^{13}C NMR Detection of Delocalized C_{10}-Allylic Cation in the Biosynthesis of Farnesyl Diphosphate

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A new signal was observed at δ 164.5 in the ^{13}C NMR spectrum obtained by the enzymatic condensation of [1-^{13}C]geranyl diphosphate with isopentenyl diphosphate by farnesyl diphosphate synthase; the signal was assigned to C-1 of a delocalized C_{10}-allylic cation on the basis of MO calculations, the ^{13}C NMR spectrum of [1-^{13}C]geraniol in magic acid (FSO_3H–SbF_5) and ^{13}C NMR spectra of the enzyme reaction under reversible inhibition conditions.

Farnesyl diphosphate (FPP) is biosynthesized from the 1′-4 condensation of geranyl diphosphate (GPP) with isopentenyl diphosphate (IPP) by FPP synthase. The prenyl chain elongation was previously proposed to proceed with the occurrence of a geranyl cation intermediate.[1] However, no tangible proof of the generation of the cation in the chain elongation was given. The present communication reports a ^{13}C NMR spectrometric detection of the delocalized C_{10}-allylic cation in the biosynthesis of FPP.

Following the procedure described in the literature,[2] FPP synthase was obtained from avian liver and purified. Purification steps included fractionation with ammonium sulfate (36–57%) followed by chromatography, first on an anion exchange column [DEAE-TOYOPEARL 650M (TOSOH)] and then on a hydroxylapatite column [Bio-Gel HTP (Bio-Rad)]. The activity of this purified FPP synthase was about 900-fold with respect to that of the ammonium sulfate fraction. [1-^{13}C]Geraniol was prepared from methyl bromo[1-^{13}C] acetate and [1-^{13}C]linalool was prepared from the [1-^{13}C] geraniol by isomerization with $AgNO_3$ following the methods described in the literature.[3] [1-^{13}C]GPP was prepared from [1-^{13}C]geraniol by phosphorylation following the method described in the literature.[4]

The condensation of [1-^{13}C]GPP with IPP by FPP synthase was checked by means of ^{13}C NMR spectral measurements.† [1-^{13}C]GPP (4 μmol) and IPP (4 μmol) dissolved in 350 μl of Tris-HCl buffer [0.05 mol dm^{-3}, pH 7.0, H_2O : D_2O = 7 : 3 (v/v)] were added to a solution of the FPP synthase (1.2 mg protein) in 100 μl of the same buffer containing $MgCl_2$ (5 mmol dm^{-3}) in an NMR tube (5 mm O.D.). ^{13}C NMR measurements of the enzyme reaction were carried out at 37 °C. Reaction mixtures made up either with the heat-inactivated FPP synthase or without IPP, but otherwise prepared identically to the above described mixture, were used for the control experiments. After 5000 scans over a period of 2 h, the ^{13}C NMR spectrum shown in Fig. 1(*a*) was observed. The signals at δ 25.9, 58.1, 62.8 and 113.0 were assigned to C-5 of FPP, C-1 of geraniol, C-1 of GPP and C-1 of linalool, respectively. In addition to these signals, a signal was observed at δ 164.5 for the first time. The peak intensity of the new signal increased with increasing the concentration of the enzyme. This signal could be seen also in a control experiment using the above reaction mixture minus IPP, but neither this new signal nor the signal of [5-^{13}C]FPP appeared in the ^{13}C NMR spectrum of the reaction mixture with heat-inactivated FPP synthase, as shown in Fig. 1(*b*).

The new signal at δ 164.5 was assigned on the basis of MO calculations and ^{13}C NMR spectral experiments as follows. It is reported that the chemical shift of the delocalized allylic cation generated in magic acid[5] (FSO_3H–SbF_5) can be estimated from the π electron density.[6] The π electron density at C-1 of the delocalized C_{10}-allylic cation generated from GPP was evaluated to be 0.61.‡ From the correlation between the π electron density and ^{13}C NMR chemical shifts,[6] the ^{13}C NMR chemical shift of C-1 of the delocalized C_{10}-allylic cation generated from geraniol in magic acid is estimated to be δ 175.5. Actually, the ^{13}C NMR spectrum of [1-^{13}C]geraniol in magic acid showed a signal at δ 177.6 assigned to C-1 of the delocalized C_{10}-allylic cation generated from [1-^{13}C]geraniol together with a signal at δ 72.2 assigned to C-1 of [1-^{13}C]geraniol.§ This assignment was confirmed by the fact that the ^{13}C NMR spectrum of [1-^{13}C]linalool in magic acid showed also the signal at δ 177.6 together with a signal at δ 124.6 assigned to C-1 of [1-^{13}C]linalool. Since the C-1 signals of geraniol and linalool in D_2O are observed at δ 58.1 and 113.0, respectively, the correction for the solvent effect between D_2O and magic acid can be considered as 11–14 ppm. This correction gives δ 163–166 for the C-1 signal of the delocalized C_{10}-allylic cation in D_2O. Thus, the signal at δ 164.5 observed in the ^{13}C NMR spectrum obtained by the enzymatic condensation is assigned to C-1 of the delocalized C_{10}-allylic cation generated from GPP.

To further confirm that the delocalized C_{10}-allylic cation occurs only in the presence of the active enzyme, the enzymatic condensation of [1-^{13}C]GPP with IPP by FPP synthase was monitored by ^{13}C NMR spectral measurements under conditions where the enzyme activity was inhibited. The FPP synthase activity was controlled with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)[7] as deactivating reagent and dithiothreitol (DTT)[8] as reactivating reagent, which are

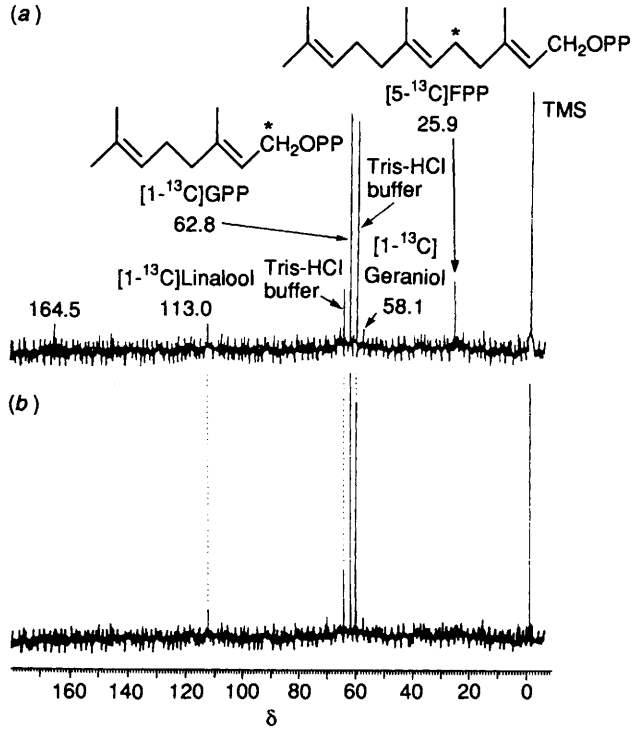

Fig. 1 ^{13}C NMR spectra of (*a*) the mixture of FPP synthase, [1-^{13}C]GPP and IPP and (*b*) the mixture of the heat-inactivated FPP synthase, [1-^{13}C]GPP and IPP. * Denotes ^{13}C.

known to interact with the cysteine residues of the enzyme.[7,9] Prior to the ^{13}C NMR measurements, the effects of the addition of DTNB and DTT on the FPP synthase activity were assayed using GPP and [4-^{14}C]IPP. When DTNB was added to a concentration of 750 μmol dm^{-3} in the reaction mixture, the enzyme activity decreased to *ca.* 10%. The activity of the deactivated enzyme was restored in 40 min by addition of DTT to a concentration of 30 mmol dm^{-3}. Therefore, it was confirmed that the FPP synthase activity can be controlled by addition of DTNB and DTT.

The activity-controlled reaction was then performed in the NMR tube under the same conditions as above and monitored by ^{13}C NMR measurements. In the absence of any activity-controlling reagent, the reaction mixture gave the ^{13}C signal at δ 164.5 as shown in Fig. 1(*a*). Addition of DTNB (750 μmol dm^{-3}) to deactivate the FPP synthase made the signal at δ 164.5 disappear, but had no effect on the intensities of the δ 25.9 (FPP, C-5) and 62.8 (GPP, C-1) signals. When the enzyme activity was restored in a 2 h incubation with DTT (30 mmol dm^{-3}), the intensity of the signal at δ 25.9 (FPP, C-5) increased, the intensity of δ 62.8 (GPP, C-1) decreased and the signal at δ 164.5 reappeared.¶ Obviously, the latest signal appears only in the enzyme reaction with active FPP synthase, suggesting that the delocalized C_{10}-allylic cation is formed in the active site of the enzyme. The cation, normally unstable at room temperature, would be stabilized by the enzyme long enough to be detected by ^{13}C NMR measurements.

In conclusion, the occurrence of the delocalized C_{10}-allylic cation in the biosynthesis of FPP was indicated by means of ^{13}C NMR measurements. This is the first example of the observation of the generation of the delocalized C_{10}-allylic cation in the biosynthesis of FPP.

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Footnotes

† All NMR experiments were performed on a 6.35-T JEOL GSX-270 spectrometer (^{13}C, 67.8 MHz) with a Fourier transform accessory, a spin decoupler and a variable temperature probe. The pulse delay used was 1.0 s. The pulse width and the data acquisition time were 4.6 μs and 0.511 s, respectively. The data points were 16384. Approximately 5000 accumulations over a period of 2 h were made to obtain satisfactory spectra. The signal to noise ratio in the region between δ 160 and 170 was *ca.* 2. The chemical shifts were relative to external Me_4Si in $CDCl_3$ (capillary).

‡ The π electron density at C-1 of the delocalized C_{10}-allylic cation generated from geraniol was evaluated on the CNDO/2 method.[10,11] The bond lengths and angles used for calculation were the values reported in the *ab initio* molecular orbital calculation of isopentenyl cations.[12] The standard geometry of the delocalized allylic cation was optimized by use of the MM2 calculation.[13]

§ Solutions of [1-^{13}C]geraniol or [1-^{13}C]linalool in 0.35 ml of SO_2ClF at −78 °C were prepared in NMR tubes (5 mm O.D.). A solution of magic acid [FSO_3H–SbF_5 = 1:1 (v/v), 0.35 ml] was added to each sample at −78 °C. ^{13}C NMR spectra of each mixture were observed at −50 °C. All chemical shifts were downfield from external Me_4Si in $CDCl_3$ (capillary).

¶ Upon addition of DTT to the DTNB-containing reaction mixture, the intensity of the C-5 signal of FPP increased 13%, while that of C-1 signal of GPP decreased 33%.

References

1 C. D. Poulter and H. C. Rilling, *Acc. Chem. Res.*, 1978, **11**, 307.
2 B. C. Reed and H. C. Rilling, *Biochemistry*, 1975, **14**, 50.
3 T. Suga, T. Hirata, T. Aoki and T. Shishibori, *Phytochemistry*, 1986, **25**, 2769.
4 V. J. Davisson, A. B. Woodside and C. D. Poulter, *Methods Enzymol.*, 1985, **110**, 130.
5 G. A. Olah and H. Mayr, *J. Am. Chem. Soc.*, 1976, **98**, 7333.
6 H. Mayr, W. Förner and P. R. Schleyer, *J. Am. Chem. Soc.*, 1979, **101**, 6032.
7 R. F. Colman, *Biochemistry*, 1969, **8**, 888.
8 W. W. Cleland, *Biochemistry*, 1964, **3**, 480.
9 R. A. Johanson and R. F. Colman, *Arch. Biochem. Biophys.*, 1981, **207**, 9.
10 J. A. Pople and M. Gordon, *J. Am. Chem. Soc.*, 1967, **89**, 4253.
11 A. Imamura and H. Fujita, *J. Chem. Phys.*, 1974, **61**, 115.
12 T. Takeda and M. Dupuis, *J. Am. Chem. Soc.*, 1983, **105**, 1713.
13 E. Osawa and Y. Mochizuki, *Quantum Chemistry Program Exchange Bull.*, 1984, **4**, 51.