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^{1}H-NMR and mass spectrometric studies of tetrahydropterins

Evidence for the structure of 6-pyruvoyl tetrahydropterin, an intermediate in the biosynthesis of tetrahydrobiopterin

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The conversion of dihydroneopterin triphosphate in the presence of 6-pyruvoyl tetrahydropterin synthase was followed by ^{1}H-NMR spectroscopy. The interpretation of the spectra of the product is unequivocal: they show formation of a tetrahydropterin system carrying a stereospecifically oriented substituent at the asymmetric C(6) atom. The spectra are compatible with formation of a (3′)-CH_3 function, and with complete removal of the 1′ and 2′ hydrogens of dihydroneopterin triphosphate. The fast-atom-bombardment/mass spectrometry study of the same product yields a $[M+H]^+$ ion at m/z 238 compatible with the structure of 6-pyruvoyl tetrahydropterin. The data support the proposed structure of 6-pyruvoyl tetrahydropterin as a key intermediate in the biosynthesis of tetrahydrobiopterin.

The biosynthetic pathway of tetrahydrobiopterin (BH_4) has been elucidated in its prominent features in the past few years by the convergent efforts of several groups [1–9]. The following sequence is now being widely accepted: GTP → 7,8-dihydroneopterin triphosphate (NH_2TP) → 6-pyruvoyl-5,6,7,8-tetrahydropterin (PPH_4) → 6-lactoyl-5,6,7,8-tetrahydropterin or 6-hydroxy-acetonoyl-5,6,7,8-tetrahydropterin → tetrahydrobiopterin (BH_4).

The enzymes involved have also been purified and characterized [4, 10–18]. While the chemical structures and some of the properties of these intermediates are known, the mechanisms by which 7,8-dihydroneopterin triphosphate (NH_2TP) and 6-pyruvoyl-5,6,7,8-tetrahydropterin (PPH_4) are formed have not yet been elucidated. The first step, the conversion of GTP to NH_2TP, is an apparently rather complicated process catalyzed by a single enzyme, GTP cyclohydrolase I, and involving ring opening of GTP, extrusion of a formyl equivalent, rearrangement of the ribosyl moiety and reclosure to form a six-membered ring [19, 20].

The second step also involves two chemically distinct processes, the elimination of triphosphate from the terminal (C3′) position of the C(6) side chain [13, 21–23], and an internal, Amadori-type rearrangement during which the N(5)-C(6) double bond of NH_2TP is reduced at the expense of oxidation of C(1′)–OH to C(1′)=O [3, 12, 21, 23–26]. The sequence of these two steps and the mechanistic details are not yet known.

A further major issue at the onset of our work was the chemical identity of PPH_4 and its properties. The intermediacy of this molecule in BH_4 biosynthesis had been proposed by several groups [6, 7, 23–25], but its high instability and the difficulties in obtaining substantial quantities of material have hindered structural studies. In fact, the only feasible procedure for its preparation requires purified 6-pyruvoyl tetrahydropterin synthase and purified NH_2TP [27], the latter also having to be prepared enzymatically from GTP [28]. Furthermore, the evidence presented for its structure was deduced from trapping experiments involving modifications of the pyruvoyl function such as reduction with $NaBH_4$ or enzymatically with NADPH to BH_4 [3, 4, 6, 8, 22, 23, 25, 26, 29]. In the present work we report detailed ^{1}H-NMR and fast-atom-bombardment/mass spectrometry (FAB/MS) experiments directly supporting the proposed structure. Parts of this work have been presented recently [30–32].

MATERIALS AND METHODS

Chemicals

NH_2TP was prepared from GTP using immobilized GTP cyclohydrolase I from *Escherichia coli* and purified as described by Smith and Nichol [27]. However, for reasons of stability, the DEAE-Sephacel column was developed with 0.2 M LiCl instead of an NH_4HCO_3 gradient. All other chemicals were of the best available quality.

Enzymes

GTP cyclohydrolase I was purified from *Escherichia coli* [19]. Since human PPH_4 synthase showed a much lower

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Abbreviations. BH_4, 6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin or tetrahydrobiopterin; BH_2, 6-(L-erythro-1,2-dihydroxypropyl)-7,8-dihydropterin or dihydrobiopterin; NH_2TP, 7,8-dihydroneopterin triphosphate; PPH_4, 6-pyruvoyl-5,6,7,8-tetrahydropterin; FAB, fast atom bombardment; MSMS, two-dimensional mass spectrometry.

Enzyme. GTP cyclohydrolase I (EC 3.5.4.16).

specific activity than the salmon enzyme [33], this enzyme was purified from salmon liver [34].

Instrumentation for ^{1}H-NMR and sample preparation

The ^{1}H-NMR spectra were recorded with a Bruker 250-MHz instrument at 27 °C. Suppression of the ^1H_2O signals was achieved by homo-gated decoupling. The sample was prepared using a buffer system optimized for PPH_4 synthase [34], which was obtained by dissolving ≈ 0.5 mg NH_2TP in ≈ 0.3 ml phosphate pH 7.6 containing 8 mM $MgCl_2$ and 1 mM dithioerythritol. This concentration of the latter is the lowest found to be necessary in order to prevent autoxidation of PPH_4 [35]. The ^{1}H-NMR spectra of a 50 mM solution of dithioerythritol in the same buffer show the expected signals of the non-exchangeable hydrogens. At concentrations < 1 mM, however, these signals appear to be broadened each to different extents and are barely detectable. This is attributed to paramagnetic broadening induced by R-S$^\cdot$ radicals formed by reaction of R-SH(D) with oxygen. The buffer was prepared by dissolving solid P_2O_5 in 99.9% D_2O, and adjusting the reading of a pH meter to pH 7.6 (calibration with aqueous standard buffer), using NaOD and solid anhydrous Na_2CO_3. In order to minimize the content of ^1H_2O, the enzyme PPH_4 synthase was dissolved in the deuterated buffer (≈ 2 ml) and the latter reduced to approximately 100 µl using a Centricon ultrafilter, the procedure being repeated four times. Similarly, NH_2TP, dithioerythritol and $MgCl_2$ were each dissolved and lyophilized twice in D_2O. Before addition of 0.36 unit enzyme (0.1 mg) to the sample, the latter was flushed gently with N_2.

Instrumentation for FAB and sample preparation

The instrument was a ZAB-HF mass spectrometer/11-250 J data system (VG Analytical Ltd, Manchester, UK) equipped with a saddle field atom gun (Ion Tech Ltd, Teddington, UK).

About 1 µg sample dissolved in 2 µl methanol was added to either 2 µl 1-thioglycerol, glycerol or *m*-nitrobenzyl alcohol (Fluka AG, Buchs, Switzerland) as a liquid matrix and bombarded with a beam of 10 keV xenon atoms. Single scans (mass range 20 – 500 Da with a scan rate of 10 s) were produced at full acceleration voltage (8 keV).

PPH_4 was produced by passing a mixture of 0.25 mM NH_2TP in 100 mM Tris/HCl pH 7.4 containing 8 mM $MgCl_2$ and 10 mM dithioerythritol through a PPH_4-synthase-immobilized Sepharose 4B column [28] at a flow rate of 1 ml/h. The enzyme reactor was kept in the dark at 30 °C, while the product was collected under N_2 in tubes cooled with solid CO_2

RESULTS AND DISCUSSION

^{1}H-NMR study of the conversion of NH_2TP to PPH_4

Fig. 1 A shows the ^{1}H-NMR spectrum of the starting material NH_2TP obtained from GTP by enzymatic conversion. GTP is still present at a concentration of ≤ 25% along with traces of ethanol. In our hands it has proved more convenient to use material of this quality since further purification invariably led to losses and breakdown, as indicated by the appearance of unidentified signals in the ^{1}H-NMR spectra. The signals of GTP and ethanol do not interfere with the interpretation of the data, the signal of ethanol having been used conveniently as internal standard. Further minor signals, e.g. at ≈ 3.05 ppm, are probably due to dithioerythritol, which

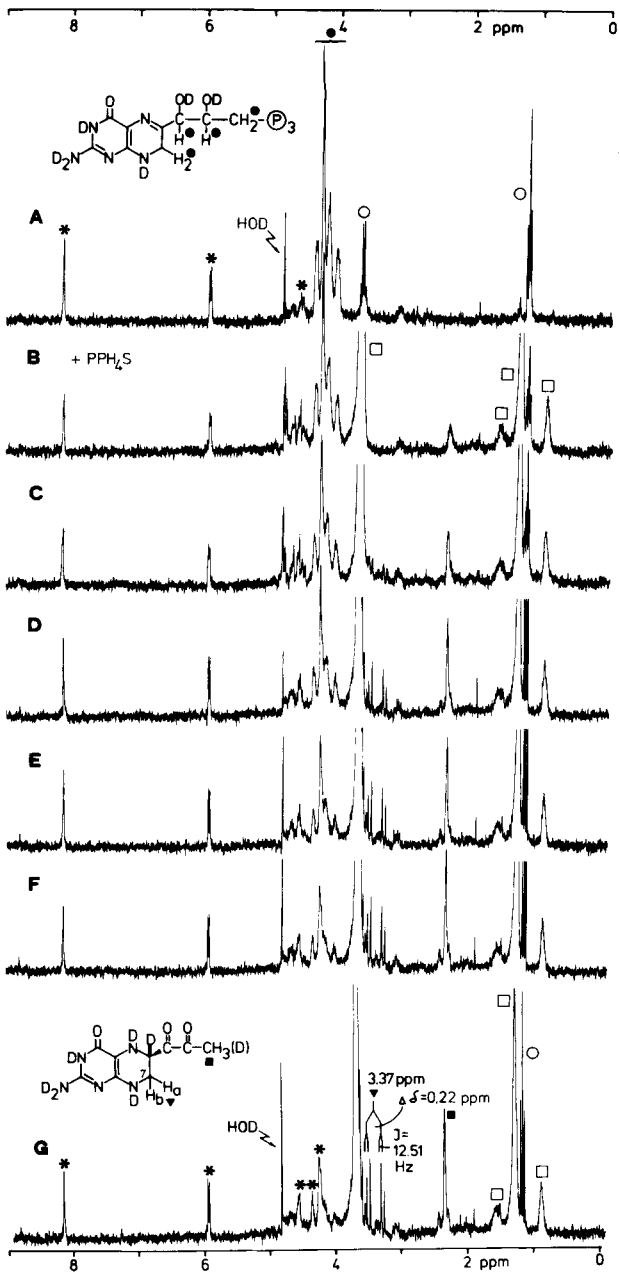


Fig. 1. *^{1}H-NMR spectrum of NH_2TP and time-dependent conversion to the product PPH_4 in the presence of PPH_4 synthase.* Spectrum A: solution of ≈ 0.5 mg NH_2TP in 0.3 ml buffer containing 8 mM $MgCl_2$ and 1 mM dithioerythritol at 27 °C, prepared as described in Materials and Methods (200 acquisitions). (*) Signals of GTP contaminant (used for the enzymatic preparation of NH_2TP); (●) signals of the nonexchangeable hydrogens of NH_2TP (see structure insert); (○) trace ethanol (impurity from the preparation of NH_2TP). Spectrum B: same as A, upon addition of 0.36 unit PPH_4 synthase (PPH_4S). Recording was initiated after ≈ 5 min and completed at ≈ 15 min, yielding an average time for the data aquisition of ≈ 10 min. (□) Signals attributed to added enzyme. Spectra C – F were obtained at 50, 90, 130, and 170 min. The final spectrum (G) was recorded after 210 min. The attribution of the (*) and (○) signals is as above. (G) (■) Signal attributed to the side-chain terminal CH_3 group of PPH_4; and (▼) AB system attributed to (7)-CH_2. For further details see text

is required in order to stabilize tetrahydropterins (unpublished results; cf. Materials and Methods).

The total activity of added PPH_4 synthase was chosen as to yield a compromise between total time required for complete

conversion and sufficient time for acquisition of a reasonable number of intermediate spectra such as shown in Fig. 1. The first spectrum (B) could be recorded after 15 min, showing essentially the signals of unmodified NH_2TP in addition to those attributed to the enzyme protein. Thereafter the signals of NH_2TP progressively disappear (C – F) and the final spectrum (G) was obtained after 210 min. This spectrum shows the following important features.

a) The signals of GTP, of ethanol and those due to the enzyme are almost unchanged, the former were thus used as standards for the estimation of signal intensities.

b) The signals of NH_2TP essentially disappeared completely.

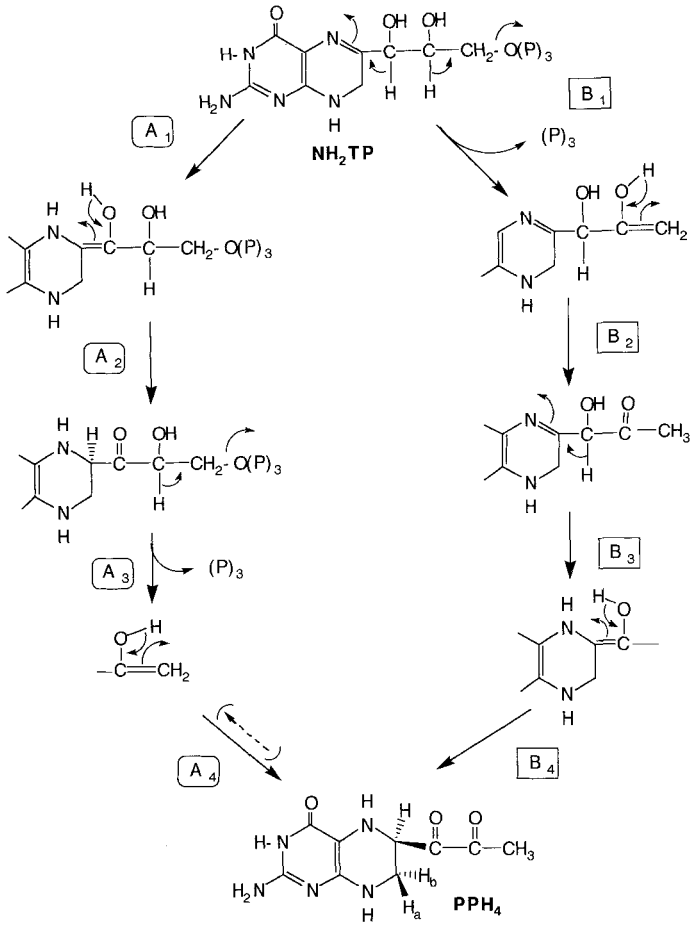
c) A main new, rather broad, signal appears at ≈ 2.3 ppm, the intensity of which corresponds approximately to 1 – 2 protons, compared to a total of 6 protons for the signals of NH_2TP (see Fig. 1 A).

d) In the region of 3.2 – 3.5 ppm a new signal can be analyzed as a typical AB system.

While there are several publications on ^{1}H-NMR spectra of pterins and biopterins at different redox states [36 – 38], we are aware of only one single report on ^{1}H-NMR of tetrahydrobiopterins in aqueous medium in the neutral pH region [39], conditions essential for studies such as those presented here. Unfortunately in this report [39] only the spectral region of 1 – 3 ppm, which provides information about the C(3′) group, was covered. We thus investigated the ^{1}H-NMR spectra of a series of dihydro- and tetrahydro-pterins in neutral aqueous solution [31], focussing on the signals of C(7) methylene and on the side-chain hydrogens. The results provide a basis for the interpretation of the spectra of the new product.

The signal at 2.3 ppm (Fig. 1 G) is typical for a CH_3 group adjacent to a carbonyl function, and coincides with the signal found at 2.34 ppm by Katoh and Sueoka for the C(3′)-CH_3 of 6-(1′-hydroxy-2′-oxo-propyl)-tetrahydropterin [39]. It is thus reasonably assigned to C(3′)-CH_3. The finding of only 40 – 60% of the expected intensity is fully compatible with earlier findings, indicating that incubations of NH_2TP with PPH_4 synthase in D_2O lead to incorporation of deuterium into the product [29]. This might originate from several processes: elimination of phosphate yields the enolate shown in Scheme 1, steps A3 or B1, and its ketonization (steps A4 or B2) can lead to incorporation of solvent hydrogen into the 3′ position. Tautomerism as in step A4 might also occur subsequent to formation of PPH_4 and could be catalyzed by PPH_4 synthase. In addition, since PPH_4 is labile under the experimental conditions used, the several low-intensity signals in the 2 – 3 ppm region are likely to arise from unidentified decay products [3, 4, 6, 8, 22, 26].

The second signal of importance is the AB system centered at 3.37 ppm (Fig. 1 G). The chemical shift, the difference in chemical shift between the H_a and the H_b signals, and the coupling constant are compatible with the presence of an asymmetric methylene function such as that at position C(7) of PPH_4 (cf. structure in Scheme 1). In fact, the C(7)-H_2 of model compounds at the tetrahydropterin level having similar C(6) side chains exhibit chemical shifts in the range 3.3 – 3.7 ppm and AB coupling constants (J_{AB}) between 12 – 13 Hz [31]. It should be pointed out that the presence of an AB signal for C(7)-H_2 demonstrates the asymmetric nature of the adjacent C(6) atom. Consequently the pterin must be in its tetrahydro form. The studies with the model compounds [31] have demonstrated that the configuration (*R* or *S*) of the C(6) side chain has strongly influenced the shape of the AB system.



Scheme 1. *Sequence of reactions involved in the conversion of NH_2TP to PPH_4.* Reactions A1 to A4 are initiated by the internal redox shuttle, while B1 to B4 start with elimination of triphosphate, written as $(P)_3$. See text for details

The finding of only a single AB system might appear trivial at first sight, it does, however, establish two points. First, protonation of the C(6)-C(1′) double bond in steps (A2) or (B4) must occur enzymatically since it is side-specific and forms the (6*R*)-derivative. This follows from the fact that in BH_4 the side chain has the 6*R* stereochemistry [40, 41]. Secondly, the absence of further similar AB systems excludes the occurrence in measurable quantities of intermediates such as that formed via step A3 during the transformation of NH_2TP to PPH_4.

A further question of mechanistic importance is whether sequences A or B (Scheme 1) occur. From a chemical point of view both possibilities are feasible, since the first steps (A1, A2 or B1, B2) lead to two intermediates in which a reactive C-H group is further acidified by introduction of a flanking keto function, thus facilitating the subsequent steps. Close inspection of the intermediate spectra B – F in Fig. 1 provides some information. In (B) the signal at 2.3 ppm [C(3′)-CH_3] is already apparent, while the AB signal around 3.4 ppm has not yet appeared. In (C) the 2.3 ppm signal has reached $\approx 50\%$, while the AB one shows barely one third of its final intensity. Clearly, the signal at 2.3 ppm could have been introduced with the protein, but this is rather improbable, since it would imply that the entire reaction has a lag phase of up to 15 min, and since the NH_2TP signals have already decreased in intensity at this point in time. A plot of the time dependence

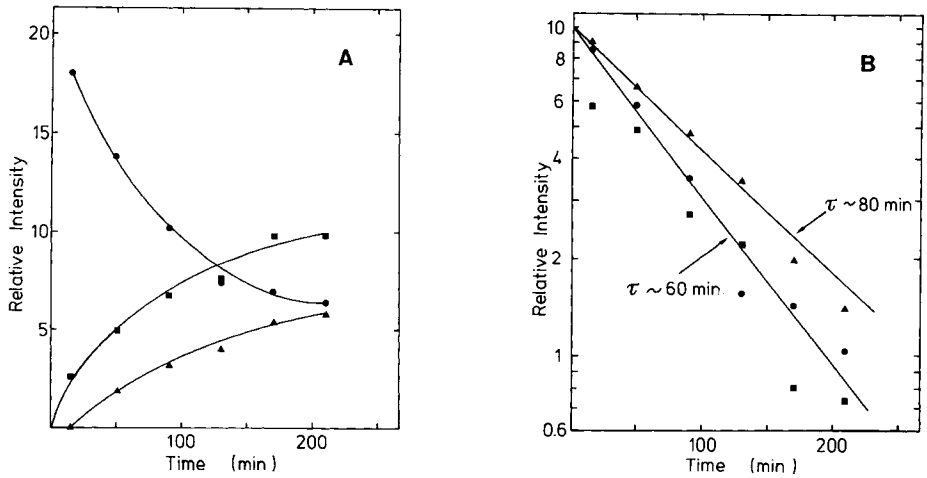


Fig. 2. *Time dependence of the intensity of some of the signals displayed in Fig. 1.* The symbols are the same as those used in Fig. 1 and reflec the intensity of the signals at 2.3 (■), 3.3 (▼), and 4.2 (●) ppm. The values shown were estimated from the peak height of these signals anc were normalized using the signals of GTP, the intensity of which was assumed to be constant during measurement. (A) Linear plot, (B logarithmic plot

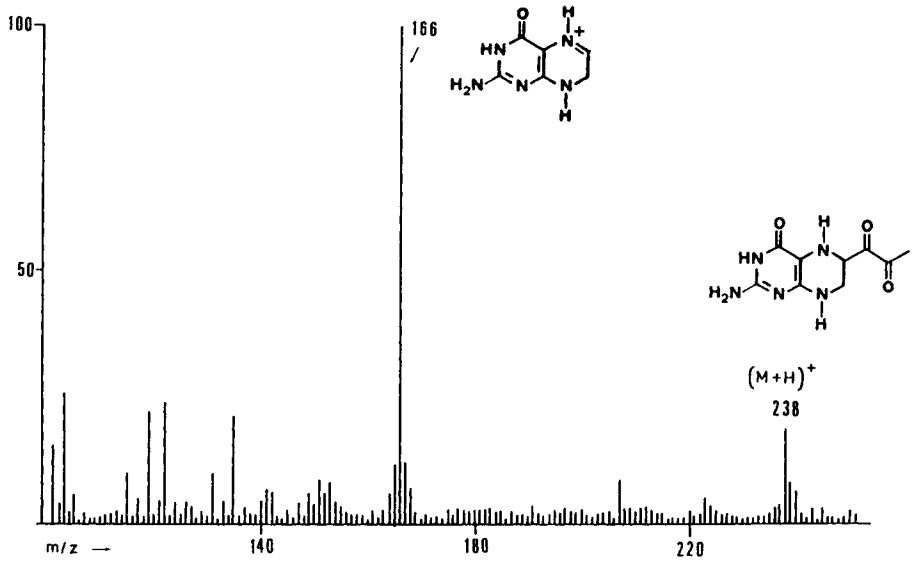


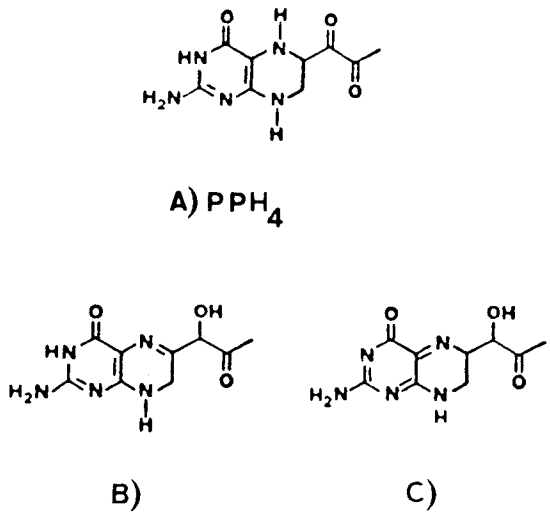
Fig. 3. *FAB spectrum of* PPH_4 (matrix thioglycerol). See text for details

of relative intensities is shown in Fig. 2 where it can be seen that the rate at which the NH_2TP signals disappear correlates with the appearance of the 2.3-ppm peak, while the rise of the AB system is somewhat slower. Taken at face value, this is in favor of the series of reactions (B1) to (B4) in Scheme 1 and agrees with the absence of additional AB signals. We would like to point out, however, that while the same trend was observed in different experiments, the quality of our data do not allow an unambiguous differentiation. The main reason for this is the uncertainty in the estimation of signal intensities, which can be problematic in such experiments; the somewhat varying resolution and peak broadening observed, e.g. in spectra (B) and (C), demonstrates the point. Such effects were observed at various occasions and also during the ^{1}H-NMR study of model compounds [31]; they probably arise from the autoxidation of tetrahydropterin species through traces of oxygen, which forms radical species leading to line broadening.

FAB/MS studies on the structure of PPH_4

In view of the low volatility, of the instability, and of the failure of conventional MS methods, FAB/MS appeared to be the method of choice for the study of the conversion product of NH_2TP. Samples of PPH_4 were obtained from the enzyme reactor and analyzed as described in Materials and Methods. The positive ion spectrum obtained using a thioglycerol liquid matrix is shown in Fig. 3; it reveals an $[M+H]^+$ ion with a relative intensity of 20% corresponding to a molecular mass of 237 Da and to the elemental composition $C_9H_{11}N_5O_3$.

The base peak at *m/z* 166 is ascribed to the $[M+H]^+$ ion of 7,8-dihydropterin ($C_6H_7N_5O$, *m* 165 Da) generated by decomposition of the only moderately stable PPH_4 either before or during analysis. The alternative route of formation, i.e. unimolecular gas-phase fragmentation of the intact protonated PPH_4 via loss of $O=C=C(OH)CH_3$, appears very unlikely since this process is of only minor importance in



Scheme 2. *Comparison of structures of different C(6)-substituted pterins having a common molecular mass*

the metastable ion decomposition under MSMS conditions [32].

Among the related compounds of special interest sepiapterin yields a $[M+H]^+$ ion at *m/z* 238 of also about 20% relative intensity. On the other hand, analysis of the parent biopterin required the use of *m*-nitrobenzyl alcohol as the liquid matrix [42] to produce an $[M+H]^+$ ion of about 10% relative intensity. This signal, at a 'chemical noise' level of about 5%, appeared to be of no use until MSMS was attempted. Analysis of (*o*-quinonoid) dihydrobiopterin (BH_2) was also found to be difficult as this molecule is probably converted to BH_4 as a stable product (conversion: *m/z* 240 → 242) during analysis. This assumption is supported by the ease of analysis of BH_4 under similar conditions, which yields the $[M+H]^+$ *m/z* 242 as the base peak. Due to the lack of well defined fragmentation patterns, the direct FAB/MS method did not allow more precise structural deductions in this case. In particular a differentiation between different isomeric structures of *m* 237 Da was not possible (a preliminary attempt towards differentiation by FAB/MSMS has been presented [32]). Sepiapterin and dihydrobiopterin can be ruled out since their chromophore is incompatible with the observed ultraviolet absorption [43]. Thus, the structure assignment of PPH_4 has two major competitors, B and C, as shown in Scheme 2. The sepiapterin tautomer (B) can also be excluded because of its discrepant chromophore [43]. The quinonoid structure C might be compatible with the absorption spectra in the ultraviolet; this is very unlikely, however, since its FAB/MS behaviour contrasts sharply with that of quinonoid BH_2, which rapidly takes up two hydrogen atoms prior or during FAB analysis, whereas in the case of PPH_4 such hydrogen uptake was not observed (Fig. 3).

In conclusion, the ^{1}H-NMR and the FAB/MS data can be safely interpreted as supporting the proposed structure for PPH_4. They are in line with the various trapping experiments reported by others [3, 4, 23, 29] and with the ultraviolet absorption spectrum reported for PPH_4 [8, 22], and demonstrate the utility of such methods for the structural elucidation and analysis of sensitive natural products.

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REFERENCES

1. Brown, G. M. (1986) in *Chemistry and biology of pteridines 1986* (Cooper, B. A. & Whitehead, V. M., eds) pp. 183–184, Walter de Gruyter, Berlin.
2. Milstien, S. & Kaufman, S. (1986) in *Chemistry and biology of pteridines 1986* (Cooper, B. A. & Whitehead, V. M., eds) pp. 169–181, Walter de Gruyter, Berlin.
3. Smith, G. K. & Nichol, C. A. (1986) *J. Biol. Chem. 261*, 2725–2737.
4. Milstien, S. & Kaufman, S. (1985) *Biochem. Biophys. Res. Commun. 128*, 1099–1107.
5. Heintel, D., Leimbacher, W., Redweik, U., Blau, N., Niederwieser, A., Curtius, H.-Ch. & Ghisla, S. (1985) in *Biochemical and clinical aspects of pteridines*, vol. 4 (Wachter, H., Curtius, H.-Ch. & Pfleiderer, W., eds) pp. 151–158, Walter de Gruyter & Co., Berlin, New York.
6. Masada, M., Akino, M., Sueoka, T. & Katoh, S. (1985) *Biochim. Biophys. Acta 840*, 235–244.
7. Heintel, D., Ghisla, S., Curtius, H.-Ch., Niederwieser, A. & Levine, R. A. (1984) *Neurochem. Int. 6*, 141–155.
8. Switchenko, A. C., Primus, J. P. & Brown, G. M. (1984) *Biochem. Biophys. Res. Commun. 120*, 754–760.
9. Smith, G. K. & Nichol, C. A. (1984) *Biochem. Biophys. Res. Commun. 120*, 761–766.
10. Blau, N. & Niederwieser, A. (1986) *Biochim. Biophys. Acta 880*, 26–31.
11. Ferre, J., Yim, J. J. & Jacobson, K. B. (1986) *J. Chromatogr. 357*, 283–292.
12. Takikawa, S., Curtius, H.-Ch., Redweik, U., Leimbacher, W. & Ghisla, S. (1986) *Eur. J. Biochem. 161*, 295–302.
13. Heintel, D., Leimbacher, W., Redweik, U., Zagalak, B. & Curtius, H.-Ch. (1985) *Biochem. Biophys. Res. Commun. 127*, 213–219.
14. Smith, G. K. (1987) *Arch. Biochem. Biophys. 255*, 254–266.
15. Sueoka, T. & Katoh, S. (1982) *Biochim. Biophys. Acta 717*, 265–271.
16. Curtius, H.-Ch., Steinerstauch, P., Leimbacher, W., Redweik, U., Takikawa, S. & Ghisla, S. (1987) in *Unconjugated pterins and related biogenic amines* (Curtius, H.-Ch., Blau, N. & Levine, R. A., eds) pp. 89–98, Walter de Gruyter, Berlin, New York.
17. Nakanishi, N., Yoshida, A., Ozawa, K. & Yamada, S. (1986) in *Enzyme 35* (Bachmann, C., Colombo, J. P., Eppenberger, H., Greengard, O., Sperling, O. & Wiesmann, U., eds) pp. 42–52, S. Karger AG, Basel.
18. Frigaira, F. A., Cotton, R. G. H. & Danks, D. M. (1981) *Biochem. J. 197*, 31–43.
19. Yim, J. J. & Brown, G. M. (1976) *J. Biol. Chem. 251*, 5087–5089.
20. Blau, N. & Niederwieser, A. (1985) *J. Clin. Chem. Clin. Biochem. 23*, 169–176.
21. Ghisla, S., Curtius, H.-Ch. & Levine, R. A. (1984) in *Biochemical and clinical aspects of pteridines*, vol. 3 (Pfleiderer, W., Wachter, H. & Curtius, H.-Ch., eds) pp. 35–52, Walter de Gruyter & Co., Berlin, New York.
22. Curtius, H.-Ch., Leimbacher, W., Niederwieser, A. & Ghisla, S. (1985) in *Biochemical and clinical aspects of pteridines*, vol. 4 (Wachter, H., Curtius, H.-Ch. & Pfleiderer, W., eds) pp. 133–141, Walter de Gruyter & Co., Berlin, New York.
23. Switchenko, A. C. & Brown, G. M. (1985) *J. Biol. Chem. 260*, 2945–2951.
24. Milstien, S. & Kaufman, S. (1983) *Biochem. Biophys. Res. Commun. 115*, 888–893.
25. Smith, G. K., Cichetti, J. A., Chandrasurin, P. & Nichol, C. A. (1985) *J. Biol. Chem. 260*, 5221–5224.
26. Quang, L. V., Katzenmeier, G., Schwarzkopf, B., Schmid, C. & Bacher, A. (1988) *Biochem. Biophys. Res. Commun. 151*, 512–517.
27. Smith, G. K. & Nichol, C. A. (1983) *Arch. Biochem. Biophys. 227*, 272–278.

28. Blau, N., Niederwieser, A., Redweik, U., Schoedon, G. & Leimbacher, W. (1988) *Pteridines and related biogenic amines* (Levine, R. A., Milstien, S., Kuhn, D. & Curtius, H.-Ch., eds) pp. 97–106, Lakeshore Publishing Co, Grosse Pointe.
29. Curtius, H.-Ch., Heintel, D., Ghisla, S., Kuster, T., Leimbacher, W. & Niederwieser, A. (1985) *Eur. J. Biochem. 148*, 413–419.
30. Ghisla, S., Takikawa, S., Steinerstauch, P., Hasler, T. & Curtius, H.-Ch. (1986) in *Chemistry and biology of pteridines 1986* (Cooper, B. A. & Whitehead, V. M., eds), pp. 299–303, Walter de Gruyter, Berlin, New York.
31. Ghisla, S., Steinerstauch, P., Hasler, T., Blau, N. & Curtius, H.-Ch. (1987) in *Unconjugated pterins and related biogenic amines* (Curtius, H.-Ch., Blau, N. & Levine, R. A., eds) pp. 67–79, Walter de Gruyter, Berlin, New York.
32. Richter, W. J., Raschdorf, F., Dahinden, R., Kuster, T., Niederwieser, A., Leimbacher, W. & Curtius, H.-Ch. (1987) in *Unconjugated pterins and related biogenic amines* (Curtius, H.-Ch., Blau, N. & Levine, R. A., eds) pp. 39–45, Walter de Gruyter, Berlin, New York.
33. Hasler, T. & Niederwieser, A. (1986) in *Chemistry and biology of pteridines 1986* (Cooper, B. A. & Whitehead, V. M., eds) pp. 319–322, Walter de Gruyter, Berlin, New York.
34. Hasler, T. & Curtius, H.-Ch. (1989) *Eur. J. Biochem. 180*, 205–211.
35. Reference deleted.
36. Schircks, B., Bieri, J. H. & Viscontini, M. (1978) *Helv. Chim. Acta 61*, 2731–2738.
37. Viscontini, M., Provenzale, R., Ohlgart, S. & Mallevialle, J. (1970) *Helv. Chim Acta 53*, 1202–1207.
38. Armarego, W. L. F. & Shou, H. (1977) *J. Chem. Soc. Perkin I*, 2529–2536.
39. Katoh, S. & Sueoka, T. (1987) *J. Biochem. (Tokyo) 101*, 275–278.
40. Bailey, S. W. & Ayling, J. E. (1978) *J. Biol. Chem. 253*, 1598–1605.
41. Furrer, H.-J., Bieri, J. H. & Viscontini, M. (1979) *Helv. Chim. Acta 62*, 2577–2580.
42. Meili, J. & Seibl, J. (1984) *Org. Mass Spectrom. 19*, 581–582.
43. Pfleiderer, W. (1978) *J. Inherited Metab. Dis. 1*, 54–60.