Biosynthesis

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Structures of Iridoid Synthase from *Cantharanthus roseus* with Bound NAD^+, NADPH, or NAD^+/10-Oxogeranial: Reaction Mechanisms

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Abstract: *Structures of the iridoid synthase nepetalactol synthase in the presence of NAD^+, NADPH or NAD^+/10-oxogeranial were solved. The 10-oxogeranial substrate binds in a transoid-O1-C3 conformation and can be reduced by hydride addition to form the byproduct S-10-oxo-citronellal. Tyr178 Oζ is positioned 2.5 Å from the substrate O1 and provides the second proton required for reaction. Nepetalactol product formation requires rotation about C1–C2 to form the cisoid isomer, leading to formation of the cis-enolate, together with rotation about C4–C5, which enables cyclization and lactol production. The structure is similar to that of progesterone-5β-reductase, with almost identical positioning of NADP, Lys146-(147), Tyr178(179), and F342(343), but only Tyr178 and Phe342 appear to be essential for activity. The transoid 10-oxogeranial structure also serves as a model for β-face hydride attack in progesterone 5β-reductases and is of general interest in the context of asymmetric synthesis.*

Terpenes and related isoprenoids are the largest class of small-molecule natural products on earth, and the most abundant by mass.[1] Most are made by enzymes that are represented by a relatively small number of folds,[2] primarily head-to-tail and head-to-head *trans*-prenyltransferases, *cis*-prenyltransferases, and a range of cyclases with one, two, or three domains. In most cases, the reactions proceed via the formation of carbocationic transition states/reactive intermediates. However, in recent work, an alternative route to the formation of terpenes—one involving reductive cyclization—was reported for iridoid biosynthesis.[3] Iridoids are monoterpenes that are produced from (C_{10}) 10-oxogeranial (**1**) by NAD(P)H-dependent reduction, followed by a cyclization step that involves either a Diels–Alder cycloaddition or a Michael addition.[3a,4] There are no X-ray structures available for any iridoid synthases (IRISs), with or without substrates or other ligands. The structures of the iridoid synthases are of interest because their products are converted into important natural products, compounds such as the anticancer drug vincristine (**2**), as well as being of mechanistic interest, not least because their amino acid sequences have high homology to the plant progesterone (**3**) 5β-reductases involved in the formation of cardiac glycoside drugs such as digitoxin (**4**). A simplified version of the biosynthesis of vincristine (**2**) from isopentenyl diphosphate (**5**) and dimethylallyl diphosphate (**6**) is shown in Scheme 1 A; the structures of other molecules discussed herein are shown in Scheme 1 B.

We expressed and purified an iridoid synthase, *Cantharanthus roseus* (1*R*, 4a*S*, 7*S*, 7a*R*) nepetalactol (**7**) synthase, from the corresponding chemically synthesized gene (Genebank accession number: K7WDL7.1). *C. roseus* is the Madagascar periwinkle and produces the anticancer indole terpenes vincristine (**2**) and vinblastine (**8**) from iridoids. Attempts to crystallize full-length protein were unsuccessful, so we attempted crystallization of two N-terminus-truncated variants, ΔN13 and ΔN25. ΔN25 crystals were obtained (details of protein expression, purification, and crystallization are given in the Supporting Information). The activity of the ΔN25 construct was (within experimental error) the same as that of the wild-type protein (Figure S1 in the Supporting Information). We then co-crystallized this IRIS with either NAD^+, NADPH, or $NAD^+ + 10$-oxogeranial and solved the structures. Full data acquisition and refinement details are given in Table S1 in the Supporting Information.

We first obtained the structures of IRIS with either NADPH or NAD^+ bound. A stereo view of the NADPH structure is shown in Figure 1 A; the NAD^+ structure is virtually identical (a 0.21 Å root mean square deviation, rmsd).The protein fold is most similar to that of progesterone 5β-reductase from *Digitalis lanata*, a plant that produces cardiac glycosides such as **4**. There is a 1.0 Å Cα rmsd over 351 residues (using the PDBe Fold Server[5]). An alignment of the active sites of the IRIS protein (+ NADPH, PDB ID: 5DBF) and the *D. lanata* 5β-reductase (+ $NADP^+$, PDB ID code 2V6G[6]) is shown in Figure 1 B. The two active-site structures are very similar and interestingly, the Tyr179 shown by site-directed mutagenesis to be essential for progesterone 5β-reductase activity[6] occupies the same position in both the IRIS (Tyr178) and progesterone 5β-reductase structures, as do the NADP cofactors and the Lys146 (Lys147 in progesterone 5β-reductase) residues, which have been proposed to be involved in progesterone 5β-reductase catalysis. There are, therefore, great similarities between the IRIS and progesterone 5β-reductase structures, with the Tyr Oζ being in close

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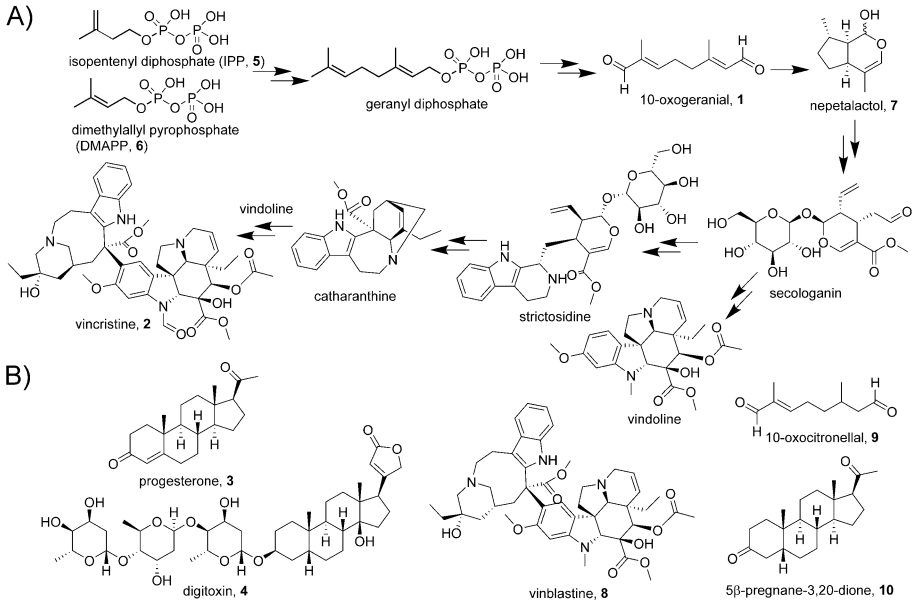

Scheme 1. A) Vincristine biosynthesis from isopentenyl diphosphate and dimethylallyl diphosphate. B) Structures of other compounds of interest.

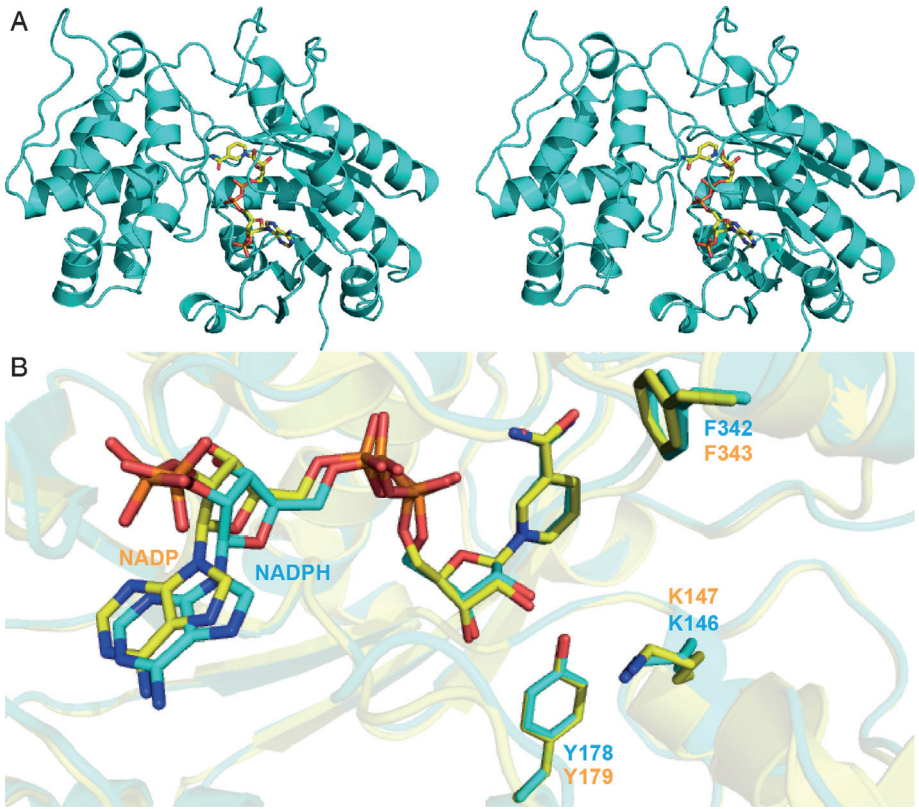

Figure 1. Structure of *C. roseus* IRIS + NADPH and a comparison with *D. lanata* progesterone 5β-reductase. A) stereo view of IRIS in complex with NADPH (PDB ID: 5DBF). B) Superimposition of IRIS + NADPH structure (cyan) and progesterone 5β-reductase (PDB ID: 2V6G[6]) in complex with $NADP^+$ (yellow).

proximity to the ribose O2 (ca. 2.8 Å) in both systems. Additional protein–ligand contacts are shown in Figures S2–S5. These structures do not, however, give any new clues to the mechanisms of catalysis.

We thus soaked 10-oxogeranial into crystals of IRIS co-crystallized with NAD^+ (to prevent 10-oxogeranial substrate reduction) and obtained an IRIS + NAD^+ + 10-oxogeranial complex structure (PDB ID: 5DBI). Attempts to obtain structures with nepetalactol or progesterone (+/−NADPH) were not successful. The bound NAD^+ and 10-oxogeranial electron-density map is shown in Figure 2A, and the active-site region, together with distances of interest, is shown in Figure 2B. This structure is essential for a detailed analysis of the IRIS (and progesterone reductase) mechanism of action. As can be seen in Figure 2 (and the ligand interaction plots in Figures S4, S5), the NAD^+ (NADH, when reduced) nicotinamide, 10-oxogeranial, and Tyr178 OζH groups are perfectly aligned for a 1,4-hydride addition. The pro-4(*S*) proton from NADH attacks the C2=C3 double bond from "above" (in Figure 2B) to generate the *trans*-enol, with the Tyr178 OζH providing the enolate hydrogen atom. This reaction is shown schematically in Figure 3A. The Tyr178 Oζ is only 2.5 Å from the 10-oxogeranial O1 in the crystal structure, suggesting that there could be a hydrogen bond present, even prior to reduction. Upon hydride attack, the tyrosinate formed can be stabilized through interaction with the ribose O2(H) (2.8 Å away in the X-ray structure, at least prior to reduction). The enol that forms upon hydride attack has a 3(*S*) center, as found in nepetalactol. However, the product of this reaction is actually *S*-10-oxocitronellal (**9**), a known[4] shunt-reaction product observed experimentally, not nepetalactol, as shown in the Figure 3A. In order for nepetalactol to be formed, there needs to be a 180° rotation about C1–C2 to form the *cisoid* isomer of **1**, which on reduction forms the *cis*-enol (Figure 3B). Rotation about C4–C5 is also required (Figure 2B and the video S1 in the Supporting

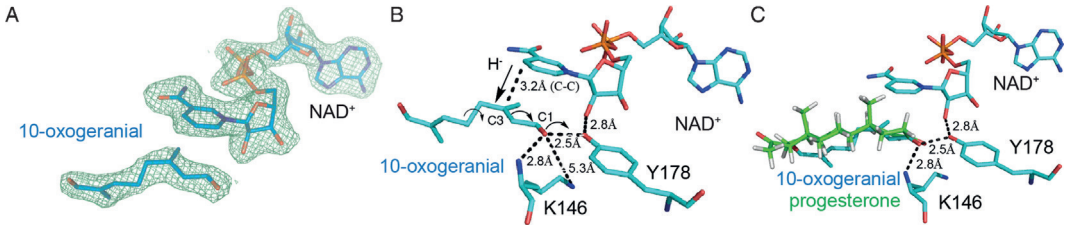

Figure 2. The active-site region of the *C. roseus* iridoid synthase from the IRIS + NAD^+ + 10-oxogeranial structure (PDB ID: 5DBI). A) Electron-density map of bound NAD^+ and substrate 10-oxogeranial ($2F_o-F_c$ map contoured at 1 sigma). B) Interaction of active-site residues K146 and Y178 with NAD^+ and 10-oxogeranial substrates. The C4–C5 rotation to enable cyclization is indicated. Dashed lines indicate distances of interest. C) Progesterone modeled into the IRIS active site.

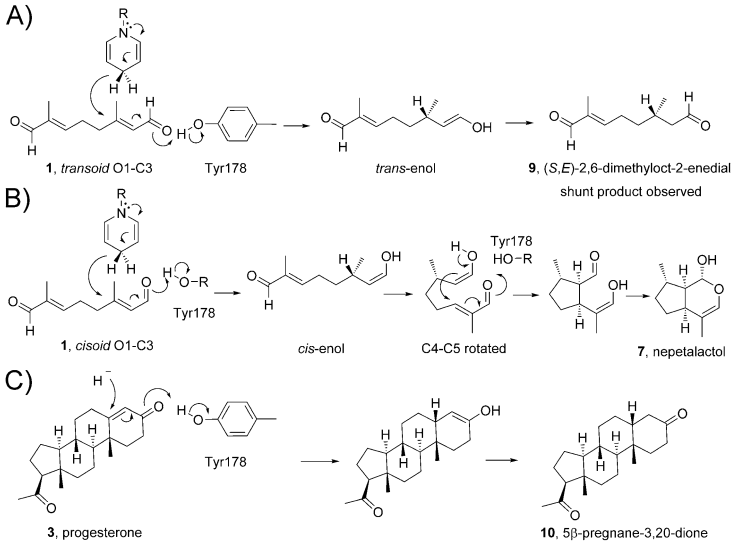

Figure 3. Proposed catalytic mechanism of iridoid synthase and progesterone reductase based on the IRIS + NAD^+ + 10-oxogeranial structure (PDB ID: 5DBI). A) *transoid* **1** gets reduced to *S*-10-oxocitronellal (**9**). B) Rotation about C1–C2 enables *cis*-enol formation, and with C4–C5 rotation, nepetalactol formation. C) Progesterone reacts as in (A).

Information), and then cyclization and lactol formation can proceed.

These results are also of interest in the context of the catalytic mechanism of the homologous progesterone 5β-reductase in plants such as *D. lanata*. The reduction of progesterone to 5β-pregnane-3,20-dione (**10**) by progesterone 5β-reductase is expected to be similar to the proposed reduction of the *transoid* form of **1** (Figure 3A), with hydride attack on the β-face at C5 (Figure 3C). When compared with the previous structures and mechanistic studies of progesterone 5β-reductase,[6] it can be seen that in IRIS, the 10-oxogeranial is immediately adjacent to Tyr178, which provides one of the protons required for catalysis, rather than occupying a more distal location, as proposed for progesterone 5β-reductase based on computational modeling. Tyr178, however, is not the only residue that has been proposed to be involved in progesterone 5β-reductase. Specifically, Lys147 was suggested to play a role[6] as a proton source. The corresponding residue in IRIS is Lys146, which together with Tyr178, is very well aligned (a 0.171 Å rmsd) between *D. lanata* progesterone 5β-reductase and the *C. roseus* IRIS. However, the lysine ε-NH_3^+ group is approximately 5.3 Å from the Tyr178 Oζ (Figure 2B), so it seems unlikely that this lysine plays an essential role in reprotonating Tyr178 after 10-oxogeranial reduction. The backbone N in Lys146 is, however, only 2.85 Å from the 10-oxogeranial O1, suggesting a hydrogen-bonding interaction, but this would probably be seen with most amino acids in this position.

Based on the structural data, it appears that the *transoid* form simply binds more strongly, and that is why it is seen crystallographically. However, it is reduced relatively slowly (in solution), which is why *S*-10-oxocitronellal (**9**) is a minor product. The *cisoid* form binds more weakly (and we do not see it), but it reacts more rapidly (in solution), which is why, under reducing conditions, nepetalactol is the major product. This is, of course, a post hoc analysis, since the X-ray structure is of the *transoid* form H-bonded to the (essential) Y178 Cζ-OH.

The similarities between the *transoid* enone fragment in 10-oxogeranial and the same *transoid* moiety in progesterone suggested that it should be possible to obtain a good model for progesterone binding simply by aligning the respective enone oxygen atoms and C3 (10-oxogeranial) and C5 (progesterone) carbon atoms. This appears to be the case, since as shown in Figure 2C, there is a very good overlap of the entire progesterone molecule with 10-oxogeranial, an observation that is expected to facilitate future work on progesterone (and other enone) reductase reactions of interest in asymmetric synthesis.

Next, we sought to determine whether there were any other residues that might have catalytic activity in both the IRIS and progesterone 5β-reductase reactions by using a SCORECONS analysis[7] of a JPRED3 alignment[8] of *C. roseus* iridoid synthase. SCORECONS is a computer program that predicts the "essential" nature of a residue for catalysis, based on its occurrence (and physicochemical similarities between different residues) in a set of aligned sequences. A score of 1000 means really essential, 0, not essential. A list of the top 20 most conserved residues is shown in Table S2. The most conserved residues are Gly residues involved in Rossman fold formation. Many of the other conserved residues are hydrophobic, and all conserved residues cluster in the N-terminal domain. Of the polar residues that might be involved in catalysis, Arg200 and Glu182 actually form a salt bridge

(2.9 Å Oε2/NH2) located approximately 10 Å from the substrate C1. Asp84 forms a salt bridge with the adenine N6. Tyr178 thus appears to be essential for catalysis in both proteins, since it is highly conserved and is positioned immediately adjacent to the 10-oxogeranial substrate.

To help test the proposed roles of this and other residues in or near the (putative) catalytic center, we produced seven IRIS single-point mutants: K146A, F149M, Y178F, Y178A, F342A, A346I, and S349F. Enzyme activity results are shown in Figure 4A and a "heat map" of the results (red = essential;

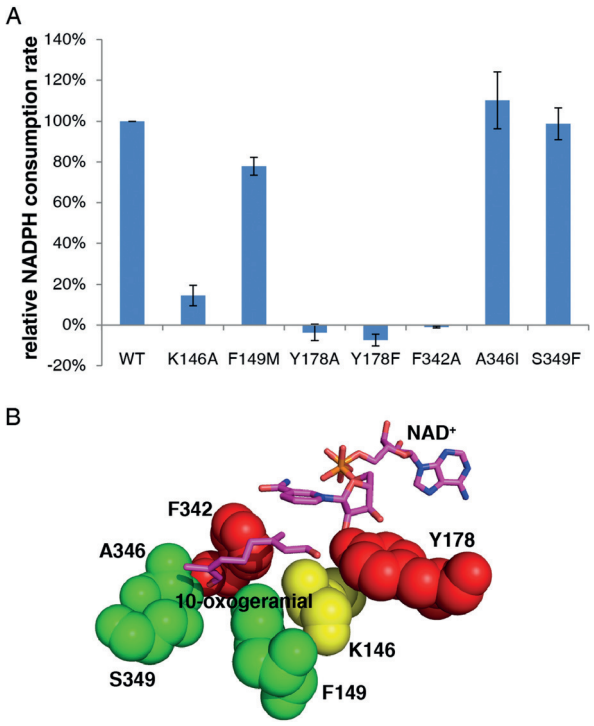

Figure 4. Mutagenesis results for *C. roseus* iridoid synthases. A) Activities of wild-type and mutant IRIS proteins for 10-oxogeranial reduction. B) "Heat map" showing the effects of mutations on catalytic activity: green = no effect; yellow = significant effect; red = major effect (no activity, within experimental error).

green = non-essential, yellow = desirable) is shown in Figure 4B. These results confirm the essential nature of Tyr178 for activity. Phe342 is also essential, and this result is of interest since it supports the role of the equivalent Phe residue in progesterone reductase in small-enol reductase activity,[3b] while Phe149 is not essential, which is consistent with its non-conserved nature. Lys146 has reduced activity, but no obvious role for this residue is suggested from the X-ray structure. It might be involved in the second half reaction, but it is clearly not absolutely essential (as also noted in progesterone reductase by Petersen et al).[3e] Ala346 and Ser349, which are close to the substrate, are also not essential for 10-oxogeranial reduction, which is again consistent their non-conserved nature. Based on Phe mutagenesis and MD simulations, Petersen et al. propose that several Phe residues (that are close to the substrate in our structure) play a dual role. In IRIS or IRIS-like reduction reactions involving small 1,4-enones, they help keep ligands inside the protein, while with large enones (e.g., progesterone) they again act as "gatekeepers", this time impeding access to the active site. Phe→Ala mutations of several PRISEs[3e] favor progesterone reduction but disfavor small enone reduction. This is consistent with what we see with IRIS, in which the F342A mutant is inactive for 10-oxogeranial reduction.

In summary, the iridoid synthase that produces nepetalactol in *C. roseus* has a structure that is very similar to that found for progesterone 5β-reductase from *D. lanata*. This iridoid synthase can produce two different products: reduction of the *transoid* substrate seen crystallographically results in formation of the side-product *S*-10-oxocitronellal, whereas reduction of the *cisoid* form, together with a C4–C5 rotation, enables cyclization and formation of the major product, nepetalactol. The Tyr178 CζOH group provides the proton required for enol formation, the Lys146 H^N can form H-bonding interactions with the substrate O1, and the tyrosinate produced in the first half reaction can be stabilized by H-bonding to the ribose O2H. There are no other acidic groups close by that appear to be involved in catalysis, although Phe342 is essential for 10-oxogeranial reduction, which is consistent with the progesterone-5β-reductase mutagenesis results with small enone substrates.[3b] Overall, these results are of broad general interest since they provide new insight into the structures and catalytic mechanisms of iridoid synthases/progesterone 5β-reductases, enzymes that are involved in the biosynthesis of important cancer and heart drugs [indole terpene alkaloids such as vincristine (**2**) and cardiac glycosides such as digitoxin (**4**)], in addition to providing structural models for the future development of asymmetric catalysts.

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