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Determination of the relative configuration of 5,6,7,8-tetrahydromethanopterin by two-dimensional NMR spectroscopy

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The relative configuration of the pterin moiety of 5,6,7,8-tetrahydromethanopterin **1**, a coenzyme isolated from methanogenic archaea, has been determined by two-dimensional NMR spectroscopy of N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin **2** to be rel-(6R; 7S; 11R). The complete proton resonance assignment of the pterin moiety of N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin **3** is described including the relative stereospecific assignment of the C(14a) methylene protons.

5,6,7,8-Tetrahydromethanopterin; Relative configuration; Heteronuclear two-dimensional NMR; Molecular modelling; Methanogenic archaea; *Methanobacterium thermoautotrophicum*

1. INTRODUCTION

5,6,7,8-Tetrahydromethanopterin **1** serves as a carrier of C_1-fragments in the metabolism of methanogenic archae [1], being an analog of tetrahydrofolic acid, which is utilized by higher organisms as a C_1 carrier [2]. In the reaction pathway of methanogenesis, CO_2 is reduced to methane in a stepwise manner [3,4]. In the course of this reaction sequence, N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin **2** as well as N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin **3** are observed as intermediates [5]. In contrast to tetrahydrofolic acid [6,7], neither the absolute nor the relative configuration of the pterin moiety of **1** have been established to date. In this communication we report the relative configuration of **1** as determined by 2D NMR of its derivative **2**.

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Abbreviations: NMR, nuclear magnetic resonance spectroscopy; TOCSY, total correlation spectroscopy; (DQF-)COSY, (double quantum filtered) correlated spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy.

2. MATERIALS AND METHODS

5,6,7,8-Tetrahydromethanopterin **1** was isolated from *Methanobacterium thermoautotrophicum* [8]. N^5,N^{10}-Methenyl-5,6,7,8-tetrahydromethanopterin **2** was prepared by enzymatic dehydrogenation of N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin **3** [9], which was synthesized from 5,6,7,8-tetrahydromethanopterin and formaldehyde by spontaneous reaction [5]. [*methylene*-^{13}C]-N^5,N^{10}-Methylene-5,6,7,8-tetrahydromethanopterin was synthesized from 5,6,7,8-tetrahydromethanopterin and [^{13}C]formaldehyde (99% ^{13}C; 20% w/w in water; Cambridge Isotope Laboratories, MA, USA). The compounds were purified to apparent homogeneity by anaerobic high performance liquid chromatography on LiChrospher 100 RP-18 (4 mm × 125 mm column, Merck, Darmstadt, Germany) in 25 mM formate pH 3, containing 30% methanol [10]. The sample of N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin analyzed by NMR was 11 mM in D_2O containing 50 mM potassium phosphate buffer pH 7.4. The sample of [*methylene*-^{13}C]-N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin was 4 mM.

NMR spectra of **2** were acquired at 293K on an AMX 600 spectrometer equipped with a broadband inverse probe. The ^{13}C-filtered TOCSY experiment [11] was recorded with 512 and 1,536 real points in ω_1 and ω_2, respectively, covering a sweep width of 2,732 Hz. The spectrum was strip-transformed (171 Hz covered by 1,024 real points) in ω_2 to enhance digital resolution. Shifted squared-sinebell apodisation (shifted by $\pi/3$ and $\pi/2$ in ω_2 and ω_1, respectively) was used in both dimensions. To validate homonuclear proton couplings taken from the one-dimensional spectrum, the spectrum of the pterin moiety was simulated with the chemical shifts and coupling constants extracted from the experimental spectrum using the program package PANIC [12]. A DQF-COSY of **3**, covering a sweep width of 3,205 Hz by 2,048 and 512 real points in t_2 and t_1, respectively, was recorded at 313K on an AMX 400 spectrometer equipped with a broadband inverse probe.

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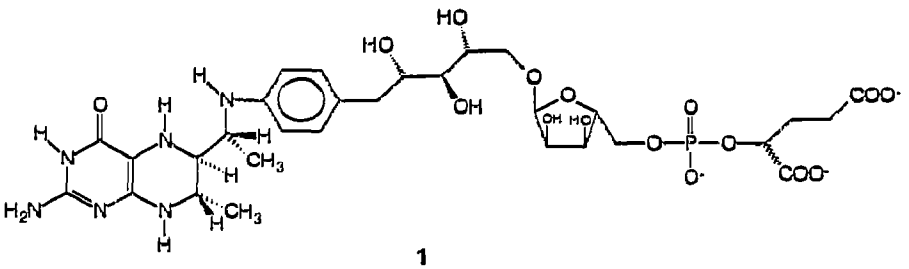

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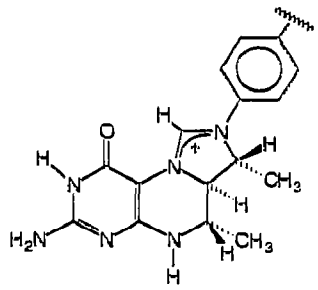

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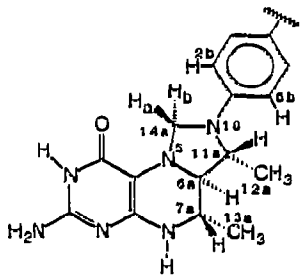

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Fig. 1. Formula of 5,6,7,8-tetrahydromethanopterin 1 and relevant fragments of N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin 2 and N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin 3. The relative configuration of the pterin moiety and the relative stereospecific assignment of the protons of C(14a) are indicated as determined in this paper. The numbering scheme for the pterin moiety was adopted from ref. [15].

A NOESY experiment of 3 with ^{13}C decoupling in t_1 and t_2 and a mixing time of 150 ms was recorded on an AMX 500 spectrometer. 2,048 and 640 real points were collected in t_2 and t_1, respectively, over a sweep width of 4,504 Hz. Chemical shifts are referenced relative to external 3-(trimethylsilyl)-propionate-d_4.

The minimum energy conformations of the four possible diastereomers of the pterin moiety were identified by Monte Carlo searches of the conformational space using the molecular modelling package MOMO [13] which employs the force field program PIMM [14]. For each diastereomer 1,000 permutations were generated by stochastical variation of the torsional angles N(5)–C(6a)–C(7a)–N(8) and N(5)– C(6a)–C(11a)–N(10) and at least 40 conformations were minimized. In each case either only one conformation was found or one conformation was found to be much lower in energy than any other.

3. RESULTS AND DISCUSSION

The resonance assignment of N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin 2 has already been published [15]. However, the configuration at the centers C(6a), C(7a) and C(11a) of the pterin moiety of 1 is still unknown. Since homo- as well as heteronuclear ^3J coupling constants are related to dihedral angles via Karplus relations [16], such coupling constants are used for the elucidation of the relative configuration at the above mentioned centers. Whilst homonuclear proton couplings of such medium-sized molecules are easily accessible from either one-dimensional or COSY spectra, heteronuclear filtered TOCSY [11] is a reliable method to accurately measure heteronuclear coupling constants, provided that all carbon atoms of interest are protonated. Since this prerequisite is fulfilled for 1 and its derivative 2, a ^{13}C-filtered TOCSY experiment of 2 was recorded to determine the heteronuclear $^3J_{CH}$ coupling constants. An expanded region of the ^{13}C-filtered TOCSY experiment is shown in Fig. 2.

The homo- as well as heteronuclear coupling constants around the C(6a)–C(7a) and C(6a)–C(11a) bonds in 2 as derived from a one-dimensional proton spectrum as well as from the ^{13}C-filtered TOCSY experiment are shown in Table I together with the appropriate dihedral angles of the minimum energy conformations of the possible diastereomers of 2, as obtained from molecular modelling [13].

The large homonuclear proton couplings observed suggest that the vicinal protons C(6a)H, C(7a)H and C(6a)H, C(11a)H are in mutual *trans* arrangements. This conclusion is confirmed by the size of all relevant heteronuclear coupling constants as well as by the observation of a NOE cross-peak between the protons C(7a)H and C(11a)H which is stronger than the C(11a)H/C(6a)H NOE cross peak, excluding the *anti*/*syn* and *syn*/*anti* configurations. Furthermore, comparison of the minimum energy conformations of the proposed configuration and of the other possible diastereomers shows that the dihedral angles obtained for the minimum energy conformation of any of the other diastereomers are in contradiction with the measured coupling constants. The respective dihedral angles are un-

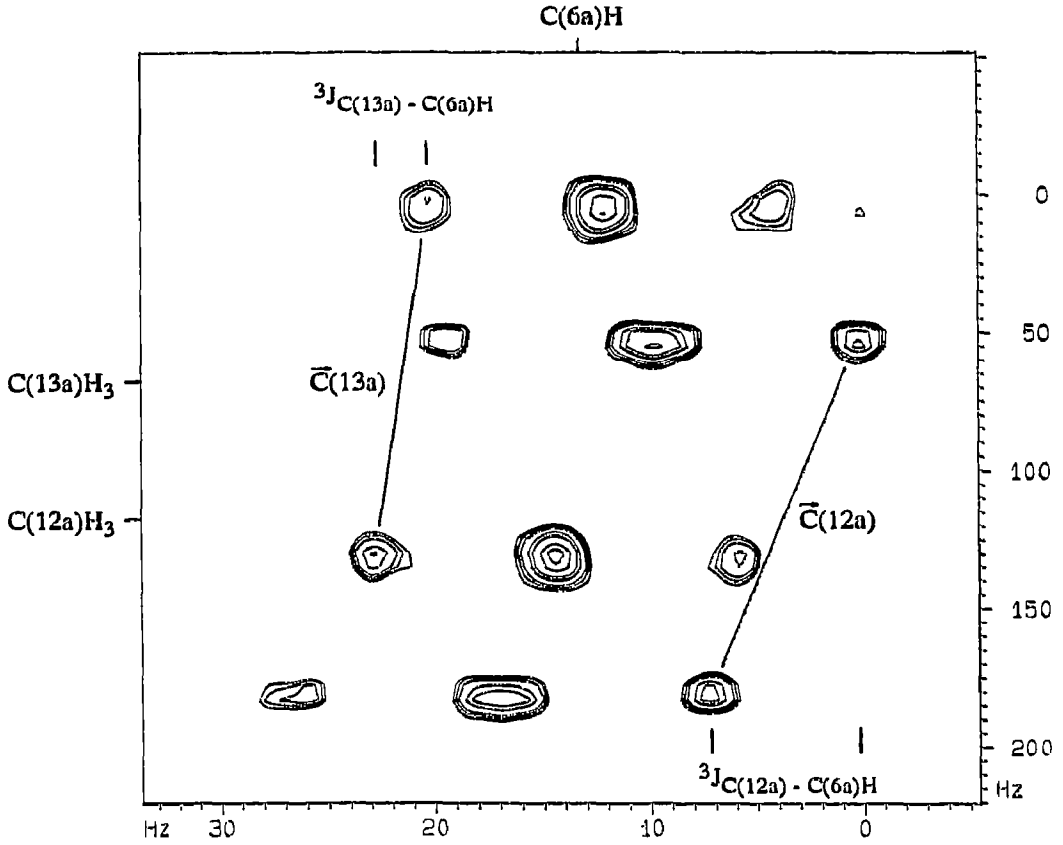

Fig. 2. Contour plot of the ^{13}C-filtered TOCSY experiment of **2** showing cross-peaks between C(6a)H (ω_2) and C(12a)H$_3$ and C(13a)H$_3$ (ω_1). The large coupling constant $^3J_{C(6a)H-C(12a)}$ = 7.2 Hz and the small coupling constant $^3J_{C(6a)H-C(13a)}$ = 2.4 Hz are clearly visible as displacements in ω_2, while the multiplet components are separated by the large $^1J_{CH}$ couplings in ω_1.

derlined in Table I. Thus the relative configuration of **2** is rel-(6R; 7S; 11R). Fig. 3 shows a drawing of the conformation of **2** as defined by the dihedral angles given in Table I.

The resonance assignment of the pterin moiety of **3** was reached in the following way: the C(12a) methyl group (1.41 ppm) and its adjacent proton C(11a)H (3.81 ppm) were identified by their COSY cross-peak and by their NOESY cross-peaks to the aromatic protons C(2b,6b)H (6.68 ppm). The remaining methyl group and its adjacent proton were assigned to C(13a)H$_3$ (1.27 ppm) and C(7a)H (3.02 ppm). C(6a)H (2.64 ppm) was identified by its COSY cross-peak to C(7a)H. In contradiction to the literature [5], the protons of the ^{13}C(14a)

Table I

Summary of experimental ^3J coupling constants and dihedral angles obtained from molecular modelling for the four relative configurations of the pterin moiety in **2**

	^3J	Dihedral angles			
		6R; 7S; 11R *anti/anti*	6R; 7R; 11S *syn/syn*	6R; 7S; 11S *anti/syn*	6R; 7R; 11R *syn/anti*
C(6a)H–C(11a)H	8.3	−146.7	13.5	−27.9	−142.4
C(6a)H–C(12a)	7.2	−22.9	−106.8	−153.8	−19.7
C(11a)H–C(7a)	6.8	−21.6	140.0	95.3	−18.6
C(6a)H–C(7a)H	9.5	176.0	61.8	175.0	56.2
C(6a)H–C(13a)	2.4	55.8	−175.1	53.9	178.0
C(7a)H–C(11a)	1.4	50.9	−64.8	51.6	−67.5

Anti and *syn* refer to the relative arrangement of C(6a)H/C(7a)H and C(6a)H/C(11a)H (in this order). The dihedral angles underlined contradict the measured coupling constants.

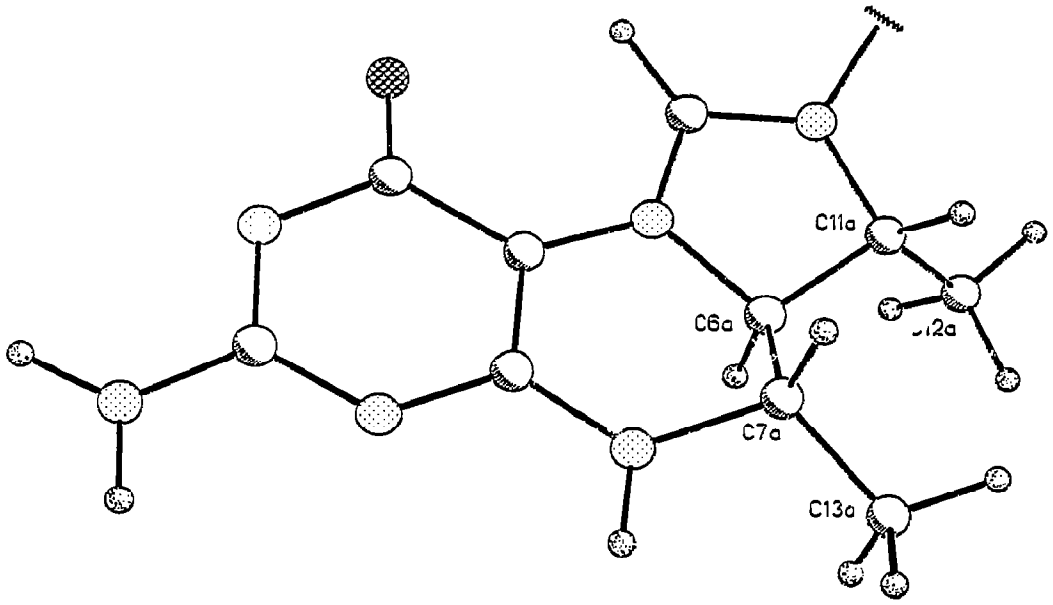

Fig. 3. Stick and ball drawing of the minimum energy conformation of **2** as determined by molecular modelling. The conformations around the C(7a)–C(13a) and C(11a)–C(12a) bonds are not truly represented because of free rotation of the methyl groups. Carbon atoms are drawn shadowed, nitrogen dotted, oxygen criss-crossed and hydrogen spotted.

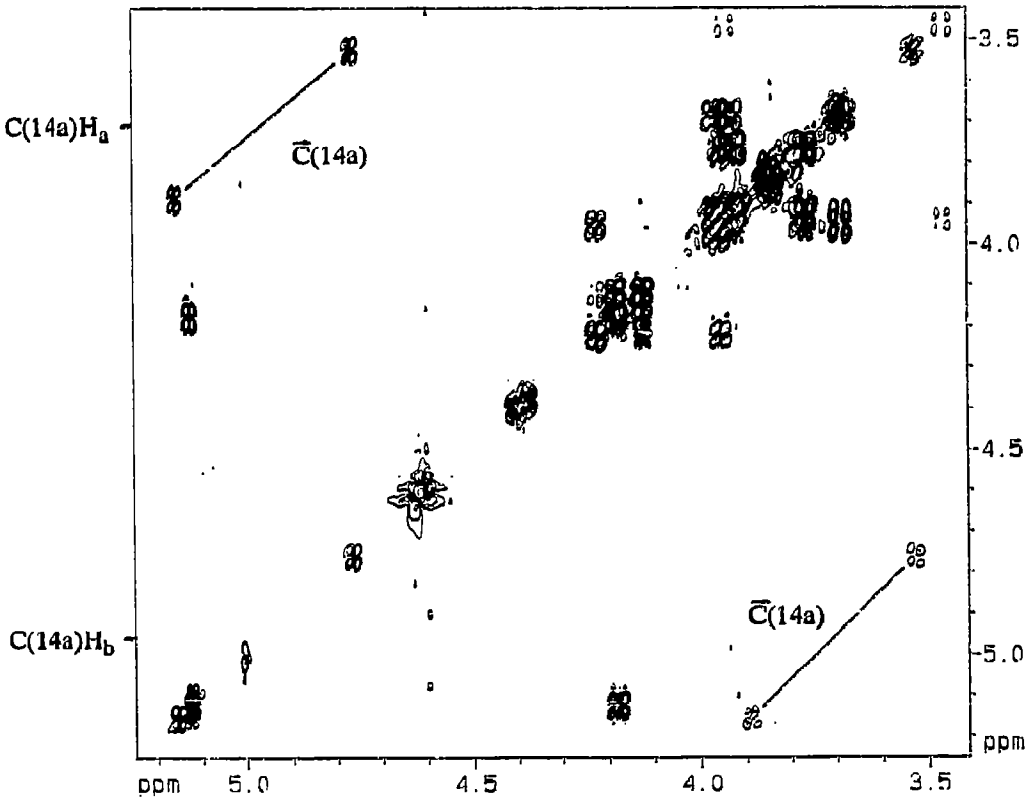

Fig. 4. Expanded region of the DQF-COSY of **3** showing cross-peaks between the $^{13}C(14a)$ methylene protons as indicated. The displacement marked is due to the direct CH-coupling and proves that both protons form a methylene group.

methylene group (3.71, 4.96 ppm) were clearly identified in the COSY spectrum by their doubled set of cross-peaks due to the direct coupling of both protons to $^{13}C(14a)$ as shown in Fig. 4.

The relative stereospecific assignment of these diastereotopic protons was derived from the observation of an NOE cross-peak between C(7a)H and C(14a)H_a at 3.71 ppm as well as C(12a)H_3 and C(14a)H_b at 4.96 ppm. This shows that C(14a)H_a and C(7a) are located on the same side of the pterin ring system and that C(12a)H_3 and C(14a)H_b are located on the other side. Therefore, C(14a)H_a is assigned the rel-(*pro*-S), and C(14a)H_b at 4.96 ppm the rel-(*pro*-R) configuration. This assignment is identical with the previously obtained assignment for N^5,N^{10}-methylene-tetrahydrofolate [17,18].

The determination of the relative configuration of **1** and the relative stereospecific assignment of the diasterotopic methylene protons of **3** open the way for investigations of the relation between structure and biological function of 5,6,7,8-tetrahydromethanopterin, which may lead to an understanding of its role in comparison to the closely related coenzyme tetrahydrofolic acid.

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