Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 16 (2006) 2475–2478
Bioorganic & Medicinal Chemistry Letters

Substrate specificity of strictosidine synthase

Elizabeth McCoy, M. Carmen Galan and Sarah E. O'Connor*

Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA, 02139, USA

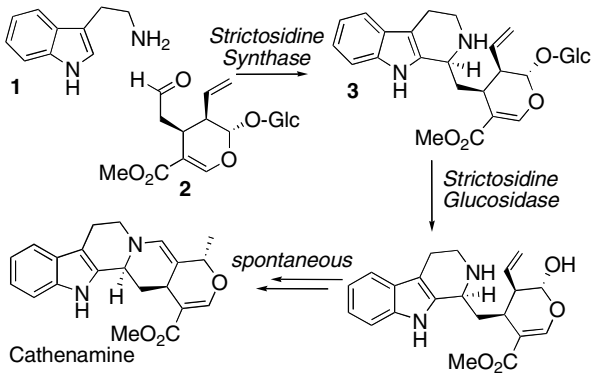
Received 6 January 2006; revised 20 January 2006; accepted 20 January 2006
Available online 14 February 2006

Abstract—Strictosidine synthase catalyzes a Pictet–Spengler reaction in the first step in the biosynthesis of terpene indole alkaloids to generate strictosidine. The substrate requirements for strictosidine synthase are systematically and quantitatively examined and the enzymatically generated compounds are processed by the second enzyme in this biosynthetic pathway.

The terpene indole alkaloids are a structurally complex class of alkaloids used for a variety of medicinal purposes such as anti-cancer and anti-hypertensive agents.[1] In the first committed step of the terpene indole alkaloid biosynthetic pathway, tryptamine (**1**) and secologanin (**2**) react to form strictosidine (**3**) by the enzyme strictosidine synthase through a stereoselective Pictet–Spengler condensation (Scheme 1).[2–5] Strictosidine is further modified by strictosidine glucosidase to yield an intermediate that rearranges in vitro to form cathenamine (Scheme 1).[6,7]

Strictosidine synthase was first isolated thirty years ago, and the enzyme has been cloned, heterologously expressed,[3,2,8–15] and structural analysis of this enzyme is in progress.[16] Although these biosynthetic enzymes may provide an opportunity for production of novel alkaloid derivatives, a firm understanding of the parameters involved in substrate recognition is needed to take advantage of this complex pathway. A systematic and quantitative analysis of strictosidine synthase substrate specificity was therefore initiated.

Enzyme activity of strictosidine synthase (*Catharanthus roseus*) was measured by HPLC, in which the amine starting material disappearance and product formation were monitored by UV. K_m and k_{cat} values were calculated for a selection of the most active amine substrates using strictosidine synthase expressed in *Escherichia coli*. This selection of substrates yielded a 40-fold variation in



Scheme 1. First committed steps of terpene indole alkaloid biosynthesis.

k_{cat} and over 100-fold variation in K_m. In all cases, product formation was assessed by the appearance of a new peak on an HPLC chromatogram that co-eluted with a chemically synthesized (mixture of diastereomers) standard and displayed the correct molecular weight by ESI mass spectrometry.[17] Non-enzymatic reactions were not observed during these assays, which were conducted at neutral pH and micromolar to low millimolar substrate concentrations.

Amine substrate specificity.[18] Notably, strictosidine synthase could synthesize alternative heterocyclic derivatives, utilizing both the 3-(2-aminoethyl)-benzofuran (**4**) and benzothiophene (**5**) analogs, though at a diminished rate relative to the tryptamine **1** substrate. Although the low activity of the thiophene substrate precluded a quantitative comparison of **4** and **5**, the rate of reaction of benzothiophene **5** is significantly

Keywords: Alkaloids; Biosynthesis; Enzyme catalysis; Glycosidase; Pictet–Spengler.
* Corresponding author. Tel.: +1 617 324 0180; fax: +1 617 324 0505; e-mail: soc@mit.edu

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.01.098

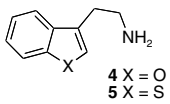
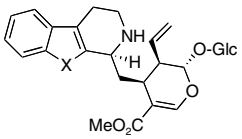
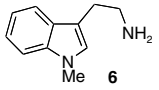
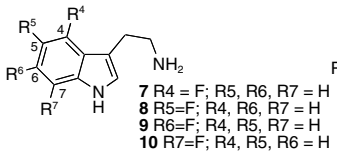
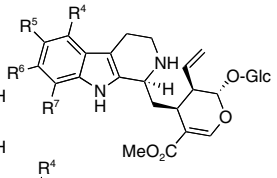
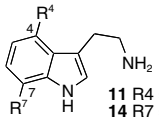
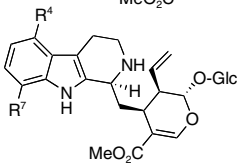
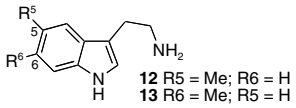
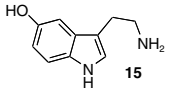
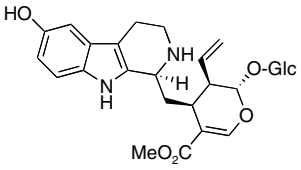
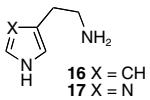
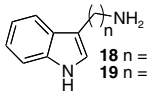
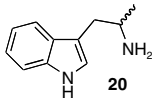
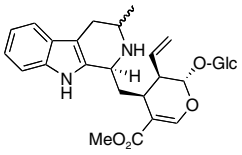
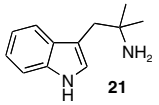
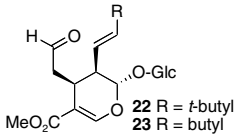
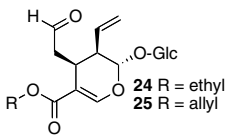
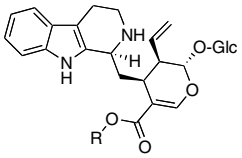
slower than that of benzofuran **4**. The benzofuran **4** exhibits a K_m value close to that of tryptamine, but displays a significantly reduced k_{cat}, suggesting that the electron-deficient nature of the benzofuran ring slows catalysis (Table 2). *N*-Methyl tryptamine **6** (Table 1) is not a competent substrate, suggesting that the enzyme tolerates only small steric perturbations at the indole nitrogen.

To further explore the effect of electron density on catalysis, the indole ring was substituted with electron-withdrawing substituents (fluoro = F) at each of the indole ring positions (Tables 1 and 2). Substitution with a fluoro moiety results in a decrease in k_{cat} in each case, again suggesting that the enzymatic reaction is inherently dependent on the electron density of the amine substrate. Although the k_{cat}/K_m ratios for all fluorinated derivatives **7**–**10** are comparable, the K_m for 4-fluoro-substituted tryptamine **7** is approximately 3- to 5-fold higher than the K_m value for the other fluorinated derivatives. However, the k_{cat} for the 4-fluoro derivative is also significantly higher than those of the other fluorotryptamine substrates, particularly those of the 5- and 6-fluoro derivatives (substrates **8** and **9**). Interestingly, the rate of the chemically catalyzed Pictet–Spengler reaction between 4- or 7-fluorotryptamine (compounds **7** or **10**) and secologanin **2** is significantly slower than that of the same reaction with 6- or 5-fluorotryptamine (compounds **8** or **9**) (data not shown). Clearly, the position of the electron-withdrawing group on the indole ring has a significant effect on enzymatic catalysis, and future experiments are directed at deciphering the reasons behind this observation.

Early qualitative studies with several tryptamine derivatives indicated that limited substitution on the indole ring was tolerated.[2] To systematically quantify the effect of indole ring substitution on K_m and k_{cat}, each position of the indole ring was substituted with a methyl group (compounds **11**–**14**, Table 1) and the kinetic parameters of active substrates were measured (Table 2). Reactivity of substrates with methyl substitutions in the 4 (compound **11**) and 7 (compound **14**) indole positions was active, while substrates with substitutions at the 5 (compound **12**) and 6 (compound **13**) positions (Tables 1 and 2) were not active (Fig. 1). The K_m for the 4-substituted tryptamine analog **11** was approximately 2-fold lower than the K_m for 7-substituted compound **14**, while substrates with methyl moieties in the 5 and 6 positions—compounds **12** and **13**—were completely inactive. Substitution with a hydroxyl group in the 5 position (compound **15**, Tables 1 and 2) did yield an active substrate, though the K_m was the highest measured in this series—a 60-fold increase compared to the native substrate tryptamine. Therefore, strictosidine synthase does not readily tolerate substitution at positions 5 and 6 of tryptamine and is most tolerant of substitutions at positions 4 and 7 (Fig. 1).

The 2-pyrrole-3-ethylamine analog (**16**) along with the isosteric histamine (**17**) were not turned over by strictosidine synthase, indicating that the benzyl moiety is absolutely required for recognition by the enzyme (Table 1).

Table 1. Substrates tested with strictosidine synthase

Unnatural substrate	Strictosidine analog
 **4** X = O **5** X = S	
 **6**	No reaction
 **7** R4 = F; R5, R6, R7 = H **8** R5=F; R4, R6, R7 = H **9** R6=F; R4, R5, R7 = H **10** R7=F; R4, R5, R6 = H	
 **11** R4 = Me; R7 = H **14** R7 =Me; R4 = H	
 **12** R5 = Me; R6 = H **13** R6 = Me; R5 = H	No reaction
 **15**	
 **16** X = CH **17** X = N	No reaction
 **18** n = 1 **19** n = 3	No reaction
 **20**	
 **21**	No reaction
 **22** R = *t*-butyl **23** R = butyl	No reaction
 **24** R = ethyl **25** R = allyl	

It was previously established that tryptophan, phenylethylamine, and tyramine are not accepted by strictosidine synthase,[2] and since pyrrole substrates are also not tolerated, we conclude that the basic indole

framework is required for recognition by this enzyme. Interestingly, the only other sequenced 'Pictet–Spenglerase' (norcoclaurine synthase), which utilizes tyrosine derived amine and aldehyde substrates, exhibits no sequence homology to strictosidine synthase.[19]

Strictosidine synthase proved to have stringent requirements for the side chain of tryptamine, as evidenced by the lack of turnover exhibited by 3-methylamine-indole (**18**) and 3-propylamine-indole (**19**) (Table 1).[20] However, 2-(1-methyl)-ethylaminoindole (±-α-methyltryptamine, **20**, Table 1) yielded a small amount of product. No product formation was observed with the bulkier α-di-methyltryptamine (**21**) (Table 1).

Aldehyde substrate specificity.[21] To investigate the aldehyde substrate specificity of strictosidine synthase, two of the key functional groups of the secologanin substrate were modified and then utilized in enzyme assays. A streamlined gram-scale isolation protocol of secologanin from a local source of *Lonicera tatarica* enabled a semisynthetic approach to yield secologanin derivatives.[22] Olefin cross-metathesis was used to introduce a variety of alkyl groups at the vinyl position of secologanin (i.e., compounds **22** and **23**, Table 1). However, these bulkier groups at the vinyl position completely prevented turnover by strictosidine synthase (Table 1). In contrast, trans-esterification at the methyl ester with larger alkyl groups (compounds **24** and **25**) gave substrates that were turned over by the enzyme to yield the corresponding strictosidine analogs, suggesting that this is a more promising position for derivatization.

While these results suggest that strictosidine synthase can produce a range of strictosidine analogs, it remained to be established whether these intermediates can be processed by the downstream terpene indole alkaloid machinery to produce novel, biologically active alkaloids. In the next step of the pathway, a dedicated glucosidase hydrolyzes the glycosidic linkage of strictosidine which rearranges in vitro to form cathenamine (Scheme 1).[6,7,23] Therefore, the first two enzymes construct the basic 5-ring framework of the corynanthe alkaloids. The corynanthe framework is an important pharmaceutical scaffold,[24–26] and derivatization of these molecules with substitutions on the indole or secologanin moieties may result in interesting new compounds with useful biological properties.

Table 2. Kinetic parameters for the most highly active amine strictosidine synthase substrates

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1}/s^{-1})
1	7.4	0.9	2030
4	7.7	0.023	50
7	42	0.35	139
8	7.1	0.043	101
9	8.9	0.056	105
10	13	0.11	141
11	80	0.19	40
14	198	0.29	24
15	1200	0.096	1.3

k_{cat} and K_m were measured using a purified *E. coli* preparation of strictosidine synthase.

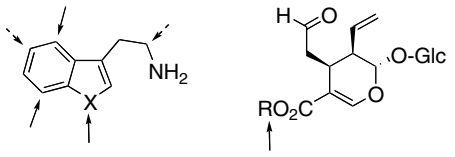


Figure 1. Solid arrows highlight the positions most amenable to substitution in the tryptamine and secologanin substrates for strictosidine synthase. Dashed arrows indicate positions for which comparatively weaker activity was observed.

All enzymatically generated strictosidine derivatives (Table 1) were incubated with the second enzyme of the pathway, strictosidine-β-glucosidase (*C. roseus*). The disappearance of the strictosidine derivative (starting material) peak by HPLC demonstrated that each of the strictosidine derivatives was processed by strictosidine glucosidase. Additionally, when the 4-fluoro and 4-methyltryptamine substrates (compounds **7** and **11**) were incubated in the presence of secologanin **2** with both enzymes, products correlating to the molecular weight of cathenamine analogs were observed when analyzed by mass spectroscopy; cathenamine is the predicted product of this deglycosylation reaction with tryptamine and secologanin.[27] While our data are consistent with the formation of cathenamine analogs as is reported in the literature,[6] isolation and spectroscopic characterization of these deglycosylated strictosidine analogs will provide further structural confirmation. These results suggest that the substrate specificities of strictosidine synthase and glucosidase are sufficiently complementary to work in tandem to produce a variety of terpene indole alkaloid intermediate analogs.

This study systematically and quantitatively probes the substrate scope of the indole amine substrate for the first committed step of the terpene indole alkaloid pathway. This enzyme catalyzes a Pictet–Spengler condensation, which, although widely utilized in alkaloid biosynthetic pathways, remains a largely unexplored enzymatic reaction. Semisynthetic efforts have also yielded secologanin derivatives that are turned over by strictosidine synthase. The dedicated glucosidase for this pathway is able to turnover strictosidine analogs generated by strictosidine synthase, suggesting that strictosidine synthase and strictosidine glucosidase can work in concert to utilize 'unnatural' substrates to generate analogs of alkaloid intermediates.

Acknowledgments

We gratefully acknowledge financial support from the Smith Family Medical Foundation, the American Chemical Society Petroleum Research Fund (41727-G4), 3M, Amgen Inc, and MIT.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.01.098.

References and notes

1. van der Heijden, R.; Jacobs, D. I.; Snoeijer, W.; Didier, H.; Verpoorte, R. *Curr. Med. Chem.* **2004**, *11*, 607.
2. Treimer, J. F.; Zenk, M. H. *Eur. J. Biochem.* **1979**, *101*, 225.
3. Mizukami, H.; Nordlov, H.; Lee, S.-L.; Scott, A. I. *Biochemistry* **1979**, *18*, 3760.
4. Treimer, J. F.; Zenk, M. H. *FEBS Lett.* **1979**, *97*, 159.
5. Kutchan, T. M. *Phytochemistry* **1993**, *32*, 493.
6. Gerasimenko, I.; Sheludko, Y.; Ma, X.; Stockigt, J. *Eur. J. Biochem.* **2002**, *269*, 2204.
7. Geerlings, A.; Ibanez, M. M.-L.; Memelink, J.; Van der Heijden, R.; Verpoorte, R. *J. Biol. Chem.* **2000**, *275*, 3051.
8. Pfitzner, U.; Zenk, M. H. *Methods Enzymol.* **1987**, *136*, 342.
9. Kutchan, T. M.; Hampp, N.; Lottspeich, F.; Beyreuther, K.; Zenk, M. H. *FEBS Lett.* **1988**, *237*, 40.
10. Pennings, E. J.; van den Bosch, R. A.; van der Heijden, R.; Stevens, L. H.; Duine, J. A.; Verpoorte, R. *Anal. Biochem.* **1989**, *176*, 412.
11. McKnight, T. D.; Roessner, C. A.; Devagupta, R.; Scott, A. I.; Nessler, C. *Nucleic Acids Res.* **1990**, *18*, 4939.
12. Bracher, D.; Kutchan, T. M. *Arch. Biochem. Biophys.* **1992**, *294*, 717.
13. Roessner, C. A.; Devagupta, R.; Hasan, M.; Williams, H. J.; Scott, I. A. *Protein Expr. Purif.* **1992**, *3*, 295.
14. Kutchan, T. M.; Bock, A.; Dittrich, H. *Phytochemistry* **1994**, *35*, 353.
15. de Waal, A.; Meijer, A. H.; Verpoorte, R. *Biochem. J.* **1995**, *306*, 571.
16. Although a structure is not yet reported, the enzyme has been crystallized. Koepke, J.; Ma, X.; Fritzsch, G.; Michel, H.; Stoeckigt, J. *Acta Crystallogr., Sect. D* **2005**, *D61*, 690.
17. The masses of product peaks that resulted from the reaction of secologanin and the following amines were: **4** ($[M+H]^+$ expect. 532.2, obsd 532.3); **5** ($[M+H]^+$ expect. 548.2, obsd 548.3); **7** ($[M+H]^+$ expect. 549.2, obsd 549.2); **8** ($[M+H]^+$ expect. 549.2, obsd 549.4); **9** ($[M+H]^+$ expect. 549.2, obsd 549.2); **10** ($[M+H]^+$ expect. 549.2, obsd 549.2); **11** ($[M+H]^+$ expect. 545.2, obsd 545.2); **14** ($[M+H]^+$ expect. 545.2, obsd 545.4); **15** ($[M+H]^+$ expect. 547.2, obsd 547.4); **20** (both diastereomers) ($[M+H]^+$ expect. 545.2, obsd 545.3). Product peaks that resulted from the reaction of tryptamine and the following aldehydes were: **24** ($[M+H]^+$ expect. 545.3, obsd 545.7); **25** ($[M+H]^+$ expect. 557.2, obsd 557.2). Synthesis of authentic standards (diastereomeric mixtures) and evaluation of chemical reaction rates was performed by reacting amine and aldehyde substrates (20 mM concentration) under aqueous conditions (150 mM maleic acid, pH 2).
18. Compounds **11** and **13** were synthesized from the corresponding commercially available indole-3-carbaldehydes as previously reported. (Harada, H.; Hirokawa, Y.; Suzuki, K.; Hiyama, Y.; Oue, M.; Kawashima, H.; Yoshida, N.; Furutani, Y.; Kato, S. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1301.) Substrates **10** and **7** were synthesized from the corresponding commercially available indoles as previously described. (Chen, Z.; Cohen, M. P.; Fisher, M. J.; Giethlen, B.; Gillig, J. R.; McCowan, J. R.; Miller, S. C.; Schaus, J. M. Preparation of *N*-(2-arylethyl)benzylamines as antagonists of the 5-HT6 receptor.: USA, 2002; p 216.) Benzofuran (**4**) and benzothiophene (**5**) derivatives were generated from reduction of the commercially available nitrile compounds. (Shafiee, A.; Mohamadpour, M. *J. Heterocycl. Chem.* **1978**, *15*, 481.) The pyrrole ethanamine compound (**16**) was synthesized as described. (Wasley, J. W. F.; Hamdan, A. *Synth. Commun.* **1985**, *15*, 71.) All other tryptamine analogs were commercially available.
19. Samanani, N.; Liscombe, D. K.; Facchini, P. J. *Plant J.* **2004**, *40*, 302.
20. Mewshaw, R. E.; Zhou, D.; Zhou, P.; Shi, X.; Hornby, G.; Spangler, T.; Scerni, R.; Smith, D.; Schechter, L. E.; Andree, T. H. *J. Med. Chem.* **2004**, *47*, 3823.
21. Synthesis of these derivatives is described in Galan, M. C.; O'Connor, S. E. *Tetrahedron Lett.* **2006**, *47*, 1563.
22. Secologanin was isolated from *L. tatarica* following a modification of a described procedure (Kinast, G.; Tietze, L. F. *Chem. Ber.* **1976**, *109*, 3640) Leaves and stems (100 g) of *L. tatarica* were ground in a blender with methanol (200 mL). The slurry stood for 50 min and was then filtered over cheesecloth. This process was repeated until the pulp was a light brown color. The methanol filtrate (1 L) was evaporated to dryness and the resulting green syrup was subjected to silica gel column chromatography (CH_2Cl_2–CH_3OH, 9:1). Fractions containing secologanin were pooled, concentrated, and the resulting solid was further purified by C18 column chromatography (H_2O–CH_3OH, 7:3) to yield purified secologanin (1 g). NMR and mass spectral data matched previously reported values.
23. Barleben, L.; Ma, X.; Koepke, J.; Peng, G.; Michel, H.; Stoeckigt, J. *Biochim. Biophys. Acta* **2005**, *1747*, 89.
24. Roquebert, J.; Demichel, P. *Eur. J. Pharmacol.* **1984**, *106*, 203.
25. Li, S.; Long, J.; Ma, Z.; Xu, Z.; Li, J.; Zhang, Z. *Curr. Med. Res. Opin.* **2004**, *20*, 409.
26. Moreno, P. R. H.; Van der Heijden, R.; Verpoorte, R. *Plant Cell Tissue Org. Cult.* **1995**, *42*, 1.
27. Substrate **7** ($[M+H]^+$ expect. 369.4, obsd 369.5) and **11** ($[M+H]^+$ expect. 365.4, obsd 365.5).