Biosynthetic Mechanism of *sn*-2,3-Di-*O*-phytanylglycerol, Core Membrane Lipid of the Archaebacterium *Halobacterium halobium*

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Abstract: The biosynthetic mechanism of the membrane core lipid of the archaebacterium *Halobacterium halobium* was studied by feeding ^{18}O-labeled glycerol, chirally deuterated glycerol, and deuterated glucose to the culture, and subsequent ^{13}C, ^{1}H, and ^{2}H NMR analyses of the isotopically enriched 2,3-di-*O*-phytanylglycerol. A ^{18}O-chase experiment showed that the oxygen atom of *sn*-C-3 of glycerol is retained ultimately in *sn*-2,3-di-*O*-phytanylglycerol, thereby suggesting its nucleophilic nature in the ether-forming reaction. Efficient and stereospecific incorporation of the C-6 of glucose and *sn*-C-3 of glycerol into the *sn*-C-1 position of the lipid strongly implied that (1) the Entner–Doudoroff pathway operates in *H. halobium* and (2) stereochemical inversion apparently takes place at the C-2 position of glycerol to form 2,3-di-*O*-alkylated lipids. Chase studies of the C-2 hydrogen further suggested an oxidation and a reduction processes are responsible for the inversion of configuration at C-2 of the glycerol moiety. A stepwise alkylation mechanism is postulated.

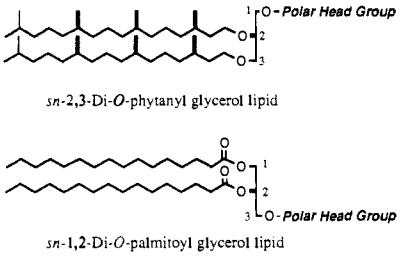
Chemical divergence of the major lipids in the plasma membrane of the usual eubacterial and eukaryotes cells and of the archaebacterial cells have attracted wide attention from chemical, biochemical, and evolutionary interests.[1] The structure of *sn*-2,3-di-*O*-alkylated glycerol, the core lipid in phospholipids and glycolipids in archaebacterial cells, having a polar head group on the *sn*-C-1 position, is the most remarkable, as illustrated in Scheme I,[2] while the major lipids of the eubacterial and eukaryotic cells mostly consist of *sn*-1,2-di-*O*-acylglycerol carrying a polar head group on its *sn*-C-3 position.

Biosynthesis of archaebacterial lipids and related metabolites have been studied extensively at various laboratories. Major contributions were made by Kates et al., who have studied the obligate halophile *Halobacterium cutirubrum*,[3] and by DeRosa et al., who have undertaken studies with the extreme acidothermophile *Sulfolobus* sp.,[4] and more recently, Poulter et al., who have reported the lipid biosynthesis of the methanogen *Methanospirillum hungatei*.[5] The latter two bacterial species actually contain an interesting 72-membered ring structure of biphytanyl diglycerol tetraether as a predominant membrane lipid, the biosynthetic precursor of which is believed to be *sn*-2,3-*O*-dialkylated glycerol.[6,7]

The most recent papers have discussed the mechanism of formation of the ether linkages of these archaebacterial membrane lipids.[5,8] Thus, by means of rather indirect approaches, the ether-bond-formation reaction is suggested to take place between a hitherto unknown intermediate of the glycerol portion and a prenyl donor, geranylgeranyl pyrophosphate being most likely. Dihydroxyacetone was previously suggested as a possible acceptor (a reactive intermediate for the glycerol moiety) by Kates et al.[9] Until now, however, the biosynthetic mechanism of the core lipid of archaebacterial cells has not yet been established in chemical terms, especially the pathway giving rise to the curious stereochemistry of the glycerol moiety and the reaction mechanism of the ether-bond formation as to whether or not the glycerol oxygens behave as nucleophiles.

We have used different approaches to study how these intriguing structural features of the archaebacterial core lipid, *sn*-2,3-di-*O*-phytanylglycerol, are elaborated and present a plausible mechanism for its biosynthesis.

Scheme I



If the ether linkages are formed by nucleophilic attack of an yet to be identified intermediate of the glycerol moiety to the precursory prenyl pyrophosphates, stereochemical inversion at the C-2 position of glycerol would not occur. The reactive intermediate of the glycerol portion for the ether-forming reaction might then be elaborated through a hitherto unknown mechanism. Clearly, the stereochemical mode of incorporation of glycerol and the fate of glycerol oxygen atoms are crucial to gain insight into the biosynthesis of the core lipid. Further, the fate of the hydrogen on C-2 of glycerol must also be important.

Thus, we have undertaken chase experiments using various isotopically labeled glycerols which show that (1) the oxygens of glycerol are in fact nucleophiles in the ether-forming reactions, (2) glycerol is incorporated into the archaebacterial core lipid stereospecifically with inversion at C-2, and (3) an oxidation–

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Table I. Summarized Results of the Feeding Experiments

substrate fed	g/L	lipid obtained (mg)	benzoate (mg)
sn-[3,3-$^2H_2$]glycerol	0.8	34	13
sn-[1,1-$^2H_2$]glycerol	0.62	35	17
[1(3)-$^2H_2$]glycerol	1.0	20	16
D-[6,6-$^2H_2$]glucose	1.0	18	17
[2-^{2}H]glycerol	0.68	24	14
(U-$^2H_8$]glycerol	0.5	22	19
[1(3)-^{18}O]glycerol	0.143[a]	20	-

[a] Nonlabeled glycerol (75 mg) was simultaneously supplemented.

reduction process is responsible for the stereochemical inversion. A part of this paper has been published in a preliminary form.[10]

Materials and Methods

Analytical and preparative thin-layer chromatography (pTLC) was carried out using precoated plates (Merck Silica gel F_{254}, 0.25-mm or 0.5-mm thickness).

Culture. The bacterial strain *Halobacterium halobium* IAM 13167 was obtained from the culture collections of Institute of Applied Microbiology, University of Tokyo, and was maintained on agar slants. Bacterial cultivation was conducted aerobically for 4 days on a rotary shaker at 37 °C equipped with a 500-mL Erlenmeyer flask containing 200 mL of the culture medium. The medium was composed of 3.3 g of peptone (Kyokuto Pharm. Ind. Co. Ltd., Tokyo, Lot No. DB5002), 10 g of $MgSO_4 \cdot 7H_2O$, 2 g of KCl, 3 g of trisodium citrate, 0.2 g of $CaCl_2$, 250 g of NaCl, and 1 L of distilled water.[11] The pH was adjusted to 7.5 before sterilization. Preculture was carried out 37 °C in a test tube containing 10 mL of the same medium as above, and the whole was used for inoculation of 200 mL of the freshly prepared medium. Each labeled precursor was dissolved in 10 mL of distilled water. The medium and the precursor solution were autoclaved separately, and 2-mL portions of the precursor solution were injected aseptically into each flask, which was then inoculated. Cells were harvested by centrifugation at 6000*g* for 30 min. The precipitate was washed with 25% aqueous NaCl solution, and the cells were collected as a precipitate. A typical yield of the cells was ca. 2 g (wet weight) from 1 L of culture.

Isolation of *sn*-2,3-Di-*O*-phytanylglycerol. Lipids were extracted and purified by the procedures described by Kates et al.[12] with slight modifications. Typically, the harvested cells (ca. 2 g) from the culture (1 L) were heated for 20 h under reflux in 40 mL of 3% methanolic HCl. To the mixture was added 10 mL of 7 N aqueous NaOH solution, and heating was continued for another 2 h. The unsaponifiable lipids were extracted 3 times with 100-mL portions of petroleum ether, and the combined organic extract was evaporated to dryness under reduced pressure. The residual brownish oil was purified by preparative thin-layer chromatography (pTLC) using *n*-hexane–diethyl ether (4:1) as solvent to give 15–20 mg (see Table I) of chromatographically homogeneous *sn*-2,3-di-*O*-phytanylglycerol, R_f 0.2 (hexane–diethyl ether 4:1), as a colorless oil: EI-MS of the *O*-TMS ether *m/z* 724 (M^+) and 709 ($M^+ - CH_3$); ^{1}H NMR 0.80–0.90 (m, $CH_3 \times 10$), 0.95–1.70 (m, $CH_2 + CH$), and 3.4–3.8 ppm (m, 9 H); ^{13}C NMR 19.7, 19.8, 22.7, 22.8, 24.4, 24.5, 24.8, 28.0, 29.9, 32.8, 36.6, 36.7, 37.1, 37.2, 37.3, 37.4, 37.5, 39.4, 63.1 (CH_2), 68.6 (CH_2), 70.1 (CH_2), 70.9 (CH_2), and 78.3 ppm (CH).

Derivatization of *sn*-2,3-Di-*O*-phytanylglycerol. (1) Benzoylation. To a solution of 15–20 mg of *sn*-2,3-di-*O*-phytanylglycerol in 1.0 mL of dry pyridine were added a few drops of benzoyl chloride and a few pieces of crystalline 4-(dimethylamino)pyridine (DMAP), and the mixture was stirred for 4 h at room temperature. To the reaction mixture was added 1 mL of water, and the whole was extracted with 50 mL of diethyl ether. The extract was washed successively with 2 N HCl (2 × 1 mL), 5% aqueous K_2CO_3 (5 × 1 mL), and brine and then dried over anhydrous $MgSO_4$. Filtration and evaporation of solvent gave a brownish oil, which was purified by pTLC (hexane–diethyl ether 8:1) to yield 13–16 mg (see Table I) of *sn*-1-*O*-benzoyl-2,3-di-*O*-phytanylglycerol: ^{1}H NMR (500 MHz) 0.8–0.9 (m, $CH_3 \times 10$), 1.0–1.6 (m, $CH_2 + CH$), 3.50 (m, 2 H), 3.55 (1 H, dd, J = 10.3 and 5.5 Hz), 3.58 (1 H, dd, 10.3 and 5.3 Hz), 3.65 (2 H, m), 3.76 (1 H, m), 4.35 (1 H, dd, J = 11.6 and 6.2 Hz), 4.47 (1 H, dd, J = 11.6 and 4.2 Hz), 7.43 (2 H, br t), 7.57 (1 H, br t), and 8.05 ppm (2 H, br d); ^{13}C NMR 19.8, 22.8, 24.6, 24.9, 28.1, 29.8, 29.9, 32.9, 37.5, 39.4, 64.7, 69.0, 70.2, 70.5, 76.7, 128.3, 129.6, and 132.9 ppm (C=O not observed).

(2) Trimethylsilylation. A small amount (200 μg) of the unlabeled or isotopically enriched lipid sample was treated with 10 μL of (trimethylsilyl)imidazole in 90 μL of ethyl acetate at 50 °C for several minutes, and an aliquot of the resulting mixture was directly introduced into the GC–MS instrument.

Phytanyl Iodide. A mixture of 44 mg of *sn*-2,3-di-*O*-phytanylglycerol, obtained from the cells grown with supplementation of [1(3)-$^2H_2$]-glycerol, and 1.5 mL of 55% HI was heated at 110 °C for 12 h.[13] After being cooled, the reaction mixture was extracted with 50 mL of ether, and the extract was washed with 5% aqueous $NaHCO_3$ and brine and then dried over anhydrous Na_2SO_4. Filtration and evaporation of solvent afforded yellow oil, which was purified by silica gel column chromatography (Merck Kieselgel 60), to give 23 mg of phytanyl iodide: EI-MS *m/z* 408 (M^+), and 281; ^{1}H NMR 0.8–0.9 (m, $CH_3 \times 5$), 1.0–1.9 (m, CH_2 and CH), and 3.2 ppm (m, 2 H); ^{2}H NMR 0.86, 1.07, 1.27, and 3.20 ppm.

Preparation of Isotopically Labeled Glycerols. [1(3)-^{18}O]Glycerol. To a 25-mL round-bottom flask containing a stirring bar were added 1.14 g of *O*-benzylglycidol prepared by the literature procedure,[14] 5 mL of freshly distilled anhydrous tetrahydrofuran (THF), 130 μL of $H_2{}^{18}O$ (Isotec Inc., 97.7 atom % enriched, Lot No. CR2114), and 30 μL of 70% perchloric acid, and the flask was stoppered. The mixture was heated for 1 h with stirring in an oil bath (80 °C bath temperature). After being cooled, the mixture was diluted with a small amount of water and extracted with 20 mL of ethyl acetate. The aqueous layer was further extracted with 10-mL portions of ethyl acetate. The combined organic extract was washed successively with saturated aqueous $NaHCO_3$ and brine and then dried over anhydrous Na_2SO_4. Filtration and evaporation of solvent under reduced pressure afforded 1.71 g of an oil, which was passed through a column of 50 g of silica gel (Merck Kieselgel 60) with hexane–acetone (2:1) as an eluent to yield 1.12 g of an oily product. Further purification was carried out on a column of 50 g of silica gel (Merck Kieselgel 60) with hexane–ethyl acetate (1:1) to give 200 mg (16.7% yield) of racemic 1-*O*-benzyl[3-^{18}O]glycerol: IR ($CHCl_3$) 3580, 3440, 2930, 2870, 1220, 1105, 1065, 1035, and 760 cm^{-1}; ^{1}H NMR 3.56 (1 H, dd, J = 6.4 and 10.1 Hz), 3.58 (1 H, dd, J = 4.2 and 10.1 Hz), 3.64 (1 H, dd, J = 5.8 and 11.3 Hz), 3.71 (1 H, dd, J = 3.7 and 11.3 Hz), 3.90 (1 H, m), 4.60 (2 H, s), and 7.30–7.37 ppm (5 H, m); ^{13}C NMR 63.93, 70.70, 71.57, 73.44, 127.67, 127.75, 128.34, and 137.50 ppm; EI-MS (di-*O*-TMS-ether) *m/z* 328 (M^+) and 313 ($M^+ - 15$). Anal. Found: C, 65.43; H, 7.88. Calcd for $C_{10}H_{14}{}^{16}O_2{}^{18}O$: C, 65.20; H, 7.66.

A mixture of 360 mg of racemic 1-*O*-benzyl[3-^{18}O]glycerol, 10 mL of ethanol, and 300 mg of 10% Pd–C (Kawaken Fine Chemicals Co. Ltd., Tokyo) was vigorously stirred overnight under a H_2 blanket at room temperature. The catalyst was then removed by filtration through a disposable filtering assembly ACRO LC-13 (Gelman Sciences), and then the solvent was removed under reduced pressure to afford 143 mg of *sn*-[1(3)-^{18}O]glycerol: ^{13}C NMR (125 MHz, CD_3OD) 65.26, 65.28, and 74.66 ppm, which was used for supplementation experiments without further purification.

Deuterium Labeled Glycerol. Deuterated glycerols used in this study are all known in the literatures and were synthesized by established methods. *sn*-[3,3-$^2H_2$]Glycerol and *rac*-[1(3)-$^2H_2$]glycerol were prepared as described by Bochovchin et al.[15] *sn*-[1,1-$^2H_2$]Glycerol was prepared according to the method described by Takano et al. from *sn*-1,2-*O*-isopropylidene[3,3-$^2H_2$]glycerol obtained as an intermediate in the above-mentioned preparation of *sn*-[3,3-$^2H_2$]glycerol.[16] Preparation of [2-^{2}H]glycerol was adopted from the method described by White and by Wohlgemuth et al.[17,18] D-[6,6-$^2H_2$]Glucose was prepared as described previously.[17,19] [U-$^2H_8$]Glycerol that was used (98.3 atom % enriched) was the product of MSD Isotopes Ltd., Canada.

Determination of Chiral Purity of the Deuterated Glycerols. A mixture of 10 mg of *sn*-[3,3-$^2H_2$]-1,2-*O*-isopropylideneglycerol prepared as an intermediate for *sn*-[3,3-^{2}H]glycerol, 5 drops of (+)-α-methoxy-α-(tri-

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fluoromethyl)phenylacetyl (MTPA) chloride, 0.5 mL of dried pyridine, and a catalytic amount of DMAP was stirred for 12 h at room temperature. The mixture was diluted with ether, and the whole was washed successively with 1 N HCl, saturated aqueous $NaHCO_3$, and brine and then dried over anhydrous Na_2SO_4. Filtration and removal of the solvent under reduced pressure afforded a brownish oil, which was purified by pTLC using hexane–ethyl acetate (4:1) as solvent. The band at R_f 0.4 was scraped, collected, and extracted with ethyl acetate. The extract was evaporated to dryness in vacuo to give 22 mg of the product: ^1H NMR (500 MHz, C_6D_6) 1.16 (3 H, s), 1.27 (3 H, s), 3.28 (1 H, dd, J = 5.86 and 8.55 Hz), 3.43 (3 H, q, J = 1.22 Hz), 3.48 (1 H, dd, J = 6.59 and 8.54 Hz), 3.81 (1 H, br t, J = 6.10 and 6.35 Hz), and 7.0–7.6 ppm (5 H, m).

A mixture of 15 mg of *sn*-[1,1-$^2H_2$]-1-*O*-benzylglycerol, 10 drops of (+)-MTPA chloride, 2.0 mL of dry pyridine, and a catalytic amount of DMAP was treated and worked up as described above to give 32 mg of *sn*-[1,1-$^2H_2$]-1-*O*-benzyl-2,3-bis-*O*-[methoxy(trifluoromethyl)phenylacetyl], glycerol which was directly analyzed by the 500-MHz ^1H NMR spectrum (acetone-d_6): 3.41 (3 H, q, J = 1 Hz), 3.49 (3 H, q, J = 1 Hz), 4.46 (2 H, s), 4.59 (1 H, dd, J = 6.3 Hz and 12.2 Hz), 4.87 (1 H, dd, J = 2.9 and 12.2 Hz), 5.64 (1 H, dd, J = 2.9 and 12.2 Hz), and 7.1–7.6 ppm (5 H, m).

Results and Discussion

Although the chemistry involved in the preparation of [1-(3)-^{18}O]glycerol was rather straightforward, the present method afforded much higher enrichment of the desired product than the previously described procedure,[20] and one feature should be emphasized. The hydrolysis of *O*-benzylglycidol in [^{18}O]H_2O–THF in the presence of catalytic $HClO_4$ proceeded by regiospecific nucleophilic attack at the terminal methylene position to give racemic [1-^{18}O]-3-*O*-benzylglycerol. The site of ^{18}O-substitution was proved by comparison of a proton-decoupled ^{13}C NMR spectrum of a mixture of substituted and unsubstituted benzylglycerol. The signal at 63.8 ppm appeared as a pair of singlets ($\Delta\delta$ = 2.47 Hz)[21] and was assigned to the hydroxymethyl carbon. Other oxymethylene carbons were observed at 71.5 and 73.4 ppm as singlets. Further, *sn*-[1(3)-^{18}O]glycerol obtained by the reductive deprotection had methylene carbon signals at 65.26 and 65.28 ppm ($\Delta\delta$ = 2.20 Hz).[21]

The chiral purity of *sn*-[3,3-$^2H_2$]- and *sn*-[1,1-$^2H_2$]glycerol was determined from 500-MHz ^1H NMR spectra of the Mosher's (+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) esters of the precursory protected glycerol derivatives,[22] i.e. *sn*-1,2-*O*-isopropylidene[3,3-$^2H_2$]glycerol and *sn*-1-*O*-benzyl-[1,1-$^2H_2$]glycerol. The diagnostic signals for *sn*-[3,3-$^2H_2$]-3-*O*-[methoxy(trifluoromethyl)phenylacetyl]-1,2-*O*-isopropylideneglycerol were a quartet, split by $^5J_{H-F}$, due to the methoxyl groups at 3.43 ppm and the corresponding counterpart signal at 3.41 ppm of the coexisting C-2 epimer. The intensity ratio was estimated to be 94:6 by integration, giving a chiral purity of 88% ee. Similarly, by comparing the signal intensities at 4.87 and 4.76 ppm (due to one of the diastereotopic protons of the (acyloxy)methylene group), the chiral purity of the di-*O*-MTPA ester of *sn*-[1,1-$^2H_2$]-1-*O*-benzylglycerol was determined to be 80% ee.

The ^{18}O-substituted glycerol and each deuterated glycerol thus obtained were fed to a culture of the halophile *Halobacterium halobium* IAM 13167. The yields of isotopically enriched lipid, and the benzoate thereof, from feeding experiments are summarized in Table I.

Ether-Forming Reaction. Very recently, the ether-forming reaction in the biosynthesis of archaebacterial *sn*-2,3-di-*O*-alkylated glycerol lipids was suggested to involve nucleophilic displacement reactions between an unknown glycerol precursor and geranylgeranyl pyrophosphate based on the facts that (1) tritiated geranylgeraniol (but not tritiated geranylgeranyl pyrophosphate) was efficiently incorporated into the membrane lipids of *M. hungatei*[5] and (2) formation of geranylgeranyl pyrophosphate or phytyl pyrophosphate was detected on a thin-layer chromatogram by chasing [^{32}P]phosphate in *H. cutirubrum*.[8] However, these results did not unambiguously show that the nucleophile is the glycerol moiety and not the phytanyl substituent. We independently tackled this problem by the ^{18}O-chase experiment using *sn*-[1-(3)-^{18}O]glycerol.

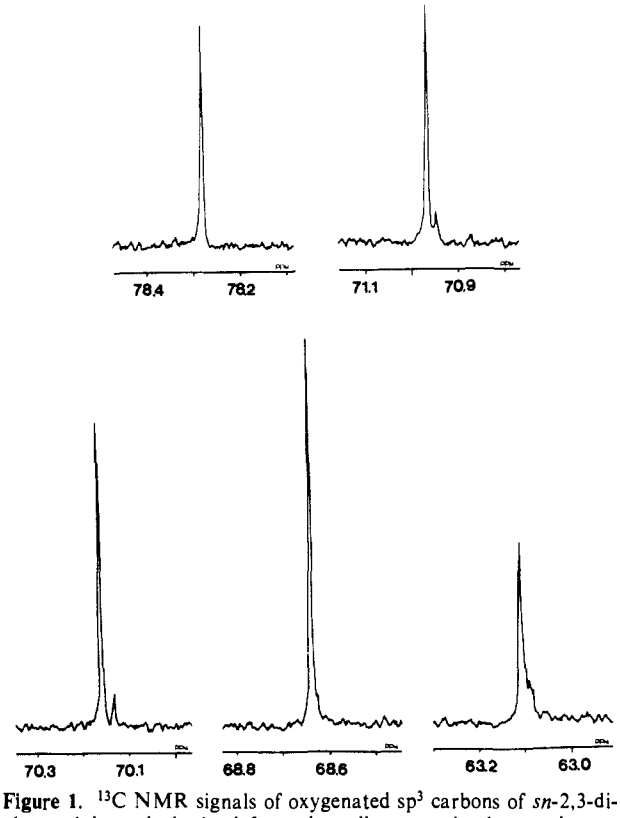

Figure 1. ^{13}C NMR signals of oxygenated sp^3 carbons of *sn*-2,3-di-*O*-phytanylglycerol obtained from the cells grown in the supplemented medium with [1(3)-^{18}O]glycerol.

Incorporation of ^{18}O into the lipid was first suggested by a significant peak at m/z 711 ($M^+ - CH_3$) in the mass spectrum of the *O*-TMS derivative of the labeled lipid obtained from the cells grown in the presence of [1(3)-^{18}O]glycerol. Unlabeled lipid had peaks at m/z 709 and 710. More definitive evidence was obtained from the ^{13}C NMR spectrum. Among the ^{13}C NMR signals of oxygenated sp^3 carbons at 63.10, 68.63, 70.16, 70.96, and 78.28 ppm, the signals at 70.96 ppm (*sn*-C-3 of the glycerol moiety) and 70.16 ppm (an oxymethylene carbon of a phytanol moiety) each had a satellite signal at ca. 2.5 Hz lower frequency (Figure 1).[21,23] Further, although the signal at 63.10 ppm (*sn*-C-1 of the glycerol moiety) was not well-resolved, it apparently was accompanied by a satellite signal as well. The satellites are ascribable to carbons directly bound to ^{18}O. Thus, the hydroxymethyl oxygen atoms of glycerol are conserved in the lipid *sn*-2,3-di-*O*-phytanylglycerol. Clearly, the carbon–oxygen bond of *sn*-C-3 of glycerol was not cleaved in the ether-bond formation, thereby strongly suggesting that the oxygen atom of glycerol does function as a nucleophile. Although this result does not necessary mean in a strict sense that the same is true at the C-2 position, it is unlikely that the mechanism of the ether-forming reaction at C-2 of glycerol is different from that at *sn*-C-3.

Stereochemistry of Glycerol Incorporation. The second question to be studied was the stereospecificity of glycerol incorporation. Chemically synthesized *sn*-[1,1-$^2H_2$]glycerol and *sn*-[3,3-$^2H_2$]-glycerol were separately fed to the culture of *H. halobium*, and two isotopically labeled samples of the membrane lipid were obtained. These samples were then benzoylated to differentiate the NMR chemical shifts of the *sn*-C-1 and *sn*-C-3 methylene groups of the glycerol moiety. The ^1H spectra of these lipid benzoates were recorded at 500 MHz, and relevant regions of the spectra are shown in Figure 2.

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(23) The signals were assigned by the long-range C–H COSY experiment.

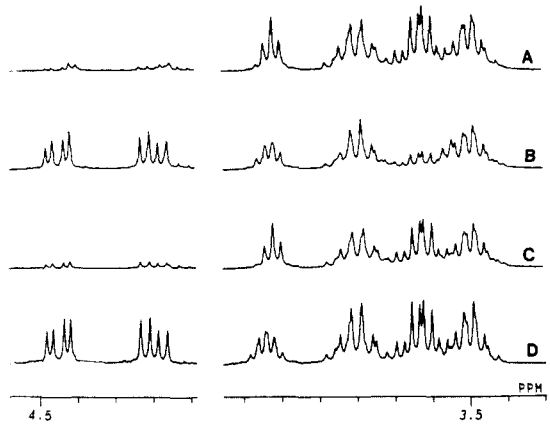

Figure 2. ^1H NMR spectra (500 MHz in $CDCl_3$) of benzoylated core lipids. The spectra are designated as follows: (A) the lipid obtained by feeding of *sn*-[3,3-$^2H_2$]glycerol, (B) the lipid obtained by feeding of *sn*-[1,1-$^2H_2$]glycerol, (C) the lipid obtained by feeding of D-[6,6-$^2H_2$]-glucose, and (D) the unlabeled control.

Significant decreases of particular signals are easily detected by simple visual inspection of the spectra of the deuterated lipid benzoates obtained from the cells grown in cultures supplemented with each deuterated substrate. These results demonstrates efficient and stereospecific incorporation of deuterium atoms into the *sn*-C-1 position of the lipid from *sn*-[3,3-$^2H_2$]glycerol and into the *sn*-C-3 position from *sn*-[1,1-$^2H_2$]glycerol, respectively. Enrichment ratios were estimated on the basis of decreases of signals in question relative to the integrated intensities of the benzoyl protons at 7.55 ppm as an intramolecular control. Thus, the C-1 position of the lipid obtained by feeding of *sn*-[3,3-$^2H_2$]glycerol was about 79% enriched, and in the case of the enantiomer, the enrichment at C-3 was approximately 68%. Estimates of the latter enrichment were less precise because of overlapping proton signals. Stereospecific incorporation of glycerol into the lipid demonstrated strict recognition of prochirality of glycerol during the biosynthesis and clearly ruled out a direct alkylation at *sn*-C-3 of glycerol and the intermediacy of dihydroxyacetone. Kates et al. previously suggested dihydroxyacetone was a possible precursor for the ether-forming reaction;[9] however, the hydroxymethyl groups of free dihydroxyacetone are indistinguishable chemically or enzymatically, which is incompatible with the present results.

It was also noteworthy that no exchange of deuterium from the methylene groups of glycerol was seen in either case. These results agree with the previous work by other groups, which reported that the tritium in [1(3)-^{3}H]glycerol was clearly retained during the formation of membrane lipids in either *Halobacterium cutirubrum*[24] or the extreme acidothermophile *Sulfolobus* sp.[25] At any rate, the atoms attached to *sn*-C-1 of glycerol remain in place during ether-bond formation in lipid biosynthesis.

This labeling pattern was further supported by ^2H NMR spectra (Figure 3). Deuterium signals due to labeling from [1(3)-$^2H_2$]glycerol were observed at 4.40 and 3.55 ppm (intensity ratio ca. 1:3), suggesting that both methylene groups of the glycerol moiety were incorporated intact. The observed higher intensity of the latter signal suggested simultaneous deuterium incorporation into the oxymethylene moiety of the phytanyl chains, since the signals due to the oxymethylene groups are overlapped in the ^2H NMR spectrum.

Enrichment into the oxymethylene moiety of phytanyl chains was verified by the fact that phytanyl iodide obtained from the treatment of the deuterium-enriched lipid with HI showed a deuterium signal from the iodomethyl group at 3.20 ppm. Since the presence of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase was reported in *H. halobium*,[26] the mevalonate pathway

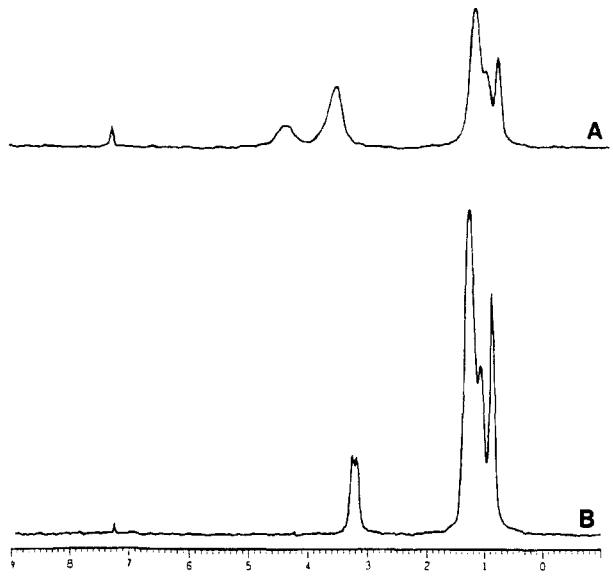

Figure 3. ^2H NMR spectra (61.48 MHz, $CHCl_3$) of (A) the benzoylated core lipid obtained by feeding of [1(3)-$^2H_2$]glycerol and (B) phytanyl iodide derived from the deuterium enriched lipid.

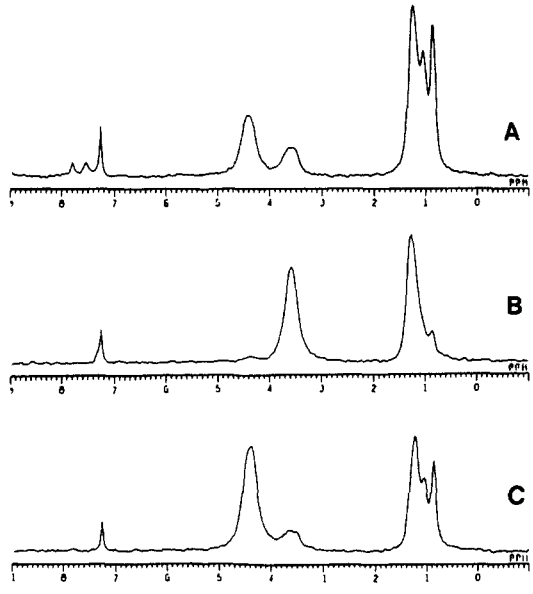

Figure 4. ^2H NMR spectra (61.48 MHz, $CHCl_3$) of benzoylated lipid obtained by feeding of (A) *sn*-[3,3-$^2H_2$]gycerol, (B) *sn*-[1,1-$^2H_2$]glycerol, and (C) D-[6,6-$^2H_2$]glucose.

in *H. halobium* is most likely to be similar to that of eubacteria and other organisms. Accordingly, the oxymethylene moiety of the phytanyl chain must be derived from the carboxyl carbon of acetate (the carbonyl carbon of acetyl CoA), and deuterium incorporation into the oxymethylene moiety is ascribable to the reduction process, facilitated by the HMG-CoA reductase. The most appropriate deuterium donor may well be a deuterated nicotinamide dinucleotide cofactor (hereafter NAD(P)H) generated presumably in the TCA cycle from the deuterated acetyl CoA.

Labeling of the methyl groups and other portions of the phytanyl moieties were also indicated by the intense signals at 0.86, 1.10, and 1.25 ppm in the ^2H NMR spectrum of the labeled phytanyl iodide, further supporting the formation of deuterated acetyl-CoA.

As shown in Figure 4, the labeling pattern observed from the *sn*-[3,3-$^2H_2$]glycerol feeding experiment was rather similar to that from the racemic [1(3)-$^2H_2$]glycerol feeding. Reversal of the signal intensities at 4.40 and 3.60 ppm suggests stereospecific incorporation of deuterium into *sn*-C-1 of the glycerol moiety of the lipid vide post and a lower level of deuterated NAD(P)H in the nicotinamide cofactor pool. The fact that methyl groups of

(24) Kates, M.; Wassef, M. K.; Pugh, E. L. *Biochim. Biophys. Acta* **1970**, *202*, 206.
(25) De Rosa, M.; Gambacorta, A.; Nicolaus, B.; Sodano, S. *Phytochemistry* **1982**, *21*, 595.
(26) Cabrera, J. A.; Bolds, J.; Shields, P. E.; Havel, C. M.; Watson, J. A. *J. Biol. Chem.* **1986**, *261*, 3578.

the phytanyl chains were efficiently enriched strongly suggests that *sn*-C-3 of glycerol is metabolized to the methyl group of acetyl-CoA as is in eubacteria and other organisms.

Complementary results were obtained by chasing *sn*-[1,1-$^2H_2$]glycerol. Thus, in the glycerol moiety of the lipid, deuterium was stereospecifically incorporated into the *sn*-C-3 position (3.60 ppm). The methyl signals were not enriched in this case. The very weak signal may be due to either slightly lowered enantiomeric purity of the substrate and/or partial scrambling during metabolism. This result further supports the proposal that *sn*-C-3 of glycerol is metabolized to the methyl group of acetyl-CoA, and the *sn*-C-1 carbon is lost by decarboxylation. Labeling of other positions of the phytanyl chains may again be explained by formation of deuterated NAD(P)H, among others, at the oxidation step of D-glyceraldehyde 3-phosphate into D-glycerate 1,3-bisphosphate.

To compare the stereochemical outcome of glucose metabolism in *H. halobium* with that of eubacteria and eukaryotes, a feeding experiment with D-[6,6-$^2H_2$]glucose was conducted. The efficient incorporation of deuterium into the benzoyloxy methylene moiety of the lipid was indicated by the intense signal at 4.40 ppm in the ^2H NMR spectrum (Figure 4) and by diminished signals at 4.35 and 4.47 ppm in the ^1H NMR spectrum (Figure 2). The phytanyl chains were also labeled similarly to the *sn*-[3,3-$^2H_2$]glycerol feeding,

With regard to the D-glucose metabolism in *Halobacterium*, operation of a modified Entner-Doudoroff pathway was previously suggested by Tomlinson et al.,[27] in agreement with the present results. If the Embden–Meyerhof–Parnas pathway were involved, deuterium enrichment in the glycerol portion of the lipid could never exceed 50% because each molecule of D-glucose is split into D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, and the latter fragment is then isomerized by triose-phosphate isomerase. The observation that the deuterium incorporation from the supplemented D-[6,6-$^2H_2$]glucose was extremely high (more than 70%), provides additional evidence for the Entner–Doudoroff pathway.[27]

The cryptic stereochemistry involved in the incorporation of glycerol into the membrane lipid of *H. halobium* has now been firmly established. In contrast to eubacteria and eukaryotes, it is the *sn*-C-1 methylene group of 2,3-di-*O*-phytanylglycerol that is derived from the *sn*-C-3 carbon of glycerol and the C-6 carbon of D-glucose.

It is well-documented that polar lipids containing a phosphate group as a polar head group at the *sn*-C-1 position of the glycerol moiety exist in archaebacterial cell membranes.[1] In addition, Kates et al. previously reported that glycerol kinase transforms glycerol into *sn*-glycerol 3-phosphate, but not into *sn*-glycerol 1-phosphate, in *H. cutirubrum*.[9] An intramolecular migration of the phosphate group in *sn*-glycerol 3-phosphate to the *sn*-1 position is untenable because the original *sn*-C-3 hydroxymethyl group would be attached to a phytanyl group in the lipid. Our results demonstrate that the *sn*-C-3 hydroxymethyl group of glycerol, which is phosphorylated first, remains attached to a polar head group (*sn*-C-1), e.g. a phosphate, in the final core lipid.

Although stereochemically feasible, an alternative pathway involving reduction of D-glyceraldehyde 3-phosphate can also be ruled out, since the present and previous studies from other laboratories showed no hydrogen was lost from either hydroxymethyl group of glycerol.[24,25] Furthermore, the ether-forming reaction must be also different from that of *O*-alkyldihydroxyacetone phosphate in mammalian cells,[28,29] in which a hydrogen of the *sn*-C-1 methylene group is stereospecifically replaced.

As already discussed, it is unlikely that the geranylgeranyl or other precursory alkyl substituents are introduced at the C-2 and *sn*-C-3 positions of glycerol through chemically different mechanisms. We suggest the oxygen atoms at C-2 and *sn*-C-3 of glycerol behave as nucleophiles to displace a pyrophosphoryl group in the phytanyl precursor. Thus, stereochemical inversion must take place at C-2 glycerol before formation of the C-2 ether bond during the biosynthesis of *sn*-2,3-*O*-diphytanylglycerol in *H. halobium*. The chemistry at the C-2 position of glycerol is crucial for further understanding of the lipid biosynthesis.

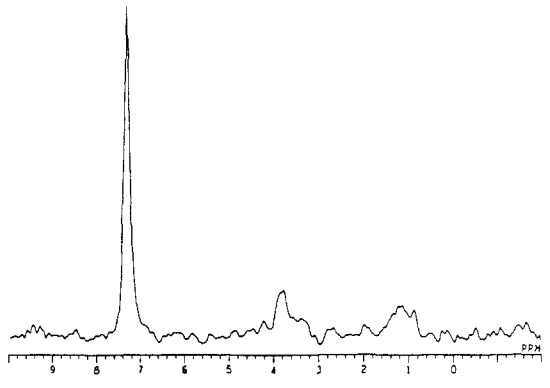

Figure 5. ^2H NMR spectrum (61.48 MHz, $CHCl_3$) of the benzoylated lipid obtained by feeding of [2-^2H]glycerol.

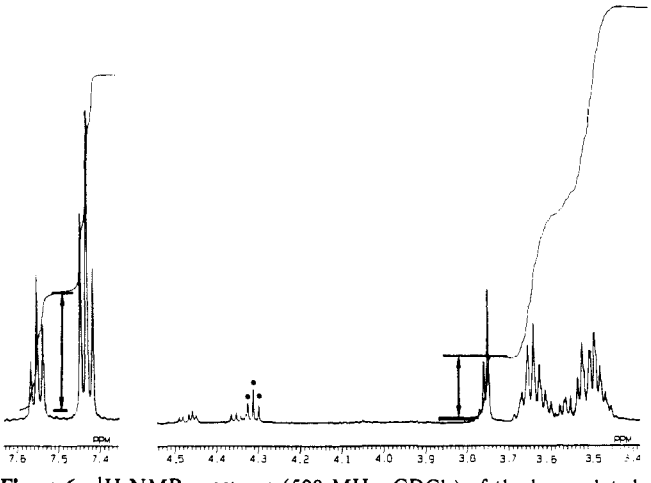

Figure 6. ^1H NMR spectrum (500 MHz, $CDCl_3$) of the benzoylated lipid obtained by feeding of [U-$^2H_8$]glycerol. The marked signals are due to impurity.

The Fate of the C-2-Hydrogen of Glycerol. Previously, DeRosa et al. reported that the tritium label in [2-^3H]glycerol was retained during biosynthesis of the membrane lipid of extreme acidothermophile *Sulfolobus* sp.,[25] while Kates et al. reported complete loss of ^3H at C-2 of glycerol during lipid biosynthesis in *Halobacterium cutirubrum*.[24] Although these somewhat contradictory observations may be due to differences in the bacterial species, it seemed worthwhile to reexamine the fate of the hydrogen of the C-2 position of glycerol.

Figure 5 displays the ^2H NMR spectrum of the benzoate derivative of the membrane core lipid, *sn*-2,3-di-*O*-phytanylglycerol, obtained from cells grown with supplementation of [2-^2H]glycerol.

As already mentioned, excellent incorporation of glycerol into the core lipid was seen under similar conditions, and deuterium enrichment ratios usually reached 65–70%. Therefore, a significant ^2H signal would have been expected in the present case if the hydrogen at C-2 of glycerol were conserved during formation of *sn*-2,3-di-*O*-phytanylglycerol. The lipid benzoate sample obtained as described above showed essentially no ^2H signal, even under conditions where the signal due to natural abundance deuterium in the $CHCl_3$ solvent appeared as a large peak. This suggests that, although glycerol is efficiently incorporated (this cannot be argued from this particular experiment), its C-2 hydrogen is eliminated in agreement with the results described previously by Kates.[24] The questions of how it is eliminated and the source of the hydrogen introduced into the C-2 position of the glycerol moiety of *sn*-2,3-di-*O*-phytanylglycerol remains.

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(28) Brown, A. J.; Snyder, F. *J. Biol. Chem.* **1983**, *258*, 4184.
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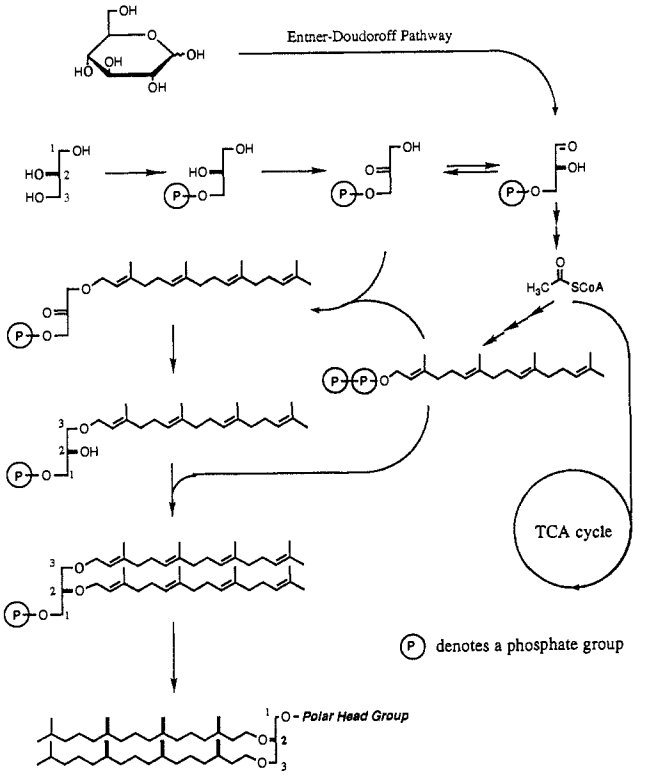
Scheme II. Plausible Biosynthetic Pathway of the Core Lipid in *Halobacterium*



We conducted another chase experiment using [U-$^2H_8$]glycerol. In this experiment, the location of label in the lipid benzoate derivative was determined from its ^1H NMR spectrum (Figure 6). It was expected that the signals due to *sn*-C-1 (a pair of double doublets at 4.35 and 4.47 ppm) and to *sn*-C-3 (again a pair of double doublets centered at 3.56 ppm) had reduced intensities since the deuterium at either C-1 or C-3 of glycerol is retained in the lipid; vide supra. But surprisingly, the signal intensity of the C-2 position in the glycerol moiety of the lipid at 3.75 ppm was only approximately 42% of the intensity of the benzoate signal at 7.55 ppm; vide supra. Deuterium cannot be derived from the C-2 position of [U-$^2H_8$]glycerol and, therefore, must be derived from either C-1 or C-3 or glycerol.

It is unlikely that the deuterium is introduced into the C-2 position of the glycerol moiety of the lipid intramolecularly since the hydrogens at C-1 and C-3 of glycerol remain in their original positions. Therefore, an intermolecular deuterium transfer must occur, and the most likely deuterium donor is deuterated NAD(P)H. The efficient formation of a deuterated NAD(P)H from [1(3)-$^2H_2$]glycerol was substantiated already. Thus, the inversion of configuration in glycerol during the biosynthesis of *sn*-2,3-di-*O*-phytanylglycerol may well involve an oxidation–reduction process.

The most likely oxidation step is the one catalyzed by glycerol 3-phosphate dehydrogenase producing dihydroxyacetone phosphate from *sn*-glycerol 3-phosphate, where a nicotinamide coenzyme seems to serve as an acceptor from the C-2 hydrogen of glycerol. It seems less likely that dihydroxyacetone phosphate is reduced back to the enantiomeric *sn*-glycerol 1-phosphate, which might in turn have played a role as a nucleophile attacking to geranylgeranyl pyrophosphates or other precursors of the phytanyl groups, since Kates et al. suggested that, barring the existence of an as yet unknown enzyme system for biosynthesis of *sn*-glycerol 1-phosphate, it may be assumed that the only glycerophosphate isomer synthesized in *H. cutirubrum* is the *sn*-glycerol 3-phosphate.[9]

To accommodate previous observations from other laboratories and our results, we have suggested a stepwise mechanism for the biosynthesis of *sn*-2,3-di-*O*-phytanylglycerol in *H. halobium*, as depicted in the Scheme II where the two crucial ether bonds are formed in different steps. We propose glycerol is phosphorylated at *sn*-C-3, and oxidation by *sn*-glycerol-3-phosphate dehydrogenase follows. The resulting dihydroxyacetone phosphate, which lies at the merging point of metabolism from glycerol and from D-glucose, reacts with a C_{20} alkyl donor such as geranylgeranyl pyrophosphate to form a mono-*O*-alkyldihydroxyacetone phosphate intermediate. This sequence can account for the stereochemical recognition of the *sn*-C-1 and *sn*-C-3 positions of glycerol. Previously, Kates et al. ruled out intermediacy of dihydroxyacetone phosphate on the basis of the assumption that a hydrogen atom of dihydroxyacetone phosphate should be washed out through a possible keto–enol tautomerization.[8,9] The observation that all the hydroxymethyl hydrogens of glycerol are retained in the lipid may be rationalized by a high turnover rate for the ether-forming reaction. This argument is supported by the extremely high incorporations of glycerol observed.

We suggest alkylated dihydroxyacetone phosphate is reduced in the next step by a dehydrogenase to generate 3-*O*-alkylglycerol 1-phosphate, followed by the second alkylation reaction. Although the stereochemistry is different, there is precedence for reduction of alkylated dihydroxyacetone phosphate.[28,29] Later hydrogenation reactions would then give rise to saturated alkyl groups in *sn*-2,3-di-*O*-phytanylglycerol.

The observation that the hydrogen at the C-2 position of glycerol is not reintroduced into the glycerol moiety of the lipid (vide supra) may be explained either by differences in cofactor usage or in the stereospecificity of the dehydrogenase. Glycerol-3-phosphate dehydrogenase was reported to utilize the *pro-S* hydrogen on the C-4 position of the dihydropyridine ring of NADH and is classified as a B-site specific dehydrogenase.[30–32] On the other hand, well-known NAD(P)H generating dehydrogenases involved in the TCA cycle are A-site specific and utilize the *pro-R* hydrogen on the C-4 position of the dihydropyridine ring.[33] Since dihydropyridine dinucleotide coenzyme is efficiently generated in *H. halobium*, most likely by the enzymes operating in the TCA cycle, one would expect a significant pool of NAD(P)H deuterated at the A site of the dihydropyridine ring when [U-$^2H_8$]glycerol, *sn*-[3,3-$^2H_2$]glycerol, or racemic [1(3)-$^2H_2$]glycerol is supplemented into the culture. Thus, hydrogen at C-2 of glycerol 3-phosphate might be removed by B-specific glycerol-3-phosphate dehydrogenase, while reduction of the C-2 position in *O*-alkyldihydroxyacetone phosphate intermediate might occur from the A site of the accumulated deuterated dihydronicotinamide cofactor. Additional experiments are required to resolve this question.

In conclusion, chase experiments clearly demonstrated that the oxygen atom at *sn*-C-1 of glycerol behaves as a nucleophile for the ether-forming reaction and that oxidation and reduction processes are responsible for the removal of the original C-2 hydrogen of glycerol as well as the intermolecular introduction of a hydrogen with inversion of configuration into the C-2 position of the glycerol moiety in the biosynthesis of the membrane core lipid of *H. halobium*. A stepwise pathway to *sn*-2,3-di-*O*-phytanylglycerol from dihydroxyacetone–phosphate has been postulated.

Acknowledgment. This work was financially supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture and by a grant from the Naito Foundation. Deuterium NMR spectra were recorded through the courtesy of Professor H. Seto and Dr. K. Furihata, the University of Tokyo, to whom we are grateful.

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(31) The generally accepted Bentley's generalization pertaining to the dehydrogenase stereospecificity suggests that the stereospecificity of a particular dehydrogenase reaction is fixed and does not depend on the source of the enzyme.[32]
(32) Bentley, R. *Mol. Asymmetry Biol.* **1978**, *2*, 1.
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