Novel Product Chemistry from Mechanistic Analysis of *ent*-Copalyl Diphosphate Synthases from Plant Hormone Biosynthesis**

Kevin Potter, Jared Criswell, Jiachen Zi, Alisha Stubbs, and Reuben J. Peters

Abstract: *An active-site water molecule coordinated by conserved histidine and asparagine residues seems to serve as the catalytic base in all ent-copalyl diphosphate synthases (CPSs). When these residues are substituted by alanine, the mutant CPSs produce stereochemically novel ent-8-hydroxy-CPP. Given the requisite presence of CPSs in all land plants for gibberellin phytohormone biosynthesis, such plasticity presumably underlies the observed extensive diversification of the resulting labdane-related diterpenoids.*

The biosynthesis of natural products has been suggested to be largely derived from hormone metabolism.[1] For example, production of labdane-related diterpenoids by plants is derived from gibberellin phytohormone biosynthesis.[2] Giberellin production is initiated by a cycloisomerization reaction which transforms the general diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP; **1**; see Scheme 1) into *ent*-copalyl diphosphate (*ent*-CPP; **2**). This reaction is catalyzed by *ent*-CPP synthases (CPSs), which are ubiquitously found in all vascular plants, thus providing a source for repeated evolution of more specialized labdane-related diterpenoid biosynthesis, as evidenced by the fact that the vast majority of the approximately 7000 known such natural products are uniquely produced by plants.[2] Intriguingly, although it is known that evolutionarily derived class II diterpene synthases can incorporate water into their reactions, thus producing a chemically altered (hydroxylated) analogue of CPP, these also all differ in stereochemistry (e.g., 8α-hydroxy-CPP, **3**).[3] Accordingly, formation of hydroxylated *ent*-CPP would represent a novel enzymatic product. Herein we report identification of the CPS catalytic base group as a water molecule activated by a histidine-asparagine dyad, and substitution of either residue by alanine leads to production of an epimeric pair of 8-hydroxylated-*ent*-CPPs (**4** and **5**).

The characteristic feature of the labdane-related diterpenoid superfamily of natural products is their biosynthetic origins in a bicyclization reaction catalyzed by class II diterpene cyclases. This feature directly results in formation of a labdadienyl hydrocarbon backbone, thus leading to the unifying nomenclature.[2] The underlying enzymatic mechanism relies on acid-base catalysis, by initiating protonation of the terminal carbon–carbon double bond and sequential cyclization by addition from the internal double bonds in a carbocationic cascade, followed by deprotonation (Scheme 1). While this sequence can simply take the form
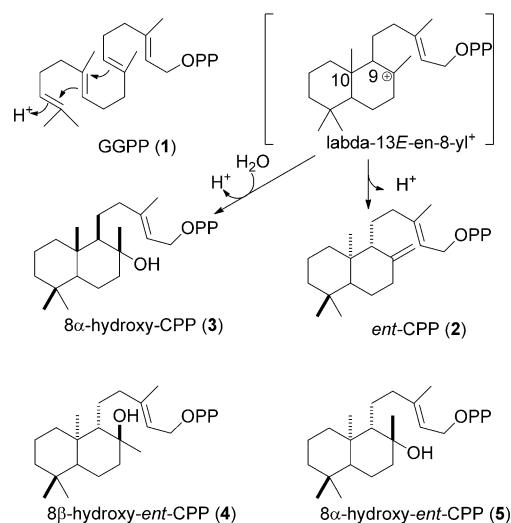
Scheme 1. Reactions mediated by known class II diterpene cycloisomerases (e.g., **2**) and cyclohydratases (i.e., **3**), along with the novel products found here (**4** and **5**).

of a cycloisomerization reaction, as indicated by the designation of CPSs as isomerases (EC 5.5.1.13), the reaction also is amendable to the addition of water prior to deprotonation, thus leading to formal designation of the relevant class II diterpene cyclases as hydratases (EC 4.2.1.133). However, these cyclohydratases also differ in their sterochemical outcome, which stems from precatalysis folding of the substrate **1** in a 'normal' pro-chair-chair conformation rather than the antipodal conformation leading to the *ent*-CPP produced by CPSs en route to the gibberellins.[2] Thus, while the CPSs produce **2** (9R,10R), all of the characterized class II diterpene cyclo-hydratases (i.e. those that produce hydroxylated analogues), despite many exhibiting clear homology to CPSs, produce **3** (9S10,S) instead.

Class II diterpene cyclases are characterized by a (D,E)xDD motif, wherein the middle aspartic acid residue acts as the catalytic acid.[4] By contrast, there does not appear to be a similarly well-conserved residue which acts as the catalytic base, perhaps in part due to the variation in substrate

[*] K. Potter, J. Criswell, Dr. J. Zi, A. Stubbs, Prof. Dr. R. J. Peters
Dept. Biochemistry, Biophysics & Molecular Biology
Iowa State University, Ames, IA 50011 (USA)
E-mail: rjpeters@iastate.edu

conformation underlying the production of different stereo-isomers of CPP, which presumably requires different relative positioning of the catalytic acid and base.[2] Thus, the catalytic base might be conserved by the stereochemical product outcome.

Building on our recently reported crystal structure for the bifunctional abietadiene synthase from *Abies grandis* (AgAS),[5] we have recently reported that histidine 348 may function as the catalytic base.[3h] However, this function is limited to the closely related bifunctional diterpene synthases from gymnosperms (all of which produce normal CPP),[6] as a histidine is not found at this position in other class II diterpene cyclases, including the only known *ent*-CPP-producing CPS from a gymnosperm, that is, the CPS from *Picea glauca* (PgCPS).[7] Intriguingly, substitutions for this histidine lead to incorporation of water into the usually catalyzed cycloisomerization reaction, with mutation to either alanine or, more specifically, aspartate, thus leading to cyclohydratase activity.[3h] However, the AgAS:H348A and H348D mutants presumably catalyze the production of **3** with the normal (9S,10S) stereochemistry, which is common among all of the previously characterized class II diterpene cyclohydratases.

Crystal structures also have been reported for the CPS from *Arabidopsis thaliana* (AtCPS).[8] Careful inspection of these revealed the presence of an active-site histidine (H263 in AtCPS), which differs in positioning from the critical histidine characterized in AgAS, but is conserved in all the known *ent*-CPP-producing CPSs, including those from the monocot rice (*Oryza sativa*; e.g., OsCPS1),[9] as well as the gymnosperm PgCPS,[7] and even a bifunctional diterpene cyclase from the moss *Physcomitrella patens* (PpCPSKS),[10] thus representing over 450 million years of evolutionary separation (Figure 1).[11]

Much as with AgAS,[3h] we initially hypothesized that this histidine might be the catalytic base. Accordingly, we substituted this histidine in AtCPS with alanine, thus creating an AtCPS:H263A mutant. Rather than the loss of catalytic activity which might have been expected, this substitution led to significant production of two novel hydroxylated products, along with some production of **2**, either from in vitro reactions with **1**, or upon expression in *E. coli* metabolically engineered to produce **1**[12] (Figure 2). The two new compounds, were
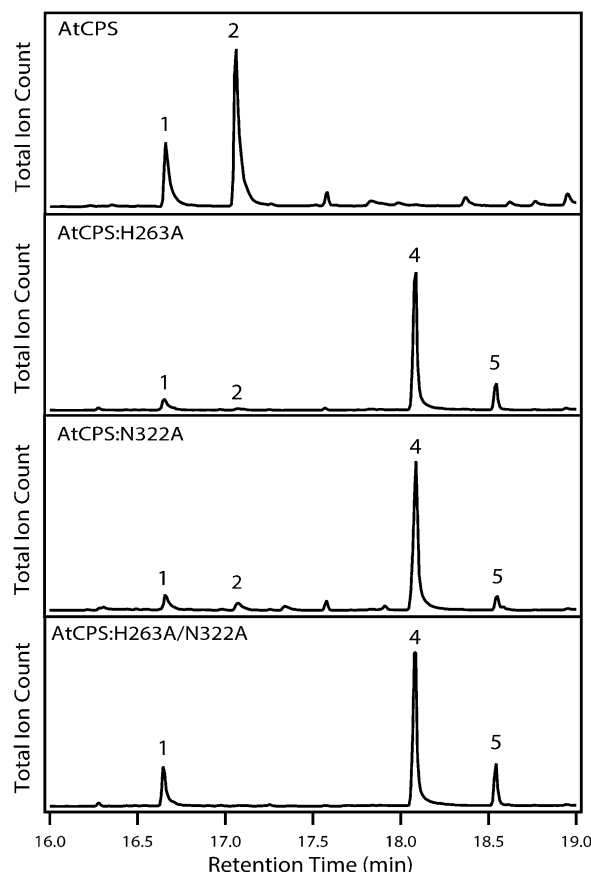
Figure 2. Effect of H263A, N322A, and H263A/N322A mutations on the AtCPS product outcome. Chromatograms from GC-MS analysis of the dephosphorylated products (numbering as in text).

detected by GC-MS, after dephosphorylation, and exhibited identical retention times and mass spectra to the previously reported pair of 8-hydroxy epimers of labda-13*E*-en-8,15-diol (see Figure S1 in the Supporting Information),[3i] thus demonstrating cyclohydratase activity. This activity was further verified by NMR analysis of the epimer corresponding to the major product **4** (see Table S1 and Figures S2 and S3).

Our previous results with AgAS suggested that it might be possible to divert AtCPS more completely to cyclohydratase activity by substitution with aspartate instead of alanine.[3h] Accordingly, we substituted aspartate for H263 in AtCPS. However, the resulting AtCPS:H263D mutant failed to exhibit any cyclohydratase activity, although it retained the ability to produce **2** (see Figure S4). This result clearly
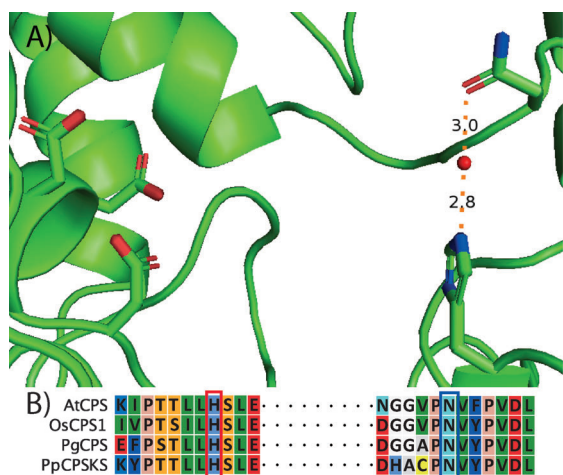

Figure 1. Identification of catalytic base group, consisting of water activated by the conserved active-site histidine and asparagine in CPSs. A) Active site of AtCPS (cartoon format) showing side chains of the identified histidine and asparagine with a bound water molecule (red sphere) as well as the aspartates of the DxDD catalytic acid motif, in stick representation. B) Conservation of the histidine and asparagine (boxed) in CPSs spanning land plant evolution, as indicated by alignment of the representative examples described in text.

differentiates the role of the critical CPS-specific histidine identified here from that previously found in AgAS.

Careful inspection of the AtCPS crystal structure revealed that H263 is hydrogen bonded to a water molecule which is also held in place by a hydrogen bond to an active-site asparagine (N322 in AtCPS). This asparagine is also well conserved in *ent*-CPP-producing CPSs (Figure 1), and is consistent with a role in catalysis. Substitution of this conserved asparagine with alanine led to cyclohydratase activity with AtCPS:N322A, as well as AtCPS:H263A/N322A, both of which similarly produce the 8-hydroxy epimers observed with AtCPS:H263A (Figure 2). These results indicate that the actual base in the cycloisomerization reaction catalyzed by CPSs is the tightly bound water (i.e., this water is activated by a catalytic His-Asn dyad, thus forming a catalytic base group). We hypothesize that, in the absence of either or both ligating residues, this water is then directly added to the labda-13*E*-en-8-yl carbocation intermediate, with the resulting oxacarbenium ion quenched by deprotonation catalyzed by H263 in the case of the AtCPS:N322A mutant, or by an additional water molecule in the case of alanine substitution for AtCPS:H263 (i.e., with H263A or H263A/N322A). To verify that the observed cyclohydratase activity was enabled by this latter hypothesized steric effect (i.e., opening up of the active site), we substituted valine and isoleucine for AtCPS:H263. While the AtCPS:H263V mutant retained some ability to make **4** as well as **2**, the AtCPSH263I mutant is largely inactive, although it does make trace amounts of **4** (see Figure S5), and is consistent with the hypothesis that cyclohydratase activity requires suitable space for an additional water molecule to bind (i.e., while sufficient steric volume might be available with these latter mutants, the hydrophobic effect of these larger aliphatic side chains makes occupation by a water increasingly unlikely).

Given that AtCPS produces *ent*-CPP, we hypothesized that the compounds observed here were actually enantiomeric, that is, *ent*- labda-13*E*-en-8,15-diols (e.g., eperu-13-en-8β,15-diol[13]), and resulted from dephosphorylation of 8-hydroxy-*ent*-CPP, rather than the previously observed normal (9*S*,10*S*) isomers (e.g., **3**). This stereochemistry was investigated by enzymatic feeding studies, thus using the previously reported specificity of the bifunctional *cis*-abienol synthase from *Abies balsamea* (AbCAS), as this has been shown to specifically react with only **3**.[3g] Based on previous work,[4,14] the endogenous class II activity of AbCAS was blocked by mutation of the aspartate which acts as the catalytic acid (i.e., the "middle" aspartate from the highly conserved DxDD motif[4]) to an alanine. The resulting AbCAS:D405A mutant does not react with **1**, but will react with **3** produced by either a previously characterized class II diterpene cyclohydratase from *Nicotiana glutinosa* (NgCLS), or the AgAS:H348D mutant (with additional D621A mutation to prevent any further reaction from its endogenous subsequently acting class I activity),[3h] but does not react with any product of the AtCPS cyclohydratase mutants (see Figure S6). These results demonstrate the expected retention of stereochemistry for the AgAS and, more critically, AtCPS cyclohydratase mutants as well. Notably, the hydroxylated products from

the AtCPS mutants then exhibit an enantiomeric configuration, and appears to be the first example of a class II diterpene cyclohydratase producing this 9*R*,10*R* stereoisomer, specifically both 8β-hydroxy-*ent*-CPP (**4**) and 8α-hydroxy-*ent*-CPP (**5**).

Although there is a substrate analogue bound in the active site of the AtCPS crystal structures, this is clearly not in a reactive conformation.[8] Nevertheless, analysis of the products of the AtCPS cyclohydratase mutants presented here provides some indication of the positioning of the water molecule relative to the labda-13*E*-en-8-yl carbocation intermediate (Scheme 2). The production of both 8-hydroxy
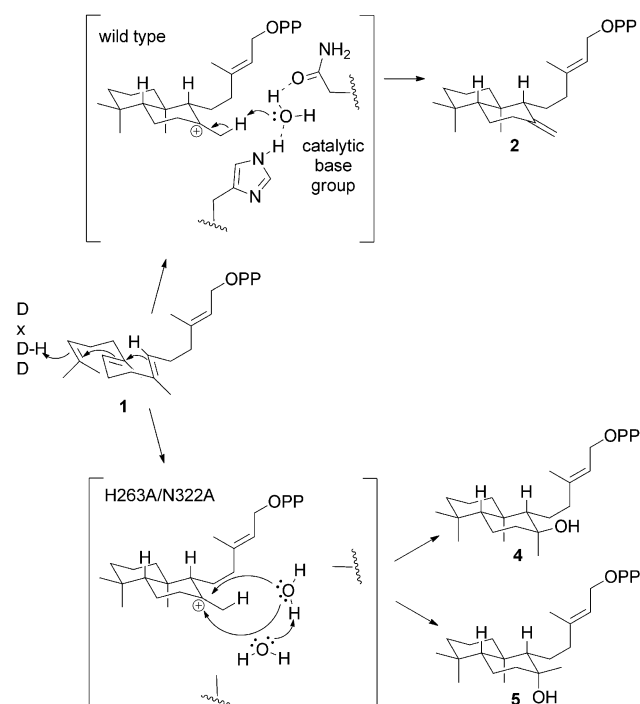

Scheme 2. The role of water in the reactions catalyzed by wild-type AtCPS or the H263A/N322A mutant.

epimers demonstrates access to both faces of this final carbocation, with the relatively greater epimeric specificity exhibited by AtCPS:N322A relative to either AtCPS:H263A or AtCPS:H263A/N322A, thus indicating that H263 hinders access to the bottom face of the labda-13*E*-en-8-yl carbocation intermediate. This access contrasts to the product outcome mediated by the AgAS:H348A mutant, which more selectively produces the 8α-epimer **3**, along with appreciable amounts of the 7,8-double-bond isomer of CPP,[3h] and thus indicates a subtle difference in relative positioning of the base in the CPSs versus gymnosperm enzymes. Intriguingly, these results suggest it may be possible to make other product variants through other active-site substitutions (e.g., the 8,9-double-bond isomer, which has not been seen before).

In any case, the ability of simple alanine substitutions, for the histidine and/or asparagine of the catalytic base group, identified here to fundamentally alter product chemical outcome demonstrates striking plasticity for the CPSs ubiq-

uitously found in all land plants. Coupled to the previously demonstrated plasticity of the subsequently acting class I diterpene synthases,[15] this highlights the ability of simple single-residue changes to generate new chemical diversity from the enzymes required for gibberellin phytohormone biosynthesis. For example, the ability of the CPS mutants, characterized here, to produce novel hydroxylated versions of CPP hold the promise of leading to the additional production of more elaborated compounds such as enantiomeric forms of *cis*-abienol, sclareol, or manoyl oxide (Scheme 3). Indeed, this plasticity presumably led to the observed extensive diversification of labdane-related diterpenoids in plants.
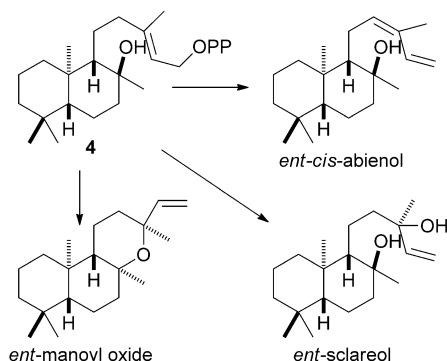
Scheme 3. Potential diterpenoids which might be produced from **4**.

Experimental Section

The recombinant AtCPS used here was expressed as a previously described pseudomature construct.[16] Mutants were generated by whole-plasmid PCR amplification with overlapping mutagenic primers of pENTR/SD/b-TOPO (Invitrogen) clones, and verified by complete gene sequencing prior to transfer by directional recombination to expression vectors (pDEST17 and pGG-DEST). The resulting constructs were heterologously expressed in the C41 Over-Express strain of *Escherichia coli* (Lucigen), much as previously described.[17] Briefly, the recombinant *E. coli* were grown in liquid NZY media to 0.6 A_{600} at 37°C, then shifted to 16°C for an hour prior to induction with 0.5 mM IPTG, followed by fermentation at 16°C. For in vitro assays, the enzymes were expressed as pDEST17 6xHis tagged constructs for ease of purification, which was accomplished much as previously described.[18] Briefly, cells from overnight fermentation were harvested by centrifugation, lysed by gentle sonication in lysis buffer (50 mM Bis-Tris, pH 6.8, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol), with the resulting lysate clarified by centrifugation (15000 $g \times$ 20 min. at 4°C). The tagged enzymes were purified over Ni-NTA His-bind resin (Novagen), in batch mode, washing with 20 mM imidazole and elution by 250 mM imidazole in column buffer (50 mM Bis-Tris, pH 6.8, 1 mM DTT). Enzymatic assays for class II activity were carried out much as previously described.[19] Enzymatic products also were investigated by expression from pGG-DEST based constructs in our previously described modular metabolic engineering system,[12] which couples production of GGPP in *E. coli* with further engineering to flux into isoprenoid metabolism,[20] as depicted in Figure 2 and Figures S1–S4. Briefly, the products resulting from 3 day fermentations of 50 mL cultures were extracted with an equal volume of hexanes, which was dried under N_2, resuspended in 1 mL fresh hexanes, and then filtered prior to analysis by gas chromatography with mass spectra detection (GC-MS), using a 3900 GC with Saturn 2100T ion trap MS (Varian) equipped with HP5ms column (Agilent), as previously described.[21]

[1] H. Y. Chu, E. Wegel, A. Osbourn, *Plant J.* **2011**, *66*, 66–79.
[2] R. J. Peters, *Nat. Prod. Rep.* **2010**, *27*, 1521–1530.
[3] a) V. Falara, E. Pichersky, A. K. Kanellis, *Plant Physiol.* **2010**, *154*, 301–310; b) A. Caniard, P. Zerbe, S. Legrand, A. Cohade, N. Valot, J. L. Magnard, J. Bohlmann, L. Legendre, *BMC Plant Biol.* **2012**, *12*, 119; c) M. Schalk, L. Pastore, M. A. Mirata, S. Khim, M. Schouwey, F. Deguerry, V. Pineda, L. Rocci, L. Daviet, *J. Am. Chem. Soc.* **2012**, *134*, 18900–18903; d) C. Sallaud, C. Giacalone, R. Topfer, S. Goepfert, N. Bakaher, S. Rosti, A. Tissier, *Plant J.* **2012**, *72*, 1–17; e) N. Günnewich, Y. Higashi, X. Feng, K. B. Choi, J. Schmidt, T. M. Kutchan, *Phytochemistry* **2012**, *91*, 93–99; f) P. Zerbe, B. Hamberger, M. M. Yuen, A. Chiang, H. K. Sandhu, L. L. Madilao, A. Nguyen, B. Hamberger, S. S. Bach, J. Bohlmann, *Plant Physiol.* **2013**, *162*, 1073–1091; g) P. Zerbe, A. Chiang, M. Yuen, B. Hamberger, J. A. Draper, R. Britton, J. Bohlmann, *J. Biol. Chem.* **2012**, *287*, 12121–12131; h) J. Criswell, K. Potter, F. Shephard, M. B. Beale, R. J. Peters, *Org. Lett.* **2012**, *14*, 5828–5831; i) F. Shephard, University of Bristol (Bristol), **2002**; j) I. Pateraki, J. Andersen-Ranberg, B. Hamberger, A. M. Heskes, H. J. Martens, P. Zerbe, S. S. Bach, B. L. Moller, J. Bohlmann, B. Hamberger, *Plant Physiol.* **2014**, *164*, 1222–1236.
[4] S. Prisic, J. Xu, R. M. Coates, R. J. Peters, *ChemBioChem* **2007**, *8*, 869–874.
[5] K. Zhou, Y. Gao, J. A. Hoy, F. M. Mann, R. B. Honzatko, R. J. Peters, *J. Biol. Chem.* **2012**, *287*, 6840–6850.
[6] F. Chen, D. Tholl, J. Bohlmann, E. Pichersky, *Plant J.* **2011**, *66*, 212–229.
[7] C. I. Keeling, H. K. Dullat, M. Yuen, S. G. Ralph, S. Jancsik, J. Bohlmann, *Plant Physiol.* **2010**, *152*, 1197–1208.
[8] a) M. Köksal, H. Hu, R. M. Coates, R. J. Peters, D. W. Christianson, *Nat. Chem. Biol.* **2011**, *7*, 431–433; b) M. Köksal, K. Potter, R. J. Peters, D. W. Christianson, *Biochim. Biophys. Acta Gen. Subj.* **2014**, *1840*, 184–190.
[9] a) S. Prisic, M. Xu, P. R. Wilderman, R. J. Peters, *Plant Physiol.* **2004**, *136*, 4228–4236; b) K. Otomo, H. Kenmoku, H. Oikawa, W. A. Konig, H. Toshima, W. Mitsuhashi, H. Yamane, T. Sassa, T. Toyomasu, *Plant J.* **2004**, *39*, 886–893.
[10] K. Hayashi, H. Kawaide, M. Notomi, Y. Sakigi, A. Matsuo, H. Nozaki, *FEBS Lett.* **2006**, *580*, 6175–6181.
[11] P. Kenrick, P. R. Crane, *Nature* **1997**, *389*, 33–39.
[12] A. Cyr, P. R. Wilderman, M. Determan, R. J. Peters, *J. Am. Chem. Soc.* **2007**, *129*, 6684–6685.
[13] a) A. Gonzalez, *Phytochemistry* **1975**, *14*, 2655–2656; b) P. Jefferies, T. Payne, *Aust. J. Chem.* **1965**, *18*, 1441; c) C. Zdero, F. Bohlmann, H. Niemeyer, *Phytochemistry* **1990**, *29*, 3247–3253.
[14] R. J. Peters, M. M. Ravn, R. M. Coates, R. B. Croteau, *J. Am. Chem. Soc.* **2001**, *123*, 8974–8978.
[15] a) C. I. Keeling, S. Weisshaar, R. P. C. Lin, J. Bohlmann, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 1085–1090; b) D. Morrone, M. Xu, D. B. Fulton, M. K. Determan, R. J. Peters, *J. Am. Chem. Soc.* **2008**, *130*, 5400–5401; c) K. Zhou, R. J. Peters, *Chem. Commun.* **2011**, *47*, 4074–4080; d) M. Xu, P. R. Wilderman, R. J. Peters, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 7397–7401; e) P. R.

Wilderman, R. J. Peters, _J. Am. Chem. Soc._ **2007**, _129_, 15736–15737.

[16] S. Prisic, R. J. Peters, _Plant Physiol._ **2007**, _144_, 445–454.

[17] R. J. Peters, J. E. Flory, R. Jetter, M. M. Ravn, H.-J. Lee, R. M. Coates, R. B. Croteau, _Biochemistry_ **2000**, _39_, 15592–15602.

[18] K. Zhou, R. J. Peters, _Phytochemistry_ **2009**, _70_, 366–369.

[19] F. M. Mann, S. Prisic, E. K. Davenport, M. K. Determan, R. M. Coates, R. J. Peters, _J. Biol. Chem._ **2010**, _285_, 20558–20563.

[20] D. Morrone, L. Lowry, M. K. Determan, D. M. Hershey, M. Xu, R. J. Peters, _Appl. Microbiol. Biotechnol._ **2010**, _85_, 1893–1906.

[21] D. Morrone, M. L. Hillwig, M. E. Mead, L. Lowry, D. B. Fulton, R. J. Peters, _Biochem. J._ **2011**, _435_, 589–595.