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Structural Diversity among Methanofurans from Different Methanogenic Bacteria

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An examination of the methanofurans isolated from a wide range of methanogenic bacteria and from *Archaeoglobus fulgidus* has revealed at least five chromatographically distinct methanofurans. Bacteria from each major genus of methanogenic bacteria have been found to contain a chemically different methanofuran. The nature of the differences in the methanofurans appears to lie in the modification of the side chain attached to the basic core structure of 4-[*N*-(γ-L-glutamyl-γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan. This was supported by the structural elucidation of the methanofuran isolated from *Methanobrevibacter smithii*, designated methanofuran-c, which was the same as the originally characterized methanofuran except for a hydroxy group at the 2 position of the 4,5-dicarboxyoctanedioic acid moiety of the molecule.

Methanofuran (Fig. 1) is a recently characterized cofactor in the conversion of CO_2 to methane by methanogenic bacteria (9–11). It is involved in the first step of CO_2 reduction, where it serves as a C_1 carrier at the formyl level of carbon oxidation (7, 10). Recent results from Wolfe and co-workers have indicated that formylmethanofuran, prepared from the methanofuran isolated from *Methanobacterium thermoautotrophicum*, can serve as an intermediate for the generation of methane in a wide range of methanogens (7). These results indicate that the structure of methanofuran is likely to be the same for all methanogens. However, as discussed in this paper, an initial chemical survey of the methanofurans from several different species of methanogens and from the recently established biochemical missing link among the archaebacteria, *Archaeoglobus fulgidus* (1, 16), has demonstrated that there are at least five different methanofurans. On analysis, each of these methanofurans appears to contain a basic core structure of 4-[*N*-(γ-L-glutamyl-γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan with different attached side chain molecules. This is supported by the structural analysis of the methanofuran from *Methanobrevibacter smithii*, which shows it to consist of this basic core structure, peptide bonded, via the terminal glutamyl residue, to the C-1 of 2-hydroxy-4,5-dicarboxyoctanedioic acid (2-hydroxyDCO) (Fig. 1).

MATERIALS AND METHODS

Bacterial strains and growth. *Methanobacterium formicicum* JF-1, *Methanobacterium bryantii* MOH, and *Methanospirillum hungatei* JF were grown in 10-liter batches on a medium modified from Schauer and Ferry (15), consisting of 1.44 g of NH_4Cl, 1.14 g of K_2HPO_4, 1.14 g of KH_2PO_4, 0.5 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.5 g of tryptone (Difco), 6.0 g of sodium formate, 2.0 g of sodium acetate, 0.01 g of $Fe(NH_4)SO_4$, 5.0 g of Na_2HCO_3, 0.001 g of resazurin, 0.46 g of NaCl, 0.092 g of $MgCl_2 \cdot 6H_2O$, 0.26 g of $CaCl_2 \cdot 2H_2O$, 0.25 g of cysteine hydrochloride, and 0.25 g of $Na_2S \cdot 9H_2O$ per liter of water. In addition, vitamin and trace mineral solutions (2) were each added to a final 2% (vol/vol) concentration. *Methanobrevibacter smithii* PS was grown in 3-liter batches on a medium modified from that of Lovley et al. (13), consisting of 0.45 g of K_2HPO_4, 0.15 g of KH_2PO_4, 0.12 g of NH_4Cl, 0.3 g of NaCl, 0.04 g of $CaCl_2 \cdot 2H_2O$, 2.5 g of $NaHCO_3$, 0.14 g of $MgCl_2 \cdot 6H_2O$, 2.5 g of sodium acetate, 5.0 g of sodium formate, 2.0 g of yeast extract, 2.0 g of tryptone, 0.0001 g of resazurin, 0.5 g of cysteine hydrochloride, and 0.5 g of $Na_2S \cdot 9H_2O$ per liter of water. Also added were antifoam C, 0.0005% (vol/vol); vitamin and trace mineral solutions (2), 1% each (vol/vol); iosbutyric, 2-methylbutyric, isovaleric, and valeric acids, each to a final concentration of 0.0005% (vol/vol); and Tween 80, 0.002% (vol/vol).

Methanococcus maripaludis JJ was provided by William B. Whitman, Department of Microbiology, University of Georgia, Athens. The organism was grown by Whitman on a complex medium as previously described (21).

Methanococcus vannielii was grown and provided as frozen cells by Edward DeMoll, National Institutes of Health, Bethesda, Md. Isolate 10-16B was grown as previously described (18). *Archaeoglobus fulgidus* was supplied as a lyophilized powder by Karl O. Setter, Department of Microbiology, University of Regensburg, Federal Republic of Germany.

Extraction and TLC analysis of methanofurans from different methanogenic bacteria. Cell extracts (0.5 to 1 ml, 6 to 35 mg of protein per ml) were prepared anaerobically under an N_2 atmosphere by diluting the cells 1:3 with degassed 50 mM (pH 7.5) phosphate buffer containing 2 mM 2-mercaptoethanol, 10 mM sodium azide, and 0.015 mg of DNase per ml. The cells were then broken by passing the cell suspension through a French pressure cell at 20,000 lb/in^2. Intact cell pellets (~0.5 g) were extracted by mixing with 0.5 ml of water and heating at 100°C for 15 min. After cooling, the resulting suspension was adjusted to pH 3 to 4 with 1 M HCl and centrifuged at 1,500 × *g* for 10 min. The resulting pellet was reextracted under the same conditions, the combined extracts were applied to a column of Dowex 50W-8X H^+ (4 by 10 mm) and washed with 1.0 ml of water, and the methanofurans were eluted with 1.0 ml of 7 M ammonia. After evaporation of the water, the resulting residue was dissolved in 100 μl of 50% ethanol for application to a precoated silica gel 60 thin-layer chromatography (TLC) plate (E. Merck AG, Darmstadt, Federal Republic of Germany). The plates were developed with solvent system 1, consisting of acetonitrile-water-formic acid (88%) (40:10:5,

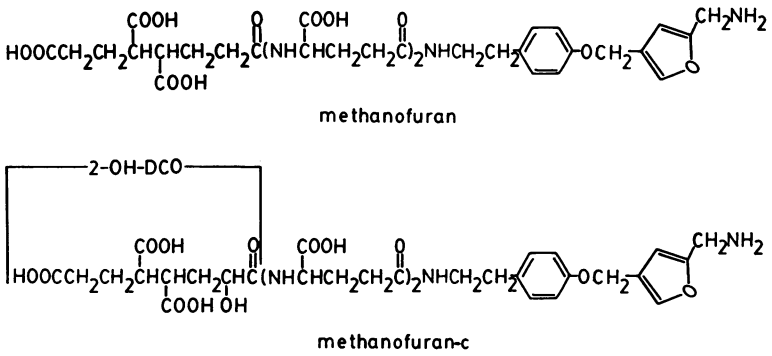

FIG. 1. Chemical structures of methanofuran and methanofuran-c.

vol/vol/vol), or system 2, consisting of *n*-butanol–acetic acid–water (12:3:5, vol/vol/vol). The methanofuran was specifically identified on the developed TLC plate as a pink band after spraying the plate with Ehrlich reagent, which consists of a solution of *p*-dimethylaminobenzaldehyde (1 g) dissolved in a mixture of concentrated HCl (25 ml) and methanol (75 ml) and heated at 100°C for 5 to 10 min (6). Yellow and purple spots were also observed with this procedure, but only the furfurylamine of the methanofuran was found to generate the pink color.

Identification of the acid cleavage products from the methanofurans. Acid hydrolysis (1 M HCl at 100°C for 15 min) of the isolated methanofurans led to the generation of the 4-aminomethyl-2-hydroxymethylfuran (F1) (10, 11, 19a), which was separated from the hydrolysis products by TLC with solvent system 2 and identified by spraying the developed plate with Ehrlich reagent (6). The known compound had an R_f of 0.31 with this solvent system. Hydrolysis of the methanofurans under the same conditions but for a period of 1 h led to the release of tyramine, which was identified on the TLC plates by ninhydrin spray. The tyramine had an R_f of 0.47 in solvent system 2.

Analysis of whole cells for DCO and 2-hydroxyDCO. The 4,5-dicarboxyoctanedioic acid (DCO) and 2-hydroxyDCO present in the methanofuran in whole cells were extracted, purified, cleaved, and assayed by gas chromatography-mass spectrometry as described previously (19).

Isolation of the methanofuran from *Methanobrevibacter smithii*. Freshly grown cells of *Methanobrevibacter smithii* (10 g, wet weight) were boiled for 10 min with 10 ml of water and centrifuged to remove the insoluble material. The pellet was reextracted by the same procedure, and the combined extracts were adjusted to pH 3.0 with HCl. The resulting precipitate was removed by centrifugation, and the clear liquid was applied to a Dowex 50W-8X H^+ column (1.5 by 3 cm), which absorbed the methanofuran. After the column was washed with 5-column volumes of water, the methanofuran was eluted with 3 M ammonia. After evaporation of the solution, the methanofuran was purified by preparative TLC with solvent system 1.

Synthesis of 2-hydroxyDCO. DCO (26 mg, 0.5 mmol), prepared by saponification of the tetramethyl ester of DCO (5) and recrystallization from water, was dissolved by warming in a mixture of 200 μl of trifluoroacetic acid and 200 μl of trifluoroacetic anhydride. The resulting solution was cooled to 0°C, and 5.1 μl of bromine (1.0 μmol) was added with shaking. The resulting red solution was then refluxed in a sealed tube for 20 min until the red color of the bromine dissipated. The resulting reaction mixture, containing the 2-bromoDCO intermediate, was evaporated to dryness, mixed with 2 ml of 1 M HCl, and heated for 12 h at 100°C. After evaporation of the acid, the sample was dissolved in methanol and reacted with an excess of diazomethane in order to form the methyl esters. Gas chromatography-mass spectrometry of the resulting methyl esters showed that they consisted of some unreacted DCO [*m/z* 287 (M^+-31), 85% base peak; *m/z* 254 (M^+-32-32), 60% base peak; and *m/z* 100 and 128 base peaks] (19), the lactone of 2-hydroxyDCO [*m/z* 217 (M^+-31), 10% base peak; *m/z* 243 (M^+-59), 30% base peak; *m/z* 211 (M^+-31-60), base peak; and *m/z* 183 (M^+-31-60-28), 51% base peak], and the dilactone of 2,7-dihydroxyDCO [*m/z* 227 (M^+-59), 96% base peak; *m/z* 139 (M^+-59-60-28), base peak]. Purification by preparative TLC on silica gel, with diethyl ether as the developing solvent, showed five bands which were made visible by the absorption of iodine vapor. One of the bands had the same R_f as the 2-hydroxyDCO isolated from *Methanobrevibacter smithii* and another had a slightly higher R_f. The eluted material from these two bands (6 mg each) gave a single peak upon gas chromatography, under conditions described previously for the tetramethyl ester of DCO, and gave a mass spectrum identical to that obtained from the 2-hydroxyDCO which was isolated from the methanofuran from *Methanobrevibacter smithii*.

RESULTS AND DISCUSSION

As determined by their R_f values, at least five chemically different methanofurans were identified in the methanogenic bacteria examined in this work (Table 1). Each compound was confirmed as a methanofuran by its reaction with Ehrlich reagent and by its cleavage to 4-aminomethyl-2-hydroxymethylfuran and tyramine by acid hydrolysis. Further proof of their close identity comes from the observation that the methanofurans isolated from *Methanobacterium thermoautotrophicum*, *Methanobacterium bryantii*, *Methanobrevibacter smithii*, and *Methanospirillum hungatei* have all been shown to complement a methanofuran-deficient, methane-generating enzyme system from *Methanobacterium thermoautotrophicum* (7).

As can be seen in Table 1, each of the methanofurans isolated from the *Methanobacterium* spp. had the same R_f, indicating that each contained a methanofuran with the same structure as the methanofuran first characterized from *Methanobacterium thermoautotrophicum* (10, 11). As further support, DCO was identified in each of these organisms as its tetramethyl ester by gas chromatography-mass spectrometry as described previously (19). The most polar methanofuran identified was isolated from *Methanosarcina barkeri* and had an R_f of only 0.131 in solvent system 1. This methanofuran

TABLE 1. Characteristics of methanofurans from different methanogens

Methanogen	R_f		Side chain present
	Solvent system 1	Solvent system 2	
Methanobacterium			
M. thermoautotrophicum	0.355	0.142	DCO
M. formicicum	0.355	0.142	DCO
Isolate 10-16B	0.355	0.142	DCO
M. bryantii	nd[a]	nd	DCO
Methanobrevibacter smithii	0.24	0.068[b]	2-HydroxyDCO
Methanococcus			
M. vannielii	0.219	0.036	2,7-DihydroxyDCO?
M. maripaludis	0.21	0.030	nd
Methanospirillum hungatei	nd	nd	No DCO or 2-hydroxyDCO
Methanosarcina barkeri	0.131	0.040	Diglutamate
Archaeoglobus fulgidus	0.131	0.04	Diglutamate

[a] nd, Not detected.
[b] An additional minor Ehrich-positive spot was detected with an R_f of 0.086.

has recently been characterized as 4-[*N*-(γ-L-glutamyl)$_4$-*p*-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan and has been given the name methanofuran-b (3). Thus, in this structure, two gamma-linked glutamic acids substitute for the DCO present in the original methanofuran.

In order to better understand which structural features are responsible for the different chemical properties in the other methanofurans, the chemical structure of the methanofuran from *Methanobrevibacter smithii*, designated here methanofuran-c, was examined. As with the other methanofurans, limited acid hydrolysis of methanofuran-c was shown to release both F1 and tyramine. The release of the F1 was maximum after 15 min of hydrolysis with 1 M HCl at 100°C, and the release of the tyramine was maximum after 1 h under the same conditions. The hydrolytic breakdown of this molecule was the same as that observed for the original methanofuran (10, 11, 19a). Both F1 and tyramine were identified by comparison with known compounds both by TLC in solvent system 1 and by gas chromatography-mass spectrometry of their diacetates (19a).

Complete acid hydrolysis (6 M HCl at 110°C for 24 h) of the methanofuran was found to release 2 mol of glutamic acid. (The glutamic acid was quantitated by analysis using a Beckmann high-pressure liquid chromatograph equipped with an ion-exchange column and a ninhydrin detection system. The methanofuran was quantitated by its absorbance at 268 nm.) Acid hydrolysis (3 M HCl at 100°C for 3 h) also released a diethyl ether-soluble component, which after reaction with diazomethane and subsequent gas chromatographic-mass spectrometric analysis, gave a mass spectrum with the following ions: *m/z* 217 (M^+-31), 10% base peak; *m/z* 243 (M^+-59), 30% base peak; *m/z* 211 (M^+-31-60), base peak; and *m/z* 183 (M^+-32-59-28), 51% base peak, indicating a molecular weight of 302. This mass corresponds to that calculated for a trimethyl ester of a monohydroxylated DCO, in which the hydroxyl group and one of the carboxylic acids is in the form of a lactone. A monohydroxylated DCO molecule is interesting in that a hydroxyl group, placed in any position in the molecule, will always lead to the formation of both 5- and 6-member lactones. Fragmentation of these different isomers would, of course, be different and could be used to establish the position of the lactone. In the case of the isolated lactone, fragments at *m/z* 159, 28% of the base peak, and *m/z* 143, 32% of the base peak, corresponding to $[OCCHCH_2CHOCOOCH_3]^+$ and $[CH_3OOCCHCH_2CH_2COOCH_3]^+$, respectively, strongly suggest a 2-hydroxyDCO with a 5-member lactone. In addition, a fragment at *m/z* 183 (M^+-$CH_2CH_2COOCH_3$), 60% of the base peak, supported the presence of a 2-hydroxyDCO with a 6-member lactone. The position of the lactone and the size of the ring, however, can be firmly established from the proton magnetic resonance spectrum of the molecule.

The proton magnetic resonance spectrum of the molecule, in addition to other resonances, showed two sets of three methyl ester resonances from 3.85 to 3.65 ppm containing a total of 18 protons, plus a triplet and multiplet at 4.97 ppm and 4.68 ppm, respectively, accounting for a total of 2 protons. The ratio of the intensities of the 4.97 and 4.68 ppm signals was found to be the same as the ratio of the two sets of methyl resonances and was found to vary from one sample preparation to the other. From the chemical shifts and splitting patterns of these protons, the 4.97 ppm signal must result from a single proton on the C-2 position of a 2-hydroxy-fatty acid ester involved in a 5-member lactone, and the 4.68 signal must arise from a single proton on the C-2 position of a 2-hydroxy-fatty acid ester involved in a 6-member lactone. The higher signal was assigned to the 5-member lactone proton based on the general rule that the C-4 protons of 5-member lactones occur at higher values than the C-5 protons of 6-member lactones (14). The change in the ratio of the intensity of these peaks can be explained by a change in the distribution between the 5- and 6-member lactones. These resonances alone, of course, did not prove that the compound was, in fact, the 2-hydroxy derivative of the DCO, since other molecules could conceivably generate this same nuclear magnetic resonance pattern. Final confirmation of the structure came from a comparison of the entire proton magnetic resonance spectrum and the mass spectrum of the unknown with those of the known; both were identical, confirming that methanofuran-c contains a 2-hydroxyDCO.

Low-resolution, fast-atom bombardment mass spectrometry of methanofuran-c established a molecular weight of 764 (M + H = 765), which is an oxygen atom higher than the original methanofuran. This result indicates that the only structural difference between these two methanofurans is the introduction of the hydroxyl group at C-2 of the DCO. The one question remaining is whether the hydroxy group is next to the peptide bond or next to the terminal carboxyl group. A biosynthetic argument would place the hydroxy group next to the peptide bond. DCO is known to be biosynthesized by the addition of two malonyl coenzyme A (CoA) units to α-ketoglutarate (19). The next to last step in the biosynthesis is postulated to involve the CoA ester of 2,3-dehydro-DCO, which is hydrogenated to the CoA ester of DCO. The 2,3-dehydro-DCO intermediate could be hydrated by the reverse addition of water to the double bond (17), which would lead directly to the formation of the CoA ester of 2-hydroxyDCO. In both biochemical schemes, the condensation of the CoA ester with the amino group of the glutamic acid would generate the peptide bond found in the final methanofurans. In the case of methanofuran-c, this would lead to the generation of the hydroxy group adjacent to the peptide bond.

At present, the structures of three different methanofurans have been established. Each consists of a central core of 4-[*N*-(γ-L-glutamyl-γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan in which a negatively charged side chain consisting of DCO, 2-hydroxyDCO, or γ-L-glutamyl is added. This is similar in principle to the addition

of γ-linked polyglutamyl residues in the side chains of folic acid and F_{420} (12). However, unlike these coenzymes that add polyanionic side chains by the addition of only glutamic acid, the methanogenic bacteria appear to have evolved different methods to accomplish this goal, at least for the methanofurans. The chromatographic data presented here also suggest that more structural variations may occur in the methanofurans. Thus, these structural differences in the methanofurans represent another example of the diversity of the methanogenic bacteria (4, 8).

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