The One-carbon Carrier Methyllofuran from *Methylobacterium extorquens* AM1 Contains a Large Number of α- and γ-Linked Glutamic Acid Residues

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*Methylobacterium extorquens* AM1 uses dedicated cofactors for one-carbon unit conversion. Based on the sequence identities of enzymes and activity determinations, a methanofuran analog was proposed to be involved in formaldehyde oxidation in Alphaproteobacteria. Here, we report the structure of the cofactor, which we termed methyllofuran. Using an *in vitro* enzyme assay and LC-MS, methyllofuran was identified in cell extracts and further purified. From the exact mass and MS-MS fragmentation pattern, the structure of the cofactor was determined to consist of a polyglutamic acid side chain linked to a core structure similar to the one present in archael methanofuran variants. NMR analyses showed that the core structure contains a furan ring. However, instead of the tyramine moiety that is present in methanofuran cofactors, a tyrosine residue is present in methyllofuran, which was further confirmed by MS through the incorporation of a 13C-labeled precursor. Methyllofuran was present as a mixture of different species with varying numbers of glutamic acid residues in the side chain ranging from 12 to 24. Notably, the glutamic acid residues were not solely γ-linked, as is the case for all known methanofurans, but were identified by NMR as a mixture of α- and γ-linked amino acids. Considering the unusual peptide chain, the elucidation of the structure presented here sets the basis for further research on this cofactor, which is probably the largest cofactor known so far.

First termed carbon dioxide reduction factor (1), methanofuran (MFR) was discovered in 1983, and its structure was identified after purification from cell extracts of *Methanothermobacter thermautotrophicus* (2). MFR, tetrahydromethanopterin (H4MPT) (3, 4), and coenzyme M (5) are cofactors that serve as the carrier molecule for one-carbon units at the oxidation states of formate, formaldehyde, and methanol, respectively, and were originally thought to be unique to the group of strict anaerobic methanogenic archaea (6). The importance of “archaeal” cofactors for one-carbon unit conversion was later extended to aerobic bacteria with the discovery of “methanogenic” enzymes in the methylocrotopic model bacterium *Methylobacterium extorquens* AM1. This bacterium harbors enzymes with sequence identity to those from methanogens, and the enzymes were active with the cofactors MFR and H4MPT purified from *M. thermautotrophicus* (7). Although H4MPT was identified as dephospho-H4MPT (7), the identity of the assumed MFR analog remained unknown in *M. extorquens* AM1 and other methyloctrophic bacteria (8–10).

In *M. extorquens* AM1, the one-carbon carrier coenzymes are involved in the oxidation of formaldehyde, which is generated from methanol (or methylamine) (11) (Fig. 1). After the enzyme-assisted binding of formaldehyde to H4MPT (12), dehydrogenation (13), and hydrolysis to formyl-H4MPT (8), the one-carbon unit is transferred to the MFR analog as shown with purified enzyme (14). Notably, and in contrast to the enzymes from methanogenic archaea, the formyl group is subsequently hydrolyzed and released as free formate by the formyltransferase-hydrolase complex (Fhc) (15). The oxidation pathway is then completed by formate dehydrogenases (16), or alternatively, formate is fed into the serine cycle (17, 18) for biomass formation via tetrahydrofolate (19).

To date, five different variants of the MFR cofactor, labeled MFR-a to MFR-e, have been identified and structurally characterized in different methanogenic archaea (2, 20–22). These MFR variants all have a common core structure consisting of an AMPF-moiety [4-[[4-(2-aminoethyl)phenoxy]-methyl]-2-furanmethanamine] linked to at least two γ-glutamic acid residues (Fig. 2A, example of MFR-a). The AMPF core structure consists of a furan derivative attached to a tyramine residue. The different MFR variants show remarkable chemical variability, as different moieties, such as the hexanetetracarboxylic acid group in MFR-a or additional glutamic acid residues (MFR-b, MFR-d, MFR-e), can be attached to the side chain of the AMPF-γ-Glu, structure. Modifications are also possible in the center of the polyglutamic acid side chain, as in the case of MFR-e, where an unusual spacer has been identified (22). In MFR-d and MFR-e, the number of glutamic acid residues is variable, ranging from 7 to 12 and from 5 to 8, respectively (22). Some work has also been dedicated to the MFR biosynthesis, and six
enzymes (MfnA–F) have been identified in *Methanoaoldococcus jannaschii* so far (23–26).

The essential role of enzymes that depend on the putative MFR analog in *M. extorquens* AM1 and other methylotrophs prompted us to identify the cofactor. We structurally characterize the cofactor from the model bacterium using high resolution MS and NMR and propose the name “methyløfuran” to take into account that it shows features not found in known MFR variants from methanogens so far.

**Experimental Procedures**

**Chemicals**—For Methyl-MFR-a was purified from *Methanothermobacter marburgensis* as described (27) and was a gift from R.K. Thauer (MPI Marburg, Germany).

Custom-synthesized polyglutamic acid peptides of the sequences E10Y and (E)20Y were obtained from ReseaChem (Burgdorf, Switzerland). The (E)20 Y peptide contained γ-linkages between the glutamic acids, whereas the E10 Y peptide was α-linked. 1-13C-Labeled l-tyrosine and U-15N-labeled l-tyrosine (purity 99%) were both ordered from Custom-synthesized polyglutamic acid peptides of the sequences E10Y and (E)20Y (crude purity) were obtained from ReseaChem (Burgdorf, Switzerland).

**Large Scale Cultivation of M. extorquens AM1—M. extorquens AM1 was grown in minimal medium containing 123 mM KH2PO4, 6.9 mM NaH2PO4, trace elements (37.6 mM Na2MoO4, 2.9 mM ZnSO4, 30.3 mM NH4Cl, 1.9 mM MgSO4, 21 mM (NH4)2SO4), buffer (13.8 mM KH2PO4, 6.9 mM Na2HPO4), trace elements (37.6 μM ZnSO4, 30.3 μM CoCl2, 12.2 μM MnCl2, 38.8 μM H3BO3, 4.0 μM Na2MoO4, 2.9 μM CuSO4, 191.9 μM CaCl2), and iron (322.4 μM Na2EDTA, 86.3 μM FeSO4). The pH was kept constant at 7.0 through the addition of 2 M ammonia, the temperature was set to 28 °C, and the aeration and stirring rate were set to 2 liters/min and 1500 rpm, respectively. For the initial identification of methyløfuran, a first batch of *M. extorquens* AM1 cell material, referred to as batch A, was produced by growing the cells in a 300 liters fermenter. The final yield was 1.2 kg cww.

A second batch of biomass, referred to as batch B, was prepared in a 3.6 liters bioreactor (Infors) filled with 1.4 liters of minimal medium as fed-batch culture. The methanol feed was controlled using a control sequence that added 6 ml of methanol to the medium as soon as the available methanol was used up by the cells, as detected by the rise in the dissolved oxygen level. After the culture had reached an A600 of 50–70, ~1.3 liters of the culture was harvested by centrifugation, leaving the rest of the culture as inoculum for the next batch. The next culture was then immediately started through the addition of 1 liter of fresh medium. This procedure was repeated 14 times, yielding in total ~340 g cdw (assuming 300 μg/ml cdw per A600 unit). Cell pellets were frozen at −20 °C until further processing.

**Extraction of Methyløfuran**—Cell material from batch A was split into 300 g cww portions. Every portion was suspended in 250 ml of 10 mM potassium phosphate buffer at pH 7 and processed independently. Cells were lysed through the addition of 69 ml of 5% hexadecyltrimethylammonium bromide solution and incubated at 30 °C for 10 min. Cell debris was removed by centrifugation. The resulting pellet was resuspended in 100 ml of 10 mM potassium phosphate buffer at pH 7 and centrifuged again. The supernatants from both steps were combined. To remove proteins, the cell extract was incubated at 75 °C for 10 min. After centrifugation, the soluble fraction was ready for purification.

Cell material from batch B was divided into six portions of ~50 g cdw each. Each portion was resuspended in 2.5 ml of water and 3 ml of methanol per g cdw and processed independently. The resuspended cell material was lysed and extracted by boiling the cells in closed bottles in a water bath at 100 °C for 10 min. After cooling on ice, the suspension was centrifuged, and the resulting pellet was re-extracted twice at 100 °C using the same amounts of water and methanol as before. The extracts were combined and concentrated to about half their volume in a rotary evaporator, thereby removing most of the methanol. After freeze-drying the extracts, the dried powder was dissolved in 50 ml of 20 mM ammonium bicarbonate buffer, and the pH was adjusted to 8.4. After ultracentrifugation at 47,000 g for 1 h, the extracts were used for purification.

**Purification of Methyløfuran**—For the purification of the cell extracts from batch A, an enzymatic assay was used for the identification of the fractions containing methyløfuran. This assay has been previously described as an alternative assay for the determination of formyltransferase activity (14). Briefly, it involves the enzymes Fhc and methenyl-H4MPT cyclohydrolase (Mch). Methenyl-H4MPT cyclohydrolase catalyzes the formation of formyl-H4MPT from methenyl-H4MPT, whereas Fhc catalyzes the transfer of the formyl group from formyl-H4MPT to methyløfuran (Fig. 1). The generation of formyl-MFR was indirectly observed through the spontaneous reduction of cytochrome c by released H4MPT, as monitored by the increase in absorbance at 550 nm. The reaction was started by the addition of fractions containing methyløfuran.

Cell extracts from batch A were first purified on a Source 15Q column (16 × 100 mm, Amersham Biosciences), equilibrated with 10 mM acetic acid at pH 5. Elution was performed using the following gradient: 30 ml of 0 mM NaCl, 10 ml of 0–0.2 M NaCl, 60 ml of 0.2 M NaCl, 150 ml of 0.2–1 M NaCl. Methyløfuran was identified in the fraction eluting at 0.4 M NaCl. A second step of purification was performed using a hydroxyapatite column (16 × 100 mm, Bio-Rad) equilibrated with 10 mM...
potassium phosphate at pH 7. The following gradient was applied: 55 ml of 10–35 mM potassium phosphate, 5 ml of 35–100 mM potassium phosphate, 50 ml of 100 mM potassium phosphate. Methylofuran eluted at 28 mM potassium phosphate. The sample was further purified using a 6-ml Resource Q column (Amersham Biosciences). A pH gradient with 10 mM acetic acid solution between pH 5 and 3 was applied, but methylofuran could not be eluted under these conditions. A linear gradient of 20 ml of 0–0.4M NaCl in 10 mM acetic acid at pH 5 was, therefore, used for elution. For further purification by HPLC, desalting was required. The sample was, therefore, loaded on a Superdex Peptide HR 10/30 column (Amersham Biosciences) and separated from salts using 30 ml of water. HPLC purification was performed on a Discovery HS F5 column (4.6 × 150 mm, Supelco) using 10 mM acetic acid, pH 3.2, as solvent A and acetonitrile for solvent B. Elution was performed at 0.7 ml/min with the following gradient: 3.5 ml of 0% B, 10.5 ml of 0–10% B, 3.5 ml of 10% B, 10.5 ml of 10–30% B. The retention time for methylofuran was 32 min. For further purification, anion exchange on a Mono Q HR 5/5 column (Amersham Biosciences) was performed using a linear gradient of 20 ml of 0–0.8 M NaCl in 10 mM potassium phosphate at pH 7. Methylofuran eluted at 0.6 M NaCl. Finally, an extraction using tetrahydrofuran (THF) was performed. The lyophilized sample was dissolved in 7 ml of THF and centrifuged. Methylofuran could be detected in the solid phase and was redissolved in water. For NMR measurements, the sample was again desalted using the Superdex Peptide column as described above and freeze-dried.

Cell extracts from batch B were purified according to a simplified protocol involving only anion-exchange chromatography. In one run 10 ml of the cell extract (corresponding to ~10 g cdw) of one portion were loaded on a 15-ml Mono Q column (GE Healthcare). Elution was performed using a linear gradient from 1% to 100% of 2 M ammonium bicarbonate at pH 8.4 within 144 ml. The flow rate was set to 4 ml/min, and 14-ml fractions were collected. The fractions containing methylofuran were identified using LC-MS at ~1.2 M ammonium bicarbonate. They were pooled and concentrated to half their volume using a rotary evaporator, thereby also removing most of the ammonium bicarbonate. Because methylofuran eluted within a rather broad peak of other compounds and the loading capacity of the column was likely exceeded, the purified sample was run a second time through the same column. For this second purification, 2.5 ml of the sample was loaded per run, and 7-ml fractions were collected. Fractions containing methylofuran were pooled and freeze-dried for NMR measurements.
Cultivation of M. extorquens AM1 in Flasks and Extraction of Methylofuran for LC-MS—For the LC-MS analysis, only small amounts of methylofuran were required, and M. extorquens AM1 was cultivated in baffled flasks for this purpose. M. extorquens AM1 cells were grown in minimal medium containing mineral salts (30.3 mM NH₄Cl, 0.81 mM MgSO₄), buffer (13.8 mM K₂HPO₄, 6.9 mM NaH₂PO₄), trace elements (15.7 μM ZnSO₄, 12.6 μM CoCl₂, 5.09 MnCl₂, 16.2 μM H₂BO₃, 1.7 μM Na₂MoO₄, 1.2 μM CuSO₄, 20.4 μM CaCl₂), iron (40.3 μM Na₂EDTA, 10.8 μM FeSO₄), and 123 mM (0.5% v/v) methanol as a carbon source. For growth on succinate, the buffer was replaced by 9.13 mM K₂HPO₄ and 11.54 mM NaH₂PO₄, and 30.83 mM succinate was used as a carbon source. A preculture (20 ml), inoculated from glycerol stock, was used for the inoculation of the main culture (100–200 ml) to an A₆₀₀ of ~0.05. All cultures were grown at 28 °C while shaking at 180 min⁻¹. After the culture reached an A₆₀₀ of ~2–3, the cells were harvested by centrifugation.

Extraction of methylofuran was performed twice, similarly to as described above, through the addition of 1 ml of water and 1.2 ml of methanol to the cell pellet and the boiling of the suspension at 100 °C for 5 min. The extract was concentrated in a vacuum concentrator to ~1.4 ml and filtered through a 10-kDa centrifugal filter unit (Amicon Ultra-4, Merck Millipore). The filtered extract was then purified using a 2-ml Mono Q column (GE Healthcare). A 24-ml linear gradient from 1 to 100% of 2M ammonium bicarbonate at pH 8.2 was applied for elution. The flow rate was set to 1 ml/min, and 1 ml fractions were collected. The fractions containing methylofuran were identified by LC-MS.

NMR Measurements—The dried samples prepared from batch A and batch B were dissolved in 500 μl of D₂O for NMR experiments performed at 298 K.

For the sample from batch A, NMR spectra were recorded with an Avance III HD spectrometer (Bruker Biospin) operating at 600.13 MHz. A one-dimensional spectrum was acquired with 20,486 scans into 16k data points across a spectral width of 7000.2 Hz with a relaxation delay of 1.17 s, and solvent suppression was achieved using a double echo Watergate scheme (i.e. W5) (28). 1H,1H COSY-DQF (29) and TOCSY (30) spectra were acquired with 128 scans into 2000 and 512 data points (in direct and indirect dimensions, respectively) across a spectral width of 5482 Hz using a relaxation delay of 1 s. The TOCSY mixing time was set to 70 ms using a MLEV-17 scheme.

Concerning the sample from batch B, NMR spectra were recorded with an Avance III HD spectrometer (Bruker Biospin) operating at 600.13 MHz and equipped with a TCI cryoprobe. A one-dimensional spectrum was acquired with 256 scans into 28,000 data points across a spectral width of 7211.5 Hz with a relaxation delay of 1 s. 1H,1H COSY-DQF and NOESY spectra were acquired into 8000 and 512 data points (in direct and indirect dimensions, respectively) across a spectral width of 7500 Hz with a relaxation delay of 1 s. The NOESY mixing time was set to 800 ms. COSY-DQF and NOESY spectra were recorded with 32 and 64 scans, respectively. The NMR data were processed using Topspin 3.2 software (Bruker Biospin). Chemical shifts were referenced with respect to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS).

LC-MS Measurements—LC-MS measurements of samples from batch B were performed on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled to a nano-2D Ultra LC system (Eksigent Technologies Inc.). For chromatographic separation, ion-pair reversed-phase HPLC using a C18 column (Dr. Maisch, Reprosil-Gold 120 C18 3 μm, 1.0 × 105 mm) was applied, as described previously (31). Solvent A was 1.7 M tributylamine reagent in 1.5 M acetic acid, pH-adjusted to pH 9.0 with ammonia, and solvent B was methanol. LC separation was performed at a flow rate of 400 nl min⁻¹, applying the following gradient of B: 0 min, 3%; 30 min, 90%; 35 min, 90%; 36 min, 3%; 45 min, 3%. Before analysis, samples were diluted 1:10 with solvent A, and 1 μl was injected. Samples were measured in negative FTMS mode at a unit resolution of 60,000 (at 400 m/z), applying nano-electrospray ionization with a spray voltage of −1.9 kV. Further source settings were a capillary temperature of 150 °C, capillary voltage of −10 V, and tube lens voltage of −100 V. The analysis of MS level two (MS-MS) was carried out on a Q Exactive Plus instrument (Thermo Fisher Scientific) coupled to an EASY-nLC 1000 (Thermo Fisher Scientific), applying an identical LC separation. For ionization, the spray voltage was set to −2.5 kV, and a capillary temperature of 250 °C was used. Fragmentation occurred in parallel reaction monitoring (PRM) mode with an isolation window of 2 Da, a collision energy of 30 eV, and a resolution of 35,000. LC-MS data analysis was performed using Xcalibur (Thermo Fisher Scientific) and the emzed framework (32).

Calculation of the Distribution of the Number of Glutamic Acid Residues in Methylofuran—The distribution of the number of glutamic acid residues of methylofuran was calculated by filtering the LC-MS data to generate mass traces with 7 ppm tolerance for all methylofuran species with 12–24 glutamic acid residues and considering charge states with 2 ≤ z ≤ 9 and natural isotopologues with up to four [¹³C]carbon atoms. The mass traces were integrated (trapezoid integration) over the elution time-window and normalized to produce the distribution. The analysis was implemented using emzed (32).

In Vivo ¹³C-Labeling of the Tyrosine Moiety—For the incorporation of labeled tyrosine, M. extorquens AM1 cells were grown in flasks containing methanol minimal medium, as described above. For ¹³C labeling, the preculture was grown in unlabeled medium and used for the inoculation of the main culture supplemented with 0.5 mM labeled tyrosine. For U-¹³C₁₅N labeling, the preculture and the main culture were both grown in medium containing 0.5 mM labeled tyrosine. After the ¹³C and U-¹³C₁₅N cultures had reached an A₆₀₀ of 2.3 and 3.8, respectively, they were harvested by centrifugation, extracted, and purified as described above. The fractions containing methylofuran were measured by LC-MS for the analysis of the labeling pattern.

Results

Identification of Methylfuran and Characterization by NMR—The discovery of a formyltransferase-hydrolase complex (FhcABCD) in M. extorquens AM1 (14, 15) and its activity with MFR from M. marburgensis suggested the presence of an MFR analog in the aerobic methylotroph. We, therefore, used an enzymatic assay we developed earlier (14) to monitor the
transfer of the formyl group and to test for the presence of the methanofuran analog from the methylothroph in fractions obtained from consecutive preparative LC separation steps (see “Experimental Procedures”). Purified active fractions were measured by $^1$H NMR for structural characterization. In the aromatic region of the one-dimensional spectrum, two doublets beside two singlets were observed (Fig. 3A), a pattern that is characteristic of the NMR signature of the APMF core structure of the methanofuran cofactors described previously (2, 15, 22). The two singlets at 7.49 ppm (1H, s) and 6.49 ppm (1H, s) corresponded to the protons annotated with 5 and 3, respectively, on the furan ring in Fig. 2B, whereas the doublets at 7.01 ppm (2H, d) and 6.78 ppm (2H, d) are characteristic of the para-disubstituted aromatic cycle of the tyramine moiety of MFRs and were assigned to protons $\delta$ and $\epsilon$, respectively. The spectrum was similar to those reported for the furan moiety and the tyramine aromatic ring in other MFRs, thereby confirming the presence of these two structures (Fig. 2B).

Furthermore, four broad resonances centered at 2.20 ppm, 2.00 ppm, 1.85 ppm, and 1.70 ppm were observed, a region of the spectrum corresponding to the side chain of the glutamic acid residues (Fig. 3B). The TOCSY and COSY spectra clearly revealed two distinct spin systems for the glutamic acid protons that corresponded to the presence of both $\alpha$- and $\gamma$-linked glutamic acids in the side chain. The spin system characterized by the $H_\gamma$ at lower field (i.e., $H_\gamma, 4.07$ ppm, $H_{\alpha,\beta}$, 1.85 ppm, and $H_{\gamma, 2.14}$ ppm) corresponded to $\alpha$-linked residues, whereas the spin system with the $H_\delta$ at higher field (i.e., $H_\delta, 3.96$ ppm, $H_{\alpha, \beta}$, 1.89 ppm, $H_{\gamma, 1.72}$ ppm, and $H_{\delta, 2.06}$ ppm) was assigned to $\gamma$-linked residues (33). By integration, it was estimated that 65 ± 10% of the glutamic acid residues would be $\gamma$-linked and 35 ± 10% $\alpha$-linked. Based on the TOCSY and NOESY spectra, a detailed description of the polyglutamic acid moiety (e.g., sequential assignment) was not possible, but the broadness and the integration strongly suggested a higher number of glutamic acid residues compared with known MFRs (Fig. 2B).

**TABLE 1**

| No. Glu | $z$ | Measured m/z | Calculated m/z | Difference | Molecular mass | Retention time |
|---------|----|--------------|----------------|------------|----------------|----------------|
| 12      | 2  | 918.3080     | 918.3116       | 3.9        | 1836.6106      | 19.50          |
| 13      | 2  | 982.8317     | 982.8329       | 1.2        | 1967.6780      | 19.58          |
| 14      | 2  | 1047.3514    | 1047.3542      | 2.7        | 2096.7174      | 19.70          |
| 15      | 2  | 1111.8716    | 1111.8755      | 3.5        | 2225.7578      | 19.79          |
| 16      | 3  | 783.9282     | 783.9288       | 0.8        | 2554.8065      | 19.85          |
| 17      | 3  | 826.9418     | 826.9430       | 1.5        | 2834.8743      | 19.95          |
| 18      | 3  | 869.9547     | 869.9572       | 2.9        | 2612.8680      | 20.04          |
| 19      | 3  | 912.9691     | 912.9714       | 2.5        | 2741.9292      | 20.14          |
| 20      | 3  | 955.9833     | 955.9856       | 2.4        | 2870.9718      | 20.20          |
| 21      | 3  | 998.9977     | 998.9998       | 2.1        | 3000.0150      | 20.27          |
| 22      | 3  | 1042.0127    | 1042.0140      | 1.2        | 3129.0600      | 20.37          |
| 23      | 3  | 1085.0263    | 1085.0282      | 1.8        | 3258.1008      | 20.44          |
| 24      | 3  | 1128.0414    | 1128.0424      | 0.9        | 3387.1461      | 20.50          |
in the order of increasing number of glutamic acid residues (Fig. 4A). Because of the acidity of the glutamic acids, the MFR species were present in different charge states with $2 \leq z \leq 9$ in the (negative ion) mass spectrum (Fig. 4B). The retention time and charge states of methylofuran were very similar to those of the $(\gamma E)_{20} Y$ polyglutamic acid standard, indicating a similar chemical structure.

From the intensity of the ions corresponding to the different species of methylofuran, a distribution showing the abundance of each species was calculated (Fig. 4C). The distribution might, however, be affected by different ionization efficiencies for the different species, although this influence was assumed to be small, especially for species differing only by a small number of glutamic acids. The distribution was roughly normal and showed the presence of methylofuran species with 12 up to 24 glutamic acid residues in their side chain. The most abundant methylofuran species contained 19 glutamic acids and made up almost a third of all the species, although the center of the distribution showed some batch-to-batch variation ($\pm 1$ glutamic acid).

Based on the exact masses of methylofurans (Table 1) and assuming a core structure linked to unmodified glutamic acids, we could determine the molecular formula to be $C_{15}H_{18}N_2O_4 + nC_5H_7NO_3$, with $n$ ranging from 12 to 24, thus with a molecular mass of up to 3387.15 Da. The molecular formula assigned to the core structure differs from the APMF core structure present in all known methanofurans ($C_{14}H_{18}N_2O_2$) (22) by CO$_2$, indicating an additional functional group(s) attached to its core structure, e.g. in the form of a carboxylic acid group.

For the further structural characterization of methylofuran, LC-MS-MS analysis was carried out. For the general characterization of the fragmentation pattern of methylofuran, analysis was performed with methylofuran containing 20 and 19 glutamic acid residues. The fragmentation of both methylofurans resulted in two distinct series of fragments separated by the mass of a glutamic acid residue (Fig. 5). One series corresponded to the loss of single glutamic acids from the side chain of methylofuran, still containing the core structure (Table 2). The other series was due to the loss of the core structure together with various numbers of glutamic acid residues, thereby generating a series of polyglutamic acids of various lengths (Table 2). Additionally, one or multiple water losses was commonly observed in both series. The observed fragmentation pattern was similar to the MS-MS analysis of the $(\gamma E)_{20} Y$ peptide standard, where the same two series of fragments were observed (Table 2). Because polyglutamic acid fragments with up to 19 residues were observed for methylofuran containing 20 glutamic acids, any modification of the glutamic acids in the side chain is very unlikely. Most importantly, a fragment corresponding to the core structure of methylofuran without any glutamic acids was also detected (Table 2). Its mass differed from APMF by the mass of CO$_2$, and the calculated mass of the neutral loss corresponded to an unmodified polyglutamic acid chain of 20 residues, which confirmed the modification being located in the core structure.

Peaks matching the mass of the formylated form of methylofuran (structure in Fig. 2C) and eluting ~25 s later than their non-formylated counterparts were also identified, although with much lower intensity (data not shown). One reason for the predominance of the non-formylated forms of methylofuran might be the fast hydrolysis of the formyl group to formate, which is catalyzed by the same enzyme complex (FhcABCD) as the formyl transfer from H$_4$MPT to methylofuran (15).
In a culture grown on succinate instead of methanol, methylfuran could also be detected and was present in similar quantities. Thus, methylfuran was produced even though no one-carbon substrate was present, an observation that is in-line with the detection of the converting enzymes under non-methylotrophic growth conditions (7, 34, 35).

**Determination of the Core Structure of Methylfuran**—From the MS data, it is evident that the core structure of methylfuran contained an additional modification corresponding to the atoms CO$_2$ compared with the APMF core structure. The simplest form of such a modification would be a carboxylic acid group. From the initial NMR data (see above), it was concluded that the furan ring and the tyramine-like aromatic ring are unmodified compared with known MFRs because resonances for all the protons were detected. Therefore, the only four possible positions left for a modification in the APMF core structure were: the two methylene groups attached to the furan moiety and the two methylene groups in the tyramine-like moiety. The two methylene groups attached to the furan ring in the $\alpha$ position of the amine and in the $\alpha$ position of the phenoxy group were assigned at 4.05 ppm and 4.81 ppm, respectively (Table 3). These chemical shifts exclude any carboxylation, as 4.81 ppm is similar to the chemical shift found for formyl-MFR-a (4.84 ppm), and 4.05 ppm is at higher field compared with the expected chemical shift of the H$_{\alpha}$ of $\alpha$ amino acids (i.e. above 5.0 ppm). Considering the two methylene groups in the tyramine-like moiety, the position next to the amine group was considered more likely to be modified, as the attachment of a carboxylic acid group at that position would result in a tyrosine residue instead of a tyramine. Furthermore,
in the biosynthesis of the methanogenic MFR, the tyramine residue is formed by the decarboxylation of tyrosine (23). In methylofuran, a tyrosine residue instead of a tyramine could, therefore, easily be explained by a modified biosynthesis where the decarboxylation step is missing. To prove this hypothesis, NMR and in vivo 13C labeling of the tyrosine moiety were applied.

Comparison of the COSY spectrum of formyl-MFR-a (Fig. 6A) with the spectrum of methylofuran (Fig. 6B, top panel) clearly demonstrates that the spin system assigned to the α and β methylene groups of the tyramine moiety (i.e. 3.26 and 2.61 ppm, respectively) is absent in the COSY spectrum of methylofuran, suggesting a structural modification in this region of the molecule. In contrast, the COSY spectrum of methylofuran reveals a unique spin system at 4.23, 2.99, and 2.67 ppm, respectively) is absent in the COSY spectrum of methylofuran, illustrating the complete connectivity of the tyrosine spin system, which is present in methylofuran instead of the tyramine moiety in MFR-a.

The presence of a tyrosine moiety instead of a tyramine in methylofuran in M. extorquens AM1 (Fig. 2B).

For the further validation of the presence of a non-decarboxylated tyrosine residue in methylofuran, in vivo incorporation of 13C-labeled tyrosine was performed through the addition of labeled tyrosine to the growth medium. The ability of M. extorquens to take up tyrosine from the medium has previously been reported (36). In a first step, tyrosine labeled with 13C at the carbonyl position was used. After extraction and purification, the labeled methylofuran was analyzed by LC-MS. In the mass spectrum, a shift of one mass unit was observed, thereby proving the presence of the carboxylic acid group (Fig. 7). The unlabeled peak was only marginally visible, and the labeling efficiency was >98%.

In addition, a labeling experiment with completely 13C- and 15N-labeled tyrosine was performed to show that the tyrosine is incorporated as a whole and without any modification of the carbon atoms. In the resulting mass spectrum, peaks corresponding to the fully labeled tyrosine were observed (data not shown). Additionally, a peak shifted by only 9 instead of 10 mass units was observed. This peak was most likely due to the exchange of the amine 15N through 14N by the activity of transaminases (36). Together, both experiments show that tyrosine is incorporated into methylofuran without decarboxylation and without any further modification of the carbon backbone.

**Discussion**

In this work we demonstrate the presence of methylofuran, which is involved in methyiotrophy, in M. extorquens AM1. Our structural characterization of the one-carbon carrier by means of mass spectrometry and NMR revealed a modified core structure compared with known archaeal MFRs that share the same APMF core structure. Instead of the tyramine residue usually present in the core structure, methylofuran contains a non-decarboxylated tyrosine residue. This modification leads to a simplified biosynthesis of methylofuran, as the tyrosine molecule can be incorporated directly, thus obviating the need for the L-tyrosine

![Figure 6. Structural elucidation of the tyrosine moiety in the core structure of methylofuran. Peaks are annotated according to Fig. 2B. Spectra were recorded at 298 K in D2O and calibrated with respect to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). A, part of the 1H,1H COSY spectrum of formyl-MFR-a, showing the connectivity of the two methylene groups of the tyramine moiety. B, part of the 1H,1H COSY (top panel) and 1H, 1H NOESY (bottom panel) spectrum of methylofuran, illustrating the complete connectivity of the tyrosine spin system, which is present in methylofuran instead of the tyramine moiety in MFR-a.](image_url)

![Figure 7. Mass spectrum of methylofuran containing a 13C-labeled carboxylic acid group in the tyrosine residue. The spectrum shows the natural abundance isotope pattern of the [M-6H]+ ion of the labeled methylofuran containing 18 glutamic acid residues (blue peaks). The green peak corresponds to a minor amount of unlabeled species still being present. The shift between the green and the first blue peak (monoisotopic peak) corresponds to the mass difference between a 13C and a 12C atom (0.1672 m/z for z = 6).](image_url)
Structure of Methylofuran in *M. extorquens* AM1

decarboxylase (MfnA) used in the archaeal MFR biosynthesis (23). The circumvention of decarboxylation is further supported by a BLASTP search (37) that revealed the absence of an MfnA ortholog in *M. extorquens* AM1. An open question is the biochemical significance of the additional carboxylic acid group and whether it is required in *M. extorquens* for the recognition and activity of the cofactor in vivo; conversely, it is also not known whether MfnA is dispensable in methanogenic archaea.

Another difference between the methylofuran in *M. extorquens* and other known MFRs is the larger number of glutamic acids in the side chain of methylofuran. The largest MFR known until now, MFR-d, contains 7–12 glutamic acid residues (22), whereas we have identified between 12 and 24 residues for methylofuran. Methylofuran is, therefore, to our knowledge the largest cofactor described so far.

In addition to containing a larger number of glutamic acids, methylofuran also shows a broader distribution of the different species. This finding raises interesting questions regarding the biological reason for the broad distribution and the large number of glutamic acid residues. The distribution might be stochastic as a result of an imperfect biosynthesis, although it might also play a role in regulation, as the large number of glutamic acids might affect the binding and recognition of the cofactor by the involved enzymes.

The last striking difference between the methylofuran from *M. extorquens* AM1 and the other known MFRs concerns the type of linkage between the glutamic acid residues. Although the other known MFRs contain exclusively γ-linked glutamic acids, we detected both α- and γ-linkages. It is currently unknown which enzyme is involved in the formation of the peptide bonds and whether side-chain elongation proceeds progressively from the core structure or is transferred en bloc.

Taken together, we here identified the methylofuran from *M. extorquens* AM1, which differs from the MFRs in methanogenic archaea described thus far. It will be interesting to also identify methylofurans in other aerobic methylotrrophic bacteria found to contain H₄MPT/methylofuran-dependent enzymes (10, 38) and to analyze the structure of the methylofuran(s) in light of the Fhc that apparently lost formyl-MFR dehydrogenase activity found in methanogenic archaea (15).

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