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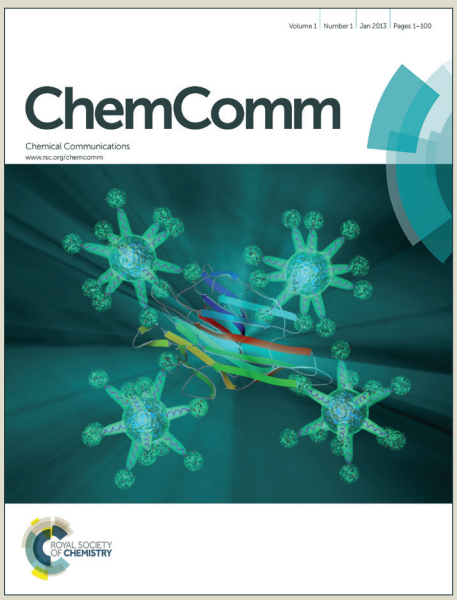

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Efficient heterocyclisation by (di)terpene synthases

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While cyclic ether forming terpene synthases are known, the basis for such heterocyclisation is unclear. Here it is reported that numerous (di)terpene synthases, particularly including the ancestral *ent*-kaurene synthase, efficiently produce isomers of manoyl oxide from the stereochemically appropriate substrate. Accordingly, such heterocyclisation is easily accomplished by terpene synthases. Indeed, the use of single residue changes to induce production of the appropriate substrate in the upstream active site leads to efficient bifunctional enzymes producing isomers of manoyl oxide, representing novel enzymatic activity.

Terpenoids form the largest class of natural products, with >45,000 known just from plants.[1] The underlying backbone structures are largely formed by terpene synthases (TPSs).[2] These enzymes catalyse formation of varied skeletal structures, almost invariably via carbon-carbon bond formation, often including cyclization, generally producing olefins.[3] However, examples of cyclic ether forming heterocyclisation are known. For example, TPSs have been identified that form the monoterpene (C10) 1,8-cineole (eucalyptol),[4] and, more recently, the diterpene (C20) manoyl oxide.[5]

TPSs are generally classified as lyases, particularly the prevalent class I enzymes that catalyse heterolytic cleavage (ionization) of allylic diphosphate ester bonds in their isoprenyl diphosphate substrates (EC 4.2.3.x).[2] However, there are examples of TPSs that act as hydrolyases, incorporating water.[3] In addition, certain TPSs catalyse heterocyclisation, forming cyclic ethers. 1,8-Cineole synthases both act as hydrolyases, incorporating water, and further utilize the resulting oxy group to form a cyclic ether. By contrast, the production of manoyl oxide proceeds from a substrate wherein oxygen is already present in the form of a hydroxyl group added during bicyclization of the general diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP, **1**) to 8α-hydroxy-copalyl diphosphate (**2**) catalysed by class II diterpene cyclases.[6] **2** is then utilized in a heterocyclisation reaction catalysed by a class I di-TPS to produce (13*R*) manoyl oxide (**3**)(Fig. 1).

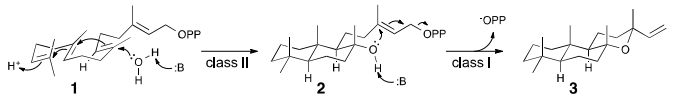

Fig. 1 Cyclization of GGPP (**1**) to 8α-hydroxy-CPP (**2**), catalysed by a class II diterpene cyclase, with subsequent heterocyclisation to manoyl oxide (**3**), catalysed by a class I di-TPS.

Previous work, based on the crystal structure of a 1,8-cineole synthase from *Salvia fruticosa*, led to partial conversion of a closely related, but more typical olefin producing sabinene synthase to incorporate water, but very little cyclic ether formation was observed.[4b] Accordingly, it is not clear what additional accommodations might be necessary for heterocyclisation. Here the formation of manoyl oxides from the relevant hydroxylated precursors, enantiomers of 8-hydroxy-copalyl diphosphate, separating the incorporation of water from heterocyclisation, was investigated, enabling elucidation of the facile ability of (di)TPSs to carry out such catalysis.

Intriguingly, previous work demonstrated that an abietadiene synthase from the gymnosperm/conifer *Abies grandis* (AgAS) will react with an analogue of **2**, 8α-hydroxy-17-nor-copalyl diphosphate, to produce an analogue of **3**, 17-nor-manoyl oxide, in a stereospecific manner, albeit with somewhat limited efficiency.[7] Nevertheless, this suggested the possibility that AgAS might be able to produce **3** from **2**. AgAS is a bifunctional di-TPS,[8] catalysing both bicyclization of **1** to copalyl diphosphate (CPP) in a class II diterpene cyclase active site, as well as subsequently catalysing further cyclisation of this to an abietane in a separate class I active site.[9] A mutation that blocks the ability of AgAS to react with **1**, D404A, has been previously reported.[9] Thus, the AgAS:D404A mutant can be used to test the ability of the AgAS class I active site to react with the products of class II diterpene cyclases co-

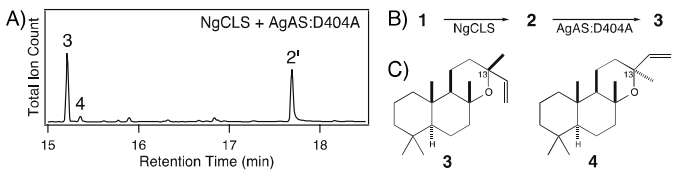

Fig. 2 The abietadiene synthase AgAS can catalyse heterocyclisation of **2** (produced by NgCLS) to **3**, along with small amounts of **4**. A) Chromatogram from GC-MS analysis of extract from AgAS:D404A co-expressed with NgCLS in *E. coli* also engineered to produce **1** (peak numbering corresponds to compound numbering described in text, with **2′** corresponding to the dephosphorylated derivative of **2**, copal-8α,15-diol). B) Reaction scheme. C) Structures of manoyl oxide (**3**) and 13-*epi*-manoyl oxide (**4**).

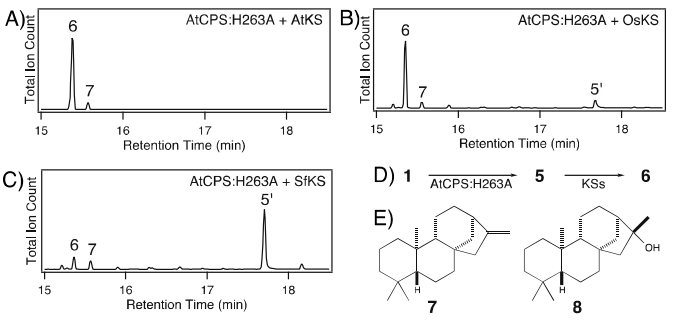

Fig. 3 Kaurene synthases (KSs) readily cyclise 8β-hydroxy-*ent*-CPP (**5**; produced by AtCPS:H263A) to *ent*-13-*epi*-manoyl oxide (**6**). Chromatograms from GC-MS analysis of extracts from A) AtKS, B) OsKS, or C) SfKS, each co-expressed with AtCPS:H263A in *E. coli* also engineered to produce **1** (peak numbering corresponds to compound numbering described in text, with **5′** corresponding to the dephosphorylated derivative of **5**, *ent*-copal-8α,15-diol). D) Reaction scheme. E) Structures of *ent*-kaurene (**7**) and 16β-hydroxy-*ent*-kaurene (**8**).

expressed in *Escherichia coli* also engineered to produce **1**,[10] via a previously described modular metabolic engineering system.[11]

Accordingly, to test the hypothesis that AgAS can cyclize **2**, AgAS:D404A was co-expressed with a class II diterpene cyclase from *Nicotiana glutinosa* that produces **2** (NgCLS).[12] This demonstrated that AgAS does react with **2** to produce the heterocycle **3**, along with smaller amounts of its C13 epimer, (13*S*) 13-*epi*-manoyl oxide (**4**)(Fig. 2). Notably, the ability of AgAS to convert **2** to **3** hinted at the possibility that other (di)TPSs might be able to catalyse such heterocyclisation when incubated with an appropriate substrate.

The recently reported (13*R*) manoyl oxide synthase from *Coleus forskohlii* is closely related to previously identified di-TPSs that produce the abietadiene isomer, miltiradiene, from both this and other plants of the Lamiaceae family.[5, 13] Interestingly, it has been previously reported that, when incubated with **2**, these miltiradiene synthases all produce predominantly **3**, along with smaller amounts **4**,[5, 13b] consistent with a somewhat broader ability of, at least these closely related, di-TPSs to catalyse such heterocyclisation. While these di-TPSs share some mechanistic similarity with AgAS in their production of abietanes, they are members of the TPS-e sub-family, while AgAS falls within the phylogenetically separate TPS-d3 sub-family.[14] Notably, the TPS-e sub-family also contains the *ent*-kaurene synthases (KSs) found in all seed plants, where they are required for gibberellin plant hormone biosynthesis, and which represent the ancestral plant class I TPS.[3, 6b]

The usual substrate for KSs is *ent*-CPP, which is enantiomeric to the CPP substrate of the abietadiene synthases described above and does not serve as a substrate for KSs. Perhaps not surprisingly then, KSs do not react with **2** (data not shown). Fortuitously, it has recently been shown that alanine substitution for either (or both) of the residues constituting the catalytic base dyad in the *ent*-CPP synthases (CPSs) also found in all seeds plants for gibberellin biosynthesis, leads to efficient production of 8β-hydroxy-*ent*-CPP (**5**),[15] the enantiomer of **2**, which was hypothesized might then be cyclised by KSs.

The ability of these ancestral plant class I TPSs to catalyse heterocyclisation was investigated with two different KSs, that from the dicot *Arabidopsis thaliana* (AtKS)[16] and the monocot *Oryza sativa* (OsKS),[17] via co-expression with the H263A mutant of the CPS from *A. thaliana* (AtCPS) that produces **5**.[15] Both AtKS and OsKS readily react with **5** to efficiently produce *ent*-13-*epi*-manoyl oxide (**6**), along with small amounts of *ent*-kaurene (**7**), arising from the residual production of *ent*-CPP by AtCPS:H263A[15] (Fig. 3). Even beyond plants, it has been shown that certain bacteria also produce gibberellin and, therefore, contain a functional KS.[10a, 10c, 18] The ability of such more divergent KSs to catalyse heterocyclisation was investigated by co-expression of the KS from *Sinorhizobium fredii* (SfKS) with AtCPS:H263A as well. Notably, while not as active as the plant KSs, SfKS also catalyses the production of **6** from **5** (Fig. 3C).

Intriguingly, bifunctional CPS/KS enzymes have been reported from both early diverging land plants,[19] representative of the ancestral TPS,[3, 6b] as well as in fungi that also produce gibberellins.[20] The catalytic histidine (i.e., H263 in AtCPS) is conserved in their class II (CPS) active sites. Accordingly, it was hypothesized that mutation of these to alanine might similarly convert these to the production of **5**, with the class I (KS) active site then serving to transform this to **6**.

This hypothesis was investigated with CPS/KS both from the bryophyte/moss, *Physcomitrella patens* (PpCPS/KS),[19a] and the fungus *Fusarium fujikuroi* (FfCPS/KS).[20b] Strikingly, the relevant H302A mutant of PpCPS/KS efficiently produces **6** from **1**, with minimal amounts of the usual major product, 16β-hydroxy-*ent*-kaurene (**8**), which presumably arises from residual production of *ent*-CPP by the altered class II (CPS) active site (Fig. 4A). Moreover, while less active overall, similar results are observed with the relevant H212A mutant of the much more divergent FfCPS/KS as well (Fig. 4B). Thus, these single residue changes are sufficient to create bifunctional *ent*-13-*epi*-manoyl oxide synthases (Fig. 4C).

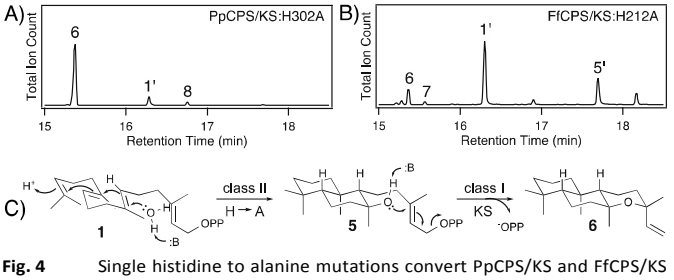

Fig. 4 Single histidine to alanine mutations convert PpCPS/KS and FfCPS/KS into bifunctional *ent*-13-*epi*-manoyl oxide synthases. Chromatograms from GC-MS analysis of extracts from A) PpCPS/KS:H302A or B) FfCPS/KS:H212A, each expressed in *E. coli* also engineered to produce **1** (peak numbering corresponds to compound numbering described in text and previous figure legends, with **1′** corresponding to the dephosphorylated derivative of **1**, (*E,E,E*)-geranylgeraniol). C) Cyclization of GGPP (**1**) to **5** catalysed by H→A mutants in class II (CPS) active sites, with subsequent heterocyclisation to **6** catalysed by the KS active sites.

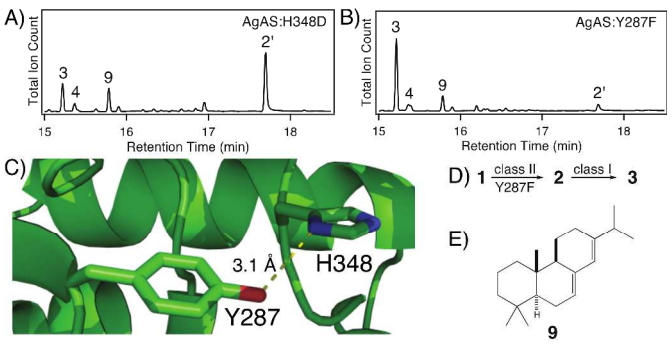

Fig. 5 A single mutation converts AgAS to a bifunctional manoyl oxide synthase. Chromatograms from GC-MS analysis of extracts from A) AgAS:H348D or B) AgAS:Y287F, each expressed in *E. coli* also engineered to produce **1** (peak numbering corresponds to compound numbering described in text and previous figure legends). C) Catalytic dyad in the class II active site of AgAS with main chain shown in cartoon format, with side chains of Y287 and H348, and dotted line indicating hydrogen bond between hydroxyl of Y287 and N(δ1) of H348, all in stick format. D) Reaction scheme. E) Structure of abietadiene (**9**).

A similar single residue change that converts the class II activity of AgAS to the efficient production of **2** has been reported (H348D).[12] In the initial study, this change was combined with a D621A mutation that blocks class I activity.[9] The results reported above suggest that the AgAS:H348D single mutant might act as a bifunctional manoyl oxide synthase.

Somewhat surprisingly, AgAS:H348D produces only small amounts of **3**, leaving large amounts of **2** unreacted, and with some residual production of abietadiene (**9**), presumably reflecting continued production of small amounts of CPP by the class II active site, along with small amounts of **4** as well (Fig. 5A). While the basis for this effect of the H348D mutation in the class II active site on the separate class I active site in AgAS is unclear, closer examination of the crystal structure available for AgAS[21] revealed that H348 is hydrogen-bonded to Y287, presumably representing a catalytic dyad (Fig. 5C). Accordingly, a Y287F mutant was constructed, and the resulting AgAS:Y287F found to more efficiently produce **3** from **1**, (Fig. 5C).

Altogether, the results reported here demonstrate that heterocyclisation is easily accommodated by the TPS reaction mechanism, as demonstrated by the range of KSs, including representatives of the ancestral plant TPSs, as well as microbial TPSs, which are able to carry out such catalysis. This reactivity provides further promiscuity that may underlie the observed diversification of terpenoid natural products. In the case of diterpenoids, this is particularly evident when coupled to the ease with which upstream class II diterpene cyclases can be converted to the production of 8-hydroxylated CPPs, which requires only single amino acid changes.[12, 15] Such plasticity is exemplified by the bifunctional manoyl oxide and *ent*-13-*epi*-manoyl oxide synthases reported here, which further represent novel enzymatic activity.

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Notes and references

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