A^2 and tyrosine A^{19}, with concomitant stabilization of the helical segment A^2–A^8. [Leu^{19}-A]insulin, in common with the A^2-substituted insulin, has a small probability for adaptation to the conformation required for receptor binding. The interaction between A^2 and A^{19} appears to be among the most important determinants for high biological activity in insulin.

Acknowledgments

We express our appreciation to Shima Joshi for technical assistance.

Supplementary Material Available

Complete synthetic details including references (6 pages). Ordering information is given on any current masthead page.

References

Blundell, T. L., Dodson, G., Hodgkin, D., & Mercola, D. (1972) *Adv. Protein Chem. 26*, 279–402.
Brown, H., Sanger, F., & Kitai, R. (1955) *Biochem. J. 60*, 556–565.
Burke, G. T., Chanley, J. D., Okada, Y., Cosmatos, A., Ferderigos, N., & Katsoyannis, P. G. (1980) *Biochemistry 19*, 4547–4556.
Danho, W., Sasaki, A., Büllesbach, E., Föhles, T., & Gattner, H.-G. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 735–746.
Ferderigos, N., Burke, G. T., Kitagawa, K., & Katsoyannis, P. G. (1983) *J. Protein Chem. 2*, 147–170.
Gilman, A., Philips, F. S., & Koelle, E. S. (1946) *Am. J. Physiol. 146*, 348–357.
Hales, C. N., & Randle, P. J. (1963) *Biochem. J. 88*, 137–146.
Katsoyannis, P. G., & Tometsko, A. (1966) *Proc. Natl. Acad. Sci. U.S.A. 55*, 1554–1561.
Katsoyannis, P. G., Tometsko, A., Zalut, C., & Fukuda, K. (1966) *J Am. Chem. Soc. 88*, 5625–5635.
Katsoyannis, P. G., Tometsko, A., Zalut, C., Johnson, S., & Trakatellis, A. C. (1967a) *Biochemistry 6*, 2635–2642.
Katsoyannis, P. G., Trakatellis, A. C., Johnson, S., Zalut, C., & Schwartz, G. (1967b) *Biochemistry 6*, 2642–2655.
Katsoyannis, P. G., Trakatellis, A. C., Zalut, C., Johnson, S., Tometsko, A., Schwartz, G., & Ginos, J. (1967c) *Biochemistry 6*, 2656–2668.
Kitagawa, K., Ogawa, H., Burke, G. T., Chanley, J. D., & Katsoyannis, P. G. (1984) *Biochemistry 23*, 1405–1413.
Lord, R. S., Gubensek, F., & Rupley, J. A. (1973) *Biochemistry 12*, 4385–4390.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Okada, Y., Chanley, J. D. Burke, G. T., & Katsoyannis, P. G. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 629–638.
Pfleiderer, G., Celliers, P. G., Stanulovich, M., Wachsmuth, E. D., Determann, H., & Braunitzer, G. (1964) *Biochem. Z. 340*, 552–564.
Sanger, F., & Tuppy, H. (1951) *Biochem. J. 49*, 481–490.
Schwartz, G., & Katsoyannis, P. G. (1976) *Biochemistry 15*, 4071–4076.
Schwartz, G., Burke, G. T., & Katsoyannis, P. G. (1981) *Int. J. Pept. Protein Res. 17*, 243–255.
Schwartz, G., Burke, G. T. Chanley, J. D., & Katsoyannis, P. G. (1983) *Biochemistry 22*, 4561–4567.

5,10-Methenyl-5,6,7,8-tetrahydromethanopterin, a One-Carbon Carrier in the Process of Methanogenesis†

Patrick Van Beelen, Johan W. Van Neck, Remi M. de Cock, Godfried D. Vogels,* Willem Guijt, and Cornelis A. G. Haasnoot

ABSTRACT: 5,10-Methenyl-5,6,7,8-tetrahydromethanopterin was isolated from *Methanobacterium thermoautotrophicum*. The structure of this compound was elucidated by various two-dimensional nuclear magnetic resonance techniques and confirmed by fast atom bombardment mass spectrometry. The structure of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin shows one additional carbon atom as compared to the parent compound methanopterin. This additional carbon atom is rapidly labeled in vivo by $^{13}CO_2$. It is shown that 5,10-methenyl-5,6,7,8-tetrahydromethanopterin is a physiologically active one-carbon carrier at the formyl level of oxidation in the methanogenic pathway from CO_2.

During short-term labeling experiments with cells of *Methanobacterium thermoautotrophicum* it was demonstrated that $^{14}CO_2$ was incorporated in substantial amounts into a so-called yellow fluorescent compound (Daniels & Zeikus, 1978). Cells of *Methanosarcina barkeri*, grown in the absence of hydrogen, incorporated $^{14}CH_3OH$ into a closely related yellow fluorescent compound. Note that in the absence of hydrogen *M. barkeri* performs the reaction $4CH_3OH \rightarrow 3CH_4 + CO_2$, whereas in the presence of hydrogen the reaction $CH_3OH + H_2 \rightarrow CH_4 + H_2O$ is performed. In the presence of hydrogen and $^{14}CH_3OH$ the yellow fluorescent compound was not labeled, which indicated that this compound plays a role in the oxidation of CH_3OH to CO_2 by *M. barkeri* (Kenealy & Zeikus, 1982).

On the basis of spectral properties and degradation studies it was erroneously assumed that the yellow fluorescent compound present in *M. thermoautotrophicum* was a carboxy-

† From the Department of Microbiology (P.V.B., J.W.V.N., R.M.d.C., and G.D.V.) and the Department of Biophysical Chemistry (W.G. and C.A.G.H.), University of Nijmegen, NL 6525 ED Nijmegen, The Netherlands. *Received January 5, 1984.* This investigation was supported by the Foundation for Fundamental Biological Research (BION) and the Netherlands Foundation for Chemical Research (SON), both subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO). A short preliminary communication has been published (Van Beelen et al., 1984).

0006-2960/84/0423-4448$01.50/0

5,6,7,8-tetrahydromethanopterin derivative (Keltjens et al., 1983b). Methanopterin was shown to be a pterin derivative and was identified as the metabolic counterpart, deficient in a one-carbon group, of the yellow fluorescent compound (Keltjens & Vogels, 1980; Keltjens et al., 1983a). The structure of methanopterin was recently elucidated by means of 2D NMR[1] techniques (Van Beelen et al., 1984a) and detailed degradation studies (Van Beelen et al., 1984b).

In this paper the structure of the compound, which was previously known as the yellow fluorescent compound or carboxy-5,6,7,8-tetrahydromethanopterin, is established to be 5,10-methenyl-5,6,7,8-tetrahydromethanopterin. It will be shown that the methenyl carbon of the compound can be specifically labeled in vivo by a short-term incubation of cells of *M. thermoautotrophicum* with $^{13}CO_2$.

Experimental Procedures

Short-Term Labeling of Cells with $^{13}CO_2$. M. thermoautotrophicum, strain ΔH, was grown as described previously (Van Beelen et al., 1984a). Fresh cells (340 g) were preincubated for 15 min under vigorous shaking and 10^5 Pa of H_2 at 60 °C. The bottle containing the thick slurry of cells was subsequently cooled, evacuated, and gassed with 10^5 Pa of H_2. An anaerobic solution of 1 M $NaH^{13}CO_2$ was prepared by adding solid $NaH^{13}CO_2$ to oxygen-free, distilled water in a glove box under an anaerobic atmosphere (H_2/N_2, 2.5./97.5 v/v), and 1.0 mL of this solution was injected into the bottle which was then incubated for about 5 min under vigorous shaking at 40 °C until 0.2 mmol of CH_4 was formed. Methane production was measured as described previously (Hutten et al., 1981). The incorporation of $^{13}CO_2$ was stopped by cooling the cells to 0 °C.

In this procedure the reduction of $^{13}CO_2$ and methanogenesis were expected to be limited by the availability of hydrogen in the thick cell suspension, and therefore only a limited incorporation of ^{13}C into methenyl-THMP was expected.

Extraction and Purification of Methenyl-THMP. The extraction and purification of methenyl-THMP and ^{13}C-labeled methenyl-THMP were performed as described previously (Van Beelen et al., 1984c). Since methenyl-THMP is only stable between pH 4 and pH 5, and then at temperatures below 80 °C, proper precautions must be taken in the isolation procedure.

Sample Preparation for NMR Measurements. The samples, dissolved in a phosphate buffer, pH 4.5, were flash evaporated 4 times from D_2O and finally dissolved in 99.75% D_2O. The pD of the samples was 4.5 (meter reading). In the sample used for ^{13}C NMR experiments the concentration of methenyl-THMP was 66 mM (10-mm tube), and in ^1H NMR experiments it was 8 mM (5-mm tube). Samples were stored at −20 °C in the dark. After completion of the NMR experiments, the samples did contain only minor impurities detectable at 240 nm by high-performance liquid chromatography (HPLC) analysis (Van Beelen et al., 1983a).

NMR Spectroscopy. ^1H NMR spectra were recorded on a Bruker WM-500 spectrometer interfaced with an Aspect-2000 computer and a real-time pulser board. The residual HDO solvent resonance was suppressed by applying the WEFT pulse sequence (Patt & Sykes, 1972; Benz et al., 1972). Chemical shifts were expressed relative to the methyl resonance of (3-trimethylsilyl)propionic acid-d_4, added as an internal calibrant.

In the 2D correlated spectroscopy (COSY) experiment the basic pulse sequence of Aue et al. (1976) was used. However, an extra 180° pulse followed by an appropriate delay was inserted prior to this pulse train in order to suppress the residual HDO resonance resulting in the following overall pulse sequence: $(180°\text{-}t_{weft}\text{-}90°\text{-}t_1\text{-}90°\text{-}t_2)_n$.

The 2D nuclear Overhauser enhancement (NOESY) spectrum was recorded by using the $(90°\text{-}t_1\text{-}90°\text{-}\tau_m\text{-}90°\text{-}t_2)$ pulse sequence with $\tau_m = 0.3$ s (Macura & Ernst, 1980). In order to reduce the solvent signal, a selective, continuous irradiation of the HDO resonance was applied at all times, except during the observation period t (Wider et al., 1983).

^{13}C NMR spectra were acquired on a Bruker WM-200 WM spectrometer (operating at 50.3 MHz) interfaced with an Aspect-2000 computer and a real-time pulser board. Chemical shifts were measured relative to methanol added as internal reference and converted to the Me_4Si scale by correcting for the CH_3OH to Me_4Si chemical shift ($\delta_{CH_3OH} - \delta_{TMS} = 49.3$). Dielectric heating of the sample caused by broad-band ^1H decoupling was minimized by a two-level decoupling scheme: during acquisition a minimal decoupling power was applied (~0.5 W), followed by a relaxation delay during which the decoupling power was switched to a lower level (~0.1 W, in order to maintain the nuclear Overhauser enhancement).

Two-dimensional heteronuclear chemical shift correlation experiments (Mandsley et al., 1977; Bodenhauser & Freeman, 1977; Bax, 1982) were performed by using the pulse sequence and phase cycling proposed by Bax (1982). The so-called "attached proton test" in ^{13}C NMR was performed as described by Patt & Shoolery (1982).

Mass Spectrometry. Samples of methenyl-THMP were desalted by using reversed-phase HPLC (Van Beelen et al., 1983a) with diluted HCl (pH 3) as mobile phase. The fractions containing methenyl-THMP were flash evaporated and dissolved in a minimal amount of glycerol.

Fast atom bombardment mass spectra were obtained on a VG Analytical Model 7070 mass spectrometer.

Results

Identification of the Side Chain of Methenyl-THMP. Methanopterin can be converted into methenyl-THMP, and it has been shown that both compounds have several structural elements in common (Keltjens et al., 1983a,b). It may therefore be expected that the NMR spectra of both compounds will reflect these similarities in structural elements.

The 500-MHz ^1H NMR spectrum of methenyl-THMP is presented in Figure 1 (top trace); for comparison purposes a 500-MHz ^1H NMR spectrum of methanopterin is shown in the bottom trace of Figure 1. The ^1H resonances of methanopterin are numbered in accordance with the previously adopted numbering of methanopterin (Van Beelen et al., 1984a; see Figure 2).

Figure 1 shows that the ^1H NMR spectra of methenyl-THMP and methanopterin indeed reflect the expected similarities in structural elements. For instance the ^1H resonances 2e, 3e, and 4e of methenyl-THMP coincide with the resonances of the α-hydroxyglutaric acid moiety in methanopterin. Similarly, the ^1H resonances marked 1d, 2d, 3d, and 4d in the methenyl-THMP spectrum agree nicely with the resonances arising from the ribofuranose moiety in methanopterin. In the latter compound this α-ribofuranose moiety is linked at carbon 5 via a phosphodiester to the α-hydroxyglutaric acid

[1] Abbreviations: NMR, nuclear magnetic resonance; methenyl-THMP, 5,10-methenyl-5,6,7,8-tetrahydromethanopterin; HPLC, high-performance liquid chromatography; WEFT, water-eliminated Fourier transform; COSY, 2D *J*-correlated NMR spectroscopy; NOESY, 2D nuclear Overhauser enhancement NMR spectroscopy; Me_4Si, tetramethylsilane; methenyl-THF, 5,10-methenyl-5,6,7,8-tetrahydrofolic acid; THF, 5,6,7,8-tetrahydrofolic acid; THMP, 5,6,7,8-tetrahydromethanopterin; 2D, two dimensional.

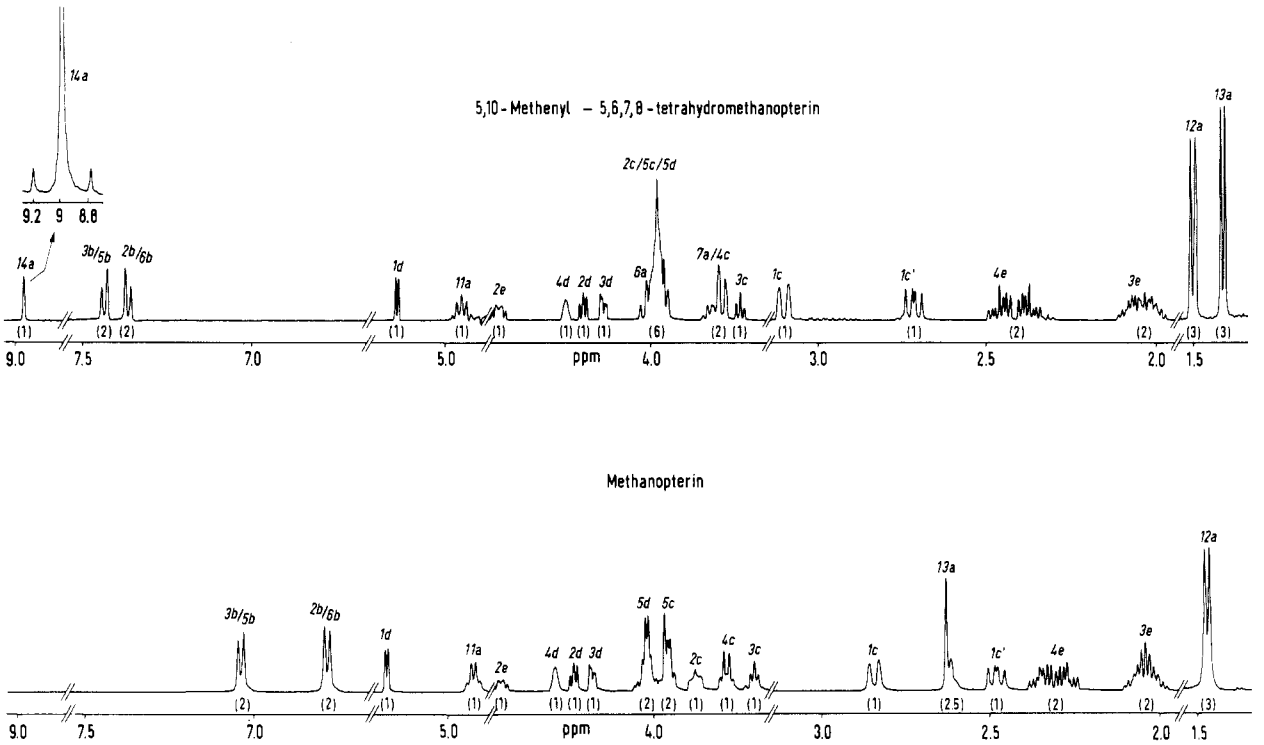

FIGURE 1: 500-MHz ^1H NMR spectra of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin (top) and methanopterin (bottom). Normalized resonance intensities are given in parentheses. The insert shows the ^{13}C satellites of resonance 14a present only in ^{13}C-labeled 5,10-methenyl-5,6,7,8-tetrahydromethanopterin. The resonances are numbered in correspondence with Figure 2.

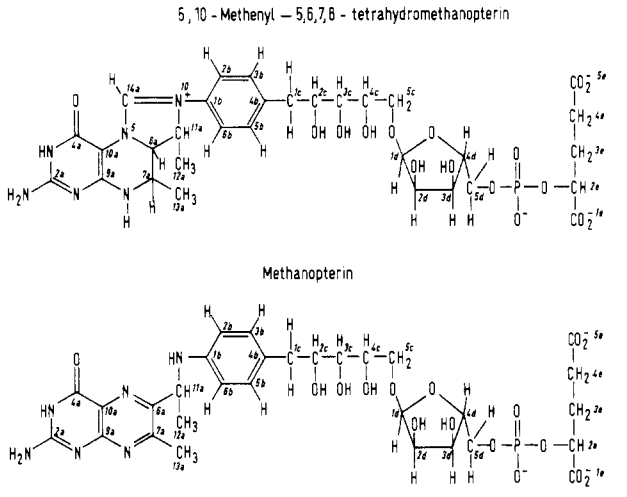

FIGURE 2: Structure of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin. The protons are numbered in accordance with the previously adopted numbering for methanopterin (Van Beelen et al., 1984a). For comparison purposes the structure of methanopterin is given. The absolute configuration of the chiral centers in both compounds is not known.

moiety (see Figure 2), and therefore, the ^1H resonances 4d, 5d, and 2e of methanopterin show a coupling with the ^{31}P nuclear resonance. A ^1H NMR spectrum of methenyl-THMP recorded under simultaneous ^{31}P broad-band decoupling (not shown) indicates that resonances 2e and 4d and some resonances in the cluster about 4 ppm are also coupled to ^{31}P. On these grounds the ^1H resonances 5d of methenyl-THMP are supposed to be located in the cluster of peaks around 4 ppm. The COSY spectrum of methenyl-THMP clearly shows a cross peak between the ^1H resonances 4d and 5d (Figure 3B) which proves that the above interpretation is correct. The presence of bound phosphate in methenyl-THMP was also shown by chemical methods (Van Beelen et al., 1984c). On these grounds it is concluded that the α-hydroxyglutaric acid, the phosphodiester, and the α-ribofuranose moieties present in methanopterin are preserved in the structure of methenyl-THMP.

On the basis of their coupling constant pattern and the chemical shift values, the ^1H resonances marked 3b/5b and 2b/6b of methenyl-THMP are assigned to the aniline moiety. On similar grounds the ^1H resonances marked 1c, 1c′, 3c, and 4c in the methenyl-THMP spectrum are assigned to the tetrahydroxypentane moiety. The COSY spectrum of methenyl-THMP reveals the connectivities between the resonances of the tetrahydroxypentane moiety and enables the assignment of the remaining resonances 2c and 5c. Figure 3A shows cross peaks between resonances 1c/1c′ and resonance 2c, which is located in the cluster of resonances around 4 ppm. Figure 3B shows the cross peaks interconnecting the 2c, 3c, 4c, and 5c resonances. The latter resonance is also located in the cluster of peaks at 4 ppm but is slightly upfield from resonances 6a and 2c. Hence, the ^1H resonances labeled 1c, 1c′, 2c, 3c, 4c, and 5c in the methenyl-THMP spectrum concur with the corresponding resonances of the tetrahydroxypentane moiety of methanopterin. The relatively small differences in chemical shift values of corresponding resonances in the two spectra are ascribed to the large difference in pD between the methenyl-THMP sample (pD 4.5) and the methanopterin sample (pD 10.25).

In summary, the α-hydroxyglutaric acid moiety, the phosphodiester moiety, the α-ribofuranose moiety, the tetrahydroxypentane moiety and the aniline moiety present in methanopterin are preserved in the structure of methenyl-THMP.

Identification of the Pterin Core of Methenyl-THMP. The COSY spectrum of methenyl-THMP (Figure 3A) shows a cross peak between methyl resonance 12a and methine resonance 11a. The chemical shift values of these resonances are almost identical with resonances 12a and 11a in the spectrum of methanopterin (Figure 1). Therefore, these resonances of methenyl-THMP are assigned to the ethyl moiety that con-

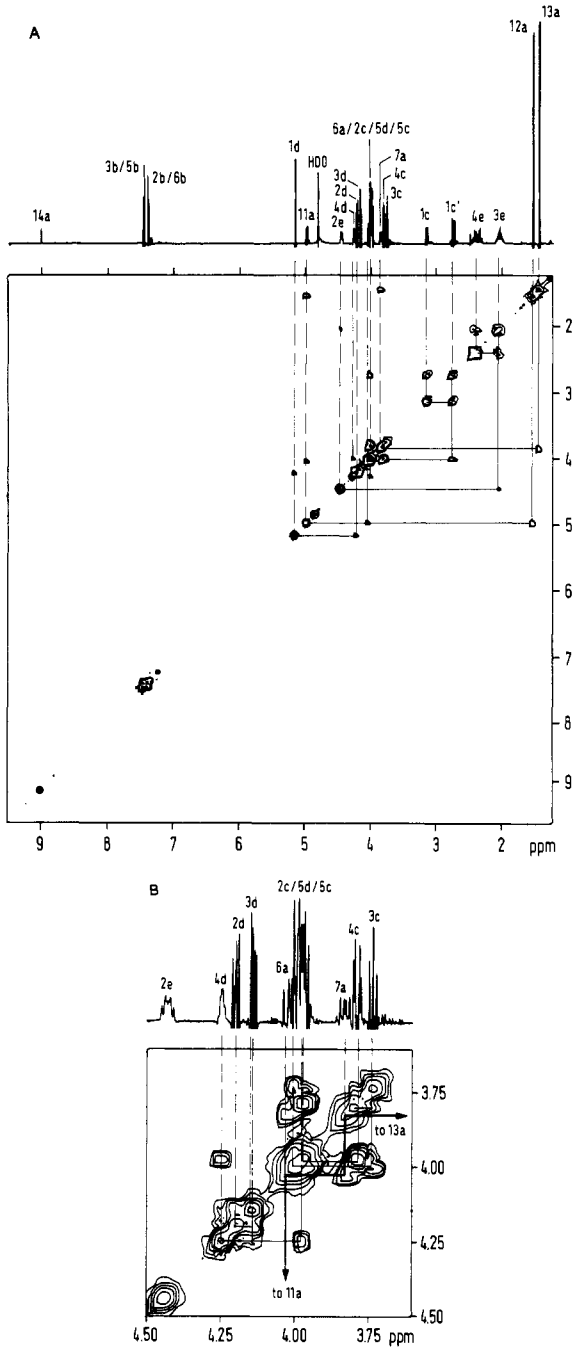

FIGURE 3: (A) Contour plot of the 500-MHz ^{1}H COSY spectrum of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin. The ^{1}H resonances are numbered according to Figure 2. (B) Expansion of (A) showing the connectivities in the 4.5–3.5 ppm region.

nects carbon 6 of the pterin moiety with the nitrogen of the aniline moiety (see Figure 2). Parts A and B of Figure 3 show that 12a, 11a, 6a, 7a, and 13a are consecutively interconnected by a series of cross peaks. Note that 12a and 13a are methyl resonances while 11a, 6a, and 7a are methine resonances. It follows that carbons 6 and 7 of the pterin moiety are sp^3 hybridized. Since the only stable pterins known to be sp^3 hybridized at carbons 6 and 7 are 5,6,7,8-tetrahydropterins (Blakley, 1969), it is concluded that methenyl-THMP is a 5,6,7,8-tetrahydropterin derivative. The protons attached to nitrogen or oxygen in methenyl-THMP (see Figure 2) are exchanged with deuterium from the solvent and are therefore not observed in the ^{1}H NMR spectrum.

In summary, all the connectivities shown in Figure 3 are in full accordance with the structure of methenyl-THMP

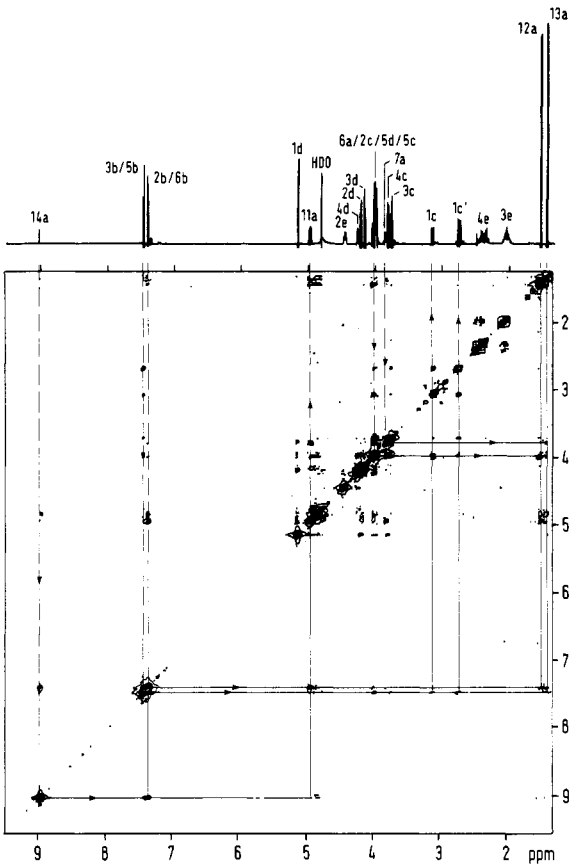

FIGURE 4: Contour plot of a 500-MHz ^{1}H NOESY spectrum of 5,10-methenyl-5,6,7,8-tetrahydropterin recorded at 0 °C and pD 4.5 (meter reading).

shown in Figure 2. All the ^{1}H resonances of methenyl-THMP are identified at this stage, except the ^{1}H resonance marked 14a (see below).

Identification of the Methenyl Moiety. In order to identify resonance 14a of the ^{1}H NMR spectrum of methenyl-THMP, a NOESY experiment was performed. Figure 4 reveals cross peaks between ^{1}H resonances 14a on the one hand and 2b/6b and 11a on the other hand, which indicates that methine proton 14a is located between the pterin and the aniline moiety. Whether methine group 14a is linked to the 5,6,7,8-tetrahydropterin moiety or to the aniline moiety or to both follows from the ultraviolet light absorption spectrum of methenyl-THMP. This spectrum shows a long wavelength absorption maximum at 336 nm (Keltjens et al., 1983b; Van Beelen et al., 1984c). However, most 5,6,7,8-tetrahydropterins do not show an absorption maximum above 305 nm, with the exception of 5,10-methenyl-5,6,7,8-tetrahydrofolic acid which shows an absorption maximum at 352 nm (Blakley, 1969). In analogy, the long wavelength absorption maximum of methenyl-THMP is ascribed to a similar conjugation between the 5,6,7,8-tetrahydropterin and the aniline moieties via a methenyl linkage (see Figure 2).

All the connectivities shown in the NOESY experiment (Figure 4) are in accordance with the proposed structure. For instance ^{1}H resonances 3b/5b show cross peaks to resonances 1c, 1c′, 3c, and 2c (located in the cluster of resonances at 4 ppm), thus indicating that the aniline and tetrahydroxypentane moieties are linked in methenyl-THMP in the same way as in methanopterin.

Cross peaks are also found between ^{1}H resonance 6a and ^{1}H resonances 12a and 13a and also between resonance 7a and resonances 12a and 13a as is expected from the structure of methenyl-THMP (Figure 2).

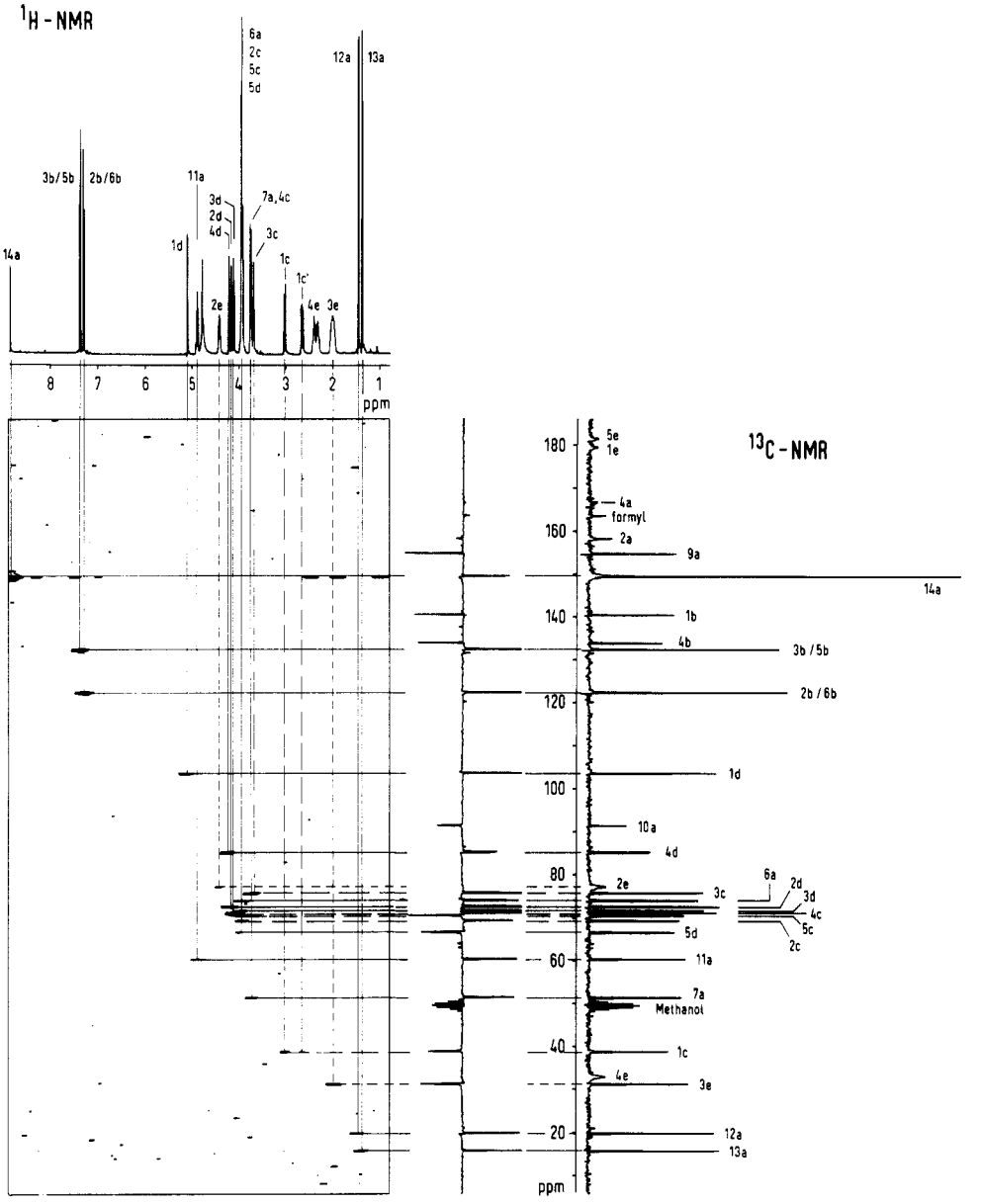

FIGURE 5: Contour plot of a 200/50.3-MHz ^{1}H–^{13}C shift-correlated 2D NMR spectrum of ^{13}C-labeled 5,10-methenyl-5,6,7,8-tetrahydromethanopterin. For reference purposes a 500-MHz ^{1}H NMR spectrum is plotted along the horizontal (f_1) axis. Along the vertical (f_2) axis the 50.3-MHz attachted proton test ^{13}C NMR spectrum and the 50.3-MHz ^{13}C{^{1}H} NMR spectrum are plotted. The connectivities between the resonances marked c and e are indicated by broken lines.

^{13}C NMR of Methenyl-THMP. In order to assign the ^{13}C NMR resonances of methenyl-THMP (shown in Figure 5 along the vertical axis), a 200/50.3-MHz ^{1}H–^{13}C shift-correlated NMR experiment was performed with ^{13}C-labeled methenyl-THMP. Most of the ^{13}C resonances show cross peaks with ^{1}H resonances and are assigned accordingly. ^{13}C resonances 1e, 2e, 4e, and 5e are broadened due to chemical exchange processes (protonation $\rightleftarrows$ deprotonation of the carboxylic acid groups, $pK_a \approx 4$–5), thereby hampering the detection of cross peaks. ^{13}C resonances 1e, 4e, and 5e are therefore assigned on the basis of the correspondence of their chemical shifts with the ^{13}C resonances of the α-hydroxyglutaric acid moiety of methanopterin. The remaining ^{13}C resonances, i.e., 4a, 2a, 9a, 1b, 4b, and 10a, do not show cross peaks to ^{1}H resonances and do not appear as "positive" signals in the ^{13}C NMR spectrum of the attached proton test. Hence, these ^{13}C resonances belong to quaternary carbon atoms and are assigned on the basis of chemical shift comparison with the corresponding ^{13}C resonances of methanopterin and methenyl-THF (Table I).

The most intense resonance in the ^{13}C NMR spectrum of ^{13}C-labeled methenyl-THMP shows a cross peak with ^{1}H resonance 14a. (The other cross peaks correlated with ^{13}C resonance 14a are artifacts due to the high intensity of this signal.) It follows that methenyl carbon 14a is enriched with ^{13}C. Moreover, the ^{13}C satellites of ^{1}H resonance 14a (Figure 1, insert) are observed in the ^{13}C-labeled sample of methenyl-THMP only. Comparison of the intensity of the ^{13}C satellites of ^{1}H resonance 14a with the total intensity of ^{1}H resonance 14a shows that methenyl carbon 14a is enriched for 6% with ^{13}C. From the ^{13}C satellites of ^{1}H resonance 14a a $^1J_{^{13}C-^1H} = 210$ Hz was inferred, which is in good agreement with the corresponding $^1J_{^{13}C-^1H} = 207$ Hz of the methenyl group of methenyl-THF (Armarego & Waring, 1980).

The ^{13}C resonance marked "formyl" in Figure 5 is not a quaternary resonance since it shows a positive peak in the ^{13}C NMR spectrum of the attached proton test. However, a cross peak to a ^{1}H resonance is not observed. This can be explained by assuming that methenyl-THMP is in equilibrium with 10-formyl-THMP just as methenyl-THF is in equilibrium with

Table I: ^1H and ^{13}C Chemical Shifts of Methenyl-THMP[a]

	methenyl-THMP			methanopterin[b]		methenyl-THF	
	$\delta(^{13}C)$	sign APT[c]	$\delta(^1H)$	$\delta(^{13}C)$	$\delta(^1H)$	$\delta(^{13}C)$[d]	$\delta(^1H)$[e]
2a	158.0	−		163.9		153.0	
4a	166.3			173.1		154.9	
6a	73.8	+	4.02	151.2		52.6	5.30
7a	51.2	+	3.8	156.5		50.4	4.65/4.12
9a	154.6	−		155.1		150.7	
10a	91.2	−		126.8		89.3	
11a	60.1	+	4.95	51.3	4.92	43.4	5.24/4.90
12a	19.5	+	1.49	20.4	1.47		
13a	15.5	+	1.41	21.1	2.63		
14a	149.3	+	8.97			146.3	9.95
(formyl	163.3	+)					
1b	140.2	−		145.5		139.3	
2b/6b	122.2	+	7.36	115.8	6.78	117.1	7.94
3b/5b	132.2	+	7.43	130.4	7.03	129.5	8.33
4b	133.6	−		129.4		130.7	
1c/1c′	38.6	−	3.10	37.0	2.84		
			2.71		2.48		
2c	69.1	+	4.0	70.5	3.87		
3c	75.5	+	3.73	74.4	3.69		
4c	70.9	+	3.8	73.1	3.77		
5c	70.3	−	4.0	69.4	3.95		
1d	103.4	+	5.14	102.4	5.17		
2d	72.4	+	4.19	71.2	4.23		
3d	71.5	+	4.13	69.9	4.17		
4d	85.0[f]	+	4.25	83.8[f]	4.28		
5d	66.4[f]	−	4.0	65.4[f]	4.02/3.99		
1e	179.3[f]			178.7[f]			
2e	76.8[f]		4.44	76.5[f]	4.44		
3e	31.0	−	2.03	30.8	2.03		
4e	32.8		2.39	33.4	2.34/2.26		
5e	181.2			182.5			

[a] The corresponding values of methanopterin and methenyl-THF are given for comparison purposes. [b] Reported by Van Beelen et al. (1984a). [c] The number of protons directly bound to the carbon atoms was determined by a so-called attached proton test (APT) (Patt & Shoolery, 1982). Primary and tertiary carbons give rise to a positive resonance intensity [marked by (+) in the table entry "sign APT"] while secondary and quaternary carbon atoms cause a negative resonance intensity. [d] Reported by Armarego & Waring (1980). [e] Reported by Khalifa et al. (1979). [f] A coupling with ^{31}P was observed.

10-formyl-THF (Blakley, 1969). Since the intensity of this formyl ^{13}C resonance is only 5% of methenyl ^{13}C resonance 14a, it follows that the intensity of the corresponding formyl ^1H resonance is only 5% of ^1H resonance 14a which explains the absence of a cross peak in Figure 5. (Note that the formyl resonance is absent in the ^1H-NMR spectrum which was recorded from a fresh sample.)

Comparison of the ^1H and ^{13}C Chemical Shifts of Methenyl-THMP with the Corresponding Values for Methanopterin and Methenyl-THF. Table I shows that the ^1H and ^{13}C chemical shifts recorded for the α-hydroxyglutaric acid moiety, the α-ribofuranose moiety, and the tetrahydroxypentane moiety of methenyl-THMP are very similar to the corresponding values for methanopterin. The ^1H and ^{13}C chemical shifts of the 5,6,7,8-tetrahydropterin moiety, the aniline moiety, and the methenyl group are quite similar to the corresponding values stated for methenyl-THF (Table I). The downfield shift of the aniline ^1H resonance of methenyl-THMP as compared to methanopterin is also observed in the *p*-aminobenzoic acid ^1H resonances of methenyl-THF as compared to folic acid (Khalifa et al., 1979; Poe, 1979).

In summary, the ^1H and ^{13}C NMR chemical shift data are in accordance with the proposed structure shown in Figure 2.

Mass Spectrometry. Fast atom bombardment mass spectrometry was performed with methenyl-THMP. The positive ion mass spectrum of natural abundance methenyl-THMP is shown in Figure 6. In the experiment performed with ^{13}C-enriched methenyl-THMP the peaks derived from this compound showed large peaks one mass unit higher as compared to Figure 6. Since methenyl-THMP was isolated by using

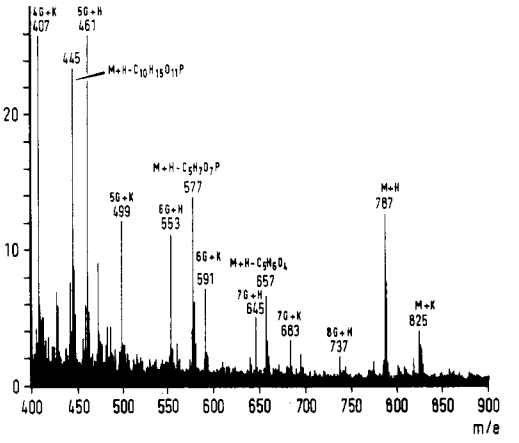

FIGURE 6: Positive ion fast atom bombardment mass spectrum of natural abundance 5,10-methenyl-5,6,7,8-tetrahydromethanopterin. *M* = mass of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin ($C_{31}H_{43}N_6O_{16}P$) and *G* = mass of glycerol ($C_3H_8O_3$).

potassium-containing buffers, the presence of glycerol clusters containing a K^+ ion instead of a H^+ ion is explained. The samples of methenyl-THMP were desalted with hydrochloric acid at pH 3. Since the bond between the tetrahydroxypentane moiety and the ribofuranose moiety is susceptible to acid hydrolysis (Van Beelen et al., 1984b) and the same may be true for the ester bonds of the phosphodiester moiety, acid hydrolysis products of methenyl-THMP are expected to be present in the mass spectrum.

Figure 7 shows that the fragmentation pattern of methenyl-THMP is in accordance with the proposed structure.

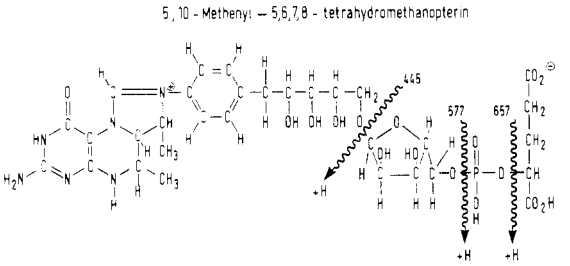

FIGURE 7: Fragmentation pattern of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin.

Discussion

The structure of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin shows one additional carbon atom as compared to methanopterin (see Figure 2). This additional carbon atom of methenyl-THMP is rapidly labeled in vivo by $^{13}CO_2$. The amount of methenyl-THMP in cells of *M. thermoautotrophicum* is about 1 μmol/g dry weight which is in the same range of concentration as coenzyme MF_{430} and coenzyme F_{420} (Van Beelen et al., 1983a), which are other coenzymes involved in methanogenesis. Since the main part of the carbon dioxide used by *M. thermoautotrophicum* is converted to methane (Schönheit et al., 1980), it would be expected that carriers of one-carbon groups in methanogenesis are present in larger amounts than those involved in other metabolic pathways. On these grounds it is plausible that methenyl-THMP is a one-carbon carrier involved in methanogenesis.

Methenyl-THMP is a physiologically active coenzyme which is very rapidly converted under hydrogen by small amounts of cell-free extracts of *M. thermoautotrophicum* and probably transformed to THMP (P. Van Beelen, unpublished results) whereas methanopterin is only slowly converted under identical conditions (Van Beelen et al., 1983b).

The resemblance between the structures of methenyl-THMP and methenyl-THF suggests the participation of a folate-like biochemistry in methanogenesis. Therefore, it was suggested that methenyl-THMP, methylene-THMP, and methyl-THMP participate in methanogenesis as one-carbon carriers at the formyl, formaldehyde, and methanol levels of oxidation, respectively (Vogels & Visser, 1983).

Acknowledgments

We thank W. J. Geerts and P. A. W. van Dael for technical assistance. NMR spectra were recorded at the Dutch National 500/200 MHz hf-NMR facility at Nijmegen. Mass spectrometry was performed by P. W. M. Wijers at the Department of Organic Chemistry at University of Nijmegen.

Registry No. Methenyl-THMP, 89455-79-8; CO_2, 124-38-9; CH_4, 74-82-8; methanopterin, 79484-89-2.

References

Armarego, W. L. F., & Waring, P. (1980) *J. Chem. Res., Miniprint*, 3910–3918.

Aue, W. P., Bartholdi, E., & Ernst, R. R. (1976) *J. Chem. Phys. 64*, 2229–2246.

Bax, A. (1982) Ph.D. Thesis, Delft, The Netherlands.

Benz, F. W., Feeney, J., & Roberts, G. C. K. (1972) *J. Magn. Reson. 8*, 114–121.

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, pp 58–99, North-Holland Publishing Co., Amsterdam.

Bodenhausen, G., & Freeman, R. (1977) *J. Magn. Reson. 28*, 471–476.

Daniels, L., & Zeikus, J. G. (1978) *J. Bacteriol. 136*, 75–84.

Hutten, T. J., De Jong, M. H., Peeters, B. P. H., Van der Drift, C., & Vogels, G. D. (1981) *J. Bacteriol. 145*, 27–34.

Keltjens, J. T., & Vogels, G. D. (1980) in *Microbial Growth on C_1 Compounds* (Dalton, H., Ed.) pp 152–158, Heyden, London, Philadelphia, Rheine.

Keltjens, J. T., Huberts, M. J., Laarhoven, W. H., & Vogels, G. D. (1983a) *Eur. J. Biochem. 130*, 537–544.

Keltjens, J. T., Daniels, L., Jansen, H. G., Borm, P. J., & Vogels, G. D. (1983b) *Eur. J. Biochem. 130*, 545–552.

Kenealy, W. R., & Zeikus, J. G. (1982) *J. Bacteriol. 151*, 932–941.

Khalifa, E., Bieri, J. H., & Viscontini, M. (1979) *Helv. Chim. Acta 62*, 1340–1344.

Macura, S., & Ernst, R. R. (1980) *Mol. Phys. 41*, 95–117.

Maudsley, A. A., Müller, L., & Ernst, R. R. (1977) *J. Magn. Reson. 28*, 463–469.

Patt, S. L., & Sykes, B. D. (1972) *J. Chem. Phys. 56*, 3182–3184.

Patt, S. L., & Shoolery, J. N. (1982) *J. Magn. Reson. 46*, 535–539.

Poe, M. (1980) *Methods Enzymol. 66*, 483–490.

Reed, L. S., & Archer, M. C. (1980) *J. Agric. Food Chem. 28*, 801–805.

Schönheit, P., Moll, J., & Thauer, R. K. (1979) *Arch. Microbiol. 123*, 105–107.

Van Beelen, P., Geerts, W. J., Pol, A., & Vogels, G. D. (1983a) *Anal. Biochem. 131*, 285–290.

Van Beelen, P., Thiemessen, H. L., De Cock, R. M., & Vogels, G. D. (1983b) *FEMS Microbiol. Lett. 18*, 135–138.

Van Beelen, P., Stassen, A. P. M., Bosch, J. W. G., Vogels, G. D., Guijt, W., & Haasnoot, C. A. G. (1984a) *Eur. J. Biochem. 138*, 563–571.

Van Beelen, P., Labro, J. F. A., Keltjens, J. T., Geerts, W. J., Vogels, G. D., Laarhoven, W. H., Guijt, W., & Haasnoot, C. A. G. (1984b) *Eur. J. Biochem. 139*, 359–365.

Van Beelen, P., De Cock, R. M., Guijt, W., Haasnoot, C. A. G., & Vogels, G. D. (1984c) *FEMS Microbiol. Lett. 21*, 159–163.

Vogels, G. D., & Visser, C. M. (1983) *FEMS Microbiol. Lett. 20*, 291–297.

Wider, G., Hosur, R. V., & Wüthrich, K. (1983) *J. Magn. Reson. 52*, 130–135.