J. Preparation of *S*-(4-Aminophenyl)-L-cysteine *S*,*S*-Dioxide. *S*-(4-Nitrophenyl)-L-cysteine *S*,*S*-dioxide (0.4 g, 1.46 mmol) was hydrogenated as described for *S*-(2-nitrophenyl)-L-cysteine *S*,*S*-dioxide. The brown crystalline product was recrystallized from hot ethanol to yield 0.31 g (87%) of light-tan crystals, which soften at 155 °C, mp 163-165 °C dec. UV (0.01 M HCl): λ_{max} = 270 nm (log ϵ = 4.19). IR (Nujol mull): ν 1139 and 1287 cm^{-1} (SO_2). ^1H NMR (D_2O, DCl): δ 3.98-4.17 (2 H, m, β-CH_2), 4.59-4.63 (1 H, dd, α-CH), 7.65-8.12 (4 H, dd, aromatic). $[\alpha]^{23}_D$ +30.2 (c = 1.175, 1 M HCl).

Acknowledgment. We are grateful to Mr. Srinagesh Koushik for the NMR spectra. We also thank Dr. Kenji Soda for helpful discussions and Dr. H. B. Dixon for advice on nomenclature. This work was supported by a grant from the National Institutes of Health (GM 42588) to R.S.P.

Biosynthesis of Archaebacterial Ether Lipids. Formation of Ether Linkages by Prenyltransferases

Donglu Zhang and C. Dale Poulter*

Contribution from the Department of Chemistry, University of Utah, Salt Lake City, Utah 84112.
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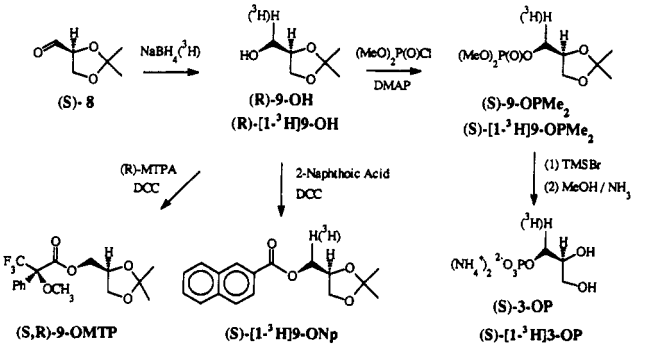
Abstract: Biosynthesis of (*R*)-2,3-di-*O*-phytanylglycerol (**1**), the core lipid of the membranes in the strict anaerobe *Methanobacterium thermoautotrophicum*, was studied in cell-free preparations. Two prenyltransferase activities were separated by ultracentrifugation. The cytosolic fraction contained activity for (*S*)-3-*O*-geranylgeranylglyceryl phosphate synthase (GGGP synthase), which catalyzes alkylation of (*S*)-glyceryl phosphate [(*S*)-**3**-OP] by geranylgeranyl diphosphate (**5**-OPP), while the pellet contained activity for (*S*)-2,3-*O*-digeranylgeranylglyceryl phosphate synthase (DGGGP synthase), which catalyzes alkylation of (*S*)-3-*O*-geranylgeranylglyceryl phosphate [(*S*)-**12**-OP] by **5**-OPP. (*S*)-**3**-OP and **5**-OPP were strongly preferred among the various compounds tested as prenyl acceptors [(*S*)-**3**-OP, (*R*)-**3**-OP, glycerol (**3**-OH), and dihydroxyacetone phosphate (**4**-OP)] and as prenyl donors [**5**-OPP, phytyl diphosphate (**6**-OPP), phytanyl diphosphate (**7**-OPP), farnesyl diphosphate (**10**-OPP), and farnesylgeranyl diphosphate (**11**-OPP)]. The products from the enzymatic reactions catalyzed by GGGP synthase and DGGGP synthase were characterized by hydrolysis of the phosphate moieties with alkaline phosphatase and comparisons of the glyceryl ethers with synthetic samples. These results demonstrate that the ether linkages in the core lipids of archaebacteria are formed in two distinct steps by a cytosolic GGGP synthase and a membrane-associated DGGGP synthase.

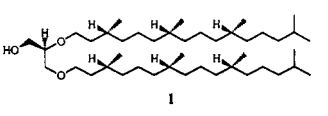
Introduction

According to a recent classification by Woese and co-workers,[1-3] all living organisms stem from the early divergence of a hypothetical progenitor into three primary kingdoms: archaebacteria, eubacteria, and eukaryotes. Designation of archaebacteria as a distinct kingdom was originally based on comparisons of ribosomal RNA sequences and is supported by several unique biochemical features, such as the construction of archaebacterial cell walls,[4] the structures of modified bases in archaebacterial tRNAs,[5] unique archaebacterial cofactors,[6] and unique structures for archaebacterial membrane lipids.[7] Archaebacteria are classified into three major phenotypes: extreme halophiles, methanogens, and thermoacidophiles,[3] which are typically confined to special ecological niches characterized by a high concentration of salt, high temperature, low pH, or absence of oxygen.

A distinctive molecular feature common to all archaebacteria is the nature of their core membrane lipids. In contrast to the membrane lipids composed of glyceryl esters of fatty acids found in eubacteria and eukaryotes, archaebacterial membranes contain isopranyl glyceryl ethers.[8,9] The two major hydrophobic components typically found in archaebacterial lipids are a C_{20} diether, (*R*)-2,3-di-*O*-(3*R*,7*R*,11*R*)-phytanylglycerol [(*R*)-**1**],[10] and a C_{40} tetraether, (*R*)-2,3-di-*O*-(3*R*,7*R*,11*R*,15*S*,18*S*,22*R*,26*R*,30*R*)-biphytanyldiglycerol [(*R*)-**2**].[11] The absolute stereochemistry at C(2) of the glyceryl moieties is opposite to that of ester or ether lipids derived from fatty acids in eubacteria and eukaryotes.[12,13]

Scheme I. Synthesis of (*S*)-**3**-OP





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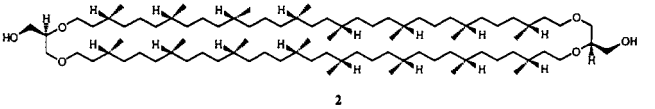

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Methanobacterium thermoautotrophicum contains approximately equal amounts of diether **1** and tetraether **2**[9] modified with phosphoserine, phosphoinositol, phosphoethanolamine, and glucosylglucose polar head groups,[14,15] along with smaller quantities of C_{15} and C_{25} diethers.[16] Incorporation experiments with labeled acetate indicated that biosynthesis of the isoprene moieties in the core lipids follows the same mevalonate pathway utilized by eubacteria and eukaryotes.[17-19]

Prenyl-transfer reactions are the major building steps in the isoprene pathway and are catalyzed by a family of prenyltransferases that mediate alkylation of electron-rich substrates (prenyl acceptors) by the hydrocarbon moieties of isoprene diphosphates (prenyl donors).[20,21] We[22] and others[19,23,24] have suggested that the ether linkages in archaebacterial core membrane lipids are likely formed by a prenyl-transfer reaction in which a glyceryl moiety serves as a prenyl acceptor. In a preliminary study with cell-free extracts, we[25] found that (*S*)-glyceryl phosphate [(*S*)-**3**-OP] was highly preferred over (*R*)-glyceryl phosphate [(*R*)-**3**-OP], dihydroxyacetone thiophosphate (**4**-OP_S), and glycerol (**3**-OH), while geranylgeranyl diphosphate (**5**-OPP) was preferred over phytyl diphosphate (**6**-OPP) and phytanyl diphosphate (**7**-OPP). We now report a full account of our studies on the biosynthetic reactions for construction of the ether linkages with cell-free extracts from *M. thermoautotrophicum* Marburg.

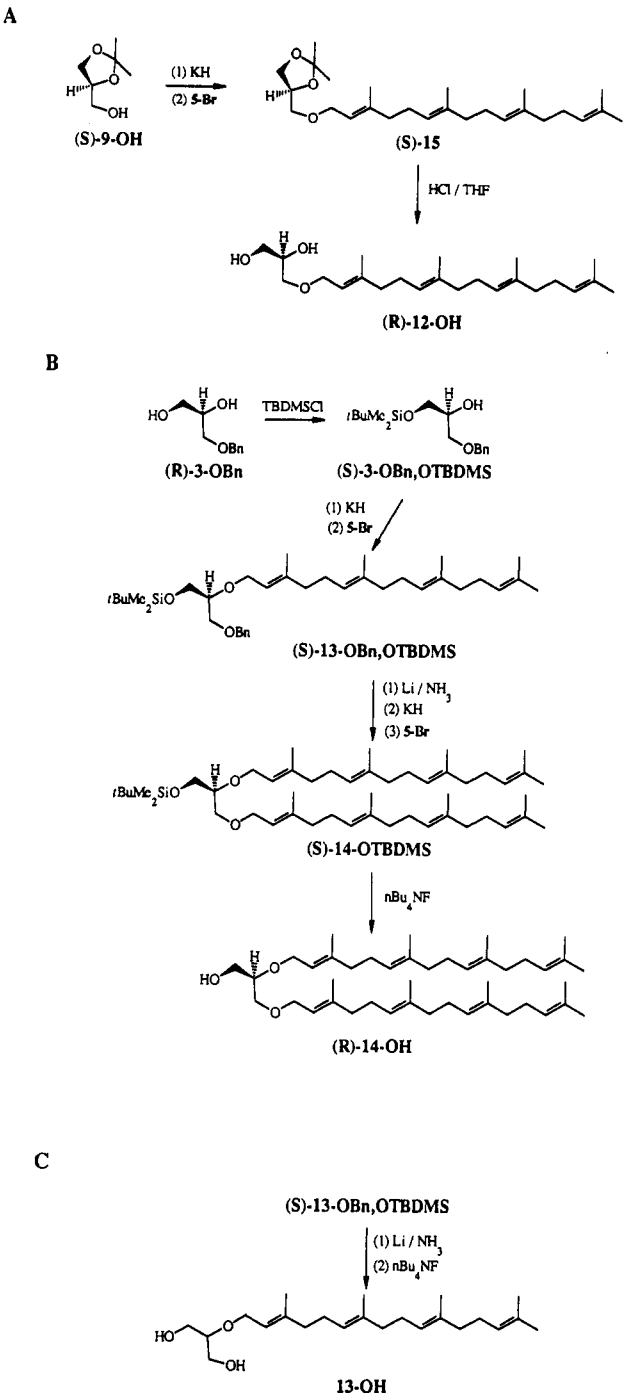
Results

Synthesis of Substrates and Products. (*S*)-Glyceryl Phosphate. Our synthesis of (*S*)-glyceryl phosphate [(*S*)-**3**-OP] is outlined in Scheme I. Optically active aldehyde (*S*)-**8** was obtained from L-arabinose in the usual manner[26] and converted to (*S*)-**3**-OP by reduction to (*R*)-**9**-OH with $NaBH_4$, followed by treatment of the alcohol with dimethyl phosphorochloridate,[27] cleavage of the methyl ester linkages with trimethylsilyl bromide, and hydrolysis of the trimethylsilyl and isopropylidene moieties. The phosphorylation sequence proceeded in an overall yield of 85%.

To ensure that the stereochemistry at C(2) in (*S*)-**8** remained intact, (*R*)-**9**-OH obtained after borohydride reduction was converted to the corresponding Mosher ester[28] (*S*,*R*)-**9**-OMTP. In addition, (*R*,*R*)- and (*R*/*S*,*R*)-**9**-OMTP were prepared. NMR spectra of the diastereomeric mixture had sharp well-resolved ^1H peaks at 3.44 and 3.47 ppm for the methoxy groups and ^{19}F peaks at 4.11 and 4.17 ppm for the trifluoromethyl moieties. In contrast, single ^1H and ^{19}F peaks at 3.44 and 4.11 ppm were seen for (*S*,*R*)-**9**-OMTP, whereas single peaks at 3.47 and 4.17 ppm were seen for (*R*,*R*)-**9**-OMTP. Within the limits of detection, (*R*,*R*)-**9**-OMTP and (*S*,*R*)-**9**-OMTP were pure diastereomers (>98%).

(*S*)-[1-^{3}H]**3**-OP was also prepared from aldehyde (*S*)-**8** as shown in Scheme I. Radiolabeled material purified by chromatography on cellulose was cospotted with synthetic (*S*)-**3**-OP and chromatographed on cellulose TLC. The plate was stained with sulfosalicylic acid/ferric chloride, sections were scraped directly into scintillation vials, and the samples were counted. The radioactivity (>99%) coeluted with unlabeled synthetic material. The specific activity of (*S*)-[1-^{3}H]**3**-OP (7 μCi/μmol) was determined from (*S*)-[1-^{3}H]**9**-ONp, the naphthoate ester of (*R*)-[1-^{3}H]**9**-OH, according to the method of Davisson et al.[29]

Scheme II. Synthesis of (A) (*R*)-**12**-OH, (B) (*R*)-**14**-OH, and (C) **13**-OH



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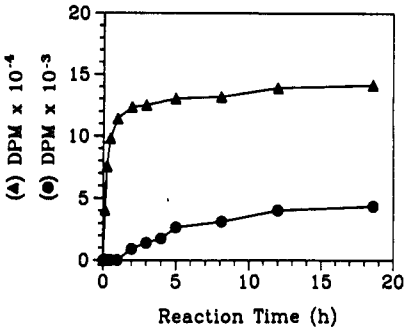

Figure 1. Progress curve for synthesis of (*S*)-**12**-OP (▲) and (*S*)-**14**-OP (●) by cell-free extracts from *M. thermoautotrophicum*. Incubation of (*S*)-[1-^{3}H]**3**-OP and **5**-OPP was at 37 °C. Samples were hydrolyzed with alkaline phosphatase, and ether extracts were analyzed by reversed-phase HPLC.

Isoprene Diphosphates. Farnesylgeraniol (**11**-OH) was prepared as described by Biellmann and Ducep.[30] Diphosphate esters of farnesol (**10**-OH), geranylgeraniol (**5**-OH), phytol (**6**-OH), phytanol (**7**-OH), and farnesylgeraniol (**11**-OH) were prepared from the alcohols by the procedure of Davisson et al.[31,32]

Glyceryl Ethers. (*R*)-3-*O*-Geranylgeranylglycerol [(*R*)-**12**-OH], 2-*O*-geranylgeranylglycerol (**13**-OH), and (*R*)-2,3-di-*O*-geranylgeranylglycerol [(*R*)-**14**-OH] were synthesized as outlined in Scheme II following procedures described by Aoki and Poulter[33] for the corresponding phytanyl ethers. For (*R*)-**12**-OH, (*S*)-**9**-OH was alkylated by geranylgeranyl bromide (**5**-Br) to give (*S*)-**15**, and the isopropylidene moiety was removed with HCl to give the monoether. In a second set of reactions, the hydroxyl group at C(1) in benzyl derivative (*R*)-**3**-OBn was selectively blocked with *tert*-butyldimethylsilyl chloride, and that at C(2) was alkylated with **5**-Br. A portion of the material was converted to **13**-OH by removal of the benzyl and *tert*-butyldimethylsilyl moieties. The remainder was treated with lithium in liquid ammonia to selectively expose the hydroxyl at C(3), which was then alkylated with **5**-Br. Treatment of (*S*)-**14**-OTBDMS with tetra-*n*-butylammonium fluoride gave diether (*R*)-**14**-OH.

Primary ether **12**-OH and secondary ether **13**-OH had characteristic patterns in their ^{1}H NMR spectra between 3.4 and 4.2 ppm. The two glyceryl methylene units in **12**-OH gave distinct multiplets centered at 3.48 and 3.65 ppm, whereas signals for the two glyceryl methylenes in **13**-OH appeared at 3.7 ppm. The C(1) methylene resonances in the geranylgeranyl units of **12**-OH and **13**-OH were at 4.0 and 4.12 ppm, respectively. Geranylgeraniol (**5**-OH), (*R*)-3-*O*-geranylgeranylglycerol [(*R*)-**12**-OH], 2-*O*-geranylgeranylglycerol (**13**-OH), and (*R*)-2,3-di-*O*-geranylgeranylglycerol [(*R*)-**14**-OH] were cleanly resolved during reversed-phase HPLC on a C_{18} column upon elution with MeOH/H_2O.

Biosynthetic Studies. Analysis of Products. A cell-free extract of *M. thermoautotrophicum* was prepared by sonication of a suspension of cells, followed by centrifugation at 10000*g* to remove cellular debris. Incubation of the clear supernatant with (*S*)-[1-^{3}H]**3**-OP and **5**-OPP at 37 °C gave a time-dependent production of radioactive material that was extractable by 1-butanol. Controls without addition of cell-free extract or with boiled extract gave no extractable radioactivity. The distribution of radioactivity following a standard incubation was measured for alcohols after hydrolysis of the phosphate moieties by alkaline phosphatase. The resulting suspension was extracted with ether, and radioactive ether-soluble products were analyzed on a C_{18} reversed-phase HPLC column which cleanly separated **3**-OH, **5**-OH, **12**-OH, **13**-OH, and **14**-OH. The results of a typical time course are summarized in Figure 1. A single product with a retention time identical to that of monoether **12**-OH was seen for incubation times up to 1 h. After 1 h, a second radioactive product with a retention time identical to that of diether **14**-OH slowly appeared. No material corresponding to monoether **13**-OH was detected. In a separate semipreparative-scale experiment, (*S*)-**3**-OP and **5**-OPP were incubated with cell-free extract for 2 h. The incubation mixture was extracted with 1-butanol. A product with a retention time identical to that of **12**-OP was detected by reversed-phase HPLC. The material was purified by HPLC and analyzed by negative ion FABMS. A base peak was detected at *m/z* 443 which corresponded to the monoanion (M − 1) of monoether **12**-OP. A high-resolution analysis gave an exact mass of 433.2565, within 1 mmu of the theoretical value for the monoanion of **12**-OP.

A large-scale incubation with 12 mg of (*S*)-**3**-OP and 50 mg of **5**-OPP at 50 °C for 5 h was followed by treatment with alkaline phosphatase and purification of ether-soluble material by HPLC. Three products were obtained. The major component (4.2 mg) was (*R*)-**12**-OH as deduced from comparisons of mass, IR, and ^{1}H and ^{13}C NMR spectra and of its optical rotation with those of an authentic synthetic sample. The minor product (ca. 200 μg) was identified as **14**-OH on the basis of its HPLC retention time, a comparison of its ^{1}H NMR spectrum with that from an authentic sample, and MS analysis of the corresponding TMS derivative, which gave a molecular ion at *m/z* 708 and a fragmentation pattern identical to that of authentic **14**-OTMS. In addition, a sample of (*S*)-[1-^{3}H]**12**-OP was prepared enzymatically from (*S*)-[1-^{3}H]**3**-OP and **5**-OPP. This material was converted to diether **14**-OP in a separate incubation with **5**-OPP and microsomal protein. These experiments establish the structures of the products of the enzymatic reactions and demonstrate that the archaebacterial diether is synthesized in two steps from (*S*)-**3**-OP and **5**-OPP via monoether (*S*)-**12**-OP.

Identification and Characterization of Enzyme Activities for Synthesis of (*S*)-12-OP and (*S*)-14-OP. The cell-free extract was subjected to centrifugation at 200000*g* for 2 h to obtain a microsomal pellet and a cytosolic supernatant. A portion of the supernatant was incubated with (*S*)-[1-^{3}H]**3**-OP and **5**-OPP for 24 h at 37 °C. The butanol-soluble materials were treated with alkaline phosphatase and analyzed by reversed-phase HPLC. A single radioactive peak that comigrated with an authentic sample of (*R*)-**12**-OH and corresponded to a 48% recovery of the radioactivity originally in (*S*)-[1-^{3}H]**3**-OP was detected. No radioactivity comigrated with (*R*)-**14**-OH. In a larger scale experiment with 10 mg of supernatant protein, 5.4 μCi of butanol-soluble material was obtained from incubation of 2.8 μmol of (*S*)-[1-^{3}H]**3**-OP (7 μCi/μmol) and 10 μmol of **5**-OPP for 4 h at 55 °C. TLC analysis of a portion of the extract on silica (1:1 ethyl acetate/pentane) indicated that 2% of the radioactivity comigrated with (*R*)-**12**-OH and 98% remained at the origin. TLC of a second sample on cellulose (60:25:5 THF/25 mM NH_4HCO_3/2-propanol) showed 1.8% of the radioactivity comigrating with (*S*)-[1-^{3}H]**3**-OP with the remainder at the solvent front. After the extract was treated with alkaline phosphatase and extracted into ether, >99% of the radioactivity comigrated with (*R*)-**12**-OH on the silica system.

Similar experiments were conducted using microsomal protein. In this case, (*S*)-**3**-OP, (*R*)-**12**-OH, and (*S*)-**12**-OP were individually incubated with **5**-OPP, and the results are summarized in Table I. No radioactivity was incorporated into (*R*)-**12**-OH or (*R*)-**14**-OH from (*S*)-[1-^{3}H]**3**-OP, nor was (*R*)-**12**-OH a substrate for formation of (*R*)-**14**-OH. However, incubation of monoether (*S*)-[1-^{3}H]**12**-OP and **5**-OPP gave a 15% conversion to diether (*S*)-**14**-OP. A control experiment established that the monoether was not a substrate for the cytosolic enzyme. These results demonstrate that diether (*S*)-**14**-OP is constructed from (*S*)-**3**-OP and **5**-OPP in two distinct steps. This first is catalyzed by a cytosolic enzyme, geranylgeranylglyceryl phosphate (GGGP) synthase, which converts the substrates to monoether (*S*)-**12**-OP. The second is catalyzed by a microsomal enzyme, digeranylgeranylglyceryl phosphate (DGGGP) synthase, which converts

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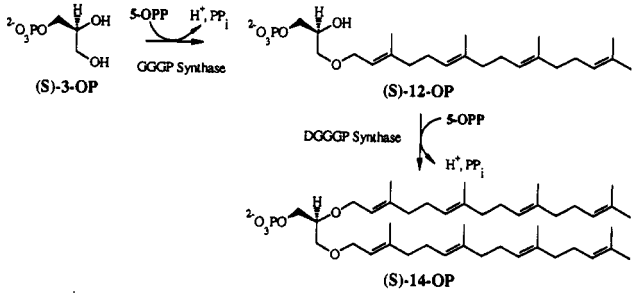
Table I. Substrate Selectivities for the Cytosolic and Microsomal Fractions from *M. thermoautotrophicum*

substrates				dpm in products[a]	
acceptor	donor	dpm	protein	(*S*)-**12**-OP	(*S*)-**14**-OP
(*S*)-[1-^{3}H] **3**-OP	**5**-OPP	3.8×10^5	supernatant	1.9×10^5	0
(*S*)-[1-^{3}H]-**12**-OP	**5**-OPP	1.0×10^6	supernatant	9.9×10^5	0
(*S*)-[1-^{3}H] **3**-OP	**5**-OPP	3.5×10^5	pellet	0	0
(*R*)-[1-^{3}H]-**12**-OH	**5**-OPP	3.2×10^5	pellet	3.2×10^5[b]	0
(*S*)-[1-^{3}H]-**12**-OP	**5**-OPP	2.5×10^6	pellet	2.1×10^6	3.2×10^5

[a] Incubations were at 37 °C for 24 h. Determined for purified alcohols after hydrolysis by alkaline phosphatase. [b] This radioactivity reflects recovery of (*R*)-[1-^{3}H]**12**-OH.

Scheme III. Biosynthesis of Core Membrane Lipids in *M. thermoautotrophicum*



(*S*)-**12**-OP and an additional molecule of **5**-OPP to (*S*)-**14**-OP. These reactions are shown in Scheme III.

Characterization of GGGP Synthase. The substrate selectivity of GGGP synthase was examined for a variety of prenyl donors and acceptors. As shown in Table II, **3**-OH and dihydroxyacetone phosphate (**4**-OP) were not substrates. (*R*)-[^{14}C]**3**-OP gave butanol-extractable radioactivity at approximately 2% of the rate observed for the *S* enantiomer. However, (*S*)-[1-^{3}H]**3**-OP was strongly selected over (*R*)-[U-^{14}C]3-OP in direct competition experiments based on ^{3}H/^{14}C isotope ratios. Among the prenyl donors examined, **5**-OPP was strongly preferred. (*R,R*)-Phytyl diphosphate [(7*R*,11*R*)-**6**-OPP] was incorporated at 18% of the maximal rate seen for **5**-OPP, and fully saturated phytanyl diphosphate [(3*RS*,7*R*,11*R*)-7-OPP] was not a substrate. In addition, the geranylgeranyl moiety was preferred over shorter (**10**-OPP) or longer (**11**-OPP) isoprenoid chains.

Like most prenyltransferases, GGGP synthase requires a divalent metal. In initial experiments, we found that addition of EDTA to buffer containing enzyme, $MgCl_2$, (*S*)-**3**-OP, and **5**-OPP stopped the reaction. Furthermore, cell-free extracts prepared from cells washed with EDTA and disrupted in metal-free buffer were inactive. These observations were used to develop a simple assay for GGGP synthase based on the difference in solubility between glyceryl phosphate and monoether **12**-OP in 1-butanol. Typically, assay mixtures containing (*S*)-[1-^{3}H]**3**-OP and **5**-OPP were incubated in buffer containing 3 mM $MgCl_2$. The reaction was terminated by addition of EDTA, and the mixture was then extracted with 1-butanol. The extent of reaction was determined from the radioactivity in the butanol extracts. Control experiments showed that 99% of the radioactivity associated with (*S*)-[1-^{3}H]**12**-OP was removed by a single extraction. In addition, the discrimination between **3**-OP and **12**-OP was excellent. In a typical run containing 2.3×10^{-2} μmol of (*S*)-[1-^{3}H]**3**-OP (ca. 1.5×10^5 dpm), background levels for controls without enzyme or with heat-inactivated enzyme were 60–200 dpm.

Discussion

The chemical structures of the core lipids of archaebacterial membranes differ from those found in eubacteria and eukaryotes in several important aspects. The archaebacterial core is a diether derivative of glycerol that contains saturated C_{20} isoprenoid hydrocarbon chains, whereas the core lipids of other organisms normally consist of fatty acid esters of glycerol.[9] The glyceryl units in archaebacterial and fatty acid core lipids have opposite absolute stereochemistries.[12,13] In addition, thermophilic archaebacterial lipids contain a unique tetraether in which the ends of the hydrocarbon chains are covalently linked to form a single molecule that spans the bilayer.[23]

Table II. Evaluation of Substrate Selectivity for GGGP Synthase[a]

acceptors[b]	donors[c]	selectivity (nmol min^{-1} mg^{-1})
(*S*)-[^{3}H]**3**-OP	**5**-OPP	0.39
(*R*)-[^{14}C]**3**-OP	**5**-OPP	0.008
[^{14}C]**3**-OH	**5**-OPP	0.002
[^{32}P]**4**-OP	**5**-OPP	<0.0001
(*S*)-[^{3}H]**3**-OP/(*R*)-[^{14}C]**3**-OP	**5**-OPP	0.39/<0.0001
(*S*)-[^{3}H]**3**-OP/[^{32}P]**4**-OP	**5**-OPP	0.39/<0.0001
(*S*)-[^{3}H]**3**-OP/[^{14}C]**3**-OH	**5**-OPP	0.39/<0.0001
(*S*)-[^{3}H]**3**-OP	(7*R*,11*R*)-**6**-OPP	0.08
(*S*)-[^{3}H]**3**-OP	(3*RS*,7*R*,11*R*)-7-OPP	<0.0001
(*S*)-[^{3}H]**3**-OP	**10**-OPP	0.009
(*S*)-[^{3}H]**3**-OP	**11**-OPP	0.05

[a] Determined in 50 mM BHDA, pH 7.2, at 37 °C. [b] 150 μM. [c] 200 μM.

In all organisms studied thus far, glyceryl phosphate arises from an ATP-driven phosphorylation of glycerol by glycerol kinase[34-36] or a reduction of dihydroxyacetone phosphate[37] by glycerol phosphate dehydrogenase. In eubacteria and eukaryotes these enzymes synthesize (*R*)-glyceryl phosphate, which serves as a substrate for the acylation steps. However, our studies show that (*S*)-glyceryl phosphate is the substrate for diether core membrane biosynthesis in *M. thermoautotrophicum*. How the *S* enantiomer is synthesized is unclear. In *Halobacterium halobium*, an extreme halophile, glycerol kinase and glycerol phosphate dehydrogenase both generate (*R*)-glyceryl phosphate,[38] although the opposite stereostructure is embedded in the diether core. Kakinuma and co-workers[24] recently reported an extensive labeling study for incorporation of exogenously supplied glycerol into the diphytanylglyceryl moiety. They found that (a) glycerol is incorporated with its hydroxymethyl moieties intact, (b) the hydrogen at C(2) is lost, and (c) the *pro-R* hydroxymethyl in glycerol becomes the hydroxymethyl moiety in diphytanylglyceryl ethers isolated from the core lipids. On the basis of the assumption that (*R*)-glyceryl phosphate is the only enantiomer synthesized de novo from glycerol in halobacteria,[38] they concluded that dihydroxyacetone phosphate is the acceptor for the first ether-forming prenyltransferase and that the carbonyl group is then reduced to give (*S*)-**12**-OP. However, the substrate selectivity we found for GGGP synthase from *M. thermoautotrophicum* revealed that (*S*)-**3**-OP is preferred to the virtual exclusion of dihydroxyacetone phosphate as the prenyl acceptor. Our results and those of Kakinuma can most economically be reconciled by a yet undetected dehydrogenase activity for reduction of **4**-OP to (*S*)-**3**-OP, which in turn serves as the substrate for GGGP synthase.

However, there is an alternate pathway for biosynthesis of the glycerol moiety in archaebacteria. DeRosa and co-workers[39] discovered that the C(2) hydrogen is *retained* in similar feeding experiments with the thermoacidophilic archaebacterium *Sulfolobus* sp. This observation was recently confirmed by Kakinuma et al.,[40] who reported that, in contrast to *H. halobium*, the *pro-S*

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hydroxymethyl of glycerol becomes the hydroxymethyl moiety in the diphytanylglyceryl core in *Sulfolobus*. It appears that the thermoacidophile possesses a kinase capable of converting glycerol directly to (*S*)-**3**-OP.

Feeding experiments with acetate and mevalonate indicate that isoprenoids in archaebacteria are assembled by the standard mevalonate pathway utilized by other organisms. In addition to hydrocarbon chains containing 1′–4-linked isoprene units like the phytanyl moieties in the membrane lipids, archaebacteia synthesize irregular isoprenoids containing 1′–1 (squalene, carotenoids) and 1′–2 (C_{50} carotenoids) structures.[23] These compounds often constitute a substantial fraction of the dry weight of the organisms.

The diphytanyl core lipid in *M. thermoautotrophicum* is assembled from (*S*)-**3**-OP and **5**-OPP in two reactions. The first, catalyzed by GGGP synthase, apparently occurs in the cytosol and yields (*S*)-**12**-OP. The second reaction is catalyzed by DGGGP synthase, an enzyme appearing in the pellet after a high-speed centrifugation, which produces (*S*)-**14**-OP, as shown in Scheme III. Presumably, the diether is the substrate for further conjugation with a variety of polar head groups to produce archaebacterial membrane lipids.

The substrate selectivity seen for GGGP synthase is typical of a prenyltransferase. All of the isoprenoid diphosphate esters were substrates for reactions with (*S*)-**3**-OP except for fully saturated **7**-OPP. The C(2)–C(3) allylic double bond is necessary to impart sufficient reactivity for production of the electrophilic carbocation that alkylates the hydroxyl group in glycerol.[21] In addition, GGGP synthase prefers GGPP over phytyl diphosphate as its allylic substrate. This trend is also typical of prenyltransferases.[21] In the isoprene pathway, the chain-elongation steps produce polyunsaturated allylic diphosphates that are, in turn, substrates for the prenyltransferases acting at branch points, such as GGGP synthase, that incorporate the isoprene chains into more complex metabolites. Processing reactions, such as hydrogenation of the double bonds, normally occur later. This was recently confirmed for core membrane lipid biosynthesis in *Halobacterium cutirubrum* by pulse-labeling experiments.[19] Although the core lipids are normally saturated in archaebacterial membranes, derivatives containing double bonds are known. For example, Moldoveanu et al.[41] recently reported mixed phytanyl–phytyl diether lipids in the membranes of *Halococcus morrhuae*, and partially saturated trigeranylgeranylglycerol is a major component of nonpolar extracts from *Caldariella acidophila*.[42]

Archaebacterial core lipids are but one example of a much larger family of naturally occurring molecules bearing heteroatoms substituted with isoprenoid residues. Other prominent examples are the dimethylallyl moieties attached to adenine in tRNAs[43] and plant cytokinins[44] and the farnesyl and geranylgeranyl chains attached to cysteine sulfhydryls in a wide variety of eukaryotic proteins.[45] A number of mixed isoprenoids are obtained by alkylation at oxygen. One example is particularly noteworthy. The brown alga *Dilophus fasciola* synthesizes (*R*)-**12**-OH.[46] The alcohol is *identical* in all aspects, including stereochemistry, to the corresponding phosphate produced by GGGP synthase in *M. thermoautotrophicum*. Algal (*R*)-**12**-OH could arise directly in a prenyl-transfer reaction with glycerol as the acceptor or from hydrolysis of (*S*)-**12**-OP. In either case, it is apparent that the enzymatic machinery needed to synthesize isoprenoid glyceryl ethers is not confined to archaebacteria.

In summary, our cell-free studies reveal that the diether core membrane lipids in archaebacteria are assembled from (*S*)-glyceryl phosphate and geranylgeranyl diphosphate in two distinct steps. The ether bonds are formed by a typical prenyl-transfer reaction between an electrophilic allylic diphosphate and an acceptor. Reduction of the double bonds in the isoprenoid chains and additional processing of the polar head group in the core lipids then occur in subsequent steps.

Experimental Section

General Procedures. Analytical TLC was performed on Merck 60 F-254 silica gel 0.25-mm glass plates visualized under UV light by iodine or by dipping in a 5% solution of phosphomolybdic acid in ethanol followed by heating and on Merck cellulose 0.1-mm glass plates visualized by iodine or by spraying with 1% sulfosalicylic acid in ethanol followed by 0.15% ferric chloride solution in ethanol. Flash chromatography was performed on 235–400-mesh silica gel (Baker) or Whatman CF-11 fibrous cellulose. Preparative silica gel TLC plates (20 × 20 cm², 1 mm) were purchased from Analtech. Reversed-phase HPLC was performed on a Waters C_{18} 10-μm Radial Pak cartridge eluted in succession with 75:25 MeOH/H_2O (48 min), 75:25 MeOH/H_2O to MeOH (4 min), and MeOH.

^1H and ^{13}C NMR spectra are referenced to TMS. ^{31}P and ^{19}F spectra are referenced to external 85% phosphoric acid and trifluoroacetic acid, respectively. ^{13}C and ^{31}P NMR chemical shifts are reported for ^1H-decoupled spectra. ^{13}C multiplicities were determined from DEPT experiments.[47] A 20-m DB-5 glass capillary column was used for analytical GC and GCMS experiments. TMS derivatives were prepared according to instructions provided by the supplier.

All solvents for chromatography were reagent grade and were glass-distilled prior to use. Anhydrous diethyl ether and THF were distilled from Na/benzophenone. Acetonitrile was distilled from P_2O_5; CH_2Cl_2, DMF, and pyridine were distilled from NaH. Methanol was distilled from Mg.

All buffers were prepared from deionized water, and pH was adjusted with 3 M HCl or KOH. Cells were disrupted with a Branson cell disrupter (Model 350). The control output was 7 and the duty cycle was 70% for the $^1/_2$-in. horn. The control output was 2.5 and the duty cycle was 70% for disruptions with the stepped microtip. Radioactivity was determined by liquid scintillation spectrometry in Opti-fluor.

Materials. *Candida mycoderma* glycerokinase, NH_4HCO_3, $Na_2H_2P_2O_7$, dihydroxyacetone, dihydroxyacetone phosphate, (*R*)-glyceryl-phosphate, calf intestinal mucosa alkaline phosphatase, DTT, 2-mercaptoethanol, Bis-Tris, glycine, and diethanolamine were purchased from Sigma. Anhydrous NH_3 was from Matheson Gas Products. *N,O*-Bis(trimethylsilyl)-2,2,2-trifluoroacetamide with 1% trimethylsilyl chloride was from Pierce. Ultrapure $(NH_4)_2SO_4$ was purchased from Schwarz/Mann Biotech. $MgCl_2$ was purchased from Mallinckrodt. Ultrapure Tris·HCl was from Bethesda Research Lab. Sodium [^{3}H]-borohydride (25 mCi, 359 mCi/mmol), adenosine 5′-[γ-^{32}P]triphosphate (250 μCi, 3 Ci/μmol), (*R*)-[U-^{14}C]glycerylphosphate (50 μCi, 171 μCi/μmol), and [U-^{14}C]glycerol (100 μCi, 20 μCi/μmol) were purchased from Amersham. (*S*)-2,3-*O*-isopropylideneglyceraldehyde and (*R*)-2,3-*O*-isopropylideneglycerol were prepared according to published procedures.[26,48] $(nBu_4N)_3HP_2O_7$, (3*RS*,7*R*,11*R*)-phytanyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate were prepared as described by Davisson et al.[31,32] [^{32}P]Dihydroxyacetone phosphate was prepared as previously described.[48] All other materials were purchased from Aldrich.

Synthesis of Substrates and Products. (*S*)-2,3-*O*-Isopropylideneglyceryl Phosphate [(*S*)-9-OP]. A solution of 50 mg of (*R*)-2,3-*O*-isopropylideneglycerol (0.38 mmol), DMAP (48 mg, 0.38 mmol), and dimethyl phosphorochloridate (0.4 mL, 1 M solution in CH_2Cl_2) in 1.5 mL of CH_2Cl_2 was allowed to stand at room temperature for 8 h.[27] Methanol (0.3 mL) was added, and solvent was removed by a gentle flow of nitrogen. Solids were removed by filtration and washed twice with ice-cold ethyl ether. Solvent was removed from the combined filtrates under vacuum to yield a colorless oil (89 mg, 98%): $[\alpha]^{25}_D$ +2.4° (*c* 2.3, MeOH); R_f = 0.26 (ethyl acetate); IR (neat) 2990, 2960, 1900, 1640, 1450, 1380, 1280, 1130, 840, 760; ^1H NMR (300 MHz, $CDCl_3$) 1.34 (3 H, s, CH_3), 1.40 (3 H, s, CH_3), 3.75 (7 H, m), 4.00 (3 H, m), 4.27 (1 H, m); ^{13}C NMR (75 MHz, $CDCl_3$) 25.8, 27.2, 55.0 (d, $^2J_{P-C}$ = 5.4 Hz, methyl on phosphate), 66.4, 68.0 (d, $^2J_{P-C}$ = 5.9 Hz), 74.0 (d, $^3J_{P-C}$ = 8.0 Hz), 109.0 (s); ^{31}P NMR (121 MHz, $CDCl_3$) −0.5; MS (CI,

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methane) 101 (18), 115 (25.5), 165 (23), 183 (62), 225 (M - CH_3, 51), 241 (M + 1, 100). HRMS calcd for $C_8H_{18}O_6P$: 241.0855. Found: 241.0841.

A solution of the material (85 mg, 0.33 mmol) in 1.5 mL of CH_2Cl_2 was treated with trimethylsilyl bromide (210 mg, 1.36 mmol).[49] After 2 h, methanol (0.5 mL) was added, and NH_3 was then bubbled through the solution. A white solid formed, solvent was removed with a gentle stream of nitrogen, and the residue was purified by flash chromatography on cellulose (R_f = 0.35, 60:35:5 THF/25 mM NH_4HCO_3/2-propanol). Organic solvent was removed under vacuum, and the aqueous residue was lyophilized to yield 58 mg of a white solid (85%): ^{1}H NMR (300 MHz, D_2O) 3.43 (1 H, dd, J = 11.8, 4.4 Hz), 3.52 (1 H, dd, J = 11.8, 4.4 Hz), 3.57–3.73 (3 H, m); ^{13}C NMR (75 MHz, D_2O) 64.9, 67.7 (d, $^2J_{P-C}$ = 7.5 Hz), 73.5 (d, $^3J_{P-C}$ = 6.7 Hz); ^{31}P NMR (121 MHz, D_2O) 4.22 (t, J_{H-P} = 6.7 Hz); FABMS (negative, glycerol) 91 (99), 153 (64), 171 (acid form M - 1, 100), 343 (2M - 1, 30). HRMS calcd for $C_3H_8O_6P$: 171.0058. Found: 171.0059.

(*S*,*R*)-1-*O*-[α-Methoxy-α-(trifluoromethyl)phenylacetyl]-2,3-*O*-isopropylideneglycerol [(*S*,*R*)-9-OMTP]. A solution of 26 mg of (*R*)-9-OH (0.2 mol), (*R*)-MTPA (47 mg, 0.2 mmol), DMAP (13 mg, 0.1 mmol), and DCC (42 mg, 0.2 mmol) in 2 mL of CH_2Cl_2 was allowed to react for 18 h. Solvent was removed at reduced pressure, and the residue was purified by preparative TLC (R_f = 0.43, 1:4 ethyl acetate/hexane) to give 45 mg (64%) of a white solid: ^{1}H NMR (300 MHz, C_6D_6) 1.20 (3 H, s, CH_3), 1.31 (3 H, s, CH_3), 3.38 (1 H, dd, J = 6.4, 8.5 Hz), 3.44 (3 H, s), 3.55 (1 H, dd, J = 6.4, 8.5 Hz), 3.90 (1 H, m), 4.02 (2 H, d, J = 5.2 Hz), 7.14 (3 H, m), 7.70 (2 H, d, J = 8.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) 25.3 (q), 26.5 (q), 55.5 (q), 65.6 (t), 66.0 (t), 72.8 (d), 109.9 (s), 123.1 (s), 125.6 (q, J_{F-C} = 286 Hz), 127.32 (d), 127.34 (d), 128.4 (d), 129.7 (s), 168.4 (s); ^{19}F NMR (282 MHz, C_6D_6) 4.11; MS (CI, methane) 115 (50), 189 (62), 291 (82), 333 (M - CH_3, 100), 349 (M + 1, 11.5). HRMS calcd for $C_{16}H_{20}O_5F_3$: 349.1262. Found: 349.1263.

(*R*,*R*)-1-*O*-[α-Methoxy-α-(trifluoromethyl)phenylacetyl]-2,3-*O*-isopropylideneglycerol [(*R*,*R*)-9-OMTP]. Following the procedure described for (*S*,*R*)-9-OMTP, (*R*)-MTPA (70 mg, 0.3 mmol), DMAP (19 mg, 0.15 mmol), DCC (62 mg, 0.3 mmol), and (*S*)-9-OH (40 mg, 0.3 mmol) yielded 70 mg (67%) of a colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) 1.21 (3 H, s, CH_3), 1.32 (3 H, s, CH_3), 3.35 (1 H, dd, J = 6.0, 8.4 Hz), 3.47 (3 H, s), 3.55 (1 H, dd, J = 6.0, 8.4 Hz), 3.84 (1 H, dd, J = 6.0, 11 Hz), 3.92 (1 H, m), 4.15 (1 H, dd, J = 6.0, 11 Hz), 7.24 (3 H, m), 7.75 (2 H, d, J = 8.1 Hz); ^{19}F NMR (282 MHz, C_6D_6) 4.16; ^{13}C NMR (75 MHz, C_6D_6) 25.4 (q), 26.8 (q), 55.4 (q), 66.0 (t), 66.3 (t), 73.2 (d), 109.7 (s), 124.2 (s), 126.1 (q, J_{F-C} = 286 Hz), 127.6 (d), 127.8 (d), 128.6 (d), 129.7 (s), 166.2 (s).

(*S*)-[1-^{3}H]Glyceryl Phosphate [(*S*)-[1-^{3}H]3-OP]. (*S*)-2,3-*O*-Isopropylideneglyceraldehyde was synthesized as described by Maloney-Huss.[26] A 52-mg (0.4 mmol) portion was immediately reduced with NaB^3H_4 (0.07 mmol, 359 μCi/μmol). After 3.5 h, $NaBH_4$ (20 mg, 0.52 mmol) was added, and the reaction was allowed to continue for 12 h. Saturated NH_4Cl was added, methanol was removed with a stream of nitrogen, and the residue was suspended in CH_2Cl_2. The organic layer was passed through a plug of $MgSO_4$, and solvent was removed with nitrogen. The material was analyzed by silica TLC (R_f = 0.5, 1:1 ethyl acetate/hexane), and 92% of the radioactivity comigrated with an authentic sample of 9-OH. Recovery of radioactivity was 5%.

The sample of (*R*)-[1-^{3}H]9-OH (0.33 mmol) was treated with 55 mg (0.7 mmol) of pyridine and 73 mg (0.5 mmol) of dimethyl phosphorochloridate as described for nonradioactive material. The product was allowed to react with 1.7 mL of a 1 M solution of trimethylsilyl bromide in CH_2Cl_2 (1.7 mmol) as previously described. The material was purified by flash chromatography on cellulose (R_f = 0.35) to give 53 mg (63%) of radioactive product (specific activity 7.0 μCi/μmol).

Phytyl Bromide (6-Br). Following the procedure of Davisson et al.,[31,32] 0.45 g (1.5 mmol) of phytol was treated with 0.45 g (1.5 mmol) of phosphorus tribromide to yield 0.37 g (69%) of a light yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) 0.28 (3 H, s, CH_3), 0.85 (6 H, s, 2 CH_3), 0.87 (6 H, s, 2 CH_3), 1.00–1.45 (16 H, br m), 1.6 (3 H, m), 2.0 (2 H, m), 4.0 [2 H, d, J = 8.3 Hz, H at C(1)], 5.5 [1 H, t, J = 8.3 Hz, H at C(2)]; ^{13}C NMR (75 MHz, $CDCl_3$) 19.8, 19.9, 22.7, 22.8, 24.6, 24.9, 25.1, 28.1, 29.9, 32.7, 32.9, 36.6, 36.7, 37.36, 37.42, 37.5, 39.4, 39.9, 120.2, 144.0.

Phytyl Diphosphate (6-OPP). Following the procedure of Davisson et al.,[31,32] 0.37 g (1.0 mmol) of 6-Br was treated with 1.9 g (2.0 mmol) of tris(tetra-*n*-butylammonium) pyrophosphate to yield 0.33 g (64%) of a white solid: ^{1}H NMR (300 MHz, D_2O) 0.73 (15 H, s, 5 CH_3), 0.85–1.5 (16 H, br m), 1.6 (3 H, m), 1.9 (2 H, br m), 4.15 (2 H, m, H at C(1)), 5.3 (1 H, m, H at C(2)); ^{13}C NMR (75 MHz, D_2O) 19.6, 19.7, 22.7, 22.8, 24.8, 25.0, 25.5, 27.9, 28.0, 32.8, 37.0, 37.2, 37.4, 37.5, 37.7, 39.5, 62.4, 120.3, 142.7; ^{31}P NMR (121 MHz, D_2O) -9.90 (d, J = 20 Hz), -5.60 (d, J = 20 Hz); FABMS (negative, glycerol) 91 (9), 97 (12), 159 (100), 177 (67), 261 (18), 277 (24), 293 (13), 295 (10), 357 (10), 455 (M - 1, 60). HRMS calcd for $C_{20}H_{41}O_7P_2$: 455.2328. Found 455.2317.

Farnesylgeranyl Chloride (11-Cl). Following the procedure of Davisson et al.,[31,32] 70 mg (0.2 mmol) of farnesylgeraniol (**11-OH**)[50] was treated with NCS (40 mg, 0.30 mmol) and dimethyl sulfide (19 mg, 0.3 mmol) to yield 74 mg (99%) of a light yellow oil: IR (CCl_4) 2960, 2910, 2850, 1660, 1440, 1380, 1250, 740; ^{1}H NMR (300 MHz, $CDCl_3$) 1.65–1.80 (18 H, s, 6 CH_3), 2.1 (16 H, m, 8 CH_2), 4.02 [2 H, d, J = 6.9 Hz, H at C(1)], 5.1 [4 H, m, H at C(6), C(10), C(14), and C(18)], 5.43 [1 H, t, J = 6.9 Hz, H at C(2)].

Farnesylgeranyl Diphosphate (11-OPP). Following the procedure of Davisson et al.,[31,32] 72 mg (0.19 mmol) of **11**-Cl was treated with tris-(tetra-*n*-butylammonium) pyrophosphate (0.5 g, 0.5 mmol). The product was purified by cellulose chromatography (R_f = 0.35, 1:9 25 mM NH_4HCO_3/THF) to yield 115 mg (39%) of a white solid: ^{1}H NMR (300 MHz, D_2O) 1.5 (18 H, m, 6 CH_3), 1.9 (16 H, br m, 8 CH_2), 4.25 [2 H, br s, H at C(1)], 4.9 [4 H, br, H at C(6), C(10), C(14), and C(18)], 5.32 [1 H, br s, H at C(2)]; ^{31}P NMR (121 MHz, D_2O) -9.63 (d, J = 19.5 Hz), -7.6 (d, J = 19.5 Hz); ^{13}C NMR (75 MHz, D_2O) 15.5, 15.6, 15.8, 17.1, 19.2, 23.5, 25.2, 26.3, 26.5, 39.3, 39.5, 64.5, 119.4, 119.5, 123.6, 123.9, 124.1, 130.1, 134.0, 134.1, 134.8, 141.6; FABMS (negative, glycerol) 159 (59.3), 177 (21.2), 231 (24), 252 (24), 261 (41.7), 277 (38.5), 293 (100), 303 (29), 341 (9.2), 360 (7.4), 517 (M - 1, 32.9). HRMS calcd for $C_{25}H_{43}O_7P_2$: 517.2484. Found: 517.2521.

(*S*)-1,2-*O*-Isopropylidene-3-*O*-geranylgeranylglycerol [(*S*)-15]. Following the procedure of Aoki and Poulter,[33] 0.211 g (1.6 mmol) of (*S*)-9-OH was added to a suspension of 0.064 g (1.6 mmol) of KH in 10 mL of THF at 0 °C. After 1 h, 0.532 g (1.5 mmol) of **5**-Br in 1 mL of THF was added within 10 min. The reaction mixture was kept on ice for 30 min and at room temperature for 10 h. The material was purified by silica gel flash chromatography (R_f = 0.35, 2:23 ethyl acetate/hexane) to give 0.33 g (52%) of a colorless oil: $[\alpha]^{25}_D$ +7.5° (*c* 0.83, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) 1.33 (3 H, s, CH_3), 1.42 (3 H, s, CH_3), 1.55–1.65 (15 H, m, 5 CH_3 on geranylgeranyl), 1.9–2.1 (12 H, br m, 6 CH_2 on geranylgeranyl), 3.38–3.68 (3 H, br m), 4.0–4.1 (3 H, m), 4.23–4.3 (1 H, m), 5.08 [3 H, br m, H at C(6), C(10), and C(14) on geranylgeranyl], 5.3 [1 H, t, J = 6.8 Hz, H at C(2) on geranylgeranyl]; ^{13}C NMR (75 MHz, $CDCl_3$) 16.1 (q), 16.6 (q), 17.8 (q), 25.5 (q), 25.8 (q), 26.4 (t), 26.7 (t), 26.87 (t), 26.93 (q), 39.7 (t), 39.81 (t), 39.87 (t), 67.1 (t), 68.1 (t), 71.1 (t), 74.9 (d), 109.5 (s), 120.7 (d), 123.9 (d), 124.3 (d), 124.5 (d), 131.4 (s), 135.1 (s), 135.4 (s), 140.6 (s); MS (EI, 70 eV) 69 (100), 93 (22), 121 (10.6), 135 (15.9), 161 (5.7), 175 (2.9), 189 (1.9), 203 (2.9), 225 (2.4), 272 (3.9), 389 (M - CH_3, 2.1), 404 (M^+, 0.7). HRMS calcd for $C_{25}H_{41}O_3$: 389.3046. Found: 389.3056.

(*R*)-3-*O*-Geranylgeranylglycerol [(*R*)-12-OH]. A solution of 0.135 g (0.29 mmol) of (*S*)-**15** in 7 mL of THF containing 6 mL of 2 N HCl[51] was heated at reflux for 4 h before addition of 4 mL of saturated $NaHCO_3$. The aqueous solution was extracted with ethyl acetate, solvent was removed at reduced pressure, and the residue was purified by silica gel flash chromatography (R_f = 0.30, 1:1 ethyl acetate/hexane) to yield 0.1 g (80%) of a colorless oil: $[\alpha]^{25}_D$ -2.3° (*c* 1.5, $CHCl_3$) (lit.[46] $[\alpha]_D$ -2.1°, $CHCl_3$); IR (CCl_4) 3590, 3454 (br), 2970, 2924, 2863, 1704, 1648, 1530, 1364, 1310, 1098, 1048, 1027, 809; ^{1}H NMR (300 MHz, $CDCl_3$) 1.5–1.7 (15 H, d, 5 CH_3), 1.9–2.1 (12 H, m, 6 CH_2), 3.1 (br, H, on hydroxyl), 3.49 (2 H, m), 3.62 (1 H, dd, J = 4.3, 11.8 Hz), 3.70 (1 H, dd, J = 4.3, 11.8 Hz), 3.85 (1 H, m), 4.0 [2 H, d, J = 6.7 Hz, H at C(1) on geranylgeranyl], 5.0–5.13 [3 H, m, H at C(6), C(10), and C(14) on geranylgeranyl], 5.30 [1 H, t, J = 6.7 Hz, H at C(2) on geranylgeranyl]; ^{13}C NMR (75 MHz, $CDCl_3$) 16.0 (q), 16.5 (q), 17.7 (q), 25.7 (q), 26.3 (t), 26.6 (t), 26.8 (t), 39.6 (t), 39.68 (t), 39.71 (t), 64.2 (t), 67.9 (t), 70.5 (d), 71.6 (t), 120.3 (d), 123.7 (d), 124.2 (d), 124.4 (d), 131.3 (s), 135.0 (s), 135.4 (s), 141.0 (s); MS (CI, methane) 69 (100), 81 (29.7), 95 (12.9), 109 (13.7), 121 (15), 135 (13.2), 149 (7.2), 163 (5.2), 175 (3), 189 (4.2), 211 (4), 225 (2.6), 253 (2.6), 267 (3.8), 273 (6.1), 351 (2.5), 365 (M + 1, 5.7).

(*S*)-1-*O*-(*tert*-Butyldimethylsilyl)-3-*O*-benzylglycerol [(*S*)-3-OTBDMS,OBn]. To an ice-cooled solution of 3.6 g (20 mmol) of (*R*)-3-*O*-benzylglycerol and 2.9 g (43 mmol) of imidazole in 15 mL of DMF was added 3.1 g (21 mmol) of *tert*-butyldimethylsilyl chloride in 5 mL of DMF over 3.5 h. The reaction mixture was kept on ice for 3 h and at room temperature for 8 h before being poured into water and then extracted with 1:4 ethyl acetate/hexane and ethyl ether. The combined organic extracts were washed with ice-cold 0.5 N HCl, saturated

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$NaHCO_3$, and saturated NaCl. Solvent was removed at reduced pressure, and the residue was purified by silica gel flash chromatography (R_f = 0.36, 15:85 ethyl acetate/hexane) to afford 5.1 g (86%) of a colorless oil: $[\alpha]^{25}_D$ +3.5° (*c* 7.7, $CHCl_3$); IR (neat) 3450 (br), 3050, 2955, 2924, 2858, 1470, 1420, 1360, 1260, 1100, 820, 735; ^1H NMR (300 MHz, $CDCl_3$) 0.10 (6 H, s, 2 CH_3 on Si), 0.92 (9 H, s, 3 CH_3 on *tert*-butyl group), 3.60–3.85 (5 H, m), 4.58 (2 H, s), 7.26 (5 H, m); ^{13}C NMR (75 MHz, $CDCl_3$) −5.32 (q), 18.5 (s), 25.9 (q), 64.0 (t), 70.7 (t), 71.0 (t), 73.5 (d), 127.59 (d), 127.62 (d), 127.64 (d), 128.3 (s); MS (EI, 70 eV) 91 (100), 131 (3.3), 180 (44.5), 207 (3.9), 261 (1.7), 297 (M^+, 43.2).

(*S*)-1-*O*-(*tert*-Butyldimethylsilyl)-2-*O*-geranylgeranyl-3-*O*-benzylglycerol [(*S*)-13-OTBDMS, OBn)]. Following the procedure described for (*S*)-**15**, 0.044 g (1.1 mmol) of KH, 0.33 g (1.1 mmol) of (*S*)-3-OTBDMS,OBn, and 0.36 g (1 mmol) of **5**-Br were combined, and the product was purified by chromatography on silica (R_f = 0.57, 2:23 ethyl acetate/hexane) to yield 0.466 g (73%) of a colorless oil: $[\alpha]^{25}_D$ −2.4° (*c* 3.2, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) 0.1 (6 H, s, 2 CH_3 on Si), 0.9 (9 H, s, 3 CH_3 on *tert*-butyl), 1.55–1.7 (15 H, m, 5 CH_3 on geranylgeranyl), 1.95–3.15 (12 H, br m, 6 CH_2 on geranylgeranyl), 3.45–3.70 (5 H, br m), 4.18 [2 H, d, *J* = 6.7 Hz, H at C(1) on geranylgeranyl], 4.58 (2 H, s, CH_2), 5.0–5.2 [3 H, m, H at C(6), C(10), and C(14)], 5.4 [1 H, t, *J* = 6.7 Hz, H at C(2) on geranylgeranyl], 7.25 (5 H, br m); ^{13}C NMR (75 MHz, $CDCl_3$) −5.2 (q), 16.0 (q), 16.5 (q), 17.7 (q), 18.2 (q), 18.5 (s), 25.2 (q), 25.9 (q), 26.37 (t), 26.44 (t), 27.8 (t), 39.6 (t), 39.7 (t), 63.0 (t), 66.9 (t), 70.2 (t), 73.5 (t), 78.5 (d), 121.2 (d), 123.9 (d), 124.2 (d), 124.4 (d), 127.46 (d), 127.50 (d), 128.3 (d), 128.5 (s), 132.0 (s), 134.9 (s), 135.2 (s), 139.7 (s); MS (CI, methane) 69 (61.9), 81 (40.9), 91 (90.9), 109 (15.5), 121 (15.1), 137 (18.9), 149 (15.7), 163 (7.4), 175 (3.3), 189 (8.6), 217 (9.1), 239 (11.4), 257 (2.2), 273 (19.4), 297 (100), 568 (M + 1, 2.0). HRMS calcd for $C_{36}H_{60}O_3Si$: 568.4312. Found: 568.4311.

(*S*)-1-*O*-(*tert*-Butyldimethylsilyl)-2-*O*-geranylgeranylglycerol [(*S*)-13-OTBDMS]. Following the procedure of Stork and Isobe,[52] 0.375 g (0.6 mmol) of (*S*)-3-OTBDMS,OBn was cleaved in 20 mL of liquid NH_3 containing 0.05 g (5 mmol) of Li at −78 °C. The product was purified by chromatography on silica (R_f = 0.36, 15:85 ethyl acetate/hexane) to afford 0.23 g (72%) of a colorless oil: $[\alpha]^{25}_D$ −13.9° (*c* 3, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) 0.1 (6 H, s, 2 CH_3 on Si), 0.95 (9 H, s, 3 CH_3 on *tert*-butyl), 1.6–1.75 (15 H, m, 5 CH_3 on geranylgeranyl), 2.1–2.3 (12 H, br m, 6 CH_2 on geranylgeranyl), 3.5–3.85 (5 H, m), 4.15 [1 H, dd, *J* = 5.9, 11.2 Hz, H at C(1) on geranylgeranyl], 4.24 [1 H, dd, *J* = 5.9, 11.2 Hz, H at C(1) on geranylgeranyl], 5.12 [3 H, m, H at C(6), C(10), and C(14) on geranylgeranyl], 5.45 [1 H, t, *J* = 5.9 Hz, H at C(2) on geranylgeranyl]; ^{13}C NMR (75 MHz, $CDCl_3$) −5.2 (q), 16.0 (q), 16.5 (q), 17.7 (q), 18.2 (q), 18.5 (s), 25.7 (q), 25.8 (q), 26.3 (t), 26.6 (t), 26.7 (t), 39.6 (t), 39.67 (t), 39.70 (t), 63.0 (t), 63.1 (t), 66.6 (t), 78.9 (d), 120.7 (d), 123.8 (d), 124.1 (d), 124.3 (d), 131.3 (s), 134.9 (s), 135.4 (s), 140.5 (s); MS (EI, 70 eV) 69 (100), 81 (99), 93 (59.8), 107 (39), 121 (62.9), 137 (99), 149 (44), 161 (10.8), 189 (11.6), 203 (31.5), 229 (5.5), 257 (3.1), 272 (8.4), 478 (M^+, 1.6). HRMS calcd for $C_{29}H_{54}O_3Si$: 478.3842. Found: 478.3849.

2-*O*-Geranylgeranylglycerol (13-OH). A solution of nBu_4NF in THF (0.2 mL, 1 M) was added to a solution of 48 mg (0.1 mmol) of (*S*)-13-OTBDMS in 5 mL of THF, and the mixture was stirred for 3.5 h at room temperature. Solvent was removed under vacuum. The product was purified by preparative TLC (R_f = 0.30, 1:1 ethyl acetate/hexane) to yield 0.036 g (94%) of a colorless oil: IR (neat) 3450 (br), 2965, 2925, 2875, 1710, 1670, 1445, 1380, 1110, 1050, 910, 730; ^1H NMR (300 MHz, $CDCl_3$) 1.5–1.7 (15 H, 5 CH_3), 1.9–2.1 (12 H, 6 CH_2), 3.5 (1 H, m), 3.67 (2 H, dd, *J* = 4.3, 11.6 Hz, H of glyceryl methylene), 3.75 (2 H, dd, *J* = 4.3 Hz, 11.6 Hz, H of glyceryl methylene), 4.13 [2 H, d, *J* = 6.7 Hz, H at C(1) on geranylgeranyl], 5.08 [3 H, m, H at C(6), C(10), and C(14) on geranylgeranyl], 5.35 [1 H, t, *J* = 6.7 Hz, H at C(2) on geranylgeranyl]; ^{13}C NMR (75 MHz, $CDCl_3$) 16.1 (q), 16.7 (q), 17.8 (q), 25.8 (q), 26.3 (t), 26.7 (t), 26.8 (t), 39.6 (t), 39.7 (t), 39.8 (t), 62.4 (t), 66.4 (t), 78.7 (d), 120.4 (d), 123.6 (d), 124.1 (d), 124.3 (d), 131.2 (s), 134.9 (s), 135.4 (s), 141.0 (s); MS (EI, 17 eV) 69 (100), 81 (99), 93 (50), 107 (25.8), 121 (32.8), 135 (37.5), 149 (9.0), 161 (6.6), 175 (2.7), 189 (3.4), 203 (6.3), 215 (1.4), 229 (2.9), 243 (1.1), 257 (1.9), 272 (2.5), 364 (M^+, 1.2). HRMS calcd for $C_{23}H_{40}O_3$: 364.2977. Found: 364.2999.

(*S*)-1-*O*-(*tert*-Butyldimethylsilyl)-2,3-di-*O*-geranylgeranylglycerol [(*S*)-14-OTBDMS]. Following the procedure described for (*S*)-**15**, 0.02 g (0.5 mmol) of KH, 0.20 g (0.42 mmol) of (*S*)-13-OTBDMS, and 0.20 g (0.56 mmol) of **5**-Br were combined to afford 0.284 g (90%) of a colorless oil. The product was purified on silica (R_f = 0.15, 1:50 ethyl acetate/hexane): ^1H NMR (300 MHz, $CDCl_3$) 0.10 (6 H, s, 2 CH_3 on Si), 0.85 (9 H, s, 3 CH_3 on *tert*-butyl), 1.55–1.75 (30 H, m, 10 CH_3 on geranylgeranyl), 1.95–2.15 (24 H, br m, 12 CH_2 on geranylgeranyl), 3.44–3.65 (5 H, m), 3.95 (2 H, d, *J* = 6.6 Hz), 4.13 (2 H, d, *J* = 6.8 Hz), 5.05 (6 H, br m), 5.36 (2 H, m); ^{13}C NMR (75 MHz, $CDCl_3$) −5.2 (q), 16.1 (q), 16.7 (q), 17.8 (q), 18.4 (q), 25.8 (q), 26.0 (q), 26.5 (t), 26.8 (t), 26.9 (t), 39.7 (t), 38.8 (t), 63.1 (t), 67.0 (t), 70.8 (t), 73.4 (t), 78.6 (d), 121.3 (d), 123.9 (d), 124.1 (d), 124.3 (d), 127.4 (d), 127.5 (d), 128.2 (d), 131.1 (s), 134.8 (s), 135.1 (s), 138.4 (s), 139.6 (s); MS (CI, methane) 69 (100), 81 (68.4), 93 (29.9), 107 (13.8), 123 (26.3), 135 (13), 149 (15.5), 161 (7.5), 171 (4.6), 189 (11.3), 207 (34.9), 217 (11.1), 273 (18.4), 477 (18.8), 545 (2.5), 750 (M + 1, 1.3). HRMS calcd for $C_{49}H_{86}O_3Si$: 750.6346. Found: 750.6344.

(*R*)-2,3-Di-*O*-geranylgeranylglycerol [(*R*)-14-OH]. Following the procedure for **13**-OH, 0.25 g (0.33 mmol) of (*S*)-**14**-OTBDMS was treated with nBu_4NF in THF to yield 0.15 g (72%) of a colorless oil (R_f = 0.22, 3:22 ethyl acetate/hexane): $[\alpha]^{25}_D$ −1.2° (*c* 0.83, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) 1.55–1.75 (30 H, m, 10 CH_3), 1.90–2.15 (24 H, br m, 12 CH_2), 3.43–3.55 (4 H, m), 3.67–3.76 (1 H, m), 3.98–4.01 [2 H, d, *J* = 6.8 Hz, H at C(1) of geranylgeranyl at glyceryl C(3)], 4.05–4.11 [1 H, dd *J* = 7.1, 12.8 Hz, H at C(1) of geranylgeranyl at glyceryl C(2)], 4.14–4.20 [1 H, dd, *J* = 7.1, 12.8 Hz, H at C(1) of geranylgeranyl at glyceryl C(2)], 5.15 (6 H, m), 5.4 (2 H, m); ^{13}C NMR (75 MHz, $CDCl_3$) 16.1 (q), 16.60 (q), 16.63 (q), 17.8 (q), 25.8 (q), 26.4 (q), 26.5 (t), 26.7 (t), 26.9 (t), 29.8 (t), 39.7 (t), 39.76 (t), 39.78 (t), 63.2 (t), 66.6 (t), 68.0 (t), 70.1 (t), 77.5 (d), 120.5 (d), 120.8 (d), 123.8 (d), 124.2 (d), 124.4 (d), 131.2 (s), 134.9 (s), 135.30 (s), 135.32 (s), 140.5 (s); MS (EI, 7 eV) 69 (100), 81 (99), 93 (89), 103 (47.6), 107 (55.5), 121 (60.2), 135 (65.6), 147 (28.1), 161 (25.4), 177 (7.8), 191 (4.7), 203 (25.6), 229 (3.1), 257 (2.3), 272 (25.8), 363 (26.5), 636 (M^+, 0.5). HRMS calcd for $C_{43}H_{72}O_3$: 636.5481. Found: 636.5479.

Cell-Free Studies. Preparation of Cell-Free Extracts. In a typical experiment 5 g of wet cells of *M. thermoautotropicum* was washed with 8 mL of buffer A (50 mM BHDA, 3 mM $MgCl_2$, 1 mM DTT, and 20 μM EDTA, pH 7.2) at 37 °C. The washed cells were suspended in 20 mL of buffer A and disrupted by sonication (8 × 30 s) with a $^1/_2$-in. horn. Cellular debris was removed by centrifugation at 8000*g* for 15 min. This preparation contained activity for GGGP synthase and DGGGP synthase. The enzymes were separated by ultracentrifugation at 200000*g* for 2 h at 4 °C. The supernatant contained GGGP synthase, and the pellet contained DGGGP synthase. Both samples could be frozen and stored at −70 °C prior to use.

Product Studies. In three separate reactions, 12 mg (57 μmol) of (*S*)-**3**-OP and 50 mg (102 μmol) of **5**-OPP were incubated with 40 mL of cell-free extract from 4.5 g of cells at 50 °C for 5 h. The combined mixtures were extracted in succession with 2:1 $CHCl_3$/MeOH and 1-butanol. The organic extracts were combined, and solvent was removed under vacuum. The residue was suspended in 3 mL of the glycine buffer, pH 10.4, and 0.7 mg of alkaline phosphatase was added. The mixture was incubated for 48 h at 37 °C. Additional 0.7-mg portions of phosphatase were added at 15 and 20 h. The hydrolysate was extracted with ethyl ether. Solvent was removed by rotary evaporation. Residual water was removed by addition of 5 mL of acetonitrile and rotary evaporation. The residue was dissolved in 5 mL of CH_2Cl_2 and filtered through a 0.45-μm filter.

Three products were purified by HPLC using a C_{18} reversed-phase column. The major product (4.2 mg) comigrated with authentic **12**-OH and gave identical IR, ^1H and ^{13}C NMR, and GC–mass [bis(TMS) derivative] spectra: $[\alpha]^{25}_D$ −2.4° (*c* 0.4, $CHCl_3$); GCMS (EI, 70 eV) *m/z* 69 (100), 81 (90.8), 93 (37.5), 103 (55.9), 109 (9.2), 117 (11.3), 121 (29.3), 130 (12.4), 135 (35.4), 137 (29.7), 147 (28.9), 161 (6.1), 164 (6.8), 175 (2.2), 187 (2.7), 189 (4.5), 205 (26), 219 (4.9), 237 (8), 257 (1.5), 272 (4.9), 289 (0.9), 361 (0.8), 508 (M^+, 0.4).

The minor product (ca. 200 μg) comigrated with authentic **14**-OH and gave an identical GC–mass spectrum (TMS derivative): MS (EI, 70 eV) *m/z* 69 (100), 81 (90.4), 93 (30.7), 109 (20), 121 (20.2), 135 (20.8), 147 (12.3), 161 (12), 175 (9.7), 189 (7.8), 203 (8.1), 217 (3.7), 229 (4.3), 240 (2.2), 257 (4.9), 272 (10.9), 285 (1.2), 299 (1.2), 313 (1.8), 353 (1.5), 361 (1.4), 368 (2.7), 385 (1.3), 410 (0.9), 435 (7), 446 (1.2), 509 (0.9), 532 (1.1), 545 (0.9), 579 (0.9), 708 (M^+, 0.3).

The third product was geranylgeraniol.

Enzymatic Synthesis of (*S*)-[1-^{3}H]-3-*O*-Geranylgeranylglyceryl Phosphate [(*S*)-[1-^{3}H]12-OP]. A 1.2-mL sample of supernatant after ultracentrifugation (10 mg of protein) containing 2.75 μmol (7 μCi/μmol) of (*S*)-[1-^{3}H]**3**-OP and 2.9 μmol of **5**-OPP was incubated at 55 °C. Additional 1.46-μmol portions of **5**-OPP were added at 0, 30, 75, and 180 min. The mixture was incubated for a total of 4 h and then extracted with water-saturated butanol. The butanol extracts were washed with deionized water, solvent was removed by rotary evaporation, and the residue was suspended in 300 μL of 20 mM NH_4HCO_3. The sample was analyzed by silica TLC. A small portion of the radioactivity (2.2%) comigrated with authentic **12**-OH (R_f = 0.28, 1:1 ethyl acetate/

(52) Stork, G.; Isobe, M. *J. Am. Chem. Soc.* **1975**, *97*, 4745–4746.

hexane), and the rest remained at the origin. A similar analysis on cellulose (R_f = 0.36, 12:7:1 THF/25 mM NH_4HCO_3/2-propanol) gave 1.8% of the radioactivity comigrating with (*S*)-**3**-OP and the remainder at the solvent front. A 10-μL sample (4×10^5 dpm) was treated with 0.1 mg of alkaline phosphatase in 400 μL of 1 M diethanolamine, 3 mM $MgCl_2$, pH 10.5, at 37 °C for 48 h. Cold **12**-OH was added, and the mixture was extracted with ether. The radioactivity in the extract (3.8×10^5 dmp) coeluted with **12**-OH on reversed-phase HPLC.

Time Course for Incubation of (*S*)-[1-^{3}H]-OP and 5-OPP with Cell-Free Extracts. A 3.5-mL portion of cell-free extract containing 30 mg of protein, 110 μg (0.53 μmol) of (*S*)-[1-^{3}H]3-OP (specific activity 7 μCi/μmol), and 2.3 mg (4.7 μmol) of **5**-OPP was incubated at 37 °C. Samples (0.2 mL) were taken at 8, 16, 32, 60, 120, 180, 240, 300, 484, 720, and 1120 min and quenched by adding 0.8 mL of 80 mM EDTA. The samples were extracted with 5.5 mL of water-saturated butanol, and the radioactivity in a 0.5-mL portion of the butanol extract was determined. Butanol was removed from the remainder of the extracts with a stream of nitrogen, and the residues were suspended in 200 μL of glycine buffer, pH 10.4. Alkaline phosphatase (50 μg) was added to each sample. The samples were incubated at 37 °C for 6 h, extracted in succession with pentane and ether, and analyzed by reversed-phase HPLC.

Assay for GGGP Synthase. Assays were run in 150 μL of 50 mM BHDA, pH 7.2, containing 3 mM $MgCl_2$, 1 mM DTT, 150 μM (*S*)-[1-^{3}H]3-OP, 200 μM **5**-OPP, and protein. The mixture was incubated at 37 °C for 10 min, and the reaction was stopped by addition of 100 μL of 0.5 M EDTA. One milliliter of saturated NaCl was added, and the solution was extracted with 3 mL of water-saturated 1-butanol by vortex mixing for 10 s. The layers were allowed to separate, and the radioactivity in a 1-mL portion of the butanol layer was measured by liquid scintillation spectrometry.

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Role of Oxonium, Sulfonium, and Carboxonium Dications in Superacid-Catalyzed Reactions[1]

Nikolai Hartz, Golam Rasul, and George A. Olah*

Contribution from the Loker Hydrocarbon Research Institute and Department of Chemistry, University of Southern California, Los Angeles, California 90089-1661.
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Abstract: Energies, electronic structures, gas-phase proton affinities, and isodesmic enthalpies (ΔH_0^{iso}) of a series of methyl-substituted oxonium and sulfonium dications and carboxonium dications (di-O-protonated carbonyl compounds) were calculated using ab initio molecular orbital theory. On the basis of computed energetics, the proton affinities and decomposition barriers were also estimated. The role of dicationic superelectrophiles in a number of superacid-catalyzed reactions is discussed.

Introduction

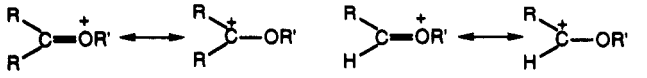
Oxonium and sulfonium ions are well-recognized intermediates in solution chemistry. Trialkyloxonium salts (Meerwein salts) are widely used alkylating agents for heteroatoms, but not for carbon nucleophiles. However, it was observed in the course of our studies that superacids can greatly enhance their reactivity. For example, Me_3O^+ and Et_3O^+, being unreactive toward benzene and toluene under aprotic or low-acidity conditions, readily alkylate them in the presence of superacids.[2]

Hydrogen–deuterium exchange experiments of isotopomeric H_3O^+ in superacids were found to indicate more rapid exchange upon increase of acidity of the media.[3a] This exchange was suggested to proceed via an associative mechanism involving a tetracoordinated oxonium dication. Similar results were reported also for the hydrosulfonium ion.[3b] On the basis of thermodynamic considerations it was subsequently suggested that H_4O^{2+} could be formed exothermically in sulfolane solutions.[4]

H_4O^{2+} and H_4S^{2+} were also subjected to several theoretical investigations.[2,3,5] It was concluded that diprotonation leads to high-lying intermediates with a considerable kinetic barrier toward dissociation. The generation of these dications in the gas phase as well as in solution was predicted to be possible despite the presence of a dipositive charge.

Whereas destabilization by Coulomb repulsion cannot be alleviated through substituent effects in the case of diprotonated water, (considering the gas-phase-isolated molecule, vide infra), it should be possible to stabilize onium dications by introducing appropriate substituents. Furthermore, related sulfonium dications should be even better suited to accommodate a dipositive charge.

In superacidic systems carboxonium ions show greatly enhanced reactivity.[6] Carboxonium ions (e.g., protonated or alkylated carbonyl compounds) which were first studied by Meerwein are highly stabilized in comparison to alkyl cations.[7] They behave as both oxonium and carbenium ions, with the former nature predominating.



R=alkyl, R'=alkyl or H

The spectroscopic properties and chemical behavior of these species fully warrant their depiction as delocalized carboxonium ions.

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