-one could not be reduced catalytically with pre-reduced platinum oxide in trifluoroacetic acid or methanol, or with palladium on charcoal in ethanol. This was not due to poisoning of the catalyst by traces of sulfur from the intermediates because the reductions were unsuccessful with rigorously purified pteridinone, and when large excesses of catalyst were used. We have no satisfactory explanation for this failure. Reduction to the tetrahydro derivative (4) with sodium borohydride, however, was successful.[5]

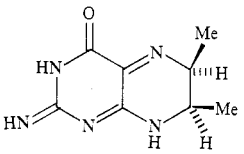
6-Methylpteridin-4(3*H*)-one, on the other hand, gave the 5,6,7,8-tetrahydro derivative (6) on hydrogenation with platinum oxide in methanol. When the reduction was carried out in trifluoroacetic acid and stopped after two equivalents of hydrogen were absorbed, a mixture of tetra- and hexahydro-pteridinone was formed. The pure hexahydro derivative (19) was obtained by allowing this reduction to proceed to completion. Alternative structures for the hexahydro product, such as the ring-opened cations of the *N*-methyltetrahydropyrazines (20) or (21), or of the *N*-hydroxymethyltetrahydropyrazines (22) or (23) derived from the bicyclic compound (19), were excluded on the grounds that the ^{1}H n.m.r. spectra in acidic solution had proton integrals consistent with two and not three H 2 protons for the singlet at 4·99 ppm [compare with the singlets at 4·30 and 4·33 ppm of the two H 2 protons in the spectra of the neutral species of *cis*- and *trans*-1,2,4a,5,6,7,8,8a-octahydroquinazolin-4(3*H*)-one].[6] Moreover, the ^{13}C n.m.r. chemical shift of C 2 was at 52·1 ppm consistent with a $-HNCH_2NH-$ structure (19) rather than $-HNCH_2OH$ structures such as (22) and (23). This deduction was arrived at from the known ^{13}C chemical shifts of 6-aminohexan-1-ol where C 1 and C 6 are at 62·9 and 43·2 ppm respectively.[7] The model shows that the oxygen adjacent to the methylene carbon atom deshields it much more strongly than an adjacent nitrogen atom. The value of 52·1 ppm observed cannot be consistent with a $-NHCH_2OH$ structure.

cis-6,7-Dimethyl- (8) and *cis*-2,6,7-trimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (10), unlike the above, were prepared as their hydrochloride salts by allowing the catalytic reduction of the respective pteridinone in acidic medium to proceed to

[4] Armarego, W. L. F., Ohnishi, A., and Taguchi, H., *Biochem. J.*, 1986, **234**, in press.
[5] Albert, A., and Matsuura, S., *J. Chem. Soc.*, 1962, 2162.
[6] Armarego, W. L. F., and Kobayashi, T., *J. Chem. Soc. C*, 1971, 238.
[7] Formacek, V., Desnoyer, L., Kellerhals, H. P., Keller, T., and Clerc, J. T., *Bruker ^{13}C Data Bank*, 1976, **1**, 139.



(1)



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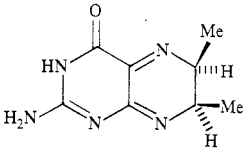

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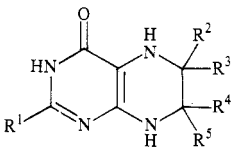
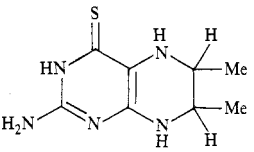


	R^1	R^2	R^3	R^4	R^5
(4)	H	H	H	H	H
(5)	H	D	D	D	D
(6)	H	Me	H	H	H
(7)	H	Me	D	H	D
(8)	H	Me	H	Me	H
(9)	H	Me	D	Me	D
(10)	Me	Me	H	Me	H



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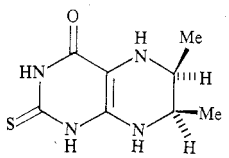

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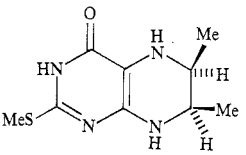

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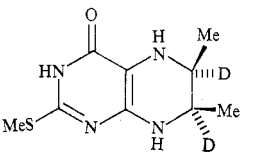

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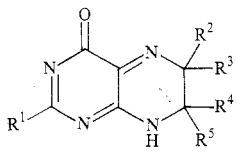
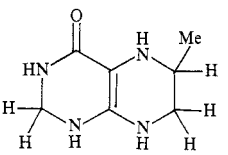
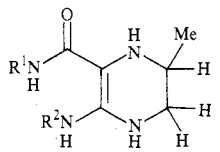


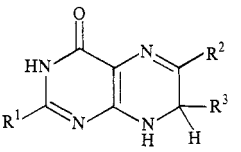
	R^1	R^2	R^3	R^4	R^5
(15)	H	H	H	H	H
(16)	H	Me	H	H	H
(17)	H	Me	H	Me	H
(18)	Me	Me	H	Me	H



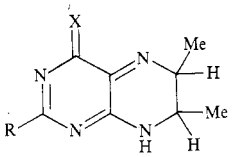
(19)



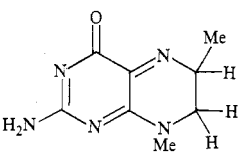
	R^1	R^2
(20)	H	Me
(21)	Me	H
(22)	H	CH_2OH
(23)	CH_2OH	H



(24)



	R	X
(25)	MeS	O
(26)	MeNH	O
(27)	SH	O
(28)	NH_2	S



(29)

completion. The *cis* stereochemistry at C 6 and C 7 was deduced from the small H 6–H 7 coupling constants (cf.[8]).

Preparation of Thiopteridines

The successful reduction of pteridin-4(3*H*)-ones to their 5,6,7,8-tetrahydro derivatives with large excesses of sodium borohydride[5] made us use this reagent to prepare 2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-thione (11). Unlike previous reactions where the 6,7-*cis* isomers were formed exclusively, this product was a 1·6 : 1·0 mixture of *cis* and *trans* isomers. As in previous reductions we were unable, however, to obtain the reduced pteridines completely free from small amounts of boric acid or sodium borate which did not interfere with enzyme activity.[4] The reduced pteridines were free from organic impurities other than solvent which adheres tenaciously. In an attempt to obtain preparations free from borate we reduced the 2-amino-6,7-dimethylpteridin-4(3*H*)-thione by hydrogenation with platinum oxide in methanol. This gave only the *cis*-5,6,7,8-tetrahydro derivative of (11) (by n.m.r.). Similar reduction in trifluoroacetic acid yielded a 2·3 : 1·0 mixture of *cis* and *trans* isomers. The sharp signals in the n.m.r. spectra of these products were an indication that they were free from platinum or platinum complexes.

6,7-Dimethyl-2-thioxo-2,3-dihydropteridin-4(1*H*)-one was reduced to the *cis*-hexahydro derivative (12) with excess sodium borohydride. However, when it was reduced catalytically with platinum oxide in methanol, 3M hydrochloric acid or trifluoroacetic acid, hydrogen uptake was not complete and the corresponding 6,7-dimethyl-2-thioxo-1,2,7,8-tetrahydropteridin-4(3*H*)-one only was formed. The 7,8-dihydro structure of this compound was deduced by analogy with the incomplete catalytic reduction products of related pteridines which were known to be the more stable 7,8-dihydro derivatives (compared with the 5,6- or 5,8-dihydro derivatives). The structures of the 7,8-dihydropteridines were deduced by unambiguous synthesis.[8] Catalytic reduction of 6,7-dimethyl-2-methylthiopteridin-4(3*H*)-one with platinum oxide in trifluoroacetic acid also gave the 7,8-dihydro derivative, but when the solvent was methanol or (D_4) methanol (with deuterium gas) reduction was complete and the *cis*-derivatives (13) and (14) were obtained respectively. These were soluble in chloroform which is unusual for oxopteridines.

Preparation of Substrates and Dihydropteridine Reductase Activity

This enzyme has a strict requirement for quinonoid dihydropteridines, e.g. structure (1), as substrates. These substrates are reduced enzymically to the respective 5,6,7,8-tetrahydropteridines at the expense of NADH which in turn is oxidized to NAD^+ (see[1]). The quinonoid species can be generated by specific oxidations (e.g. with peroxidase–hydrogen peroxide or air, potassium ferricyanide, bromine) of the corresponding 5,6,7,8-tetrahydropteridines. Although many of them can be identified by their characteristic u.v. spectra, and by their reactions and n.m.r. spectra at low temperature, they are short lived. They either rearrange to the more stable tautomers, the 7,8-dihydropteridines (24), or undergo degradation depending on the nature and position of the substituents.[1] Neither the tetrahydro nor the 7,8-dihydro derivatives, nor the parent pteridines, are substrates for the enzyme. Consequently,

[8] Armarego, W. L. F., and Schou, H., *J. Chem. Soc., Perkin Trans. 1*, 1977, 2529.

when a tetrahydropteridine is oxidized by the specific oxidants and the transient species formed can be enzymically reduced back to the original tetrahydropteridine at the expense of NADH we can presume that the transient species have a quinonoid structure such as (1).

In order to ascertain that the transient species were substrates we had to show that (i) maximum enzyme activity (i.e. initial rates of oxidation of NADH) was observed at least immediately after addition of enzyme, (ii) at a set concentration of substrate and NADH, enzyme activity increased linearly with increase in enzyme concentration, (iii) at a constant enzyme and NADH concentration but varying substrate concentration, the initial rates against substrate concentrations were hyperbolic, i.e. indicative of saturation kinetics, and (iv) that Lineweaver–Burk plots of the data in (iii), i.e. reciprocal of initial velocity against reciprocal of concentration yielded the kinetic parameters (apparent Michaelis constants K_m and maximum velocities V_{max}). We had shown that the tetrahydropteridin-4(3*H*)-ones (4), (6) and (8) were oxidized by peroxidase–hydrogen peroxide to the transient species (15)–(17) respectively.

Table 1. Kinetic parameters of human brain dihydropteridine reductase in 0·1 M Tris–HCl buffer pH 7·4 at 25°

7,8-Dihydropteridine-4(6*H*)-one	K_m (μM)	V_{max}[A]	V/K	NADH (μM)
2-Amino-6,7-dimethyl, (1)	24·7(±0·6)	199(±2·1)	8·06	95
2,6,7-Trimethyl, (18)	38·8(±0·7)	10·1(±0·1)	0·26	111
6,7-Dimethyl-2-methylthio, (25)	85·5(±0·04)	83·1(±0·3)	0·97	106
6,7-Dimethyl-2-methylamino, (26)	127(±5)	150(±4)	1·18	117
2-Amino-6,8-dimethyl,[B] (29)	349(±26)	24·3(±2·6)	0·06	108

[A] Calculated on μM NADH oxidized per min per mg of protein.

[B] Formed from 6,8-dimethyl-5,6,7,8-tetrahydropterin hydrochloride (Armarego, W. L. F., and Milloy, B. A., *Aust. J. Chem.*, 1977, **30**, 2023), ϵ_{270} 14450 in 4 mM HCl (Pfleiderer, W., and Mengel, R., *Chem. Ber.*, 1971, **104**, 2293), in the assay.

These transient species were substrates for dihydropteridine reductase and we had determined their kinetic parameters.[4] We report an extension of these studies in which we have examined the viability of the derivatives (18), (25), (26) and (29) as substrates for dihydropteridine reductase. The results are summarized in the form of kinetic parameters in Table 1. The parameters of the known substrate 2-amino-6,7-dimethyl-7,8-dihydropteridin-4(6*H*)-one (1), were redetermined under identical conditions and were for comparison. The V/K values have been included in Table 1 because they are a better index of the overall efficiency of the enzyme with a particular substrate than the K_m and V_{max} values (see below). When we oxidized the related *cis*-2,6,7-trimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (10) with peroxidase–hydrogen peroxide we found that the quinonoid species (18) was formed at a relatively slow rate which was rate limiting. However, when the oxidant was replaced by three molar, or more, equivalents of potassium ferricyanide (with respect to the pteridine), the oxidation was complete within five seconds. By using the latter oxidant we showed, by the above four criteria, that the transient quinonoid dihydropteridine (18) formed was a substrate (Table 1). Similarly we have shown that the quinonoid pteridines (25) and (26) derived from oxidation of 2-methylthio- (13) and 2-methylamino- (kindly supplied by Dr P. Waring) 6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one respectively are also substrates (Table 1). Kaufman[9] had previously demonstrated

that the methylamino compound (26) was a substrate by measuring only *one* initial rate. This rate was 36% of the initial rate of the parent compound (1) measured under identical conditions (compare with Table 1; V/K values are a good index of substrate reactivity in these cases, the larger the value the better the substrate). The two quinonoid species (25) and (26) had typical u.v. spectra (cf.[1]). We had previously shown that the latter rearranged to the 7,8-dihydro tautomer with a half life of 34·6 min (pH 7·6 Tris–HCl buffer, k_{obs} $2{\cdot}0\times10^{-2}$ min^{-1}) and when the rate was compared with that of the 6,7-dideuterated derivative the k_H/k_D value was 9·0.[10] In the present study the 2-methylthio analogue (25) rearranged similarly, but with a half life of 0·9 min (pH 7 Tris–HCl buffer, k_{obs} 0·75 min^{-1}) and when compared with the rate of rearrangement of the deuterated quinonoid dihydropterin derived from (14) the k_H/k_D value was 18. This further confirms the quinonoid structures (25) and (26) because these rearrangements are known to proceed with very large deuterium isotope effects.[1] The 7,8-dihydro compounds formed were slowly further oxidized to the respective pteridines by air.

The thioxopteridinone (12) was oxidized by peroxidase–hydrogen peroxide or potassium ferricyanide as observed by the u.v. spectral changes. However, no dihydropteridine reductase active species could be detected, and the t.l.c. and u.v. spectral properties of the final oxidation product were similar to those of the original 6,7-dimethyl-2-thioxo-2,3-dihydropteridin-4(1*H*)-one. This experiment demonstrates that either the quinonoid species (27) were formed in very small (steady state) concentrations or were not formed at all. It should be pointed out that 6-methyl-5,6,7,8-tetrahydro-lumazine [the 2-oxygen analogue of (12)] has been oxidized to 6-methyl-7,8-dihydropteridin-2(3*H*),4(6*H*)-dione [related to the 2-oxygen analogue of (27)] which was not a substrate or inhibitor of the enzyme. We examined the oxidation of the tetrahydrothiopterin (11) with the above two oxidants, and the t.l.c. properties of the products after various intervals of time were similar. The intermediate (28) proved to be a substrate for the enzyme according to the first three criteria above. However, attempts to determine the kinetic parameters were unsatisfactory because it was not possible to reproduce the initial rate traces to within the accepted standard deviation. This is possibly due to the facile oxidation of the 4-thio group to give the respective disulfide which may or may not be enzyme active and/or dismutation between the species (28) and the unchanged 4-thioxo compound (11) in the early stages of oxidation.

The ability of 6,8-dimethyl-7,8-dihydro(6*H*)pterin (29) to act as a substrate for dihydropteridine reductase was also examined in order to find out the effect of methyl substitution at N 8. This compound proved to be a substrate, albeit the poorest of the active pteridines that were studied, but satisfied the criteria for substrate activity stated above.

We conclude that the enzyme can tolerate various substituents in the 2-position, as well as an 8-methyl group, in the quinonoid dihydropteridine substrates with a 4-oxo or 4-thioxo group. The results exclude the possibility that the tautomer with the exocyclic double bond at position 2 [e.g., structure (2)] is a substrate at the active site because such a tautomer cannot be formed when the 2-position is unsubstituted [cf. compounds (15)–(17)], and when it is substituted with a methyl

[9] Kaufman, S., *J. Biol. Chem.*, 1964, **239**, 332.
[10] Armarego, W. L. F., and Waring, P., *J. Chem. Soc., Perkin Trans. 2*, 1982, 1227.

(18) or a methylthio (25) group. The tautomer with a double bond between C 8a–N 8 [e.g., structure (3)] is also excluded on the grounds that its presence cannot be detected in the ^{1}H n.m.r. spectra in neutral solution,[3] kinetic evidence[10] and because the 8-methyl pterin (29) cannot form this tautomer at pH 7·3. The oxidation of 6,8-dimethyl-5,6,7,8-tetrahydropterin to yield (29) should produce a cation with the positive charge on N 8 if it formed a tautomer such as (3); but at neutral pH this should deprotonate at N 3 to form the neutral species (29). The evidence that the pteridine substrates are not in the protonated states on the enzyme had been presented by us previously.[4] The above study also demonstrates that the predominant tautomer in aqueous solution at pH ~ 7 [structure (1), with the endocyclic C 2–N 3 double bond] is the active substrate for dihydropteridine reductase.

Experimental

Microanalyses were determined by the Australian National University Analytical Unit. Analytical specimens were dried at 25° and 0·3 mmHg in the presence of potassium hydroxide unless otherwise stated. ^{1}H and ^{13}C n.m.r. spectra were measured on a Jeol FX 90Q spectrometer operating at 34°. Sodium 3-(trimethylsilyl)propionate was used as internal standard for the ^{1}H n.m.r. spectra (δ values are in ppm and J values in Hz). Kinetic measurements were performed on a Unicam SP1800 double-beam spectrometer and Rikadenki B-281H recorder with 5 mV across the slide wire to produce a maximum pen movement corresponding to 0·1 absorbance units. For higher sensitivity a Cary 219 spectrometer adjusted to produce maximum pen movement of 0·01 absorbance units was used and accurate absorbance for concentration measurements were performed on a Perkin–Elmer Lambda 1 single-beam spectrometer. The cell holders were kept at constant temperature (25°) with recirculating water in a Coolnics thermostat. The mass spectra were measured at 16 eV on a Hitachi M-70 mass spectrometer. Thin-layer chromatography (t.l.c.) was run on Silica gel 60 F_{254} (Merck) and eluted with BuOH/AcOH/H_2O 20 : 3 : 7. The spots were revealed by u.v. light (at 250 and 365 nm) and staining with iodine vapour. I.r. spectra (KBr disc) were measured on a Unicam SP 1050.

*Synthesis of Pteridin-4(3*H*)-ones*

5,6-Diaminopyrimidin-4(3*H*)-one, m.p. 238–243° (dec.), was prepared by desulfurizing the respective 2-thio derivative[11] and was recrystallized from water (Found: C, 33·9; H, 5·6; N, 39·8. $C_4H_6N_4O.0·8H_2O$ requires C, 34·2; H, 5·5; N, 39·9%). The ^{1}H n.m.r. spectrum had δ (D_2O): 7·76; and ϵ_{278} 8912 (pH 7). Pteridin-4(3*H*)-one, prepared from the above pyrimidinone and glyoxalbis(sodium bisulfite),[11] was purified by alumina (Brockman II, B.D.H.) column chromatography and eluted with 18 mM aqueous ammonia. The main fraction (monitored by t.l.c.) was evaporated, the residue dissolved in hot water (charcoal), the pH was adjusted to 3–4 and cooled. The solid was collected, crystallized from a large volume of acetic acid and sublimed at 320°/0·9 mmHg (Found: C, 49·0; H, 2·7; N, 38·1. $C_6H_4N_4O$ requires C, 48·7; H, 2·7; N, 37·8%). ^{1}H n.m.r. (1 M DCl): 8·97, d, J 2·3, H 6 or 7; 9·05, d, J 2·3, H 7 or 6; 9·10, s, H 2; (1 M NaOD): 8·46, s, H 2; 8·74, d, J 2·2, H 6 or 7; 8·85, d, J 2·2, H 7 or 6. (*6,7-D_2)-Pteridin-4(3*H*)-one*, m.p. 303–355° (dec.), was similarly prepared by using (1,2-D_2)-glyoxalbis(sodium bisulfite)[12] and sublimed at 230°/0·7 mmHg (Found: C, 48·2; H + D, 4·2; N, 37·5; *m/e* 149. $C_6H_2D_2N_4O$ requires C, 48·0; H + D, 4·2; N, 37·3%; M^+ 150). ν_{max} 3090 (3030), 2925 (2885), 1724 (1724), 1615 (1617), 1545 (1550), 1434 (1468), 1378 (1398), 1322 (1320), 1268 (1280), 1210 (1228), 1182 (1163), 1150 (1108) 1013 (1032), 1000, 990, 925 (923), 836 (827), 710 (720) and 652 (607) cm^{-1}, the values for the unlabelled ^{1}H-containing compound above being the ones in parentheses. The ^{1}H n.m.r. spectrum indicated *c.* 3·7% contamination with the ^{1}H-containing compound. 5,6,7,8-Tetrahydropteridin-4(3*H*)-one[5] (4), prepared by reduction of pteridin-4(3*H*)-one with $NaBH_4$, always contained small amounts of

[11] Albert, A., Brown, D. J., and Cheeseman, G., *J. Chem. Soc.*, 1951, 474.
[12] Armarego, W. L. F., Randles, D., and Taguchi, H., *Biochem. J.*, 1983, **211**, 357.

borate (green-edged flame test with H_2SO_4/EtOH) although it gave one spot on t.l.c. (Found: C, 41·5; H, 5·7; N, 31·8. $C_6H_8N_4O.0·1NaH_2BO_3.H_2O$ requires C, 41·2; H, 5·6; N, 32·0%). The formula weight is consistent with the reported ϵ_{289} value of 8511 (pH 7·0) for the anhydrous neutral species; ^{1}H n.m.r. (1 M DCl): 3·69, m, H 6,6,7,7 and 8·59, s, H 2. Similar reduction of (6,7-D_2)-pteridin-4(3*H*)-one with $NaBD_4$ in D_2O gave (6,6,7,7-D_4)-pteridin-4(3*H*)-one (5) (Found: C,41·4; H+D, 8·55; N, 31·5; *m/e* 156, base peak. $C_6H_4D_4N_4O.0·02NaH_2BO_3.H_2O$ requires C, 41·0; H+D, 8·1; N, 31·9%; M^+, 156]. The u.v. spectra and t.l.c. properties were identical with the above. The ^{1}H n.m.r. spectrum indicated that it was better than 95% deuterated on C 6 and C 7.

6-Methylpteridin-4(3*H*)-one[13] (150 mg) in methanol (300 ml) and platinum oxide (250 mg pre-reduced in 10 ml of methanol) were shaken with hydrogen (25°/720 mmHg) for 4·2 h, a further quantity of platinum oxide (100 mg pre-reduced in 10 ml of methanol) was added and shaking was continued for a further 1·5 h. The solution was filtered and evaporated to dryness. The residue (144 mg) was recrystallized from water (10 parts) to give 6-methyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one (6), m.p. >211° (dec.) (Found: C, 45·9; H, 6·3; N, 29·9. $C_7H_{10}N_4O.H_2O$ requires C, 45·65; H, 6·6; N, 30·4%) which was pure by t.l.c., and had u.v. spectra as reported.[5] Reduction with $NaBH_4$ (instead of KBH_4 as reported)[5] also gave the 6-methyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one which could not be freed for small amounts of borate (flame test) (Found: C, 36·4; H, 5·4; N, 24·0. $C_7H_{10}N_4O.0·76NaH_2BO_3.0·1H_2O$ requires C, 36·3; H, 5·1; N, 24·2%). The ^{1}H n.m.r. spectrum [1 M DCl): 1·48, d, *J* 6·3, 6-Me; 3·39, q, J_{gem} −14·4, J_{vic} 9·4, H 7$_{ax}$; ~ 3·7, m, H 6; 3·77, q, J_{gem} −14·5, J_{vic} 3·0, H 7$_{eq}$; 8·2, s, H 2] indicates a conformation similar to that of 6-methyl-5,6,7,8-tetrahydropterin, i.e. the methyl group is predominantly equatorial.[8] When 6-methylpteridin-4(3*H*)-one (200 mg) in trifluoroacetic acid (5 ml) and platinum oxide (50 mg pre-reduced in 2·5 ml of trifluoroacetic acid) was shaken with hydrogen (25°/720 mmHg), the reduction was very rapid, and after 2 h the catalyst was filtered off, the filtrate was added to a cold solution of methanol (1·8 ml) and 7 M methanolic HCl (0·4 ml) followed by dry ether (60 ml) whereby the colourless hydrochloride separated. It was collected and washed with ether (3×15 ml) by centrifugation and dried *under vacuum*. *6-Methyl-2,3,5,6,7,8-hexahydropteridin-4(1H)-one* (19) *hydrochloride* (242 mg) was recrystallized from methanol containing a few drops of methanolic HCl and ether, and had m.p. 213–218° (effervescence) (Found: C, 35·5; H, 6·1; Cl, 21·6; N, 23·1. $C_7H_{12}N_4O.1·45HCl.H_2O$ requires C, 35·2; H, 6·5; Cl, 21·5; N, 23·4%). ϵ_{258} (inflex) 158 (pH 0); ϵ_{293} 1054 (pH 7·3) and ϵ_{286} 1776 (pH 13·2). ^{1}H n.m.r. (1 M DCl): 1·62, d, *J* 6·2, 6-Me; 3·61, q, J_{gem} −14·2, J_{vic} 10·6, H 7$_{ax}$; 3·95, q, J_{gem} −14·2, J_{vic} 4·4, H 7$_{eq}$; ~4·0, m, H 6; 4·99, s, H 2. ^{13}C n.m.r. (proton coupled) (D_2O, dioxan internal standard 67·6 ppm downfield from Me_4Si): 16·0, q, *J* 131, 6-Me; 44·7, m, C 7; 49·2, m, C 6; 52·1. t, *J* 159, C 2; 157·6, s, C 8a; 162·9, s, C 4. *6-Methyl-(6,7-D_2)-5,6,7,8-tetrahydropteridin-4(3H)-one* (7) was prepared as above but by using $NaBD_4$ in D_2O and contained borate (flame test), m.p. 213–215° (dec.) [Found: C, 44·5; H+D, 7·3; N, 29·3; *m/e* 168 (100%), 153 (75%), 139·3 (metastable). $C_7H_8D_2N_4O.0·1NaH_2BO_3.0·9H_2O$ requires C, 44·0; H+D, 7·3; N, 29·5%; M^+ 168, M^+ −15 (Me) 153]. The u.v. spectra and t.l.c. properties were identical with the above and the ^{1}H n.m.r. spectrum indicated better than 95% deuteration. The concentration of aqueous solutions were determined from ϵ_{290} 9685 (pH 7·0).

6,7-Dimethylpteridin-4(3*H*)-one[13] [500 mg, ^{1}H n.m.r. (1 M DCl): 2·78, s, 7-Me or 6-Me; 2·81, s, 6-Me or 7-Me; 9·23, s, H 2] in 3M HCl (25 ml) and platinum oxide (100 mg pre-reduced in 25 ml of 3 M HCl) were shaken with hydrogen as above (5·25 h). The catalyst was filtered off and the filtrate evaporated to dryness. The colourless residue was dissolved in methanol (23 ml) and methanolic HCl (7 M, 18 ml) was added. The cis-*6,7-Dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one* (8) *hydrochloride* (500 mg) that separated on cooling, was collected and dried under vacuum, m.p. >219° (dec.) (Found: C, 36·7; H, 6·1; Cl, 25·5; N, 21·3. $C_8H_{12}N_4O.1·87HCl.0·7H_2O$ requires C, 36·7; H, 6·1; Cl, 25·5; N, 21·3%). U.v. spectrum had ϵ_{262} 6680 (pH 0·0) and ϵ_{292} 9025 (pH 7·0). ^{1}H n.m.r. (0·5 M DCl): 1·28, d, *J* 6·6, 6-Me or 7-Me; 1·35, d, *J* 6·6, 7-Me or 6-Me; 3·85 and 3·93, m, *J* 6·6 and 3·3, H 6 and 7; 8·09, s, H 2. *6,7-Dimethyl-(6,7-D_2)-5,6,7,8-tetrahydropteridin-4(3H)-one* (9) *hydrochloride*, m.p. >218° (dec.) was similarly obtained by using 3 M DCl in D_2O and D_2 gas [Found: C, 34·3; H+D, 6·6; Cl, 25·2; N, 19·7; *m/e* 182

[13] Albert, A., Brown, D. J., and Cheeseman, G., *J. Chem. Soc.*, 1952, 4219.

(87%), 167 (100), 152 (75), 138 (55). $C_8H_{10}D_2N_4O.2\,HCl.1{\cdot}4H_2O$ requires C, 34·3; H + D, 6·7; Cl, 25·3; N, 20·0%; M^+ 182, $M^+ - 15$ (Me) 167, $M^+ - 30$ (2Me) 152, $M^+ - 44$ 138]. Its ^1H n.m.r. spectrum indicated better than 95% deuterium incorporation at C6 and C7.

5,6-Diamino-2-methylpyrimidin-4(3*H*)-one sulfate[14] (0·5 g) and diacetyl (0·5 ml) in water (10 ml) was boiled under reflux for 1·5 h, the pH was adjusted to 4 and cooled. The *2,6,7-trimethylpteridin-4(3H)-one* (138 mg, 55%) that separated was washed with ethanol, dried and had m.p. >241° (dec.) (Found: C, 54·5; H, 5·3; N, 28·5. $C_9H_{10}N_4O$ requires C, 54·8; H, 5·5; N, 28·4%). ^1H n.m.r. (D_2O): 2·54, s, 2-Me; 2·66, s, 6-Me or 7-Me; 2·68, s, 7-Me or 6-Me. Platinum oxide (50 mg) in 3 M hydrochloric acid (5 ml) was pre-reduced by shaking with hydrogen at 20°/720 mmHg, the trimethylpteridinone (50 mg) was added, and hydrogenation was continued until completion (9·5 h). Filtration and evaporation of the filtrate to dryness gave a solid (70 mg) which was dissolved in 7 M methanolic hydrogen chloride and a small volume of ether was added to cause the separation of cis-*2,6,7-trimethyl-5,6,7,8-tetrahydropteridin-4(3H)-one* (10) *hydrochloride* (30 mg), m.p. 210·5–211·5° (dec.) (Found: C, 37·2; H, 6·3; Cl, 23·7; N, 19·1. $C_9H_{14}N_4O.2\,HCl.1{\cdot}4H_2O$ requires C, 37·1; H, 6·5; Cl, 23·9; N, 19·2%). ϵ_{263} 8280 (4 mM HCl). ^1H n.m.r. (D_2O): 1·26, d, *J* 6·6, 6-Me or 7-Me; 1·31, d, *J* 6·6, 7-Me or 6-Me; 2·34, s, 2-Me; 3·81, m, *J* 6·6 and 3·3, H6 and 7.

cis-6,7-Dimethyl-2-methylamino-5,6,7,8-tetrahydropteridin-4(3*H*)-one hydrochloride supplied by Dr P. Waring had ϵ_{270} 13 800 (4 mM HCl).

Syntheses of Thiotetrahydropteridines

6,7-Dimethyl-2-thioxo-2,3-dihydropteridin-4(1*H*)-one[15,16] [0·5 g, ^1H n.m.r. (2 M DCl): 2·64, s, 6-Me or 7-Me; 2·68; s, 7-Me or 6-Me (1 M NaOD): 2·47, s, 6-Me and 7-Me] in 1 M sodium carbonate (5 ml) was reduced by boiling under reflux with sodium borohydride (6 g, added in small portions) until the u.v. spectrum in 1·5 M hydrochloric acid indicated complete reduction (15 h), and cooled. The solid (0·26 g) was collected, washed with water (centrifuge) and dried. The solid (0·15 g) was dissolved in methanol (15 ml), ethanol (15 ml) was added, the solution was filtered and cooled. The yellow solid was collected, washed with small volumes of methanol and dried to give colourless cis-*6,7-dimethyl-2-thioxo-3,4,5,6,7,8-hexahydropteridin-4(1H)-one* (12) (28 mg), m.p. >259° (dec.) (Found: C, 41·7; H, 5·6; N, 23·3; S, 13·2. $C_8H_{12}N_4OS.0{\cdot}5CH_3OH.0{\cdot}1H_2O.0{\cdot}18NaH_2BO_3$ requires C, 41·6; H, 6·0; N, 22·8; S, 13·1%). ^1H n.m.r. (0·01 M NaOD): 1·07, d, *J* 6·7, 6-Me or 7-Me; 1·08, d, *J* 6·7, 7-Me or 6-Me; 3·24, octet, *J* 6·7 and 3·2, H6 or 7; 3·55, octet, *J* 6·7 and 3·2, H7 or 6. ϵ_{282} 5342 (pH 0). Reduction with platinum oxide in 3 M hydrochloric acid, methanol or trifluoroacetic acid (5–11 h) gave the 6,7-dimethyl-2-thioxo-3,4,7,8-tetrahydropteridin-4(1*H*)-one [^1H n.m.r. (5 M DCl): 1·55, d, *J* 7·1, 7-Me; 2·53, s, 6-Me; 4·94, q, *J* 7·1, H7] which was oxidized by air in the solid state and in solution at room termperature to give the parent pteridinone.

6,7-Dimethyl-2-methylthiopteridin-4(3*H*)-one, m.p. >255° (dec.) was prepared in 63% yield by boiling the preceding thioxo compound (5 g) in 0·5 M sodium hydroxide (50 ml) with methyl iodide (4·42 ml) for 15 min, cooling, filtering and recrystallizing the product from propan-1-ol [lit.[15,17] m.p. 283° (dec.) prepared by condensation of 5,6-diamino-2-methylthiopyrimidin-4(3*H*)-one and diacetyl] (Found: C, 49·0; H, 4·6; N, 25·4; S, 14·1. Calc. for $C_9H_{10}N_4OS$: C, 48·6; H, 4·5; N, 25·2; S, 14·4%). ^1H n.m.r. (0·1 M NaOD): 2·54, s, MeS; 2·60, s(br), 6-Me and 7-Me and (2 M DCl): 2·70; s, 6-Me and 7-Me; 2·83, s, MeS. Attempts to prepare the tetrahydro derivative as above required very large amounts of sodium borohydride and gave a very impure product that released methanethiol readily on attempted purification. Smaller amounts of borohydride were required when propan-1-ol was used but purification was difficult. cis-*6,7-Dimethyl-2-methylthio-5,6,7,8-tetrahydropteridin-4(3H)-one* (13) (67 mg), m.p. >115° (dec.), was obtained when the methylthiopteridinone (50 mg) in methanol (5 ml) was hydrogenated with pre-reduced platinum oxide (45 mg in 5 ml methanol) at 20°/720 mmHg (7·5 h). The catalyst was filtered off and the filtrate was evaporated (Found: N, 17·0; S, 9·5. $C_9H_{14}N_4OS.5{\cdot}6H_2O$ requires N, 17·1, S, 9·8%). ^1H n.m.r. ($CDCl_3$): 1·18, d, *J* 6·6, 6-Me or 7-Me; 1·22, d, *J* 6·6, 7-Me or 6-Me;

[14] Albert, A., Brown, D. J., Wood, H. C. S., *J. Chem. Soc.*, 1954, 3832.
[15] Schneider, H.-J., and Pfleiderer, W., *Chem. Ber.*, 1974, **107**, 3377.
[16] Gal, E. M., *J. Am. Chem. Soc.*, 1950, **72**, 3532.
[17] Angier, R. B., and Curran, W. V., *J. Am. Chem. Soc.*, 1959, **81**, 5650.

2·49, s, MeS; 3·49, octet, *J* 6·6 and 3·0, H6 or 7; 3·74, octet, *J* 6·6 and 3·0, H7 or 6. ϵ_{274} 6710 (1·5 M HCl). Similar reduction in CD_3OD and D_2 gas gave the *6,7-dideutero derivative* (14) which had >96% deuterium incorporation (by n.m.r.). Reduction with platinum oxide in trifluoroacetic acid gave *6,7-dimethyl-2-methylthio-7,8-dihydropteridin-4(3H)-one* which was isolated as the hydrochloride: ^{1}H n.m.r. (5 M DCl): 1·57, d, *J* 7·1, 7-Me; 2·58, s, MeS; 2·95, s, 6-Me; 4·99, q, *J* 7·1, H7.

2,5,6-Triaminopyrimidin-4(3*H*)-thione, prepared by direct thiation of the respective pyrimidin-4(3*H*)-one sulfate as before,[18] was very impure and was purified by placing it (2 g, in 150 ml water) on a Dowex 50W×4 (400 ml, 20–50 mesh, H^+ form) column, washing it with water (1·5 l.), 0·5 M hydrochloric acid (0·5 l.) and 1·5 M hydrochloric acid (8 l.), and was eluted with 2 M hydrochloric acid (10 l., followed by the u.v. spectral band at 310 nm). Evaporation gave a residue which was triturated with the latter solvent (*c.* 4 ml), filtered off and dried at 100° for 2 h to give the colourless *thione hydrochloride* (1·32 g), m.p. >300° (dec.) (Found: C, 21·1; H, 3·9; Cl, 30·6; N, 30·6; S, 13·8. $C_4H_3N_5S.2HCl$ requires C, 20·9; H, 3·9; Cl, 30·8%; N, 30·4; S, 13·9; lit.[18] reported the free base). This hydrochloride (200 mg) was dissolved in water (5 ml), the pH was adjusted to 4 and diacetyl (0·2 ml) was added and boiled under reflux (70 min). The solid (150 mg) that separated on cooling was collected and recrystallized from water to give *2-amino-6,7-dimethylpteridin-4(3H)-thione* (140 mg), m.p. >290° (dec.) (Found: C, 45·9; H, 4·4; N, 33·3; S, 14·5. $C_7H_9N_5S.0·15H_2O$ requires C, 45·8; H, 4·5; N, 33·4; S, 15·3%). ^{1}H n.m.r. (1 M DCl): 2·66, s, 6-Me or 7-Me; 2·68, s, 7-Me or 6-Me. The thione (50 mg) was reduced with sodium borohydride in sodium carbonate as above except that at the end of the reaction (12 h, under nitrogen) 7 M methanolic hydrogen chloride was added (ice bath) and evaporated to dryness. The residue (2·33 g) was passed through a Dowex 50W×8 (2 ml, 100–200 mesh) column, washed with 0·5 M to 1·5 M hydrochloric acid, and eluted with 2 M hydrochloric acid. Evaporation of the last eluate gave a mixture of cis- and trans-*2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3H)-thione* (11) *hydrochloride* (1·6 : 1·0 by n.m.r.) (60 mg), m.p. >211° (dec.) (Found: C, 26·3; H, 4·7; Cl, 23·4; S, 9·5. $C_8H_{13}N_5S.1·6HCl.0·8\ NaCl.0·8\ H_3BO_3$ requires C, 26·3; H, 4·7; Cl, 23·3; S, 8·8%); ^{1}H n.m.r. (2 M DCl at 270 MHz): *cis* isomer 1·33, d, *J* 6·8, 6-Me or 7-Me; 1·41, d, *J* 6·8, 7-Me or 6-Me; 3·98, m, *J* 6·8 and 3·3, H6 or 7; 4·05, m, *J* 6·8 and 3·3, H7 or 6; *trans* isomer 1·42, d, *J* 6·6, 6-Me or 7-Me; 1·53, d, *J* 6·6, 7-Me or 6-Me; 3·56, m, *J* 6·6 and 8·3, H6 or 7; 3·74, m, *J* 6·6 and 8·3, H7 or 6, assigned by proton decoupling. Catalytic reduction with platinum oxide in trifluoroacetic acid (11 h) also gave the *cis* and *trans* mixture (2·3 : 1·0 by n.m.r.) of the tetrahydro-pteridinthione hydrochloride, whereas catalytic reduction in methanol gave exclusively the *cis* isomer (by n.m.r.).

Dihydropteridine Reductase Assay

The assays were performed in cuvettes in the thermostated cell holders of a double-beam spectrometer set at 340 nm. Stock solutions containing 1 M Tris–HCl (100 μl), 2 mM potassium ferricyanide (100 μl) and water (670 μl) were placed in each cuvette. The substrate in 4 mM hydrochloric acid (100 μl, final concentration 0·1 to ~ $1·5K_m$) was added to each cuvette and allowed to equilibrate (*c.* 1 min). NADH (30 μl, with final concentrations listed in Table 1) was added simultaneously to the cuvettes by using two platinum buckets which were then moved up and down (10 times) into the assay solution to ensure complete mixing. One of the buckets also contained human brain dihydropteridine reductase[19] (3–8 μl, 0·118–0·314 μg). Soon after mixing the pen recorder was activated. The initial rates of the reaction from at least five different concentrations of pteridine substrates were calculated from the rate traces and using ϵ_{340} 6200 for the extinction coefficient of NADH. The K_m and V_{max} values (see Table 1) were computed from the initial rates and concentrations of substrates by means of the Cornish–Bowden program.[20] In preliminary runs we showed that at the highest concentrations of potassium ferricyanide and NADH the rate of oxidation of the latter was neligible compared with the enzymic rate. Also the largest non-enzymic rate of oxidation of NADH by the highest concentration of quinonoid dihydropteridine was small (<5%) compared with the enzymic rate,

[18] McCormack, J. J., and Mautner, H. G., *J. Org. Chem.*, 1964, **29**, 3370.
[19] Armarego, W. L. F., and Waring, P., *Biochem. Biophys. Res. Commun.*, 1983, **113**, 895.
[20] Cornish-Bowden, A., and Endrenyi, L., *Biochem. J.*, 1981, **193**, 1005.

and both these non-enzymic rates are compensated in the double beam of the spectrometer, i.e. the loss in NADH concentration was small and the concentration of NADH was still at or near saturating levels.

The rate of rearrangement of quinonoid dihydropteridines to the 7,8-dihydropteridines were determined by generating the quinonoid species from the respective 5,6,7,8-tetrahydropteridines with ferricyanide as above and observing the rate of change of absorbance at 340 nm, a wavelength at which the difference in ϵ values between $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ is very small compared with the total change in absorbance during the rearrangement.

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