

Facilitation of Diphosphate Group Elimination from Geranyl Diphosphate by Magnesium Ion Chelation in Cyclic Monoterpenoid Biosynthesis

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The role of divalent metal ions in the biosynthesis of cyclic monoterpenoids has been investigated by an analysis of the ^{31}P and ^{13}C NMR spectra of geranyl diphosphate (GPP) in the presence and in the absence of Mg^{2+} . This study revealed that Mg^{2+} binds to the diphosphate moiety of GPP in the ratio of 1:1, equidistantly from both phosphorus atoms, and that Mg^{2+} chelation weakens the C(1)–O(5) bond of GPP, facilitating elimination of the diphosphate group.

Cyclic monoterpenoids such as limonene and α -terpineol are biosynthesized from geranyl diphosphate (GPP) in higher plants,^{1,2} and divalent metal ions such as Mg^{2+} or Mn^{2+} are known to be essential for this to take place.³ Although, earlier, the need for divalent metal ions in this reaction had been studied,^{3–5} their precise role in cleavage of the diphosphoric group from the GPP molecule was unknown. We report results for a study using ^{31}P and ^{13}C NMR spectral measurements on the structure of the chelate Mg^{2+} and GPP and the role of the former in cleavage of the C–OP bond. The diamagnetic Mg^{2+} ion was selected for the NMR measurements since paramagnetic ions such as Mn^{2+} would broaden the GPP resonances.

Results and Discussion

MO calculations predicted stabilization of the GPP– Mg^{2+} chelate:⁶ Fig. 1 shows the total energy of GPP coordinated with Mg^{2+} and the two-centre energy between C(1) and O(5). Following a circular orbital of radius 0.2 nm centred on O(4) of GPP, Mg^{2+} was made to move from point (a) in the direction of O(3). The total energy has a minimum value at an O(3)– Mg^{2+} distance of 0.2 nm. Since the O(4)– Mg^{2+} distance is always 0.2 nm, the most stable structure corresponds to that in which Mg^{2+} is located at an equivalent distance from both O(3) and O(4), *i.e.* point (b) in Fig. 1. Furthermore, the increase in the two-centre energy between C(1) and O(5) by the approach of Mg^{2+} to O(3) indicates that chelation of Mg^{2+} to the diphosphate moiety weakens the C(1)–O(5) bond of GPP.

Chelation of Mg^{2+} to the diphosphate moiety of GPP is expected to cause a change in the electron density of the phosphorus atoms. This change was observed by measuring the ^{31}P NMR spectra of a 7.5 mmol dm⁻³ solution of GPP at pH 7.0 and 25 °C with addition of various concentrations of Mg^{2+} . Addition of Mg^{2+} to GPP caused both peaks assignable to P_α and P_β of the diphosphoric group to shift downfield, as shown in Fig. 2. The shifts were 0.68 and 1.58 ppm for the signals of P_α and P_β , respectively, at a mole ratio of Mg^{2+} to GPP of 1:1. Further addition of metal ion, however, had practically no effect on the resonance signals. On the basis of the variation of the ^{31}P NMR chemical shift as shown in Fig. 2, the degree of association of Mg^{2+} to GPP was evaluated;^{7,8} these are 91% for P_α and 93% for P_β at an equimolar ratio of Mg^{2+} to GPP. Only a small increase in these values was observed with further addition of Mg^{2+} , thus confirming that GPP binds to Mg^{2+} in the mole ratio of 1:1.

Chelate formation between GPP and Mg^{2+} was further investigated by measuring the ^{31}P NMR spectra at low temperatures. The spectral measurements of the mixture were carried out over a temperature range from 9 to –35 °C. As the tempera-

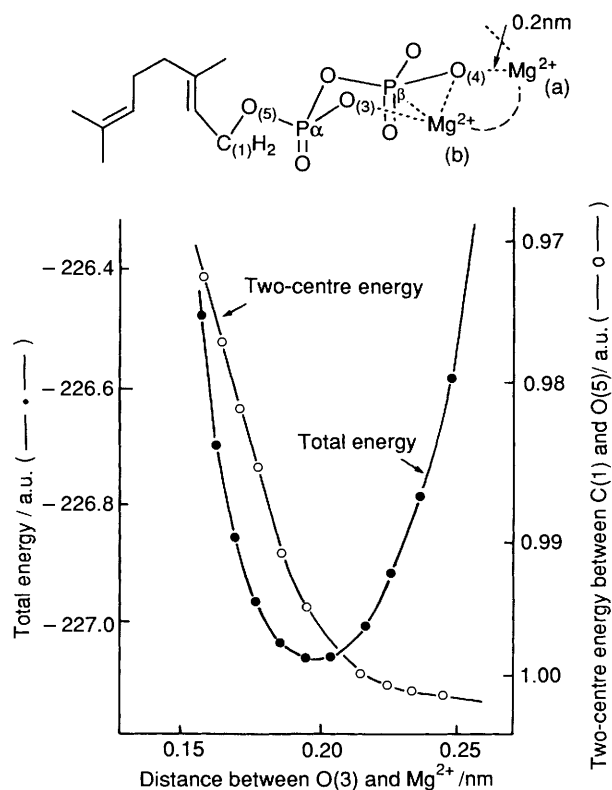


Fig. 1 Total energy of GPP coordinated with Mg^{2+} and two-centre energy between C(1) and O(5)

ture was lowered, both the P_α and P_β signals in the spectrum of a GPP: Mg^{2+} = 1:0.5 mixture broadened and finally each broad signal was split into two peaks (Fig. 3). The coalescence points were –14 and 9 °C for the P_α and P_β signals, respectively. The chemical shifts in the spectrum of GPP: Mg^{2+} = 1:1 were similar to the lower field peaks of the split signals in the case of GPP: Mg^{2+} = 1:0.5. On the other hand, the chemical shifts of GPP without Mg^{2+} at –35 °C were identical with the upper field peaks of the split signals in the case of the GPP: Mg^{2+} = 1:0.5 mixture. Thus, for a half molar ratio of Mg^{2+} to GPP, the latter exchanges between a bound and an unbound state with Mg^{2+} at temperatures above those of coalescence. On the basis of the coalescence points and the chemical shift differences of the split peaks,⁹ the free energy of activation ΔG^\ddagger for the exchange reactions was calculated. The energies are 50.6 J mol⁻¹ for P_α and 53.1 J mol⁻¹ for P_β , respectively. The similarity between both values indicates that Mg^{2+} binds equidistantly from both O(3) and O(4).

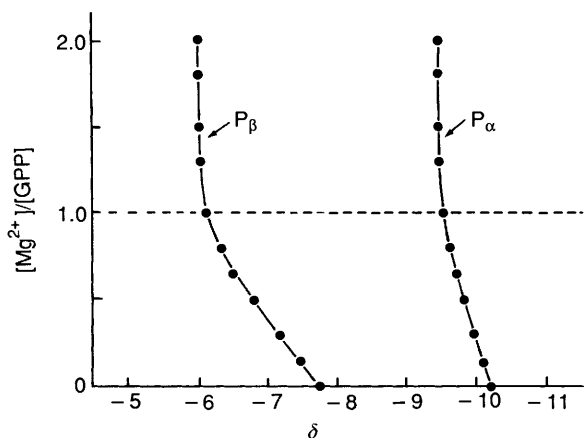


Fig. 2 ^{31}P NMR chemical shifts of the mixture of GPP and Mg^{2+} as a function of the ratio of Mg^{2+} to GPP

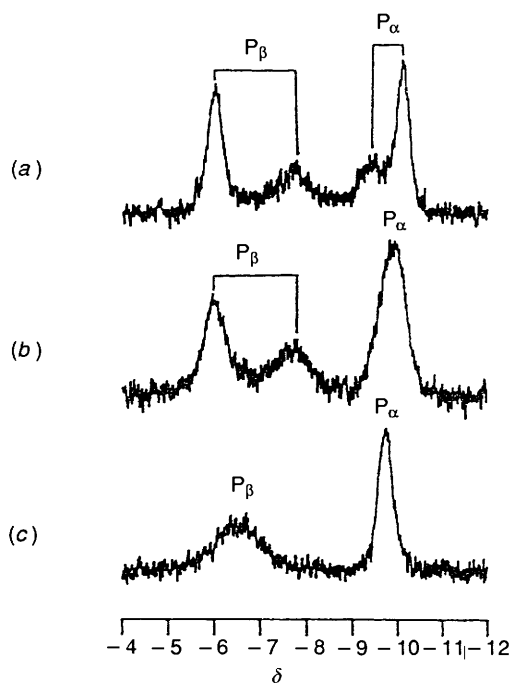


Fig. 3 ^{31}P NMR spectra of the mixture of GPP with Mg^{2+} at pH 7.0 and subzero temperatures: (a) $6.94 \text{ mmol dm}^{-3}$ GPP and $3.47 \text{ mmol dm}^{-3}$ Mg^{2+} , at -35°C ; (b) at -14°C ; (c) at 9°C

Besides equidistant coordination of Mg^{2+} to the diphosphate O(3) and O(4) atoms, weakening of the C(1)–O(5) bond by Mg^{2+} chelation was also predicted from the MO calculations. Such a bond weakening must be accompanied by a lowering of the electron density at the C(1) atom. This was established by comparing the ^{13}C NMR spectra of $[1-^{13}\text{C}]$ GPP in the absence and in the presence of Mg^{2+} . In the absence of Mg^{2+} , the signal for C(1) appeared as a doublet at δ 65.48. When Mg^{2+} was added to give a GPP: Mg^{2+} ratio of 1:1, the signal shifted downfield to δ 65.62. The result suggests that coordination of Mg^{2+} to GPP lowers the electron density of C(1).

Thus, Mg^{2+} binds to the diphosphate moiety of GPP in a mole ratio of 1:1 and equidistantly from both O(3) and O(4). Such ion chelation weakens the C(1)–O(5) bond of GPP. At the enzymatic level, Mg^{2+} chelation to the diphosphate moiety of GPP is expected to facilitate the elimination of the diphosphate group, with the consequent formation of a carbocation that can readily cyclize into cyclic monoterpenoids.

Experimental

NMR Measurements.—The solutions were prepared in water for ^{31}P NMR measurements and in $[^2\text{H}_2]$ water for ^1H and ^{13}C NMR measurements. Solutions of GPP (7.5 mmol dm^{-3}) for measurements at 25°C were made up in *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES)–NaOH buffer (0.1 mol dm^{-3}), pH 7.0. Appropriate amounts of a 0.2 mol dm^{-3} solution of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ were added to the GPP solution so as to obtain the desired Mg^{2+} :GPP concentration ratio. For measurements at lower temperatures, the GPP- Mg^{2+} solutions (4 cm^3) were emulsified with heptane (4 cm^3) and sorbitan tristearate (0.1 g).¹⁰ This allowed us to obtain spectra at temperatures as low as -40°C . ^{31}P NMR spectra (202.47 MHz) were obtained on a JEOL GSX-500 spectrometer equipped with a variable-temperature controller. Field-frequency lock was achieved with external $[^2\text{H}_6]$ acetone. Chemical shifts δ are given in ppm downfield from external phosphoric acid. ^1H and ^{13}C NMR spectra were measured on a JEOL GSX-270 spectrometer (^1H 270 MHz; ^{13}C 67.94 MHz). Chemical shifts δ are given in ppm using 3-(trimethylsilyl)[2,3- $^2\text{H}_4$]propionic acid sodium salt (TSP) as internal standard. Coupling constant values J are given in Hz.

(E)-3,7-Dimethylocta-2,6-dienyl Diphosphate (Geranyl Diphosphate).—Following the reported method,¹¹ (*E*)-3,7-dimethylocta-2,6-dien-1-ol (562 mg, 3.6 mmol) was treated with *N*-chlorosuccinimide (529 mg, 3.96 mmol) and dimethyl sulfide (266 mg, 4.32 mmol). The resulting geranyl chloride was phosphorylated with tristetraethylammonium hydrogen diphosphate (7.55 g, 8.37 mmol). The crude product was purified on a silica gel column with isopropyl alcohol–25% ammonia solution–water (6:3:1) and crystallized by addition of a saturated aqueous lithium chloride to give the diphosphate (289 mg, 24% yield); $\delta_{\text{H}}(\text{D}_2\text{O})$ 5.47 (1 H, t, J 7.3, $\text{C}=\text{CHCH}_2\text{O}$), 5.22 [1 H, t, J 7.3, $(\text{CH}_3)_2\text{C}=\text{CH}$], 4.49 (2 H, t, J 6.8, CH_2O), 2.15 (4 H, m, CH_2CH_2), 1.73 (3 H, s, CH_3), 1.70 (3 H, s, CH_3) and 1.64 (3 H, s, CH_3); $\delta_{\text{C}}(\text{D}_2\text{O})$ 145.6 (C-3), 136.5 (C-7), 126.9 (C-6), 122.7 (d, $J_{\text{C,P}}$ 9.8, C-2), 65.5 (d, $J_{\text{C,P}}$ 5.8, C-1), 41.6 (C-4), 28.4 (C-5), 27.6 (C-8), 19.8 (C-10), 18.4 (C-9); $\delta_{\text{P}}(\text{H}_2\text{O})$ -7.76 (1 P, d, $J_{\text{P,P}}$ 20, P_β) and -10.12 (1 P, d, $J_{\text{P,P}}$ 21, P_α).

MO Calculations.—The total energy and two-centre energy were obtained from the CNDO/2 calculation.¹² The bond lengths and angles were the values reported from an X-ray analysis.¹³ Optimization of the molecular structure was made by use of the MM2 method for the carbon chain portion.

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