Substrate Specificity and Diastereoselectivity of Strictosidine Glucosidase, a Key Enzyme in Monoterpene Indole Alkaloid Biosynthesis

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Abstract

Strictosidine glucosidase (SGD) from *Catharanthus roseus* catalyzes the deglycosylation of strictosidine, an intermediate from which thousands of monoterpene indole alkaloids are derived. The steady state kinetics of SGD with a variety of strictosidine analogs revealed the substrate preferences of this enzyme at two key positions of the strictosidine substrate. Additionally, SGD from *C. roseus* turns over both strictosidine and its stereoisomer vincoside, indicating that although this enzyme prefers the naturally occurring diastereomer, the enzyme is not completely diastereoselective. The implications of the substrate specificity of SGD in metabolic engineering efforts of *C. roseus* are highlighted.

Monoterpene indole alkaloids (MIA) are a large class of pharmaceutically valuable and structurally complex natural products.[1] Directed biosynthesis studies have shown that the MIA pathway can produce a variety of "unnatural" alkaloids by utilizing non-natural substrate analogs.[2] This inherent flexibility suggests that MIA biosynthesis could provide a robust platform for metabolic engineering. However, not all substrate analogs are likely to be incorporated into the pathway with equal efficiency. If substrate specificity of individual biosynthetic enzymes correlates with rate limiting steps *in vivo*, then enzymes having a low catalytic efficiency for a non-natural substrate could be reengineered to improve turnover of the analog.[3] Therefore, evaluation of enzyme substrate specificity is critical for biosynthetic engineering efforts. In MIA biosynthesis, the central biosynthetic precursor strictosidine **1** is deglycosylated by strictosidine glucosidase (SGD) to yield a reactive intermediate that rearranges to form the wide variety of MIA (Scheme 1).[4] Here we evaluate the substrate specificity of SGD from *Catharanthus roseus* with a variety of strictosidine analogs to determine whether SGD could act as a bottleneck in the production of novel alkaloids from unnatural strictosidine analogs.

SGD was assayed with strictosidine analogs **2–9** (Table 1).[5–8] An HPLC assay was used to monitor both strictosidine disappearance and deglycosylated product formation. Since all kinetic data appeared to fit a sigmoidal rather than a Michaelis-Menten curve, kinetic constants were obtained from a sigmoidal fit to the data (Figure 1).[9,10] SGD from *C. roseus* has been reported to form aggregates consisting of 4 to 12 monomers;[4a] although a sigmoidal fit has not been previously reported for *C. roseus* SGD, the oligomeric state of the enzyme is compatible with the cooperative mechanism suggested by the sigmoidal curve.

The catalytic efficiencies ($V_{max}/K_{0.5}$) of indole substituted strictosidine derivatives **2–9** varied by less than an order of magnitude from the naturally occurring strictosidine **1**. Strictosidine analogs with methyl groups in the 9, 10, 11, and 12 positions (compounds **2–5**) demonstrated that steric effects did not disrupt enzyme activity dramatically. Not surprisingly, replacement of methyl groups with larger methoxy substituents (compounds **6, 7**) resulted in a small increase in the $K_{0.5}$ values. Electronic perturbations did not appear to impact the turnover profoundly; catalytic efficiencies of fluorinated strictosidine analogs (**8, 9**) did not vary significantly from **1**.

The recently reported crystal structure of SGD from *Rauwolfia serpentina* (PDB code 2JF6) indicates that whereas the glucose moiety of **1** is buried within the enzyme active site, the indole portion of strictosidine points toward the surface of the enzyme.[11] The results of these substrate specificity studies suggest that the active site of SGD has not evolved to discriminate against substitutions on the indole ring. Notably, 5 methyl and 6 methyl tryptamine are not turned over by the enzyme strictosidine synthase (Scheme 1) to form strictosidine analogs **3** and **4**, respectively.[5] The specificity of the early stages of the MIA pathway therefore appears to be controlled in large part by strictosidine synthase and not by SGD. Any significant differences observed in incorporation of indole-substituted tryptamine substrates into alkaloid products are not likely to be due to the substrate specificity of SGD.

Whereas the modestly sized indole substituents had a moderate effect on SGD catalysis, replacement of the methyl ester of strictosidine with the significantly larger pentynyl ester[12] (compound **10**, Figure 2) resulted in a significant decrease in turnover by SGD. No formation of deglycosylated product was observed under the assay conditions used for strictosidine analogs **2–9**, although deglycosylation was observed when the SGD concentration was increased from approximately 0.6 nM to 50 µM. The ester appears to be positioned more deeply in the interior of SGD compared to the indole moiety,[11] and steric clashes of the pentynyl group with the surrounding enzyme residues may impede effective catalysis. Mutation of SGD may improve turnover of this substrate.

Notably, the slow turnover rate of **10** parallels the results of feeding studies in which *C. roseus* hairy root cultures were grown in the presence of the precursor to **10**, the pentynyl ester of secologanin. In these feeding studies,[3a,12] a significant accumulation of pentynyl strictosidine **10** was observed, a result consistent with slow deglycosylation of **10** by SGD (Figure 3, black trace). Therefore, the enzyme substrate specificity can predict bottlenecks in precursor directed biosynthesis. Despite slow deglycosylation by SGD, **10** is nevertheless converted by *C. roseus* into several downstream alkaloid products; one alkaloid (serpentine) is shown in Figure 3 (red trace).[12] The production levels of these alkaloids could be expected to increase further if a reengineered variant of SGD that more effectively deglycosylates **10** is incorporated into the MIA pathway.

Strictosidine synthase, the enzyme that forms strictosidine **1** from tryptamine and secologanin (Scheme 1), is absolutely stereoselective. The vincoside diastereomer **11** is not formed enzymatically in any known MIA pathway (Figure 2).[1] Although SGD from *Rauwolfia serpentina* does not turn over the vincoside diastereomer[4b] we observed that the *C. roseus* enzyme does in fact turn over the "non-natural" vincoside[13] as evidenced by an LC-MS assay and a glucose detection assay.[14] Steady state kinetic constants were not obtained for this substrate because vincoside **11** spontaneously formed the lactam **12** during the course of the assay, thereby complicating kinetic analysis.[15] However, the rate of deglycosylation of **11** appeared to be qualitatively slower than that of strictosidine **1**. Inspection of the SGD sequence from *R. serpentina*, which has 70% amino acid identity with *C. roseus* glucosidase,[4] did not reveal any obvious differences in the strictosidine binding site,[11] so the structural basis for the difference in diastereoselectivity between the two enzymes remains to be determined.

SGD catalyzes deglycosylation of strictosidine analogs containing indole substituents at relatively high catalytic efficiencies. In contrast, a significant decrease in catalysis was noted after the methyl ester of strictosidine was replaced with a pentynyl ester (compound **10**), a result consistent with accumulation of **10** in feeding studies. Despite the slow turnover of **10** by SGD, **10** is nevertheless converted by *C. roseus in vivo* into several downstream alkaloid products.[12] We envision that the levels of these alkaloids will increase if a reengineered variant of SGD that more effectively deglucosylates **10** is incorporated into the MIA pathway. Surprisingly, SGD from *C. roseus* appears to turnover vincoside, the diastereomer of strictosidine, indicating that the stereoselectivity of the MIA pathway is not maintained by SGD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

1. a) van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R. Curr. Med. Chem 2004;11:607. [PubMed: 15032608] b) O'Connor SE, Maresh JJ. Nat. Prod. Rep 2006;23:532. [PubMed: 16874388]

2. McCoy E, O'Connor SE. J. Am. Chem. Soc 2006;128:14276. [PubMed: 17076499]

3. Recent examples of enzyme engineering in the MIA pathway are: a) Chen S, Galan MC, Coltharp C, O'Connor SE. Chem. Biol 2006;13:1137. [PubMed: 17113995] b) Bernhardt P, McCoy E, O'Connor SE. Chem. Biol 2007;14:888. [PubMed: 17719488] c) Loris EA, Panjikar S, Ruppert M, Barleben L, Unger M, Schübel H, Stöckigt J. Chem. Biol 2007;14:979. [PubMed: 17884630]

4. a) Geerlings A, Ibañez MM-L, Memelink J, van der Heijden R, Verpoorte R. J. Biol. Chem 2000;275:3051. [PubMed: 10652285] b) Gerasimenko I, Sheludko Y, Ma X, Stöckigt J. Eur. J. Biochem 2002;269:2204. [PubMed: 11985599]

5. McCoy E, Galan MC, O'Connor SE. Bioorg. Med. Chem. Lett 2006;16:2475–2478. [PubMed: 16481164]

6. Strictosidine glucosidase (SGD) was expressed in *Escherichia coli* as an N-terminal maltose-binding protein (MBP) or C-terminal 6-His tag fusion using a codon optimized synthetic gene previously described in reference [5]. The MBP fusion was used for the determination of all kinetic constants.

7. Synthesis of strictosidine analogs has been previously described in reference [5]. Briefly, strictosidine analogs **1, 2, 5, 8,** and **9** were synthesized enzymatically by incubating strictosidine synthase with secologanin and the corresponding tryptamine analog followed by purification via preparative HPLC. Strictosidine analogs **3, 4, 6** and **7** were synthesized as diastereomeric mixtures chemically by incubating secologanin and the corresponding tryptamine analog in pH 2, 100 mM maleic acid. The strictosidine diastereomers were purified by preparative HPLC. Exact masses and representative NMR data of the purified products are reported in the Supplementary Material.

8. Quantitative SGD kinetic assays were performed at the reported optimal pH of the enzyme (pH 6, citrate phosphate buffer, see reference [4]). Assays were conducted at 37°C in the presence of 0.61 nM SGD. Nine or more different substrate concentrations were used for each analog, and six time points were measured for each substrate concentration. An HPLC assay was used to monitor strictosidine consumption at 238 nm with naphthalene acetic acid as an internal standard. The concentration of all strictosidine analogs was determined with a standard curve. A Logistic curve using Origin was used to fit the data, and R^2 values ranged from 0.979–0.999.

9. Hammes GG, Wu C-W. Annu. Rev. Biophys. Bioeng 1974;3:1. [PubMed: 4371650]

10. A sigmoidal curve was observed for SGD expressed as both an N-terminal maltose binding protein fusion and as a C-terminal His-tag fusion indicating that a specific affinity tag does not alter the kinetic parameters significantly.

11. Barleben L, Panjikar S, Ruppert M, Koepke J, Stöckigt J. Plant Cell 2007;19:2886. [PubMed: 17890378]

12. See Galan MC, McCoy E, O'Connor SE. Chem. Comm 2007:3249–3251. [PubMed: 17668090].Briefly, a pentynyl secologanin derivative (500 μM) was incubated with a *C. roseus* hairy root culture. After two weeks, alkaloids were extracted from the cell cultures and analyzed by MS and NMR to demonstrate that the pentynyl ester had been incorporated into the MIA biosynthetic pathway.

13. Vincoside and deuterated vincoside were synthesized by incubating tryptamine or β,β-D_2 tryptamine with secologanin in pH 2, 100 mM maleic acid for 12 hours at 37°C followed by purification via preparative HPLC. MS and NMR data for this compound are shown in the Supplementary Material.

14. To further validate the activity of SGD, the use of a glucose detection reagent was used to validate that glucose was produced in the presence of SGD.Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland R. Anal. Biochem 1997;253:162. [PubMed: 9367498]

15. Patthy-Lukáts Á, Kocsis Á, Szabó L, Podányi B. J. Nat. Prod 1999;62:1492. [PubMed: 10579859]
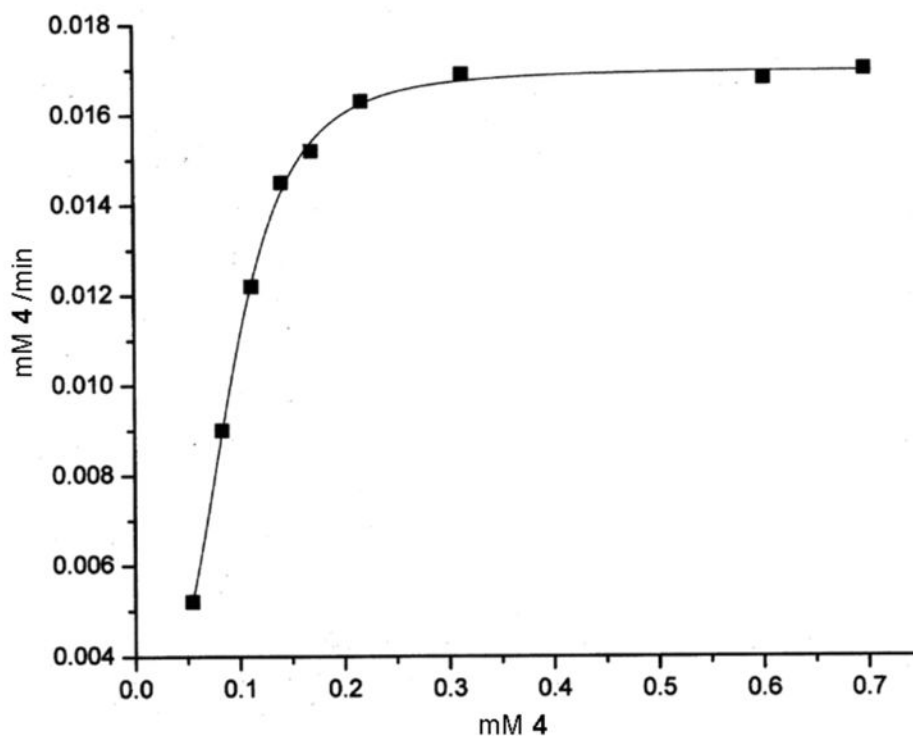
Figure 1.
Representative kinetic data for SGD. The kinetic data for all substrates tested (substrate **4** shown above) fit a sigmoidal curve. The equation used was $y = A_2 + (A_1 - A_2)/[1+ (x/x_o)^p]$. A_2 was used as the V_{max} value, and x_o was used as $K_{0.5}$. All substrates had a Hill coefficient of 1.8 or greater.
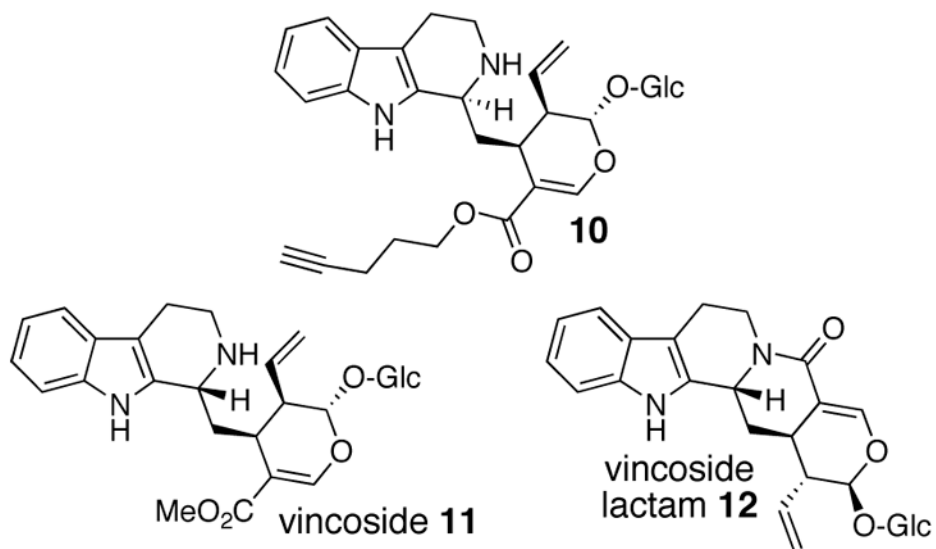
Figure 2.
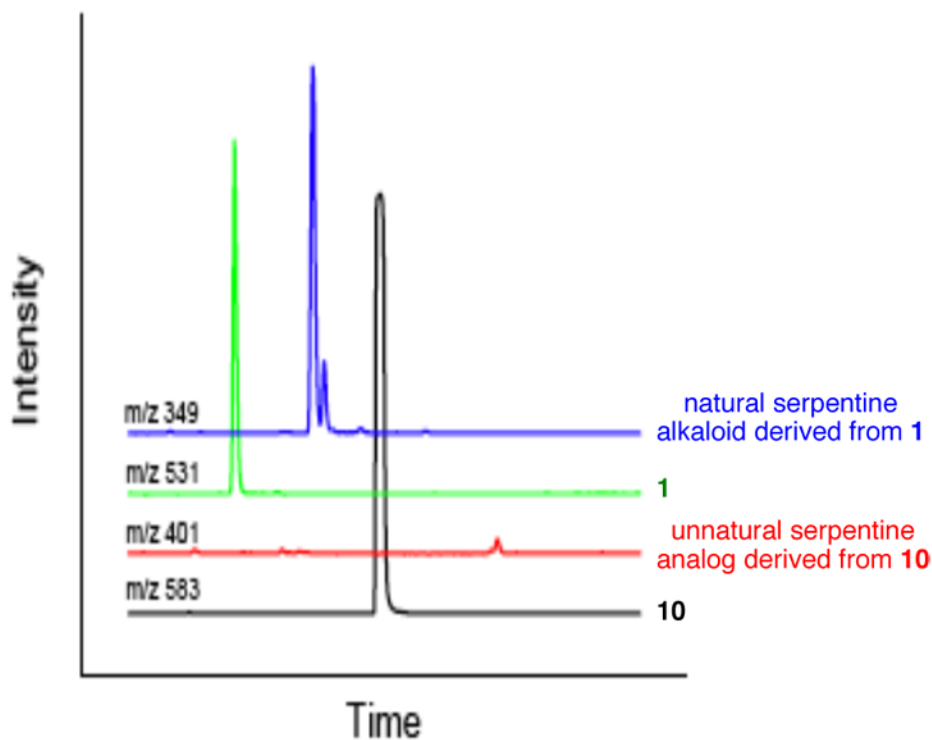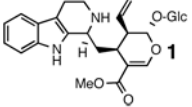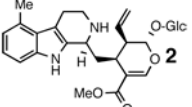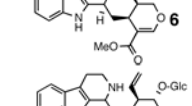Structures of additional analogs **10** and **11** turned over by SGD. Compound **12** forms spontaneously from **11**.
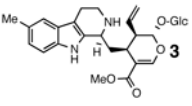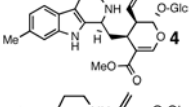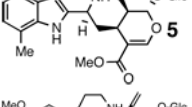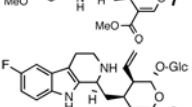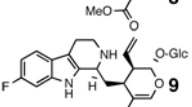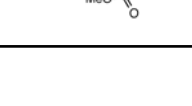
Figure 3.
LC-MS traces of extracts from *C. roseus* root culture incubated with a pentynylated secologanin substrate analog. More pentynyl strictosidine analog **10** (*m/z* 583, black trace) accumulates relative to the final alkaloid analog product pentynyl serpentine (*m/z* 401, red trace). In contrast, less natural strictosidine **1** (*m/z* 531, green trace) is observed relative to natural serpentine alkaloid (*m/z* 349, blue trace). See reference [12] for detailed structural characterization of the alkaloid products.
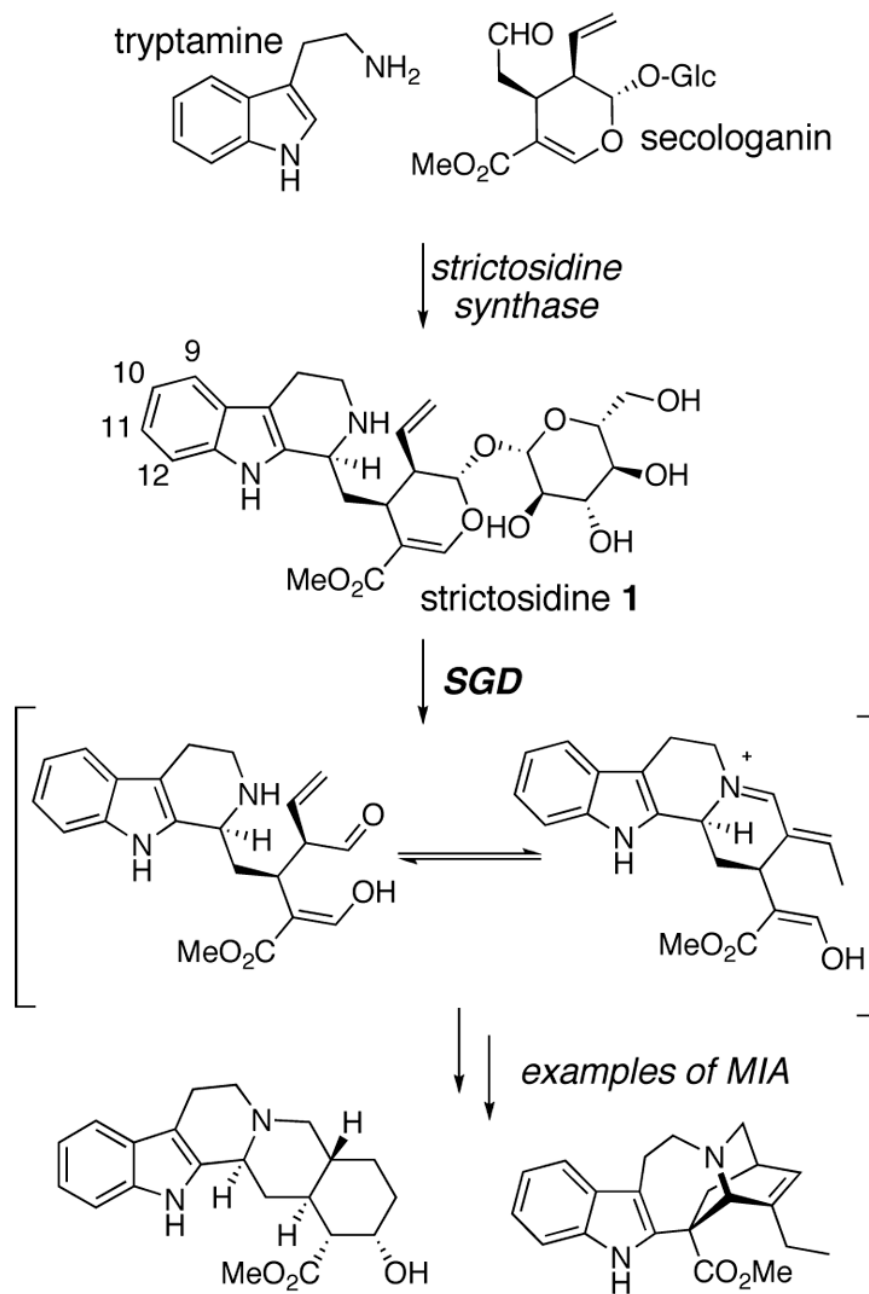
Scheme 1.
Strictosidine **1** is deglycosylated by strictosidine glucosidase (SGD) to form a reactive intermediate that is the precursor for thousands of monoterpene indole alkaloid products.

Table 1

Steady state kinetic parameters for strictosidine analogs **1–9**. See reference [8] for a description of the enzyme assay.

Analogs	$K_{0.5}$ (mM)	V_{max} (mM/min)	relative V_{max}/$K_{0.5}$
	0.22	0.078	1
	0.22	0.018	0.2
	0.071	0.016	0.6
	0.092	0.017	0.5
	0.14	0.016	0.3
	0.37	0.035	0.3
	0.43	0.037	0.2
	0.15	0.038	0.7
	0.12	0.032	0.7