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Enzymatic Syntheses of ^{13}C-enriched Geranylgeranyl Diphosphate and Casbene from ^{13}C-labeled Isopentenyl Diphosphate

Qiulong Huang, Kexue Huang and A. I. Scott *

Department of Chemistry, Texas A & M University, College Station, TX 77843

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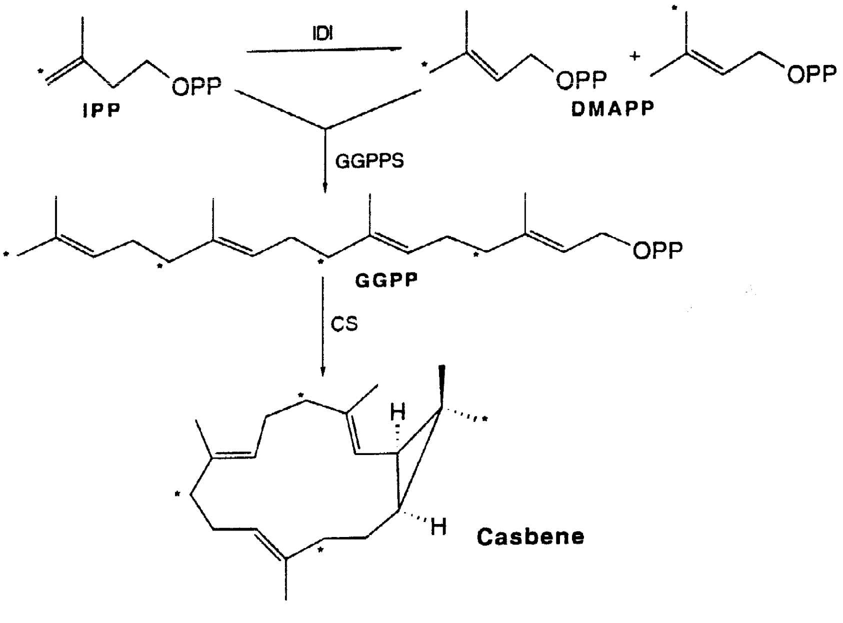
Abstract: Geranylgeranyl diphosphate and casbene were synthesized in high yields from [4-^{13}C]-3-methyl-3-butenyl diphosphate, using coupled enzyme reactions. © 1998 Elsevier Science Ltd. All rights reserved.

Geranylgeranyl diphosphate (GGPP),[1] the universal precursor of diterpenoids, is synthesized from isopentenyl diphosphate and dimethylallyl diphosphate, geranyl diphosphate or farnesyl diphosphate, all three reactions catalyzed by geranylgeranyl diphosphate synthase. Although farnesyl diphosphate has been synthesized enzymatically in high yield,[2] to the best of knowledge, the enzymatic synthesis of geranylgeranyl diphosphate from non radioactive material has not been reported. Among the macrocyclic diterpene hydrocarbons formed from GGPP is casbene, found in the young castor bean seedling (*Ricinus communis* L.).[3] Casbene, which is produced in response to fungal attack, serves the castor bean as a phytoalexin and exhibits antifungal and antibacterial activity.[4] Casbene synthase (CS, EC 4.6.1.7) catalyzes the cyclization of GGPP to form casbene in a single enzymatic step.[5] (Scheme 1). We here report the enzymatic syntheses of geranylgeranyl diphosphate and of casbene using ^{13}C-labeled isopentenyl diphosphate (IPP).

Scheme 1



The synthesis of [4-^{13}C]-3-methyl-3-butenyl diphosphate followed known reactions (Scheme 2). An aldol condensation between acetone and formaldehyde gave 1-hydroxy-3-butanone(**1**),[6] which was protected by triisopropylsiliyl chloride (TIPS-Cl) and then reacted with ^{13}C-methyl-triphenylphosphine iodine. The alcohol (**4**) was activated and bis-phosphorylated according to the method of Davisson et al[7] to afford labeled isopentenyl diphosphate (**5**).[8]

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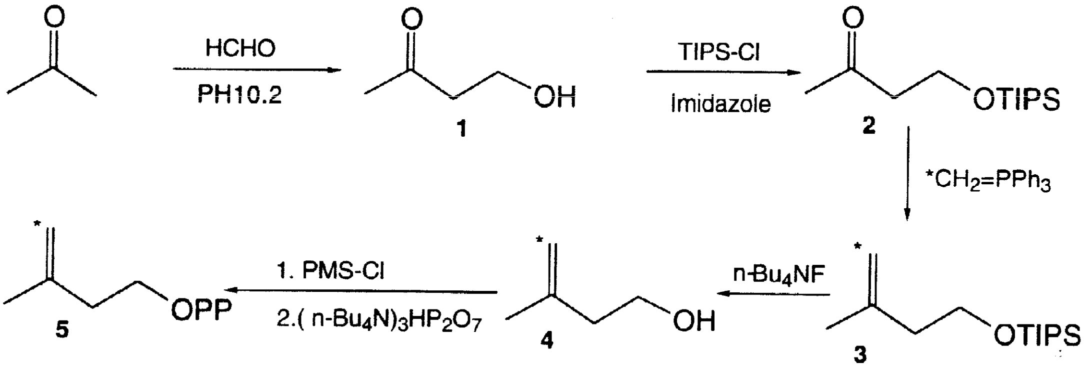
Scheme 2



Isopentenyl diphosphate:dimethylallyl diphosphate (DMAPP) isomerase (IDI) catalyzes the 1,3-allylic rearrangement reaction converting IPP into DMAPP *via* a postulated two-base cationic mechanism, resulting in the observed antarafacial transposition of hydrogen.[9] In our studies, the IPP isomerase was prepared in a crude cell free extract according to Hahn et al[10]. The sp^2 hybridized carbon in IPP is converted into sp^3 carbon in DMAPP, thereby providing a very convenient method to monitor the reaction by carbon-13 NMR. IPP shows a signal at 110 ppm (C-4) and DMAPP at 24.5 ppm (C-3 methyl) (Figure 1). We found that when the reaction time was extended to 16 hours, a new signal appeared at 16.6 ppm, due to the scrambling of label during the enzyme catalyzed reaction. A similar phenomenon has been observed by Ogura,[11] presumbly due to some degree of freedom of rotation of the carbon-carbon bond (C_2-C_3), (not totally free) in the initial carbocation, before the second base acts upon it to remove a proton, a result supportive of the cationic mechanism.

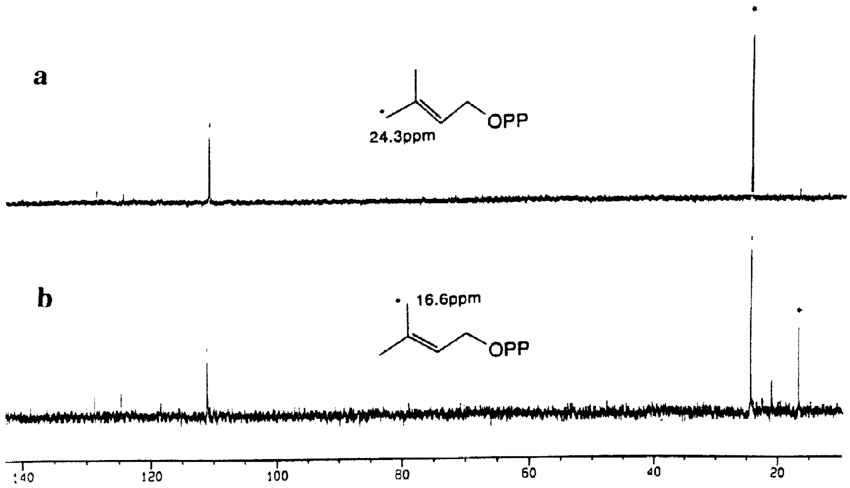


Figure 1. ^{13}C-NMR (in 10% D_2O) of isomerization of IPP with isomerase. (a). After 1 hour incubation at 37°C. (b). After 16-hr incubation.

In the coupled enzymatic synthesis of geranylgeranyl diphosphate, we used GGPP synthase, which was obtained according to the method of Meth et al[12] with modification. A typical reaction was conducted as follows: In a 1.5 ml centrifuge tube, 100 μl IPP (ca. 1 mg IPP), 100 μl isomerase, 300 μl GGPP synthase and 500 μl 25 mM phosphate buffer (pH 7.2, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF) were mixed and incubated at 37°C overnight. The supernatant was checked by NMR for residual IPP and DMAPP, the precipitate extracted with deuterated methanol and the extract analyzed by NMR. In our experiment, when the expression of GGPP synthase was high, no residual IPP and DMAPP were observed and the GGPP produced by coupled reactions showed the correct labeling pattern (Figure 2). There were four additional signals between 15.9-18.0 ppm. A DEPT NMR experiment showed these are all assignable to methyl groups, formed by scrambling during the isomerization. The data are consistent with the scrambled methyls of GGOH.[13] Experiments in which DMAPP, GPP or FPP was incubated with IPP in the absence of isomerase gave the expected signals for GGPP. It was found that even in the presence of excess DMAPP, GPP or FPP, some unreacted IPP was detected. Thus, the concentrations of IPP and of the appropriate chain starters are critical. In the coupled reactions using isomerase, the concentrations of IPP and DMAPP are regulated by the isomerase, so all the IPP is consumed. We also found that inorganic pyrophosphatase improved the efficiency of GGPP synthase, by removing product inhibition. A large scale reaction from 100 mg IPP monitored by ^{13}C NMR afforded 31.4 mg GGPP (74% overall) after isopropanol-acetonitrile extraction and flash column chromatography.

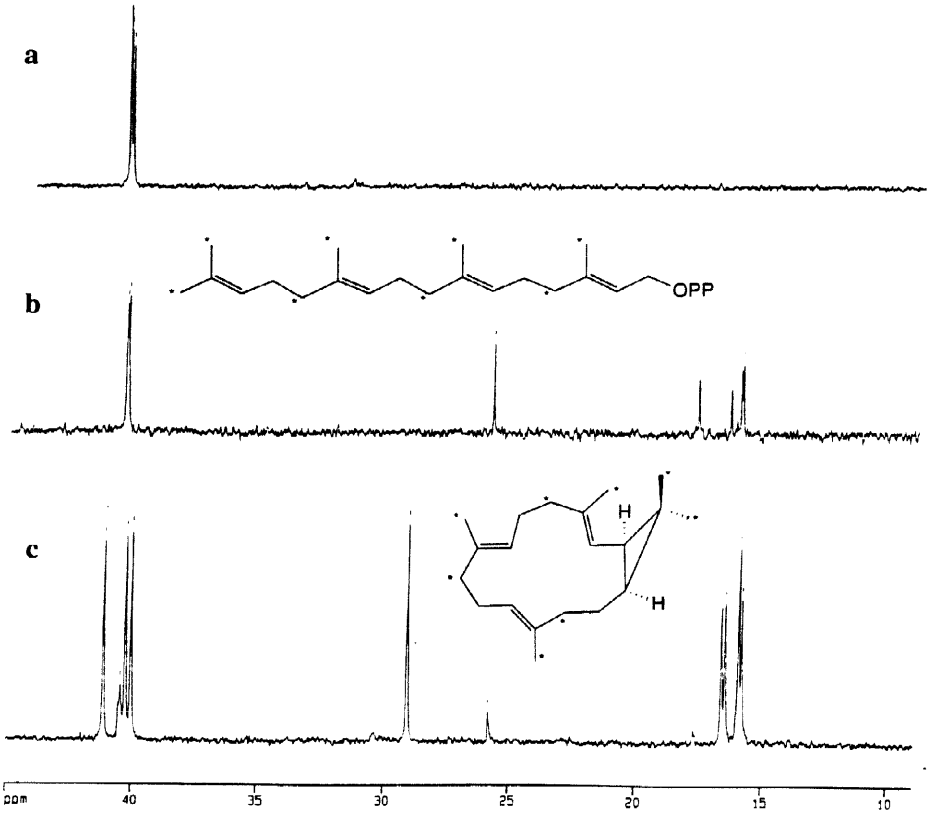


Figure 2. ^{13}C-NMR of GGPP and casbene. (a). GGPP from DMAPP and IPP by GGPP synthase. (b). GGPP from IPP by coupled isomerase and GGPP synthase. (c). Casbene from IPP by isomerase, GGPP synthase and casbene synthase.

Synthesis of casbene Enzymatic syntheses of casbene have been achieved by several groups.[3,14] However, the yields were low and casbene had to be separated from other hydrocarbons. Since GGPP is now easily synthesized from the coupled enzyme reaction, we explored coupling with a third enzyme, casbene synthase

(CS). In this experiment, the CS was a crude cell-free extract obtained according to the procedure of Huang et al,[15] which,when coupled with the isomerase and GGPP synthase, afforded casbene almost exclusively (Figure 2). We see from the spectrum that there are eight major signals, four (41.1, 40.3, 40.0 and 29.1 ppm) due to the normal prenylation/cyclization, and the other four (16.6, 16.5, 15.9 and 15.8 ppm) due to the scrambling introduced by the isomerase. The NMR data are consistent with those reported by Crombie et al,[16] showing the characteristic *gem*-methyl carbons (29.1 and 16.6 ppm) The product was extracted from the enzymatic reaction with deuterated methanol (instead of $CDCl_3$), in order to detect any residual GGPP. The NMR of the mixture showed a small amount of residual GGPP (25.8 ppm). There was no detectable IPP or DMAPP, leading to an estimated overall yield of greater than 80% based on the starting IPP.

Summary Carbon-13 labeled GGPP and casbene have been synthesized by coupled enzymatic reactions and the process monitored conveniently by ^{13}C-NMR. The successful large-scale synthesis of GGPP provides optimism for the large-scale synthesis of casbene, or other cyclic hydrocarbons, such as taxadiene, the important intermediate in the biosynthesis of taxol. The synthesis of analogs of GGPP and their cyclization with various diterpenoid cyclases are under investigation.

Acknowledgment. Our special thanks go to Dr. C. D. Poulter for his generous gifts of the plasmids for IPP isomerase and GGPP synthase.

References and Notes

1. Abbreviations used are: DMAPP, dimethylallyl diphosphate; DTT, dithiothreitol; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; PMSF, phenylmethylsulfonyl fluoride.
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8. ^{1}H NMR of **5** (D_2O, pH 8.0) δ 5.0-4.5 (2H, overlapped by peaks from H_2O), 3.92 (2H, dt, J=6.4 Hz, J=6.3 Hz), 2.29 (2H, dt, J=6.4 Hz, J=6.0 Hz), 1.67 (3H, d, J=5.7 Hz). ^{13}C NMR δ 143.3 (d, J=70 Hz), 110.8, 63.4, 37.3, 21.1.
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