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Communication

Recycling Upstream Redox Enzymes Expands the Regioselectivity of Cycloaddition in Pseudo-Aspidosperma Alkaloid Biosynthesis

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ABSTRACT: Nature uses cycloaddition reactions to generate complex natural product scaffolds. Dehydrosecodine is a highly reactive biosynthetic intermediate that undergoes cycloaddition to generate several alkaloid scaffolds that are the precursors to pharmacologically important compounds such as vinblastine and ibogaine. Here we report how dehydrosecodine can be subjected to redox chemistry, which in turn allows cycloaddition reactions with alternative regioselectivity. By incubating dehydrosecodine with reductase and oxidase biosynthetic enzymes that act upstream in the pathway, we can access the rare pseudoaspidosperma alkaloids pseudo-tabersonine and pseudo-vincadifformine, both *in vitro* and by reconstitution in the plant *Nicotiana benthamiana* from an upstream intermediate. We propose a stepwise mechanism to explain the formation of the pseudo-tabersonine scaffold by structurally characterizing enzyme intermediates and by monitoring the incorporation of deuterium labels. This discovery highlights how plants use redox enzymes to enantioselectively generate new scaffolds from common precursors.

Alkaloid-producing plants in the Apocynaceae family have evolved cyclases that catalyze the cycloaddition of a highly reactive substrate, dehydrosecodine (**1**), into distinct alkaloid scaffolds.[1,2] We and others recently discovered and characterized the only known enzymes that catalyze cycloaddition of **1**: tabersonine synthase (*Ti*TabS and *Cr*TS), which catalyzes the formation of (−)-tabersonine (**2**) (precursor to anticancer drugs vinblastine and vincristine), catharanthine synthase (*Cr*CS), which catalyzes formation of (+)-catharanthine (**3**) (precursor to vinblastine and vincristine), and coronaridine synthase (*Ti*CorS), which catalyzes formation of (−)-coronaridine (**4**) (precursor to antiaddiction agent ibogaine) (Figure 1).[2−5] *Ti*TabS/*Cr*TS and *Cr*CS directly yield **2** and **3** from **1** by a formal [4+2] cycloaddition,[2,6] while *Ti*CorS initially forms a hitherto uncharacterized unstable intermediate, which is then enzymatically reduced to **4**. The **1** substrate could, in principle, undergo alternative cycloaddition reactions to yield additional scaffolds, but extensive mutagenesis of these cyclases did not result in an expansion of the enzymatic product profile.[2] Here we show that the cyclase *Ti*CorS can, in addition to generating **4**, also produce the alternative pseudo-aspidosperma (Ψ-aspidosperma)-type alkaloid pseudo-tabersonine (Ψ-tabersonine) (**5**) (Figure 1). We show the mechanistic basis behind this transformation by first characterizing the unstable intermediate produced by the cyclase *Ti*CorS. This intermediate can be intercepted by a reductase to generate **4** or by both a reductase and an oxidase to generate the alternative scaffold **5**. Deuterium labeling provides evidence for the mechanism of these enzymatic transformations. In short, the chemical reactivity of **1** is exploited by both a cyclase and a pair of redox enzymes that can isomerize the alkene moieties, which in turn facilitates new cyclization regioselectivity.

Tabernanthe iboga, a plant that produces **2** and **4** via enzymes *Ti*TabS and *Ti*CorS, respectively,[3] also produces Ψ-aspidosperma-type alkaloid 20-*epi*-ibophyllidine (Figure 1).[7−9] We hypothesized that **5** would be the precursor to 20-*epi*-ibophyllidine.[10] The natural product **5**, rarely observed in nature, has only been isolated from *Tabernaemontana calcarea*, a species closely related to *T. iboga*.[11,12] To identify *T. iboga* enzymes that could form (+)-Ψ-tabersonine **5**, we performed coupled *in vitro* biochemical assays in which the unstable **1** substrate is enzymatically generated from the upstream intermediate stemmadenine acetate (**6**). **6** is first oxidized by the flavin-dependent enzyme precondylocarpine acetate synthase (PAS)[5] to generate precondylocarpine acetate (**7**) and then undergoes a 1,4-iminium reduction by the medium chain alcohol dehydrogenase dihydroprecondylocarpine acetate synthase (DPAS) (Figure 1).[13] The resulting reduced unstable product, dihydroprecondylocarpine acetate (**8**), undergoes elimination of an acetoxy group to yield **1**, which is then captured by one of the cyclases (Figure 1).

Our initial hypothesis was that a dedicated cyclase in *T. iboga* would catalyze isomerization and cyclization of **1** to **5**. The *T. iboga* transcriptome, which contains the two previously identified cyclases *Ti*TabS and *Ti*CorS (81% sequence identity, Figure S1), does not harbor any additional cyclase homologues that might have alternative cyclization specificity. However, to our surprise, we observed that under certain conditions, **5**,

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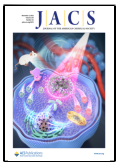

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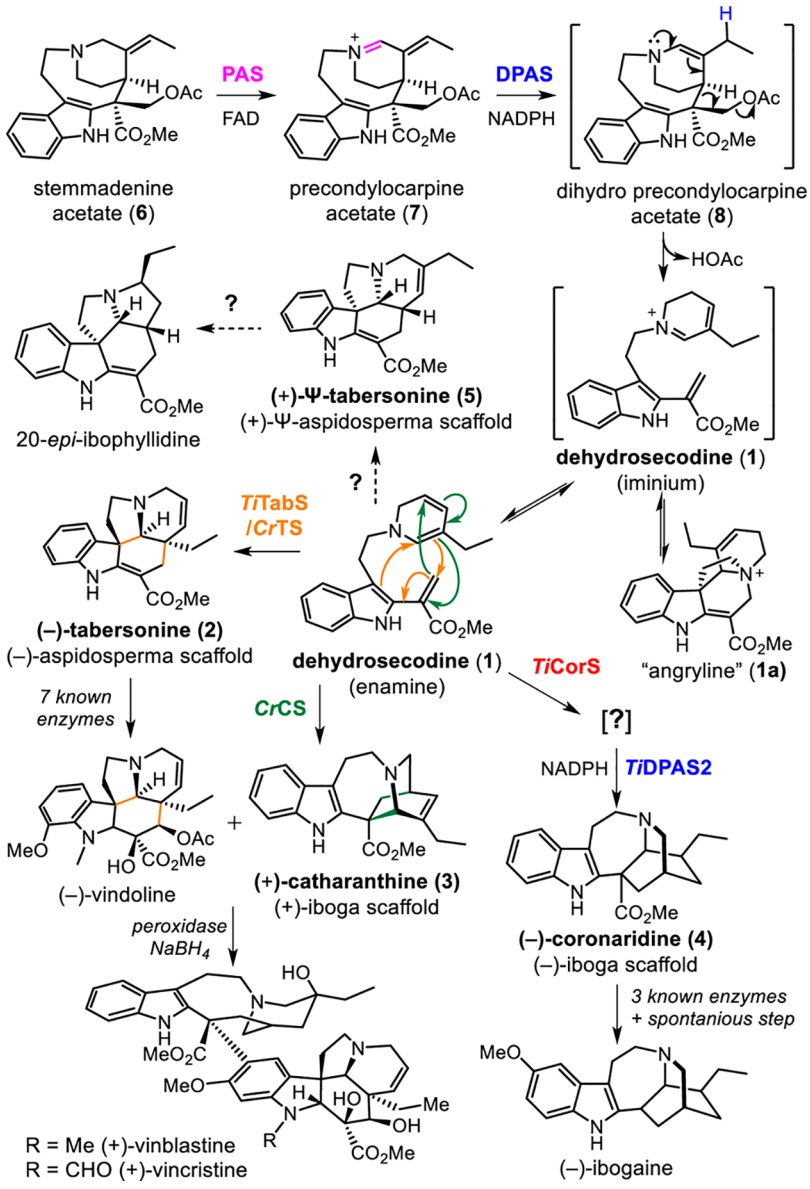

Figure 1. Dehydrosecodine (**1**)-derived alkaloids produced by the Apocynaceae family of plants.

rather than **4**, was formed in assays using *Ti*CorS (Figure 2a). Therefore, *Ti*CorS appears to be involved in production of both **4** and **5**.

In vitro assays using heterologously produced proteins (Figure S2) were used to probe the conditions that led to a switch in product selectivity. We first noted that the use of specific homologues of reductase DPAS and oxidase PAS with *Ti*CorS led to changes in the product profile. *T. iboga* has two homologues of the reductase DPAS (*Ti*DPAS1, *Ti*DPAS2, Figure S3) and three homologues of PAS (*Ti*PAS1, *Ti*PAS2, *Ti*PAS3, Figure S4).[3] Additionally, a PAS homologue (*Cr*PAS)[5] from the taxonomically related plant, *Catharanthus roseus*, which produces vinblastine, is also available and was tested. Most importantly, the pH of the reaction was critical, with **5** formation being observed at pH 7.5 and **4** production observed at pH 9.5 (Figures S5 and S6). Overall, **5** formation was favored at pH 7.5, with *Ti*PAS1−3 or *Cr*PAS, *Ti*DPAS1. and *Ti*CorS, **2** production was favored at pH 7.5, with *Ti*PAS1−3 or *Cr*PAS, *Ti*DPAS1, and *Ti*TabS, and **4** formation was favored at pH 9.5 with *Ti*PAS1−3, *Ti*DPAS2, and *Ti*CorS (Figure 2a and Figures S5 and S6). Additionally, we also could produce the over-reduced version of **5**, pseudo-vincadifformine (Ψ-vincadifformine) (**9**), by using *Ti*PAS1−3 (instead of CrPAS), *Ti*DPAS1, and *Ti*CorS (Figure 2a and Figure S7).

To further substantiate the results obtained *in vitro*, we reconstituted the biosynthetic enzymes reported here, leading to the production of **2**, **5**, and **9** in *Nicotiana benthamiana*. Enzymes were transiently expressed in *N. benthamiana* leaves, disks were excised from the transformed leaf tissue, and these disks were placed in buffer containing **6** (Figure 2b). We observed the production of **2**, **5**, and **9** in the extracts of the leaf disks using this expression system (Figure 2b). **4** was not detected in any of the enzyme combinations tested in *N. benthamiana*, presumably because of the higher pH conditions required for formation of this product. Additionally, the selectivity observed for the PAS homologues was not observed *in planta*, because *N. benthamiana* harbors an endogenous enzyme that is able to oxidize substrate **6** (Figure 2b).[5]

An acid-stable isomer of dehydrosecodine, angryline (**1a**), can also be isolated and directly used in cyclization assays

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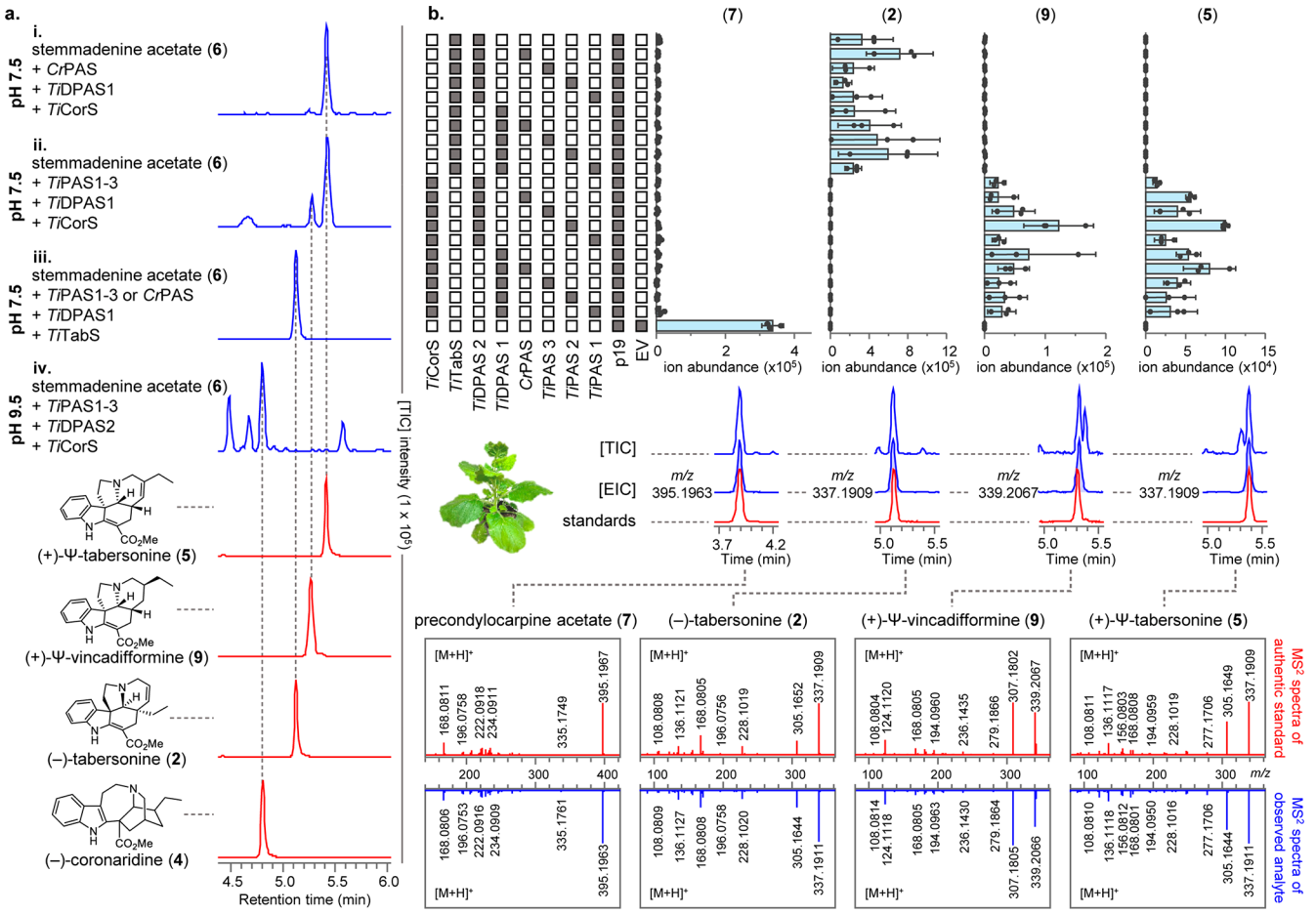

Figure 2. Biosynthesis of Ψ-tabersonine (**5**). (a) *In vitro* production of (i) (+)-Ψ-tabersonine (**5**), (ii) (+)-Ψ-vincadifformine (**9**), (iii) (−)-tabersonine (**2**), and (iv) (−)-coronaridine (**4**). (b) Reconstitution of Ψ-tabersonine (**5**) biosynthesis in *N. benthamiana* from stemmadenine acetate (**6**). Pathway enzyme combinations (filled gray boxes) transiently expressed in *N. benthamiana* and harvested leaf disks fed with stemmadenine acetate (**6**) and the resulting levels of precondylocarpine acetate (**7**), (−)-tabersonine (**2**), Ψ-vincadifformine (**9**), and Ψ-tabersonine (**5**) (bars represent ± S.E.). Representative TIC and EIC for the products observed in *N. benthamiana* reconstitution along with authentic standards and MS^2 fragmentation spectra are shown.

(Figure 1).[2] **1a** must be used at a pH value above 8.5, where it will open to generate the reactive **1**. When **1a** was used in enzymatic assays in place of **6** (pH 9.5), we could observe formation of **5**, **2**, **4**, and **9** (Figure S8). Notably, both PAS (*Ti*PAS1−3, *Cr*PAS) and *Ti*DPAS1 were required for the formation of **5** and **9**, indicating that these enzymes are required for the formation of the Ψ-aspidosperma scaffold. PAS was not required for **4** production, which was favored when reductase *Ti*DPAS2 was used (Figures S9 and S10).

To investigate the mechanism by which *Ti*CorS can act, we first set out to characterize the initial, unstable product that is released from *Ti*CorS in the absence of reductase (Figure 1). We optimized conditions under which this intermediate could be isolated and reductively trapped this compound with $NaBH_4$. NMR analysis showed that the compound was 16-carbomethoxycleavamine (**10**), suggesting that the initial cyclization product of *Ti*CorS is 16-carbomethoxycleaviminium (**11**) (Figure 3 and Figure S11). When the *Ti*CorS product was reduced with $NaBD_4$, the deuterium label was incorporated at carbon 21, **10-d**, which would be expected for 1,2-reduction of **11** (Figure 3). Notably, the crystal structure of related cyclase *Cr*CS (70.8% sequence identity, Figure S1) showed that CS initially forms (+)-16-carbomethoxycleaviminium (**11a**),[14] which then subsequently cyclizes to **3**, though unlike *Ti*CorS, the intermediate is not released from the active site.[2] Moreover **3** can open to form **11a** under acidic conditions (Figure S12).[1,2,15] We used CD spectroscopy to show that *Ti*CorS generates (−)-16-carbomethoxycleavamine (**10**), which is the opposite enantiomer that is generated by *Cr*CS (Figure S13).

With knowledge of the cyclization product of *Ti*CorS, we proposed a mechanism for the formation of **4**. After release from the active site of *Ti*CorS, **11** undergoes a 1,4-reduction by *Ti*DPAS2, which in turn would facilitate a second cyclization to form **4** (Figure 4). We speculate that at higher pH values, *Ti*DPAS2 favors 1,4-reduction, which when followed by tautomerization,[16] primes the substrate to cyclize to **4**.

However, the mechanism by which **11** could be transformed into the **5** scaffold was still not clear. Therefore, we performed the reaction in the presence of isotopically labeled NADPH (pro-(*R*)-NADPD) to determine where the deuteride is incorporated in **5**.[13,17,18] We incubated **6** with *Cr*PAS, *Ti*DPAS1, and *Ti*CorS or *Ti*TabS, along with pro-(*R*)-NADPD, which is required for DPAS reduction (Figure S14). With *Ti*TabS, we saw formation of the **2** product with a mass consistent with incorporation of one deuterium, as expected from the action of DPAS acting on **7** (Figure 4).[13] However, when *Ti*CorS was substituted for *Ti*TabS, the

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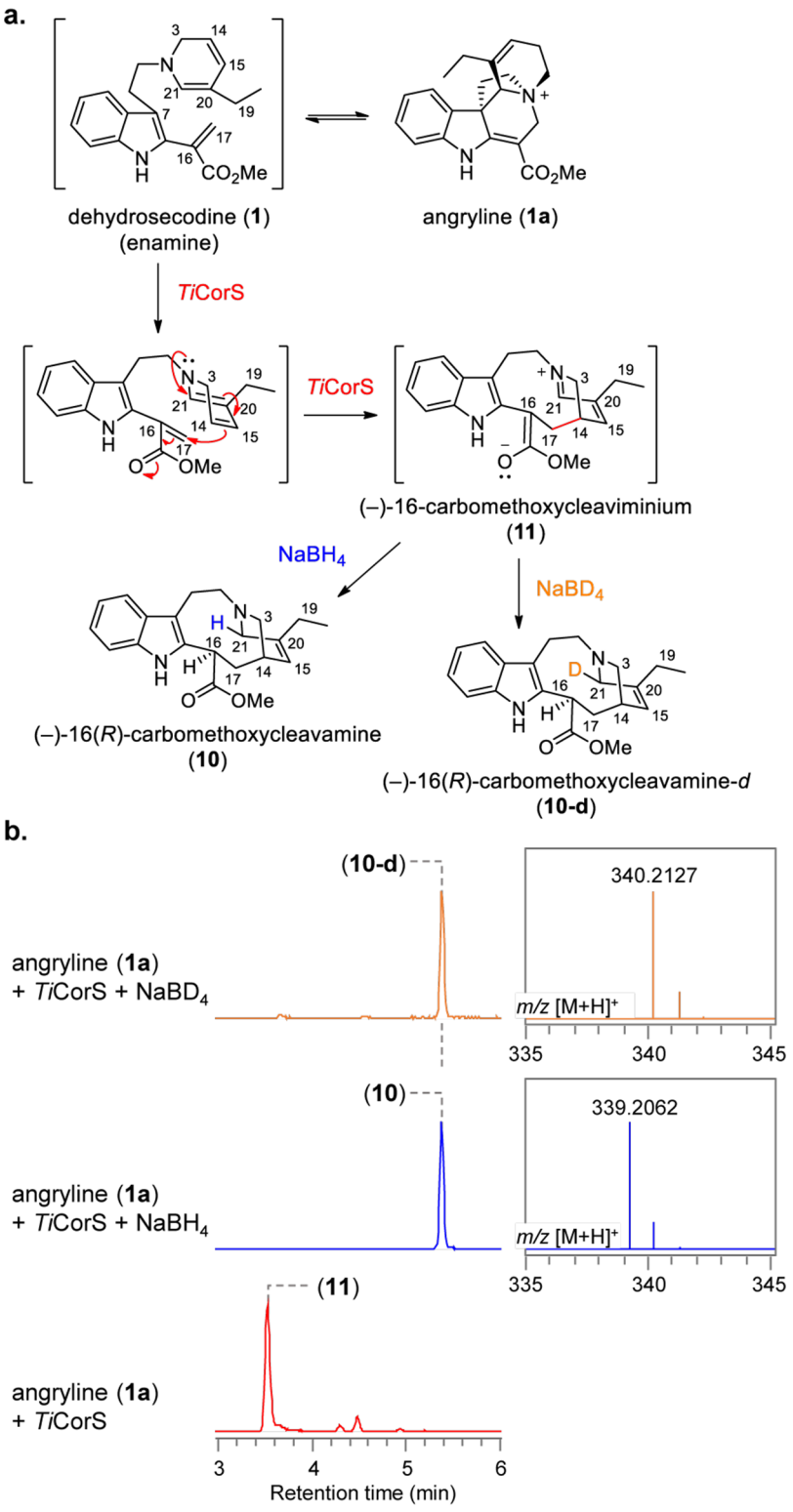

Figure 3. Formation of 16-carbomethoxycleavamine (**10**). (a) *Ti*CorS generates 16-carbomethoxycleaviminium (**11**), which could be trapped by $NaBH_4$ to generate 16-carbomethoxycleavamine (**10**) or $NaBD_4$ to generate 16-carbomethoxycleavamine-*d* (**10-d**). (b) TIC and MS spectra representing the formation of 16-carbomethoxycleaviminium (**11**) and 16-carbomethoxycleavamine (**10**) or 16-carbomethoxycleavamine-*d* (**10-d**).

resulting **5** product had a mass consistent with incorporation of two deuterium atoms (Figure 4 and Figure S14), clearly demonstrating that formation of **5** from **6** requires two reduction steps. Furthermore, the **9** product showed a mass incorporation of three deuterium atoms (Figure 4 and Figure S14). To corroborate these results, we also incubated the trapped isomer of **1**, **1a** with PAS, *Ti*DPAS1, the labeled NADPD cofactor, and *Ti*TabS or *Ti*CorS. The **2** product produced by *Ti*TabS had no deuterium incorporation, as expected; formation of **5** and **9** from **1a** showed incorporation of one and two deuterium atoms, respectively, and was strictly

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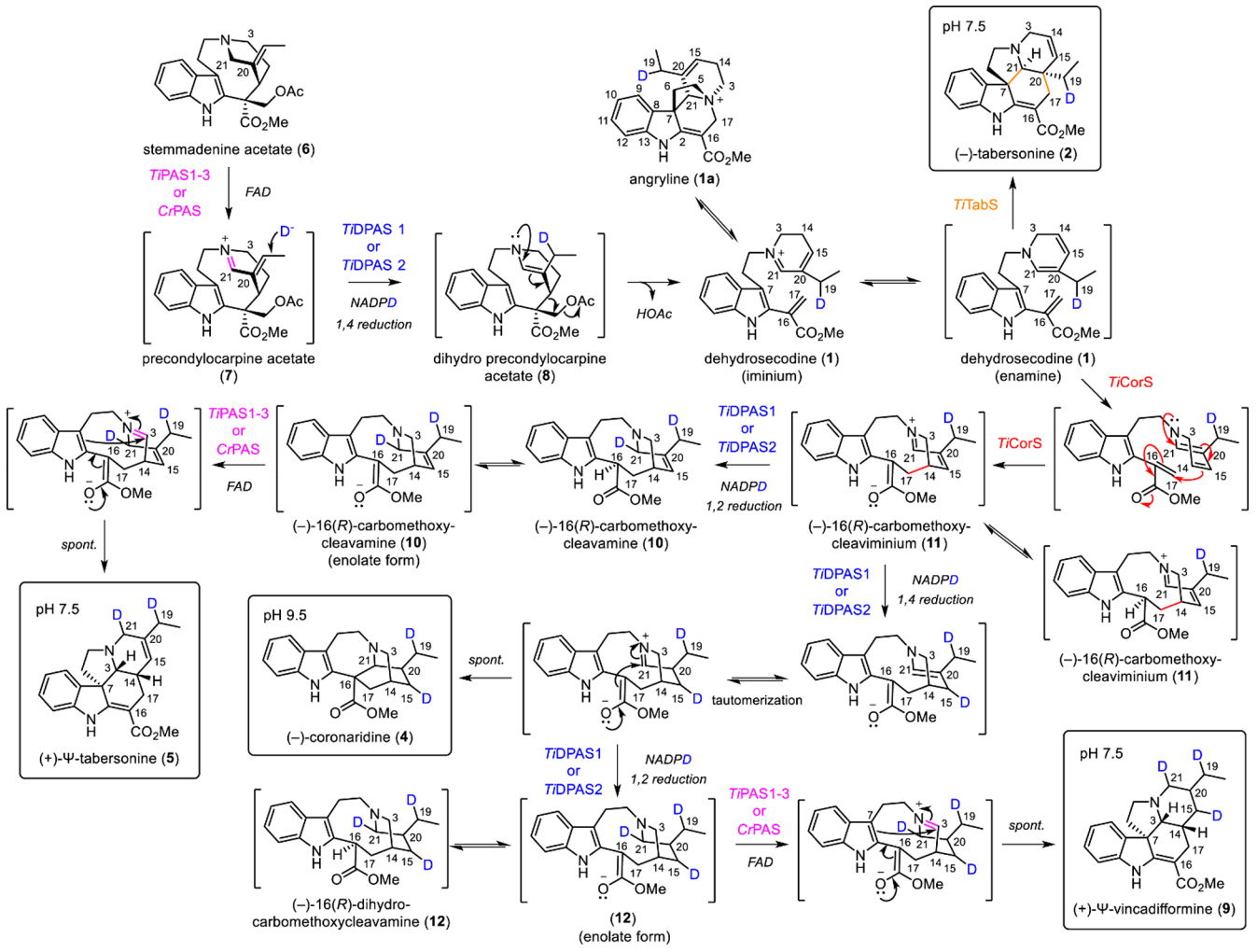

Figure 4. Proposed mechanism for the formation of (−)-iboga and (+)-Ψ-aspidosperma alkaloids. Biosynthesis of (−)-iboga alkaloid (−)-coronaridine (**4**) and Ψ-aspidosperma alkaloids (+)-Ψ-tabersonine (**5**) and (+)-Ψ-vincadifformine (**9**) with deuterium (blue) tracers using isotopically labeled NADPD.

dependent upon the presence of both reductase, *Ti*DPAS1, and oxidase, PAS (Figure S15).

We isolated isotopically labeled **2**, **5**, and **9** and showed that the deuterium labels were incorporated at carbon 19 for **2** as expected,[13] and carbons 19 and 21 for **5**, carbons 19, 21, and 15 for **9** (Figure 4). We performed CD spectroscopy of these isolated products and assigned the stereochemistry as (+)-Ψ-tabersonine (**5**) and (+)-Ψ-vincadifformine (**9**), which is the expected stereochemistry based on the downstream ibophyllidine products (Figure S16). Finally, we incubated the chemically 1,2-reduced *Ti*CorS product, **10**, with the oxidase PAS and observed formation of **5** (Figure 4 and Figure S17), indicating that cyclization occurs after PAS-catalyzed oxidation.

Together, this evidence strongly supports a mechanism for the formation of **5** (Figure 4). *Ti*CorS cyclizes **1** to **11** and releases it from the active site, where it is reduced to **10** via 1,2-reduction by *Ti*DPAS1 and then reoxidized by PAS. The resulting intermediate is then primed to spontaneously cyclize to form **5**. **9** forms by PAS oxidation of the doubly reduced (−)-16-dihydrocarbomethoxycleavamine (**12**) (Figure 4 and Figure S7). The switch between **4** and **5** is ultimately controlled by whether DPAS catalyzes a 1,4-reduction or 1,2-reduction. The changes in the assay pH conditions or protein−protein interactions may be responsible for favoring 1,2-reduction over 1,4-reduction. Alternatively, an as yet undiscovered reductase that generates **4** at physiological pH may be responsible for the biosynthesis of this compound in *T. iboga*.

We hypothesized that the (+)-16-carbomethoxycleavamine (**10a**) intermediate generated from opening of **3** could serve as a precursor to (−)-Ψ-tabersonine (**5a**).[19] However, oxidation of (+)-16-carbomethoxycleavamine (**10a**) by PAS yielded only a small amount of product, which, although having a mass and retention time consistent with (+)-Ψ-tabersonine **5**, could not be fully characterized (Figure S17). Thus, PAS may only recognize one 16-carbomethoxycleavamine enantiomer.

Here we show how redox transformations of dehydrosecodine (**1**) enable cycloaddition reactions with alternative regioselectivity to form (−)-coronaridine (**4**), (+)-Ψ-tabersonine (**5**), or (+)-Ψ-vincadifformine (**9**). Notably, these redox enzymes, DPAS and PAS, which transform stemmadenine acetate (**6**) into dehydrosecodine (**1**), are recruited from upstream in the biosynthetic pathway. Therefore, this discovery highlights how plants can recycle enzymes for use in more than one pathway step. Future studies are required to establish how the recruitment of these upstream enzymes is controlled. Nevertheless, the detailed chemical analyses described here provide a compelling hypothesis for the

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mechanism by which these redox reactions and subsequent cyclizations expand the number of scaffolds produced from the versatile dehydrosecodine (**1**) intermediate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c08107.

Additional experimental details, assays, materials, and methods, and NMR spectra described in this study (PDF)

Accession Codes

GenBank accession numbers: MH213134 (*Cr*PAS), MK840850 (*Ti*PAS1); MK840851 (TiPAS2); MK840852 (*Ti*PAS3); MK840853 (*Ti*TabS); MK840854 (*Ti*CorS); MK840855 (*Ti*D-PAS1); MK840856 (*Ti*DPAS2).

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Notes

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■ ABBREVIATIONS

Cr, *Catharanthus roseus*; *Ti*, *Tabernanthe iboga*; *Cr*PAS, precondylocarpine acetate synthase; *Ti*PAS1, precondylocarpine acetate synthase 1; *Ti*PAS2, precondylocarpine acetate synthase 2; *Ti*PAS3, precondylocarpine acetate synthase 3; *Ti*DPAS1, dihydro-precondylocarpine acetate synthase 1; *Ti*DPAS2, dihydroprecondylocarpine acetate synthase 2; *Cr*CS, catharanthine synthase; *Cr*TS, tabersonine synthase; *Ti*TabS, tabersonine synthase; *Ti*CorS, coronaridine synthase; FAD, flavin adenine dinucleotide; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); TIC, total ion chromatogram; EIC, extracted ion chromatogram; S.E., standard error

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