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Characterization of santalene synthases using an inorganic pyrophosphatase coupled colorimetric assay

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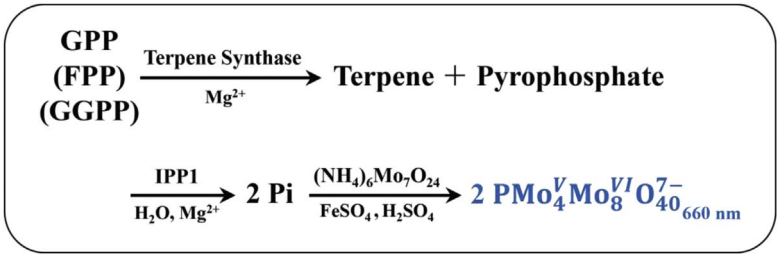
ABSTRACT

We developed a colorimetric assay using yeast inorganic pyrophosphatase (IPP1) as a coupling enzyme to measure the activities of terpene synthases. IPP1 hydrolyzes pyrophosphate, the byproduct of terpene synthase catalyzed reactions, into orthophosphate, which can then be quantitated by reacting with molybdic acid to form a blue color compound. As a proof of concept, this method was used to quantitatively characterize three santalene synthases, SaSSy and SspiSSy involved in sandalwood oil biosynthesis, and a phylogenetically distant SanSyn from *Clausena lansium*. Our study provided the kinetic parameters of all three santalene synthases and demonstrated the validity of the enzyme couple colorimetric assay by the comparison of this assay with the existing GC-MS (Gas Chromatography-Mass Spectrometry) method.



Introduction

Terpenoid (also called isoprenoid, terpene) is a class of natural products composed of two isoprene units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) as building blocks. Depending on the number of the repetitive basic building blocks, terpenoid can be classified into monoterpenes, sesquiterpenes, and diterpenes etc. [1]. The biosynthesis of terpenes involves catalytic activities of a large group of terpene synthases and downstream modifications such as oxidoreduction, hydroxylation, acetylation by a variety of other enzymes including desaturase [2], cytochrome P450 [3], acetyltransferase [4] etc.. Consequently, terpene is one of the largest and structurally most diverse groups of natural products, playing key metabolic, structural, regulatory and defensive roles in all kingdoms of life. Terpenoids originated from different organisms impact many aspects of human life, such as medicine [5,6], flavor and fragrances [7], and nutrition [8].

There are two IPP and DMAPP biosynthetic pathways: the mevalonic acid (MVA) and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. DMAPP and IPP condense to form the all-*trans* isoprenoid diphosphates, geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) in reactions catalyzed by the Head-to-Tail *trans*-prenyl transferases enzymes geranyl diphosphate synthase (GPPS), farnesyl diphosphate synthase (FPPS), and geranylgeranyl diphosphate synthase (GGPPS) respectively. GPP, FPP and

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GGPP can be further processed by the ε Head-to-Head prenyl transferases to make C_{30} and C_{40} hydrocarbon species which are the precursors of steroids and carotenoids etc., or alternatively, they can be converted by terpene cyclases to form various monoterpenes, sesquiterpenes, and diterpenes with different ring structures.

Class I terpene synthases share similarity in their catalytic mechanisms: e.g. containing an aspartate rich $DD(X)_nD$ and an NSE/DTE motif involved in binding the trinuclear Mg^{2+}, which triggers the departure of the pyrophosphate group of the substrate, leaving a carbon cation intermediate in the hydrophobic pocket of the active site. Many of the terpene cyclases are product promiscuous generating multiple, in some cases, up to hundreds of structurally and stereochemically diverse products. To measure the enzyme activities and kinetic parameters of these enzymes are therefore challenging. Chromatographic methods in combination with mass spectrometry (LC-MS, GC-MS) are commonly used to characterize and quantitate the formation of individual products and the sum of the products is used to determine the overall activity. The large number of products, some with very diverse structures, some with subtle structural differences and some with low yield, result in the measurement of this type of enzymes with current methods oftentimes inaccurate. Furthermore, the LC-MS and GC-MS methods require expensive equipment and authentic standards that are oftentimes commercially unavailable.

Sandalwood oil is highly valued for its pleasant odor and used widely in perfumery and cosmetics. The therapeutic effects of its active components have also been documented in literature [9–11]. The biosynthetic pathway requiring a Class I terpene synthase, santalene synthase, has recently been characterized [12] (Fig. 1). Two recent publications on the same santalene synthase, SaSSy by two different research groups [13,14] reported a 40-fold difference of the k_{cat} value and 17-fold difference of k_{cat}/K_m. Such discrepancy could be due to the difference in qualities of the recombinant proteins or alternatively due to the inaccuracy of numerical calculation of the integral of small peaks in chromatographic elution profile since both groups used GC-MS method to characterize the enzyme.

Inorganic pyrophosphatase has been used in quantitating DNA and RNA synthesis [15] and very recently terpene synthesis [16]. In this study, yeast inorganic pyrophosphatase (IPP1) coupled assay was used to determine the activities and kinetic parameters of santalene synthases. Two santalene synthases SaSSy and SspiSSy from *Santalum*, and SanSyn from a tropical fruit *Clausena lansium* (Wampee) were cloned, expressed, and purified from *Escherichia coli* respectively. The three enzymes differ in product specificity. SanSyn forms predominantly α-santalene, which accounts for > 90 % of its overall products, a percentage significantly higher (~3 fold) than that of SaSSy or SspiSSy [17]. Chromatography-based methods are limited by the availability and purity of standards. The volatile properties of products make product enrichment difficult. Thus at the low end of substrate concentration in kinetic assay, it is hard to accurately measure product formation. This is probably another main reason that causes the controversial kinetic data in the literature. The IPP1-coupled method, however, measures the formation of pyrophosphate, thus low amount of activity can be detected by concentrating the reaction mixture. Furthermore, this method can be applied broadly to all kinds of terpene synthases [18] and prenyltransferases [19] some of which are of great interest as drug targets. The colorimetric assay has its advantage in supporting high throughput screening.

Materials and methods

Gene syntheses, plasmid construction and yeast strains

SaSSy (Uniprot # E3W202), SspiSSy (Uniprot # E3W204) and SanSyn (Uniprot # E5LLI1) were codon optimized and synthesized by General Biosystems, and inserted into pET-28a vector under T7 promoter with a C-terminal His_6 tag to form pET28a-SaSSy, pET28a-SspiSSy and pET28a-SanSyn plasmids respectively. The yeast inorganic pyrophosphatase (IPP1, Uniprot # P00817) gene was amplified by yeast colony PCR using forward primer (5′-ACTTTAAGAAGGAGATATACCATGACCTACACTACCAGACA-3′), reverse primer (5′-ATCTCAGTGGTGGTGGTGGTGCTCGAGTTAAACAGAACCGGAGATGAAG-3′), and *Saccharomyces cerevisiae* BY4742 wild type strain as template. The PCR product was then inserted into pET-28a vector using Gibson assembly cloning method [20] to form pET28a-IPP1 plasmid.

In order to obtain α-santalene as standard in GC-MS analysis, yeast genetic engineering and fermentation was performed. The MVA pathway genes were overexpressed by strong inducible GAL1 promoter. SanSyn gene was amplified using forward primer (5′-TGACATAACTAATTACATGACTTAATCATCCAATTTAACTGGATCT-3′) and reverse primer (5′-TTAACGTCAAGGAGAAAAAACTATACATGTCAACACAACA

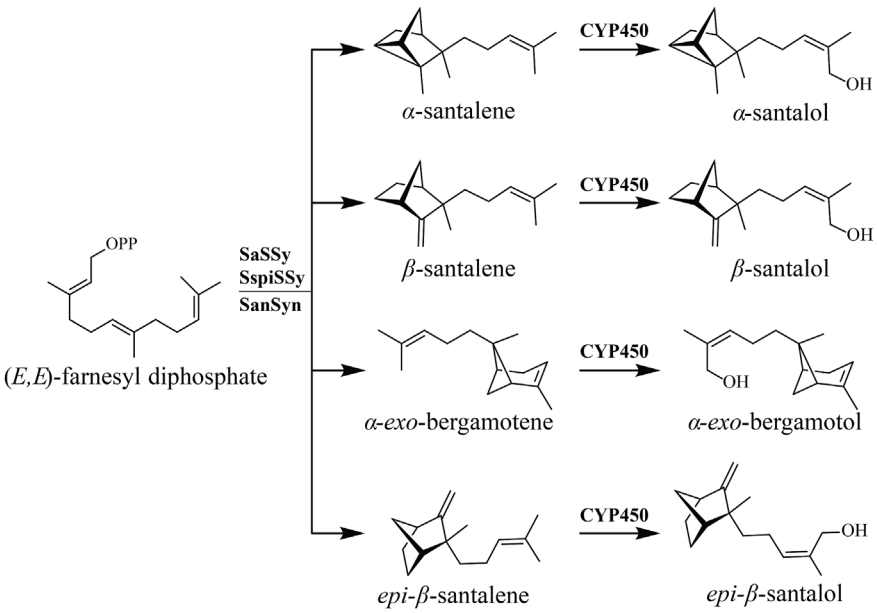

Fig. 1. The biosynthetic pathway of sandalwood oil. Specific hydroxylation at santalene and bergamotene is catalyzed by CYP450 monooxygenase (SaCYP736A167) to produce santalol and bergamotol [12].

AGTTTCATCTG-3′). The PCR product was inserted into pRS426 high copy 2μ plasmid under the control of a GAL1 promoter using Gibson assembly cloning method to form recombinant plasmid pRS426-GAL1p-SanSyn, which was transformed into the MVA pathway modified yeast strain, resulting in Santa 1.0 strain (unpublished).

Protein expression and purification

SaSSy

Recombinant SaSSy protein was produced and purified with some modifications to a previously described protocol [13]. pET28a-SaSSy was transformed into *Escherichia coli* BL21 (DE3) cells. The transformant was grown in LB medium containing kanamycin (50 μg/mL) at 37 °C in a shaker incubator at 220 rpm, and induced for the expression of SaSSy with 0.2 mM isopropyl *β*-D-1-thiogalactopyranoside (IPTG) overnight at 20 °C. Typically, cells from 1 L culture were lysed with a French press (Panda plus, Niro Soavi) at 14,000 psi in 120 mL buffer A (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 1 mM $MgCl_2$) containing 1 mM dithiothreitol (DTT), protease inhibitor cocktails (Roche), and 30 mM imidazole. The lysate was then centrifuged at 14,000 × *g* 30 min at 4 °C. The supernatant was collected and subjected to 1% of streptomycin sulfate precipitation to remove nucleic acids. The protein solution was filtered and applied to a 5 mL Ni-NTA high-trap column (GE). The column was washed with 10 column volume (CV) buffer A containing 30 mM imidazole, and eluted with buffer A containing 100 mM imidazole. The 40 mL eluate containing SaSSy was then dialyzed against 3 L dialysis buffer B (25 mM HEPES, pH 7.4, 10% glycerol, 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT) overnight. The resulting protein solution was then concentrated using a YM30 centricon (Millipore) and aliquoted in eppendorf tubes, flash-frozen with liquid nitrogen and stored at −80 °C freezer for later enzymatic assays. The specific activity of SaSSy is 67 nmol· min^{-1}· mg^{-1}.

SspiSSy

pET28a-SspiSSy was transformed into *E. coli* ArcticExpress cells (Agilent). The transformant was grown in LB medium with kanamycin (50 μg/mL) and gentamicin (15 μg/mL) until OD_{600} reached 0.6 to 0.8, and induced for the expression of SspiSSy with 0.2 mM IPTG overnight at 16 °C. Typically, cells from 4 L culture were lysed with a French press in 170 mL same lysis buffer used for SaSSy purification, centrifuged to remove cell debris and unbroken cells and applied to a 5 mL pre-equilibrated Ni-NTA high-trap column. The column was then washed with 12 CV buffer A containing 30 and 50 mM imidazole, and eluted with 100 mM imidazole. The eluate with 100 mM imidazole containing SspiSSy was pooled and dialyzed against 2 L buffer C (20 mM sodium phosphate, pH 7.4, 1 mM $MgCl_2$, 1 mM DTT) to remove salt. The resulting protein solution was then loaded onto a 5 mL Q Sepharose column (GE). The Q column was then eluted with 200 mL linear salt gradient from 0.1 to 0.3 M NaCl in buffer C. Fractions containing SspiSSy of high purity examined by SDS-PAGE were collected, dialyzed, concentrated and stored at −80 °C freezer as described in the purification of SaSSy. The specific activity of SspiSSy is 96 nmol·min^{-1}· mg^{-1}.

SanSyn

pET28a-SanSyn was transformed into *E. coli* ArcticExpress cells. The transformant was inoculated into 5 mL medium and grown overnight as described for SspiSSy. The culture was then expanded to 200 mL medium containing tryptone 10 g/L, yeast extract 10 g/L, NaCl 10 g/L, $K_2HPO_4{\cdot}3H_2O$ 1.31 g/L, KH_2PO_4 2.5 g/L, pH 7.2, and incubated with shaking at 220 rpm at 37 °C. When OD_{600} reached 1.2, this starter culture was diluted into a 3.0 L bioreactor (eppendorf) with an initial volume of 2.0 L. The fermentation medium is consisted of tryptone 16.3 g/L, yeast extract 23 g/L, NaCl 12 g/L, $K_2HPO_4{\cdot}3H_2O$ 1.31 g/L, KH_2PO_4 2.5 g/L, pH 7.2. Glucose and $MgSO_4{\cdot}7H_2O$ were sterilized separately and added to the fermentation medium in an initial concentration of 5 and 0.65 g/L respectively. The initial agitation speed and flow rate of aeration was set at 200 rpm and 2.0 L/min. The dissolved oxygen concentration was maintained above 30% saturated. When OD_{600} of the culture reached approximately 25, the temperature was adjusted to 20 °C, and IPTG was added to a final concentration of 1 mM. During induction, glucose was maintained at 0.6 g/L, and pH at 7.2–7.4. After induction for 12 h, the cells were harvested by centrifugation at 8000 × *g* for 10 min at 4 °C, and the pellet was frozen at −20 °C. Typically, cells (wet weight ~28 g) from 500 mL culture were lysed with a French press in the same lysis buffer and applied to a 5 mL pre-equilibrated Ni-NTA high trap column. The column was then washed with buffer A containing 30 and 45 mM imidazole, and eluted with 100 mM imidazole. Eluate containing SanSyn was pooled and dialyzed against 2 L dialysis buffer C. The resulting protein was subjected to anion exchange chromatography as described for the purification of SspiSSy. The protein collected from Q column was concentrated, and loaded onto Superdex 200 (300 mL) and eluted with buffer B. SanSyn was eluted as a monomer. Purified SanSyn was then flash frozen with liquid nitrogen and stored at −80 °C freezer for later enzymatic assays. The specific activity of this enzyme is 69 nmol·min^{-1}· mg^{-1}.

IPP1

pET28a-IPP1 was transformed into *E. coli* BL21 (DE3) cells. A single colony from the transformant plate was inoculated into a 5 mL LB medium starting culture containing kanamycin (50 μg/mL) and grown overnight at 37 °C. The culture was then expanded to 1 L and continued to grow at 220 rpm 37 °C until OD_{600} 0.8. Cells were induced for the expression of IPP1 with 0.1 mM IPTG overnight at 16 °C, pelleted by centrifugation (8000 × *g*), and lysed with a French press in 180 mL lysis buffer of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl and 2 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysate was centrifuged for 20 min at 15,000 × *g* to remove the cell debris, and nucleic acids were precipitated and discarded by the slow addition of 1.25% streptomycin sulfate. The protein was precipitated with $(NH_4)_2SO_4$ (90% saturation, 4 °C) and desalted by Sephadex G-25 with the elution buffer containing 30 mM Tris-HCl, pH 7.6, 3 mM pyrophosphate. The protein solution (40 mL) was loaded onto a 40 mL DEAE-Sepharose column (Sigma-Aldrich resins, home-packed column). The protein was then eluted with 30 mM Tris-HCl, pH 7.6, 1 mM pyrophosphate containing three step gradients (120 mL each) 20, 100 and 200 mM NaCl respectively. The 100 mM NaCl fractions contain homogenous IPP1 visualized by 12% SDS-PAGE and therefore were collected and subjected to buffer exchange (to remove pyrophosphate) by $(NH_4)_2SO_4$ precipitation and Sephadex G-25. The final storage buffer is 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% glycerol. IPP1 with a concentration of 1.8 mg/mL was aliquoted, flash-frozen with liquid nitrogen and stored at −80 °C freezer for later use.

SDS electrophoresis

Proteins of different sources were denatured using Laemmli loading buffer and heated at 100 °C for 5 min before applied to gel electrophoresis [21]. 10 and 12% polyacrylamide gel were used to separate santalene synthase and IPP1 respectively. The electrophoresis was run at a constant voltage (200 V) for 80 min before subjected to Coomassie staining. The gels were photographed with a gel imaging system (BaiJing).

IPP1 activity assay

An online IPP1 activity assay protocol [22] was used with slight modification. Mainly the pH of the assay buffer was changed from 9.0 to 7.4, which is the optimal condition for santalene synthase activity. The calculated specific activity was 400 units/mg.

Establishment of pyrophosphate standard curve

Sodium pyrophosphate was diluted in 25 mM HEPES, pH 7.4, 10% (v/v) glycerol, 5 mM DTT and 10 mM $MgCl_2$ to a series of concentrations (100, 50, 25, 5, 1 and 0 μM). Note that this buffer is the assay buffer for optimal santalene synthase activity. 10 μL IPP1 (0.1 mg/mL) was added to 100 μL pyrophosphate standards and the reaction mixture was incubated at 25 °C for 10 min. The reaction was then stopped by heat inactivation in boiling water bath for 2 min and cooled to room temperature, followed by centrifugation at 13,000 × *g*, for 5 min. 105 μL supernatant was transferred to a new eppendorf tube. A colorimetric solution was freshly prepared: 5 mL Solution A (10% Ammonium molybdate in 10N H_2SO_4) was added to 40 mL deionized water. 2.5 g of $FeSO_4{\cdot}7H_2O$ was then dissolved and the total volume of the solution was adjusted to 50 mL with deionized water. 105 μL colorimetric solution was then added to the IPP1 reaction solution (1:1 vol), mixed and incubated at 25 °C for 10 min. 200 μL mixture was transferred to 96-well plate for A_{660} measurement using a Tecan M200 plate reader. A_{660} at 0 concentration of pyrophosphate was subtracted to obtain ΔA_{660} at various concentrations of pyrophosphate. The standard curve was plotted with assays in triplicate.

FPP quantitation

The substrate of santalene synthase, FPP was purchased from Sigma-Aldrich and dissolved in distilled water. To determine FPP concentration, a calf intestinal alkaline phosphatase (CIP, New England Biolabs) coupled assay was performed. A typical reaction (105 μL) contains 1 μL CIP (10 Units), and various concentrations of FPP in the range of 10–200 μM, Cutsmart buffer provided by New England Biolabs. The control is assay without alkaline phosphatase. The reaction mixture was incubated at 37 °C for 2 h. 105 μL colorimetric molybdate solution was added to stop the reaction. 200 μL was taken out from the mixture into a 96 well plate for A_{660} measurement. The A_{660} of enzyme free sample was subtracted to obtain ΔA_{660}, which was then used to calculate FPP concentration using the pyrophosphate standard curve.

Qualitative analyses of santalene synthase enzyme products using GC-MS

500 μL reaction mixture containing 0.3 μM enzyme (SaSSy, SspiSSy or SanSyn), 60 μM FPP in 25 mM HEPES, pH 7.4, 10% glycerol, 5 mM DTT, 10 mM $MgCl_2$ was set up in eppendorf tube and overlaid with 500 μL hexane to trap volatile products. After incubated for 2 h at 30 °C water bath, the reaction mixtures were vortexed for 2 min and centrifuged to separate the organic layer for GC-MS detection [13]. GC-MS was carried out on a Shimadzu QP2010 GC-MS system operating in ion scan mode (scan range: m/z 41 to 250). Samples were analyzed on an Rxi-1ms (30 m × 0.25 mm ID × 0.25 μm df) column. The injector was operated in splitless mode with the injector temperature maintained at 250 °C. Helium was used as the carrier gas with a flow rate of 1.48 mL/min. The oven program for the Rxi-1ms column was: ramp of 10 °C min^{-1} from 50 to 100 °C, 5 °C min^{-1} to 180 °C, held 3 min, 10 °C min^{-1} to 250 °C, held 5 min. All mass spectra were compared with the NIST11 library and the literature [13].

IPP1-coupled santalene synthase activity assay

Time-dependent santalene synthase activity assay

A typical assay (100 μL) contains 25 mM HEPES, 10% (v/v) glycerol, 5 mM DTT, 10 mM $MgCl_2$ and 60 μM substrate (FPP). 0.2 μM SaSSy/SspiSSy or SanSyn was added and incubated at 30 °C for 0, 3, 6, 9, 12 min. Reactions were stopped by heat inactivation in boiling water bath, followed by exactly the same protocol described above using IPP1 as coupling enzyme. A_{660} at 0 time point was subtracted from A_{660} at each of the time points to obtain ΔA_{660} which was then used to calculate the amount of pyrophosphate produced.

Dose-dependent santalene synthase activity assay

Dose-dependent assay (100 μL) contains 25 mM HEPES, 10% (v/v) glycerol, 5 mM DTT, 10 mM $MgCl_2$ and 60 μM FPP. Reaction time was 5 min. Enzyme concentrations range from 0.1 to 0.5 μM.

IPP1-coupled assays for kinetic parameters

To measure the kinetic parameters of santalene synthases, a modified protocol was used [13]. A typical assay (2.5 mL) contains 25 mM HEPES, 1% (v/v) glycerol, 100 mM KCl, 0.5 mM *tris* (2-carboxyethyl) phosphine (TCEP), 1 mM $MgCl_2$ and the FPP with a series of concentrations (0.3, 0.6, 1.2, 1.8, 3.0, 4.8, 6.0, 9.6 and 12 μM). The reaction was initiated by the addition of 50 nM santalene synthase. 1 mL of reaction mixture was immediately removed and heat inactivated in boiling water for 2 min and used later as blank. At the end of the reaction (5 min) another 1 mL reaction mixture was stopped in the same way. The samples including blanks were then lyophilized overnight using a lyophilizer (CHRIST). Deionized water was added to adjust the volume to 100 μL. 10 μL IPP1 (0.1 mg/mL) was then added and followed by steps as previously described. The A_{660} of the blank for each assay was correspondingly subtracted. Resulting ΔA_{660} was converted to pyrophosphate concentration referring to the standard curve, and the reaction velocity could then be calculated. The hyperbolic curves of reaction velocities versus substrate concentrations were then analyzed using Prism5 software to extract kinetic parameters.

All assays were performed in triplicate.

Authentic standard preparation

Yeast Santa 1.0 cells were grown for the production of α-santalene in a double phase fermentation system in the 3.0 L bioreactor (eppendorf). The organic phase contained 150 mL dodecane on the top of 1 L culture medium and was used on purpose for capturing α-santalene. Roughly 100 mL organic phase was recovered after 72 h fermentation. Most of the dodecane was removed by distillation using a rotor vac heated in an oil bath under negative pressure (−0.1 MPa). The headspace temperature was set at 150 °C. Roughly 5 mL crude distilled product was obtained and 1 mL was spotted on a glass-backed plate precoated with silica (GF254) (20 × 20 cm) and subjected to thin layer chromatography (TLC) separation, using hexane as developing solvent. The Silica gel plate was stained with $KMnO_4$. Individual staining spots were scraped off the plate separately and dissolved in hexane. GC-MS method was used to locate α-santalene to one of the TLC spots. Hexane was readily removed by rotary evaporation resulting in 700 μL concentrated α-santalene standard.

Authentic standard quantitation using ^1H NMR

5, 10, 20, 40, 60 μL α-santalene standard obtained as described above was diluted into 500 μL $CDCl_3$ spiked with 10 μL of 10% 2,5-norbornadiene (SPC Scientific) (v/v, diluted into $CDCl_3$). A Bruker AVANCE III HD 400 MHz Nuclear Magnetic Resonance Spectrometer equipped with a 5 mm BBO probe and a Z-axis pulse field gradient was used. The key acquisition parameters for 1D ^1H NMR spectra were as follows: 32 scans, a relaxation delay of 1 s, 90° pulse width of 9.2 μs at a transmitter power of 15.00 W, temperature of 25 °C, acquisition time of 4.09 s, and spectral width 8012.82 Hz with 297.8 K acquired data points. The free induction decay signal was locked using the $CDCl_3$ solvent and then optimized for magnetic field homogeneity (shimmed). The 90° pulse width was calibrated prior to the experiment. All spectra were collected with samples in 5 mm NMR tubes and processed using NMR software MestReNova. The α-santalene concentrations were determined using the following equation:

$$\frac{4n_s}{I_s} = \frac{n_x}{I_x},$$

where:

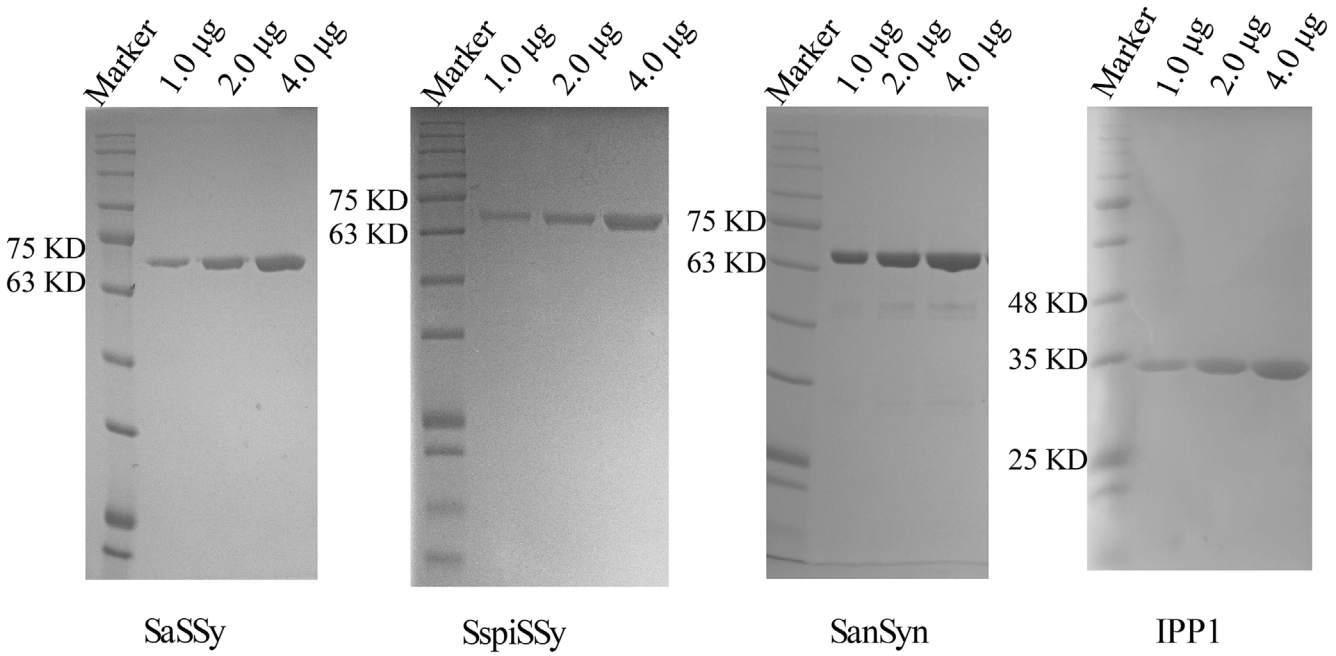

Fig. 2. SDS-PAGE analyses of santalene synthases and IPP1. Purified proteins of different quantities as indicated were separated on 10% SDS gels (for SaSSy SspiSSy, and SanSyn) and a 12% gel for IPP1.

n_x unknown amount of substance of α-santalene
n_s amount of substance of 2,5-norbornadiene
I_x integral of ^1H peaks at C_{12} of α-santalene (defined as 1)
I_s integral of ^1H peaks at $C_{1,3\ 4,5}$ of 2,5-norbornadiene

Correlation of IPP1 coupled assay with GC-MS assay for santalene synthases

0.5 μM SaSSy was added to 350 μL reaction mixture containing 25 mM HEPES, pH 7.4, 10% (v/v) glycerol, 0.5 mM TCEP, 10 mM $MgCl_2$, and 60 μM FPP to initiate the reaction. At 0 time point, 100 μL was removed to a new tube and heat inactivated in boiling water for 2 min for later use as a blank for IPP1 coupled assay. At 30 min, 200 μL reaction mixture was transferred into two eppendorf tubes, 100 μL into each tube. One tube was immediately heat inactivated in boiling water for 2 min and subjected to IPP1 assay as described above. Since heating may result in the evaporation of the volatile terpenoid products, reaction in the other tube was immediately stopped by the addition of 100 μL 0.5 M EDTA (pH 8.0) for the purpose of later GC-MS analysis. 200 μL hexane containing 100 μM α-humulene (Sigma-Aldrich) as internal standard was added and vortexed for 2 min. After centrifuged at 15,000 × *g* for 10 min, the organic phase was removed for GC-MS analysis. The method of GC-MS analysis was as described above. The santalene authentic standard obtained by fermentation was diluted with hexane to establish a series of concentrations (3–118.5 μM) (each spiked with 100 μM α-humulene as an internal control to calibrate the detector response factor). The integral of the α-santalene TIC (total ion count) peak was normalized by the α-humulene internal standard. A santalene standard curve was thus established by plotting the numerical integration against the corresponding α-santalene concentration.

Results and discussion

Sequence alignment and protein structure modeling

SaSSy and SspiSSy from *Santalum album* L. and *Santalum spicatum* respectively are highly homologous to each other with 95% sequence identity but exhibit evolutionary distance from the *Clausena lansium* (Wampee) SanSyn, with only 31% sequence identity based on sequence alignment (Fig. S1). SaSSy and SspiSSy belong to TPS-b subfamily and SanSyn belongs to TPS-a subfamily [13] (Fig. S2). Regardless of the evolutionary distance, the calculated 3-D structures of SaSSy and SanSyn are quite similar (Fig. S3A). Computer modeling revealed that the structure of SanSyn is different from SaSSy in several key residues at the active site that determines the size and compatibility of the hydrophobic pocket and probably the deprotonation position of intermediate during the reaction (Fig. S3B). This is likely the reason why SanSyn forms uniformly α-configuration santalene (> 90%). These key residues include Thr467 in the NSE/DTE motif of SaSSy replaced by a Gly in SanSyn and a Glu297 residue at the bottom of the hydrophobic pocket in SanSyn that is absent in SaSSy. Such findings provide basis for enzymatic engineering to change the composition and odors of sandalwood oil produced by microbial fermentation.

Protein purification

Three santalene synthases SaSSy, SspiSSy and SanSyn and *S. cerevisiae* inorganic pyrophosphatase were purified to near homogeneity (Fig. 2). The expression levels of the three santalene synthases varied, with SaSSy the highest, requiring Ni-NTA, one step of chromatographic purification, SanSyn the lowest, requiring Ni-NTA, Q sepharose and superdex 200 gel filtration, three steps of chromatography and SspiSSy medium high, requiring Ni-NTA and Q sepharose, two steps of purification. The typical yield of SaSSy, SspiSSy and SanSyn were 8, 1.2, 1.8 mg/L cell culture (Note that for the expression of SanSyn a bioreactor allowing much higher cell density was used). The IPP1 expression was robust. The yield was 18 mg/L cell culture (Tables S1–S4).

Qualitative assessment of products formed by santalene synthases

The GC elution profiles and mass spectra of the reaction products formed by the three santalene synthases were shown in Fig. 3. SaSSy, SspiSSy both produced mainly four products, α-santalene, α-*exo*-bergamotene, *epi*-β-santalene and β-santalene respectively. The ratios of four products are also consistent with a previous report [13]. By contrast, SanSyn produced predominantly one product, α-santalene as is also consistent with the literature [13,14].

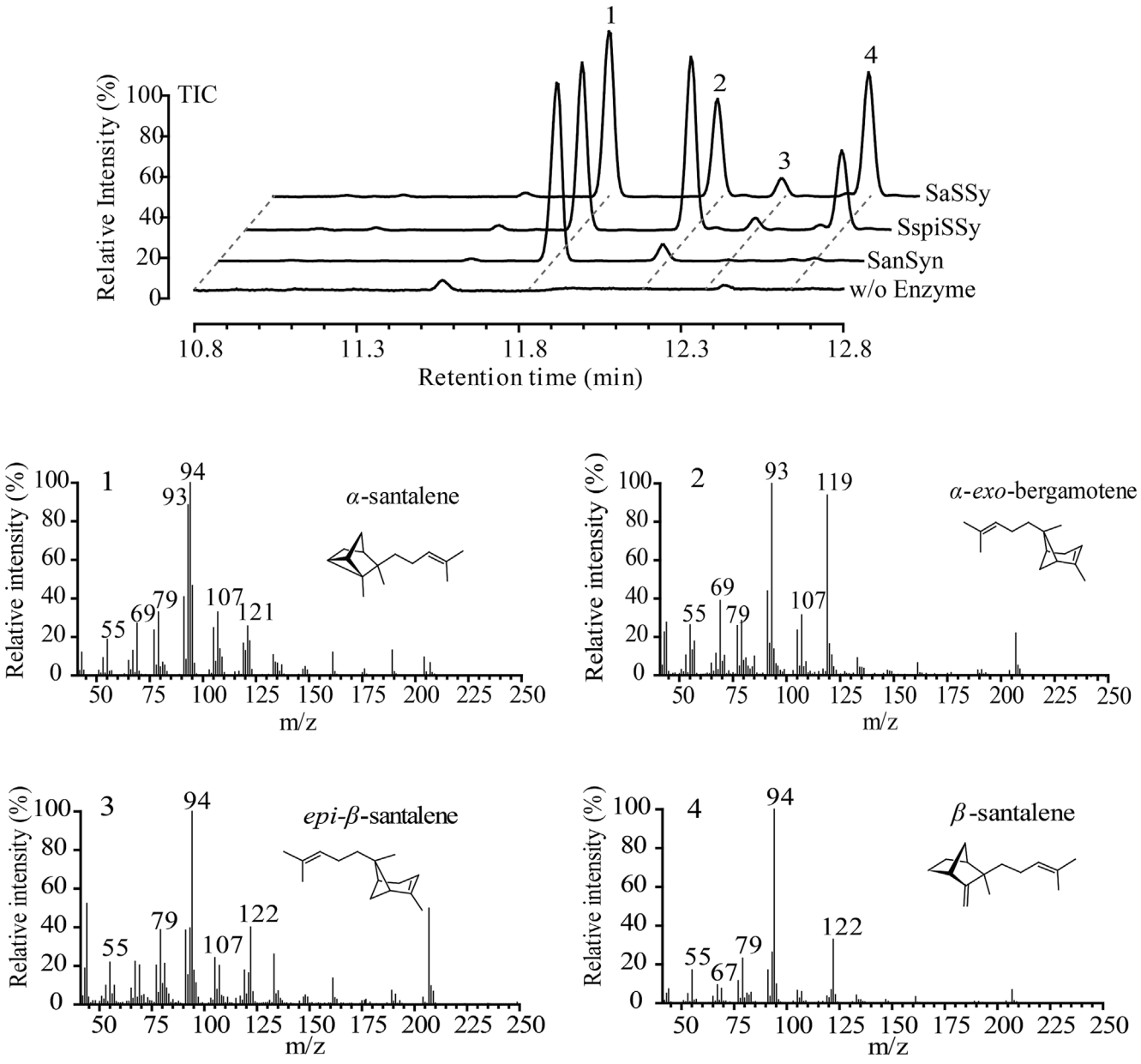

Fig. 3. GC-MS chromatogram of *in vitro* assays with recombinant santalene synthases; SaSSy, SspiSSy, and SanSyn using *(E,E)*-FPP as substrate. Peaks: (1) *α*-santalene, (2) *α-exo*-bergamotene, (3) *epi-β*-santalene, (4) *β*-santalene. Mass spectral data of the main compounds are shown.

The use of IPP1 to establish a pyrophosphate standard curve

The activity of our purified IPP1 is comparable to the commercial yeast IPP1 with a specific activity of 400 units per mg. One unit liberates 1 μmole inorganic orthophosphate per min at pH 7.4 and 25 °C. The assay condition at pH 7.4 was chosen for convenience in couple of santalene synthase activity assay. It was reported that yeast IPP1 is active in a broad pH range [23,24], providing the possibility of using it as a coupling enzyme for assays to measure the activities of diverse terpenoid synthases. In the presence of excess IPP1 (1 μg), the increase of ΔA_{660} is proportional to the pyrophosphate concentrations (1–60 μM) (Fig. 4A). The slope is two-fold that of the phosphate standard curve (Fig. 4B). This indicates that pyrophosphate was completely converted to orthophosphate, which reacted with ammonium molybdate to form phosphomolybdic acid with characteristic absorbance at 660 nm. Fig. 4A was then used as a standard curve to quantitate pyrophosphate in IPP1-coupled santalene synthase activity assays.

Time and dose dependent enzyme activities

Next, we set up end point activity assays for all three santalane synthases. Pyrophosphate formation was measured with the coupling IPP1 enzyme. Time-dependent linear increase of absorbance at 660 nm was observed for all three enzymes indicating time-dependent formation of pyrophosphate (Fig. 4C–E). The formation of pyrophosphate is also enzyme dose-dependent. Increase of the amount of enzyme is proportional to the increase of pyrophosphate formation as measured by A_{660}, confirming the validity of the IPP1-coupled assays (Fig. 4F–H).

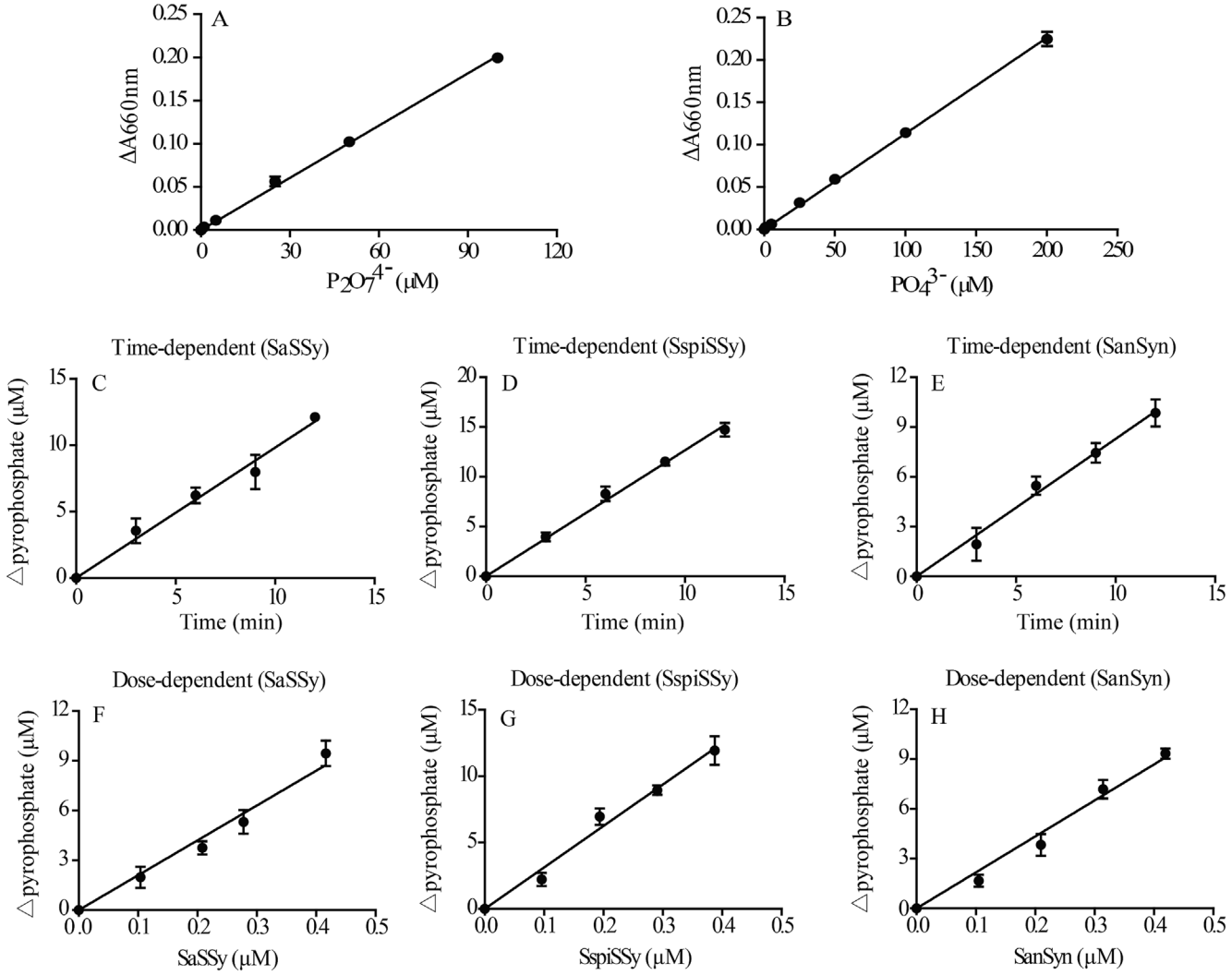

Fig. 4. Time & dose dependent assay of santalene synthases. (A) The standard curve of pyrophosphate in IPP1 aided molybdic acid assay. (B) The standard curve of phosphate in molybdic acid assay. The time dependent IPP1 coupled assay for (C) SaSSy, (D) SspiSSy and (E) SanSyn. The dose dependent IPP1 coupled assay for (F) SaSSy, (G) SspiSSy and (H) SanSyn.

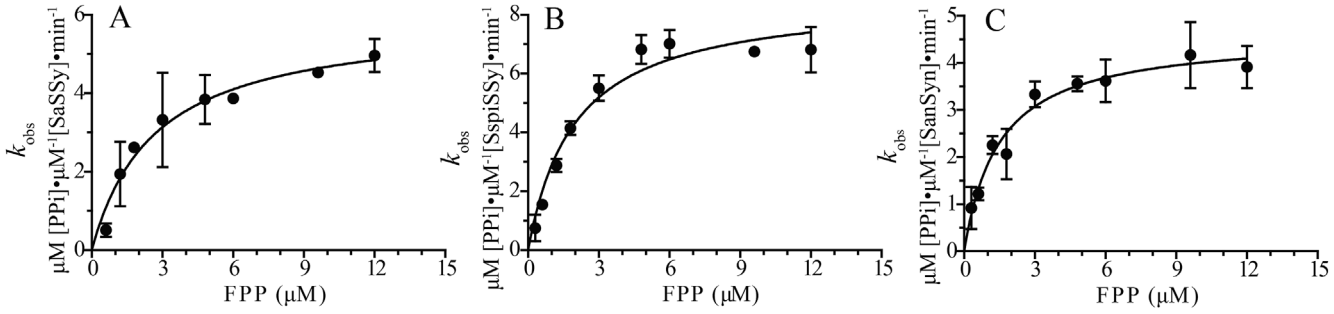

Fig. 5. Michaelis–Menten kinetic analysis of santalene synthases. 50 nM (A) SaSSy, 50 nM (B) SspiSSy, and 50 nM (C) SanSyn are subjected to IPP1 coupled colorimetric assay with varying concentrations of FPP.

Kinetic parameter measurement

We performed enzyme activity assays of the three enzymes by varying the concentrations of substrates using IPP1-coupled method (Fig. 5). Reaction velocities against substrate concentrations exhibited hyperbolic curves, which were fit to extract Michaelis–Menten constants. The K_m values for SaSSy, SspiSSy and SanSyn agree very well with previous reports [13,14] and are listed in Table 1. The k_{cat} values

Table 1
Kinetic parameters of three santalene synthases measured by IPP1 coupled colorimetric assay.

Enzyme	SaSSy	SspiSSy	SanSyn
k_{cat} (min^{-1})	4.4 ± 0.2	6.4 ± 0.2	4.5 ± 0.2
K_m (μM)	2.7 ± 0.9	2.0 ± 0.4	1.5 ± 0.4

for SaSSy, SspiSSy and SanSyn are 4.4 ± 0.2, 6.4 ± 0.2, 4.5 ± 0.2 min^{-1} respectively (Table 1). The k_{cat} numbers for SaSSy reported by two other groups are 0.5 min^{-1} and 0.34 S^{-1} (20 min^{-1}) [13,14] (Table 2). Reaction conditions are very similar. Both groups used GC-MS method. The impurity of "authentic standards" obtained by different groups may account for such big differences in turn-over numbers. The k_{cat} of SspiSSy was reported to be 2.6 S^{-1}, 7.6 times of the k_{cat} of SaSSy by the same authors (Table 2) [13]. It is worth noting that SaSSy and SspiSSy are highly homologous to each other (95% identity) (Fig. S1). Such difference in turn-over number between two nearly identical enzymes is intriguing. The protocol used for kinetic parameter measurement in the same paper is also contradictory to the k_{cat} number they observed [13]. Our data however shows that the catalytic activity of SspiSSy is only slightly (40%) higher than SaSSy (Table 2). In these assays, a larger volume of reaction mixture followed by lyophilization to concentrate phosphate product was critical since molybdic acid based colorimetric assay fails to reproducibly quantitate phosphate at the low end of concentrations. The volatile property of santalene and other terpenes hampers the usage of concentrating steps, when using GC-MS and other chromatography-based methods to directly quantitate the terpenoid products. Such problems can be bypassed using the IPP1-coupled assay. To our best knowledge, this is the first time that the kinetic parameters of SanSyn being reported. It is of great interest that the kinetic parameters of SanSyn are similar to those of santalene synthases from sandalwood. A potential application of SanSyn in engineered microbial fermentation to produce valuable sandalwood oil thus seems equally feasible to the usage of a sandalwood counterpart, and the advantage of this is the chance of having dominant α-santalol as the final product.

Authentic standard preparation and quantitation

Since the activities of recombinant proteins prepared by different research groups could vary, we decided to use traditional GC-MS method to measure the activities of our preparation of enzymes and to demonstrate the accuracy of the IPP1 coupled assay. Towards this end, we needed to obtain authentic standard as a reference for direct quantitation of the enzymatic products using GC-MS. A genetically engineered yeast strain Santa 1.0 (described in "Materials and methods") was fermented for the production of α-santalene, which was then enriched using oil-bathed rotary evaporation and TLC separation as described in the "Materials and methods" section. The resulting fermentation extract spiked with 1 μL (9.83 μmoles) internal standard 2,5-norbornadiene were subjected to ^{1}H NMR. The C_{12} hydrogen of α-santalene (labeled in blue in Fig. 6A inset) exhibits a characteristic chemical shift at 5.20 ppm (Fig. 6A). Four hydrogens of 2,5-norbornadiene (labeled in red in Fig. 6A inset) share a characteristic chemical shift at 6.75 ppm (Fig. 6A). The ratio of the integrals of these two signals can be used to calculate the molar ratio between α-santalene with concentration to be determined and 2,5-norbornadiene with known concentration. As an example, using the representative spectrum (sample containing 20 μL fermentation extract) in Fig. 6A, a 1.00: 2.65 M ratio between α-santalene and 2,5-norbornadiene could be determined. Samples containing different amount of fermentation extract (5, 10, 20, 40, 60 μL) spiked with 2,5-norbornadiene were analyzed. Determined concentrations of α-santalene are proportional to the amount of fermentation extract added (Fig. 6B). The stock of fermentation extract was calculated to be 1340 ± 6 mM.

Correlation of IPP1 coupled assay with GC-MS assay for santalene synthases

With the known concentration of α-santalene in the fermentation extract, we set up GC-MS experiments in order to obtain the proportional relationship between the integral of TIC peak and α-santalene concentration as described in "Materials and methods" section (Fig. 7). In the GC elution profile (Fig. 7A), the TIC peaks corresponding to α-santalene, the residual solvent, dodecane, and the internal control, α-humulene (to calibrate the detector response factor) are all clearly visible. The Mass spectra of dodecane and α-humulene are also presented (Fig. 7A). Plotting the numerical integration of TIC peak against the α-santalene concentration generates a standard curve (Fig. 7B) that can be later referred to for product formation estimation in the enzyme reaction. To correlate IPP1 couple assay with the existing GC-MS method, we performed both assays and measured the formation of products. Results using both methods agree with each other. In the given reaction conditions, the four major products formed by SaSSy including three santalenes and bergamotene sum up to be 12.5 ± 0.1 nmoles, whereas IPP1-coupled assay estimates the formation of 11.5 ± 0.5 nmoles pyrophosphate in the 350 μL reaction system.

In this study, we aimed to develop new assays for santalene synthases and to reconcile the controversial kinetic data in the literature. Our results demonstrate that IPP1-coupled method is valid and has advantage in determining the overall activities and kinetic constants regardless of the product variation. Other advantages include low cost with no need of expensive equipment and authentic standards, environmentally friendly, fast and sensitive by easily concentrating the products. This method could potentially be applied to the study of other terpene synthases and prenyltransferases, and used for high-throughput screening for inhibitors of drug targets in this enzyme family.

Table 2
Comparison of kinetic parameters obtained using different methods.

Enzyme	SaSSy			SspiSSy	
	Christopher et al. 2011 [13]	Prabhakar et al. 2015 [14]	This study	Christopher et al. 2011 [13]	This study
k_{cat} (min^{-1})	20.4	0.50 ± 0.03	4.4 ± 0.2	156	6.4 ± 0.2
K_m (μM)	1.4 ± 0.3	0.59 ± 0.24	2.7 ± 0.9	1.4 ± 0.3	2.0 ± 0.4
k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)	2.4×10^5	1.4×10^4	2.7×10^4	1.9×10^6	5.3×10^4

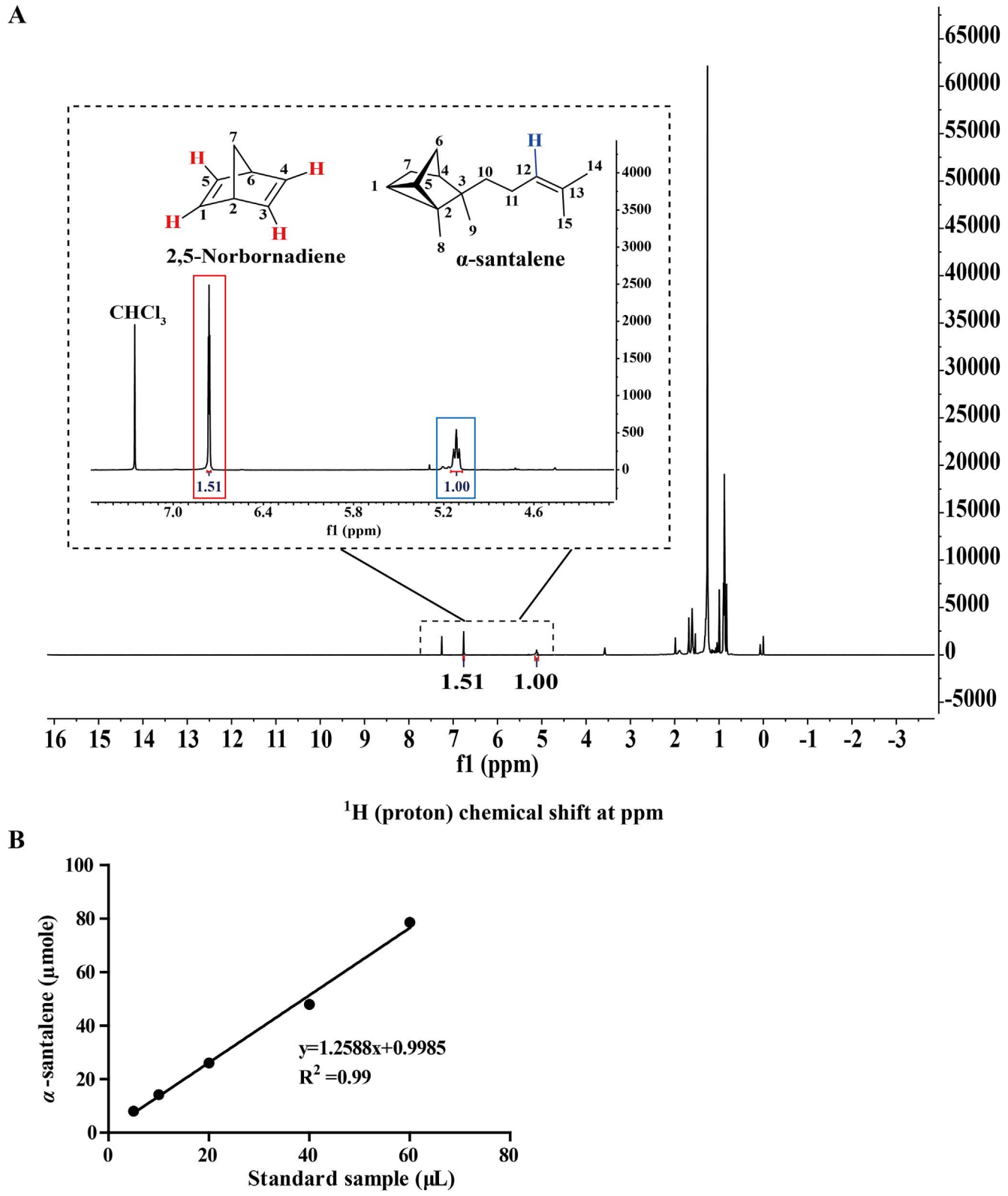

Fig. 6. Quantitation of α-santalene in the fermentation extract. (A) ^{1}H NMR spectrum of α-santalene standards (20 μL) spiked with 9.83 μmoles 2,5-norbornadiene. (B) The standard curve for α-santalene contents against the volumes of fermentation extract.

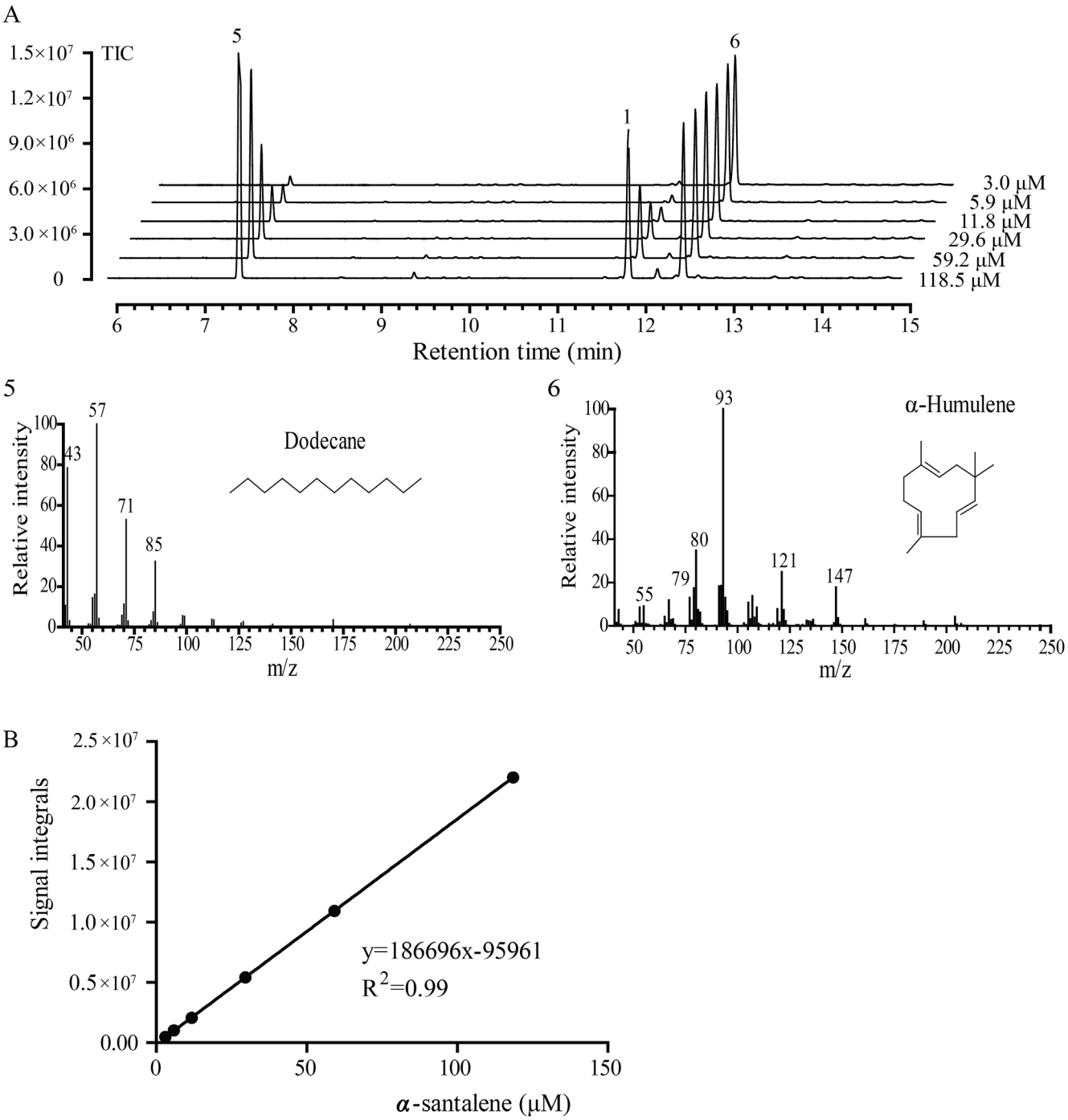

Fig. 7. GC-MS result of α-santalene standards. (A) GC-MS chromatogram of fermentation extract diluted by hexane with 100 μM α-humulene as the internal standard. (1) α-santalene; (5) dodecane; (6) α-humulene. (B) The standard curve for the numerical integrals of TIC signals against α-santalene concentration.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2018.02.002.

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