

Supporting Information

Chemoselective Derivatization of Alkaloids in Periwinkle

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1. Chemistry

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 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 μ m). Extracts were concentrated under reduced pressure at $< 40^{\circ}\text{C}$. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian 500 MHz and Bruker 600 MHz spectrometers equipped with Sun workstations. For ^1H and ^{13}C NMR spectra recorded in CD_3OD , chemical shifts (δ) are given in ppm relative to solvent peaks (^1H , $\delta = 3.32$; ^{13}C , $\delta = 49.1$) as an internal standard.

2. Kinetic assay for strictosidine synthase with **1a-e.**

Strictosidine synthase was overexpressed and purified from *E. coli* as previously described.¹² To obtain kinetic values for secologanin analogs **1a-e**, strictosidine synthase (100 nM) was incubated with tryptamine **2**, secologanin analog **1a-e** in 100 mM NaH_2PO_4 (pH 6.8) buffer at 30°C . Tryptamine **2** concentration was held constant at 2 mM (well above K_m value of 7 μM).¹² Reaction rates were measured at 7 secologanin analog concentrations ranging from 50 μM - 1.2 mM. 1-Naphthalene-acetic acid (140 μM) was used as an internal standard. The reactions were quenched with NaOH aq. (2 equiv.) after a given time point and then directly injected onto the HPLC (Hibar RT 250-4LiCHrosorb) using a 22 to 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. Kinetic data were run in triplicate. Kinetic parameters were obtained using Origin 7.0 software (Origin lab) (Figure 1). A negative control performed in the absence of enzyme ensured that negligible background reaction occurred under the assay conditions. All enzymatic products resulting from reaction of strictosidine synthase, tryptamine and secologanin analogs **1a-e** were characterized by high-resolution mass spectrometry (Table S1). Additionally, authentic standards for **3c** and **3d** were chemically synthesized, structurally characterized by ^1H NMR and showed to co-elute with the enzymatic product. The synthesis and characterization of **3c** is described below. Synthesis and characterization of **3d** is described in O'Connor, S.E. *et al.* (*Chem. Biol.* 2006, **13**, 1137-1141- reference 16 in manuscript).

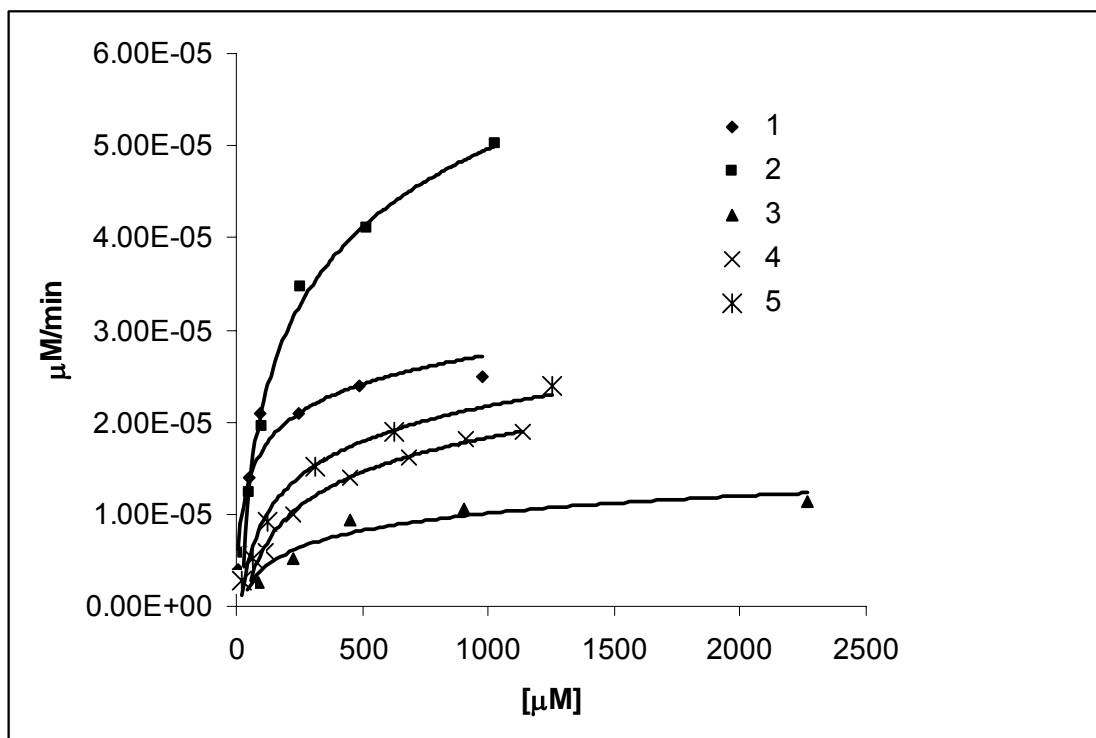


Figure 1. Michaelis Menten plot for secologanin analogues **1-5** obtained using a purified *E. coli* preparation of strictosidine synthase.

Secologanin analog concentration determination.

Secologanin **1a** and secologanin analogs **1b-e** have a very low extinction coefficient, which hinders accurate quantification of secologanin stock solution concentration. To address this issue, secologanin analogs **1a-e** were reacted with benzoic hydrazide which has a known extinction coefficient (238 nm = 6×10^6 [benzoic hydrazide (mM)]). Seven different dilutions of a stock solution of benzoic hydrazide were prepared (19 mg, 98% in 1 mL of DMSO). The samples were analyzed by HPLC (Hibar C18 column) with a water (0.1% TFA)/ MeCN gradient of 10-50% acetonitrile over 20 minutes with diode array detection (retention time of the compound was found to be 3.3 min). The concentration of the secologanin stock solutions could then be determined by reaction of 10 μ L of this stock solution with 10 μ L of the benzoic hydrazide stock solution (3-fold estimated concentration of secologanin analog) in phosphate buffer (100 mM, pH= 5) in a total volume of 100 μ L. Ten μ L of each sample was analyzed by HPLC. The decrease in A238 at 3.3 min was correlated to the concentration of secologanin derivative that reacted with the hydrazide. Concentration for analog **1e** was estimated due to the instability of the NHBoc group at the ester position under these reaction conditions.

Strictosidine propargyl ester analog 3c.

Tryptamine **2** hydrochloride (2.04 mg, 12.8 μ mol) and propargyl secologanin **1c** (2 mg, 4.8 μ mol) were incubated in 50 mM phosphate citrate pH 5 buffer at 30°C for 5 days. The crude mixture was subjected to preparative HPLC purification on a Grace vydac column (C18 monomeric, 100Å, H₂O (0.1% TFA)/MeCN, gradient 20-60% MeCN over 20 min, R.T. 14 min.) to give 1 mg (30%) of propargyl strictosidine **3c**. From the spectroscopic data we observed a chemical shift assigned to H-15 at 3.12 ppm. Vicinal coupling constants of $J_{15, 14S} = 4.5$ Hz and $J_{5, 14R} = 11.5$ Hz were observed, suggesting that C-15 is in the S configuration as in strictosidine.²⁷ Full NMR characterization is shown below. The compound was then used as an authentic standard. ¹H NMR (CD₃OD, 500MHz): δ 7.59 (s, 1H, H-17), 7.41 (d, 1H, *J*_{9,10} 7.7, H-9), 7.32 (d, 1H, *J*_{12,11} 8.3, H-12), 7.09 (dd, 1H, *J*_{11,10} 7.0, H-11), 7.00 (dd, 1H, H-11), 5.68 (m, 1H, H-19), 5.86 (d, 1H, *J*_{21,20} 9.6, H-21), 5.43 (dd, 1H, *J*_{18z,19} 16.7, H-18Z), 5.34 (dd, 1H, *J*_{18E,19} 17.4, H-18E), 4.68 (d, 1H, *J*_{1',2'} 7.7, H-1'), 4.47 (m, 1H, H-3), 4.23 (m, 1H,

H-23b), 4.05 (m, 1H, H-23a), 3.87 (dd, 1H, $J_{6a',6b'}$ 1.9, $J_{6a',5'}$ 13.5, H-6a'), 3.66 (m, 1H, H-6b'), 3.50-3.20 (m, 4H, H-2', H-3', H-4', H-5'), 3.47 (m, 1H, H-5 β), 3.18 (m, 1H, H-5 α), 3.12 (dd, 1H, $J_{15, 14S}$ 4.5, $J_{5 14R}$ 11.5, H-15), 2.96 (m, 1H, H-6 β), 2.71 (m, 1H, H-6 α), 2.28 (dd, 1H, $J_{14R, 14S}$ 14.7, H-14S), 2.06 (dd, 1H, H-14R), 1.30 (s, 1H, H-25).
 ESI(C₂₉H₃₄N₂O₉): calculated m/z 555.2 [M+H]⁺, found m/z 555.2 [M+H]⁺.

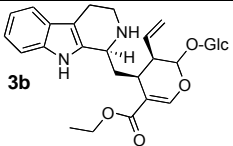
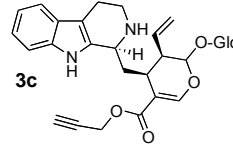
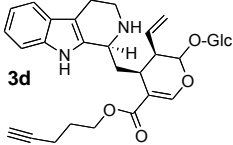
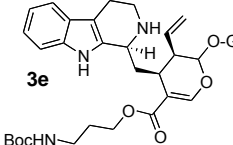
Compound structure	Molecular Formula	m/z expected	m/z found
	C ₂₈ H ₃₆ N ₂ O ₉	545.2494	545.2491
	C ₂₉ H ₃₄ N ₂ O ₉	555.2337	555.2340
	C ₃₁ H ₃₈ N ₂ O ₉	583.2656	583.2639
	C ₃₄ H ₄₇ N ₃ O ₁₁	674.3283	674.3287

Table S1. High-resolution mass spectrometry data for enzymatically generated strictosidine analogs.

3. Feeding experiments

Feeding experiments with hairy root cultures

C. roseus hairy root cultures (provided by Prof. J. Shanks (Iowa State) and Prof. Lee-Parsons (Northeastern)) were sub-cultured in 25 mL of half strength Gamborg's media and vitamins (pH = 5.7) and grown for 7 days at 26°C shaking at 50 rpm. Secologanin pentynyl analog **1d** was dissolved in water, syringe filtered through a 0.2 µm filter to sterilize and was added to half strength Gamborg's media and vitamins (pH = 5.7) media to a final concentration of 1 mM on day 7. Flasks of roots (25 mL) were cultured for an additional 14 days. Autoclaved water was added to the cultures weekly to account for evaporation. Roots were removed from the media, and plant material was extracted as described below. LC-MS analysis over the course of the culture indicated that **1d** did not significantly degrade in the media.

Feeding experiments with seedlings

Secologanin pentynyl analog **1d** was added to MS media supplemented with vitamins (Sigma) (50 mL, pH = 5.7) with 2g/L phytoigel. The final concentration of analog in the media was 1 mM. The supplemented media was added to a square plate and allowed to solidify. *C. roseus* seeds (Horizonherbs) were sterilized for 30 seconds in ethanol, 10 minutes in 10% bleach, filtered under vacuum and washed 3 times with filter sterilized water. The seeds were then germinated on solid MS media and allowed to grow for 2 weeks. Seedlings were grown in the dark for 7 days and then under incandescent light with 16 hours light and 8 hours dark. Seedlings were then transplanted to MS media containing 500 µM **1d** and cultured for an additional week.

Alkaloid extraction

The plant material was ground with a mortar and pestle in methanol (3 x 30 mL). The methanol extract was filtered and concentrated under vacuum to yield a yellow solid which was then sonicated for 30 minutes in 3% aqueous HCl. The HCl solution was extracted with hexanes (3 x 30 mL) to remove lipids and other hydrophobic material. A saturated solution of NH₄OH was then used to adjust the pH of the solution to

approximately 8. This solution was then extracted with methylene chloride (3 x 30 mL) to remove the alkaloids into the organic layer. The combined methylene chloride fractions were washed with brine, dried over sodium sulfate and concentrated to yield a yellow alkaloid extract (2-10 mg). The extract was dissolved in methanol (1 mL) for further analysis by HPLC and LC-MS.

LC-MS analysis

The alkaloid mixtures were diluted 1/1000 with methanol for mass spectral analysis. 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 performed on an Acquity Ultra Performance BEH C18, 1.7 μ m, 2.1 X 100 mm column on a gradient of 10-60 % acetonitrile/water 0.1% formic acid over 20 minutes at a flow rate of 0.25 mL/min. The capillary and sample cone voltages were 2000 and 30 V, respectively. The desolvation and source temperature were 350 and 100 °C. The cone and desolvation gas flow rates were 20 and 700 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 the mass spectrum of peaks of interest was smoothed and centered with TOF mass correction, locking on the reference infusion of ajmalicine (m/z 353.1865, 1nM solution at 20 μ L/min) reference. All data from analog treated samples were compared to a negative control from a culture containing only tryptamine.

Analog purification by preparative HPLC and NMR analysis

Alkaloid root extracts were purified on a 10 X 20 mm Vydac reverse phase column using a gradient of 20-60% acetonitrile/ water (0.1%TFA or 0.1% formic acid) over 25 minutes. The mixture was monitored at 238 nm and fractions containing the alkaloid analogs of interest were combined and concentrated. Isolated alkaloids were analyzed by NMR on a Bruker AVANCE-600 NMR spectrometer equipped with a 5 mm $^1\text{H}\{^{13}\text{C},^{31}\text{P}\}$ cryo- probe.

Fosmidomycin inhibition

Fosmidomycin (Toronto Research Chemicals) was added to 10 day old hairy root cultures at a final concentration of 100 μ M or 1 mM in the presence and absence of **1d** (1 mM). Experimental controls included addition of **1d** (1 mM) in the absence of fosmidomycin and addition of secologanin **1a** (1 mM) to a fosmidomycin treated culture. Product levels were measured by integration of the peaks corresponding to the analog and natural alkaloids using MassLynx software. It was assumed that the substitution of the methyl ester with a longer alkyl moiety does not significantly affect the ionization of the compounds.

Biotinylation of hairy root cell culture extract.

Biotin PEO azide (reference 16 in manuscript) (0.5 mg, 0.00125 mmol), copper sulfate pentahydrate (5 equiv), ascorbic acid (10 equiv.) were added to a stirring solution of cell culture extract (2 mg) in water (0.5 mL). The reaction mixture was allowed to stir overnight at room temperature.

Captavidin purification

Crude biotinylated root extracts dissolved in citrate phosphate buffer (50 mM, pH 4.0, 1.0 mL) were added to a cartridge containing Captavidin agarose (Molecular Probes, 5 mL suspension) that had been previously equilibrated with the same buffer. The tube was gently rocked at room temperature. After 20 minutes, the eluant was filtered and the agarose washed twice with citrate phosphate buffer (2 mL), filtrates were collected and concentrated under reduced pressure and were used in subsequent binding-release cycles. Sodium carbonate (50 mM, pH 10.0, 1 mL) was added to the cartridge and the slurry was allowed to shake for another 20 minutes at room temperature. The biotinylated compounds were then eluted from the column, and the column washed twice with the sodium carbonate buffer (2 mL) to ensure that all biotinylated molecules were eluted. Upon re-equilibration of the agarose column, the binding-release cycle was repeated 4 times. Control cultures lacking **1d** were also subjected to the click reaction conditions and failed to yield these compounds, suggesting that these compounds are derived from **1d**.

4. Tabulated ^1H NMR data for isolated alkaloid analogs

Strictosidine pentynyl analog 3d

^1H NMR (CD_3OD , 500MHz): δ 7.58 (s, 1H, H-17), 7.48 (d, 1H, $J_{9,10}$ 7.8, H-9), 7.32 (d, 1H, $J_{12,11}$ 8.5, H-12), 7.15 (dd, 1H, $J_{11,10}$ 7.0, H-11), 7.06 (dd, 1H, H-11), 6.01 (m, 1H, H-19), 5.86 (d, 1H, $J_{21,20}$ 8.9, H-21), 5.38 (dd, 1H, $J_{18z,19}$ 17.4, H-18Z), 5.38 (dd, 1H, $J_{18E,19}$ 17.4, H-18E), 4.81 (d, 1H, $J_{1',2'}$ 7.6, H-1'), 4.33 (dd, 1H, $J_{3, 14S}$ 3.1, $J_{3, 14R}$ 10.4, H-3), 4.19 (m, 1H, H-23b), 4.08 (m, 1H, H-23a), 4.01 (dd, 1H, $J_{6a',6b'}$ 2.1, $J_{6a',5'}$ 2.8, H-6a'), 3.65 (dd, 1H, $J_{6b',5'}$ 11.6, H-6b'), 3.50-3.20 (m, 4H, H-2', H-3', H-4', H-5'), 3.47 (m, 1H, H-5 β), 3.18 (m, 1H, H-5 α), 3.09 (dd, 1H, $J_{15, 14S}$ 3.9, $J_{5, 14R}$ 11.0, H-15), 2.83 (m, 1H, H-6 β), 2.75 (m, 1H, H-6 α), 2.21 (dd, 1H, $J_{14R, 14S}$ 13.0, H-14S), 1.95 (dd, 1H, H-14R), 2.10 (m, 2H, H-25), 1.74 (s, 1H, H-27), 1.61 (m, 2H, H-24).

Ajmalicine pentynyl analog 4d

^1H NMR (CD_3OD , 600MHz): δ 7.63 (m, 1H, H-17), 7.56 (m, 1H, H-9), 7.38 (dd, 1H, J 8.6, 4.6 H-12), 7.23 (dd, 1H, J 8.6, H-11), 6.98 (td, 1H, J 8.4, 2.6, H-10), 4.56 (qd, 1H, J 5.9, 3.2, H-19), 4.23 (m, 1H, H-23b), 4.02 (m, 1H, H-23a), 3.42 (brd, 1H, J 13.5 H-3), 3.19 (dt, 1H, J 12.6, 3.6, H-14 α), 3.20-3.00 (m, H-5 β , H-6 β), 2.93 (dd, 1H, J 16.9, 3.2, H-21 β), 2.80-2.58 (m, 2H, H-6 α , H-5 α), 2.45 (m, 1H, H-15), 2.30 (t, 1H, J 16.9, H-21 α), 2.20 (tt, 1H, J 3.2, 3.2, 12.0, H-20), 1.40 (m, 3H, H-14 β), 1.28 (s, 1H, H-24), 1.12 (m, 1H, H-18).

Serpentine pentynyl analog 5d

^1H NMR (CD_3OD , 600MHz): δ 8.50-8.20 (m, 1H, H-6), 8.39 (m, 1H, H-5), 8.37 (dd, 1H, J 8.3, 1.6, H-9), 7.76 (ddd, 1H, J 9.1, 6.3, <2, H-11), 7.73-7.70 (m, 2H, H-12, H-17), 7.46 (m, 1H, H-10), 4.90-4.75 (m, 3H, H-21 β , H-14 α , H-19), 4.60 (m, 1H, H-21 α), 4.23 (m, 1H, H-23b), 4.02 (m, 1H, H-23a), 3.10 (dd, 1H, J 17.4, H-14 β), 2.96 (1H, dddd, J 10.9, 6.0, 1.2, H-15), 2.82 (tt, 1H, J 12.4, 3.8, H-20), 1.40 (d, 3H, J 6.7, H-18), 1.28 (s, 1H, H-24).

5. LC-MS data

5A. Pentynyl hairy root extract: serpentine **5a** and **5d**

5B. Pentynyl hairy root extract: ajmalicine **4a** and **4d**

5C. Pentynyl hairy root extract: strictosidine **3a** and **3d**

5D. Pentynyl hairy root extract: biotintylated serpentine **5f**

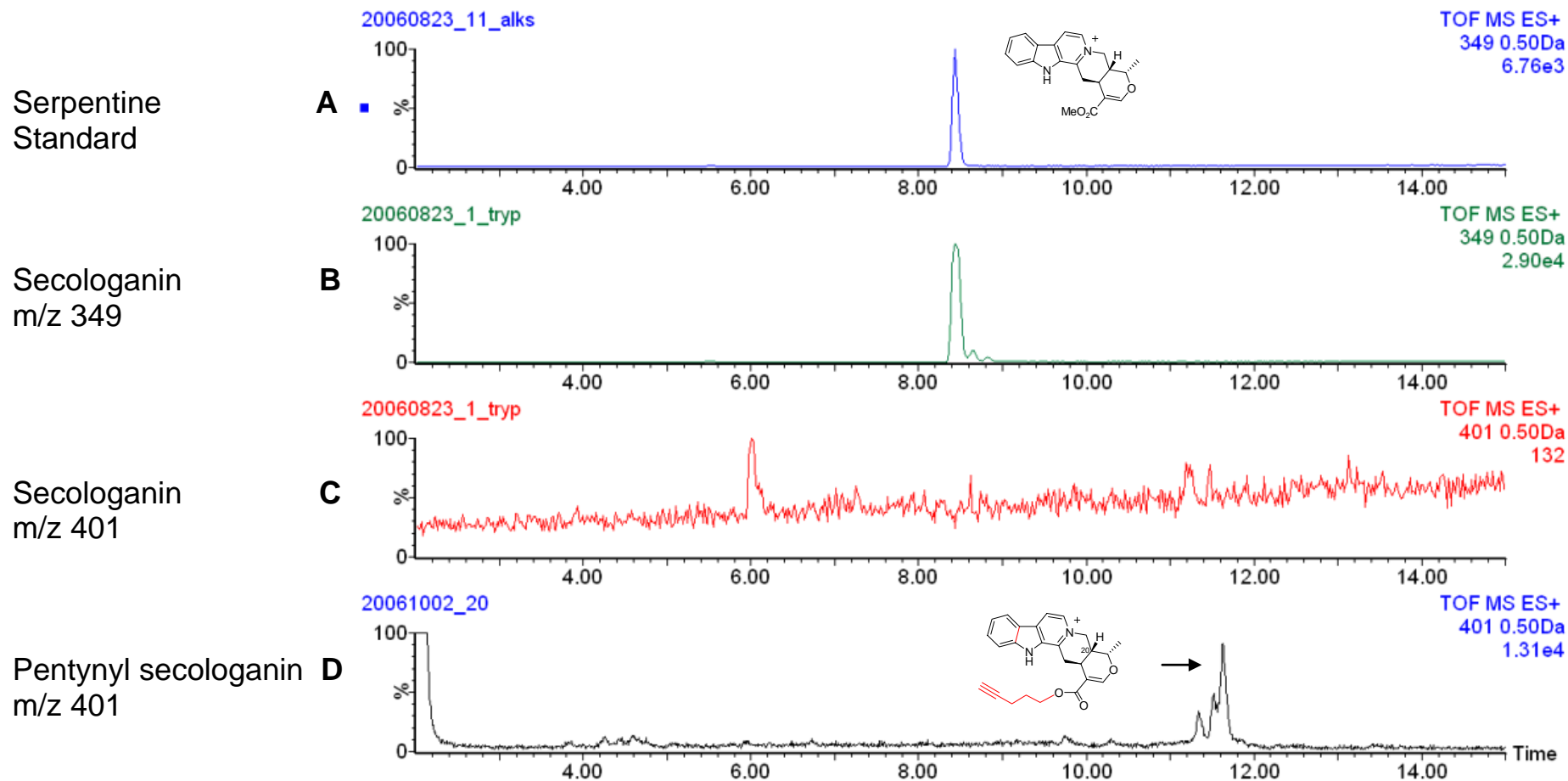
5E. Pentynyl hairy root extract: biotintylated ajmalicine **4f**

5F. Ajmalicine, pentynyl ajmalicine and biotintylated ajmalicine: captavidin column

5G. Negative control: hairy root extracts treated with CuSO₄ and ascorbic acid

5A. Pentynyl hairy root extract: serpentine 5a and 5d

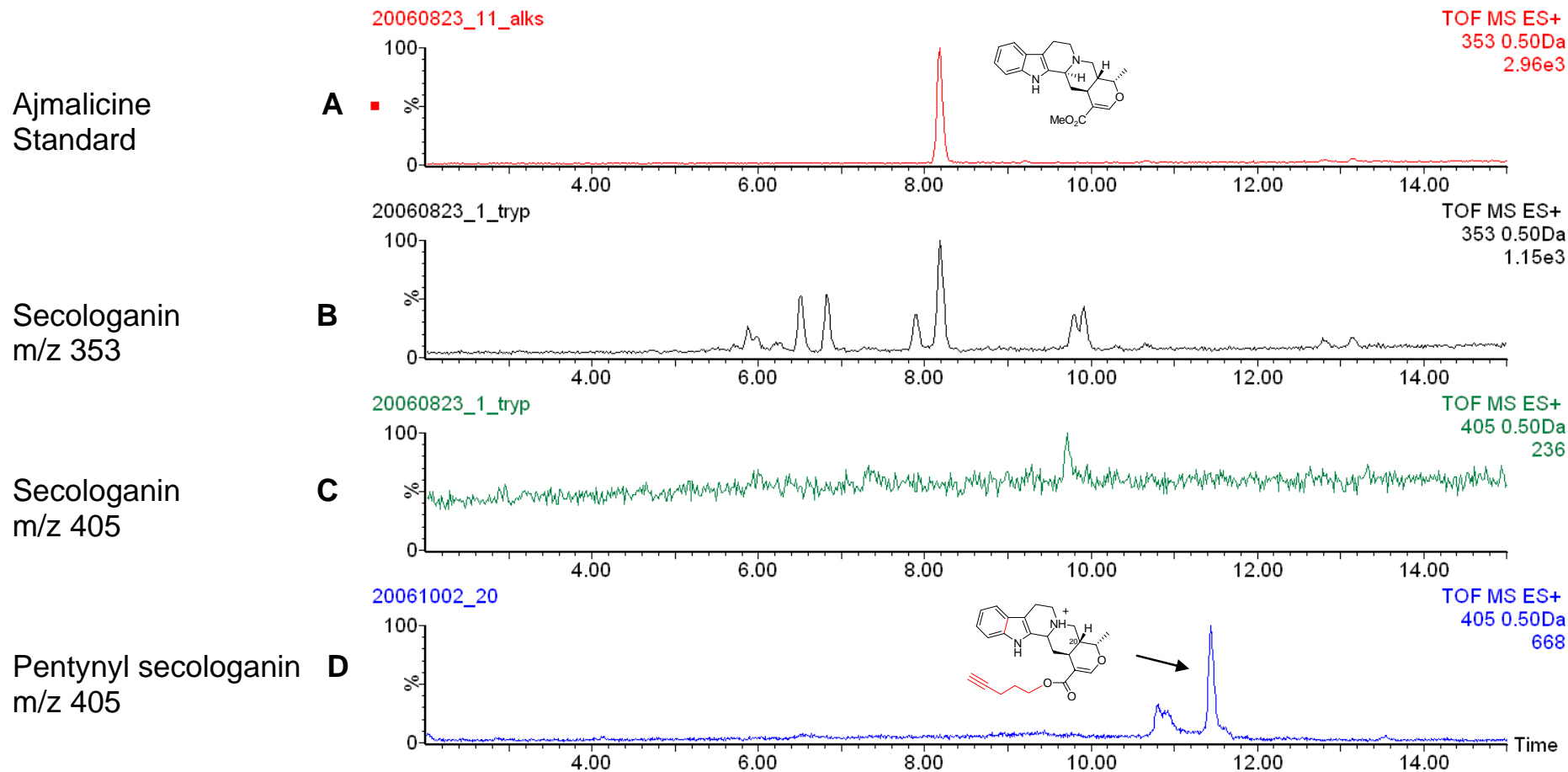
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A Serpentine **5a** standard. **B** Naturally occurring serpentine **5a** in hairy root culture. **C** No pentynyl serpentine **5d** in hairy roots lacking **1d**. **D** Appearance of pentynyl serpentine **5d** in hairy roots supplemented with **1d**.

5B. Pentynyl hairy root extract: ajmalicine **4a** and **4d**

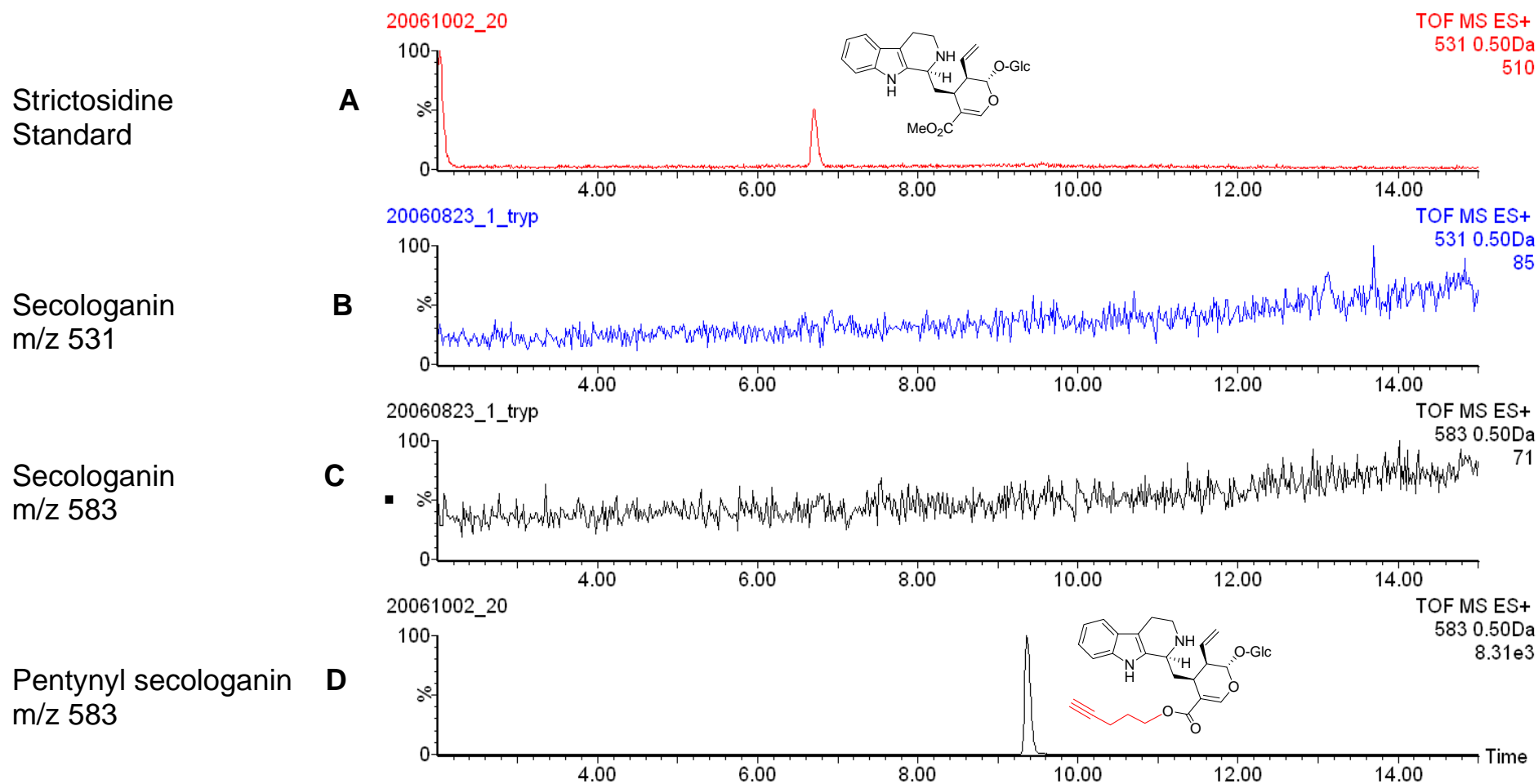
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A Ajmalicine **4a** standard. **B** Naturally occurring ajmalicine **4a** in hairy root culture. **C** No pentynyl ajmalicine **4d** in hairy roots lacking **1d**. **D** Appearance of pentynyl ajmalicine **4d** in hairy roots supplemented with **1d**.

5C. Pentynyl hairy root extract: strictosidine **3a** and **3d**

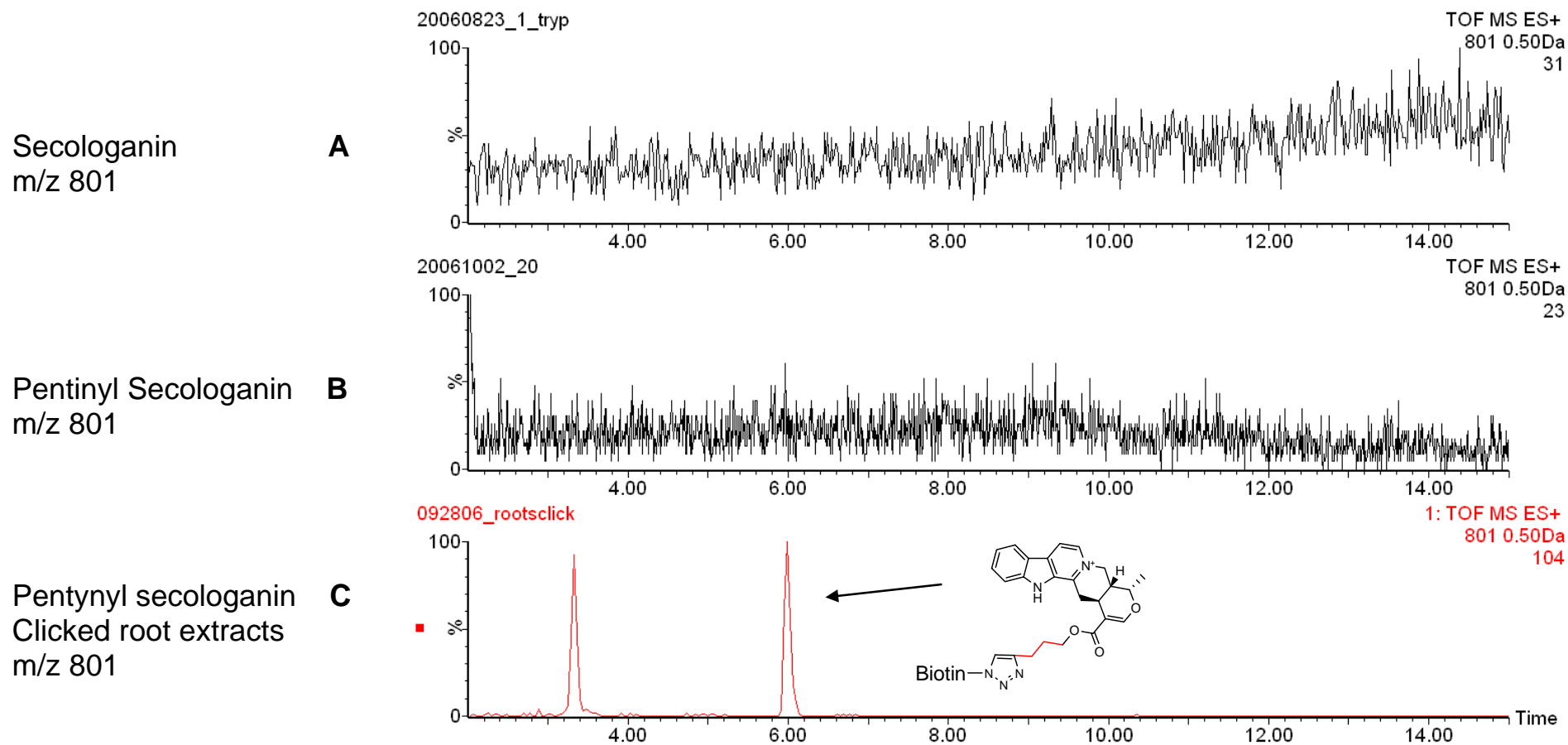
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A Strictosidine standard. **B** Strictosidine **3a** is rapidly metabolized and does not accumulate. **C** No pentynyl strictosidine **3d** in hairy roots lacking **1d**. **D** Appearance of pentynyl strictosidine **3d** in hairy roots supplemented with compound **1d**.

5D. Pentynyl hairy root extract: biotinylated serpentine 5f

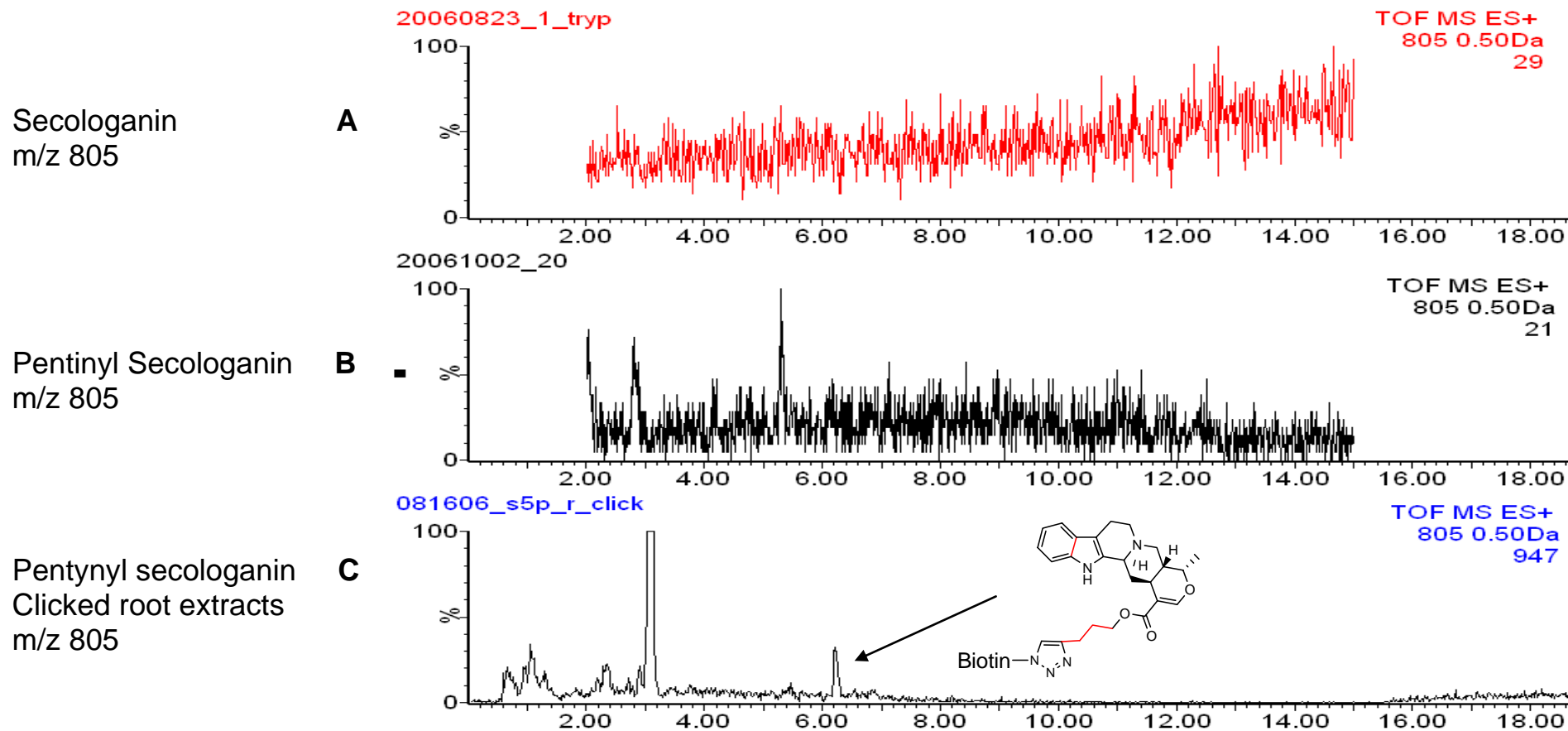
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A Absence of biotinylated serpentine in hairy roots lacking **1d**. **B** Absence of biotinylated serpentine in hairy roots fed with **1d** before click chemistry. **C** Appearance of biotinylated serpentine in hairy roots fed with **1d** after click chemistry.

5E. Pentynyl hairy root extract: biotinylated ajmalicine 4f

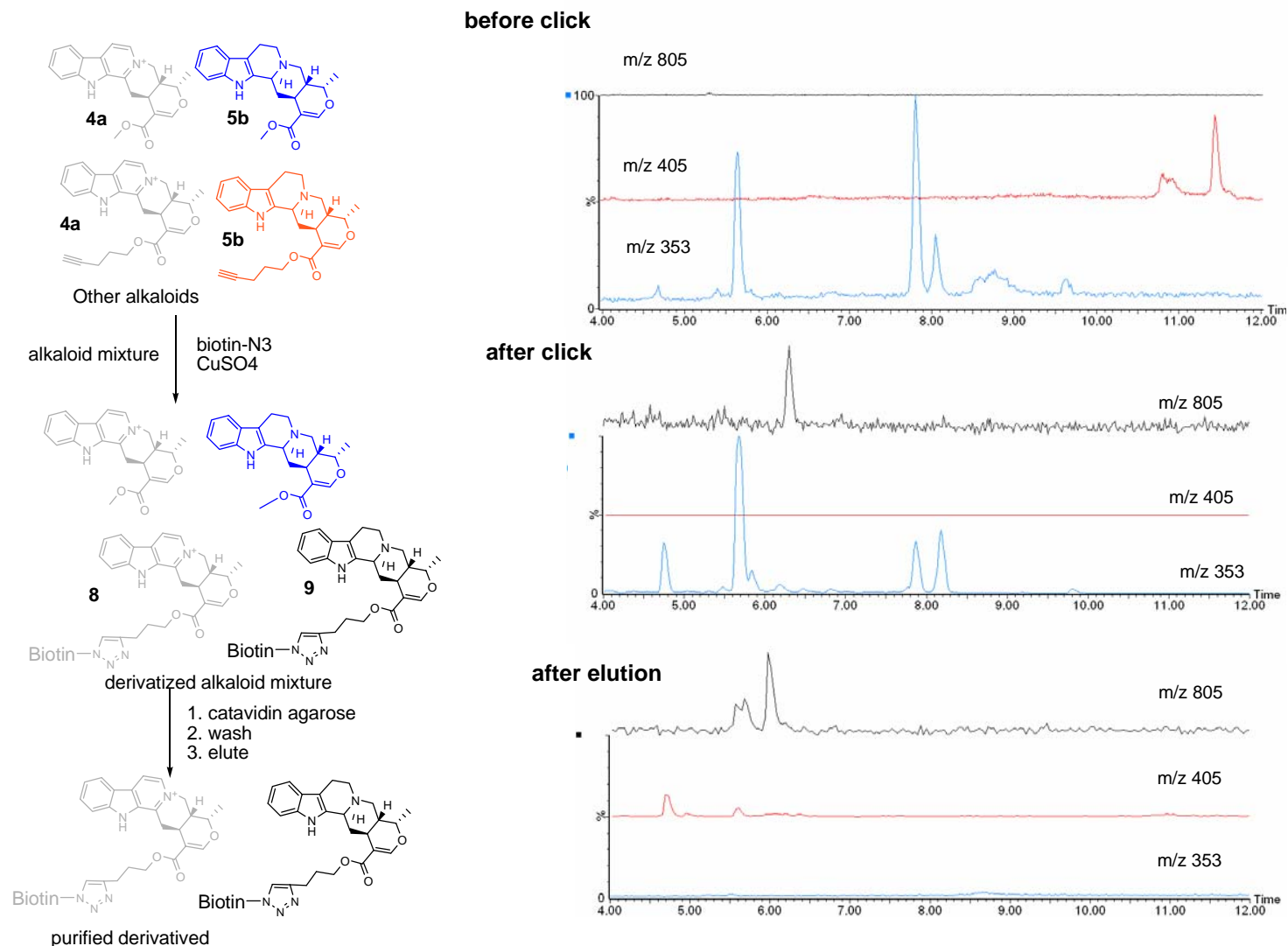
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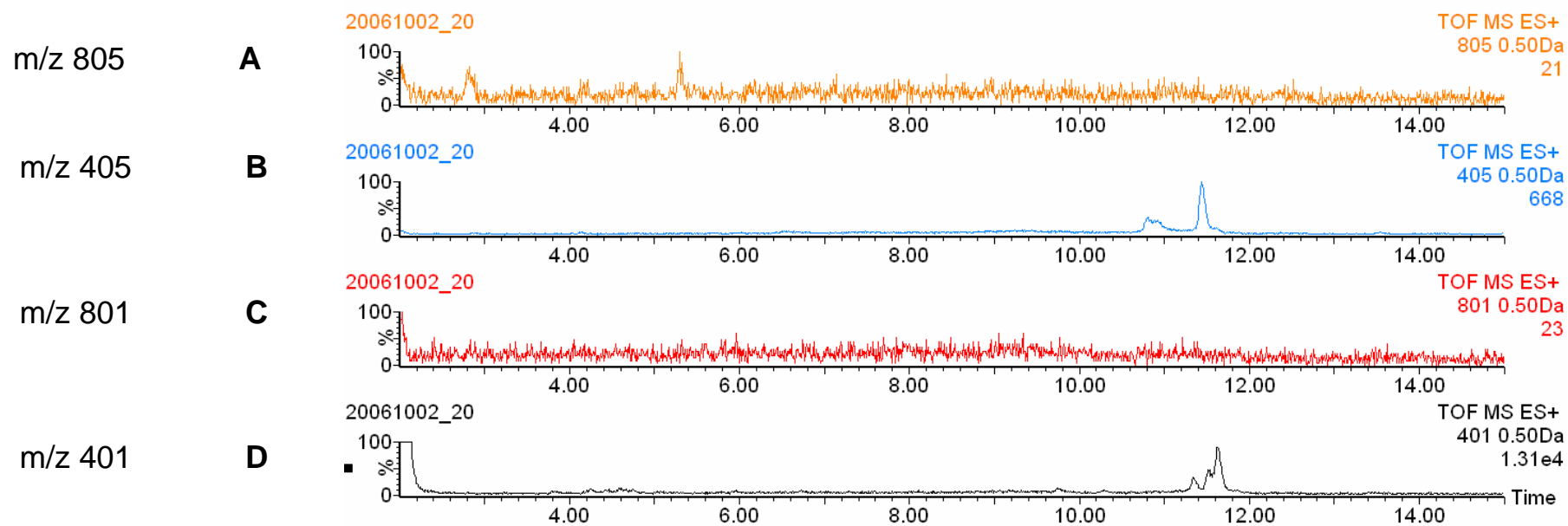
A Absence of biotinylated ajmalicine in secologanin fed hairy roots. **B** Absence of biotinylated serpentine in hairy roots fed with **1d** before click chemistry. **C** Appearance of biotinylated ajmalicine in hairy roots fed with **1d** after click chemistry.

5F. Ajmalicine, pentinyl ajmalicine and biotinylated ajmalicine: captavidin column

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Extracted LC-MS traces for derivatives of ajmalicine **4**. **A** The native alkaloid **4a** ($[M+H]^+=353$) and the derivative **4d** ($[M+H]^+=405$) can both be observed before the 'click' reaction **B** After incubation with biotin azide **8** and CuSO₄ **4d** disappeared and a new compound corresponding to the dition of biotin **4e** ($[M+H]^+=805$) appeared **C** After elution of the compounds from the CaptAvidin resin, the natural alkaloid **4a** is no longer observed.

5G. Hairy Root extracts treated with CuSO_4 and ascorbic acid in water – control

Treatment of hairy root extracts with ascorbic acid and copper sulphate in water (conditions used for click chemistry) showed no change in alkaloid profile.