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Brief Communication

Structure-Based Engineering of Strictosidine Synthase: Auxiliary for Alkaloid Libraries

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SUMMARY

The highly substrate-specific strictosidine synthase (EC 4.3.3.2) catalyzes the biological Pictet-Spengler condensation between tryptamine and secologanin, leading to the synthesis of about 2000 monoterpenoid indole alkaloids in higher plants. The crystal structure of *Rauvolfia serpentina* strictosidine synthase (STR1) in complex with strictosidine has been elucidated here, allowing the rational site-directed mutation of the active center of STR1 and resulting in modulation of its substrate acceptance. Here, we report on the rational redesign of STR1 by generation of a Val208Ala mutant, further describing the influence on substrate acceptance and the enzyme-catalyzed synthesis of 10-methyl- and 10-methoxystrictosidines. Based on the addition of strictosidine to a crude strictosidine glucosidase preparation from *Catharanthus* cells, a combined chemoenzymatic approach to generating large alkaloid libraries for future pharmacological screenings is presented.

INTRODUCTION

The Pictet-Spengler condensation is a well known reaction required for the chemical synthesis of alkaloids [1] and represents the first dedicated step of indole alkaloid biosynthesis (Figure 1). Characterization of this reaction at the enzymatic level has so far been carried out for three structurally diverse and therapeutically potent alkaloid families. The first of these consists of about 6000 isoquinoline structures, including morphine and codeine; the second is known as the monoterpenoid indole alkaloids, comprising about 2000 structurally complex and important members, including the anticancer agents vinblastine and vincristine; the third is a small family that encompasses the monoterpenoid isoquinolines such as the emetic drug emetine. Strictosidine synthase catalyzes the Pictet-Spengler condensation of tryptamine and secologanin and represents one of the most highly characterized enzymes of alkaloid biosynthesis, as strictosidine plays an important role as a central precursor in the biosynthesis of almost all plant monoterpenoid indole alkaloids (Figure 1). Biochemical studies have clearly demonstrated a pronounced substrate specificity and complete enantioselectivity of the enzyme, leading exclusively to 3α-(*S*)-strictosidine [2].

Strictosidine synthase has been detected and biochemically characterized from cell suspension cultures of the Apocynaceae plant *Catharanthus roseus* [2, 3], the Indian medicinal plant *Rauvolfia serpentina* [4], as well as from the Rubiaceae *Ophiorrhiza pumila* [5]. The cDNA of STR1 has been cloned from *R. serpentina* and has been shown to be actively expressed in *Escherichia coli*, yeast, and insect cells [6, 7]. Moreover, to our knowledge, it is the first example of an enzyme involved in alkaloid biosynthesis being overexpressed and purified in milligram quantities. Amino acid sequence comparison has yielded limited information concerning the reaction mechanism and specific substrate acceptance of STR1. In order to analyze the molecular structure of the synthase, the structure of the native enzyme as well as its complexes with its natural substrates tryptamine and secologanin have been recently determined, providing the first, to our knowledge, information on the active site and preliminary insight into the mechanism of the synthase [8–10].

In order to design STR1 mutants that can accept various derivatives of tryptamine and produce corresponding strictosidine derivatives, we solved the crystal structure of *Rauvolfia*-STR1 in complex with its product 3α-(*S*)-strictosidine. Based on the structure obtained, we carried out rational site-directed mutagenesis experiments; this strategy provided the opportunity to modulate the substrate specificity of STR1, with the aim to develop combinatorial biomimetic approaches for the generation of novel and large alkaloid libraries, containing thousands of new products, by engineering the wild-type enzyme. The current paper thus describes the crystal structure of STR1 in complex with strictosidine and mutational studies for

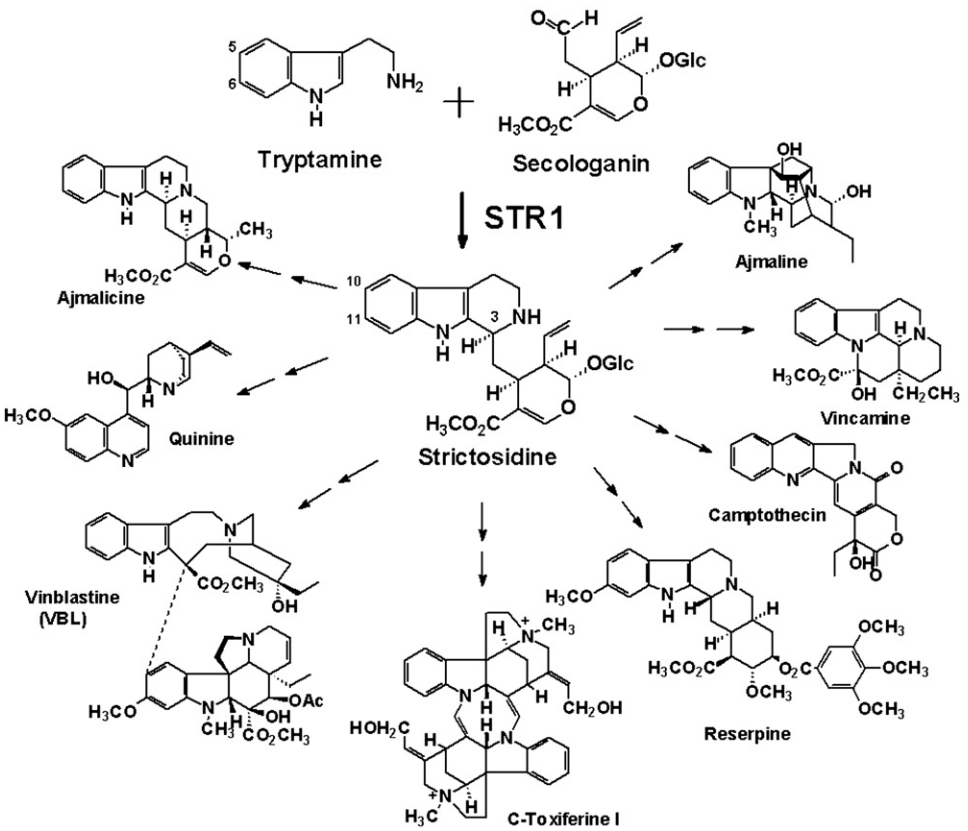

Figure 1. Strictosidine as the Central Intermediate in Indole Alkaloid Biosynthesis

Strictosidine synthase (STR1) catalyzes the enantioselective condensation of tryptamine and secologanin, leading to 3-α(S)-strictosidine—a central reaction in the biosynthesis of the entire family of monoterpenoid indole alkaloids in plants.

modulation of STR1 activity, exemplifying how a divergent biomimetic approach can be used to generate large sets of novel alkaloids. In addition, the structure of the enzyme complex also provides information on residues interacting with the remaining part of the strictosidine molecule, which is important for the future generation of additional enzyme mutants.

RESULTS AND DISCUSSION

Crystal Structure of the STR1-Strictosidine Complex

The crystal structure of the native *Rauvolfia*-STR1 has been previously solved [10]. The overall architecture of STR1 resembles a six-bladed β propeller fold. All six blades are radially arranged around a pseudo 6-fold-symmetry axis, and each blade contains a twisted, four-stranded antiparallel β sheet. The active site is located near the pseudo 6-fold-symmetry axis (Figure 2A). The crystal structure of STR1 in complex with strictosidine at a resolution of 3.0 Å provides further detailed information on the architecture of the active site (Figure 2A). As shown in Figure 2B, the indole ring of the tryptamine unit is located in the hydrophobic pocket, which is lined by 6 residues (Phe226, Val208, Val167, Trp149, Tyr151, including Gly210). The 5 hydrophobic residues are either invariant or conservatively substituted by other hydrophobic amino acids in the enzyme from different plant species.

The tryptamine part of the strictosidine molecule is stacked between 2 aromatic residues, Tyr151 and Phe226, which we have called the "indole sandwich." The sandwich appears to play an important role in keeping the indole ring in place via π-π interaction with the aromatic residues and via van der Waals interactions with the 3 other hydrophobic amino acids, Val208, Val167, and Trp149. All of these residues are conserved in presently known STR sequences (see the Supplemental Data available with this article online). The latter three amino acids shield the steric positions 10 and 11 of strictosidine (5 and 6 positions, respectively, in tryptamine), in particular, the Val208 residue (Figure 2C). Tryptamine derivatives with bulky groups at these positions therefore act as poor substrates. In fact, four substrate specificity studies with a number of putative tryptamine derivatives, performed with strictosidine synthase from *Catharanthus* [3, 11] and *Rauvolfia* [4, 10], underlined such an effect. Those tryptamine derivatives with relatively small substituents (fluoro, hydroxyl) at these positions are, in principle, accepted, whereas the substrate 6-methoxytryptamine showed much lower transformation (~2% relative enzyme activity

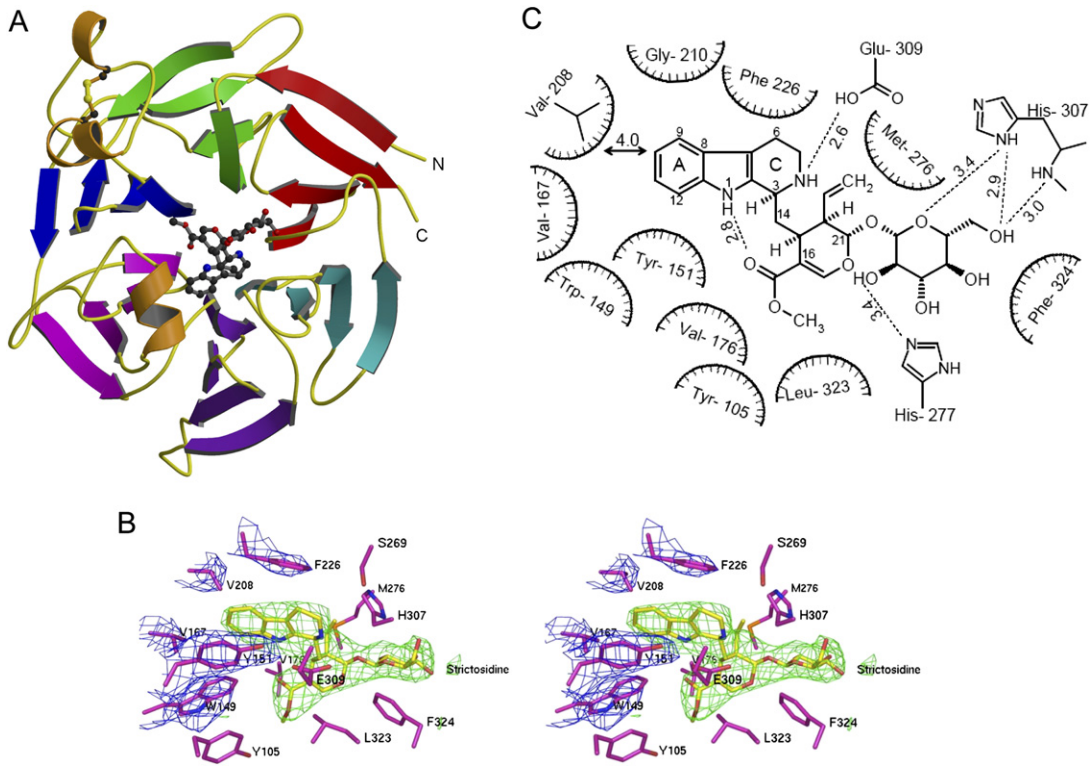

Figure 2. Strictosidine at the Active Site of STR1

(A) Ribbon diagram of the STR1-strictosidine complex. Each blade of the propeller is shown in a different color. The strictosidine is shown in ball and stick.

(B) Stereo view of the strictosidine-binding site region of the STR1-strictosidine complex. Residues within 4 Å of strictosidine are shown in purple, and strictosidine is shown in yellow. The ($F_o - F_c$) SIGMAA-weighted electron density of strictosidine contoured at 3.1 σ is shown in green. The ($F_o - F_c$) SIGMAA-weighted electron densities of Val208A and that of other hydrophobic residues around the indole part of the strictosidine molecule, contoured at 2 σ, are shown in blue. Tyr151 and Phe226 represent the indole sandwich.

(C) 2D representation of the STR1-strictosidine complex highlighting Val208 and the hydrogen bond network in a distance of < 4.1 Å.

compared to tryptamine), and 5-methoxy- or 5-methyltryptamine is not accepted by native STR1. Positions 5 and 6 of tryptamine seem to be, however, crucial for pharmacological activity, and a number of valuable drugs, such as vinblastine, vincristine, reserpine, or quinine, harbor a methoxy group at these positions.

Mutational Studies for Modulation of STR1 Activity

Based on the STR1-strictosidine complex, it was noted that a methoxy group at the 5 position of tryptamine would be in close proximity to Val208. In order to design a novel STR1 mutant that can, in contrast to the wild-type, accept 5-methoxy- or 5-methyltryptamine as a substrate, the bulky Val208 was replaced by Ala. The resulting mutant, STR1-Val208Ala, exhibits conversion of 5-methoxy- and 5-methyltryptamine to the corresponding substituted strictosidine (Table 1) in the presence of secologanin, which was confirmed by using HPLC, LC-MS, ^{1}H-, and ^{13}C-NMR (Supplemental Data). Kinetic and turnover studies also showed more efficient conversion of 6-methyl- and 6-methoxytryptamine by the mutant compared to the native enzyme (Supplemental Data). Remarkably, the K_M value of 5-hydroxytryptamine is much lower for the mutant than the wild-type STR1. For other mutants of STR1, their activities, and their future use to generate an expanded N-heteroyohimbine alkaloid library, see the Supplemental Data.

Substitution of amino acid Val208 highlights the critical role of this residue in substrate recognition at the indole moiety of strictosidine (Table 1). A different approach (saturation mutagenesis) with strictosidine synthase from *C. roseus* also points to an important role for the corresponding valine residue in substrate recognition (S. O'Connor, personal communication). The new STR1 mutant Val208Ala retains its enantioselectivity. ^{1}H-NMR analysis of the 10-methylstrictosidine lactam-tetraacetate obtained from methylated strictosidine by base-catalyzed lactamization and acetylation showed that one of the four acetyl signals was significantly shifted to a higher field (1.18 ppm compared to the remaining three signals between 1.86 and 2.05 ppm, see Supplemental Data), clearly indicating the 3α-(S)-configuration [2]. Moreover, the complex structure of STR1-strictosidine provides knowledge about residues interacting with the remaining part of strictosidine, which is important for the design of mutants, and takes the secologanin part into consideration.

Table 1. Kinetic Data for Conversion of Tryptamine and Its 5- and 6-Substituted Derivatives for the Wild-Type and Mutant STR1

Substrates	Enzymes	K_M (mM)	K_{cat} (s^{-1})	K_{cat}/K_M ($mM^{-1}s^{-1}$)
Tryptamine	Wild-type	0.072 (±0.02)	10.65	147.92
	Val208Ala	0.219 (±0.04)	54.09	246.99
5-Methyl-tryptamine	Wild-type	n.d.	—	—
	Val208Ala	0.281 (±0.13)	6.56	23.35
5-Methoxy-tryptamine	Wild-type	n.d.	—	—
	Val208Ala	3.592 (±1.12)	79.66	22.18
6-Methyl-tryptamine	Wild-type	0.393 (±0.17)	2.32	5.90
	Val208Ala	0.762 (±0.21)	10.95	14.37
6-Methoxy-tryptamine	Wild-type	0.962 (±0.15)	5.32	5.53
	Val208Ala	0.307 (±0.01)	16.66	54.27
5-Fluoro-tryptamine	Wild-type	0.259 (±0.10)	37.46	144.63
	Val208Ala	1.302 (±0.13)	21.24	16.31
6-Fluoro-tryptamine	Wild-type	0.136 (±0.05)	23.37	171.84
	Val208Ala	0.356 (±0.09)	13.63	38.29
5-Hydroxy-tryptamine	Wild-type	2.255 (±1.76)	562.14	249.29
	Val208Ala	0.844 (±0.34)	18.12	21.47

Data are the average of 2–3 independent experiments. n.d. = not detectable.

Chemoenzymatic Approach

Strictosidine and its derivatives are source material for preparative structure modification. The enzyme that follows STR1 in the biosynthesis of the indole alkaloid family is strictosidine glucosidase (SG). SG delivers the highly reactive strictosidine aglycone that enters all the pathways to the 2000 monoterpenoid indole alkaloids and, in addition, can serve as an efficient building block for biomimetic generation of novel alkaloids [12, 13]. The biomimetic approach adopted here encompasses both the enzymatic and chemical reactions in "one pot" (see Experimental Procedures). It is based on the observation that, in the presence of NH_4^+ ions, N-heteroyohimbine alkaloids are formed in enzyme assays [14]. The variability of this reaction and the biochemical potential of reactive alkaloid intermediates allows the insertion of various substituents into the heteroyohimbine structure by way of a simple reaction that can be systematically applied to preparative structure modifications. The combinatorial approach has proven to be successful with different substituted primary amines (Figure 3) by using a crude enzyme extract from *C. roseus* cell suspensions starting with strictosidine. Cultivated *Catharanthus* cells synthesize a great number of indole alkaloids [15] and exhibit significant expression of SG. The versatility of this system is further exemplified by the sole requirement of the SG enzyme, making it theoretically possible to use any indole alkaloid-producing cell system, including *Rauvolfia*. The approach can now be significantly extended by STR1 and SG mutants from *R. serpentina*, by using a combination of X-substituted tryptamine derivatives and various substituted primary amines.

SIGNIFICANCE

The family of monoterpene indole alkaloids is characterized by an enormous structural variety and a wide range of pharmacological qualities. Several of them have long-standing medical applications. Due to their structural complexity, the extraction and isolation of these alkaloids from plant material is very poor, but purification from the natural source remains economically more efficient than total synthesis. The development of new production strategies for alkaloids of pharmaceutical interest is therefore a significant objective. Moreover, the generation of novel alkaloid derivatives is expected to deliver a higher number of biologically valuable compounds.

In the current approach, an easily executable biomimetic concept is presented that allows the insertion of various substitutions into the heteroyohimbine structure from a strictosidine starting point. The biochemical potential pertaining to reactive strictosidine aglyca can be systematically used for preparative structure modifications.

The crystal structure of STR1 in complex with its product provides a structural understanding of the observed substrate preference and its rational modulation. With the STR1 mutant Val208Ala, we present a re-engineered enzyme that, in contrast to a mutant

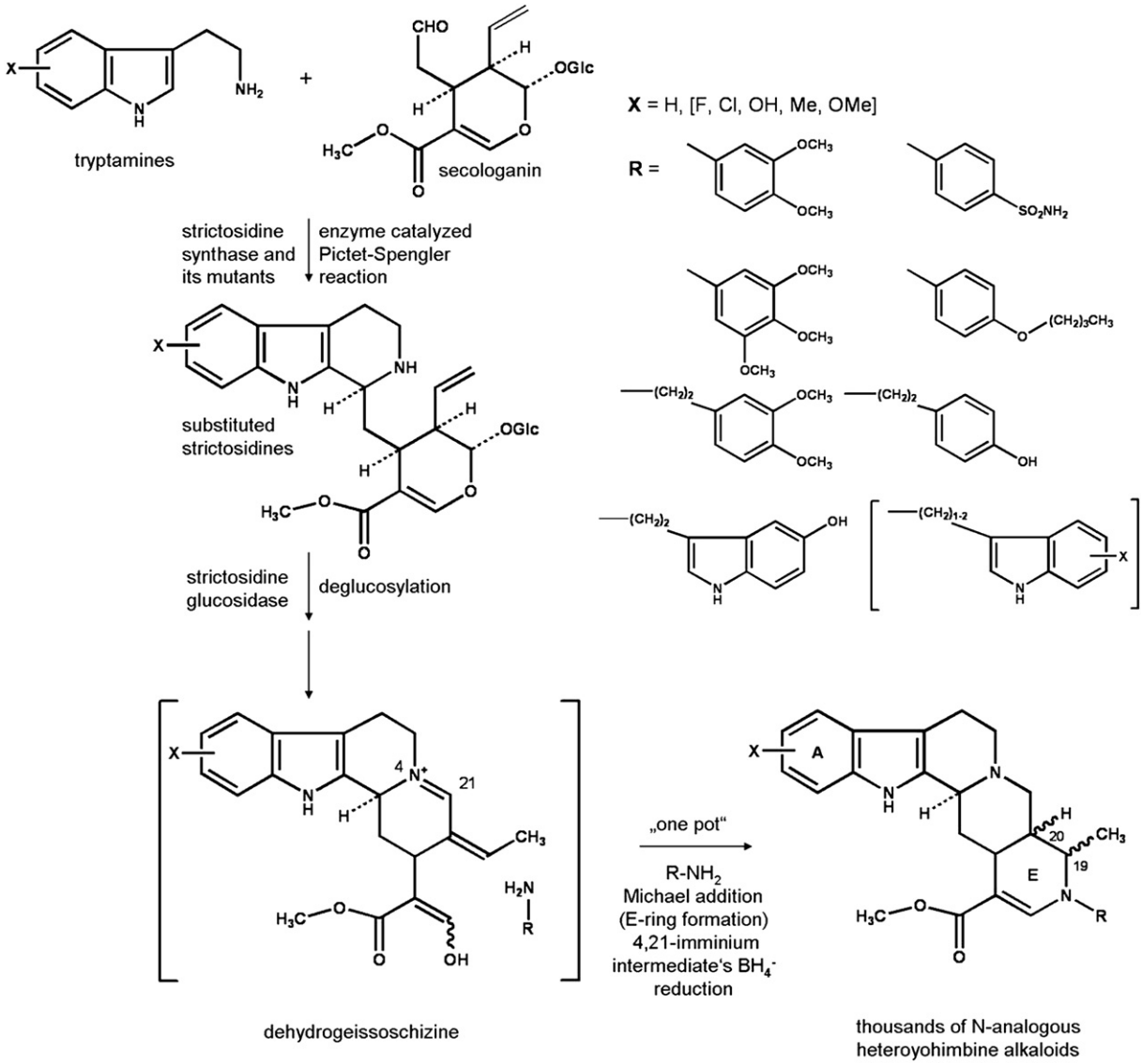

Figure 3. Chemoenzymatic Synthesis of Novel N-Analogous Heteroyohimbine Alkaloids

The illustrated combination of X with mono- and disubstitution and with R substitution (including R-X substitution) delivers thousands of new alkaloids of the heteroyohimbine type. Each "one-pot" experiment leads to four diastereomeric alkaloids (at positions 19 and 20; residue R in brackets has not yet been tested in the biomimetic approach).

accepting secologanin analogs [16], now broadens the substrate specificity for tryptamine derivatives and thus the amount of strictosidine derivatives. Strictosidine can be used as source material for further chemoenzymatic modifications.

Based on this example, we show that calculation of the substrate acceptance and engineering of novel alkaloid derivatives is practicable for the indole part of the strictosidine molecule and will be an attractive strategy for the future generation of large libraries of novel indole alkaloid analogs of potential biological value.

EXPERIMENTAL PROCEDURES

X-Ray Crystallography

The STR1 crystals were soaked for 10 min in a solution containing 25% glycerol and 3 mM strictosidine prior to flash cooling at 100K in a liquid nitrogen stream. X-ray data were collected by using synchrotron radiation at the X13 beamline of European Molecular Biology Laboratory-Hamburg. The complete data were collected to 3.0 Å resolution. The data were processed by using the programs DENZO and SCALEPACK [17]. The R_{merge} at the highest-resolution bin is 75.8%, and one of the reasons for the high value is that the data have 11.8-fold redundancy. However, the $I/\sigma(I)$ at this bin is 3.7, and with this improved signal-to-noise ratio, we used data to 3.0 Å. As the data were nonisomorphous with the native data, the structure of the STR1-strictosidine complex

structure was solved by using the molecular replacement protocol of Auto-Rickshaw, an automated crystal structure determination pipeline [18]. The high-resolution structure of STR1 (PDB code: 1FP8) was used as a search model. Within the pipeline, the program MOLREP [19] was used for molecular replacement, and the rigid-body, positional, as well as B factor refinement were performed by using the program CNS [20]. Strictosidine was built in the residual electron density by using the graphics program COOT [21]. Subsequent refinement procedures were carried out by using the program REFMAC5 [22] (see the data collection and refinement statistics in the Supplemental Data).

"One-Pot" Biomimetic Synthesis of N-Analogous Alkaloids

For a typical experiment, 100 mg (0.19 mmol) strictosidine—generated as described previously [23]—was incubated in 3 ml 0.1 M KPi buffer (pH 7.0) in the presence of 500 mg (3.1 mmol) tryptamine hydrochloride (as primary amine compound) and 10 mg crude enzyme from *C. roseus* cell suspension at 30°C for 5 hr. The resulting precipitation was separated and dissolved immediately in 5 ml methanol and reduced by excess $NaBH_4$ for 4 hr. The reaction mixture was diluted with 30 ml water and extracted four times with ethyl acetate. The organic solvent was combined and evaporated, and the remaining residue was purified by preparative TLC in a chloroform-methanol-ammonia (100:3:0.1) solvent system. The four possible C-19/C-20 stereoisomers were identified with a molecular weight of 494 each; R_f values and yields (%) were A, 0.77 (9); B, 0.72 (4); C, 0.58 (6); and D, 0.46 (16) (see the Supplemental Data).

Supplemental Data

Supplemental data describing X-ray data collection statistics and refinement of the enzyme complex strictosidine synthase-strictosidine, spectroscopic product identification (MS, NMR), determination of C-3 stereochemistry of 10-methylstrictosidine, isolation of crude enzyme for biomimetic synthesis and enzyme assay with purified STR1, the turnover rate of STR1 wild-type compared to the mutant Val208Ala, preparation of 10-methylstrictosidine (HPLC trace), sequence alignment of strictosidine synthase from different plant species, and data on additional mutants are available at http://www.chembiol.com/cgi/content/full/14/9/979/DC1/.

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Accession Numbers

Coordinates of the STR1-stricosidine complex have been deposited in the Protein Data Bank with accession code 2v91.