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Studies on the non-mevalonate isoprenoid biosynthetic pathway. Simple methods for preparation of isotope-labeled (*E*)-1-hydroxy-2-methylbut-2-enyl 4-diphosphate

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Abstract—(*E*)-1-Hydroxy-2-methylbut-2-enyl 4-diphosphate (**6**) has been recently detected as an intermediate of isoprenoid biosynthesis via 1-deoxy-D-xylulose 5-phosphate. The compound was synthesized in two steps from 2-methyl-2-vinyloxirane with an overall yield of 72%. We also report a method affording [1-^{3}H]-**6** and [1-$^{2}H_{1}$]-**6** from the same starting material in four steps where the hydrogen isotope is introduced in the last reaction step from deuterated or tritiated sodium borohydride. The radiochemical yield of [1-^{3}H]-**6** was 15%. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Terpenoids serve a wide variety of physiological functions. With more than 30,000 reported representatives which are all assembled from two simple 5-carbon intermediates, i.e. isopentenyl diphosphate (IPP, **7**) and dimethylallyl diphosphate (DMAPP, **8**), they constitute one of the largest groups of natural products.[1]

For several decades, the mevalonate pathway has been believed to be the exclusive source for IPP and DMAPP. Following the seminal discoveries by Rohmer, Arigoni and their co-workers,[2–4] a second pathway for IPP and DMAPP has been rapidly unfolding during the last decade (for review see Refs. 5 and 6). This non-mevalonate pathway is now known to coexist with the mevalonate pathway in higher plants and to serve as the exclusive source for terpenes in many eubacteria.

The non-mevalonate pathway starts with the condensation of D-glyceraldehyde 3-phosphate (**2**) and pyruvate (**1**) affording 1-deoxy-D-xylulose 5-phosphate (**3**)[7,8] which has been shown earlier to serve as a biosynthetic precursor of the vitamins B_1 (thiamine) and B_6 (pyridoxal) (Scheme 1).[9–11] **3** is converted into 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**5**) by the catalytic action of four enzymes specified by the *ispCDEF* genes (for a review, see Ref. 5). The cyclic diphosphate **5** is reductively opened by IspG protein to yield (*E*)-1-hydroxy-2-methylbut-2-enyl 4-diphosphate (**6**),[12,13] which is converted to IPP (**7**) as well as DMAPP (**8**) by IspH protein.[14] The reactions catalyzed by IspG and IspH protein may involve additional proteins which remain to be identified.

This paper reports simple methods for the synthesis of isotope-labeled **6**.

2. Results

Commercially available 2-methyl-2-vinyloxirane (**9**) was converted into (*E*)-4-chloro-2-methylbut-2-en-1-ol (**14**) by treatment with $TiCl_4$ at −80°C (Scheme 2). Reaction of **14** with tris(tetra-*n*-butylammonium) pyrophosphate afforded **6**. The overall yield of the simple two step procedure is 72%. The product is identical with **6** synthesized by different routes[15–19] and with the natural product obtained by biotransformation of [U-$^{13}C_5$]1-deoxy-D-xylulose by an *E. coli* strain engineered for the overexpression of the *xylB* (specifying D-xylulokinase) and *ispCDEFG* genes.[12] The product contains 3% of the Z isomer as shown by NMR analysis.[15]

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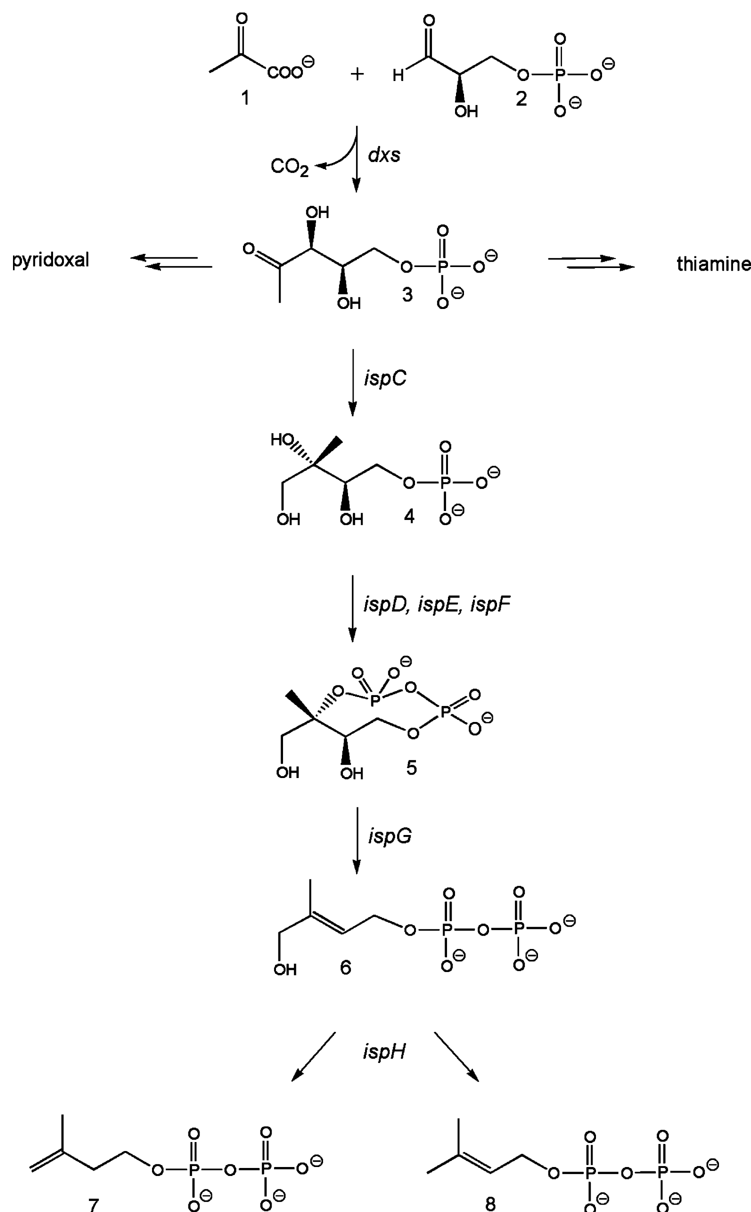


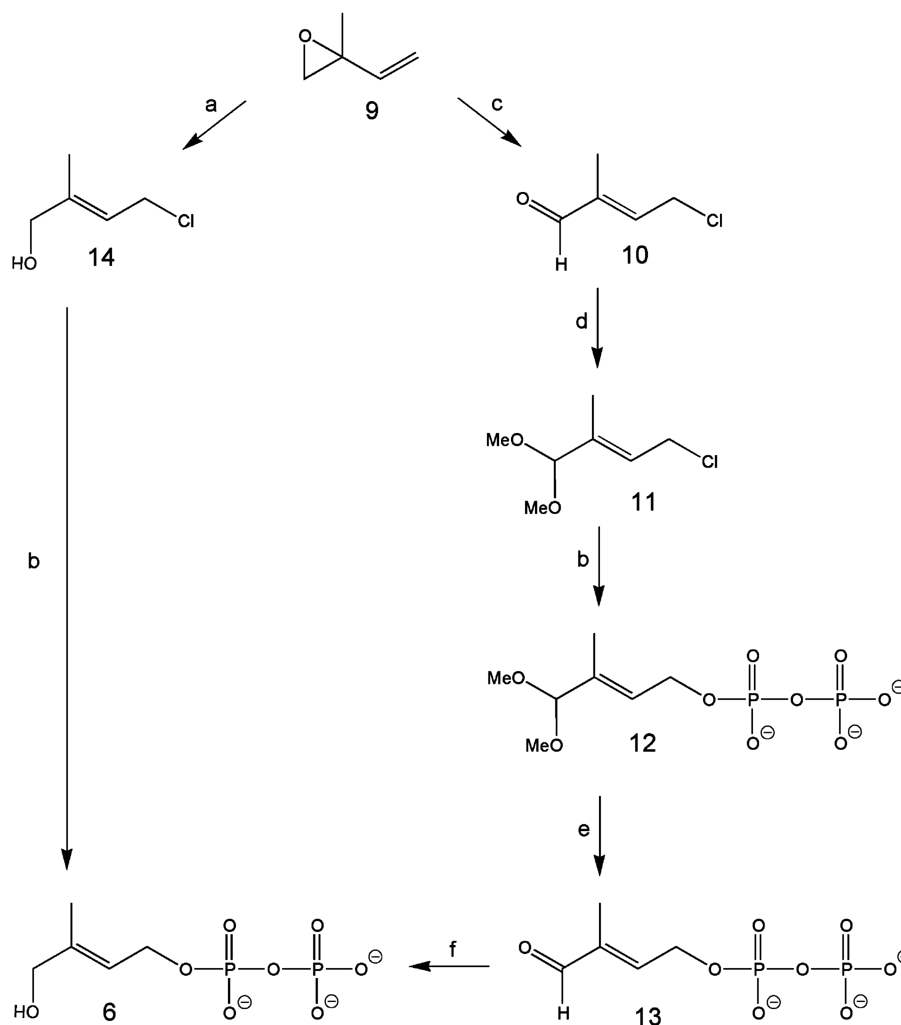
Scheme 1. Biosynthesis of IPP (**7**) and DMAPP (**8**) via 1-deoxy-D-xylulose 5-phosphate (**3**).

In an alternative reaction sequence designed for incorporation of hydrogen isotopes (deuterium or tritium), (*E*)-4-chloro-2-methyl-2-buten-1-al (**10**) was obtained from 2-methyl-2-vinyloxirane **9** by a modification of published procedures;[20] the Z isomer was not formed in detectable amounts. The aldehyde **10** was protected by conversion into the acetal **11** which was then reacted with tris(tetra-*n*-butylammonium) pyrophosphate[21] affording the diphosphate **12**. Acid hydrolysis of **12** afforded the aldehyde **13** which was converted into **6** by reduction with sodium borohydride. The overall yield for the conversion of **9** to **6** was 23%.

The reduction of **13** with NaB^2H_4 afforded $[1\text{-}^2H_1]$-**6** as confirmed by ^1H and ^{13}C NMR spectroscopy. The reduction of **13** with tritiated sodium borohydride afforded $[1\text{-}^3H]$-**6** with a radiochemical yield of 15%. Since the reduction of **13** proceeds under very mild conditions, the reaction can be scaled down to volumes of a few microliters. The tritium-labeled product can then be obtained at maximum specific activity.

3. Discussion

The enzymes of the non-mevalonate pathway are essential in numerous pathogenic bacteria including Enterobacteria and *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and in *Plasmodium* spp., the causative agents of malaria.[5] Tuberculosis and malaria are each responsible for more than a million deaths per year worldwide. Whereas the morbidity and mortality caused by bacterial and protozoal parasites is relatively low in developed countries, that situation is bound to progressively become worse by the rapidly escalating resistence of pathogens against all currently used



Scheme 2. Synthesis of (*E*)-1-Hydroxy-2-methylbut-2-enyl 4-diphosphate (**6**). *Reagents and conditions*: (a) $TiCl_4$, CH_2Cl_2, −80°C; (b) tris(tetra-*n*-butylammonium) hydrogendiphosphate, MeCN, 25°C, (c) $CuCl_2$, ethylacetate, 80°C; (d) $HC(OMe)_3$, *p*-TsOH, 25°C; (e) HCl, pH 2; (f) $NaBH_4$, 25°C.

antimicrobial agents. The development of novel antiinfective agents therefore appears urgent. Fosmidomycin, an inhibitor of *ispC* gene catalyzing the conversion of **3** into **4** has been shown to cure the infection of mice by *Plasmodium vinckei*, thus establishing the non-mevalonate pathway as a potential therapeutic target.[22] The enzymes of the pathway are also essential in plants, and their study could be conducive to novel herbicides.

Recent studies have shown that **6** stimulates the proliferation of human γδ T lymphocytes.[13] Thus, the compound may signal the presence of microorganisms in animal tissue to the innate immune system. Apparently, the non-mevalonate pathway intermediate is perceived as a non-specific marker of microbial infection by the mammalian immune system. The detection of molecular patterns common to a wide variety of pathogenic agents by the innate branch of the immune system is currently of major focus of immunological research.

Radiolabeled samples of **6** could serve as valuable reagents for further biochemical studies of the non-mevalonate pathway as well as for the investigation of T lymphocyte stimulation by **6**. They may also be useful as tools for the screening of compound libraries for antiinfective and herbicide activity.

The present study was designed to provide synthetic routes for the preparation of **6** labeled with ^{2}H, ^{3}H, ^{32}P or ^{33}P. Hydrogen isotopes can be introduced via deuterated or tritiated borohydride in the transformation of **13** to **6**. Since the one step reaction occurs at room temperature under mild conditions, it can be performed with very small amounts, thus enabling the synthesis of [1-^{3}H]-**6** of maximum specific activity.

The preparation of **6** in a single reaction step from **14** and pyrophosphate appears appropriate for the preparation of ^{32}P- or ^{33}P-labeled from commercially available radiolabeled pyrophosphate. The reaction conditions are mild, and the reaction can therefore be scaled down to very small volumes in order to obtain **6** with maximum specific activity which could serve to monitor and characterize receptors for **6** supposed to be present on the surface of γδ T lymphocytes.

4. Experimental

4.1. (*E*)-4-Chloro-2-methylbut-2-en-1-ol (14)

$TiCl_4$ (285 mg, 1.5 mmol, 164.5 μL) was dissolved in 3 mL of dry CH_2Cl_2 under N_2. The solution was cooled to −80 to −90°C, and a solution of 84 mg of 2-methyl-2-vinyloxirane (**9**) (98.2 μL, 1 mmol) in 0.4 mL of CH_2Cl_2 was added in drops with stirring. After 90 min, the reaction mixture was quenched by adding 5 mL of 1 N HCl. After warming to room temperature, the phases were separated and the aqueous layer was extracted four times with 20 mL of diethyl ether. The combined organic phases were dried over $MgSO_4$. Evaporation of the solvent and purification by flash chromatography (pentanes/diethyl ether 1:1 v/v) afforded 93 mg of pure product. ^1H NMR (CD_3CN, 500 MHz): δ 5.62 (tq, J=8.2 Hz; 1.58, 1H), 4.16 (d, J=8.2 Hz, 2H), 3.89 (s, 2H), 1.64 (d, J=1.60, 3H); ^{13}C NMR (CD_3CN, 125 MHz): δ 143.2, 119.9, 66.8, 41.5, 13.4.

4.2. (*E*)-1-Hydroxy-2-methylbut-2-enyl 4-diphosphate (6) from 14

A solution containing 227 mg (0.25 mmol) of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate in 400 μL of MeCN was added slowly at room temperature to a solution of **14** (25 mg, 0.21 mmol) in 250 μL of MeCN affording an orange–red solution. After 2 h, the solvent was removed under reduced pressure. The orange-colored oil was dissolved in 3 mL of H_2O, and the solution was passed through a column of DOWEX 50 WX8 (1×4 cm, NH_4^+ form) that has been equilibrated with 20 mL of 25 mM NH_4HCO_3. The column was developed with 20 mL of 25 mM NH_4HCO_3. Fractions were combined and lyophilized to yield 0.19 mmol of pure product (90%). ^1H NMR (D_2O, 500 MHz): δ 5.52 (tq, J=6.9 Hz, 1H), 4.39 (t, J=7.2 Hz, 2H), 3.88 (s, 2H), 1.57 (s, 3H); ^{13}C NMR (D_2O, 125 MHz): δ 141.6, 122.7 (d, J=8.0 Hz), 68.6, 64.3 (d, J=5.1 Hz), 15.1; ^{31}P NMR: δ −3.49 (d, J=22.3 Hz), −7.17 (d, J=22.0 Hz).

4.3. (*E*)-4-Chloro-2-methyl-2-buten-1-al (10)[20]

A mixture containing 1.17 mL (12 mmol) of 2-methyl-2-vinyloxirane (**9**), 1.6 g (12 mmol) of $CuCl_2$ and 510 mg (12 mmol) of LiCl in 10 mL of ethylacetate was heated to 80°C for 30 min. The reaction was terminated by adding 50 g of ice. The mixture was filtered through a sintered glass funnel. CH_2Cl_2 (100 mL) was added, and the organic phase was collected. The aqueous layer was extracted twice with 100 mL of CH_2Cl_2. The combined organic phase was dried over anhydrous $MgSO_4$, filtered, and concentrated. The crude product was purified by chromatography over silica gel (3×37 cm; eluent, CH_2Cl_2) affording 755 mg of a yellow liquid (6.4 mmol, 53%). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.46 (s, 1H), 6.53 (t, J=7.5 Hz, 1H), 4.28 (d, J=7.5 Hz, 2H), 1.80 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 194.3, 145.7, 141.1, 38.6, 9.2

4.4. (*E*)-4-Chloro-2-methyl-2-buten-1-aldimethylacetal (11)

A solution containing 200 mg (1.68 mmol) of **10**, 650 μL (6.1 mmol) of $HC(OMe)_3$ and 7 mg of *p*-toluene sulfonic acid was stirred for 3 h at room temperature. The crude mixture was purified by chromatography over silica gel (eluent, *n*-hexane/ethylacetate, 7:3) to yield 250 mg of a colorless liquid (1.35 mmol, 90%). ^1H NMR ($CDCl_3$, 500 MHz): δ 5.78 (t, 1H, J=7.9), 4.51 (s, 1H), 4.18 (d, J=7.9 Hz, 2H), 3.23 (s, 6 H), 1.64 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 139.0, 125.0, 107.1, 53.9, 40.7, 11.4. Anal. calcd for $C_7H_{13}ClO_2$: C, 51.07; H, 7.96. Found: C, 51.52; H, 8.02%.

4.5. 3-Formyl-2-buten-1-diphosphate triammonium salt (13)

To a solution of **11** (25 mg, 0.15 mmol) in 250 μL of MeCN, a solution of 0.162 g (0.18 mmol) of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate in 400 μL of MeCN was added slowly at room temperature affording an orange–red solution. After 2 h, the solvent was removed under reduced pressure. The orange-colored oil was dissolved in 3 mL of H_2O, and the solution was passed through a column of DOWEX 50 WX8 (1×4 cm, NH_4^+ form) that has been equilibrated with 20 mL of 25 mM NH_4HCO_3. The column was developed with 20 mL of 25 mM NH_4HCO_3. Fractions were combined and lyophilized. The residue was dissolved in 2 mL of water. The solution was acidified to pH 3 with aqueous HCl. After 2 min, the solution was neutralized and directly used in the next step. ^1H NMR (D_2O, 500 MHz): δ 9.29 (s, 1H), 6.79 (t, 1H, 5.6 Hz), 1.65 (s, 3H); ^{13}C NMR (D_2O, 125 MHz): δ 198.6, 152.7 (d, J=7.5 Hz), 138.4, 62.7 (d, J=4.9), 8.5

4.6. (*E*)-[1-$^2H_1$]1-Hydroxy-2-methylbut-2-enyl 4-diphosphate ([1-$^2H_1$]-6) from 13

A solution (volume, 0.55 mL) containing 11 μmol of **13** (triammonium salt) and 0.3 mg (7.4 μmol) of NaB^2H_4 was stirred for 60 min at 0°C. The solution was acidified to pH 2 by addition of 1 M HCl and was neutralized after 2 min by addition of 1 M NaOH and lyophilized to yield 8.8 μmol (80%). ^1H NMR (D_2O, 500 MHz): δ 5.52 (tq, J=6.9 Hz, 1H), 4.39 (t, J=7.2 Hz, 2H), 3.86 (s, 1H), 1.57 (s, 3H); ^{13}C NMR (D_2O, 125 MHz): δ 141.5, 122.7 (d, J=8.0 Hz), 68.3 (t, J=22.6 Hz), 64.3 (d, J=5.1 Hz), 15.1; ^{31}P NMR: δ −3.49 (d, J=22.3 Hz), −7.17 (d, J=22.0 Hz)

4.7. (*E*)-[1-^3H]1-Hydroxy-2-methylbut-2-enyl 4-diphosphate ([1-^3H]-6) from 13

A solution (volume, 1 mL) containing 100 mM Tris hydrochloride, pH 8, 14 μmol (5 mCi) of $NaBH_3T$ and 14 μmol of **13** (triammonium salt) was stirred for 30 min at room temperature. The solution was acidified to pH 2 by addition of 1 M HCl and was neutralized after 2 min by addition of 1 M NaOH (yield: 0.75 mCi, 48% based on **11**). The radiochemical purity was determined by reversed-phase ion-pair HPLC using a column of

Multospher 120 RP 18-AQ-5 (4.6×250 mm) which was developed with a gradient of 7–50% (v/v) methanol in 10 mM tetra-*n*-butylammoniumhydrogen phosphate, pH 6.0 (flow rate, 1 mL min^{-1}, retention volume, 30.5 mL). The effluent was monitored by online liquid counting.

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