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7-Methylpterin in methanogenic bacteria

(7-Methylpterin; *Methanobacterium thermoautotrophicum*)

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1. SUMMARY

A blue fluorescent compound was extracted and purified from cells of *Methanobacterium thermoautotrophicum*. The compound was identified as 7-methylpterin on the basis of its (physico-) chemical properties and by comparison with 7-methylpterin prepared by organic synthesis. The compound is present in all methanogenic bacteria studied so far and it provides methanogenic bacteria the characteristic blue fluorescence observed upon fluorescence microscopy.

2. INTRODUCTION

Methanogenic bacteria can be tentatively identified by fluorescence microscopy [1,2]. Depending on the excitation and barrier filters employed either a green fluorescence due to the presence of coenzyme F_{420}, a 5-deazaflavin derivative [3], or a blue fluorescence is observed [2]. The presence of blue fluorescent compounds, called F_{342} [4,5], F_{340} [6], F_{350} [2] and methanopterin [7] was described previously. Methanopterin itself does not fluoresce at physiological conditions [7].

In this paper the extraction and purification of the most prominent blue fluorescent compound that is identical to F_{342} [4] and F_{350} [2] is described. The compound could be identified as 2-amino-4-hydroxy-7-methylpteridine (7-methylpterin) (Fig.1) by its (physico-) chemical properties and by comparison with the synthetic product.

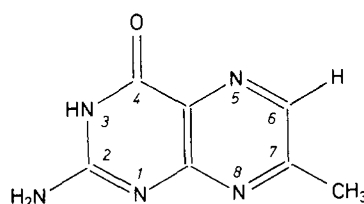

Fig.1. Structure of 7-methylpterin.

3. MATERIALS AND METHODS

3.1. Materials

DEAE-Sephadex A-25, QAE-Sephadex A-25 and Sephadex G-25 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Kieselgel-60 and cellulose thin-layer chromatography (TLC) plates (0.25 mm) were from Merck (Darmstadt, FRG). Pterin-6-carboxylic acid was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 7-Methylpterin and pterin-7-carboxylic acid were prepared as described by Mowat et al. [8].

3.2. *Extraction and purification of 7-methylpterin from* Methanobacterium thermoautotrophicum

Methanobacterium thermoautotrophicum, strain ΔH (DSM 1053) was mass-cultured in a 12 or 300

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l fermentor in a defined mineral medium as described by Schönheit et al. [9] and as modified by Van Beelen et al. [5]. Extraction of whole cells and the first purification step employing DEAE-Sephadex A-25 column chromatography was performed as described previously [7]. At this step the blue fluorescent 7-methylpterin was eluted from the column by washing with 25 mM phosphate buffer (pH 7). 7-Methylpterin containing fractions were pooled, lyophilized to dryness and subsequently dissolved in a minimal amount of 15% aqueous ethanol; 5-ml portions were applied to a Sephadex G-25 column (80 × 3 cm) equilibrated with 15% aqueous ethanol. The blue fluorescent material was collected, flash evaporated to remove ethanol, and lyophilized. The material was dissolved in 10 mM NH_4HCO_3 that was adjusted to pH 9 with concentrated ammonia and applied to a QAE-Sephadex A-25 (HCO_3^- form) column (25 × 2 cm) equilibrated and washed with 10 mM NH_4HCO_3 (pH 9) until all 7-methylpterin was removed. Eventually, the compound was further purified by high pressure liquid chromatography (HPLC).

3.3. *Purification of the alkaline permanganate oxidation product*

One –2 mg of the pterin isolated from *M. thermoautotrophicum* was dissolved in 1 ml 0.1 M NaOH containing 16 mg $KMnO_4$. The solution was heated for 2 h at 80°C. The reaction was stopped with 0.2 ml ethanol and MnO_2 formed was removed by centrifugation. The green fluorescent supernatant was flash evaporated to dryness and dissolved in 10 mM NH_4HCO_3 (pH 9). The solution was applied to a QAE-Sephadex (HCO_3^- form) A-25 column (25 × 1 cm). The unreacted material was eluted from the column by a linear gradient of NH_4HCO_3 (0.5 l, 10 – 500 mM). The oxidation product that stuck to the top of the column could be removed by washing with 0.1 M NH_4HCO_3. The fluorescent material was collected and flash evaporated several times after repeated dissolution in distilled water to remove NH_4HCO_3.

3.4. *Chromatographic methods*

Thin-layer chromatography was performed on Kieselgel-60 or cellulose plates. The plates were developed with solvent systems as indicated in RESULTS AND DISCUSSION. Fluorescent spots were detected with a UVL-21 Black-ray lamp emitting at 366 nm.

HPLC was executed as described previously [5] except that a linear gradient of 0–50% methanol in 15 min was used.

3.5. *Spectroscopic assays*

Ultraviolet-visible light absorption spectra were obtained with a Cary 118 recording spectrophotometer. ^{1}H-NMR spectra were recorded with a Bruker WH 90 spectrophotometer operating at 90 MHz and 25°C with a Bruker WM 500 spectrophotometer operating at 500 MHz and 27°C. Sample preparation and the recording of the spectra were executed as described previously [7].

4. RESULTS AND DISCUSSION

The compound presumed to be 7-methylpterin was extracted and purified as described in section 3.2. The ultraviolet-visible light absorption spectra of this compound at different pH values (Fig.2) show all the characteristics of an (oxidized) pterin

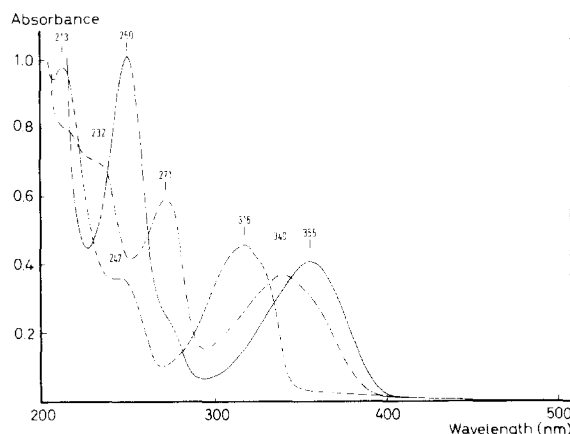

Fig.2. Ultraviolet-visible light absorption spectra of 7-methylpterin isolated from *Methanobacterium thermoautotrophicum*. The spectra were recorded at pH 1.2 (- - - - - -), pH 7.1 (– · – · –) and pH 12.2 (———). The ultraviolet-visible light spectra of synthetic 7-methylpterin recorded at the same pH values were identical.

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Table 1

Thin-layer chromatography and high pressure liquid chromatography of pterins

Compound	R_f-values [a]					Retention time (min) HPLC
	A	B	C	D	E	
7-Methylpterin						
Authentic	0.64	0.48	0.79	0.49	0.81	9.37
Synthetic	0.64	0.48	0.79	0.49	0.81	9.37
$KMnO_4$ Oxidation product	0.27	0.21	0.79	0.49	0.62	– [b]
Pterin-7-carboxylic acid	0.27	0.21	0.79	0.49	0.62	–
Pterin-6-carboxylic acid	0.30	0.30	0.68	0.45	0.59	–

[a] TLC plates and solvents: (A) Kieselgel-60, 1-butanol/acetic acid/water (4/1/1, by vol.); (B) cellulose, 2-butanol/formic acid/water (6/2/1, by vol.); (C) Kieselgel-60, 80% aqueous ethanol; (D) cellulose, 3% (w/v) ammonium chloride; (E) cellulose, 2-propanol/1% ammonia (2/1, v/v).

[b] Pterin carboxylic acids showed no retention.

[10]. Moreover, the observed ratio $A_{249}/A_{355} = 2.52$ of the maximal absorbances around 250 nm and around 360 nm in 0.1 M NaOH is indicative of the presence of a 7-substituted pterin [11]. Furthermore, the ultraviolet-visible light absorption spectra of the naturally occurring (Fig.2), and the

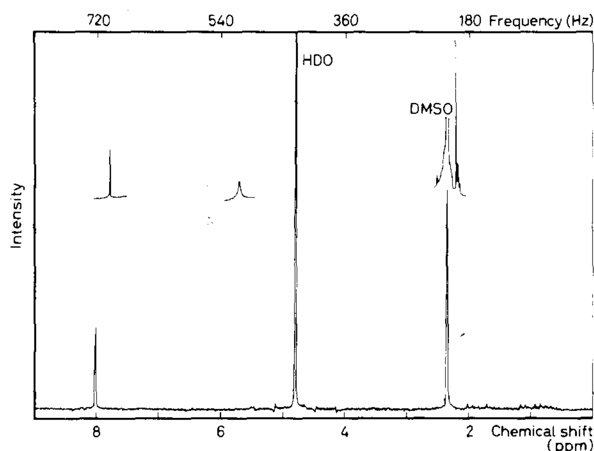

Fig.3. 90 MHz ^{1}H-NMR spectrum of 7-methylpterin isolated from *Methanobacterium thermoautotrophicum* and recorded in 1 M NaOD; spectral width, 1080 Hz; pulse width, 5.3 μs; number of scans, 900. The inset shows details of the 500 MHz ^{1}H-NMR spectrum recorded in DMSO (d_6) at pH 8 under the following conditions: spectral frequency, 500. 1395 MHz; spectral width, 5000 Hz; acquisition time 1.638 s; pulse width, 16 μs; number of scans, 64.

synthetic compound were identical. Both compounds were chromatographically indistinguishable (Table 1).

The 90 MHz ^{1}H-NMR spectrum (Fig.3) recorded in 1 M NaOD revealed two singlets situated at $\delta = 8.0$ ppm and $\delta = 2.3$ ppm showing a proton ratio of 1 : 2.7. A non-integral ratio was obtained since protons attached to a methyl group were exchanged for deuterium during sample preparation, viz. lyophilization from D_2O. This was evidenced by the fact that the proton ratio varied for different sample preparations. Moreover, in the 500 MHz high-resolution ^{1}H-NMR spectrum (Fig.3, inset) the high-field signal was split into 3 singlets accounting for CH_3-, CH_2D- and CHD_2-moieties. The position of the low-field and high-field signals were dependent on the pH value of the sample, indicating that a vinologous proton and a methyl group are attached to an aromatic moiety. A broadened signal was observed at $\delta = 5.3$ ppm (2H) when the spectrum was recorded in DMSO (d6) (Fig.3, inset) and that was absent in the spectrum recorded in D_2O. This indicates that the amino protons at C-2 were exchanged for deuterium. These results apply to 7-methylpterin as well as to 6-methylpterin. Ultraviolet-visible light spectral data pointed to the first possibility.

Definite proof could be obtained by chemical oxidation of the compound. Alkaline per-

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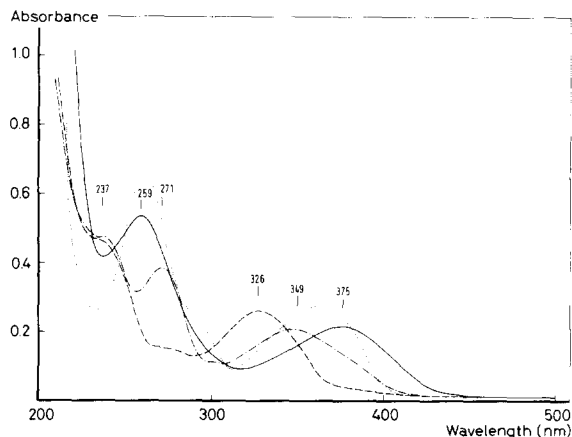

Fig.4. Ultraviolet-visible light absorption spectra of pterin-7-carboxylic acid prepared from isolated 7-methylpterin. The spectra were recorded at pH 1.3 (— — —), pH 6.4 (–·–·–) and pH 12.2 (———). The ultraviolet-visible light spectra of synthetic pterin -7-carboxylic acid recorded at the same pH values were identical. The spectrum of pterin -6-carboxylic acid (······) was recorded at pH 12.2.

manganate oxidation of the isolated compound yielded a green fluorescent product showing identical chromatographic (Table 1) and ultraviolet-visible light spectroscopic (Fig.4) properties as pterin-7-carboxylic acid. These properties differed from those observed for the blue fluorescent pterin-6-carboxylic acid (Table 1, Fig.4).

7-Methylpterin is the main blue fluorescent compound present in *M. thermoautotrophicum* and the molar content even exceeds those of other ethanol-extractable coenzymes analyzed previously [5]. The presence of 7-methylpterin could be demonstrated by TLC and HPLC in all strains of methanogenic bacteria examined thus far by us, and belonging to the genera *Methanobacterium, Methanobrevibacter, Methanococcus, Methanospirillum* and *Methanosarcina* (unpublished). The compound is identical to the formerly described F_{350} [2] and F_{342} [5] and presumably also F_{340} [6]. In other respects, 7-methylpterin is rather unique in living organisms, contrary to the much more common 6-methylpterin.

The question concerning its function in methanogenic bacteria is still open. *M. thermoautotrophicum*, and probably all methanogens, contain two other pterin derivatives, viz. methanopterin [7] and carboxy-5,6,7,8-tetrahydromethanopterin (c-THMP) [12], that are involved in the first steps of reduction of CO_2 to methane [12,13]. The latter two compounds are also 7-methyl-substituted pterins, but they contain a $-CH(CH_3)$-group substituted at C-6 of the pterin moiety [P. van Beelen, unpublished]. Possibly 7-methylpterin functions in the biosynthesis or biodegradation of methanopterin and C-THMP.

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