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Directed Biosynthesis of Alkaloid Analogs in the Medicinal Plant *Catharanthus roseus*

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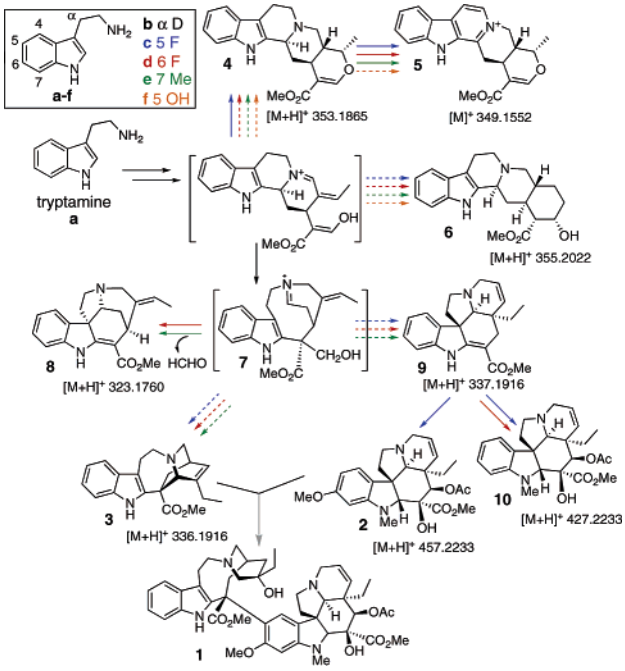
Terpene indole alkaloids (TIA) are plant natural products with diverse structures and biological activities.[1] A highly branched biosynthetic pathway is responsible for the production of approximately 130 different alkaloids in Madagascar periwinkle (*C. roseus*) from a common biosynthetic intermediate.[1] The most well-known of these alkaloids in the anticancer agent vinblastine **1**, resulting from the dimerization of **2** and **3** (Scheme 1).[2] Although numerous biosynthetic pathways can incorporate unnatural starting materials to yield novel natural products,[3] it was not clear whether the eukaryotic TIA pathway could utilize unnatural substrates to make new alkaloids. Here we describe feeding tryptamine analogs to *C. roseus* to demonstrate that the enzymes of this pathway can turnover unnatural substrates and that the electronic and steric properties of the non-natural substrates impact how these substrates partition among the branches of the TIA pathway. We use both differentiated *C. roseus* (seedlings) and hairy root culture. Seedlings produce a greater variety of TIA, while root cultures produce a smaller subset of TIA in greater quantities. High resolution (HR) LC−MS is used in conjunction with the extensive knowledge of the *C. roseus* metabolome to predict the structures of the analogs. Several of the most abundant alkaloid analogs are isolated and subjected to NMR spectroscopy. Our work demonstrates that the TIA biosynthetic machinery can be used to produce many novel alkaloid structures and also highlights the potential of this pathway for future metabolic engineering efforts.

To assess the formation of unnatural alkaloids in *C. roseus*, a hairy root culture was cultivated in liquid media supplemented with the tryptamine analogs **a**−**f** (1 mM).[4] Alkaloids were extracted from the roots after 3 weeks of growth and assessed by HRLC−MS. Comparison of cultures incubated with tryptamine **a** and the deuterated analog **b** allowed us to identify a subset of TIA compounds, **4**−**6**, into which the exogenous substrate was incorporated (Scheme 1). Co-injection with authentic standards of **4**−**6** further validated these assignments.

Hairy roots cultured in media supplemented with tryptamine analogs **c**−**f** were then analyzed. HRLC−MS identified derivatives with molecular weights corresponding to the addition of a fluorine, hydroxyl, or methyl group on the indole ring of **4**−**6** (Supporting Information). None of the compounds assigned as alkaloid analogs were observed in extracts derived from cultures incubated with tryptamine **a**. Formation of these compounds, therefore, is strictly dependent on the presence of the specific unnatural substrate (Figure 1, Supporting Information).

The intensities of the mass spectrometry signals assigned to the alkaloid derivatives suggest that in roots grown on media supplemented with **c**, **d**, and **e**, the major products correspond to products derived from the unnatural starting material, with the parent (natural) alkaloids present in lower quantities. However, in extracts of roots treated with **f**, the analogs were minor products compared to the parent alkaloids. This may be due to the low catalytic efficiency of this substrate for certain enzymes in the pathway or the uptake of **f** into the root culture may be less efficient.

Scheme 1. Major Biosynthetic Pathways of *C. roseus*[a]



[a] Dashed arrows signify LC−MS evidence that the analog was incorporated. Solid arrows have NMR evidence or MS/MS data to support the structural assignment. **1**, only found in mature plants, is not observed in these experiments.[5]

Although HRMS data allows assignment of molecular formula, *C. roseus* produces numerous alkaloids with identical molecular weights. For example, although serpentine is the major product at *m*/*z* 349 (as evidenced by coelution with authentic standard), diastereomers of serpentine are also produced. Therefore, NMR analysis is required to fully validate structural assignment. Hairy root culture extracts treated with **c**, **d**, and **e** were fractionated by preparative HPLC and several of the most abundant analogs were purified in milligram quantities. ^{1}H-NMR spectra was used to assign **5-c** and **5-d** as fluorinated serpentine, **5-e** as methylated serpentine, and **4-c** as fluorinated ajmalicine (Supporting Information). Interestingly, since yohimbine **6** was the major product with $m/z = 355$ in control cultures, we expected NMR analysis to confirm that a methylated product with $m/z = 369$ was a derivative of **6**. However, ^{1}H-NMR analysis revealed a spectrum inconsistent with any known *C. roseus* alkaloid with this expected mass, suggesting that unexpected alkaloids may result when unnatural substrates are used. Efforts are underway to determine the structure of this alkaloid analog.

An additional major product was observed in roots treated with **d** and **e**. These product analogs, with *m*/*z* of 337 and 341,

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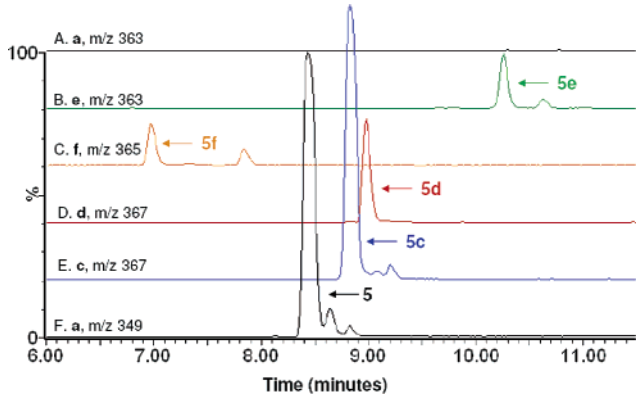

Figure 1. LC-MS extracted traces of *m*/*z* corresponding to analogs of **5a**−**f** from hairy root cultures treated with analogs **a** and **c**−**f**. Trace A shows the absence of a methylated serpentine analog in control cultures treated with **a**.

respectively, correlate to a native alkaloid with *m*/*z* of 323. Known *C. roseus* alkaloids with this molecular weight correspond to "off pathway" products suspected to result from the deformylation of preakuammicine **7**, a proposed central intermediate along the way to vindoline **2** and catharanthine **3** (Scheme 1).[6] NMR analysis was used to assign the structures as akuammicine **8** analogs; fluorinated **8-d** and methylated **8-e** akuammicine analogs were isolated and characterized by ^{1}H-NMR. In contrast, **8** or analogs thereof are not accumulated in significant amounts in roots treated with **a**, **b**, or **c**.

We suspect that the presence of akuammicine derivative **8** represents a bottleneck in the production of alkaloid derivatives derived from 6-fluoro (**d**) and 7-methyl (**e**) tryptamine owing to the substrate specificity of downstream enzymes. Alternatively, the preakuammicine **7** derivatives resulting from compounds **d** and **e** may be chemically unstable and easily deformylated to yield the nonproductive dead-end product **8**.

Root cultures do not produce the entire array of TIA found in differentiated *C. roseus* plants (Scheme 1). Most notably, vindoline **2**, the precursor of the pharmaceutically important bisindole alkaloid vinblastine **1**, is never produced in hairy root or cell suspension culture.[7] To determine if analogs **c**−**f** could be incorporated into the vindoline **2** pathway, *C. roseus* seedlings were germinated and grown on media containing compounds **c**−**f**. Although a complex mixture of alkaloids were observed, LC−MS experiments strongly suggested that unnatural substrates **c**−**f** were incorporated into a wide range of structures in these seedlings (Supporting Information).

Several pieces of evidence suggest that seedlings treated with compounds **c** and **d** produce derivatives of vindoline **2**. Molecular formula data obtained by LC−MS indicate that compounds with a molecular weight consistent with fluorinated **2** and the demethoxy-vindoline derivative **10** were produced in seedlings cultured with analog **c** (Supporting Information). The corresponding fluorinated **2** derivative was not observed in seedlings cultured with analog **d**, but a molecular mass corresponding to fluorinated demethoxyvindoline **10** was observed (Scheme 1). Although authentic fluorinated standards of these compounds are not available, the slightly longer retention times relative to the natural standards are consistent with the relative retention times of characterized fluorinated TIA such as **5c** (Figure 1) and previously characterized TIA intermediates.[8] Moreover, no other alkaloid with molecular formulas matching that of **2** and **10** have been reported to be isolated from *C. roseus*,[1] lending support to our proposed assignments evidenced by exact mass. Furthermore, the putative vindoline analogs yielded an MS/MS fragmentation pattern identical to that of a vindoline **2** standard (Supporting Information).

The fluorinated derivatives **c** and **d** appeared to be widely incorporated into additional alkaloids by the TIA machinery (Supporting Information). For example, analogs with a parent *m*/*z* 337 were also observed and may be derivatives of catharanthine **3** and tabersonine **9**, compounds that are commonly found in *C. roseus* seedlings. The methylated derivative **e** also appeared to be incorporated, although it did not appear that the conversion of **9** to **2** occurred. The hydroxylated substrate **f** was much less widely incorporated. As in the roots, a major product of the seedlings treated with **d** and **e** is fluorinated **8-d** and methylated **8-e** akuammicine, respectively, as evidenced by co-injection with the characterized compounds from the root cultures. Compounds **c**−**e** were incorporated into at best approximately 50% of the total alkaloids, and hydroxylated analogs derived from **f** comprised only minor products in the extracts.

Since seedlings produce complex mixtures of alkaloids in relatively low yields, NMR analysis of seedling extracts was not performed at this time. Therefore, although the MS data allows an assignment of the molecular formula of the analogs, definitive structural assignments of these analogs cannot be made. However, it is clear from the MS data that a variety of alkaloid analogs are being produced when seedlings are treated with analogs **c**−**f**.

Manipulation of biosynthetic pathways is a powerful way to make natural-product-like compounds that are not easily accessible via total synthesis. However, at the outset of this study, it was unclear whether the TIA pathway had the capacity to produce novel alkaloids. There is very limited precedence for precursor directed biosynthesis in plants,[9] and our understanding of the enzymatic transformations in the TIA pathway is limited. Our findings reported here demonstrate that *C. roseus* can produce an array of alkaloid analogs. Electronically and sterically modified starting materials partition differently among the branches of the pathway and may lend insight into the mechanism or specificity of downstream enzymes. Incorporation of additional substrate analogs and evaluation of the biological activities of the isolated compounds are currently underway.

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Supporting Information Available: Detailed experimental procedures, tabulated exact masses, spectroscopic data for compounds **4c**, **5c**−**e**, **8d**,**e** and extracted LC−MS chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Van der Heijden, R.; Jacobs, D. I.; Snoeijer, W.; Hallard, D. V. R. *Curr. Med. Chem.* **2004**, *11*, 607−628.
(2) Bensch, K. G.; Malawista, S. E. *Nature* **1968**, *218*, 1176−1177.
(3) Chartrain, M.; Salmon, P. M.; Robinson, D. K.; Buckland, B. C. *Curr. Opin. Biotechnol.* **2000**, *11*, 209−214.
(4) Bhadra, R.; Vani, S.; Shanks, J. V. *Biotechnol. Bioeng.* **1993**, *41*, 581−592.
(5) Datta, A.; Srivastava, P. S. *Phytochemistry* **1997**, *46*, 135−137.
(6) (a) Battersby, A. R.; Bhatnagar, A. K. *Chem. Commun.* **1970**, 193−195. (b) Scott, A. I.; Qureshi, A. A. *J. Am. Chem. Soc.* **1969**, *91*, 5874−5876. (c) El-Sayed, M.; Choi, Y. H.; Frederich, M.; Roytrakul, S.; Verpoorte, R. *Biotechnol. Lett.* **2004**, *26*, 793−798.
(7) Vazquez-Flota, F.; de Luca, V.; Carrillo-Pech, M.; Canto-Flick, A.; De Lourdes, M. M. *Mol. Biotechnol.* **2002**, *22*, 1−8.
(8) McCoy, E.; Galan, M. C.; O'Connor, S. E. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2475−2478.
(9) (a) Hagan, D. O.; Robins, R. J.; Wilson, M.; Wong, C. W.; Berry, M. *J. Chem. Soc., Perkin Trans.* **1999**, *1*, 2117−2120. (b) Prince, C. L.; Shuler, M. L.; Yamada, Y. *Biotechnol. Prog.* **1997**, *13*, 506−510.

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