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Importance of the isopropylidene terminal of geranylgeranyl group for the formation of tetraether lipid in methanogenic archaea

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Abstract—Labeling experiments using several deuterated lipids were pursued to study the biosynthesis of macrocyclic isoprenoidal lipids of thermophilic methanogenic archaea, *Methanothermobacter thermautotrophicus*. The isopropylidene terminal of geranylgeranyl group of monomeric precursor appeared to be important for the C–C bond formation at the hydrophobic end in the macrocyclic lipids. A mechanism involving a radical trigger at the allylic methyl group is proposed for this C–C bond formation.

Archaea, which have been attracting considerable attention from both biochemical and evolutionary aspects, are distinct from bacteria and eukarya, and are now classified in the third independent domain.[1] The most characteristic biochemical marker of archaea is found in the chemical structure of its core membrane lipids. Archaeal cell membrane lipids are composed of saturated isoprenoid chains linked to a glycerol molecule at the *sn*-2 and 3-positions by ether linkage. The isoprenoid hydrocarbon chains of lipid molecule are frequently joined at the hydrophobic end to form a macrocyclic structure as large as 36- and 72-membered rings as shown in Figure 1.[2]

The biosynthesis of these macrocyclic lipids is believed to proceed in a straight manner via digeranylgeranylglyceryl phosphate derived from *sn*-1-glycerol phosphate and geranylgeranyl diphosphate.[3] However, nothing is known about the mechanistic details of the crucial carbon–carbon bond(s) formation leading to

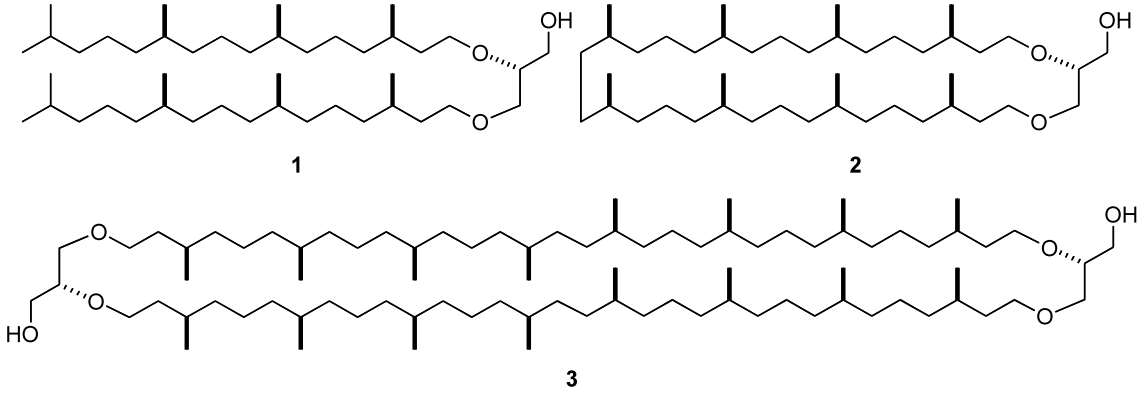

Figure 1. Structure of typical core lipids of archaeal membrane.

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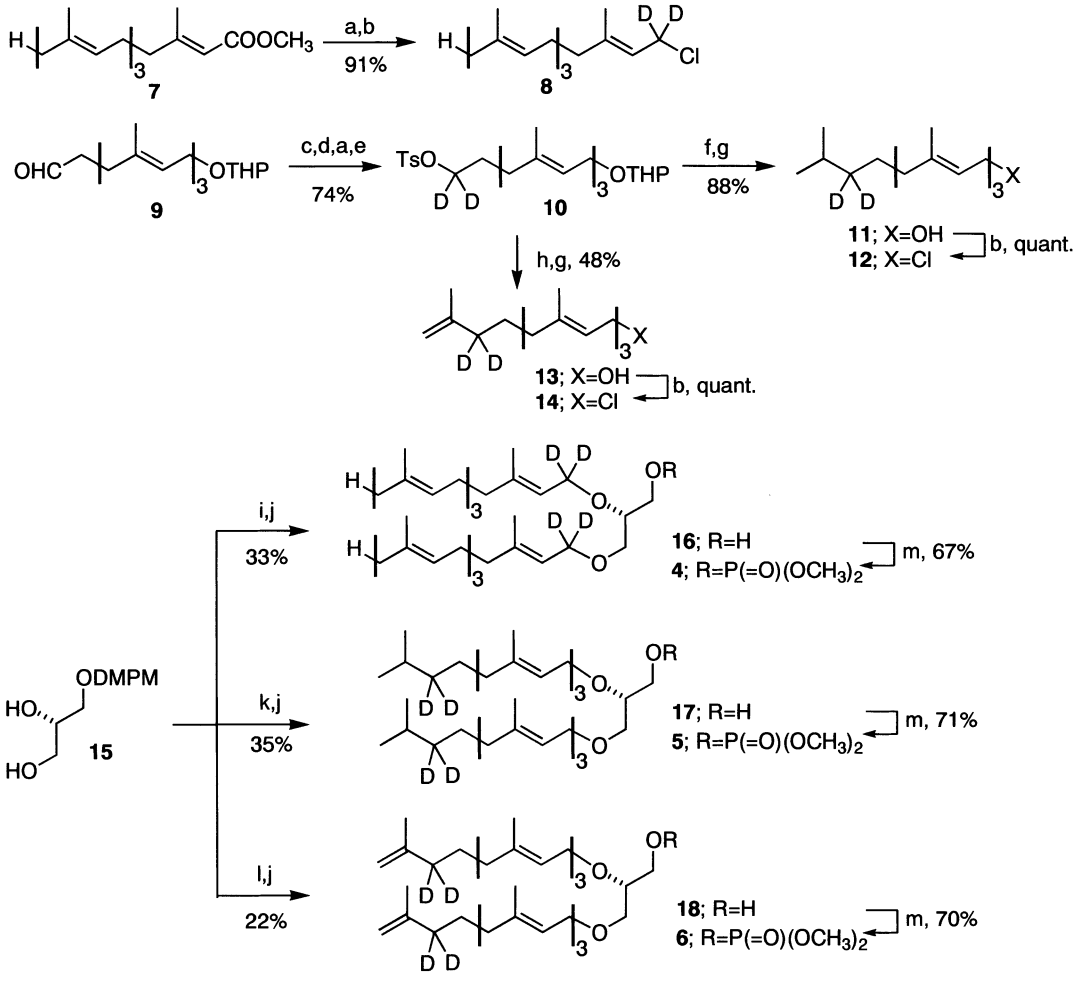
these macrocyclic structures. We recently proposed a speculative mechanism for the C–C bond formation reaction in the macrocyclic lipid biosynthesis based on the feeding experiments of mevalonate-d_9 in thermophilic methanogen.[4] Thus, the C–C bond forming reaction might occur by intermolecular acid catalyzed condensation at the saturation stage after isomerization of double bond. We then undertook feeding experiments of deuterium labeled digeranylgeranylglycerol analogs in order to figure out whether or not the proposed mechanism is feasible for the C–C bond formation. Described here are the results of these feeding experiments of several deuterated lipids to thermophilic methanogen, *Methanothermobacter thermautotrophicus*, which possesses both diphytanylglycerol **1** and a 72-membered lipid **3**, to get insight into the biosynthetic pathway of the macrocyclic lipids.

The first issue to be solved was to specify appropriate deuterated substrates which were certainly incorporated into the macrocyclic lipids. However, several attempts of feeding deuterated digeranylgeranylglyceryl phosphate, deuterated digeranylgeranylglycerol, and deuterated *sn*-3-*O*-geranylgeranylglycerol, all of which seemed to be plausible biosynthetic precursors, were unsuccessful. This may be due to their poor solubility in a medium and/or low permeability through membrane. Finally we came to find the dimethyl ester of digeranylgeranylglyceryl phosphate **4** was incorporated into both diphytanylglycerol **1** and 72-membered lipid **3** in the feeding experiments of *M. thermautotrophicus*. Therefore, we further synthesized additionally modified deuterated lipids **5** and **6** in order to investigate a role of the terminal double bonds of geranylgeranyl groups. Although its biosynthetic significance was unknown, bisdihydrogeranylgeranylglycerol lipid such as **5** was isolated from thermophilic archaea, *Thermococcus* S 557.[5]

The synthesis of deuterated lipids **4**–**6** was straightforward as shown in Scheme 1. Thus, ester **7** was converted into deuterated allylic alcohol **8** in high yield by reduction with $LiAl^2H_4$. Conversion of **8** into deuterated lipid **16** was carried out according to the method previously described by us.[6] Finally, **16** was phosphorylated with dimethyl phosphorochloridate to give



Scheme 1. Synthesis of deuterated lipids **4**–**6**. *Reagents:* (a) $LiAlD_4$/ether, (b) $(CH_3)_2S$, NCS/CH_2Cl_2, (c) PDC/DMF, (d) CH_2N_2/ether, (e) TsCl/py, (f) ^{i}PrMgBr, Li_2CuCl_4/THF, (g) TsOH/MeOH, (h) isopropenylMgBr, Li_2CuCl_4/THF, (i) NaH/DMSO; **8**, (j) NaH/DMSO; **12**, (k) NaH/DMSO; **14**, (l) $(CH_3O)_2P(=O)Cl$, DMAP/py.

deuterated substrate **4** in high yield. In the case of **5** and **6**, the known aldehyde **9**[7] was converted into deuterated tosylate **10** by a conventional way. Coupling reaction of **10** with either isopropylmagnesium bromide or isopropenylmagnesium bromide in the presence of copper catalyst gave, after acid hydrolysis, **11** and **13** in high yield, respectively. Transformation of **11** and **13** into **5** and **6** was carried out as described above, respectively.[8]

The feeding experiments of the synthesized deuterated lipids **4**–**6** to *M. thermoautotrophicus* JCM 10044^T (= DSM 1053) were carried out as already reported.[9] Deuterated substrates **4**–**6** were aseptically and anaerobically added to the culture (5–7 L) in a final concentration of 10 mg/L. After respective cultivation under a 80% H_2 and 20% CO_2 gas mixture for 5 days at 65°C, the cells were harvested by centrifugation and the lipids were separately purified through extraction and solvolysis of the polar head groups according to the standard manner.[10] Repetitive chromatography afforded diphytanylglycerol **1** (ca. 1 mg) and the 72-membered lipid **3** (ca. 2 mg) in a pure form. These core lipids were further converted to their corresponding benzoates, which were subsequently analyzed by ^{2}H NMR. The ^{2}H NMR spectra of these lipid benzoates are shown in Figures 2 and 3.

At a glance, no deuterium incorporation was observed in the feeding experiment of **6** (Fig. 2A and Fig. 3A). This suggests that, contrary to our previous speculation, the double bond migration cannot be a trigger for C–C bond formation in the biosynthesis of archaeal macrocyclic lipids. On the other hand, when **4** was used as substrate, deuterium was apparently incorporated into

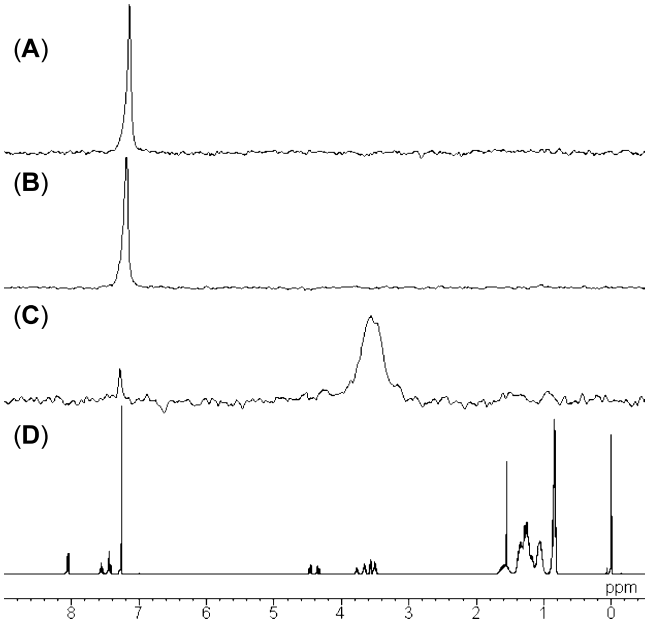


Figure 2. ^{2}H NMR spectra (60 MHz, CCl_4–$CHCl_3$) of biosynthesized 72-membered lipid benzoates by feeding with (A); **6** (30,000 scans), (B); **5** (30,000 scans), and (C); **4** (60,000 scans), to *M. thermautotrophicus*. (D); ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of authentic 72-membered lipid benzoate.

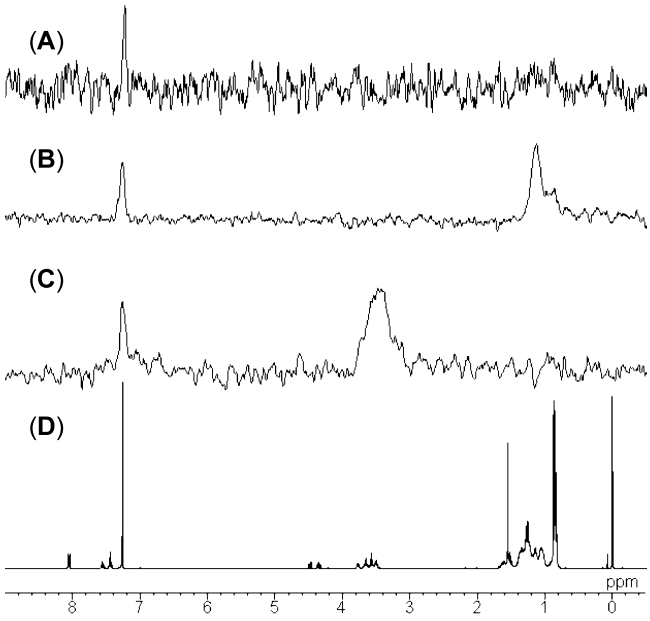


Figure 3. ^{2}H NMR spectra (60 MHz, CCl_4–$CHCl_3$) of biosynthesized 2,3-di-*O*-phytanyl-*sn*-glycerol benzoates feeding with (A); **6** (50,000 scans), (B); **5** (50,000 scans), and (C); **4** (50,000 scans), to *M. thermautotrophicus*. (D); ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of authentic 2,3-di-*O*-phytanyl-*sn*-glycerol benzoate.

both diphytanylglycerol **1** and 72-membered lipid **3** as shown in Figure 2C and Figure 3C. Interestingly enough, the substrate **5** without the double bond at far end was incorporated into diphytanylglycerol **1**, but not into the 72-membered lipid **3** (Fig. 2B and Fig. 3B).[11] These results clearly indicated that the presence of the Δ^{14} double bond of digeranylgeranyl groups was crucial for the formation of macrocyclic lipids and that hydrogenation of the double bond at the far end appeared to be a branching point leading either to the diphytanylglycerol lipid or to the 72-membered lipid. Since we assume that the methyl phosphate function of these substrates may well be hydrolyzed by certain phospholipases into phosphate anion, the actual substrate for the macrocyclic lipid biosynthesis in the cell appears to be digeranylgeranylglyceryl phosphate or its equivalent.

As already reported, two of the three hydrogens of C-16 methyl group of geranylgeranyl moiety were retained during the C–C bond formation reaction in the macrocyclic lipid biosynthesis.[4] This observation was a key feature to rule out some higher oxidized states such as an aldehyde or a carboxylate as an intermediate for the C–C bond formation. Further, essentially no loss of hydrogen at C-13, -14 and C-17 positions of geranylgeranyl group was observed during macrocyclic lipid biosynthesis.[4] Taking into account all of these observations together, therefore, it is strongly suggested that the allylic C-16 position of geranylgeranyl groups is directly activated by a responsible enzyme and the activated species are combined together to form a new C–C bond.

It should be worth noting that similar C–C bond forming reaction was reported in the biosynthesis of

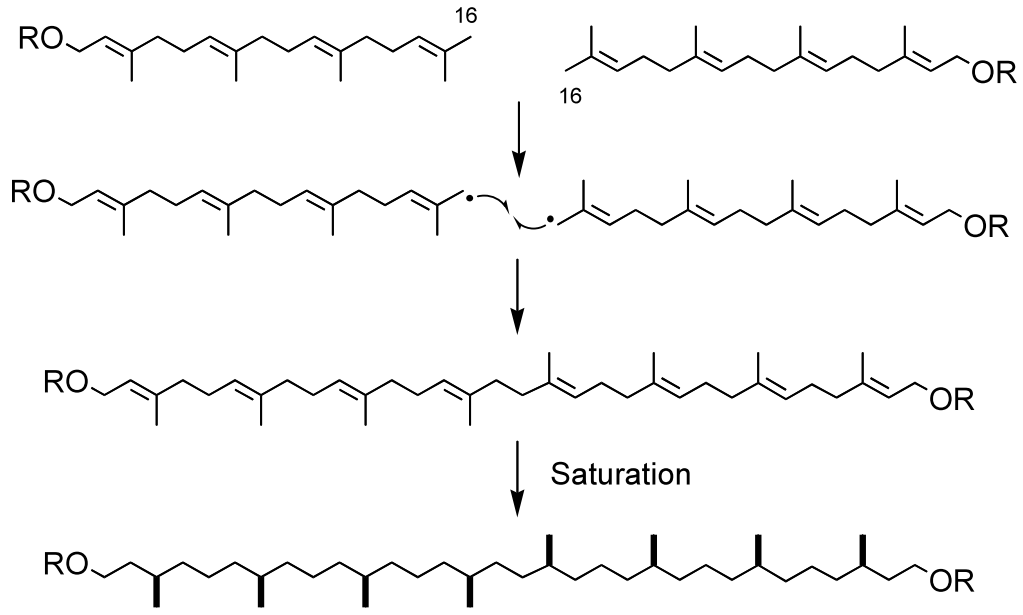


Figure 4. Proposed mechanism of the C–C bond forming reaction in the biosynthesis of macrocyclic lipids in methanogenic archaea.

diabolic acid [(15*R*,16*R*)-15,16-dimethyltriacontanedioic acid] found in anaerobic bacteria, *Butyrivibrio fibrisolvens*. Although it was not proved, Arigoni et al. proposed a radical mechanism involving cobalamin intermediates for this reaction based on the labeling experiments.[12] Interestingly in both methanogen and bacteria, a carbon–carbon bond is formed by activation of unreactive sp^3-carbon centers under strictly anaerobic conditions. Therefore, the most intriguing issue as to creating this type of critical C–C bond in the present archaeal case may also be explained mechanistically by a radical trigger reaction through a relatively stable allylic radical species as shown in Figure 4. In this scenario, it appears to be reasonable to observe actual involvement of double-bond migration to certain extent as described previously.[4]

Acknowledgements

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8. Physico-chemical data of **4**–**6**. **4**; colorless oil: ^{1}H NMR ($CDCl_3$): δ 1.60 (18H, s), 1.66–1.68 (12H, m), 1.97–2.13 (24H, m), 3.47–3.54 (2H, m), 3.69 (1H, quint, $J=5.0$ Hz), 3.76 (3H, d, $J=2.0$ Hz), 3.79 (3H, d, $J=2.0$ Hz), 4.05–4.22 (2H, m), 5.11 (6H, m), 5.32 (1H, s), 5.34 (1H, s). ^{2}H NMR ($CHCl_3$): δ 4.00, 4.13. ^{13}C NMR ($CDCl_3$): δ 16.05, 16.56, 17.72, 25.73, 26.37, 26.41, 26.65, 26.78, 39.65, 39.70, 39.72, 54.26 (d, $J=5.8$ Hz), 54.31 (d, $J=5.8$ Hz), 66.06 (m), 67.15 (d, $J=5.8$ Hz), 68.64, 76.01 (d, $J=7.4$ Hz), 120.34, 120.42, 123.71, 124.07, 131.13, 134.82, 135.22, 140.42, 140.50. ^{31}P NMR ($CDCl_3$): δ 1.52. Anal. calcd for $C_{45}H_{73}{}^2H_4O_6P$: C, 72.15; H+^{2}H, 10.36. Found: C, 71.78; H+^{2}H, 10.66. **5**; colorless oil: ^{1}H NMR ($CDCl_3$): δ 0.86 (12H, d, $J=6.8$ Hz), 1.35 (4H, t, $J=7.8$ Hz), 1.51 (2H, quint, $J=6.6$ Hz), 1.58–1.67 (18H, m), 1.91–2.09 (20H, m), 3.48–3.54 (2H, m), 3.70 (1H, quint, $J=5.1$), 3.77 (3H, d, $J=1.6$), 3.79 (3H, d, $J=1.6$), 4.01–4.22 (6H, m), 5.07–5.11 (4H, m), 5.31–5.37 (2H, m). ^{2}H NMR ($CHCl_3$): δ 1.10. ^{13}C NMR ($CDCl_3$): δ 15.87, 15.98, 16.52, 22.58, 25.51, 26.30, 26.33, 26.55, 27.67, 37.66 (quint, $J=18.8$ Hz), 39.58, 39.68, 39.80, 54.20 (d, $J=3.3$ Hz), 54.24 (d, $J=3.3$ Hz), 66.71, 67.04 (d, $J=5.8$ Hz), 67.83, 68.63, 76.00 (d, $J=7.4$ Hz), 120.38, 120.46,

123.61, 123.73, 135.18, 140.25, 140.34. ^{31}P NMR ($CDCl_3$): δ 1.17. Anal. calcd for $C_{45}H_{77}{}^2H_4O_6P$: C, 71.77; H+^{2}H, 10.84. Found: C, 71.91; H+^{2}H, 11.23. **6**; colorless oil: ^{1}H NMR ($CDCl_3$): δ 1.50 (4H, t, J=7.6 Hz), 1.60 (12H, s), 1.66–1.71 (12H, m), 1.93–2.09 (20H, m), 3.47–3.54 (2H, m), 3.69 (1H, quint, J=5.0 Hz), 3.76 (3H, d, J=2.0 Hz), 3.79 (3H, d, J=2.0 Hz), 4.01 (2H, d, J=6.8 Hz), 4.06–4.22 (4H, m), 4.66–4.70 (4H, m), 5.09–5.12 (4H, m), 5.31–5.37 (2H, m). ^{2}H NMR ($CHCl_3$): δ 1.95. ^{13}C NMR ($CDCl_3$): δ 15.93, 16.03, 16.53, 22.42, 25.77, 26.36, 26.40, 26.60, 36.60 (quint, J=18.8 Hz), 39.21, 39.63, 39.72, 54.26 (d, J=3.3 Hz), 54.32 (d, J=3.3 Hz), 66.78, 67.11 (d, J=5.8 Hz), 67.89, 68.70, 76.07 (d, J=7.4 Hz), 109.69, 120.44, 120.53, 123.73, 124.19, 134.80, 135.18, 140.30, 140.39, 145.93. ^{31}P NMR ($CDCl_3$): δ 1.21. Anal. calcd for $C_{45}H_{73}{}^2H_4O_6P$: C, 72.15; H+^{2}H, 10.36. Found: C, 71.90; H+^{2}H, 10.63.

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