step was now rate limiting. This observation prompted the search for accumulation of the putative amino intermediate **17** by UV–vis and NMR spectroscopy and HPLC isolation of a species that has the anticipated structure. **17** is quantitatively chemically competent but under present conditions kinetically at best marginally competent in subsequent enzyme conversion to anthranilate by anthranilate synthase.

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REFERENCES

Asano, Y., Lee, J. J., Shieh, T. L., Spreafico, F., Kowal, C., & Floss, H. G. (1985) *J. Am. Chem. Soc. 107*, 4314.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Dardenne, G. A., Larsen, P. O., & Wieczorkowska, E. (1975) *Biochim. Biophys. Acta 381*, 416.
Ganem, B. (1978) *Tetrahedron 34*, 3353.
Gibson, F. (1968) *Biochem. Prep. 12*, 94.
Goncharoff, P., & Nichols, B. P. (1984) *J. Bacteriol. 159*, 57.
Haslam, E. (1974) *The Shikimate Pathway*, p 104, Halstead, New York.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Levin, J. G., & Sprinson, D. B. (1964) *J. Biol. Chem. 239*, 1142.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Nagano, H., & Zalkin, H. (1970) *J. Biol. Chem. 245*, 3097.
Paluh, J. L., Zalkin, H., Betsch, D., & Weith, H. L. (1985) *J. Biol. Chem. 260*, 1889.
Pawlak, J. L., & Berchtold, G. A. (1987) *J. Org. Chem. 52*, 1765.
Policastro, P. P., Au, K. G., Walsh, C. T., & Berchtold, G. A. (1984) *J. Am. Chem. Soc. 106*, 2443.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074.
Teng, C.-Y. P., & Ganem, B. (1984) *J. Am. Chem. Soc. 106*, 2463.
Teng, C.-Y. P., Ganem, B., Doktor, S. Z., Nichols, B. P., Bhatnagar, R. K., & Vining, L. C. (1985) *J. Am. Chem. Soc. 107*, 5008.
Weiss, U., & Edwards, J. M. (1980) *The Biosynthesis of Aromatic Compounds*, Chapters 7–12, Wiley, New York.
Zalkin, H. (1985) *Methods Enzymol. 113*, 287.
Zalkin, H., & Hwang, L. H. (1971) *J. Biol. Chem. 246*, 6899.

Stereochemical Analysis of Prenyltransferase Reactions Leading to (*Z*)- and (*E*)-Polyprenyl Chains†

Michio Ito, Masaki Kobayashi, Tanetoshi Koyama,* and Kyozo Ogura*
Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Sendai, 980 Japan
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ABSTRACT: A feasible method was developed to determine the stereochemical direction of the C–C bond formation with respect to the face of the double bond of isopentenyl diphosphate in the prenyltransferase reactions. This method was applied to the reactions of undecaprenyl diphosphate synthase and heptaprenyl diphosphate synthase, which catalyze (*Z*)-prenyl chain elongation and (*E*)-prenyl chain elongation, respectively. In both cases, the C–C bond formation was found to take place at the *si* face of the double bond with elimination of one of the hydrogens of C-2 in a syn fashion.

Among a variety of biochemical reactions, cryptic stereochemistry involved in the conversion between prochiral molecules is interesting from a viewpoint of comparative biochemistry as well as enzymatic reaction mechanism. The chain elongation reactions of isoprenoid biosynthesis have to some extent been studied from such a viewpoint.

Prenyltransferase is the enzyme that catalyzes the chain elongation by sequential condensation of IPP[1] with allylic diphosphates to give a product with a certain chain length and configuration. More than 10 enzymes with different specificities have so far been separated from various organisms.

Since the stereochemistry of farnesyl diphosphate synthase reaction was first studied by Cornforth et al. (1966a), the stereochemistry of the hydrogen elimination from C-2 of IPP has been well elucidated with various prenyltransferases

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[1] Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, (2*E*,6*E*)-farnesyl diphosphate; Tris, tris(hydroxymethyl)aminomethane; MS, mass spectrometry; NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; CD, circular dichroism; HPLC, high-performance liquid chromatography.

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(Poulter & Rilling, 1981). Until very recently, however, pig liver farnesyl diphosphate synthase was the only enzyme that was studied with respect to the stereochemical direction of the C–C bond formation (Cornforth et al., 1966b). Therefore, as far as the C–C bond formation is concerned, there was no reason to assume that the same stereochemical relation might hold widely for prenyltransferases. In particular, the stereochemistry of enzymatic C–C bond formation leading to (*Z*)-prenyl chains has been an interesting problem to be solved. We developed a method of stereochemical analysis and applied it as follows: first, to determine the stereochemical course of the reaction of undecaprenyl diphosphate synthase, which catalyzes the formation of (*Z*)-prenyl chains; second, to determine the stereochemistry of heptaprenyl diphosphate synthase reaction resulting in the formation of (*E*)-prenyl chains.

Part of the study of undecaprenyl diphosphate synthase has been reported as a preliminary communication (Kobayashi et al., 1985).

Experimental Procedures

Materials. Potato acid phosphatase and alkaline phosphatase of calf intestine were obtained from Boehringer Mannheim GmbH. FPP was synthesized from (2*E*,6*E*)-farnesol purchased from Aldrich Chemical Co. by diphosphorylation according to Davisson et al. (1985). Undecaprenyl diphosphate synthase and heptaprenyl diphosphate synthase were purified from *Bacillus subtilis* according to the method of Takahashi et al. (1980, 1982).

*(E)- and (Z)-[4-^{2}H]Isopentenyl Diphosphate (**1** and **2**).* A solution of 3-methylbut-3-en-1-ol (27.0 g) in 60 mL of dry carbon tetrachloride was cooled with ice and stirred during addition of a solution of 50 g of bromine in 100 mL of carbon tetrachloride. After the solvent was removed under reduced pressure, 80 mL of 5 M KOH in methanol was added dropwise to the residual brown oil. After the mixture was stirred for 15 h at room temperature, 1 L of diethyl ether was added, and the white precipitates formed were removed by filtration. The filtrate was washed with water and with saturated NaCl solution, dried over anhydrous $MgSO_4$, and distilled under reduced pressure: yield, 29.23 g (58.5%); bp (2.5 mm) 58–60 °C. The *E* and *Z* isomers of 4-bromo-3-methylbut-3-en-1-ol were separated from each other by applying them at a 6.4 × 120 cm silica gel column, which was eluted with a solvent system of hexane–ethyl acetate (1:1 v/v).

A solution of 6.8 g of (*E*)-4-bromo-3-methylbut-3-en-1-ol and 17.3 g of dry *N*,*N*,*N*′*N*′-tetramethylethylenediamine in 170 mL of absolute diethyl ether was placed in a 300-mL three-necked flask. The solution was cooled to −78 °C on a dry ice-acetone bath, and 32 mL of 1.6 M solution of *n*-butyllithium in hexane (Mitsuwa's Pure Chemical) was added dropwise under vigorous stirring for 1.5 h. Then, 76 mL of 1.3 M solution of *tert*-butyllithium in pentane (Aldrich) was added gradually, and the resulting bright yellow mixture was stirred vigorously for 2 h. Deuterium oxide (99.8%, Stohler Isotope Chemicals, 18 mL) was added, and the mixture was stirred further for 3 h at room temperature. The mixture was extracted with diethyl ether and the extract was washed with dilute HCl until the aqueous layer became acidic. The extract was then washed with saturated NaCl solution, dried over anhydrous $MgSO_4$, and distilled in vacuo.

(*E*)-[4-^{2}H]-3-Methylbut-3-en-1-ol: yield, 1.56 g (43.2%); NMR δ 1.74 (3 H, s), 2.22 (2 H, t), 3.61 (2 H, t), 4.70 (1 H, s).

The *Z* isomer was also obtained by a method similar to that described above: yield, 0.71 g (55.1%); MS *m/z* 87 (M^+, $C_5H_9{}^2HO$), 69, 68, 57 (base peak), 41; NMR δ 1.74 (3 H, s), 2.22 (2 H, t), 3.61 (2 H, t), 4.74 (1 H, s).

The deuterium contents of these alcohols were both 93% as determined by mass spectrometry. Both of the alcohols were diphosphorylated as usual (Kandutch et al., 1964).

*Enzymatic Synthesis of (4S,8S,2E,6E)- and (4R,8R,2E,6E)-[4,8-$^2H_2$]Farnesol (**S-4** and **R-4**).* The incubation mixture contained, in a final volume of 1 L, 20 mmol of Tris-HCl buffer, pH 7.7, 10 mmol of $MgCl_2$, 7 mmol of 1,4-dithiothreitol, 300 μmol of DMAPP, 300 μmol of **1**, and 100 mg of protein of farnesyl diphosphate synthase from pig liver (Holloway & Popják, 1967). Two flasks containing the above mixture were kept at 37 °C for 24 h and then the contents adjusted to pH 9 with Tris-HCl buffer. To hydrolyze the diphosphate esters, 2000 units of alkaline phosphatase was added and the mixture incubated again at 37 °C for 36 h. The product was extracted with pentane and purified with a 0.9 × 25 cm column of silica gel. (4*S*,8*S*,2*E*,6*E*)-[4,8-$^2H_2$]Farnesol (*S*-**4**) was eluted with a solvent system of pentane–diethyl ether (1:1 v/v): yield, 17.8 mg (26.4% based on **1**); MS *m/z* 224 (M^+, $C_{15}H_{24}{}^2H_2O$), 206, 193, 181, 163, 137, 94, 82, 69 (base peak), ^{2}H content, 92% NMR δ ($CDCl_3$) 1.61 (6 H, s), 1.69 (6 H, s), 2.04 (6 H, m), 4.14 (2 H, d), 5.10 (2 H, br t), 5.41 (1 H, br t); $[\alpha]_{320}$, +3.0 ± 1.3°.

(4*R*,8*R*,2*E*,6*E*)-[4,8-$^2H_2$]Farnesol (*R*-**4**) was also obtained by a similar incubation with DMAPP and **2** as substrates: yield, 15.6 mg (47.6% based on **2**); ^{2}H content, 92%; $[\alpha]_{320}$, −3.8 ± 0.5°.

*Ozonolysis of (4S,8S,2E,6E)- and (4R,8R,2E,6E)-[4,8-$^2H_2$]Farnesol (**S-4** and **R-4**) to (S)- and (R)-[3-^{2}H]Levulinic Acid (**S-5** and **R-5**).* (4*S*,8*S*,2*E*,6*E*)-[4,8-$^2H_2$]Farnesol (*S*-**4**, 17.8 mg) was ozonized in 2 mL of ethyl chloride at −78 °C with a stream of ozone in oxygen until a pale blue color persisted in the solution. Removal of the solvent at room temperature left the ozonide, to which 0.5 mL of 98% formic acid and 0.1 mL of 30% H_2O_2 were added. After the mixture had been allowed to stand for 4 h at room temperature, the peroxides were decomposed by gradual addition of powdered $FeSO_4$ until the evolution of gas ceased. A small amount of diethyl ether was added, and the mixture was dried over anhydrous $MgSO_4$. Filtration of the mixture through a glass filter and evaporation of the solvent gave (*S*)-[3-^{2}H]levulinic acid (*S*-**5**).

Ethereal diazomethane was added dropwise to a solution of *S*-**5** in diethyl ether at 0 °C, and the mixture was kept at 4 °C for 12 h. The mixture was dried over anhydrous $MgSO_4$. Filtration of the mixture through a glass filter and evaporation of the solvent gave methyl (*S*)-[3-^{2}H]levulinate: yield, 2.71 mg (13.1%); MS *m/z* 131 (M^+, $C_6H_9{}^2HO_3$), 116, 100, 89, 72, 43 (base peak), ^{2}H content, 92%; $[\alpha]_{320}$, +63.3 ± 17.3° (diethyl ether, *c* 0.23).

Methyl (*R*)-[3-^{2}H]levulinate was also obtained similarly from *R*-**5**: yield, 2.91 mg (16.0%); ^{2}H content, 92%; $[\alpha]_{320}$, −82.8 ± 6.2° (diethyl ether, *c* 0.27).

Enzymatic Synthesis of (2Z,6Z,10Z,14Z,18Z,22Z,26Z,-30E,34E)-[4,8,12,16,20,24,28-$^2H_7$]Decaprenol and (2Z,6Z,10Z,14Z,18Z,22Z,26Z,30Z,34E,38E)-[4,8,12,16,-20,24,28,32-$^2H_8$]Undecaprenol. The alcohols with the *S* configurations were synthesized as follows: Five flasks each containing, in a final volume of 1 L, 100 mmol of Tris-HCl buffer, pH 8.5, 500 μmol of $MgCl_2$, 50 mmol of NH_4Cl, 5 g of Triton X-100, 25 μmol of FPP, 100 μmol of **1**, and 200 mL of a solution of undecaprenyl diphosphate synthase from *B. subtilis* (Takahashi & Ogura, 1982) were kept at 37 °C for 24 h. After incubation, about 2 g of KOH was added to stop the enzymatic reaction. About 500 g of $(NH_4)_2SO_4$ was

added, and the products were extracted with 1-butanol. The solvent was removed on a rotary evaporator, and the residue was treated as usual with potato acid phosphatase for hydrolysis (Fujii et al., 1982). The polyprenols formed were purified by silica gel TLC with a solvent system of benzene–ethyl acetate (9:1), followed by HPLC with a 2.6 × 500 mm column of Hitachi gel 3011 and a solvent system of methanol–hexane (4:1). [$^2H_7$]Decaprenol: yield, 5.2 mg (5.7% based on **1**); MS, cf. Figure 2A; ^2H content, 93%; $[\alpha]_{320}$, +5.9 ± 1.2°. [$^2H_8$]Undecaprenol: yield, 14.2 mg (15.9% based on **1**); MS, cf. Figure 2B; ^2H content, 93%; $[\alpha]_{320}$, +6.3 ± 0.5°.

The alcohols with the *R* configurations were synthesized in the same way as described above except that **2** instead of **1** was used as the homoallylic substrate. Yield: [$^2H_7$]decaprenol, 4.3 mg (4.7% based on **2**); [$^2H_8$]undecaprenol, 9.8 mg (11.3% based on **2**).

Enzymatic Synthesis of (all-E)-[4,8,12,16-$^2H_4$]Heptaprenol. The alcohol that has the *S* configurations was synthesized as follows: The incubation mixture contained, in a final volume of 1 L, 100 mmol of Tris-HCl buffer, pH 7.0, 10 mmol of $MgCl_2$, 25 μmol of FPP, 25 μmol of **1**, and 200 mL of a solution of heptaprenyl diphosphate synthase prepared from *B. subtilis* (Takahashi et al., 1980). Nine reaction mixtures were made and they were incubated at 37 °C for 20 h. The procedures for the extraction and purification of the products were similar to those described in the last section: yield, 9.1 mg (29.2% based on **1**); MS *m/z* 498 (M^+, $C_{35}H_{54}{}^2H_4O$), 480, 411, 343, 275, 206, 137, 69 (base peak); ^2H content, 93%.

The alcohol with the *R* configurations was synthesized in the same way as described above except that **2** instead of **1** was used as the homoallylic substrate: yield, 6.8 mg (21.8% based on **2**); MS *m/z* 498 (M^+, $C_{35}H_{54}{}^2H_4O$), 480, 411, 343, 275, 206, 137, 69 (base peak); ^2H content, 92%.

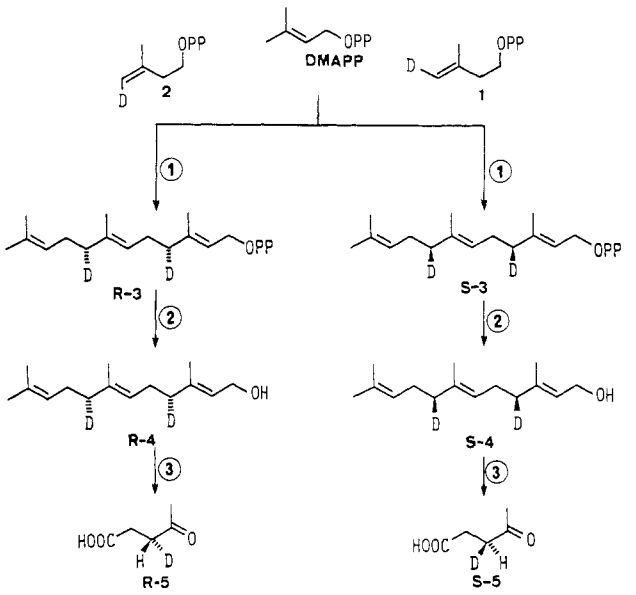
Ozonolysis of Polyprenols. The enzymatically synthesized polyprenols were ozonized in 3 mL of dichloromethane–pentane solution (3:1 v/v) at −78 °C with a stream of ozone in oxygen until a pale blue color persisted in the solution, and then the solvent was removed on a rotary evaporator. To the resulting ozonide was added 0.5 mL of 98% formic acid and 0.1 mL of 30% H_2O_2, and the mixture was allowed to stand at room temperature for 4 h. The peroxides were decomposed by gentle addition of powdered $FeSO_4$ until the evolution of gas ceased. The mixture was dried over anhydrous $MgSO_4$. After filtration of the mixture through a glass filter, the solvent was removed by evaporation. [3-^2H]Levulinic acid thus obtained was subjected to CD analysis. Methyl [3-^2H]levulinate was obtained by the treatment of the acid with diazomethane.

Spectroscopic Measurements. A Shimadzu-LKB gas chromatograph–mass spectrometer, Type 9000, was used. The contents of ^2H were calculated from the relative intensities of the molecular ions in each mass spectrum by comparison with those of the corresponding nonlabeled authentic samples. The potential of the ionizing electron beam was 70 eV. CD spectra were measured in methanol with 10-mm light path on Jasco CD spectrograph, Type J-400X. ORD spectra were measured in diethyl ether with a light path of 2 mm on Jasco ORD spectrograph, Type ORD–UV-5.

Results

Method of Stereochemical Analysis. Cornforth et al. (1966b) have determined the steric face of the C–C bond formation in the farnesyl diphosphate synthase reaction to be *si* by identifying the absolute configuration of [2-^2H] succinic acid derived from (2*E*,6*E*)-[4,8,12-$^2H_3$]farnesol biosynthesized from (2*R*,3*R*)-[2-^2H]mevalonic acid via six steps of enzymatic reactions using a crude homogenate of pig liver. It is generally difficult to obtain enzyme systems capable of converting mevalonate into polyprenyl products other than farnesol so efficiently that the method of Cornforth et al. can be applied. Now, however, it is preferable to analyze the stereochemistry of prenyltransferase reactions using purified enzymes, since purified preparations of various prenyltransferases have become available.

Scheme I: Synthesis of (*S*)- and (*R*)-[3-^2H]Levulinic Acid (*S*-**5** and *R*-**5**)[a]



[a] Key: 1, farnesyl diphosphate synthase from pig liver; 2, alkaline phosphatase; 3, O_3.

Therefore, we devised a method of analysis that could be applied directly to prenyltransferase reactions on the basis of the following strategy. A prenyltransferase reaction with an allylic diphosphate and (*E*)- or (*Z*)-[4-^2H]IPP (**1** or **2**) would give on ozonolysis (*S*)- or (*R*)-[3-^2H]levulinic acid (*S*-**5** or *R*-**5**), which could be correlated with a specimen derived from the chiral (2*E*,6*E*)-[4,8-$^2H_2$]farnesol (*S*-**4** or *R*-**4**) synthesized from DMAPP and **1** or **2** using pig liver farnesyl diphosphate synthase whose stereochemistry of reaction is known (Cornforth et al., 1966b; Scheme I).).

Substrates **1** and **2** were synthesized by diphosphorylation of the corresponding alcohols synthesized as follows: 3,4-Dibromo-3-methylbutan-1-ol obtained by bromination of 3-methylbut-3-en-1-ol was treated with KOH to give (*E*)- and (*Z*)-4-bromo-3-methylbut-3-en-1-ol, which were separated from each other by silica gel chromatography. The bromo derivatives were converted into the corresponding lithium compounds, which were then quenched with ^2H_2O to give (*E*)- and (*Z*)-[4-^2H]-3-methylbut-3-en-1-ol.

It is a key to this study to know the relation between the configuration of [3-^2H]levulinic acid and its optical rotation. It is also important to know whether the optical rotation value would be sufficient to distinguish between the enantiomers obtained in only small amounts because limited amounts of enzyme are available.

In order to obtain (*S*)- and (*R*)-[3-^2H]levulinic acid (*S*-**5** and *R*-**5**), ^2H-labeled chiral farnesols were synthesized on a scale of 2-L incubation (Scheme I). A 10-mg quantity of (4*S*,8*S*,2*E*,6*E*)-[4,8-$^2H_2$]farnesol (*S*-**4**) was obtained from **1** and DMAPP as substrates by the action of pig liver farnesyl diphosphate synthase followed by the treatment with alkaline phosphatase, and it was subjected to ozonolysis to afford (*S*)-[3-^2H]levulinic acid (*S*-**5**). Similarly the *R* isomer (*R*-**5**)

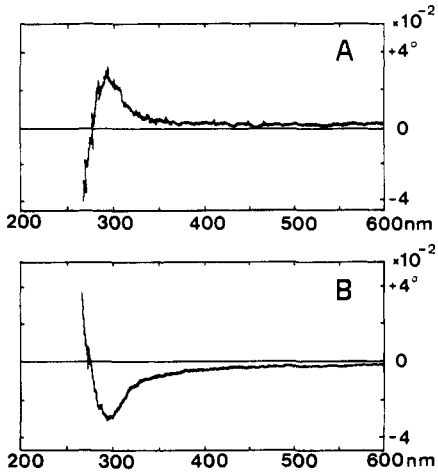


FIGURE 1: ORD curves of the [3-^{2}H]levulinic acids derived from (2*E*,6*E*)-[4,8-$^2H_2$]farnesols: (A) (*S*)-[3-^{2}H]levulinic acid (*S*-**5**) derived from (4*S*,8*S*,2*E*,6*E*)-[4,8-$^2H_2$]farnesol (*S*-**4**) (diethyl ether, *c* 0.23); (B) (*R*)-[3-^{2}H]levulinic acid (*R*-**5**) from (4*R*,8*R*,2*E*,6*E*)-[4,8-$^2H_2$]-farnesol (*R*-**4**) (diethyl ether, *c* 0.27).

was also synthesized enzymatically from **2** in place of **1**. As shown in Figure 1, the ORD spectra of the enantiomers *S*-**5** and *R*-**5** indicated that they were clearly distinguishable from each other, showing positive and negative Cotton effects, respectively. They could be differentiated by CD spectroscopy as well.

Stereochemistry of Undecaprenyl Diphosphate Synthase Reaction. The method was first applied to the stereochemical analysis of the reaction catalyzed by undecaprenyl diphosphate synthase, which occurs widely in bacteria as an enzyme responsible for the synthesis of the glycosyl carrier lipid essential for the synthesis of cell wall components (Allen, 1985). Large-scale incubations were made with **1** or **2** and FPP as substrates with partially purified undecaprenyl diphosphate synthase obtained from *B. subtilis* (Takahashi & Ogura, 1982). The enzymatic reaction products were treated with acid phosphatase by the method of Fujii et al. (1982) to give the corresponding alcohols, which were then purified by TLC and HPLC. They were identified as [$^2H_7$]decaprenol and [$^2H_8$]undecaprenol by mass spectroscopy (Figure 2). It is noteworthy that the main fragmentation of both [$^2H_7$]- and [$^2H_8$]prenols begins at and continues from the ω end. Thus, the first three main fragments eliminated after loss of H_2O (69, 68, and 68) contain no deuterium, but the subsequent C_5 units lost contain deuterium. The polyprenols were combined and subjected to ozonolysis. The CD spectrum of the levulinic acid derived from the products of the incubation of **1** and FPP as substrates showed a positive Cotton curve (Figure 3A), indicating that the configuration was *S*. On the contrary, the spectrum of the levulinic acid derived from the products of the incubation of **2** and FPP showed a negative Cotton curve (Figure 3B), indicating that the configuration was *R*. Mass spectral analysis showed that the deuterium contents of the levulinic acids were both approximately 65%. This low value is not a result of deuterium loss but is accounted for by the dilution with the nonlabeled levulinic acid derived from the (*all-E*)-farnesyl moiety of the polyprenols. Therefore, it was evident that the C–C bond formation in the undecaprenyl diphosphate synthase reaction took place at the *si* face of the double bond of IPP.

The present observations, taken in conjunction with the previous finding that the 2-*pro-S* hydrogen is lost during the reaction of this enzyme (Takahashi & Ogura, 1982), indicate that the enzymatic synthesis of (*Z*)-polyprenyl chains proceeds in such a stereochemical mode that the C–C bond is formed on the same side as the C–H bond is cleaved (Scheme IIB). This mode of reaction is similar to that of farnesyl diphosphate

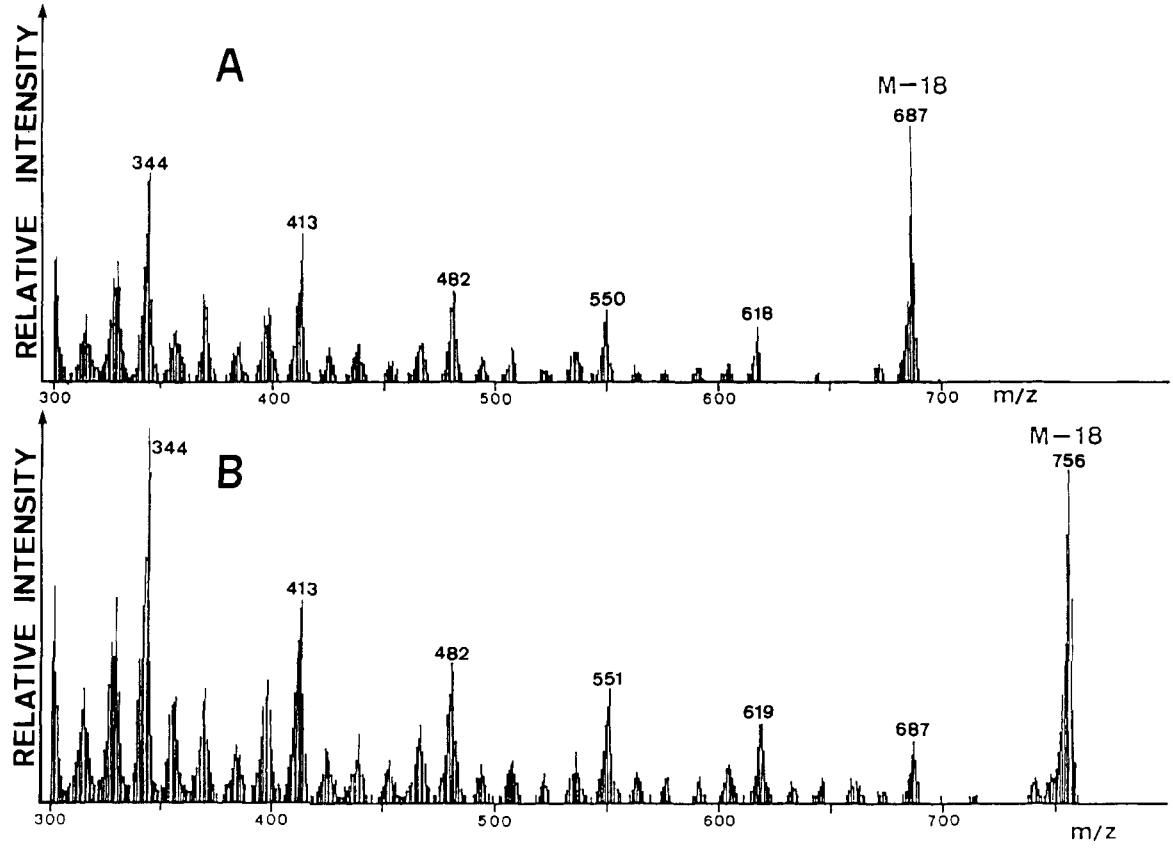


FIGURE 2: Mass spectra of the polyprenols derived from **1** and FPP as substrates by the action of undecaprenyl diphosphate synthase: (A) [$^2H_7$]decaprenol; (B) [$^2H_8$]undecaprenol.

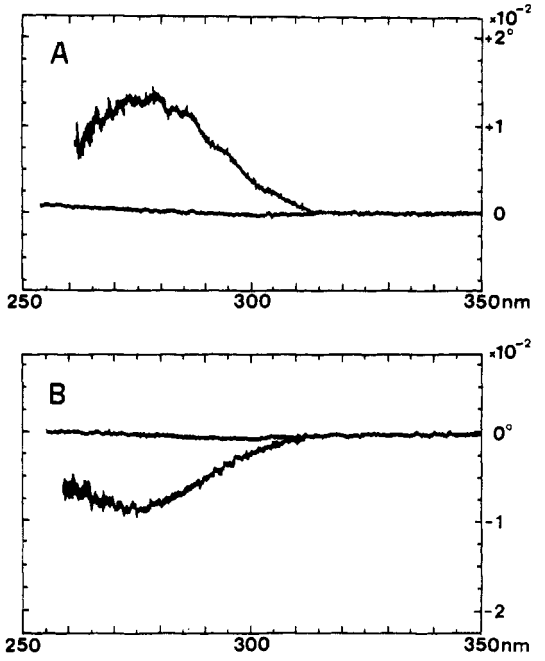


FIGURE 3: CD spectra of the [3-^{2}H]levulinic acids resulting from ozonolysis of [$^2H_7$]decaprenol and [$^2H_8$]undecaprenol: (A) [3-^{2}H]-levulinic acid derived from the polyprenols obtained by the incubation of **1** and FPP as substrates (methanol, *c* 0.25); (B) [3-^{2}H]levulinic acid derived from the polyprenols obtained by the incubation of **2** and FPP as substrates (methanol, *c* 0.21).

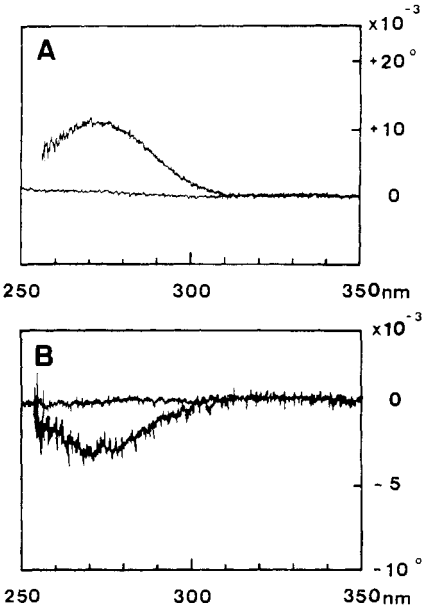


FIGURE 4: CD spectra of the [3-^{2}H]levulinic acids resulting from ozonolysis of [$^2H_4$]heptaprenols: (A) [3-^{2}H]levulinic acid derived from [$^2H_4$]heptaprenol obtained by the incubation of **1** and FPP as substrates (methanol, *c* 0.27); (B) [3-^{2}H]levulinic acid derived from [$^2H_4$]-heptaprenol obtained by the incubation of **2** and FPP as substrates (methanol, *c* 0.16).

synthase in terms of syn addition–elimination, though farnesyl diphosphate synthase catalyzes the formation of an *E* chain with loss of the 2-*pro-R* hydrogen (Cornforth et al., 1966a).

Stereochemistry of Heptaprenyl Diphosphate Synthase Reaction. We were also interested in the study of the stereochemistry of the C–C bond formation leading to an (*E*)-polyprenyl chain catalyzed by heptaprenyl diphosphate synthase, which is one of the two polyprenyl diphosphate synthases of *B. subtilis* (Takahashi et al., 1980) and is responsible for the synthesis of menaquinone-7. Large-scale incubations were made with **1** and FPP as substrates with partially purified heptaprenyl diphosphate synthase. The [$^2H_4$]heptaprenol obtained by acid phosphatase treatment (Fujii et al., 1982) of the synthase reaction product was subjected to ozonolysis. The CD spectrum of the resulting levulinic acid showed a positive Cotton effect (Figure 4A), indicating that the configuration was *S*. On the other hand, the spectrum of the levulinic acid derived from the enzymatic reaction starting with **2** and FPP showed a negative Cotton curve as shown in Figure 4B, indicating that the configuration was *R*. Therefore, it was evident that the C–C bond formation in the heptaprenyl diphosphate synthase reaction also took place at the *si* face of the double bond of IPP (Scheme IIC).

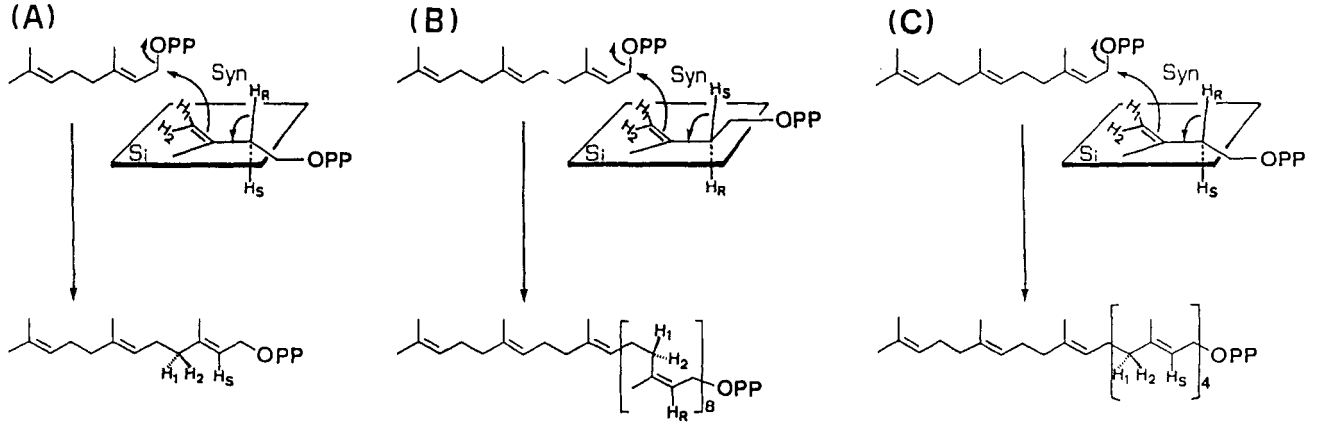
Since previous work in this laboratory (Takahashi et al., 1980) has shown that the 2-*pro-R* hydrogen of IPP is eliminated during the reaction of this synthase, it is concluded that the stereochemical course of this bacterial synthase reaction is exactly the same as that of the farnesyl diphosphate synthase reaction of pig liver (Cornforth et al., 1966b).

Recently, we demonstrated that the stereochemistry of the reaction catalyzed by farnesyl diphosphate synthase of pumpkin fruit is the same as that of pig liver enzyme (Ito et al., 1986; Scheme IIA). Namely, the stereochemical mode of "*si* addition–*pro-R* elimination" is common to the three (*all-E*)-prenyl diphosphate synthases of different organisms, from bacteria to higher organisms.

Discussion

It is interesting that the direction of the C–C bond formation is the same as that of the C–H bond to be cleaved at the

Scheme II: Stereochemistry of Reactions Catalyzed by Farnesyl Diphosphate Synthase from Pig Liver (Cornforth et al., 1966b) and Pumpkin Fruit (Ito et al., 1986) (A), by Undecaprenyl Diphosphate Synthase from *B. subtilis* (B), and by Heptaprenyl Diphosphate Synthase from *B. subtilis* (C)



2-position of IPP in the prenyltransferase reactions irrespective of the configuration of the double bond to be formed. Cunningham and Overton (1975) have shown that in a synthetic model system there is a stereochemical preference for the syn relationship between the newly formed C–C bond and the allylic hydrogen atom that is lost during an S_E2' reaction. Similar is the case for the stereochemistry of prenyltransferase reactions thus far revealed by us and others. However, this consistency does not necessarily warrant the S_E2' nature for the prenyltransferase reaction, because the stereospecificity in enzymatic reaction may be a result of how the enzyme binds the substrates. Actually, the proton addition and elimination at C-4 and from C-2 of IPP are in an anti relation in the reaction catalyzed by isopentenyl diphosphate isomerase (Clifford et al., 1971), though this reaction is also regarded formally as an S_E2' reaction. Poulter and Rilling [(1978) and references cited therein] have confirmed the electrophilic character of farnesyl diphosphate synthase reaction and suggested that prenyltransferase reactions take place by an ionization–condensation–elimination mechanism. They have also made a reasonable suggestion that the substrates may bind to the enzyme in such a way that the departing pyrophosphate ion will assist stereospecific removal of the proton from C-2 of IPP.

It is interesting from a standpoint of comparative biochemistry that the *si* addition of the C–C bond formation is common to all the four enzymes so far investigated. It was once generally accepted that the 4-*pro-S* hydrogen of mevalonic acid, which is biogenetically equivalent to the *pro-R* hydrogen at C-2 of IPP, is lost in the formation of (*E*)-prenyl residues, whereas the 4-*pro-R* hydrogen (equivalent to the 2-*pro-S* hydrogen of IPP) is lost in the formation of (*Z*)-prenyl residues (Cornforth et al., 1966a; Archer et al., 1966; Poulter & Rilling, 1981). Recently, however, some papers have appeared reporting that (*Z*)-prenyl resideus are formed in some higher plants with retention of the 4-*pro-R* hydrogen of mevalonic acid (Jedlicki et al., 1972; Suga et al., 1983 a,b, 1986; Banthorpe et al., 1985). It would be very interesting to investigate the stereochemical relationship between the newly formed C–C bond and the C–2 hydrogen atom that is "unusually" eliminated in the reaction catalyzed by prenyltransferases from such kinds of higher plants.

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REFERENCES

Allen, C. M. (1985) *Methods Enzymol. 110*, 281–299.
Archer, B. L., Barnard, D., Cockbain, E. G., Cornforth, J. W., Cornforth, R. H., & Popják, G. (1966) *Proc. R. Soc. London, Ser. B 163*, 519–523.
Banthrope, D. V., Bunton, C. A., Cori, O., & Francis, M. J. O. (1985) *Phytochemistry 24*, 251–252.
Clifford, K., Cornforth, J. W., Mallaby, R. & Phillips, G. T. (1971) *J. Chem. Soc., Chem. Commun.*, 1599–1600.
Cornforth, J. W., Cornforth, R. H., Donninger, C., & Popják, G. (1966a) *Proc. R. Soc. London, Ser. B 163*, 492–514.
Cornforth, J. W., Cornforth, R. H., Popják, G., & Yengoyan, L. (1966b) *J. Biol. Chem. 241*, 3970–3987.
Cunningham, I. M., & Overton, K. H. (1975) *J. Chem. Soc., Perkin Trans. 1*, 2140–2149.
Davisson, V. J., Woodside, A. B., & Poulter, C. D. (1985) *Methods Enzymol. 110*, 130–144.
Fujii, H., Koyama, T., & Ogura, K. (1982) *Biochim. Biophys. Acta 712*, 716–718.
Holloway, P. W., & Popják, G. (1967) *Biochem. J. 104*, 57–70.
Ito, M., Koyama, T., & Ogura, K. (1986) *Chem. Lett.*, 101–104.
Jedlicki, E., Jacob, G., Faini, F., Cori, O., & Bunton, C. A. (1972) *Arch. Biochem. Biophys. 152*, 590–596.
Kandutch, A. A., Paulus, H., Levin, E., & Bloch, K. (1964) *J. Biol. Chem. 239*, 2507–2515.
Kobayashi, M., Ito, M., Koyama, T., & Ogura, K. (1985) *J. Am. Chem. Soc. 107*, 4588–4589.
Popják, G., & Cornforth, J. W. (1966) *Biochem. J. 101*, 553–568.
Poulter, C. D., & Rilling, H. C. (1978) *Acc. Chem. Res. 11*, 307-313.
Poulter, C. D., & Rilling, H. C. (1981) in *Biosynthesis of Isoprenoid Compounds* (Porter, J. W., & Spurgeon, S. L., Eds.) Vol. 1, pp 165–171, Wiley, New York.
Suga, T., Hirata, T., Aoki, T., & Shishibori, T. (1983a) *J. Am. Chem. Soc. 105*, 6178–6179.
Suga, T., Aoki, T., Hirata, T., & Saragai, Y. (1983b) *Chem. Lett.*, 1467–1470.
Suga, T., Hirata, T., Aoki, T., & Kataoka, T. (1986) *J. Am. Chem. Soc. 108*, 2366–2371.
Takahashi, I., & Ogura, K. (1982) *J. Biochem. (Tokyo) 92*, 1527–1537.
Takahashi, I., Ogura, K., & Seto, S. (1980) *J. Biol. Chem. 255*, 4539–4543.