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Biosynthesis of the 5-(Aminomethyl)-3-furanmethanol Moiety of Methanofuran

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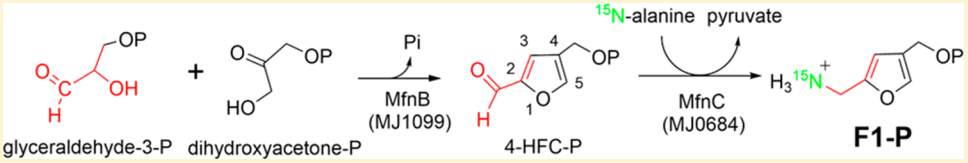
Supporting Information



ABSTRACT: We have established the biosynthetic pathway and the associated genes for the biosynthesis of the 5-(aminomethyl)-3-furanmethanol (F1) moiety of methanofuran in the methanogenic archaeon *Methanocaldococcus jannaschii*. The recombinant enzyme, derived from the MJ1099 gene, was shown to readily condense glyceraldehyde 3-phosphate (Ga-3P) and dihydroxyacetone-P (DHAP) to form 4-(hydroxymethyl)-2-furancarboxaldehyde phosphate (4-HFC-P). The recombinant purified pyridoxal 5′-phosphate-dependent aminotransferase, derived from the MJ0684 gene, was found to be specific for catalyzing the transamination reaction between 4-HFC-P and [^{15}N]alanine to produce [^{15}N] 5-(aminomethyl)-3-furanmethanol-P (F1-P) and pyruvate. To confirm these results in cell extracts, we developed sensitive analytical methods for the liquid chromatography−ultraviolet−electrospray ionization mass spectrometry analysis of F1 as a 7-nitrobenzofurazan derivative. This method has allowed for the quantitation of trace amounts of F1 and F1-P in cell extracts and the measurement of the incorporation of stable isotopically labeled precursors into F1. After incubation of cell extracts with [1,2,3-$^{13}C_3$]pyruvate and DHAP, 4-([$^{2}H_2$]hydroxymethyl)-2-furancarboxylic acid phosphate (4-HFCA-P) or 4-([$^{2}H_2$]hydroxymethyl)-2-furancarboxaldehyde phosphate (4-HFC-P) was found to be incorporated into F1-P. 4-HFCA-P and 4-HFC-P were confirmed in cell extracts after removal of the phosphate. The low level of incorporation of [1,2,3-$^{13}C_3$]pyruvate into F1-P in these experiments is explained by the fact that the labeled pyruvate must first be converted into Ga-3-P through gluconeogenesis before being incorporated into 4-HFC-P. Cell extracts incubated with 4-HFC-P and a mixture of [^{15}N]aspartate, [^{15}N]glutamate, and [^{15}N]alanine produced [^{15}N]F1-P. We also demonstrated that aqueous solutions of methylglyoxal or pyruvate heated with dihydroxyacetone led to the formation of 4-HFC and 4-HFCA, suggesting a possible prebiotic route to this moiety of methanofuran.

Methanofuran (MF) is the first in a series of coenzymes involved in the biochemical reduction of carbon dioxide to methane.[1−3] This process, known as methanogenesis, is known to be conducted only by the methanogenic archaea that produce more than 400 million tons of methane each year as an essential part of the global carbon cycle.[4] At present, only one gene has been identified as encoding an enzyme involved in methanofuran biosynthesis. This gene, MJ0050 in the methanogenic archaeon *Methanocaldococcus jannaschii*, encodes a tyrosine decarboxylase that produces the tyramine moiety[5] found in all known MFs[6] (Figure 1A,B). Homologues of this gene are found in all currently sequenced methanogenic genomes available in public database. Recently, the homologue in *Methanococcus maripaludis* (MMP0131) was demonstrated to be an essential gene.[7] Analysis of methanogenic genomes for genes associated with homologues of the MJ0050 gene has yet to reveal the presence of any additional gene(s) encoding methanofuran biosynthetic enzymes.

Here we establish a pathway for the biosynthesis of the 5-(aminomethyl)-3-furanmethanol (F1) moiety of methanofuran (Figure 1, red). Our approach has made extensive use of bioinformatics and bioanalytical and chemical methods coupled with biochemical reasoning. This approach was necessary because the usual method for pathway identification, based on genetic methods for determining genes associated with a given pathway, was not feasible in *M. jannaschii* in part because of the absence of metabolite uptake.[8]

On the basis of the structure of the first chemically characterized MF-a (Figure 1A), it is expected that a minimum of at least 10 enzymes are required for MF biosynthesis from primary metabolites. 1,3,4,6-Hexanetetracarboxylic acid (HTCA), a component of the MF side chain (Figure 1A), requires at least 14 separate enzymatic steps for its biosynthesis.[9] HTCA, however, is not a required component of methanofuran because this moiety is replaced with two γ-linked glutamates in methanofuran-b,[10] by a 2-hydroxy-HTCA in methanofuran-c,[6] or by a series of γ-linked glutamates in the methanofuran present in *M. jannaschii* (Figure 1B) denoted methanofuran-d, which has recently been characterized in our lab.

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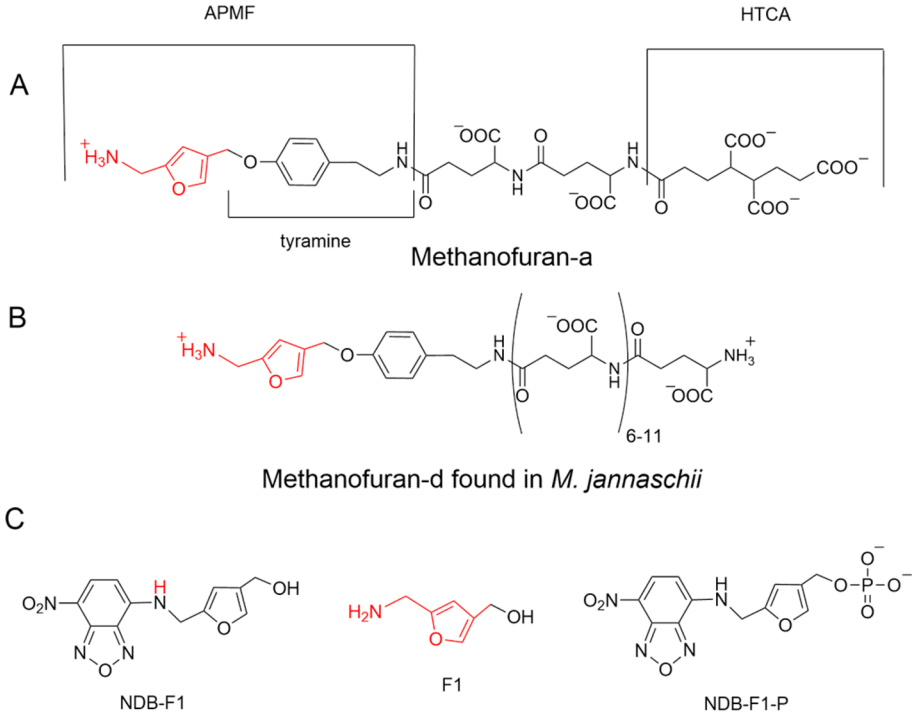

Figure 1. Chemical structures of the first characterized methanofuran-a (A), the methanofuran-d from *M. jannaschii* (B), and NDB-F1, F1, and NDB-F1-P (C). The F1 moiety of methanofuran is colored red. The red hydrogen in NDB-F1 is expected to be acidic and thus the one ionized in forming the $[M - H]^-$ ion.

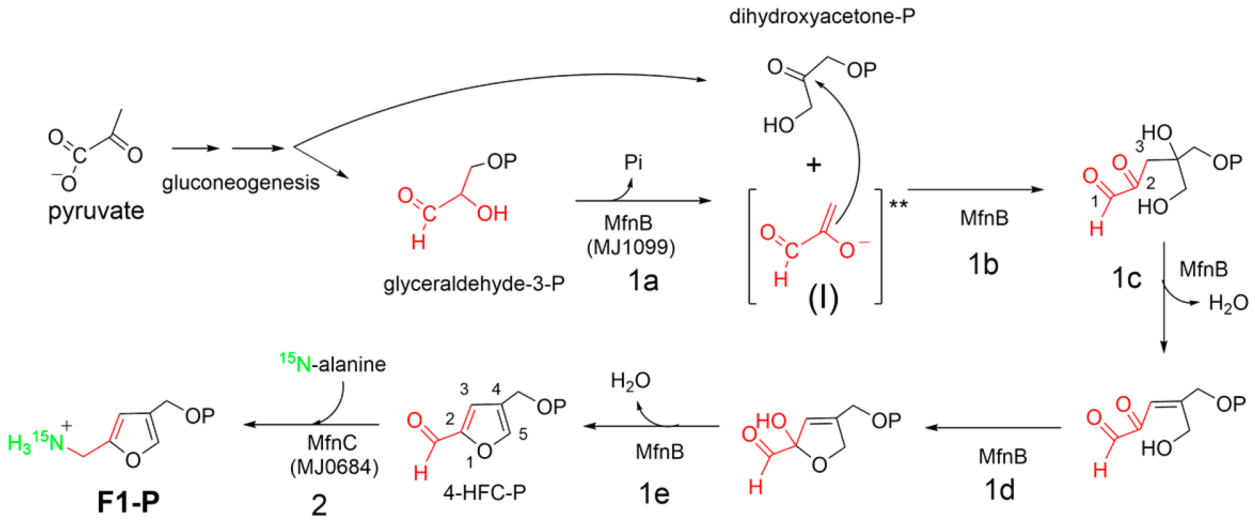

Figure 2. Proposed biosynthetic route to F1-P, the furan precursor to the methanofurans. Red indicates the atoms incorporated from glyceraldehyde 3-phosphate, and green indicates the nitrogen incorporated from L-alanine. Steps 1a−1e represent the proposed chemical steps required for converting Ga-3-P and DHAP to 4-HFC-P and are all catalyzed by a single enzyme, MfnB. Two asterisks denote a proposed intermediary (I) derived by the elimination of phosphate from Ga-3-P that may be involved in the initial aldol condensation reaction.

Here we specifically address the formation of F1-P in *M. jannaschii* and propose that cell extracts condense glyceraldehyde 3-phosphate (Ga-3-P) produced from pyruvate, with dihydroxyacetone-P (DHAP) to form 4-(hydroxymethyl)-2-furancarboxaldehyde (4-HFC-P). This reaction is catalyzed by the product of the MJ1099 gene, which we name 4-HFC-P synthase (MfnB). Then, 4-HFC-P is transaminated to F1-P, the precursor to F1 in MF (Figure 2) catalyzed by 4-HFC-P:alanine aminotransferase (MfnC), the product of the MJ0684 gene. Support for this pathway comes from the identification of 4-HFC-P and F1-P in *M. jannaschii* cell extracts and the demonstration that 4-HFC-P is converted into F1-P in cell extracts of *M. jannaschii*. We have made extensive use of the 7-nitrobenzofurazan-F (NBD-F) amine reagent[11] to facilitate the analysis of F1 as the NBD derivative (Figure 1C), using both high-performance liquid chromatography (HPLC) and liquid chromatography−ultraviolet−electrospray ionization mass spectrometry (LC−UV−ESI−MS). Our work represents the first progress on MF biosynthesis since our first papers[12,13] and those from Bacher's lab[14,15] appeared 18−23 years ago.

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MATERIALS AND METHODS

Chemicals. 5-(Hydroxymethyl)-2-furancarboxaldehyde (5-HFC), furfurylamine (FA), tetrabutylammonium phosphate, methyl 3-furoate, pyridoxal 5′-phosphate hydrate, ATP, furaldehyde, methylglyoxal, oxaloacetic acid, and alkaline phosphate from bovine intestinal mucosa (13 mg/mL, >4000 units/mg) were obtained from Sigma-Aldrich. [^{15}N]-L-Glutamate, [^{15}N]-L-asparate, and [^{15}N]-L-alanine each with 98% ^{15}N were also obtained from Sigma-Aldrich. 4-Fluoro-7-nitrobenzofurazan (NBD-F) was obtained from Alfa Aesar (Ward Hill, MA). Methyl 3-furancarboxylate was obtained from TCI America. 4-(Hydroxymethyl)furan-2-carboxylic acid (4-HFCA) and 5-(hydroxymethyl)furan-2-carboxylic acid (5-HFCA) were prepared by the oxidation of the corresponding aldehydes to carboxylic acids with Ag_2O in base.[16] [1,3,3,3-$^2H_4$]Methylglyoxal was prepared, purified, and assayed as previously described.[17] Sodium [1,2,3-$^{13}C_3$]pyruvate (99% ^{13}C) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). A sample of aminoacetone was supplied by C. Watanabe (Texas A&M University, College Station, TX).[18]

The chemical syntheses of 4-HFCA, 5-(hydroxymethyl)-2-furancarboxylic acid (5-HFCA), 4-([$^2H_2$]hydroxymethyl)-2-furancarboxylic acid-P ([$^2H_2$]-4-HFCA-P), 4-HFC, 4-([$^2H_2$]-hydroxymethyl)-2-furancarboxaldehyde ([$^2H_2$]-4-HFC), 4-(hydroxymethyl)-2-furancarboxaldehyde phosphate (4-HFC-P), 4-([$^2H_2$]hydroxymethyl)-2-furancarboxaldehyde phosphate ([$^2H_2$]-4-HFC-P), 5-(aminomethyl)-2-furanmethanol (5-AFM), 3-(aminomethyl)-2-furanmethanol (3-AFM), and 5-(aminomethyl)-3-furanmethanol (F1) and the chemical reaction of pyruvate with dihydroxyacetone to form 4-HFCA and 5-HFCA and methylglyoxal and dihydroxyacetone to form 4-HFC and 5-HFC are described in the Supporting Information.

TLC Analysis and Preparative TLC Purification of Intermediates. TLC solvent system 1 consisted of acetonitrile, water, and 88% formic acid [80:20:10 (v/v/v)]; solvent system 2 consisted of 5% of 88% formic acid in acetonitrile (v/v), and solvent system 3 consisted of methyl acetate. We visualized F1, F1-P, FA, and 4-{[4-(2-aminoethyl)phenoxy]methyl}-2-furanmethanamine (APMF) by spraying the plate with 2% ninhydrin in ethanol and then heating the plate to 140 °C. The same procedure was used to detect furans containing aminomethyl groups using the Ehlrich spray detection reagent.[6] Furans containing a carbonyl group conjugated with the furan were readily detected by their UV absorbance on the TLC plates. R_f values of different compounds in different solvents were as follows: solvent system 1, 0.63 for F1, 0.53 for hypoxanthine (used as an internal UV marker to identify the position of F1 on the TLC plate), and 0.72 for furfurylamine (FA, 2-aminomethylfuran); solvent system 2, 0.12 for F1, 0.28 for FA, 0.52 for 4-HFCA, and 0.60 for 5-HFCA; solvent system 3, 0.56 for NBD-F1, 0.56 for NBD-2-AFM, 0.63 for NBD-NH_3, 0.70 for NBD-tyramine, 0.47 for 4-HFC, and 0.58 for 5-HFC.

A Search for a Suitable Derivative for the Analysis of F1. As previously described, F1 can be assayed in biological samples by GC−MS of its diacetate derivative.[13] This procedure is very time-consuming and does not allow for the accurate quantitation of F1 in biological samples. Direct analysis of F1 by HPLC is difficult because of the short wavelength at which F1 absorbs (215 nm), common to many compounds, and because F1 is not retained by reverse phase HPLC columns. Considering these difficulties, we developed a more sensitive procedure that involves the preparation of a UV-absorbing and fluorescent derivative of F1 that allows the measurement of the small amount of F1 in cell extracts and incubation mixtures. Several different derivatives were tried, but the NBD derivative was selected for the following reasons. (1) The NBD derivative is easily prepared and purified by preparative TLC prior to HPLC and LC−UV−ESI−MS analysis. (2) The NBD-F1 derivative has an absorbance peak at 480 nm in addition to fluorescence (excitation at 480 nm and emission at 542 nm). (3) The NBD derivative forms an intense $(M - H)^-$ ion at m/z 289.1 in negative ion ESI−MS that greatly increases the sensitivity of detecting this molecule and also allows for the measurement of stable isotope incorporation. The reason for this increased ionization efficiency in the negative ion appears to be due to the acidity of the hydrogen on the nitrogen bridging the NBD and the furan in the NBD-F1 derivative (red hydrogen in Figure 1C). The $(M - H)^-$ ion at m/z 289.1 for the NBD-F1 derivative could be detected with 12-fold greater sensitivity than the MH^+ ion at m/z 291.1. LC−ESI−collision-induced dissociation−MS/MS (LC−ESI−CID−MS/MS) of this NDB-F1 in positive and negative ionization modes was used to confirm that the F1 isolated from the cells was identical to that obtained from the synthetic NDB-F1 sample. The increased sensitivity in detecting the NBD derivative of furfurylamine by ESI−MS was shown by direct infusion of the dilute solutions of this compound into the AB Sciex 3200 Q TRAP mass spectrometer. A downside of this analysis was that no fragment ion that established the position at which the label was incorporated into F1 could be identified.

HPLC Analysis of Intermediates. The chromatographic separation and analysis of intermediates and derivatives of intermediates were performed on a Shimadzu HPLC system with a C_{18} reverse phase column (Phenomenex Kinetex, 100 mm × 4.0 mm, 2.6 μm particle size) equipped with a photodiode array detector (PDA) and a fluorescence detector. The elution profile consisted of 5 min at 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% sodium azide) and 5% methanol followed by a linear gradient from 50 to 100% methanol over 28 min at a rate of 0.5 mL/min. Quantitation of NDB-F1 was based on the measured fluorescence intensity (excitation at 480 nm and emission at 542 nm) and/or the absorbance at 480 nm. In this chromatographic system, 4-(hydroxymethyl)-2-furancarboxylic acid (4-HFCA) eluted at 3.99 min, 5-(hydroxymethyl)-2-furancarboxylic acid (5-HFCA) at 4.96 min, NDB-NH_3 at 7.67 min, NDB-ethylamine at 10.85 min, NBD-F1 at 11.23 min, the NBD derivative of 5-(aminomethyl)-2-furanmethanol (5-AFM) at 11.36 min, and NBD-APMF at 20.24 min.

LC−UV−ESI−MS Analysis of Intermediates. Analysis was performed with an AB Sciex 3200 Q TRAP mass spectrometer system. The liquid chromatograph was an Agilent 1200 Series model. For the analysis of NBD-F1, the Phenomenex Kinetex (100 mm × 4.0 mm, 2.6 μm particle size) column was used and the injection volume was 15 μL. Solvent A was water with 25 mM ammonium acetate, and solvent B was methanol. The flow rate was 0.5 mL/min. Gradient elution was employed in the following manner [t (minutes) and %B]: 0.01 and 5, 10 and 65, 15 and 65, and 15.01 and 5, respectively. The column effluent was passed through a variable-wavelength detector set at 480 nm and then into the Turbo Spray ion source. Quantitation in LC−UV−ESI−MS analyses was based on the absorbance at 480 nm, thereby avoiding problems arising from ion suppression occurring in the mass spectrometer. ESI was employed at −4500 V and 600 °C. Curtain gas, gas 1, and gas 2 flow pressures were 35, 70, and 60 psi, respectively. Desolvation, entrance, and collision cell entrance potentials were −40, −12, and −22.5 V, respectively.

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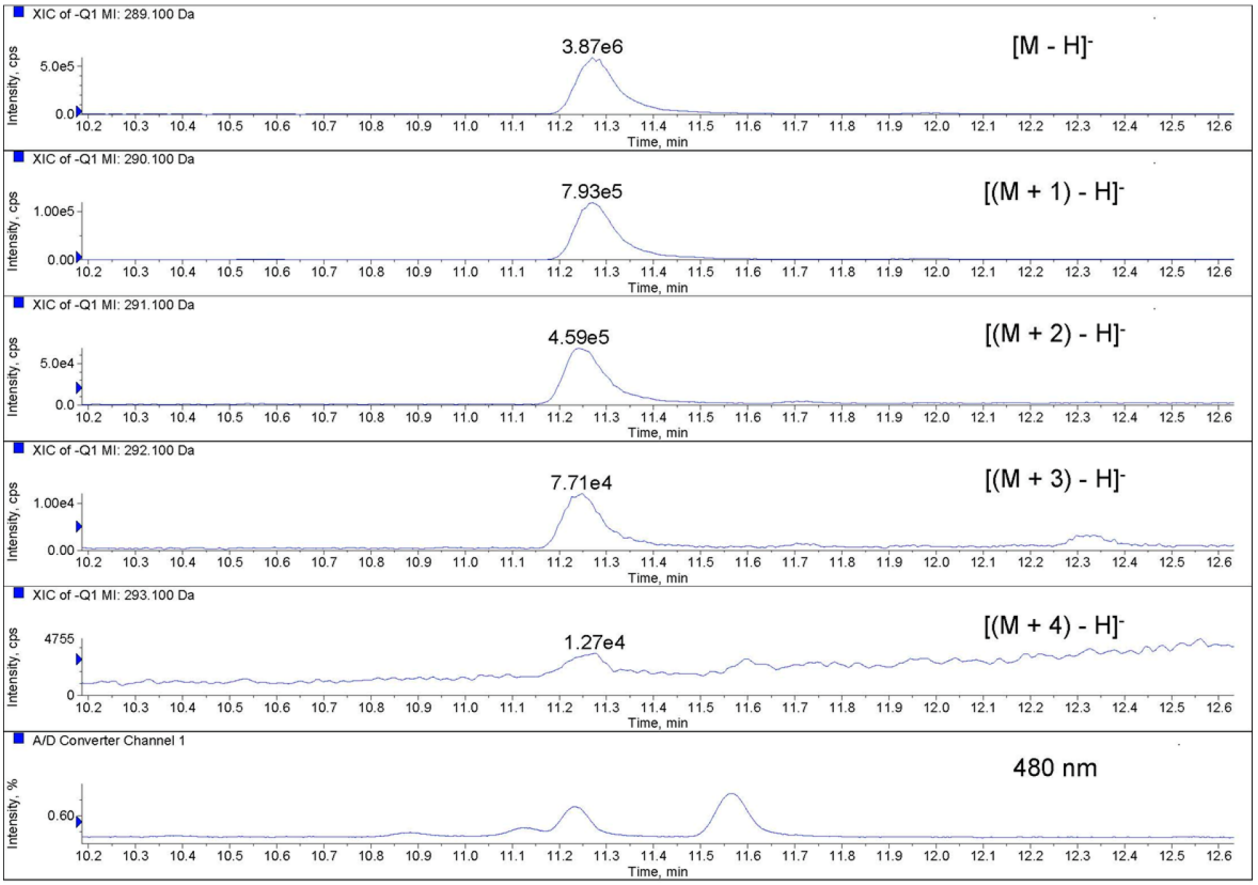

Figure 3. LC−UV−ESI−MS data for the analysis of NBD-F1 from experiment 4 reported in Table 1. The first five traces represent the intensities of the $(M - H)^-$, $(M + 1 - H)^-$, $(M + 2 - H)^-$, $(M + 3 - H)^-$, and $(M + 4 - H)^-$ ions at m/z 289.1, 290.1, 291.1, 292.1, and 293.1, respectively, and the bottom trace is the recorded absorbance at 480 nm from the NBD derivative. The values of isotope abundance were calculated from the peak areas recorded above each peak. The notations +1, +2, +3, and +4 represent the nominal mass increase as a result of the incorporation of ^1H and/or ^{15}N. The UV peak at 11.23 min is NBD-F1, and that at 11.56 min is unknown. From the differences in the measured isotopic ion intensities recorded for the samples, corrected for the amount found in unlabeled samples, the calculated isotopic incorporations reported in Table 1 were obtained.

Selected ion monitoring (SIM) was used to measure intensities for each ion from m/z 289.1 to 296.1 with a dwell time of 50 ms per ion.

Cell Extracts of *M. jannaschii*. Cell extracts of *M. jannaschii* were prepared by sonication under argon and stored as previously described under anaerobic conditions at −20 °C and had ∼30 mg/mL protein.[19] The buffer used in the extraction consisted of 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES/K^+), 10 mM $MgCl_2$, and 20 mM DTT (pH 7.5), kept under argon.

Incubation of Precursors with *M. jannaschii* Cell Extracts, Isolation of F1, and Formation and Purification of NBD-F1. In a typical incubation, cell extracts (25 or 50 μL) were incubated under argon with substrates and cofactors being added as 0.1 M aqueous solutions adjusted to pH 7.0. Samples were contained in 1 mL screw top glass bottles (1 cm × 2.5 cm) sealed with TFE/silicone septa. The volume(s) of the substrate solutions used was one-tenth the cell extract volume used. In some cases, the argon atmosphere was substituted with hydrogen at 20 psi. After being mixed, the samples were incubated for 15 min at 70 °C in a water bath after which 1.5 volumes of methanol was mixed with the sample. After centrifugation (16000*g* for 5 min), the liquid was separated from the pellet and the methanol evaporated with a stream of nitrogen gas. The sample was then diluted with 100 μL of water, and 10 μL of 0.1 M glycine buffer (pH 10.8) containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$ was added along with 1 μL of alkaline phosphatase (13 mg/mL, >4000 units/mg of protein). The resulting sample was incubated for ∼2 h at 37 °C to hydrolyze the phosphate monoesters.

In some experiments, where a phosphorylated product was not expected, another more detailed method of purifying the F1 was employed. Here 50 μL of cell extract or incubation mixture was mixed with 7 μL of 2 M trichloroacetic acid (TCA) and the protein precipitate was separated by centrifugation (16000*g* for 5 min). The resulting clear extract was diluted 3-fold with water and applied to a small Dowex 50W-8X-H^+ column (1 mm × 5 mm). The column was washed with 400 μL of water, and the amino-containing compounds were eluted with 200 μL of 6 M ammonium hydroxide. After evaporation of the solvent, F1 was purified by preparative TLC using solvent system 1 where it had the same R_f (0.63) as a known sample of F1. The identity and position of the samples on the TLC plate could be established either with Ehlrich reagent spray or with ninhydrin spray. To quantitatively recover the F1 from the sample, only the portion of the TLC plate containing the known F1 sample was sprayed, so the exact position of the compound isolated from the cells could be determined without exposure of this portion of the TLC plate to the spray reagent. In some cases, the samples were spiked with hypoxanthine, which runs a little lower (R_f = 0.57) on the plate than F1 and can be easily visualized on the TLC plate under UV

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light. The area of the plate containing the F1 was removed, and the F1 was eluted with 50% methanol. The R_f of F1 was larger that than of the major amino-containing compound present in these cell extracts, which was β-glutamate (R_f = 0.55).[20]

In either procedure, the final sample containing the F1 was evaporated to dryness and dissolved in 10 μL of water and 10 μL of 0.1 M borate buffer (pH 9.3) and then adjusted to pH 9.3, if required, by the addition of small amounts (~1−3 μL) of 1 M sodium hydroxide. To this solution was added 10 μL of 0.1 M NDB-F in tetrahydrofuran (THF) and the sample incubated at 60 °C for 30 min to form the NDB derivative. Preparative TLC of the entire reaction mixture using solvent system 3, prior to LC−UV−ESI−MS analysis, was used to purify the NDB-F1 derivative present in the sample. NBD-F1 (R_f = 0.52) eluted just below the NBD-ammonia spot that was present in every sample and served as a useful marker. This area of the TLC plate was removed, and the NBD-F1 was eluted with methyl acetate, the solvent evaporated, and the sample placed in a 50% methanol/water mixture for LC−UV−ESI−MS analysis.

Quantitation of F1 Production. A known sample of NBD-ethylamine was prepared by the reaction of ethylamine with NBD-F followed by purification of the product by preparative TLC. The absorbance of the prepared solution was measured, and from this measurement, the concentration was determined using an extinction coefficient of 2.8×10^4 M^{-1} cm^{-1} calculated from the reported UV spectrum of *N*-methyl-4-amino-7-nitrobenzofurazan.[21] This sample was then used to calibrate the LC−UV peak areas obtained from the LC−UV−ESI−MS system. A representative example of such data for the analysis of NBD-F, from experiment 4 in Table 1 is shown in Figure 3.

Identification of 4-HFCA-P and/or 5-HFCA-P in *M. jannaschii* Cell Extracts. A *M. jannaschii* cell extract (100 μL) was mixed with 200 μL of methanol; the sample was centrifuged to remove the proteins, and the clear supernatant was separated and the methanol evaporated. The sample was then enzymatically dephosphorylated as described above and the whole sample evaporated and purified by preparative TLC using 5% formic acid (88%) in acetonitrile. The area of the plate containing the 4-HFCA and 5-HFCA was removed, and the compounds were eluted and analyzed by HPLC with PDA detection and by LC−ESI−MS. HPLC with PDA detection was not sensitive enough to clearly establish the presence of 4-HFCA because of the presence of so many other UV-absorbing compounds. LC−UV−ESI−MS using this solvent gradient but with 1% formic acid in solvents A and B was thus used for the analysis. Only the material isolated from the plate with an R_f corresponding to that for 4-HFCA contained 4-HFCA. 5-HFCA was detected in the area corresponding to the 5-HFCA isomer. The 4- and 5-isomers were further distinguished by the differences in the values of the relative abundance of specific product ions measured in the MRM mode. The values of abundance (relative to the *m*/*z* 97 abundance) of *m*/*z* 69 and 79 were <2 and 38%, respectively, for 4-HFCA and 39 and 10%, respectively, for 5-HFCA. The enhanced product ion (-EPI) spectra of 4-HFCA, 2-HFCA, and 5-HFCA are shown in Figure S1 of the Supporting Information. The LC−ESI−MRM−MS data for these compounds are shown in Figures S2−S4 of the Supporting Information.

Identification of 4-HFC and/or 4-HFC-P in *M. jannaschii* Cell Extracts as the *O*-(4-Nitrobenzyl)-hydroxylamine Derivative. To 100 μL of *M. jannaschii* cell extract was added 10 μL of 0.1 M *O*-(4-nitrobenzyl)-hydroxylamine in 1 M sodium acetate buffer (pH 4), and the samples were then heated at 100 °C for 15 min. The resulting suspension was centrifuged (16000*g* for 5 min) to remove the insoluble material. The soluble material was separated from the pellet, and 50 μL of 0.1 M glycine buffer (pH 10.8) containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$ was added along with 1 μL of alkaline phosphatase (13 mg/mL, >4000 units/mg of protein). After incubation at 37 °C for 2 h, the sample was concentrated to ~30 μL with a stream of nitrogen gas and extracted three times with 200 μL of methyl acetate. All of the extracted material was then purified by preparative TLC using methyl acetate as the eluting solvent where the 4-HFC *O*-(4-nitrobenzyl)-hydroxylamine derivative had an R_f of 0.59. The area of the plate containing the desired compound was removed, and the 4-HFC *O*-(4-nitrobenzyl)-hydroxylamine derivative eluted with methyl acetate. Known samples prepared from 4-HFC and 5-HFC were prepared in the same manner, and each showed similar electron impact spectra (70 eV) with the expected M^+ at *m*/*z* 278. The samples were analyzed by HPLC using two different columns. Using the Phenomenex Kinetex column, with the solvent system described above, the major peak eluted at 26.6 min and had an absorbance maximum at 275 nm. Using the Shimadzu HPLC system with a C18 reverse phase column (Varian Pursuit XRs, 250 mm × 4.6 mm, 5 μm particle size) and an elution profile consisting of 5 min at 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% NaN_3) and 5% methanol followed by a linear gradient to 20% sodium acetate buffer and 80% methanol over 40 min at a rate of 0.5 mL/min, the major peak eluted at 37.8 min and had an absorbance maximum at 275 nm. On either column, minor additional peaks at approximately the same retention times and with the same absorbance spectra were seen. These additional peaks resulted from the presence of the *cis* and *trans* oximes as well as the two different planar isomers around the carbon on the oxime and C-2 of the furan.

Cloning, Recombinant Expression, and Confirmation of Overexpression of the MJ1099 and MJ0684 Gene Products in *Escherichia coli*. Gene MJ1099 (UniProt entry Q58499) and gene MJ0684 (UniProt entry Q58097) were amplified by PCR from *M. jannaschii* genomic DNA using oligonucleotide primers MJ1099-Fwd (5′-GGTCATATGATACTATTAGTAAG-3′) and MJ1099-Rev (5′-GCTGGATCCTTACTTACAAAG-3′) and MJ0684-Fwd (5′-GGTCATATGCTATCAAAAAGG-3′) and MJ0684-Rev (5′-GACGGATCCTCACTCAAAAATCTC-3′). PCR amplification was performed as described previously[14] using an annealing temperature of 50 °C for MJ1099 and 55 °C for MJ0684. Purified PCR products were digested with NdeI and BamHI restriction enzymes and ligated into compatible sites in plasmid pT7-7 for pMJ1099 and pMJ0684. The sequences of the resulting plasmids of pMJ1099 and pMJ0684 were verified by the DNA sequencing facility at the University of Iowa, and the resulting pMJ1099 and pMJ0684 were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene). The transformed cells were grown in LB Broth, Miller medium (200 mL) supplemented with 100 μg/mL ampicillin at 37 °C while being shaken until they reached an OD_{600} of 1.0. Recombinant protein production was induced by the addition of lactose to a final concentration of 28 mM. After being cultured for an additional 4 h, the cells were harvested by centrifugation (4000*g* for 5 min) and frozen at −20 °C. Induction of the desired protein was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis of total cellular proteins.

E. coli cell pellets (~1 g) containing these recombinant proteins were resuspended in 3 mL of extraction buffer [50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES/K^+) (pH 7.0), 10 mM $MgCl_2$, and 20 mM DTT] and

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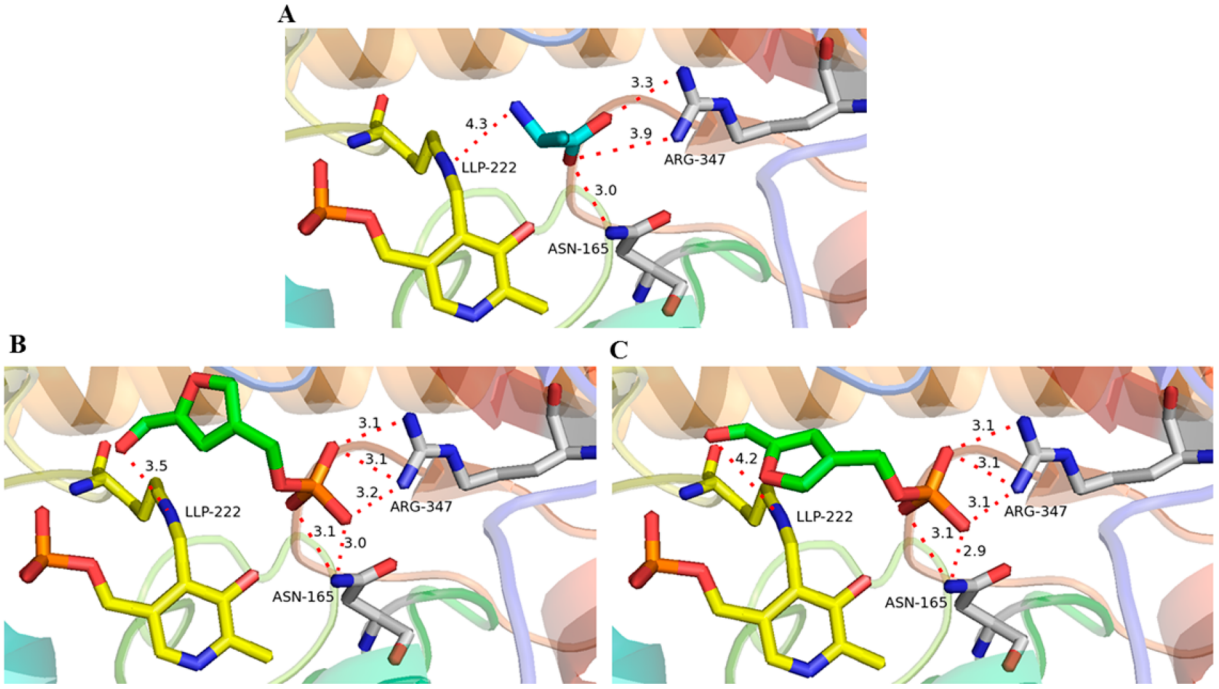

Figure 4. Molecular docking of alanine and 4-HFC-P into MfnC: (A) alanine (cyan carbon atoms), (B) one possible orientation of 4-HFC-P (green carbon atoms), and (C) another possible orientation of 4-HFC-P docked into the active site of MfnC. The indicated measured distances are given in angstroms. The Schiff base PLP bound to the ϵ-amino group of Lys222 (LLP) is colored yellow. Arg347 and Asn165 carbon atoms are colored white, nitrogen atoms blue, oxygen atoms red, and phosphorus atoms orange.

lysed by sonication followed by centrifugation (16000*g* for 10 min) to remove insoluble material.

Purification and Identification of Recombinant Proteins. *E. coli* cell extracts were prepared as described above. The majority of *E. coli* proteins contained in the extract were removed by heating the cell lysate to 80 °C for 10 min, followed by centrifugation (16000*g* for 10 min) to remove insoluble material. The MJ1099-derived protein 4-HFC-P synthase (MfnB) and the MJ0684-derived protein [4-HFC-P:alanine aminotransferase (MfnC)] were purified by anion exchange chromatography on a MonoQ HR column (1 cm × 8 cm) with a linear gradient from 0 to 1 M NaCl in 25 mM Tris/HCl (pH 7.5) over 55 min at a rate of 1 mL/min. MfnB eluted in a single peak centered at 0.40 M NaCl, and MfnC eluted in a single peak centered at 0.43 M NaCl. Purified MfnB and MfnC were >95% pure as judged by SDS−PAGE with Coomassie staining. Protein concentrations were determined by Bradford analysis.[19] The identity of the purified enzyme was verified by matrix-assisted laser desorption ionization mass spectral analysis of the tryptic peptides derived from the excised protein band as described in the Supporting Information.

Testing MfnB To Catalyze the Production of 4-HFC-P or 4-HFCA-P. To test whether the gene product of MJ1099 is the enzyme catalyzing the condensation of Ga-3-P and DHAP to produce 4-HFC-P or pyruvate and DHAP to produce 4-HFCA-P, the following procedure was followed. In a 100 μL reaction volume, 5 μg of enzyme (MfnB) was incubated with 1 mM DHAP and 1 mM Ga-3-P or pyruvate in 50 mM MES buffer (pH 6.5) at 70 °C for 20 min. The reaction mixture was cooled to room temperature, and 90 μL of the resulting sample was transferred to another reaction vessel; 10 μL of glycine buffer [1 mM ZnOAc and 1 mM $MgCl_2$ (pH 9.4)] and 1 μL of phosphatase (0.2 unit/μL) were added. The reaction mixture was incubated at 37 °C for 30 min, and the resulting samples were analyzed by HPLC and LC−MS for 4-HFC or 4-HFCA.

To separate and analyze 4-HFC-P, 4-HFC, and 4-HFCA derivatives from the enzymatic reaction catalyzed by 4-HFC-P synthase, the same HPLC system was used but was equipped with a Varian Pursuit C_{18} reverse phase column (XRs, 250 mm × 4.6 mm, 5 μm particle size). The elution profile consisted of 5 min at 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% NaN_3) and 5% methanol followed by a linear gradient to 45% sodium acetate buffer and 55% methanol over 40 min at a rate of 1.0 mL/min. 4-HFC-P, 4-HFC, and 4-HFCA were quantitated by their absorbance at 280 nm. In this chromatographic system, 4-HFC-P eluted at 4.3 min, 4-HFC eluted at 10.3 min, and 5-HFC eluted at 10.7 min.

Transamination of [$^2H_2$-*hydroxymethyl*]-4-HFC-P to [$^2H_2$-*hydroxymethyl*]F1-P in *M. jannaschii* Cell Extracts with Purified 4-HFC-P:Alanine Aminotransferase (MfnC). To 25 μL of *M. jannaschii* cell extract were added 5 μL of a 1.4 mM solution of [$^2H_2$-*hydroxymethyl*]-4-HFC-P, 5 μL of a 0.1 M solution of ^{15}N-labeled aspartate, glutamate, and alanine, and 1 μL of 1 mM PLP, and the sample was incubated under argon for 30 min at 70 °C. The biopolymers were then removed by precipitation after the addition of 60 μL of methanol followed by centrifugation (16000*g* for 5 min). After separation of the clear methanol/water layer, the methanol was evaporated and the sample diluted with 50 μL of water. The monophosphate esters were then enzymatically hydrolyzed as described above. We isolated the F1 present in the sample by passing the sample through a Dowex 50W-8X-H^+ column (1 mm × 5 mm), washing the column with water, and eluting the F1 with 6 M aqueous NH_4OH. The F1 contained in the evaporated eluted sample was converted into the NBD derivative and purified as described above.

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For the assay of purified MfnC, 25 μL of a 0.2 mg/mL solution of the protein dissolved in 25 mM TES buffer (pH 7.5) was incubated with the substrates and assayed for product as indicated above for the cell extracts. Other purified recombinant PLP-containing enzymes (see Discussion) were assayed by the same procedure.

Characterization of the Transamination Reaction between Alanine and α-Ketoglutarate Catalyzed by Purified MfnC. To examine the substrate specificity of MfnC, we tested its ability to catalyze a transamination reaction between alanine and α-ketoglutarate. Thus, a 20 μL solution of the recombinant enzyme (0.6 mg/mL) was mixed with 20 μL of extraction buffer and 5 μL portions of 0.1 M [^{15}N]-L-alanine and 0.1 M α-ketoglutarate in water. The sample was then incubated at 70 °C for 30 min, and the resulting clear extract was diluted 3-fold with water and applied to a small Dowex 50W-8X-H^+ column (2 mm × 10 mm). The column was washed with 400 μL of water, and the amino-containing compounds were eluted with 400 μL of 6 M NH_4OH. The eluted amino acids were converted into their trifluoroacetyl methyl ester (TM) derivatives and assayed by GC−MS as previously described.[13] This method of analysis allowed for the determination of the extent of glutamate formation as well as any ^{15}N incorporation.

Molecular Docking of Alanine and 4-HFC-P into MfnC. To further examine the active site of 4-HFC-P:alanine aminotransferase's ability to function with alanine and 4-HFC-P as substrates, molecular docking of the alanine and 4-HFC-P was performed. The alanine structure was acquired from the Protein Data Bank (PDB), and 4-HFC-P was generated using the ProDRG server.[22] The X-ray crystal structure of MfnC[23] is deposited as PDB entry 2Z61. Molecular docking is a computer modeling technique that can be used to visualize how proposed ligands fit into the active site of a protein. We use this method to show that alanine and 4-HFC-P are both able to orient in the active site for the transfer of the amino group from alanine to 4-HFC-P via PLP. Preparation of the protein and substrate files for molecular docking were prepared using AutoDock Tools version 1.5.6.[24] AutoDock Vina[25] was used to perform the molecular docking of the substrates (alanine and 4-HFC-P) into MfnC. The parameters for molecular docking were a box size of 20 Å × 20 Å × 20 Å that was centered within the coordinates of (50.280, 27.702, 25.397) and was used for each substrate with an output of nine generated poses of each substrate in the active site of MfnC.

Of the nine generated poses for alanine that were generated, the first pose was used because it had the lowest energy (−3.6 kcal mol^{-1}), and this orientation could be stabilized by the conserved Asn165 and Arg347 (Figure 4A). Of the nine generated poses of 4-HFC-P, the second pose (−5.2 kcal mol^{-1}) and the sixth pose (−5.0 kcal mol^{-1}) were chosen because they each were oriented in the active site so that the phosphate was stabilized by Asn165 and Arg347. Additionally, the aldehyde of 4-HFC-P is close to the nitrogen of the Schiff base-bound Lys222-PLP to receive the amino group donated from pyridoxamine phosphate (Figure 4B,C). In this case, two poses of 4-HFC-P were chosen because it is unknown if there are additional forces to define the orientation of the furan ring in the active site as either away from PLP (Figure 4B) or toward PLP (Figure 4C).

RESULTS AND DISCUSSION

Methanofuran (MF) is one of six coenzymes that are required to catalyze the biochemical reduction of carbon dioxide to methane[1−3] and is the only coenzyme known to contain a furan ring. Earlier work on the biosynthesis of F1 in growing cells of a rumen isolate 10-16B,[26] a methanogen similar to *Methanobrevibacter ruminatium*, showed that two labeled acetates were each incorporated as a unit into F1 (Figure 5). The methyl

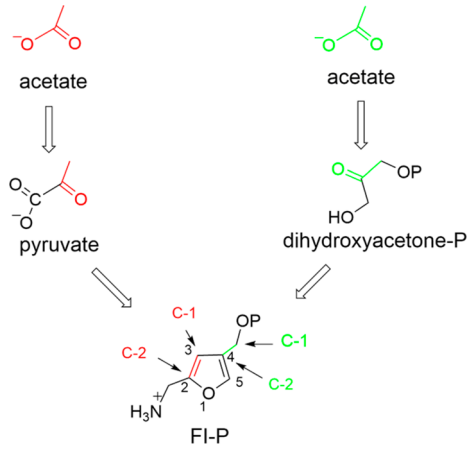

Figure 5. Originally observed incorporation of labeled acetate into F1-P. The red and green colors represent the different positions where the two carbon units of acetate are incorporated.

group of one of the acetates was incorporated at C-3, and its carboxyl carbon was incorporated at C-2 of F1-P (Figure 5, red). The other acetate methyl group was incorporated at the C-4 hydroxymethyl group, and its carboxyl carbon was incorporated at C-4 (Figure 5, green).[13] These data were consistent with pyruvate, or a metabolite derived from pyruvate, and DHAP serving as precursors to F1-P.

Bioinformatics Identification of the 4-HFC-P Synthase (MfnB). The first step in the sequence of reactions leading to F1-P would involve an aldol condensation of the methyl group of a three-carbon unit with the ketone in DHAP. To identify a possible aldolase to catalyze this step, we searched the *M. jannaschii* genome for such an enzyme. We had considered this reaction to be catalyzed by either the enzyme encoded by MJ0644, which was weakly homologous with 4-hydroxy-2-oxoglutarate aldolase or 4-carboxy-4-hydroxy-2-oxoadipate aldolase, or MJ1099, which is annotated as a hypothetical enzyme. However, we have failed to identify any enzymatic reaction catalyzed by the gene product of MJ0644. A PSI-BLAST search[27] indicated that the MJ1099 gene product contains a class I aldolase domain. In addition, the homologues of the MJ1099 gene were distributed in all methanogens and methylotrophic bacteria, which require methanofuran and methanopterin as coenzymes to oxidize formaldehyde.[28,29] This further suggested that the MJ1099 gene product had a possible role in the biosynthesis of these cofactors. To establish if the gene product of MJ1099 was responsible for this 4-HFC-P biosynthesis, we cloned and heterologously expressed this gene in *E. coli*, and the enzymatic activity of the purified enzyme was tested *in vitro*.

Identification of the Reaction Catalyzed by MfnB. Our results showed the gene product of MJ1099 catalyzed the aldol condensation between Ga-3-P and DHAP to produce 4-HFC-P. 4-HFC-P was then treated with phosphatase, and the resulting 4-HFC was confirmed by co-elution with the synthetic known 4-HFC by HPLC (Figure 6). The 4-HFC was also confirmed by LC−ESI−MS/MS. Although our isotope labeling showed that pyruvate was a precursor to 4-HFC-P, the MJ1099 gene product was not able to use pyruvate as a substrate, indicating in the *M.*

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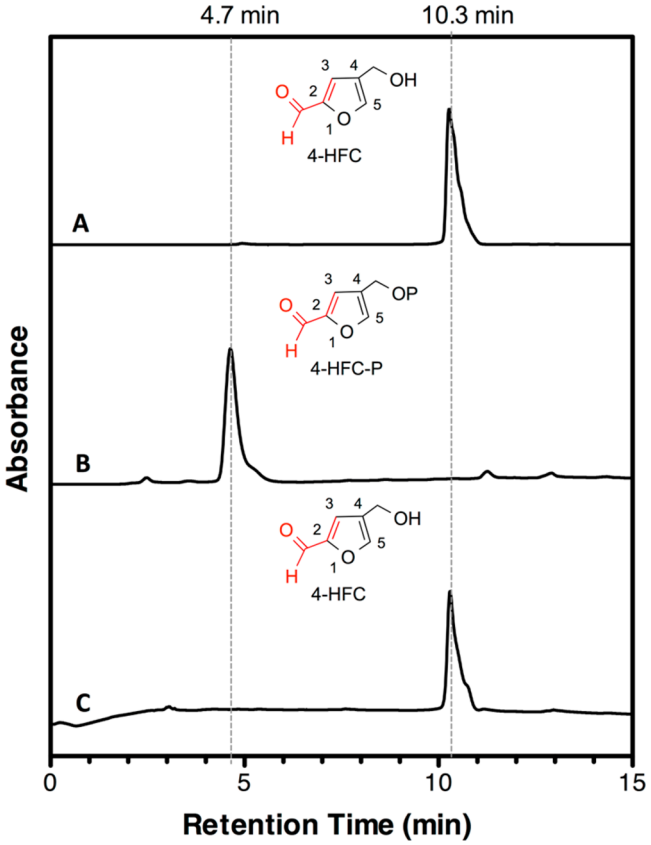

Figure 6. (A) HPLC analysis of synthetic 4-HFC. (B) Enzymatic reaction product generated by MfnB. (C) Resulting product from the MfnB reaction treated with phosphatase.

jannaschii cell extract, pyruvate must be converted to Ga-3-P and/or DHAP via gluconeogenesis, which then condense with DHAP to form 4-HFC-P. Possible enzyme-bound intermediates that would be required to generate 4-HFC-P by the enzyme are shown in Figure 2 as steps 1a−1e. The types of reactions involved in each step would be described as phosphate elimination, aldol condensation, dehydration, cyclization, and dehydration, respectively. Surprisingly, the formation of 4-HFC-P and all of these reactions must proceed in the active site of this single enzyme.

As shown in step 1a of Figure 2, we propose that the first step in the mechanism of this enzyme is the conversion of a Ga-3-P to a bound form of methylglyoxal. Two well-studied enzymes are known to produce methylglyoxal: triosephosphate isomerase[30,31] and methylglyoxal synthase.[32] These enzymes have different protein folds, but each has the same catalytically important conserved Glu, His, and Lys residues that are located in the active sites enantiomorphic to each other. MfnB, however, must have a much more complex active site that must function without a catalytic histidine residue because MfnB contains no histidine. The enolate formed by the elimination of phosphate is expected to undergo the condensation with a bound DHAP as the second step in the reaction.

Identification and Characterization of the 4-HFC-P:Alanine Aminotransferase (MfnC). The second step in the pathway to F1-P (Figure 2, step 2) is the transamination of 4-HFC-P to F1-P. Having previously recombinantly produced 12 of the 21 PLP-dependent enzymes annotated in the *M. jannaschii* genome, we selected six of the gene products that were annotated as aminotransferases to test for their ability to catalyze step 2 in Figure 2. The enzymes selected were derived from the MJ0001, MJ0684, MJ0959, MJ1008, MJ1341, and MJ1391 genes. Only the protein encoded by the MJ0684 gene was found to catalyze this reaction, and it was found to use only alanine as the amino group donor. The enzyme incubated with [$^2H_2$-*hydroxymethyl*]-HFC-P and labeled [^{15}N]alanine, [^{15}N]glutamate, and [^{15}N]-aspartate, or [^{15}N]alanine, with unlabeled glutamate, and aspartate, under the standard assay conditions each produced F-1-P with 93% of the molecules containing $^2H_2$ and ^{15}N. This result clearly showed that MfnC catalyzed the desired reaction with only alanine serving as the amino group donor. The remaining 7% of the molecules that were increased by m/z 2 can be explained from the 2% of ^{14}N in the labeled alanine used and 2% ^{1}H in the deuterium in the NaB$^2H_4$ used to prepare the substrate.

MfnC's ability to transaminate α-ketoglutarate with alanine was tested to determine its substrate specificity for the amino acceptor. MfnC was shown to poorly catalyze the transamination between [^{15}N]alanine and α-ketoglutarate. After a 30 min incubation with these substrates, only ∼0.2% of the α-ketoglutarate had been converted into [^{15}N]glutamate. GC−MS analysis showed that the recovered alanine, as the TM derivative, had the same enrichment as the starting alanine.

The inability of MfnC to use α-ketoglutarate supports the finding that it used a non-amino acid substrate as the amino group acceptor. The X-ray crystal structure was determined previously, which demonstrates that MfnC was missing an amino acid in the active site to use the annotated substrate aspartate.[33] The homologue to *mfnC* in *M. maripaludis* (MMP1072) was recently shown to be an advantageous but nonessential gene for this methanogen.[7] These findings suggest that another PLP-dependent enzyme present in these cells can also catalyze this transamination reaction. This occurrence of catalytic promiscuity and/or loss of substrate specificity is quite typical of PLP-dependent enzymes.[34]

Molecular Docking of Alanine and 4-HFC-P into MfnC. The X-ray crystal structure of the MJ0684-derived enzyme has been reported previously[23,33] and in many ways is like kynureninase (EC 3.7.1.3), a PLP-dependent enzyme that catalyzes the hydrolytic cleavage of L-kynurenine to anthranilic acid and L-alanine.[35] In addition to the use of alanine as the amino donor, another interesting aspect of this enzyme is that it uses an aldehyde as an amino group acceptor. Other examples in which aldehydes serve as acceptors in transamination reactions include a homotaurine:2-oxoglutarate aminotransferase (GabT), which is involved in homotaurine metabolism,[36] γ-aminobutyrate transaminase,[37] and acetylornithine δ-aminotransferase, or ornithine δ-aminotransferase, involved in ornithine biosynthesis.[38]

PLP-dependent transaminases typically proceed through a ping-pong mechanism, where both substrates have to bind to the same area of the active site to facilitate the transfer of the amino group from the donor through PLP to the acceptor.[39,40] The molecular docking studies were conducted with the two known substrates: alanine and 4-HFC-P. Figure 4 compares the binding of L-alanine (Figure 4A) and two possible orientations of 4-HFC-P in the active site (Figure 4B,C). Asn165 and Arg347 were previously identified as interacting with the amino donor carboxyl moiety by Yang[23] when he examined the X-ray crystal structure and showed that several amino acids are conserved in aspartate aminotransferases and are not present in the *M. jannaschii* structure. These data led him to propose that the enzyme was a kynurenine aminotransferase.[35] The distances

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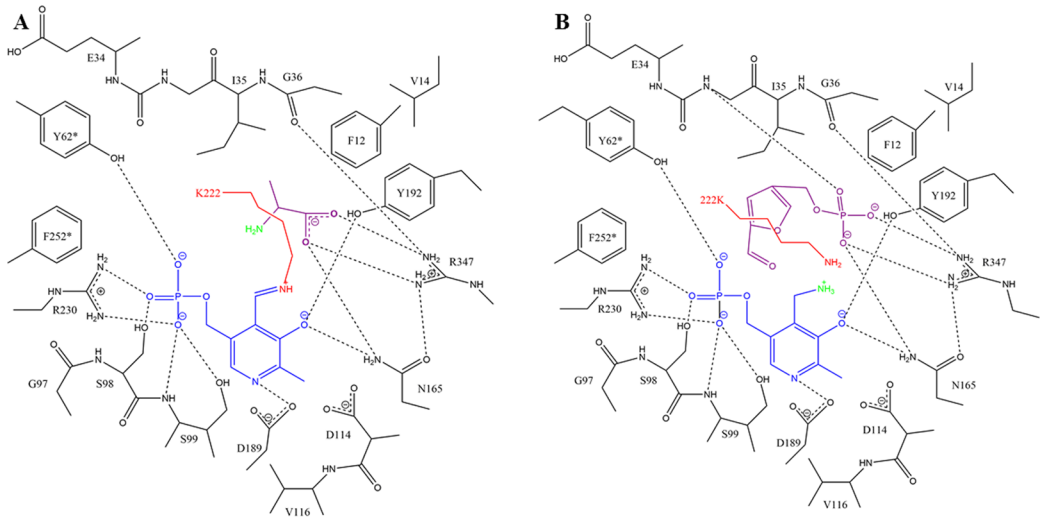

Figure 7. Schematic of the MfnC active site with bound alanine (A) and bound 4-HFC-P after transfer of the alanine α-amino group from alanine to PLP (B). The PLP is colored blue, and Lys222 is colored red. The two substrates are colored purple, alanine (A) and 4-HFC-P (B). The dotted lines represent salt bridges in the active site to stabilize PLP and substrates.

measured between the oxygen and the amino groups of Asn165 and Arg347 are all <4 Å, suggesting that the position of the substrate is sufficiently close to participate in a salt bridge with Asn165 and Arg347. The binding of the alanine α-carboxylate via a salt bridge to Arg347 is as expected for an aminotransferase.

The acetylornithine aminotransferase (AcOAT) from *Thermus thermophilus* HB8 has been shown to have an additional amino acid, glutamate, in the active site to compensate for the lack of an additional carboxyl group moiety used to stabilize the active site.[39] However, unlike AcOAT,[39] in which a glutamate carboxylate from AcOAT substitutes for the absent carboxylate in the substrate acetylornithine, in *M. jannaschii* MfnC the phosphate group of 4-HFC-P performs this function. This phosphate group is fulfilling the same function as the additional glutamate carboxylate of AcOAT for the non-amino acid substrate (4-HFC-P). Further examination of the MfnC active site shows that it does not contain residues that would indicate phosphate binding, but with the phosphate moiety serving the same function as the carboxyl moiety of an amino acid, 4-HFC-P would bind in the same position as the amino acid substrate (alanine) (Figure 4). The molecular docking of 4-HFC-P supports this hypothesis, as the oxygen atoms from the phosphate are within 4 Å of Asn165 and Arg347 to be properly stabilized in the active site and the bound substrate is positioned for the transfer of the amino group from PLP to 4-HFC-P to produce F1-P (Figure 4A,B).

Figure 4 shows the distances of the nitrogen of the Lys222-PLP (LLP) Schiff base that transfers the amino group from the amino donor (alanine) to the aldehyde acceptor (4-HFC-P) to be <5 Å, suggesting that the atoms are sufficiently close to hydrogen bond and transfer the amino group between substrates.

Figure 7 shows the two-dimensional active site with the PLP bound in a Schiff base with Lys222 and alanine stabilized by Asn165 and Arg347 for the transfer of the amino group from alanine (green, Figure 7A) to 4-HFC-P. In Figure 7B, the deaminated alanine has left the active site and 4-HFC-P has bound with the phosphate stabilized by the same amino acids for the carboxyl moiety of alanine. The amino group on PLP is then poised to be transferred to 4-HFC-P to generate F1-P (Figures 2 and 7B).

Identification of 4-HFCA-P and 4-HFC-P in *M. jannaschii* Cell Extracts. The strongest piece of evidence for the presence of a given pathway occurring in an organism is the identification of pathway intermediates in the cells. 4-HFCA-P and 4-HFC-P were identified in *M. jannaschii* cell extracts supporting their involvement in the biosynthesis of F1. 4-HFCA-P was extracted from the cell extracts with methanol, and after treatment with phosphatase, the 4-HFCA was isolated by preparative TLC using solvent system 2. 4-HFCA was identified in this fraction on the basis of the following analytical methods. (1) HPLC analysis identified the presence of the compound on the basis of its retention time and UV absorbance spectrum compared with those of a known. (2) LC–ESI–MS using two different solvent systems each followed by MRM compared to a known sample of 4-HFCA confirmed the identity of the observed peak as 4-HFCA (Figures S2–S4 of the Supporting Information). (3) The R_f of the sample from the cells in TLC solvent systems 2 and 3 was the same as the known 4-HFCA, further supporting the identification of the peak as 4-HFCA.

4-HFC-P was extracted from the cell extracts with methanol, and after treatment with phosphatase, the 4-HFC *O*-(4-nitrobenzyl)-hydroxylamine oxime derivative was prepared and isolated by preparative TLC using solvent system 2. Mass spectral analysis showed an M^+ ion at m/z 278, the same as that observed from a known sample. Final confirmation of the structure was based on TLC R_f, HPLC retention times, and the observed ratios of different *cis* and *trans* oxime isomers produced compared to a known sample. These results strongly indicate that 4-HFCA-P and 4-HFC-P are present in cell extracts and are possible intermediates in F1-P biosynthesis. Our results do, however, show that 4-HFCA-P does not have to be produced by these cells to make F1-P, yet the cells can produce F1-P from 4-HFCA-P. One possibility is that 4-HFCA-P can be produced by an alternate route, perhaps from pyruvate and DHAP, and is reduced to 4-HFC-P that is a common intermediate in both pathways. Alternately, 4-HFCA-P, produced by the oxidation of aldehyde in 4-HFC-P, is used for some other purpose by the cells. However, because 5-HFCA-P was also detected in the cell extracts (Figure S1 of the Supporting Information) this compound may be present in the cells only as a result of spontaneous chemical reactions[41] that would be expected to be

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Table 1. Experimental Data for the Concentrations of F1 and F1-P in Cell Extracts of *M. jannaschii* and the Incorporation of Labeled Precursors into F1 Catalyzed by the Same Extracts

experiment	experiments performed[a]	concentration (μM) of F1 present in cell extract (extent of labeling)[b]
1	cell extract with no phosphatase treatment	0.32 (100% no label)
2	cell extract with phosphatase treatment	0.92 (100% no label)
3	cell extract incubated with [$^{13}C_3$]pyruvate (7.1 mM), DHAP (7.1 mM), a ^{15}N-labeled amino acid mixture[c] (each amino acid at 7.1 mM), and ATP (7.1 mM)	6.4 (86% no label, 11.5% ^{15}N, 0.73% $^{13}C_2$, 1.6% $^{13}C_3$, 0.14% ^{15}N and $^{13}C_3$)
4	cell extract incubated with [$^2H_2$-*hydroxymethyl*]-4-HFCA-P (88 μM), ATP (8.8 mM), and a ^{15}N-labeled amino acid mixture[c] (each amino acid at 8.8 mM) under H_2	41 (86.5% no label, 3.6% ^{15}N, 8.3% $^2H_2$, 1.8% ^{15}N and $^2H_2$)
5	cell extract incubated with [$^2H_2$-*hydroxymethyl*]-4-HFC-P (200 μM)	63 (2.3% no label, 97.7% $^2H_2$)
6	cell extract incubated with [$^2H_2$-*hydroxymethyl*]-4-HFC (4.3 mM) and a ^{15}N-labeled amino acid mixture[c] (8.3 mM)	16 (9.5% no label, 1.1% ^{15}N, 53.5% $^2H_2$, 36.5% $^2H_2$ and ^{15}N)[c]

[a]Extracts (25−50 μL) were incubated with the indicated substrates for 15 min at 70 °C. F1 was isolated and converted into the NBD derivative as described in the text. [b]Concentrations are calculated from the observed areas of the absorbance (A_{480}) recorded from the NBD-F1 peak in the separated samples obtained during LC−UV−ESI−MS analysis. The isotopic intensities of the $(M - H)^-$ ion at m/z 289.1 were used to measure the isotopic incorporations. The reported intensities have been corrected for the values of natural abundance observed in unlabeled samples and are reported as the mole fraction of the molecules with the indicated number of incorporated isotopes. [c]The labeled amino acids consisted of an equimolar mixture of [^{15}N]alanine, [^{15}N]glutamate, and [^{15}N]aspartate.

even more rampant in thermophiles. The fact that such reactions do occur is confirmed by our demonstration that 4- and 5-HFCA are produced by heating pyruvate and dihydroxyacetone (DHA) (see below).

Detection of F1 and F1-P in Cell Extracts and Incorporation of Labeled Precursors into F1-P by Cell Extracts of *M. jannaschii*. Using the isolation and derivization NBD procedure for F1 but without phosphatase treatment, cell extracts were found to contain 0.32 μM free F1 (Table 1, experiment 1). This free F1 could be a precursor of methanofuran or could have arisen by the hydrolysis of methanofuran, which is present in the cell extracts at a concentration of 1−6 nmol/mg of protein, or ∼90 μM.[10,42] Treatment of the cell extracts with phosphatase increased the measured concentration of F1 in the cell extract from 0.32 to 0.92 μM, indicating that F1-P was present at a concentration of ∼0.6 μM in the cell extract (Table 1, experiment 2). Because F1-P cannot arise by the hydrolysis of methanofuran because methanofuran contains no phosphate, this indicates that F1-P is likely an intermediate in methanofuran biosynthesis.

By incubating cell extracts with several possible stable isotope-labeled precursors followed by quantifying the amount of F1-P produced and the incorporation of the label precursors by mass spectrometry, we established the biosynthetic route to F1-P. Incubation with [$^{13}C_3$]pyruvate, DHAP, ATP, and ^{15}N-labeled glutamate, aspartate, and alanine led to a 7-fold increase in the amount of F1, with 11.5% of the molecules having one ^{15}N, 0.73% having $^{13}C_2$, 1.6% having $^{13}C_3$, and 0.14% having both ^{15}N and $^{13}C_3$ (Table 1, experiment 3). The increase in the concentration of F1-P from 0.92 to 6.4 μM was also observed and indicated a large increase in the amount of F1-P. At first glance, these data would seem to indicate that pyruvate is not a precursor to F1 because was little incorporation of label from [$^{13}C_3$]pyruvate. The cell extracts, however, contained unlabeled 4-HFCA-P, 4-HFC-P (see above), and probably also pyruvate. Therefore, the low level of incorporation of $^{13}C_3$ can be explained by reduction of the measured level of incorporation after mixing with these unlabeled precursors. Accordingly, the fact that some incorporation was observed is fully consistent with pyruvate being a precursor of F1. The presence of 0.73% of the molecules with $^{13}C_2$ is explained by cleavage of pyruvate back to [$^{13}C_2$]-acetyl-CoA and $^{13}CO_2$, catalyzed by pyruvate oxidoreductase.[43] The reverse reaction, with unlabeled CO_2, produces [$^{13}C_2$]-pyruvate. The low level of incorporation of ^{15}N from added labeled amino acids can likewise be explained by dilution with the unlabeled amino acids in the extract. Among these are α-glutamate, β-glutamate, aspartate, and alanine that function as osmolites.[20] These amino acids are found at high concentrations in *M. jannaschii* cell extracts (∼0.1 M) causing the amount of ^{15}N incorporated to decrease. In addition, possible equilibration of the label by transamination reactions with other α-keto acids present in the extract[44] would further reduce the level of incorporation.

The possible involvement of 4-HFCA-P in the biosynthesis of F1-P was confirmed by the incorporation of deuterated 4-HFCA-P as well as ^{15}N from ^{15}N-labeled amino acids into F1-P by cell extracts after incubation with ATP under hydrogen (Table 1, experiment 4, and Figure 3). A further increase in the concentration of F1 from 6.4 to 41 μM could be explained by ATP phosphorylating the 4-HFCA-P carboxylic acid group followed by its reduction to 4-HFC-P from hydrogen via F_{420}, NADH, FMN, and FAD, reduced by the hydrogenases present in these cells.[45] The identity of this reductant is currently unknown. Incubation of the aldehyde [$^2H_2$-*hydroxymethyl*]-4-HFC-P led to the incorporation of both deuteriums into F1-P (Table 1, experiment 5) and a further increase in the concentration of F1-P to 63 μM. Cell extracts were also found to convert 4-HFC to F1 (Table 1, experiment 6), but the increase in the concentration of F1 was 4-fold smaller.

Incubation of the cell extracts with [1,3,3,3-$^2H_4$]methylglyoxal and DHAP, and aminoacetone and DHAP (all with added L-glutamate), failed to produce labeled F1 or to significantly increase the amount of F1-P in the cell extract. When these experiments were conducted with [^{15}N]glutamate, ∼20% ^{15}N was always observed to be incorporated into F1. This incorporation likely arose from the transamination of F1 and/or F1-P not directly from [^{15}N]glutamate, but with one or more amino acids labeled via the transamination reaction with the 4-HFC-P present in the cell extract. Including [^{15}N]alanine, [^{15}N]aspartate, and [^{15}N]glutamate in the incubation mixture increased the level of incorporation of ^{15}N to 31%, indicating that alanine or aspartate was a better amino group donor than glutamate.

Prebiotic Synthesis of 4-HFCA, 5-HFCA, 4-HFC, and 5-HFC. To test if 4-HFCA can be formed nonenzymatically, we heated solutions containing sodium pyruvate and DHA and

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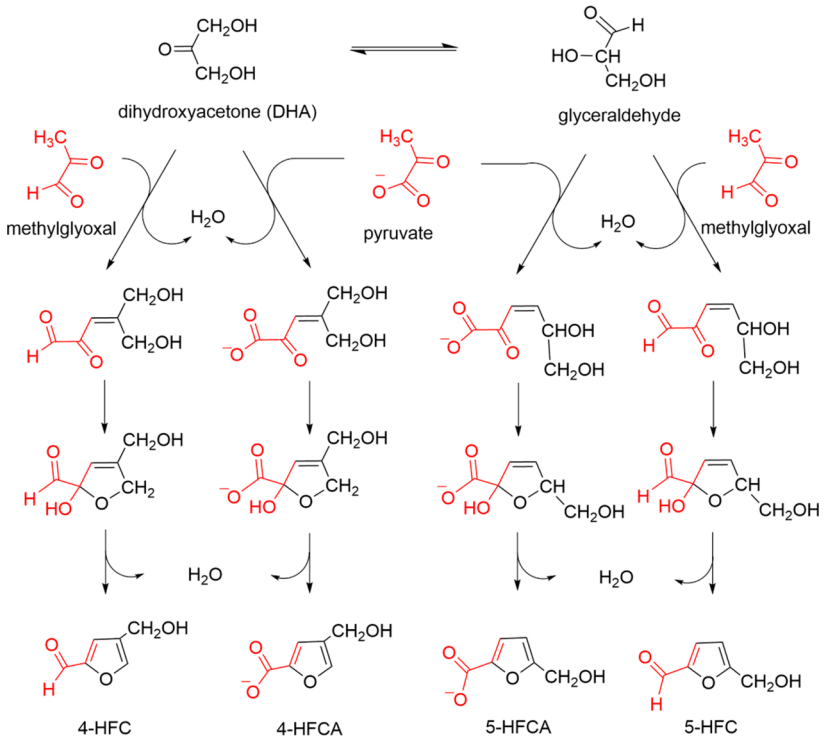

Figure 8. Possible chemical/prebiotic mechanism for the formation of 4-HFCA, 5-HFCA, 4-HFC, and 5-HFC from pyruvate, methylglyoxal, and DHA.

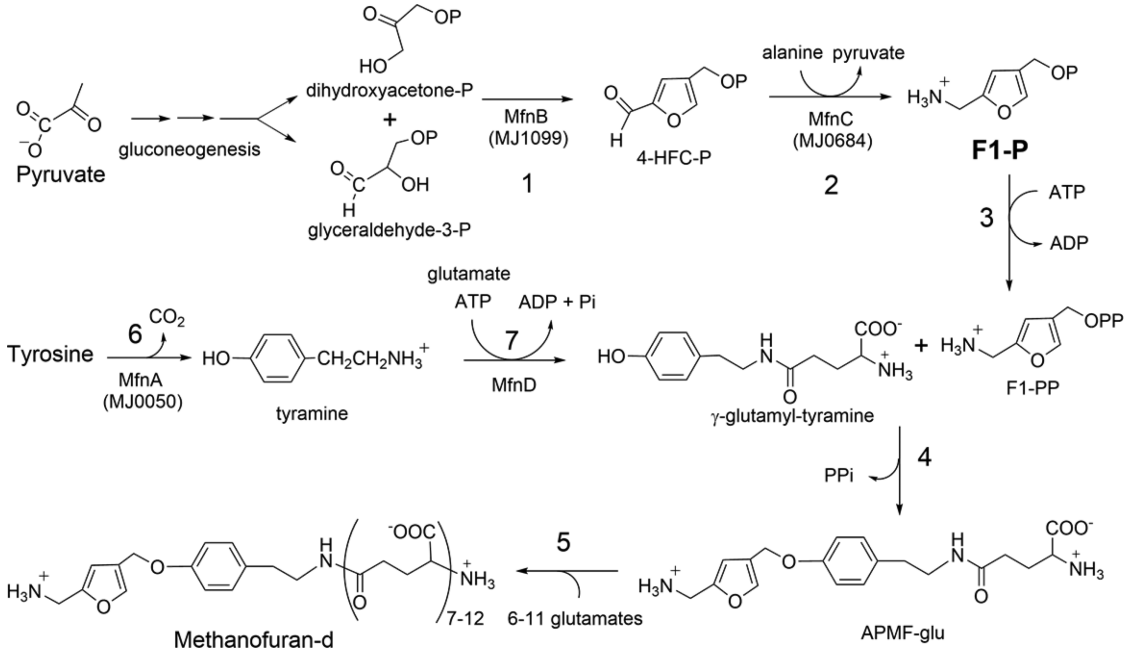

Figure 9. Proposed last steps in the biosynthesis methanofuran from *M. jannaschii*.

measured the production 4-HFCA and 5-HFCA. After heating the samples, we purified the products from the reaction mixture by preparative TLC. HPLC and LC−UV−ESI−EPI−MS analysis was conducted to identify these products. The three EPI spectra (A, B, and E) shown in Figure S5 of the Supporting Information are known samples of 4-HFCA, 3-HFCA, and 5-HFCA, respectively. Spectra B and C are for the 4-HFCA and 5-HFCA, respectively, recovered from the reaction. These data clearly show that both furans are synthesized as products of the chemical reaction of pyruvate and DHA and give us a hint about the possible prebiotic origin of 4-HFCA. The reaction yield for the HFCA isomers was found to be ∼0.1% for 4-HFCA and ∼0.05% for 5-HFCA. Similar results were obtained for the synthesis of 4-HFC and 5-HFC from heating a sample of methylglyoxal and DHA (data not shown). Possible chemical mechanisms accounting for the production of these compounds are shown in Figure 8 and are similar to the biosynthetic pathway proposed here.

The biochemical route(s) for the formation of the functional part of contemporary coenzymes is generally quite complex and

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most times has little relationship to possible prebiotic routes to the same compound. Thus, coenzyme M, mercaptoethanesulfonic acid, the simplest coenzyme, is formed from PEP and sulfite in a five-step process in the methanogens,[9] where a simple prebiotic route would consist of the addition of sulfite to ethylene sulfide. The demonstration here that a simple chemical condensation of pyruvate or methylglyoxal with dihydroxyacetone can produce intermediates in the biosynthesis of the furan ring in methanofuran may help support the notion of the early evolution of this coenzyme.

Final Steps in Methanofuran Biosynthesis. The exact route and precursor condensing F1-P with tyramine to form 4-{[4-(2-aminoethyl)phenoxy]methyl}-2-furanmethanamine (APMF) is unknown. As observed in thiamin,[46] folate,[47] methanopterin,[48] and terpenoid biosynthesis, such a condensation can occur by the displacement of pyrophosphate from a pyrophosphate ester (Figure 9, step 4). The pyrophosphate ester can be generated either by transfer of a pyrophosphate to the alcohol acceptor as seen in folate and methanopterin biosynthesis[49] or by the phosphorylation of a monophosphate as seen in thiamin biosynthesis.[46] Because F1-P is the likely precursor, it would have to be first phosphorylated to F1-PP (Figure 8, step 3). Recently, we have identified a novel tyramine-glutamate ligase (MfnD) that catalyzes the addition a γ-linked glutamate to tyramine (unpublished result). In this case, condensation with F1-PP likely occurs after γ-glutamyltyramine is produced (Figure 9, step 4). Next, the remaining γ-linked glutamates would be sequentially added to form the final coenzymes (Figure 9, multiple step 5).

Concluding Remarks. Methanofuran is one of six coenzymes that are required to catalyze the biochemical reduction of CO_2 to methane and is the only coenzyme known to contain a furan ring. Here we present a proposed pathway for the biosynthesis of this furan ring from primary metabolites that are consistent with the metabolite identification and labeling data. We also identified the gene product of MJ1099 as encoding an aldolase that catalyzes the formation of 4-HFC-P from Ga-3-P and DHAP, which we have named 4-HFC-P synthase (MfnB). Further analysis of the MfnB mechanism is currently being undertaken in our lab to improve our understanding of how the multistep mechanism can proceed with a single enzyme.

The gene product of MJ0684 encodes the transaminase responsible for the conversion of 4-HFC-P to F1-P using alanine as the amino donor, which we have named 4-HCF-P:alanine aminotransferase (MfnC), which has also been identified. This work lays the foundation for the identification of all the remaining genes encoding all the enzymes in methanofuran biosynthesis.

■ ASSOCIATED CONTENT

Supporting Information

Additional information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

Ga-3-P, glyceraldehyde 3-phosphate; F1, 5-(aminomethyl)-3-furanmethanol; NBD, 7-nitrobenzofurazan; NBD-F, 4-fluoro-7-nitrobenzofurazan; F1-P, 5-(aminomethyl)-3-furanmethanol -P; F1-PP, 5-(aminomethyl)-3-furanmethanol-PP; DHAP, dihydroxyacetone-P; 4-HFCA-P, 4-(hydroxymethyl)-2-furancarboxylic acid-P; 4-HFCA, 4-(hydroxymethyl)-2-furancarboxylic acid; 4-HFC, 4-(hydroxymethyl)-2-furancarboxaldehyde; MfnC, 4-HFC-P:alanine aminotransferase; MF, methanofuran; HTCA, 1,3,4,6-hexanetetracarboxylic acid; 5-HFC, 5-(hydroxymethyl)-2-furancarboxaldehyde; 4-HFC-P, 4-(hydroxymethyl)-2-furancarboxaldehyde-P; FA, furfurylamine; 5-HFCA, 5-(hydroxymethyl)-2-furancarboxylic acid; 5-AFM, 5-(aminomethyl)-2-furanmethanol); 3-AFM, 3-(aminomethyl)-2-furanmethanol; APMF, 4-{[4-(2-aminoethyl)phenoxy]methyl}-2-furanmethanamine; GC−MS, gas chromatography−mass spectrometry; ESI, electrospray ionization; CID, collision-induced dissociation; PDA, photodiode array detector; SIM, selected ion monitoring; 3-HFCA, 3-(hydroxymethyl)-2-furancarboxylic acid; MRM, multiple-reaction monitoring; EPI, enhanced product ion; LIT, linear ion trap; TCA, trichloroacetic acid; THF, tetrahydrofuran; PLP, pyridoxal 5′-phosphate; TM, trifluoroacetyl methyl; DHA, dihydroxyacetone.

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