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Hydrolysis of isoprenyl diphosphates with the acid phosphatase from *Cinnamomum camphora*

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Abstract. Direct observations of the enzymatic hydrolysis of C_{10} acyclic allylic isoprenyl diphosphates by an acid phosphatase from the leaves of *Cinnamomum camphora* (camphor tree) were made using ^1H and ^{31}P NMR spectrometers. The measurements indicated that the allylic primary diphosphates, geranyl diphosphate and neryl diphosphate, were hydrolyzed to their corresponding alcohols in a sequential manner via their corresponding monophosphates, whereas the allylic tertiary diphosphate, linalyl diphosphate, was hydrolyzed only to its corresponding monophosphate.
Key words. *Cinnamomun camphora*; ^1H NMR; ^{31}P NMR; hydrolyses; acid phosphatase; geranyl diphosphate; nerly diphosphate; linalyl diphosphate.

Acyclic allylic isoprenyl diphosphates, such as geranyl diphosphate (**1**), neryl diphosphate (**2**) and linalyl diphosphate (**3**), have been suggested as precursors for the biosynthesis of monoterpenoids[1-4]. However, the isoprenyl diphosphates may be hydrolyzed by a phosphatase before their utilization as precursors for the biosynthesis of cyclic monoterpenoids, because the cyclic monoterpene synthase is present together with a phosphatase in intact plants. In fact, C_{10} isoprenyl alcohols, such as geraniol in *Pelargonium roseum*[5], nerol in *Helichrysum arenarium*[5], and linalool in *Cinnamomum comphora* var. *linalooliferum*[5,6], have been found to be major components of the essential oils of those plants; but there are few records of the corresponding phosphate compounds being isolated as natural products.

Many reports on the isolation of phosphatases from plants have been published, but nevertheless, little is known about the process of isoprenyl diphosphate hydrolysis in vivo[7]. In connection with studies on isoprenoid biosynthesis, direct observations were made of enzymatic hydrolyses of isoprenyl diphosphates by an acid phosphatase from the leaves of *Cinnamomum camphora* (camphor tree), using ^1H and ^{31}P NMR spectrometers.

Materials and methods

Monitoring of the enzymatic hydrolysis with ^1H and ^{31}P NMR spectrometers. ^1H NMR spectra were obained on the FT NMR spectrometer (270 MHz) with TSP-d_4 as an internal standard. The measurements were carried out at 30 °C with a 5-mm NMR tube containing the enzyme preparation (0.5 U) and the acyclic allylic diphosphate (10 mM) dissolved in D_2O at pH 7. The spectra were monitored at 5-min-intervals with a pulse width of 4.3 μs corresponding to a 45° flip angle, a spectral width of 3000 Hz, a 5-s repetition rate and 60 scans.

^{31}P NMR measurements were carried out on an FT NMR spectrometer at 30 °C with a 10-mm NMR tube containing the enzyme preparation (0.5 U) and the acyclic allylic diphosphate (10 mM) dissolved in a 0.1 M citrate buffer soln at pH 6.6. Chemical shifts are referred to 85% H_3PO_4 as external reference. The spectra were monitored at 5-min-intervals with a pulse width of 11.5 μs corresponding to a 45° flip angle, a spectral width of 5000 Hz, a 2-s repetition rate and 120 scans.

Preparation of the acyclic allylic diphosphates and their corresponding monophosphates. Geranyl diphosphate (**1**) and neryl diphosphate (**2**) were prepared from their corresponding alcohols, geraniol and nerol, by chlorination, followed by phosphorylation[8].

1: ^1H NMR (D_2O) $\delta = 1.64$ (3H, s, 10-Me), 1.70 (3H, s, 8-Me), 1.73 (3H, s, 9-Me), 2.13 (4H, m, 4- and 5-H_2), 4.48 (2H, t, J = 6.6 Hz, 1-H_2), 5.23 (1H, t, J = 6.5 Hz, 6-H), and 5.47 (1H, t, J = 6.5 Hz, 2-H); ^{31}P NMR (D_2O, ^1H complete decoupling) $\delta = -7.7$ (1P, d, J = 20.4 Hz, P_β) and −10.2 (1P, d, J = 20.4 Hz, Pα). **2:** ^1H NMR (D_2O) $\delta = 1.64$ (3H, s, 10-Me), 1.70 (3H, s, 8-Me), 1.78 (3H, s, 9-Me), 2.16 (4H, m, 4- and 5-H_2), 4.47 (2H, t, J = 6.8 Hz, 1-H_2); 5.21 (1H, t, J = 6.8 Hz, 6-H), and 5.48 (1H, t, J = 6.8 Hz, 2-H); ^{31}P NMR (D_2O, ^1H complete decoupling) $\delta = -7.8$ (1P, d, J = 20.7 Hz, P_β) and −10.2 (1P, d, J = 20.7 Hz, Pα).

(±)-Linalyl diphosphate (**3**), geranyl monophosphate (**4**), neryl monophosphate (**5**), and linalyl monophosphate (**6**) were prepared from their corresponding alcohols, (±)-linalool, geraniol, and nerol by phosphorylation with a dioxane diphosphate complex[9,10].

3: ^1H NMR (D_2O) $\delta = 1.57$ (3H, s, 9-Me), 1.63 (3H, s, 10-Me), 1.68 (3H, s, 8-Me), 1.78 (2H, m, 5-H_2), 2.06 (2H, m, 4-H_2), 5.16

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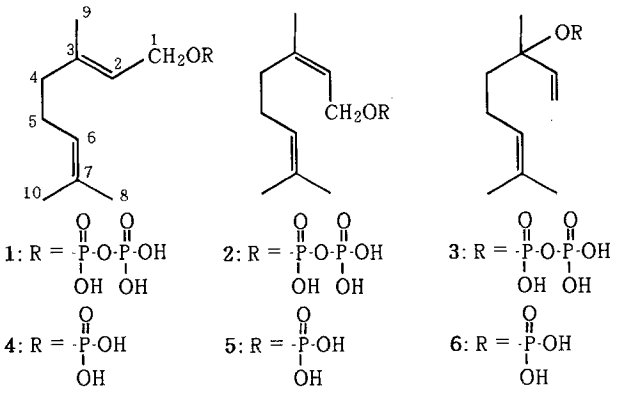
(1H, dd, J = 10.9 and 1.1 Hz, 1-H(*trans*)), 5.24 (1H, m, 6-H), 5.27 (1H, dd, J = 17.4 and 1.1 Hz, 1-H (*cis*)), and 6.09 (1H, dd, J = 17.4 and 10.9 Hz, 2-H)); ^{31}P NMR (D_2O, ^{1}H complete decoupling) $\delta = -10.2$ (1P, d, J = 20.8 Hz, P_β) and -14.5 (1P, d, J = 20.8 Hz, Pα). **4:** ^{1}H NMR (D_2O) $\delta = 1.64$ (3H, s, 10-Me), 1.71 (6H, s, 8- and 9-Me), 2.10 (4H, m, 4- and 5-H_2), 4.31 (2H, dd, J = 6.6 and 5.4 Hz, 1-H_2), 5.23 (1H, t, J = 6.6 Hz, 6-H), and 5.44 (1H, t, J = 6.6 Hz, 2-H); ^{31}P NMR (D_2O, ^{1}H complete decoupling) $\delta = 2.4$ (1P, s). **5:** ^{1}H NMR (D_2O) $\delta = 1.64$ (3H, s, 10-Me), 1.70 (3H, s, 8-Me), 1.77 (3H, s, 9-Me), 2.16 (4H, m, 4- and 5-H_2), 4.30 (2H, t, J = 6.8 Hz, 1-H_2), 5.22 (1H, t, J = 6.7 Hz, 6-H), and 5.46 (1H, t, J = 6.7 Hz, 2-H); ^{31}P NMR (D_2O, ^{1}H complete decoupling) $\delta = 2.3$ (1P, s). **6:** ^{1}H NMR (D_2O) $\delta = 1.52$ (3H, s, 9-Me), 1.63 (3H, s, 10-Me), 1.68 (3H, s, 8-Me), 1.72 (2H, m, 5-H_2), 2.03 (2H, m, 4-H_2), 5.08 (1H, dd, J = 10.7 and 1.5 Hz, 1-H (*trans*)), 5.19 (1H, dd, J = 17.6 and 1.5 Hz, 1-H (*cis*)), 5.24 (1H, m, 6-H), and 6.11 (1H, dd, J = 17.6 and 10.7 Hz, 2-H)); ^{31}P NMR (D_2O, ^{1}H complete decoupling) $\delta = -3.2$ (1P, s).

Purification of the acid phosphatase. All steps were carried out at 4 °C unless otherwise stated. The leaves of *C. camphora* (50 g) were frozen with liquid N_2, ground in a mortar, and homogenized with isoluble polyvinylpolypyrrolidone (50 g) and cold 0.1 M Tris-HCl buffer (300 ml; pH 7.6) containing 0.1 M sucrose, 5 mM dithiothreitol, and 10 mM $MnCl_2$. The homogenate was squeezed through eight layers of gauze to remove debris. The filtrate was centrifuged at 100,000 *g* for 30 min and then at 100,000 *g* for 90 min to give a cell-free extract. The enzyme activity was assayed[11] by measuring the amount of phenol formed by the reaction in an enzyme solution (2 ml; pH 5.8) containing 4.25 mM phenyl phosphate and 3.3 mM 4-aminoantipyrine.

The cell-free extract (60 ml) was fractionated with 80% saturated ammonium sulfate, and the precipitate was dissolved in a minimum volume of 10 mM Tris-HCl buffer (pH 7.6). The solution was desalted by passing through Sephadex G-25, and then fractionated by chromatography on a DEAE-cellulose column (1.6 × 30 cm). The phosphatase fractions were eluated at a flow rate of 20 ml/h with a linear concentration gradient of 10 to 300 mM Tris-HCl buffer (pH 7.6; a total volume of 350 ml). The active fraction thus obtained was further fractionated by chromatography on a Sephadex G-150 (1.2 × 80 cm) with 10 mM Tris-HCl buffer (pH 7.6) at a flow rate of 5 ml/h.



Formulas 1-6.

As shown in table 1, the enzyme preparation was purified 93-fold against the ammonium sulfate fraction.

Properties of the enzyme

The molecular weight of the acid phosphatase was estimated to be approximately 120 kDa by gel filtration chromatography with a Sephadex G-150 column (1.2 × 80 cm). On the basis of SDS polyacrylamide gel electrophoresis[12] in a gel containing 7.5% (w/v) polyacrylamide and 0.1% (w/v) SDS, the molecular weight was confirmed to be 120 kDa.

The optimum pH for the enzyme activity was measured in buffer soln (0.1 M acetate buffer for pH 4.75 to 6.45 and 0.1 M citrate buffer for pH 6.65 to 9.70) containing 5 mM phenyl phosphate and 0.1 U of the enzyme preparation. The optimum pH for the enzyme preparation was 5.8; no activity was found in the pH range of 7.5 to 9.7.

Thermal stability. Aliquots of enzyme preparation (2 ml; 0.1 U) dissolved in 0.1 M citrate buffer (pH 5.8) were separately heated for 10 min at 30, 40, 50, 60, 70, 80 and 90 °C and then immediately cooled to 0 °C. To each portion of enzyme solution, phenyl phosphate was added to give a final concentration of 5 mM, and then the mixture was incubated at 37 °C for 1 h. The activities of the heat-treated enzyme preparations were 99, 95, 91, 59, 32, 9 and 1%, respectively, of the activity of the non-treated enzyme preparation.

Effect of fluoride ions on the enzymatic hydrolysis. The enzyme activity was assayed for the enzyme solution (2 ml; 0.1 U) dissolved in 0.1 M citrate buffer (pH 5.8) containing 5 mM phyenyl phosphate and various concentrations (0.5, 1.0, 2.0, 4.0, and 5.0 mM) of potassium fluoride. The mixture was incubated at 37 °C for 1 h and then the enzyme activity was assayed; the activities of the enzyme preparations were 68, 59, 34, 12 and 2%, respectively, of that of the enzyme preparation in the absence of potassium fluoride.

Results and discussion

A phosphatase was isolated from the leaves of the camphor tree and purified. Details are shown in table 1. The properties of the phosphatase, such as thermal stability, optimum pH and the effect of fluoride ion on the activity, were quite similar to those of the acid phosphatase (EC 3.1.3.2) isolated from sweet potato[13], pea seeds[14], and tobacco leaves[15], but different from those of the bornyl diphosphate hydrolase isolated from sage[16]. The observations indicate that the phosphatase from the camphor tree is an acid phosphatase.

The process of the enzymatic hydrolysis of the acyclic allylic diphosphates, **1**–**3**, was observed directly by monitoring the reaction with ^{1}H and ^{31}P NMR measurements. Figure 1 shows the ^{31}P NMR spectral change in the process of the enzymatic hydrolysis of

Table 1. Purification of the phosphatase from *C. camphora*.

	Total protein mg	Total activity μmol/min	Specific activity μmol/min/mg	Purification fold
Ammonium sulfate	94.2	120	1.27	1
Sephadex G-25	46.8	138	2.97	2.3
DEAE-cellulose	1.35	72.9	54	43
Sephadex G-150	0.71	84.1	118	93

geranyl diphosphate (**1**) in a 0.1M citrate buffer solution at pH 6.6. The intensities of the signals at δ −9.0 and −10.5 due to the diphosphoric group of **1** decreased during the first 15-min-incubation, and a new signal at δ 2.0 due to the phosphoric group of the corresponding monophosphate (**4**) appeared. In turn, a signal due to inorganic phosphate appeared at δ 1.2 and the intensity of the signal increased as more incubation time elapsed. These observations indicate that **1** was first hydrolyzed to **4**, and then further hydrolysis of the resultant product occurred gradually. Quite similar changes in the intensities of the ^{31}P NMR signals were observed for the hydrolysis of neryl diphosphate (**2**) with the phosphatase. Hydrolyses of **1** and **2** could not be monitored by ^{1}H NMR, because of the overlap of the methyl signals of the allylic diphosphate with the methyl signals of the corresponding products.

Figure 2 shows the ^{1}H NMR spectral changes in the enzymatic hydrolysis of linalyl diphosphate (**3**) at pH 7 in a non-buffer solution. The intensity of the C-9 methyl signal of **3** at δ 1.57 decreased during the first 15 min of the incubation, and a new peak at δ 1.52 assignable to the C-9 methyl protons of linalyl monophosphate (**6**)

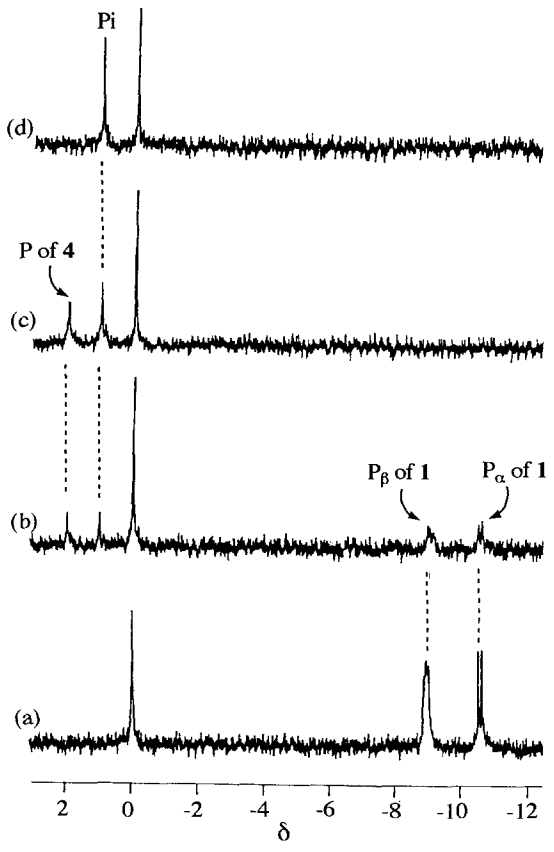


Figure 1. ^{31}P NMR spectra obtained during the hydrolysis of geranyl diphosphate (**1**) by the acid phosphatase in a 0.1 M citrate buffer soln at pH 6.6. The spectra (*a*)–(*d*) correspond to those measured at 0, 15, 60, and 120 min, respectively, after starting the incubation. Pi denotes inorganic phosphate.

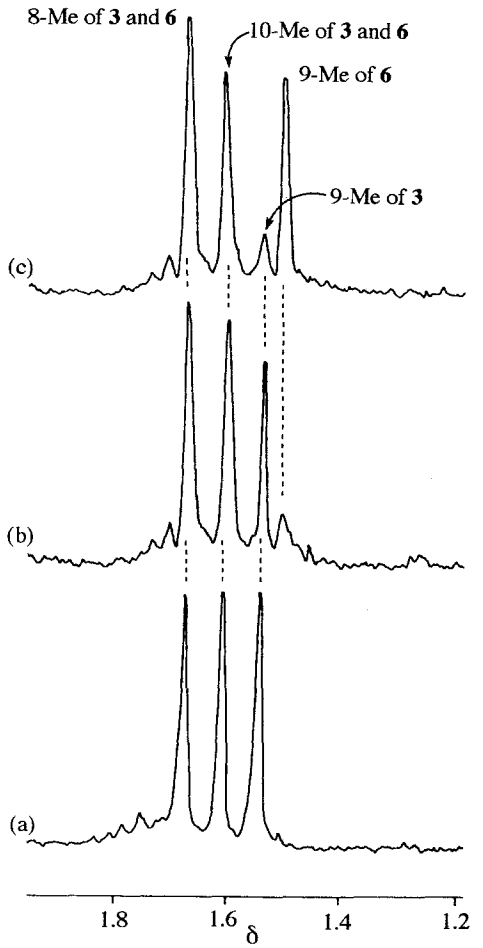


Figure 2. Methyl resonance region of the ^{1}H NMR spectra obtained during the hydrolysis of linalyl diphosphate (**3**) by acid phosphate in D_2O at pH 6.5. The spectra (*a*)–(*c*) correspond to those measured at 0, 15, and 30 min, respectively, after starting the incubation.

Table 2. Hydrolysis of the acyclic allylic phosphates, **1–6**, with the acid phosphatase.

Substrates	K_m	V_{max}	V_{max}/K_m
	mM	μmol/min	
1	1.3	0.38	0.29
2	0.64	0.31	0.48
3	5.9	0.28	0.047
4	1.3	0.059	0.040
5	1.1	0.085	0.077
6	-	0.00	-

appeared. The intensity of the new signal increased strikingly after 30-min-incubation, and the C-9 methyl signal of **3** disappeared. The process of the enzymatic hydrolysis of **3** at pH 7 in a 0.1 M citrate buffer solution was also monitored by ^{31}P NMR measurements. The intensities of the signals at δ −14.5 and −10.2 due to the diphosphoric group of **3** decreased during the first 15 minutes of the incubation. New signals at δ −3.2 and 1.2, due to the phosphorus atoms of the corresponding monophosphate and to inorganic phosphate, respectively, appeared. Although the signals of the diphosphoric group of **3** disappeared during the 60-min-incubation, the intensity of the signal for the phosphoric group of **6** did not decrease even after a further 60-min-incubation. These observations show that linalyl diphosphate (**3**) was hydrolyzed to its corresponding monophosphate (**6**), but no further hydrolysis of the monophosphate occurred.

The K_m and V_{max} values for the enzymatic hydrolyses of acyclic allylic diphosphates and monophosphates were evaluated on the basis of the change in the intensities of the ^{31}P signals for diphosphates, monophosphates, and inorganic phosphate in the ^{31}P NMR spectra, and are shown in table 2. The V_{max} values for the hydrolyses of the acyclic diphosphates, **1–3**, to the corresponding monophosphates, **4–6**, were similar in all cases, and were five or more times higher than those for the hydrolysis of the monophosphates to their corresponding alcohols. This shows that the P–O bond of the diphosphate is hydrolyzed more rapidly than the P–O bond of the monophosphate. On the other hand, the K_m values for the primary diphosphates, **1** and **2**, were similar to those for the primary monophosphates, **4** and **5**, but were smaller than those for the tertiary phosphates, **3** and **6**. This shows that the affinity of the acid phosphatase for the primary phosphates is stronger than its affinity for the tertiary phosphates.

In conclusion, it can be stated that the acid phosphatase prepared from the leaves of the camphor tree hydrolyzed sequentially the phosphoric ester bonds of the primary isoprenyl diphosphates, **1** and **2**, to their corresponding alcohols. The hydrolysis pattern of the tertiary isoprenyl diphosphate, **3**, differed from that observed for the primary isoprenyl diphosphates; the diphosphate (**3**) was hydrolyzed only to its corresponding monophosphate. Although it is interesting to consider these results in relation to the regulation of monoterpene biosynthesis such as camphor and linalool in the camphor trees[6,17], further investigations will be necessary to understand all the multi-enzyme reactions involved. The direct monitoring of enzymatic reactions with the NMR spectrometer may be useful for studies of these multi-enzyme pathways in vivo.

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