Biosynthesis of Methanofuran in Methanobacterium thermoautotrophicum*

Wolfgang Eisenreich and Adelbert Bacher
From the Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, D-8046 Garching, Federal Republic of Germany

The $^{13}$C NMR signals of methanofuran were assigned by two-dimensional $^1$H and $^{13}$C NMR experiments. On this basis, the incorporation of $^{13}$C-labeled acetate and pyruvate into methanofuran by growing cells of Methanobacterium thermoautotrophicum was analyzed by one- and two-dimensional $^{13}$C NMR experiments. The data were analyzed by a retrobiosynthetic approach based on a comparison of labeling patterns in a variety of metabolites. The data show that the furan ring is formed by condensation of two molecules from the pyruvate/triose pool. The tetracarboxylic acid moiety is assembled from ketoglu tarate, two molecules of acetyl-CoA, and one molecule of carbon dioxide.

Methanogenic bacteria have developed a unique metabolic strategy which enables them to generate energy by the formation of methane from carbon dioxide and hydrogen (for review see Daniels et al., 1984; Jones et al., 1987; Thauer, 1990). This unusual metabolism has been studied in considerable detail and has been shown to involve a variety of hitherto unknown coenzymes (DiMarco et al., 1990). The primary acceptor for carbon dioxide is the novel coenzyme, methanofuran (1, Fig. 1), which is characterized by the presence of an aminomethylfuran system. Carbon dioxide is bound at the aminomethyl group of the coenzyme and is immediately reduced to the formate level (Karrasch et al., 1989; Börner et al., 1989). The formate moiety is then transferred to tetrahydrodianopterin, which is structurally and functionally similar to tetrahydrofolate (Breitung and Thauer, 1990). Subsequent reduction steps involving the deazaflavin type coenzyme $F_3$ and coenzyme M lead to the formation of methane and the generation of energy.

Methanofuran has been isolated and characterized by Wolfe and his coworkers (Leigh et al., 1984). The structure has been elucidated by a combination of mass spectrometry, NMR spectroscopy, and chemical degradation. However, a detailed NMR study has not yet been performed.

White studied the biosynthetic pathway of methanofuran in rumen isolate 10-16B and in Methanobrevibacter smithii by incorporation of $^1$H- and $^{13}$C-labeled acetate (White, 1987, 1988). The isotope distribution in biosynthetic methanofuran was monitored by mass spectrometry. Whereas this method has the advantage of high sensitivity, it is not always possible to unequivocally assign $^{13}$C-isotope enrichments to individual atoms in the target molecule. In contrast, unequivocal $^{13}$C assignments for each individual carbon atom can be obtained by NMR spectroscopic techniques. This paper reports on a detailed NMR investigation of methanofuran and on the incorporation of $^{13}$C-labeled acetate and pyruvate.

EXPERIMENTAL PROCEDURES

Chemicals—$[1-^{13}$C]Acetate, $[2-^{13}$C]acetate, and $[1,2-^{13}$C$_2$]acetate were purchased from Cambridge Isotope Laboratories (Woburn, MA). $[1,2-^{13}$C$_2$]Pyruvate was obtained from Isotec Inc. (Miamisburg, OH). Other chemicals were of the highest purity available.

Microorganism—Methanobacterium thermoautotrophicum Marburg (DSM 2133) was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Federal Republic of Germany. The strain was subcultured at weekly intervals in serum bottles under an atmosphere of H$_2$/CO$_2$ (4:1, v/v) which were incubated at 65 °C.

Bacterial Culture—M. thermoautotrophicum was grown on mineral salt medium under an atmosphere of H$_2$/CO$_2$ as described earlier (Eisenreich et al., 1991a). $^{13}$C-Labeled acetate and pyruvate were added to logarithmically growing cultures to a final concentration of 4.0 and 2.1 mM, respectively. The pH was continuously adjusted to 6. Cells were harvested at the end of exponential growth phase.

NMR Spectroscopy—NMR Spectra were recorded at 8.46 Tesla on a Bruker AM 360 spectrometer at 27 °C. Methanofuran was measured in 80% H$_2$O/20% D$_2$O (pH 3.6) or in 99.9% D$_2$O (pH 3.6). pH values are uncorrected glass electrode readings.

All $^{13}$C NMR spectra were recorded under identical conditions. Data acquisition and processing parameters were as follows: 64,000 data set, 30° pulse width (2 μs), 2.5-s scan interval, 14.7-kHz spectral range, composite pulse decoupling, 1-Hz line broadening.

Two-dimensional double quantum filtered COSY, distortionless enhancement by polarization transfer, and INADEQUATE experiments were performed according to standard Bruker software (DIS887). Phase-sensitive two-dimensional TOCSY and spin-locked NOE (ROESY) spectroscopy were performed according to Braunschweiler and Ernst (1983) and Bothner-By et al. (1984). The H-detected multiple quantum $^1$H-$^{13}$C chemical shift correlation experiments were performed according to Bax and co-workers (Bax and Subramanian, 1986; Bax and Summers, 1986). Samples were not rotated during two-dimensional experiments. Water suppression was achieved by phase-coherent presaturation.

Data acquisition and processing parameters for two-dimensional experiments were as follows. COSY: 32 scans per $τ_1$ increment, 1.6-s relaxation delay, 512 × 2056 raw data matrix size zero-filled to 2056 in $τ_1$ and processed with 3 Hz Gaussian in the $f_1$ dimension and 90°-shifted sine bell filtering in the $f_2$ dimension. TOCSY: 24 scans per $τ_1$.

*This work was supported by the Fond der Chemischen Industrie and the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
increment, 2.9 s relaxation delay, 62 ms MLEV-17 mixing period preceded and followed by 2.5 ms trim pulses; 90° pulse width, 60 μs; 512 × 2056 raw data matrix size, zero-filled to 10256 in f1 and processed with 2 Hz Gaussian in the f1 dimension and 90°-shifted sine bell filtering in the f2 dimension. ROESY: 64 scans per τ1 increment, 1.0 s relaxation delay, 200 ms continuous wave spin lock period; 90° pulse width, 60 μs; 360 × 1024 raw data matrix size, zero-filled to 1024 in τ1 and processed with 2 Hz Gaussian in the f1 dimension and 90°-shifted sine bell filtering in the f2 dimension. 'H-13C HMBC: 64 scans per τ1 increment, start of coherence experiment 123 ms after bilinear rotation decoupling pulse, 3.5 ms delay period for evolution of JCH corresponding to a coupling constant of 145 Hz; '3C decoupling during acquisition by the globally optimized alternating-phase rectangular pulses sequence; 500 × 2048 raw data matrix size, zero-filled to 1024 in τ1 and processed with 10 Hz Gaussian in f1 and 90°-shifted squared sine filtering in f2. 'H-13C HMBC: 86 scans per τ1 increment, 0.8 s relaxation delay, 3.5 ms delay for suppression of 13C, 60 ms delay period for evolution of long range couplings; 200 × 1024 raw data matrix size, zero-filled to 512 words in τ1 and processed with 90°-shifted sine bell filtering in f1. INADEQUATE: 128 scans per τ1 increment, 2.0 s relaxation delay, Ernst-type phase cycle, 3.6 ms delay for evolution of 13C, 128 × 512 raw data matrix size, zero-filled to 512 words in τ1 and processed with 60°-shifted sine bell filtering in f1 and f2.

High Performance Liquid Chromatography—Methanofuran was analyzed by reversed-phase HPLC using a column of Nucleosil 10 C4 (4.55 × 260 mm) and an eluent containing 12% methanol and 100 mM ammonium formate. The effluent was monitored by photometry (220 nm) and by refractometry. The retention volume of methanofuran was 9.2 ml.

Isolation of Methanofuran—Cell paste of M. thermoautotrophicum (100 g) was suspended in 50% aqueous acetone (400 ml) at -10°C. The suspension was centrifuged, and the residue was again extracted as described until the supernatant was colorless (approximately 10 times). The supernatants from the extraction procedures were combined and concentrated to dryness under reduced pressure. The residue was dissolved in 80 ml of water and placed on a column of QAE Sephadex A-25 (HCO3 form, 3.250 mm) and an eluent containing 12% methanol and 100 mM ammonium formate. The effluent was monitored by photometry (220 nm) and by refractometry. The retention volume of methanofuran was 9.2 ml.

Purification of Methanofuran—Crude methanofuran was purified by preparative HPLC using a column of Lichrosorb RP18 (16 × 250 mm) with an eluent containing 22% methanol and 30 mM formic acid. The eluent was monitored by photometry (220 nm) and by refractometry. Methanofuran had a retention volume of 500 ml. Fractions were combined and evaporated to dryness under reduced pressure. Preparative HPLC purification was repeated using the same column with an eluent containing 33% methanol and 60 mM formic acid. The retention volume was 145 ml. Fractions were combined, evaporated to dryness under reduced pressure, and dried under vacuum.

Isolation of Nucleosides and Amino Acids—The residual cell mass after extraction of methanofuran was treated with 1 M NaOH to hydrolyze RNA. Nucleosides were isolated from the supernatant as described (Eisenreich et al., 1984). The protein-containing residue was hydrolyzed in 6 M HCl, and amino acids were isolated as described earlier (Eisenreich et al., 1984; 1986).
### Biosynthesis of Methanofuran

#### TABLE I

**Assignment of the $^1$H NMR signals of methanofuran**

| Position | Chemical shift $^a$ ppm | DQF-COSY | TOCSY | ROESY |
|----------|-------------------------|-----------|--------|--------|
| NH(d)    | 8.11                    | 2d        | 2d,4d,3d,3d' | 2e,3d,5d,7d,2d |
| NH(c)    | 8.07                    | 2c        | 2e,4c,3c,3c' | 4d,3c,3c',2c,4c |
| NH(b)    | 7.93                    | 8b        | 8b,7b     | 4c,8b,7b |
| 5a       | 7.70                    |           |          |        |
| 3b/5b    | 7.21                    | 2b/6b     | 2b/6b    | 7b,8b  |
| 2b/6b    | 6.98                    | 3b/5b     | 3b/5b    |        |
| 3a       | 6.70                    |           |          |        |
| 7a       | 4.99                    |           |          |        |
| 6a       | 4.26                    |           |          |        |
| 2d       | 4.24                    | 3d,3d',NH(d) | NH(d),4d,3d,3d' | 3d,3d',4d,NH(d) |
| 2c       | 4.19                    | 3c,3c',NH(c) | NH(c),4c,3c,3c' | 3c,3c',4c,NH(c) |
| 8b       | 3.43                    | 7b,NH(b)  | NH(b),7b | 3b,4c,8b NH(b) |
| 7b       | 2.77                    | 8b        | 8b        | 3b,8b   |
| 5e       | 2.59                    | 6e        | 6e,3e,7e,2e | 6e       |
| 4e       | 2.59                    | 3e        | 3e,3e,7e,2e | 3e       |
| 4d       | 2.40                    | 3d,3d'    | 3d,3d',NH(d),2d | 2d,NH(c) |
| 7e       | 2.40                    | 6e        | 5e,4e,3e,6e | 6e       |
| 2e       | 2.35                    | 3e        | 5e,4e,3e,6e | 3e,NH(d) |
| 4c       | 2.27                    | 3c,3c'    | 3c,3c',NH(c),2c | 3c,3c',2c,NH(b),NH(c),3b,5b |
| 3d       | 2.20                    | 3d',2d,4d | 4d,3d',NH(d),2d | 3d',2d,NH(d) |
| 3c       | 2.09                    | 3c',2c,4c | 4c,3c',NH(c),2c | 3c',2c,NH(c),8b |
| 3d'      | 1.98                    | 3d,3d,4d  | 4d,3d,4d,NH(d),2d | 3d,2d,NH(d) |
| 3c'      | 1.90                    | 3c,2c,4c  | 4c,3c,NH(c),2c | 3c,5c,NH(c),8b |
| 3e       | 1.83                    | 2e,4e     | 5e,4e,7e,2e | 2e,4e   |
| 8e       | 1.83                    | 7e,5e     | 5e,6e,7e,2e | 7e,5e   |

$^a$ Referenced to external 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt, in D$_2$O (pH 3.6).

---

**Fig. 2.** Part of the phase-sensitive 2D-ROESY spectrum of methanofuran obtained with a 200-ms spin-lock period. Displayed peaks are opposite in phase relative to the diagonal signals. Other connectivity patterns indicated in Table I could be observed by inspection of the two-dimensional matrix at a lower contour level. The one-dimensional $^1$H NMR spectrum of methanofuran is shown at the axes.

Additional confirmation of $^{13}$C assignments was obtained by an INADEQUATE experiment performed with a sample of methanofuran from a growth experiment with [1,2-$^{13}$C$_2$] acetate (Fig. 4 and Table II). Carbon pairs biosynthetically derived from acetate gave strong $^{13}$C-$^{13}$C correlation signals. This experiment corroborated the assignments of 10 pairs of directly adjacent carbon atom pairs which had been jointly incorporated from the double-labeled precursor. It should be noted that some of the $^{13}$C-$^{13}$C couplings in Fig. 4 are nonlinear as a consequence of the relatively small chemical shift differences between the coupled carbon atoms. As a consequence, the satellites are not symmetrical with respect to the unlabeled central peak.

The carbon atom pairs 1c/1d (179.09 ppm) and 9e/10e (180.97 and 181.00 ppm) could not be assigned on the basis of two-dimensional correlation methods due to signal overlapping. However, a detailed analysis of $^{13}$C-$^{13}$C coupling satellites in the one-dimensional $^{13}$C NMR spectrum obtained from the fermentation experiment with [1,2-$^{13}$C$_2$]acetate revealed $^{13}$C-$^{13}$C coupling from C-5e to the signal at 181.00 ppm, which was therefore assigned as C-10e.

**Biological Studies—** M. thermoautotrophicum (Marburg strain) was grown autotrophically in a mineral salt medium at 65 °C. The culture medium was gassed with a mixture of H$_2$/CO$_2$ (80:20, v/v). The growing cultures were supplemented with $^{13}$C-labeled acetate (4.0 mM) or pyruvate (2.1 mM). The pH was kept at 6.0 to facilitate the uptake of the organic supplements.

After a culture period of 50–100 h, the cells were harvested by centrifugation. Methanofuran was obtained from the cell mass by repeated extraction with acetone/water. The raw material was purified by anion-exchange chromatography followed by preparative reversed-phase HPLC.

$^{13}$C NMR spectra of methanofuran were recorded at 90.6 MHz (Fig. 5). The acquisition parameters were the same throughout the study. Integrals of $^{13}$C NMR signals were determined. Relative $^{13}$C abundances were calculated for all carbon atoms of methanofuran analyzed by comparison of signal integrals with the integrals of a natural abundance sample. The carbon atom with the lowest relative $^{13}$C enrichment in each compound was arbitrarily assigned a value of 1.0 under the assumption that at least one carbon atom in each compound was derived exclusively from CO$_2$ and not incorporated from the double-labeled precursor.
from acetate or pyruvate. This assumption has been shown to be valid in studies with \textit{M. thermoautotrophicum} but need not be correct in studies with other anaerobic microorganisms (Strauss et al., 1992).

The data are summarized in Table III. The carboxylic groups 1c, 1d, 5e, and 6e of methanofuran were unlabeled in all experiments. Thus, these carbon atoms were obligatorily derived from CO\textsubscript{2} and could not be contributed by either acetate or pyruvate.

Some of the \textsuperscript{13}C signals of methanofuran were closely adjacent even at pH 3.6. Due to signal overlap, it was sometimes impossible to obtain separate enrichment values for overlapping signals. This presented no problems in cases where all overlapping signals were in the natural abundance range. For example, neither of the overlapping signals of C-1c and C-1d acquired significant amounts of label from any of the precursors studied. It follows that these carbon atom were derived from carbon dioxide.

The problem of signal overlap was more prominent in the sample obtained from \textit{[1,2-\textsuperscript{13}C\textsubscript{2}]-acetate} because contiguously labeled carbon pairs appear as multiplets containing a central signal corresponding to the singly labeled species and satellites due to \textsuperscript{13}C-\textsuperscript{13}C coupling. However, the signal overlap did not significantly reduce the biosynthetic information for two reasons. (i) The information obtained from \textit{[1,2-\textsuperscript{13}C\textsubscript{2}]-acetate}, on one hand, and from \textit{[1-\textsuperscript{13}C\textsubscript{1}]-acetate}, on the other hand, is redundant to a considerable extent. (ii) The overlapping multiplets in the one-dimensional \textsuperscript{13}C NMR spectrum of the sample obtained from double-labeled acetate could be resolved by the INADEQUATE experiment shown in Fig. 4. In this double quantum experiment, only pairs of labeled carbon atoms are detected. The $f_2$ dimension corresponds to the chemical shift of single carbon atoms, but the $f_1$ dimension represents the sum of the chemical shifts of two contiguous and \textsuperscript{13}C-labeled carbon atoms. Thus, even if the signals overlap in the $f_1$ dimension, they are clearly separated in the $f_2$ dimension. By this experimental approach, each acetate moiety which had been incorporated into methanofuran as an intact unit could be determined unequivocally.

The experiment with \textit{[1-\textsuperscript{13}C]-pyruvate} needs specific consideration, because these data were not redundant with the other experiments. Luckily, several cases of signal overlap concerned pairs of atoms which were both unlabeled (e.g. 9e/10e, 1c/1d, 4e/5e). The only uncertainty with biosynthetic relevance concerned the unresolved groups of signals for le, 5c, and 5d in the methanofuran signal from \textit{[1,2-\textsuperscript{13}C\textsubscript{2}]-acetate} into the furan ring, and three pairs were incorporated into the tetracarboxylic moiety. Each of the glutamate moieties showed one pair of carbon atoms from acetate. About 60--80\% of the \textsuperscript{13}C signal intensity of these
Biosynthesis of Methanofuran

Fig. 3. Part of the HMQC spectrum of methanofuran. The one-dimensional $^1$H NMR and $^{13}$C NMR spectra are shown at the axes.

Fig. 4. Part of the INADEQUATE spectrum of methanofuran from the growth experiment with [1,2-13C$_2$]acetate. The one-dimensional $^{13}$C NMR spectrum of methanofuran from the fermentation with [1,2-13C$_2$]acetate is shown at the top.

carbon atoms was located in the satellites arising by $^{13}$C-$^{13}$C coupling as shown by evaluation of $^{13}$C signal integrals. These carbon atom pairs were also visualized by a two-dimensional INADEQUATE experiment (Fig. 4 and Table II) and by a $^{13}$C TOCSY experiment (data not shown). The results of all labeling experiments are summarized in Fig. 6. Joint incorporation of contiguous carbon atom pairs are shown by bold lines.

We have described earlier the labeling patterns of amino acids and nucleosides biosynthesized in *M. thermoautotrophicum* from $^{13}$C-labeled acetate and pyruvate (Eisenreich *et al.*, 1991a, 1991b). The glutamate moieties of methanofuran virtually duplicate the labeling pattern of glutamate (6, Fig. 7) isolated from cell protein. Similarly, the labeling pattern of the 4-hydroxyphenylethylamine moiety repeats the labeling pattern of tyrosine (Eisenreich *et al.*, 1991a). This correspondence of labeling patterns is biosynthetically trivial but documents the validity of the experimental approach.

The labeling patterns of amino acids and nucleosides described earlier allow the reconstruction of central metabolite pools such as pyruvate (2) and dicarboxylic acids by a retrobiosynthetic approach (Fig. 7). For example, the labeling pattern of precursors from the triose/pyruvate pool could be reconstructed from the labeling pattern of ribose (3) and amino acids (Eisenreich and Bacher, 1991a, 1991b). This analysis served as a basis for the interpretation of the labeling pattern of methanofuran by a pattern-recognition approach.

The labeling pattern of the furan moiety suggests the incorporation of two triose modules. More specifically, a plausible reaction mechanism can be written with dihydroxyacetone phosphate (7) and pyruvate (2) as precursors (Fig. 8). These findings are in agreement with the results obtained by mass spectrometric studies (White, 1988).

Three contiguous pairs of carbon atoms are incorporated into the tetracarboxylic subunit of methanofuran. Notably, one of the incorporated acetate modules contributes the carboxylic group 10e. This finding is firmly documented by the two-dimensional INADEQUATE and $^{13}$C TOCSY experiments.

Both [1-13C]acetate and [2-13C]acetate can contribute their label to each of the carbon atoms 2e and 3e. The relative $^{13}$C enrichments of these carbons was about 2.5 and 4.4 in the experiments with [1-13C]- and [2-13C]acetate. These values represent about half the enrichment in other labeled carbon atoms in each respective experiment. The data signify that the acetate moiety must have passed through an inherently symmetrical intermediate such as succinate (4, Fig. 7).
The proposed reaction sequence does not predict whether the biosynthetic pathway proceeds at the level of the free acid or starts from a carboxamide-type precursor. In either case, a synthesis concept in organic chemistry. For details see Fig. 6.

The arrows indicate reconstructed precursors in analogy to the retrobiosynthetic approach (from Eisenreich and Bacher, 1991a, 1991b). The arrows indicate reconstructed precursors in analogy to the retrobiosynthetic approach (from Eisenreich and Bacher, 1991a, 1991b). The arrows indicate reconstructed precursors in analogy to the retrobiosynthetic approach (from Eisenreich and Bacher, 1991a, 1991b). The arrows indicate reconstructed precursors in analogy to the retrobiosynthetic approach (from Eisenreich and Bacher, 1991a, 1991b).

The observed labeling pattern suggests the following biosynthetic pathway (Fig. 9). An aldol-type reaction between 2-ketoglutarate (5) and acetyl-CoA yields the hydroxy acid 8. Elimination of water yields the unsaturated acid 9 which adds a second molecule of acetyl-CoA. Reductive carboxylation via agreement with this hypothesis, the carbon atoms 1e-4e and 9e virtually duplicate the labeling pattern of 2-ketoglutaric acid (5). It should be noted that the formation of 2-ketoglutarate from pyruvate via succinate is well established in M. thermoautotrophicum (Fuchs and Stupperich, 1978, 1980).

The carbon atoms 6e and 7e are derived from an acetate module, and thus one would expect that the carbon atoms 6e-8e are formed via pyruvate as intermediate. However, this hypothesis can be ruled out since the carboxylic group 8e is not labeled from C-1 of pyruvate. It follows that the carboxylate group 8e is derived obligatorily from carbon dioxide.

The proposed reaction sequence does not predict whether the biosynthetic pathway proceeds at the level of the free acid or starts from a carboxamide-type precursor. In either case, a nonsymmetrical distribution of label would result as a consequence of the prochiral character of the meso-tetracarboxylic acid.

\[ \text{Biosynthesis of Methanofuran} \]

| Position | \(^{13}C\) chemical shift | \(^{13}C/^{12}C\) Coupling | Relative \(^{13}C\) enrichment |
|----------|--------------------------|--------------------------|----------------------------|
|          | ppm                      | Hz                       | %                          | [1-\(^{13}C\)]acetate | [2-\(^{13}C\)]acetate | [1-2-\(^{13}C\)]acetate | [1-\(^{13}C\)]pyruvate |
| 10e      | 181.00                   | 53.1                     | 78                         | 6.1                      | 1.0                      | 1.0                      | 1.0                      |
| 9e       | 180.97                   | 10.6                     | 1.2                        | 1.2                      | 1.0                      | 1.0                      | 1.0                      |
| 8e       | 179.94                   | 1.6                      | 1.2                        | 1.2                      | 1.0                      | 1.0                      | 1.0                      |
| 1c       | 179.09                   | 1.4                      | 1.1                        | 1.1                      | 1.0                      | 1.0                      | 1.0                      |
| 1d       | 179.09                   | 1.2                      | 1.2                        | 1.2                      | 1.0                      | 1.0                      | 1.0                      |
| 1e       | 177.15                   | 1.5                      | 1.5                        | 1.5                      | 1.0                      | 1.0                      | 1.0                      |
| 5c       | 177.05                   | 1.2                      | 1.2                        | 1.2                      | 1.0                      | 1.0                      | 1.0                      |
| 5d       | 176.99                   | 1.3                      | 1.3                        | 1.3                      | 1.0                      | 1.0                      | 1.0                      |
| 1b       | 158.18                   | 1.3                      | 1.3                        | 1.3                      | 1.0                      | 1.0                      | 1.0                      |
| 2a       | 149.39                   | 73.5                     | 73                         | 5.7                      | 1.3                      | 5.2                      | 1.0                      |
| 5a       | 145.17                   | 1.4                      | 1.4                        | 1.4                      | 1.0                      | 1.0                      | 1.0                      |
| 4b       | 134.56                   | 3.4                      | 3.4                        | 3.4                      | 1.1                      | 3.1                      | 1.1                      |
| 3b/5b    | 132.26                   | 76.6                     | 76                         | 7.6                      | 9.3                      | 4.3                      | 1.0                      |
| 4a       | 124.21                   | 1.0                      | 1.0                        | 1.0                      | 4.3                      | 4.3                      | 1.0                      |
| 2b/6b    | 117.46                   | 3.1                      | 3.1                        | 3.1                      | 4.4                      | 4.4                      | 1.0                      |
| 3a       | 113.58                   | 1.3                      | 1.3                        | 1.3                      | 4.2                      | 4.2                      | 1.0                      |
| 7a       | 63.99                    | 1.5                      | 1.5                        | 1.5                      | 5.0                      | 5.0                      | 1.0                      |
| 2c       | 55.68                    | 1.1                      | 1.1                        | 1.1                      | 1.0                      | 1.0                      | 1.0                      |
| 2d       | 55.53                    | 1.1                      | 1.1                        | 1.1                      | 1.0                      | 1.0                      | 1.0                      |
| 4e       | 50.53                    | 1.1                      | 1.1                        | 1.1                      | 1.0                      | 1.0                      | 1.0                      |
| 5e       | 50.45                    | 4.3                      | 4.3                        | 4.3                      | 6.1                      | 6.1                      | 1.0                      |
| 6b       | 42.72                    | 1.2                      | 1.2                        | 1.2                      | 8.8                      | 8.8                      | 1.0                      |
| 6a       | 37.90                    | 1.2                      | 1.2                        | 1.2                      | 3.8                      | 3.8                      | 1.0                      |
| 7b       | 35.79                    | 1.3                      | 1.3                        | 1.3                      | 7.4                      | 7.4                      | 1.0                      |
| 2e       | 35.54                    | 2.7                      | 2.7                        | 2.7                      | 4.4                      | 4.4                      | 1.0                      |
| 4e       | 34.58                    | 2.2                      | 2.2                        | 2.2                      | 3.6                      | 3.6                      | 1.0                      |
| 7e       | 34.22                    | 2.2                      | 2.2                        | 2.2                      | 4.6                      | 4.6                      | 1.0                      |
| 4d       | 33.97                    | 2.6                      | 2.6                        | 2.6                      | 4.3                      | 4.3                      | 1.0                      |
| 3e       | 29.35                    | 2.3                      | 2.3                        | 2.3                      | 3.6                      | 3.6                      | 1.0                      |
| 3d       | 29.29                    | 2.3                      | 2.3                        | 2.3                      | 3.6                      | 3.6                      | 1.0                      |
| 3e       | 28.42                    | 2.3                      | 2.3                        | 2.3                      | 4.5                      | 4.5                      | 1.0                      |
| 6e       | 27.40                    | 2.3                      | 2.3                        | 2.3                      | 4.5                      | 4.5                      | 1.0                      |

\(^{13}C/^{12}C\) couplings observed in the spectrum of the biosynthetic sample from the [1,2-\(^{13}C\)]acetate fermentation indicating the incorporation of intact acetate units.

1 Fraction of \(^{13}C/^{12}C\) coupled satellites in the sample biosynthesized from [1,2-\(^{13}C\)]acetate.

2 Average due to signal overlapping.

3 Signals are degenerate by inherent molecular symmetry, but the carbon atoms have different biosynthetic origins.

4 ND, not determined due to signal overlapping.
The labeling pattern of the furan module reflects the incorporation of two molecules from the triose/pyruvate pool. A plausible reaction mechanism starting from dihydroxyacetone phosphate (7) and pyruvate (2) suggests the formation of the ring system by an aldol condensation (Fig. 8). The amino methyl group could then be formed by reductive amination of the carboxylic group.

The labeling pattern of the tetracarboxylic acid indicates its formation from one ketoglutarate, two acetyl-CoA, and one CO₂. This suggests the sequence of reactions shown in Fig. 9. It should be noted that α-ketosuberate is biosynthesized in methanogenic bacteria by a similar condensation mechanism involving ketoglutarate and two acetyl-CoA molecules (White, 1989).

It is surprising that the carboxylic group 8e is derived from CO₂ and not from pyruvate. However, reductive carboxylation is a frequent reaction in methanogenic bacteria and has been demonstrated with acetate, pyruvate, and succinate as substrate (Fuchs and Stupperich, 1978, 1980; Stupperich and Fuchs, 1984a, 1984b).

White studied the incorporation of ¹³C-labeled acetate into methanofuran by mass spectrometry (White, 1987, 1988). The advantages of this method are obvious. The sensitivity exceeds the NMR experiment by far, and as a consequence, purification of metabolites can be achieved efficiently by online gas chromatographic separation. However, in contrast to ¹³C NMR analysis, it is not always possible to determine the enrichment of each individual carbon atom.

In agreement with our data, White obtained evidence for the formation of the furan ring system from two three-carbon units (White, 1988). On the other hand, he suggested the formation of the tetracarboxylic moiety from 2-ketoglutarate, and two malonate molecules (White, 1987). The incorporation of malonate into the tetracarboxylic acid subunit of methanofuran is ruled out unequivocally by the NMR analysis reported in this paper.

Acknowledgment—We thank Angelika Kohlhe for help with the preparation of the manuscript.

REFERENCES

Bax, A. & Subramanian, S. (1986) J. Magn. Reson. 67, 565–569
Bax, A. & Summer, M. F. (1986) J. Am. Chem. Soc. 108, 2095–2094
Beale, J. M., Lee, J. P., Nakagawa, A., Omura, S. & Hoss, G. (1986) J. Am. Chem. Soc. 108, 331–332
Börner, G., Karrasch, M., & Thauer, R. K. (1969) FEBS Lett. 244, 1–25
Bothner-Bry, A., Stephens, R. L., Lee, J. T., Warren, C. D. & Jeanloz, R. W. (1984) J. Am. Chem. Soc. 106, 811–813
Braunschweiler, L. & Ernst, R. (1983) J. Magn. Reson. 53, 521–528
Breitung, J. & Thauer, R. K. (1990) FEBS Lett. 275, 226–230
DiMarco, A. A., Bohik, T. A. & Wolfe, R. S. (1990) Annu. Rev. Biochem. 59, 355–394
Eisenreich, W. & Bader, A. (1991b) J. Biol. Chem. 266, 23840–23849
Eisenreich, W., Schwarzkopf, B. & Bacher, A. (1991a) J. Biol. Chem. 266, 6922–6931
Fuchs, G. & Stupperich, E. (1978) Arch. Microbiol. 118, 121–125
Fuchs, G. & Stupperich, E. (1980) Arch. Microbiol. 127, 267–272
Jones, W. J., Nagle, D. P., Jr., & Whitman, W. B. (1987) Microbiol. Rev. 51, 135–177
Karrasch, M., Börner, G., Ennelli, M. & Thauer, R. K. (1989) FEBS Lett. 253, 226–230
Leight, J. R., Rinehart, K. L., Jr., & Wolfe, R. S. (1984) J. Am. Chem. Soc. 106, 3920–3940
Strauss, G., Eisenreich, W., Bacher, A. & Fuchs, G. (1992) Eur. J. Biochem. 205, 535–585
Stupperich, E. & Fuchs, G. (1984a) Arch. Microbiol. 139, 8–13
Stupperich, E. & Fuchs, G. (1984b) Arch. Microbiol. 139, 14–20
Thauer, R. K. (1990) Biochem. Biophys. Acta 1018, 250–259
White, R. H. (1987) Biochemistry 26, 3155–3167
White, R. H. (1988) Biochemistry 27, 4415–4420
White, R. H. (1989) Arch. Biochem. Biophys. 270, 691–697