Available online at www.sciencedirect.com
SCIENCE DIRECT®
Archives of Biochemistry and Biophysics 448 (2006) 150–155
ABB
www.elsevier.com/locate/yabbi

Amorpha-4,11-diene synthase: Mechanism and stereochemistry of the enzymatic cyclization of farnesyl diphosphate

Sarah Picaud [a], Per Mercke [a], Xiaofei He [b], Olov Sterner [c], Maria Brodelius [a], David E. Cane [b], Peter E. Brodelius [a,*]

[a] *Department of Chemistry and Biomedical Science, University of Kalmar, SE-39182 Kalmar, Sweden*
[b] *Department of Chemistry, Brown University, Providence, Rhode Island 02912-9108, USA*
[c] *Department of Organic Chemistry, Lund University, P.O. Box 124, SE-22100 Lund, Sweden*

Received 4 June 2005, and in revised form 15 July 2005
Available online 11 August 2005

Abstract

Recombinant amorpha-4,11-diene synthase from *Artemisia annua*, expressed in *Escherichia coli*, was incubated with the deuterium-labeled farnesyl diphosphates, (1*R*)-[1-^{2}H]FPP, (1*S*)-[1-^{2}H]FPP, and [1,1-$^2H_2$]FPP. GC–MS analysis of amorpha-4,11-diene formed from the deuterated FPPs shows that the deuterium atoms are retained in the product. Furthermore, analysis of the MS-spectra obtained with the differently labeled substrate indicates that the H-*1si*-proton of FPP is transferred during the cyclization reaction to carbon 10 of amorphadiene while the H-*1re*-proton of FPP is retained on C-6 of the product. Proton NMR and COSY experiments proved that the original H-*1si*-proton of FPP is located at C-10 of amorpha-4,11-diene as a result of a 1,3-hydride shift following initial 1,6-ring closure. The results obtained support the previously suggested mechanism for the cyclization of farnesyl diphosphate by amorph-4,11-diene synthase involving isomerization of FPP to (*R*)-nerolidyl diphosphate (NPP), ionization of NPP, and C-1,C-6-ring closure to generate a bisabolyl cation, followed by a 1,3-hydride shift, 1,10-ring closure to generate the amorphane skeleton, and deprotonation at either C-12 or C-13 to afford the final product (1*S*,6*R*,7*R*,10*R*)-amorpha-4,11-diene.

Keywords: Sesquiterpenes; Amorpha-4,11-diene synthase; Enzyme mechanism; Deuterated substrate; GC–MS; Proton NMR; COSY

Sesquiterpenoids are a structurally diverse class of isoprenoids found in plants, fungi, and some bacteria, which play a variety of physiological and ecological roles, including plant–plant, plant–insect, and plant–pathogen interactions. The structural diversity and stereochemical complexity of the C_{15}-isoprenoid skeletons of sesquiterpenoids are remarkable. More than 300 types of cyclic sesquiterpenes have been characterized to date and each is derived from the common acyclic precursor, farnesyl diphosphate (**1**, FPP).[1] Sesquiterpene synthases are key branch point enzymes in the biosynthesis of these compounds. Several sesquiterpene synthases have been cloned from plants and the deduced amino acid sequences show high identity/similarity to one another [1–9]. Numerous plant sesquiterpene synthase sequences have been recognized on the basis of this sequence similarity, but the nature of the cyclization products is as yet unknown. Our understanding of the relationship between protein structure and product selectivity of highly homologous sesquiterpene synthases is still limited.

We recently reported the molecular cloning and biochemical characterization of amorpha-4,11-diene synthase from *Artemisia annua*, which catalyzes the cyclization of farnesyl diphosphate to (1*S*,6*R*,7*R*,10*R*)-amorpha-4,11-

* Corresponding author. Fax: +46 480 446262.
E-mail address: peter.brodelius@hik.se (P.E. Brodelius).

[1] *Abbreviations used:* ADS, amorpha-4,11-diene synthase; AMU, atomic mass unit; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; NPP, nerolidyl diphosphate; IMAC, immobilized metal affinity chromatography.

0003-9861/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2005.07.015

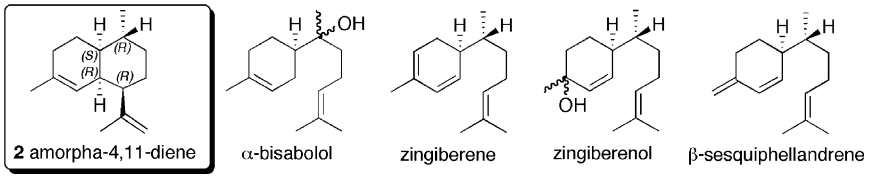

Fig. 1. Amorpha-4,11-diene (**1**) and bisabolane-type co-products of the ADS-catalyzed cyclization of FPP in the presence of Mg^{2+}.

diene (**2**) [8,10]. Studies by Bouwmeester et al. [11] established that amorphadiene synthase mediates a key step in the biosynthesis of the antimalarial endoperoxide artemisinin. Furthermore, these authors established the structure and stereochemistry of enzymatically produced amorpha-4,11-diene [11]. We have previously proposed a mechanism for the formation of amorpha-4,11-diene (**2**) [10] that posits an initial 1,6-ring closure to generate a bisabolyl cation intermediate, based on the generation of a number of bisabolane-type by-products in the enzymatic reaction including α-bisabolol (1.6% of total sesquiterpenoids), (+)-zingiberene (0.2%), zingiberenol (0.4%), and (+)-β-sesquiphellan- drene (1.8%) [10] (Fig. 1). The formation of these products may be explained by premature quenching of the cyclization cascade that leads to amorphadiene (**2**). On the other hand, no germacrane-type sesquiterpenes could be detected in the enzymatic assay mixture. Furthermore, the acyclic monoterpene substrate, geranyl diphosphate (GPP), is converted to the cyclic compounds α-terpineol, limonene, and terpinen-4-ol by ADS, in contrast to the action of tobacco *epi*-aristolochene synthase, which only produces the acyclic products linalool and myrcene when incubated with GPP (B.T. Greenhagen, S.G. Koenig, L. Olofsson, P.E. Brodelius, J.P. Noel, J. Chappell, unpublished) (Fig. 2). The cyclization of FPP to *epi*-aristolochene by the tobacco enzyme has been shown to involve the intermediacy of the 10-membered ring germacrene A [12].

The enzymatic cyclization of FPP to other 4,10-dimethyl-7-isopropyloctalins such as cubenene [13], *epi*-cubenol [13,14], γ-cadinene [15], and δ-cadinene [16] has been suggested or shown to proceed through a common helminthogermacradienyl intermediate, which is formed by an initial 1,10-ring closure (Fig. 3). We now describe experiments with recombinant amorpha-4,11-diene synthase using deuterium-labelled FPPs, that confirm the proposed intermediacy of a bisabolyl cation in the cyclization reaction and and the operation of a 1,3-hydride shift during the carbocationic cyclization cascade.

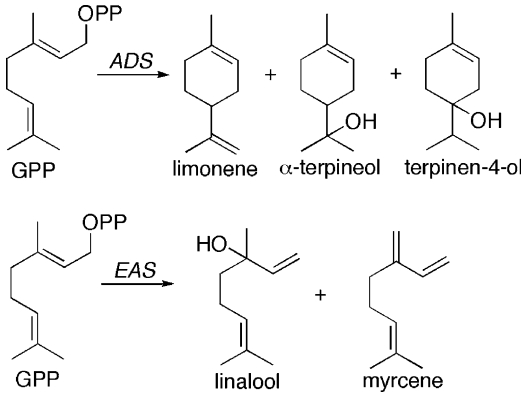

Fig. 2. Cyclization or solvolysis of the anomalous substrate geranyl diphosphate by ADS or *epi*-aristolochene synthase (EAS).

Materials and methods

Reagents

[1-^{3}H]FPP (0.59 TBq/mmol) was purchased from Amersham-Pharmacia Biotech. IPTG, FPP, and kanamycin were obtained from Sigma. All other biochemicals and reagents were purchased from commercial sources. (1*R*)-[1-^{2}H]FPP and (1*S*)-[1-^{2}H]FPP, synthesized as described by Cane et al. [17], were a gift from Dr. W. König through Dr. H. Bouwmeester. [1,1-$^2H_2$]FPP was synthesized as previously described [13,17]. Semisynthetic amorpha-4,11-diene, synthesized from artemisinic acid, was kindly supplied by Dr. Ben Jansen.

Production of recombinant ADS

For production of recombinant ADS, *E. coli* BL21 (DE3) cells were transformed with bacterial expression

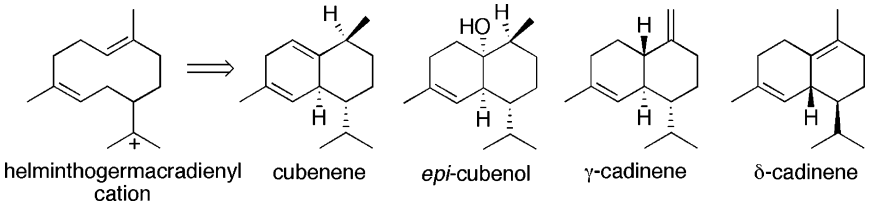

Fig. 3. Formation of 4,10-dimethyl-7-isopropyl octalin sesquiterpenes via a helmintho-germacradienyl cation (1,10-ring closure).

vector pET28 carrying the ADS cDNA. A single colony was used to inoculate LB medium (10 ml) containing kanamycin (50 μg/ml), which was cultivated at 37 °C overnight. An aliquot (2 ml) of this culture was added to fresh LB medium (200 ml) containing kanamycin (50 μg/ml). Bacteria were grown at 37 °C to $OD_{600} = 0.5$. Induction of ADS production was achieved by addition of 0.2 mM isopropyl β-D-thiogalacto-pyranoside. The cells were maintained for 4 h at 30 °C and then harvested by centrifugation (2000*g*, 10 min). Subsequently, the bacteria were suspended in 20 mM sodium phosphate buffer, pH 6.5, containing 10% (v/v) glycerol and 0.5 M NaCl (100 ml). After another centrifugation and re-suspension of the cells in the same buffer (20 ml), sonication of the cells was carried out for 2 min in 5-s pulses with 5 s between pulses on ice (Vibracell power sonicate setting 12–15 W). The cell lysate was centrifuged at 10,000*g* for 30 min at 4 °C and the supernatant, containing the soluble recombinant enzyme was filtered through a 0.45 μm non-pyrogenic sterile filter. The crude enzyme preparation obtained was immediately used or frozen in liquid nitrogen.

Standard enzyme activity assay

ADS activity was measured as previously described using ^{14}C-labeled FPP [10].

Production of deuterium-labeled amorpha-4,11-diene

The deuterium labeling experiments were conducted in large-scale incubations. Crude enzyme extract (40 ml) was supplemented with 250 μM $MnCl_2$ and 14.4 μM deuterium-labeled substrate (total amount ~500 μg). The mixture was incubated in glass tubes (5 × 8 ml) overnight at 30 °C. The reaction mixture was extracted twice with *n*-pentane (2 × 8 ml) and the pentane extracts were pooled and passed over a small glass column filled with 250 mg of silica gel 60 (Merck; size: 0.2–0.5 μm) into a new glass tube. The column was rinsed with additional pentane (2 × 1 ml), which was collected in the same tube. The five fractions obtained were pooled and the pentane phase was concentrated by evaporation under a stream of helium gas to around 500 μl.

GC–MS analysis of enzymatic products

The quality and purity of the extracts from the enzymatic reactions were analyzed by capillary GC on an Agilent 6890 gas chromatograph equipped with a HP-5M5 5% phenyl methyl silohexane capillary column (0.25 μm film thickness, 0.25 mm id × 30 m) and an Agilent 5973 Network Mass Selective Detector. The GC injection port was operated at 100 °C. The oven temperature was programmed from 100 to 200 °C at a rate of 5 °C/min and from 200 to 250 °C at a rate of 25 °C/min. The final temperature was maintained for 26 min. Helium was used as a carrier gas and samples (1.0 μl) were injected in split mode. Mass spectra were measured in electron impact mode at 70 eV with scanning from *m*/*z* 34 to 400 at 3.93 scans s^{-1}.

NMR experiments

^{1}H NMR spectra (500 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse, multinuclear 5-min probe head sine-shaped equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$. COSY experiments were recorded with gradient enhancements using sine-shaped gradient pulses. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). The isolation and structure determination based on the ^{1}H NMR data of amorpha-4,11-diene (1β,6β,7β,10αH-cadina-4,11-diene) is reported in [19]. For clarity, since the chemical shift previously reported for 5-H is incorrect, the ^{1}H chemical shifts are given here as well: 5.07 5-H, 4.88 13-Hα, 4.65 13-Hβ, 2.56 6-H, 1.99 3-Hα, 1.95 7-H, 1.83 3-Hβ, 1.77 1-H, 1.74 12-H_3, 1.68 9-Hβ, 1.60 14-H_3, 1.57 2-Hα, 1.50 8-Hα, 1.40 10-H, 1.32 2-Hβ, 1.25 8-Hβ, 0.96 9-Hα, 0.87 15-H_3.

Protein concentrations

Protein concentrations were determined according to Bradford [19] using BSA as standard.

Results and discussion

Production of deuterium-labeled amorpha-4,11-diene

We have recently reported the production and characterization of recombinant ADS in *E. coli* [10]. The crude enzyme preparation was used to produce amorpha-4,11-diene from the deuterium-labeled substrates (1*R*)-[1-^{2}H]FPP (**1c**), (1*S*)-[1-^{2}H]FPP (**1b**), or [1, 1-^{2}H]FPP (**1a**). The enzymatic reactions were run in the presence of Mn^{2+} rather than Mg^{2+} since the number and amount of by-products are reduced when Mn^{2+} is used as cofactor [10], thereby facilitating the NMR analysis of labeled amorphadiene. Analysis of the product by GC–MS showed that the yield of amorphadiene was around 96%.

GC–MS analysis of products

Table 1 and Fig. 4 show some fragment ions observed in the electron impact mass spectra of amorpha-4,11-diene derived from FPP, (1*R*)-[1-^{2}H]FPP (**1c**), (1*S*)- [1-^{2}H]FPP (**1b**), and [1,1-$^2H_2$]FPP (**1a**). The molecular ion ($[M]^+$) of amorphadiene obtained from [1,1-$^2H_2$]FPP shifts by two atomic mass units (AMUs) relative to

Table 1
Mass spectrometric parent and fragment ions from un-labeled and deuterated amorpha-4,11-diene

	Ions (m/z)[a]			
	M^+	**a**	**b**	**c**
Amorpha-4,11-diene (**2**) generated from non-labeled FPP	204	189	121	93
[$^2H_2$]Amorpha-4,11-diene (**2a**) generated from [1,1-$^2H_2$]FPP (**1a**)	206	191	123	94
[^{2}H]Amorpha-4,11-diene (**2b**) generated from (1*S*)-[1-^{2}H]FPP (**1b**)	205	190	122	93
[^{2}H]Amorpha-4,11-diene (**2c**) generated from (1*R*)-[1-^{2}H]FPP (**1c**)	205	190	122	94

[a] Ions shown in Fig. 3.

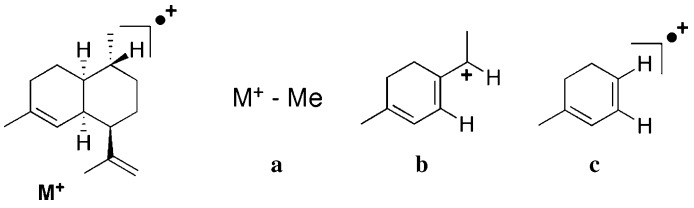

Fig. 4. Some mass spectrometric fragment ions from amorpha-4,11-diene.

unlabeled amorphadiene, establishing that both deuterium atoms of the labeled FPP substrate are retained in the enzyme-generated sesquiterpene product. In agreement with this, the molecular ion ([M]$^+$) of amorphadiene obtained from (1*R*)-[1-^{2}H]FPP or (1*S*)-[1-^{2}H]FPP shifts by 1 AMU. Furthermore, the [M-methyl] ion (ion **a**) (shift 2 and 1 AMU, respectively, for the doubly deuterated and singly deuterated substrates) shows that no deuterium is present in any of the methyl groups (i.e., C-13, -14 or -15). Ion **b**, arising from cleavage of both the C-6/C-7 and C-9/C-10 bonds, retains the deuterium atoms of all three substrates (shift 2 or 1 AMU). Finally, ion **c**, formed by cleavage of the C-6/C-7 and C-1/C-10 bonds, is shifted by 1 AMU for (1*R*)-[1-^{2}H]FPP and [1,1-$^2H_2$]FPP while ion **c** from amorphadiene (**2b**) derived from (1*S*)-[1-^{2}H]FPP does not retain any deuterium label. From the information obtained from ions **b** and **c**, we can conclude that the H-*1si*-proton of FPP has been transferred to C-10 of amorphadiene during the cyclization reaction. Together these observations are all consistent with our originally suggested mechanism for the ADS-catalyzed cyclization reaction (Fig. 5).

NMR of deuterium-labeled amorpha-4,11-diene

To study further the fate of the hydrogen atoms from C-1 of farnesyl diphosphate we have carried out cyclizations using (1*R*)-[1-^{2}H]FPP (**1c**), (1*S*)-[1-^{2}H]FPP (**1b**), or [1,1-$^2H_2$]FPP (**1a**) and analyzed each of the resulting labeled samples of amorpha-4,11-diene by ^{1}H NMR (Figs. 5 and 6). The full assignment of each of the protons in the ^{1}H NMR spectrum of amorpha-4,11-diene has previously been reported [18], and the chemi-

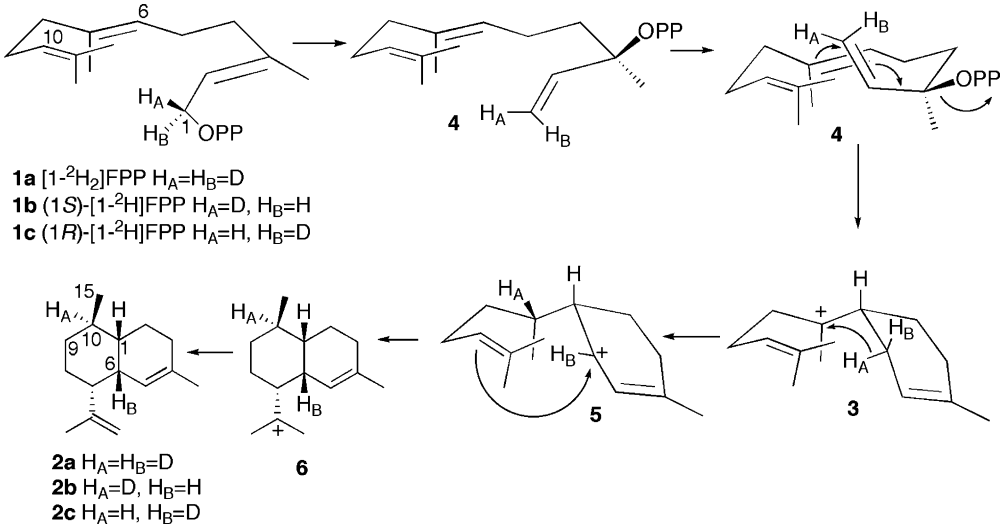

Fig. 5. Mechanism for the formation of amorpha-4,11-diene (**7**) from FPP (**1**) by ADS. OPP denotes diphosphate moiety.

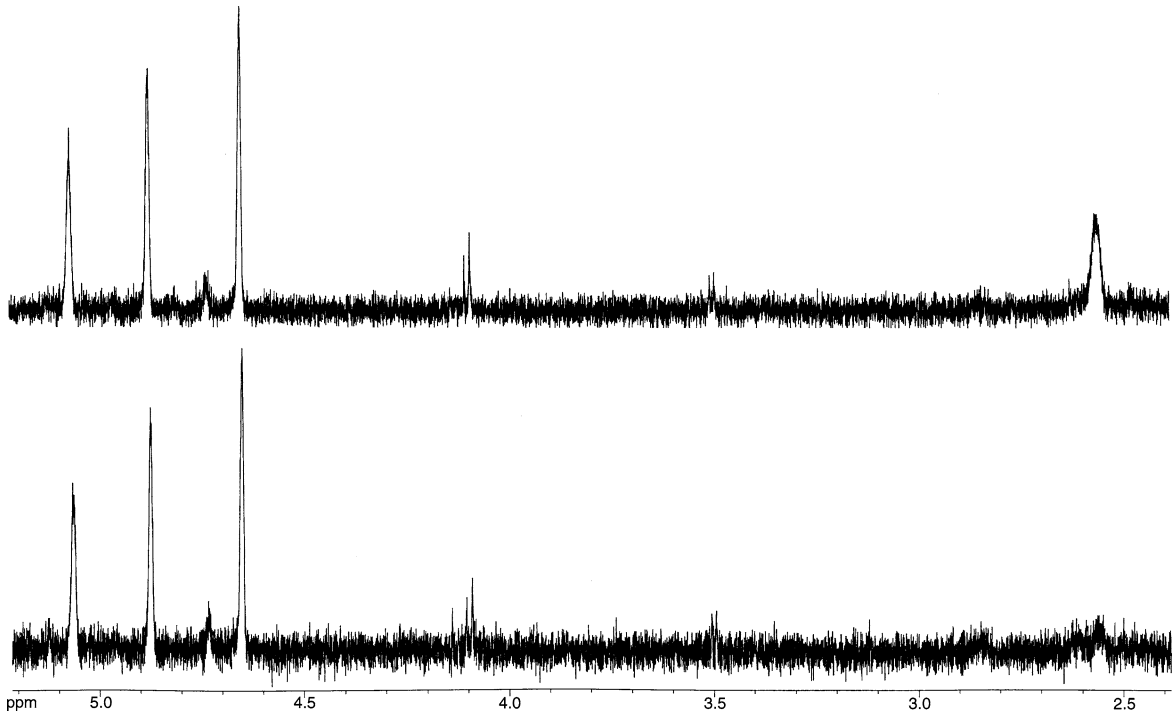

Fig. 6. Sections of the NMR spectra of amorphadiene **2b** derived from (1*S*)-[1-^{2}H]FPP (top) and amorphadiene **2c** derived from (1*R*)-[1-^{2}H]FPP (bottom), showing the presence of a peak at a chemical shift of 2.56 due to the presence of a proton at C-6 in the former and and its absence in the latter.

cal shifts are given in the experimental section for reference. Amorphadiene (**2c**) derived from (1*R*)-[1-^{2}H]FPP (**1c**) did not display a signal for H-6 but showed a signal corresponding to H-10. Although, this H-10 signal appeared as a complex multiplet at ~1.40 ppm overlapping additional signals due to impurities, the presence of H-10 was evident in the corresponding COSY spectrum which showed a clear correlation between H-10 and the H-15 methyl doublet at 0.87 ppm. On the other hand, amorphadiene (**2b**) derived from (1*S*)-[1-^{2}H]FPP (**1b**) displayed a proton signal for H-6 but no visible signal for H-10; similarly there was no H-10/H-15 cross-peak in the COSY experiment. Finally, the [6,10-$^2H_2$]amorpha-4,11-diene (**2a**) generated from [1,1-$^2H_2$]FPP (**1a**) had no signal for H-6 in the proton spectrum and no H-10/H-15 methyl cross-peak in the corresponding COSY spectrum. Furthermore, the H-9β resonance is more split in the product ^{1}H NMR spectrum of amorphadiene (**2c**) derived from (1*R*)-[1-^{2}H]FPP (**1c**), due to coupling to H-10, than either of the corresponding signals in amorphadienes **2b** and **2a**, derived from (1*S*)-[1-^{2}H]FPP and [1,1-$^2H_2$]FPP, respectively, each of which carries a deuterium at C-10. Fig. 6 shows a downfield section of the ^{1}H NMR spectra of **2b** and **2c** which clearly demonstrates the presence of 6-H in **2b** and its absence in **2c**. Due to the signal overlap in the region where H-10 would appear, this signal is not clearly visible in the ^{1}H NMR spectrum. However, the presence or absence of 2D COSY correlation peaks between H-10 and H-15 as discussed above clearly demonstrates that H-10 is absent in **2b** and present in **2c**.

Mechanism of ADS

The NMR results clearly support our originally proposed mechanism for the cyclization of FPP to amorpha-4,11-diene, as outlined in Fig. 5. After ionization of FPP, the paired diphosphate anion is recaptured at C-3 to form (3*R*)-nerolidyl diphosphate (**4**, NPP), which can undergo rotation about the C-2–C-3 bond to generate the corresponding *cisoid* conformer. Ionization of NPP followed by C-1,C-6-ring closure (FPP numbering) generates the bisabolyl cation (**3**), which undergoes a 1,3-hydride shift to give intermediate **5** in which the original H-*1si*-proton of FPP is transferred to C-7 (FPP numbering, corresponding to C-10 of the ultimately derived amorphadiene). The carbocationic intermediate **5** can then further cyclize by electrophilic attack on C-10 (FPP numbering), thereby generating the amorphenyl cation **6**. Deprotonation at either C-12 or C-13 of the amorphenyl cation affords the product (1*S*,6*R*,7*R*,10*R*)-amorpha-4,11-diene (**2**). The labeling results rule out an alternative generation of intermediate **5** by a pair of consecutive 1,2-hydride shifts and firmly exclude the cyclization mechanism previously suggested by Akhila et al. [20]. While to our knowledge this is the first demonstration of a 1,3-hydride shift involving a bisabolyl cation intermediate, Croteau has previously established a completely

analogous 1,3-hydride shift of an α-terpinyl cation in the formation of β-phellandrene [21].

The biosynthesis of a number of other 4,10-dimethyl-7-isopropyloctalins has been described. (+)-Epicubenol (Fig. 3) is formed from FPP by a crude enzyme preparation from *Streptomyces* sp. LL-B7 [13] as well as by a cell-free extract from cultivated cells of the liverwort *Heterocyphus planus* [14]. The liverwort extract also produced cubenene. Nabeta et al. [15] have further separated the *H. planus* extract into two fractions, one producing a mixture of epicubenol and cubenene and the other producing (−)-γ-cadinene. It was concluded that both epicubenol and cubenene were produced by a single sesquiterpene synthase. Finally, recombinant cadinene synthase from cotton produces (+)-δ-cadinene as a single product from FPP [16]. The enzymatic formation of each of these 4,10-dimethyl-7-isopropyloctalins has been suggested or shown to proceed via a helminthogermacradienyl cation, which is formed through an initial 1,10-ring closure, followed by a 1,3-hydride shift. After a second cyclization to form a bicyclic cation, the cadinenes are formed by deprotonation, while formation of both epicubenol and cubenene requires an additional 1,2-hydride shift [13,14].

The order in which the 1,6- and 1,10-cyclizations (FPP numbering) occur determines the location of the positive charge in the final carbocationic intermediate and therefore the regiochemistry of termination of the cyclization cascade. The initial 1,10-cyclization pathway and coupled 1,3-hydride shift lead to the formation of products with an isopropyl side chain. The subsequent 1,10-cyclization generates a bicyclic carbocation intermediate in which the positive charge is initially at a position corresponding to the original C-7 of FPP (C-10 using the amorphadiene numbering), leading to formation of 4,10-dimethyl-7-isopropyloctalins. By contrast, the ADS reaction involves initial 1,6-ring closure to a bisabolyl cation intermediate followed by a 1,3-hydride shift and a subsequent 1,10-cyclization, leading to the generation of a carbocation at C-11, and resulting in the formation of amorpha-4,11-diene with an isopropenyl side chain. In spite of the superficial structural similarity between amorpha-4,11-diene and the 4,10-dimethyl-7-isopropyloctalin sesquiterpenes, the ADS-catalyzed reaction therefore clearly involves an initial 1,6-cyclization followed by a 1,10-ring closure.

Acknowledgments

Financial support from University of Kalmar, The Crafoord Foundation, the Carl Tryggers Foundation, and the European Union (Marie Curie Scholarship to S.P.) is acknowledged, as well as a grant from the US National Institutes of Health (GM30301 to D.E.C.).

References

[1] P.J. Facchini, J. Chappell, Proc. Natl. Acad. Sci. USA 89 (1992) 11088–11092.
[2] X.Y. Chen, Y. Chen, P. Heinstein, V.J. Davisson, Arch. Biochem. Biophys. 324 (1995) 255–266.
[3] K. Back, J. Chappell, J. Biol. Chem. 270 (1995) 7375–7381.
[4] J. Crock, M. Wildung, R. Croteau, Proc. Natl. Acad. Sci. USA 94 (1997) 12833–12838.
[5] S.M. Colby, J. Crock, B. Dowdle-Rizzo, P.G. Lemaux, R. Croteau, Proc. Natl. Acad. Sci. USA 95 (1998) 2216–2221.
[6] C.L. Steele, J. Crock, J. Bohlmann, R. Croteau, J. Biol. Chem. 273 (1998) 2078–2089.
[7] P. Mercke, J. Crock, R. Croteau, P.E. Brodelius, Arch. Biochem. Biophys. 369 (1999) 213–222.
[8] P. Mercke, M. Bengtsson, H.J. Bouwmeester, M.A. Posthumus, P.E. Brodelius, Arch. Biochem. Biophys. 381 (2000) 173–180.
[9] S. Picaud, M. Brodelius, P.E. Brodelius, Phytochemistry 66 (2005) 961–967.
[10] S. Picaud, L. Olofsson, M. Brodelius, P.E. Brodelius, Arch. Biochem. Biophys. 436 (2005) 215–226.
[11] H.J. Bouwmeester, T.E. Wallaart, M.H.A. Janssen, B. van Loo, B.J.M. Jansen, M.A. Posthumus, C.O. Schmidt, J.-W. De Kraker, W.A. König, M.C.R. Franssen, Phytochemistry 52 (1999) 843–854.
[12] K.A. Rising, C.M. Starks, J.P. Noel, J. Chappell, J. Am. Chem. Soc 122 (2000) 1861–1866.
[13] D.E. Cane, M. Tandon, J. Am. Chem. Soc. 117 (1995) 5602–5603.
[14] K. Nabeta, K. Kigure, M. Fujita, T. Nagoya, T. Ishikawa, H. Okuyama, T. Takesawa, J. Chem. Soc. Perkin Trans. 1 (1995) 1935–1939.
[15] K. Nabeta, M. Fujita, K. Komuro, K. Katayama, T. Takasawa, J. Chem. Soc., Perkin Trans. 1 (1997) 2065–2070.
[16] C.R. Benedict, J.-L. Lu, D.W. Pettigrew, J. Liu, R.D. Stipanovic, H.J. Williams, Plant Physiol. 125 (2001) 1754–1765.
[17] D.E. Cane, J.S. Oliver, P.H.M. Harrison, C. Abell, B.R. Hubbard, C.T. Kane, R. Lattman, J. Am. Chem. Soc. 112 (1990) 4513–4524.
[18] F. Bohlmann, T. Gerke, J. Jakupovic, R.M. King, H. Robinson, Phytochemistry 23 (1984) 1183–1184.
[19] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[20] A. Akhila, R.S. Thakur, S.P. Popli, Phytochemistry 26 (1987) 1927–1930.
[21] R.E. LaFever, R. Croteau, Arch. Biochem. Biophys. 301 (1993) 361–366.