198. ^{15}N-NMR. Spectra of Pterins and Folic Acid Derivatives[1])

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Summary

^{15}N-Chemical shifts of a series of 5,6,7,8-tetrahydropterins, 7,8-dihydropterins and pterins have been measured in acidic solution by means of a probe for 20 mm sample tubes. Included are the relevant data of folic acid (**11**), 5,6,7,8-tetrahydrofolic acid (**5**) and *N*(5,10)-metheno-5,6,7,8-tetrahydrofolic acid (**6**). The different oxidation states are clearly reflected in the chemical shifts of N(5) and N(8). Assignment of the nitrogen resonances was achieved by protonation effects (discrimination between N(1) and N(3)) and with the aid of alkyl substitution at C(6) and C(7), to distinguish between N(5) and N(8).

Introduction. – ^{1}H- and ^{13}C-NMR. spectroscopy have proven useful as non-destructive tools in structure determination and conformational analysis of pterins, folic acid and their 7,8-dihydro- and 5,6,7,8-tetrahydro-derivatives [3–5]. Since the biological activity of this class of compounds is largely associated with the nitrogen atoms, ^{15}N-NMR. data are of particular interest and promise further insight into the structure of these molecules in solution.

The solubility of many of the title compounds, however, is limited and, hence, observation of ^{15}N-spectra in natural isotope abundance with standard instrumentation in most cases exceeds an acceptable time. In addition, the instability of some of the hydrogenated pterins does not permit very long acquisition times. ^{15}N-isotope enrichment, on the other hand, is rather difficult from the preparative point of view. Recent advances in instrumentation, however, have facilitated the observation of ^{15}N-NMR. spectra in natural abundance [6]. Besides the application of higher field strengths, large diameter sample probes have helped to achieve a considerable increase in the signal-to-noise ratio [7]. This has led us to the construction of a probe for 20 mm o.d. sample tubes, which is used in a *Varian* XL-100-15 spectrometer system. The gain in sensitivity as compared with the standard 12 mm probe is of the order of 3–4, thus reducing the experimental time for accumulation of spectral information by a factor of 10–20. This was essential in the study of the title compounds. As an example, the spectrum of 6,7-dimethyl-5,6,7,8-tetrahydropterin dication (**1**) is illustrated in *Figure 1.*

[1]) ^{15}N-NMR.- spectroscopy, part 4; part 3: [1]. Pterins, part 66; part 65 [2].

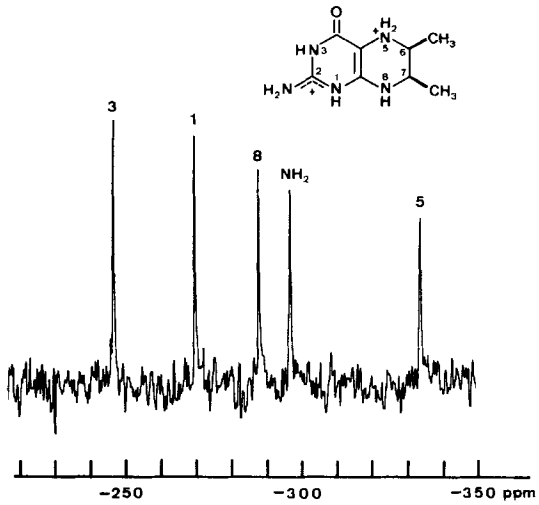
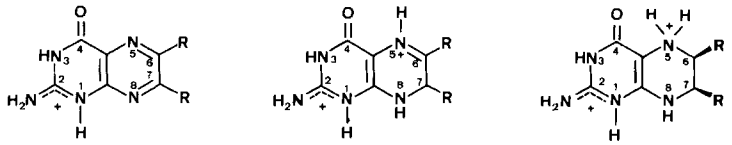


Fig. 1. ^{15}N-*spectrum of* **1** *in* CF_3COOH (0.7M) proton noise-decoupled, 3,500 pulses

Results. – The structures of the protonated title compounds *(Scheme 1)* are well established by ^{1}H- and ^{13}C-NMR. data [3] [5a, b], pK_a measurements [8], and X-ray crystallographic results [9] [10].

Scheme 1. *Structures of pterins, dihydro- and tetrahydropterins in TFA*

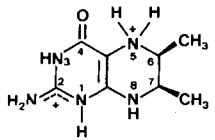
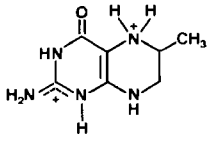
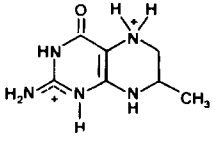
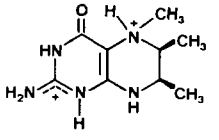
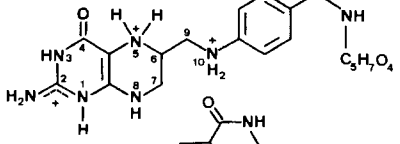
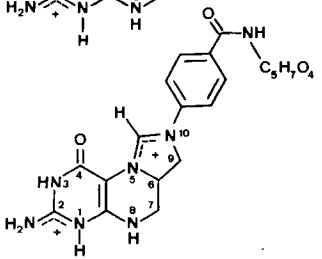


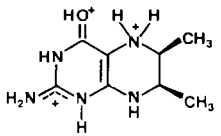
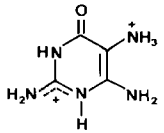
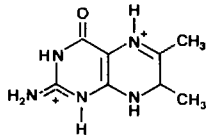
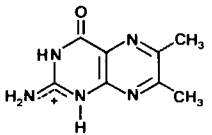
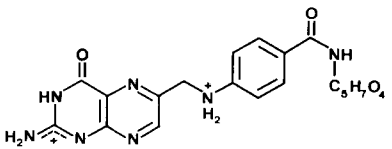
Most protons attached to the nitrogen atoms are susceptible to ready exchange with solvent protons. Assignment techniques based on $^{15}N, H$-coupling constants are, therefore, not very promising. This leaves us with the assignment by spectral comparison which requires that all compounds are subjected to the same kind of medium effects. In particular, protonation states and tautomeric forms should be the same. Trifluoroacetic acid (TFA) was the solvent of choice, because all compounds of interest were soluble in concentrations >0.5 M and stable over the observation period (1–5 h). Their structures in TFA correspond to those given in *Scheme 1.* All signals of dihydro- and tetrahydropterins exhibit nuclear *Overhauser* enhancement upon proton irradiation, whereas the pyrazine N-atoms of the pterins could only be detected with the inverse decoupler gating technique [11].

The chemical shifts, relative to nitromethane as an external standard, are summarized in *Table 1.* The negative sign denotes that the corresponding N-atom absorbs at lower frequency than the standard molecule.

Characteristic changes in the chemical shifts of 5,6-dimethyl-5,6,7,8-tetrahydropterin (**1**) were observed for different protonation states *(Tab. 2).*

Table 1. ^{15}N-*Chemical shifts*[a]) *of 5,6,7,8-tetrahydropterins, 7,8-dihydropterins and pterins*

Compound		N(1)	N(3)	N(5)	N(8)	NH_2	N(10)	Amide	Solvent
	(**1**)	−269.4	−246.3	−333.2	−287.4	−296.5			TFA
	(**2**)	−269.0	−246.5	−332.9	−299.6	−297.5			TFA
	(**3**)	−268.1	−247.7	−346.3	−286.1	−297.3			TFA
	(**4**)	−270.1	−246.2	−330.6	−288.8	−295.9			TFA
	(**5**)	−267.7	−242.8	−335.5	−303.6	−295.5	−319.7	−261.4	6N HCl
	(**6**)	−268.3	−245.5	−251.3[b])	−300.7	−298.1	−245.5[b])	−262.1	TFA

Compound		N(1)	N(3)	N(5)	N(8)	NH_2	N(10)	Amide	Solvent
	(7)	−263.2[b])	−257.9[b])	−337.2	−265.2[b])	−288.1			FSO_3H
	(8)	−255.8	−242.8	−350.0	−302.9	−298.3			H_2O (dihydrochloride)
	(9)	−266.5	−245.6	−194.1	−294.0	−296.3			H_2O (dihydrochloride)
	(10)	−262.3	−236.7	−84.5[b])	−79.3[b])	−296.7			TFA
	(11)	−264.1	−238.0	−78.5	−58.3	−294.8	−325.5	−262.2	TFA

[a]) δ [ppm] rel. to ext. CH_3NO_2.
[b]) Assignment arbitrary.

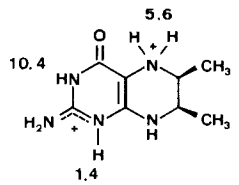
Table 2. *^{15}N-Chemical shifts*[a]) *of 6,7-dimethyl-5,6,7,8-tetrahydropterin*

Species	N(1)	N(3)	N(5)	N(8)	NH_2
Monocation in DMSO (Dihydrochloride + 1 mol-equiv. NaOH)	−227.9	−240.1	−332.1	−288.0	−298.8
Addition of					
0.2 mol-equiv. CF_3COOH	−230.5	−240.2	−332.3	−288[b])	−298.5
0.4 mol-equiv. CF_3COOH	−235.5	−239.9	−332.1	−288.3	−297.8
0.6 mol-equiv. CF_3COOH	−238.3	−240.2	−333[b])	−289.2	−297.6
2.0 mol-equiv. CF_3COOH (+ 1 ml H_2O)[c])	−243.3	−240.9	−333.5	−288.1	−298.1
2.0 mol-equiv. CF_3COOH + 1 ml H_2O + 1 ml 6N HCl	−256.7	−240.9	−333.5	−288.6	−296.0
Dihydrochloride in DMSO	−249.3	−241.0	−332.8	−289.6	−296.7

[a]) δ [ppm] rel. to ext. CH_3NO_2.
[b]) Broadened.
[c]) H_2O added to increase solubility.

Discussion. – 1. *5,6,7,8-Tetrahydropterins.* The chemical shift assignment of N(5) and N(8) in the spectrum of 6,7-dimethyl-5,6,7,8-tetrahydropterin dication (**1**) is based on a comparison with the 6-methyl- and 7-methyltetrahydropterins **2** and **3**. The chemical shifts of N(1), N(3) and the NH_2 group are scarcely influenced by different methyl substitution at C(6) and C(7). In contrast, N(5) and N(8) show pronounced substituent effects. δ (N(8)) in 6,7-dimethyltetrahydropterin (**1**) differs from δ (N(8)) in 6-methyltetrahydropterin (**2**) by a 12.2 ppm shift towards higher frequencies. A comparable β-substituent effect [12] of 13.1 ppm is found for δ (N(5)) in 6,7-dimethyltetrahydropterin (**1**) relative to 7-methyltetrahydropterin (**3**). The corresponding γ-substituent effects [12] are comparatively small (1.3 and 0.2 ppm). From the remaining 3 nitrogen resonances in **1**, the one at lowest frequencies is assigned to the NH_2 group. Its value of −296.5 ppm is reproduced within 2 ppm in all cases where the pyrimidine moiety has the tautomeric form given in *Scheme 1*. In addition, it compares well with the value for guanosine in DMSO/TFA (−296.8 ppm) [6a]. The discrimination between N(1) and N(3) was made with the aid of their protonation behaviour. The pK_a values of 6,7-dimethyl-5,6,7,8-tetrahydropterin dihydrochloride are given in *Scheme 2* [8].

Scheme 2. *pK_a values of 6,7-dimethyl-5,6,7,8-tetrahydropterin dication* (**1**)



Addition of one mol-equivalent NaOH to the dihydrochloride will thus abstract the proton at position N(1). A comparison of the ^{15}N-NMR. spectra of the dication (in TFA, *Tab. 1*) and the monocation (in DMSO with one mol-equivalent NaOH, *Tab. 2*) reflects this very clearly. Whereas the chemical shifts of 4 N-atoms remain

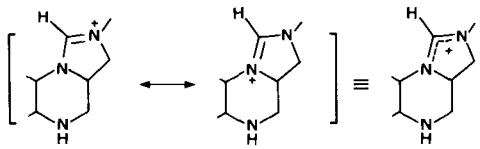
fairly constant, one line is shifted towards higher frequencies by about 40 ppm. This resonance is assigned to N(1). It is shifted back towards lower frequencies upon addition of increasing amounts of TFA and finally aqueous HCl-solution. The chemical shift for N(1) thus obtained (−256.7 ppm) and the value observed for the dihydrochloride in DMSO (−249.3 ppm) when compared with the TFA solution (−269.4 ppm) indicate the presence of N(1),N(5)-diprotonated and N(5)-monoprotonated substrate in rapid prototropic equilibrium.

α-Methyl substitution is generally not a reliable assignment criterion for protonated amines [12]. The α-substituent shift (~8 ppm for neutral species) rarely exceeds 2–3 ppm and is, therefore, of the magnitude of solvent shifts. This is demonstrated by comparing the data of 5,6,7-trimethyltetrahydropterin dication (**4**) and 6,7-dimethyltetrahydropterin dication (**1**).

Starting from the assigned spectrum of 6-methyltetrahydropterin dication (**2**), the analysis of the spectrum of 5,6,7,8-tetrahydrofolic acid in hydrochloric acid (trication **5**) is straightforward. The resonances of the pterin moiety do not deviate by more than 4 ppm from those of **2**. The amide resonance lies at −261.4 ppm, the *p*-aminobenzoic acid N-resonance at −319.7 ppm.

N(5,10)-metheno-5,6,7,8-tetrahydrofolic acid is of considerable biological interest because of its coenzyme status [13]. The nitrogen atoms N(5) and N(10) participate in an imidazolinium system as represented by the canonical formulae of *Scheme 3*.

Scheme 3



Only 6 of the 7 N-resonances are observed in the spectrum of the dication **6**. It seems unlikely that the missing N-resonance is lost because of signal nulling due to unfavorable NOE, since several spectra have been measured under different conditions. It is much more probable, by comparison of signal intensities, that the missing line (N(5) or N(10)) coincides with the N(3) resonance. Five chemical shifts are very similar to those of 5,6,7,8-tetrahydrofolic acid. The resonance at −251.3 ppm (either N(5) or N(10)) is in the range of imidazolinium resonances [14], as expected for structure **6**.

2. *7,8-Dihydropterins.* 6,7-Dimethyl-7,8-dihydropterin, unlike 7,8-dihydrofolic acid, is stable in TFA solution. It is known from X-ray data that in the monocationic form N(5) is protonated rather than N(1) [10]. The ^{15}N-NMR. spectral data suggest that in TFA solution the N(1),N(5)-dication **9** is formed because the resonances of the pyrimidine moiety compare well with those of the pterin and tetrahydropterin dications. The N(5) shift is rather typical for an iminium-type N-resonance [14], and a full NOE enhancement is observed.

3. *Pterins.* 6,7-Dimethylpterin monocation (**10**) exhibits an NH_2 chemical shift very similar to its 5,6,7,8-tetrahydro derivative **1**. The resonances of N(1) and N(3) are both shifted to higher frequencies, probably as a consequence of the adjacent aromatic pyrazine ring. The assignment of N(5) and N(8) is arbitrary.

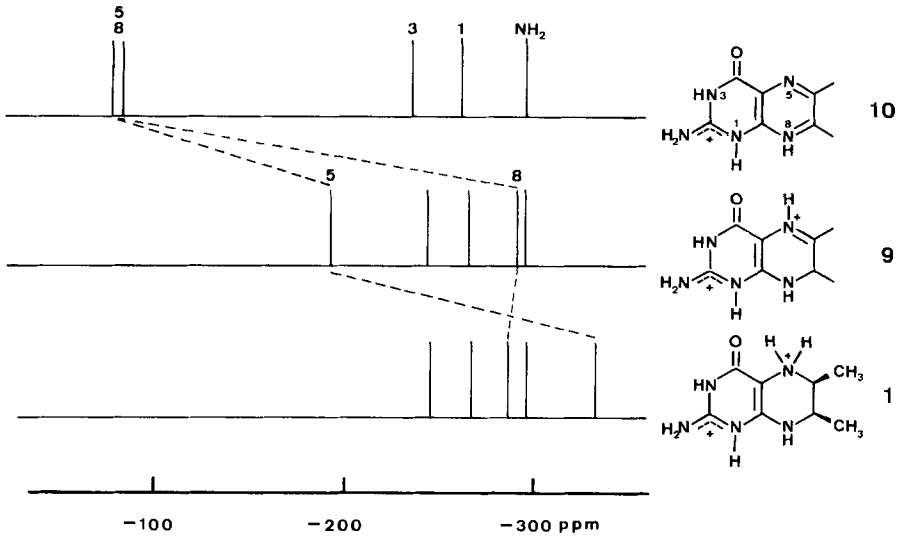


Fig. 2. *Schematic* ^{15}N*-spectra* (in TFA) *of the pterin* **10**, *the 7,8-dihydropterin* **9** *and the 5,6,7,8-tetrahydropterin* **1**

In folic acid, the difference in the chemical shifts between the 2 aromatic N-atoms N(5) and N(8) is much more pronounced. Assuming that the different shifts for each of the 2 N-atoms in 6,7-dimethylpterin (**10**) and folic acid (**11**) are caused by β- and γ-substituent effects, the high-frequency signal (−58.3 ppm) can be assigned to N(8), the one at −78.5 ppm to N(5). This assumption holds in comparison with substituted pyridines where the increments for β- and γ-substitution are 9 ppm and 4 ppm respectively, towards lower frequencies. It should be noted that the sign of the β-substituent effect in the pyrazine ring is opposite to the corresponding effect in the tetrahydropyrazine ring. The rest of the signals of **11** are readily assigned on the basis of the already mentioned data.

A comparison of the N-chemical shifts in TFA of 6,7-dimethylpterin (**10**), its 7,8-dihydro (**9**) and 5,6,7,8-tetrahydro (**1**) derivatives is given in *Figure 2*. The resonances of the pyrimidine part are scarcely influenced, whereas those of the pyrazine ring show large low-frequency shifts in the dihydro and tetrahydro-derivatives.

In conclusion, ^{15}N chemical shift data are a valuable new parameter to characterize the structure of pterins and, in particular, the different oxidation and protonation states.

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Experimental Part

The ^{15}N-NMR. spectra were measured on a *Varian* XL-100-15 spectrometer equipped with a home-built probe head for 20 mm o.d. sample tubes. The new probe was constructed using a *Varian* 4415 probe and removing the *Dewar* part. Receiver coil, spinner turbine and matching networks were

redesigned. A single coil configuration was chosen to obtain a shorter 90° pulse length. The line-width of the nitrate signal, taken as a measure for resolution, is less than 0.6 Hz and compares well with the commercial 12 mm probe. Sensitivity is better by a factor of 3–4. An external fluorine lock accessory is used for field-frequency stabilization. The probe requires a minimal sample volume of 7.5 ml. Probe temperature is 40° under proton noise-decoupling conditions.

The chemical shifts were determined relative to external $NH_4{}^{15}NO_3$ and then converted to the proposed nitromethane standard [15]. Typical acquisition parameters include a spectral width of 5,000 Hz, 0.4 s acquisition time, and pulse delays of 4 s at a 40° flip angle. Noise-decoupled spectra of approximately 0.7M solutions require 1,000 free induction decays for protonated N-atoms.

The synthesis and purification of the compounds investigated are described: **1** [16] [17], **2** [17], **3** [18], **4** [4a], **5** [5c], **6** [19], **7** [16] [17], **8** [20], **9** [21], **10** [21], and **11** [22].

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