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Sesquiterpenes produced by truncated taxadiene synthase

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Abstract

Soluble, highly active N-terminal truncated taxadiene synthase catalyzes the formation of an isomeric mixture of taxadienes from geranylgeranyl diphosphate. Farnesyl diphosphate was also found to be a good substrate, producing four sesquiterpenes which were characterized. The dual activities of taxadiene synthase and product inhibition caused by sesquiterpene metabolites make it imperative that active GGDP synthase be present in order for multi-enzyme systems to follow the taxol pathway in vitro.

In the biosynthesis of the diterpenoid taxol, the first committed step is the conversion of geranylgeranyl diphosphate(GGDP) into taxadiene, catalyzed by a single enzyme, taxadiene synthase (TS).[1] Attempts to express the full length amino-acid sequence of this enzyme in *E. coli* led to intractable inclusion bodies. Truncation of the N-terminal end produced soluble highly active enzyme (TS79H).[2] Using this overexpressed truncated taxadiene synthase, we have found that the enzyme is somewhat nonspecific, producing a mixture of taxa-4(5), 11(12)-diene (**1**) (87%) and taxa-4(20), 11(12)-diene (**2**) (13%) from geranylgeranyl diphosphate (GGDP) (Scheme 1).[3,4]

Diterpene cyclases use GGDP as substrate, while sesquiterpene cyclases utilize farnesyl diphosphate (FDP). In general these classes of cyclase have rigorous substrate requirements. Although diterpene cyclases are theoretically capable of binding and turning over smaller substrates such as FDP, it is uncommon to find a diterpene cyclase which exhibits dual or triple substrate activity. In a mutagenetic analysis of taxadiene synthase, we changed the histidine-579 residue to aspartate, the preferred residue for most FDP cyclases, and compared the activity of the resulting enzyme to that of truncated wild-type taxadiene synthase. To our surprise, the latter synthase readily accepts FDP, producing larger quantities of the same product mixture as that produced by the mutant, as shown by GC analysis.[5,6]

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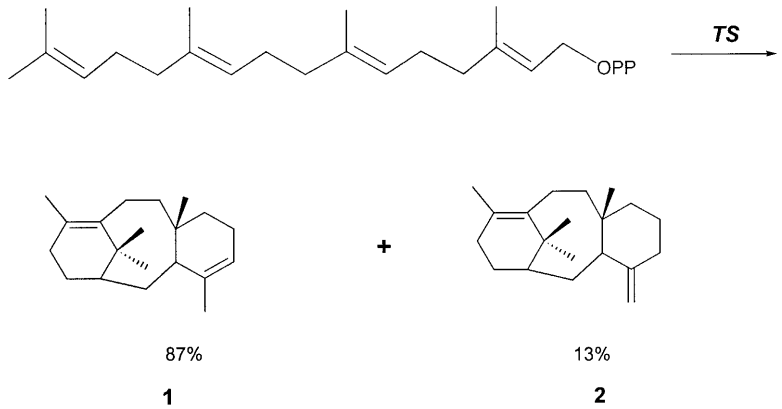
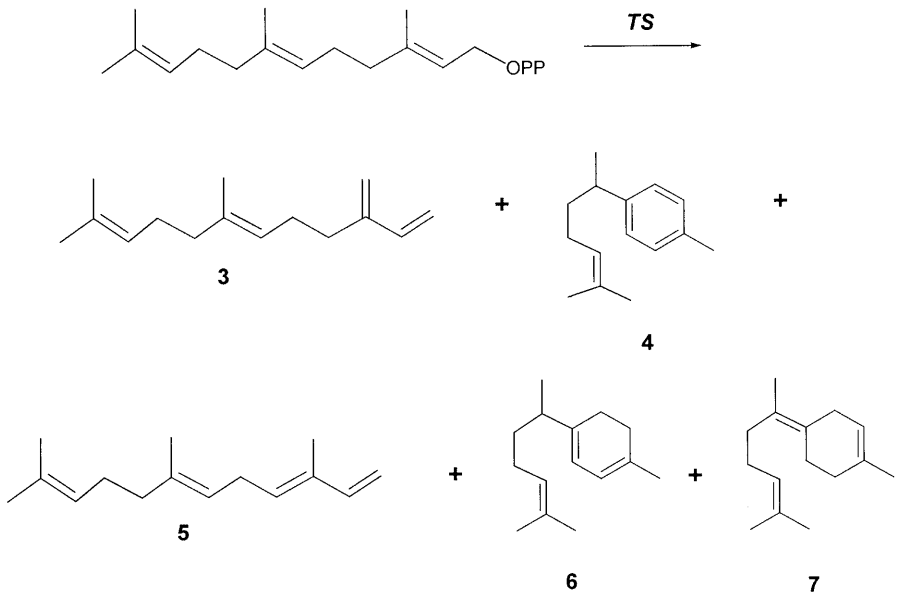


Scheme 1.

Five major peaks present in the ratio 21:4:16:28:31 were separated by preparative GC and characterized as *E*-β-farnesene (**3**), α-curcumene (**4**), *E*,*E*-α-farnesene (**5**), γ-curcumene (**6**), and *E*-γ-bisabolene (**7**) (Scheme 2).[7]



Scheme 2.

All five compounds were detected in a fresh extract, with **4** as the minor component. On standing in air, **4** became dominant while **6** and **7** were not detectable, indicating that **4** is an oxidation product of these metabolites.

Although there is no report whether the native enzyme isolated from the Pacific yew accepts FDP, we have demonstrated that truncated taxadiene synthase accepts FDP as substrate, producing a mixture of the four sesquiterpenes (excluding α-curcumene, **4**), [8] and the thioredoxin fusion taxadiene synthase was also found to utilize FDP as substrate.[9] It is therefore possible that native taxadiene synthase accepts FDP. Homology search shows that the C-terminal domain of taxadiene synthase shares 44% identity with that of *E*-α-bisabolene synthase from *Abies grandis*, indicating a possible common origin of the sesquiterpenoid and diterpenoid cyclases which would be responsible for the cross reactivity. Coincubation of equal concentrations of FDP and GGDP provided evidence that GGDP is not the preferred substrate of the cyclase.[10] The dual activity of taxadiene synthase makes this enzyme a very interesting target in the engineering of the taxol pathway. In addition, we found strong inhibition caused by the sesquiterpene products when performing kinetic studies with purified enzymes.[11] It is now clear that in order to synthesize taxol efficiently, an active form of GGDP synthase should be present in multi-enzyme syntheses in vitro, to avoid entering the sesquiterpene manifold.

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References

1. Lin, X.; Hezari, M.; Koepp, A. E.; Floss, H. G.; Croteau, R. *Biochemistry* **1996**, *35*, 2968.
2. The N-terminal sequence of the native taxadiene synthase is unknown, and the full-length premature protein is 862 residues. TS79H was generated by removing 78 residues from the N-terminus, expressed as N-terminal His-tagged protein. Huang, Q.; Roessner, C. A.; Scott, A. I, to be published.
3. Huang, Q.; Roessner, C. A.; Scott, A. I. *219th ACS National Meeting*, (March 26–30, 2000), Abstracts, part 2, p. 85.
4. Williams, D. C.; Wildung, M. R.; Jin, A. Q.; Dalal, D.; Oliver, J. S.; Coates, R. M.; Croteau, R. *Arch. Biochem. Biophys.* **2000**, *379*, 137.
5. The standard incubation is as follows: to 500 ml buffer composed of 30 mM HEPES, pH 8.5, 10% glycerol, 5 mM sodium bisulfite, 5 mM sodium ascorbate, 10 mM KF, 2 mM DTT and 2 mM β-CD, a crude cell lysate from 4 liters of cell culture and 1 g of crude FDP, which was prepared from *trans*,*trans*-farnesol according to the method of Davisson (Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, *51*, 4768), were added, and $MgCl_2$ was added to a concentration of 5 mM. The well-mixed solution was kept at 30°C overnight. The resulting mixture was heated at 90°C for ten minutes. After centrifugation, the pellet and supernatant were both extracted with hexane (200 mL×3). The combined hexane was evaporated and the residue passed through a silica gel column. Fractions with $R_f = 0.6–0.9$ (hexane) were collected and analyzed by GC. Around 45 mg of product mixture was obtained.
6. Analytical GC was performed using an SGE 25 m×0.25 mm ID BP1 column, 20 psig He, temperature programmed 60°C for 1 min, to 280°C at 15°C/min. Capillary retention times for components **3**–**7** were 8.54, 8.85, 8.99, 9.05, and 9.24. Preparative GC utilized a 5% OV101 on Chromosorb G-HP, 60 ml/min N_2, 185°C isothermal.
7. Metabolites were identified by comparison of GC, MS, ^1H and ^{13}C spectra with data obtained from synthetic compounds and literature sources. Components **3** and **5** were prepared by dehydration of *E*-nerolidol. Compo-

nent **4** was prepared by deoxygenation of gossonol (Elzen, G. W.; Williams, H. J.; Vinson, S. B. *J. Chem. Ecol.* **1984**, *10*, 1251) by the method of Small et al. (Small, G. H.; Minella, A. E.; Hall, S. S. *J. Org. Chem.* **1975**, *40*, 3151). Component **6** was prepared by dehydration of β-bisabolol (Frater, G.; Muller, U. *Helv. Chim. Acta* **1989**, *72*, 653). Component **7** was prepared by the method of Faulkner et al. (Faulkner, D. J.; Wolinsky, L. E.; *J. Org. Chem.* **1975**, *40*, 389). For dehydration, 100 mg alcohol in 1 ml of dry pyridine was treated with 1 equiv. $POCl_3$, stirred at rt overnight, poured into water, and extracted with ether. The extract was washed with 1 M HCl and water, dried over $MgSO_4$ and evaporated. Products were purified by preparative GC. ^1H spectra for several components were from Andersen, N. H.; Syrdal, D. D. *Phytochemistry* **1970**, *9*, 1325. ^{13}C spectra for **4**: 144.6, 135.1, 131.3, 128.9, 126.8, 124.6, 39.0, 38.5, 26.2, 25.7, 22.5, 21.0, 17.7. ^{13}C spectra for **6**: 139.0, 131.3, 131.1, 124.8, 118.8, 117.8, 40.2, 35.0, 31.6, 26.6, 26.1, 25.7, 23.0, 19.5, 17.7.

8. Further truncation at the N-terminus shows that the relative activities of truncated forms vary for both GGDP and FDP as substrate, but the ratio of products always stays the same.
9. Huang, K.; Huang, Q.; Wildung, M. R.; Croteau, R.; Scott, A. I. *Protein Expression and Purification* **1998**, *13*, 90.
10. Larger amounts of the sesquiterpene products than taxadienes (1.7:1.0) are generated when equal concentrations of FDP and GGDP were coincubated with TS79H at 30°C for 16 hours.
11. Kinetic studies have been done on TS79H using GGDP as substrate, revealing a K_m of 2.40 μM, and a K_{cat} of 3.04 min^{-1}. However, kinetic data for FDP substrate could not be measured on purified truncated taxadiene synthase, due to inhibition from FDP products, which is supported by the fact that products from FDP actually kill the enzyme when a 1:1 mixture of GGDP and FDP was used.