^1H NMR ($CDCl_3$) δ 6.23 (m, 1 H), 5.93 (dd, J_1 = 17 Hz, J_2 = 10 Hz, 1 H), 5.3-5.0 (m, 2 H), 2.1 (s. 3 H), 2.86 (s, 3 H), 1.2 (s, 6 H); ^{13}C NMR ($CDCl_3$) δ 220.71 (s), 155.78 (s), 143.1 (d), 120.43 (d), 113.63 (t), 50.03 (s), 28.0 (q), 23.55 (q), 21 (q); IR (film) 2980, 2920, 1685, 1625, 1470, 1450, 1382, 1367, 1108, 1028 cm^{-1}.

Reactions of Trisubstituted Sulfone (7g) with Base. (a) Potassium *tert*-Butoxide. To a stirred solution of 107 mg (0.28 mmol) of **7g** in 4 mL of THF, at 0 °C, was added 63 mg (0.56 mmol) of potassium *tert*-butoxide, in one solid portion. The resultant mixture was stirred at 0 °C overnight and than at room temperature for 1 h. The solution was then diluted with 20 mL of ether and treated with 1 mL of saturated ammonium chloride. The aqueous phase was extracted with an additional ether portion, and the combined organics were washed with brine, dried over magnesium sulfate (anhydrous), and then concentrated under reduced pressure. This affords 70 mg of pale yellow oil, NMR analysis of which reveals a mixture of 53% (syn/anti)-2-methyl-2,4-dipentyl-1,3-dithietane 1,1,3,3-tetraoxide (**19g**) and 45% (*E*/*Z*)-6-methyl-6-dodecene (**8g**). Chromatography of this crude oil on silica gel (petroleum ether) provided 20 mg (39%) of **8g** with an *E*/*Z* ratio of 2.2:1 (obtained from 300-MHz integral comparison of the vinyl methyl region[31]): ^1H NMR ($CDCl_3$) δ 5.12 (br t, 1 H), 2.04-1.90 (m, 4 H), 1.67 (br s, 0.94 H, CH_3 of *Z* isomer), 1.57 (br s, 2.06 H, CH_3 of *E* isomer), 1.45-1.1m (m, 12 H), 0.95-0.84 (m, 6 H); ^{13}C NMR ($CDCl_3$) δ 135.34 (s), 135.09 (s), 125.30 (d). 124.57 (d); IR (film) 2960, 2938, 2880, 2860, 1465, 1382, 1130, 1115 cm^{-1}. Further elution (7:3 petroleum ether-methylene chloride) provided 40 mg (46%) of **19g** as an oil (1.6:1 mixture of isomers[32]): ^1H NMR ($CDCl_3$) δ 5.51 (t, *J* = 7.5 Hz, 0.6 H), 5.45 (t, *J* = 7.5 Hz, 0.4 H), 2.35-1.15 (m, 4 H), 1.96 (s, 1.8 H), 1.82 (s, 1.2 H), 1.57-1.27 (m, 12 H), 0.95-0.85 (m, 6 H); ^{13}C NMR ($CDCl_3$) δ 105.47 (s), 105.13 (s), 100.33 (d), 99.19 (d), 32.41 (t), 31.36 (t), 30.88 (t), 30.80 (t), 30.78 (t), 26.24 (t), 26.09 (t), 25.84 (t), 24.62 (t), 24.24 (t), 24.0k (t), 22.06 (t), 22.00 (t), 16.97 (q), 13.70 (q), 13.74 (q), 13.67 (q); IR (film) 2970, 2940, 2880, 2860, 1470, 1450, 1340, 1160, 1110, 922 cm^{-1}. Anal. Calcd for $C_{13}H_{26}S_2O_4$: C, 50.29; H, 8.44; S, 20.65. Found: C, 50.31; H, 8.63; S, 20.70.

(b) *n*-Butyllithium (2 equiv). To a stirred solution of 127 mg (0.334 mmol) of **7g** in 4 mL of THF, at -78 °C, was added 0.42 mL (0.667 mmol) of a 1.6 M solution of *n*-butyllithium in hexane. The bath was allowed to slowly warm to room temperature and stir overnight. Workup (ammonium chloride-ether as above) gave a colorless oil which was analyzed by NMR to contain **7g**, **19g**, and **8g** in a ratio of 2:1:2.5, respectively. The yields calculated based on weight obtained are as follows: **7a**, 24%; **19g**, 11.6%; **8g**, 30%: (34.4% unaccounted for).

(c) *n*-Butyllithium (3 equiv). Procedure was identical with (b) above. Affords a crude oil, which by NMR contains **7g**, **19g**, and **8g** in a ratio of 0:1:1, respectively. Actual yields based on weight: **7g**, <1%; **19g**, 37%; **8g**, 37%; (25% unaccounted for). Chromatography as in (a) above lead to isolated yields: **19g**, 15%; **8g**, 29%.

(d) Tetrabutylammonium Fluoride. To a stirred solution of 63 mg (0.166 mmol) of **7q** at room temperature was added dropwise 0.66 mL (0.662 mmol) of a 1 M solution of tetrabutylammonium fluoride in THF. The resultant solution was stirred for 3 h, diluted with 30 mL of ether and washed with water (2×) and then brine. The organic layer was dried over magnesium sulfate (anhydrous) and concentrated under reduced pressure to afford 43 mg of pale yellow oil. NMR analysis of this residue reveals >90% **19g** (actual yield = 83%). The experiment was repeated starting at -78 °C, warming to room temperature over 1.5 h, and then stirring for 1 h. The yield of **19g** was 92% with barely a trace of **8g** visible by thin-layer chromatographic analysis (silica-methylene chloride).

Acknowledgment. We are indebted to the Donors of the Petroleum Research Fund, administered by the American Chemical Society, for the support of this research.

(25) Two compounds (**6k** and **7e**) were made from the α-phenyl reagent, $CF_3SO_2CH(C_6H_5)SO_2CH_3$, which was prepared from benzyl triflone anion and methanesulfonic acnhydride: G. J. Boudreaux, Ph.D. Thesis, Brandeis University, 1985.
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Stereochemistry of Hydrogen Elimination in the Biosynthesis of Polyprenols in Higher Plants[1]

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Abstract: In the biosynthesis of polyprenols in the leaves of *Mallotus japonicus*, the elimination of the C(4) prochiral hydrogen of mevalonate during the formation of their (*E*)-prenyl chain followed Cornforth's basic principle for isoprenoid biosynthesis, but the formation of their (*Z*)-prenyl chain involved, contrary to the basic principle, the elimination of the *pro*-4*S* hydrogen of mevalonate. The reversed hydrogen elimination during the formation of the (*Z*)-prenyl chain was confirmed by tracer experiments using geranylgeranyl pyrophosphate and stereospecifically ^3H-labeled isopentenyl pyrophosphate as a prerequisite substrate. The biological formation of the (*Z*)-prenyl chain of polyprenols was demonstrated to result from the successive addition of isopentenyl pyrophosphate to geranylgeranyl pyrophosphate. The elimination of the reversed hydrogen was found to be a common occurrence in the formation of the (*Z*)-prenyl chain of polyprenols in the leaves of 11 other higher plants examined hitherto.

The basic principle of the mechanism in the biological formation of prenyl pyrophosphate was established by the studies on the biosynthesis of squalence in yeast and mammalian enzymes[2-4] and of polyprenol in rubber latex.[5] The principle involves the stereochemical picture that the *pro*-4*S* hydrogen of mevalonic acid (MVA) is lost in the formation of an (*E*)-prenyl unit, while the *pro*-4*R* hydrogen is eliminated in the formation of a (*Z*)-prenyl

(1) This paper forms Part 1 in the series "The Biosynthesis of Isoprenoids in Higher Plants" and in part has been presented in preliminary forms (a-c) and communications (d and e): (a) the ACS/CSJ Chemical Congress, Honolulu, HI, April 1979. (b) 23rd Symposium on the Cnemistry of Natural Products, Nagoya, Japan, Oct 1980. (c) 2nd U.S.-Japan Seminar on the Biosynthesis of Natural Products, Honolulu, HI, Sept 1982. (d) Suga, T.; Hirata, T.; Aoki, T.; Shishibori, T. *J. Am. Chem. Soc.* **1983**, *105*, 6178-6179. (e) Suga, T.; Aoki, T.; Hirata, T.; Saragai, Y. *Chem. Lett.* **1983**, 1467-1470.

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Table I. Radioactivities and $^3H/^{14}C$ Atom Ratios in the Malloprenols Biosynthesized from (4*R*)- and (4*S*)-[2-^{14}C,4-^3H]MVAs with the Leaves of *M. japonicus*

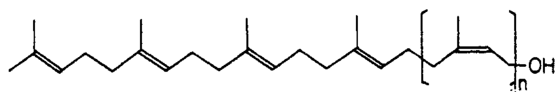
MVA used ($^3H/^{14}C$ ratio)	compd[a]	obsd				^3H:^{14}C expected atom ratio	
		^3H, dpm	^{14}C, dpm	$^3H/^{14}C$ ratio	^3H:^{14}C atom ratio[b]	A[c]	B[d]
(4*R*)-[2-^{14}C,4-^3H]MVA							
(4.22)	MPL-9	1045	246	4.25	(9.1 ± 0.2):9	4:9	9:9
	MPL-10	2602	600	4.34	(10.3 ± 0.2):10	4:10	10:10
	MPL-11	5666	1343	4.22	(11.0 ± 0.1):11	4:11	11:11
(3.44)	MPL-10	8108	2264	3.58	(10.4 ± 0.1):10	4:10	10:10
	MPL-11	7681	2200	3.49	(11.2 ± 0.1):11	4:11	11:11
(13.2)	MPL-10	3797	291	13.1	(9.9 ± 0.2):10	4:10	10:10
	MPL-11	4745	359	13.2	(11.0 ± 0.2):11	4:11	11:11
(4*S*)-[2-^{14}C,4-^3H]MVA							
(13.2)	MPL-9	658	1133	0.58	(0.40 ± 0.06):9	5:9	0:9
	MPL-10	981	3909	0.25	(0.19 ± 0.02):10	6:10	0:10
	MPL-11	922	4703	0.20	(0.17 ± 0.01):11	7:11	0:11
(5.4)	MPL-9	135	4717	0.03	(0.05 ± 0.02):9	5:9	0:9
	MPL-10	88	548	0.16	(0.30 ± 0.02):10	6:10	0:10
	MPL-11	43	1167	0.37	(0.07 ± 0.01):11	7:11	0:11
(13.2)	MPL-10	2185	7073	0.31	(0.24 ± 0.01):10	6:10	0:10
	MPL-11	5471	2196	0.25	(0.21 ± 0.01):11	7:11	0:11

[a] MPL denote malloprenol. [b] Normalized atom ratios. The deviations were calculated on the ground of the standard deviation in the counting of the radioactivity of each samples. [c] Calculated by expecting that the (*E*)- and the (*Z*)-prenyl units are formed by loss of the *pro*-4*S* and *pro*-4*R* hydrogen atoms of MVA, respectively, following the doctorinal isoprenoid pathway. [d] Calculated by expecting that the (*E*)-prenyl units result from the usual loss of the *pro*-4*S* hydrogen atom of MVA, whereas the (*Z*)-prenyl units do from the unusual loss of the *pro*-4*S* hydrogen atom.

unit. It has generally been accepted that this stereochemical picture is involved in the biosynthesis of all the isoprenoids.[6-13] However, the hydrogen elimination that breaks the basic principle had been observed in the formation of the (*Z*)-prenyl unit of nerol[14-17] and (2*Z*,6*E*)-farnesol,[14] although it is still ambiguous whether these (2*Z*)-prenols resulted from the isomerization of the corresponding (2*E*)-prenols or the direct formation from C_5 precursors without any isomerization.[18-20] We have now established that the hydrogen elimination opposed to Conforth's principle occurs during the elongation of the (*Z*)-prenyl units in the biosynthesis of polyprenols in higher plants and here wish to report the results.

Results and Discussion

Polyprenols present in the leaves of *Mallotus japonicus* Muell Arg. (subclass Dilleniidae) had been previously established to be homologues as shown in structures **1–4** and named malloprenol-9,



1, $n = 5$; **2**, $n = 6$; **3**, $n = 7$; **4**, $n = 8$

-10, -11, and -12, respectively. These malloprenols had been demonstrated to be biosynthesized by successive cis condensation of isoprene residues with (2*E*,6*E*,10*E*)-geranylgeranyl pyrophosphate (GGPP) by comparison of incorporations of (all-*E*)-di-, (all-*E*)-tri-, (all-*E*)-tetra-, and (all-*E*)-pentaprenyl [1,1-$^3H_2$]-pyrophosphates with those of their corresponding 2*Z* isomers into the malloprenols.[21]

The labeling pattern in the (*E*)- and (*Z*)-prenyl units of the malloprenols was examined by the incorporation of double-labeled (4*R*)- and (4*S*)-[2-^{14}C,4-^3H]MVAs. The $^3H/^{14}C$ ratios of the malloprenols biosynthesized from these precursors were as shown in Table I. If the malloprenols are biosynthesized from double-labeled MVA following Cornforth's basic principle that the (*E*)- and (*Z*)-prenyl units are formed by loss of the *pro*-4*S* and *pro*-4*R* hydrogens of MVA, respectively,[2-5] the $^3H/^{14}C$ atom ratios in the malloprenols are expected to be as given in column A of Table I. This table shows the inconsistency of the expected atom ratios with the ratios observed for the malloprenols. On the other hand, the observed $^3H/^{14}C$ atom ratios were in good agreement with the atom ratios given in column B, which were calculated by expecting that the (*E*)-prenyl units result from the usual loss of the *pro*-4*S* hydrogen of MVA, whereas the (*Z*)-prenyl units

Table II. Radioactivities and Molar Ratios of the Degradation Products from the Malloprenols Biosynthesized from (4*S*)-[2-^{14}C,4-^3H]MVA

compd[a]	obsd			ACE:LA expected molar ratio	
	sp act, dpm/mmol		ACE:LA molar ratio[b]		
	ACE[a]	LA[a]		A[c]	B[d]
MPL-10	2.29×10^5	2.47×10^5	1:(9.7 ± 0.6)	1:9	1:3
MPL-11	4.52×10^5	4.75×10^5	1:(10.5 ± 0.1)	1:10	1:3
MPL-11	5.33×10^5	5.53×10^5	1:(10.4 ± 0.8)	1:10	1:3

[a] MPL, ACE, and LA denote malloprenol, acetone, and levulinic acid, respectively. [b] The deviations were calculated from the standard deviation in the radioactivity of each sample. [c] Calculated from the expectation that the (*E*)- and (*Z*)-prenyl units are equivalently formed from external MVA. [d] Calculated from the expectation that external MVA cannot participate in the biosynthesis of the (*Z*)-prenyl units.

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Table III. Radioactivities and $^3H/^{14}C$ Atom Ratios in the Malloprenols Biosynthesized from (2*S*)- and (2*R*)-[2-^3H,4-^{14}C]IPPs with the Leaves of *M. japonicus*

		obsd				^3H:^{14}C expected atom ratio	
IPP used ($^3H/^{14}C$ ratio)	compd[a]	^3H, dpm	^{14}C, dpm	$^3H/^{14}C$ ratio	^3H:^{14}C atom ratio[b]	A[c]	B[d]
(2*S*)-[2-^3H,4-^{14}C]IPP (5.92)	MPL-9	3100	545	5.69	(8.6 ± 0.1):9	4:9	9:9
	MPL-10	3550	632	5.62	(9.5 ± 0.3):10	4:10	10:10
	MPL-11	3350	563	5.95	(11.1 ± 0.2):11	4:11	11:11
	MPL-12	3130	493	6.35	(12.9 ± 0.2):12	4:12	12:12
(2*R*)-[2-^3H,4-^{14}C]IPP (5.59)	MPL-9	163	287	0.57	(0.91 ± 0.03):9	5:9	0:9
	MPL-10	146	560	0.26	(0.47 ± 0.02):10	6:10	0:10
	MPL-11	153	745	0.21	(0.40 ± 0.01):11	7:11	0:11
	MPL-12	64	417	0.15	(0.33 ± 0.02):12	8:12	0:12

[a] MPL denote malloprenol. [b] Normalized atom ratios. The deviations were calculated on the grounds of the standard deviation in the counting of the radioactivity of each samples. [c] Calculated by expecting that the (*E*)- and the (*Z*)-prenyl units are formed by loss of the *pro*-2*R* and *pro*-2*S* hydrogen atoms of IPP, respectively, following the doctorinal isoprenoid pathway. [d] Calculated by expecting that the (*E*)-prenyl units result from the usual loss of the *pro*-2*R* hydrogen atom of IPP, whereas the (*Z*)-prenyl units does from the unusual loss of the *pro*-2*R* hydrogen atom.

result from the unusual loss of the *pro*-4*S* hydrogen. These observations clearly indicate the occurrence of the *pro*-4*S* hydrogen elimination of MVA during the biological formation of the (*Z*)-prenyl chain of the malloprenols.

Loss of the 4-tritium might be due to compartmentalization[23] such that the (*E*)-prenyl residue is formed in a part of the plant to which external MVA is readily accessible and the (*Z*)-prenyl residue is formed in a part of the plant to which external MVA cannot efficiently penetrate. In order to clarify this point, the distribution of the radioactivity was examined by determining the labeling pattern in the malloprenols biosynthesized from (4*S*)-[2-^{14}C,4-^3H]MVA. The radioactive malloprenol-10 and -11 were degraded to ^{14}C-labeled acetone and levulinic acid by $KMnO_4$–$NaIO_4$ oxidation. The molar ratios of the acetone to the levulinic acid were in good agreement with the ratios calculated from the expectation that the (*E*)- and (*Z*)-prenyl units are equivalently formed from external MVA, as shown in Table II. These examinations demonstrate that compartmentalization does not affect the incorporation of label in the biosynthesis of the malloprenols in the plant. Thus, these feeding experiments of MVA obviously indicate that the formation of the (*E*)-prenyl chain follows Cornforth's basic principle,[2-5] but the formation of the (*Z*)-prenyl chain involves, contrary to the basic principle, the elimination of the *pro*-4*S* hydrogen of MVA.

The stereochemistry of hydrogen elimination was further examined by feeding experiments of the direct precursors, (2*S*)- and (2*R*)-[2-^3H,4-^{14}C]isopentenyl pyrophosphates, with the leaves of *M. japonicus*. The radioactivities of the malloprenols (**1–4**) biosynthesized were as shwon in Table III. If the malloprenols are formed from the double-labeled isopentenyl pyrophosphate (IPP) following Cornforth's basic principle that the (*E*)- and (*Z*)-prenyl units are formed by loss of the *pro*-2*R* and *pro*-2*S* hydrogens of IPP, respectively, the $^3H/^{14}C$ atom ratios in the malloprenols are expected to be as given in column A of Table III. The atom ratios observed for the malloprenols were inconsistent with those expected. The observed atom ratios were in good agreement with those given in column B, which were calculated on the assumption that the (*E*)-prenyl units result from loss of the *pro*-2*R* hydrogen atom of IPP, whereas the (*Z*)-prenyl units result from loss of the *pro*-2*R* hydrogen atom. These observations clearly indicate occurrence of the *pro*-2*R* hydrogen elimination of IPP (equivalent to the *pro*-4*S* hydrogen of MVA) during the biological formation of the (*Z*)-prenyl chain of the malloprenols.

The mode of the hydrogen elimination in the formation of the (*Z*)-prenyl units was furthermore examined by incubating GGPP and IPP with an enzyme system of *M. japonicus*. The radioactivities of a mixture of malloprenol-9 (**1**) to -12 (**4**) were as shown in Table IV. When (2*S*)-[2-^3H,4-^{14}C]IPP was used as a precursor, the $^3H/^{14}C$ ratio in the IPP was retained in the resulting malloprenols; this indicates no loss of the tritium atom in the biological formation of the (*Z*)-prenyl units. When the 2*R*-^3H-labeled isomer was used as a precursor, on the other hand, great loss of the tritium atom was observed in the malloprenols. These observations indicate that the *pro*-2*R* hydrogen of IPP is eliminated during the formation of the (*Z*)-prenyl chain of the malloprenols by successive addition of IPP to GGPP.

We next investigated the stereochemistry of the hydrogen elimination during the biological formation of the polyprenols in 11 other higher plants, which were selected from all seven subclasses belonging to dicotyledons in Takhtajan's classification[24] of flowering plants. These 11 higher plants are as follows: *Magnolida glandiflora* L. (subclass Magnoliidae), *Nandina do-*

Table IV. Radioactivities and $^3H/^{14}C$ Ratios in the Malloprenols Biosynthesized from Geranylgeranyl Pyrophosphate and (2*S*)- and (2*R*)-[2-^3H,4-^{14}C]IPPs with the Enzyme Preparation of *M. japonicus*

	malloprenols biosynthesized		
IPP used ($^3H/^{14}C$ ratio)	^3H, dpm	^{14}C, dpm	$^3H/^{14}C$ ratio[a]
(2*S*)-[2-^3H,4-^{14}C]IPP (9.90)	717	69	10.5 ± 1.3
(2*R*)-[2-^3H,4-^{14}C]IPP (7.61)	913	980	0.93 ± 0.07

[a] The deviations were calculated from the standard deviation in the radioactivity of each sample.

Table V. Content of Polyprenols and Composition of Polyprenol Homologues in Several Higher Plants

plants	content of polyprenols, mg/g of leaves	composition, % C_{45}	C_{50}	C_{55}	C_{60}	C_{65}
subclass Magnoliidae						
Magnolia glandiflora	0.19	1	6	61	32	
subclass Rununcuilidae						
Nandina domestica	0.54		20	72	8	
subclass Hamamelididae						
Alnus serrulatoides	0.69		13	70	17	
Betula platyphylla	0.63	13	42	43	2	
subclass Caryophyllidae						
Mirabilis jalapa	0.44	10	18	51	21	
subclass Dilleniidae						
Aleurites cordata	0.68	5	24	57	14	
Cleome spinosa	0.51	20	41	33	6	
Euphorbia pulcherrima	1.08	11	25	53	11	
Mallotus japonicus	1.81	10	40	43	7	
Triadica sebifera	0.63	7	46	43	4	
subclass Rosidae						
Aesculus turbinata	0.67		4	32	55	9
subclass Asteridae						
Solanum melongena	0.06		4	42	54	

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Table VI. Radioactivities and $^3H/^{14}C$ Atom Ratios in the Polyprenols Biosynthesized from (4*R*)-[2-^{14}C,4-^3H]MVA with the Leaves of Several Higher Plants

(4*R*)-[2-^{14}C,4-^3H]MVA, $^3H/^{14}C$ ratio	compd[a]	obsd ^3H, dpm	obsd ^{14}C, dpm	obsd $^3H/^{14}C$ ratio	obsd $^3H:^{14}C$ atom ratio[b]	$^3H:^{14}C$ expected atom ratio A[c]	$^3H:^{14}C$ expected atom ratio B[d]
3.57	MGPL-9	1218	336	3.63	(9.2 ± 0.2):9	4:9	9:9
	MPGL-10	1923	496	3.88	(10.9 ± 0.2):10	4:10	10:10
2.76	NDPL-10	4242	1602	2.65	(9.6 ± 0.1):10	4:10	10:10
	NDPL-11	7343	2798	2.62	(10.4 ± 0.1):11	4:11	11:11
3.72	ASPL-11	2400	678	3.54	(10.5 ± 0.1):11	4:11	11:11
4.38	BPPL-10	9520	2107	4.52	(10.3 ± 0.1):10	4:10	10:10
	BPPL-11	4572	1041	4.39	(11.0 ± 0.1):11	4:11	11:11
3.91	MJPL-9	26640	6571	4.05	(9.3 ± 0.2):9	4:9	9:9
	MJPL-12	8791	2287	3.84	(11.8 ± 0.2):12	4:12	12:12
3.03	ACPL-10	456	159	2.87	(9.5 ± 0.1):10	4:10	10:10
	ACPL-11	900	306	2.94	(10.7 ± 0.1):11	4:11	11:11
	ACPL-12	1061	348	3.05	(12.1 ± 0.1):12	4:12	12:12
2.19	CSPL-11	13632	6441	2.12	(10.6 ± 0.1):11	4:11	11:11
3.58	EPPL-10	3976	1111	3.58	(10.0 ± 0.1):10	4:10	10:10
4.13	TSPL-10	11617	2970	3.91	(9.5 ± 0.1):10	4:10	10:10
	TSPL-11	1073	270	3.97	(10.6 ± 0.1):11	4:11	11:11
	TSPL-12	594	142	4.18	(12.1 ± 0.1):12	4:12	12:12
4.55	ATPL-13	1372	303	4.53	(12.9 ± 0.1):13	4:13	13:13
2.76	SMPL-10	13634	4970	2.74	(9.9 ± 0.1):10	4:10	10:10
	SMPL-11	1233	452	2.73	(10.9 ± 0.1):11	4:11	11:11

[a] MGPL, NDPL, ASPL, BPPL, MJPL, ACPL, CSPL, EPPL, TSPL, ATPL, and SMPL denote polyprenols obtained from *M. gradiflora*, *N. domestica*, *A. serrulatoides*, *B. Platyphylla*, *M. jalapa*, *A. cordata*, *C. spinosa*, *E. pulcherrima*, *T. sebifera*, *A. turbinate*, and *S. melongena*, respectively. [b] Normalized atom ratios. The deviations were calculated from the standard deviation in the radioactivity of each sample. [c] Calculated from the expectation that the (*E*)- and the (*Z*)-prenyl units are formed by loss of the *pro*-4*S* and *pro*-4*R* hydrogen atoms of MVA, respectively. [d] Calculated from the expectation that both the (*E*)- and the (*Z*)-prenyl units are formed by loss of the *pro*-4*S* hydrogen atom of MVA.

Table VII. Radioactivities and $^3H/^{14}C$ Atom Ratios in the Polyprenols Biosynthesized from (4*S*)-[2-^{14}C,4-^3H]MVA with the Leaves of Several Higher Plants

(4*S*)-[2-^{14}C,4-^3H]MVA, $^3H/^{14}C$ ratio	compd[a]	obsd ^3H, dpm	obsd ^{14}C, dpm	obsd $^3H/^{14}C$ ratio	obsd $^3H:^{14}C$ atom ratio[b]	$^3H:^{14}C$ expected atom ratio A[c]	$^3H:^{14}C$ expected atom ratio B[d]
14.1	MGPL-9	218	209	1.04	(0.66 ± 0.03):9	5:9	0:9
	MGPL-10	274	303	0.90	(0.64 ± 0.02):10	6:10	0:10
11.8	NDPL-10	307	325	0.94	(0.80 ± 0.01):10	6:10	0:10
	NDPL-11	342	658	0.52	(0.48 ± 0.01):11	7:11	0:11
13.6	ASPL-11	198	255	0.78	(0.63 ± 0.11):11	7:11	0:11
12.8	BPPL-10	105	350	0.30	(0.23 ± 0.01):10	6:10	0:10
	BPPL-11	132	156	0.85	(0.73 ± 0.01):11	7:11	0:11
11.6	MJPL-9	623	3663	0.35	(0.27 ± 0.01):9	5:9	0:9
	MJPL-12	1791	4995	0.73	(0.76 ± 0.01):12	8:12	0:12
13.5	ACPL-10	563	2359	0.24	(0.18 ± 0.06):10	6:10	0:10
	ACPL-11	502	1477	0.34	(0.28 ± 0.08):11	7:11	0:11
	ACPL-12	824	1925	0.43	(0.38 ± 0.07):12	8:12	0:12
13.4	CSPL-11	2744	6884	0.40	(0.33 ± 0.01):11	7:11	0:11
9.76	EPPL-10	219	2381	0.09	(0.09 ± 0.01):10	6:10	0:10
12.7	TSPL-10	165	1137	0.15	(0.11 ± 0.01):10	6:10	0:10
	TSPL-11	86	1187	0.07	(0.06 ± 0.01):11	7:11	0:11
	TSPL-12	15	476	0.03	(0.03 ± 0.01):12	8:12	0:12
12.1	ATPL-13	92	181	0.51	(0.55 ± 0.01):13	9:13	0:13
11.8	SMPL-10	809	4177	0.19	(0.16 ± 0.01):10	6:10	0:10
	SMPL-11	290	489	0.59	(0.55 ± 0.01):11	7:11	0:11

[a-d] These correspond to *a–d* in Table VI.

mestica Thunb. (subclass Ranuncuilidae), *Alnus serrulatoides* Call. and *Betula platyphylla* Sukatchev var. *japonica* Hara (subclass Hamamelididae), *Mirabilis jalapa* L. (subclass Caryophyllidae), *Aleurites cordata* Muell. Arg., *Cleome spinosa* L., *Euphorbia pulcherrima* Willd., and *Triadica sebifera* Small (subclass Dilleniidae), *Aesculus turbinate* Blume (subclass Rosidae), and *Solanum melongena* L. (subclass Asteridae). The leaves of these 11 plants contain C_{45}, C_{50}, C_{55}, and C_{60} homologues of the polyprenols as in the case of *M. japonicus*, as shown in Table V. The structures of these polyprenols were confirmed by comparison of their IR, ^1H and ^{13}C NMR and MS spectra, HPLC, and reversed-phase TLC with those of the malloprenols. The leaves of *A. turbinate* contained a C_{65} homologue in addition to a series of C_{45}, C_{50}, C_{55}, and C_{60} homologues.

The labeling pattern in the (*E*)- and (*Z*)-prenyl units of each polyprenol was examined by incorporation of (4*R*)- and (4*S*)-[2-^{14}C,4-^3H]MVAs. The results were as shown in Tables VI and VII. The observed $^3H/^{14}C$ atom ratios were not in agreement with the atom ratios expected from Cornforth's basic principle as given in column A of Tables VI and VII, but the observed $^3H/^{14}C$ atom ratios were in good agreement with those expected for loss of the *pro*-4*S* hydrogen of MVA as given in column B of Tables VI and VII. This agreement indicates occurrence of the *pro*-4*S* hydrogen elimination of MVA during the formation of the (*Z*)-prenyl chain of the polyprenols in all the higher plants examined in the same way as the biosynthesis of the malloprenols. These findings established that such a reversed hydrogen elimination is common to the biological formation of the (*Z*)-prenyl chain of the polyprenols in higher plants.

Consequently, it has been demonstrated that the elongation of the (*Z*)-prenyl chain in the biosynthesis of the polyprenols takes place by successive addition of IPP to GGPP, as well as this chain-elongation involves the elimination of the *pro*-2*R* hydrogen of IPP, which is equivalent to the *pro*-4*S* hydrogen of MVA. Thus,

the hydrogen elimination opposed to Cornforth's principle was established to be a common occurrence in the formation of (*Z*)-prenyl chain of polyprenols in higher plants. A mode of hydrogen elimination in the formation of the (*Z*)-prenyl chain was first reported in the biosynthesis of rubber latex where the rubber chain exhibits an all-*Z* conformation; the *pro*-4*R* hydrogen of MVA was eliminated in the biosynthesis.[5] The mode of *pro*-4*R* hydrogen elimination was also reported in the biosynthesis of free and esterified betulaprenols-6 to -9 in the woody tissue of *Betula verrucosa*, while, in the cases of free betulaprenols-10 to -13 in the woody tissue and free betulaprenols-6 to -13 in the leaf tissue of the same plant, their modes of hydrogen elimination had not been clarified.[25] Recently, the stereochemistry of hydrogen elimination in the biosynthesis of polyprenols in microorganism was established[26] to be in agreement with the elimination expected from Cornforth's principle. In the biosynthesis of C_{10} and C_{15} prenols in higher plants, it has been reported[27] that there is no obligatory chemical link between the prochirality of the leaving hydrogen and the geometry of the newly formed prenyl unit. Although the precise arrangement of an enzyme–substrate complex in the prenylation is not known, some interpretations have been made;[4,27-30] these define the orientation of C-1 of the allylic substrate with respect to the double bond of IPP. The C-1 of the allylic residue which has just released inorganic pyrophosphate binds to C-4 of IPP from the *si* face of its double bond. For orientations that permit the formation of the (*Z*)-prenyl units, only the proton originating from the *pro*-4*R* hydrogen of MVA must be lost from C-2 of IPP by the syn elimination.[4,28,29] On the contrary, the inverse loss of the *pro*-2*R* hydrogen of IPP, which is equivalent to the *pro*-4*S* hydrogen of MVA, may be caused by (i) the addition of the allylic residue to the *re* face of the double bond of IPP, followed by the syn elimination of the corresponding hydrogen atom, or (ii) the addition of the allylic residue to the *si* face of the double bond of IPP, followed by the anti elimination of the hydrogen atom. The reversed hydrogen elimination in the polyprenol biosynthesis seems to imply that the spatial arrangement of the active site in the enzyme participating in the biosynthesis of the (*Z*)-prenyl chain of the polyprenols in the leaves of higher plants may be in a diastereomeric relationship with that of the active site in the enzyme in mammals and microorganisms. An approach to solve this point is now in progress.

Experimental Section

Preparative TLC was carried out by use of silica-gel plates (Merck GF_{254}, 0.75 mm thick). Reversed-phase TLC was carried out on a paraffin-impregnated kieselguhr with acetone–water (8:1, v/v) saturated with paraffin. The reversed-phase TLC plates were prepared by dipping a kieselguhr plate (Merck G, 0.25 mm thick) into a solution of 5% liquid paraffin in hexane. Polyprenols were visualized as colored spots on the TLC plates by spraying with a *p*-anisaldehyde–H_2SO_4 reagent, followed by heating on a hot plate, and/or by spraying with a 2′,7′-dichlorofluorescein–EtOH reagent.[31,32] HPLC was performed on a Waters Radial-pak C_{18} column or a TSK-gel LS-410 column with MeOH as solvent. TLC radiochromatographic measurements were carried out on an Aloka radiochromato-scanner (JTC-203). Radioactivities of ^3H- or ^{14}C-labeled samples were measured by a Packard Tri-Carb liquid scintillation spectrometer (Model 3330) with counting efficiencies of 45% and 75%, respectively, in Bray's scintillant.[33] Radioactivities of ^3H,^{14}C-labeled samples were assayed with the same spectrometer by a spillover method[34] in the same scintillant. Counting efficiencies of ^3H and ^{14}C were 35% and 57%, respectively, and the maximum overlap of ^{14}C into ^3H channel was about 10%. The counting error was about 3% for ^3H and about 4% for ^{14}C.

DL-[5-$^3H_2$]MVA (6.7 mCi/mmol) and DL-[2-^{14}C]mevalonolactone (20 mCi/mmol) were products of the Radiochemical Centre, Amersham. NaB^3H_4 (335 mCi/mmol) was product of New England Nuclear Boston, MA.

Composition of Polyprenols in Several Higher Plants. The leaves (100 g of each) of the plants, such as *Magnolia glandiflora*, *Nandina domestica*, *Alnus serrulatoides*, *Betula platyphylla*, *Mirabilis jalapa*, *Aleurites cordata*, *Cleome spinosa*, *Euphorbia pulcherrima*, *Mallotus japonicus*, *Triadica sebifera*, *Aesculus turbinate*, and *Solanum melongena*, were collected at the beginning of July in the suburbs of Hiroshima city and immersed in MeOH (300 mL) at room temperature for 2 weeks. The MeOH extract was concentrated to 30 mL, diluted with H_2O (30 mL), and then extracted with hexane (4 × 30 mL). Removal of the solvent from the hexane extract gave an oily substance, which was subjected to preparative TLC with benzene to give a mixture of polyprenols. The composition of polyprenols in the mixture was analyzed by means of HPLC with a Radial-pak C_{18} column. The content of polyprenols in the leaves and the composition of polyprenols are shown in Table V.

Separation of each homologue of the polyprenols was carried out on the same HPLC system as above. The spectroscopic data of C_{50}-polyprenol are as follows: IR (liquid) 3320, 1002 (OH), 1660, and 840 cm^{-1} (C=C); MS (70 eV), *m/z* (relative intensity) 698 (1, M^+), 680 (4, M − H_2O), and 69 (100); ^1H NMR ($CDCl_3$) δ 1.60 (12 H, s, (*E*)-C-(CH_3)=CH), 1.68 (18 H, s, (*Z*)-C(CH_3)=CH), 1.75 (3 H, s, C(3)-CH_3), 2.3–2.1 (36 H, CH_2CH_2), 4.08 (2 H, d, *J* = 7.3 Hz, CH_2OH), 5.12 (10 H, br s, >C=CH), and 5.44 (1 H, t, *J* = 7.3 Hz, C(2)H); ^{13}C NMR ($CDCl_3$) δ_C 136.0 (C(3)), 124.5 (C(2)), 59.0 (C(1)), 25.6 and 17.6 (methyl carbons of ω-terminal), 23.4 (six methyl carbons attached to *Z* double bond), and 16.0 (three methyl carbons attached to *E* double bond).

Synthesis of (4*R*)- and (4*S*)-[2-^{14}C,4-^3H]MVAs. According to the methods described in the literature,[35] a mixture of (3*R*,4*R*)- and (3*S*,4*S*)-[4-^3H]mevalonolactones (1.9 mg, 3.0 mCi) was obtained by reduction of *N*-(diphenylmethyl)-*trans*-3,4-epoxy-3-methylpentanamide (120 mg) with NaB^3H_4 (11 mg, 100 mCi), followed by hydrolysis with 1 M NaOH and then lactonization with 1 M HCl. A part of the [4-^3H]mevalonolactone was then mixed with (3*RS*)-[2-^{14}C]mevalonolactone to give a mixture possessing a $^3H/^{14}C$ ratio as shown in Tables I, VI, and VII. Each mixture was hydrolyzed with 0.01 M KOH to give potassium (4*R*)-[2-^{14}C,4-^3H]mevalonate.

Following the same procedure as above, a mixture of (3*R*,4*S*)- and (3*S*,4*R*)-[4-^3H]mevalonolactones (1.5 mg, 2.6 mCi) was prepared from *N*-(diphenylmethyl)-*cis*-3,4-epoxy-3-methylpentanamide (120 mg) and NaB^3H_4 (11 mg, 100 mCi). The ^3H-labeled mevalonolactone was mixed with (3*RS*)-[2-^{14}C]mevalonolactone, followed by hydrolysis with 0.01 M KOH to give potassium (4*S*)-[2-^{14}C,4-^3H]mevalonate.

Synthesis of (2*S*)- and (2*R*)-[2-^3H,4-^{14}C]IPPs. The (4*R*)-[2-^{14}C,4-^3H]MVA (71.4 μCi for ^{14}C) and (4*S*)-[2-^{14}C,4-^3H]MVA (12.7 μCi for ^{14}C) were converted into the corresponding (2*S*)-[2-^3H,4-^{14}C]IPP (3.58 μCi for ^{14}C) and (2*R*)-[2-^3H,4-^{14}C]IPP (1.8 μCi for ^{14}C), respectively, by use of a cell-free extract prepared from pig liver[36] in 5–10% yield based on MVA used.

Feeding of (4*R*)- and (4*S*)-[2-^{14}C,4-^3H]MVAs to the Leaves of Several Higher Plants. Aqueous solution (0.3 mL) of potassium (4*R*)- and (4*S*)-[2-^{14}C,4-^3H]mevalonates was fed, separately, to the leaves of *M. japonicus* through their petioles over 1 h. After uptake of the tracer, water was taken up over 70 h, and then the plant materials were cut into small pieces and extracted with MeOH (3 × 100 mL). The MeOH solution was concentrated to 50 mL and extracted with hexane (4 × 20 mL). After removal of the solvent, the hexane extract was subjected to preparative TLC with benzene to give a crude oily substance, which was further purified by TLC with hexane–ethyl acetate (4:1, v/v) to give a mixture of polyprenols. This mixture was subjected to reversed-phase TLC to give malloprenol-9, -10, and -11. Their radioactivities are shown in Table I. Analogous experiments with (4*R*)- and (4*S*)-[2-^{14}C,4-^3H]-MVAs were done several times, as shown in Tables I and VIII.[37] The levels of incorporation of the MVAs into the malloprenols were 0.01–0.2%.

Feeding experiments of (4*R*)- and (4*S*)-[2-^{14}C,4-^3H]MVAs with other plants, such as *M. glandiflora*, *N. domestica*, *A. serrulatoides*. *B. platyphylla*, *M. jalapa*, *A. cordata*, *C. spinosa*, *E. pulcherrima*, *T. sebifera*,

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A. turbinate, and *S. melongena*, were carried out in the same procedure as described in the case of *M. japonicus*. The weight of the leaves used and the radioactivities and $^{3}H/^{14}C$ ratios of the substrates used are shown in Table VIII.[37] The radioactivities of the polyprenols biosynthesized are shown in Tables VI and VII. The levels of incorporation of the MVAs into the polyprenols were 0.01–0.3%.

Degradation of Malloprenols. Following the reported procedure of the $KMnO_4$–$NaIO_4$ oxidation,[38] a suspension of the radioactive malloprenol-10 (22.5 mg) and $NaIO_4{\cdot}H_2O$ (430 mg) in water (4 mL) was stirred for 10 min at room temperature. The mixture was neutralized with sodium carbonate under cooling to −10 °C. To the solution, $KMnO_4$ (30 mg) dissolved in water (1 mL) was added drop by drop, and then the mixture was stirred at 5 °C for 100 h under a nitrogen atmosphere. After reduction of the remaining oxidant with sodium hydrogen sulfite, the mixture was steam-distilled. The distillate was treated with a 2,4-dinitrophenylhydrazine hydrochloride solution as usual to give a yellow powder, which was subjected to TLC with hexane–ethyl acetate (4:1, v/v) to give acetone (2,4-dinitrophenyl)hydrazone (1.0 mg).

The aqueous residue of the steam distillation was acidified to pH 2.0 with 2 M HCl and was extracted with ether by a liquid–liquid continuous extractor to give an acidic product. The acidic product was subjected to preparative TLC on silica gel with hexane–ethyl acetate (4:1, v/v) to give levulinic acid (1.3 mg), which was purified as *p*-bromophenacyl derivative (1.8 mg). Degradation of the radioactive malloprenol-11 was also carried out in a similar manner as above. Homogeneity of the acetone (2,4-dinitrophenyl)hydrazone and the *p*-bromophenacyl levulinate was checked by the HPLC analysis with TSK-gel LS-410 column. The precise weights of these radioactive derivatives were determined from the peak area of HPLC by use of the standard curves which had been prepared with corresponding authentic samples. The specific radioactivities of the products are shown in Table II.

Feeding of (2*S*)- and (2*R*)-[2-^{3}H,4-^{14}C]IPPs to the Leaves of *M. japonicus*. A solution of (2*S*)-[2-^{3}H,4-^{14}C]IPP ($^{3}H/^{14}C$ = 5.92, 0.96 μCi for ^{14}C) or (2*R*)-[2-^{3}H,4-^{14}C]IPP ($^{3}H/^{14}C$ = 5.59, 0.84 μCi for ^{14}C) in water (1 mL) was separately fed to the leaves of *M. japonicus* (50 g) through their petioles over 1 h. After uptake of the tracer, water was taken up over 70 h. The plant materials were extracted with MeOH. The MeOH solution, after removal of the solvent, was treated with hexane. The hexane extract was subjected to preparative TLC on silica gel and then reversed-phase TLC to give malloprenol-9–12. The radioactivities of the malloprenols are shown in Table III. The levels of incorporation of the IPPs into the malloprenols were 0.01–0.1%.

Enzyme Preparation from the Leaves of *M. japonicus*. The fresh leaves (20 g) were frozen with liquid nitrogen, ground in a mortar, and then slurried with insoluble polyvinylpyrrolidone (20 g) in 0.1 M potassium phosphate buffer (pH 7.5, 200 mL). After filtration through several layers of cheesecloth, the filtrate was centrifuged at 30000*g* for 20 min to give a supernatant. To the supernatant, ammonium sulfate was added over a period of 30 min until 40% saturation was obtained. Precipitated protein was collected by centrifugation (25000*g*, 20 min) and resuspended in 0.1 M Tris-HCl buffer (pH 7.5, 30 mL). The suspended material, after dialysis against the same buffer for 15 h, was centrifuged at 30000*g* for 20 min. The resultant supernatant (0.88 mg of protein/mL) was used as an enzyme preparation.

Incubation of (2*S*)- and (2*R*)-[2-^{3}H,4-^{14}C]IPPs with the Enzyme Preparation of *M. japonicus*. The enzyme preparation (1 mL, 0.88 mg of protein) of *M. japonicus* was added to a solution of (2*R*)-[2-^{3}H.4-^{14}C]IPP ($^{3}H/^{14}C$ = 9.90, 0.027 μCi for ^{14}C) or (2*S*)-[2-^{3}H,4-^{14}C]IPP ($^{3}H/^{14}C$ = 7.61, 0.023 μCi for ^{14}C), geranylgeranyl pyrophosphate (10 μM), $MgCl_2$ (10 mM), iodoacetamide (10 mM), and Triton X-100 (2%) in 1 mL of Tris-HCl buffer (0.1M, pH 7.5). The mixture was incubated at 35 °C for 90 min and then, after acidification with 1 M HCl (0.8 mL), heated at 50 °C for 30 min. The reaction mixture, after addition of 1 M NaOH (1.2 mL), was extracted with hexane. The hexane-soluble material was subjected to preparative TLC with hexane–ethyl acetate (4:1, v/v) to give a mixture of malloprenol-9–12. The radioactivities of the malloprenol mixture are shown in Table IV. The levels of incorporation of the IPPs into the malloprenol mixtures were 0.5–2.0%.

Acknowledgment. We acknowledge the invaluable assistance of T. Yoshioka in the feeding experiments of the MVAs into *M. jalapa*. We thank Drs. A. Yasui and Y. Fujita of the Central Research Laboratories of Kuraray Co., Ltd., for a gift of the sample of geranylgeraniol and Dr. Kohji Karasawa and Kiyosi Hashimoto of the Botanical Garden of Hiroshima City for providing the leaves of *B. platyphylla*. The present work was supported in part by Grant-in-Aids for Scientific Research No. 247027 and 347024, Special Project Research No. 56209019, and Co-operative Research No. 57340031 from the Ministry of Education, Science and Culture.

Supplementary Material Available: Table of the weight of the leaves used and radioactivities and $^{3}H/^{14}C$ ratios of the substrates used for feeding to the leaves of several higher plants (1 page). Ordering information is given on any current masthead page.

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