Chemoselective derivatization of alkaloids in periwinkle†

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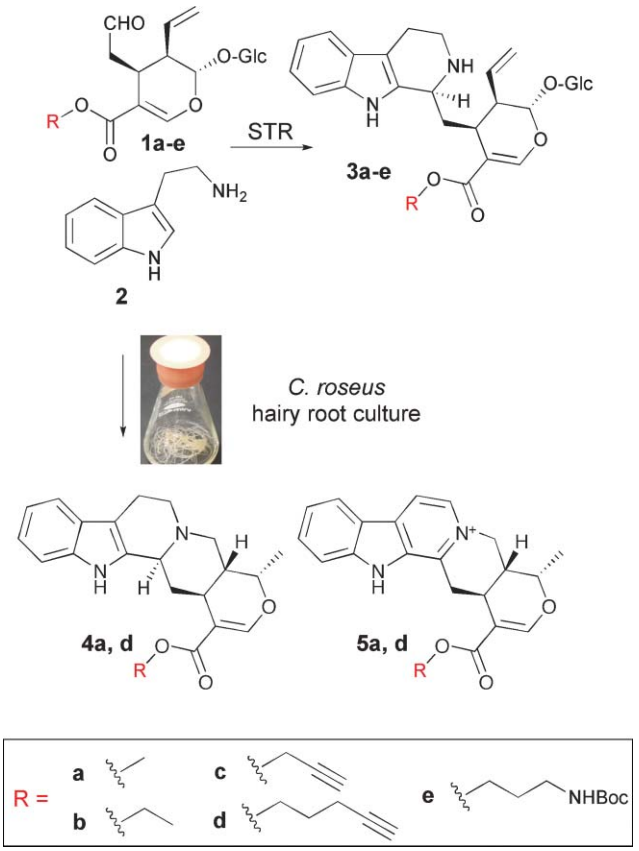
The terpene indole alkaloid biosynthetic pathway can utilize a secologanin substrate analog containing a handle for functionalization, and the resulting non-natural alkaloids can be chemoselectively derivatized in crude extracts of plant tissue.

Terpene indole alkaloids (TIA) are a family of plant-derived compounds that have important medicinal properties.[1] The TIA are a particularly diverse class of natural products, comprising approximately 2000 members with a range of chemical structures and a wealth of biological activities.[1,2] However, the difficulties encountered in identifying and cloning plant enzymes have hindered biosynthetic and engineering studies of plant natural product pathways in comparison to microbial systems.[3–5] Here we report that a substrate analog containing a chemoselective handle could be incorporated into the TIA pathway and allowed derivatization of the alkaloids in plant cell extracts (Scheme 1).

All TIA use the starting substrates secologanin **1a** and tryptamine **2** to form the central biosynthetic intermediate of the pathway strictosidine **3a** (Scheme 1).[6] We have recently shown that analogs of tryptamine **2** can be incorporated into the alkaloids produced by *Catharanthus roseus* (Madagascar periwinkle) hairy root culture including the heteroyohimbine alkaloid ajmalicine (raubasine) **4a**[7] and serpentine **5a**[8] (Scheme 1).[9] Encouraged by these results, we set out to explore the incorporation of the more complex iridoid terpene secologanin substrate into the TIA pathway.

We assessed the turnover of a series of secologanin analogs **1b–e** prepared semi-synthetically from isolated secologanin[10,11] with the first committed enzyme of the pathway, strictosidine synthase (STR) (Scheme 1, Table 1). STR catalyzes the formation of the central intermediate **3a** *via* a stereoselective Pictet–Spengler condensation of secologanin **1a** and tryptamine **2**.[12–15] Any secologanin derivative must be turned over by STR to be successfully incorporated into any TIA. Secologanin derivatives **1b–e** were assayed with a recombinant preparation of the enzyme. Ethyl ester derivative **1b** showed modest changes in K_m and V_{max} compared to the natural substrate **1a**, resulting in an overall slight decrease in V_{max}/K_m. Propargyl ester **1c** and pentynyl ester **1d**, analogs which contain a rigid alkyne chain, and compound **1e**, derivatized with a protected amine, exhibited an increase in K_m and a decrease in V_{max}. An STR mutant that exhibits selectivity for analog **1d** over the natural secologanin substrate **1a** has also been reported.[16]



Scheme 1 Terpene indole alkaloid (TIA) pathway. Strictosidine synthase (STR) catalyzes formation of **3** from **1** and **2**. Alkaloids **4** and **5** are derived from later *C. roseus* enzymes that act on **3**.

We next evaluated whether one of these secologanin substrate analogs could be turned over by later enzymes of the TIA pathway. Secologanin pentynyl derivative **1d** was chosen for further analysis since this analog contains an alkyne group that can be chemoselectively modified with an azide using Huisgen's 1,3-dipolar cycloaddition reaction ("click chemistry").[17] This

Table 1 Kinetic constants for compounds **1a–e** with STR

Compound	K_m (μM)	V_{max} (μM min^{-1} × 10^{-5})	V_{max}/K_m (relative)
1a	42 ± 6	3 ± 0.03	1
1b	106 ± 4	6 ± 0.02	0.8
1c	215 ± 10	1.5 ± 0.04	0.1
1d	313 ± 15	2.5 ± 0.05	0.1
1e	250 ± 20	2.7 ± 0.03	0.2

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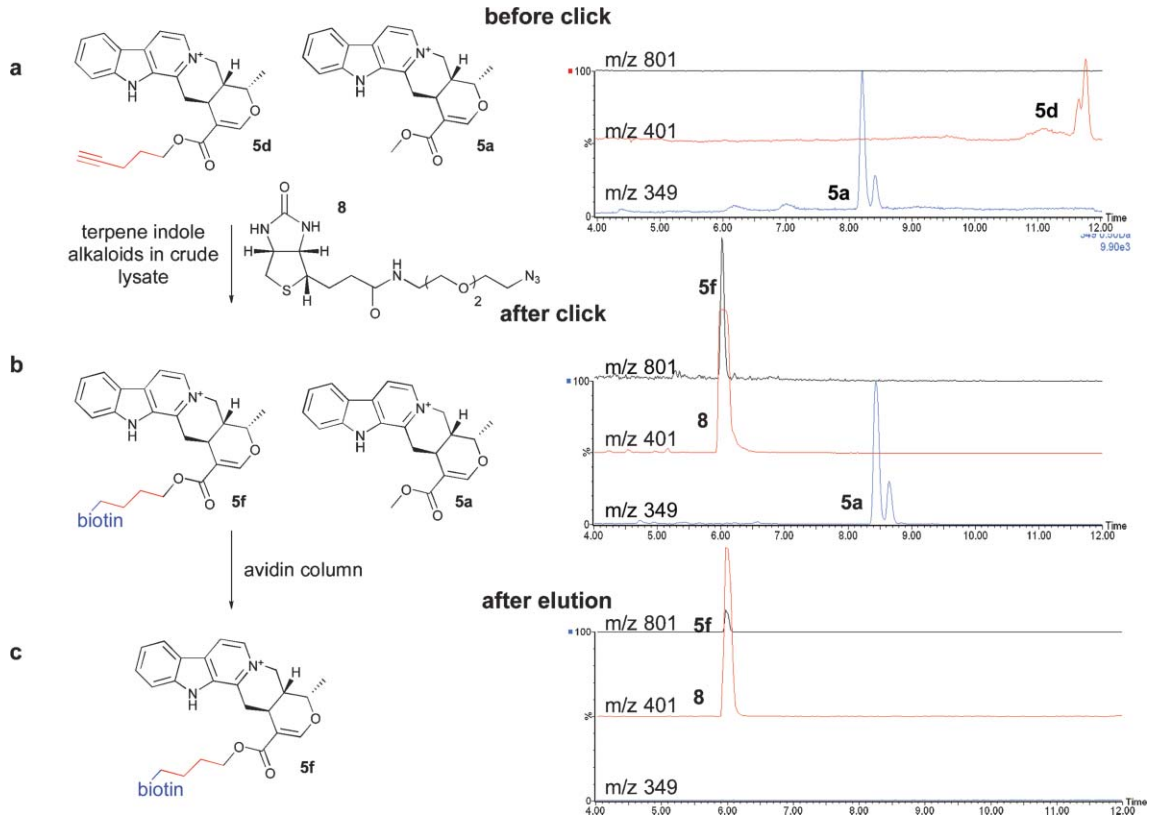

Scheme 2 Click chemistry in crude plant cell extracts. The crude alkaloid mixture can be derivatized with biotin azide **8** and purified by captavidin agarose. This scheme shows extracted LC–MS traces for derivatives of serpentine **5**. **a**. The native alkaloid **5a** ($[M]^+$ 349) and the derivative **5d** ($[M]^+$ 401) can both be observed before the 'click' reaction. **b**. After incubation with biotin azide **8** and $CuSO_4$ **5d** disappeared and a new compound corresponding to the addition of biotin **5e** ($[M]^+$ 801) appeared. **8** is also observed in the $[M + H]^+ = 401$ chromatogram. **c**. After elution of the compounds from the captavidin resin, the natural alkaloid **5a** ($[M]^+$ 349) is no longer observed.

functional group, if accepted by the pathway, could be used as a "tag". For example, we envision that if used in conjunction with a tryptamine substrate derivatized with a photo-affinity or other reactive group that can covalently modify proteins, **1d** could be used as a label to facilitate identification and isolation of *C. roseus* biosynthetic enzymes.[18] Alternatively, the alkyne group could be used to simplify purification and identification of alkaloids from the plant lysate, in which case the modified pentynyl group could be subsequently removed from the final alkaloid product after isolation and purification.[19] Although microbial natural products are often exported from the cell into the media,[20] most plant natural products are retained in the cell, and purification of the desired compounds from the plant cellular components presents a challenging problem.[21]

Feeding experiments with *C. roseus* hairy root cultures were used to probe the incorporation of the secologanin analog **1d** *in vivo*. Substrate **1d** (500 μM) was incubated with *C. roseus* hairy root liquid cultures for 7 days. High-resolution mass spectrometry analysis of extracts of the *C. roseus* cultures suggested that secologanin analog **1d** was incorporated into several alkaloid biosynthetic pathways. Molecular weights consistent with the novel derivatives pentynyl serpentine **5d** ($C_{25}H_{25}N_2O_3$: expected $[M]^+$ 401.1865; observed 401.1860) and pentynyl ajmalicine **4d** ($C_{25}H_{29}N_2O_3$: expected $[M + H]^+$ 405.2173; observed 405.2151) along with pentynyl strictosidine **3d** ($C_{31}H_{38}N_2O_9$: expected $[M + H]^+$ 583.2656; observed 583.2639) were observed in crude extracts of *C. roseus* cultured in the presence of **1d** (Scheme 1, Supporting Information). These derivatives could be purified from the crude extracts and ^1H NMR analysis of these isolated compounds supported structural assignments of **4d** and **5d** (Supporting Information).

Differentiated plants produce alkaloids not found in cell culture; for example, the bis-indole alkaloid vinblastine is not produced in hairy root culture.[22] To investigate whether analog **1d** was incorporated into other branches of the TIA pathways, **1d** was also cultured with *C. roseus* seedlings. However, no additional compounds were observed in the seedling tissue, suggesting that modifications to the methyl ester of secologanin bias partitioning of this non-natural substrate into the heteroyohimbine (*i.e.* ajmalicine **4**) branch of the TIA pathway.

Production of the natural substrate secologanin **1a** can be suppressed with the DOXP reductoisomerase inhibitor fosmidomycin.[23] Therefore, culturing *C. roseus* with fosmidomycin could minimize the formation of natural alkaloids relative to the desired non-natural alkaloids. We used LC–MS to compare the levels of **4a** and **5a** with the levels of **4d** and **5d** in both control experiments (cultured without fosmidomycin) and in cultures containing either 100 μM or 1 mM fosmidomycin. When *C. roseus* was co-cultured with fosmidomycin and **1d**, we observed a 1.5 to 2 fold increase in the ratio of unnatural to natural alkaloids when compared to control cultures lacking fosmidomycin (Table 2). These data suggest that by suppressing the biosynthesis of the natural

Table 2 Ratio of amount of alkaloid derived from **1d** over amount of alkaloid derived from **1a** in the presence and absence of fosmidomycin. Fosmidomycin inhibits secologanin **1a** biosynthesis

	Ratio of *m*/*z* unnatural / *m*/*z* natural	
Culture condition	401/349	405/353
1d	0.13 ± 0.02	0.08 ± 0.02
1d and 0.1 mM fosmidomycin	0.12 ± 0.03	0.11 ± 0.04
1d and 1 mM fosmidomycin	0.21 ± 0.11	0.17 ± 0.05

secologanin substrate **1a** the amount of non-natural alkaloids relative to natural alkaloids can be increased.

Having demonstrated that **1d** could be incorporated into the alkaloid pathway, we next showed that derivatization of the alkyne moiety *via* copper catalyzed 1,3-dipolar cycloaddition was compatible with the alkaloid chemical structure. Azide biotin labeling reagent **8**[16] was incubated with the crude root extracts in the presence of copper sulfate and ascorbic acid. LC–MS analysis showed that the peaks corresponding to the derivatives **4d** and **5d** disappeared, and compounds exhibiting the expected 400 Da increase in mass correlating to the addition of the biotin moiety became apparent (**4f** (Supporting Information) $C_{41}H_{53}N_8O_7S$: expected $[M + H]^+$ 801.3758; observed 801.3798; **5f** (Scheme 2 and Supporting Information) $C_{41}H_{57}N_8O_7S$: expected $[M]^+$ 805.4065; observed 805.4060).

Captavidin, a derivative of avidin which permits tight binding to biotin at low pH and dissociation of the biotin complex at basic pH,[24] was used to capture biotinylated compounds from the crude extract. Compounds corresponding to both natural and non-natural alkaloids were present prior to incubation with biotin azide **8** (Scheme 2a). After incubating this crude extract with biotin azide **8** and copper sulfate, the peaks corresponding to the non-natural alkaloids disappeared, and compounds corresponding to biotin derivatized compounds became apparent (Scheme 2b). This extract was then incubated with the captavidin derivatized agarose. Gratifyingly, LC–MS analysis revealed that natural alkaloids **4–5** were no longer present in the elution fraction, but that the derivatized alkaloids were still observed, suggesting that the alkaloids were modified as expected with biotin and that this is a viable way to detect non-natural metabolites containing this functional group (Scheme 2c).

In summary, the first committed enzyme of this plant pathway, strictosidine synthase, can turnover a variety of secologanin analogs derivatized at the methyl ester functionality. We demonstrated that one of these iridoid terpene analogs is successfully incorporated into a branch of the terpene indole alkaloid pathway, and moreover, that an orthogonal chemical handle can be used to derivatize the alkaloid analogs. Although plant metabolic pathways present a number of challenges compared to microbial pathways, we show that directed biosynthesis is a viable strategy to produce novel alkaloids in *C. roseus* plant cell culture and that functionalized substrate analogs can be used to facilitate the derivatization of these unnatural products.

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