Supplementary Information for

Genetic Engineering of Plant Cell Culture to Produce Unnatural Alkaloids

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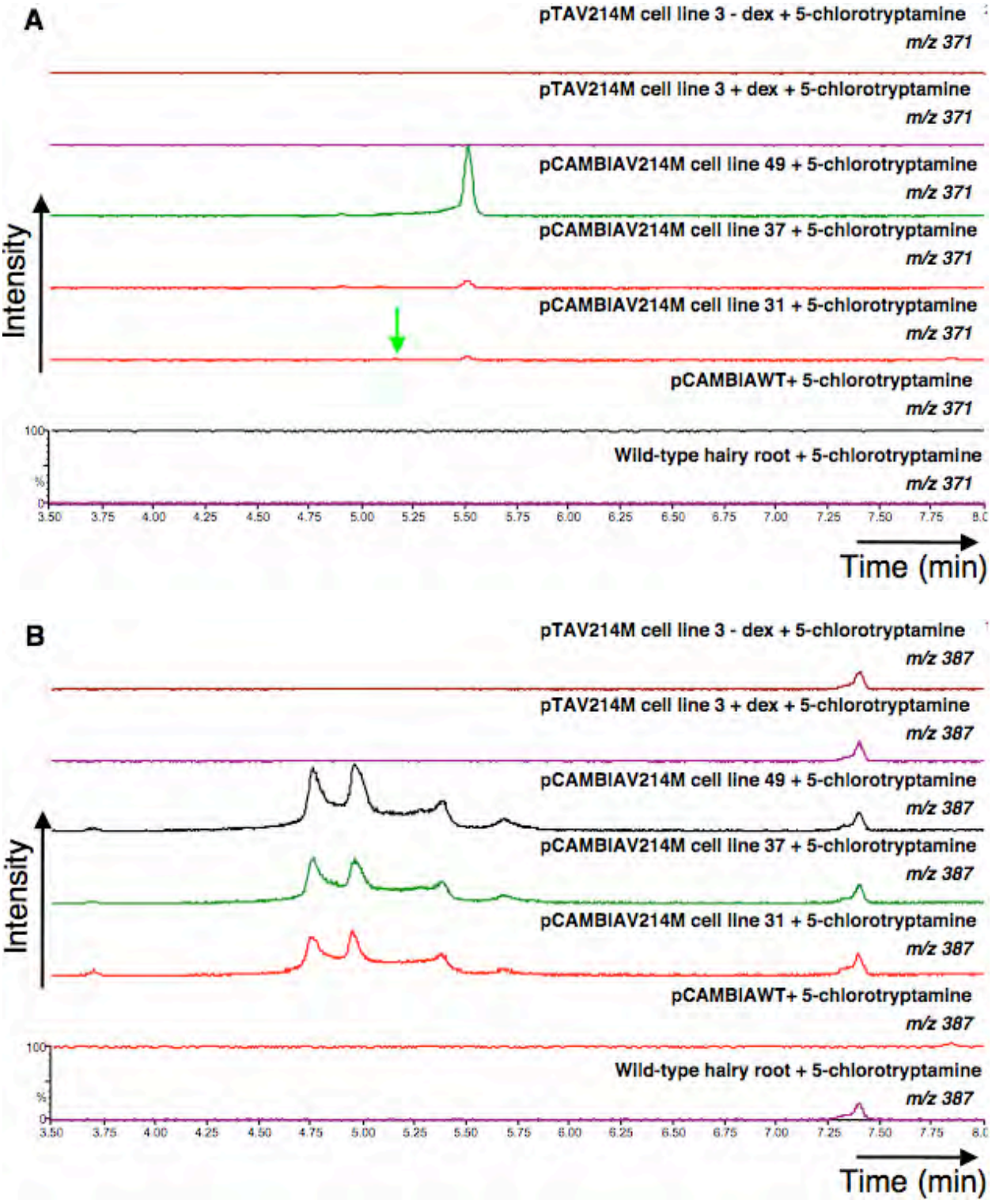

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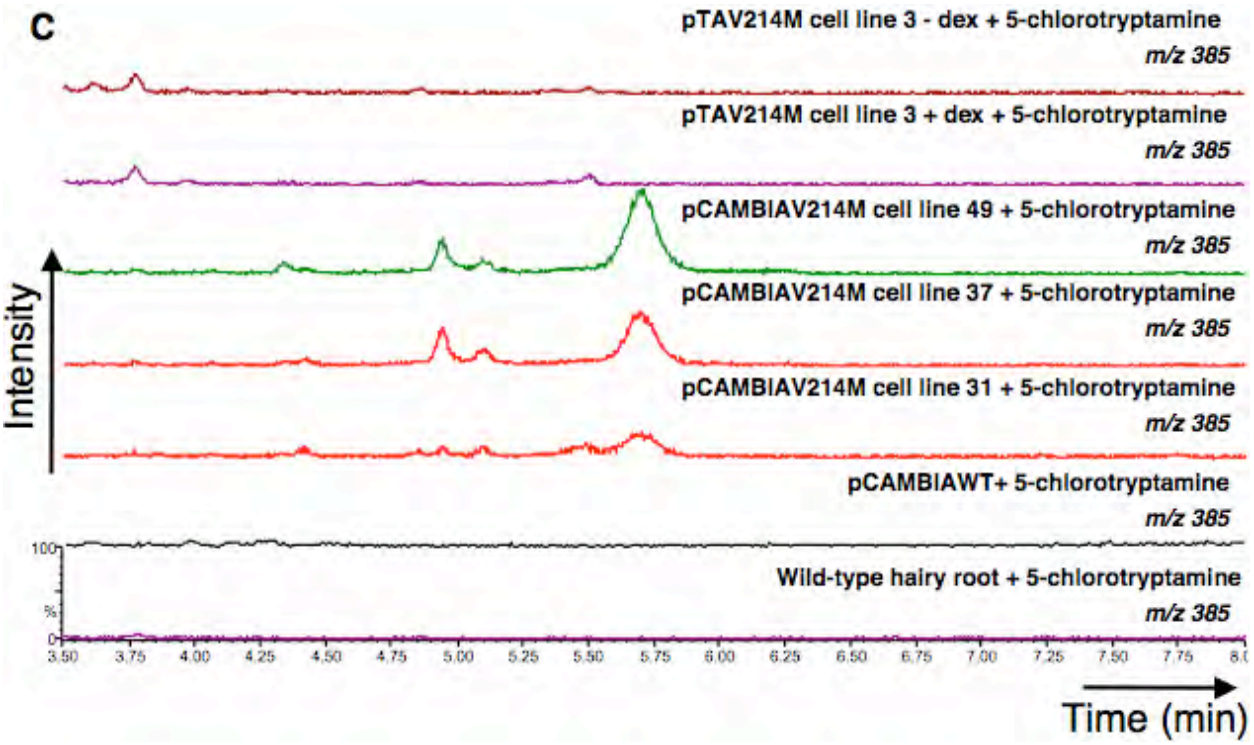

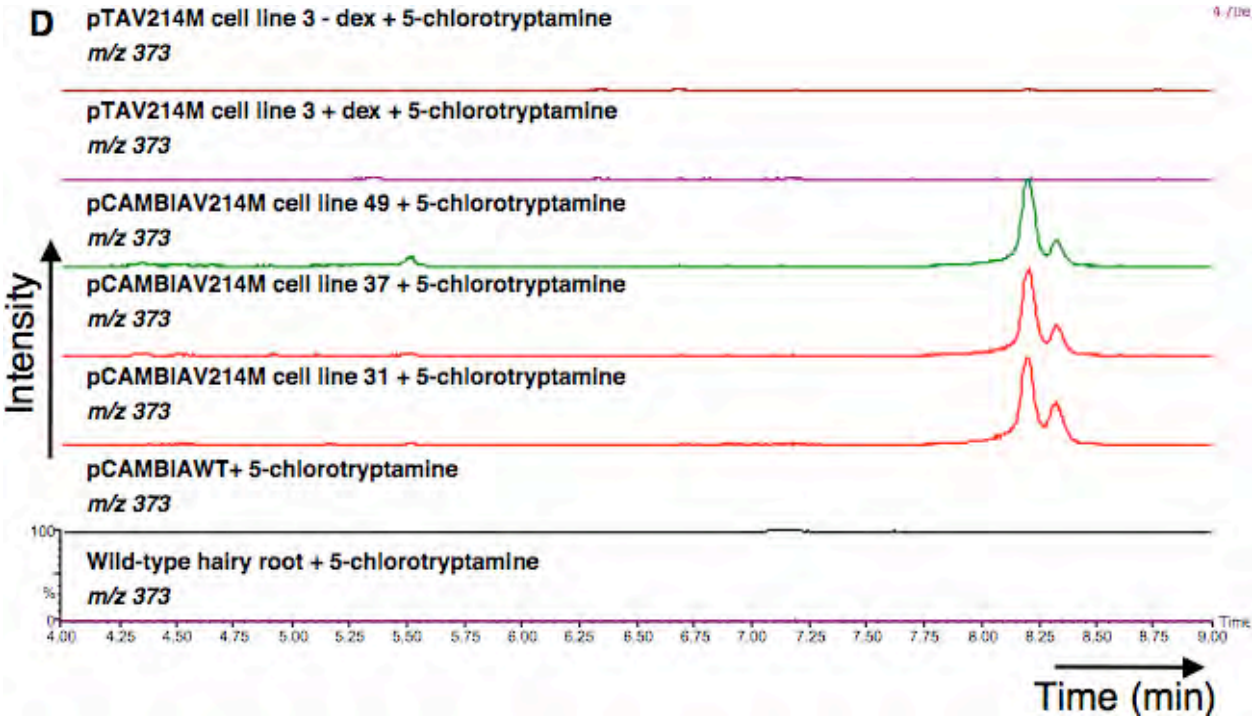

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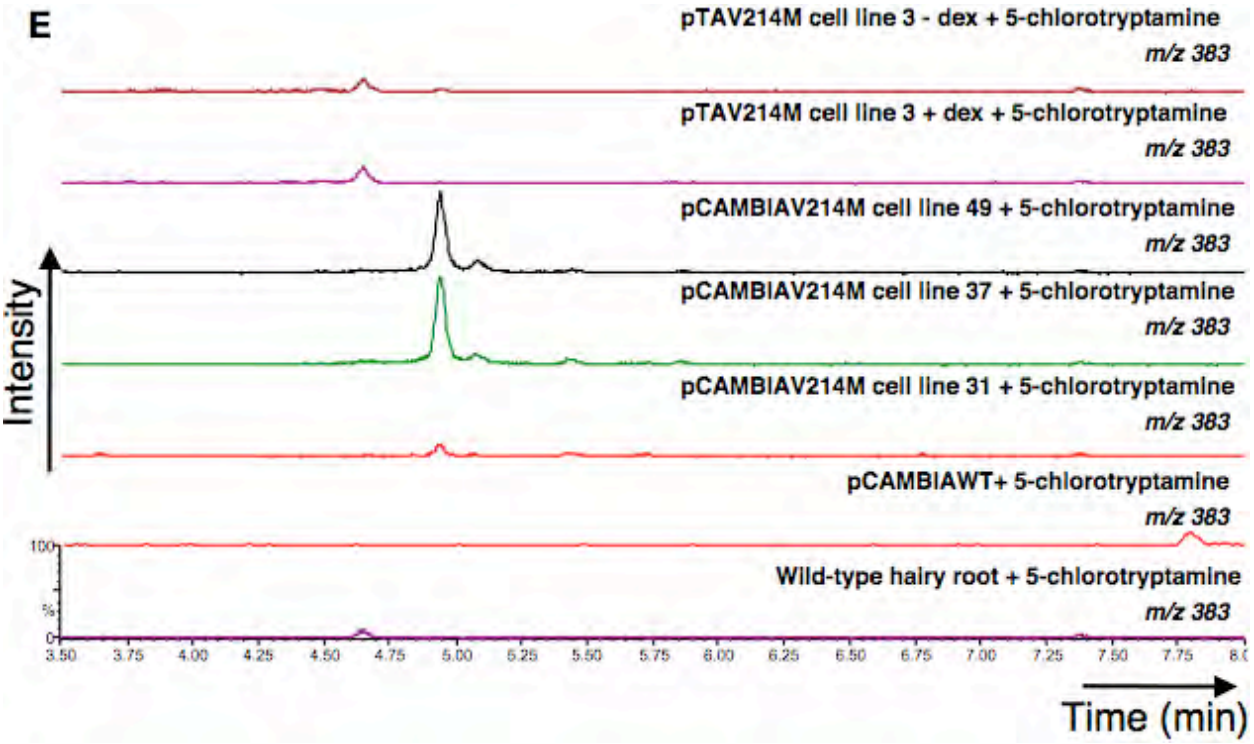

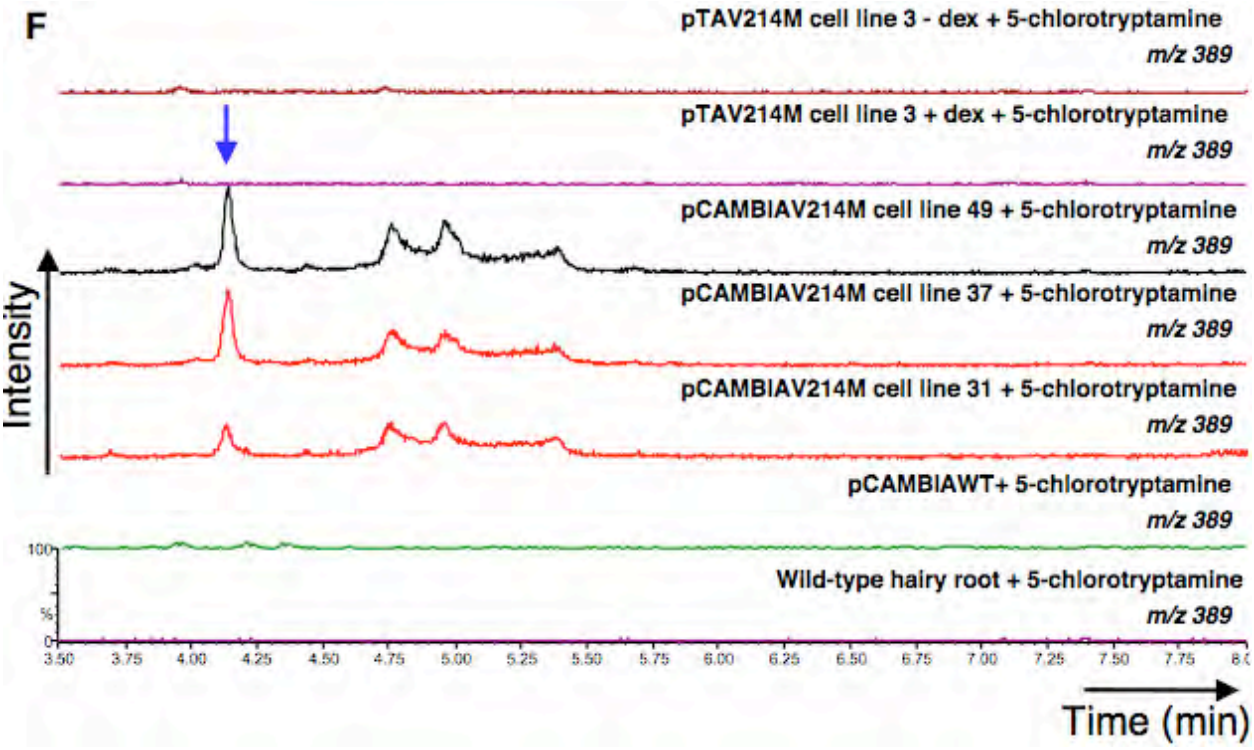

Figure S1, part I, A-F. LC-MS traces of selected alkaloid analogs formed by feeding 5-chlorotryptamine **2a** to 2-week-old pCAMV214M hairy roots (cell lines 31, 37 and 49).

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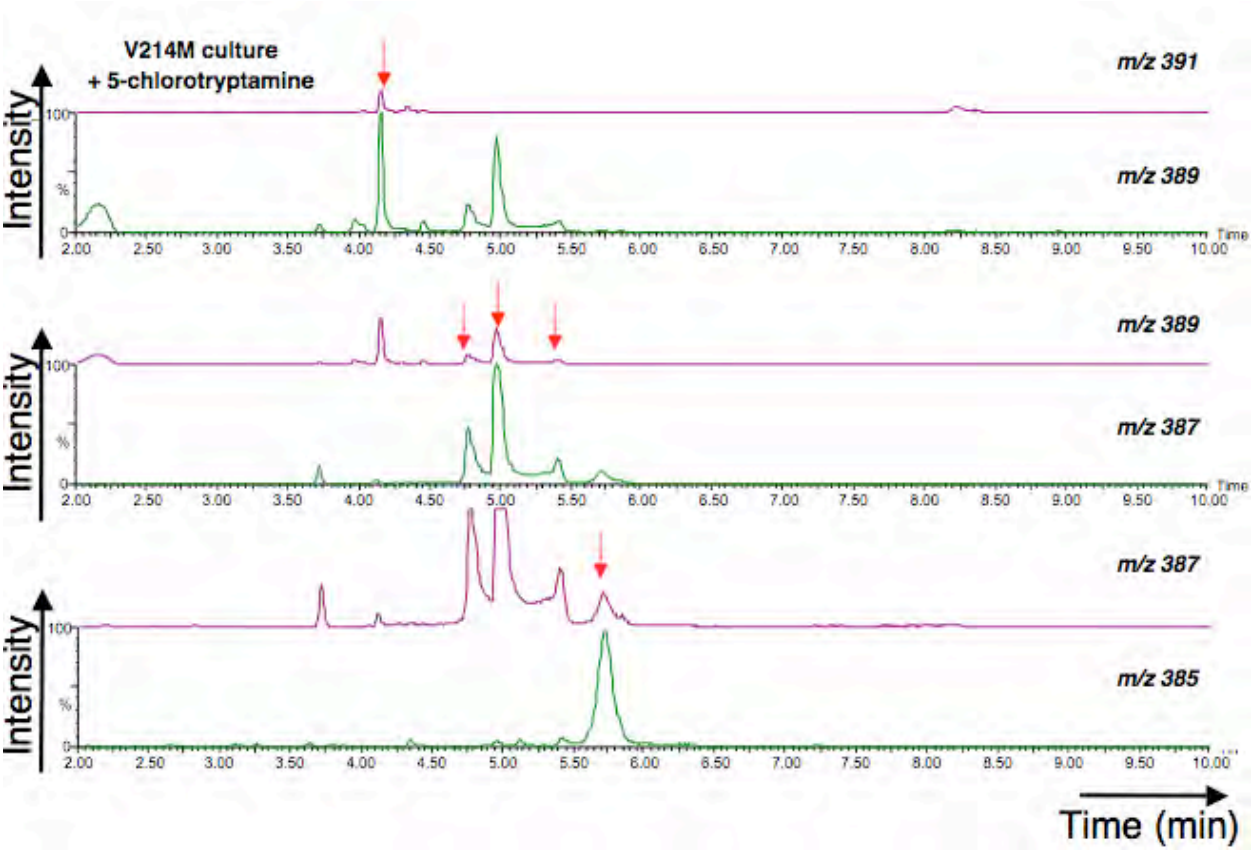
Formation of chlorinated analogs (**S4A,** *m/z* 371, 337+34; **S4B**, *m/z* 387, 353+34; **S4C**, *m/z* 385, 351+34; **S4D**, *m/z* 373, 339+34; **S4E**, *m/z* 383, 349+34; and **S4F**, *m/z* 389, 355+34) upon feeding of 5-chlorotryptamine **2a** to hairy roots. These analogs (*m/z* M+34) are absent in pTAV214M cultures (cell lines 3) fed with 5-chlorotryptamine **2a** either with or without the glucocorticoid inducer, dexamethasone, as well as in wild-type and pCAMWT cultures fed with 5-chlorotryptamine **2a**.



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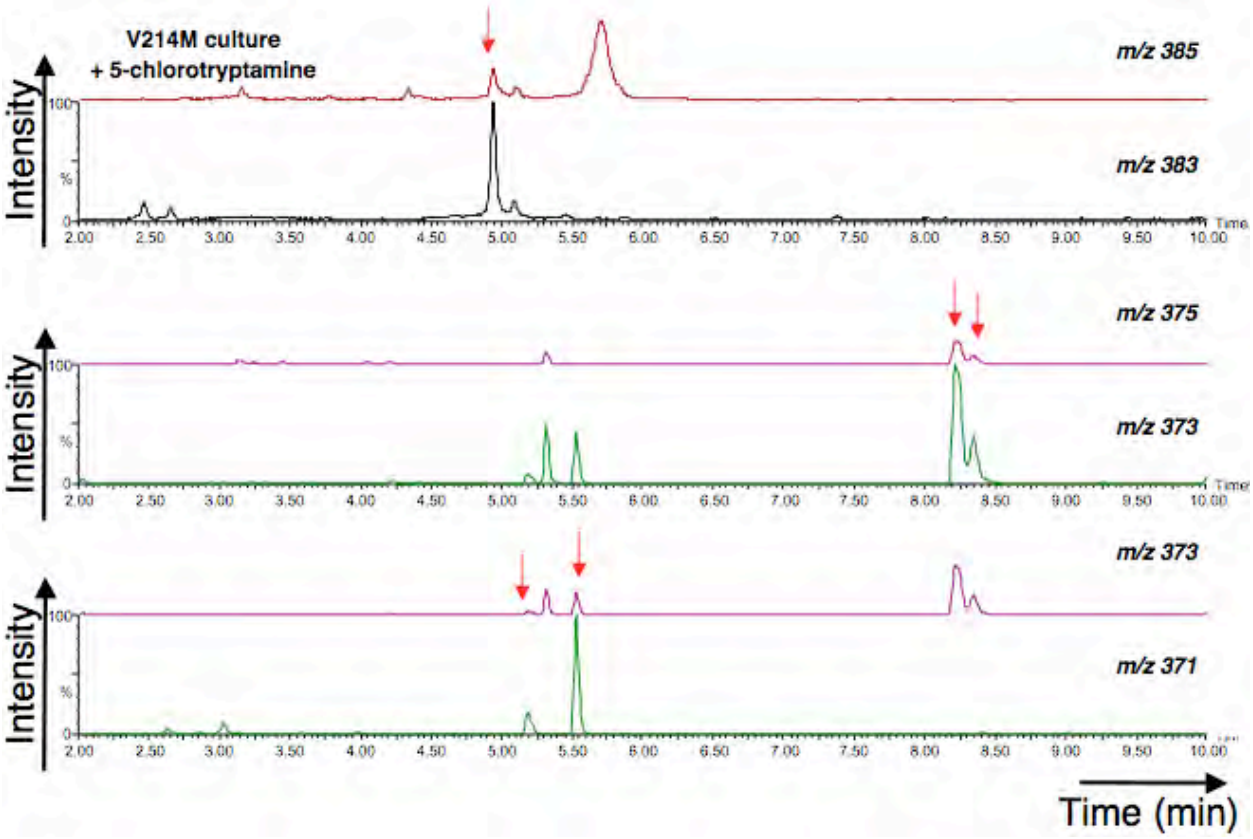

Figure S1, part II. LC-MS traces of selected alkaloid analogs formed by feeding 5-chlorotryptamine **2a** to 2-week-old pCAMV214M hairy roots showing characteristic isotopic signature of chlorine. Intensities are normalized pairwise.

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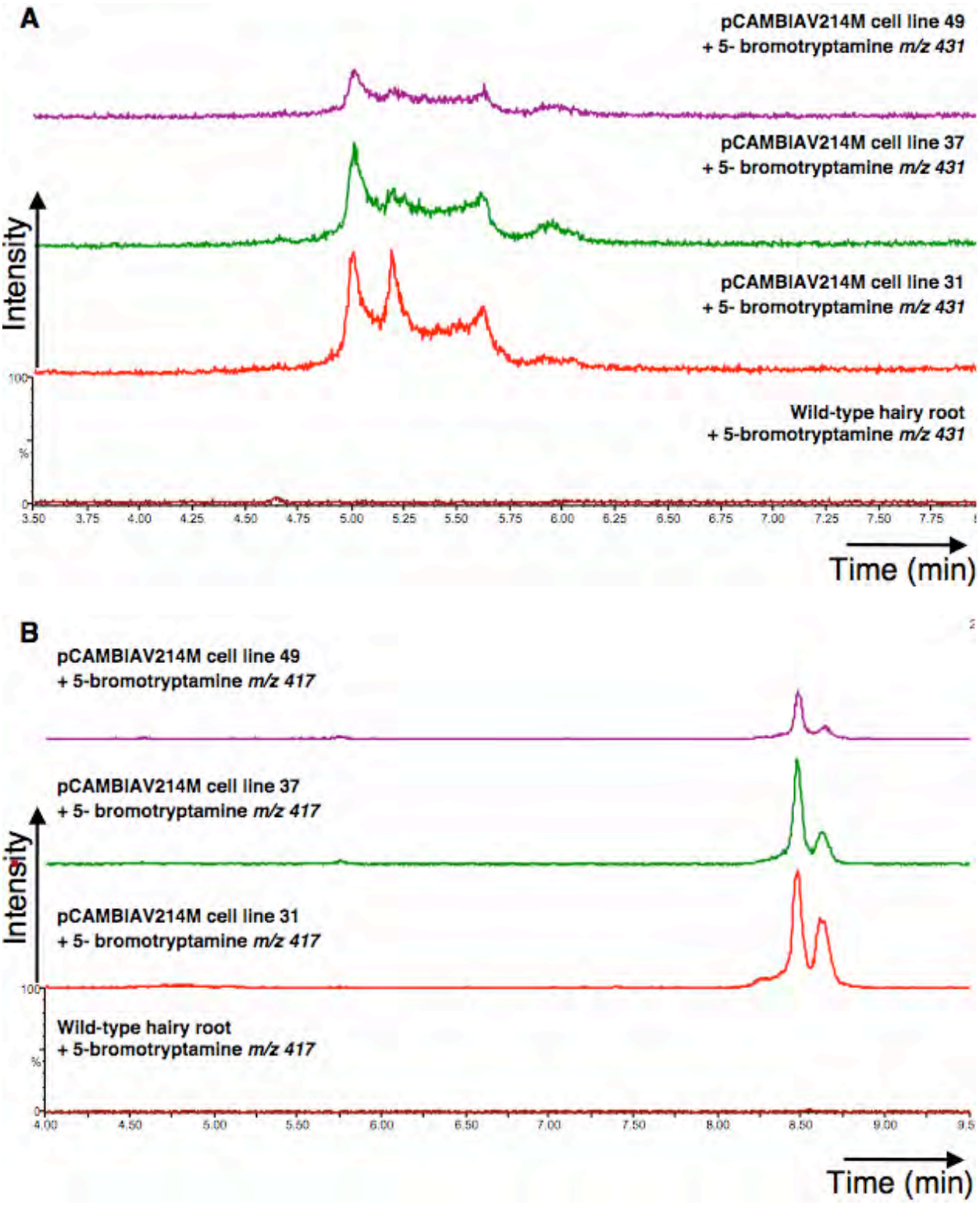

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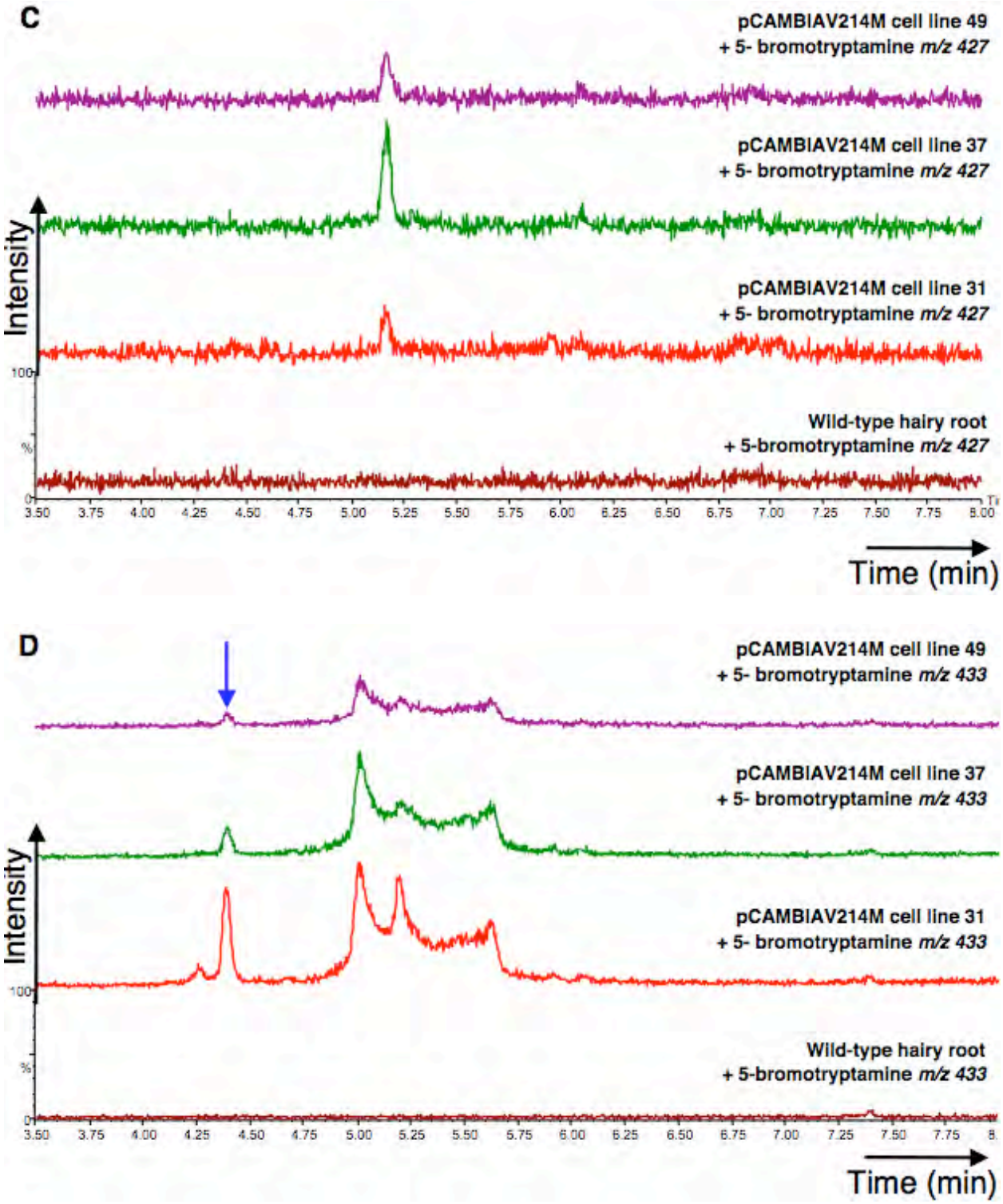

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Figure S1, part III, A-D. LC-MS traces of selected alkaloid analogs formed by feeding 5-bromotryptamine **2c** to 2-week-old pCAMV214M hairy roots (cell lines 31, 37 and 49). Formation of brominated analogs (**S6A,** *m/z* 431, 353+78; **S6B,** *m/z* 417, 339+78; **S6C,** *m/z* 427, 349+78; and **S6D,** *m/z* 433, 355+78) upon feeding of 5-bromotryptamine **2c** to hairy roots. These analogs (*m/z* M+78) are absent in wild-type culture fed with 5-bromotryptamine **2c**.

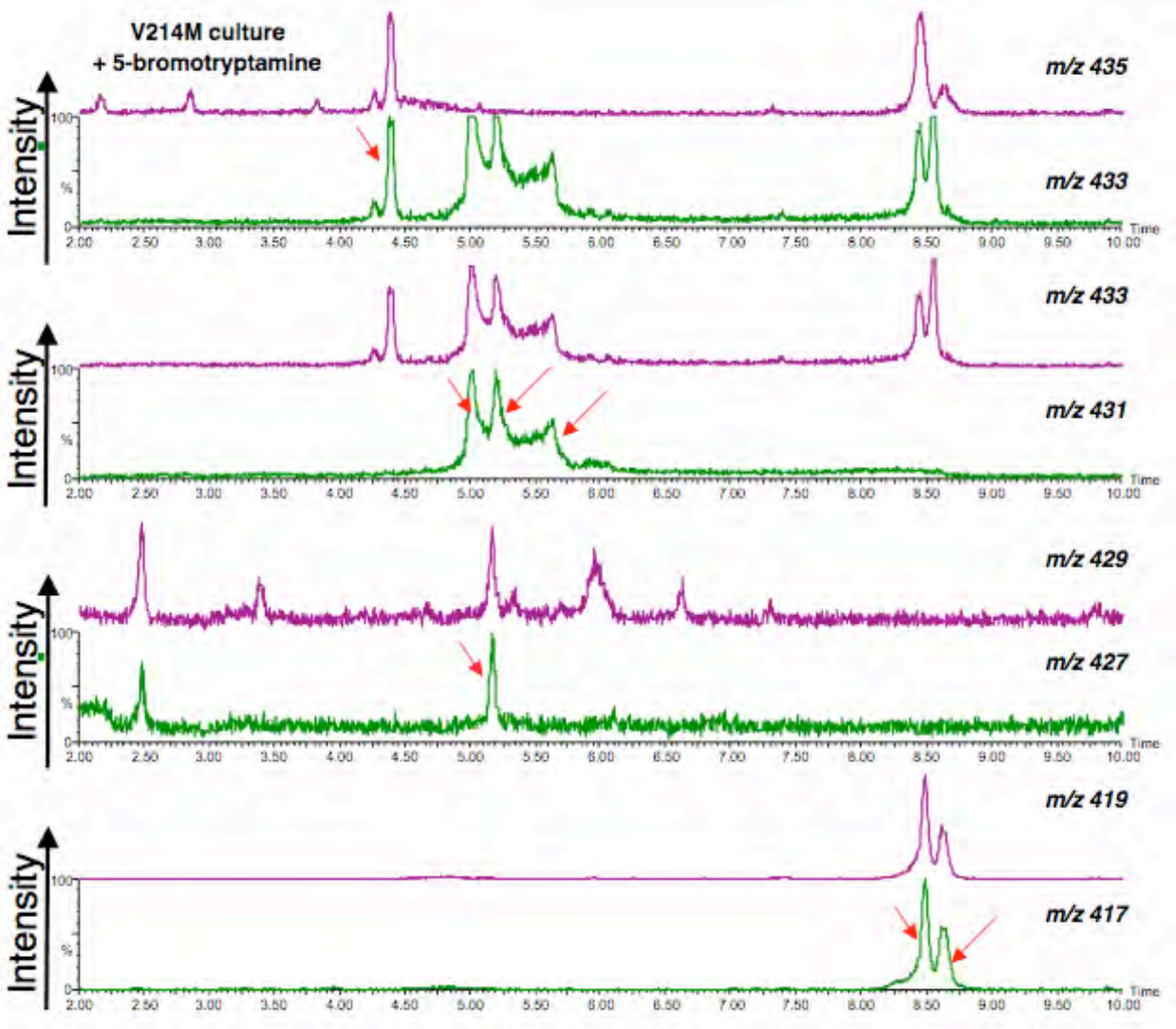

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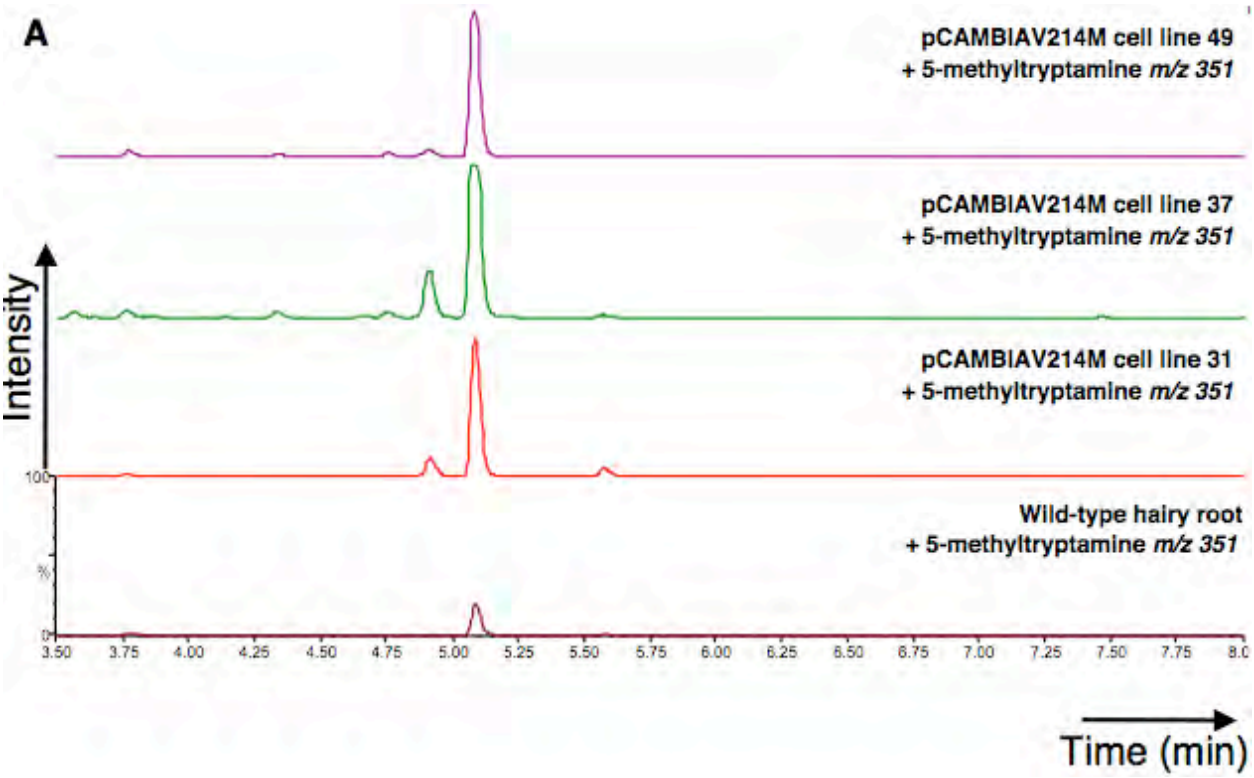
Figure S1, part IV. LC-MS traces of selected alkaloid analogs formed by feeding 5-bromotryptamine **2a** to 2-week-old pCAMV214M hairy roots showing characteristic isotopic signature of bromine. Intensities are normalized pairwise.



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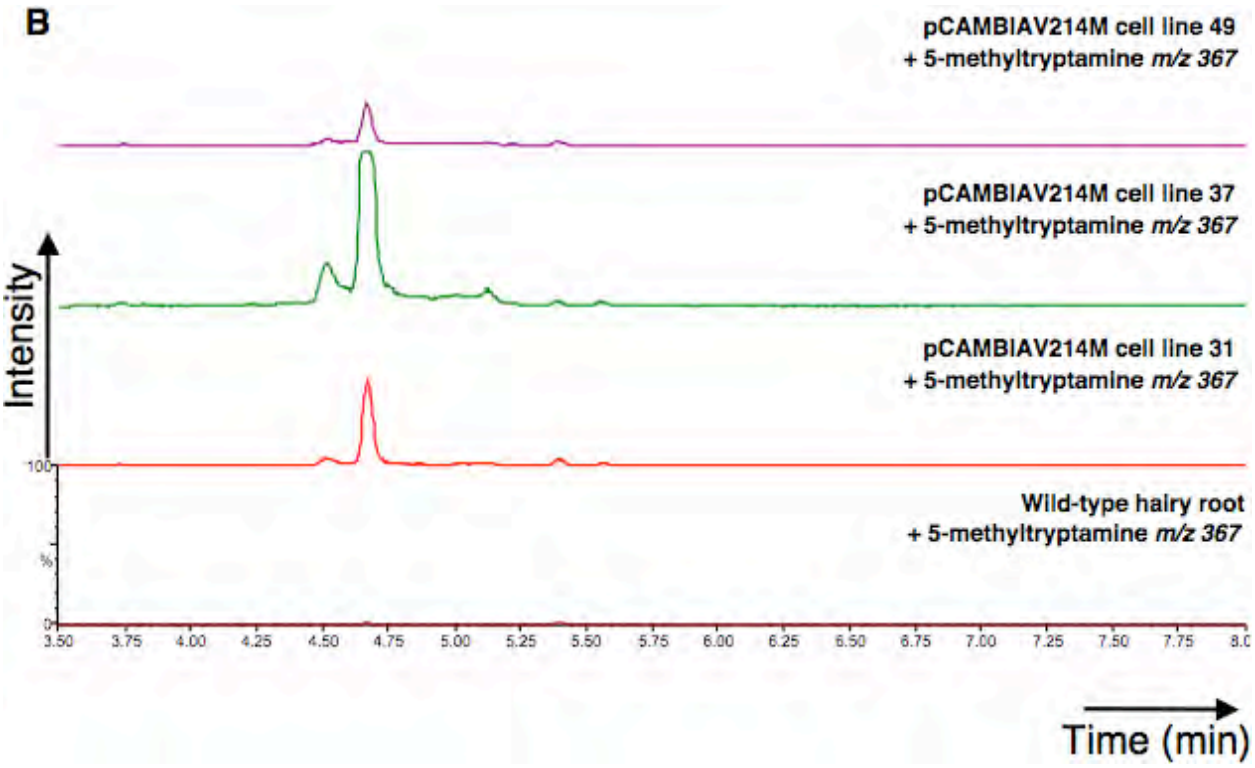

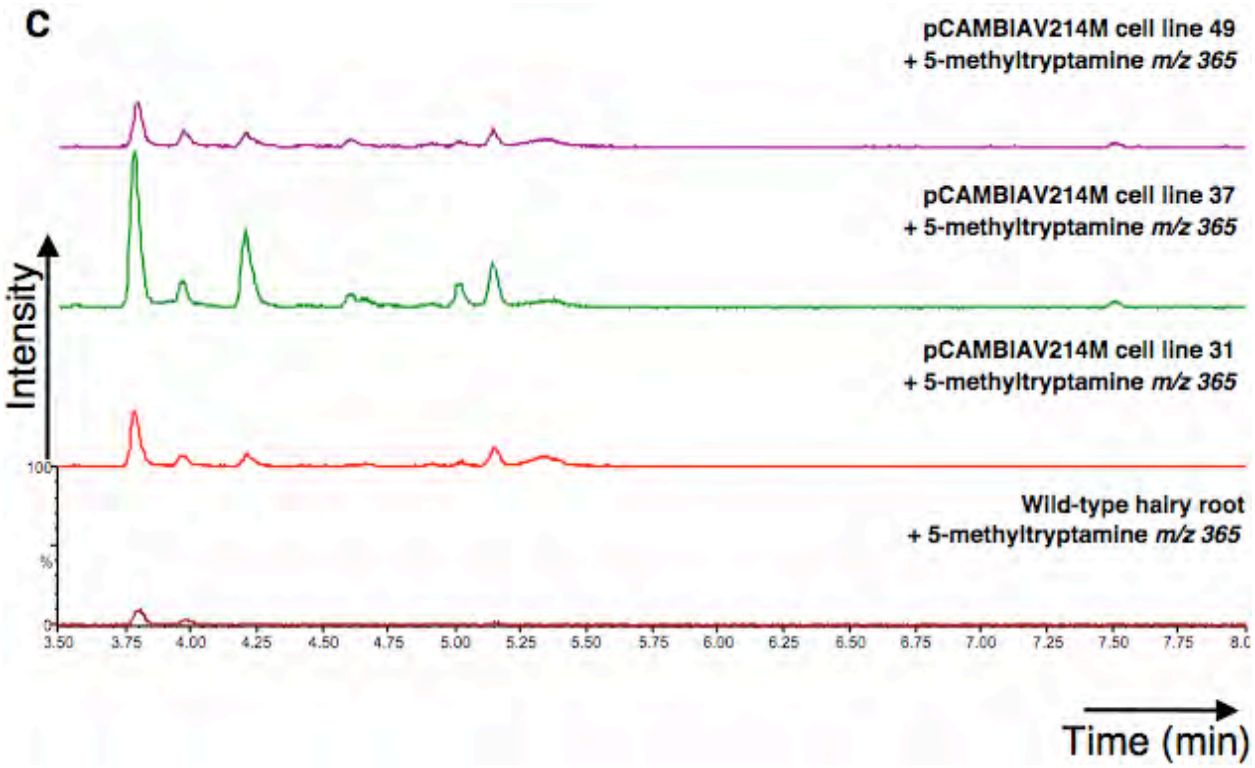

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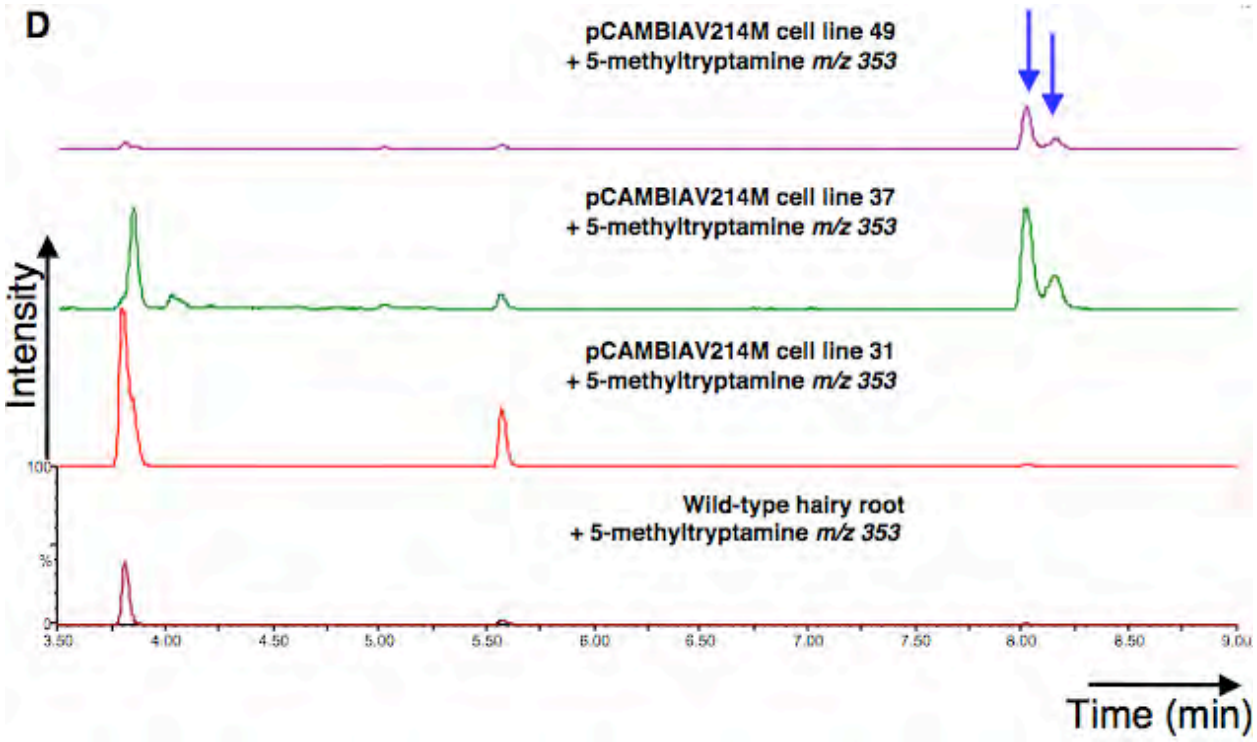

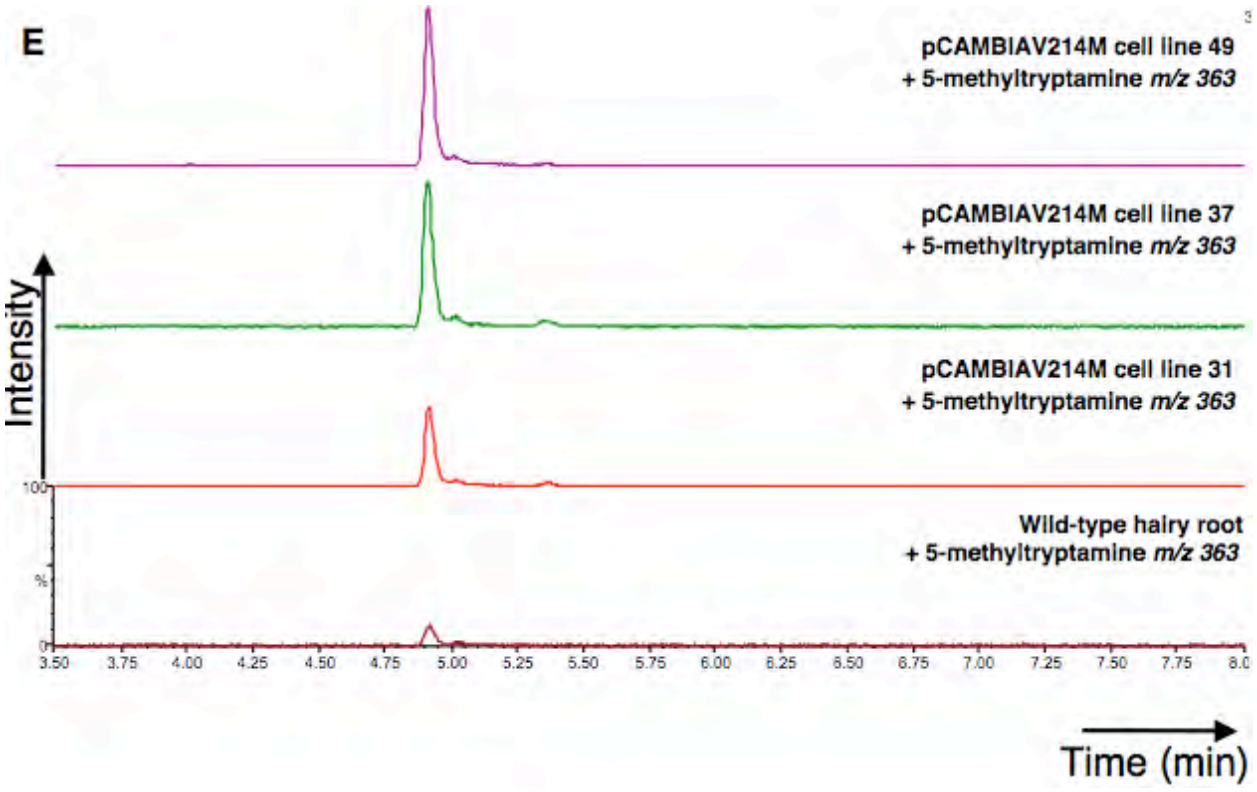

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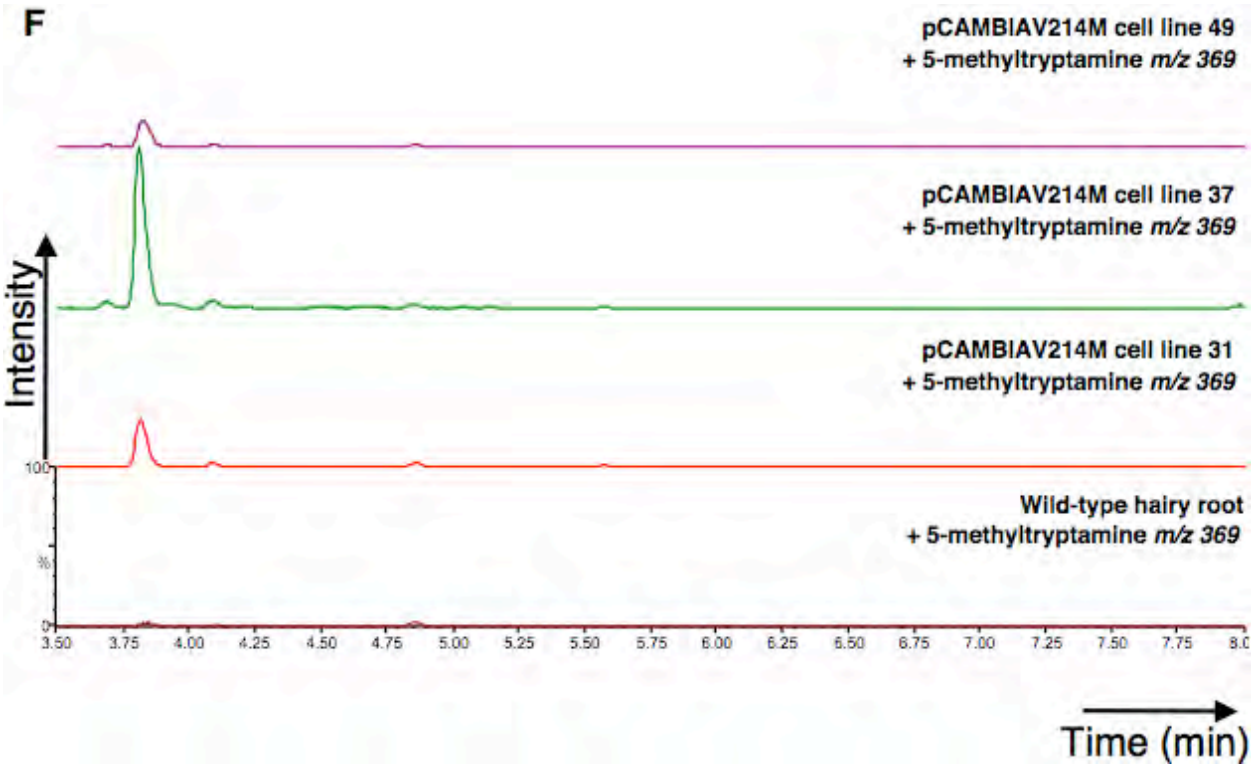

Figure S1, part V, A-F. LC-MS traces of selected alkaloid analogs formed by feeding 5-methyltryptamine to 2-week-old pCAMV214M hairy roots (cell lines 31, 37 and 49). Formation of methylated analogs (**S8A,** *m/z* 351, 337+14; **S8B**, *m/z* 367, 353+14; **S8C**, *m/z* 365, 351+14; **S8D**, *m/z* 353, 339+14; **S8E**, *m/z* 363, 349+14; and **S8F**, *m/z* 369, 355+14) upon feeding of 5-methyltryptamine **2b** to hairy roots. Note that some of these analogs (*m/z* M+14) are still present, though in significantly lower quantities, in wild-type culture fed with 5-methyltryptamine **2b**. Definitive assignment of the structure of these compounds as originating from **2b** depends on more detailed structural characterization.

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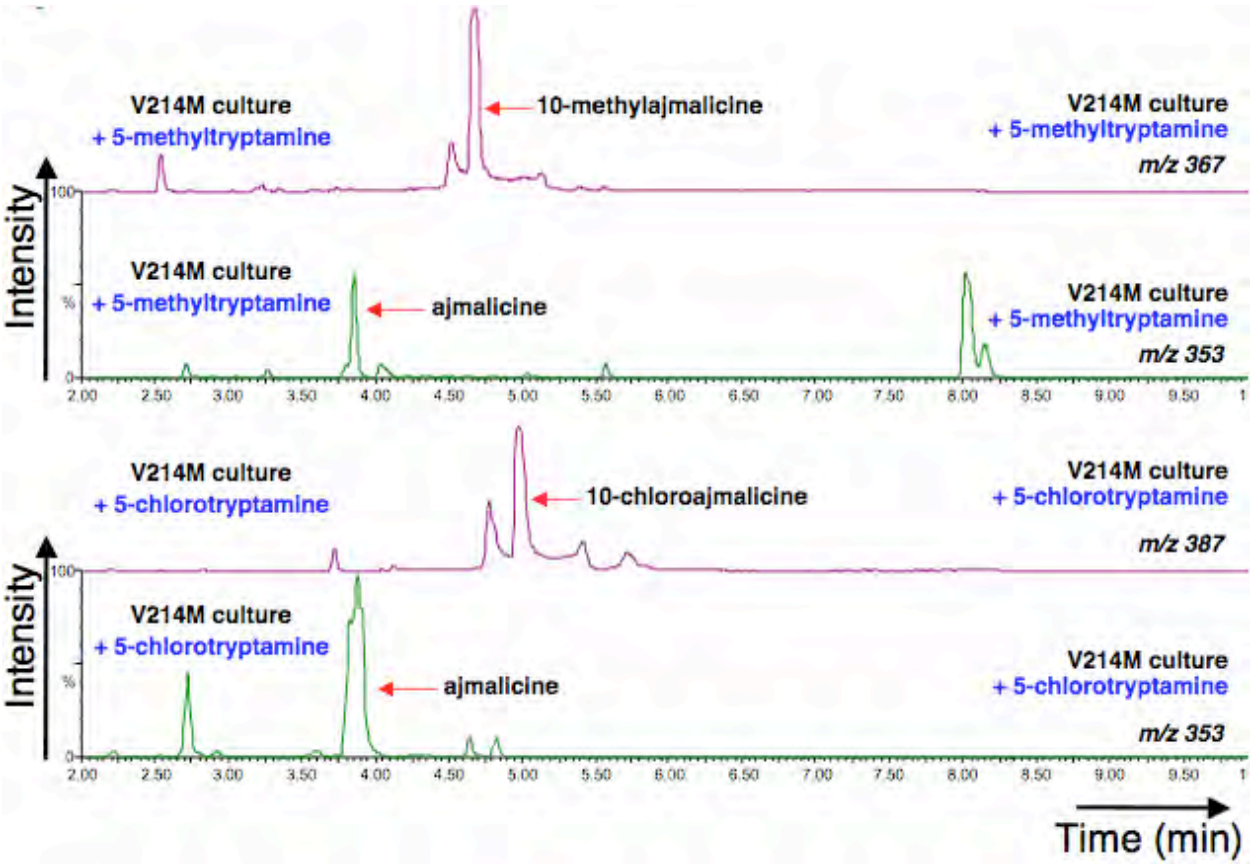

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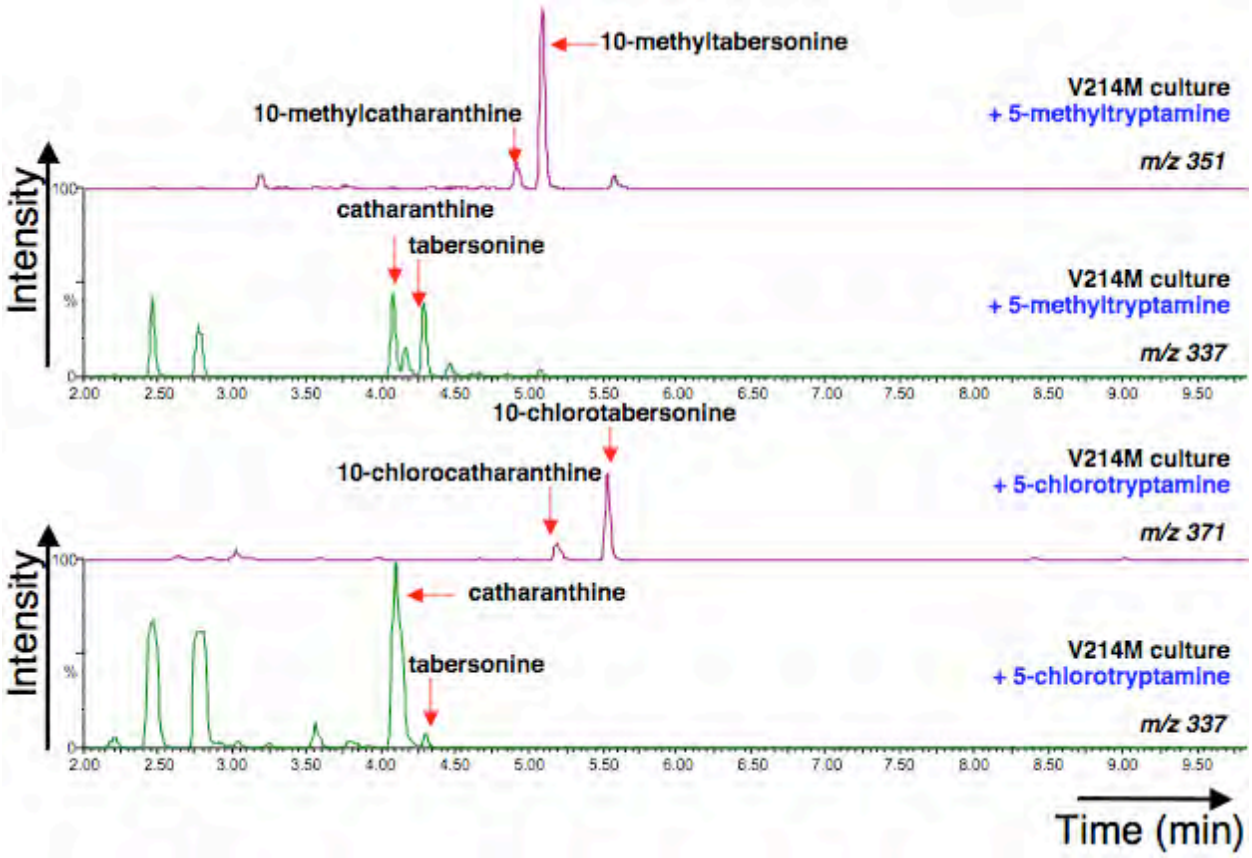

Figure S2. LC-MS traces of selected alkaloid analogs formed by feeding either 5-chlorotryptamine or 5-methyltryptamine to 2-week-old pCAMV214M hairy roots showing relative production levels of natural alkaloids (ajmalicine **4** and tabersonine **5**) compared to unnatural chlorinated/methylated alkaloids. Intensities are normalized pairwise. The ratio of natural catharanthine to natural tabersonine (green traces) changes depending on whether **2a** or **2b** is fed to the cultures.

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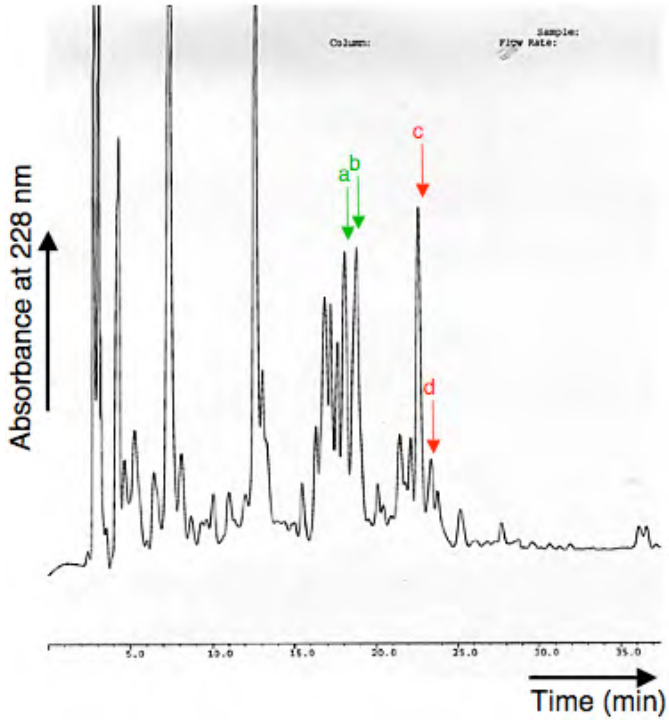

Figure S3, part I. HPLC trace of alkaloids from pCAMV214M hairy root (cell line 31) fed with 5-chlorotryptamine **2a** (0.5 mM). Fractions a, b, c and d, referred to below, were collected and analyzed.

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II

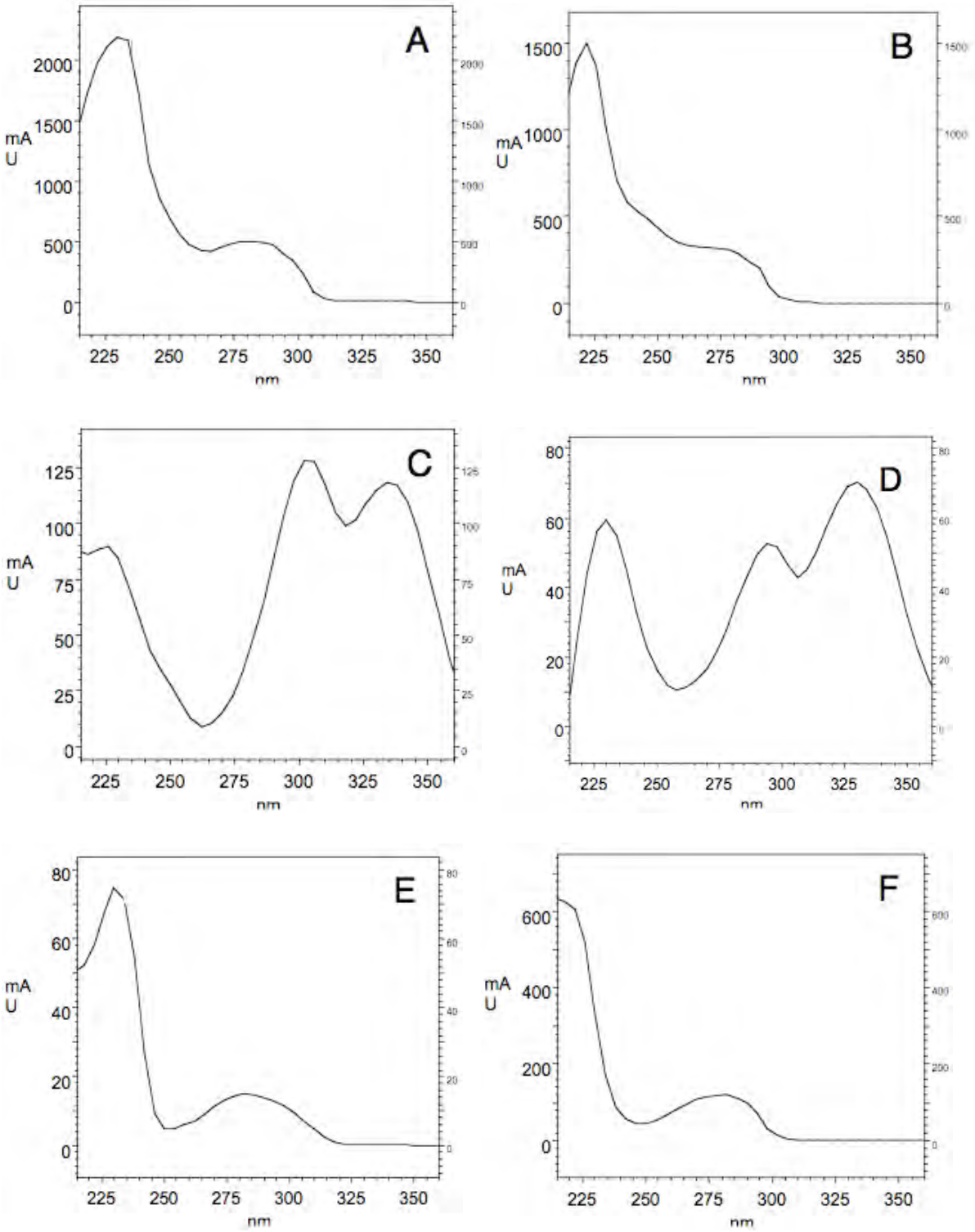

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Figure S3, part II. UV spectra of alkaloid standards and isolated alkaloids from pCAMV214M hairy root (cell line 31) fed with 5-chlorotryptamine (0.5 mM). **A.** 10-chloroajmalicine **4a** (fraction c in **Figure S3, part I**). **B.** ajmalicine standard**. C.**10-chlorotabersonine **5a** (fraction d in **Figure S3, part I**). **D.** tabersonine standard. **E.** Unknown chlorinated alkaloid with *m/z* 371 with UV spectra comparable to that of catharanthine **7** (fraction d in **Figure S3, part I**). **F.** catharanthine standard.

Figure S3, part III. ^{1}H NMR and ^{13}C-^{1}H HSQC spectra of 10-chloroajmalicine **4a**. All alkaloids were isolated from pCAMV214M hairy root (cell line 31) fed with 5-chlorotryptamine **2a** (0.5 mM).

Tabulated NMR data for 10-chloroajmalicine **4a** (Fraction c in **Figure S3, part I**)
^{1}H NMR (600 MHz, CD_3OD): δ 7.64 (d, *J* = 1.8 Hz, 1H), 7.53 (d, *J* = 1.8 Hz, 1H), 7.39 (d, *J* = 9.0 Hz, 1H), 7.17 (dd, *J* = 1.8, 8.4 Hz, 1H), 4.87 (m, 1H), 4.59 (qd, *J* = 3.6, 6.6 Hz, 1H), 3.89 (dd, *J* = 5.7, 12.6 Hz, 1H), 3.79 (s, 3H), 3.70 (dd, *J* = 3.0, 12.0 Hz, 2H), 3.45 (dd, *J* = 1.2, 1.8 Hz, 1H), 3.25 (m, 1H), 3.22 (m, 1H), 3.15 (dd, *J* = 4.2, 16.2 Hz, 1H), 2.84 (br t, *J* = 11.4 Hz, 1H), 2.26 (m, 1H), 1.53 (m, 1H), 1.28 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (500 MHz, CD_3OD): δ 156.20, 124.05, 118.88, 114.07, 73.61, 63.03, 55.67, 54.54, 51.88, 40.94, 32.28, 30.29, 20.30, 14.74 (note that quaternary carbon peaks are not observed in ^{1}H-^{13}C HSQC ;ESI-MS (*m/z)*: $[M]^+$ calcd. for $C_{21}H_{23}N_2O_3Cl$, 387.1490; found, 387.1483.

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NMR data for 15-chlorotabersonine **5a** (Fraction d in **Figure S3, part I**)

^{1}H NMR (500 MHz, CD_3OD): δ 7.68 (d, J = 2.0 Hz, 1H), 7.26 (dd, J = 2.0, 8.0 Hz, 1H), 7.03 (d, J = 8.0 Hz, 1H), 6.06 (d, J = 10.5 Hz, 1H), 6.00 (dd, J = 4.0, 10.5 Hz, 1H), 4.21 (d, J = 10.0 Hz, 1H), 3.80 (s, 3H), 3.67 (m, 1H), 3.57 (m, 1H), 3.17 (m, 1H), 2.94 (m, 1H), 2.34 (d, J = 17.5 Hz, 1H), 1.83 (d, J = 7.0 Hz, 1H), 1.78 (dd, J = 2.5, 7.0 Hz, 2H), 1.67 (d, J = 6.50 Hz, 1H), 1.14 (t, J = 7.50, 1H), 0.73 (t, J = 7.5 Hz, 3H); ESI-MS (*m/z)*: $[M]^+$ calcd. for $C_{21}H_{23}N_2O_2Cl$, 371.1526; found, 371.1528.

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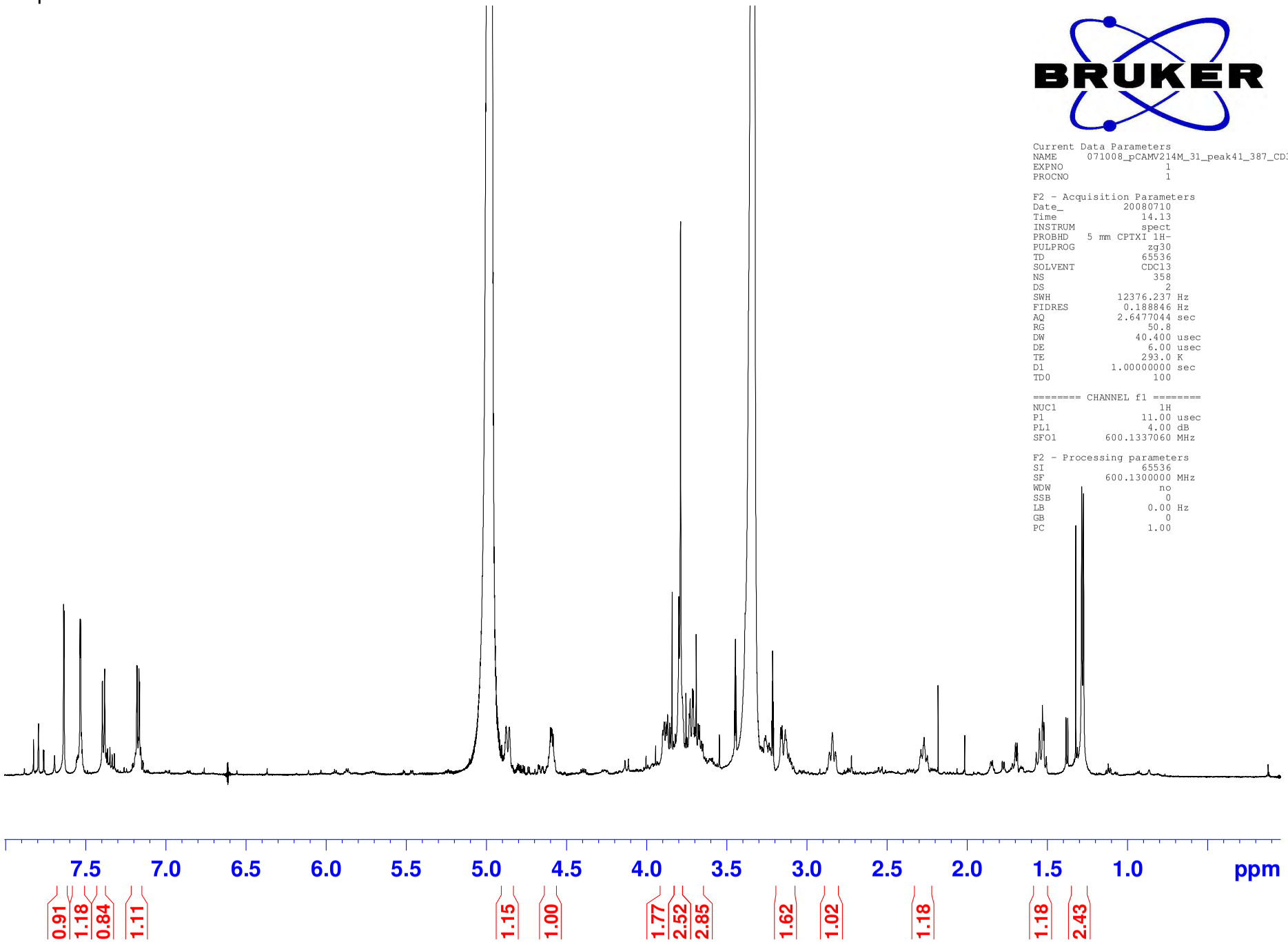
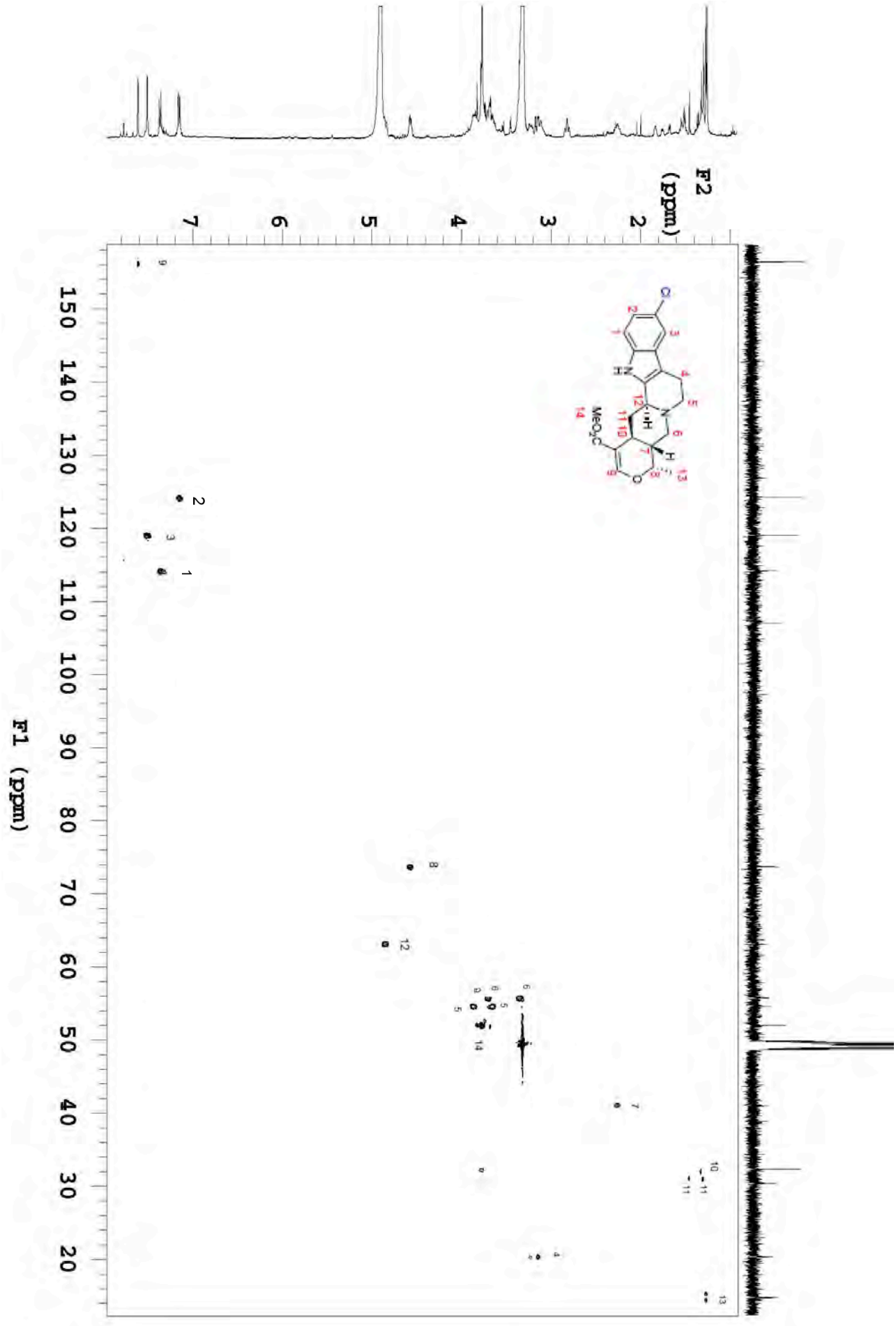
Compound 4a





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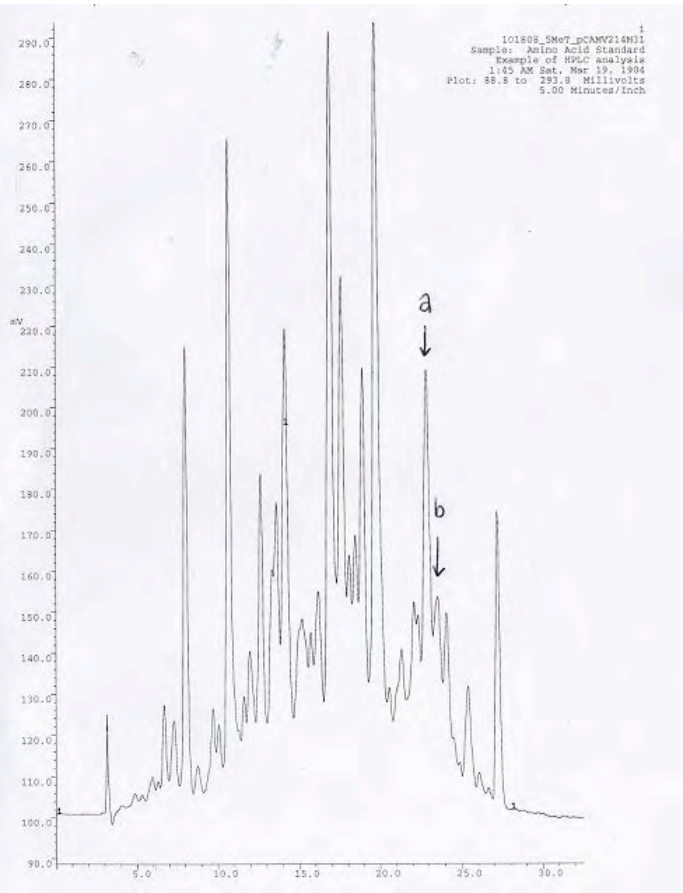

Figure S4, part I. HPLC trace of alkaloids from pCAMV214M hairy root (cell line 31) fed with 5-methyltryptamine **2b** (0.5 mM). Fractions a and b, referred to below, were collected and analyzed.

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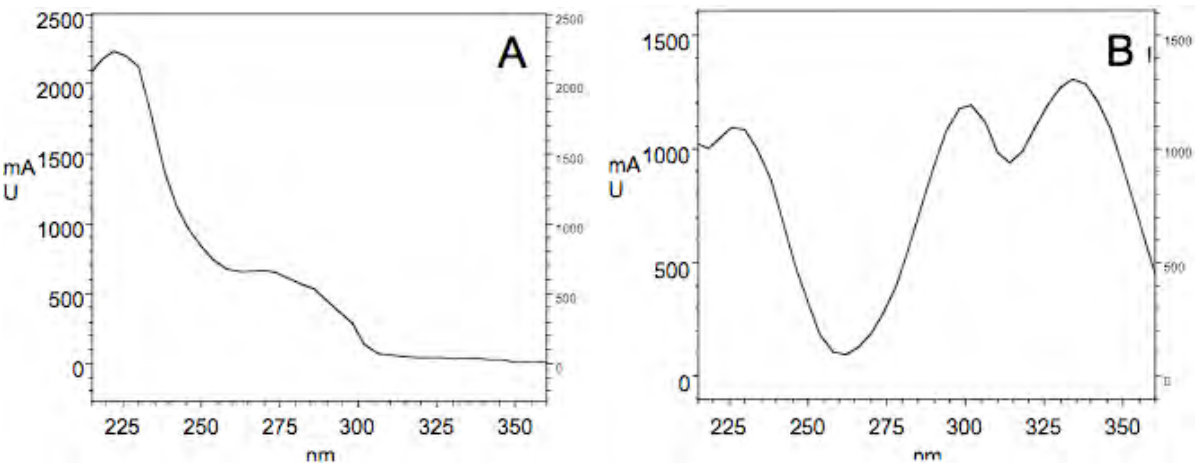

Figure S4, part II. UV spectra of isolated alkaloids from pCAMV214M hairy root (cell line 31) fed with 5-methyltryptamine (0.5 mM). **A.** 10-methylajmalicine **4b** (fraction a in **Figure S4, part I**). **B.** 10-methyltabersonine **5b** (fraction b in **Figure S4, part I**).

Figure S4, part III. ^{1}H NMR, ^{13}C-^{1}H HSQC, and ^{13}C-^{1}H HMBC spectra of 10-methyltabersonine **5b.** The alkaloid was isolated from pCAMV214M hairy root (cell line 31) fed with 5-methyltryptamine **2b** (0.5 mM).

Tabulated NMR data for 10-methylajmalicine **4b** (Fraction a in **Figure S4, part I**)

^{1}H NMR (500 MHz, CD_3OD): δ 7.60 (d, J = 1.5 Hz, 1H), 7.01 (d, J = 9.0 Hz, 1H), 6.93 (dd, J = 4.5, 8.0 Hz, 1H), 4.81 (dd, J = 4.0, 11.0 Hz, 1H), 4.57 (dd, J = 4.0, 6.5 Hz, 1H), 3.89 (m, 1H), 3.77 (s, 3H), 3.66 (m, 2H), 3.45 (m, 1H), 3.25 (m, 1H), 3.22 (m, 1H), 3.13 (m, 1H), 2.84 (m, 1H), 2.41 (s, 3H), 2.25 (m, 1H), 1.52 (m, 1H), 1.26 (d, J = 6.5 Hz, 3H); ESI-MS (*m/z*): $[M]^+$ calcd. for $C_{22}H_{27}N_2O_3$, 367.2022; found, 367.2015.

NMR data for 10-methyltabersonine **5b** (Fraction b in **Figure S4, part I**)

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^{1}H NMR (600 MHz, CD_3OD): δ 7.40 (s, 1H), 7.08 (d, *J* = 8.0 Hz, 1H), 6.93 (d, *J* = 7.9 Hz, 1H), 6.06 (d, *J* = 10.0 Hz, 1H), 6.00 (dd, *J* = 4.4, 10.1 Hz, 1H), 4.22 (d, *J* = 15.4 Hz, 1H), 4.01 (d, *J* = 14.0 Hz, 1H), 3.93 (s, 1H), 3.80 (s, 3H), 3.67 (m, 1H), 3.57 (m, 1H), 2.92 (d, *J* = 16.6 Hz, 1H), 2.42 (m, 1H), 2.33 (s, 3H), 2.19 (br s, 2H), 1.29 (s, 1H), 1.19 (m, 2H), 0.71 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (500 MHz, CD_3OD): δ 135.49, 130.89, 122.80, 122.62, 111.49, 72.15, 54.30, 51.98, 51.78, 44.44, 30.96, 28.80, 21.25, 7.81 (note that quaternary carbon peaks are not observed in ^{1}H-^{13}C HSQC); ESI-MS (*m/z)*: $[M]^+$ calcd. for $C_{22}H_{27}N_2O_2$, 351.2073; found, 351.2064.

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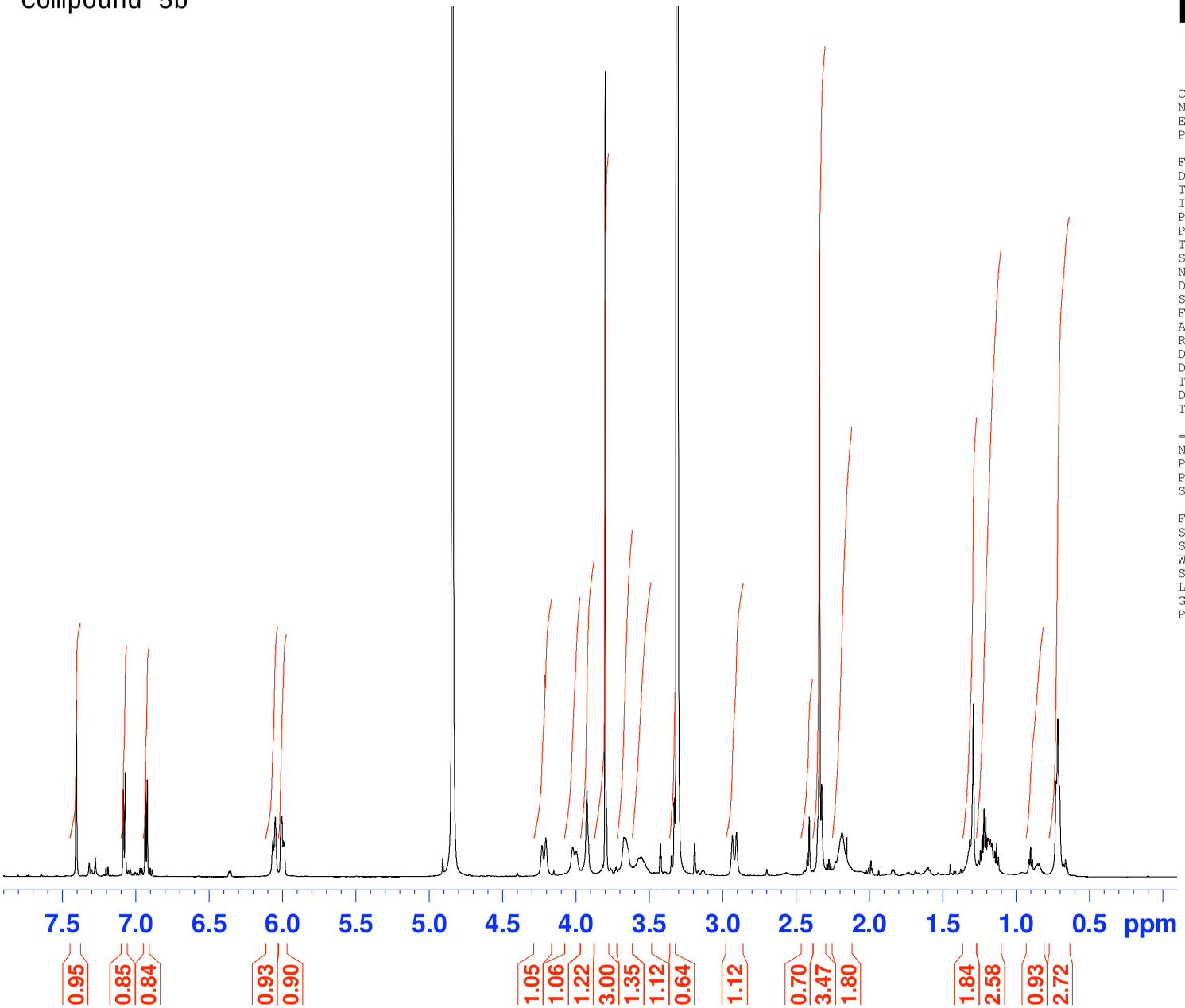
Compound 5b

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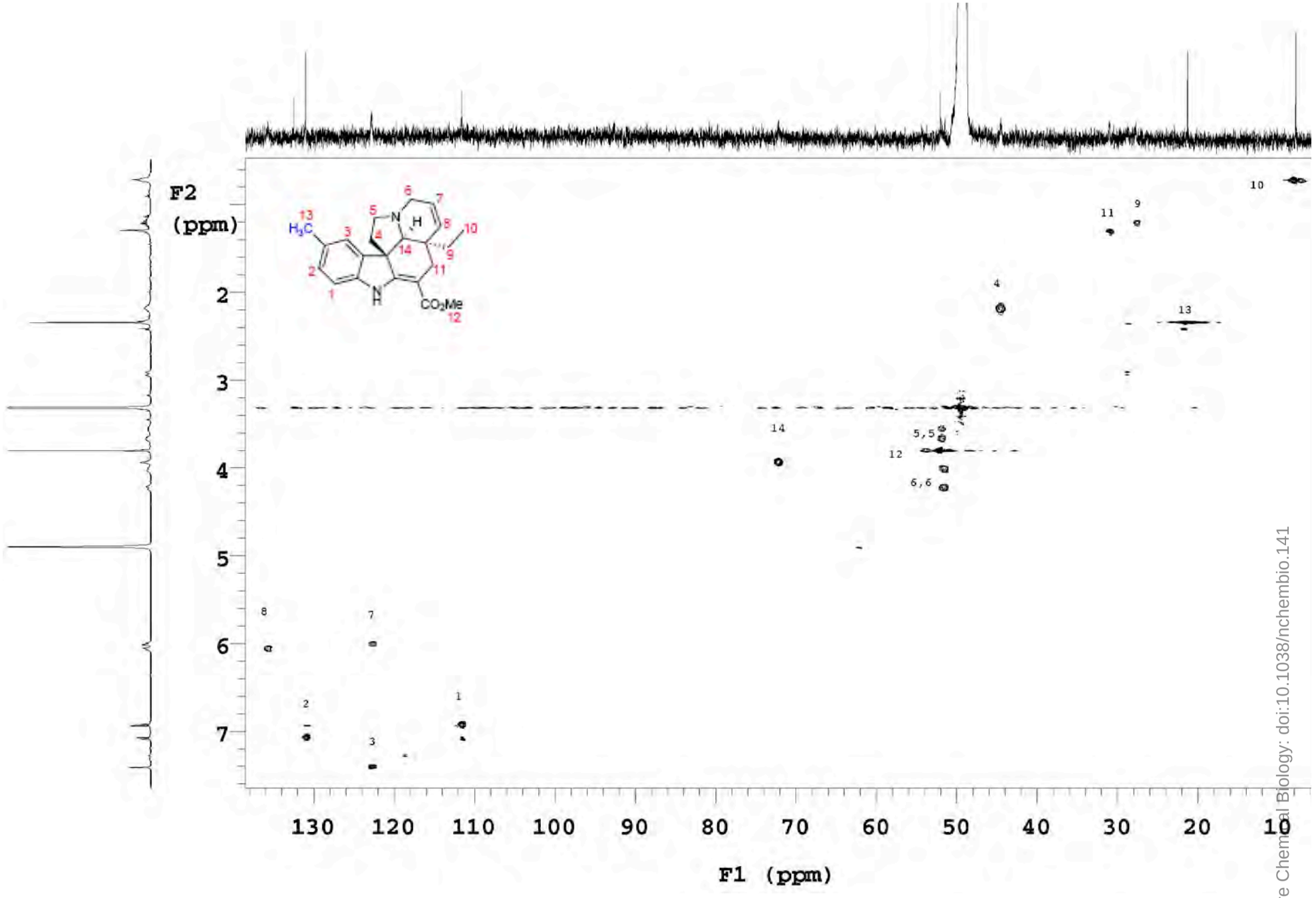

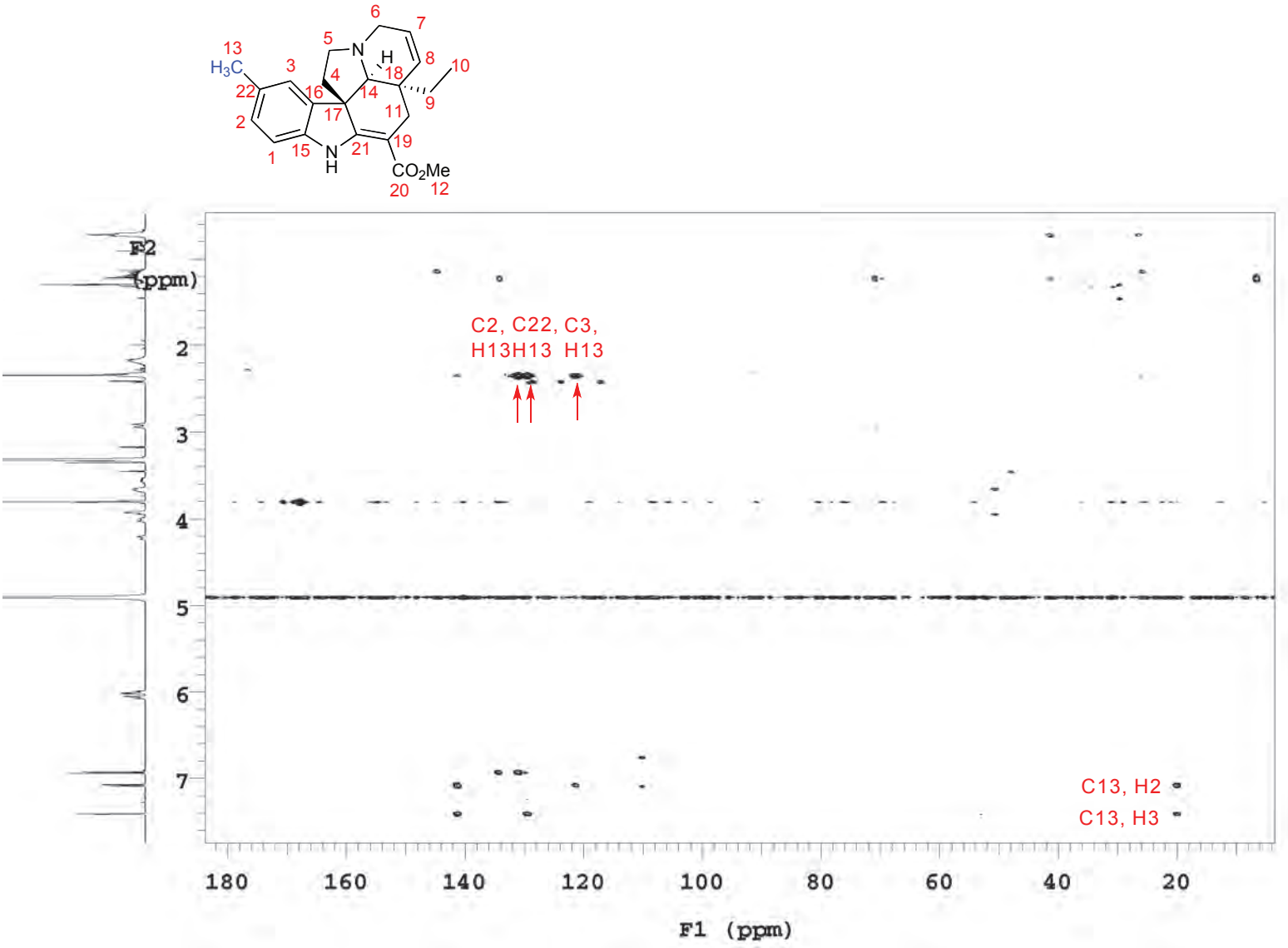

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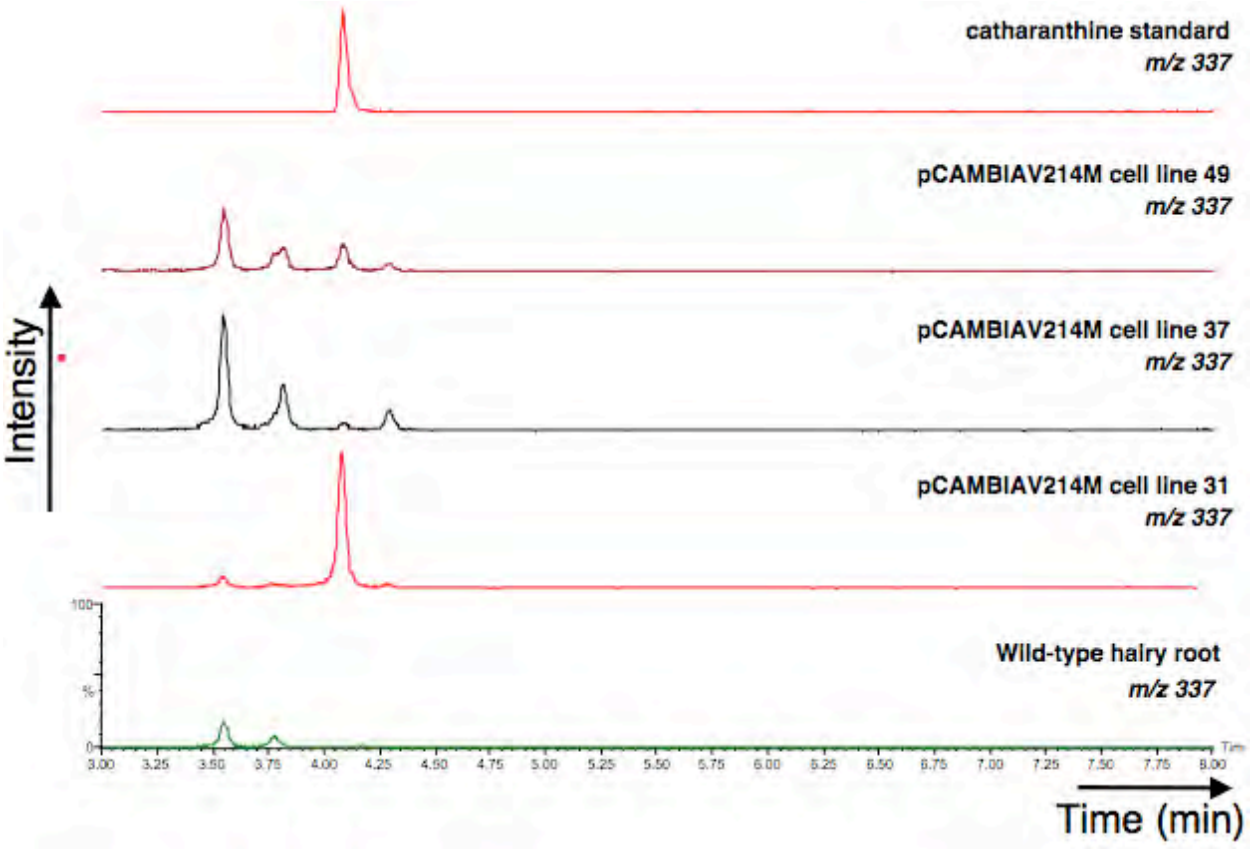

Figure S5. LC-MS trace of catharanthine production in transgenic hairy roots. Hairy roots that do not express the V214M mutant produce only trace amounts of catharanthine **7**. All lines expressing V214M displayed enhanced production of **7.**

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REAL-TIME RT-PCR OF TRANSGENIC HAIRY ROOTS

HAIRY ROOT LINES	WT	pTAV214M 3 + IND	pTAV214M 3 - IND	pTAV214M 26 + IND	pTAV214M 26 - IND	pCAMV214M 31	pCAMV214M3 7	pCAMV214M4 9
FOLD CHANGE	1.00	0.44	1.10	1.75	1.34	28.16	16.04	9.11

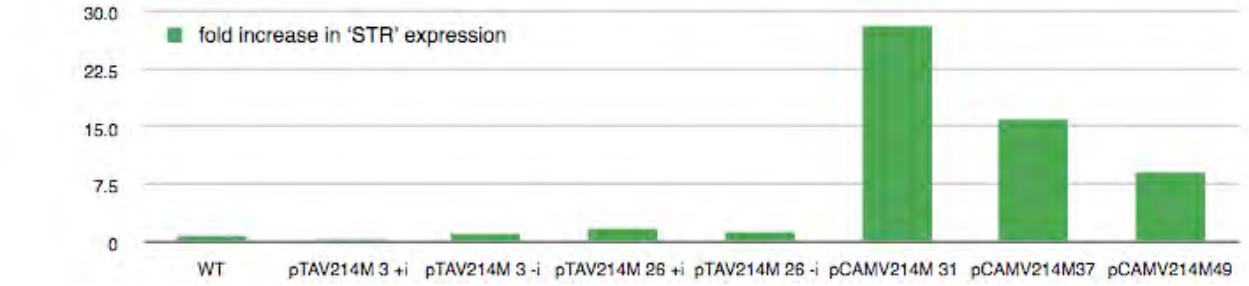

Figure S6. Real-time RT-PCR analysis shows relative expression levels of 'STR' (sum total of both wild-type and V214M mutant) in transgenic hairy roots. All chosen pCAMV214M hairy root lines (cell line 31, 37 and 49) produce STR mRNA at levels approximately 10 to 30 fold higher than the expression level of the endogenous STR observed in "wild type" culture transformed with *A. rhizogenes* lacking plasmid. pTAV214M hairy roots (cell line 3 and 26) do not show significant changes in mRNA expression levels of the enzyme either with (+ i) or without (- i) treatment with the glucocorticoid inducer, dexamethasone (10 μM).

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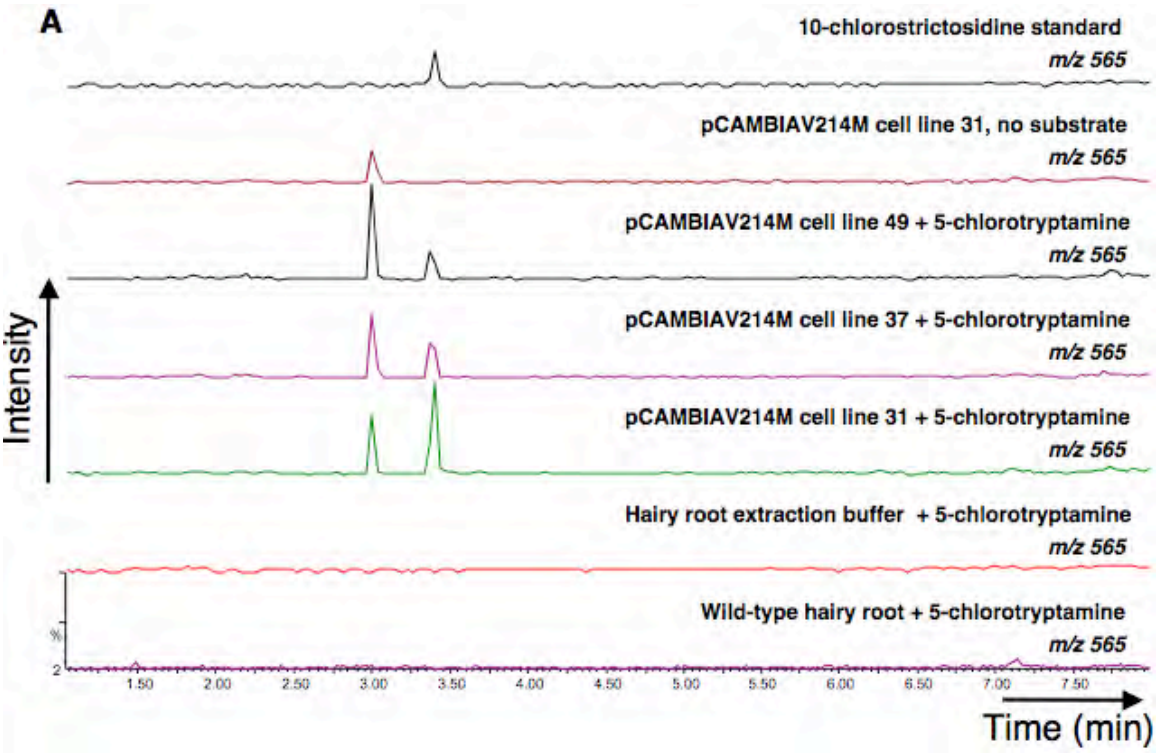
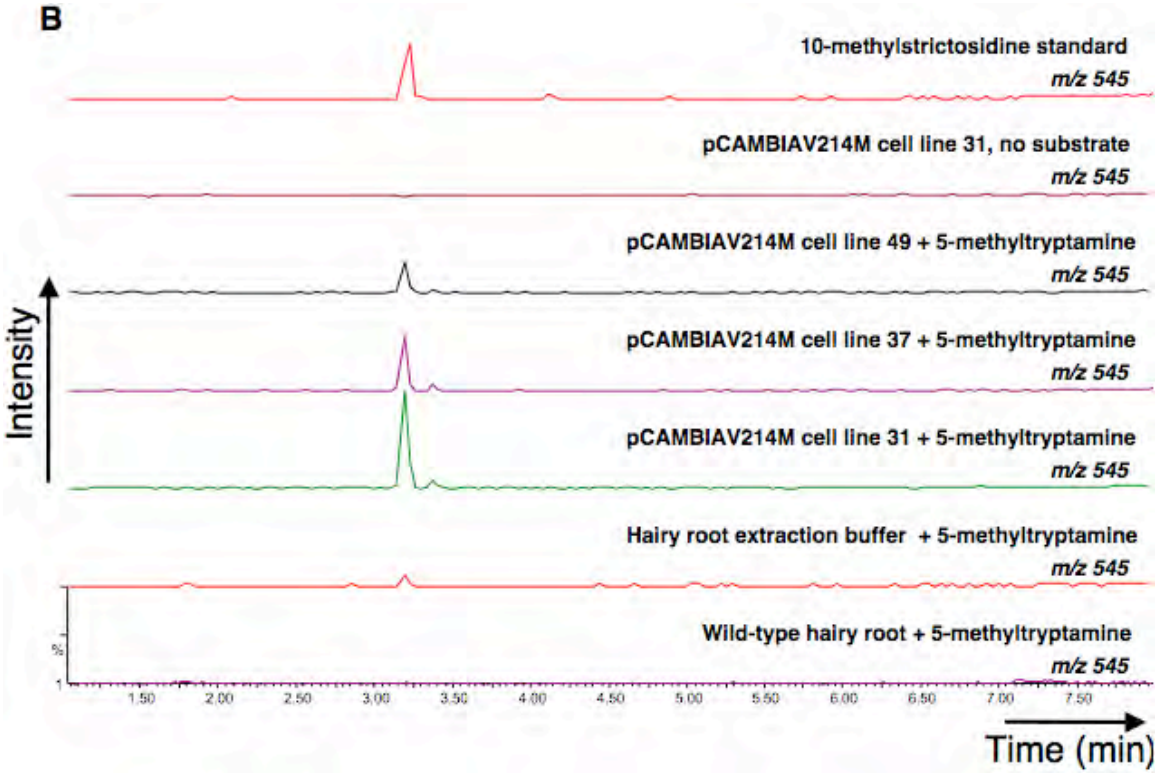
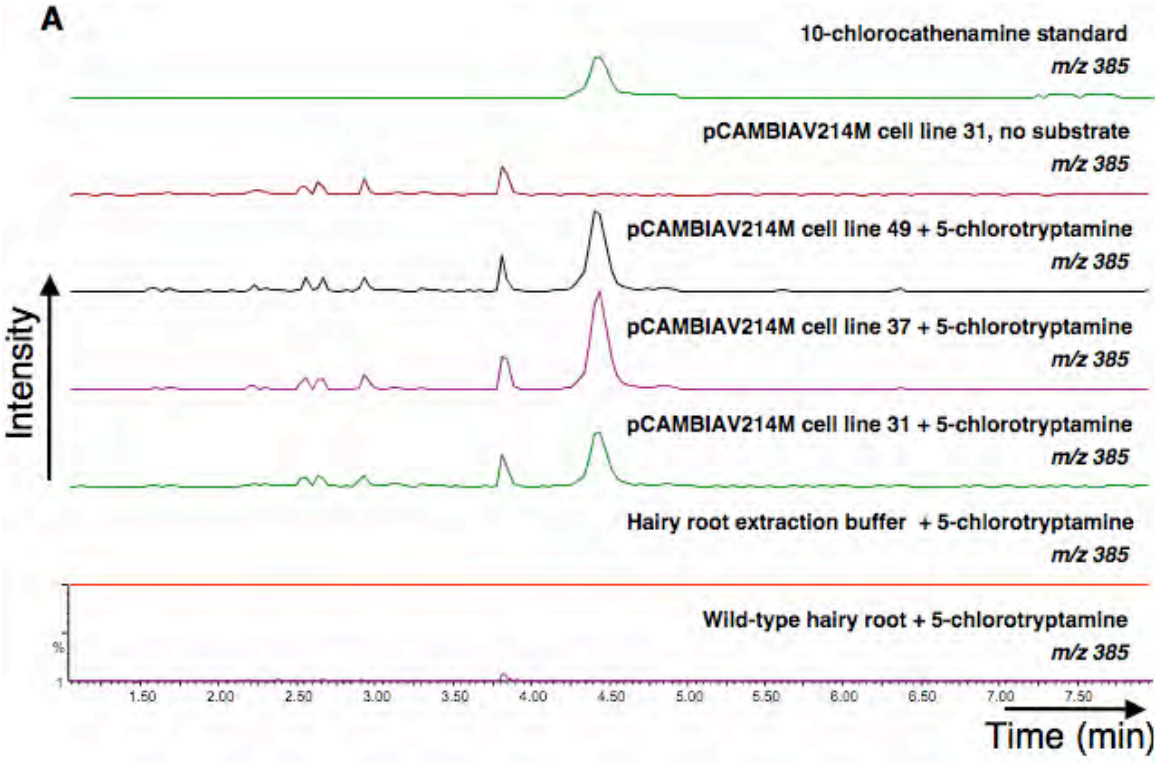




Nature Chemical Biology: doi:10.1038/nchembio.141

Figure S7, part I, A (top) and S18B (bottom). LC-MS traces of an *in vitro* enzymatic assay of pCAMV214M hairy root (cell lines 31, 37 and 49) lysates with 1 mM secologanin and 1 mM 5-chlorotryptamine **1a** (**A**) or 5-methyltryptamine **1b** (**B**). *m/z* 565 corresponds to 10-chlorostrictosidine **3a** and *m/z* 545 corresponds to 10-methylstrictosidine **3b**.



Nature Chemical Biology: doi:10.1038/nchembio.141

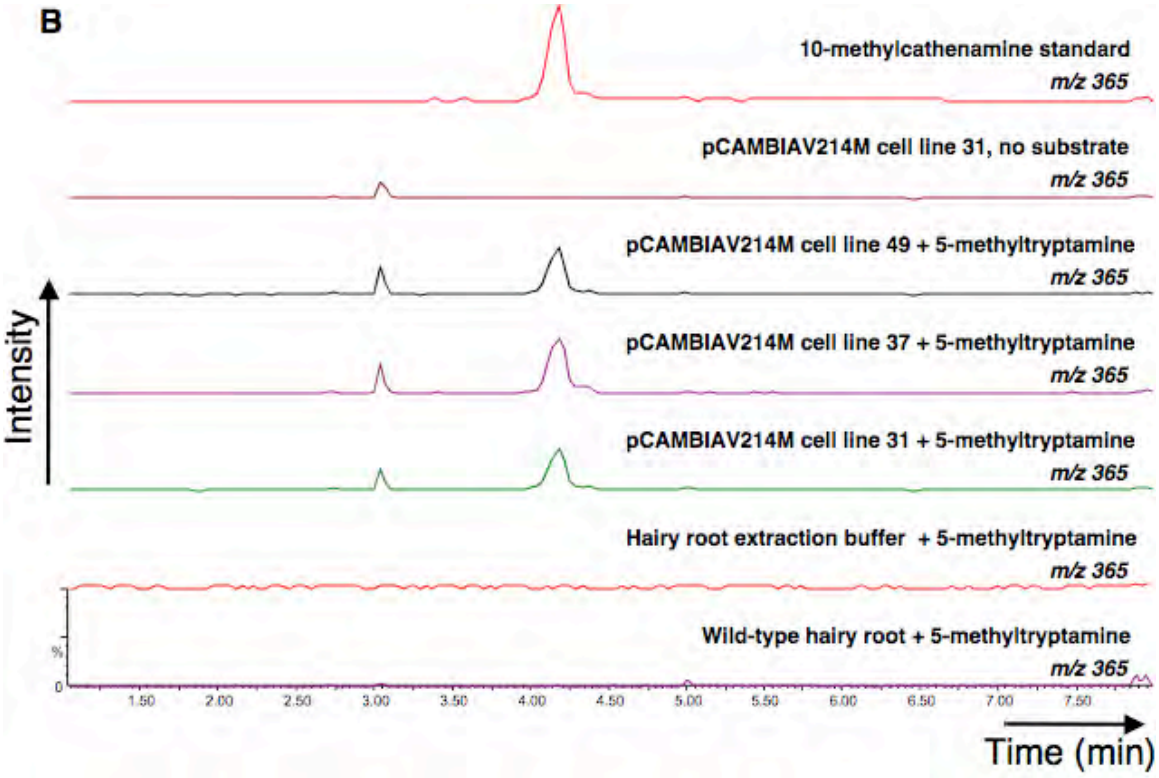


Figure S7, part II, A (top) and S19B (bottom). LC-MS traces of an *in vitro* enzymatic assay of pCAMV214M hairy root (cell lines 31, 37 and 49) lysates with 1 mM secologanin and 1 mM 5-chlorotryptamine **2a** (top) or 5-methyltryptamine **2b** (bottom). *m/z* 385 corresponds to 10-chlorocathenamine (deglycosylated strictosidine **3a**) and *m/z* 365 corresponds to 10-methylcathenamine (deglycosylated strictosidine **3b**).

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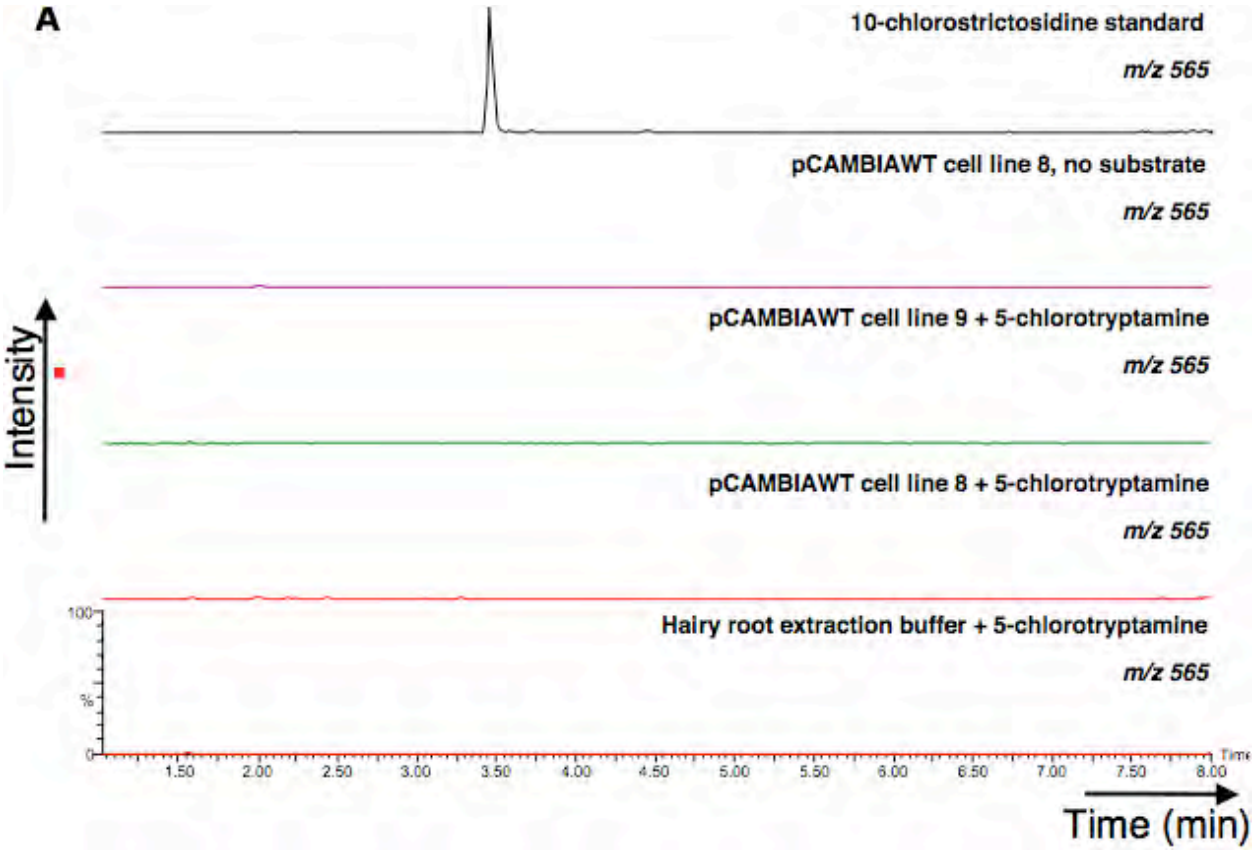

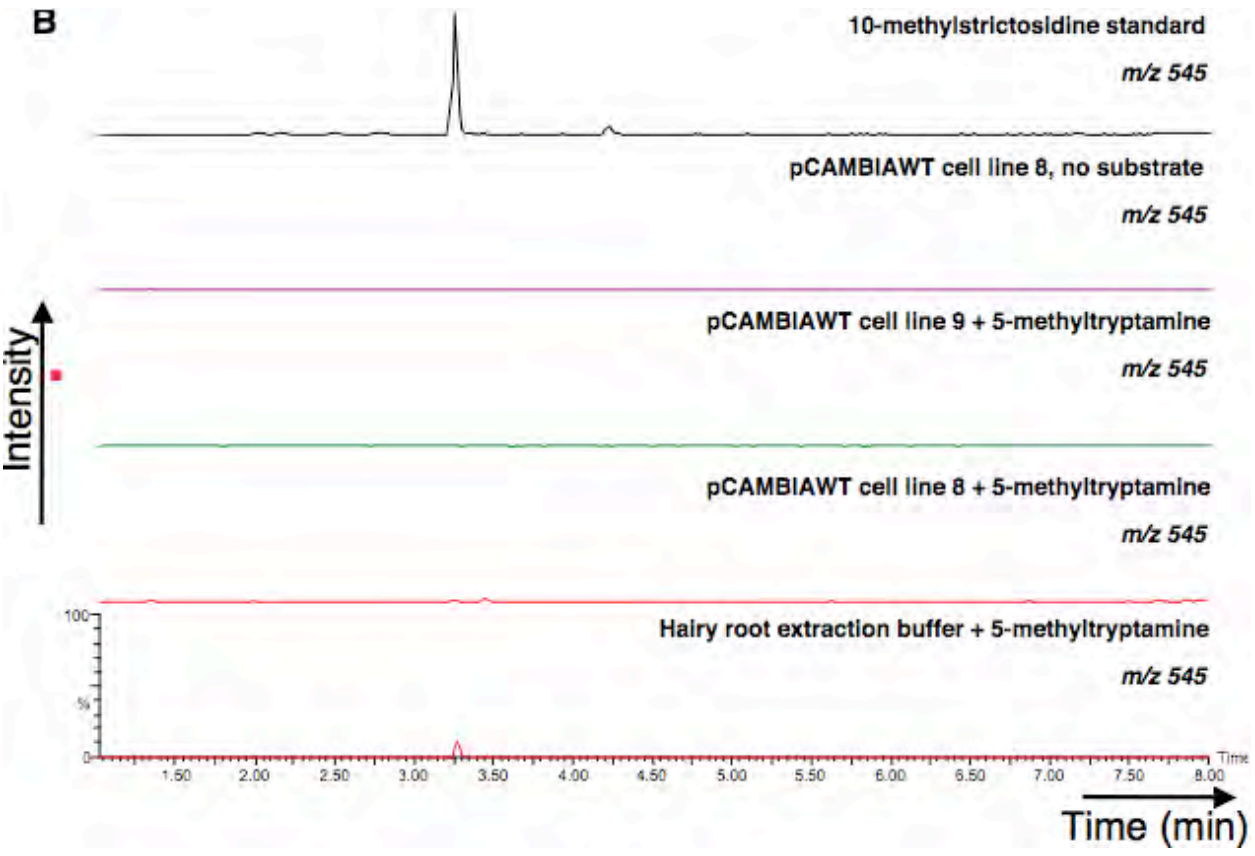

Nature Chemical Biology: doi:10.1038/nchembio.141

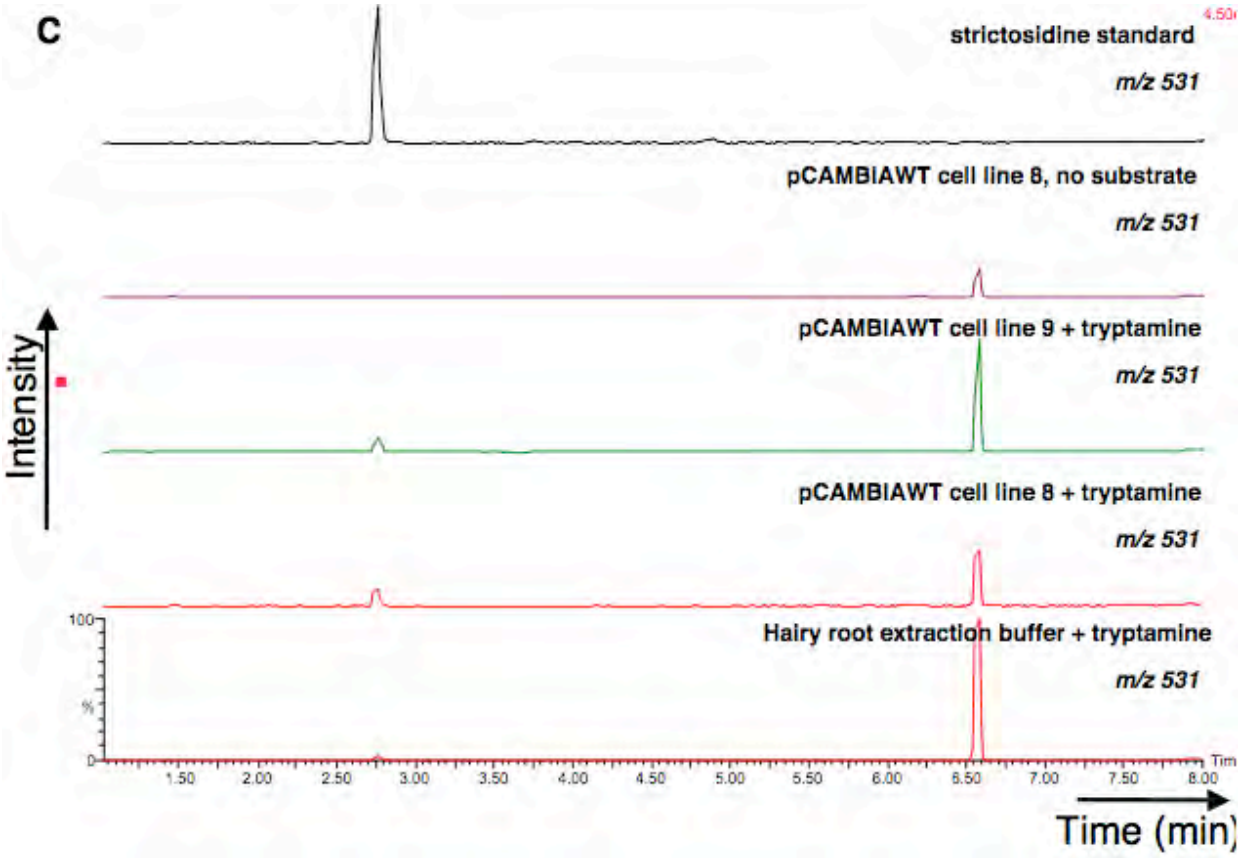

Figure S7, part III, A-C. LC-MS traces of an *in vitro* enzymatic assay of pCAMWT hairy root (cell lines 8 and 9) lysates with 1 mM secologanin and 1 mM 5-chlorotryptamine **1a** (**A**), 5-methyltryptamine **1b** (**B**) or tryptamine (**C**). *m/z* 565 corresponds to 10-chlorostrictosidine **3a**, *m/z* 545 corresponds to 10-methylstrictosidine **3b** and *m/z* 531 corresponds to strictosidine.

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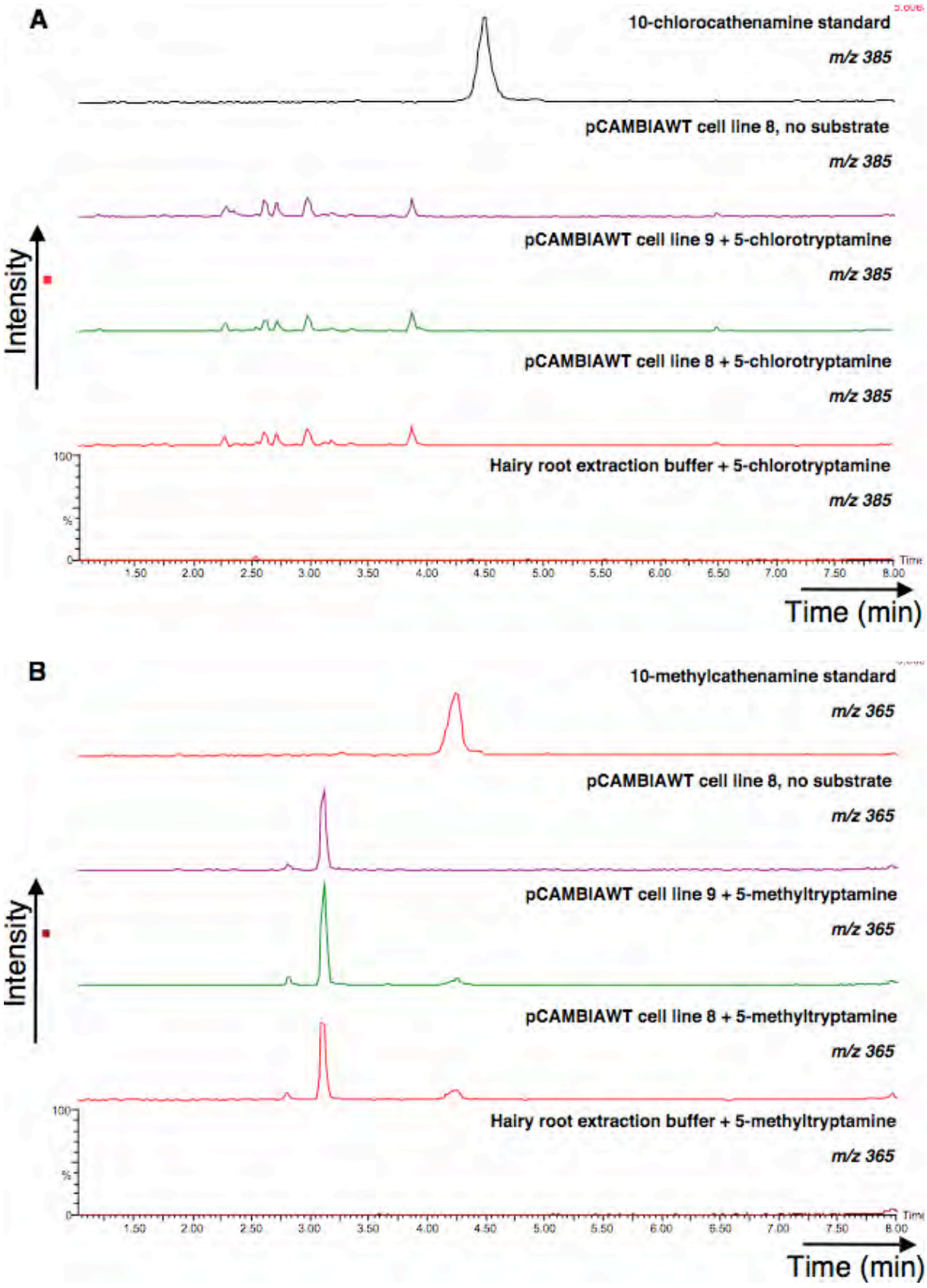


Nature Chemical Biology: doi:10.1038/nchembio.141

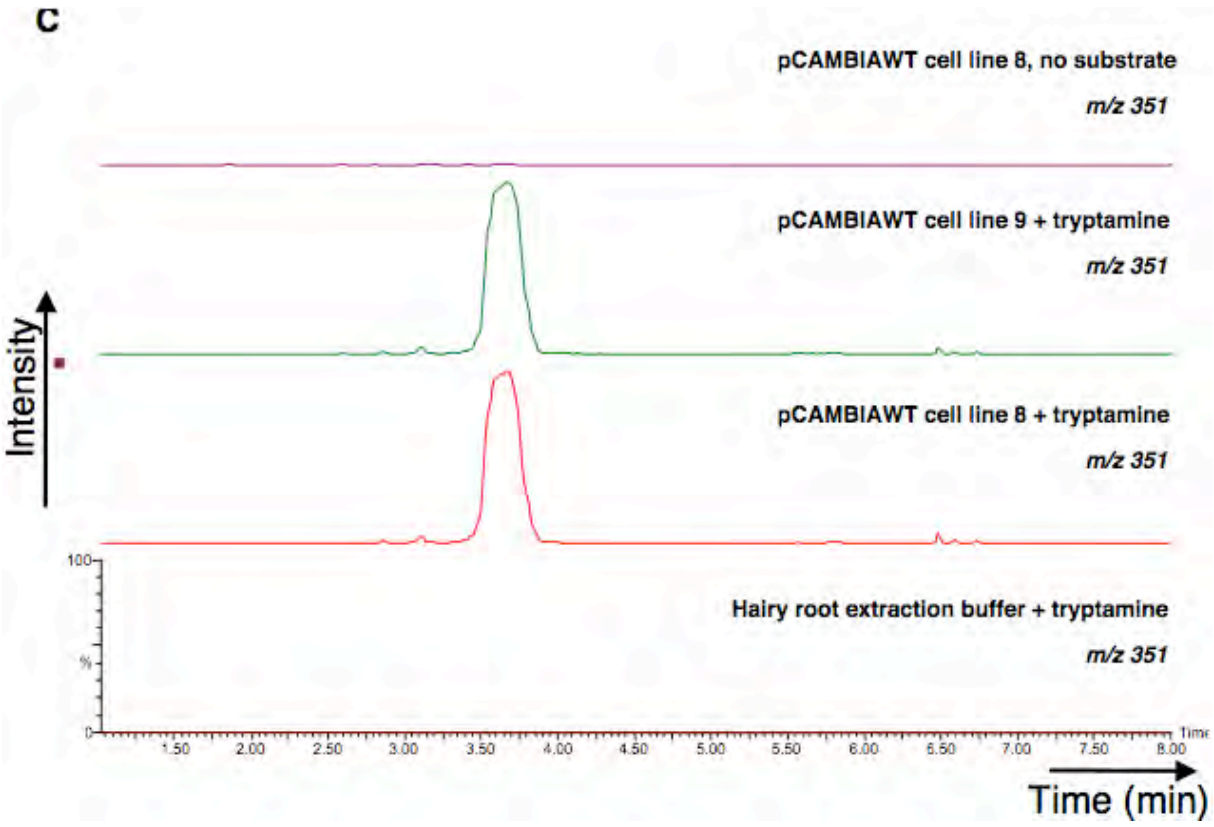
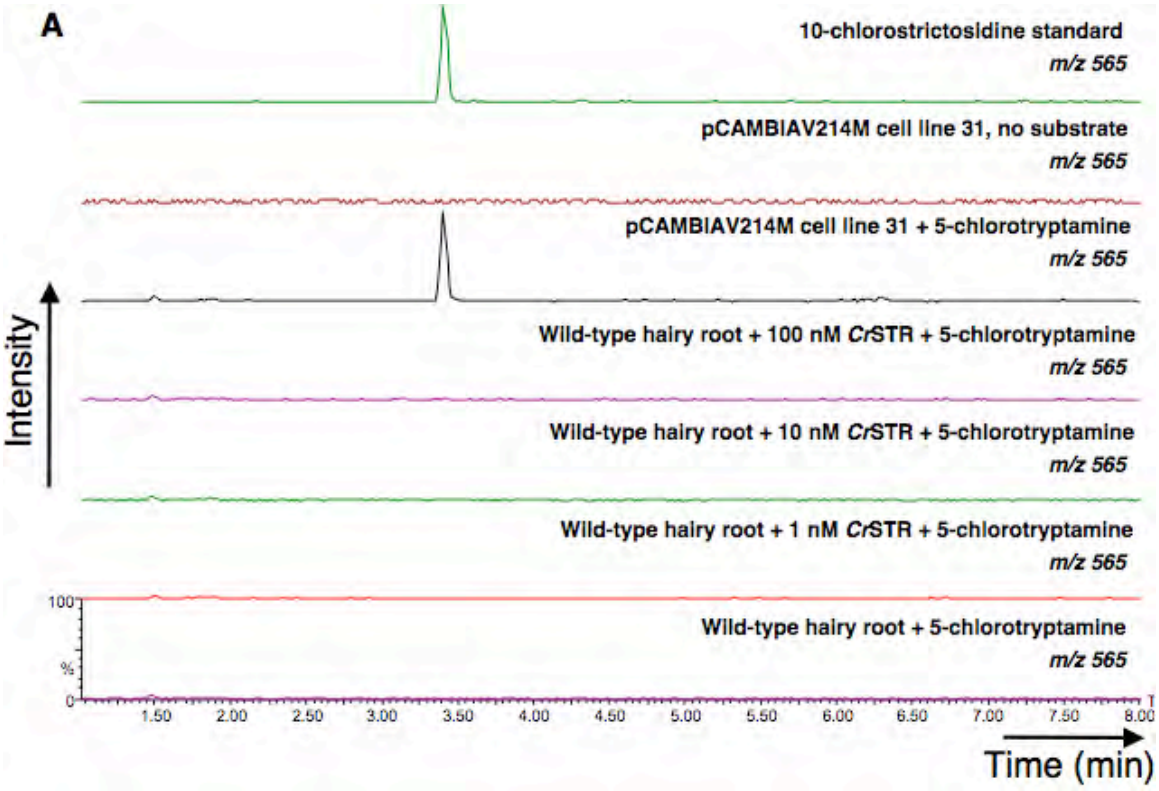
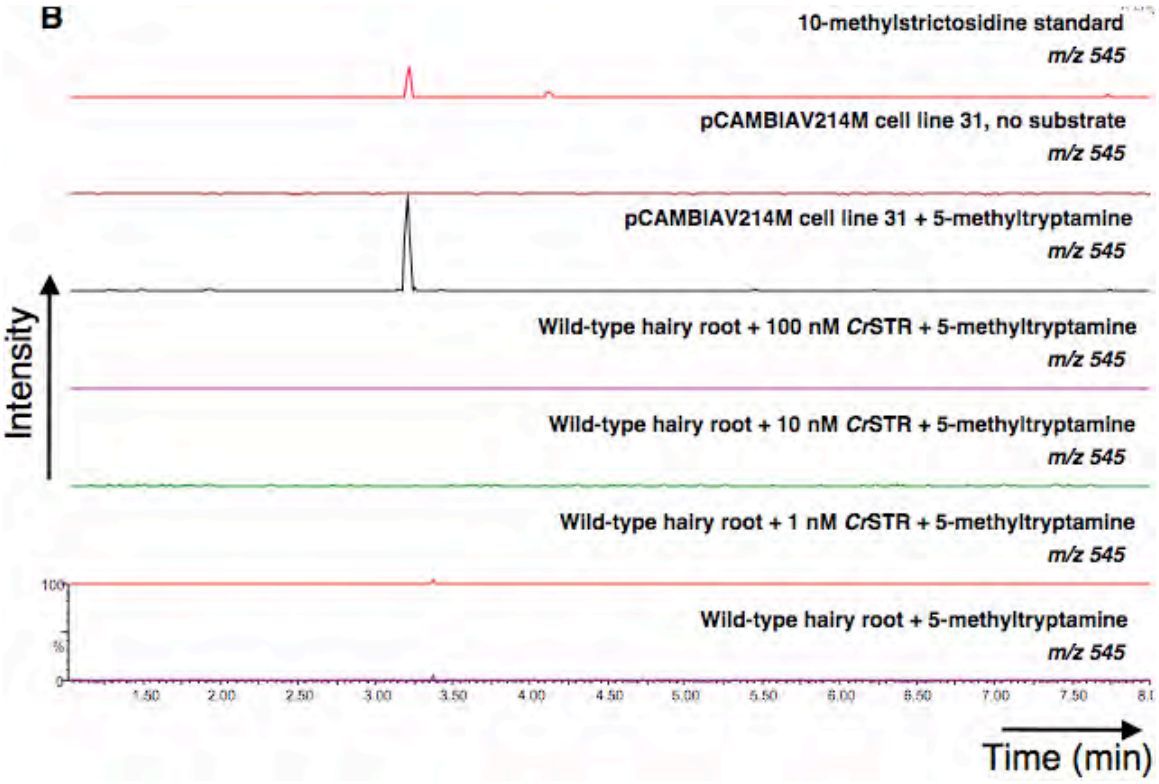


Figure S7, part IV, A-C. LC-MS traces of an *in vitro* enzymatic assay of pCAMWT hairy root (cell lines 8 and 9) lysates with 1 mM secologanin and 1 mM 5-chlorotryptamine **1a** (**A**), 5-methyltryptamine **1b** (**B**) or tryptamine **(C**). *m/z* 385 corresponds to 10-chlorocathenamine, *m/z* 365 corresponds to 10-methylcathenamine and *m/z* 351 corresponds to cathenamine.

Nature Chemical Biology: doi:10.1038/nchembio.141





Nature Chemical Biology: doi:10.1038/nchembio.141

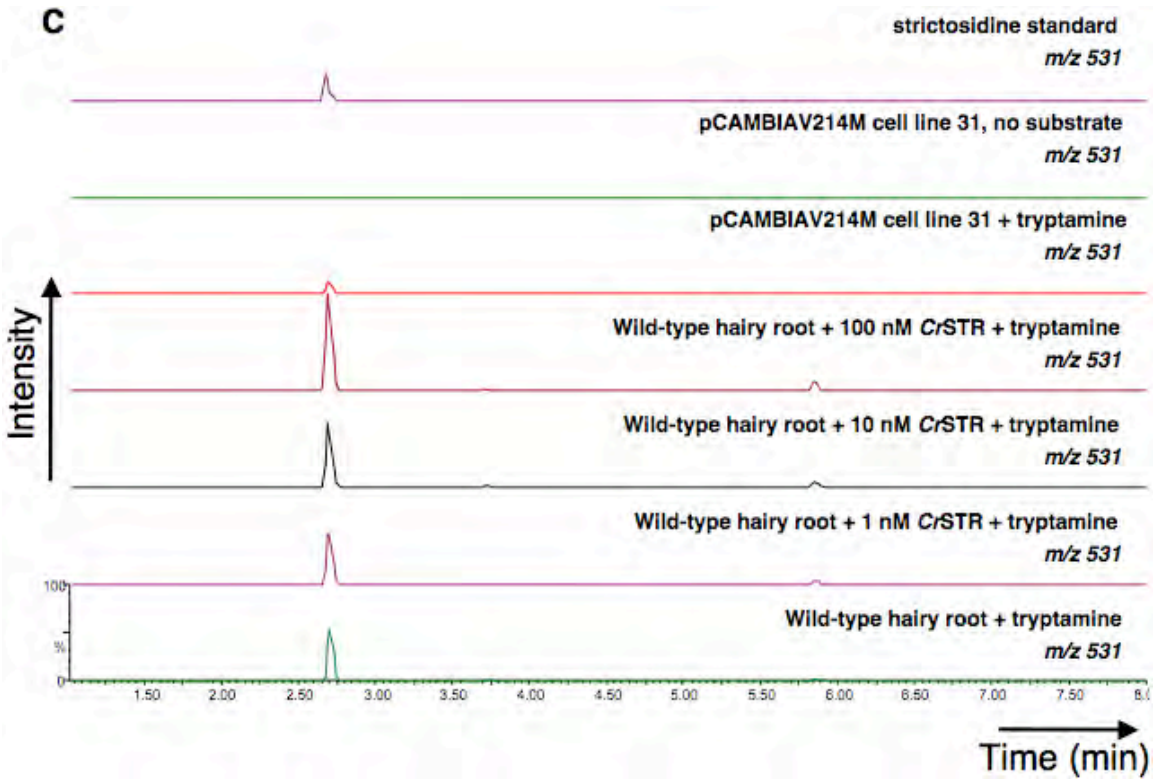


Figure S7, part V, A-C. LC traces of the *in vitro* enzymatic assay of wild-type hairy root lysates 'spiked' with 1 nM, 10 nM or 100 nM recombinant wild-type strictosidine synthase (CrSTR) in the presence of 1 mM secologanin and 1 mM 5-chlorotryptamine (**A**), 5-methyltryptamine (**B**), or tryptamine (**C**). *m/z* 565, 10-chlorostrictosidine **3a**; *m/z* 545, 10-methylstrictosidine **3b**; and *m/z* 351, strictosidine **3**.

2. Supplementary Tables

Table S1. High resolution MS data for alkaloids observed in hairy root extracts.

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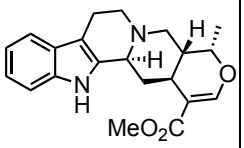
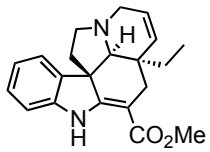
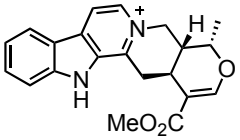
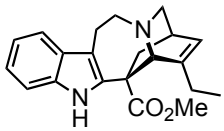
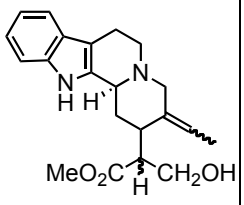
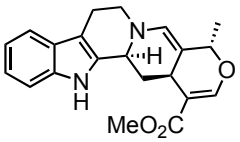
			a	b	c
	**Ajmalicine**	**4**	**4a***	**4b*****	**4c*****
	Expected [M+H]	353.1865	387.1490	367.2022	431.0970
	Observed [M+H]	353.1856	387.1483	367.2015	431.0983
	Accuracy (ppm)	-2.5	2.1	-1.9	3.0
	RT (min)	3.85	4.95	4.67	5.19
	**Tabersonine**	**5**	**5a***	**5b*****	**5c**
	Expected [M+H]	337.1916	371.1526	351.2073	
	Observed [M+H]	337.1922	371.1528	351.2064	not obs.
	Accuracy (ppm)	1.8	0.5	-2.6	
	RT (min)	4.28	5.51	5.07	
	**Serpentine**	**6**	**6a*****	**6b*****	**6c*****
	Expected [M+H]	349.1552	383.1162	363.1709	427.0657
	Observed [M+H]	349.1548	383.1177	363.1699	427.0650
	Accuracy (ppm)	-1.1	3.9	-2.8	-1.6
	RT (min)	4.07	4.94	4.91	5.17
	**Catharanthine**	**7**	**7a****	**7b*****	**7c**
	Expected [M+H]	337.1916	371.1526	351.2073	
	Observed [M+H]	337.1916	371.1531	351.2059	not obs.
	Accuracy (ppm)	0.0	1.3	-4.0	
	RT (min)	4.09	5.17	4.90	
	**Isositsirikine**		***	***	***
	Expected [M+H]	355.2022	389.1636	369.2178	433.1127
	Observed [M+H]	355.2020	389.1632	369.2182	433.1118
	Accuracy (ppm)	-0.6	1.0	1.1	-2.1
	RT (min)	2.99	4.13	3.82	4.38
	**Cathenamine**		***	***	
	Expected [M+H]	351.1709	385.1319	365.1865	
	Observed [M+H]	351.1724	385.1328	365.1860	not obs.
	Accuracy (ppm)	4.3	2.3	-1.4	
	RT (min)	4.52	5.70	5.32	

RT = retention time, LC gradient 10-60% acetonitrile/water (0.1% TFA) in 13 minutes

*Assigned based on exact mass, retention time, UV spectra, ^{1}H-NMR, and known alkaloid profile of *C. roseus*.

** Assigned based on exact mass, retention time, UV spectra, and alkaloid profile.

*** Assigned based on exact mass, retention time and alkaloid profile.

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3. Supplementary Methods

a. Construction of Plant Expression Vectors Containing STR Mutant Gene

The native strictosidine synthase (STR) gene with the complete signal sequence for correct localization (accession number X61932) was obtained by reverse-transcription PCR amplification of mRNA isolated from *C. roseus* hairy root culture (Qiagen, Rneasy kit). Site-directed mutagenesis was then performed to introduce the Val214Met mutation using overlapping mutagenic primers (5' gaaagagctacatATGcccggcggtgcag 3' and 5' ctgcaccgccgggCATatgtagctctttc 3') (Stratagene, Quikchange). For constitutive STR mutant expression, the mutant STR gene was ligated into the NcoI/BstEII site downstream of the CaMV 35S promoter in the pCAMBIA 1305.1 vector (Broothaerts et al. *Nature.* **433**, 629-633 (2005)) to yield the construct pCAMV214M. A construct harbouring the *wild-type* STR under control of the constituitive promoter, pCAMWT, was also generated as a control. For inducible STR mutant expression, the mutant STR gene was ligated into the XhoI/SpeI site downstream of the GAL4-UAS promoter in the pTA7002 vector (Aoyama, T. & Chua, N.-H. *Plant J.* **11**, 605-612 (1997)) to yield the construct pTAV214M. Both pCAMBIA 1305.1 and pTA7002 also contain a hygromycin plant selection marker and a kanamycin bacterial selection marker. Complete constructs for both constitutive and inducible expression were sequenced to ensure plasmid integrity.

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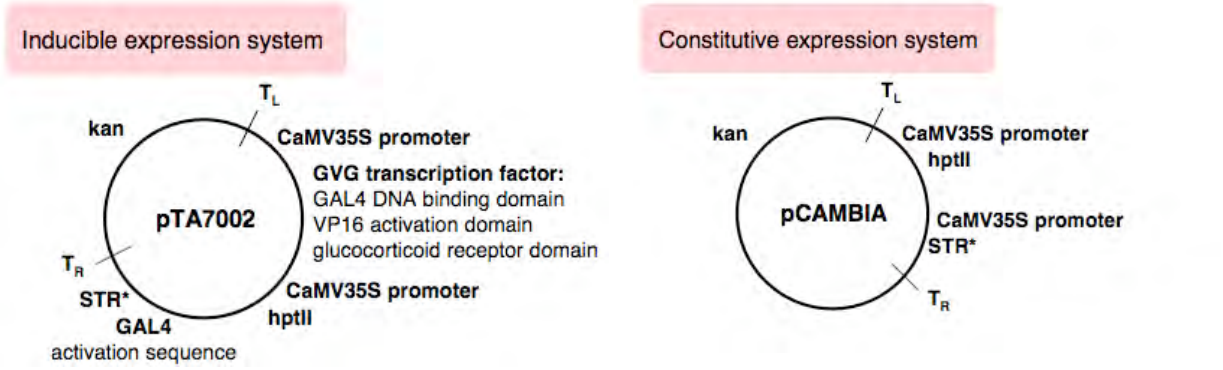

Schematic diagrams of strictosidine synthase (STR) mutant inducible expression system (left) and constitutive expression system (right) constructs.

b. Generation of Transgenic Hairy Root Cultures

The constructs containing the STR Val214Met mutant gene, as well as the construct containing the *wild-type* STR gene, were each transformed into *Agrobacterium rhizogenes* ATCC 15834 via electroporation (1mm cuvette, 1.25 kV). Transformation of *C. roseus* seedlings with the generated *Agrobacterium* strains was performed as previously reported (Hughes, E.H. et al. *Biotechnol. Prog.* **18**, 1183-1186 (2002)). Briefly, 250-300 *C. roseus* seedlings (Vinca Little Bright Eyes, Nature Hills Nursery) were germinated aseptically on Gamborg's B5 media (full strength basal salts, full strength vitamins, 30 g/L sucrose, pH 5.7) and grown in a 16-hour light 8-hour dark cycle at 26 °C for three weeks. Seedlings were then wounded with extra-fine forceps at the stem tip, and transformed 3-5 μL of *A. rhizogenes* from a freshly grown liquid culture were inoculated on the wound.

Hairy roots appeared at the wound site 2-3 weeks after infection for about 80% of the seedlings infected. After hairy roots reached 1-4 cm in length (usually about six

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weeks after infection), they were excised and transferred to Gamborg's B5 solid media (half strength basal salts, full strength vitamins, 30 g/L sucrose, 6 g/L agar, pH 5.7) containing hygromycin for selection (three weeks on 0.01 mg/mL in first selection and three weeks on 0.03 mg/mL in second selection) and the antibiotic cefotaxime (0.25 mg/mL in both selections) to remove remaining bacteria. All cultures were grown in the dark at 26°C. After the solid media selection process, hairy roots were subcultured at least once in media lacking both hygromycin and cefotaxime prior to adaptation to liquid culture.

To adapt the line to liquid culture, approximately 200 mg of hairy roots (typically five 3-4 cm long stem tips) from each line that grew successfully on solid media were transferred to 50 mL of half-strength Gamborg's B5 liquid media. The cultures were grown at 26°C in the dark at 125 rpm. All lines were maintained on a 21-28 day subculture cycle depending on the growth rate of each line.

Below, the solid media selection and liquid media adaptation processes are summarized. Hairy root transformants were screened for survival in solid media supplemented with hygromycin and subsequently for fitness and fast growth in liquid media. The number of transformants decreased significantly after solid media selection for each of the constructs transformed. Adaptation to liquid media further eliminated slow growing lines. After the selection and adaptation processes, four to eight hairy root lines from each STR mutant that grew the most rapidly in liquid media were chosen for alkaloid production and mutant enzyme expression assays.

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Hairy roots selection and adaptation processes.

Plasmid	# transformed hairy roots	# hairy roots after solid media selection	# hairy roots after liquid media adaptation
pCAMV214M	240	53	29
pCAMWT	280	74	5*
pTAV214M	270	44	15

* only 5 (out of 74) lines were selected for adaptation to liquid media

c. Verification of Transferred DNA (T-DNA) Integration by Genomic DNA Analysis

To verify the integration of transferred DNA (T-DNA) into the plant genome, the genomic DNA from transformed hairy roots was isolated (Qiagen Dneasy kit) and then subjected to PCR amplification using T-DNA specific primers with STR primers serving as a positive control (see below). Specifically, primers for PCR amplification were designed to amplify the complete STR gene (STR_for and STR_rev), an 800bp region of the selection marker HPT gene (HPT_for and HPT_rev), and a 500bp region of the CaMV 35S promoter (CaMV 35S_for and CaMV 35S_rev) (see below).

PCR primers for verification of T-DNA integration of transformed hairy roots.

	Primer Name	Primer Sequence (‘5 → 3’)
1	STR_for	CCATGGCAAACTTTTCTGAATCTAAATCC
2	STR_rev	GGTCACCCTAGCTAGAAACATAAGAATTTC
3	HPT_for	GCCTGAACTCACCGCGACGTC
4	HPT_rev	CCTCCAGAAGAAGATGTTGGC
5	CaMV 35S_for	TAGAGGACCTAACAGAAC
6	CaMV 35S_rev	CCGTGTTCTCTCCAAATG
7	STRV214M_for	CCTTATTATTGAAAGAGCTACATATG
8	STRV214M_rev	GCTAGAAACATAAGAATTTCCCTTG

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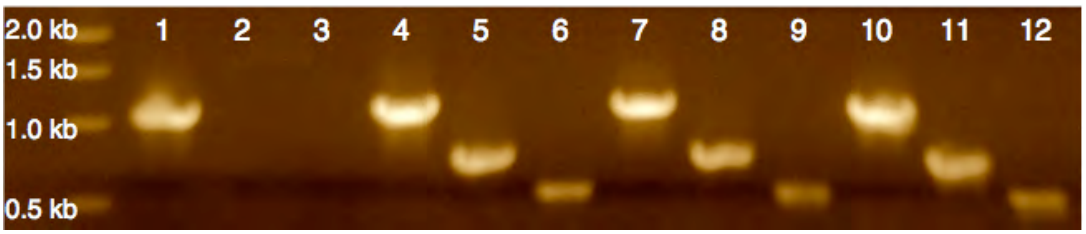

1% agarose gel of PCR amplification of pCAMV214M and wild-type hairy root genomic DNA.

Lane: template, primers.

1: wild-type, STR

2: wild-type, HPT

3: wild-type, CaMV35S

4: pCAMV214M cell line 31, STR

5: pCAMV214M cell line 31, HPT

6: pCAMV214M cell line 31, CaMV35S

7: pCAMV214M cell line 37, STR

8: pCAMV214M cell line 37, HPT

9: pCAMV214M cell line 37, CaMV35S

10: pCAMV214M cell line 49, STR

11: pCAMV214M cell line 49, HPT

12: pCAMV214M cell line 49, CaMV35S

PCR amplification of genomic DNA from all of the selected transformed lines (pCAMV214M_31: lanes 4-6, pCAMV214M_37: lanes 7-9, and pCAMV214M_49: lanes 10-12) was successful for all three sets of primers. PCR-amplification of hairy root transformed with *A. rhizogenes* lacking the pCAMBIA vector (provided by Professor Jacqueline Shanks (Iowa State) and Professor Carolyn Lee-Parsons (Northeastern)) genomic DNA was successful only when STR specific primers were used (lanes 1-3). These results indicated that STR mutant was successfully incorporated into the *C. roseus* genome in all chosen lines.

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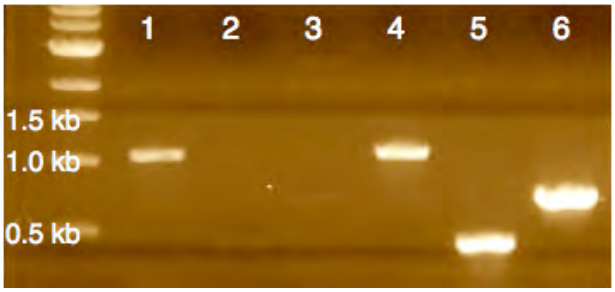
Additionally, a primer pair was designed to match the point mutation corresponding to the V214M sequence change at the 3' end of the forward primer (STRV214M_for and STRV214M_rev). These primers readily amplified a 500bp region of the mutant gene. However, the mismatch at the 3' end prevented efficient amplification of the native STR gene in wild type hairy root cultures (see below).



1% agarose gel of PCR amplification of pCAMV214M and wild-type hairy root genomic DNA demonstrating V214M point mutation

Lane: template, primers

1: wild-type, STR

2: wild-type, STRV214M (specific for V214M mutation)

3: wild-type, HPT

4: pCAMV214M cell line 31, STR

5: pCAMV214M cell line 31, STRV214M (specific for V214M mutation)

6: pCAMV214M cell line 31, HPT

d. Evaluation of Alkaloid Production in Engineered C. roseus

Transgenic hairy root lines were evaluated for alkaloid production after substrate analog feeding. Transformed hairy roots were initially grown in half-strength Gamborg's B5 liquid media supplemented with the corresponding tryptamine analog at 0.5 mM concentration. Co-cultivation with tryptamine analogs 5-chlorotryptamine **2a**, 5-

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methyltryptamine **2b** and 5-bromotryptamine **2c** at this relatively high concentration at the initial growth phase resulted in growth retardation. Hairy roots fragmented and became brown a few days after addition of the tryptamine analogs. To address this problem, tryptamine analogs (and 10 μM dexamethasone for the inducible expression system) were added to the liquid culture towards the end of the log phase and the beginning of the stationary phase (usually after three weeks). After one week of co-cultivation with the substrate, hairy roots were ground with a mortar, pestle and 106 μm acid washed glass beads in methanol (10 mL/g of fresh weight hairy roots) from the harvested tissue. The crude natural product mixtures were filtered and subsequently subjected to LC-MS analysis. In total, three hairy root lines transformed with *A. rhizogenes* harboring the pCAMV214M plasmid and two hairy root lines with pTAV214M plasmid were evaluated for alkaloid production. Additionally, hairy roots transformed with wild-type *A. rhizogenes* lacking the plasmid were also evaluated.

These crude alkaloid mixtures were diluted 1/100 with methanol for mass spectral analysis. Samples were ionized by ESI with a Micromass LCT Premier TOF Mass Spectrometer. The LC was performed on Acquity Ultra Performance BEH C18, 1.7 μm, 2.1 x 100 mm column on a gradient of 10-60% acetonitrile/water (0.1% TFA) over 13 minutes at a flow rate of 0.6 mL/min. The capillary and sample cone voltages were 1300 and 60 V, respectively. The desolvation and source temperature were 300 and 100 °C. The cone and desolvation gas flow rates were 60 and 800 L/hour. Analysis was performed with MassLynx 4.1. Accurate mass measurements were obtained in W-mode. The spectra were processed using the Mass Lynx 4.1 mass measure, in which

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the mass spectrum of peaks of interest was smoothed and centered with TOF mass correction, locking on the reference infusion of reserpine.

Several new compounds were formed by transgenic root lines transformed with pCAMV214M as evidenced by LC-MS analysis (**Figure S1, part I, part II** feeding with 5-chlorotryptamine **2a**; **Figure S1, part III, part IV** feeding with 5-bromotryptamine **2c;** and **Figure S1, part V** feeding with 5-methyltryptamine **2b**). Control experiments indicated that these compounds were not present when the tryptamine analog substrate was absent from the media. Additionally, hairy root cultures infected with wild-type *A. rhizogenes* lacking the expression vector ("wild type hairy root") or *A. rhizogenes* harboring the inducible plant expression construct (pTAV214M) did not produce these compounds when cultivated under same conditions (**Figure S1, S2**). Moreover, hairy root cultures infected with *A. rhizogenes* harboring the constitutive *wild-type* STR expression construct (pCAMWT) also did not produce these compounds under same conditions (**Figure S1, part I**). Note that under this normalized intensity scale, a peak with mass and UV absorbance consistent with 10-chlorocatharanthine (*m/z* 371) cannot be clearly observed with this injection. (A trace of a more concentrated injection can be seen on **Figure S1, part II**.)

e. Purification and Isolation of Alkaloids from Transformed Hairy Roots Supplemented with 5-chlorotryptamine 2a and 5-methyltryptamine 2b

Root tips (10-15) from transformed hairy roots (line 31) were subcultured in 150 mL Gamborg's B5 liquid media and grown at 26°C in the dark at 125 rpm for three weeks prior to supplementing the media with either 5-chlorotryptamine **2a** or 5-

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methyltryptamine **2b**. After one week of co-cultivation, hairy roots were extracted as described above in methanol (10 mL/g of fresh weight hairy roots). Alkaloid extracts were filtered, concentrated under vacuum and redissolved in 20% acetonitrile/water (0.1% TFA) (1 mL/g of fresh weight hairy roots).

For 5-chlorotryptamine **2a** feeding, the redissolved mixture was purified on a 10 x 20 mm Vydec reverse phase column using a gradient of 20-60% acetonitrile/water (0.1% TFA) over 35 minutes. Alkaloids were monitored at 228 nm and fractions containing the alkaloid analogs of interest, as determined by the characteristic isotopic distribution expected for chlorinated molecules ($^{35}Cl/^{37}Cl$) from LC-MS analysis, were combined and concentrated under vacuum (**Figure S3, part I**).

Isolated alkaloids were analyzed by LC-MS (same parameters as above), analytical HPLC, and where possible, NMR (Bruker AVANCE-600 NMR spectrometer equipped with a 5mm 1H{13C,31P} cryo-probe).

Fractions a-d (**Figure S3, part I**) were characterized by LC-MS, compared to characteristic UV spectra of known alkaloids and, when greater than 1 mg quantities were obtained in sufficient purity, also by ^{1}H NMR and ^{1}H-^{13}C HSQC. Chlorinated alkaloids generally displayed longer retention times than the natural alkaloids. Fraction **a** corresponded to ajmalicine **4**; fraction **b** corresponded to catharanthine **7**; fraction **c** corresponded to 10-chloroajmalicine **4a**; and fraction **d** appeared to be a mixture of 15-chlorotabersonine **5a** and another chlorinated alkaloid also with *m/z* 371.

For 5-methyltryptamine **2b** feeding, similar procedures were performed to feed and isolate alkaloids from transgenic hairy roots. One notable difference was in the

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purification step: since methylated alkaloids are generally more hydrophilic than chlorinated alkaloids, the redissolved alkaloid mixture was purified on a 10 x 20 mm Vydec reverse phase column using a gradient of 20-50% acetonitrile/water (0.1% TFA) over 35 minutes.

Alkaloids were monitored at 228 nm and fractions containing the alkaloid analogs of interest, as determined by LC-MS analysis, were combined and concentrated under vacuum (**Figure S4**). All isolated alkaloids were characterized by LC-MS, compared to characteristic UV spectra of known alkaloids and, when greater than 1 mg quantities were obtained in sufficient purity, also by ^{1}H NMR and ^{1}H-^{13}C HSQC. Fraction **a** appeared to be a mixture of 10-methylajmalicine **4b** and another compound with *m/z* 379; fraction **b** contained a mixture of 15-methyltabersonine **5b** and a compound with *m/z* 353. Fraction b was further purified on a 10 x 20 mm Vydex reverse phase column using an isocrat 28% acetonitrile/water (0.1% TFA) to obtain 15-methyltabersonine **5b** in over 85% purity.

f. Verification of Expression of Mutant STR Enzyme by Real Time RT-PCR

Real time RT-PCR was used to assess the expression levels of STR (sum total of both mutant V214M STR and native STR). Expression levels in hairy roots infected with *A. rhizogenes* lacking the pCAMV214M STR mutant construct were compared to expression levels in hairy roots harboring pCAMV214M. mRNA from transformed hairy roots was isolated and purified from contaminant DNA using Qiagen RNeasy Plant Mini Kit and Rnase-free DnaseI, respectively. The resulting mRNA was then reverse-

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transcribed to cDNA using Qiagen QuantiTect Reverse transcription kit and then subjected to PCR with specific primers (see below), Qiagen SYBR Green PCR kit and a Biorad DNA Engine Opticon 2 system. The threshold-cycle (C_T) was determined as the cycle with a signal higher than that of the background plus 10 x standard deviation (SD). *C. roseus* 40S ribosomal protein S9 (Rps9), a house keeping gene, was used to adjust the amount of the total mRNA in all samples. Real time RT-PCR was performed in triplicate and the data are pictured as the relative expression levels of STR (sum total of both V214M mutant and native STR) mRNA in transgenic hairy roots as well as hairy roots lacking the pCAMBIA plasmid (**Figure S6**). Hairy root lines containing the pTAV214M construct also failed to show expression of the V214M mutant STR both in the presence and absence of dexamethasone, the inducer.

PCR primers for real-time RT-PCR of transformed hairy roots. Primers were designed using GenScript web tool (http://www.genscript.com/ssl-bin/app/primer)

Primer Name	Primer Sequence ('5 → 3')	Amplicon size
Homol_STR_for	GTCCAAGATGGCCGAGTTAT	140
Homol_STR_rev	TATGTCCTCCCACACAATGG	
STR_V214M_for	TATTATTGAAAGAGCTACATATG	134
STR_V214M_rev	CTCTGCACTGCCTTTCTTG	
Rbps9_for	TTGAGCCGTATCAGAAATGC	122
Rbps9_rev	CCCTCATCAAGCAGACCATA	

g. In vitro Enzymatic Assay of Transformed Hairy Roots Expressing STR Mutant

After at least three rounds of subculture in liquid media, transformed hairy roots were subjected to an enzymatic assay to evaluate the expression of the STR mutant *in*

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vitro. In the constitutive expression system (hairy roots transformed with pCAMV214M), hairy roots subcultured for three weeks were harvested and ground with a mortar, pestle and 106 μm acid washed glass beads in extraction buffer (0.1 M phosphate buffer, 2 mM EDTA, 4 mM DTT, 0.1 μM pepstatin, 0.1 μM leupeptin, 1% PVP, pH 7.2) at 4 °C. The hairy root extracts were centrifuged for 10 min at 13000 rpm at 4 °C to remove cell debris, concentrated, and assayed for STR V214M enzymatic activity without further purification. Total protein concentration in the extracts was quantified using Biorad Protein Assay Solution.

A typical reaction mixture contained 0.1 M phosphate buffer pH 7, 2.5 mM DTT, 1 mM secologanin, and 1 mM tryptamine analog **2a**-**c**. Approximately 20 μg of crude enzyme was added to 100 μL reaction mixture and the reaction was incubated at 30 °C for 4 hours. To quench the reaction, 625 μL of methanol was added to 50 μL of the reaction mixture and the resulting solution was centrifuged, filtered through a 0.2 μm filter and then subjected to LC-MS analysis. Samples were ionized by ESI with a Micromass LCT Premier TOF Mass Spectrometer. The LC was performed on an Acquity Ultra Performance BEH C18, 1.7 μm, 2.1 x 100 mm column on a gradient of 10-60% acetonitrile/water (0.1% TFA) over 7 minutes at a flow rate of 0.6 mL/min. In total, three lines (pCAMV214M_31, pCAMV214M_37, and pCAMV214M_49) with constitutive mutant STR expression were assayed for enzymatic activity. Lysate from hairy roots overexpressing *wild-type* strictosidine synthase and hairy roots infected with *A. rhizogenes* lacking plasmid (“wild type hairy root”) were used as negative controls. Additionally, any non-enzymatically catalyzed background reaction that occurred simply

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in the presence of the hairy root extraction buffer was also measured (no enzyme control) (**Figure S7**).

LC-MS analysis indicated the formation of the corresponding 10-chloro and 10-methylstrictosidine analogs **3a** and **3b** in samples with crude lysate from pCAMV214M hairy roots in all chosen lines (**Figure S7, part I**). The strictosidine analog peaks co-eluted with chemically synthesized standards (Yerkes *et al. Bioorg. Med. Chem. Lett.* **18**, 3095-3098 (2008).). Additionally, substantial levels of deglycosylated strictosidine (cathenamine) analogs were formed in these crude extracts (**Figure S7, part II**). Deglycosylated strictosidine, or cathenamine, is the next biosynthetic intermediate of the pathway, and is formed after strictosidine is deglycosylated by the enzyme strictosidine glucosidase (SGD). Cathenamine analogs were observed as evidenced by formation of a product with the correct molecular weight and retention time, indicating that the endogenous SGD of the hairy roots efficiently deglycosylated the strictosidine analogs **3a** and **3b** (**Figure S7, part II**). No downstream alkaloid biosynthesis (i.e. **4**, **5** or **6**) was observed in the hairy root lysates; biosynthetic enzymes downstream of SGD are labile and extreme care must be taken to preserve the activity of these enzymes in cell free lysates (Yerkes and O'Connor, unpublished results). Strictosidine and cathenamine analogs were not observed in negative controls. Samples with crude lysates from hairy roots transformed with wild-type *A. rhizogenes*, samples lacking hairy root extracts and samples without tryptamine analogs each failed to produce strictosidine analogs **2a** and **2b** and the corresponding deglycosylated strictosidine (cathenamine) analogs. Some background reaction occurred in the absence of hairy root extract with substrate **2b**, as

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evidenced by formation of a small amount of **3b** in the control reaction that lacked hairy root lysate. However, formation of **3b** was significantly enhanced upon addition of lysate derived from transformed hairy root lines containing the V214M gene (**Figure S7, part I**). The corresponding strictosidine and cathenamine analogs were not formed when 5-bromotryptamine **2c** was used as the tryptamine analog substrate (data not shown). This was expected, as the catalytic efficiency of the Val124Met enzyme for 5-bromotryptamine is significantly lower than for 5-chloro and 5-methyltryptamine (Bernhardt and O'Connor, unpublished results).

For the inducible system, 10 μM or 30 μM dexamethasone (an artificial glucocorticoid receptor ligand required for activation of gene expression in pTA7002) was added to two or three-week old hairy roots in liquid culture. Since previous reports suggest that inducible protein expression peaks approximately 72 hours after induction, hairy roots were harvested at this point and processed in the same manner as described for the constitutive system. Despite various expression and induction conditions, we did not observe the formation of strictosidine analogs **3a** or **3b** when the crude lysates from two chosen lines (pTAV214M cell lines 3 and 26) were incubated with tryptamine analogs **2a** or **2b** (data not shown). These results were consistent with the real time reverse transcriptase PCR data that indicated that negligible expression of the V214M mutant is observed in these lines.

To verify that the production of unnatural strictosidine analogs was specifically due to overexpression of the STR V214M mutant and not a result of other genetic changes related to the *Agrobacterium*-mediated transformation, lysate from hairy roots

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overexpressing *wild-type* strictosidine synthase ("pCAMWT") was incubated with either 5-chlorotryptamine **2a,** 5-methyltryptamine **2b** or tryptamine using conditions described above. Formation of 10-chlorostrictosidine **3a** or 10-chlorocathenamine was not observed when lysates from pCAMWT control hairy roots were incubated with 5-chlrotryptamine **2a** (**Figure S7, part III, IV**). While the formation of 10-methylstrictosidine **3b** was not observed, a small amount of 10-methylcathenamine formed when lysates from pCAMWT control hairy roots were incubated with 5-methyltryptamine **2b** (**Figure S7, part IV**). This suggests that 10-methylstrictosidine **3b** could form from a chemical reaction (background, as was also observed in the no enzyme control (hairy root extraction buffer)) and was subsequently deglycosylated by strictosidine glusosidase. Formation of both strictosidine and cathenamine was observed when lysates from pCAMWT control hairy roots were incubated with tryptamine suggesting that enzymes remain active under the assay conditions (**Figure S7, part III, IV**). These results confirm that genetic changes—unrelated to the expression of the engineered STR—that occur as a result of the plant transformation do not give rise to the formation of unnatural strictosidines.

To ensure that a large amount of *wild-type* strictosidine synthase could not turn over 5-chlorotryptamine **2a** and 5-methyltryptamine **2b**, various concentrations (1 nM, 10 nM and 100 nM; wild type enzyme k_{cat}/K_m 208333 $M^{-1}s^{-1}$ for tryptamine; no detectable activity for **2a**, **2b** or **2c**) of recombinant wild-type strictosidine synthase (*Cr*STR) (for preparation and concentration determination of the enzyme see reference 10) were added to lysates of control hairy root cultures lacking V214M expression. Assay

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conditions were identical to those used in the previous section. Formation of 10-chlorostrictosidine **3a** or 10-methylstrictosidine **3b** was not observed when lysates of control hairy roots were incubated with 5-chlorotryptamine **2a** and 5-methyltryptamine **2b**, respectively, even in the presence of 100 nM recombinant *Cr*STR (**Figure S7, part V**).

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