Stabilisation of transition states prior to and following eudesmane cation in aristolochene synthase†

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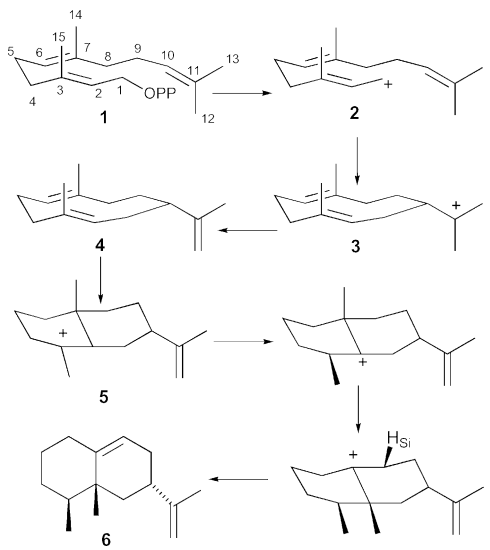
Received 21st March 2006, Accepted 8th May 2006
First published as an Advance Article on the web 25th May 2006
DOI: 10.1039/b604147g

The mechanistic details of the cyclisation of farnesylpyrophosphate (FPP) by aristolochene synthase (AS) from *Penicillium roqueforti* have only recently begun to emerge, mainly through the analysis of the reaction products generated by AS-mutants. The reaction proceeds through several intermediates including germacrene A and eudesmane cation. Previous work suggested that the side chain of phenylalanine 178 promoted the conversion of eudesmane cation to aristolochene. We now report that the catalytic function of this residue during the conversion of eudesmane cation to aristolochene is mainly due to the large size of its side chain, which facilitates the hydride shift from C2 to C3, rather than its aromatic character. In addition, F178 appears to control the regioselectivity of the final deprotonation step and, together with F112, helps stabilise the developing positive charge on C1 after the expulsion of pyrophosphate from the substrate. These results complete a screen of likely active-site aromatic residues and establish their respective roles in the conversion of FPP to aristolochene.

Introduction

Sesquiterpene synthases catalyse the cyclisation of the universal acyclic precursor farnesyl pyrophosphate (FPP, **1**) in marine and terrestrial plants, fungi, bacteria and insects, to generate more than 300 distinct sesquiterpene products, many of which have important antibiotic, antifungal or neurotoxic activities. Many sesquiterpene cyclases share a common protein structure known as the terpenoid fold.[1,2] Plant sesquiterpene cyclases show significant amounts of homology and may be the products of divergent evolution from a common precursor.[3] Many fungal enzymes on the other hand show little sequence similarity among themselves and with their plant counterparts, nevertheless, many appear to share the same fold.[1,4–7] All these enzymes serve as high fidelity templates for their common substrate and subtly control conformation and stereochemistry of the intermediates during the cyclisation reactions.[2,8] They represent a masterpiece of evolution in achieving exquisite chemical and stereochemical selectivity within a common protein fold for the conversion of a shared substrate to many different products.

Aristolochene synthase (AS) from *Penicillium roqueforti* is a monomeric enzyme that catalyses the Mg^{2+}-dependent cyclisation of **1** to the bicyclic sesquiterpene (+)-aristolochene **6** (Scheme 1), the precursor of fungal toxins such as PR-toxin, sporogen-AO1, phaseolinone, gigantenone, phomenone and bipolaroxin.[9] The molecular details of the AS catalysed reaction have only recently begun to emerge. The crystal structures of epi-aristolochene synthase from *N. tabacum*[6] and trichodiene synthase from *F. sporotrichioides*,[1] which, unlike the structure of AS,[7] were obtained in the presence of substrate analogues, suggest that the pyrophosphate group of **1** binds tightly to the enzyme through two Mg^{2+} binding sites at the entrance to the active site,[10] while the aliphatic tail of the substrate binds in a deep hydrophobic cleft of the enzyme. Recent work supported the proposal[11] that AS catalyses the cyclisation of FPP to aristolochene *via* the intermediate *S*-germacrene A **4** (Scheme 1).[12] The conformation of **1** bound in the enzyme's active site is known to be a critical determinant of the reaction pathway leading to attack of C1 of farnesyl cation **2** by the double bond at C10–C11 subsequent to



Scheme 1 Mechanism of aristolochene synthase.

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† Electronic supplementary information (ESI) available: total ion chromatograms from GC-MS analyses of the products of AS-F178C, AS-F178V, AS-F178Y, AS-F112A and ASF112AF178V. Mass spectral analysis of the sesquiterpene products (aristolochene, germacrene A, valencene, α-farnesene, β-farnesene, α-selinene, β-selinene, selina-4,11-diene) and of authentic reference samples. GC-traces for the determination of the stereochemistry of germacrene A by heat induced Cope rearrangement and comparison with authentic β-elemenes. See DOI: 10.1039/b604147g

the expulsion of pyrophosphate (Scheme 1).[8,13] The intermediate germacrene A then undergoes a further cyclisation to form the bicyclic eudesmane cation **5** through protonation of the C6–C7 double bond by Y92.[12] The positive charge on **5** is stabilised through interaction with the π-system of the indole ring of W334.[14] Successive 1,2-hydride shift and methyl migration followed by loss of H_{Si} on C8 finally results in the generation of (+)-aristolochene.[15]

Previous work suggested that the conversion of eudesmane cation **5** to aristolochene was facilitated by the bulky aromatic side chain of F178.[16] This residue is ideally placed to stabilise the developing positive charge on C2/C3 of eudesmane cation (Fig. 1). Its replacement with valine in AS-F178V led to the accumulation of the intermediate germacrene A **4** and the production of selinenes **10**, **11**, and **12** as well as the linear α-**8** and β-farnesene **9**, which are the result of erroneous deprotonation (Scheme 2).[16] These observations were interpreted to suggest that F178 fulfilled a dual role during AS catalysis. The presence of F178 is required for the conversion of farnesyl cation to germacrene A, most likely by stabilising the developing positive charge on C11 of **3**. In addition, F178 promotes the conversion of eudesmane cation **5** to aristolochene through either the stabilisation of the cations on C2 and C3, through interaction of the aromatic phenyl ring with the positive charge, or through induction of the hydride shift from C2 to C3 of **5**. Here we report experiments that confirm the function of F178 during AS catalysis and suggest that the large size of F178 rather than its aromaticity promotes the formation of aristolochene during AS catalysis.

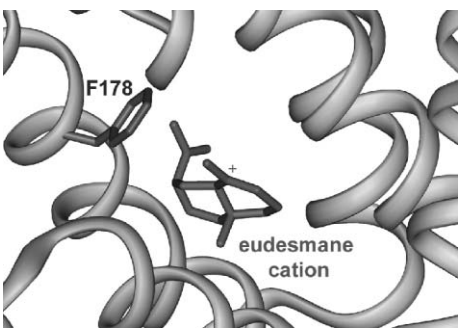
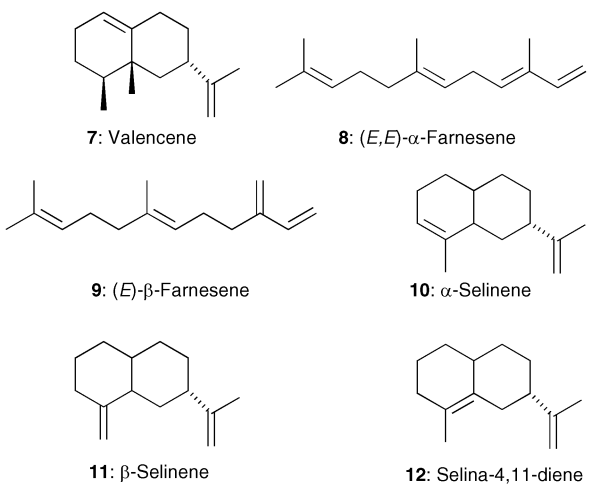


Fig. 1 Sketch indicating the proximity of F178 to eudesmane cation **5**, which was modelled into the active site of the X-ray crystal structure of the *apo*-enzyme of AS.[7]



Scheme 2 Sesquiterpene hydrocarbons produced by AS-mutants.

Results and discussion

In the previously characterised AS-F178V,[16] which produced 50% germacrene A **4** together with smaller amounts of sesquiterpenes **7–12** (Table 1), the smaller, non-aromatic isopropyl group replaced the bulky benzyl group. To distinguish between the importance of size and aromaticity of residue 178, cDNAs for AS-F178C, AS-F178I, AS-F178Y, AS-F178W were generated by site directed mutagenesis from a cDNA of wild type AS isolated from *P. roqueforti*. Mutants were expressed in *E. coli* BL21(DE3) cells and purified as previously described.[8] The steady-state kinetic parameters of the mutants were measured by incubating them with [1-^{3}H]-FPP and determining the amount of the hexane extractable, tritiated products. The assay was based on that reported previously[12] but optimised to ensure a linear dependence of the rates on protein concentrations. AS is known to aggregate at increased concentrations of protein and its activity is only a linear function of protein concentration for a narrow concentration range.[10] Kinetic assays for AS and all mutant proteins were carried out within the carefully established linear range. For the measurements of the steady-state kinetic parameters reported here, the optimal incubation times were carefully determined to ensure linearity of turnover with reaction time. Under these optimised conditions the Michaelis constant (K_M = 8.7 ± 1.67 μM) and the turnover number (k_{cat} = 0.55 ± 0.03 s^{-1}) were increased compared to those previously reported (k_{cat} = 0.03 ± 0.01 s^{-1}; K_M = 2.3 ± 0.5 μM).[12,17] Overall the catalytic efficiency was increased

Table 1 Relative amounts of hexane extractable products and kinetic parameters for wild-type and mutants of AS

	Relative product distribution (%)								Kinetic parameters		
AS	**4**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	K_M (μM)	k_{cat} ($s^{-1} \times 10^3$)	k_{cat}/K_M (M^{-1} s^{-1})
WT	7.5	91.5	0.4						8.7 ±1.7	550.0 ±30	63,218 ± 12,825
F178C	67.2	8.8	20.2				3.8		5.9 ±0.3	9.9 ±1.2	1,678 ± 221
F178V[a]	54.1	10.8	5.2	2.7	9.2	5.7	9.1	2.1	2.0 ±0.01	6.3 ±0.3	3,150 ± 151
F178I	54.0	43.0	3.0						0.9 ±0.2	4.1 ±0.5	4,556 ± 1,155
F178Y	10.7	86.4	2.7						9.9 ±3.3	52.0 ±1.0	5,253 ± 1,754
F178W	9.6	88.3	2.1						4.7 ±2.5	79.0 ±8.0	16,809 ± 9,102
F112A	12.5			53.1	34.3				19.7 ±0.7	2.3 ±0.3	116.8 ± 11.0
F112A-178V	37.1			22.3	40.6				46.3 ±3.8	1.4 ±0.01	30.2 ± 2.5

[a] Data from Forcat & Allemann.[16]

approximately 5-fold under the optimised conditions (k_{cat}/K_M = 63,218 ± 12,825 $M^{-1}s^{-1}$), relative to that reported previously.[12] The kinetic parameters for the mutant enzymes were also determined using the same assay conditions. The Michaelis constants of the mutants were generally slightly reduced when compared to the wild type enzyme. Surprisingly, the bigger reductions were observed for non-aromatic side chains. The reduction in the catalytic efficiencies of the mutants was largely a consequence of reduced turnover numbers (Table 1).

GC-MS analysis of the hexane extractable products generated in incubations of the mutant enzymes with FPP indicated that AS-F178Y and AS-F178W, in which the benzyl group of residue 178 was replaced with another aromatic side chain, generated products very similar to those of the wild type enzyme in that the main product was aristolochene (86.4 and 88.3%, respectively) (Fig. 2 and Table 1). Germacrene A **4** and valencene **7** were formed as side-products. These products were identified by comparison of their GC-retention times and of their mass spectra with those of the products formed by AS or with authentic samples (ESI†). However, in good agreement with the postulated role of residue 178 affecting a step after the formation of germacrene A **4**, AS-F178C and AS-F178V produced significantly reduced amounts of the wild type product aristolochene, but increased amounts of the intermediate germacrene A. Unlike AS-F178V, AS-F178C produced only a small amount of β-selinene **11**, but no α-selinene **10** or selina-4,11-diene **12** (ESI†). This observation together with the product distribution observed for AS-F178V suggested that F178 also affected a step after the formation of eudesmane cation **5**. The absence of reaction products originating from deprotonation of C1 may indicate that the hydride shift from C2 to C3 and the methyl shift from C7 to C2 occur simultaneously, thereby preventing the formation of an intermediate with a positive charge on C3.

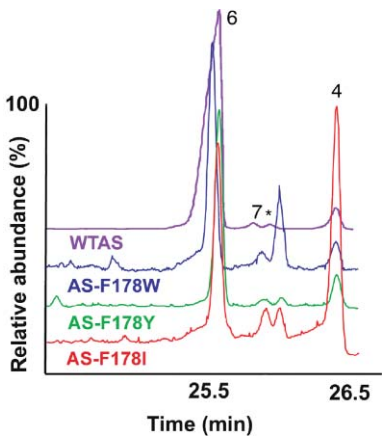


Fig. 2 Total ion chromatograms from GC-MS analyses of the hexane extractable products of FPP utilisation by AS (purple), AS-F178W (blue), As-F178Y (green) and AS-F178I (red). * is a non-terpene contaminant.

The formation of the selinenes, which result from deprotonation of eudesmane cation **5** prior to the 1,2-hydride shift from C2 to C3, was not observed when the benzyl group of F178 was replaced with the 2-butyl group of isoleucine (Fig. 2). These results strongly suggest that the size of residue 178 rather than its aromatic character is central to its mechanistic function in the events that control the conversion of eudesmane cation to aristolochene. It is worthy of note that AS-F178C produced increased proportions of valencene **7** when compared to the wild type enzyme. Since **7** is most likely produced by loss of the proton on C6 of the final carbocation, F178 must also be involved in preventing erroneous deprotonation in the last step of the synthesis of aristolochene. No residue has previously been reported to be involved in this step of AS catalysis.

The X-ray structure of AS,[7] which indicated the proximity of F178 to C2/C3 of eudesmane cation (Fig. 1), together with the results presented here suggests that this residue primarily promotes the hydride shift from C2 to C3. Inspection of the X-ray structure also indicated that F178 is ideally placed between C3 and C11 of germacrene A (Fig. 3) to first support conversion of farnesyl cation to germacrene A and then to promote the hydride shift.

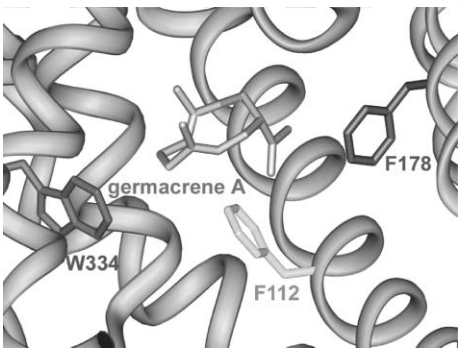


Fig. 3 Relative orientations of W334, F112 and F178 relative to germacrene A **4** bound in active site of AS. **4** was docked into the active site cavity in the X-ray crystal of the *apo*-enzyme.[7]

An additional function of F178 during formation of germacrene A has been described previously.[16] The results obtained in the analysis of AS-F178V suggested that residue 178 could be involved in the stabilisation of carbocation **3**, or the transition state preceding it, through interactions between the cation and the π-system of the ring. Alternatively, the size of the ring could be involved in folding the substrate into a reactive conformation. The observation that AS-F178I did not however produce α- or β-farnesene suggested the involvement of other residues in the stabilisation of germacryl cation **3**. Analysis of the structure of AS[7] indicated that the benzyl group of F112 was placed between W334 and F178 to potentially assist F178 during these early steps of catalysis and to stabilise the developing charge on **3** (Fig. 3). In order to define the role of F112 in the catalytic mechanism, AS-F112A was produced and its products determined. GC-MS analysis revealed the production of a small amount of germacrene A, in addition to the formation of the linear products, **8** and **9** (Table 1, ESI†).

It had been previously proposed that F112 helped define the stereochemistry on C10 of germacrene A **4**.[7] However, co-injection of β-elemene generated by the heat-induced Cope rearrangement[12,18] of germacrene A **4** produced by AS-F112A with a racemic sample of β-elemene, revealed that both wild-type AS and AS-F112A produced the same stereoisomer of **4** (ESI†). The presence of α- and β-farnesene suggested that intermediate **2** accumulated during catalysis by AS-F112A, leading to deprotonation from C4 and C15. Residue 112, therefore, most likely contributes significantly to the stabilisation of the transition state following farnesyl cation **2**.

Once **4** is synthesised, W334 and F112 are involved in the formation of **5**. When F112 was replaced by alanine, the reaction terminated at **4**, indicating that W334 was not able to induce the production of **5** in the absence of F112. Hence, the side products **10**, **11** and **12** were not formed. The absence of selinenes in catalysis by ASF112A suggested that **10**, **11**, and **12** were genuine products formed by ASF178V through anomalous deprotonations of **5** rather than products of an acid-catalysed rearrangement of **4** during workup.[10] To confirm the proposed roles of both F178 and F112, the double mutant AS-F112AF178V was produced and its products determined by GC-MS analysis. The products generated by the double mutant were the same as those produced by AS-F112A supporting the proposed roles of residues 112 and 178, namely the stabilisation of transition states prior to and following eudesmane cation **5**. As had been observed for ASF178V, germacrene A was also the major product generated by AS-F112AF178V. The double mutants did however not produce any selinenes since in the absence of an aromatic side chain on residue 112 eudesmane cation was not formed. These observations further confirm that the selinenes produced by ASF178V and ASF178C are genuine reaction products.[10]

Conclusions

The site directed mutagenesis study of aristolochene synthase from *P. roqueforti* reported here provides further evidence that germacrene A is an intermediate in the production of aristolochene from FPP as suggested previously (Scheme 1).[12] F178 is a critical residue that controls the efficient formation of germacrene A and its conversion to aristolochene through both its large size and the aromaticity of its side chain. The 1,2-hydride shift that initiates the conversion of eudesmane cation **5** to aristolochene appears to require the presence of a bulky side chain on residue 178 implying that steric effects initiate this reaction. Whether the subsequent methyl shift from C7 to C2 is also promoted by steric bulk, for instance of residue Y92, or whether it is induced by proton loss from C8 is currently being actively investigated.

Experimental

Materials

All chemicals were purchased from Fluka or Sigma. Oligonucleotides were purchased from Alta Biosciences (University of Birmingham). Pfu polymerase and restriction endonuclease DpnI were obtained from Promega and New England Biolabs, respectively. Competent *E. coli* BL21(DE3) and ultracompetent XL10-Gold® were purchased from Stratagene. The miniprep kit was purchased from Qiagen and used according to the supplier's instructions. HP-Q-sepharose for protein purification was obtained from GE-Healthcare. Farnesyl pyrophophate was synthesised according to a published protocol by Dr D. J. Miller and Mr Fanglei Yu.[19] [1-^{3}H]-FPP (20 mC mmol^{-1}) was purchased either from Sigma or from Tocris and diluted with cold FPP to a working specific activity of 50 μC μmol^{-1}. Eco-scint scintillation fluid was obtained from National Diagnostics.

A reference sample of germacrene A from soldier cephalic secretion of a subterranean termite species was provided by Larry Cool, Forest Products Laboratory, University of Berkeley, California, Berkeley, USA. Valencene was a gift from De Monchy Aromatics Ltd. Prof. Koenig, University of Hamburg, Germany, provided us with a racemic sample of α- and β-elemene and standards of α- and β-selinene and selina-4,11-diene. β-(*E*)-Farnesene was a gift from John A. Pickett, FRS, and Lynda Ireland, BBSRC-Institute for Arable Crops, Rothamsted, UK. (*E*,*E*)- and (*E*,*Z*)-α-farnesene were obtained from Silvia Dorn, Institute of Plant Science ETH-Zurich, Zurich, Switzerland, while aristolochene was generated by incubation of FPP with WTAS.

Production of AS-mutants

The following mixture was prepared: 2 μl of a solution containing 10 μM of both forward and reverse primers in sterile water, 100 ng of cDNA template, 1 μl of 10 mM dNTPs, 5 μl of Pfu Polymerase buffer, 1 μl of Pfu polymerase and 40 μl of sterile water. The PCR amplification reaction was carried out following the Promega recommended thermal cycling conditions. The mutagenic primers were as follows: 5′-CCGACTTTCGTG**TGC**ATGCGCGCGCAG and 5′-CTGCGCGCGCAT**GCA**CACGCCAGTCGG for AS-F178C, 5′-CCGACTTTCGTG**ATT**ATGCGCGCGCAG and 5′-CTGCGCGCGCAT**AAT**CACGCCAGTCGG for AS-F178I, 5′-CCGACTTTCGTG**TGG**ATGCGCGCGCAG and 5′-CTGCGCGCGCAT**CCA**CACGCCAGTCGG for AS-F178W, and 5′-GACTGCTGACCGTTCTC**GCC**CTTATCGATGATGTTCTTG and 5′-CAAGAACATCATCGATAAG**GGC**GAGAACGGTCAGCAGTC for AS-F112A (altered nucleotides in bold). Plasmids were purified from overnight cultures using the Qiagen miniprep kit as described by the manufacturer. All DNA sequences were confirmed by sequence analysis using an Applied Biosystems 3700 automated DNA sequencer (Functional Genomics Laboratory, University of Birmingham).

For protein production, *E. coli* BL21 (DE3) cells were transformed with the cDNA constructs, grown at 37 °C in LB medium with 0.3 mM ampicillin until they reached an OD_{600} of 0.6 and protein production was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside for 3.5 h. Cells were harvested by centrifugation at 8000 g for 10 min and resuspended in 20 mM Tris, pH 8, 5 mM EDTA and 5 mM 2-mercaptoethanol. Proteins were then extracted from the inclusion bodies and purified following the protocol described previously.[12]

Characterisation by GC-MS of sesquiterpene products

500 μl incubations were set up containing 25 μM purified protein, 2 mM FPP, 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 15% glycerol at 25 °C. Reactions were terminated by addition of 200 μl of 100 mM EDTA (pH 7.25). Products were extracted into *n*-hexane (3 × 3 ml) and passed through 1.5 g silica gel columns. Pooled fractions were concentrated to 10–25 μl by a rotary evaporator and analyzed by GC-MS using a ThermoQuest Finnigan GC 8000 gas chromatograph equipped with a 30 m ZB5 column and a MD 1000 mass spectrometer. Splitless injections of 2 μl were performed at 110 °C. The column temperature was increased from 50 °C to 150 °C at a rate of 4 °C min^{-1} and maintained for 15 min at 150 °C.

Determination of the absolute configuration of germacrene A

The absolute configuration of germacrene A **4** produced by aristolochene synthase and its mutants was determined using a

GC equipped with a 30 m (0.2 mm) heptakis(*O*-TBDMS-2,3-di-*O*-methyl)-β-cyclodextrin (50% in OV17) chiral column. Splitless injections with an injector temperature of 250 °C induced the Cope rearrangement of the enzymatically produced germacrene A. The stereochemistry of the resulting β-elemenes was confirmed by comparison with authentic samples.

Steady-state kinetics

Optimal enzyme concentration for the kinetic assays was determined for each mutant, incubating 100 μM FPP (200 μC μmol^{-1}) with increasing concentrations of protein (from 20 nM to 3 μM). For the determination of the appropriate incubation time, a solution of 100 μM FPP (200 μC μmol^{-1}) was assayed with the maximum concentration of protein that, following the procedure described above, was found to be in linear relationship to the formation of products. Kinetic reactions were set up by incubating increasing concentrations of FPP (200 μC μmol^{-1}) with the appropriate concentration of each mutant enzyme. All kinetic studies were carried out at 30 °C using the buffer described for the characterization of products in a final volume of 250 μl. The reactions were terminated by the addition of 200 μl of 100 mM EDTA (pH 7.25).

Acknowledgements

This work was supported by the BBSRC through a studentship to SF and grant 6/B17177 (RKA). We thank Dr Miller and Mr Fanglei Yu for the synthesis of FPP.

References

1 M. J. Rynkiewicz, D. E. Cane and D. W. Christianson, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 13543–13548.
2 C. A. Lesburg, J. M. Caruthers, C. M. Paschall and D. W. Christianson, *Curr. Opin. Struct. Biol.*, 1998, **8**, 695–703.
3 J. Bohlmann, G. Meyer-Gauen and R. Croteau, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 4126–4133.
4 M. J. Rynkiewicz, D. E. Cane and D. W. Christianson, *Biochemistry*, 2002, **41**, 1732–1741.
5 C. A. Lesburg, G. Zhai, D. E. Cane and D. W. Christianson, *Science*, 1997, **277**, 1820–1824.
6 C. M. Starks, K. Back, J. Chappell and J. P. Noel, *Science*, 1997, **277**, 1815–1820.
7 J. M. Caruthers, I. Kang, M. J. Rynkiewicz, D. E. Cane and D. W. Christianson, *J. Biol. Chem.*, 2000, **275**, 25533–25539.
8 A. Deligeorgopoulou and R. K. Allemann, *Biochemistry*, 2003, **42**, 7741–7747.
9 R. H. Proctor and T. M. Hohn, *J. Biol. Chem.*, 1993, **268**, 4543–4548.
10 B. Felicetti and D. E. Cane, *J. Am. Chem. Soc.*, 2004, **126**, 7212–7221.
11 D. E. Cane and Y. S. Tsantrizos, *J. Am. Chem. Soc.*, 1996, **118**, 10037–10040.
12 M. J. Calvert, P. R. Ashton and R. K. Allemann, *J. Am. Chem. Soc.*, 2002, **124**, 11636–11641.
13 M. J. Calvert, S. E. Taylor and R. K. Allemann, *Chem. Commun.*, 2002, 2384–2385.
14 A. Deligeorgopoulou, S. E. Taylor, S. Forcat and R. K. Allemann, *Chem. Commun.*, 2003, 2162–2163.
15 D. E. Cane, P. C. Prabhakaran, J. S. Oliver and D. B. McIlwaine, *J. Am. Chem. Soc.*, 1990, **112**, 3209–3210.
16 S. Forcat and R. K. Allemann, *Chem. Commun.*, 2004, 2094–2095.
17 D. E. Cane, P. C. Prabhakaran, J. S. Oliver and D. B. Mc Ilwaine, *J. Am. Chem. Soc.*, 1990, **112**, 3209–3210.
18 J.-W. d. Kraker, M. C. R. Franssen, A. d. Groot, W. A. Koenig and H. J. Bowmeester, *Plant Physiol.*, 1998, **117**, 1381–1392.
19 V. J. Davisson, A. B. Woodside, T. R. Neal, K. E. Stremler, M. Muehlbacher and C. D. Poulter, *J. Org. Chem.*, 1986, **51**, 4768–4779.