New Phytologist

***New Phytologist* Supporting Information**

Article title: Redirecting tropane alkaloid metabolism reveals pyrrolidine alkaloid diversity in *Atropa belladonna*

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Fig. S2 A proposed subnetwork of decorated aromatic tropane esters in *A. belladonna* root.
Fig. S3 Modified pyrrolidine alkaloids are decreased in *PPAR*-silenced *A. belladonna* roots.
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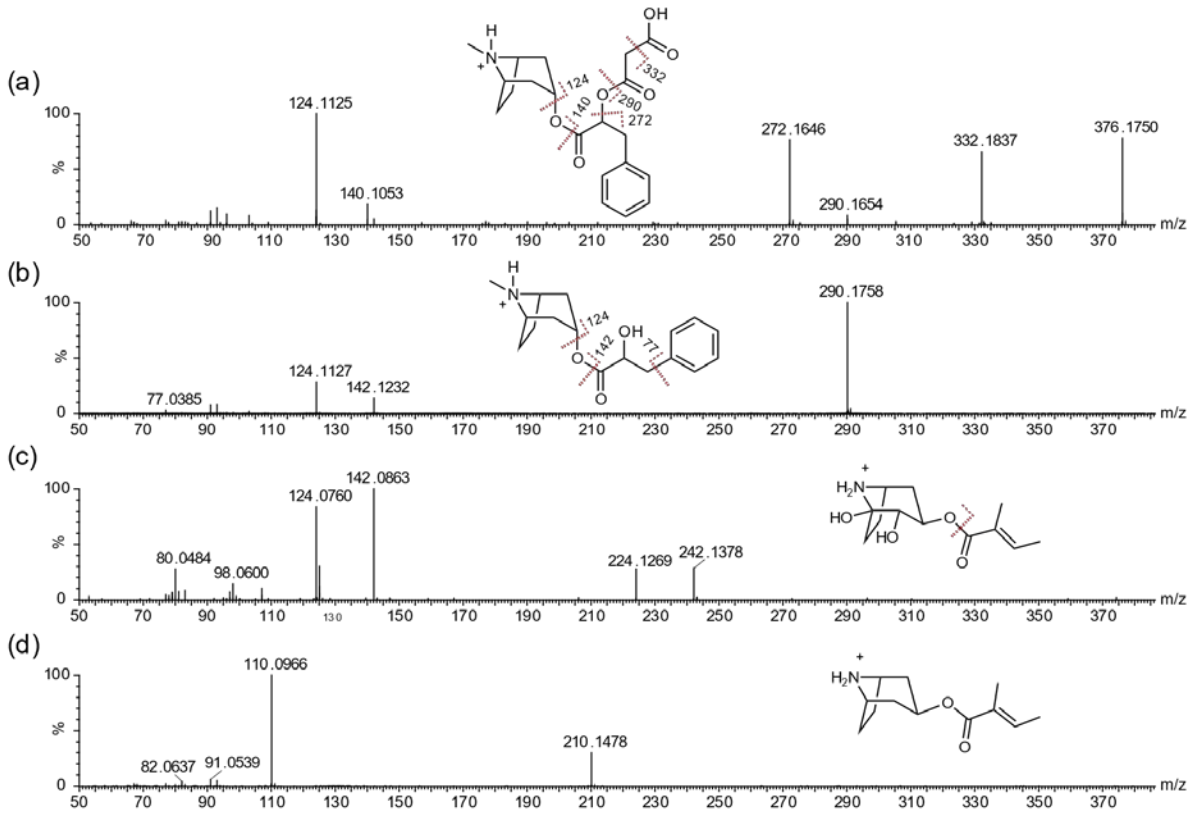

Fig. S1. Tandem mass spectra and proposed fragmentation of representative tropane and nortropane alkaloids. Proposed fragmentation position is represented by red dashed lines with the bottom of the dashed "L" pointing towards the proposed fragment. Panels (a) and (b) show the tandem mass spectra for the tropanes, *m/z* 376.17 (T10) and *m/z* 290.17 (T7), respectively while panels (c) and (d) show the tandem mass spectra for the nortropanes, *m/z* 242.14 (T15) and *m/z* 210.15 (T13), respectively. *It should be noted that in either case hyoscyamine could be the source of the *m/z* 290.17 fragment in panels (a) and (b). *m/z* corresponds to the mass to charge ratio.

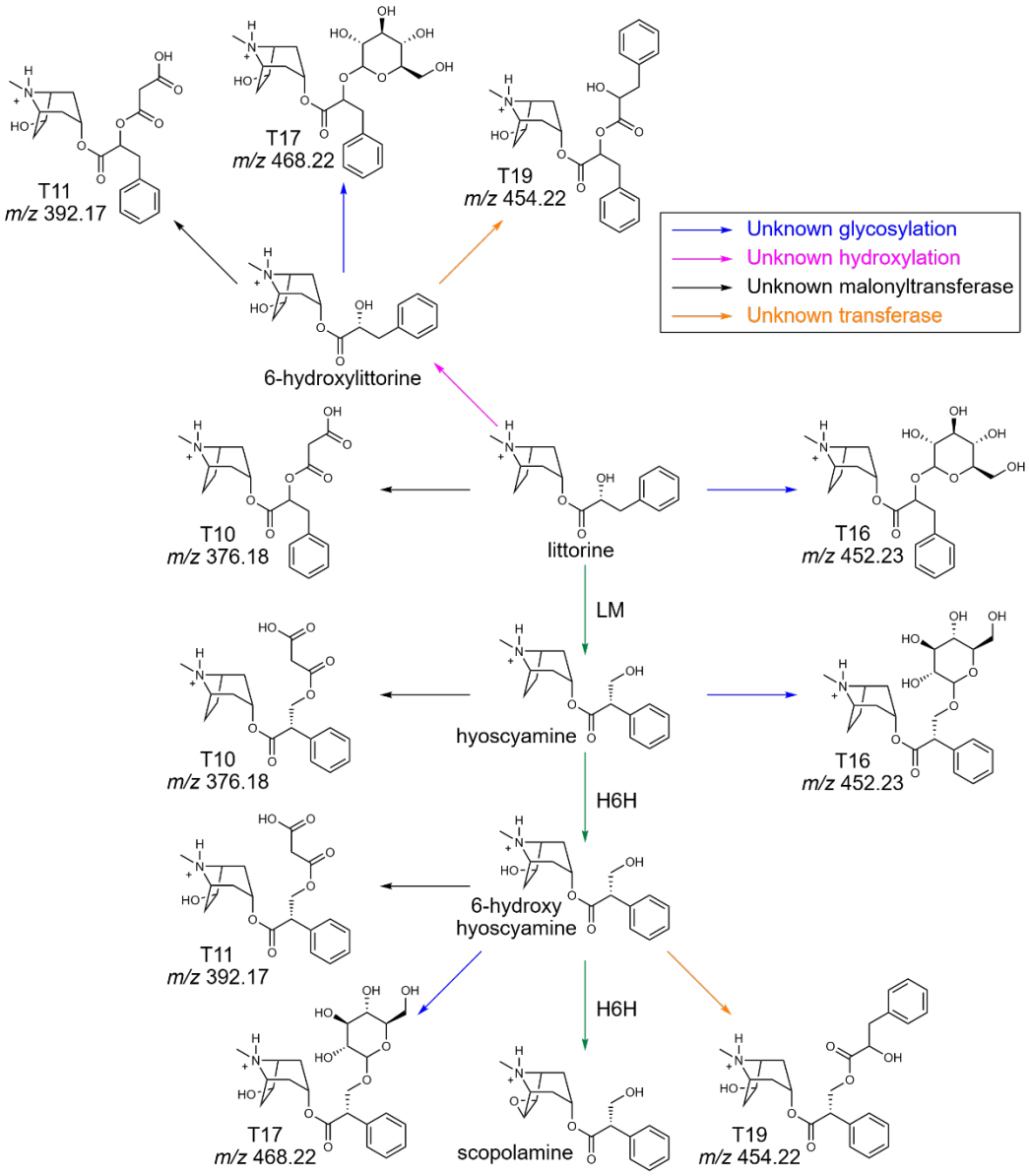

Fig. S2 A proposed subnetwork of decorated aromatic tropane esters in *A. belladonna* root. Proposed enzymatic reactions are color-coded based on the chemical modification occuring. Dark-blue arrows represent unknown glycosylations, pink represent unknown hydroxylations, light blue represent unknown malonylations, and orange represent unknown transferase reactions. Black arrows indicate characterized tropane alkaloid pathway steps. Enzymatic abbreviations are as follows: LM, littorine mutase; H6H, hyoscyamine-6β-hydroxylase. Chemical structures are inferred from tandem mass spectra found in Table S8. For simplicity, hydroxylations are shown at the 6 position of the tropane core, but this decoration could occur at a different position. Metabolite structures are displayed in the $[M+H]^+$ form. The stereochemistry of the sugar moieties are unresolved. *m/z* corresponds to the mass to charge ratio.

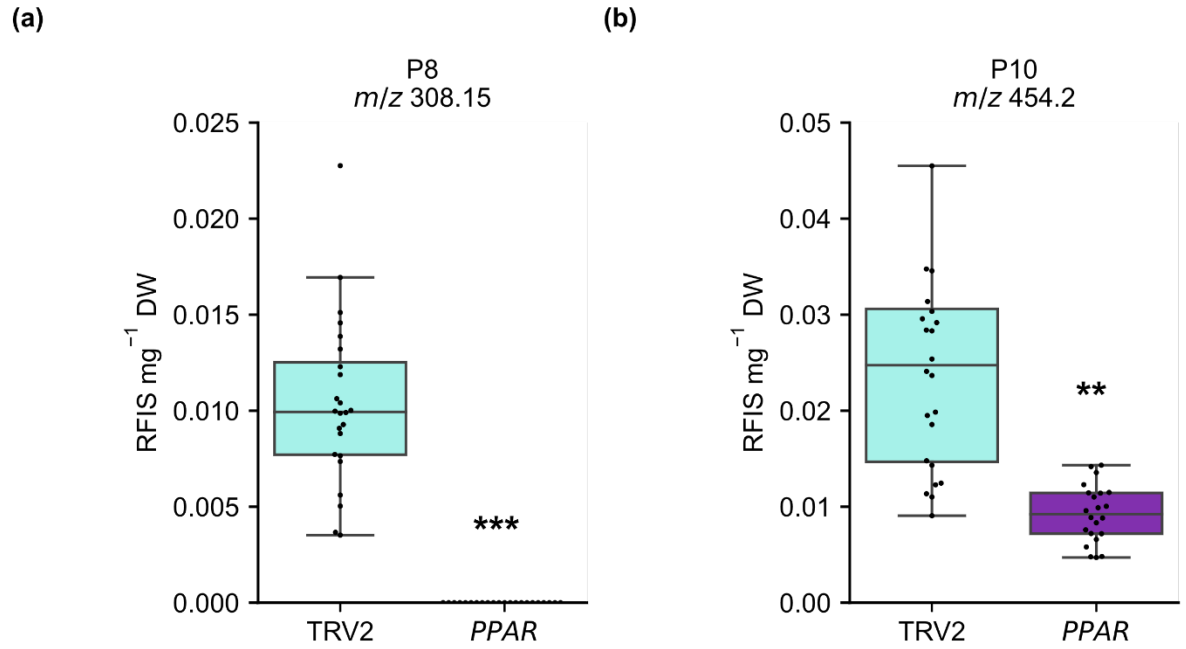

Fig. S3 Modified pyrrolidine alkaloids are decreased in *PPAR*-silenced *A. belladonna* roots. Metabolite abundances of the *m/z* 292.15 (P1 and P2) products with hydroxy and hexose additions, (a). P8 (*m/z* 308.15) and (b) P10 (*m/z* 454.21), respectively, in TRV2 empty vector controls (n = 24) (left, cyan) and phenylpyruvic acid reductase-silenced (*PPAR*) root samples (n = 22) (right, purple). Data are shown as response factor normalized to the internal standard (RFIS) per mg dry weight. Box and whisker plots are displayed with horizontal lines indicating upper extreme, upper quartile, median, lower quartile, and lower extreme in order from top to bottom. The dots on the box and whisker plots represent individual samples. Statistical significance as determined by a Welch's t-test (***, $P < 0.001$; **, $P < 0.01$). *m/z* corresponds to the mass to charge ratio.

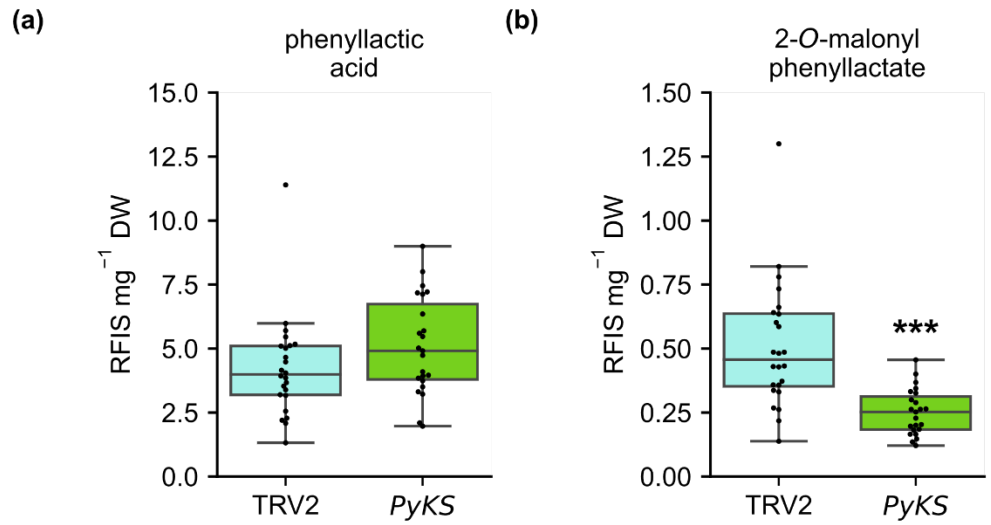

Fig. S4 Abundance of phenyllactic acid and 2-*O*-malonylphenyllactate in *PyKS*-silenced *A. belladonna* roots. (a) phenyllactic acid and (b) 2-*O*-malonylphenyllactate, respectively, in TRV2 empty vector controls (n = 24) (left, cyan) and *PyKS*-silenced lines (n = 23) (right, green). Data are shown as response factor normalized to the internal standard (RFIS) per mg dry weight. Box and whisker plots are displayed with horizontal lines indicating upper extreme, upper quartile, median, lower quartile, and lower extreme in order from top to bottom. The dots on the box and whisker plots represent individual samples. Statistical significance as determined by a Welch's t-test (***,P < 0.001).

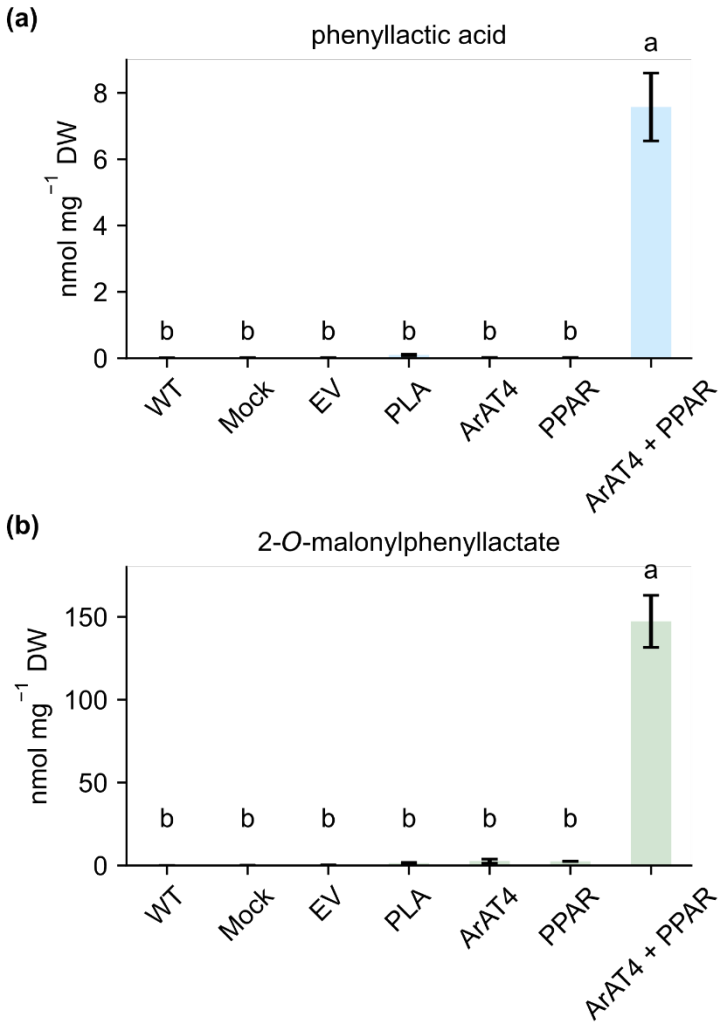

Fig. S5 Phenyllactic acid production through transient expression in *Nicotiana benthamiana*. Transient expression of *ArAT4* and *PPAR* in *N. benthamiana* results in the production of (a) phenyllactic acid (blue) and (b) 2-*O*-malonylphenyllactate (green). Data are presented as mean (n = 3) ± SE. There is a statistical difference in the mean metabolite (phenyllactic acid and 2-*O*-malonylphenyllactate) level (nmol per mg of dry weight) between different transient expression samples (One-Way ANOVAs, (a) F= 54.5, (b) F=86.4, $P < 0.001$). Difference in letters (a vs. b) indicates a statistical difference as determined by Tukey HSD ($P < 0.001$). 2-*O*-malonylphenyllactate production occurs through an unknown *N. benthamiana* malonyltransferase. Samples are defined as follows: WT; uninfiltrated *N. benthamiana* leaf, Mock; *N. benthamiana* leaf infiltrated with infiltration media, EV; *N. benthamiana* leaf infiltrated with an empty vector control, PLA; *N. benthamiana* leaf infiltrated with phenyllactic acid, ArAT4; *N. benthamiana* leaf infiltrated with phenylalanine:4-hydroxyphenylpyruvate aminotransferase, PPAR; *N. benthamiana* leaf infiltrated with phenylpyruvic acid reductase, ArAT4 + PPAR; *N. benthamiana* leaf infiltrated with phenylalanine:4-hydroxyphenylpyruvate aminotransferase and phenylpyruvic acid reductase.

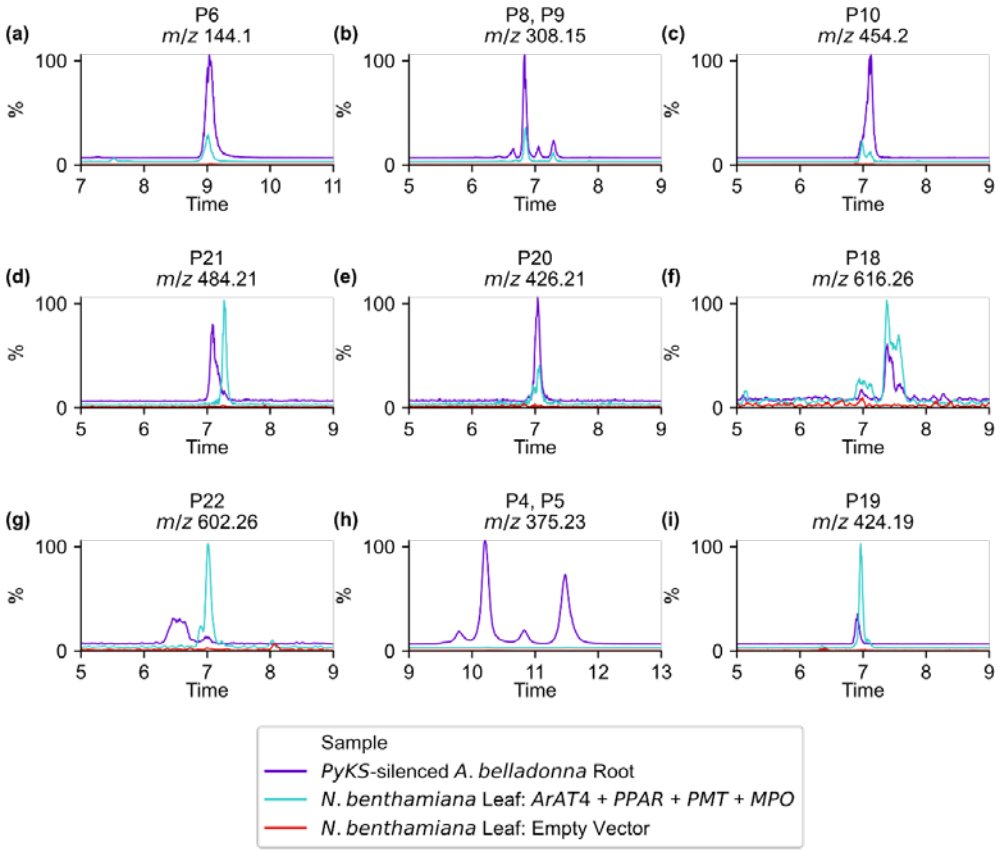

Fig. S6 Conserved enzymatic activity results in pyrrolidine alkaloid modifications in *N. benthamiana*. Chromatograms showing production of modified P1 and P2 (*m/z* 292.15) products in *N. benthamiana* (cyan), the co-retention of these products with metabolites in *A. belladonna PyKS*-silenced root samples (purple), and a negative empty vector control showing the lack of product in *N. benthamiana* without P1 and P2 production (red). The P1 or P2 (*m/z* 292.15) hydrolysis product (a) P6, *m/z* 144.1) and the P1 or P2 (*m/z* 292.15) metabolites containing an additional hydroxy (b) P8, P9; *m/z* 308.15), hexose (c) P10, *m/z* 454.2), sedoheptulose (d) P21, *m/z* 484.22), reduced pentose (e) P20, *m/z* 426.21), dihexose (f) P18, *m/z* 616.26), and hexose with a phenyllactate moiety (g) P22, *m/z* 602.26) are produced in *N. benthamiana* and co-retain with the *PyKS*-silenced *A. belladonna* root sample metabolites. Some of these metabolites show co-retention with minor isomers, including P22, P21, and P10. The P4 and P5 (*m/z* 375.23) dipyrrolidine metabolites (h) are not produced at detectable levels in *N. benthamiana* plants transiently expressing *ArAT4*, *PPAR*, *PMT*, and *MPO*. Although a metabolite corresponding to the P1 or P2 (*m/z* 292.15) isomers with a pentose addition (i) P19, *m/z* 424.19) is produced in *N. benthamiana* plants transiently expressing *ArAT4*, *PPAR*, *PMT*, and *MPO*, this metabolite does not co-retain with the metabolite accumulating in *A. belladonna PyKS*-silenced root suggesting that these metabolites are distinct isomers. Enzyme abbreviations are as follows: PMT2; putrescine methyltransferase, MPO; methylputrescine oxidase, ArAT4; phenylalanine:4-hydroxyphenylpyruvate aminotransferase, PPAR; phenylpyruvic acid reductase. *m/z* corresponds to the mass to charge ratio.

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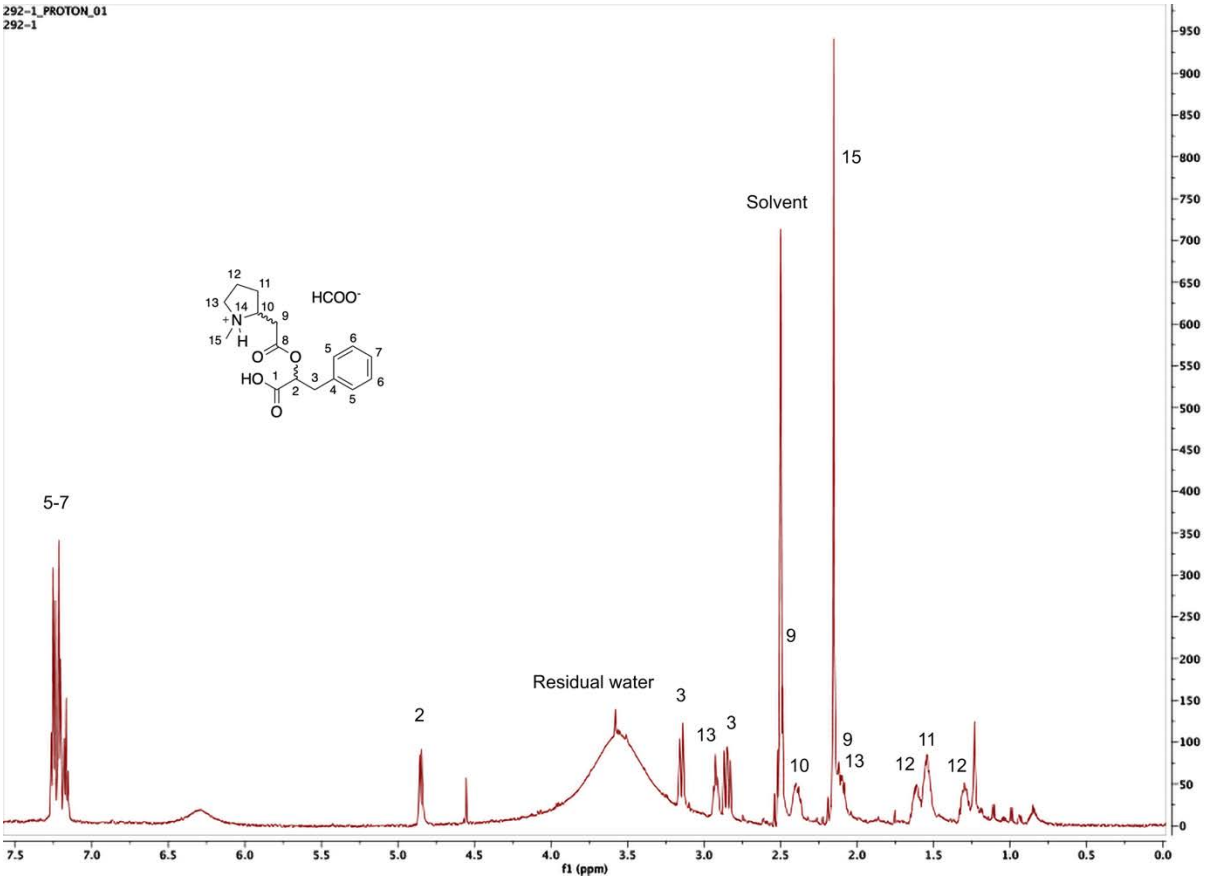
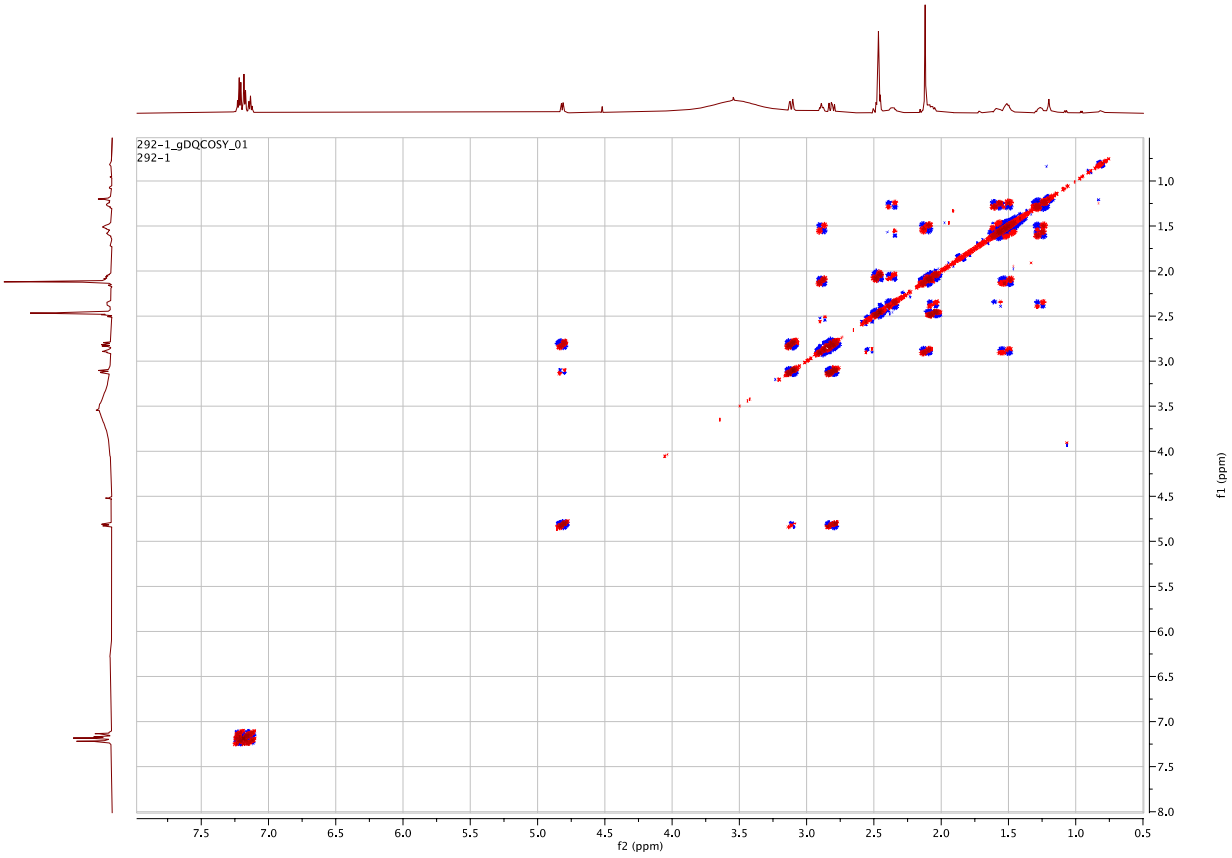
Dataset S1. NMR spectra of pyrrolidine metabolites P1 and P2

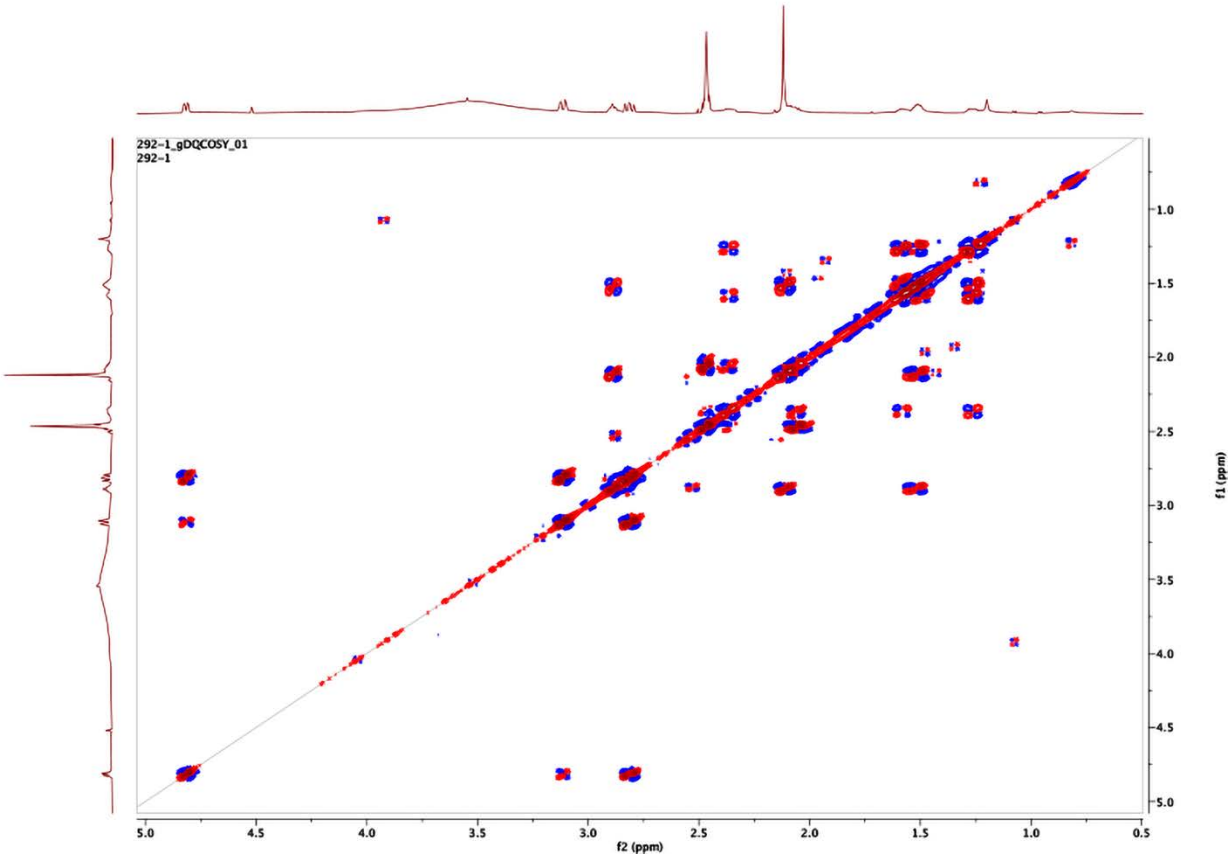
(a) 1D proton spectrum of pyrrolidine metabolite P1, in DMSO-d_6



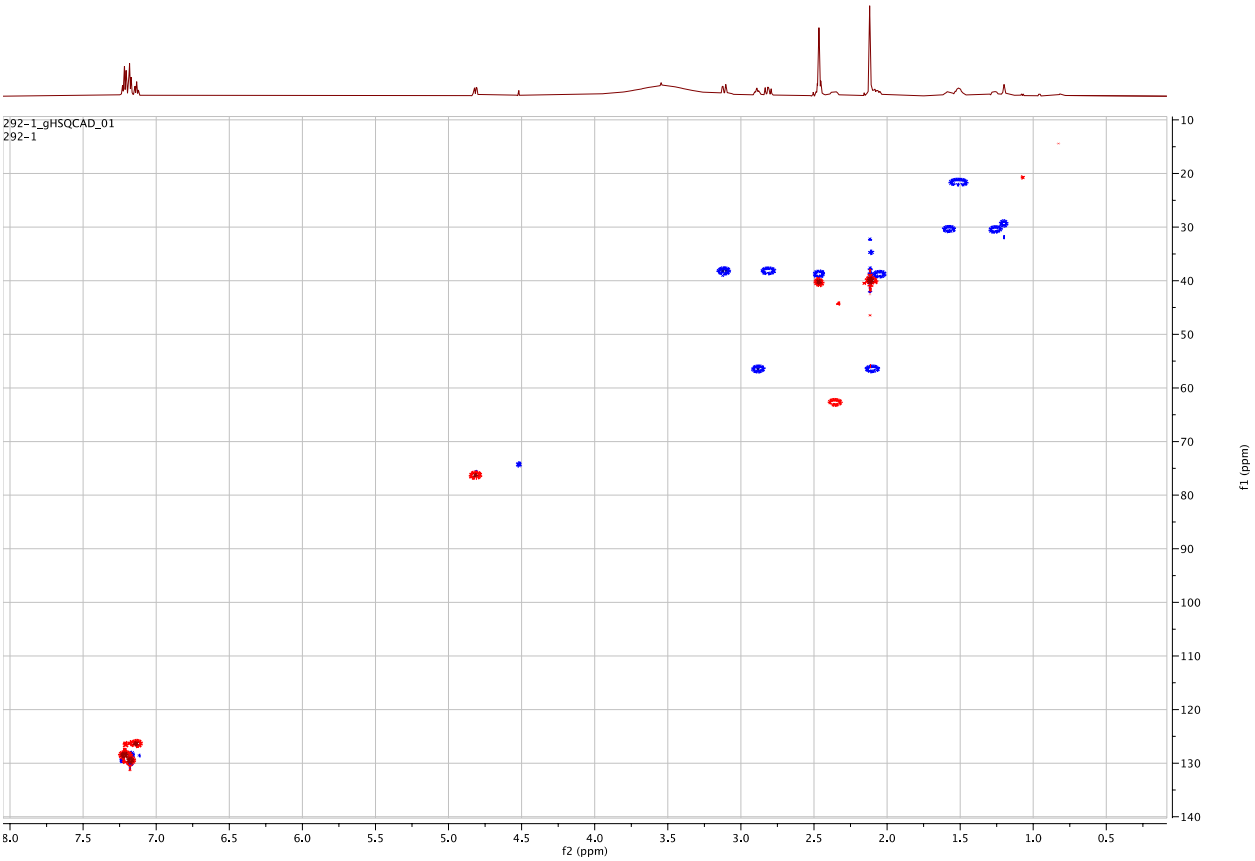
(b) ^{1}H-^{1}H DQF-COSY of pyrrolidine metabolite P1



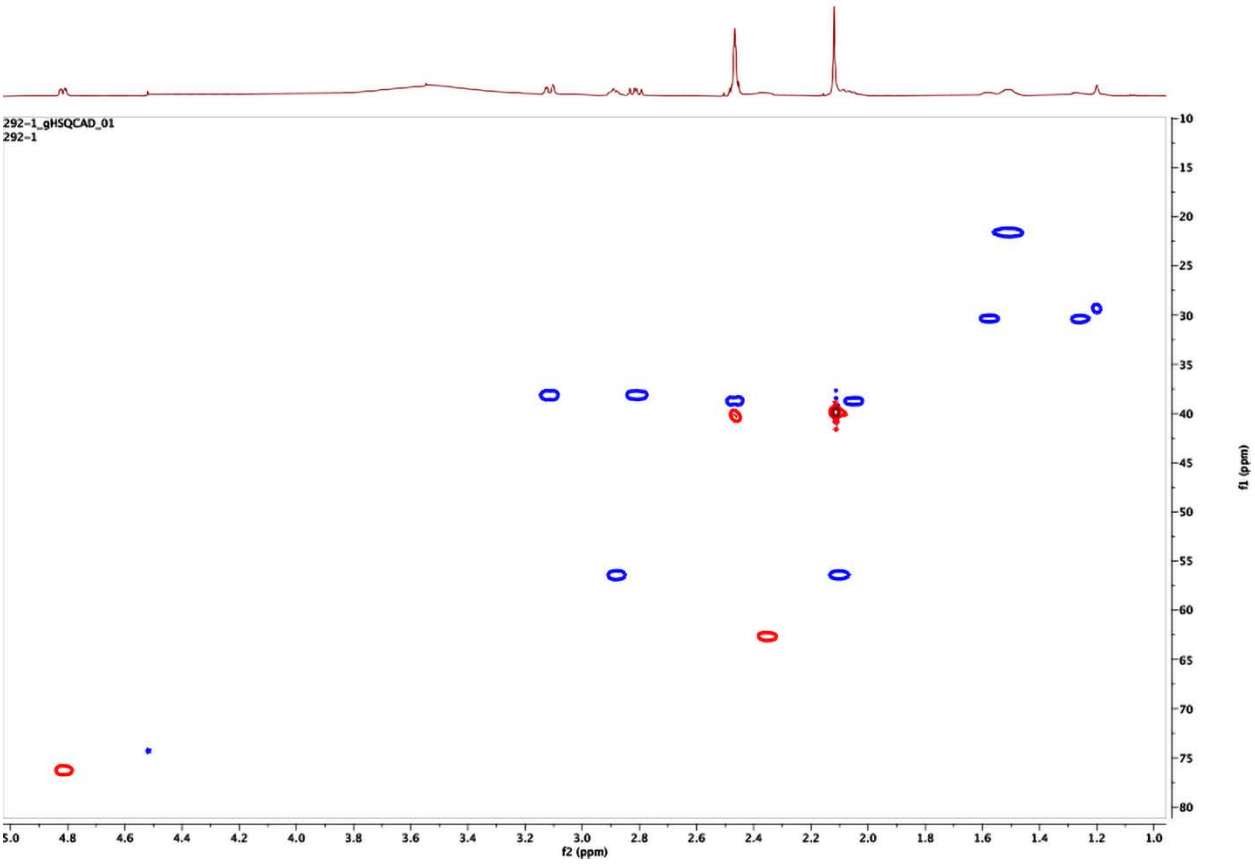
(c) ^{1}H-^{1}H DQF-COSY of pyrrolidine metabolite P1 (phenyl region excluded)



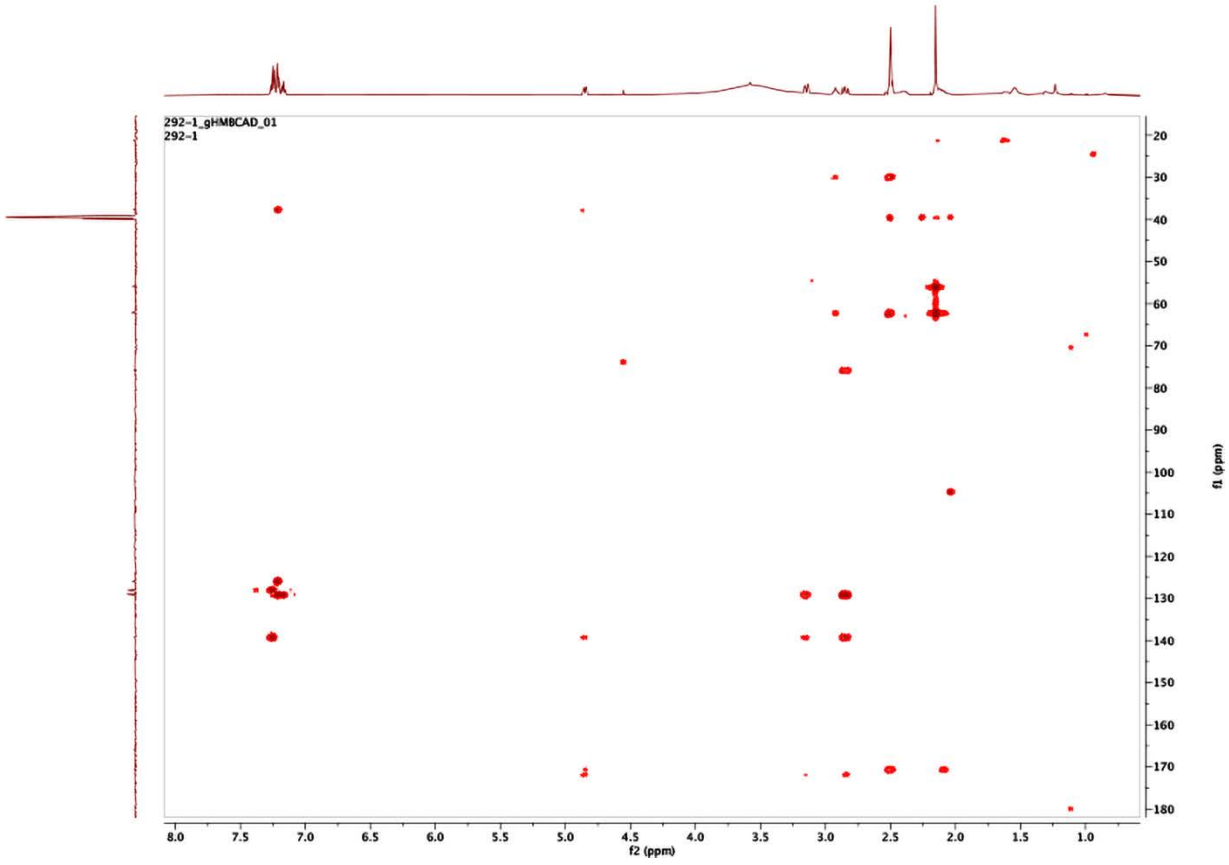
(d) HSQC of pyrrolidine metabolite P1



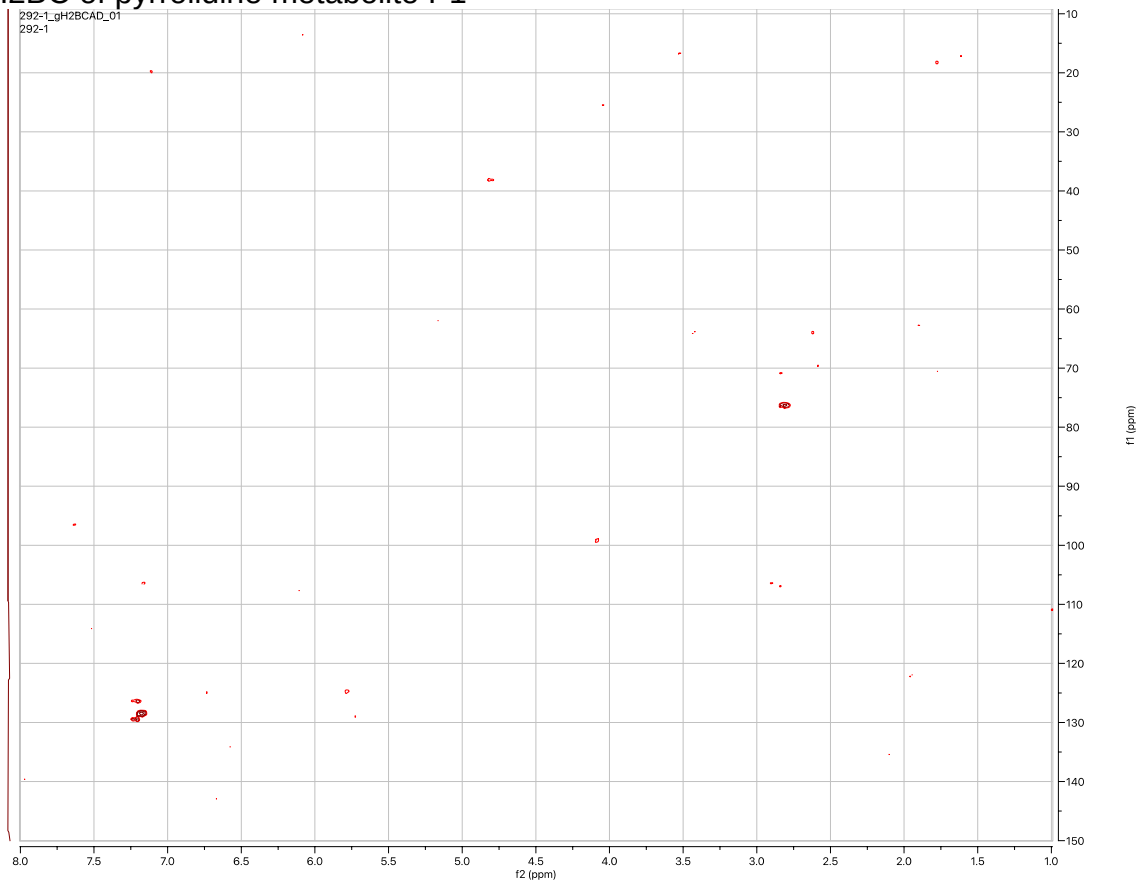
(e) HSQC of pyrrolidine metabolite P1 (excluding phenyl ring region)



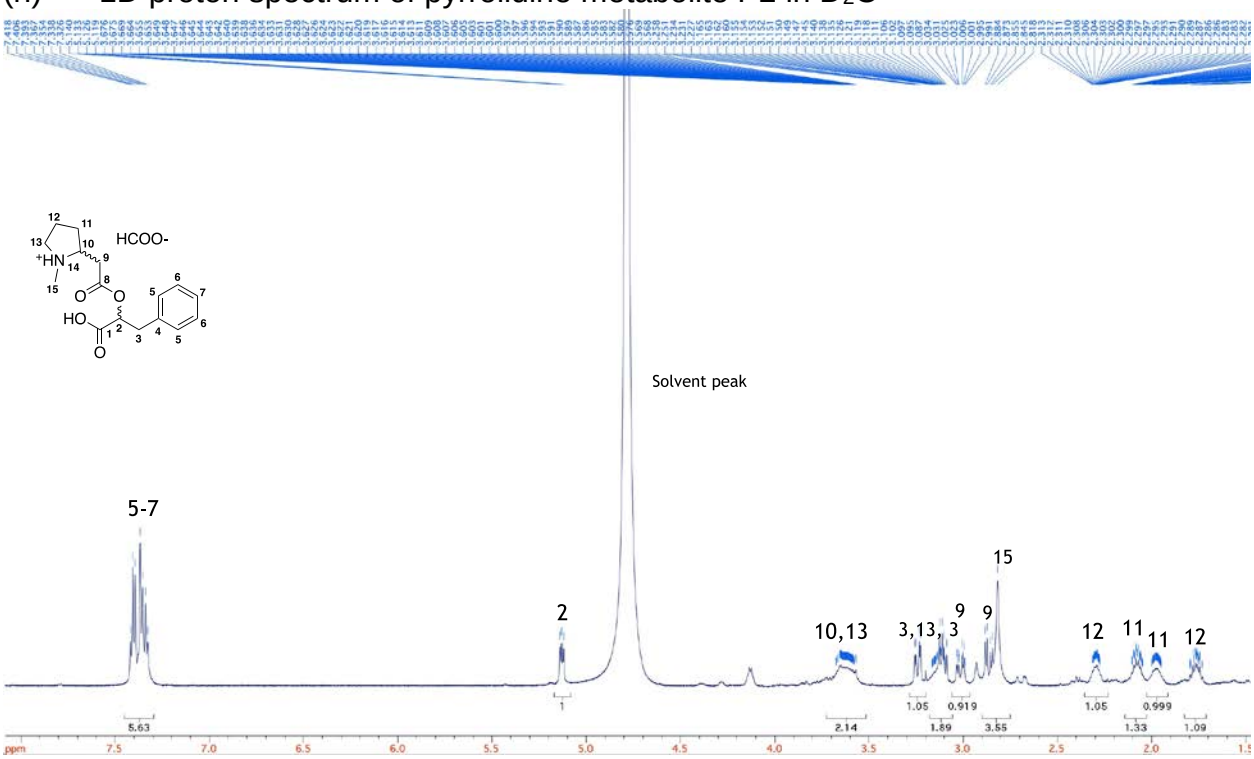
(f) HMBC of pyrrolidine metabolite P1



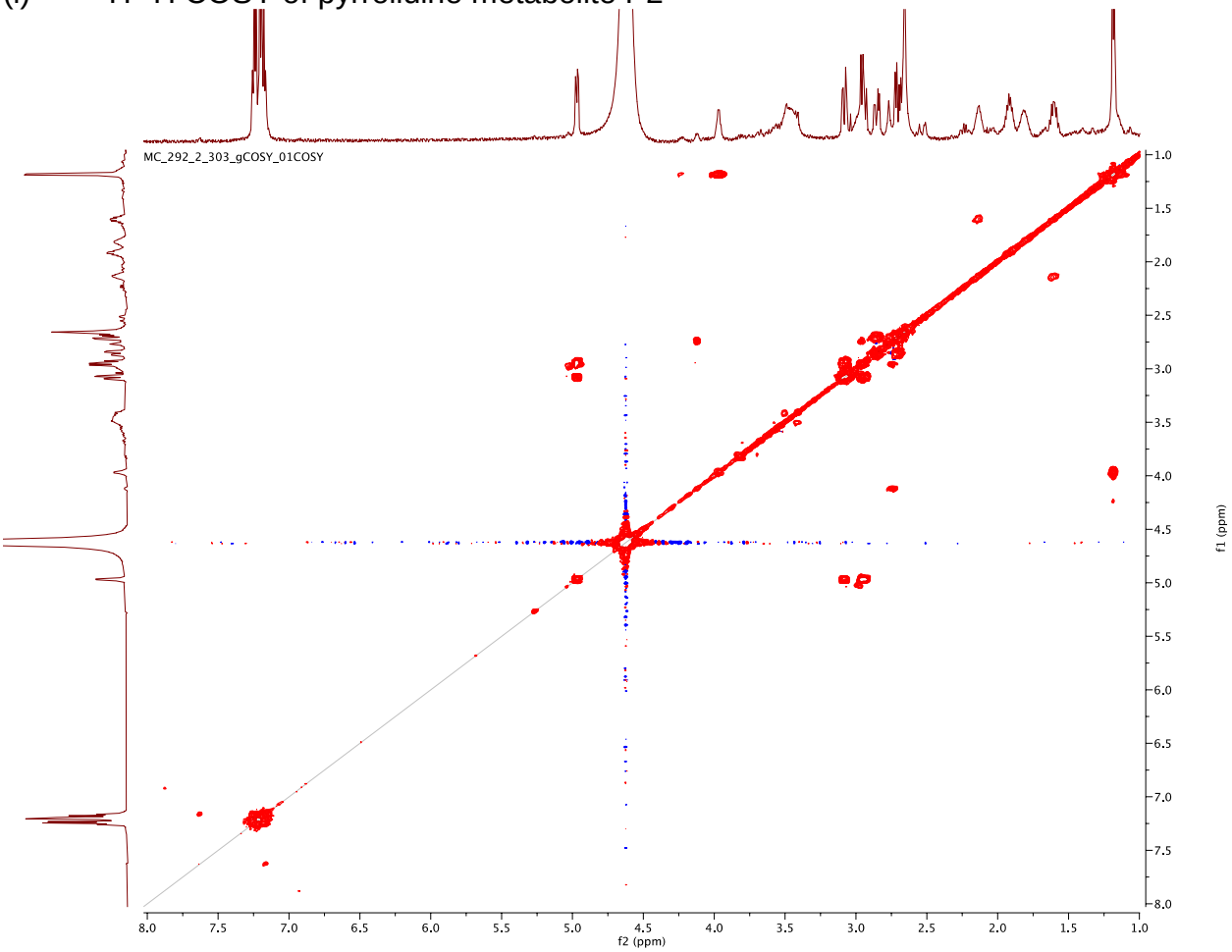
(g) H2BC of pyrrolidine metabolite P1



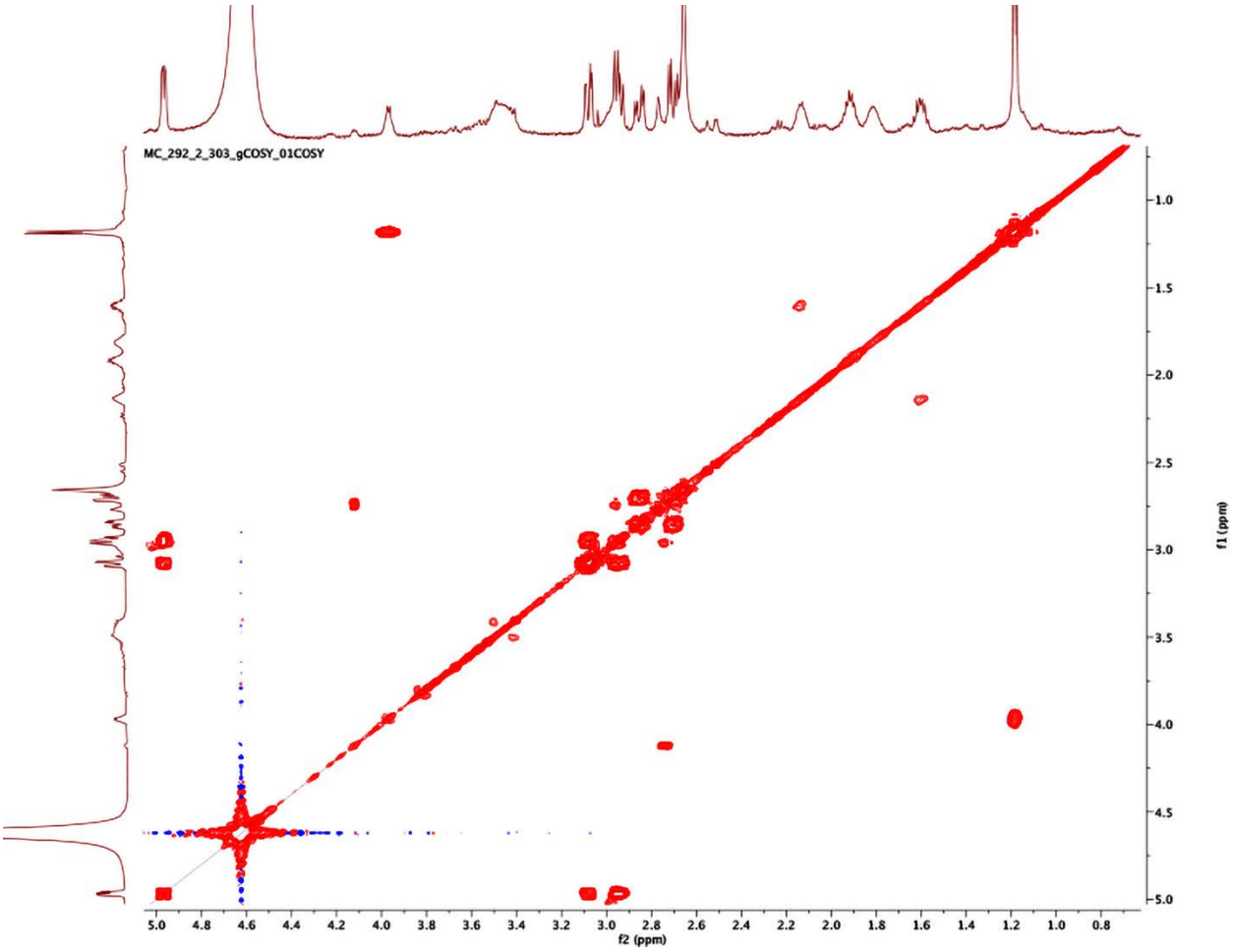
(h) 1D proton spectrum of pyrrolidine metabolite P2 in D_2O



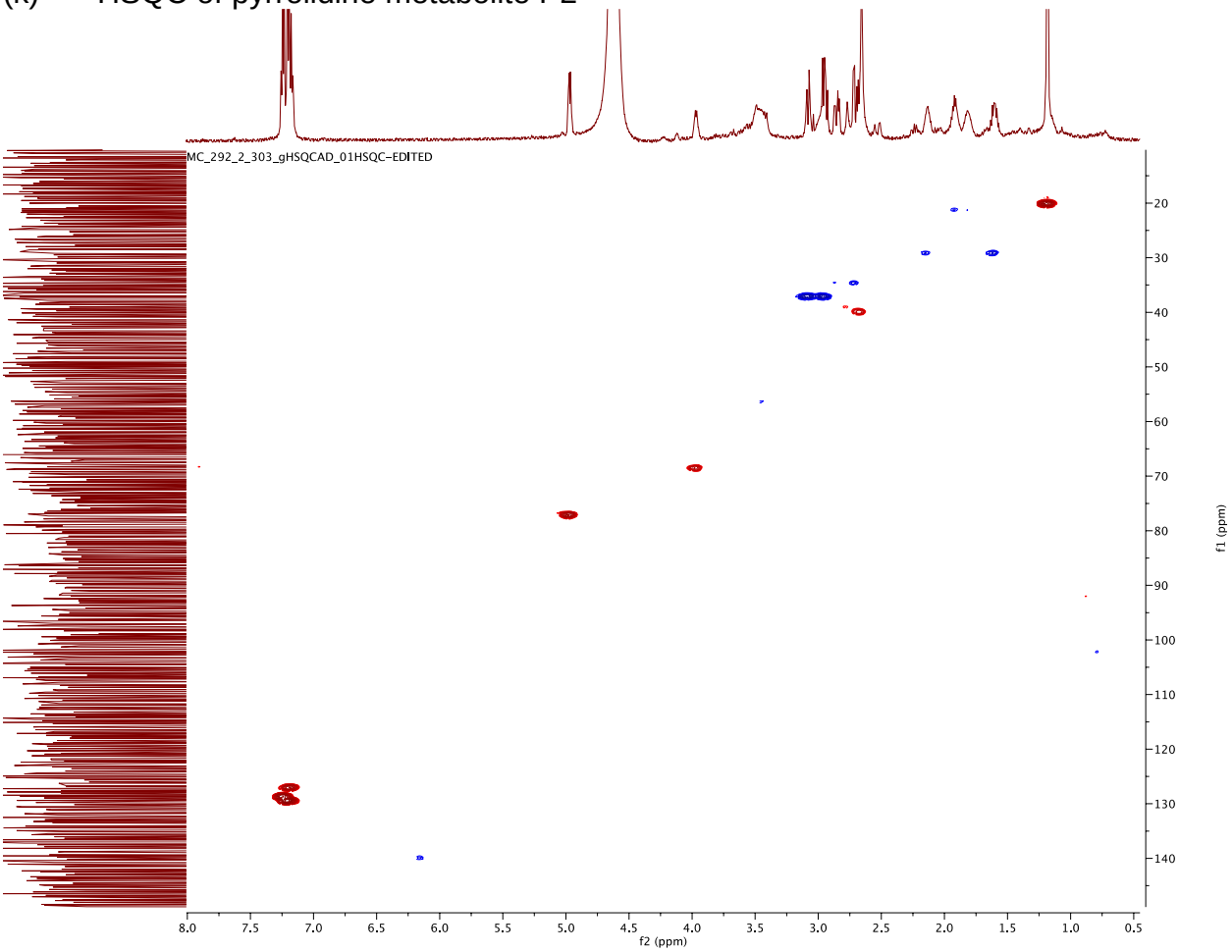
(i) ^{1}H-^{1}H COSY of pyrrolidine metabolite P2



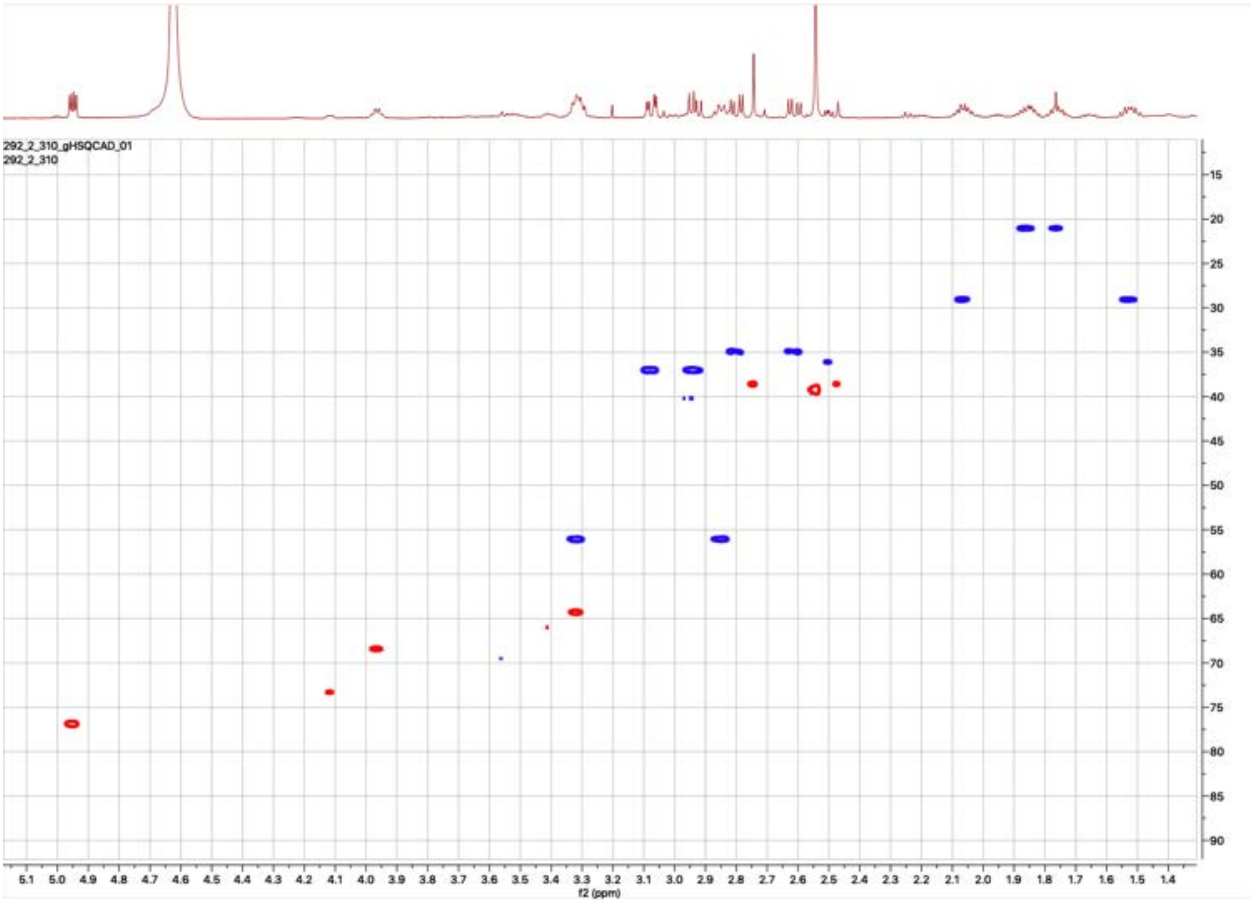
(j) ^{1}H-^{1}H COSY of pyrrolidine metabolite P2 (phenyl ring region excluded)



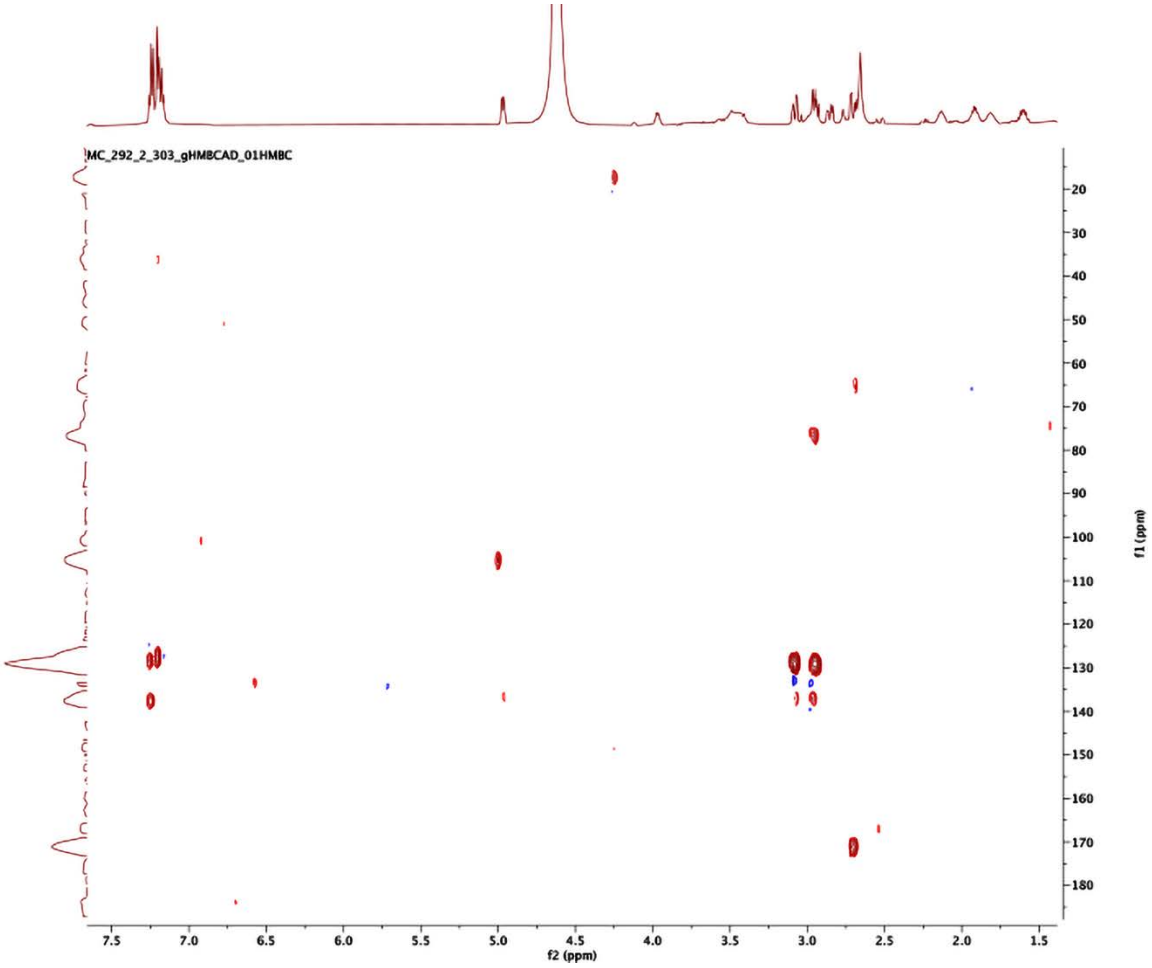
(k) HSQC of pyrrolidine metabolite P2



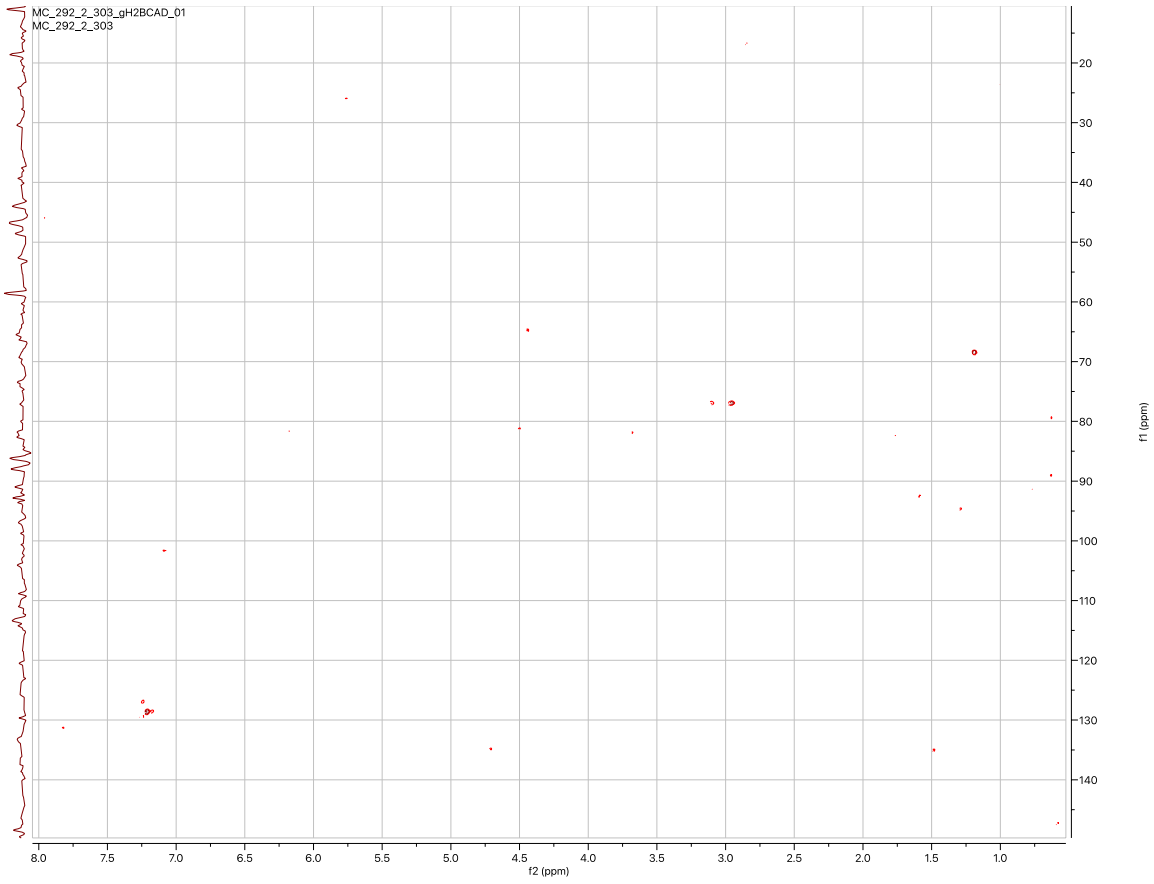
(l) HSQC of pyrrolidine metabolite P2 (phenyl region excluded)



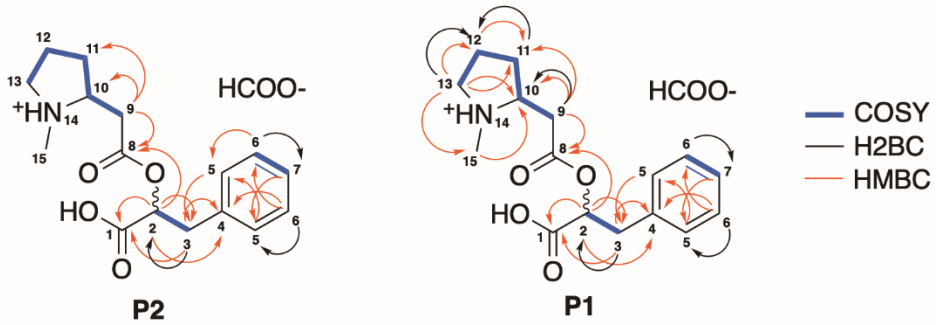
(m) HMBC of pyrrolidine metabolite P2



(n) H2BC of pyrrolidine metabolite P2



(o) Spin-spin correlations determined from COSY, H2BC, and HMBC 2D-NMR experiments for pyrrolidine metabolites P2 and P1.



Materials and Methods S1

Purification and structural characterization of P1 and P2 (*m/z* 292.15) metabolites

Virus-induced gene silencing in *A. belladonna* was used as previously described to generate bulk, silenced *AbPyKS* material for pyrrolidine extraction and purification (Bedewitz *et al.*, 2018). Several flats of *Atropa belladonna* were silenced and harvested 6-weeks post silencing to generate enough material for metabolite purification. Roots were washed, flash-frozen in liquid nitrogen, and stored at -80°C at the time of harvest. Metabolites were extracted from 50 g of bulk, silenced *AbPyKS* powdered roots in 500 ml of extraction solvent (20% methanol and 0.1% formic acid in water) for 3 hours on an orbital shaker at room temperature.

To purify P1 and P2 metabolites, the extract from bulk-silenced *A. belladonna* plants was fractionated using a Waters 2795 Alliance HT Separations Module equipped with a Thermo Scientific™ Acclaim™ 120 C18 column (4.6 mm x 150 mm, 5 µm particle size) and LKB 2211 Superrac Fraction Collector. The gradient in Table S5 was used for the initial separation of the crude metabolite extract, with a flow rate of 1.0 ml min^{-1}, a column temperature of 45°C, and an injection volume of 100 µl. Fractions were collected every 25 s and screened for purity on a Waters Xevo G2-XS Q-TOF mass spectrometer equipped with a Shimadzu LC-20AD HPLC system, assessing abundance using signal for *m/z* 292.15 ($[M+H]^+$) in positive ion mode. Separation of P1 and P2 isomers proceeded as described above except that the LC-MS method was modified to an isocratic method (Table S6) and fractions were collected every 20 s. Fractions were screened for purity as described above, and pure fractions were pooled and the solvent evaporated under vacuum in a centrifugal concentrator.

Samples were dissolved in 300 µl of DMSO-d_6 (P1) or D_2O (P2) and were transferred to solvent-matched Shigemi tubes for NMR analysis. NMR analysis was performed at the Max T. Rogers NMR facility at Michigan State University, using a Varian Inova 600 MHz NMR spectrometer under control of VnmrJ software. Proton (^1H) spectra were acquired at 600 MHz, with carbon (^{13}C) spectra being acquired at 125 MHz. Double quantum-filtered gradient correlation spectroscopy (dqqCOSY), gradient heteronuclear single

quantum coherence (gHSQC), gradient heteronuclear multiple-bond correlation (gHMBC), and gradient heteronuclear two-bond correlation (H2BC) spectra were also collected for both P1 and P2. NMR data were processed using MestReNova v.14.1.1 software. Spectra in DMSO-d_6 are referenced to the residual solvent signals at δ = 2.5 ppm (^{1}H) and 39.7 ppm (^{13}C); spectra in D_2O are uncorrected, as the residual water peak signal can shift with concentration or pH.

Purification and structural elucidation of 2-*O*-malonylphenyllactate

Frozen *A. belladonna* root tissue pooled from several 10 month-old plants (450 g) was extracted in four equal batches in methanol : water (1:1, v/v) by pulverizing in a blender until thawed and free-flowing (total volume ~1.4 l). The solution was filtered under vacuum using Whatman No. 1 filter paper and Celite® 545 filter aid. 500 ml of the filtrate was dried in aliquots under vacuum. The residue was resuspended and extracted in 8 ml 20% methanol containing 1 mM ammonium formate (pH = 7.6). Following mixing, insoluble material was removed by centrifugation at 10,000 × ***g*** for 10 min, the supernatant was transferred to 15 ml conical tubes and stored at ~2°C.

An anion exchange purification method was developed for purifying 2-*O*-malonylphenyllactate. SupelcleanTM LC-SAX (Part No. 57017, Cl- counterion, 3 ml, bed wt. 500 mg) solid phase extraction (SPE) cartridges were conditioned with 4 ml of MilliQ water, methanol and conditioning solvent (80% water: methanol with 1 mM ammonium formate, pH 7.6) in sequence. Approximately 4 ml of extract was loaded onto the column, followed by several washings; first, 4 ml of conditioning solvent. second, 4 ml of 80% water: methanol (4:1, v/v) with 0.35 % formic acid; third, 4 ml of 50% water methanol (1:1, v/v) with 0.35% formic acid; fourth, 4 ml methanol with 0.088% formic acid. The 2-*O*-malonylphenyllactate was eluted into a 15 ml centrifuge tube using 4 ml water : methanol (1:1, v/v) containing 0.1 M sulfuric acid. The eluate was transferred to 50 ml centrifuge tubes and diluted to 90% water: methanol (9:1, v/v, 0.02 M sulfuric acid) by adding 16 ml of water. The sample was further purified using J.T. Baker Bakerbond^{TM} Octadecyl SPE cartridges (Part. No. 7020-03, C18, 3 mL, bed wt. 500 mg). The SPE cartridges were conditioned with 4 ml of MilliQ water, methanol and

MilliQ water in sequence. All 20 ml of diluted eluate from the anion exchange step was loaded onto the column. The column was washed with 4 ml of 0.088% aqueous formic acid to remove sulfuric acid. The 2-*O*-malonylphenyllactate was eluted using 4 ml of methanol, the sample was transferred to a Pyrex culture tube, and then the solvent was evaporated to dryness under vacuum. The sample was then resuspended in 500 µl of 10% methanol in water and loaded onto an Oasis^{TM} WCX SPE cartridge (Part. No.186004646, WCX, 6 ml, bed wt. 500 mg) that had been washed with 7 ml of methanol and 7 ml of water, and then conditioned with 10% methanol in water. The column was washed with 9 ml of water, 9 ml of 10% methanol in water, and 2-*O*-malonylphenyllactate was eluted in 20% methanol in water. The sample was transferred to a Pyrex culture tube and evaporated to dryness under vacuum.

The sample was dissolved in 300 µl of acetonitrile-d_3 (99.96 atom % D) and transferred to solvent-matched Shigemi tubes for NMR analysis. ^{1}H, ^{13}C and gHSQC NMR experiments were performed using a Bruker Avance 900 spectrometer equipped with a TCI triple resonance probe. All spectra were referenced to non-deuterated solvent signals (δH = 1.94 and δC = 118.70 ppm).

References

Bedewitz MA, Jones AD, D'Auria JC, Barry CS. 2018. Tropinone synthesis via an atypical polyketide synthase and P450-mediated cyclization. *Nature Communications* **9**: 5281.