Supporting information.

Title: Charting the Undiscovered Metabolome with Synthetic Multiplexing

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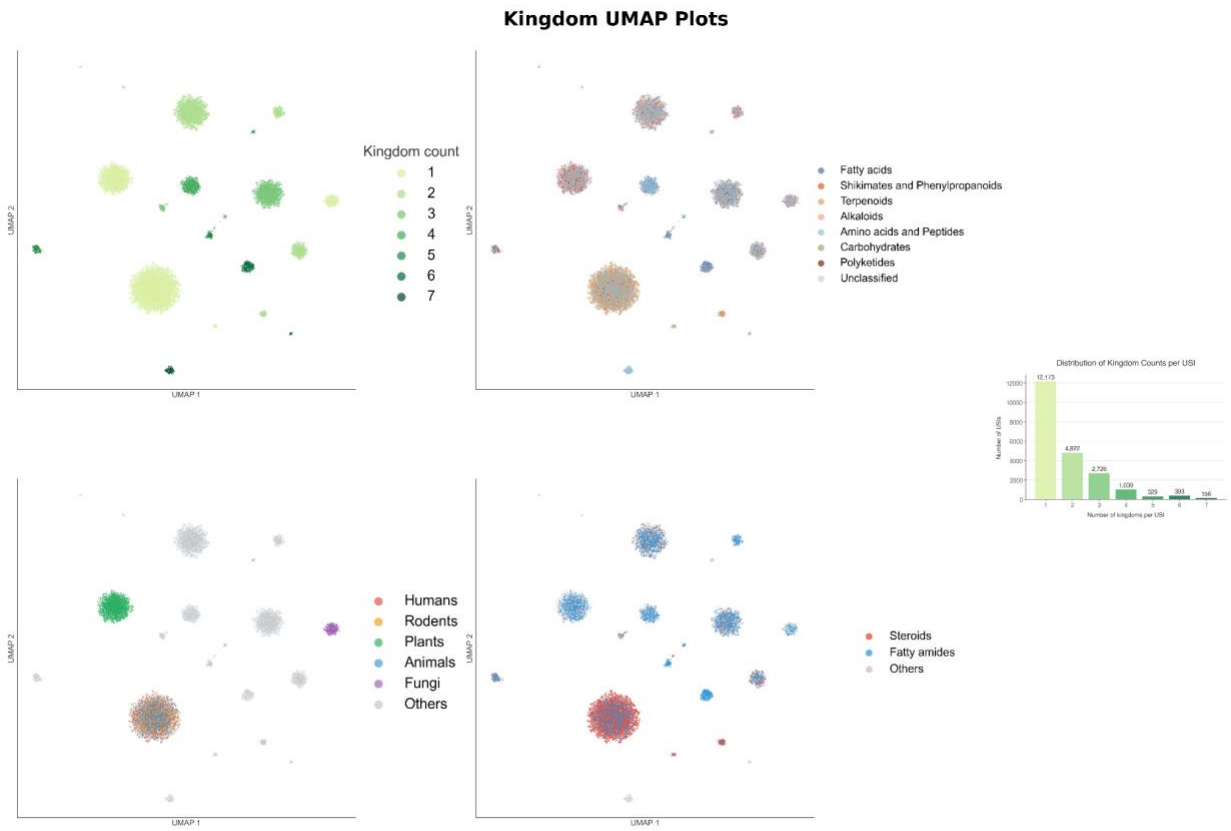
Supplementary Table 1 List of compounds used in reactions - Google Sheets

lists all reactants used in the multiplex synthesis experiments. Each row corresponds to a distinct compound and includes detailed chemical and structural information. The table provides the following columns: compound_name (the name of the derived or related reactant), SMILES (the original Simplified Molecular Input Line Entry System representation), corrected_SMILES (curated SMILES strings adjusted for syntax and structure accuracy), formula (the molecular formula), neutralized_formula (the formula standardized to a neutral charge state), exact_mass (monoisotopic mass), np_class and np_superclass (natural product classification levels), np_pathway (the biosynthetic or chemical pathway classification), inchikey (a standardized InChIKey for compound identification), and 2d_inchikey (an InChIKey truncated to represent 2D connectivity only). This table serves as a comprehensive reference for all chemical entities involved in the multiplex synthetic reactions performed in MS\MS spectral library creation.

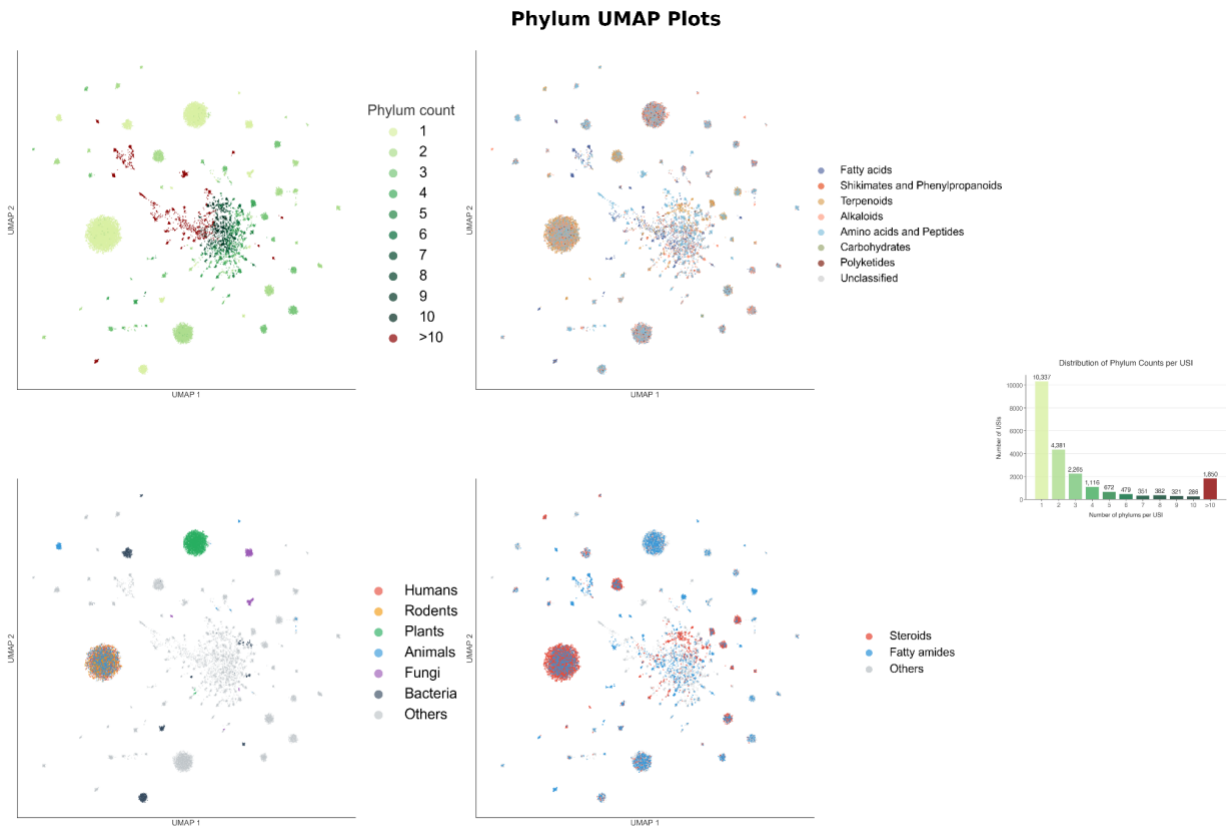
Supplementary Table 2 **Synthesis_Reaction_record_Final - Google Sheets**
provides a comprehensive list of the 4,000 chemical reactions conducted as part of this study. Each reaction is uniquely identified by a Reaction_ID and described by its Reaction_name, with additional details on the sequential synthetic steps (Step_2, Step_3, and Step_4). The table includes direct access to experimental records via the Signal_notebook_link (which can be only accessed by UCSD user credentials) where all reactions are documented; an example of this document is Signal notebook record example. Analytical information is provided through the Run_on_MS (mass spectrometry) and the corresponding MassIVE_ID for raw data access. The Processing_software column indicates the tools used for library creation. The table also tracks spectra curation into libraries, indicating whether a Library_Created, This table serves as a detailed experimental and analytical record supporting reproducibility and data transparency.

Supplementary Table 3 **Supplementary Table 3 - Google Sheets**

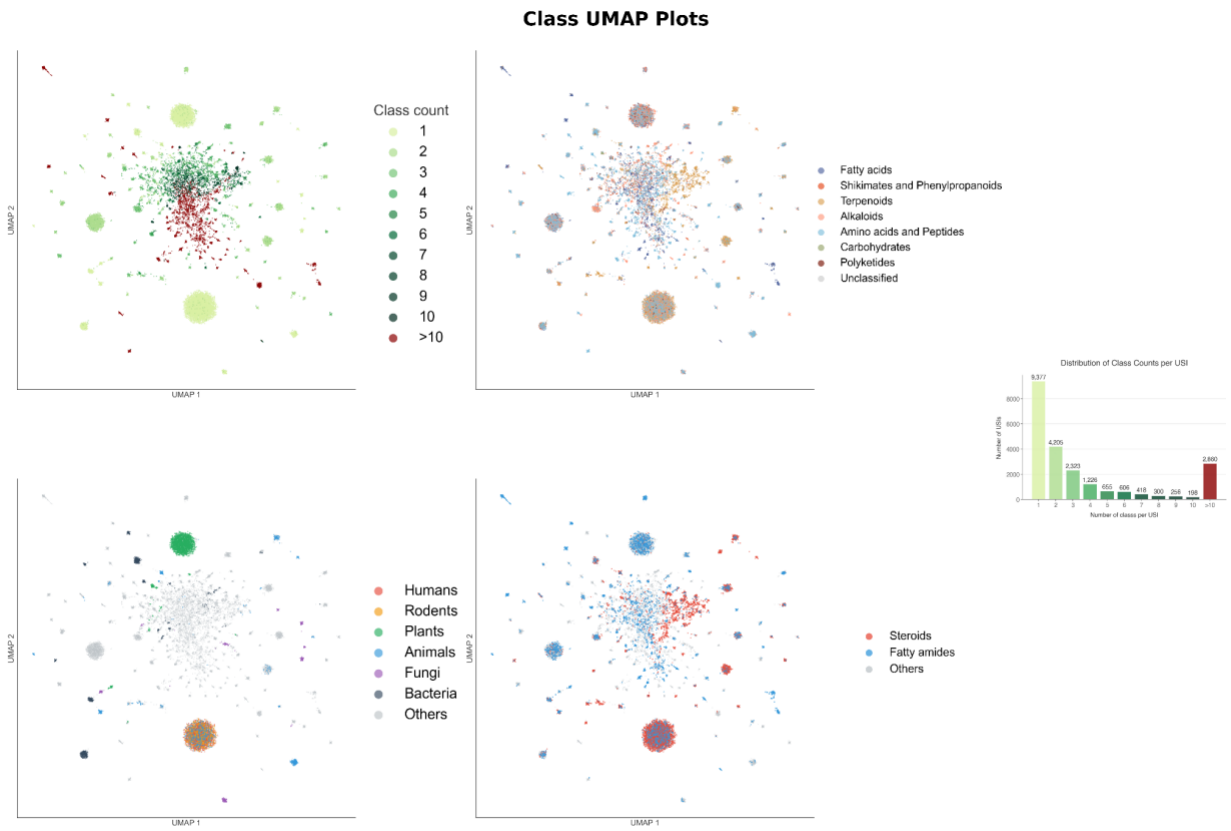
contains the results of the overlap analysis between the multiplexed synthetic MS/MS library generated in this study and several major public structural databases, including PubChem, HMDB, GNPS, PubChemLite, NORMAN, and FooDB.



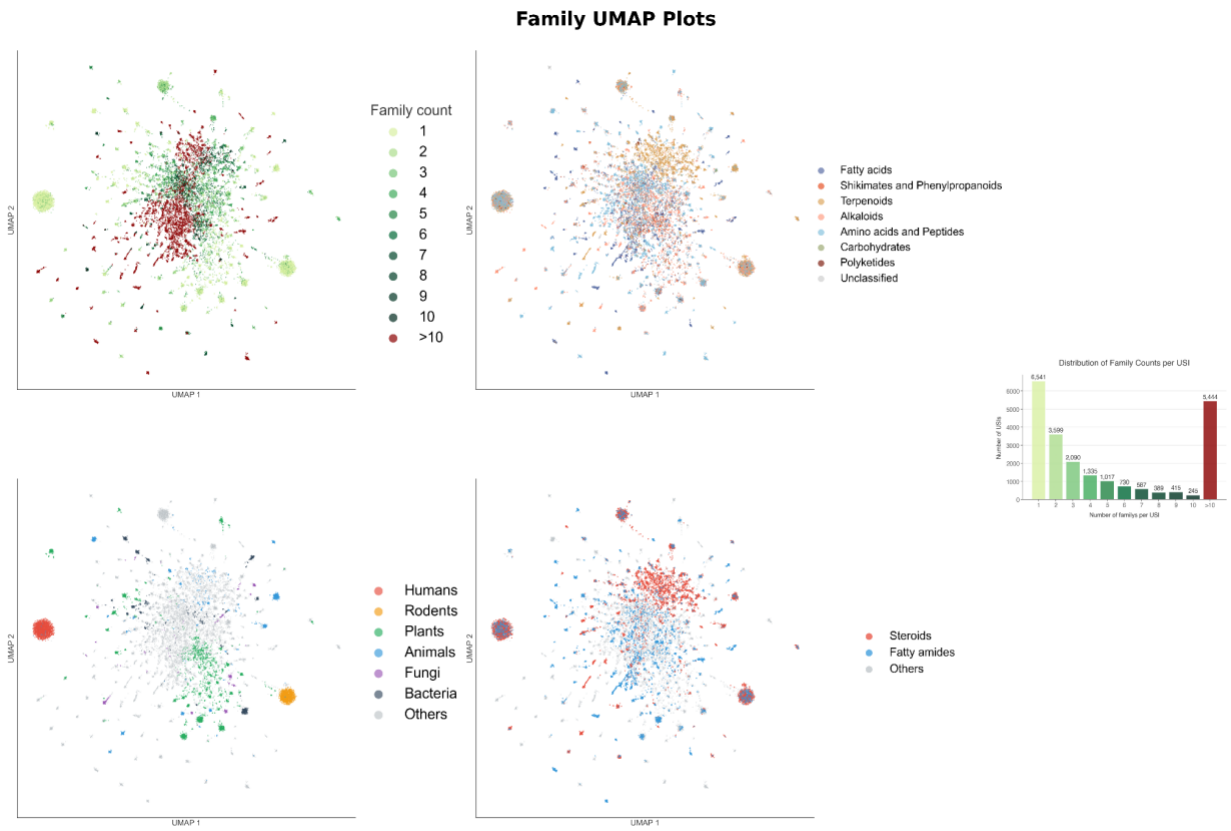
Supplementary Figure 1 | UMAP visualizations of the kingdom distribution of matched spectra across public datasets.



Supplementary Figure 2 | UMAP visualizations of the phylum distribution of matched spectra across public datasets.



Supplementary Figure 3 | UMAP visualizations of the class distribution of matched spectra across public datasets.



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88 **Supplementary Figure 4 | UMAP visualizations of the family distribution of matched**
89 **spectra across public datasets.**

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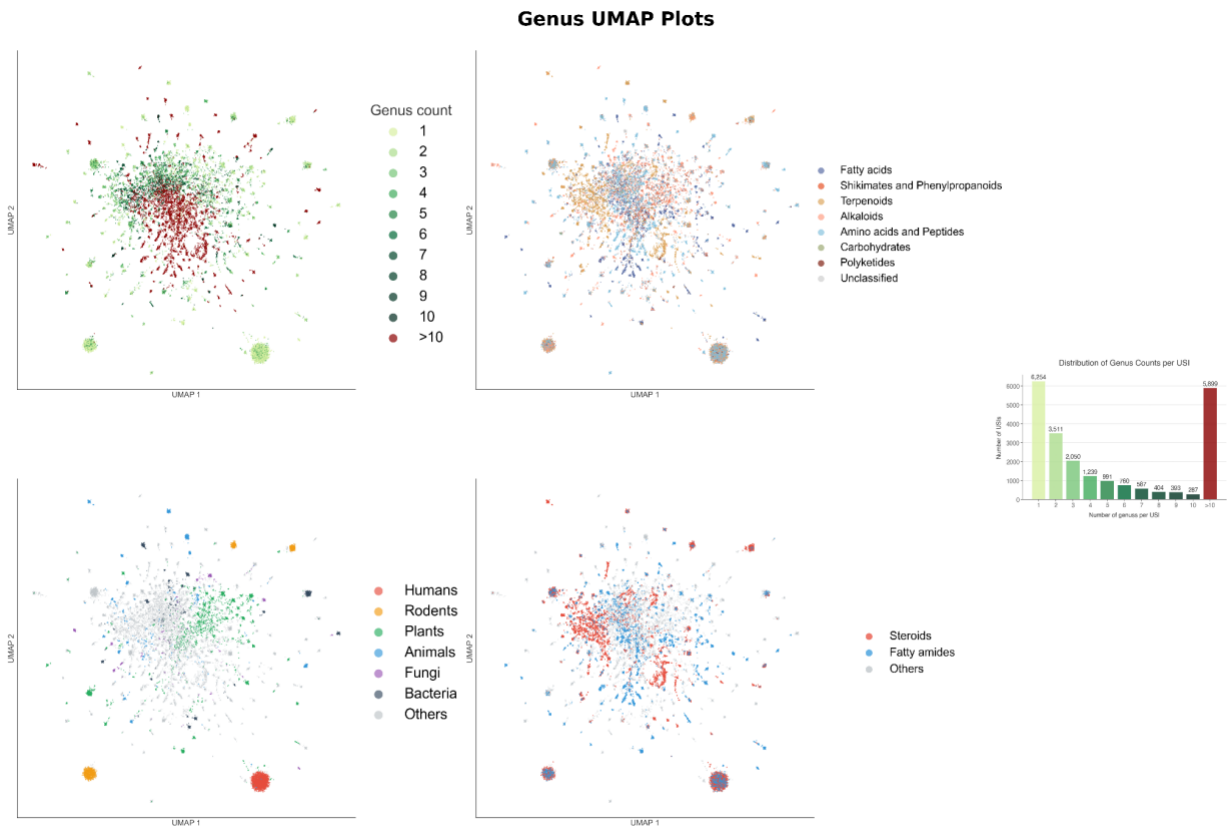
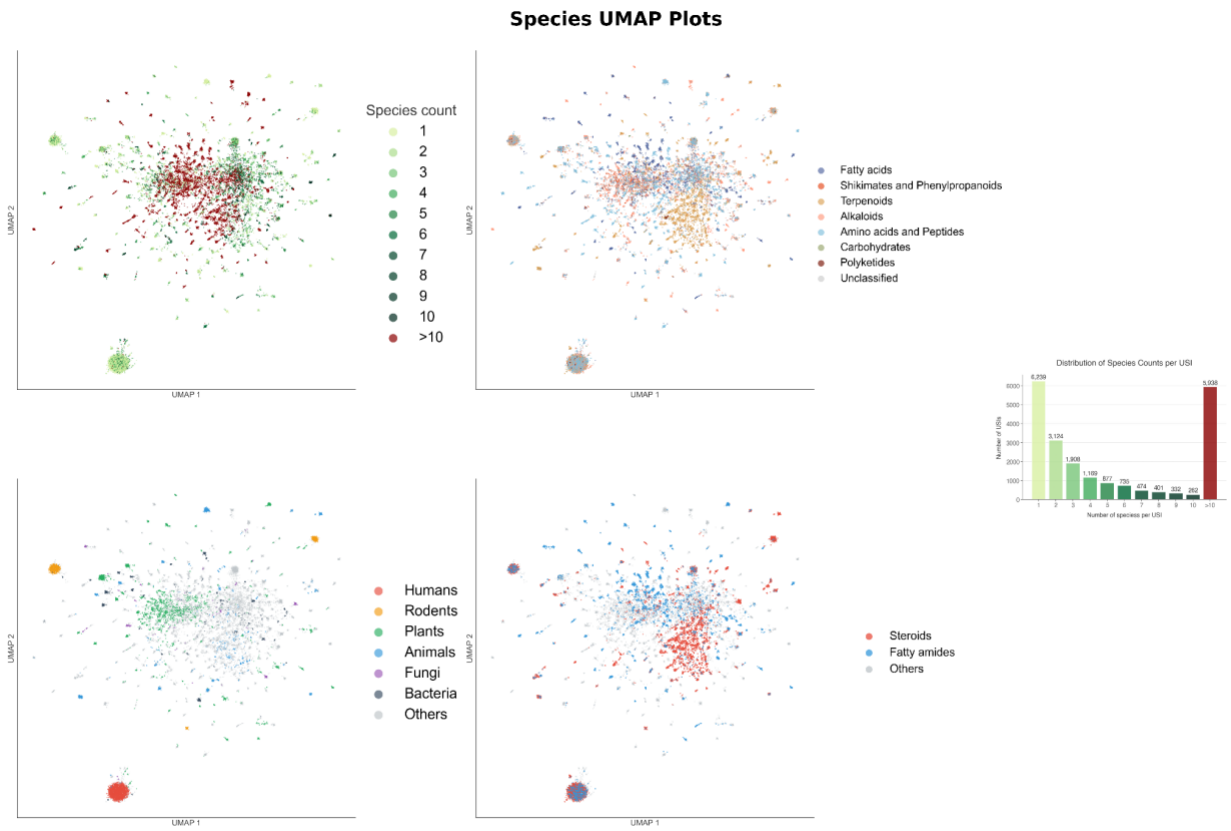
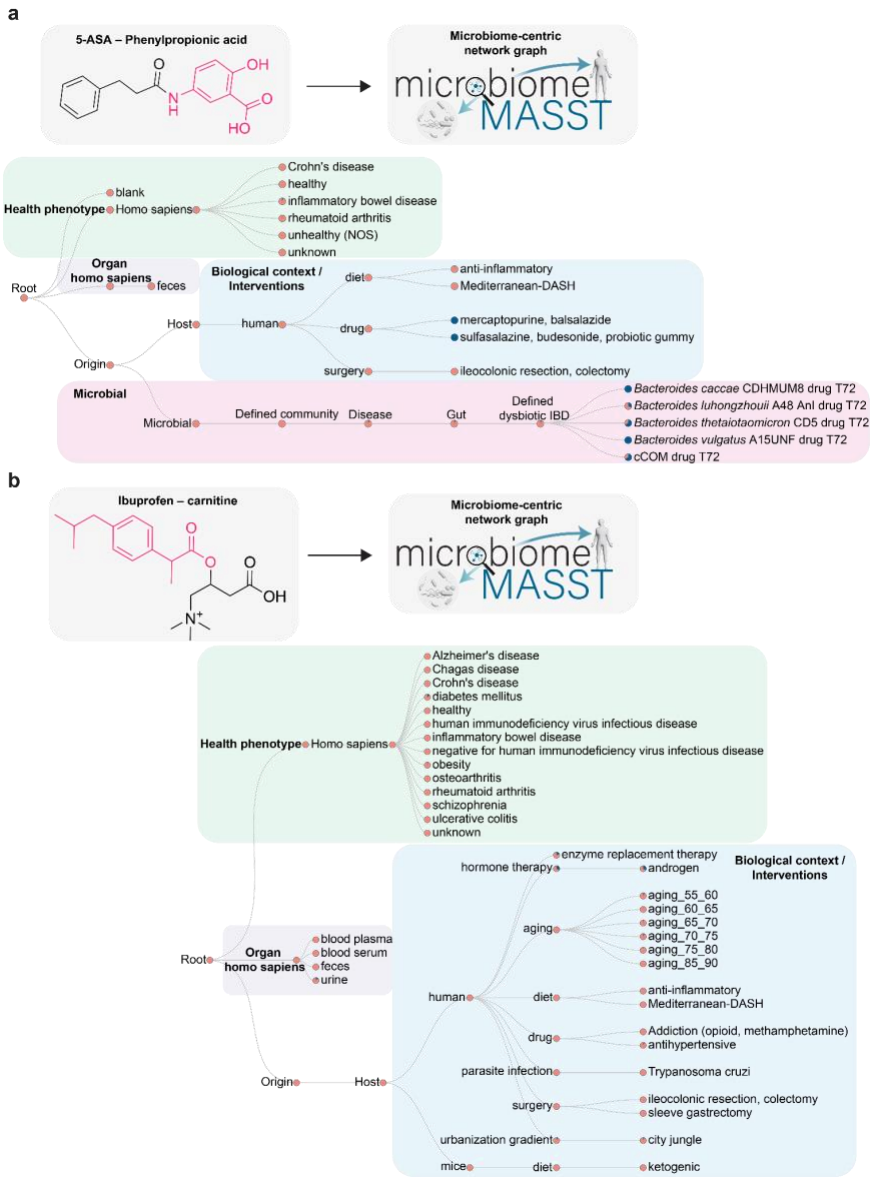


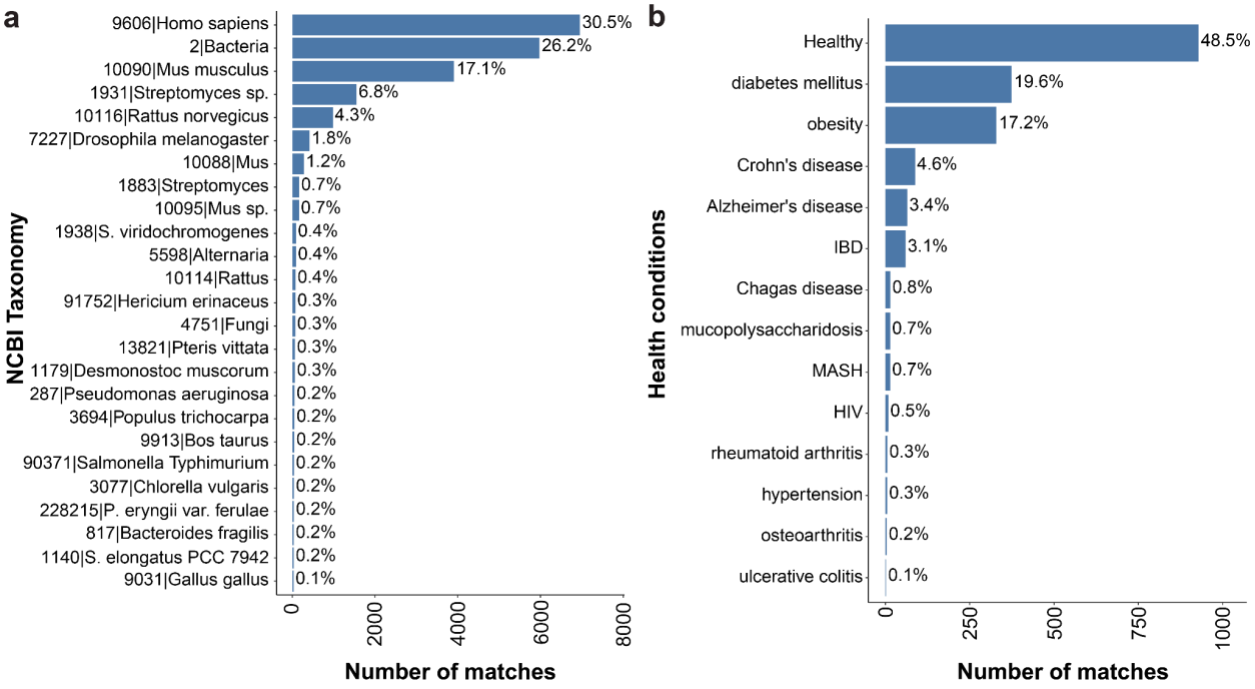
Supplementary Figure 5 | UMAP visualizations of the genus distribution of matched spectra across public datasets.



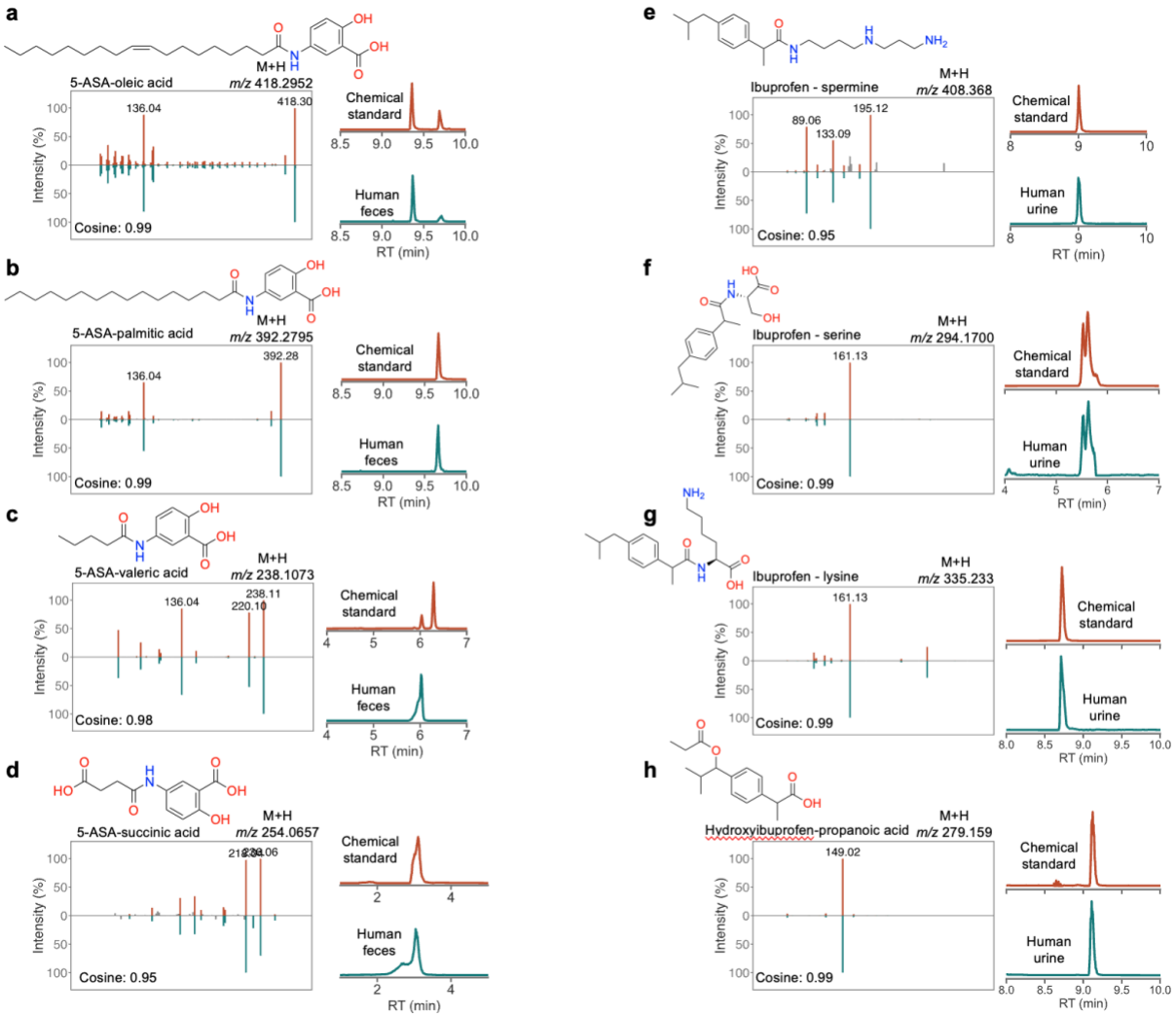
Supplementary Figure 6 | UMAP visualizations of the species distribution of matched spectra across public datasets.



Supplementary Figure 7 | The distribution of 5-ASA-phenylpropionic acid and ibuprofen-carnitine in host biology. a) The universal spectrum identifier (USI) of 5-ASA-phenylpropionate retrieved from the GNPS2 library was used as input in microbiomeMASST. MicrobiomeMASST results showed that 5-ASA-phenylpropionate was detected in bacterial culture supplemented with the substrates 5-ASA and phenylpropionate, exclusively observed in humans under diets, drugs, surgery interventions, and in health conditions including Crohn's disease, inflammatory bowel disease, and rheumatoid arthritis. **b)** Ibuprofen-carnitine was detected in several health conditions including obesity, osteoarthritis, and ulcerative colitis, across several biological contexts such as aging and parasite infection, and found in blood, feces, and urine.



Supplementary Figure 8 | Prevalence of succinic acid conjugates across taxonomy and health conditions. a) Distribution of succinic acid conjugates matched across NCBI Taxonomy using the PanReDU database. The bars show the number of matches. On the right side of the bars, the percentage of all matches is shown. **b)** Distribution of succinic acid conjugates matched across health conditions. The bars show the number of matches and the right labels show the percentage of only human matches. IBD, inflammatory bowel disease; MASH, metabolic dysfunction-associated steatohepatitis; HIV, human immunodeficiency virus.



Supplementary Figure 9 | Structure validation of 5-ASA and ibuprofen drug metabolites. MS/MS spectral matching and retention time alignment of **a)** 5-ASA-oleic acid, (should be interpreted as a 5-ASA-C18:1 fatty acid conjugate as other isomers are possible) **b)** 5-ASA-palmitic acid (C16:0 fatty acid), **c)** 5-ASA-valeric acid, (C5:0 fatty acid) **d)** 5-ASA-succinic acid, **e)** ibuprofen-spermidine, **f)** ibuprofen-serine, **g)** ibuprofen-lysine, and **h)** hydroxyibuprofen-propanoic acid.

Quantification

Quantification of Ibuprofen carnitine and 5-ASA phenylpropionic acid in Urine sample and fecal sample respectively (scripts used for quantification are available (abubakerpatan/Quantification-Script: Script used for quantification).

The LC-MS/MS method used for the analyses of the method validation and quantification was the same as previously described in LC-MS/MS data collection. The analytical method was performed according to the International Conference on Harmonization (ICH) guidelines[124] for ibuprofen

carnitine and 5-ASA phenylpropionic acid. The method was validated based on the evaluation of the following parameters: specificity, precision (repeatability and intermediate precision), linearity, limit of detection (LOD), limit of quantification (LOQ), and accuracy. A matrix match calibration curve was created by spiking pool urine (ibuprofen carnitine) and pool fecal (5-ASA-phenylpropionic acid) into calibrates to create a matrix match calibration curve for quantitation. Detailed information regarding the methodology used for each of them is described below. The validation was performed using Rise Plus Urobiome samples of human urine MSV000096359 that would contain the ibuprofen carnitine compound and Crohn's cohort MSV000099375 that contains the 5-ASA phenylpropionic acid. Peak area for ibuprofen carnitine and 5-ASA phenylpropionic acid was extracted using Skyline[125] (version 23.1). The method employed reached the acceptance criteria specified for each parameter of ibuprofen carnitine (Table 1), and 5-ASA phenylpropionic acid (Table 2). For quantification in biological samples, one sample of the Crohn's cohort and one sample of the Rise Plus Urobiome study with the highest peak area was injected in the validated method (samples were resuspended in 100 µL of 50/50 MeOH/H_2O containing 1 µM of sulfamethazine). For the calculation of the amounts in the samples, it was estimated that 200 uL of urine sample and 54 mg fecal samples would be the starting material, and the extraction yield was also extrapolated to 100%.

Specificity

The specificity was determined by injecting a blank solution containing only the internal standard (sulfadimethazine), and an injection of a solution containing all the ibuprofen carnitine (n=3). The relative standard deviation (RSD) was calculated based on each peak's retention time in the Rise Plus Urobiome and fecal samples. The MS and MS/MS spectra confirmed the specificity and identity of these compounds. The retention times of the peak of interest were as follows: Ibuprofen carnitine, 2.09 min and 5-ASA-phenylpropionic acid 4.52 min. These compounds didn't show interferences compared to the solution containing only the mixture of standards.

Linearity

The linearity of the method was determined by calibration curves in concentration ranges comprising each compound at the samples of interest. A stock solution containing 1mM of each Ibuprofen carnitine and 5-ASA phenylpropionic acid was prepared in 50/50 MeOH/H_2O, followed by serial dilutions to get the concentration range mentioned in (Table 1) and (Table 2) and used to acquire calibration curves for all the compounds simultaneously. From this solution, 7 points were prepared with levels ranging from 10nM to 1uM for Ibuprofen carnitine and 100nM to 2uM for 5-ASA phenylpropionic acid with each spike with urine matrix Ibuprofen carnitine and fecal matrix for 5-ASA phenylpropionic acid. Each concentration level was injected in triplicate and the analytical curves were built based on the nominal concentrations, and the average between the ratios of each compound and the internal standard used (Ratio = $A_{compound}/A_{IS}$). A polynomial equation was obtained for each curve, and the correlation coefficients (R) were calculated for each compound. The R coefficients are available in (Table 1) and (Table 2).

Limit of detection and limit of quantification

LODs and LOQs were estimated by the mean of the slopes (S) and the standard deviation of the y-intercept (y). These limits were calculated by the following equations: LOD = (3.3*y)/S and LOQ = (10*y)/S. All the slopes, intercepts, LODs, and LOQs are shown in (Table 1) and (Table 2).

Accuracy and Precision

The accuracy and precision of the method was determined by recovery analyses. For this, known amounts of the solution containing the standards were spiked to the sample P1-D-6_2_5753 for 5-ASA-phenylpropionic acid and sample STD_SPK_urine sample for Ibuprofen carnitine solutions in two different concentrations (low and high) considering the predetermined calibration curve and concentration range. Three replicates for each level were injected and analyzed in the validated method. The accuracy was determined by the difference between the theoretical and experimental concentration values and the values were within the acceptance range of 80–120% and the precision by coefficient variation (CV).

Multiplex synthesis

Multiplex syntheses were conducted utilizing standard chemical methodologies. The reaction parameters, such as temperature, reagent concentration, solvent composition, and reaction duration, were systematically refined to ensure consistent reaction efficiency across all targets. Subsequent adjustments to the method were implemented based on the solubility of substrates, their chemical reactivity, and sensitivity. The polarity of solvents and buffer systems were modified to enhance substrate dissolution and stability, while the concentrations of catalysts were optimized to improve reaction yields. These adjustments guaranteed that the multiplex synthesis was carried out efficiently, achieving high yield and selectivity.

Amidation (a). A carboxylic acid containing compound (1 eq.) and 2 mL of DMF were added to a 20 mL scintillation vial with a magnetic stir bar. To this solution, solid EDC (1.2 eq.) and neat DIPEA (1.5 eq.) were subsequently added, and the solution was stirred at 23°C. After 15 minutes, a mixture containing each amine (0.01 eq.), DMAP (0.2 eq.) were added, and the reaction was stirred for 14 hours. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Amidation (b). A carboxylic acid containing compound (1 eq.) and 2.5 mL of anhydrous THF were added to a 20 mL scintillation vial with a magnetic stir bar The solution was cooled to 0°C with an ice bath. To this solution, ethyl chloroformate (1.2 eq.) and Et_3N (1.2 eq.) were added, and the solution was stirred at 0°C for 1.5 hours. A mixture containing 0.01 eq of each amine was prepared in 2.5 mL of water with 1.5 eq of NaOH. After 1.5 hours of stirring, the amine mixture was added to the activated carboxylic acid mixture. The reaction was then stirred for 3 h and allowed to gradually warm to 23°C, yielding a clear, homogeneous solution. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Amidation (c). An amine containing compound (1 eq.) and 2.5 mL of anhydrous DCM were added to a 20 mL scintillation vial with a magnetic stir bar The solution was cooled to 0°C with an ice bath. To the cooled solution, Et_3N or pyridine (1.2 eq.) were subsequently added, and a mixture of acyl chlorides (0.01 eq. per compound) was added dropwise to the mixture. The mixture was

stirred for 2 to 3 hours and allowed to gradually warm to 23 °C. The mixture was then concentrated *in vacuo,* and an LC-MS/MS sample was then prepared from the concentrated mixture.

Amidation (d). A carboxyl compound (1 eq.) was dissolved in 2.5 mL of DMF in a scintillation vial with a magnetic stir bar. To the solution, (10 eq.) of ghosez's reagent was added.This was then stirred until the reaction mixture turned yellow, then (2 eq.) of neat pyridine and the amine containing compounds were added. The reaction mixture was then stirred 2-3 hr. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Esterification (a). A carboxylic acid compound (1 eq.) and 2 mL of DMF were added to a 20 mL scintillation vial with a magnetic stir bar. To this solution, solid EDC (1.2 eq.) was subsequently added, and the solution was stirred at 23°C. After 15 minutes, A mixture containing 0.01 eq of each alcohol , and DMAP (0.5 eq.) were added, and the reaction was stirred for 14 hours. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Esterification (b). An alcohol compound (1 eq.) and 2.5 mL of anhydrous DCM were added to a 20 mL scintillation vial with a magnetic stir bar. The solution was cooled to 0°C with an ice bath. To this solution, either Et_3N or pyridine (1.2 eq.) were added. While stirring at 0°C, a mixture containing 0.01 eq of each acyl chloride was added dropwise to the mixture. The mixture was stirred for 2-3 h and allowed to gradually warm to 23°C. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Esterification (c). A carboxyl compound (1 eq.) was dissolved in ethanol in a round bottom flask with a magnetic stir bar. To the solution, a few drops of sulfuric acid were added. The solution was refluxed in an oil bath for 2-3 h and allowed to gradually warm to 23°C. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Esterification (d). A carboxyl compound (1 eq.) was dissolved in 2.5 mL of DMF in a scintillation vial with a magnetic stir bar. To the solution,10 eq. of Ghosez's Reagent was added and stirred until the reaction mixture turned yellow. Then 2 eq. of neat pyridine, and the OH containing compounds was added. The reaction mixture was stirred for 2-3 hr. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Esterification (e). A carboxylic acid compound (1 eq.) and 2.5 mL of anhydrous THF were added to a 20 mL scintillation vial with a magnetic stir bar. The solution was cooled to 0°C with an ice bath. To this solution, ethyl chloroformate (1.2 eq.) and neat Et_3N (1.5ED eq.) were added. The solution was then stirred at 0°C for 1.5 hours. After 1.5 hours, a solution containing 0.01 eq of each alcohol was added dropwise to the activated carboxyl mixture. The mixture was then stirred for an additional 2-3 h at 23°C. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Acetylation (a). In a 20 mL scintillation vial, 1 eq. of the compound was added along with 2.5 mL of anhydrous DCM and a magnetic stir bar. The solution was cooled to 0°C in an ice bath, and 1.2 eq of anhydrous sodium carbonate was added along with 0.3 eq. of DMAP. Then, 1.2 eq. of acetic anhydride was added to the reaction mixture dropwise. The mixture was then stirred for another hour at 0°C, then allowed to warm to 23°C and stirred overnight. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Acetylation (b). To a 20 mL scintillation vial, 1 eq. of an alcohol compound was added along with 2.5 mL of anhydrous DCM. and a magnetic stir bar. Then, 2 eq. of EDC, 2 eq. of acetic acid, and 3 eq. of triethylamine were added to the mixture. The reaction was then stirred at 23°C for 5-6 hours. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Sulfation. In a 20 mL scintillation vial, 1 eq. of an alcohol was added along with 2.5 mL of anhydrous DCM and a magnetic stir bar. Then, 1.5 eq. of pyridine sulfur trioxide was added under dry argon. The reaction was then stirred for 1-3 hours at 23°C The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Methylation. In a 20 mL scintillation vial, 1 eq. of an alcohol, carboxylic acid, or amine was added along with 2.5 mL of anhydrous DMF. Then, 1.5-2 eq. of sodium carbonate and 1.5-2 eq. of methyl iodide were added. The reaction was then left to stir overnight at 23°C. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Oxidation. In a 20 mL scintillation vial, 1 eq. of an alcohol was added along with 2.5 mL of DCM. Then, 0.04 eq. of TEMPO, 0.1 eq. of NaBr, and 2 eq. of sodium hypochlorite were added dropwise over 15 minutes. The reaction was then stirred for 2-3 hours. The mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Oxidation of PUFAs using H_2O_2. 1.4 eq. of free fatty acids were added to a 20 mL scintillation vial with 1mL of ethanol. The solution was then mixed with 1 eq. of 120 mM hydrogen peroxide in water solution. After 30 minutes, the reaction was divided into two 1 mL aliquots. In the first, 0.1 mL of 1M KOH in water was added. This aliquot was then incubated for 30 minutes at 40°C in a tube rotator. Afterwards, 100 µL of formic acid was added to obtain an acidic pH of 4. In the second aliquot, 1 mL of acidified water with formic acid (pH=4) was added.

Boc deprotection (a). A boc protected compound (1 eq.) was dissolved in 2 ml of Dioxane and cooled to 0°C with an ice bath.10 eq. of 4M HCl in Dioxane solution was added dropwise while stirring 0°C. The reaction was allowed to warm up to 23°C and stirred for 1-3 hours at room temperature. The reaction mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Boc deprotection (b). A boc protected compound (1 eq.) was dissolved in 2 ml of DCM and cooled to 0°C with an ice bath. Then, 10 eq. of TFA was added dropwise while stirring 0 °C. The reaction was then allowed to warm to 23°C and stirred for an additional 1-3 hours. The reaction mixture was then concentrated *in vacuo* and an LC-MS/MS sample was prepared.

Glucuronidation. Each substrate (30 mg, 1 eq.) was dissolved in toluene (3 mL). Acetobromo-α-D-glucuronic acid methyl ester (300 mg, 10 eq.) and silver carbonate (290 mg) were then added sequentially. The mixture was stirred vigorously and maintained under controlled heating for different time intervals 24 h or 3 days at 75°C, and up to 5 days at 90°C depending on the substrate, to achieve optimal conversion.

After completion, the reaction mixture was cooled to 23°C, filtered to remove solids, and concentrated under reduced pressure. The resulting residue was redissolved in methanol (0.5 mL) and an equal volume of 1 M aqueous LiOH, and the solution was stirred at room temperature for 28 h. The solvent was then evaporated, and the mixture was neutralized with 1 M acetic acid (0.5 mL). The crude products were subjected directly to LC–MS/MS analysis.

Reductive amination. An amine compound (1 eq.) and a carbonyl compound (1 eq.) were mixed in anhydrous DCM (5 mL) and then treated with sodium triacetoxyborohydride (1.4 eq.) and AcOH (1 eq.). The mixture was stirred at 23 °C under argon for 24 h. The reaction mixture was then

quenched by adding 1 M NaOH, and the product was extracted with DCM. The extract was then washed with brine and dried with Na_2SO_4. The solvent was then evaporated.

Hydrolysis. A mixture of methyl esters (1 eq.) was dissolved in THF:H2O (5 mL: 5 mL) and cooled to 0°C with an ice bath for 15 min before adding solid LiOH (5 equiv) in one portion. The reaction was then allowed to warm to 23°C and stirred 12 h. Excess hydroxide was then quenched with 1 M HCl. The product was extracted with ethyl acetate (5 mL × 3) and the combined organic extracts were washed with brine (5 mL), dried over sodium sulfate, filtered, and concentrated *in vacuo*.

Ring opening. In a 250 ml round bottom flask with an inert argon atmosphere, δ-decalactone (1 eq.) And sodium ethoxide (21% ethanol solution, 1.2 eq.) were added while stirring at room temperature.The reaction was left to stir overnight, then glacial acetic acid (1 eq.) was added to neutralize the NaOEt. The mixture was then concentrated under reduced pressure. Saturated brine (100 mL) was then added to the obtained residue, and the mixture was extracted with DCM (100 ml). The organic layer was then washed with saturated aqueous sodium bicarbonate solution (100 ml) and saturated brine (100 mL). The organic layer was then dried over anhydrous sodium sulfate and concentrated under reduced pressure.

Acid chloride formation. To a solution containing a carboxylic acid (1 eq.) in anhydrous Dichloromethane (DCM) (2.5 ml) oxalyl chloride was added(2 eq), followed by 2 drops of DMF. The mixture was stirred at 23°C for 30 min, then excess oxalyl chloride and dichloromethane were removed *in vacuo.*

Glycosylation. To a solution of a hydroxyl (1 eq.) containing compound in 3 mL of toluene, and (10 eq.) of acetobromo-sugar methyl ester was added, followed by the addition of (2 eq.) of silver carbonate. The reaction mixture was refluxed and stirred at 75°C for more than 24 hours. After reflux, the mixture was cooled to room temperature, filtered, and evaporated to dryness under vacuum. The resulting solid residue was dissolved in 10 mL of methanol, and (6 eq.) of 1 M aqueous LiOH were added. The mixture was stirred at 23°C for 28 hours. After the solvent removal, the product was neutralized with 0.5 mL of 1 M acetic acid. The reaction crudes were injected into the LC-MS/MS system and used for further analysis.