Rapid Identification of Enzyme Variants for

Reengineered Alkaloid Biosynthesis in Periwinkle

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Supplemental Data

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1. *STS construct and expression in yeast*

A synthetic gene encoding *Catharanthus roseus* strictosidine synthase (Geneart, codon usage optimized for expression in yeast) in pMCR (kan^R) was used as a template for cloning the strictosidine synthase gene into the yeast expression plasmid pGAL-MF (Dualsystems Biotech AG). pGAL-MF encodes a mating factor alpha signal-peptide directly upstream of the multiple cloning site. This yeast signal sequence enables secretion of the heterologously expressed protein into the culture medium during expression. pGAL-MF contains a galactose-inducible promoter and confers resistance to ampicillin (bacteria) or growth on uracil deficient medium (yeast, deficient in biosynthesis of uracil).

Standard electroporation protocols (http://www.fhcrc.org/science/labs/gottschling /yeast/ytrans.html) were used to transform the plasmid pSTSMF-FLAG into electrocompetent *S. cerevisiae* BJ5465 (BJ5465 lacks the ability to synthesize uracil). *S. cerevisiae* is maintained in yeast-peptone-dextrose medium (YPD, composition per liter: 10 g yeast extract, 20 g peptone and 2% w/v glucose). Plasmid-containing yeast were maintained on a minimal medium depleted of uracil (SCMM-U, *S. cerevisiae* minimal medium, composition per liter: 6.7 g yeast extract without amino acids and 1.92 g yeast supplemental media without uracil) supplemented with 2% (w/v) glucose.

Transformed *S. cerevisiae* BJ5465 harboring pSTSMF-FLAG was grown overnight in SCMM-U supplemented with 2% glucose. The cells were recovered from an overnight culture (OD_{600} ~ 10) and washed in SCMM-U medium without any carbon source to remove the glucose. The overnight culture was then diluted with SCMM-U medium containing 2% (w/v) raffinose and 2% (w/v) galactose to yield a culture with an

OD_{600} = 1. The culture was incubated at 25 °C for 24-72 h at 225 rpm. The cells were then removed by centrifugation and the supernatant was concentrated and dialyzed against a buffer at pH 7.0 (the enzyme was confirmed to be stable in 50 mM (TBS) Tris·HCl and 150 mM sodium chloride; 10 mM MOPS (morpholinopropane sulphonic acid); 50 mM sodium phosphate; and 10 mM BES (*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid). The enzyme was either subjected to further purification or could be stored after the addition of glycerol (25%) at -20 °C.

2. *Kinetic analysis of STS mutants*

Representative assay data using the Val214Met mutant and 5-methyltryptamine are shown in Figure S1.

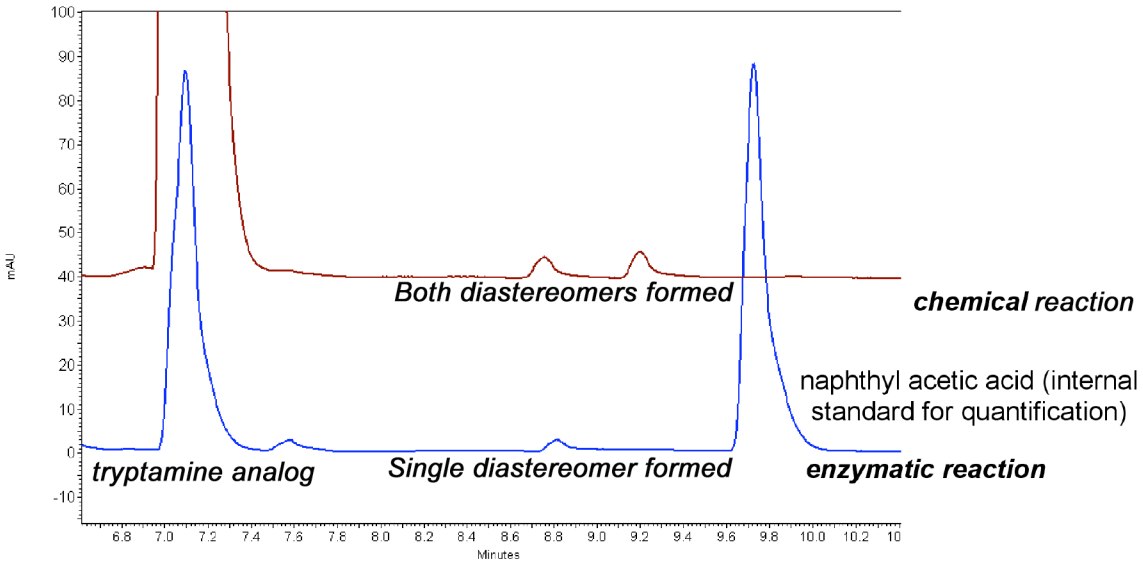


Figure S1. HPLC trace (280 nm) of a representative spectrum used in analysis of 5-methyltryptamine kinetics using the Val214Met mutant. The top trace (brown) represents the chemical reaction control, which shows formation of two peaks corresponding to the two diastereomers, 10-methylstrictosidine **8l** and 10-methylvincoside, respectively. The

blue trace represents the enzymatic reaction with Val214Met and 5-methyltryptamine **6l** in which only one diastereomer is formed. Naphthyl acetic acid (NAA) is added as an internal standard. 5-methyltryptamine (rt = 7.1 min), secologanin (rt = 7.6 min, low absorbance at 280 nm), 3*S*-diastereomer of strictosidine analog (rt = 8.8 min), 3*R*-diastereomer of strictosidine (vincoside) analog (rt = 9.2 min), NAA (rt = 9.8 min).

3. *Saturation mutagenesis protocol*

Two flanking primers (pGALMF_fwd, pGALMF_rev; Table S1) were designed using the Integrated DNA Technologies Inc. web tool (http://www.idtdna.com/SciTools/SciTools.aspx). Complementary mutagenic primers were designed to incorporate the NNK (MNN, reverse sense) degenerate codon that encodes for 32 sequence possibilities (**N** = A, C, G, T; **K** = G, T; **M** = A, C) (Table S2), effectively reducing the number of possible repetitive substitutions.

Table S1. PCR primers for saturation mutagenesis.

Primer name	Primer sequence (5'->3')
pGALMF_fwd	GGGGTATCTTTGGATAAAAGAGAGGCTGAAGCTGAATTCGATATC
pGALMF_rev	GGGAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGAC
V171Sat_fwd	CTTCACTGACNNKTCCTCTATTC
V171Sat_rev	GAATAGAGGAMNNGTCAGTGAAG

V214Sat_fwd	GAAGGAGTTGCACNNKCCAGGTGGTGCTG
V214Sat_rev	CAGCACCACCTGGMNNGTGCAACTCCTTC
F232Sat_fwd	GTTGTTGCTGAGNNKTTGTCCAACAGAATC
F232Sat_rev	GATTCTGTTGGACAAMNNCTCAGCAACAAC
E315Sat_fwd	GAAGGTGAACACTTCNNKCAAATTCAAGAGC
E315Sat_rev	GCTCTTGAATTTGMNNGAAGTGTTCACCTTC

All PCR reactions were carried out using Expand High-Fidelity PCR Kit (Roche Biosciences, Inc.). The conditions described in the manufacturers' instructions were followed, except that 1.0 μl DMSO was added to each 50 μl PCR reaction. The thermocycling program started with denaturation (94 ºC, 3 min), followed by 29 cycles of denaturation (94 ˚C, 30 s), annealing (55 ˚C, 30 s) and extension (72 ˚C, 1 min) and ending with extension (72 ˚C, 7 min). Increased product yield was sometimes achieved by lowering the annealing temperature to 52 ˚C.

PCR products were either purified by agarose gel (1%) electrophoresis or spin-column purification (Qiagen PCR clean-up kit). If gel purification was used, excised gel bands were extracted and purified using GenElute Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO). In the first PCR reaction, the forward flanking primer (pGALMF_fwd) was added in equimolar concentration to the reverse mutagenic primer. The reverse flanking primer (pGALMF_rev) was likewise added with the forward mutagenic primer in a

second PCR reaction. The two resulting PCR fragments both contained the degenerate codon (Figure S2). The PCR products were purified by spin-column (after digestion by *Dpn* I to remove parental plasmid DNA) and then utilized in the third splicing-overlap extension PCR reaction to produce the full-length mutated gene. The full-length gene was purified by agarose (1%) gel electrophoresis and the 1.1-kb DNA band was excised and purified.

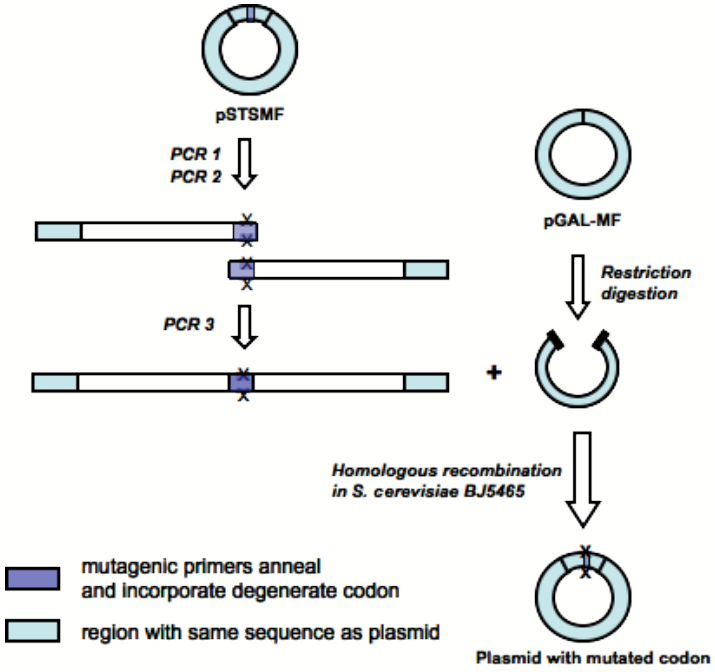


Figure S2. Saturation mutagenesis approach to construct mutant libraries. Three PCR reactions were used to assemble the mutagenized gene with regions that flank the plasmid. Homologous recombination after transformation of the linearized plasmid and mutant gene in *S. cerevisiae* BJ5465 afforded the mutagenized plasmid.

The pGAL-MF plasmid was digested with *Hind* III and *Xho* I restriction enzymes in the recommended buffer and purified by agarose gel electrophoresis. The vector and the mutated gene were then added in ratios ranging from 1:8 - 1:4 to electrocompetent *S.*

cerevisiae BJ5465 cells, subjected to electroporation, diluted in 1 mL, 1 M ice-cold sorbitol and then plated onto two Petri dishes (15-cm diameter) with SCMM-U agar medium and 2% (w/v) glucose. The plates were incubated at 30 °C and colonies appeared after 48-72 hours. The 42-45 base pair overlap between the vector and the gene allowed homologous recombination in *S. cerevisiae*, which produced a plasmid with the mutated inserted gene (Figure S2). The recombined plasmid allowed growth on the uracil-deficient (selection) media.

4. *Colorimetric assay for strictosidine synthase activity*

The colorimetric assay utilizes visualization of a pigment that is formed when strictosidine glucosidase acts upon strictosidine in the presence of excess tryptamine. This pigment was described for action of secologanin synthase, strictosidine synthase and strictosidine glucosidase on loganin as previously described (A. Geerlings, F. J. Redondo, J. Memelink, A. Contin, R. van der Heijden, R. Verpoorte, *Biotechnol. Tech.* **1999**, *13*, 605-608.). Evidence for the structure of the chromophore, however, has not been reported.

LC-MS analysis indicated that a compound with a mass consistent with deglucosylated strictosidine (*m/z* 351) was observed in the assay mixture, as would be expected when strictosidine glucosidase is added to strictosidine. Deglucosylated strictosidine was not observed when either STS or SG was left out of the assay mixture (Figure S3). Additionally, a mass corresponding to deglucosylated strictosidine plus addition of tryptamine and loss of water (ESI-MS $C_{31}H_{33}N_4O_2$: calculated *m/z* 493.2604

$[M+H]^+$, found *m/z* 493.2579 $[M+H]^+$) was also observed, suggesting that a tryptamine adduct was formed from the excess tryptamine present in the assay mixture (Figure S3).

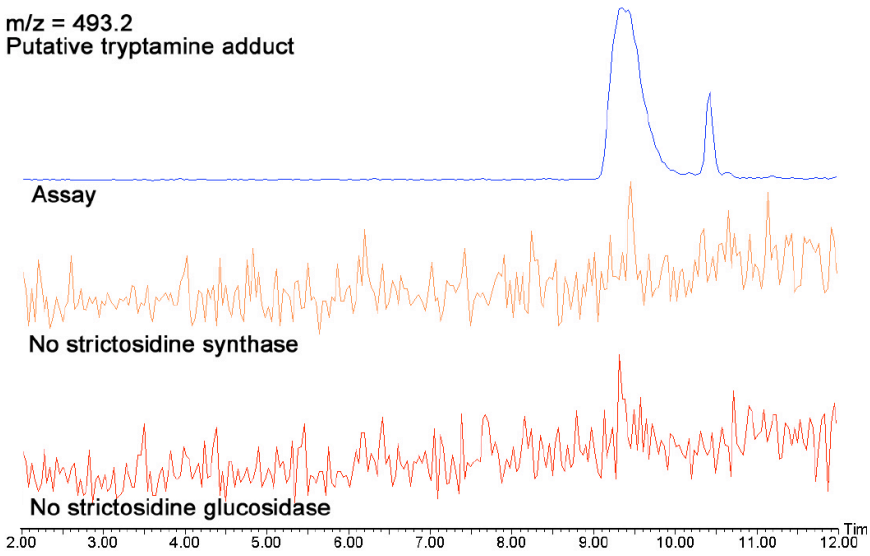
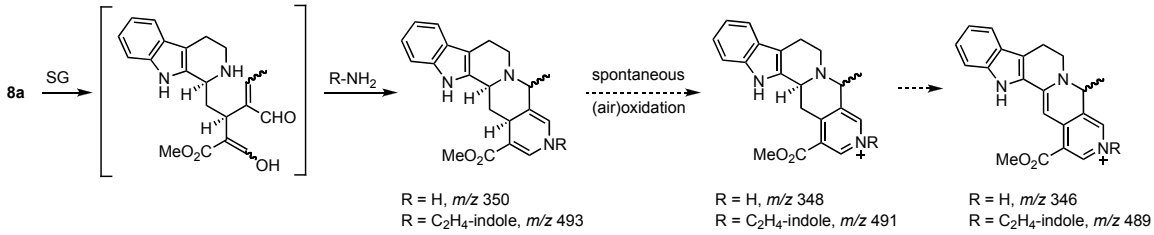


Figure S3. Formation of adduct (*m/z* 493) is strictly dependent on the presence of both strictosidine synthase and strictosidine glucosidase. LC-MS traces extracted at *m/z* 493 show that a compound corresponding to a tryptamine adduct (*m/z* 493) is formed in the assay (top trace). This compound is not observed in assay controls lacking either strictosidine synthase (middle trace) or strictosidine glucosidase (bottom trace). The second (smaller) peak is the 493-isotope of *m/z* 491 that is also formed as an adduct.

In the presence of excess ammonium sulfate and no productive enzymatic pathway, a dihydropyridine moiety has been shown to be formed from deglycosylated strictosidine (Heinstein *et al.* J. Med. Plant Res. **1979**, *37*, 349-357). The dihydropyridine has been shown to oxidize to the pyridine (Heinstein *et al.* J. Med. Plant Res. **1979**, *37*, 349-357) and could oxidize further to extend conjugation. As the chromophore forms

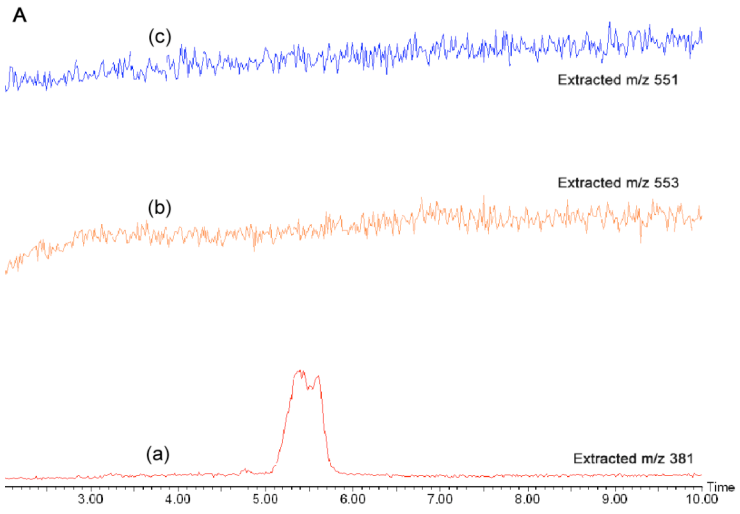
over the course of several hours, LC-MS analysis shows two compounds forming with two and four mass units less than the putative adduct. With the tryptamine substrate, these masses correspond to *m/z* 491 (ESI-MS $C_{31}H_{31}N_4O_2$: calculated *m/z* 491.2447 $[M]^+$, found *m/z* 491.2458 $[M]^+$) and *m/z* 489 (ESI-MS $C_{31}H_{29}N_4O_2$: calculated *m/z* 489.2291 $[M]^+$, found *m/z* 489.2290 $[M]^+$).



NMR-evidence for tryptamine adducts and pyridininium protons: *m/z* 491: NMR (600 MHz, CH_3CN) δ ppm: 9.50 (s, indole-NH), 9.26 (s, indole-NH), 7.78 (s, 1H, pyridinium-H), 7.57 (d, 1H, *J* 7.7, Ar-H, cosy: indole a), 7.43-7.40 (m, 3H, Ar-H, cosy: indole a,b), 7.20 (dt, 1H, Ar-H, *J* 0.9, 7.7 cosy: indole a), 7.13 (t, 1H, *J* 0.8, 7.8, Ar-H, cosy: indole a), 7.07 (t, 1H, *J* 0.8, 7.9, Ar-H, cosy: indole b), 7.06 (d, *J* 1.5, pyrrole-H), 6.94 (t, 1H, *J* 0.8, 7.9, Ar-H, cosy: indole b), 6.82 (s, 1H, pyridinium-H); *m/z* 489: NMR (600 MHz, CD_3Cl) δ ppm: 9.86 (s, indole-NH), 9.14 (s, indole-NH), 8.44 (s, 1H, pyridinium-H), 7.82 (s, 1H, pyridinium-H), 7.61 (d, 1H, Ar-H), 7.45 (t, 1H, Ar-H), 7.37 (t, 1H, Ar-NH), 7.30-7.16 (m, 4H, Ar-H, pyrrole-H), 6.91 (t, 1H, Ar-H), 6.74 (t, 1H, Ar-H).

To verify that a tryptamine adduct is responsible for the formation of color, we added SG to a purified solution of 5*R*-hydroxymethyl strictosidine **8m** (containing no tryptamine **6a** or analog of **6a**). This resulted in the formation of *m/z* 383 (Figure S4A). Upon addition of 2'-*R*-hydroxymethyltryptamine **6m** and incubation overnight (18 h) a color formed and LC-MS analysis of this reaction showed the formation of two new compounds with *m/z* 553 and 551, which can be explained by presence of two

equivalents of tryptamine analog (*m/z* M+60, where M is *m/z* 493 or 491) (Figure S4B). The control reaction (containing no **6m**) was not colored and did not contain a mass of the putative degradation product of dehydrogeissoschizine **10**, 5,6-dehydroflavopereirine (data not shown). Representative data with the Phe232Leu mutant, SG and tryptophanol **8m** are shown in Figure S4.



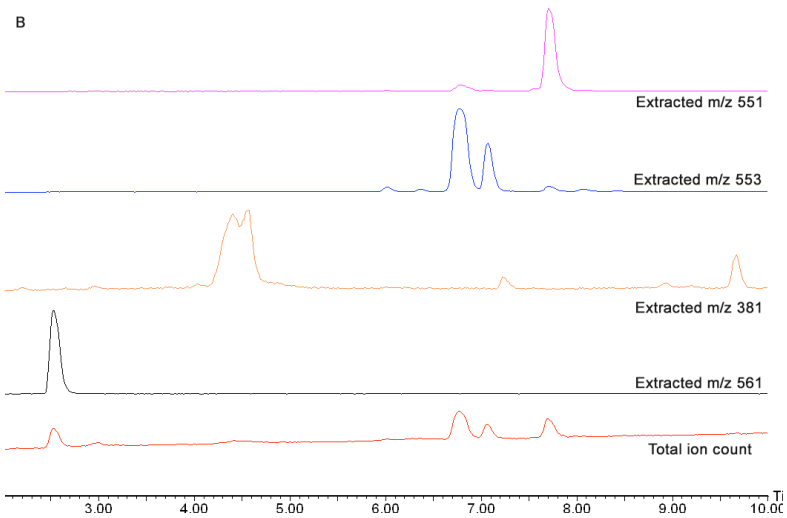


Figure S4. Experiment to test formation of tryptamine adduct. (A) Purified 5*R*-hydroxymethyl strictosidine **8m** (*m/z* 561, 531+30) and strictosidine glucosidase are reacted for 3 h. Under these conditions only the corresponding deglycosylated strictosidine analog is present (*m/z* 381, 351+30) (trace a) and no adducts with **6m** (*m/z* 553 or 551, corresponding to 493+60 or 491+60, respectively) are observed (traces b and c). (B) After addition of **6m** to a reaction containing **8m** SG and *m/z* 381, two new masses are formed on the expense of *m/z* 381: *m/z* 553 and *m/z* 551 (two top traces). These two products appear to be tryptamine adducts of *m/z* 381 (NMR evidence). The total ion count (lower trace) is clean and no mass corresponding to the putative dihydroflavopereirine degradation product of **10** is observed.

5. *Screening in 96-well plates*

Assays of saturation mutagenesis libraries were conducted in 96-well plates. In all 96-well plate screens, assay solution (50 µl) was added as a 2-fold concentrated solution to yeast minimal media containing enzyme activity (50 µl) to yield the following final concentrations: secologanin **7** (1 mM), tryptamine **6a** or tryptamine analog (2 mM),

strictosidine glucosidase (in excess so as to not limit display of strictosidine synthase activity; determined empirically for each glucosidase preparation) and sodium phosphate buffer (25 mM, pH 7.0). The plates were incubated at 30 °C for up to 72 hours and scanned with a photo scanner periodically to monitor color formation. Active strictosidine synthase variants produced a yellow color in the wells of the screening plate (Figure S5). The yellow pigment is insoluble and visualization can be facilitated by addition of an organic co-solvent (acetonitrile 5-10% v/v). This modification will likely be crucial for adaptation to colorimetric screening by spectrophotometry. Wells on the expression plate corresponding to color formation on the screening plate were recultured in SCMM-U medium supplemented with 2% glucose. Plasmid was extracted from yeast using the Zymoprep Plasmid Extraction Kit according to the manufacturer's instructions (Zymo Research, www.zymoresearch.com). Plasmid was transformed into and isolated from *E. coli* TOP10 (cleaner DNA isolation) and then submitted for sequencing analysis of the gene by custom designed primers. The screening plate of the Phe232 saturation library with *R*-tryptophanol is shown below (Figure S5).

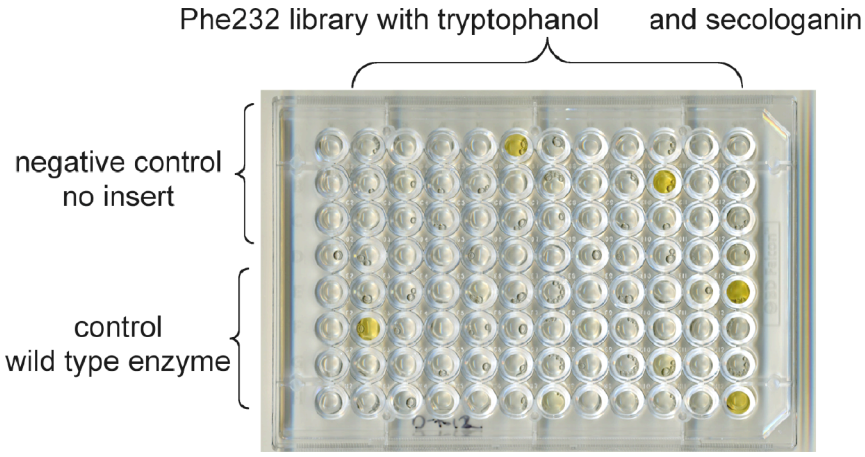


Figure S5. Assay plate that shows color formation from the Phe232 saturation library incubated with 2'*R*-hydroxymethyltryptamine **6m**. The first column shows the controls

(rows 1-4: no-insert control, rows 5-8: wild-type strictosidine synthase control). These wells show no color-formation, as expected, after 72 h incubation. All other wells contained different members of the Phe232 saturation library. However, after 24 h incubation several reactions showed color formation significantly higher than any background color-intensity. The plasmid from each of these wells was sequenced, and shown to encode either Phe232Leu or Phe232Met mutations.

6. *Enzymatic synthesis of strictosidine analogs*

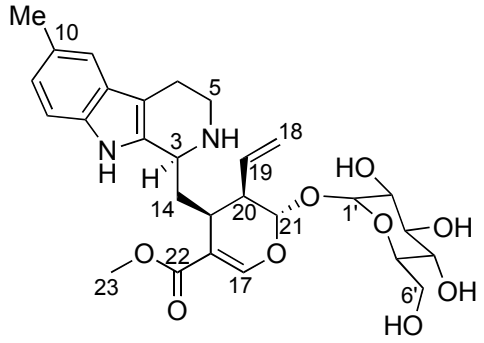
Preparation of crude cell extracts for synthesis

The STS-encoding gene was inserted into the commercially available pET-28a(+) vector between the *Nco* I and *Xho* I restriction sites, as described in the Experimental Procedures. The resulting construct was transformed into *Escherichia coli* TOP10 for plasmid maintenance and then introduced to *E. coli* BL21(DE3) for protein expression. A single colony of plasmid-containing bacteria was inoculated in Luria-Bertani broth (LB-media, supplemented with 30 μg/ml kanamycin) and incubated overnight (37 °C, 200 rpm). The seed culture was then inoculated (1% v/v) in fresh LB-media (4 L in 2.8-L Fernbach flasks, 30 μg/ml kanamycin) and incubated until $OD_{600\ nm}$ = 1 (~ 2.5 h, 37 °C, 200 rpm). At this point, the culture was chilled to 4 °C, IPTG was added (1 mM final concentration) to induce expression, and the culture was incubated (18 °C, 200 rpm) for a total of 24 h. The cell material was recovered by centrifugation (3000*g*, 4 °C, 15 min) to an approximate wet-weight of 7 g/L culture. The cells were resuspended in five volumes sodium phosphate buffer (100 mM, pH 7.0) and lysed by passing the suspension twice through a french (hydraulic) press. The DNA was sheared using a 22-gauge syringe

needle and the crude cell extract was recovered by centrifugation (10000*g*, 4 °C, 120 min). The cell extract was used directly for synthesis.

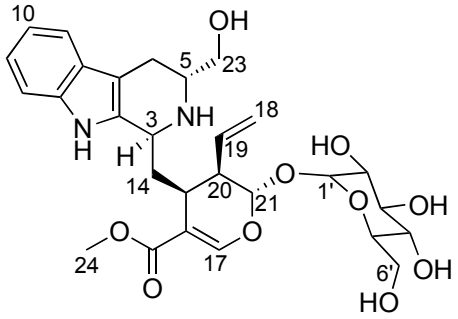
The Experimental Procedures section contains a tabulation of high-resolution mass spectrometry and NMR spectroscopy data for strictosidine analogs ***8l-n****.*

10-methyl strictosidine (**8l**)



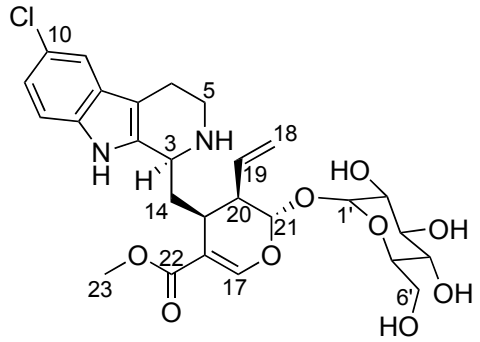
5-methyltryptamine hydrochloride (105 mg, 0.5 mmol, 1 eq) and secologanin (194 mg, 0.5 mmol, 1 eq) were dissolved in 225 mL stirred aqueous buffer (25 mM sodium phosphate, pH 7.0). Val214Met STS crude extract prepared as described above was then added (25 mL, 10% v/v) and the reaction was continued for 72 h at 30 °C. Water was removed using a rotary evaporator and methanol was added (two volumes) to precipitate the protein. After sonication and filtration, the solvent was evaporated to afford a yellow solid. The solid was dissolved in 40 mL methanol and 5 mg product was purified by preparative HPLC (reverse-phase column, 1 mL injections, gradient of 80:20 to 10:90 water-0.1% TFA:acetonitrile over 20 min). Analytically pure 10-methylstrictosidine (**8l**) was characterized by NMR (dr >99.5%).

5-R-hydroxymethyl strictosidine (**8m**)



2'-*R*-hydroxymethyl tryptamine (190 mg, 1 mmol, 1 eq) was added as a solution in acetonitrile (25 mL, 5% v/v final concentration of organic solvent) and secologanin was added as a powder (388 mg, 1 mmol, 1 eq) to 450 mL stirred aqueous buffer (25 mM sodium phosphate, pH 7.0). Cell-extracts containing Phe232Leu STS prepared as described above was then added (25 mL, 5% v/v) and the reaction was continued for 46 h at 30 °C. Most of the water was removed using a rotary evaporator and methanol was added (two volumes) to precipitate the protein. Filtration and evaporation of the remaining solvent afforded a yellow oil. The oil was dissoved in 40 mL methanol and purified by preparative HPLC (Reverse-phase column, 1 mL injections, gradient of 80:20 to 40:60 water-0.1% TFA:acetonitrile over 10 min). Analytically pure 5-*R*-hydroxymethyl strictosidine **8m** was obtained as a pale-yellow amorphous solid (190 mg, 0.34 mmol, 43%, dr >99.5%).

10-chlorostrictosidine (**8n**)



5-chlorotryptamine hydrochloride salt (230 mg, 1 mmol, 1 eq) and secologanin (388 mg, 1 mmol, 1 eq) were dissolved in 450 mL stirred aqueous buffer (25 mM sodium phosphate, pH 7.0). Val214Met STS crude extract prepared as described above was then added (25 mL, 5% v/v) and the reaction was continued for 72 h at 30 °C. Water was removed using a rotary evaporator and methanol was added (two volumes) to precipitate the protein. After sonication and filtration, the solvent was evaporated to afford a yellow solid. The solid was dissolved in 40 mL methanol and 10 mg product was purified by preparative HPLC (reverse-phase column, 1 mL injections, gradient of 80:20 to 10:90 water-0.1% TFA:acetonitrile over 15 min). Analytically pure 10-chlorostrictosidine **8n** was characterized by NMR (dr >99.5%).

7. *Feeding studies with C. roseus*

5-*R*-hydroxymethyl strictosidine (**8m**) supplemented hairy root culture:

Strictosidine analog **8m** was synthesized as described above. Differentiated hairy root cell cultures of *C. roseus* were inoculated in half-strength Gamborg medium (400 mL) and grown for two weeks (32 ºC, 60 rpm). 5*R*-hydroxymethyl strictosidine **8m** (0.5 mM, 110 mg as filter-sterilized solution in water) was then added. After one week, the medium was separated from the hairy roots and the roots were washed in water several times. Hairy root cultures were ground with a mortar and pestle in methanol (3 x 30 mL). The media or the root extract was acidified (pH 3) with hydrochloric acid and extracted with hexanes. The aqueous layer was then basified (pH 9) with ammonium hydroxide and extracted with dichloromethane. After drying the organic layer under sodium sulfate, the solvent was evaporated to give a yellow oil. The oil was dissolved in methanol and analyzed by LC/MS and contained *m/z* $[M+H]^+$ = 385.2 as the major species. This compound was isolated using preparative HPLC with the following gradients: 20-40% increasing gradient of acetonitrile in water with 0.1% TFA for 15 minutes; then, a gradient of 30-40% over 10 minutes until the compound was >80% pure. Isolated yield was: *m/z* 385.2 (2.0 mg, 5 µmol). Characterization of this compound by HRMS and NMR is reported in the Experimental Procedures section.

*Putative ajmalicine **1** (or isomer of **1**) analog formation:*

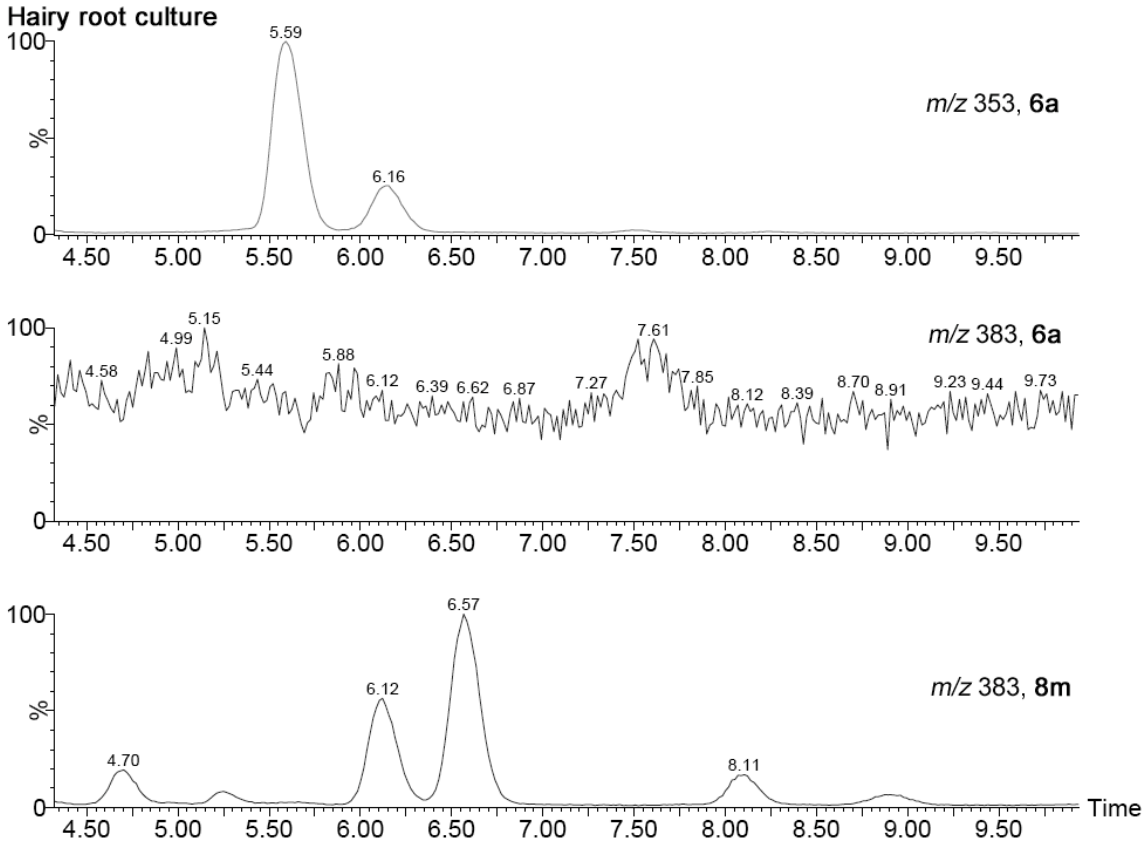


Figure S6. LC-trace of putative ajmalicine **1** (or isomer of **1**) analog. Peaks for *m/z* 383 (C22H27N2O4 calc *m/z* 383.1971 $[M+H]^+$, observed *m/z* 383.1955 $[M+H]^+$) exist in the **8m**-supplemented culture that are not present in the control (supplemented with **6a**). The corresponding natural MIA ajmalicine **1** (*m/z* 353) is present in the control culture.

*Unknown alkaloid (**15m**) formed when hairy roots are supplemented with **8m**:*

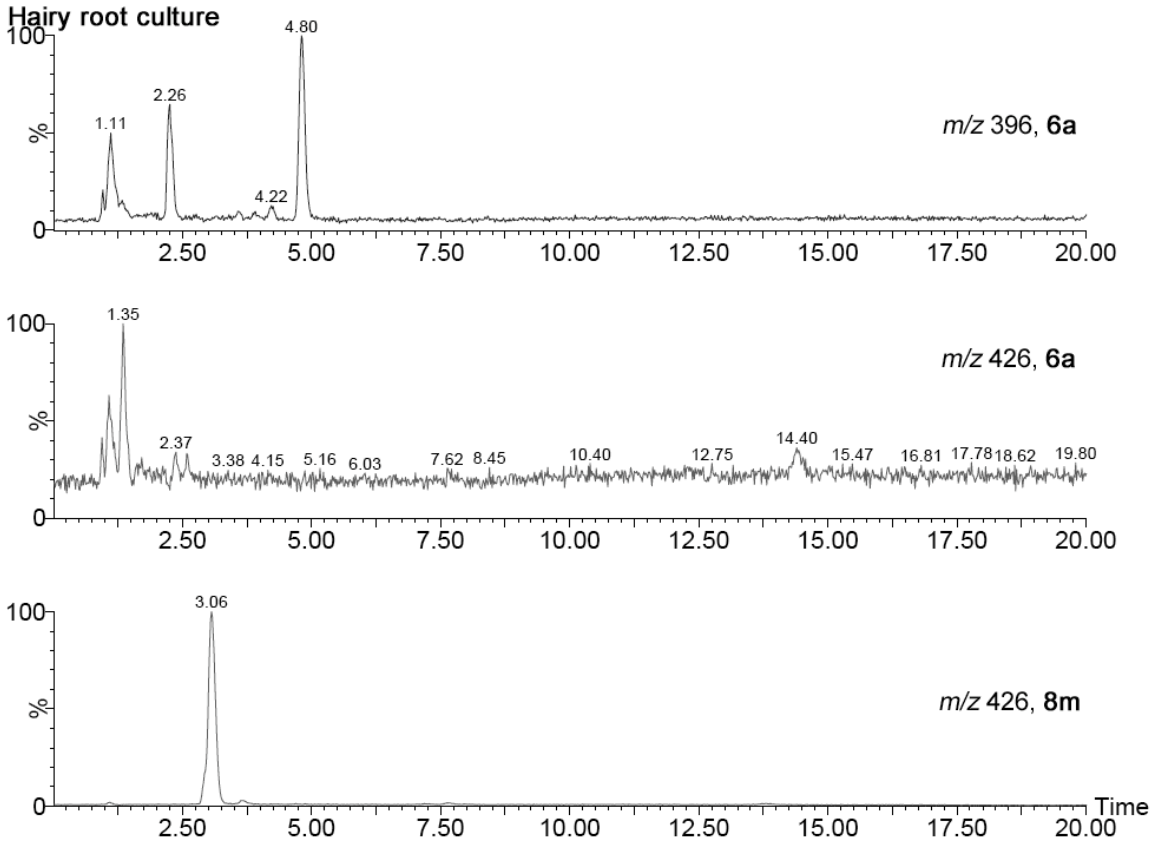


Figure S7. LC-trace of unknown compound **15m** (retention time, 3.1 min), which is formed when hairy roots are supplmented with **8m**. NMR spectroscopy shows that the compound is an indole alkaloid. The alkaloid with *m/z* 426 does not exist in the control culture (supplemented with **6a**). Interestingly there is a peak at 4.8 min in the control culture (top trace) that could correspond to a natural alkaloid; however, this peak is an isotope signal for unidentified compound *m/z* 395.

10-bromostrictosidine (**8o**) supplemented hairy root culture

Strictosidine analog **8o** was synthesized on small scale together with its vincoside diastereomer. Briefly, 10-bromostrictosidine hydrochloride was added to secologanin in malate buffer (10 mM, pH 2.0) and reacted overnight. The reaction was filter-sterilized and added to hairy root cultures as described above (final volume 20 mL half-strength Gamborg media, compound concentration 0.5 mM). After seven days the hairy roots were extracted into methanol, filtered and injected into the LC-MS. Assignments as brominated compounds are based on isotopic signals of ^{79}Br and ^{81}Br incorporated in the alkaloid analog. Bromine-substituted analogs are expected to have later elution times (more hydrophobic) than the natural MIA alkaloids. Natural alkaloids are assigned by comparison to authentic standards.

Analog of m/z *353 (natural alkaloid* m/z *353 from* ***6a*** *is ajmalicine* ***1****):*

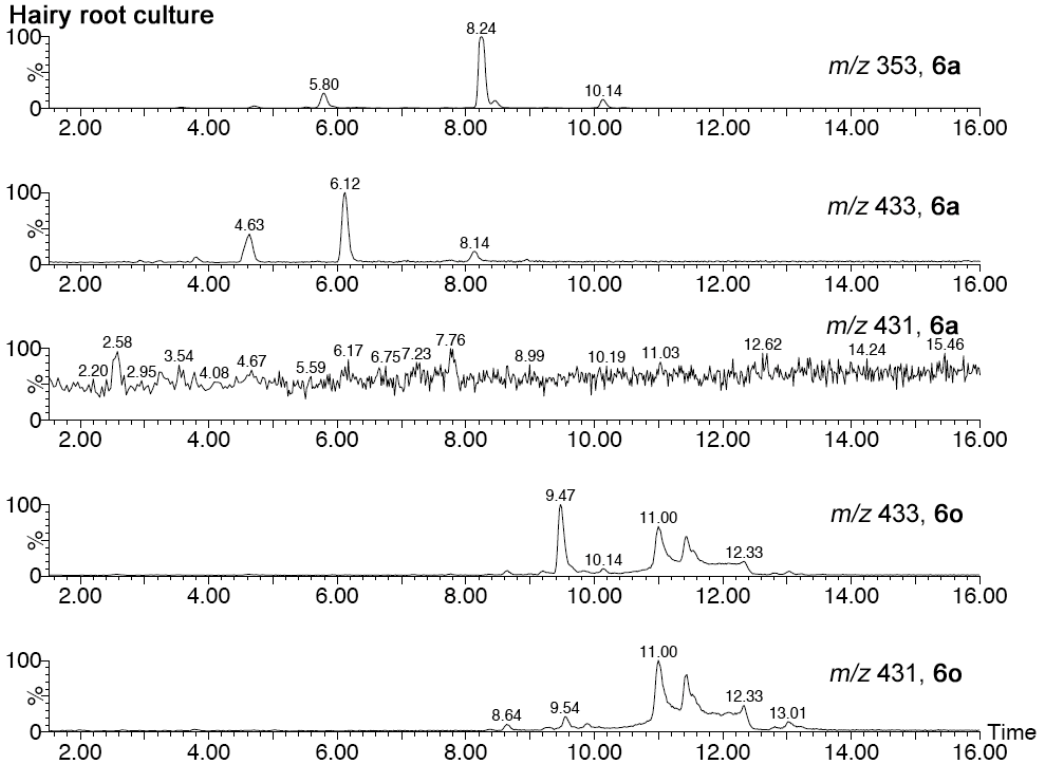


Figure S8. LC-trace of proposed brominated ajmalicine analogs (structural isomers likely) that have retention times of 11.0 min and 11.4 min. The control culture (supplemented with **6a**) does not contain compounds at *m/z* 431 and 433 at these retention times. Furthermore, the isotopic signal from ^{79}Br and ^{81}Br (*m/z* 431 and 433, respectively) provide some evidence for a preliminary assignment as **1o** (or an isomer of **1o**). The top trace indicates that ajmalicine has a retention time of 8.2 min (in the LC-method used) and this peak co-elutes with an authentic standard (not shown).

A*nalog of* m/z *355 (natural alkaloid* m/z *355 at 6.5 min from* ***6a*** *is yohimbine):*

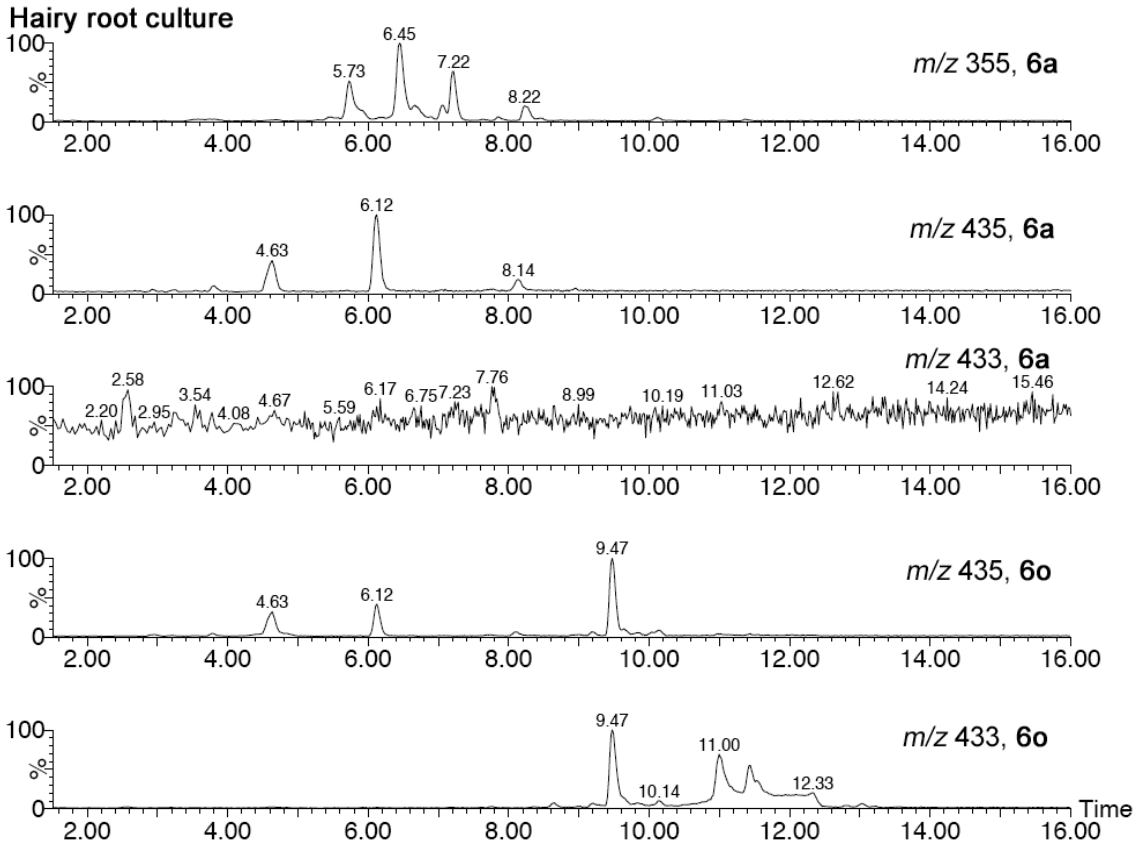


Figure S9. LC-trace of putative brominated yohimbine-type alkaloid **12** analog (retention time, 9.5 min). Based on the presence of the two characteristic isotope signals expected from a brominated molecule, we assign *m/z* 435 and *m/z* 433 at 9.5 min as any structural isomer of yohimbine (**12o**). The corresponding signals are not observed in the control culture. The natural MIA yohimbine elutes at 6.5 min using this LC-method (not shown).

Analog of m/z *323 (natural alkaloid* m/z *323 at 8.1 min from* ***6a*** *is akuammicine (J. Amer. Chem. Soc. (2006) 128, 14276-14277):*

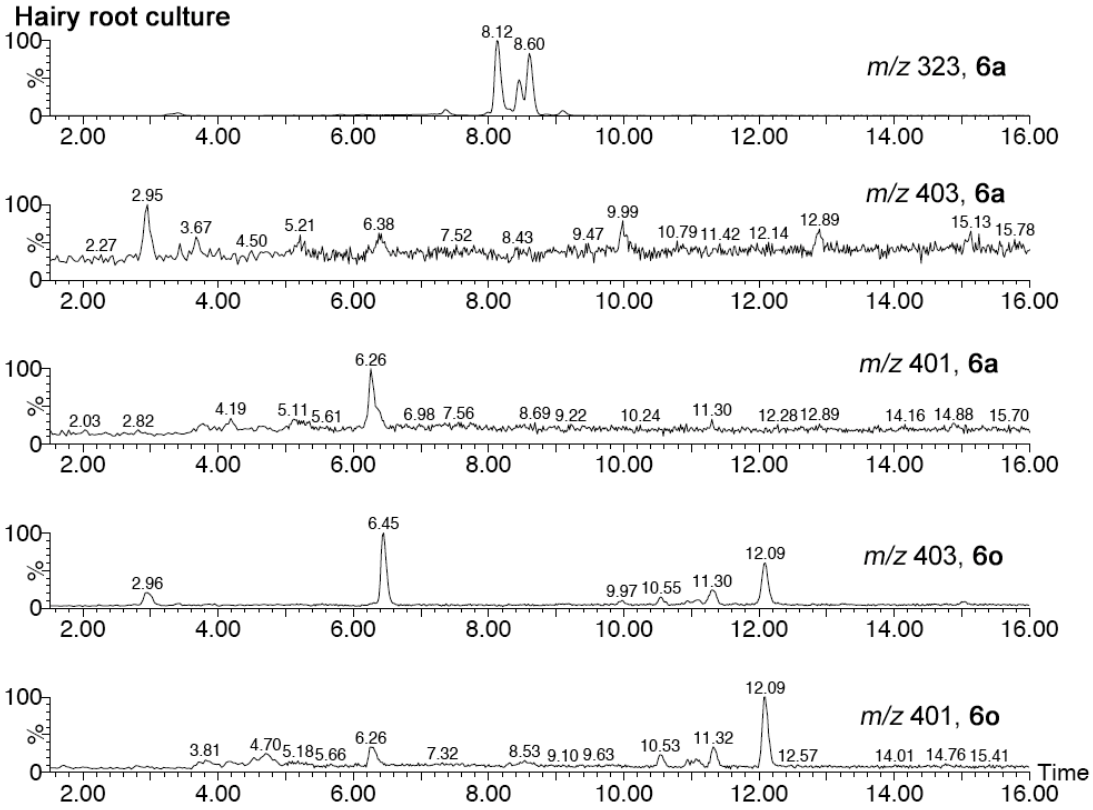


Figure S10. LC-trace of putative brominated alkaloid analog of *m/z* 323. In *J. Amer. Chem. Soc. (2006) 128, 14276-14277*, NMR evidence is presented for compound **13** eluting at 8.1 min (top trace). Based on the presence of the two characteristic isotope signals expected from a brominated molecule, we assign *m/z* 401 and *m/z* 403 at 12.1 min as any structural isomer of akuammicine (**12o**). The corresponding signals are not observed in the control culture.