

Metabolic soft spot and pharmacokinetics: functionalization of C-3 position of an Eph-ephrin antagonist featuring a bile acid core as an effective strategy to obtain oral bioavailability in mice

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Supplementary Material

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S.1 *In vitro* phase I liver metabolism in MLM

In phase I metabolic stability assays, samples were prepared in 100 mM phosphate buffered saline (PBS), pH 7.4, containing a NADPH-generating system (5 mM MgCl₂, 10 mM glucose-6-phosphate, 2 mM NADP⁺, 1.5 U/mL of glucose-6-phosphate dehydrogenase) and 1 mg/mL mouse liver microsomes. After incubation under stirring (5 min, 37°C), 100 µM UniPR129 and UniPR500 stock solutions in DMSO were added to start the reaction. Final DMSO percentage in samples was kept at 1%. At fixed time points (t = 0, 15, 30, 45, 60 min), single aliquots of reaction mixture were withdrawn, processed with two vol. of ACN containing 100 nM UniPR126, centrifuged (16000 g, 10 min, 4 °C) and the supernatant was directly injected in the HPLC-ESI-MS/MS system.

Results were reported as percentage (%) of compound remaining *vs.* time. We verified the absence of unspecific binding to MLM proteins by means of control samples in which UniPR compounds were incubated in the absence of a NADPH generating system.

S.2 *In vitro* phase II liver metabolism: glucuronidation in MLM

8 mM Uridin-di-phosphoglucuronic acid (UDPGA), 100 µM saccharic-acid-1,4-lactone, 10 mM magnesium chloride (MgCl₂), 1 mM ethylenediamino-tetracetic acid (EDTA) and 500 µM 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS) solutions were prepared independently in 100 mM TRIS buffer, pH = 7.7. Assay samples contained: (i) 15 µl of MLM (final protein concentration = 1 mg/ml); (ii) 30 µl of each co-factor solution; (iii) 132 µL of TRIS buffer. After pre-incubation for 5 min at 37 °C, UniPR129 and UniPR500 stock solutions in DMSO was added to each sample. Aliquot sampling, quenching of reaction and centrifugation steps occurred as detailed in section S.1.

S.3 *In vitro* phase II liver metabolism: sulfonation in HLS₉ and MLS₉ fractions

5 mM 3-phosphoadenosin-5-phosphate (PAPS), 2.5 mM MgCl₂ and 1 mM dithiothreitol (DTT) solutions were prepared separately in 100 mM PBS, pH = 7.4. Assay samples contained: (i) 15 µl of HLS₉ or MLS₉ to reach a final protein concentration of 1 mg/ml (ii) 50 µl of MgCl₂ and (iii) 50 µl of DTT solution in PBS. UniPR compounds stock solution in DMSO was spiked into the samples (final compound concentration: 1 µM). 15 µl of PAPS solution were also added and each sample was incubated at 37 °C. Aliquot sampling and sample processing steps occurred as detailed in section S.1.

S.4 *In vivo* dosing of UniPR129 and UniPR500 in mouse plasma: HPLC-ESI-MS/MS method

Calibration standards (CS, concentration range: 1000-2 nmol/L) were prepared by spiking blank mouse plasma with stock solutions of UniPR129 and UniPR500 in DMSO (final DMSO concentration = 1%). Specificity of the method was checked comparing HPLC-MS/MS chromatograms of blank plasma samples to those of UniPR129 and UniPR500 at the Lowest Limit of quantification (LLOQ). Within-run accuracy and precision were checked employing QC samples representative of the whole calibration range (HQC = 800 nmol/l; MQC = 400 nmol/l; LQC = 5 nmol/l) with n = 3 replicates at each QC concentration level. Between-run accuracy and precision were checked employing QC samples at n=3 concentration levels in n = 3 analytical runs over three days. The acceptance criteria for accuracy were to get back-calculated concentrations ± 15% of the nominal ones, except for the LLOQ, which had to be ± 20%. The acceptance criteria for precision, expressed as relative standard

deviation (%RSD), was not to exceed 15%, except for the LLOQ, where it had to be not more than 20%. All plasma samples (CS, QC and unknown) from the fast PK experiments were all processed by addition of a double volume of ACN containing 100 nM UniPR126 as internal standard, centrifuged (16000 g, 10 min, 4 °C) and the supernatant was analyzed by HPLC-ESI-MS/MS. Recovery and Matrix effect were measured on LQC and HQC samples (n=3). Matrix effect was determined by comparison of the analyte/internal standard ratios in QC samples prepared as pure standards in buffered solution, processed by two vol of ACN with the analyte/IS ratios in QC samples prepared in biological matrix (mouse plasma), processed by ACN addition before the spiking with the analyte. Recovery was determined by comparison of the analyte/IS ratios in QC samples prepared by spiking the analyte in mouse plasma samples before ACN addition with the analyte/IS ratios in QC samples prepared in biological matrix (mouse plasma), pre-processed by ACN addition.

Chromatographic traces were acquired on a Thermo Accela UHPLC system (Thermo, USA), employing a Synergy Fusion C₁₈ 80 Å RP-column (2.0 x 100 mm, 4 µm; Phenomenex, USA) in gradient elution. Gradient was as follows: eluent A: ACN; eluent B: MilliQ water, both added with 0.1% v/v HCOOH. t = 0 min: 5%A; t = 0-3 min: 5-95%A; t = 3-6 min: 95%A; t = 6-7 min: 95-5%A; t = 7-10 min: 5%A. Total run time: 10 min. Flow rate: 0.35 ml/min; injection volume: 10 µl. The UHPLC system was coupled to a Thermo TSQ Quantum Access Max Triple Quadrupole mass spectrometer (Thermo, USA), with a heated electrospray (H-ESI) ion source. Ion source voltage was set at 3500 V; Capillary temperature at 270 °C; sheath gas (N₂) at a pressure of 35 psi and auxiliary gas (N₂) at a pressure of 15 psi; Argon was employed as collision gas at a pressure of 1.5 mtorr. Mass spectrometer operated in positive ion (ESI⁺) and in Multiple Reaction Monitoring (MRM) mode following the parent-product transitions of UniPR compounds. Xcalibur software v. 2.2 (Thermo, USA) was employed for both acquisition and processing of HPLC-MS/MS traces. Tube lens voltages (TL) and collision energies (CE) for each transition were optimized by Flow Injection Analysis (FIA). UniPR129: $m/z = 577.4 [M+H]^+ \rightarrow m/z = 358.2, 202.1, 184.1$ (TL = 111.9 V; CE = 26, 29, 31 eV, respectively); UniPR500: $m/z = 590.4 [M+H]^+ \rightarrow m/z = 572.6, 389.4, 184.1$ (TL = 99.4 V; CE = 21, 22, 36 eV); UniPR126 (IS): $m/z = 563.4 [M+H]^+ \rightarrow m/z = 545.8, 187.9, 146.1$ (TL = 81.8 V; CE = 20, 25, 48 eV); UniPR141 (IS): $m/z = 586.4 [M-H]^- \rightarrow m/z = 374.3, 228.1, 167.1$ (TL = 134 V; CE = 42, 37, 40 eV, respectively); verapamil: $m/z = 455.3 [M+H]^+ \rightarrow m/z = 303.2, 165.1, 150.1$ (TL = 85 V; CE = 26, 27, 34 eV); diclofenac sodium salt: $m/z = 294.0 [M-H]^- \rightarrow m/z = 250.0, 214.1, 178.1$ (TL = 34 V; CE = 15, 24, 34 eV).

S.5 *In vitro* phase II metabolite profiling: HPLC-HR-MS method

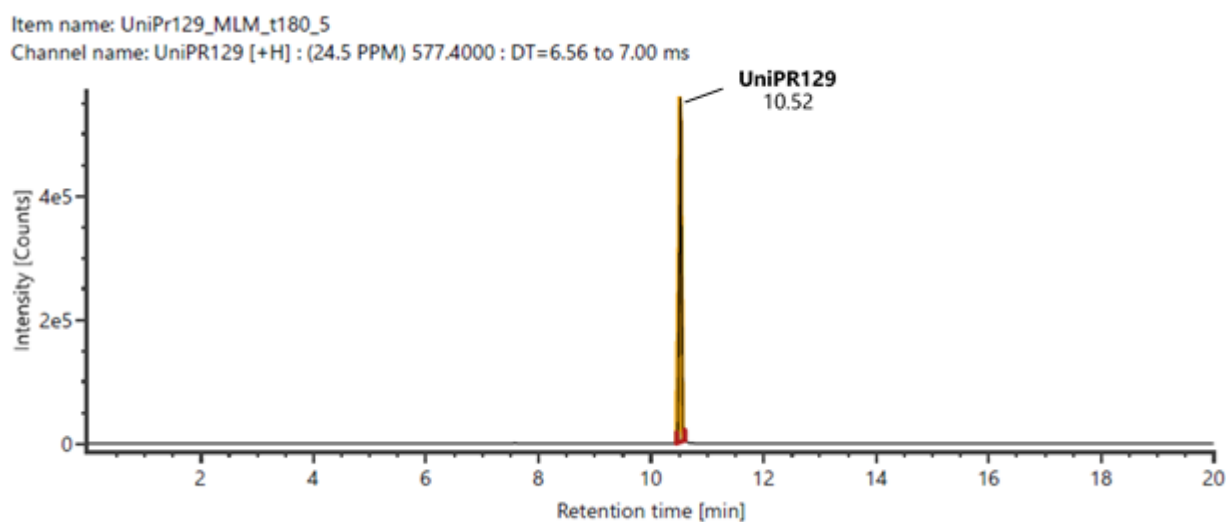
The Thermo LTQ-Orbitrap mass spectrometer was weekly calibrated in ESI⁺ mode by Pierce ESI Positive Ion Calibration solution (Thermo, USA) and in ESI⁻ mode by Pierce ESI Negative Ion Calibration solution (Thermo, USA) following manufacturer instructions. Tolerance for accurate mass measurements was set at 3 ppm in the mass range $m/z = 50-2000$ amu. A linear gradient elution on a Phenomenex Synergy Fusion C₁₈ 80 Å RP-column (2.0 x 100 mm, 4 µm; Phenomenex, USA) was chosen for phase II metabolite profiling. Eluent A was ACN; eluent B was Milli-Q water. Both phases were acidified by 0.2% HCOOH. Gradient was as follows: t = 0-3 min: 5%A; t = 3-33 min: 5-95%A; t = 33-38 min: 95%A; t = 38-39 min: 95-5%A; t = 39-45 min: 5%A. Total run time: 45 min. Flow rate: 0.20 ml/min; injection volume: 5 µL. HR-MS scans were performed in Full Scan mode ($m/z = 100-1600$ amu). Capillary temperature was set at 275 °C; sheath gas pressure was 20 psi; auxiliary gas pressure

was 5 psi; sweep gas pressure was 5 psi; ESI source voltages were set at 3000 V in ESI⁺ and 2100 V in ESI⁻; capillary voltage was set at 13 V in ESI⁺ and 35 V in ESI⁻; tube lens voltage was set at 85 V in ESI⁺ and -110 V in ESI⁻. In Full Scan mode, resolution was set at 100 000.

S.6 UniPR129 *In vitro* phase I metabolite profiling

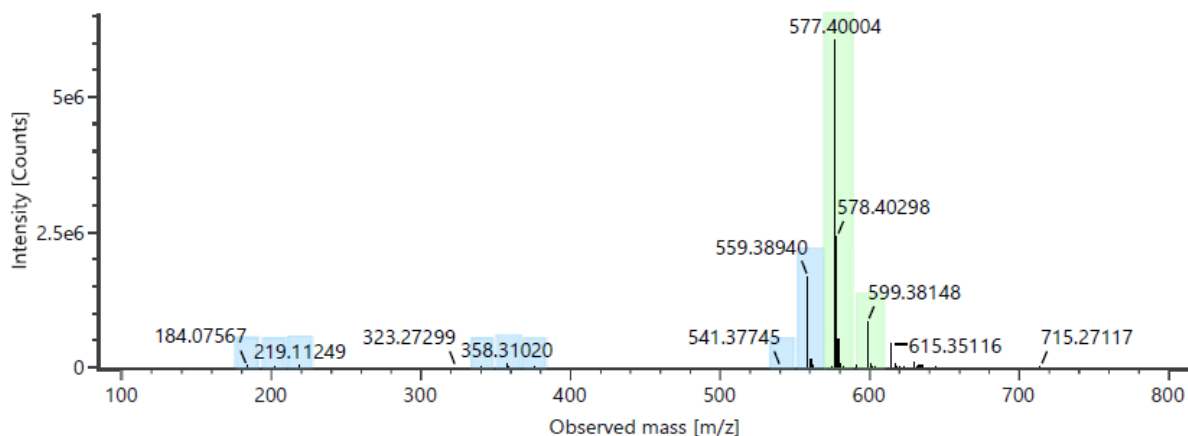
Figure S1. a-c. Parent compound UniPR129 in MLM

a. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 577.40$ [M+H]⁺ and RT = 10.52 min.

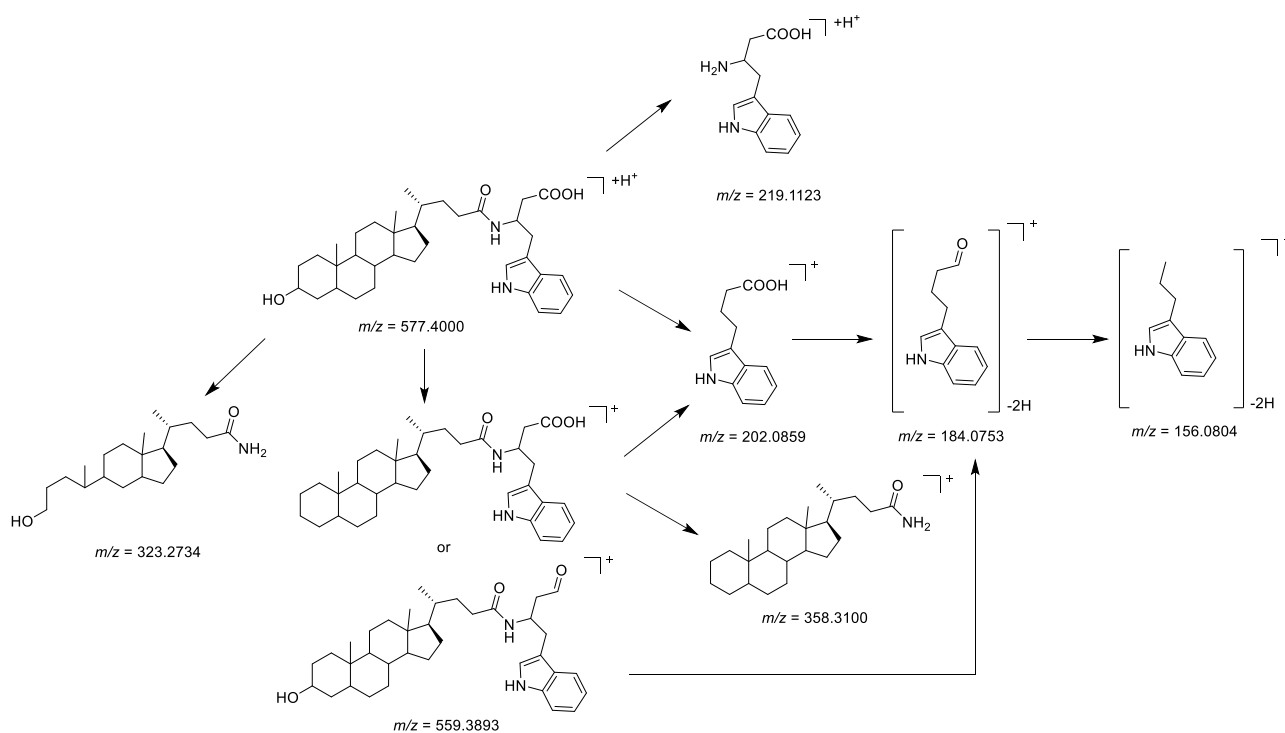


b. Experimental high resolution mass value in ESI⁺ for parent compound UniPR129.

Item name: UniPr129_MLM_t180_5 Channel name: Low energy : Time 10.5237 +/- 0.0205 minutes : Drift Times: 6.78 +/- 0.22, 7.12 +/- 0.22 ms
Item description:



c. High resolution high energy (MSE) MS spectrum in ESI⁺ of UniPR129 together with tentative fragmentation pattern. The base peak at $m/z = 559.4$ corresponds to the loss of water (-18). Fragment ion at $m/z = 202.1$ is compatible with the 4-(indol-3-yl)butanoic acid fragment, while product ions at $m/z = 184.1$ and 156.1 could account for consecutive loss of water (-18 with respect to 202.1) and carbonyl (-28 with respect to 156.1) group. One or more of these three ion products are in the MS/MS spectrum of UniPR129 metabolites M1-5, M9 and M11.



Component name: UniPR129, Observed m/z : 577.4000,
Observed RT (min): 10.52

Channel name: product ions from precursor ion id = 0

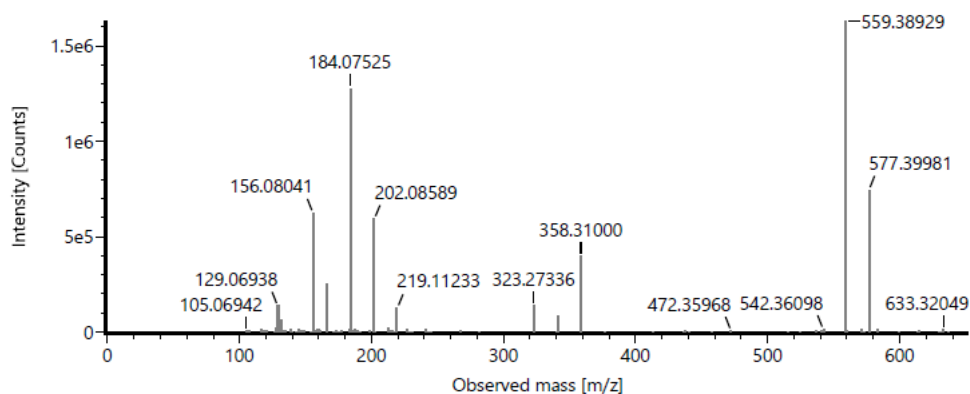
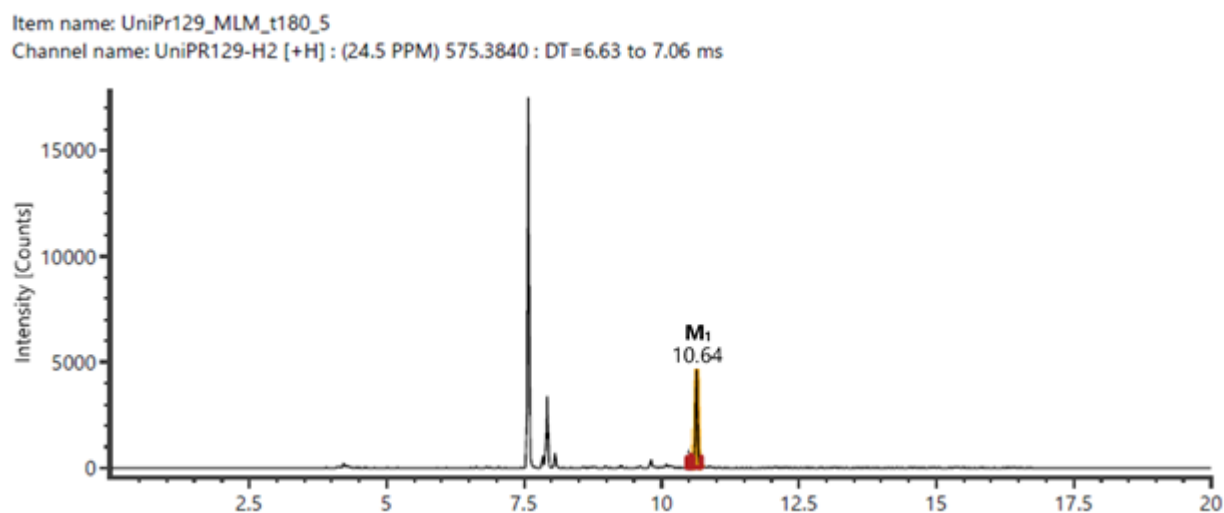


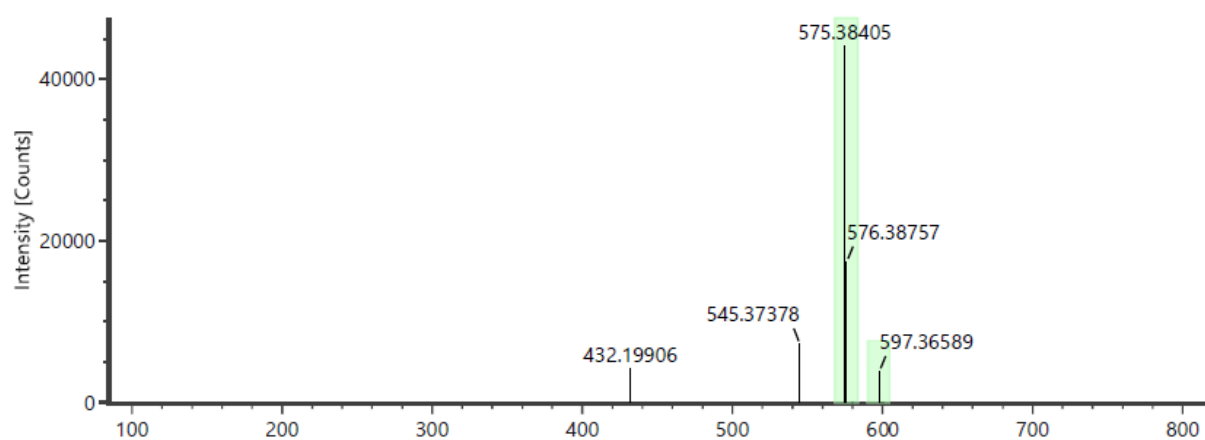
Figure S2. *a-d. Metabolite M1 in MLM.*

a. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 575.38$ [M+H]⁺ and RT = 10.64 min.

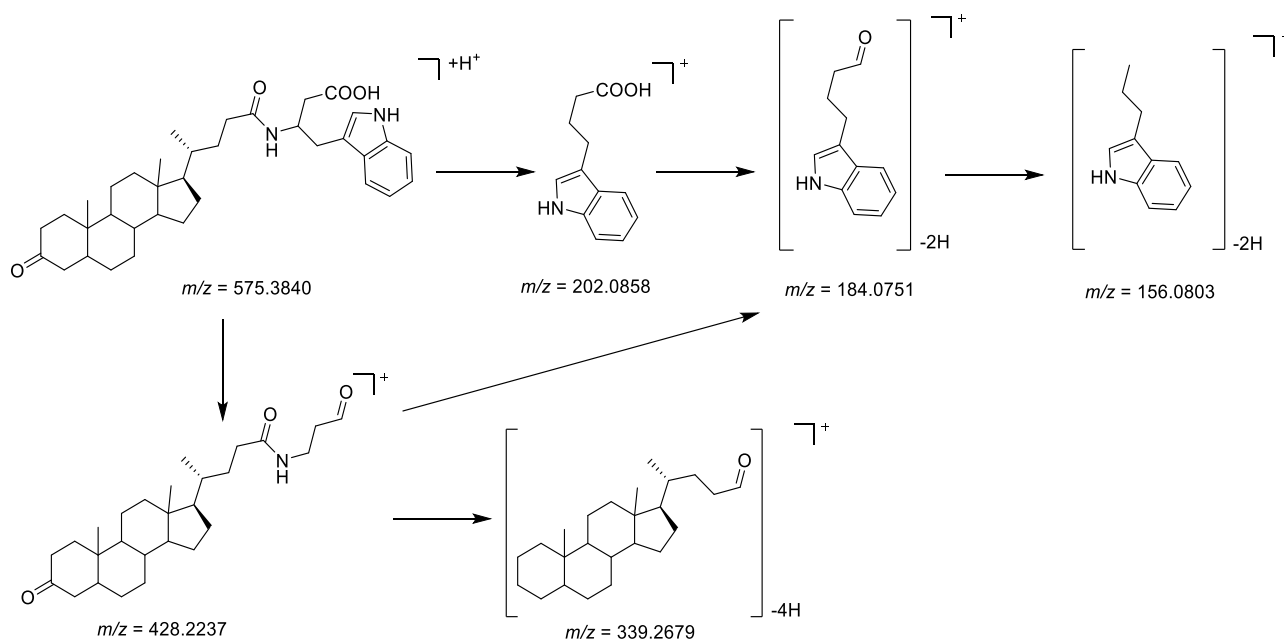


b. Experimental high resolution mass value for metabolite **M1** in ESI⁺.

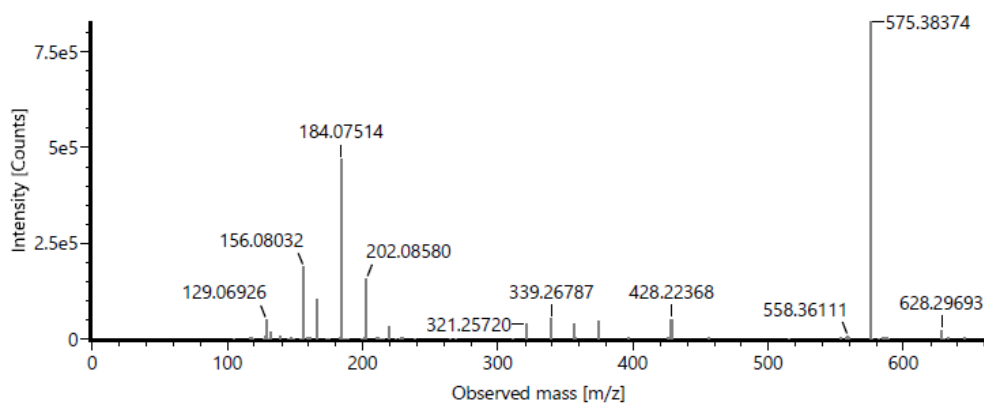
Item name: UniPr129_MLM_t180_5 Channel name: Low energy : Time 10.6418 +/- 0.0205 minutes : Drift Times: 6.84 +/- 0.22, 7.12 +/- 0.22 ms
Item description:



c. High resolution high energy (MS^E) MS spectrum in ESI^+ of **M1** together with tentative fragmentation pattern. In the MS/MS spectrum, peculiar fragments are present at $m/z = 428.2$, which is compatible with the loss of the 4-(indol-3-yl)-butanoyl fragment, and at $m/z = 339.3$, which likely derived from the further loss of the 3-aminopropanoyl group and the loss of the 3-keto group.



Component name: UniPR129-H2, Observed m/z : 575.3838, Channel name: product ions from precursor ion id = 8
Observed RT (min): 10.64



d. Metabolic soft spots on UniPR129 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M1**, UNIFI software returns as the most likely soft spots C-1-21 of the steroidal moiety; in particular, the oxidation of the 3-hydroxyl to a 3-keto group could be regarded as the most reasonable transformation.

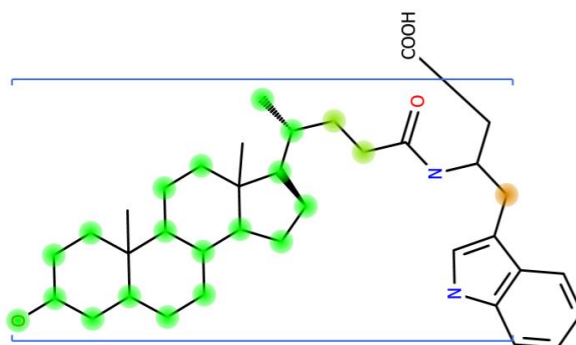
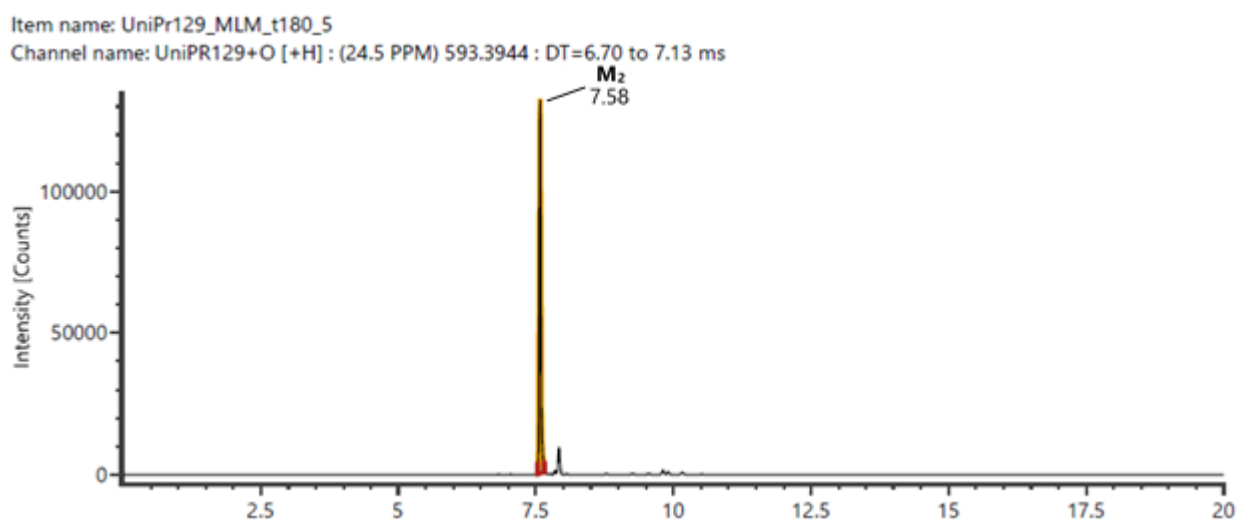


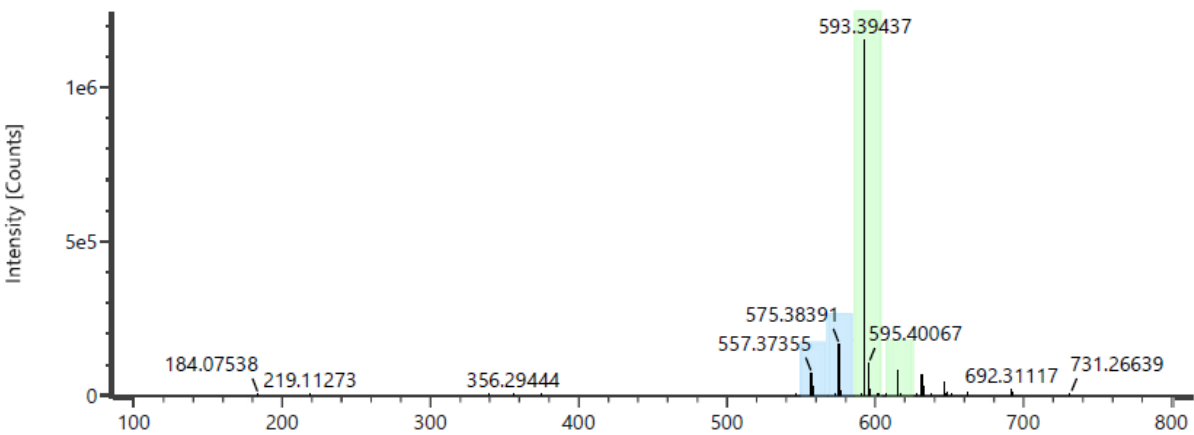
Figure S3. a-d. Metabolite **M2** in MLM.

a. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 593.39$ [M+H]⁺ and RT = 7.58 min.

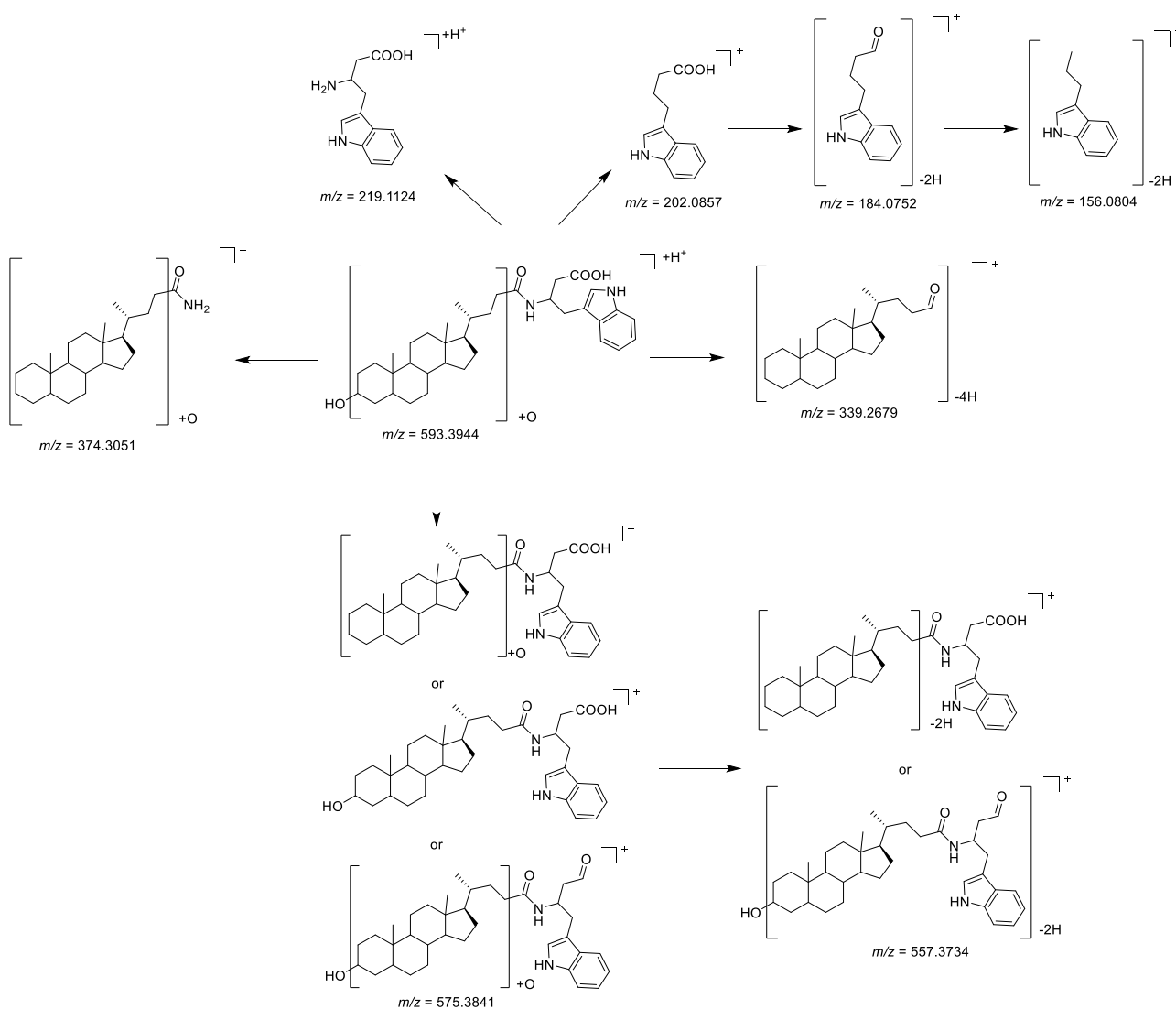


b. Experimental high resolution mass value for metabolite **M2 in ESI+.**

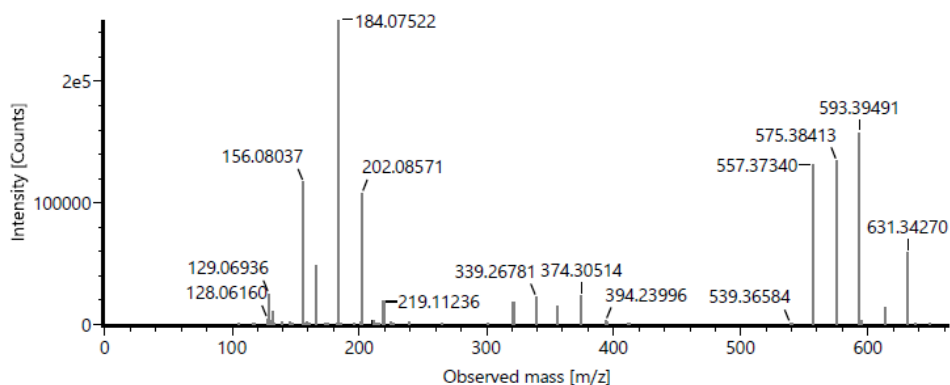
Item name: UniPr129_MLM_t180_5 Channel name: Low energy : Time 7.5796 +/- 0.0205 minutes : Drift Times: 6.92 +/- 0.22, 7.24 +/- 0.22 ms
Item description:



c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M2** together with tentative fragmentation pattern. The characteristic fragment ions are $m/z = 575.4$ and 557.4 , which derived from sequential loss of a hydroxyl group (-16 with respect to 593.4) and water (-18 with respect to 575.4), respectively.



Component name: UniPR129+O, Observed m/z : 593.3944, Channel name: product ions from precursor ion id = 10
Observed RT (min): 7.58



d. Metabolic soft spots on UniPR129 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M2**, UNIFI software returns as the most likely soft spots the C-atoms in the whole steroidal moiety except for C-4-7.

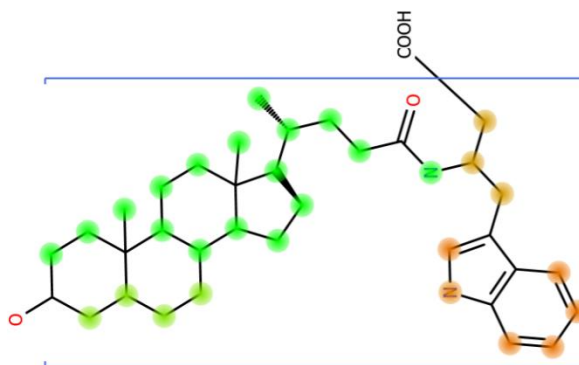
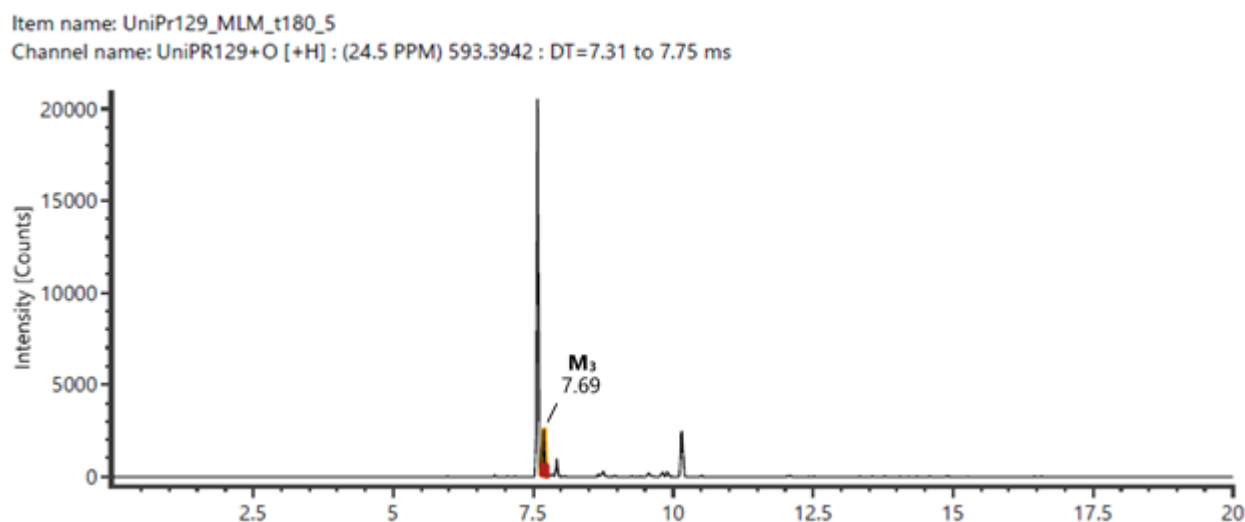


Figure S4. a-d. Metabolite **M3** in MLM.

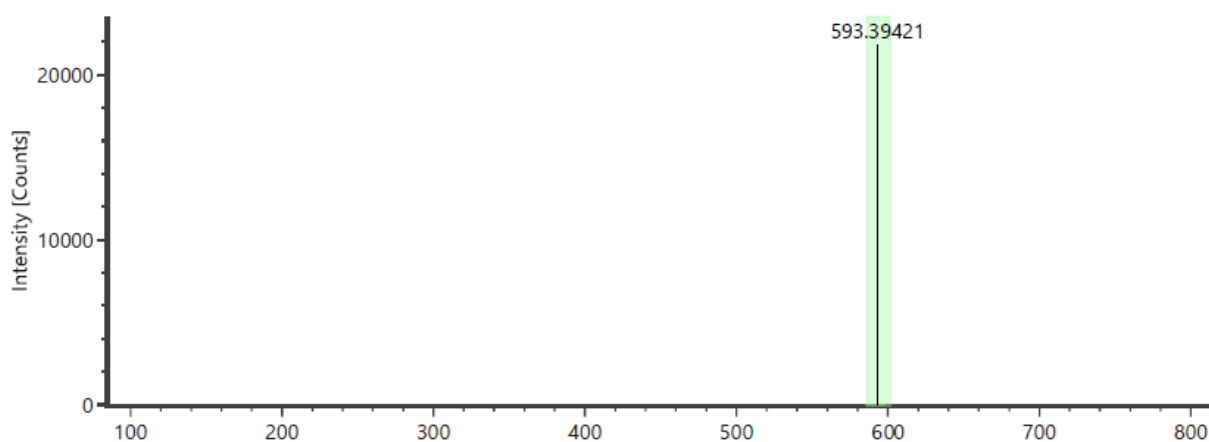
a. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 593.39$ [M+H]⁺ and RT = 7.69 min.



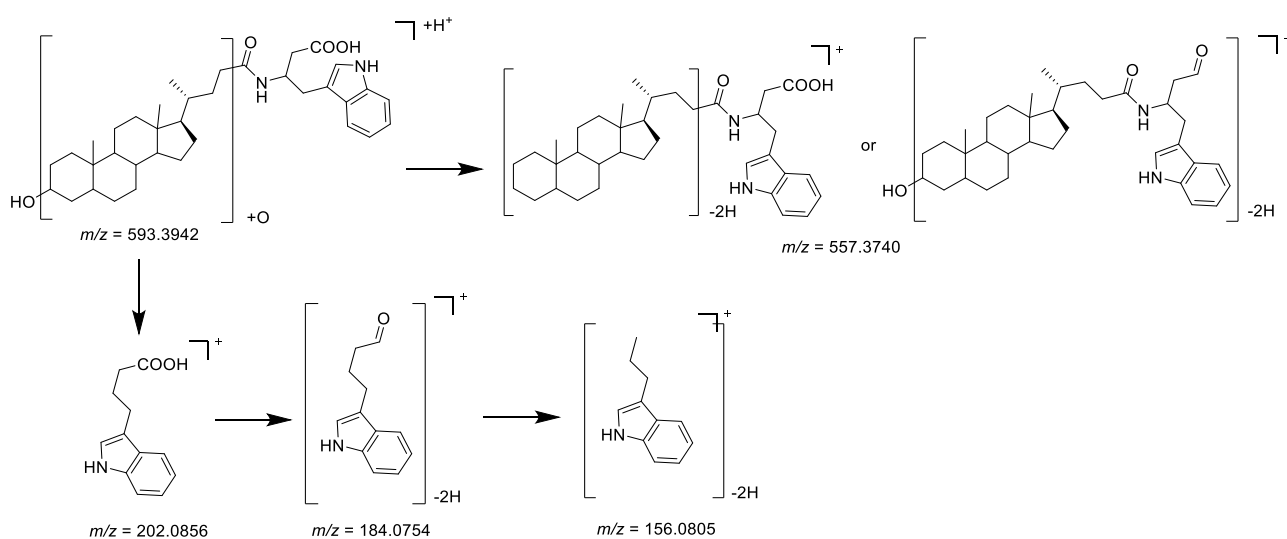
b. Experimental high resolution mass value for metabolite **M3 in ESI⁺.**

Item name: UniPr129_MLM_t180_5
Item description:

Channel name: Low energy : Time 7.6910 +/- 0.0205 minutes : Drift Times: 7.53 +/- 0.22 ms

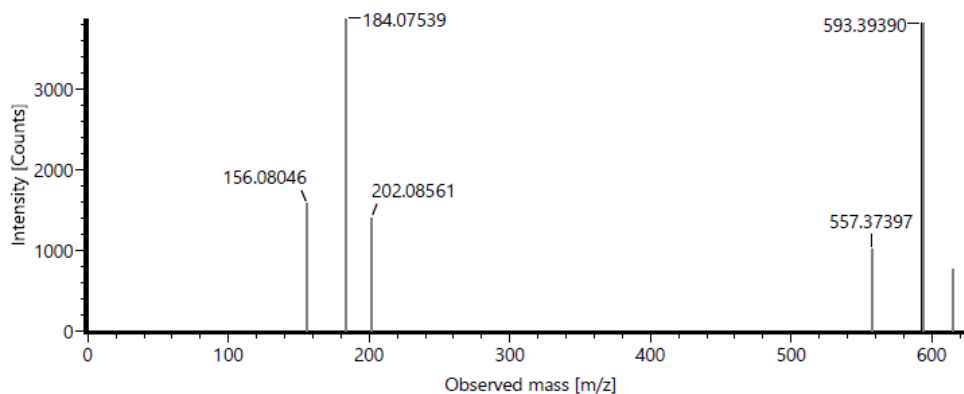


c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M3 together with tentative fragmentation pattern. **M3** shares the main product ions in the MS/MS spectrum (m/z = 557.4, 202.1, 184.1 and 156.1) with **M2**.**



Component name: UniPR129+O, Observed m/z:
593.3942, Observed RT (min): 7.69

Channel name: product ions from precursor ion id = 1171



d. Metabolic soft spots on UniPR129 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M3**, UNIFI software returns as the most likely soft spots the C-atoms in the whole steroidal moiety.

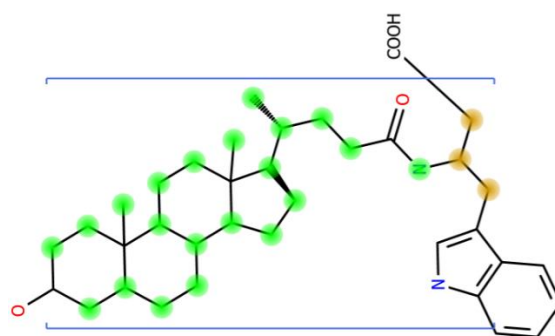
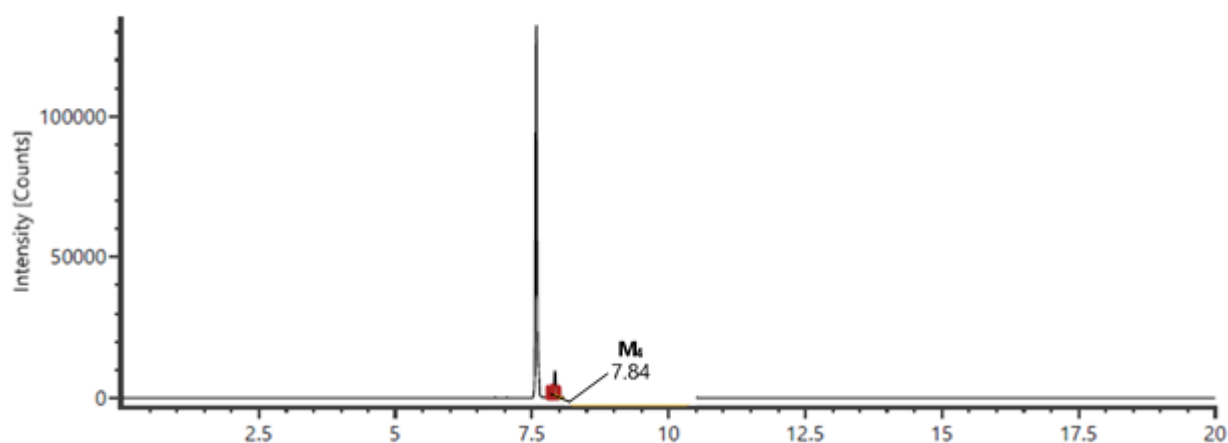


Figure S5. a-d. Metabolite **M4** in MLM.

a. Extracted ion chromatogram (XIC) in ESI⁺ at m/z = 593.39 [M+H]⁺ and RT = 7.84 min.

Item name: UniPr129_MLM_t180_5

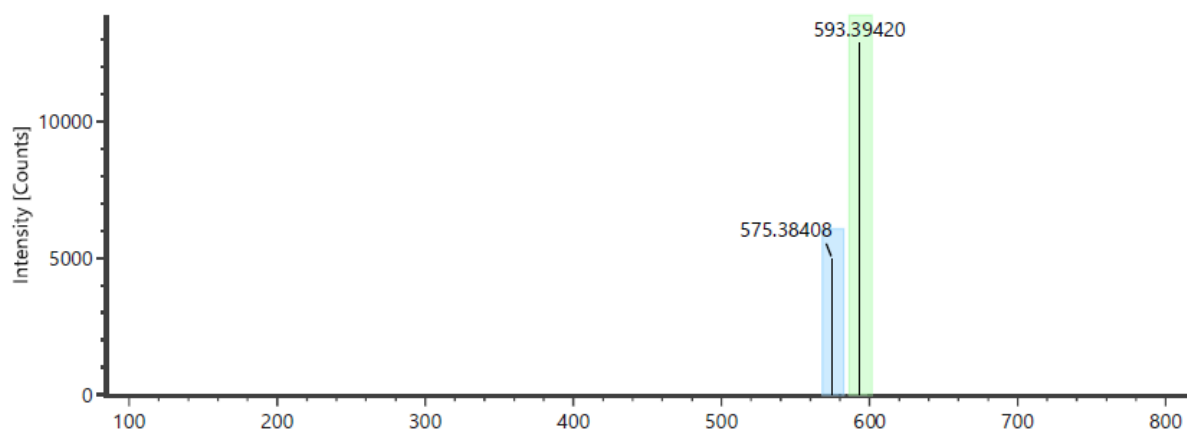
Channel name: UniPR129+O [+H] : (24.5 PPM) 593.3942 : DT=6.68 to 7.11 ms



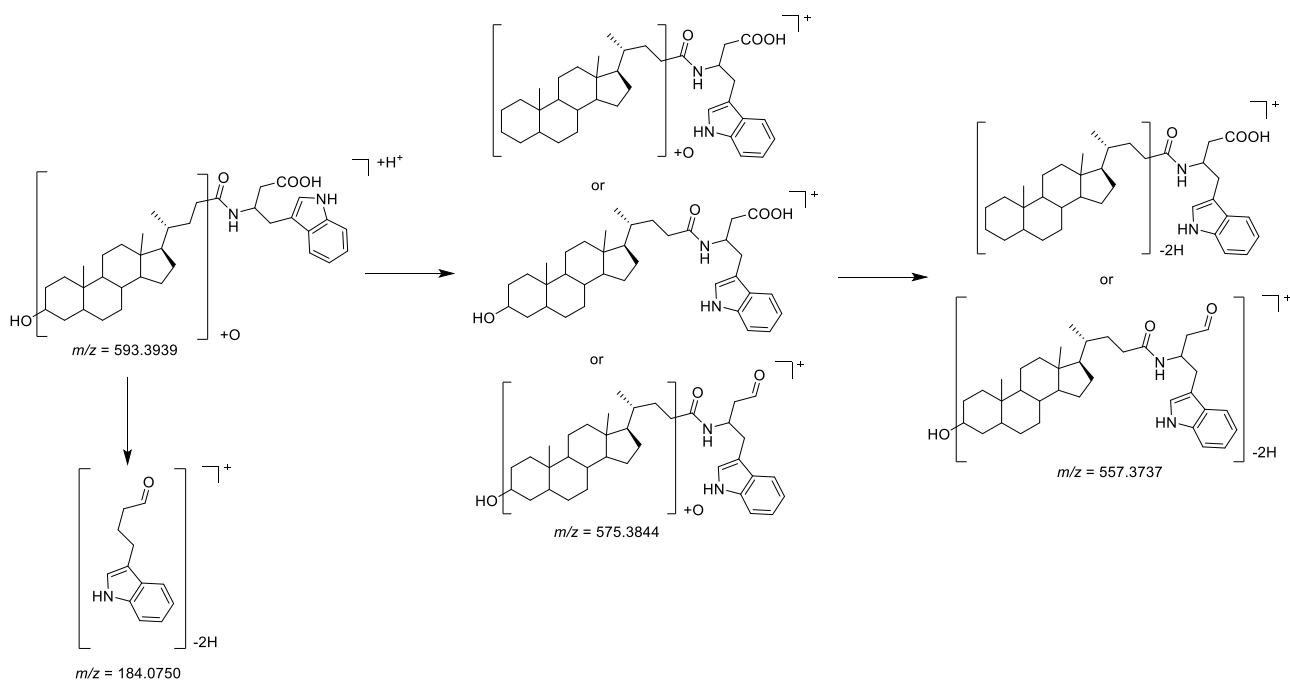
b. Experimental high resolution mass value for metabolite **M4** in ESI⁺.

Item name: UniPr129_MLM_t180_5
Item description:

Channel name: Low energy : Time 7.8389 +/- 0.0205 minutes : Drift Times: 6.90 +/- 0.22 ms

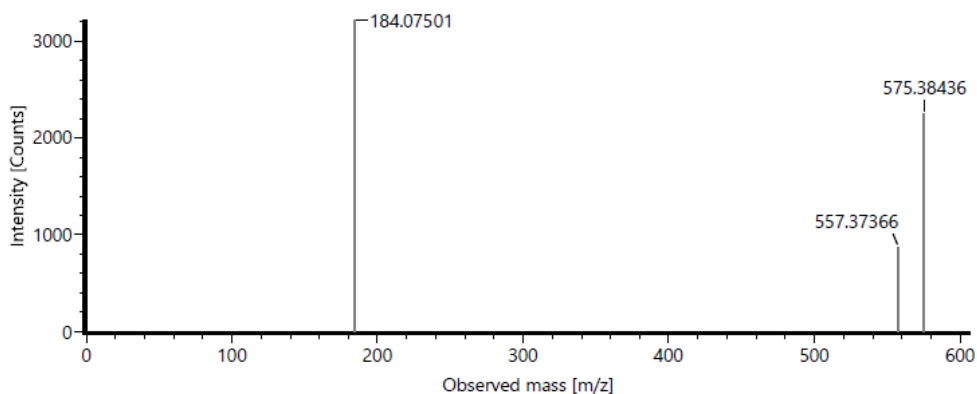


c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M4** together with tentative fragmentation pattern. Product ions at m/z = 557.4 and 184.1 are the same reported for **M3** and **M2**.



Component name: UniPR129+O, Observed m/z:
593.3942, Observed RT (min): 7.84

Channel name: product ions from precursor ion id = 1784



d. Metabolic soft spots on UniPR129 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M4**, UNIFI software returns as the most likely soft spots the C-atoms in the whole steroidal moiety, as for **M3**.

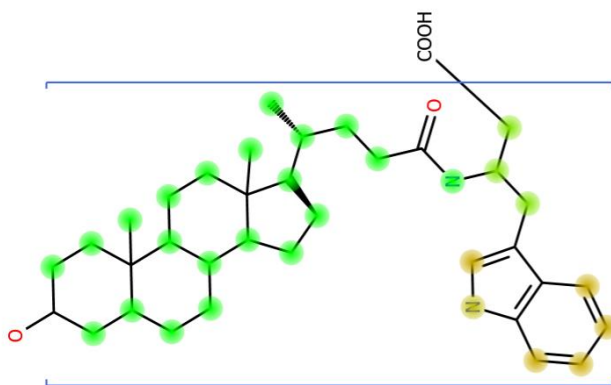
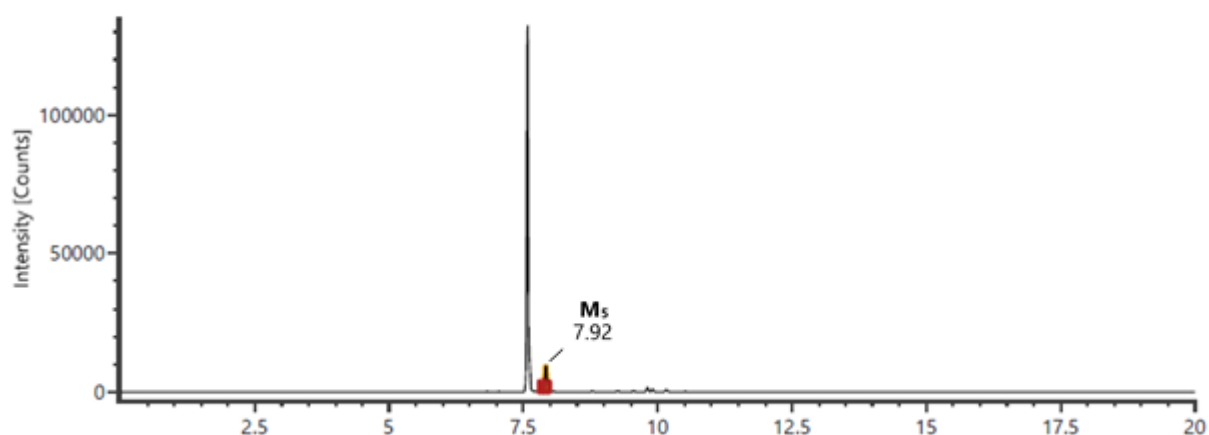


Figure S6. a-d. Metabolite **M5** in MLM.

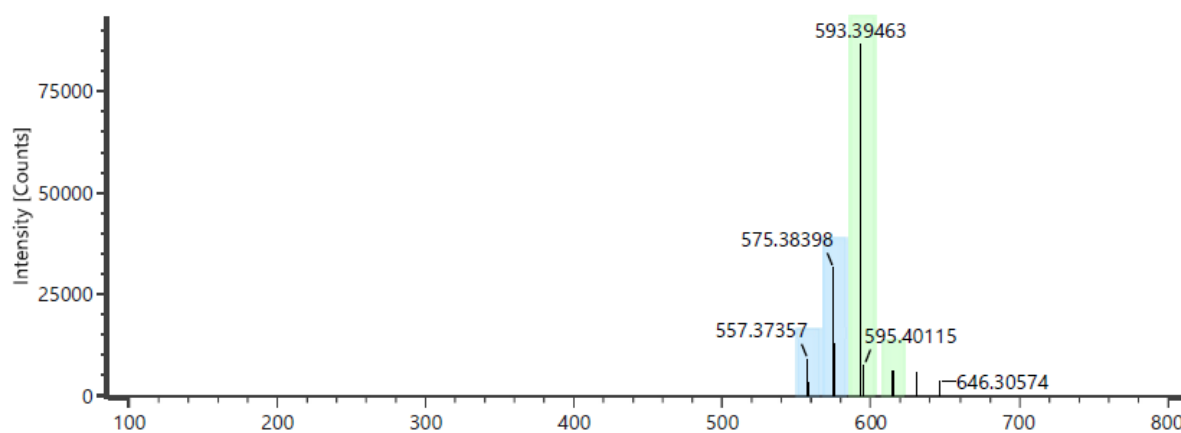
a. Phase I metabolite **M5** in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 593.39$ [M+H]⁺ and RT = 7.92 min.

Item name: UniPr129_MLM_t180_5
Channel name: UniPR129+O [+H] : (24.5 PPM) 593.3946 : DT=6.70 to 7.14 ms

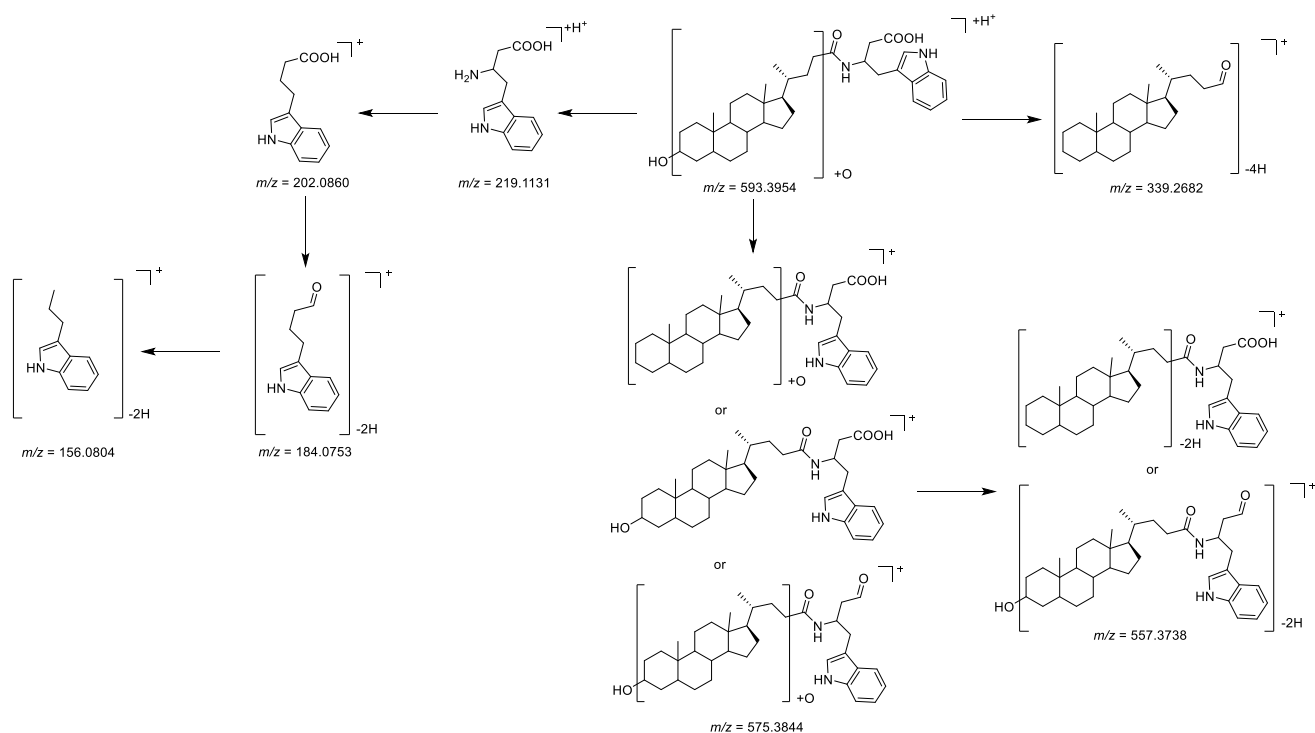


b. Experimental high resolution mass value for metabolite **M5 in ESI⁺.**

Item name: UniPr129_MLM_t180_5 Channel name: Low energy : Time 7.9206 +/- 0.0205 minutes : Drift Times: 6.92 +/- 0.22, 7.19 +/- 0.22 ms
Item description:

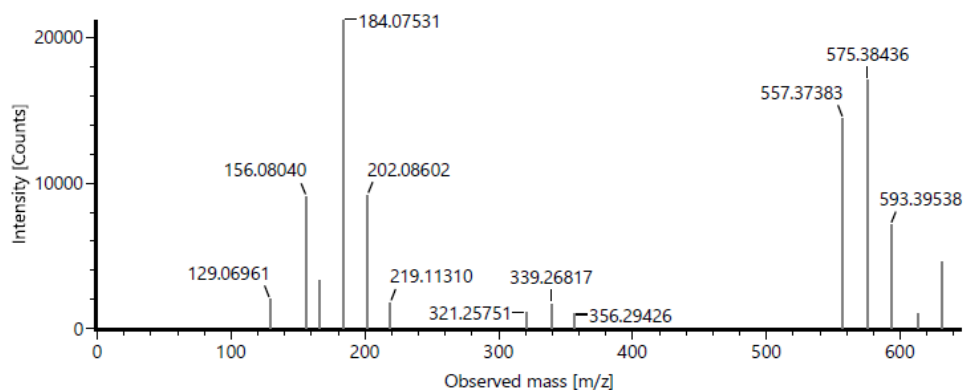


c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M5 together with tentative fragmentation pattern. All fragment ions in the MS/MS spectra have already been reported for **M2**.**



Component name: UniPR129+O, Observed m/z:
593.3946, Observed RT (min): 7.92

Channel name: product ions from precursor ion id = 343



d. Metabolic soft spots on UniPR129 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M2**, UNIFI software returns as the most likely soft spots C-atoms in the whole steroidal moiety with the exception of C-4-7, C-22 and C-23.

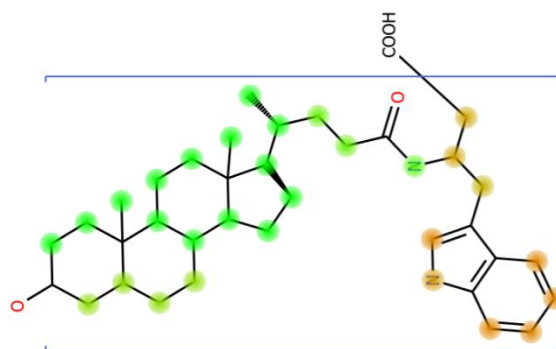
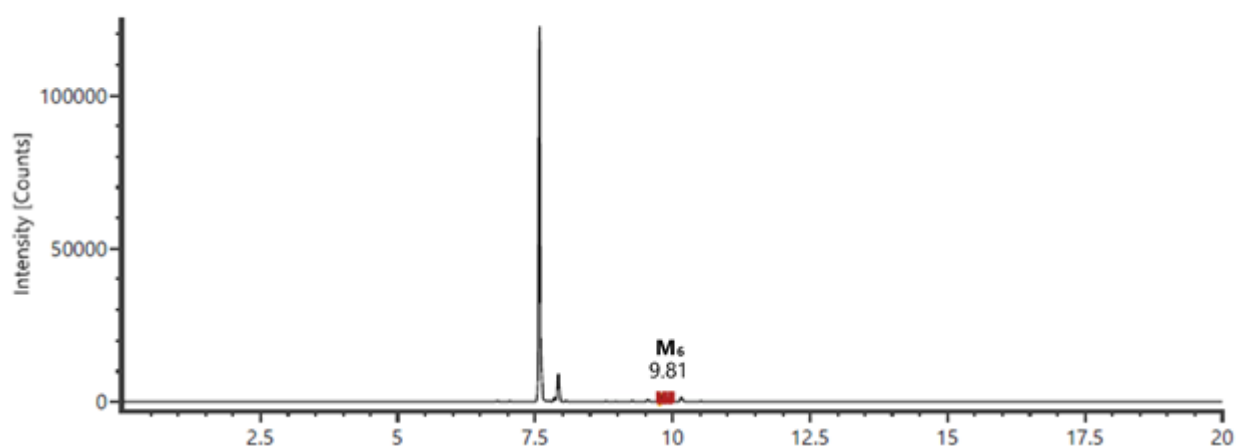


Figure S7. a-d. Metabolite **M6** in MLM.

a. Phase I metabolite **M6** in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 593.39$ [M+H]⁺ and RT = 9.81 min.

Item name: UniPr129_MLM_t180_5

Channel name: UniPR129+O [+H] : (24.5 PPM) 593.3934 : DT=6.81 to 7.24 ms

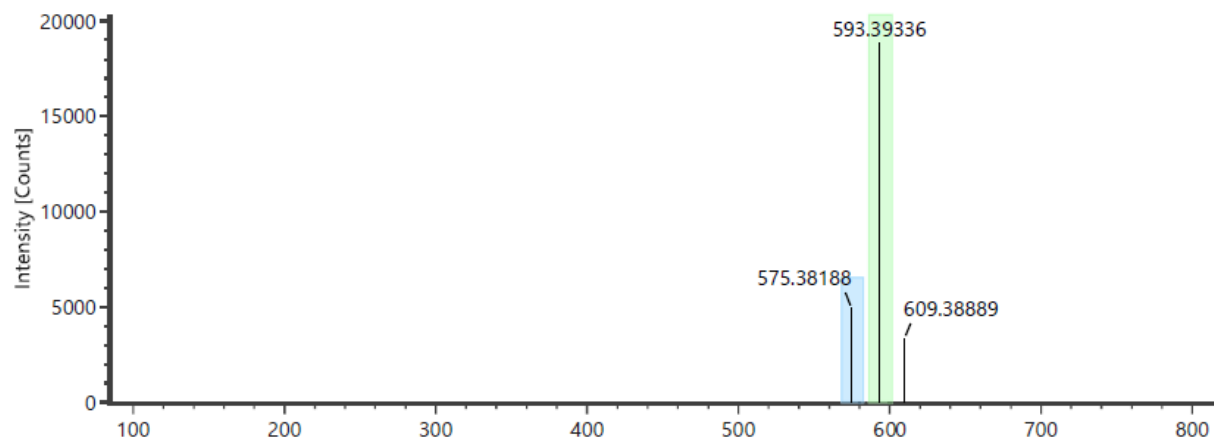


b. Experimental high resolution mass value for metabolite **M6 in ESI⁺.**

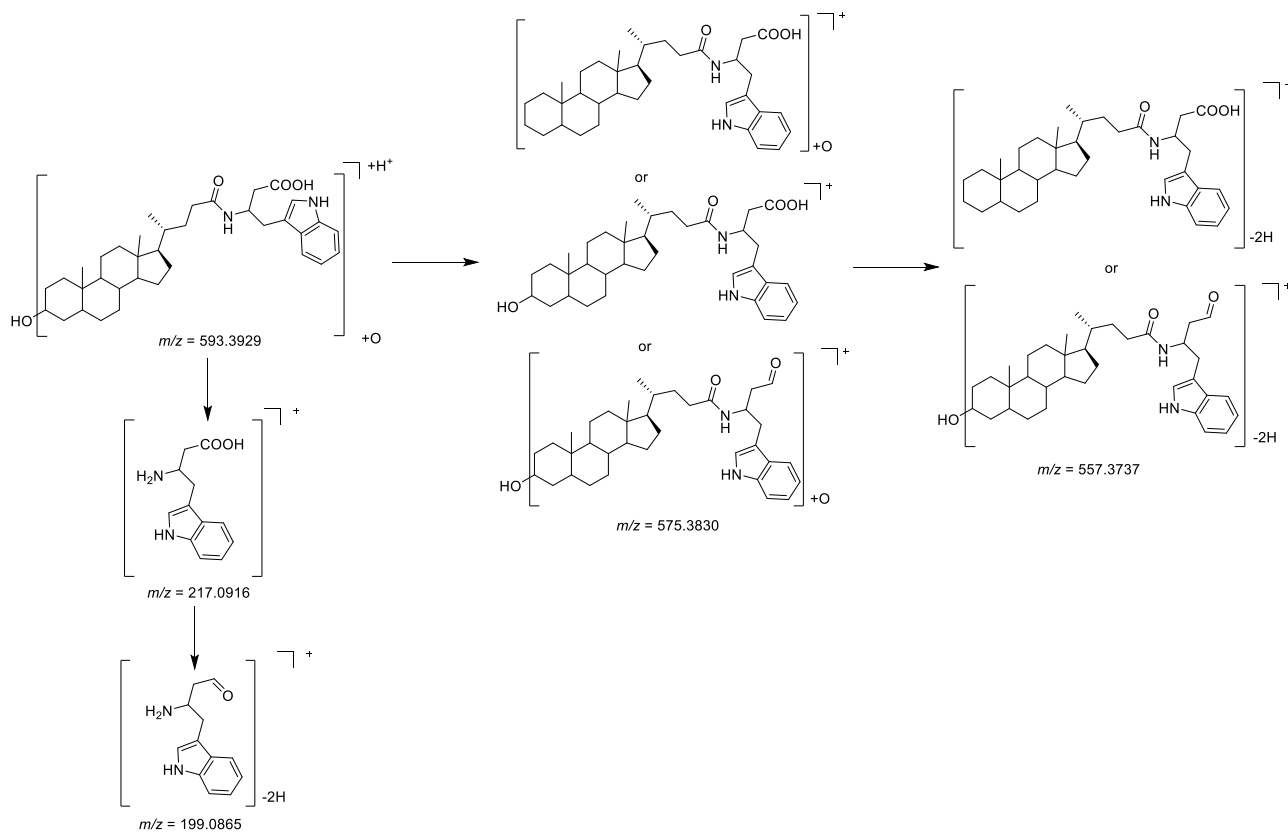
Item name: UniPr129_MLM_t180_5

Channel name: Low energy : Time 9.8155 +/- 0.0205 minutes : Drift Times: 7.03 +/- 0.22 ms

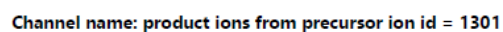
Item description:



c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M6 together with tentative fragmentation pattern. In addition to the product ion at m/z = 557.4, m/z = 217.1 consist of the L- β -homotryptophan moiety and m/z = 199.1 derived from the previous ion after loss of water (-18).**



Channel name: product ions from precursor ion id = 1301



Channel name: product ions from precursor ion id = 1301

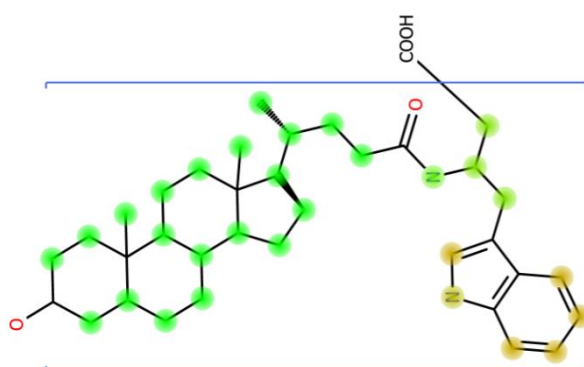
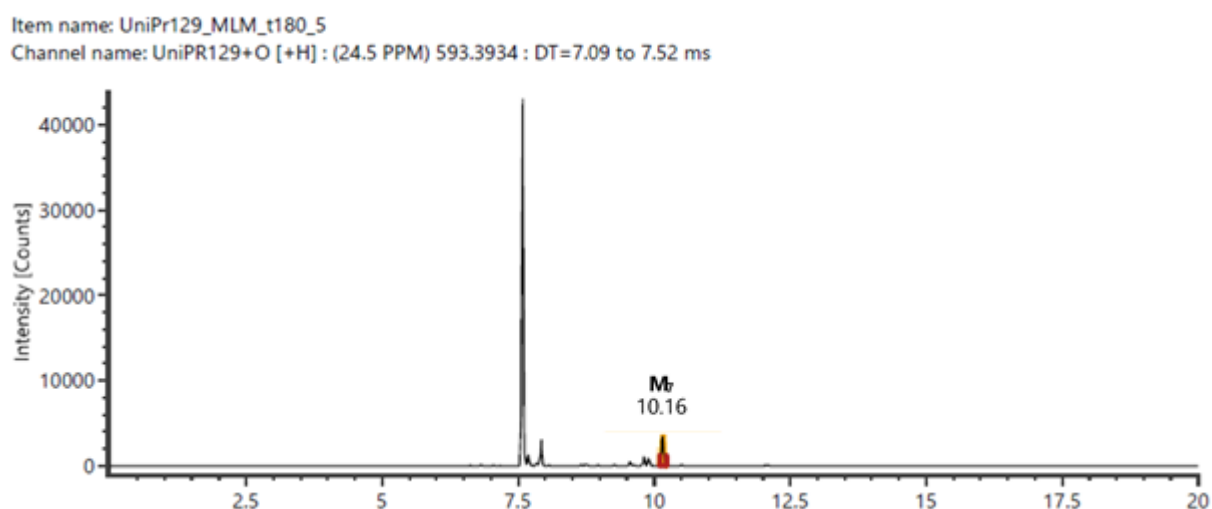


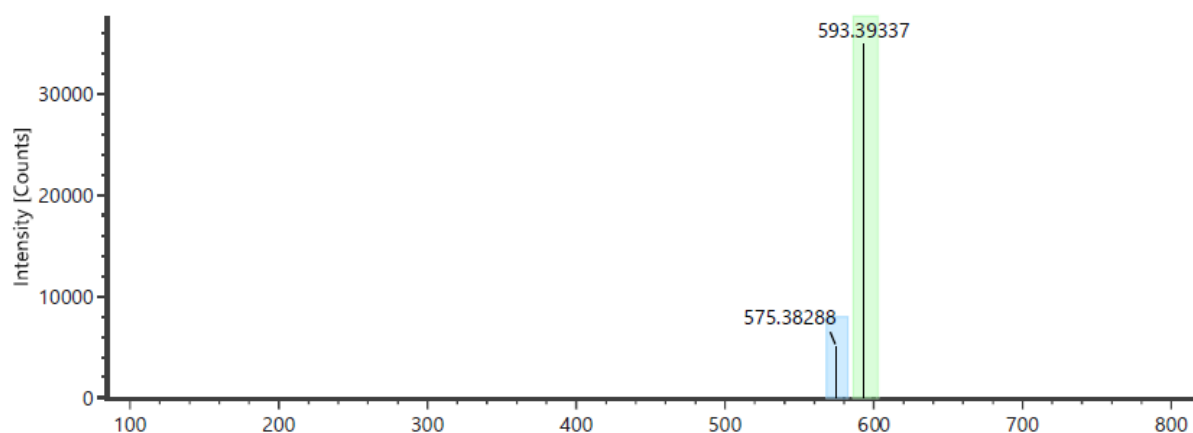
Figure S8. a-d. Metabolite M7 in MLM.

a. Phase I metabolite **M7** in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 593.39$ [M+H]⁺ and RT = 10.16 min.

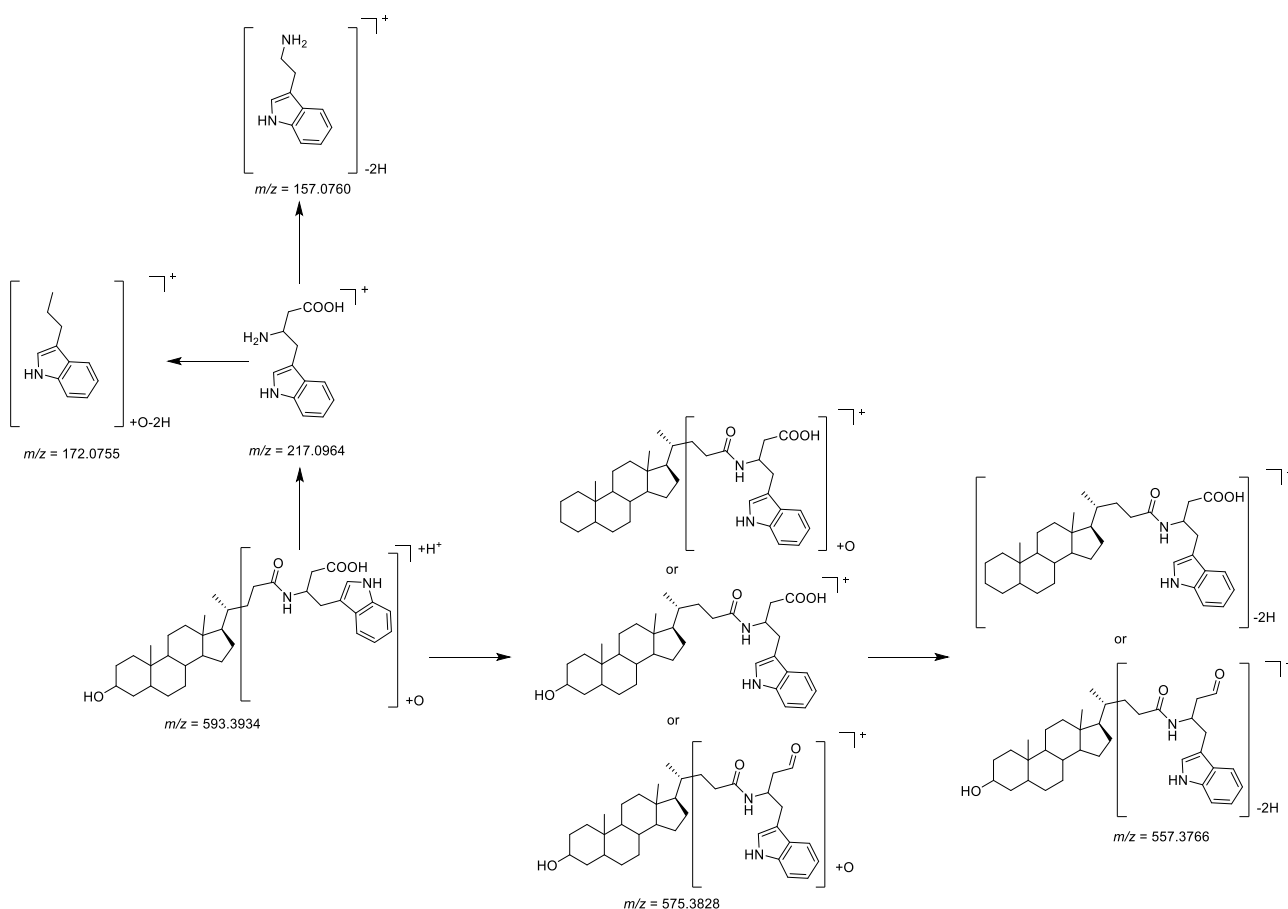


b. Experimental high resolution mass value for metabolite **M7** in ESI⁺.

Item name: UniPr129_MLM_t180_5 Channel name: Low energy : Time 10.1578 +/- 0.0205 minutes : Drift Times: 7.30 +/- 0.22 ms
Item description:

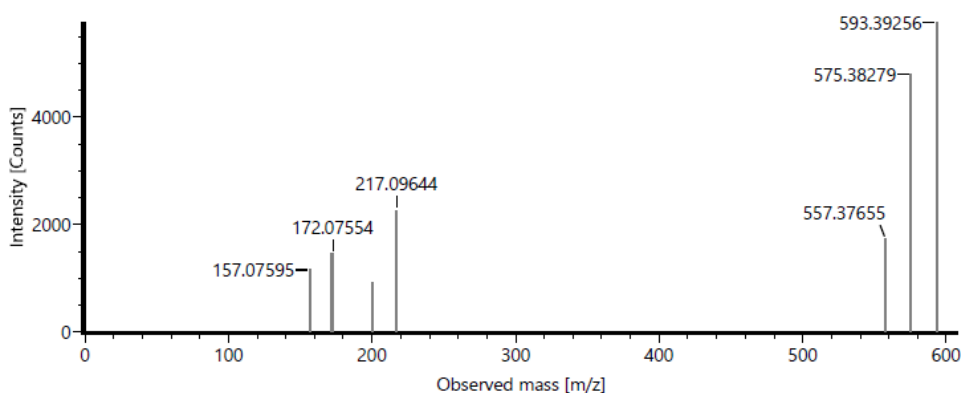


c. High resolution high energy (MS^E) MS spectrum in ESI^+ of **M7** together with tentative fragmentation pattern. Ion fragment at $m/z = 557.4$ has been found in all previously reported M+O metabolites and ion product at $m/z = 217.1$ is the same reported for **M6**. A peculiar peak at $m/z = 172.1$, however, could account for the oxidation of the propyl-indolic moiety.



Component name: UniPR129+O, Observed m/z : 593.3934, Observed RT (min): 10.16

Channel name: product ions from precursor ion id = 761



d. Metabolic soft spots on UniPR129 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M7**, UNIFI software returns as the most likely soft spot the C-atom of the propyl-indolic moiety of UniPR129.

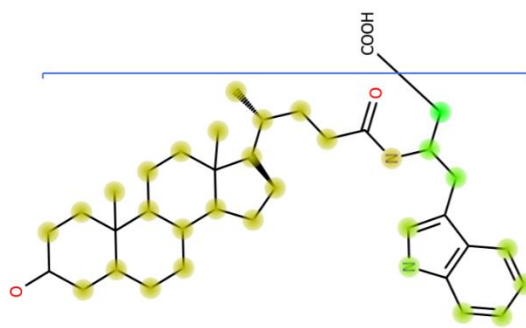
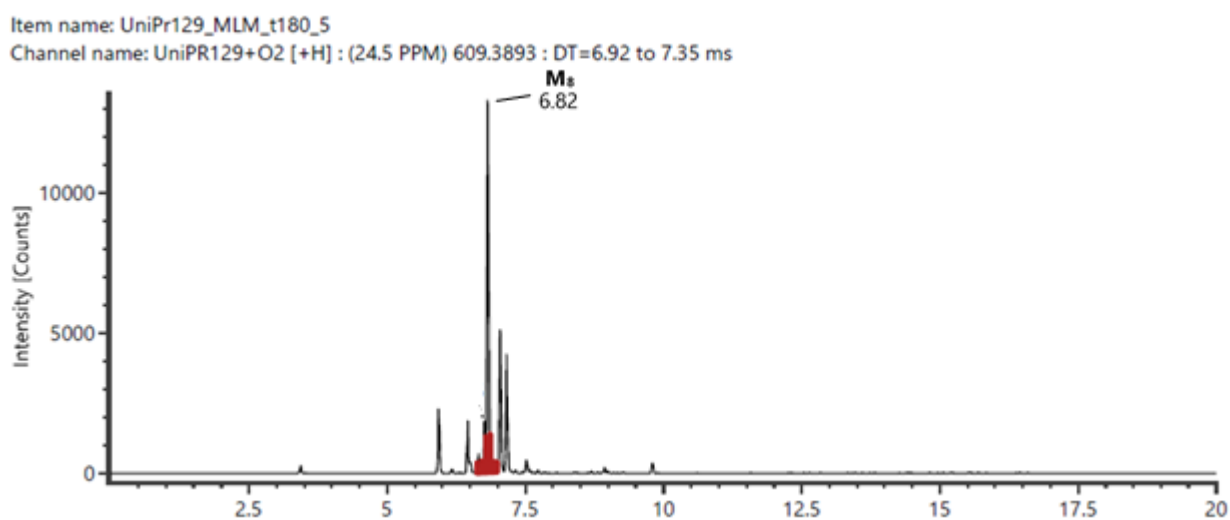


Figure S9. a-c. Metabolite **M8** in MLM.

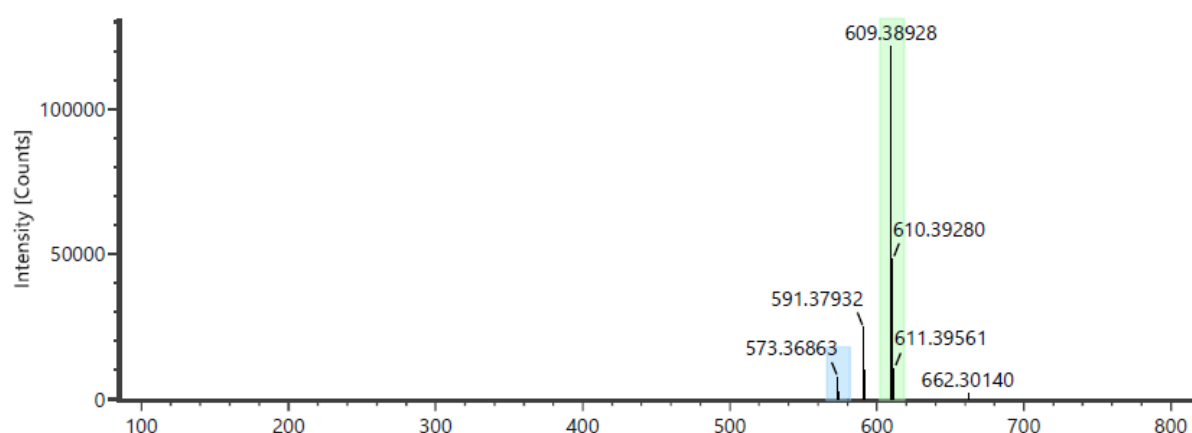
a. Phase I metabolite **M8** in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 609.39$ [M+H]⁺ and RT = 6.82 min.



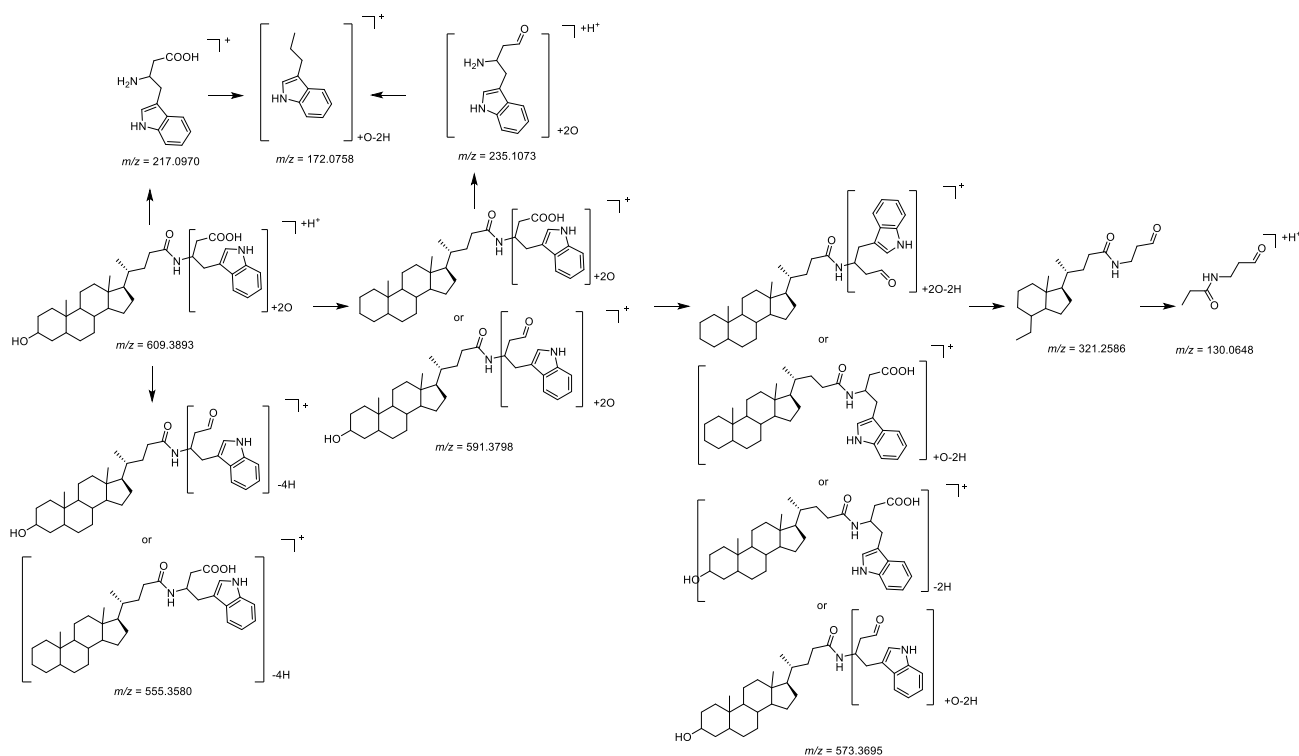
b. Experimental high resolution mass value for metabolite **M8 in ESI⁺.**

Item name: UniPr129_MLM_t180_5
Item description:

Channel name: Low energy : Time 6.8226 +/- 0.0205 minutes : Drift Times: 7.13 +/- 0.22 ms



c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M8 together with tentative fragmentation pattern. MS/MS spectrum shows a base peak at $m/z = 591.4$, compatible with the loss of water (-18); other characteristic peaks are at $m/z = 573.4$ (further dehydration, -18) and at $m/z = 555.36$ (loss of three water molecules with respect to $m/z = 609.36$, -54). Fragment ions at $m/z = 172.1$ and at $m/z = 235.1$ could account for a double oxidation on the L- β -homotryptophan; however, UNIFI software could not predict likely sites for oxidation.**



Component name: UniPR129+O2, Observed m/z:
609.3893, Observed RT (min): 6.82

Channel name: product ions from precursor ion id = 243

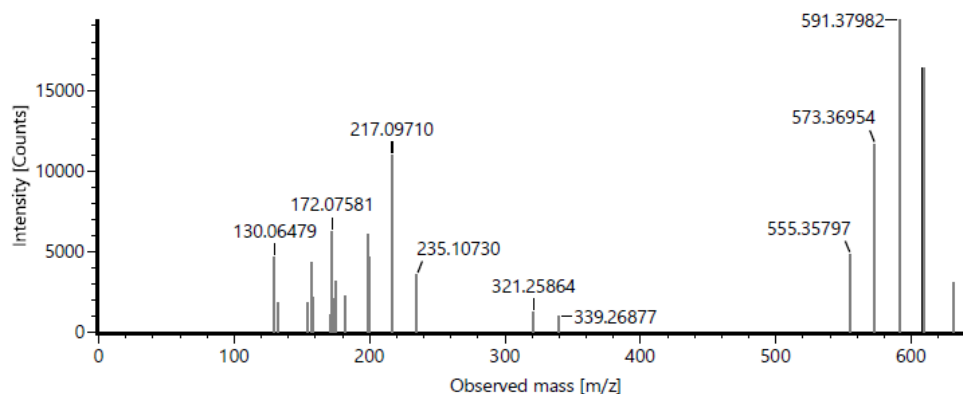
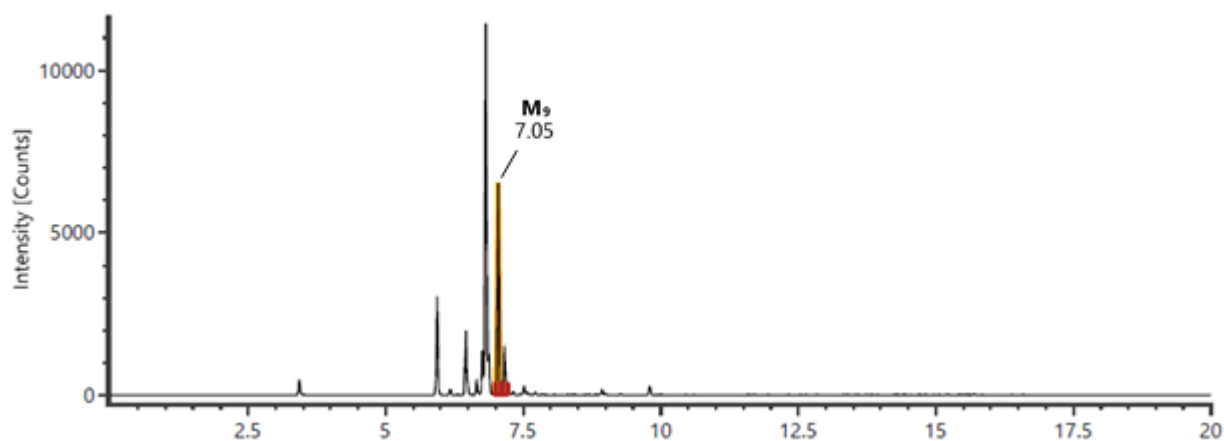


Figure S10. a-c. Metabolite M9 in MLM.

a. Phase I metabolite **M9** in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 609.39$ [M+H]⁺ and RT = 7.05 min.

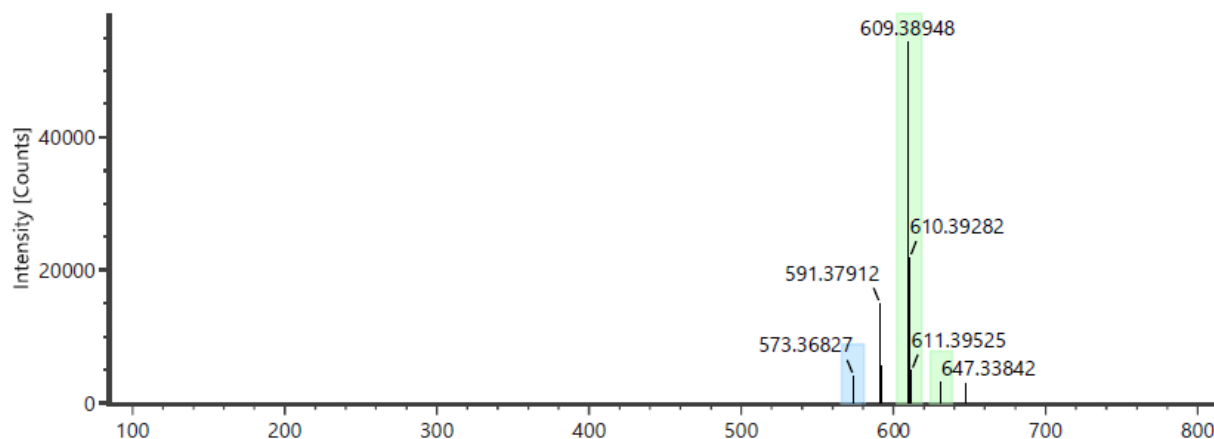
Item name: UniPr129_MLM_t180_5

Channel name: UniPR129+O2 [+H] : (24.5 PPM) 609.3895 : DT=6.77 to 7.20 ms

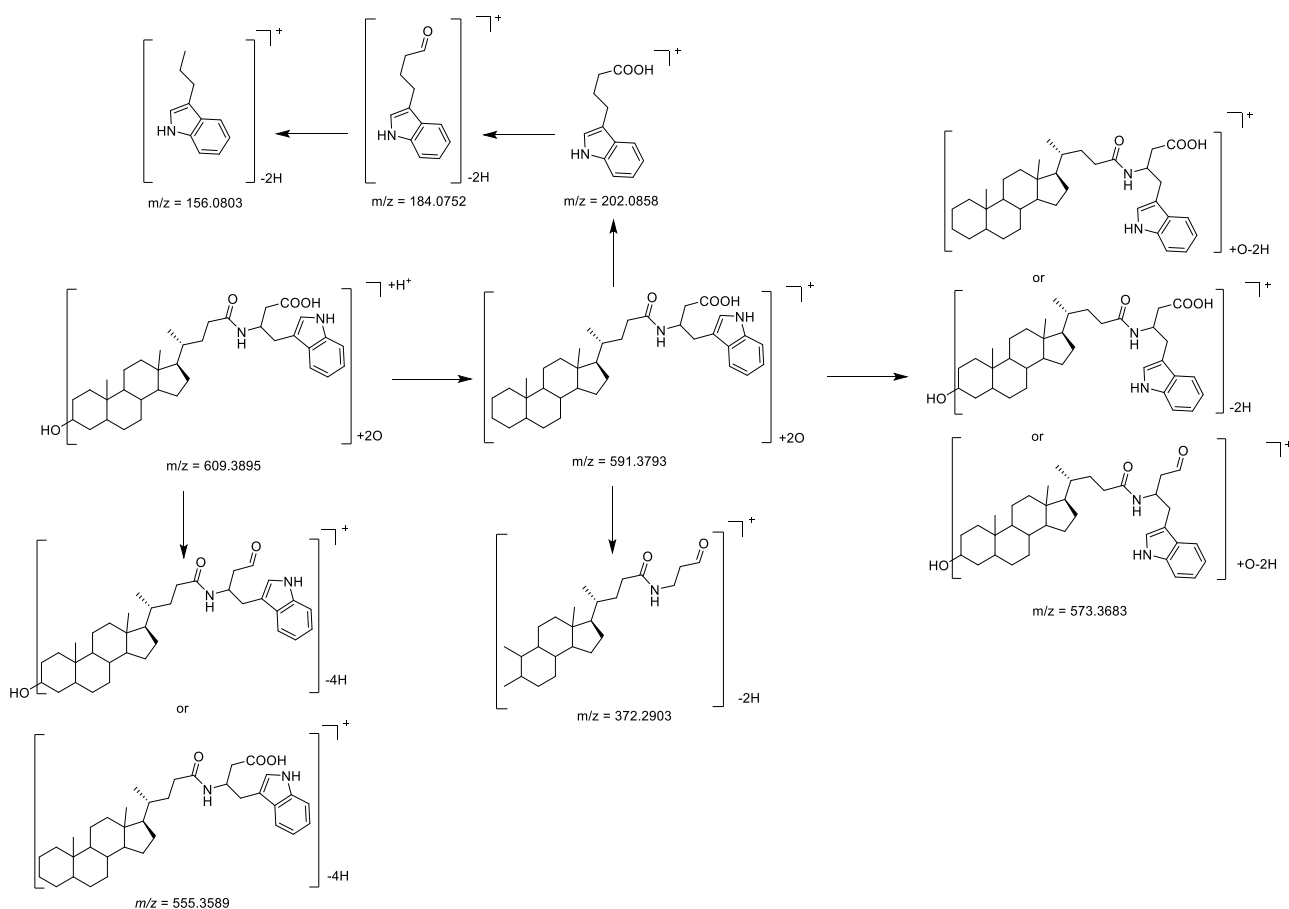


b. Experimental high resolution mass value for metabolite **M9 in ESI⁺.**

Item name: UniPr129_MLM_t180_5 Channel name: Low energy : Time 7.0474 +/- 0.0205 minutes : Drift Times: 6.98 +/- 0.22, 7.28 +/- 0.22 ms
Item description:



c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M9 together with tentative fragmentation pattern. In the MS/MS spectrum, product ions at m/z = 573.4 and 555.4 are compatible with the loss of two and three water molecules, respectively. Despite no fragment suggests oxidation to occur on the L- β -homotryptophan portion, in contrast with **M8**, UNIFI software cannot return the most likely sites of oxidation on the steroidal moiety.**



Component name: UniPR129+O2, Observed m/z:
609.3895, Observed RT (min): 7.05

Channel name: product ions from precursor ion id = 521

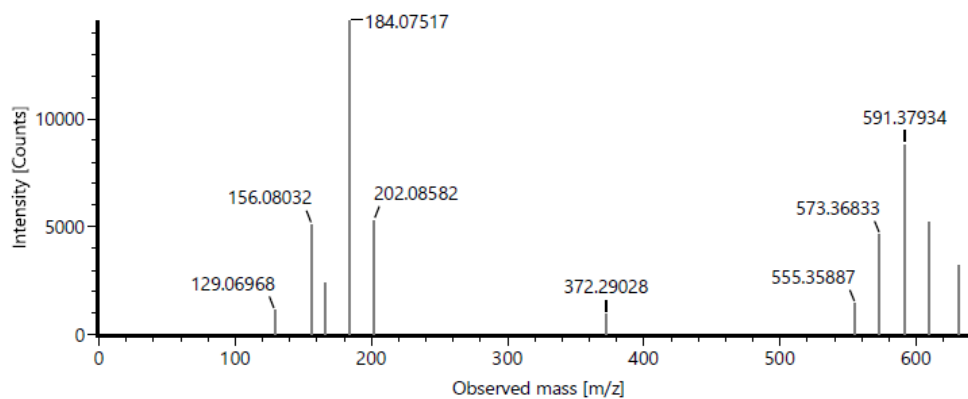
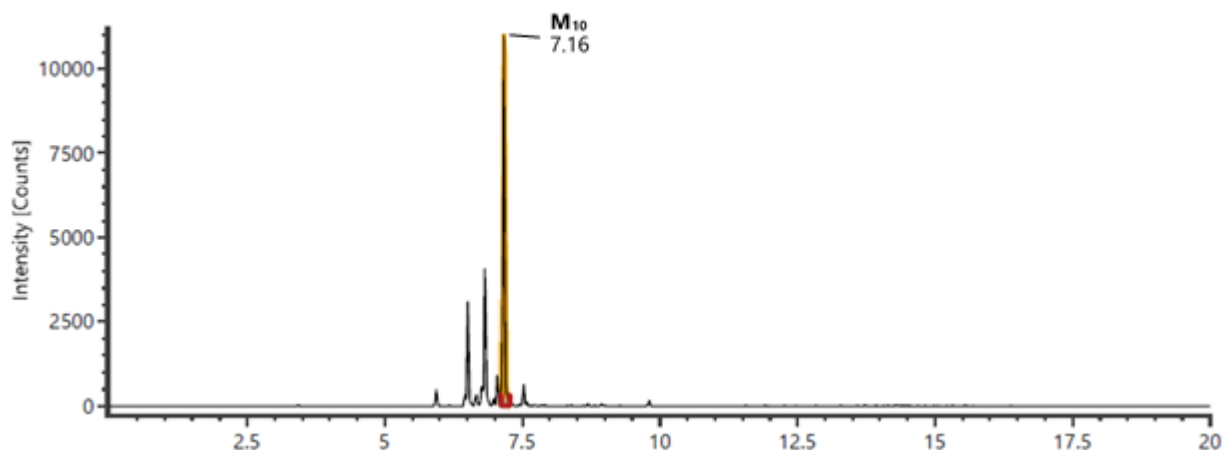


Figure S11. a-c. Metabolite M10 in MLM.

a. Phase I metabolite **M10** in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 609.39$ [M+H]⁺ and RT = 7.16 min.

Item name: UniPr129_MLM_t180_5

Channel name: UniPR129+O2 [+H] : (24.5 PPM) 609.3893 : DT=7.26 to 7.69 ms

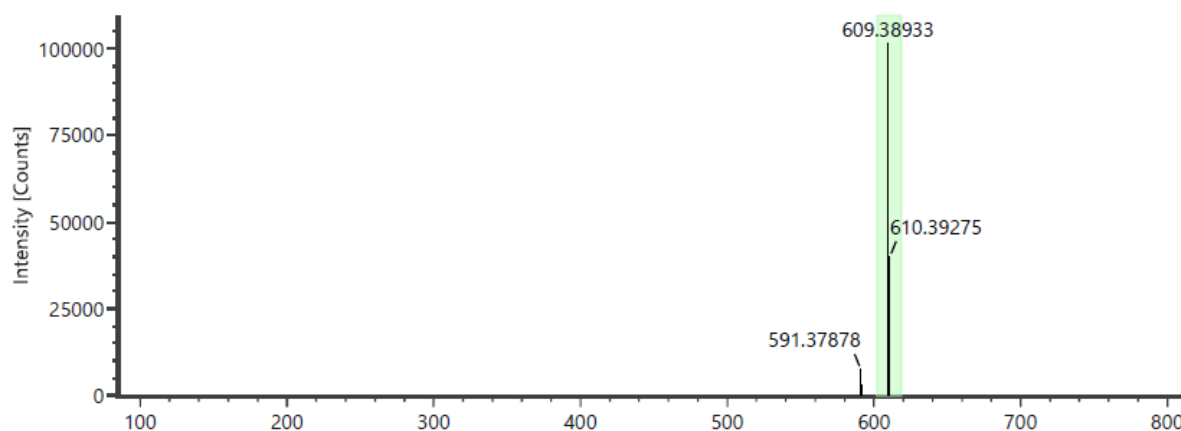


b. Experimental high resolution mass value for metabolite M10 in ESI⁺.

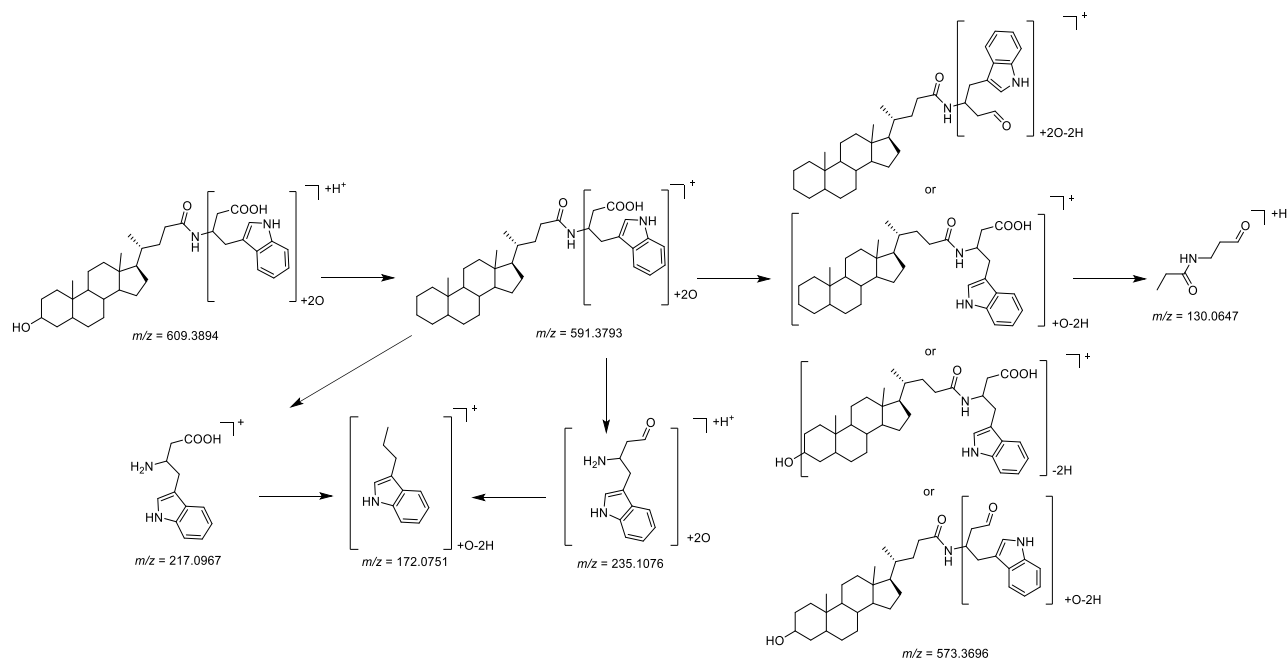
Item name: UniPr129_MLM_t180_5

Channel name: Low energy : Time 7.1641 +/- 0.0205 minutes : Drift Times: 7.47 +/- 0.22 ms

Item description:



c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M10** together with tentative fragmentation pattern. All the ions in the MS/MS spectrum are shared with **M8**; in particular, likewise **M8**, fragment ion at $m/z = 235.1$ could account for a double oxidation on the L- β -homotryptophan portion. However, UNIFI software cannot predict likely sites for oxidation.



Component name: UniPR129+O2, Observed m/z:
609.3893, Observed RT (min): 7.16

Channel name: product ions from precursor ion id = 295

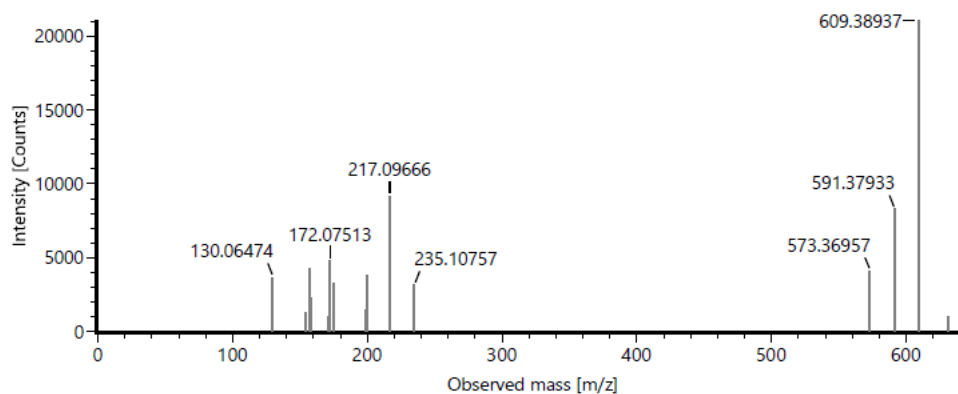
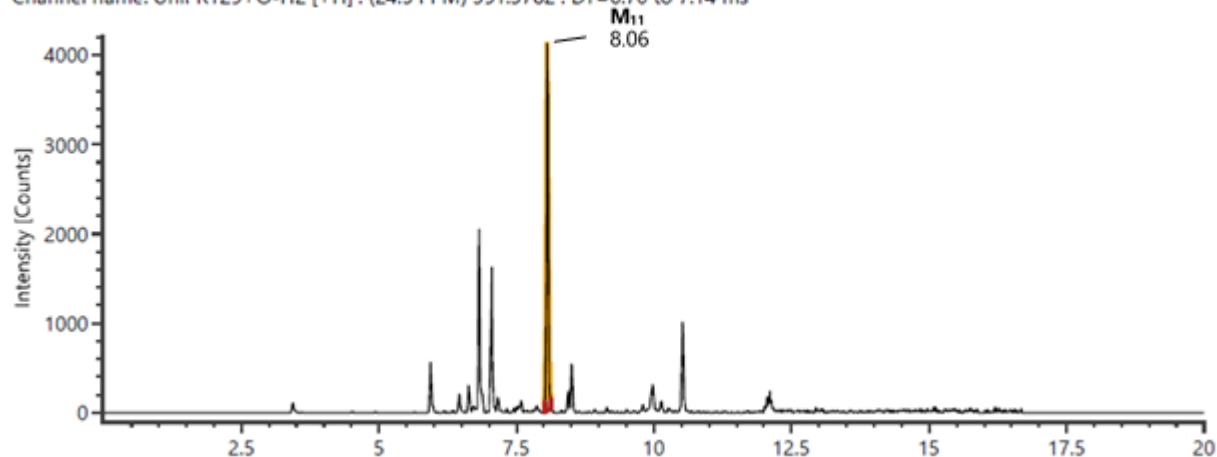


Figure S12. a-d. Metabolite **M11** in MLM.

a. Phase I metabolite **M11** in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 591.38$ [M+H]⁺ and RT = 8.06 min.

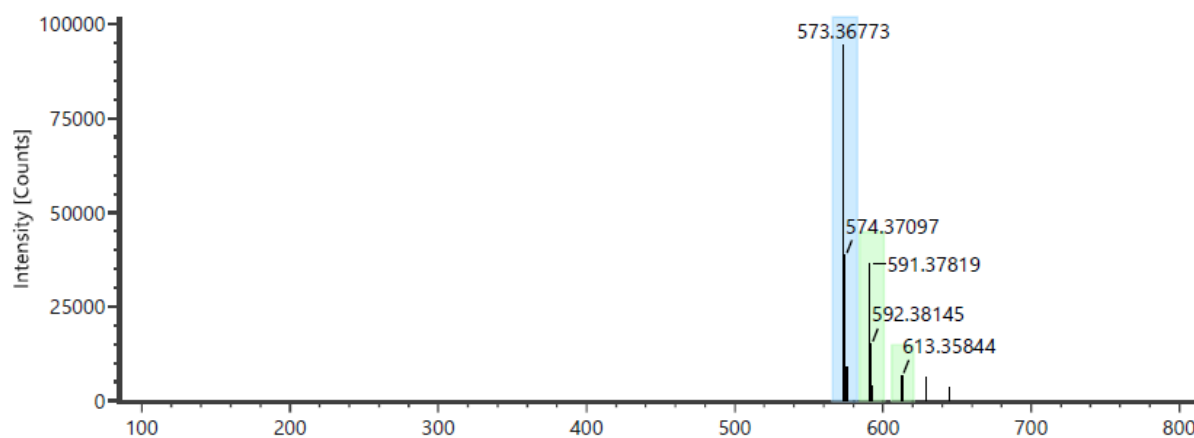
Item name: UniPr129_MLM_t180_5

Channel name: UniPR129+O-H2 [+H] : (24.5 PPM) 591.3782 : DT=6.70 to 7.14 ms

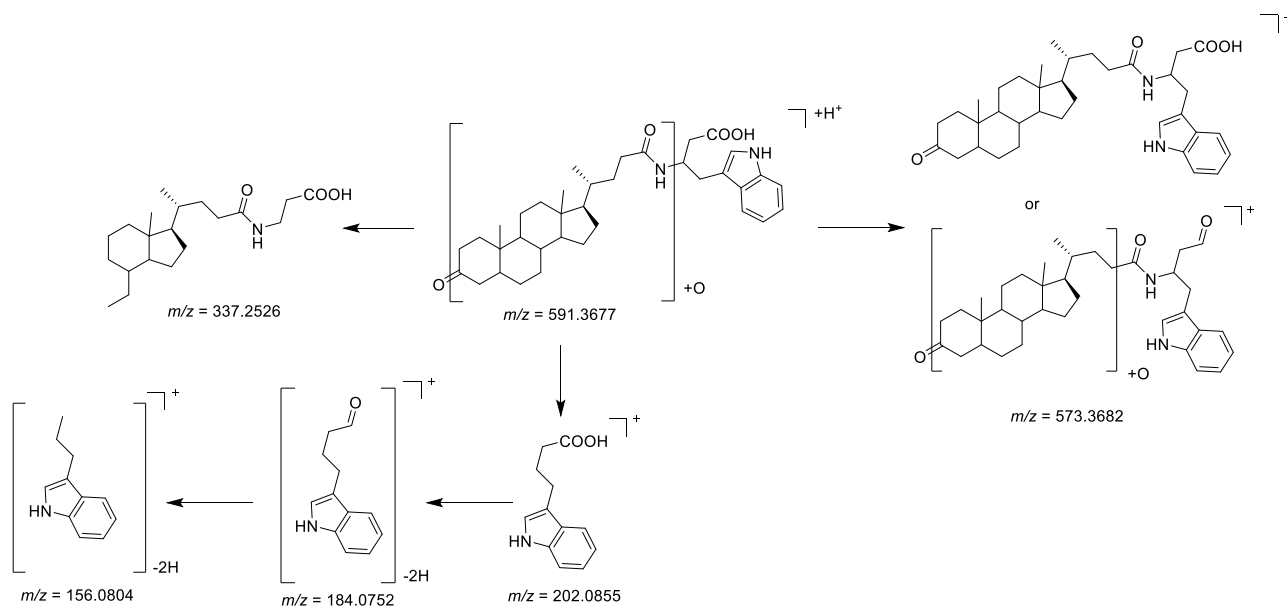


b. Experimental high resolution mass value for metabolite **M11 in ESI⁺.**

Item name: UniPr129_MLM_t180_5 Channel name: Low energy : Time 8.0647 +/- 0.0205 minutes : Drift Times: 6.92 +/- 0.22, 7.08 +/- 0.22 ms
Item description:

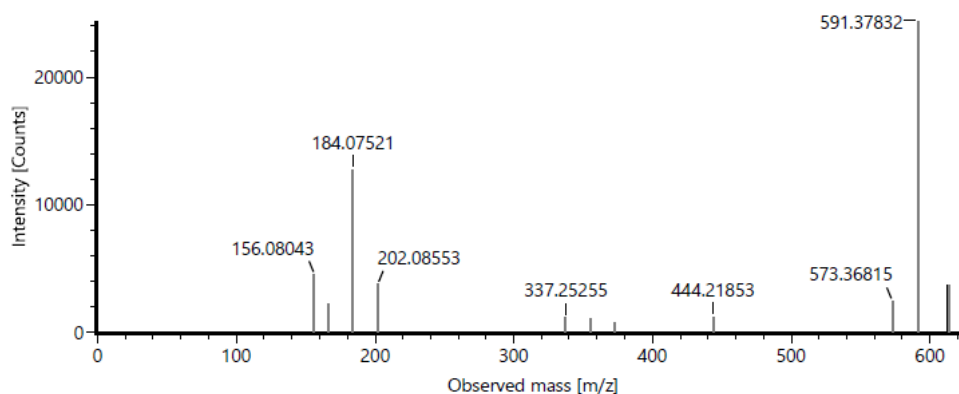


c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M11 together with tentative fragmentation pattern. The three product ions at m/z = 202.1, 184.1 and 156.1 prompt to exclude the oxidation to occur on L- β -homotryptophan group. Moreover, the low intense fragment ion at m/z = 337.2 allows to speculate that oxidation involves a C-atom on the A- or B-ring of the steroidal core.**

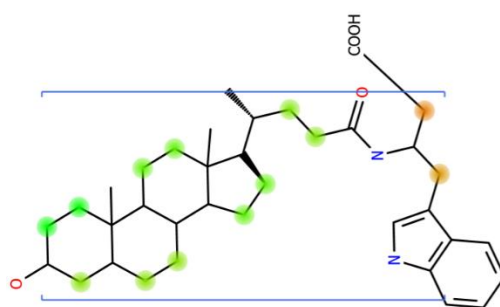


Component name: UniPR129+O-H2, Observed m/z:
591.3785, Observed RT (min): 8.06

Channel name: product ions from precursor ion id = 455



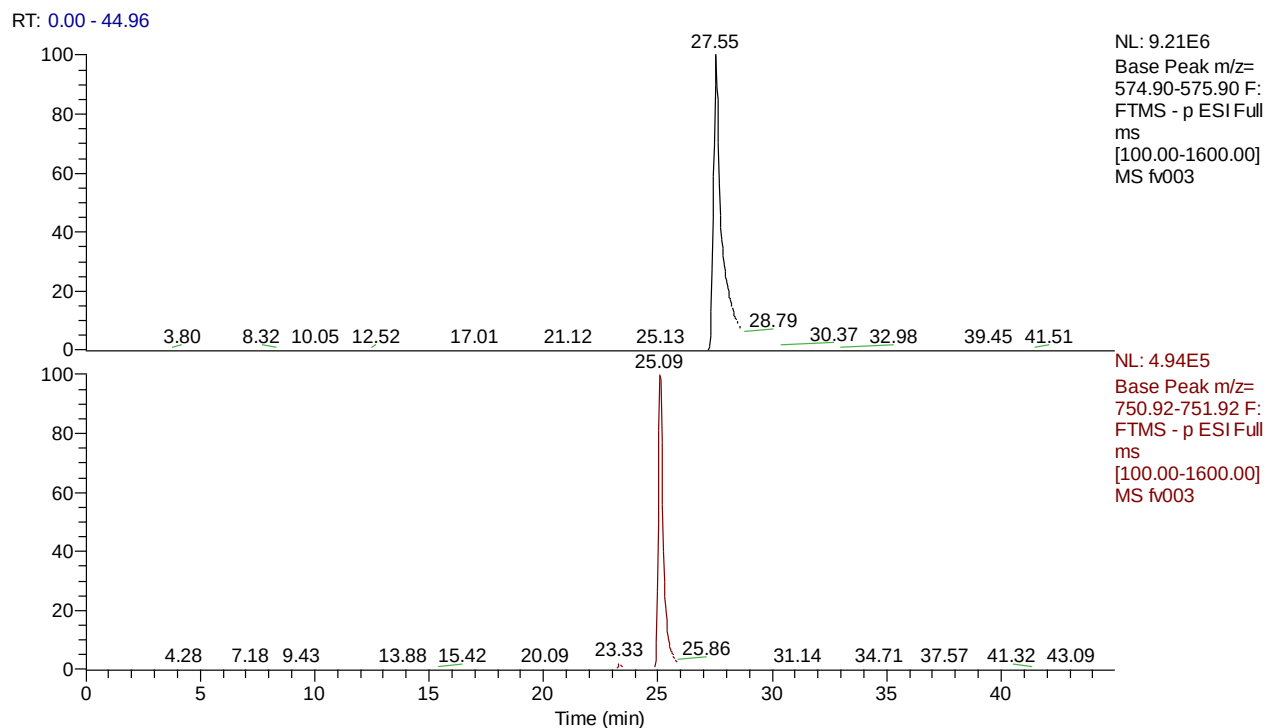
d. Metabolic soft spots on UniPR129 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M11**, UNIFI software returns as the most likely soft spots C1 or C2 of the steroidal core.



S.7 UniPR129 *In vitro* phase II metabolite profiling

Figure S13. *a-b.* Metabolite **M12** in *MLS₉* fraction.

a. Phase II conjugate of UniPR129 with glucuronic acid **M12** in mouse liver microsomes. Upper trace: Extracted ion chromatogram (XIC) in ESI⁻ at $m/z = 575.40$ [M-H]⁻ corresponding to parent UniPR129; lower trace: XIC in ESI⁻ at $m/z = 751.42$ [M-H]⁻ corresponding to the glucuronide.



b. Experimental high resolution mass values and isotopic distribution in ESI⁻ for metabolite **M12** (upper) if compared to calculated value (lower spectrum).

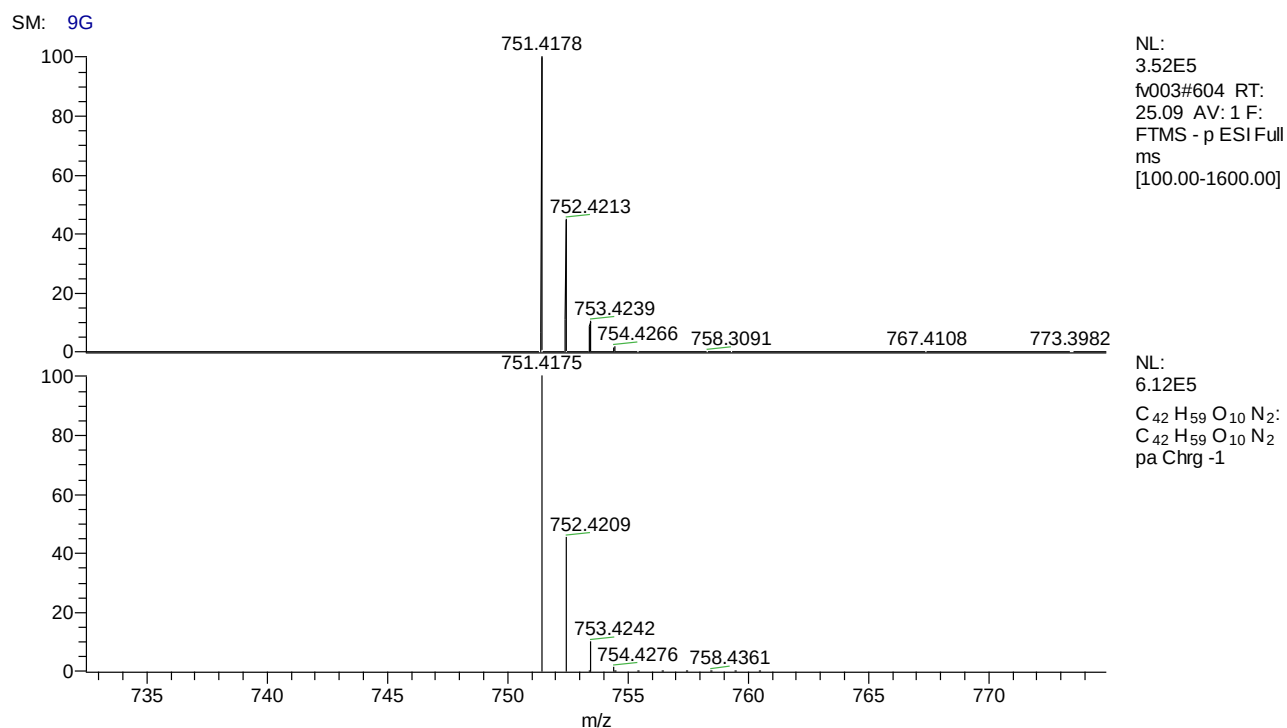
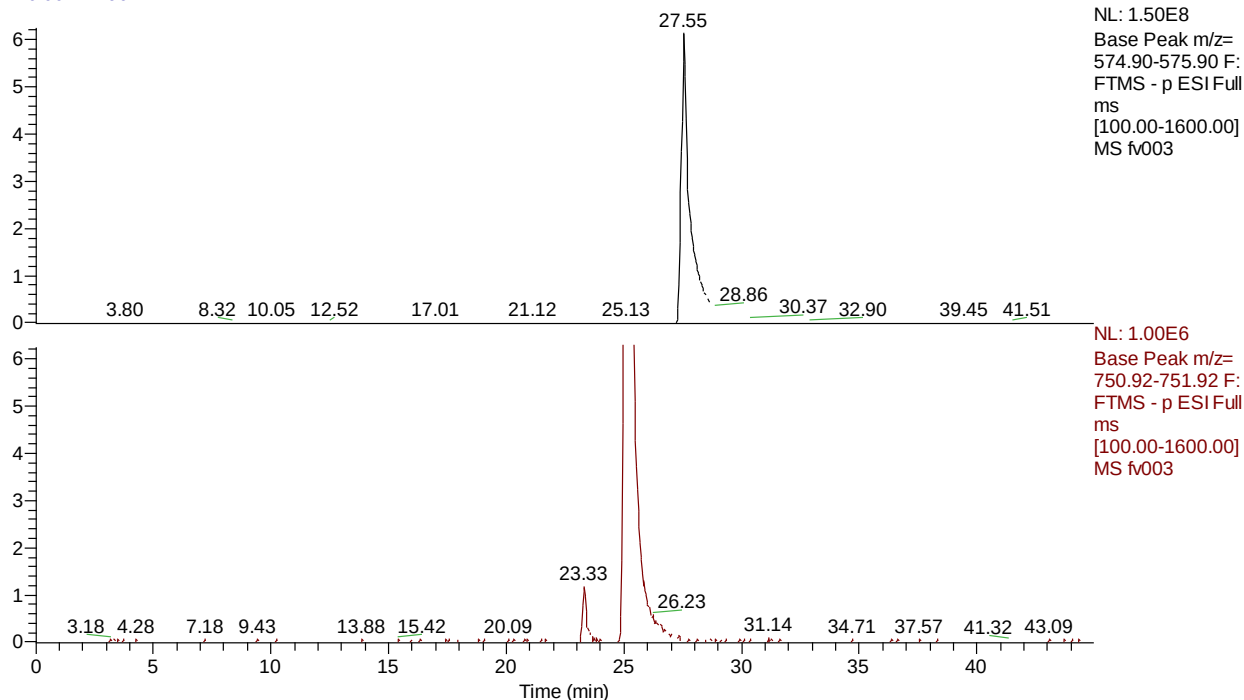


Figure S14. a-b. Metabolite M13 in MLS₉ fraction.

a. Phase II conjugate of UniPR129 with glucuronic acid **M13** in mouse liver microsomes. Upper trace: Extracted ion chromatogram (XIC) in ESI⁻ at $m/z = 575.40$ [M-H]⁻ corresponding to parent UniPR129; lower trace: XIC in ESI⁻ at $m/z = 751.42$ [M-H]⁻ corresponding to the conjugate with glucuronic acid.

RT: 0.00 - 44.96



b. Experimental high resolution mass values and isotopic distribution in ESI⁻ for metabolite **M13** (upper) if compared to calculated value (lower spectrum).

SM: 9G

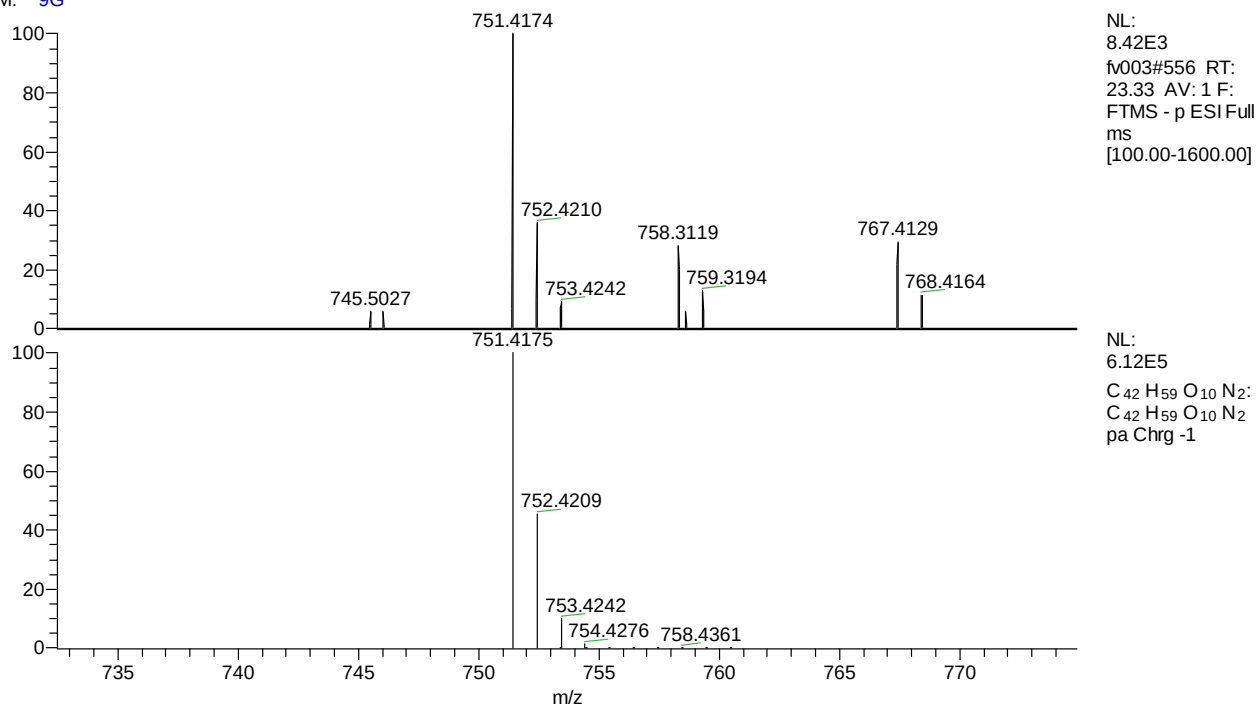
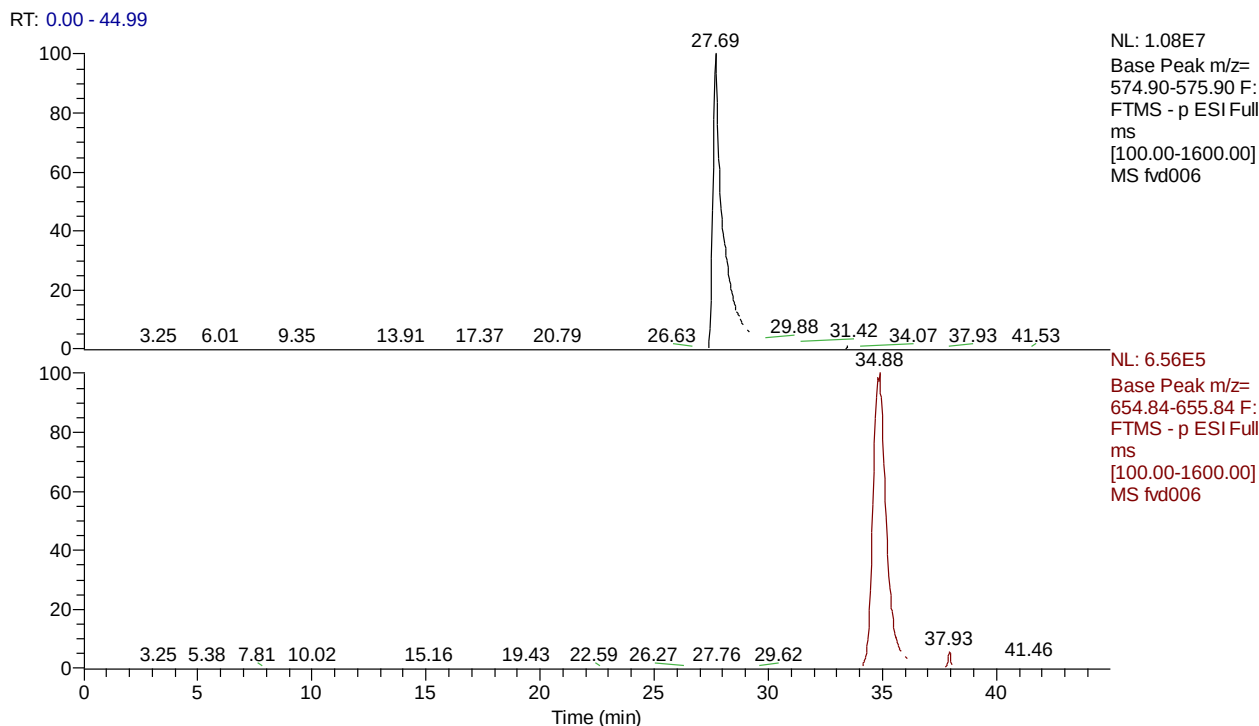
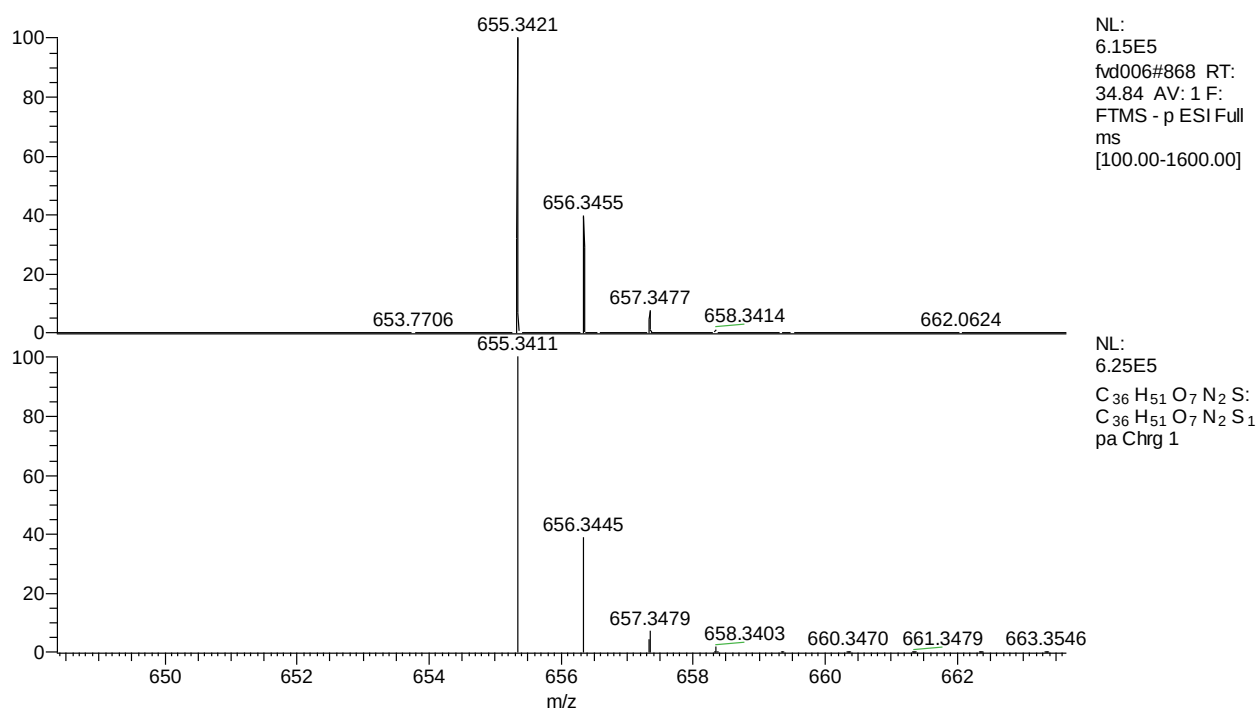


Figure S15. a-b. Metabolite M14 in HLS₉ fraction.

a. Phase II conjugate of UniPR129 with active sulphate **M14** in human liver S₉ fraction. Upper trace: Extracted ion chromatogram (XIC) in ESI⁻ at $m/z = 575.40$ [M-H]⁻ corresponding to parent UniPR129 ; lower trace: XIC in ESI⁻ at $m/z = 655.34$ [M-H]⁻ corresponding to the conjugate with active sulphate.



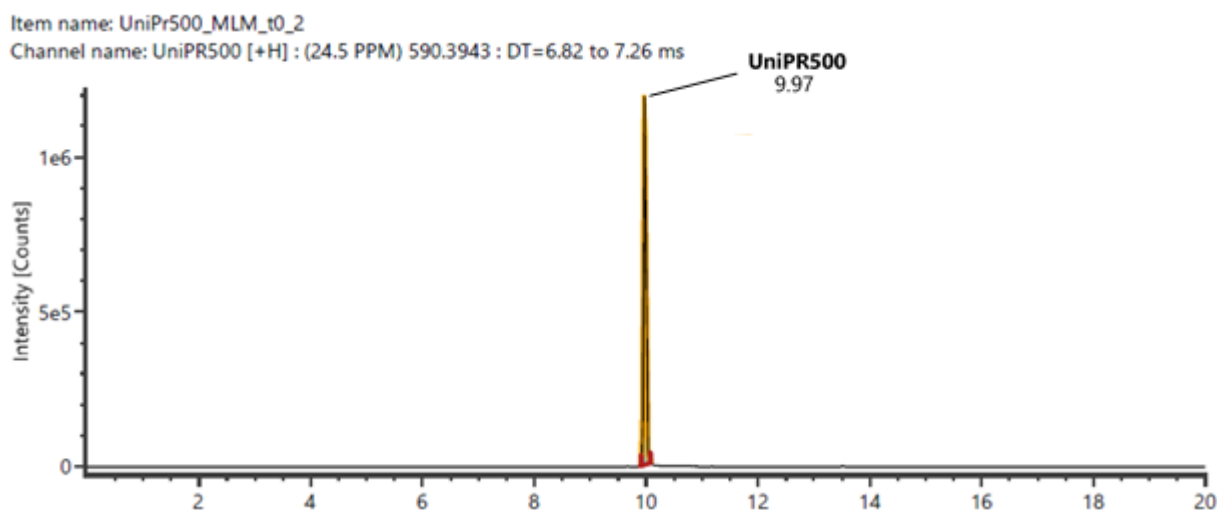
b. Experimental high resolution mass values and isotopic distribution in ESI⁻ for metabolite **M14** (upper) if compared to calculated value (lower spectrum).



S.8 UniPR500 *In vitro* phase I metabolite profiling

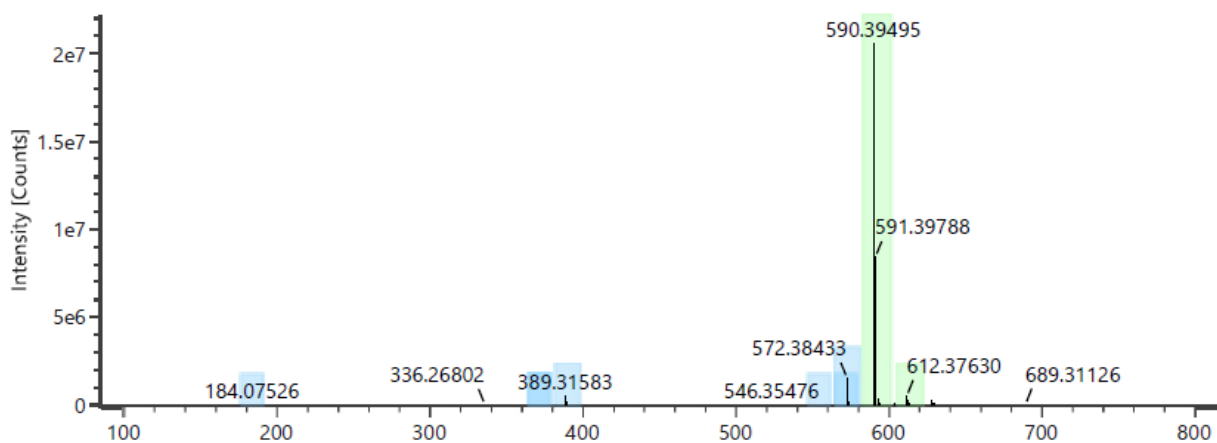
Figure S16. a-c. Parent compound UniPR500 in MLM.

a. UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 590.39$ [M+H]⁺ and RT = 9.97 min.

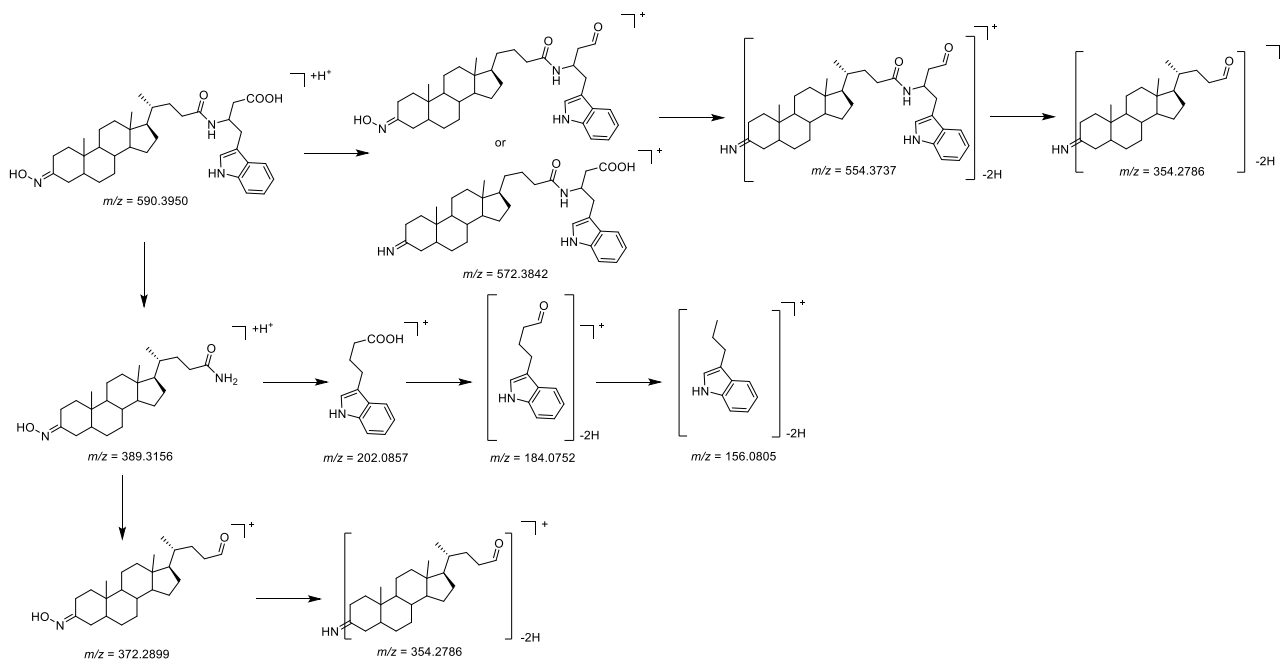


b. Experimental high resolution mass value in ESI⁺ for UniPR500.

Item name: UniPr500_MLM_t180_4 Channel name: Low energy : Time 9.9682 +/- 0.0212 minutes : Drift Times: 7.06 +/- 0.22, 7.32 +/- 0.22 ms
Item description:



c. High resolution high energy (MS^E) MS spectrum in ESI^+ of UniPR500 together with tentative fragmentation pattern. The most intense signal, other than that of molecular ion, is represented by $m/z = 572.4$ that accounts for loss of water (-18). A peculiar ion fragment at $m/z = 389.3$ is compatible with the loss of the 4-(indol-3-yl)-butanoyl group, liberated as the $m/z = 202.1$ ion. The ion triad $m/z = 202.1$, 184.1 and 156.1, already reported for UniPR129, is found in the MS/MS spectrum of several metabolites of UniPR500.



Component name: UniPR500, Observed m/z : 590.3950,
Observed RT (min): 9.97

Channel name: product ions from precursor ion id = 0

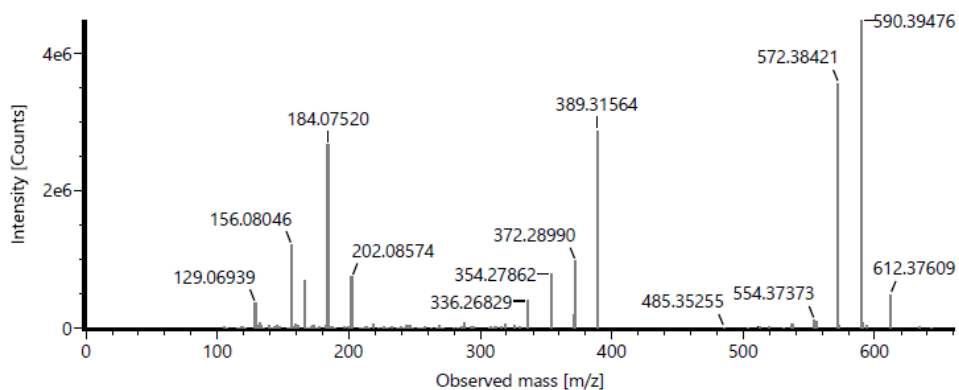
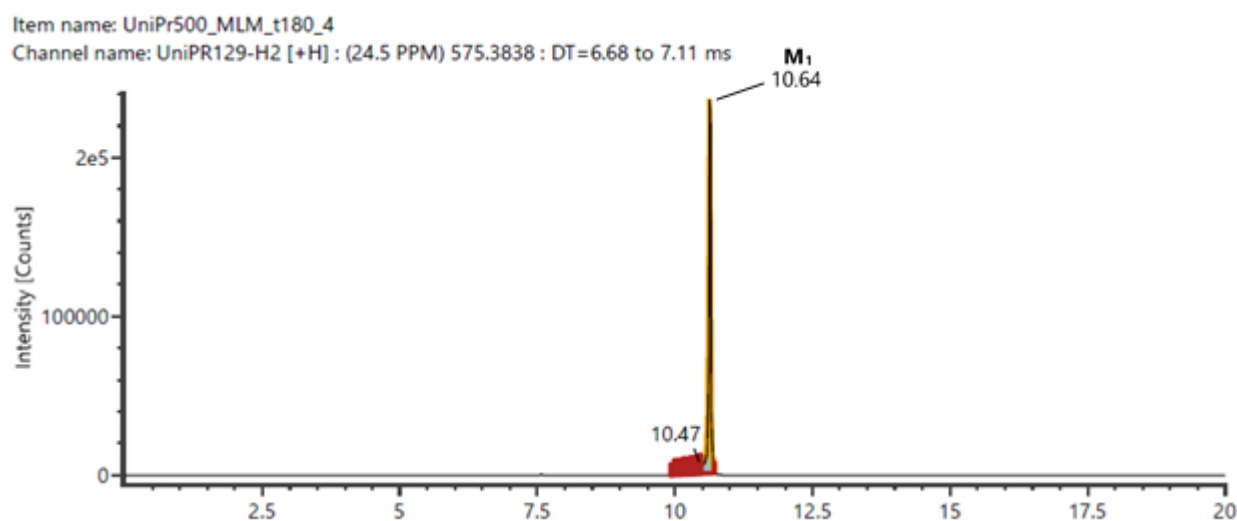


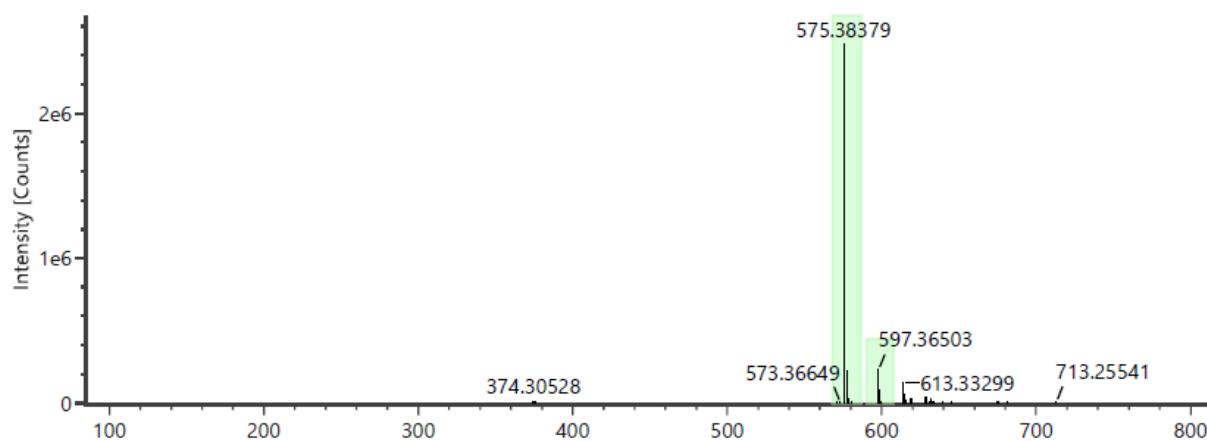
Figure S17. a-d. Metabolite M1 in MLM.

a. Phase I Metabolite **M1** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 575.38$ [M+H]⁺ and RT = 10.64 min.

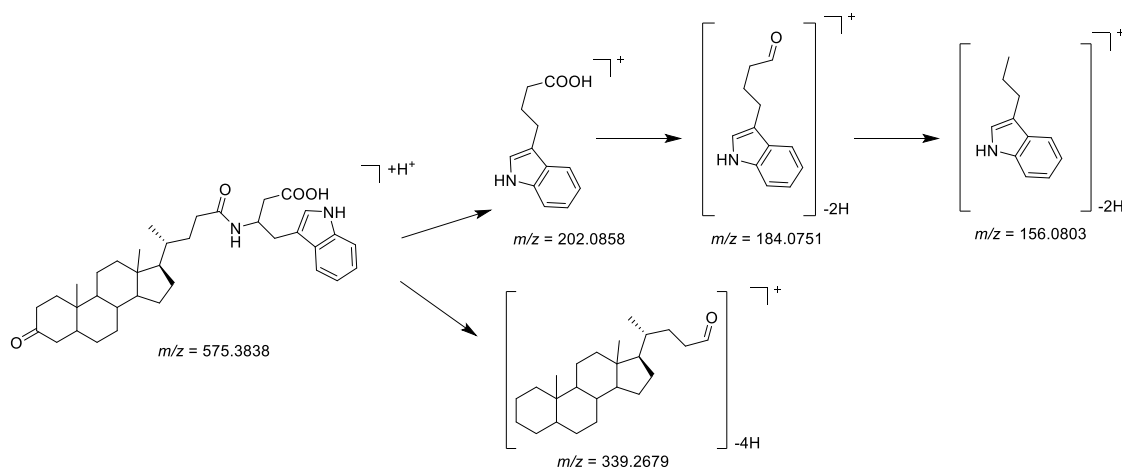


b. Experimental high resolution mass value in ESI⁺ for metabolite **M1**.

Item name: UniPr500_MLM_t180_4 Channel name: Low energy : Time 10.6402 +/- 0.0212 minutes : Drift Times: 6.90 +/- 0.22, 7.21 +/- 0.22 ms
Item description:



c. High resolution high energy (MS^E) MS spectrum in ESI^+ of 3-keto metabolite **M1** together with tentative fragmentation pattern. Considering the exact mass, the RT and the MS/MS spectrum, this metabolite reasonably corresponds to the M1 reported for UniPR129.



Component name: UniPR129-H2, Observed m/z : 575.3838, Channel name: product ions from precursor ion id = 8
Observed RT (min): 10.64

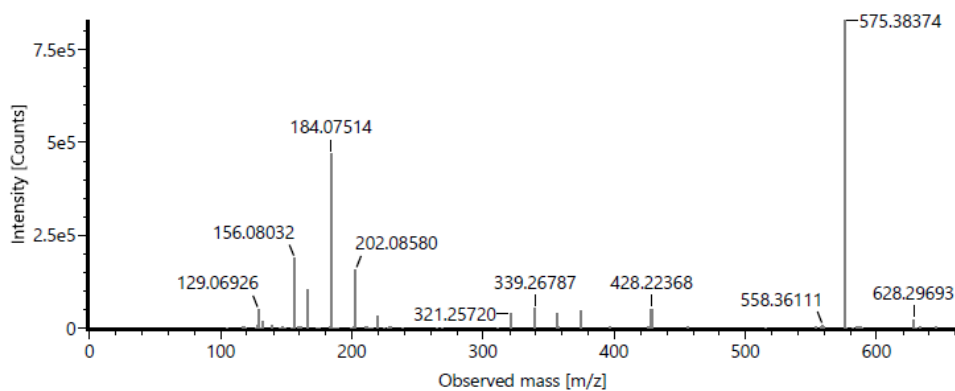
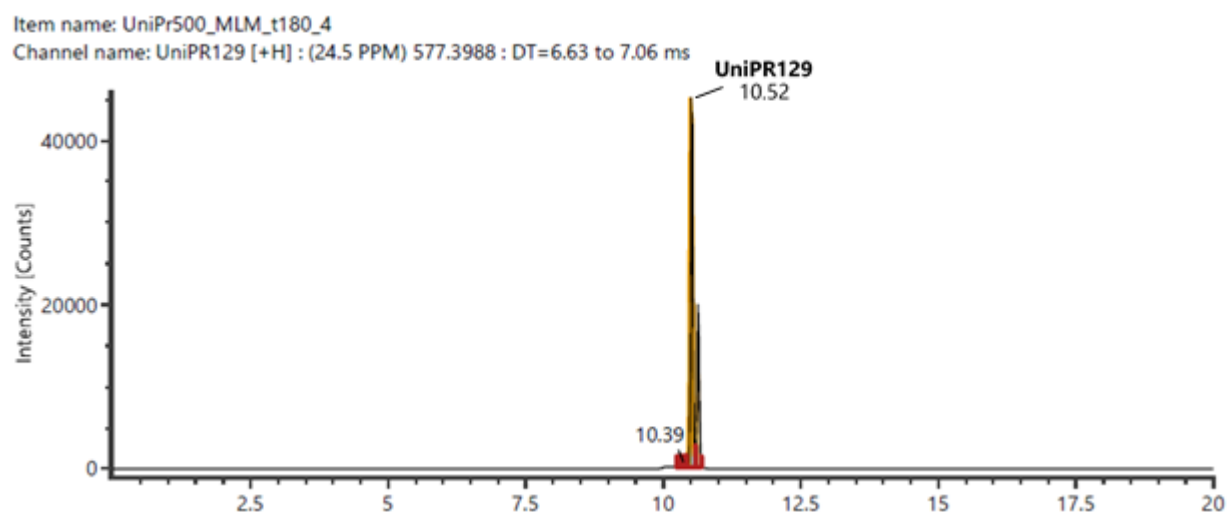


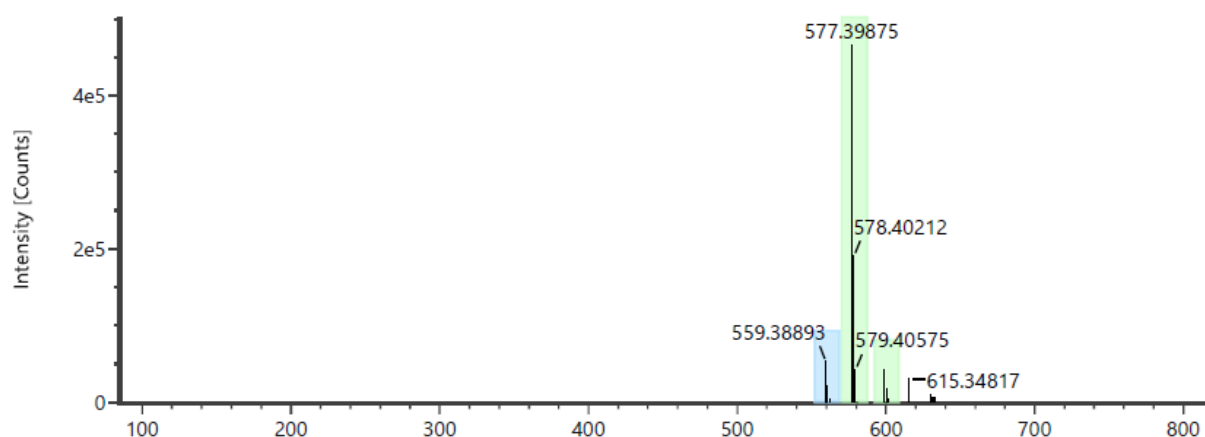
Figure S18. *a-c. UniPR129 derived from UniPR500 in MLM.*

a. UniPR129 derived from UniPR500 in mouse liver microsomal incubations. Extracted ion chromatogram (XIC) in ESI⁺ at m/z = 577.40 [M+H]⁺ and RT = 10.52 min.

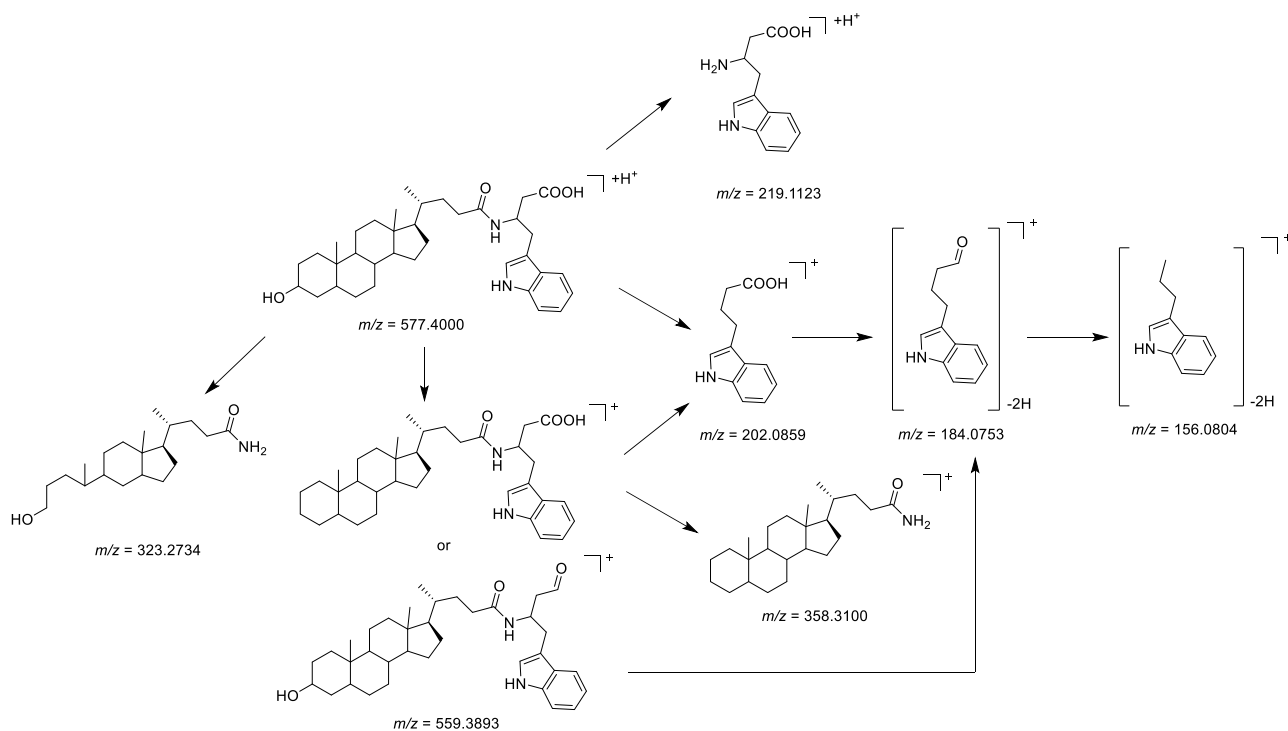


b. Experimental high resolution mass value in ESI⁺ for UniPR129.

Item name: UniPr500_MLM_t180_4 Channel name: Low energy : Time 10.5202 +/- 0.0212 minutes : Drift Times: 6.84 +/- 0.22, 7.17 +/- 0.22 ms
Item description:



c. High resolution high energy (MS^E) MS spectrum in ESI^+ of UniPR129 together with tentative fragmentation pattern.



Component name: UniPR129, Observed m/z : 577.4000,
Observed RT (min): 10.52

Channel name: product ions from precursor ion id = 0

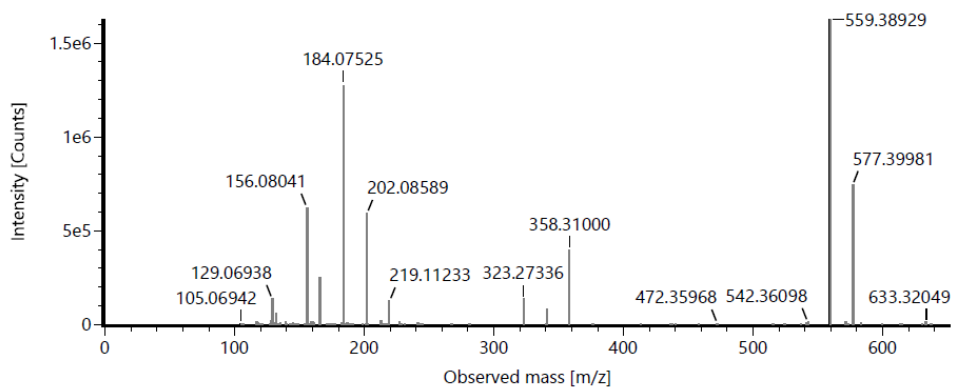
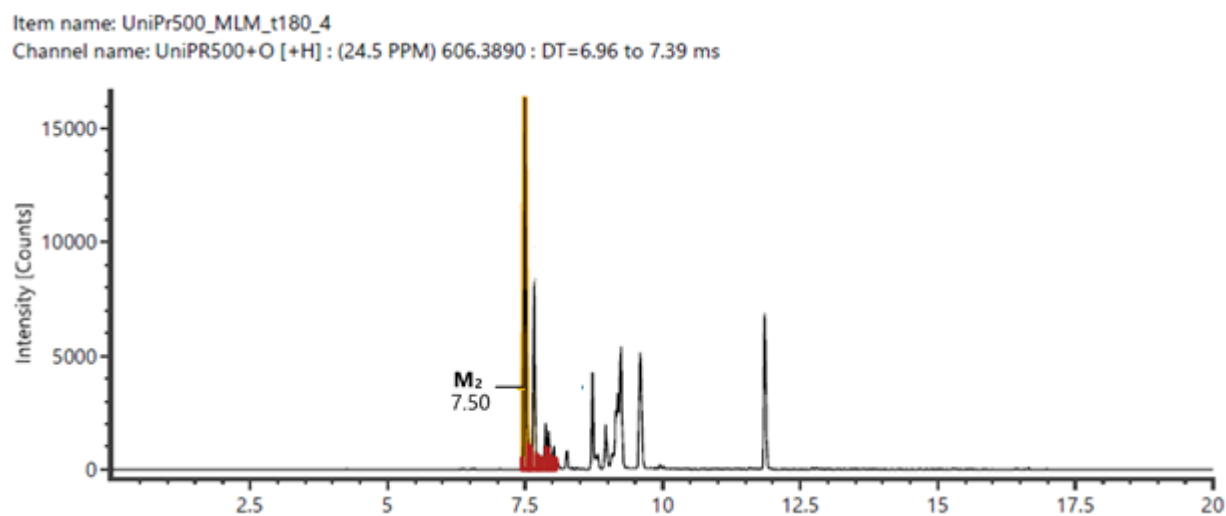
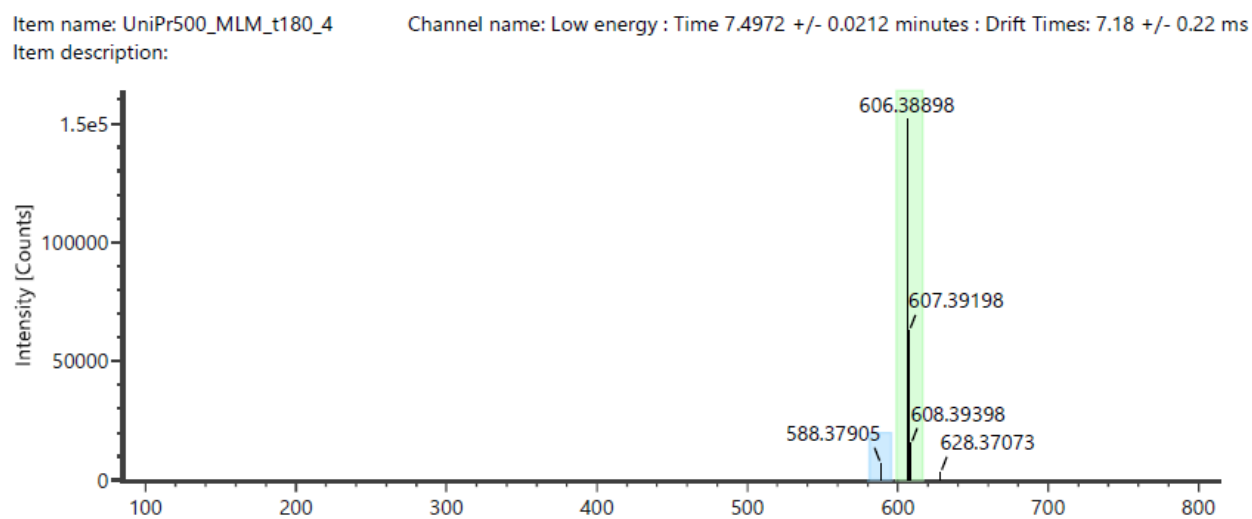


Figure S19. a-d. Metabolite **M2** in MLM.

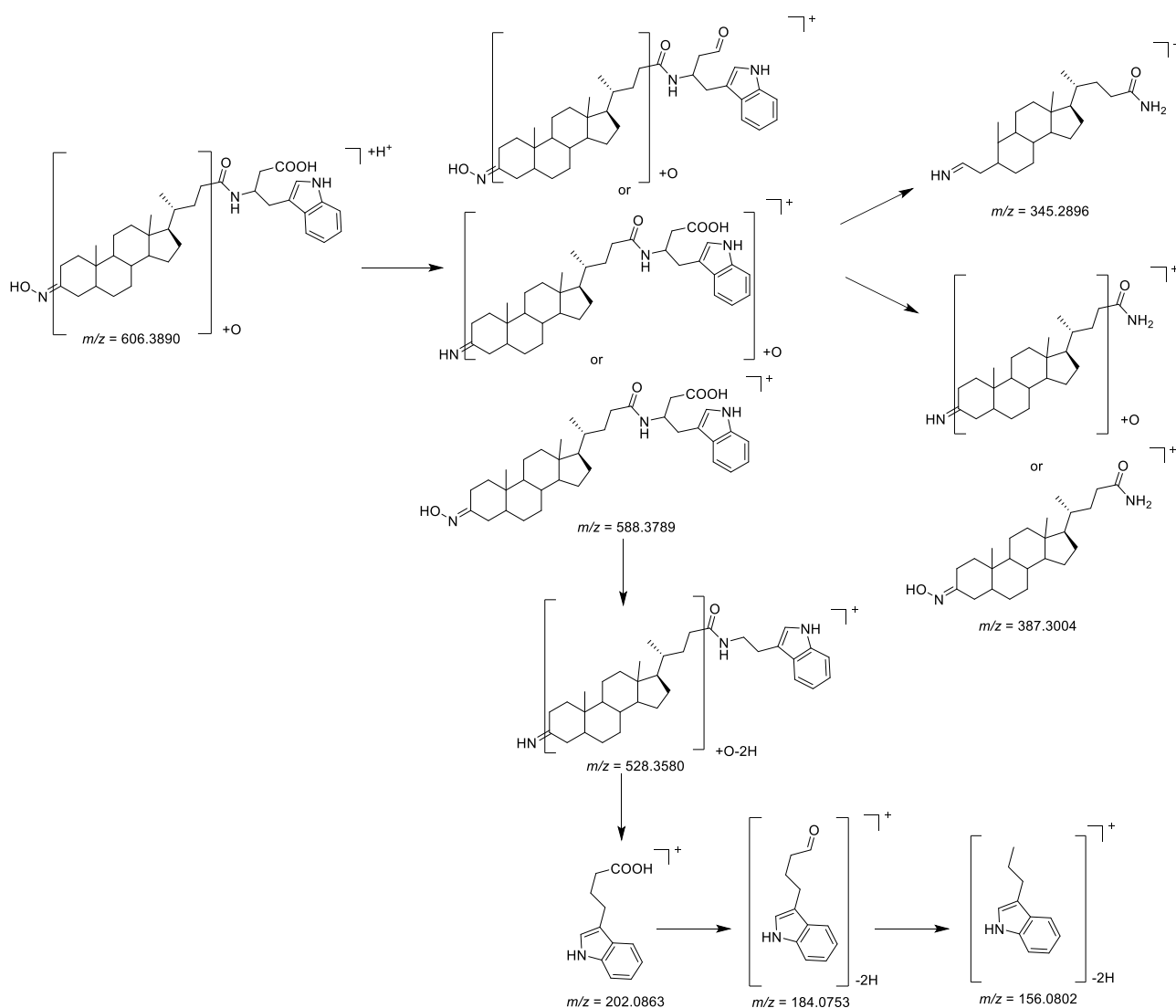
a. Phase I Metabolite **M2** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 606.39$ [M+H]⁺ and RT = 7.50 min.



b. Experimental high resolution mass value in ESI⁺ for **M2**.

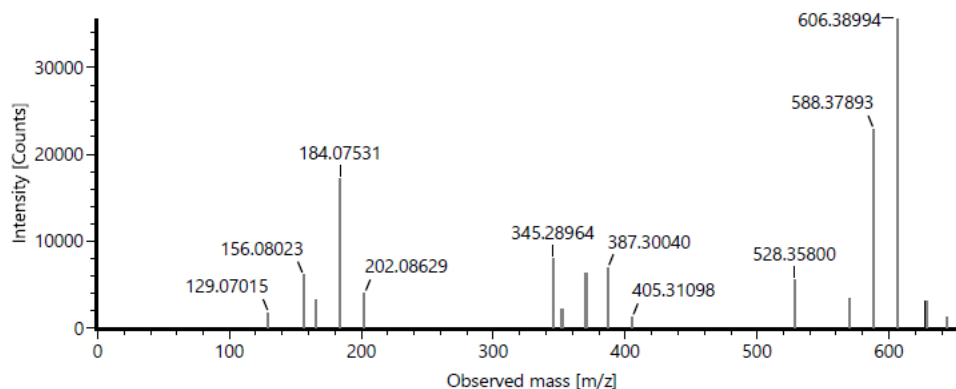


c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M2** together with tentative fragmentation pattern. The most intense fragment ion in the MS/MS spectrum is at $m/z = 588.4$, which could be due to a loss of water (-18); peak at $m/z = 528.36$ is compatible with the loss of the methyl-carboxy group and a water molecule (-60) with respect to $m/z = 588.4$. The further loss of the 4-(indol-3-yl)-butanoyl group could account for the generation of the peculiar fragment at $m/z = 345.2$, which, in turn, liberates the fragment ion at $m/z = 345.2$, probably by losing a hydroxy-ethyl group from the A-ring of the steroidal core. These two product ions suggest oxidation to occur on C-1 or C-2 of the steroid.



Component name: UniPR500+O, Observed m/z:
606.3890, Observed RT (min): 7.50

Channel name: product ions from precursor ion id = 208



d. Metabolic soft spots on UniPR500 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M2**, UNIFI software returns as the most likely soft spots C-1-23 of the steroidal core.

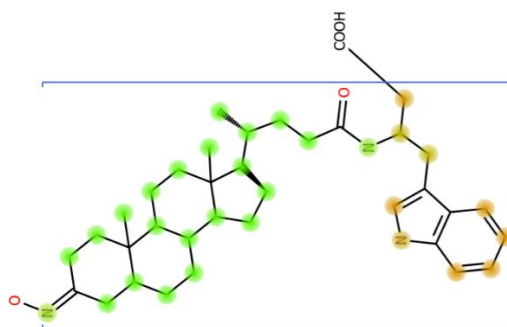
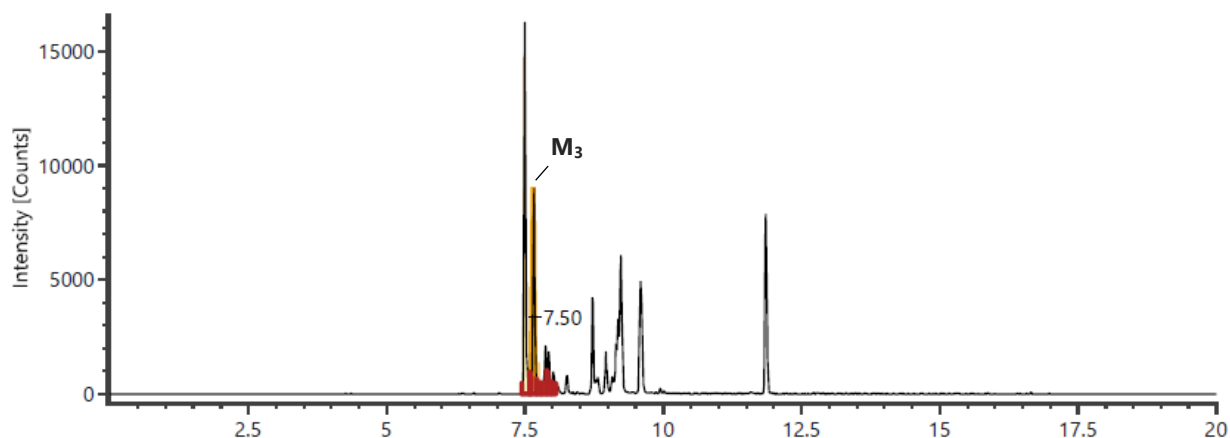


Figure S20. a-d. Metabolite **M3** in MLM.

a. Phase I Metabolite **M3** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 606.39$ [M+H]⁺ and RT = 7.66 min.

Item name: UniPr500_MLM_t180_4

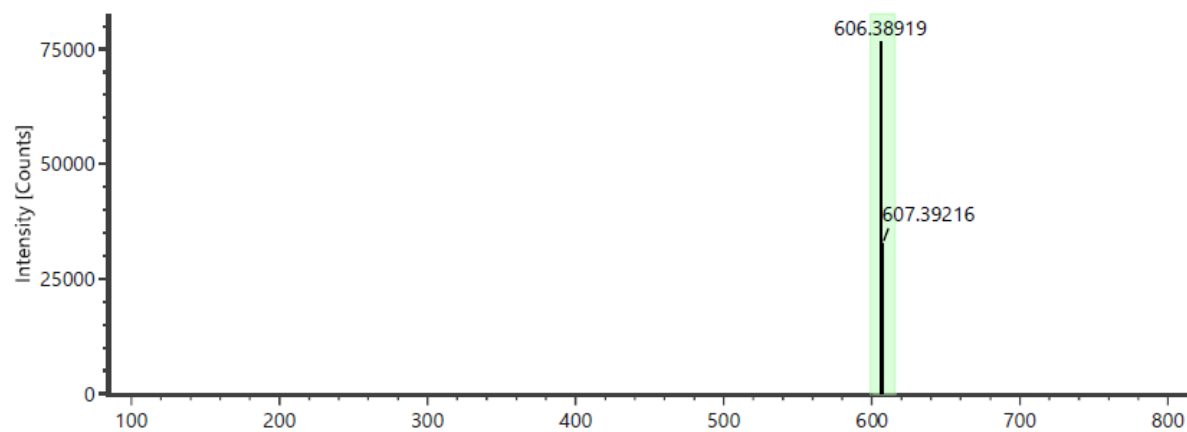
Channel name: UniPR500+O [+H] : (24.5 PPM) 606.3892 : DT=6.90 to 7.33 ms



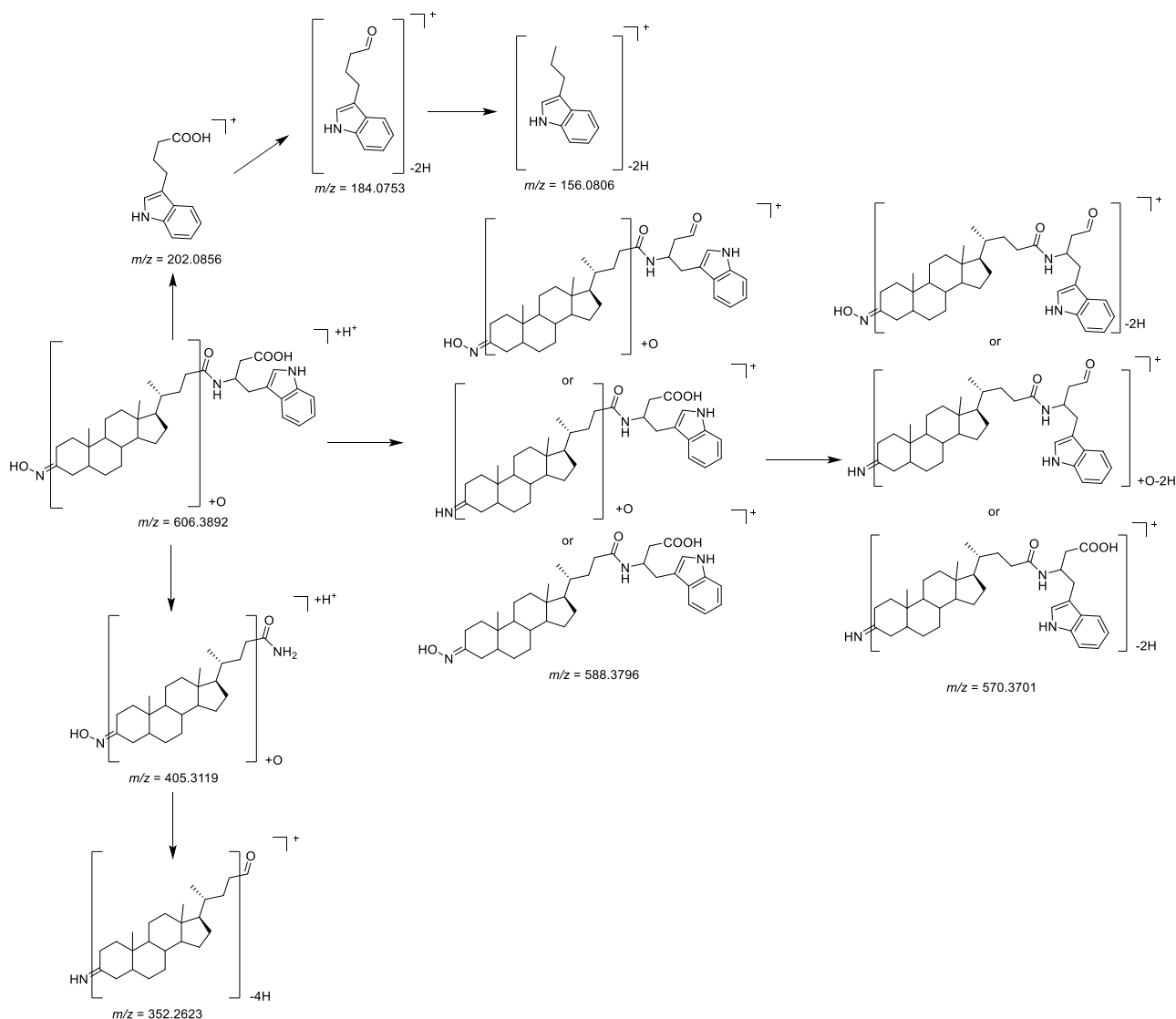
b. Experimental high resolution mass value in ESI⁺ for M3.

Item name: UniPr500_MLM_t180_4
Item description:

Channel name: Low energy : Time 7.6649 +/- 0.0212 minutes : Drift Times: 7.11 +/- 0.22 ms

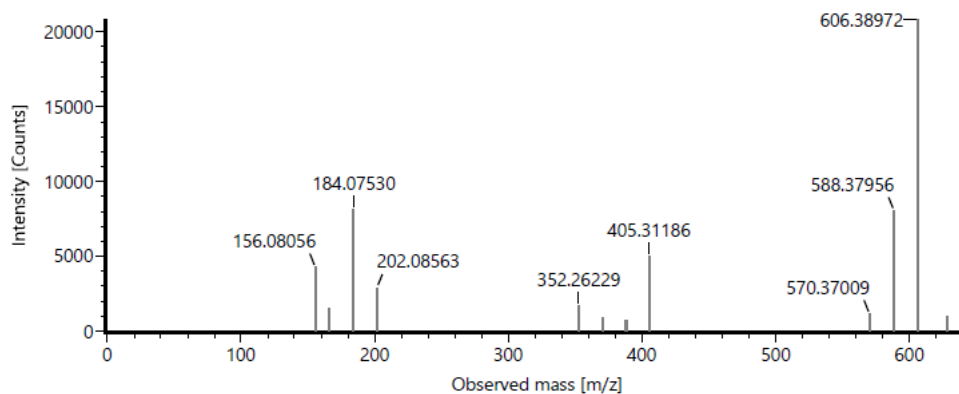


c. High resolution high energy (MS^E) MS spectrum in ESI^+ of **M3** together with tentative fragmentation pattern. Fragment ions at $m/z = 588.4$ and 570.4 have already been reported for **M3**. A characteristic peak is found in the MS/MS spectrum at $m/z = 405.3$, which could account for the loss of the 4-(indol-3-yl)butanoxy fragment from 570.4 , and suggests oxidation to occur on the steroidal moiety. Another peculiar ion peak is at $m/z = 352.3$, which is compatible with the loss of the amino group and two water molecules from 405.3 .



Component name: UniPR500+O, Observed m/z:
606.3892, Observed RT (min): 7.66

Channel name: product ions from precursor ion id = 415



d. Metabolic soft spots on UniPR500 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M3**, UNIFI software returns as the most likely soft spots C-1-23 of the steroidal core, likewise **M2**.

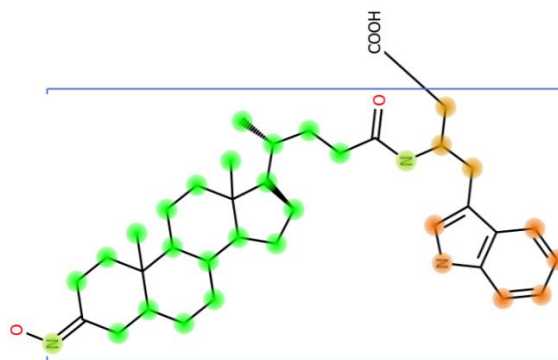
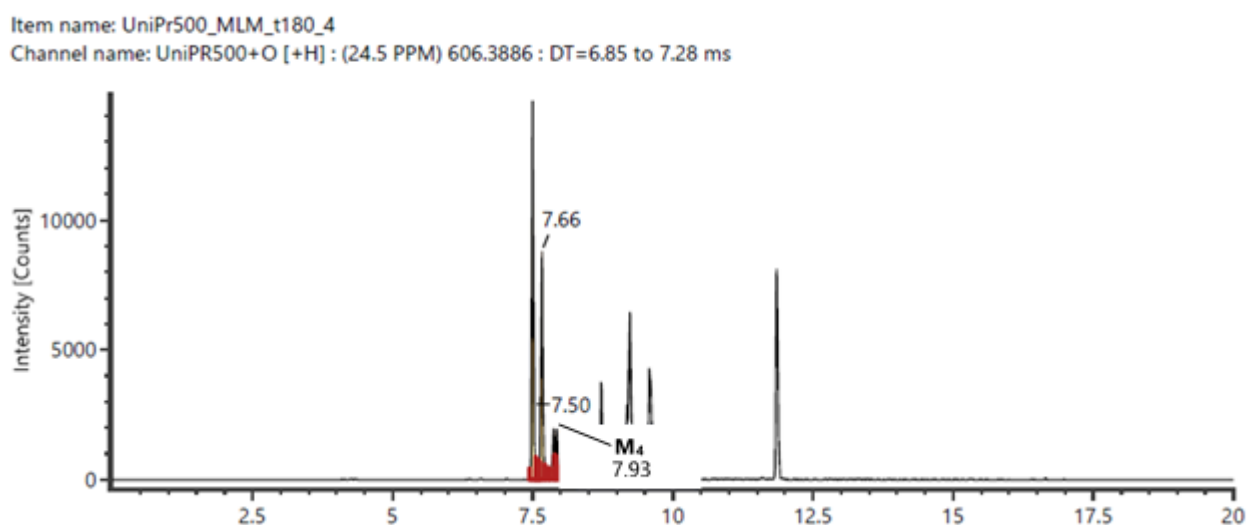
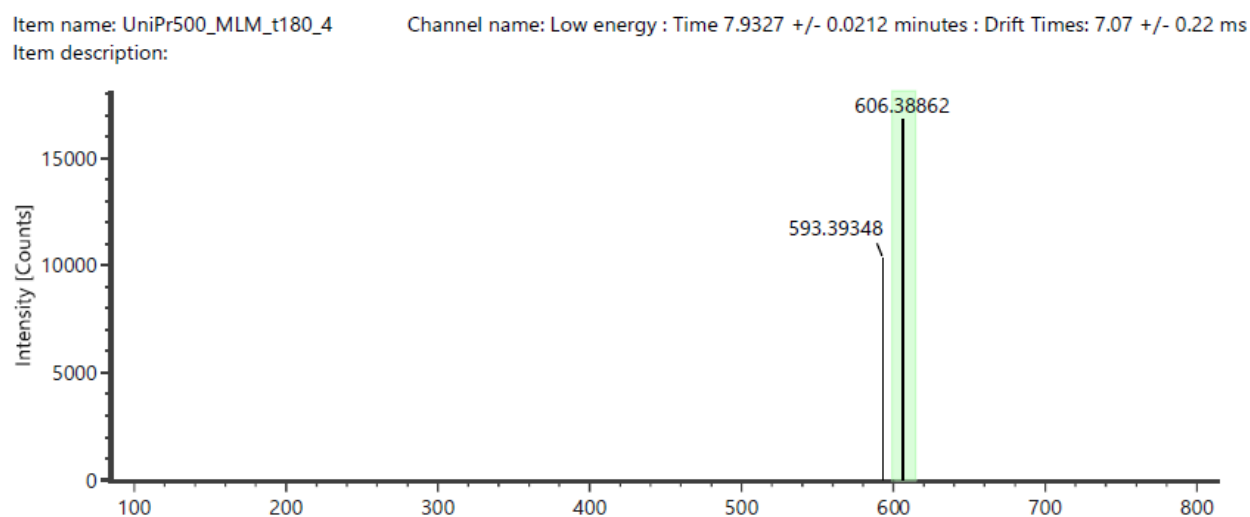


Figure S21. *a-d. Metabolite M4 in MLM.*

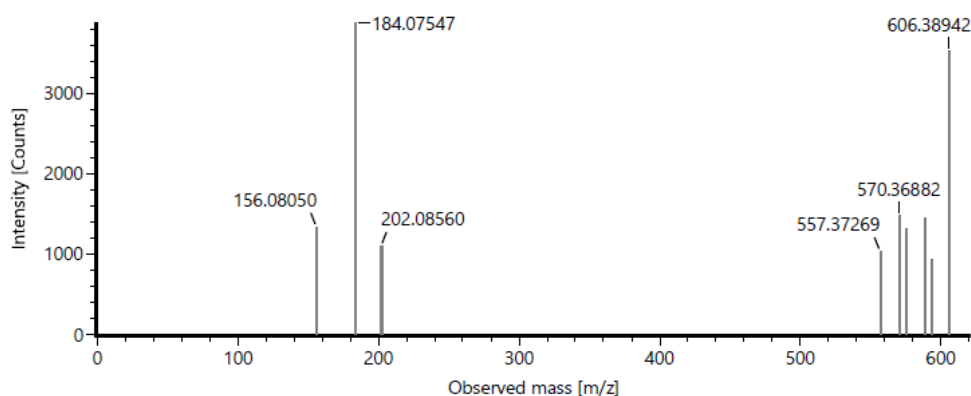
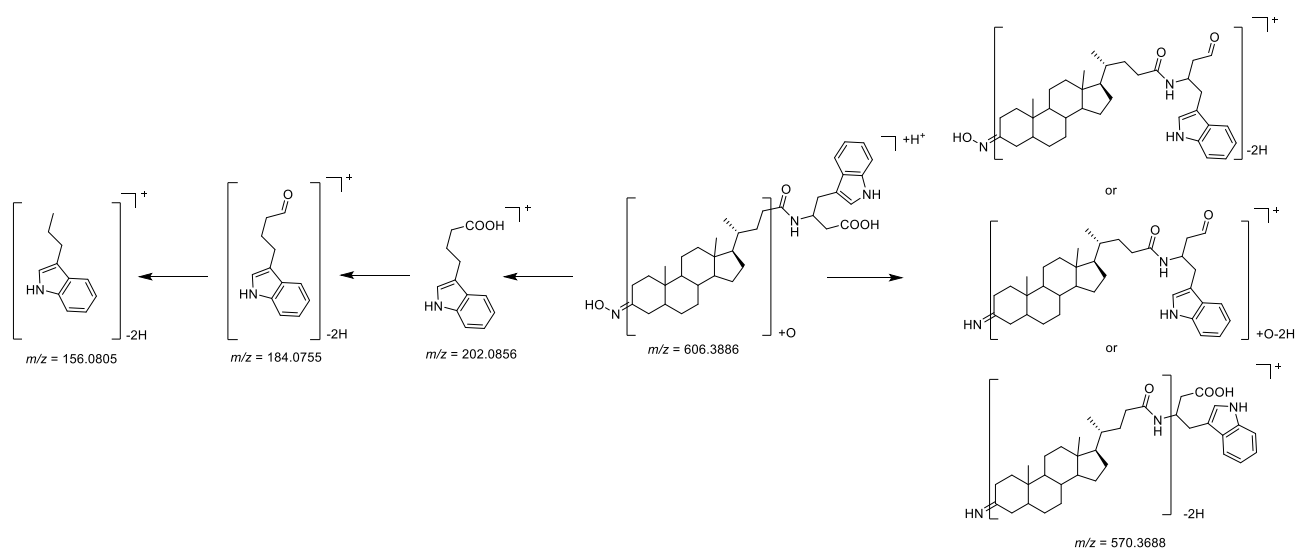
a. Phase I Metabolite **M4** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 606.39$ [M+H]⁺ and RT = 7.93 min.



b. Experimental high resolution mass value in ESI⁺ for **M4**.



c. High resolution high energy (MS^E) MS spectrum in ESI^+ of **M4** together with tentative fragmentation pattern. The characteristic ion of the MS/MS spectrum is at $m/z = 557.4$, possibly deriving from $m/z = 570.5$ after loss of the oxime moiety (-31) and a water molecule (-18).



d. Metabolic soft spots on UniPR500 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M4**, UNIFI software returns as the most likely soft spots C-1, C-2 and C-4-23 of the steroidal core.

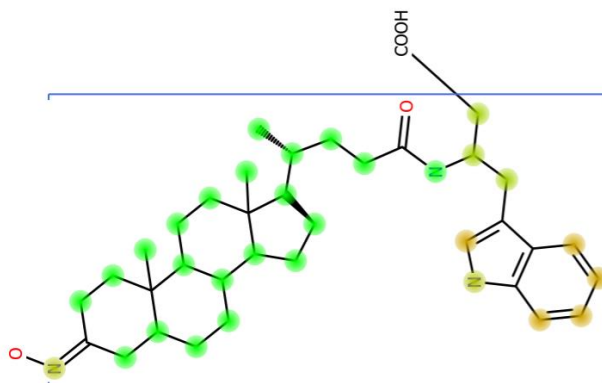
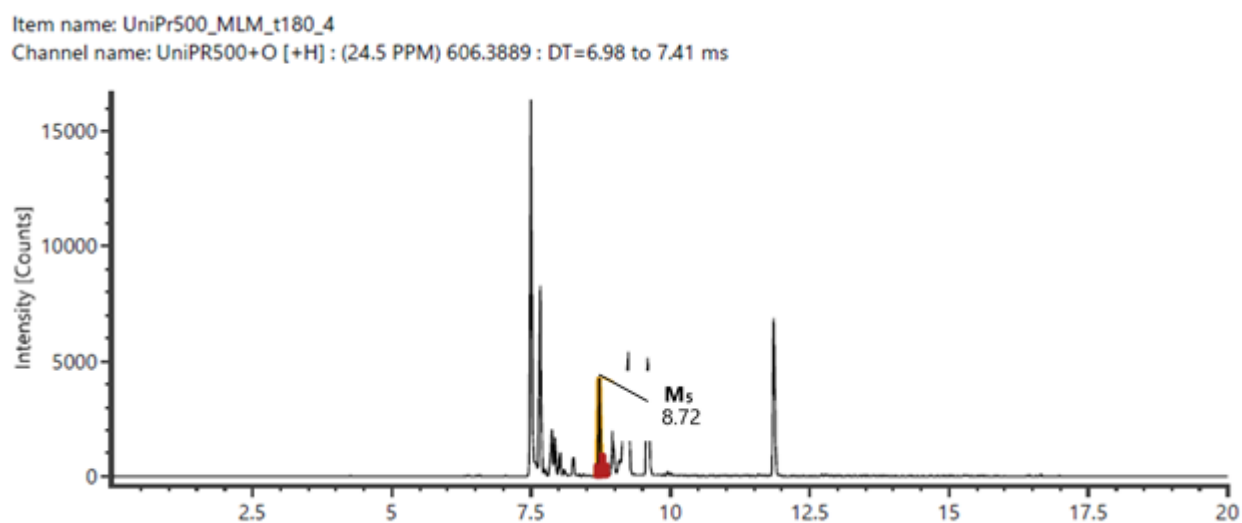


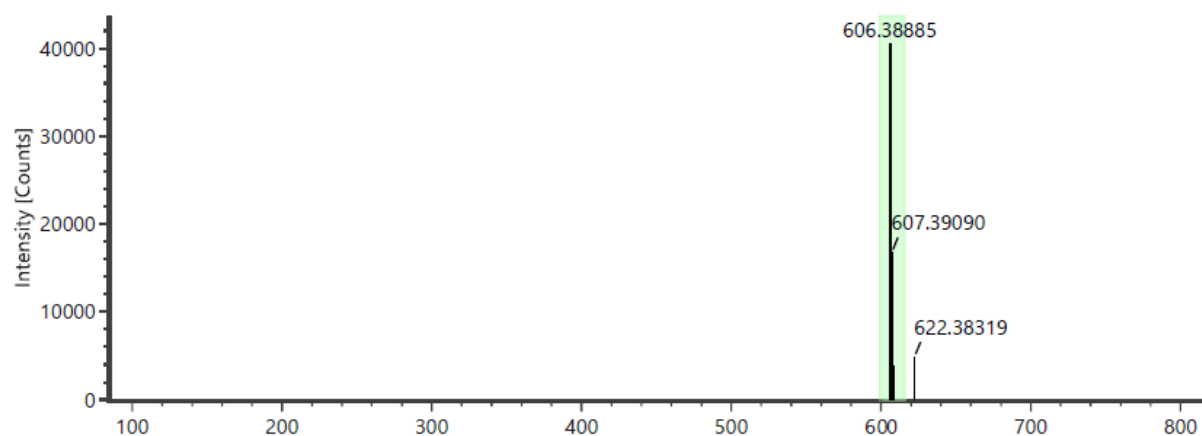
Figure S22. a-d. Metabolite **M5** in MLM.

a. Phase I Metabolite **M5** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 606.39$ [M+H]⁺ and RT = 8.72 min.

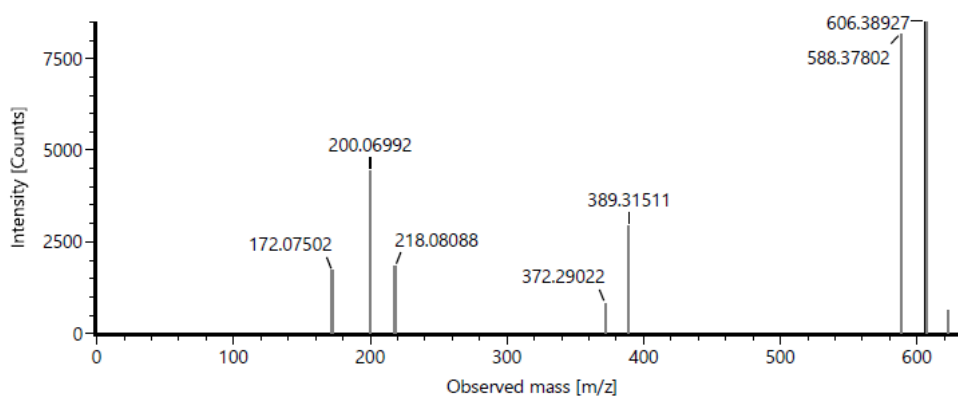
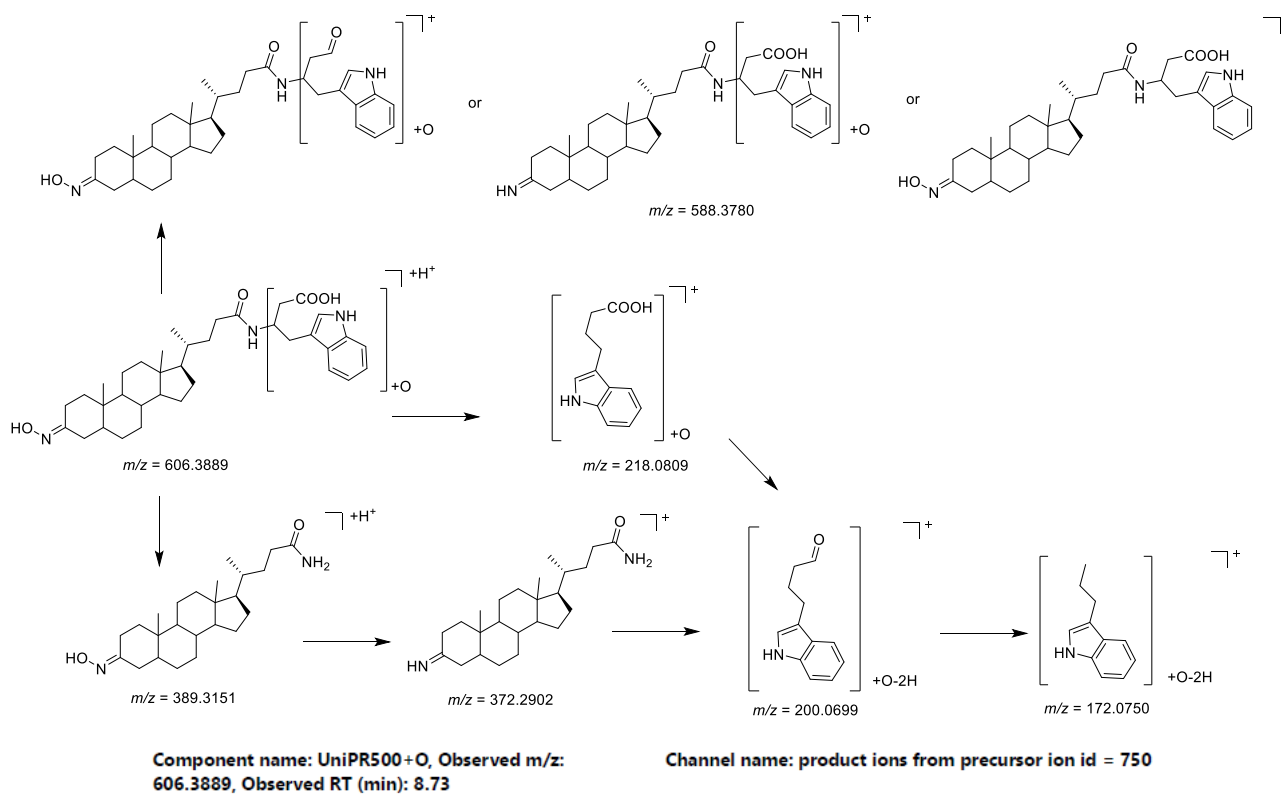


b. Experimental high resolution mass value in ESI⁺ for **M5**.

Item name: UniPr500_MLM_t180_4 Channel name: Low energy : Time 8.7266 +/- 0.0212 minutes : Drift Times: 7.19 +/- 0.22 ms
Item description:



c. High resolution high energy (MS^E) MS spectrum in ESI^+ of **M5** together with tentative fragmentation pattern. While ion peaks at $m/z = 588.4$, 389.3, 372.3 and 172.07 have been previously described for M3, two peculiar fragment ions are present in the MS/MS spectrum at $m/z = 218.1$, which is compatible with the 4-(indol-3-yl)butanoic acid carrying and hydroxyl group; and at $m/z = 200.1$, which accounts for a loss of water of the previous one. Those two ion signals, together with the one at $m/z = 172.07$, suggest oxydation to occur on the L- β -homotryptophan.



d. Metabolic soft spots on UniPR500 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M5**, UNIFI software returns as the most likely soft spots the C atoms of the propyl-indolic moiety of UniPR500.

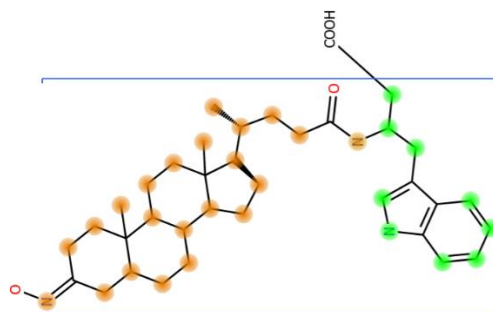
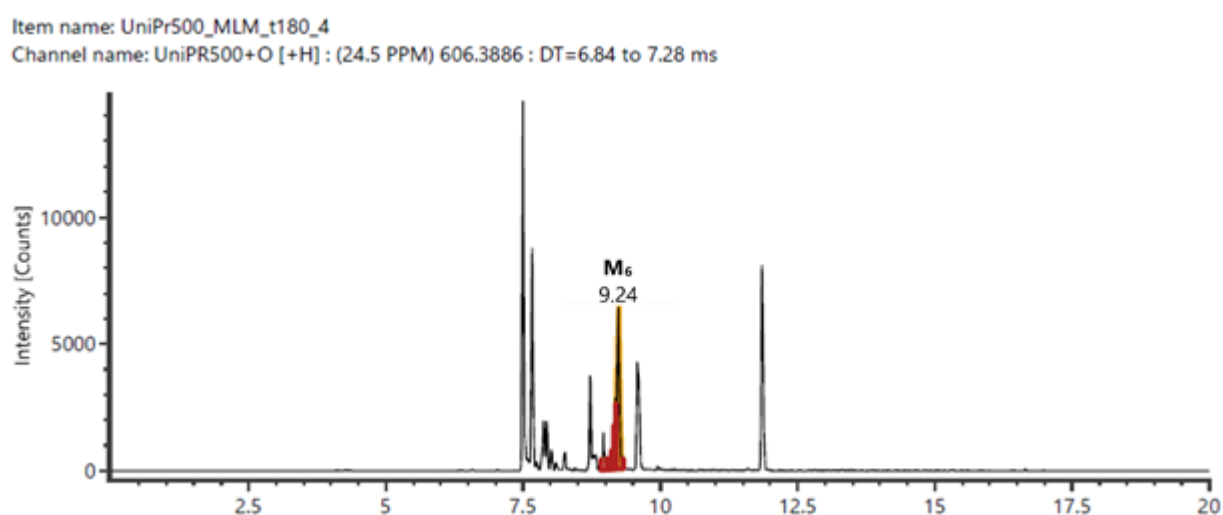


Figure S23. a-d. Metabolite **M6** in MLM.

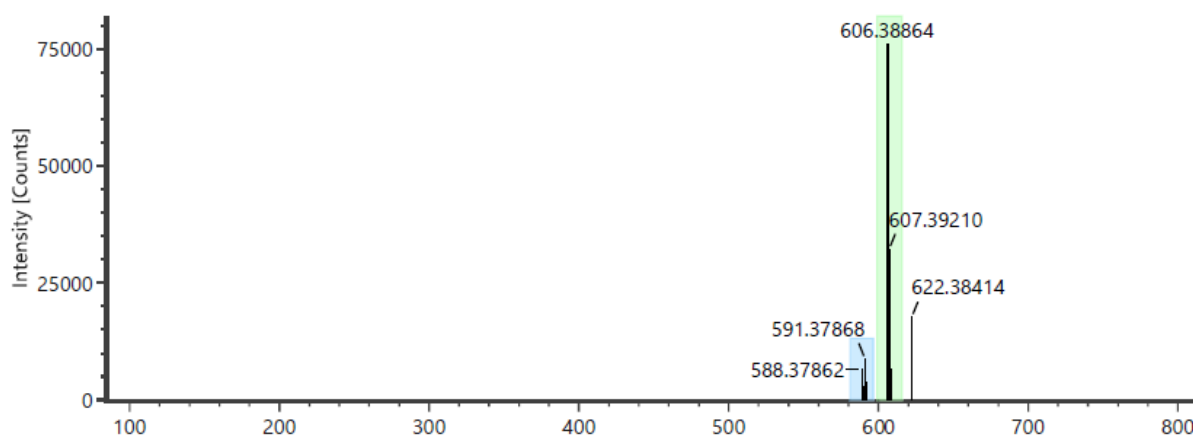
a. Phase I Metabolite **M6** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 606.39$ [M+H]⁺ and RT = 9.24 min.



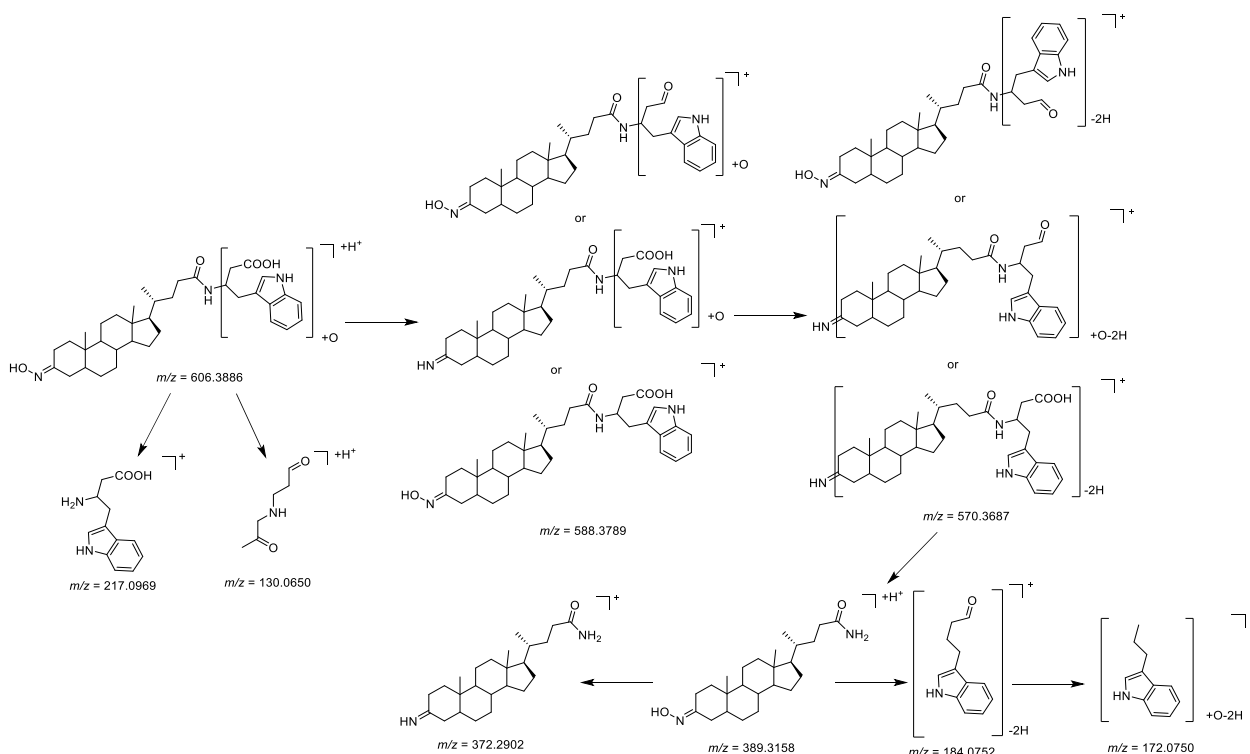
b. Experimental high resolution mass value in ESI⁺ for M6.

Item name: UniPr500_MLM_t180_4
Item description:

Channel name: Low energy : Time 9.2355 +/- 0.0212 minutes : Drift Times: 7.06 +/- 0.22 ms

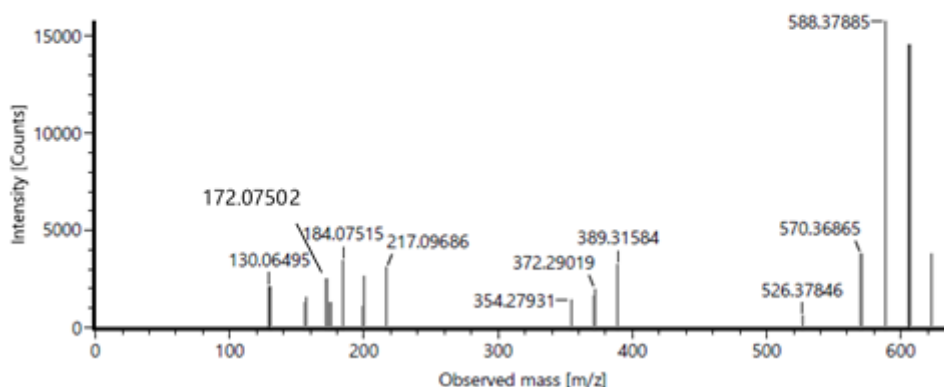


c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of M6 together with tentative fragmentation pattern. The base peak at $m/z = 588.4$ is compatible with dehydration (-18); fragment ion at $m/z = 570.4$ probably derived from the further loss of water and corresponds to the $m/z = 575.4$ reported for UniPR129 M7. Product ions at $m/z = 389.3$, 372.3 and 354.3 are shared with UniPR500, while peaks at $m/z = 217.1$ and 172.1 - the latter accounting for oxidation on the 3-propyl-indole - are the same found in the MS/MS spectrum of UniPR129.



Component name: UniPR500+O, Observed m/z:
606.3886, Observed RT (min): 9.24

Channel name: product ions from precursor ion id = 418



d. Metabolic soft spots on UniPR500 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M6**, UNIFI software returns as the most likely soft spots the propyl-indolic moiety of UniPR129.

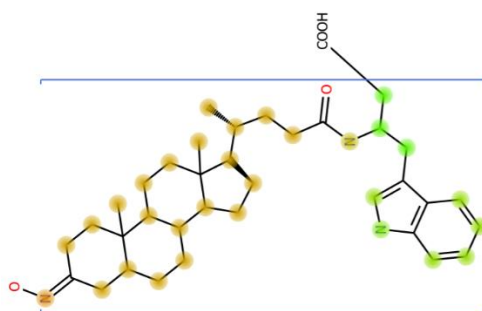
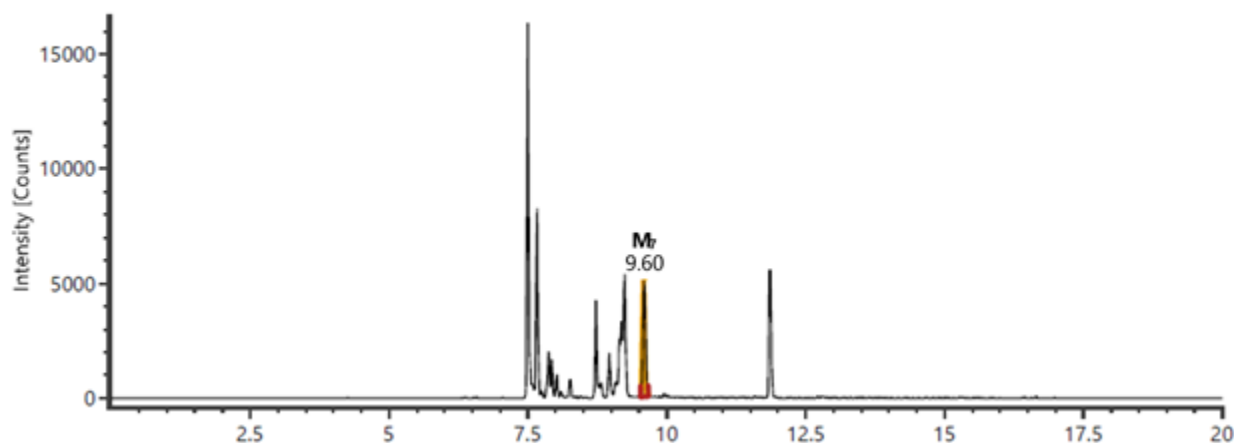


Figure S24. a-d. Metabolite **M7** in MLM.

a. Phase I Metabolite **M7** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 606.39$ [M+H]⁺ and RT = 9.60 min.

Item name: UniPr500_MLM_t180_4

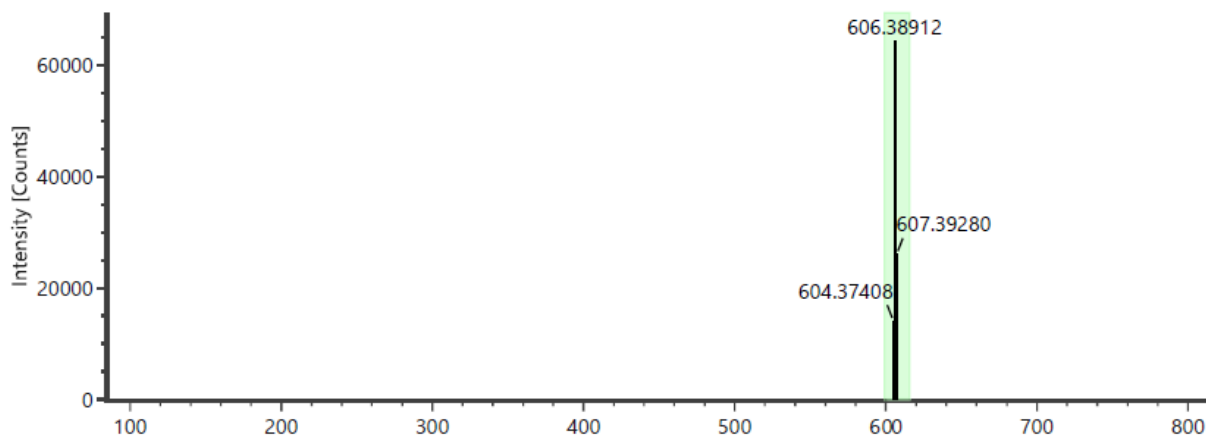
Channel name: UniPR500+O [+H] : (24.5 PPM) 606.3891 : DT=6.97 to 7.40 ms



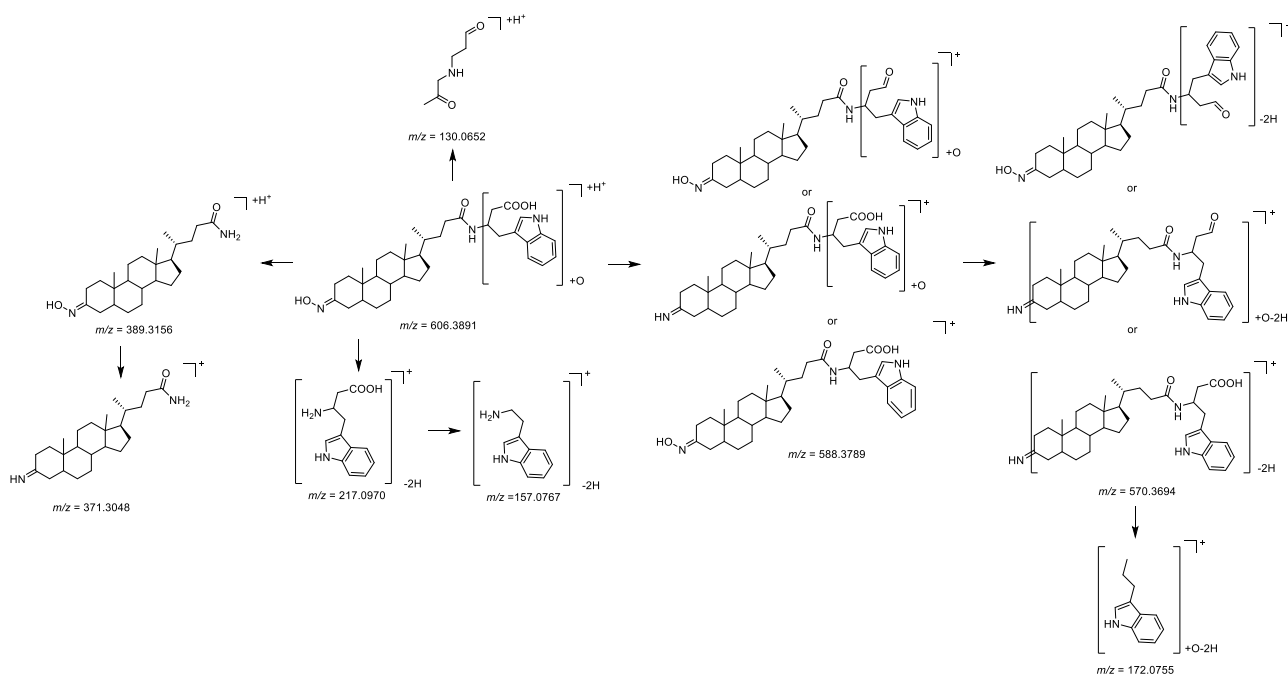
b. Experimental high resolution mass value in ESI⁺ for M7.

Item name: UniPr500_MLM_t180_4
Item description:

Channel name: Low energy : Time 9.5922 +/- 0.0212 minutes : Drift Times: 7.18 +/- 0.22 ms

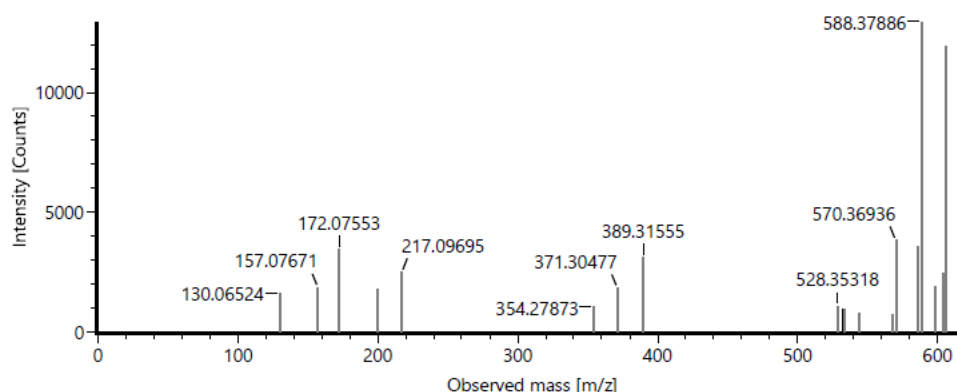


c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of M7 together with tentative fragmentation pattern. Ion peaks at m/z = 588.4, 570.4, 389.3, 217.1 and 130.1 were also found in M3 MS/MS spectrum. Peculiar fragment ion at m/z = 371.3 derived from m/z = 389.3 *via* dehydration; characteristic ion peak at m/z = 157.1 is compatible with the loss of a hydroxyl group (-17) from m/z = 172.1.



Component name: UniPR500+O, Observed m/z:
606.3891, Observed RT (min): 9.59

Channel name: product ions from precursor ion id = 500



d. Metabolic soft spots on UniPR500 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M7**, UNIFI software returns as the most likely soft spots (i.e. the propyl-indole) found for **M5**.

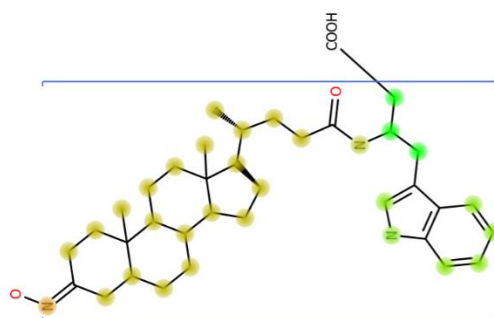
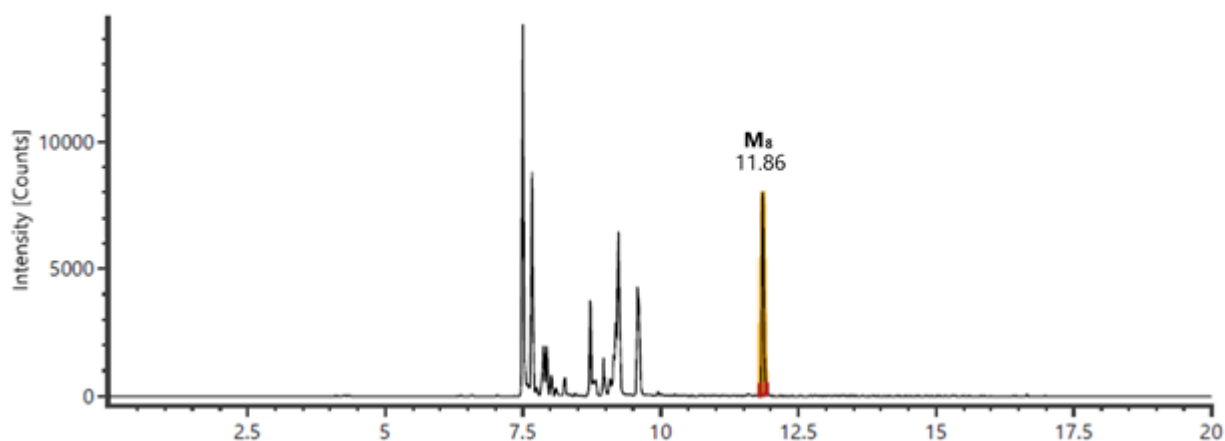


Figure S25. a-d. Metabolite **M8** in MLM.

a. Phase I Metabolite **M8** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 606.39$ [M+H]⁺ and RT = 11.86 min.

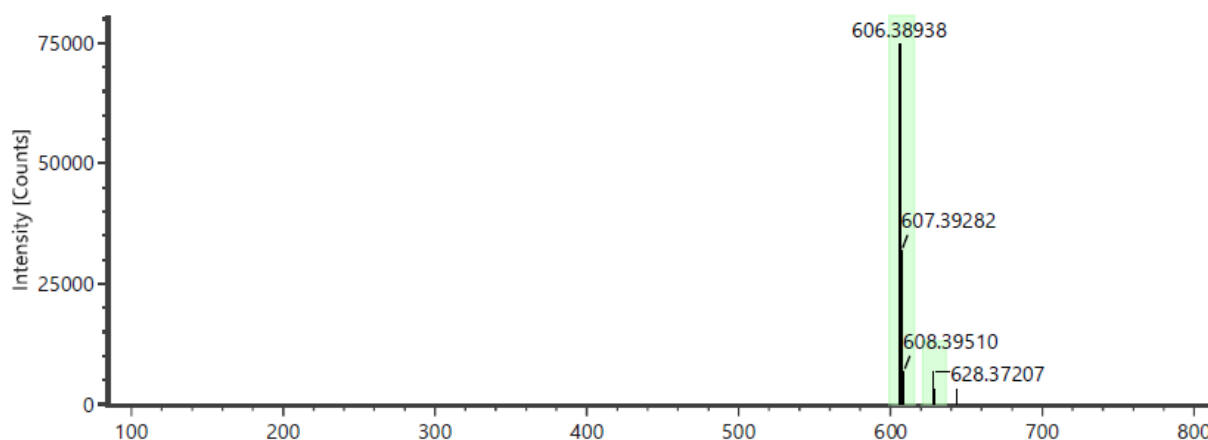
Item name: UniPr500_MLM_t180_4

Channel name: UniPR500+O [+H] : (24.5 PPM) 606.3894 : DT=6.86 to 7.29 ms

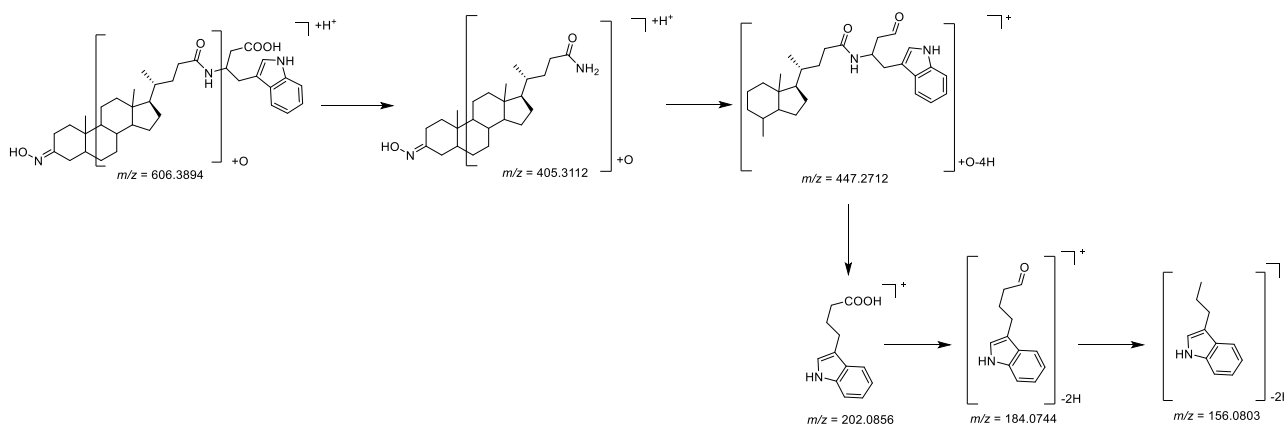


b. Experimental high resolution mass value in ESI⁺ for M8.

Item name: UniPr500_MLM_t180_4 Channel name: Low energy : Time 11.8575 +/- 0.0212 minutes : Drift Times: 7.07 +/- 0.22, 7.30 +/- 0.22 ms
Item description:

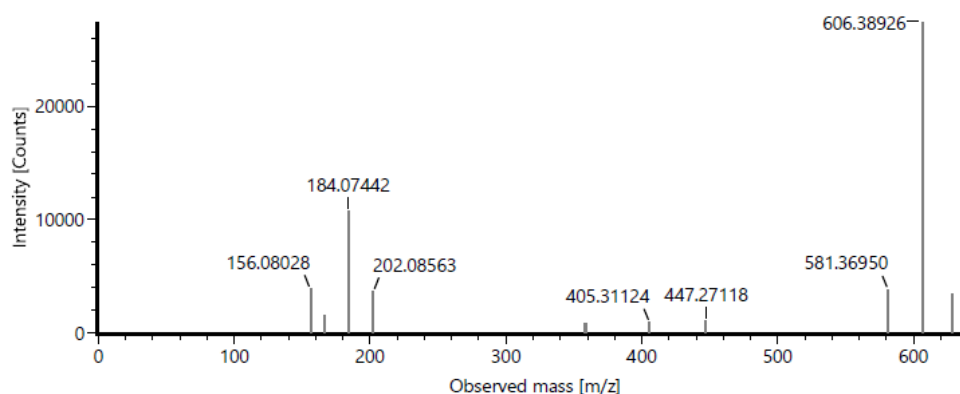


c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of M8 together with tentative fragmentation pattern. In the MS/MS spectrum, peak at $m/z = 405.3$ is the same found for M4. Characteristic fragment ion at $m/z = 447.3$ is compatible with the breaking of the steroidal core and the concomitant dehydroxylation of the carboxylic group.



Component name: UniPR500+O, Observed m/z : 606.3894, Observed RT (min): 11.86

Channel name: product ions from precursor ion id = 429



d. Metabolic soft spots on UniPR500 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M8**, UNIFI software returns as the most likely soft spots C-7-23 of the steroidal core.

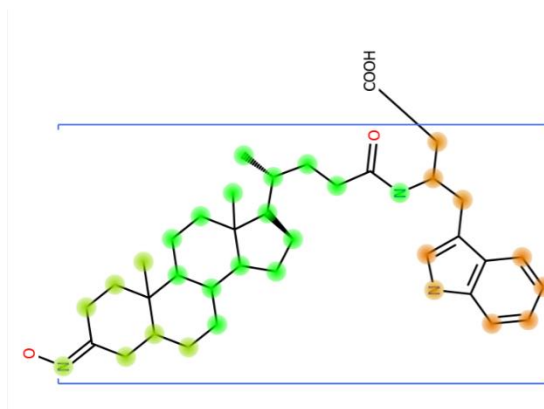
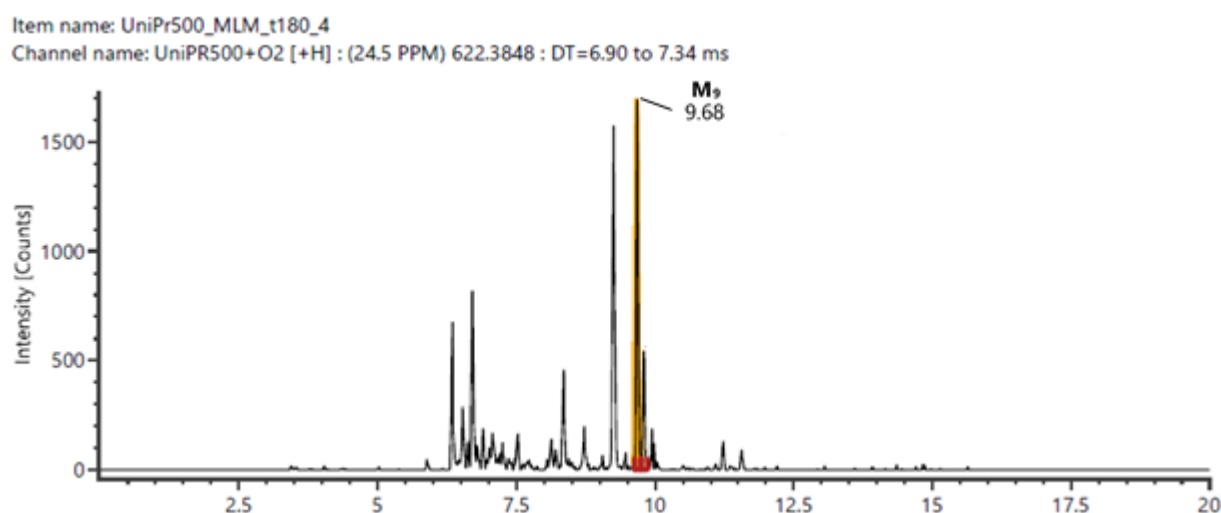
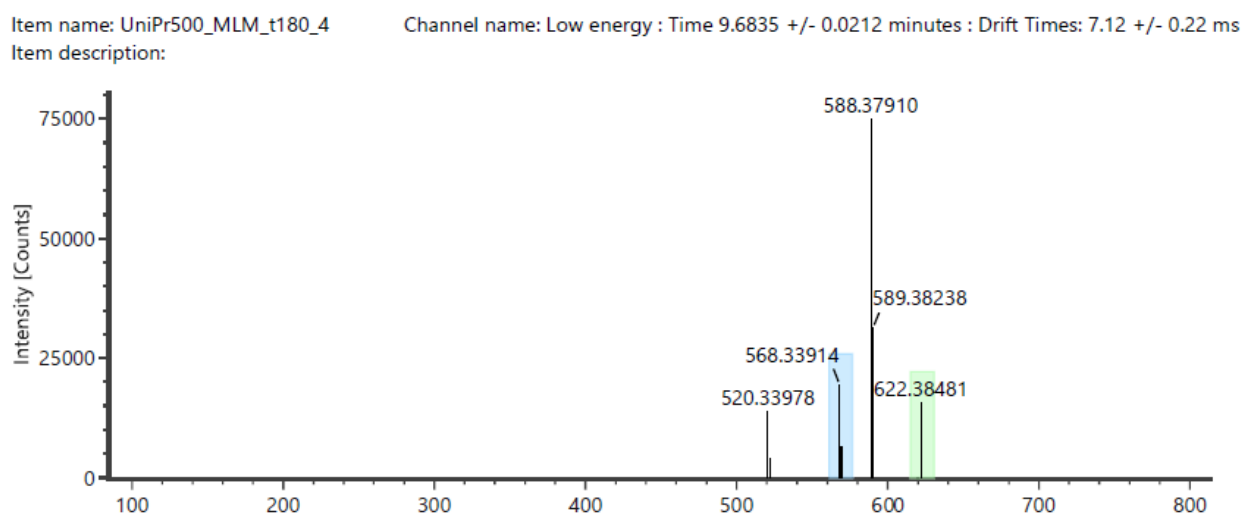


Figure S26. a-c. Metabolite **M9** in MLM.

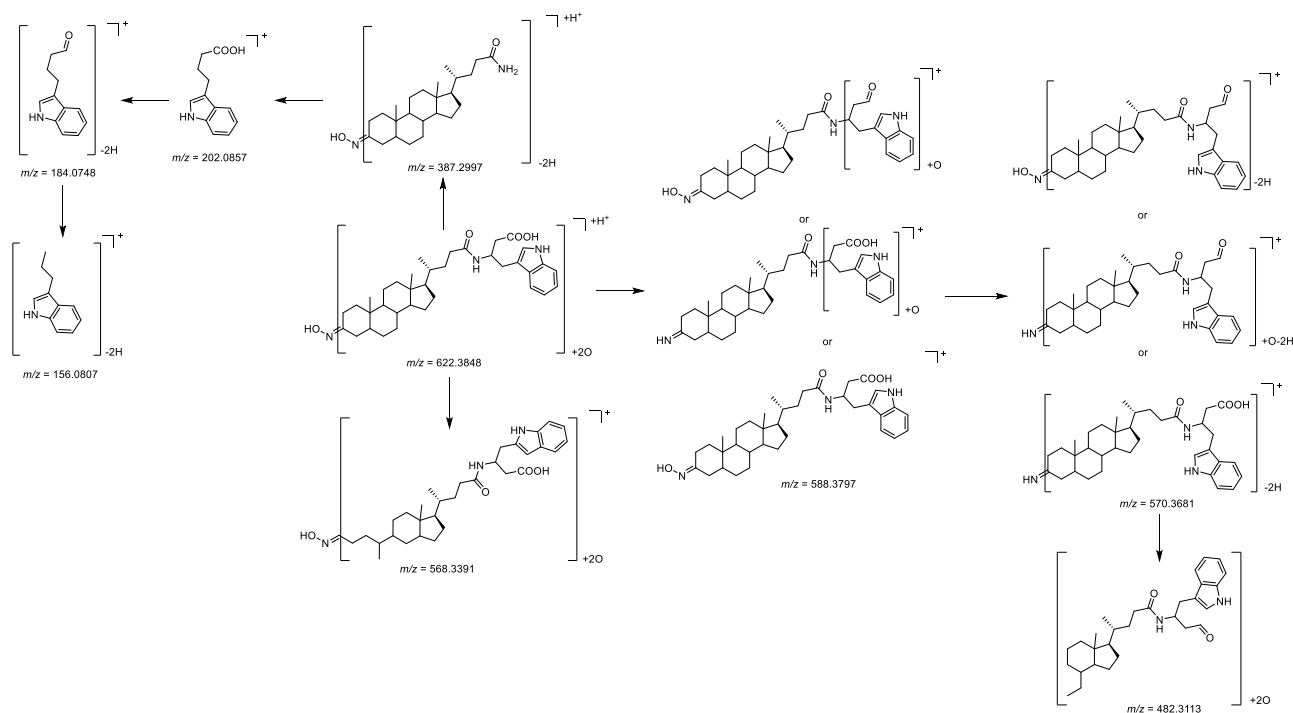
a. Phase I Metabolite **M9** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 622.38$ [M+H]⁺ and RT = 9.68 min.



b. Experimental high resolution mass value in ESI⁺ for **M9**.



c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M9** together with tentative fragmentation pattern. Fragment ions at $m/z = 588.4$ and 570.4 have been already described for **M7**, while $m/z = 387.3$ has been reported for **M2**. Peak at $m/z = 568.3$ could derive from a partial disruption of the steroid, which is further enhanced in the fragment ion at $m/z = 482.3$, which is compatible with a concomitant dehydroxylation of the carboxylic moiety. However, UNIFI software could not predict likely sites for oxidation.



Component name: UniPR500+O2, Observed m/z : 622.3848, Observed RT (min): 9.68

Channel name: product ions from precursor ion id = 1767

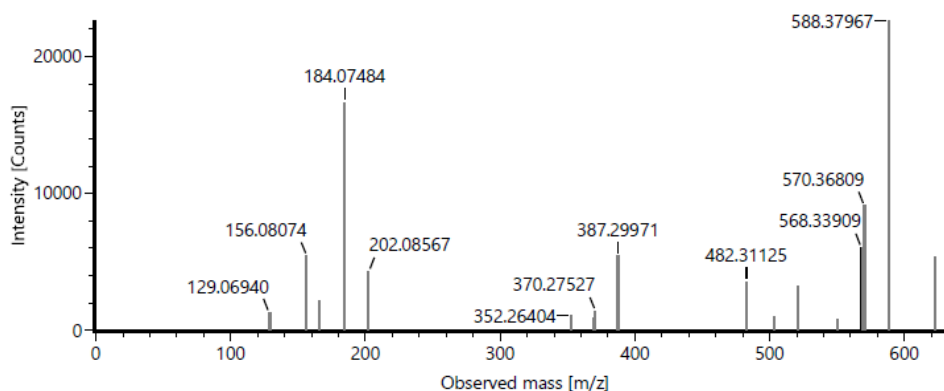
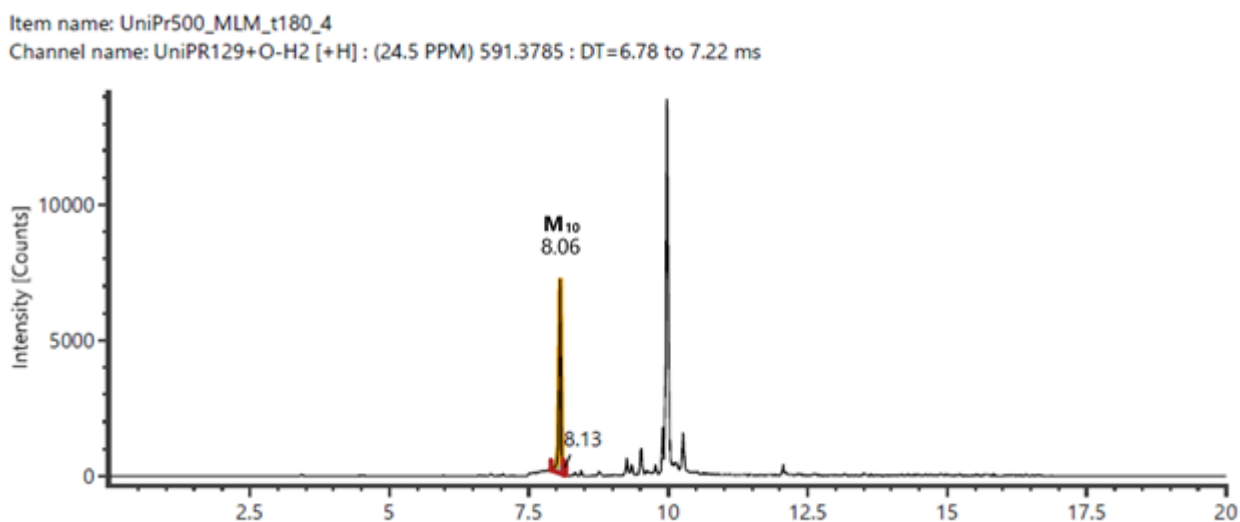


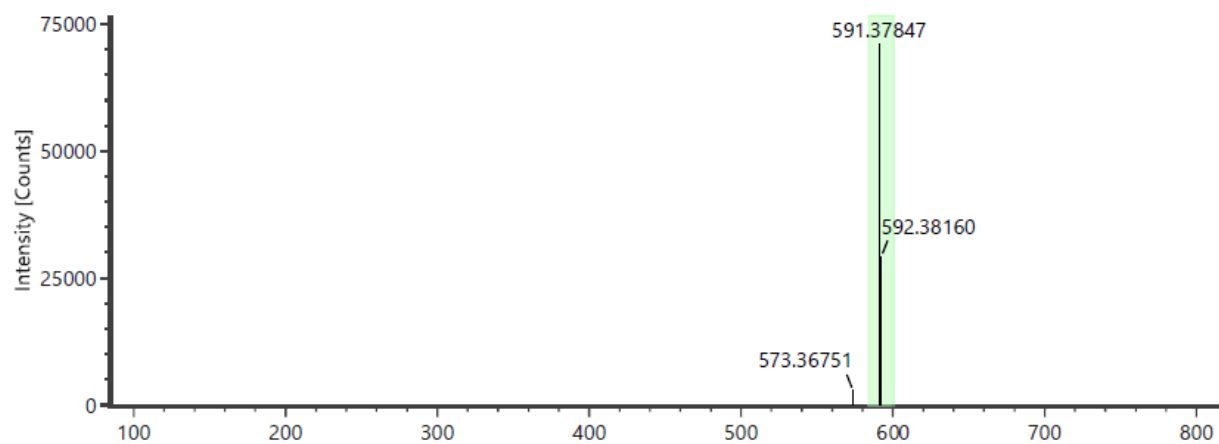
Figure S27. a-d. Metabolite M10 in MLM.

a. Phase I Metabolite **M10** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 591.38$ [M+H]⁺ and RT = 8.06 min.

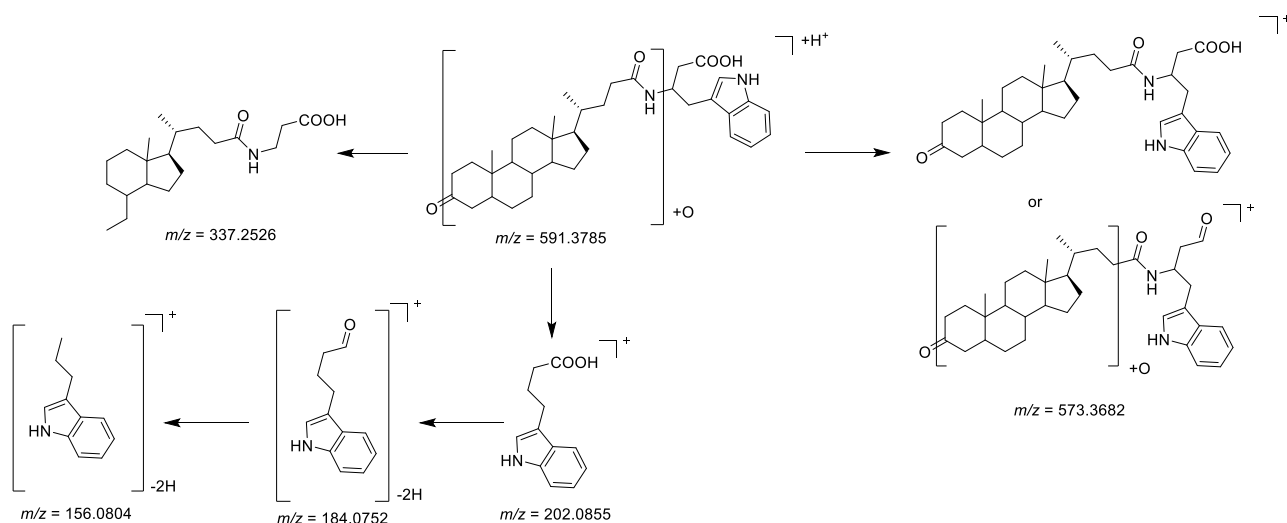


b. Experimental high resolution mass value in ESI⁺ for **M10**.

Item name: UniPr500_MLM_t180_4 Channel name: Low energy : Time 8.0607 +/- 0.0212 minutes : Drift Times: 7.00 +/- 0.22 ms
Item description:

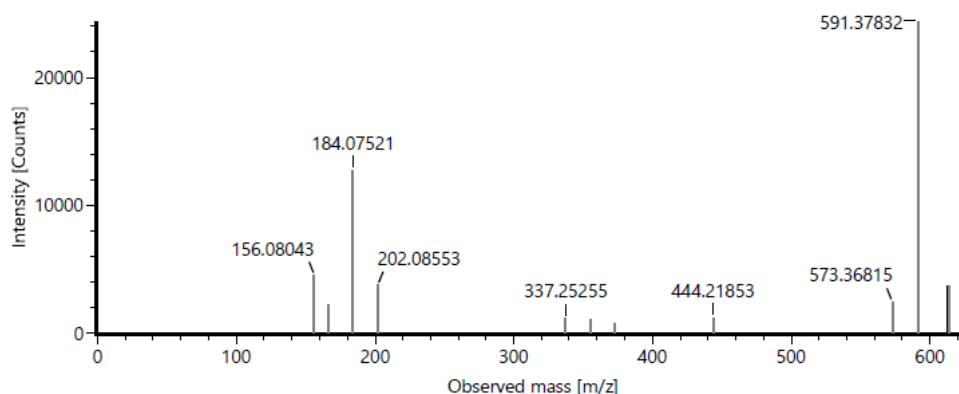


c. High resolution high energy (MS^E) MS spectrum in ESI^+ of **M10** together with tentative fragmentation pattern. The three product ions at $m/z = 202.1$, 184.1 and 156.1 prompt to exclude the oxidation to occur on L- β -homotryptophan group. Moreover, the low intense fragment ion at $m/z = 337.2$ allow to speculate that oxidation involves a C-atom on the A- or B-ring of the steroidal core.



Component name: UniPR129+O-H2, Observed m/z : 591.3785, Observed RT (min): 8.06

Channel name: product ions from precursor ion id = 455



d. Metabolic soft spots on UniPR129 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M10**, UNIFI software returns as the most likely soft spots C1 or C2 of the steroidal core.

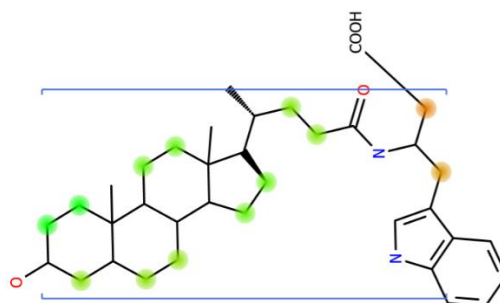
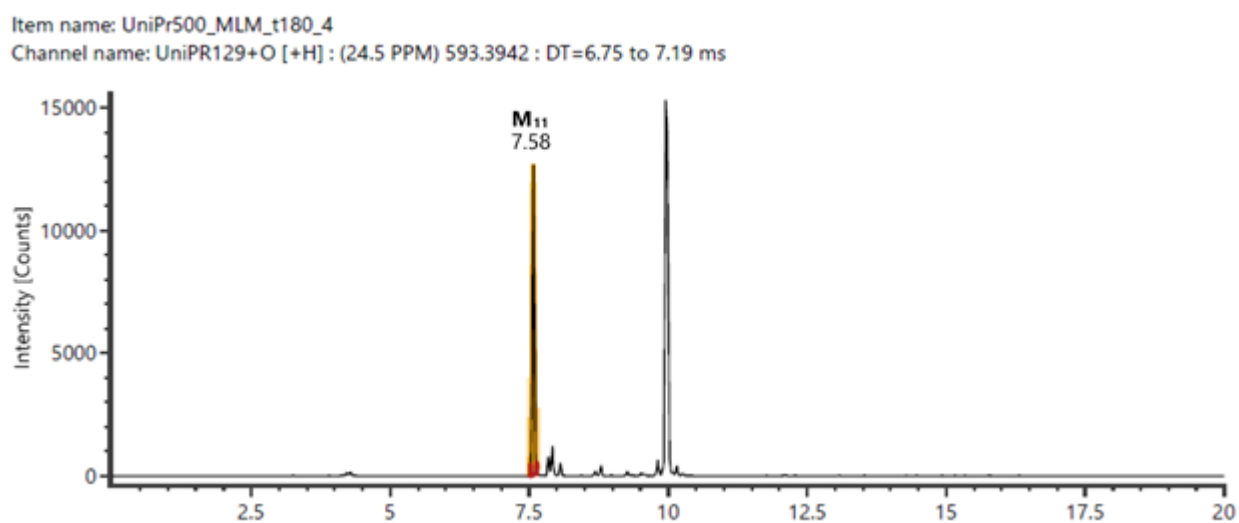


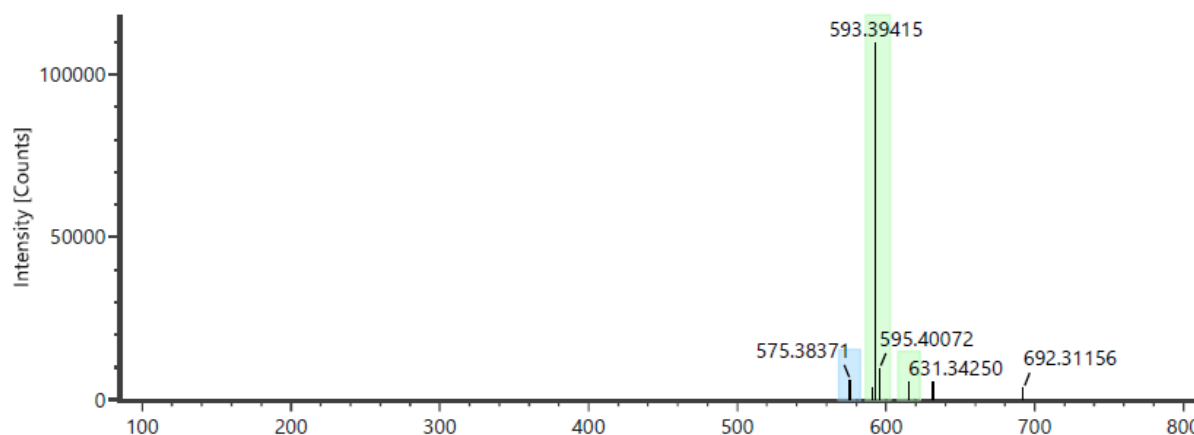
Figure S28. a-d. Metabolite **M11** in MLM.

a. Phase I Metabolite **M11** derived from UniPR500 in mouse liver microsomes. Extracted ion chromatogram (XIC) in ESI⁺ at $m/z = 593.39$ [M+H]⁺ and RT = 7.58 min.

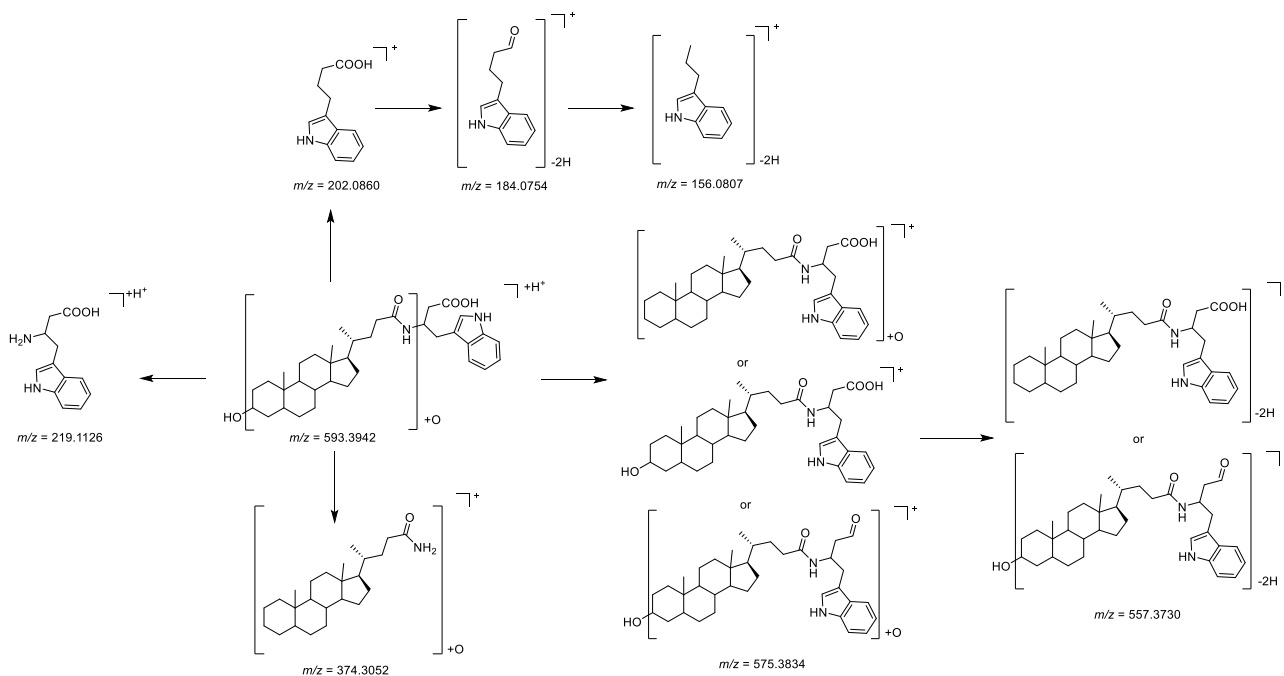


b. Experimental high resolution mass value in ESI⁺ for **M11.**

Item name: UniPr500_MLM_t180_4 Channel name: Low energy : Time 7.5771 +/- 0.0212 minutes : Drift Times: 6.97 +/- 0.22, 7.30 +/- 0.22 ms
Item description:

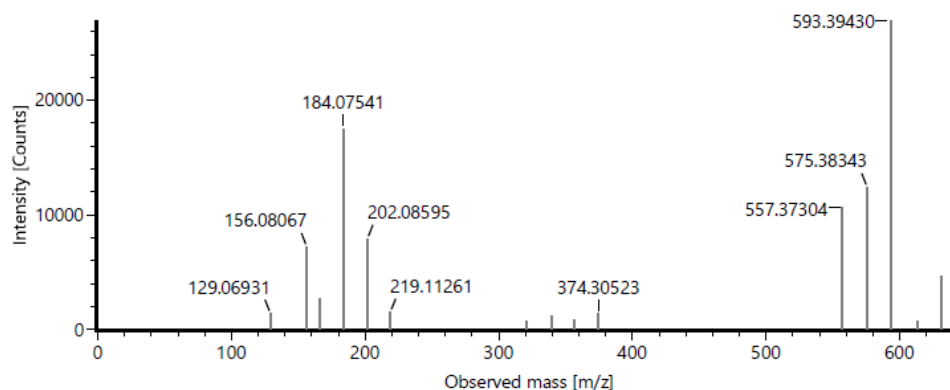


c. High resolution high energy (MS^E) MS spectrum in ESI⁺ of **M11 together with tentative fragmentation pattern. In the MS/MS spectrum three characteristic fragments can be found: the peak at m/z = 575.4, compatible with the loss of water; product ion at m/z = 374.3 derived from parent 593.4 by dehydration and loss of the 4-(indol-3-yl)butanoic acid fragment; product ion at m/z = 219.1 consisting of the L- β -homotryptophan group. The other fragment ions recall the ones from **M4** fragmentation.**

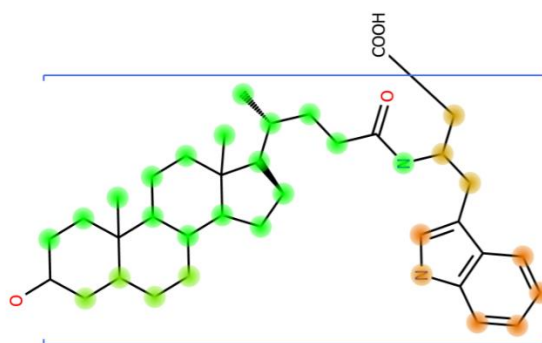


Component name: UniPR129+O, Observed m/z:
593.3942, Observed RT (min): 7.58

Channel name: product ions from precursor ion id = 295



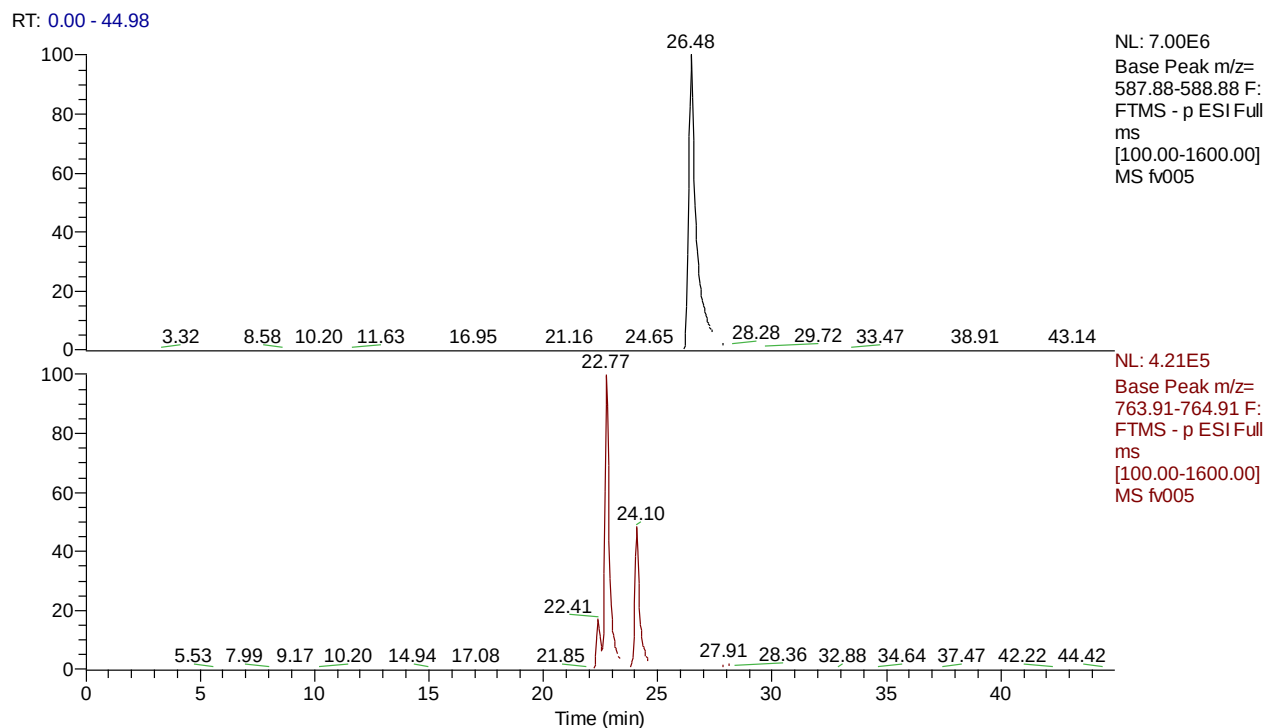
d. Metabolic soft spots on UniPR500 reported with a color-scale: the most probable sites of modification are highlighted in bright green. Based on the MS/MS spectrum of **M11**, UNIFI software returns as the most likely soft spots C-7-23 of the steroidal core.



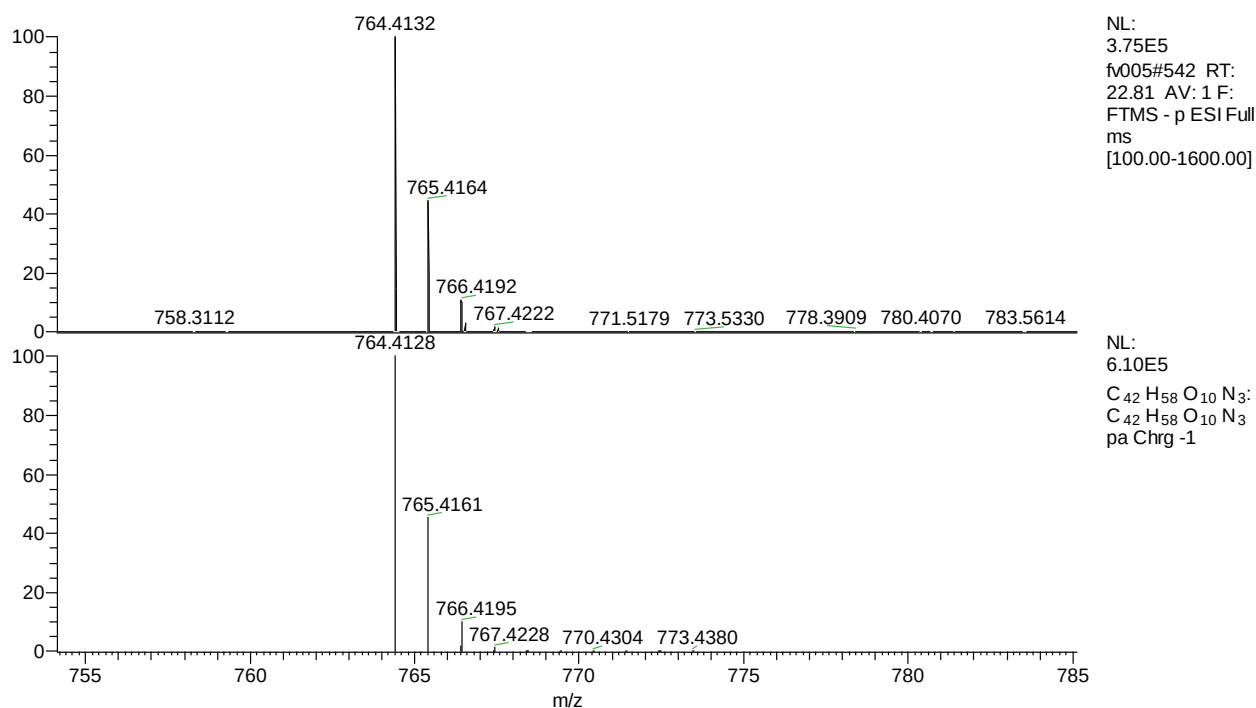
S.9 UniPR500 *In vitro* phase II metabolite profiling

Figure S29. a-d. Metabolites **M12-M14** in *MLS₉* fraction.

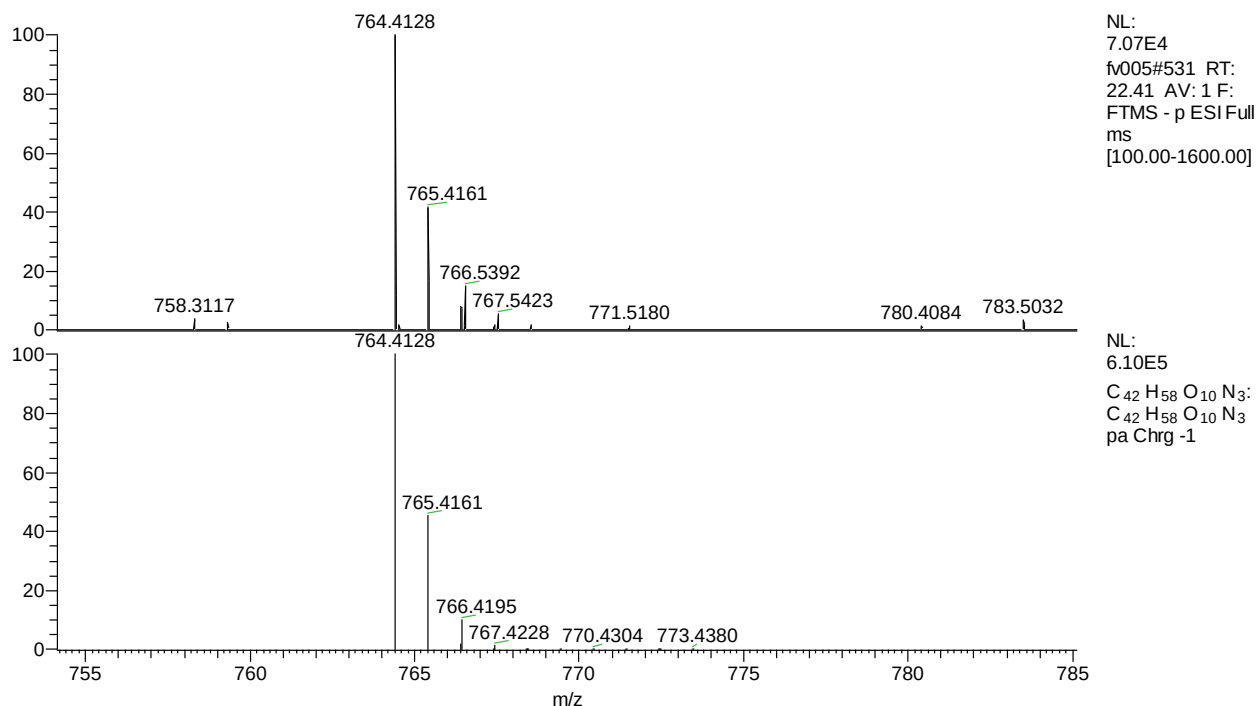
a. Phase II conjugates of UniPR500 with glucuronic acid **M12-M14** in mouse liver microsomes. Upper trace: Extracted ion chromatogram (XIC) in ESI⁻ at $m/z = 588.38$ [M-H]⁻ corresponding to parent UniPR500; lower trace: XIC in ESI⁻ at $m/z = 764.41$ [M-H]⁻ corresponding to the glucuronides.



b. Experimental high resolution mass values and isotopic distribution in ESI⁻ for metabolite **M12** (upper, RT = 22.77 min) if compared to calculated value (lower spectrum).



c. Experimental high resolution mass values and isotopic distribution in ESI⁻ for metabolite **M13** (upper, RT = 22.41 min) if compared to calculated value (lower spectrum).



d. Experimental high resolution mass values and isotopic distribution in ESI⁻ for metabolite **M14** (upper, RT = 24.10 min) if compared to calculated value (lower spectrum).

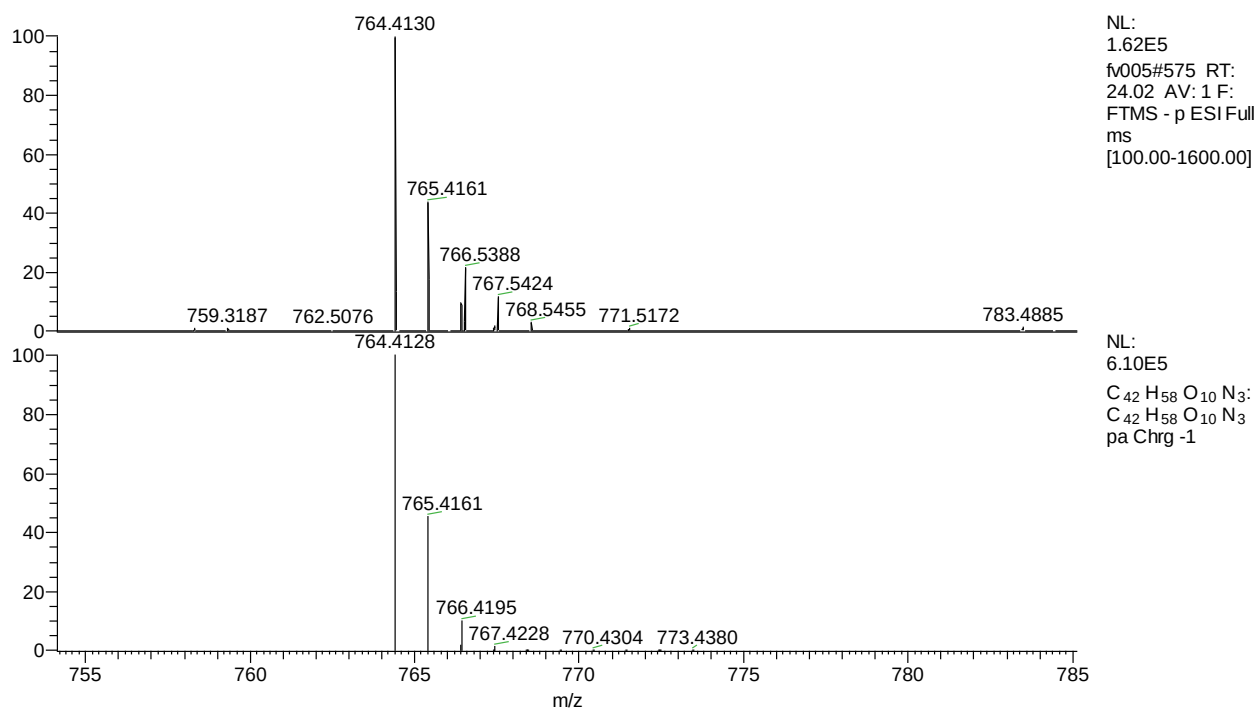
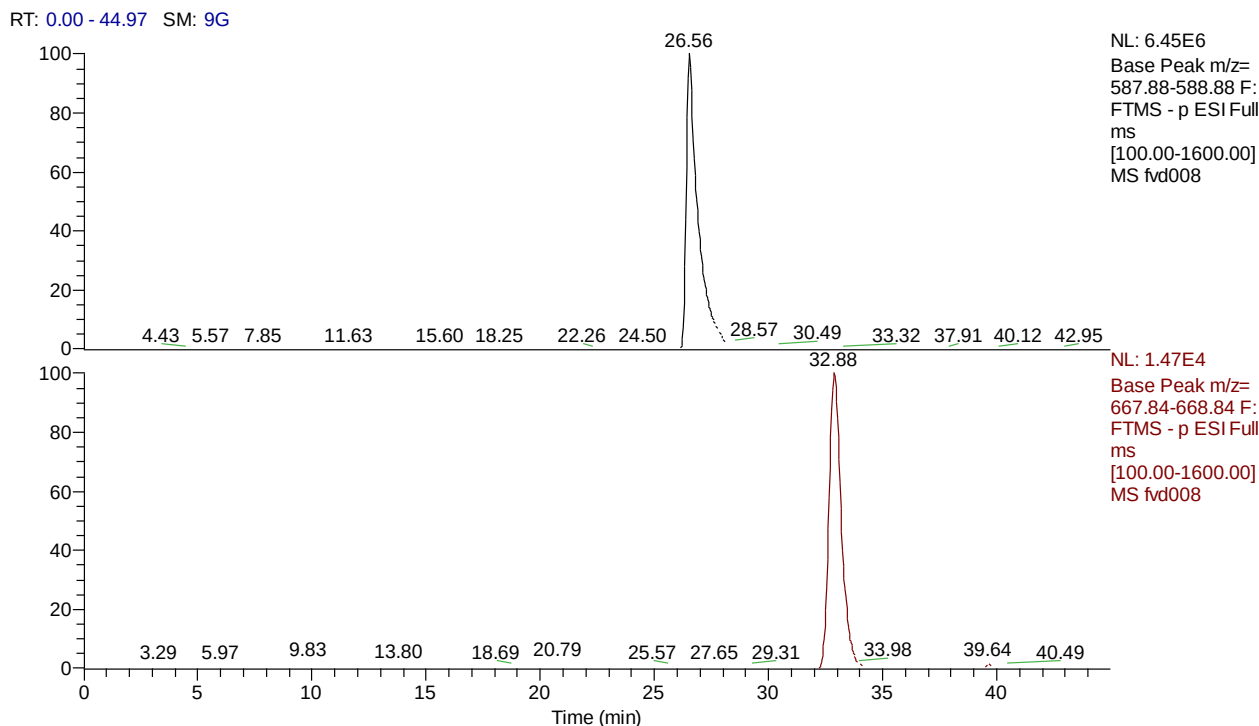


Figure S30. a-b. Metabolite M15 in HLS₉ fraction.

a. Phase II conjugate of UniPR500 with active sulphate **M15** in human liver S₉ fraction. Upper trace: Extracted ion chromatogram (XIC) in ESI⁻ at $m/z = 588.38$ [M-H]⁻ corresponding to parent UniPR500; lower trace: XIC in ESI⁻ at $m/z = 668.34$ [M-H]⁻ corresponding to the conjugate with active sulphate.



b. Experimental high resolution mass values and isotopic distribution in ESI⁻ for metabolite **M15** (upper, RT = 32.9 min) if compared to calculated value (lower spectrum).

