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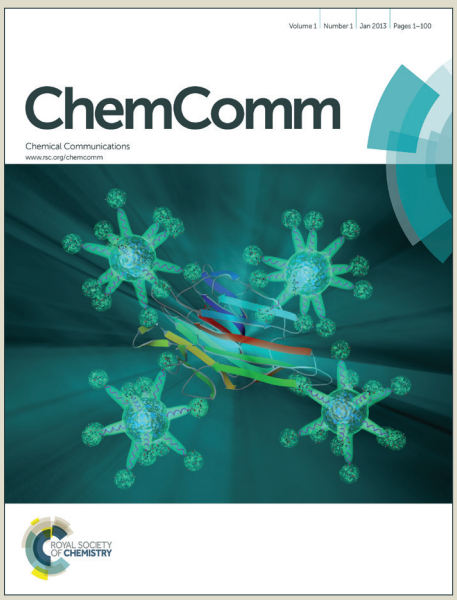

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Discovery of a P450-catalyzed step in vindoline biosynthesis: a link between the aspidosperma and eburnamine alkaloids

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Franziska Kellner[a], Fernando Geu-Flores[b], Nathaniel H. Sherden[a], Stephanie Brown[a], Emilien Foureau[c], Vincent Courdavault[c], and Sarah E. O'Connor*[a]

Here we report the discovery of a cytochrome P450 that is required for the biosynthesis of vindoline, a plant-derived natural product used for semi-synthesis of several anti-cancer drugs. This enzyme catalyzes the formation of an epoxide that can undergo rearrangement to yield the vincamine-eburnamine backbone, thereby providing evidence for the long-standing hypothesis that the aspidosperma- and eburnamine-type alkaloids are biosynthetically related.

Monoterpene indole alkaloids (MIAs) are a group of structurally diverse and pharmaceutically important alkaloids produced by a number of plant families. The plant *Catharanthus roseus* is the natural source of vinblastine (**6a**) and vincristine (**6b**), two MIAs that are used as chemotherapy drugs (Figure 1A).[1] These compounds are produced commercially by extracting the two monomeric precursor molecules catharanthine (**5**) (an iboga type alkaloid) and vindoline (**4**) (an aspidosperma type alkaloid) from *C. roseus* and then coupling these compounds chemically.[2] Development of new methods to cost-effectively produce natural products is an active, on-going area of investigation. However, these approaches require a full understanding of the enzymes that comprise the biosynthetic pathway. Here we identify a missing enzyme in vindoline biosynthesis, a cyctochrome P450 named 16-methoxytabersonine 3-oxygenase (16T3O, CYP71D1), which catalyzes the epoxidation of 16-methoxytabersonine (**2**). Furthermore, isolation and characterization of the 16T3O product reveals that a structural rearrangement takes place in the absence of the enzyme that acts immediately downstream of 16T3O. This rearrangement generates a compound closely related to the pharmacologically important alkaloid vincamine (**10**) (an eburnamine type alkaloid). Therefore,

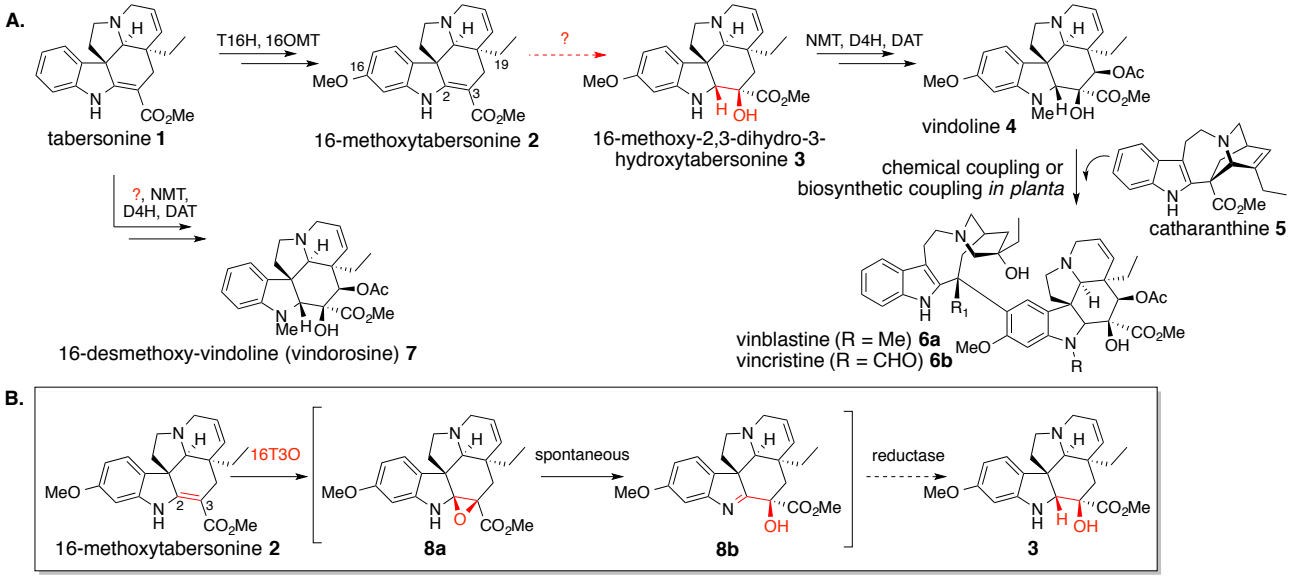

FIGURE 1. Vindoline (4) and vindorosine (8) biosynthesis in *Catharanthus roseus*. A. Vindoline (**4**) can be further converted to the anticancer agents vinblastine (**6a**) and vincristine (**6b**). **B.** Two-step conversion of 16-methoxytabersonine (**2**) to 16-methoxy-2,3-dihydro-3-hydroxytabersonine (**3**) is part of the vindoline biosynthetic pathway. The net hydration is proposed to take place first by epoxide formation, spontaneous rearrangement to the imine alcohol, and then reduction of the imine to form the final product.

the discovery of 16T3O also provides experimental evidence for the biosynthetic relationship between the aspidosperma and eburnamine alkaloids.

In vindoline biosynthesis, the biosynthetic intermediate tabersonine (**1**) is subjected to the action of a series of tailoring enzymes (Figure 1A). Briefly, tabersonine (**1**) is hydroxylated by the cytochrome P450 T16H,[3] and then methylated by 16OMT[4] to form 16-methoxytabersonine (**2**). The next reaction, which yields the product 16-methoxy-2,3-dihydro-3-hydroxytabersonine (**3**), is a formal hydration of 16-methoxytabersonine (**2**), but the enzyme(s) that are responsible for this step are unknown. Subsequently, a methyltransferase, NMT,[5] catalyzes methylation of the indoline nitrogen, after which the resulting product is further hydroxylated by D4H[6] and then acetylated by DAT[7] to ultimately yield vindoline (**4**).

Chemical studies suggest that the poorly understood 16-methoxytabersonine hydration reaction to yield **3** likely proceeds in two steps.[8–11] First, the C2,C3 alkene of 16-methoxytabersonine (**2**) is converted to the epoxide (**8a**), which then spontaneously opens to form the corresponding imine alcohol (**8b**) (Figure 1B). In the second step, the imine alcohol (**8b**) is reduced to form the desired product, 16-methoxy-2,3-dihydro-3-hydroxytabersonine (**3**). Based on this model chemistry, we hypothesized that the biosynthetic process of the hydration likely requires one oxidizing enzyme to catalyze formation of the epoxide/imine alcohol, followed by a second reducing enzyme to catalyze the reduction of the imine.

Since many epoxidation reactions are catalyzed by cytochrome P450s,[12] we selected five cytochrome P450 genes (Supplementary Information (SI)) from an RNA-seq dataset that were upregulated in response to an alkaloid elicitor (methyl jasmonate) and/or showed similar expression profiles to other MIA biosynthetic genes. The expression of these five P450s was transiently downregulated in *C. roseus* plants using virus induced gene silencing (VIGS). Extracts of silenced leaves were analyzed by LC-MS to assess whether vindoline (**4**) levels were reduced as a result of gene downregulation. Silencing of one of the candidates (CYP71D1, subsequently named 16-methoxytabersonine 3-oxidase, 16T3O) led to a substantial decrease in vindoline levels (SI, Figure S1A). Additionally, a compound with a mass and retention time identical to 16-methoxytabersonine (**2**)) (SI), the expected substrate of 16T3O, was observed in high levels compared to the negative control, suggesting that much less of the 16T3O substrate is consumed in silenced tissue. Successful downregulation of *16T3O* was confirmed by qRT-PCR, which showed a 68% reduction in transcript levels (SI, Figure S1B). Additionally, analysis of silenced tissue revealed that a compound with a mass corresponding to 16-desmethoxy-vindoline (vindorosine, *m/z* 427 (**7**), Figure 1A) was decreased compared to negative controls (SI, Figure S1A). Therefore, *16T3O* also appears to play a role in the biosynthesis of this vindoline shunt product in *C. roseus* by directly using tabersonine as a substrate.[13]

16T3O is localized to the endoplasmic reticulum (ER) in *C. roseus* cell cultures (SI, Figure S2A-D). This is consistent with the classical localization pattern of type II P450s, in which the globular catalytic domain is exposed to the cytosol, thereby facilitating substrate binding as well as product release in this cellular compartment. The upstream vindoline biosynthetic enzymes T16H and 16OMT also reside in this location, suggesting that the first three steps of vindoline biosynthesis occur in the cytosol.[13,14]

To rigorously characterize the 16T3O product that is ultimately converted to vindoline (**4**), an enzymatic reaction with 16-methoxytabersonine (**2**) and 16T3O was performed on a large scale. *16T3O* was introduced into a strain of yeast (WAT11)[15] harbouring *cpr* (the cytochrome P450 reductase that is required for proper functioning of P450 enzymes) on a high copy plasmid, and then cultured in the presence of 16-methoxytabersonine (**2**) for 48 hours (SI). The resulting product was isolated from the media, purified and characterized by IR, HRMS, and $^{1}H/^{13}C/^{15}N$ NMR (SI). We anticipated that we would observe either epoxide **8a**, or the imine alcohol **8b** that forms when the epoxide spontaneously opens (Figure 1A). However, a detailed inspection of the NMR data† revealed that the enzymatic product had rearranged to form the vincamine-type product **9** (Figure 2).[16] Previous reports indicate that 2-imine, 3-alcohols on the tabersonine framework can undergo facile acid catalyzed rearrangement to the eburnamine-vincamine skeleton (Figure 2).[17–20] Therefore, while we could not observe the epoxide directly, we can infer from this previous work that the epoxide is

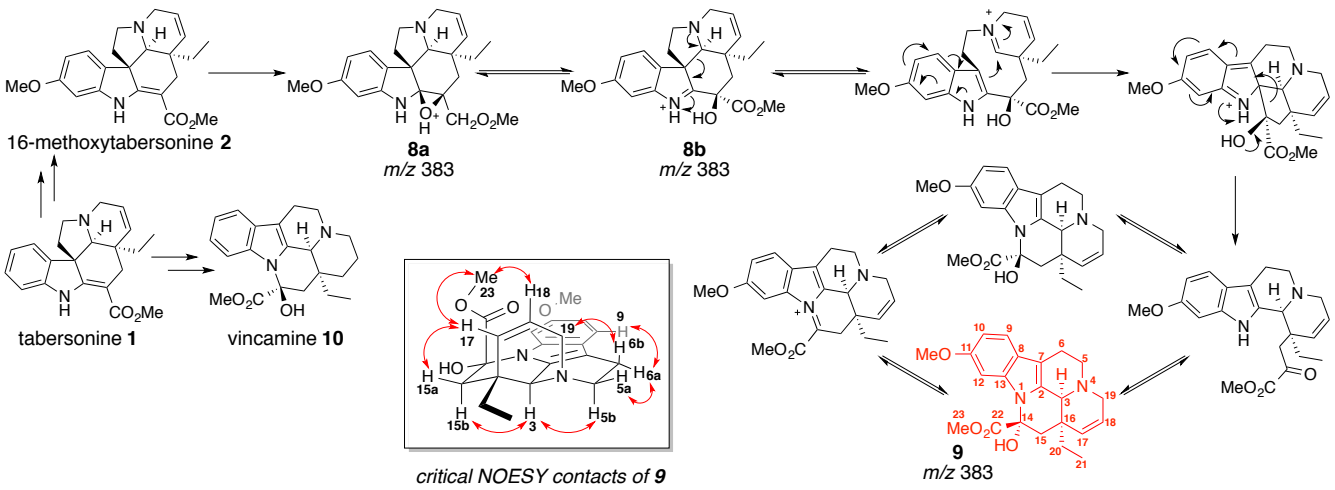

FIGURE 2. Characterization of the T3O enzymatic product. The epoxide intermediate rearranges to form vincamine (**10**) type compounds. NMR characterization of the isolated 16T3O product indicated that it rearranges to form product **9**. Key NOESY contacts for **9** are shown.

formed initially. Importantly, Wenkert first proposed this rearrangement in the biosynthesis of the pharmaceutically important vincamine (**10**) from the tabersonine/aspidosperma skeleton.[19] Therefore, the structure of this enzymatic product suggests that a 16T3O homolog, in the absence of the downstream reductase that reduces imine alcohol **8b** (Figure 1B), is likely to be involved in the formation of the pharmaceutically important vincamine-related alkaloids in other plants.[21–23]

Conclusions

Here we report the discovery of the cytochrome P450 16T3O, one of the missing enzymes in the vindoline biosynthetic pathway. For a complete elucidation of vindoline biosynthesis from tabersonine, now only one enzyme remains to be discovered, namely, a reductase that reduces the imine **8b** to form 16-methoxy-2,3-dihydro-3-hydroxytabersonine (**3**). *In planta* silencing experiments suggest that 16T3O is responsible for the biosynthesis of both vindoline (**4**) as well as the shunt product 16-desmethoxy-vindoline (vindorosine) (**7**). Tabersonine (**1**) appears to be the common starting point for several different alkaloids, with now four enzymes known to act on this substrate: T16H1,[3,13] T16H2,[13] T19H,[24] and 16T3O.

In *C. roseus*, an as-yet undiscovered partner reductase traps the 16T3O product to form **3**. Our work demonstrates that in the absence of this reductase, the 16T3O product spontaneously rearranges to form **9**, a member of the eburnamine class of MIAs. The previously reported, extensive work on this rearrangement in model chemical reactions leads us to infer that 16T3O initially catalyzes the formation of epoxide **8a**, which first opens to imine alcohol **8b** and then rearranges to **9**. The discovery that the action of 16T3O triggers a rearrangement to **9** strongly suggests that a 16T3O homolog is involved in the biosynthesis of vincamine and related alkaloids. Therefore, the discovery of this enzyme can be used as a starting point to elucidate the biosynthetic pathway of vincamine, a compound with demonstrated medicinal properties (vasodilator)[25] in producer plants such as *Vinca minor*.

Notes and references

[a] Department of Biological Chemistry, The John Innes Centre, Norwich, NR4 7UH UK, sarah.oconnor@jic.ac.uk

[b] Copenhagen Plant Science Centre and Section for Plant Biochemistry, Department of Plant and Environmental Sciences, University of Copenhagen, DK-1870, Frederiksberg, Denmark

[c] Université François Rabelais de Tours, EA2106 "Biomolécules et Biotechnologies Végétales"; 37200 Tours, France

† Spectral data for the 16T3O product **9**: ^{1}H NMR (400 MHz $CDCl_3$): δ 7.31 (d, $^{3}J_{9,10}$ = 8.5 Hz, 1H, H_9), 7.04 (d, $^{4}J_{12,10}$ = 2.3 Hz, 1H, H_{12}), 6.77 (dd, $^{3}J_{10,9}$ = 8.6 Hz, $^{4}J_{10,12}$ = 2.3 Hz, 1H, H_{10}), 5.48 (dt, $^{3}J_{18,17}$ = 10.3 Hz, $^{3}J_{18,19}$ = 3.2 Hz, 1H, H_{18}), 5.28 (br. d, $^{3}J_{17,18}$ = 10.3 Hz, 1H, H_{17}), 3.96 (br s, 1H, H_3), 3.79 (s 3H, *Ar*-OMe), 3.50 (s 3H, H_{23}), 3.38 (dd, $^{2}J_{5a,5b}$ = 13.9 Hz, $^{3}J_{5a,6a}$ = 7.0 Hz, 1H, H_{5a}), 3.25 (dd, $^{2}J_{5b,5a}$ = 11.0 Hz, $^{3}J_{5b,6b}$ = 6.4 Hz, 1H, H_{5b}), 3.09–3.01 (comp. m, 1H, H_{6a}), 3.05 (app. t, *J* = 2.9Hz, 2H, H_{19}), 2.63 (d, $^{2}J_{15a,15b}$ = 14.2 Hz, 1H, H_{15a}), 2.48 (ddd, $^{2}J_{6b,6a}$ = 16.3 Hz, $^{3}J_{6b,5b}$ = 6.2 Hz, $^{3}J_{6b,5a}$ = 2.1 Hz, 1H, H_{6b}), 2.15 (d, $^{2}J_{15b,15a}$ = 14.2 Hz, 1H, H_{15b}), 1.84 (dq, $^{2}J_{20a,20b}$ = 15.0 Hz, $^{3}J_{20a,21}$ = 7.3 Hz, 1H, H_{20a}), 1.54 (dq, $^{2}J_{20b,20a}$ = 14.8, $^{3}J_{20b,21}$ = 7.4 Hz, 1H, H_{20b}), 0.96 (app. t, $^{3}J_{21,20}$ = 7.6 Hz, 3H, H_{21}); ^{13}C NMR (100 MHz $CDCl_3$): δ 172.1 (C_{22}), 156.1 (C_{11}), 137.7 (C_{13}), 131.2 (C_2), 126.9 (C_{17}), 125.6 (C_{18}), 123.2 (C_8), 118.4 (C_9), 109.4 (C_{10}), 106.4 (C_7), 97.4 (C_{12}), 84.1 (C_{14}), 57.2 (C_3), 55.7 ($C_{Ar\text{-}OMe}$), 52.7 (C_{23}), 49.8 (C_5), 46.0 (C_{15}), 43.7 (C_{19}), 38.5 (C_{16}), 35.2 (C_{20}), 16.7 (C_{22}), 8.4 (C_{23}); ^{15}N NMR (40 MHz) (partial): δ 139.1 (N1); IR ($CDCl_3$ deposited thin film on ZnSe) vmax 3427 (OH), 2958 (aliphatic C–H stretching), 2875 (aliphatic C–H stretching), 1741 (ester C=O stretching), 1633, 1570, 1489, 1361, 1272, 1256 (ester C–O–C), 1203, 1172, 1144, 1105, 1031, 830 (trisubstituted phenyl C–H bending), 783 (trisubstituted phenyl C–H bending), 725 (trisubstituted phenyl C–H bending) cm–1; HRMS (EI) m/z calc'd for $C22H27N2O4^{+}$ $[M+H]^{+}$: 383.1965, found 383.1961 (–1.04 ppm); MS^2 (EI) *m/z* 365.1824 (37), 336.1563 (43), 174.0911 (100).

Electronic Supplementary Information (ESI) available: All Experimental Procedures, Supplementary Tables 1-3, Supplementary Figures 1-2. See DOI: 10.1039/c000000x/

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