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Redesign of a Central Enzyme in Alkaloid Biosynthesis

Brief Communication

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Summary

Plant alkaloids exhibit a diverse array of structures and pharmaceutical activities, though metabolic engineering efforts in these eukaryotic pathways have been limited. Strictosidine synthase (STR) is the first committed step in the biosynthesis of over two thousand terpene indole alkaloids. We describe a rational redesign of the STR binding pocket to selectively accommodate secologanin substrate analogs. The mutant is selective for a substrate that can be chemoselectively derivatized. Evidence that this substrate can be processed by later steps of the terpene indole alkaloid pathway is provided. The work demonstrates that the central enzyme of this alkaloid pathway can be redesigned and that the pathway can turn over the unnatural intermediate that is generated. Modulation of the substrate specificity of enzymes of this complex pathway is therefore likely to enable metabolic engineering efforts of these alkaloids.

Introduction

Strictosidine synthase (STR) catalyzes a Pictet-Spengler reaction [1] between secologanin **1** and tryptamine **2** (Figure 1A) [2–4]. The resulting product, strictosidine **3**, is a common biosynthetic precursor for more than two thousand terpene indole alkaloids (TIAs) [5–8]. The TIAs are a diverse, structurally complex class of alkaloids with important biological activities, including, most notably, the anticancer agents vinblastine and vincristine [9].

Redesigning the substrate specificity of this central enzyme is an important step toward re-engineering the TIA pathway to produce "unnatural" natural products with novel or improved biological properties. Although STR can accommodate certain analogs of tryptamine and secologanin [10, 11], it would be desirable if the substrate selectivity for this enzyme could be expanded, or if the enzyme could be engineered to be selective for unnatural substrates. Here we describe how the STR binding pocket can be mutated to selectively accommodate secologanin substrate analogs. Specifically, the binding pocket was altered to selectively accommodate a substrate equipped with a chemoselective functional group.

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Results and Discussion

Secologanin is a key substrate in the terpene indole alkaloid biosynthetic pathway and may be the rate-limiting substrate in vivo [12]. The rearrangement of the densely functionalized secologanin molecule is responsible for the diversity of alkaloid structures [13–15]. Secologanin has also been used as an enantiomeric template to chemically synthesize alkaloid natural products [16, 17].

Secologanin analogs **4–8** modified at the methyl ester (R_1) and vinyl (R_2) positions [18] were assayed with a family of STR (*C. roseus*) mutants (Figures 2A and 2B). STR mutants were designed based on both sequence homology among STR homologs and the recently reported crystal structure of STR from *R. serpentina* (Figure 2A) [11]. The mutant enzymes were screened with substrate analogs **4–8** under competitive assay conditions containing saturating concentrations of tryptamine (2 mM) and a 1:1 mixture of secologanin (2.5 mM) and secologanin analog (2.5 mM). Product ratios and the relative initial rates of product formed under these competitive assay conditions were compared by integration of product peaks by HPLC at 280 nm.

The most favorable increase in selectivity was observed with the pentynyl secologanin derivative **4** and mutant D177A. The rate of wild-type strictosidine **3** to unnatural product formation **9** (Figure 1B) was 5.4:1 in favor of the wild-type product. However, the ratio changed to 0.66:1 strictosidine **3**:strictosidine analog **9** with the D177A mutation, indicating that the mutant preferentially turns over the bulkier, more hydrophobic substrate analog (Figure 3). Surprisingly, the somewhat shorter alkyne analog **5** displayed much lower activity than secologanin **1** in this competition assay (relative rate ratio was 75:1 strictosidine:strictosidine analog) with STR, though mutation to D177A did improve the turnover of this substrate in the competition assay (relative rate ratio was 46:1 strictosidine:strictosidine analog). D177A also exhibited enhanced specificity for the more hydrophilic analog **6** (Figure 2B), showing a ratio of 1.7:1 strictosidine **3**:strictosidine analog compared to 8.8:1 for wild-type in the competition assay. The specificity for analog **4** was also moderately increased by the mutation of D177 to G (a ratio of 1.5:1 strictosidine **3** to analog **9**).

High-resolution mass spectrometry analysis confirmed the identity of all enzymatic products. To further validate the identity and stereochemistry of the enzymatic product **9**, an authentic standard was chemically synthesized. The stereochemistry of the standard was validated by NMR data that showed a chemical shift at 3.09 ppm with vicinal coupling constants of $J_{15,\ 14S}$ = 3.9 Hz and $J_{5,\ 14R}$ = 11.0 Hz that was assigned to H-15 (Figure 1A), suggesting that C-15 is in the *S* configuration, as in strictosidine [19]. A signal at 4.33 ppm ($J_{3,\ 14S}$ = 3.1 Hz and $J_{3,\ 14R}$ = 10.4 Hz) assigned to H-3 (Figure 1A) is also in agreement with the strictosidine stereoisomer; the signal for H-3 of the diastereomer vincoside (C-15 = *R* stereoisomer) should appear further down field and $J_{3,\ 14S} > J_{3,\ 14R}$ [19, 20]. Strictosidine and vincoside

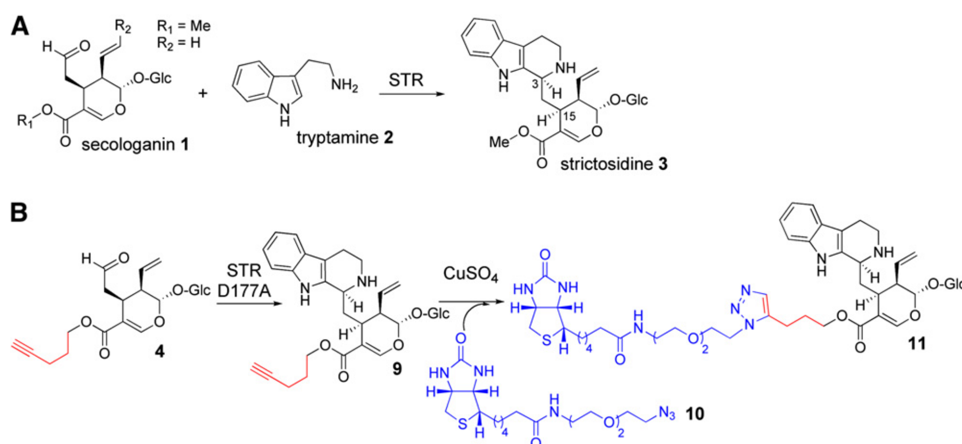

Figure 1. Reaction of Strictosidine Synthase

(A) Reaction catalyzed by strictosidine synthase (STR).

(B) Reaction of D177A mutant and chemoselective derivatization.

analogs are readily separable by HPLC, and since this standard displayed the identical HPLC retention time as the enzymatic product, we conclude that the mutated enzyme retains same stereoselectivity as wild-type STR.

The crystal structure of STR [11] validates that D177 is proximal to the methyl ester of secologanin (Figure 4). When compound **4** is modeled into the secologanin-binding pocket, the pentynyl group contacts D177; mutating the aspartate residue to alanine presumably creates a larger pocket to accommodate the pentynyl group. Steady-state kinetic analysis with **4** revealed that the K_m of D177A (169.4 ± 30.0 μM) is ~2-fold lower than the K_m with STR (312.6 ± 15.7 μM). The V_{max} value for D177A with **4** (6×10^{-5} ± 0.22 μmol/min) is similar to that of wild-type STR (5×10^{-5} ± 0.05 μmol/min). Therefore, the mutation doubled the catalytic efficiency (V_{max}/K_m) of the enzyme with **4**.

When W153, a residue also proximal to the methyl ester of secologanin, was mutated to phenylalanine, an increased selectivity favoring the natural substrate secologanin **1** compared to analog **4** was observed (11:1 strictosidine **3**:analog **9**). W153H and W153G mutants displayed a ratio of 2.7:1 and 2.2:1. The phenylalanine side chain may disrupt the local structure to favor the smaller methyl ester. However, all W153 mutants exhibited low activity relative to the wild-type enzyme with both natural and nonnatural substrates (Figure S1, see the Supplemental Data available with this article online), thus we hesitate to provide a conclusive structural explanation for this observation. Additionally, the propargyl functionality of analog **5** is more rigid than the pentynyl ester **4** and may disrupt the local environment at W153, accounting for the reduced catalytic efficiency of substrate **5** with both STR and D177A (Figure S2). The longer, more flexible pentynyl ester of **4** may avoid perturbing the local environment around W153.

Although a single point mutation could switch the selectivity of STR for the methyl ester analogs, secologanin analogs modified at the vinyl position were not accepted with any point mutations tested (**7–8**; Figure 2B) (data not shown). The vinyl group faces into the interior of the enzyme and appears to be more tightly

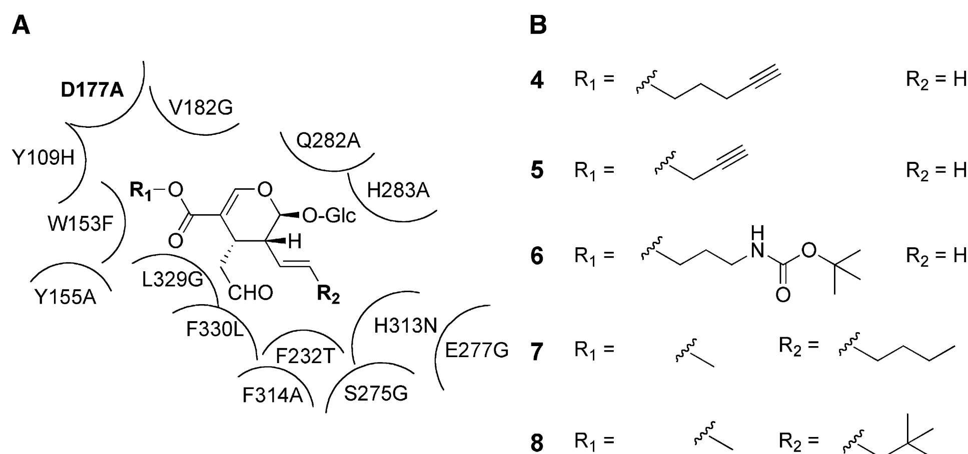

Figure 2. Mutations of Strictosidine Synthase and Secologanin Substrate Analogs

(A) Residues mutated in the secologanin binding pocket. *C. roseus* amino acid numbering is used.

(B) Secologanin substrate analogs.

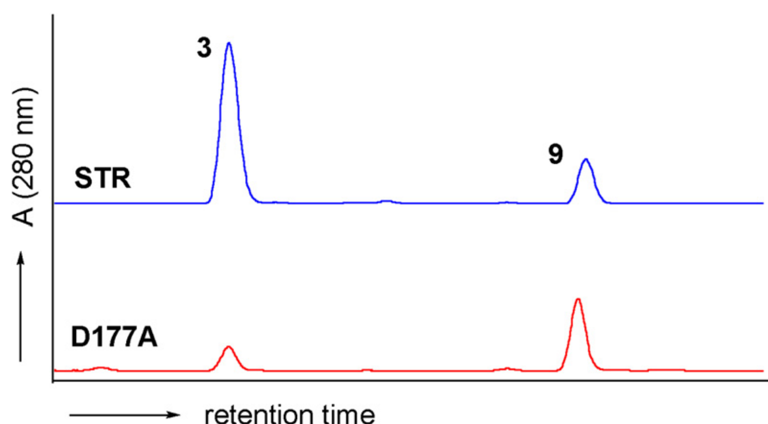

Figure 3. Competition Assay with **1** and **4** to Yield **3** and **9**

$[M+H]^+$ of **9** expected: 583.2656, found: 583.2639.

packed by surrounding residues. More extensive protein mutations will be required to accommodate these substrates.

Substrate analog **4** and the resulting enzymatic product **9** contain a terminal alkyne that can be chemoselectively modified with an azide derivative under aqueous conditions using copper-catalyzed cycloaddition, or click chemistry [21]. The copper-(I)-catalyzed triazole formation from azides and terminal acetylenes is a powerful reaction, due to its reliability, its specificity, and the compatibility of the reactants with aqueous reaction conditions [21]. This mutant enzyme facilitates introduction of an orthogonal chemical group that will allow derivatization of the product.

To demonstrate that the enzymatic product could in fact be derivatized, we used copper-catalyzed click chemistry to add an azido-derivatized biotin to the terminal alkyne of the strictosidine analog **9** (Figure 1B). The azide biotin-labeling reagent **10** was prepared from commercially available biotin amine via the diazo transfer reaction [22] and then incubated with the pentynyl-strictosidine **9** derivative in the presence of copper sulfate and ascorbic acid in water to yield **11** in high yields via the copper-catalyzed 1,3-dipolar cycloaddition (Figure 1B; Supplemental Data).

To our knowledge, this is the first example of modification of the substrate specificity of an enzyme in this pathway and the first example of re-engineering of a "Pictet-Spenglerase." Modulating the substrate specificity of the biosynthetic enzymes constitutes an essential step forward for metabolic engineering efforts in the TIA pathway. To our knowledge, there is no available literature precedence that would indicate that the later steps of this plant pathway can turn over alternative substrates. However, preliminary data showed that secologanin analog **4** was incorporated into the heteroyohimbine TIA biosynthetic pathway [5] when **4** was incubated with *C. roseus* hairy root cell cultures [23]. High-resolution mass spectrometry indicated that the cell cultures produced a new compound with the expected molecular weight of a pentynyl serpentine analog ($[M]^+$ expected: 401.1865, found: 401.1860). This compound was not observed in controls cultured in the absence of substrate **4**, suggesting that the formation of the new product is strictly dependent on the presence of the "unnatural" substrate (Figure S3).

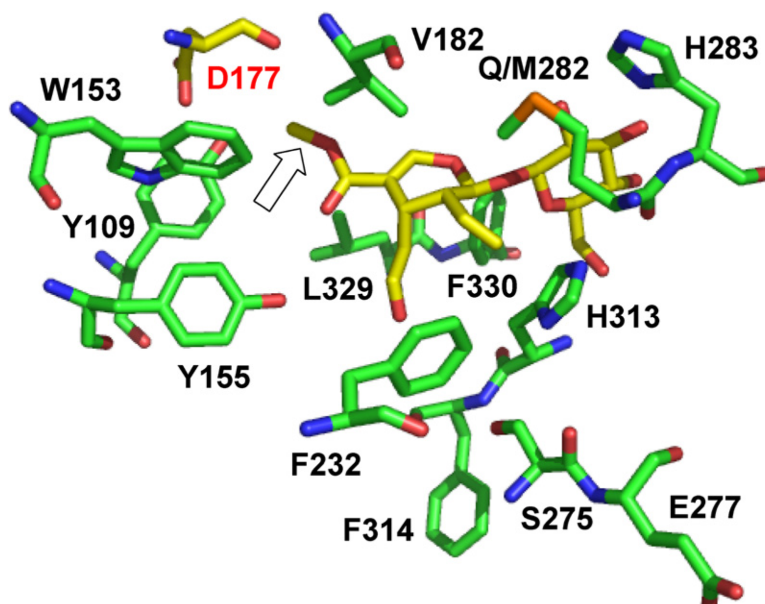

Figure 4. Crystal Structure of Strictosidine Synthase with Secologanin

The ester of secologanin (R_1) is highlighted with an arrow. Glutamine 282 is a methionine residue in the *R. serpentina* enzyme. Structure taken from PDB file 2FPC [11].

Significance

Utilizing the biosynthetic machinery of *C. roseus* to make alkaloid analogs is an attractive strategy to obtain novel "natural" products. Understanding the substrate specificity of the biosynthetic enzymes and re-engineering them to manipulate their activity and selectivity is key to realizing this goal. We have demonstrated that the selectivity of STR, the central enzyme of this alkaloid pathway, can be tuned with a single point mutation. Based on structural information, we were able to modulate the enzyme activity to accept secologanin analogs containing a chemical tag that can be selectively derivatized at a later stage via copper-catalyzed cycloaddition. Moreover, we show evidence that this analog can be processed by later steps of the terpene indole alkaloid pathway. Notably, the biological activity of natural products is often improved by modifications to the structures. For example, a fluorinated semisynthetic derivative of the TIA vinblastine is showing promise in clinical trials [24]. The terpene indole alkaloid biosynthetic pathway may provide a convenient, economical means of obtaining additional derivatives of terpene indole alkaloids that show improved biological activity.

Experimental Procedures

Mutant Construction, Protein Expression, and Purification

Strictosidine synthase was expressed and purified as previously described [10]. Briefly, enzymes were expressed in *E. coli* BL21(DE3) and subjected to a one-step affinity purification step by amylose column (New England Biolabs). The Stratagene QuikChange Site-Directed Mutagenesis Kit was used to incorporate the desired mutations into the expression plasmid. Mutagenesis primers were synthesized by Integrated DNA Technologies (Table S1). Each mutant was sequenced to confirm the desired amino acid mutation. Expression and purification were analyzed by SDS-PAGE. Protein concentration was determined using Bio-Rad Protein Assay microassay protocol, absorbance at 280 nm, and by scanning densitometry of an SDS-PAGE.

Activity Assay for Strictosidine Synthase and Mutants

Strictosidine synthase and mutant activity was measured using an HPLC assay. Protein (100 nM) was incubated with tryptamine, secologanin, in 100 mM NaH_2PO_4 (pH 6.8 at 30°C). An aliquot of the reaction (20 μl) was directly injected onto the HPLC (Hibar RT 250-4LiCHrosorb) using a 22%–95% acetonitrile/water gradient. The absorbance of tryptamine and strictosidine was monitored at 280 nm. A standard curve of strictosidine was used to convert absorbance units to millimoles.

To obtain qualitative activity data, concentrations of 2 mM tryptamine and 2.5 mM secologanin were utilized and assayed for four time points to obtain a linear rate (Figure S1).

Competitive assays contained tryptamine (2 mM) and a 1:1 mixture of secologanin (2.5 mM) and secologanin analog (2.5 mM). The relative ratios of product formed were compared by integration of product peaks at 280 nm.

To obtain kinetic values for secologanin analog **4**, the tryptamine concentration was held constant at 2 mM, and reaction rates were measured at seven secologanin analog concentrations (50 μM to 1.2 mM). 1-Naphthalene-acetic acid (140 μM) was used as an internal standard to ensure accurate injection volume. The reactions were quenched with NaOH (2 equiv.). All kinetic assays were performed in triplicate. Kinetic parameters were obtained using Origin 7.0 software (Origin lab). A negative control performed in the absence of enzyme ensured that negligible background reaction occurred under the assay conditions. The identity of the alternate product was validated by mass spectrometry, and coelution with an authentic standard. A diastereomeric mixture of the authentic standard was synthesized, and the S diastereomer was purified and characterized by NMR spectroscopy. This demonstrated that the mutant enzyme yielded the S stereocenter. For the enzymatic product **9**, high-resolution mass spectrometry yielded m/z 583.2639 $[M+H]^+$ (expected m/z 583.2656 $[M+H]^+$).

General Chemical Methodology

Chemicals were purchased from Aldrich and used without further purification. All reactions were performed under anhydrous conditions and monitored by TLC on Kieselgel 60 F254 (EMD). Detection was carried out by examination under UV light (254 nm) and by charring with cerium molybdate stain. Flash chromatography was performed on silica gel (Sorbent Technologies, 60 μ, mesh 32–63 μm). Extracts were concentrated under reduced pressure at <40°C. ^{1}H NMR (1D, 2D), and ^{13}C NMR spectra were recorded on a Varian Merc300 MHz and Varian 500 MHz spectrometers equipped with Sun workstations. For ^{1}H and ^{13}C NMR spectra recorded in CD_3OD, chemical shifts (δ) are given in ppm relative to solvent peaks (^{1}H, δ = 3.32; ^{13}C, δ = 49.1) as an internal standard. The LC/MS analysis of samples was performed on a Waters Acquity UPLC system ionized by ESI with a Micromass LCT Premier TOF Mass Spectrometer as a detector. The LC was run on a BEH C18, 1.7 μm, 2.1 × 100 mm column. The capillary and sample cone voltages were 2000 and 100 V. The desolvation and source temperatures were 350°C and 100°C. The cone and desolvation gas flow rates were 20 and 700 liters/hr. Analysis was performed with MassLynx 4.1.

Pentynyl 5-Ethenyl-4-(2-Oxoethyl)-6-[3,4,5-Trihydroxy-6-(Hydroxymethyl) Oxan-2-yl]oxy-5,6-Dihydro-4H-Pyran-3-Carboxylate (4)

Secologanin (50 mg, 0.13 mmol) was dissolved in 4-pentyn-1-ol (4 ml), sodium bicarbonate (3 equiv.) was added, and the mixture was stirred at 90°C for 4 hr. Completion of the reaction was determined by MS (ESI). The reaction was filtered and concentrated to a minimum volume, and the crude product was subjected to flash column chromatography (CH_2Cl_2/MeOH, 9/1), followed by C18 chromatography (MeOH/H_2O, 8/2) and preparative HPLC (H_2O [0.1% TFA]/MeCN, gradient 20%–50% MeCN over 20 min) to give the desired compound. ^{1}H NMR (CD_3OD, 500 MHz): 9.68 (s, 1H), 7.48 (s, 1H), 5.77–5.66 (m, 1H), 5.56 (d, 1H, *J*5.8), 5.40 (dd, 1H, *J*5.8, 10.6), 5.30 (t, 1H, *J*10.9), 4.79 (d, 1H, *J*7.7), 4.70 (m, 1H), 4.22 (m, 1H), 3.90 (dd, 1H, *J*2.1, 12.3), 3.66 (m, 1H), 3.60–3.48 (m, 1H), 3.45 (m, 1H), 3.40–3.18 (m, 2H), 3.04 (m, 1H), 2.76 (m, 1H), 2.38–2.24 (m, 2H), 2.10–1.98 (m, 1H), 1.88 (s, 1H), 1.74 (m, 1H), 1.68–1.58 (m, 2H). ESI($C_{21}H_{28}O_{10}$): calculated m/z 441.2 $[M+H]^+$, found m/z 441.2 $[M+H]^+$.

Strictosidine Pentynyl Ester Analog (9)

Tryptamine hydrochloride (2.04 mg, 12.8 μmol) and pentynyl secologanin (2.8 mg, 6.4 μmol) were dissolved in an aqueous solution of maleic acid (0.5 ml, 100 mM [pH = 2]), and the reaction was stirred at ambient temperature overnight. Upon disappearance of the starting materials, the crude mixture was subjected to preparative HPLC purification on a Grace vydac column (C18 monomeric, 100A, H_2O [0.1% TFA]/MeCN, gradient 20%–60% MeCN over 20 min, RT, 14 min) to give 1 mg (30%) of the desired stereoisomer. ^{1}H NMR (CD_3OD, 500 MHz): δ7.58 (s, 1H, H-17), 7.48 (d, 1H, *J9,10* 7.8, H-9), 7.32 (d, 1H, *J12,11* 8.5, H-12), 7.15 (dd, 1H, *J11,10* 7.0, H-11), 7.06 (dd, 1H, H-11), 6.01 (m, 1H, H-19), 5.86 (d, 1H, *J21,20* 8.9, H-21), 5.38 (dd, 1H, *J18z,19* 17.4, H-18Z), 5.38 (dd, 1H, *J18E,19* 17.4, H-18E), 4.81 (d, 1H, *J1′,2′* 7.6, H-1′), 4.33 (dd, 1H, *J3, 14S* 3.1, *J3, 14R* 10.4, H-3), 4.19 (m, 1H, H-23b), 4.08 (m, 1H, H-23a), 4.01 (dd, 1H, *J6a′,6b′* 2.1, *J6a′,5′* 2.8, H-6a′), 3.65 (dd, 1H, *J6b′,5′* 11.6, H-6b′), 3.50–3.20 (m, 4H, H-2′, H-3′, H-4′, H-5′), 3.47 (m, 1H, H-5β), 3.18 (m, 1H, H-5α), 3.09 (dd, 1H, *J15, 14S* 3.9, *J5 14R* 11.0, H-15), 2.83 (m, 1H, H-6β), 2.75 (m, 1H, H-6α), 2.21 (dd, 1H, *J14R, 14S* 13.0, H-14S), 1.95 (dd, 1H, H-14R), 2.10 (m, 2H, H-25), 1.74 (m, 1H, H-27), 1.61 (m, 2H, H-24). ESI ($C_{31}H_{38}N_2O_9$): calculated m/z 583.2656 $[M+H]^+$, found m/z 583.2639 $[M+H]^+$.

Supplemental Data

Supplemental data describing evaluation of secologanin concentration, synthesis of biotin azide, and synthesis of biotinylated strictosidine are available at http://www.chembiol.com/cgi/content/full/13/11/1137/DC1/.

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