Electronic Supplementary Material (ESI) for Environmental Science: Processes & Impacts.

Supplementary Information I (SI-I)

Combining predictive and analytical methods to elucidate pharmaceutical biotransformation in activated sludge

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S1. Literature Search

Supplementary Table S1. Detailed overview on results from the literature search. Number of TPs, precision and TP vs Parent ratios were calculated only for the TPs found through suspect screening.

Authors	Year	Substance class	Number of compounds	Inital concentration	Experimental setup	Analytical instrument	Analytical method	Screening type	Prediction type	Total predicted TPs	Total number of found TPs	Number of found TPs with suspect screening only	Precision	TP vs parent ratio	Reference
Damian E. Helbling, Juliane Hollender, Hans-Peter E. Kohler, Heinz Singer and Kathrin Fenner	2010	pharmaceuticals and pesticides	12	**100 µg/L**	batch reactor	HPLC-MS Orbitrap	dd-MS2 with inclusion list	target and non-target	UM-PPS	1039	26	21	2.02%	2.2	10.1021/es100970m
Damian E. Helbling, Juliane Hollender, Hans-Perter E. Kohler and Kathrin Fenner	2010	general amides, pesticides and pharmaceuticals	30	**100 µg/L**	batch bottle reactor	HPLC-MS Orbitrap	dd-MS2 with inclusion list	target and non-target	UM-PPS	NA	53	NA	NA	1.8	10.1021/es101035b
Susanne Kern, Rebekka Baumgartner, Damian E. Helbling, Juliane Hollender, Heinz Singer, Martin J. Loos, René P. Schwarzenbach and Kathrin Fenner	2010	pharmaceuticals and biocides	8	**100 µg/L**	batch reactor	HPLC-MS Orbitrap	dd-MS2 with inclusion list	suspect	UM-PPS	two generations	12	12	NA	1.5	https://doi.org/10.1039/C0EM00238K
Carsten Prasse, Manfred Wagner, Ralf Schulz, and Thomas A. Ternes	2011	pharmaceuticals	2	**4 µg/L**	batch bottle reactor	HPLC-MS Orbitrap and 1D/2D NMR	dd-MS2/3 top 2 without inclusion list	suspect and non-target	UM-PPS	56	9	5	8.93%	4.5	https://doi.org/10.1021/es103732y
Elisabeth Müller, Walter Schüssler, Harald Horn, Hilde Lemmer	2013	pharmaceuticals	1	**10 mg/L**	batch bottle reactor	LC-MS Q-Trap hybrid triple-quadrupole	additional GC-MS/MS	suspect	UM-PPS	NA	2	2	NA	2.0	https://doi.org/10.1016/j.chemosphere.2013.02.070
Sebastian Huntscha, Thomas B. Hofstetter, Emma L. Schymanski, Stephanie Spahr, and Juliane Hollender	2014	benzotriazoles	3	**0.5-2.4 mg/L**	batch bottle reactor	UHPLC-MS Orbitrap	dd-MS2 with inclusion list	suspect and non-target	UM-PPS	three generations	13	NA	NA	4.3	https://doi.org/10.1021/es405694z
Thomas Letzel, Anne Bayer, Wolfgang Schulz, Alexandra Heermann, Thomas	2015	pharmaceuticals	5	**1-50 µg/L**	batch reactor and waste water samples	RPLC-ESI-QqTOF-MS, RPLC-	full-scan with MS/MS	target, suspec and non-target	EAWAG-BBD/PPS	132	6	6	4.55%	1.2	https://doi.org/10.1016/j.chemos

Lucke, Giorgia Greco, Sylvia Grosse, Walter Schüssler, Manfred Sengl, Marion Letzel						HILIC-ESI-TOF-MS, RPLC-ESI-QqQ-MS									phere.2015.06.083
Tina Kosjek, Noelia Negreira, Miren López de Alda, Damià Barceló	2015	pharmaceuticals	1	**1 mg/L**	batch bottle reactor	UHPLC-MS Orbitrap	dd-MS2 top 5	suspect and non-target	UM-PPS	NA	9	8	NA	9.0	https://doi.org/10.1016/j.chemosphere.2014.04.081
Pablo Gago-Ferrero, Emma L. Schymanski, Anna A. Bletsou, Reza Aalizadeh, Juliane Hollender, Nikolaos S. Thomaidis	2015	pharmaceuticals	173	**unspiked**	waste water samples	UHPLC-QTOF-MS	dd-MS2	suspect and non-target	Metabolite Predict	284	47	13	4.58%	0.3	https://doi.org/10.1021/acs.est.5b03454
Rebekka Gulde, Ulf Meier, Emma L. Schymanski, Hans-Peter E. Kohler, Damian E. Helbling, Samuel Derrer, Daniel Rentsch, and Kathrin Fenner	2016	pharmaceuticals, pesticides and wastewater-derived chemicals	19	**120 µg/L**	batch bottle reactor	HPLC-MS Orbitrap	dd-MS2 with inclusion list	suspect and non-target	EAWAG-BBD/PPS, metaprint2D	170	101	33	19.41%	5.3	https://doi.org/10.1021/acs.est.5b05186
Vasiliki G.Beretsou, Aikaterini K. Psoma, Pablo Gago-Ferreroa, Reza Aalizadeh, Kathrin Fenner, Nikolaos S. Thomaidis	2016	pharmaceuticals	1	**2 mg/L**	batch bottle reactor	UHPLC-QTOF-MS	dd-MS2 with inclusion list	suspect and non-target	EAWAG-BBD/PPS, Metabolite Predict	NA	14	12	NA	14.0	https://doi.org/10.1016/j.watres.2016.07.029
Thomas Letzel, Sylvia Grosse, Wolfgang Schulz, Thomas Lucke, Angela Kolb, Manfred Sengl, and Marion Letzel	2016	pharmaceuticals	1	**10-40 µg/L**	batch reactor and waste water samples	RPLC-ESI-QqTOF-MS, RPLC-HILIC-ESI-TOF-MS, RPLC-ESI-QqQ-MS	full-scan with MS/MS	target, suspec and non-target	EAWAG-BBD/PPS	62	4	3	4.84%	4.0	10.1021/bk-2016-1241.ch006
Tina Kosjek, Noelia Negreira, Ester Heath, Miren López de Alda, Damià Barceló	2018	pharmaceuticals	1	**1 mg/L**	batch bottle reactor	UHPLC-MS Orbitrap	dd-MS2 top 5	suspect	EAWAG-BBD/PPS	14	11	4	28.57%	11.0	http://dx.doi.org/10.1016/j.scitotenv.2017.08.061
Stefan Achermann, Per Falås, Adriano Joss, Cresten B. Mansfeldt, Yujie Men, Bernadette	2018	micropollutants	93	**6 mg/L**	batch reactor	HPLC-MS Orbitrap	dd-MS2 with inclusion list	suspect	EAWAG-BBD/PPS	219	75	75	34.25%	0.8	10.1021/acs.est.8b02763

Vogler, and Kathrin Fenner															
Michael T. Zumstein, Damian E. Helbling	2019	pharmaceuticals	6	**100 µg/L**	batch reactor	HPLC-MS Orbitrap	dd-MS2 with inclusion list	suspect	EAWAG-BBD/PPS	2 or 3 generations	16	16	NA	2.7	https://doi.org/10.1016/j.watres.2019.02.024
Tjasa Gornik, Ana Kovacic, Ester Heath, Juliane Hollender, Tina Kosjek	2020	pharmaceuticals	1	**1 µg/L - 1 mg/L**	batch reactor and pilot WWT bioreactor	HPLC-MS Orbitrap	dd-MS2 top 7 with inclusion list	suspect and non-target	EAWAG-BBD/PPS	3 generations	10	NA	NA	10.0	https://doi.org/10.1016/j.watres.2020.115864
Rebecca A. Trenholm, Brett J. Vanderford, Narasimman Lakshminarasimman, Drew C. McAvoy and Eric R. V. Dickenson	2020	micropollutants	3	**1 mg/L**	batch bottle reactor	LC-QTOF-MS	no MS2	suspect	EAWAG-BBD/PPS	40	9	9	22.50%	3.0	10.1061/(ASCE)EE.1943-7870.0001691
Xuebing Wang, Nanyang Yu, Jingping Yang, Ling Jin, Huiwei Guo, Wei Shi, Xiaowei Zhang, Liuyan Yang, Hongxia Yu, Si Wei	2020	pesticides and pharmaceuticals	60	**unspiked**	waste water samples	LC-QTOF-MS and LC-quadrupole-MS/MS	dd-MS2 top 20	suspect	EAWAG-BBD/PPS	NA	57	21	NA	1.0	https://doi.org/10.1016/j.envint.2020.105599
Gang Wu, Jinju Geng, Yufei Shi, Liye Wang, Ke Xu, Hongqiang Ren	2020	pharmaceuticals	1	**500 µg/L**	batch bottle reactor	LC-QTOF-MS	dd-MS2	suspect	EAWAG-BBD/PPS, Pathpred	NA	4	4	NA	4.0	https://doi.org/10.1016/j.watres.2020.116158
Juliet Kinyua, Aikaterini K. Psoma, Nikolaos I. Rousis, Maria-Christina Nika, Adrian Covaci, Alexander L. N. van Nuijs and Nikolaos S. Thomaidis	2021	psychoactive substances	2	**2 mg/L**	batch reactor	LC-QTOF-MS	DIA MS2	suspect	EAWAG-BBD/PPS, Metabolite Predict	NA	10	10	NA	5.0	10.3390/metabo11020066
Wenwen Cai, Pu Ye, Bin Yang, Zhouqi Shi, Qian Xiong, Fangzhou Gao, Yousheng Liu, Jianliang Zhao, Guangguo Ying	2021	fungicides	2	**500 µg/L**	batch bottle reactor	HPLC-QTOF-MS	dd-MS2 with inclusion list	suspect and non-target	EAWAG-BBD/PPS, PathPred	36	10	3	8.33%	5.0	https://doi.org/10.1016/j.jes.2020.11.007
Yeowool Choi, Junho Jeon, Sang Don Kim	2021	triphenyl phosphate	1	**100 µg/L**	waste water samples	UHPLC-MS Orbitrap	dd-MS2 top 5	target, suspec and non-target	EAWAG-BBD/PPS	NA	29	4	NA	29.0	https://doi.org/10.1016/j.watres.2021.117201
A. B. Martínez-Piernas, P. Plaza-Bolaños, A. Agüera	2021	pharmaceuticals	20	**unspiked**	waste water samples	LC-QTOF-MS	DIA MS2	suspect	EAWAG-BBD/PPS	262	5	5	1.91%	0.3	https://doi.org/10.1016/j.jhazmat.

															2021.125080
Aikaterini K.Psoma, Nikolaos I. Rousis, Eleni N. Georgantzi, Nikolaos S. Thomaidis	2021	pharmaceuticals	4	**0.2-2 mg/L**	batch bottle reactor	UHPLC-QTOF-MS	dd-MS2 with inclusion list and DIA MS2	suspect and non-target	EAWAG-BBD/PPS	NA	22	NA	NA	5.5	https://doi.org/10.1016/j.scitotenv.2020.144677
Rebekka Gulde, Moreno Rutsch, Baptiste Clerc, Jennifer E. Schollée, Urs von Gunten, Christa S.McArdell	2021	micropollutants	87	**10 µg/L**	batch bottle reactor and waste water samples	HPLC-MS Orbitrap	dd-MS2 with inclusion list	suspect and non-target	O3-PPS	NA	84	NA	NA	1.0	https://doi.org/10.1016/j.watres.2021.117200
Yangping Zhang, Haifeng Zhang, Juan Wang, Zhiyong Yu, Hongyan Li, Min Yang	2021	pesticides	30	**unspiked**	waste water and river samples	LC-QTOF-MS	DIA MS2	suspect and target	Metabolite pilot	1400	20	18	1.29%	0.7	https://doi.org/10.1016/j.scitotenv.2021.147978
Stephanie L. Rich, Michael T. Zumstein, and Damian E. Helbling	2022	micropollutants	40	**100 µg/L**	batch bottle reactor	HPLC-MS Orbitrap	dd-MS2 with inclusion list	suspect	EAWAG-BBD/PPS	227	46	46	20.26%	1.2	https://doi.org/10.1021/acs.est.1c06429

S2. Prediction of Transformation Products

Transformation products were predicted using EAWAG-PPS and enviPath. The pathway prediction algorithm in enviPath is different from the original algorithm implemented in EAWAG-PPS (Supplementary Figure S1).

S2.1 Prediction with EAWAG-PPS

EAWAG-PPS was run in Batch mode (https://test.eawag-bbd.ethz.ch/rxnmulti/

access upon request) using the parameters specified in Supplementary Supplementary Table **S**.

Supplementary Table S2. Settings used for TP prediction in the EAWAG-PPS/BBD

Parameter	Value
Relative reasoning	True
Immediate rules	True
Likelihood cutoff	Neutral
Recursion depth	3

S2.2 Prediction with enviPath models

The TP predictions with enviPath were performed using a Python script available on GitHub (https://github.com/FennerLabs/TP_predict/blob/main/TP_prediction/find_best_TPs.py). The following relative reasoning models were used:

- ML–ECC–BBD
- ML–ECC–BBD+SOIL
- ML–ECC–BBD+SLUDGE
- ML–ECC–BBD+SOIL+SLUDGE

The relative reasoning models are publicly available at https://envipath.org/package/308fc905-f84d-410b-b3ca-ed888d59dd33.

The predicted pathways were stored for each model separately as a data package on enviPath for visualization:

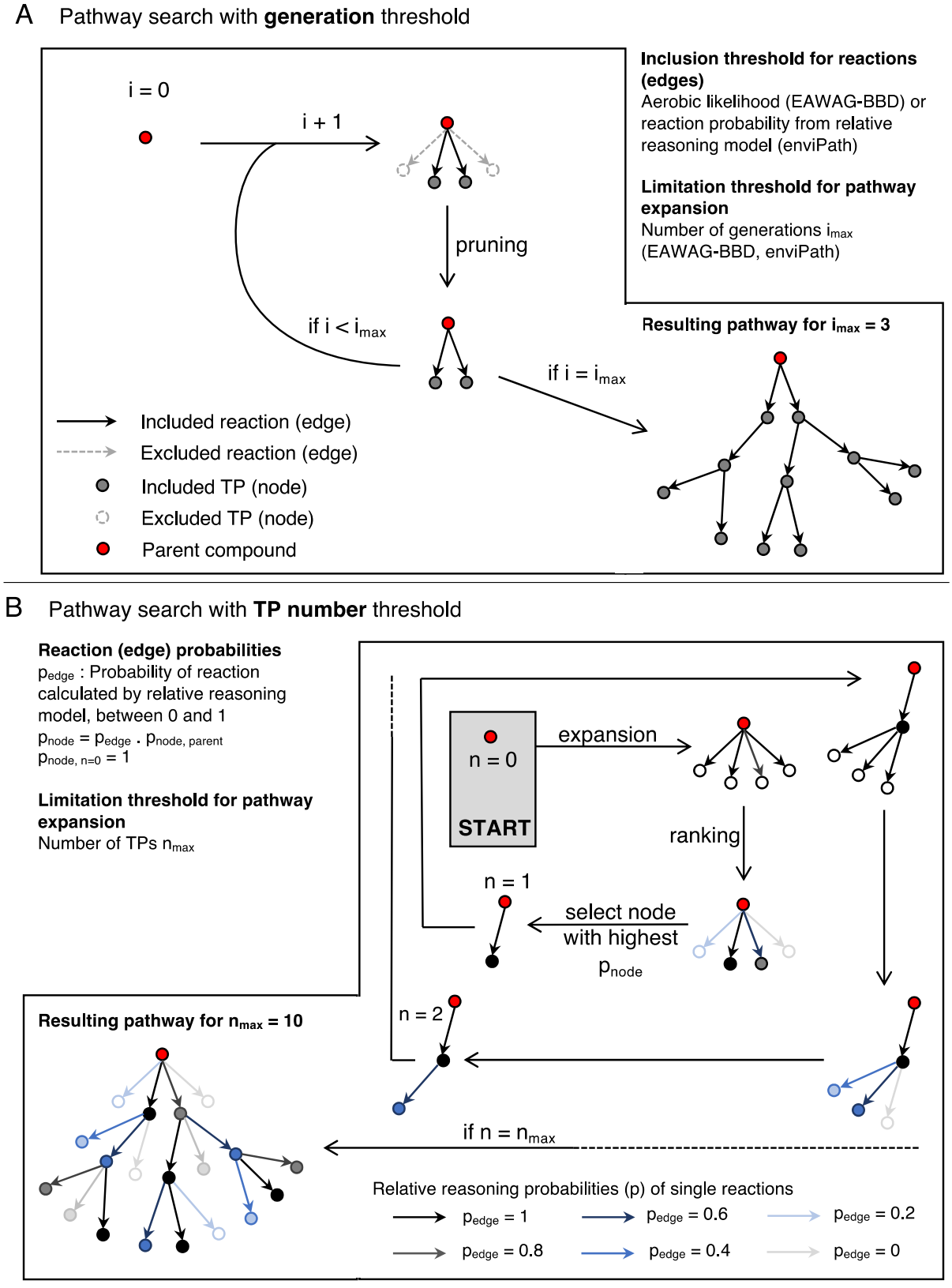
- Package for results using BBD - ML - ECC - 2022 model: https://envipath.org/package/0915fad3-b889-4aa8-ac98-0707b717be57
- Package for results using BBD+SOIL - ML - ECC - 2022 model:

https://envipath.org/package/80cf58b1-21e2-4c28-9cc6-dc69c6445bdf

- Package for results using BBD+SLUDGE - ML - ECC - 2022 model: https://envipath.org/package/7d64aa85-2e3c-413f-a538-4d5f2bfd4662
- Package for results using BBD+SOIL+SLUDGE - ML - ECC - 2022 model: https://envipath.org/package/11f2acd5-5209-4d49-ad77-93f6f6965886

S2.3 Suspect list compilation

To compile the suspect list from the TP prediction output, the corresponding python script on GitHub was used (https://github.com/FennerLabs/TP_predict/tree/main/File_conversion/Prediction_output_to_mass_list). The following programming languages and libraries were used: Python version 3.6.13, *RDKit* version 2020.09 (used for mass calculation), *Pandas* version 1.1.5 (used for export as csv files), and *PubChemPy* version 1.0.4 (used for CAS number search).

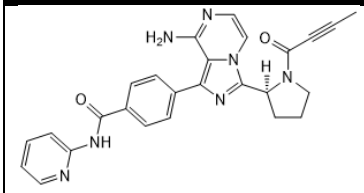
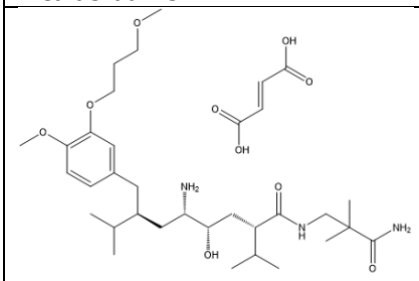
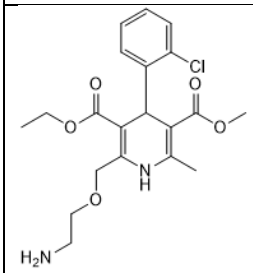
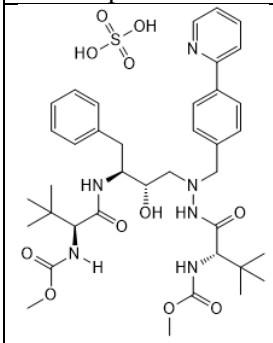
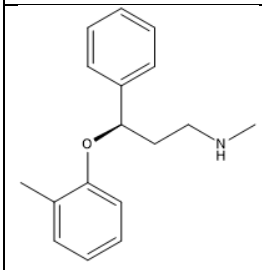


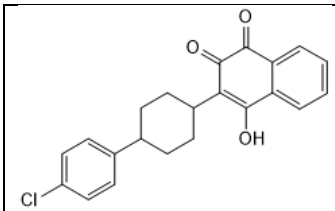
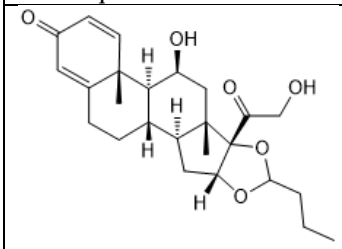
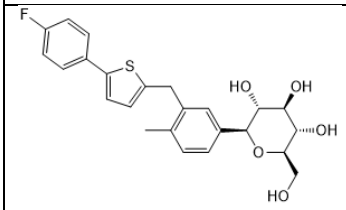
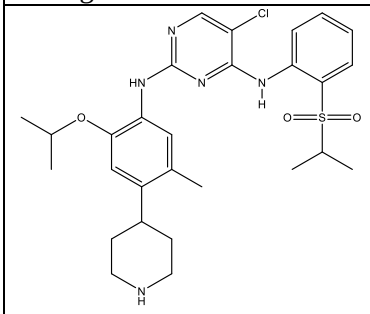
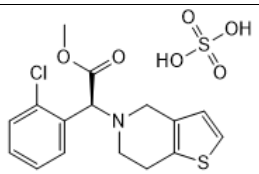
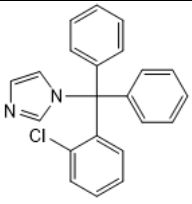
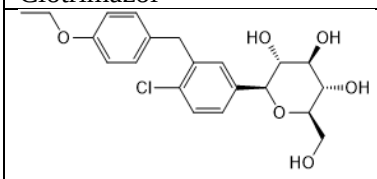
Supplementary Figure S1. Pathway search algorithms used for TP prediction. A In EAWAG-PPS, a breadth-first graph expansion explores predicted nodes one generation after the other. Newly generated nodes are pruned based on the inclusion criterion. When the generation threshold i_{max} is reached, the network expansion stops. In enviPath, this type of expansion is available with a relative reasoning threshold probability for node pruning, but not used in this work. B The probabilities assigned to each reaction in enviPath allow for a weight-biased graph traversal, where nodes with high probability are added to the network before nodes with low probability. This type of pathway search yields the top k TPs with the highest probability to be observed according to the relative reasoning model. The maximum number of TPs to be found is specified as n_{max}. In this work, the weight-biased graph expansion is used for the enviPath models.

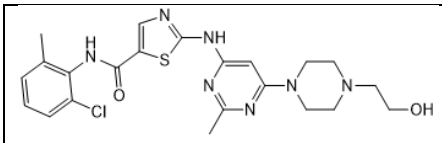
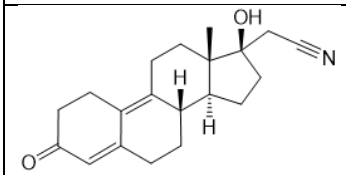
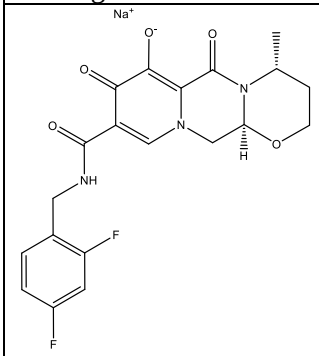
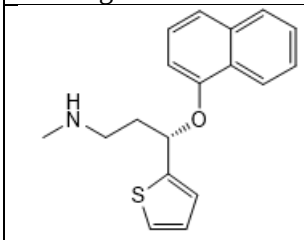
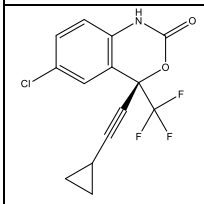
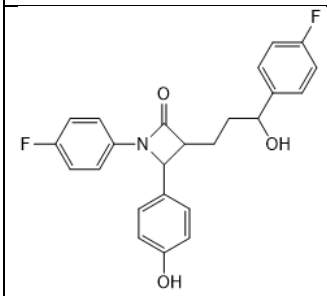
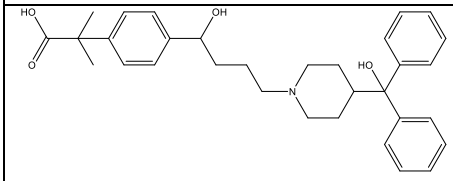
S3. Biotransformation Experiments

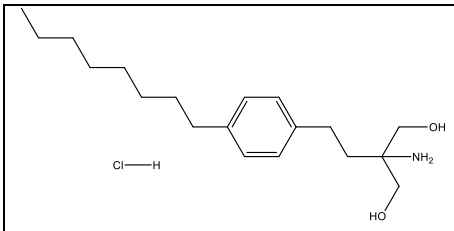
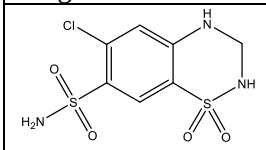
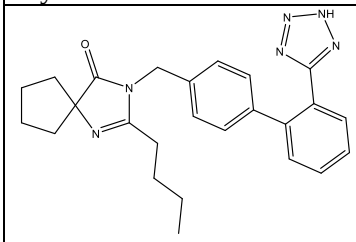
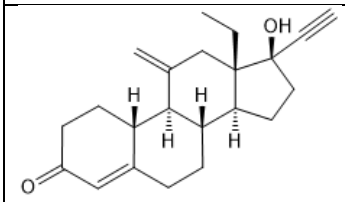
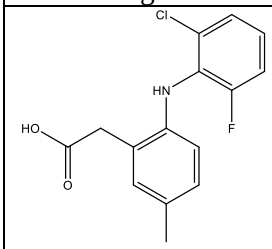
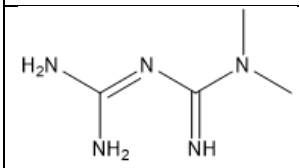
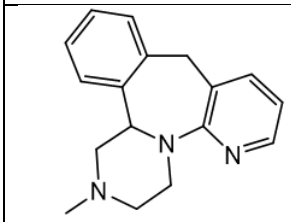
S3.1 Test compounds

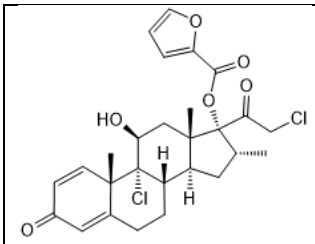
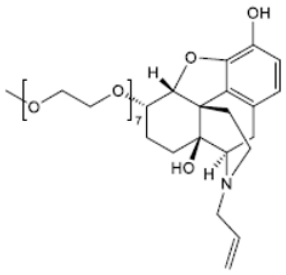
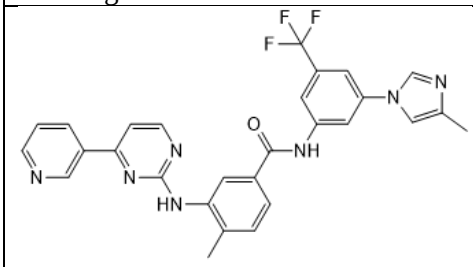
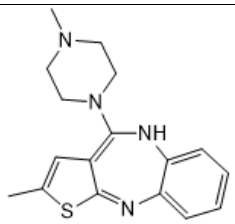
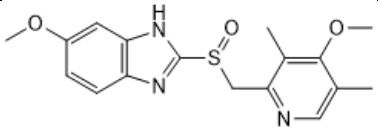
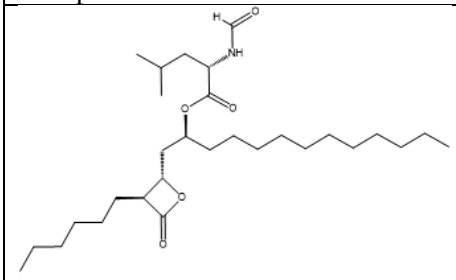
Supplementary Table S3. Selected APIs for the biotransformation tests, short name for identification, drug class, main manufacturing company and properties.

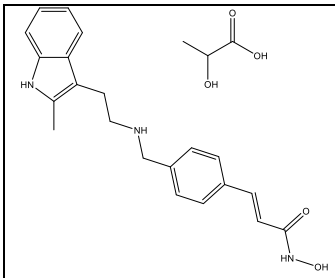
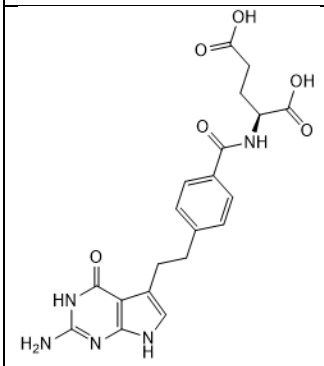
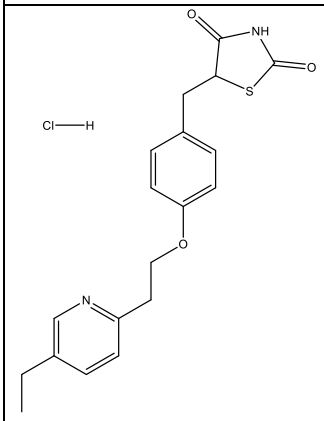
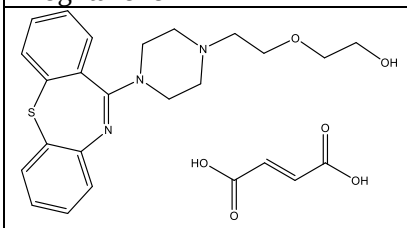
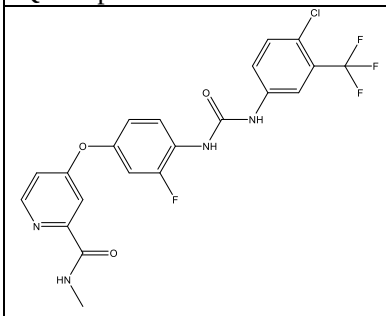
API	Short name	Drug class	log Koc*	log P**	Charge**
 Acalabrutinib	Aca	Antineoplastic	4.40	2.56	neutral
 Aliskiren	Ali	Antihypertensive	NA	3.12	cation
 Amlodipine	Aml	Cardiovascular	1.57	1.64	cation
 Atazanavir	Ata	Antiretroviral	4.51	4.54	neutral
 Atomoxetine	Atm	Psychostimulant	3.56	3.81	cation

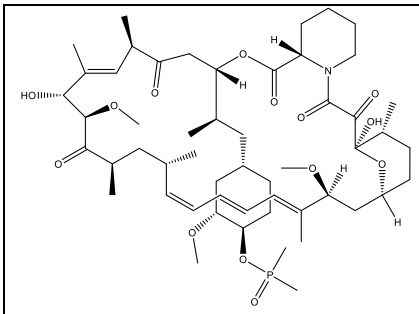
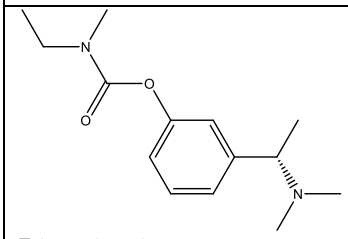
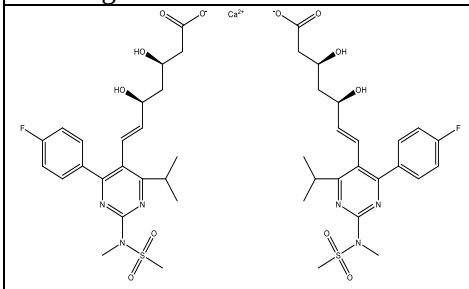
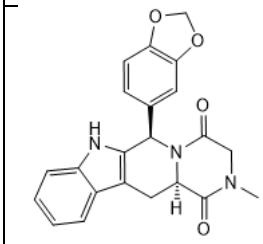
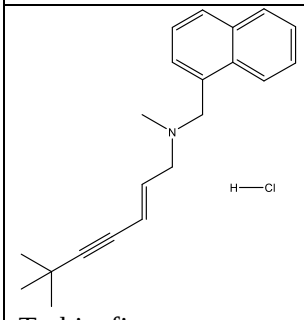
 Atovaquone	Atv	Antimalarial	4.49	5.00	anion
 Budesonide	Bud	Corticosteroid	4.73	2.73	neutral
 Canagliflozin	Can	Antidiabetic	4.37	3.52	neutral
 Ceritinib	Ceri	Antineoplastic	5.39	5.81	cation
 Clopidogrel	Clp	Antiplatelet	3.17	4.03	neutral
 Clotrimazol	Clt	Antifungal	3.92	5.84	neutral-cation
 Dapagliflozin	Dap	Antidiabetic	2.31	2.11	neutral

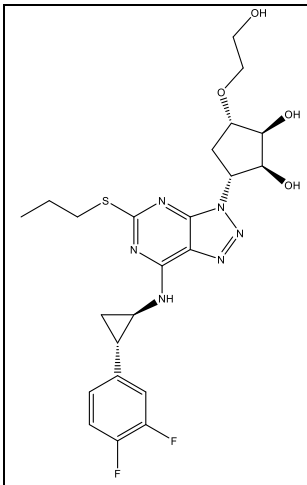
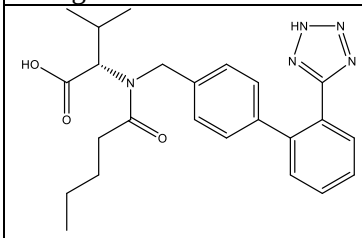
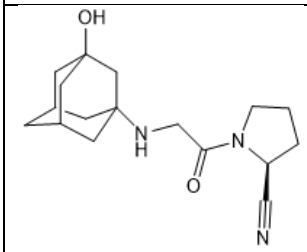
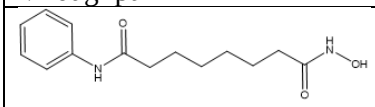
 Dasatinib	Das	Antineoplastic	2.99	4.01	neutral-cation
 Dienogest	Die	Contraceptive	5.18	2.31	neutral
 Dolutegravir	Dol	Antiviral	NA	1.10	neutral
 Duloxetine	Dul	Antidepressant	3.47	4.20	cation
 Efavirenz	Efa	Antiviral	3.17	5.15	neutral
 Ezetimibe	Eze	Antilipemic	4.92	4.56	neutral
 Fexofenadine	Fex	Antihistamine	5.20	2.94	zwitterion

 Fingolimod	Fin	Multiple Sclerosis Agents	3.28	4.06	cation
 Hydrochlorothiazide	Hyd	Diuretics	1.72	-0.58	neutral
 Irbesartan	Irb	Angiotensin II Receptor Antagonists	4.33	4.47	neutral-anion
 Keto-desogestrel	Ket	Contraceptive	5.35	3.60	neutral
 Lumiracoxib	Lum	Anti-inflammatory	3.14	4.31	anion
 Metformin	Met	Antidiabetic	0.98	-0.92	cation
 Mirtazapine	Mir	Antidepressant	2.81	3.21	neutral-cation

 Mometasone	Mom	Corticosteroid	4.96	5.06	neutral
 Naloxegol	Nal	Opioid Antagonists	4.94	1.36	neutral-cation
 Nilotinib	Nil	Antineoplastic	5.40	5.36	neutral-cation
 Olanzapine	Ola	Antipsychotic	2.71	3.39	neutral-cation
 Omeprazole	Ome	Antiulcer	2.97	2.43	neutral
 Orlistat	Orl	Weight Loss	4.32	8.11	neutral

 Panobinostat	Pan	Antineoplastic	1.79	-3.85	zwitterion
 Pemetrexed	Pem	Antineoplastic	NA	0.43	anion
 Pioglitazone	Pio	Hypoglycemic Agents	4.14	3.40	neutral-anion
 Quetiapine	Que	Antipsychotic	3.05	2.81	neutral-cation
 Regorafenib	Reg	Antineoplastic	5.18	4.49	neutral

 Ridaforolimus	Rid	Antineoplastic	NA	7.25	neutral
 Rivastigmine	Riv	Alzheimer Disease Agents	2.87	2.41	cation
 Rosuvastatin	Ros	Antilipemic	1.54	1.92	anion
 Tadalafil	Tad	Erectile Dysfunction	4.79	1.64	neutral
 Terbinafine	Ter	Antifungal	3.27	5.53	cation

 Ticagrelor	Tic	Antithrombotic	3.23	2.28	neutral
 Valsartan	Val	Angiotensin II Receptor Antagonists	2.77	5.27	cation
 Vildagliptin	Vil	Antidiabetic	2.96	-0.22	cation
 Vorinostat	Vor	Antineoplastic	3.09	2.00	anion

* Obtained from OPERA v2.7 (https://github.com/kmansouri/OPERA), **Values calculated with ChemAxon software (www.chemicalize.com) for pH 7

The spike mixture containing all APIs (20 mL, 10 mg/L (± 8%) for each parent, in EtOH) was prepared gravimetrically by adding each stock solution (about 160 mg each, 1 g/L in MeOH or EtOH) of the respective substance.

S3.2 Preparation of test reactors

Activated sludge (4 L, total suspended solids (TSS) 2.8 g/L, and 1.27 mg/L dissolved O_2 at 15.4 °C at the time of collection) was collected from the ARA-Neugut (Dübendorf, Switzerland) and immediately transported to Eawag laboratories. The activated sludge (3 x 700 mL) was centrifuged for 1 min at 2'000 rpm and afterwards the supernatant (3 x 400 mL) was separated. The remaining volume (3 x 300 mL) was used for the incubations with high biomass (HB, TSS 7.1 g/L). The supernatant (600 mL) was then poured to native sludge (150 mL, not centrifuged) to prepare the incubations with dilute biomass (DB, TSS 0.6 g/L). Sludge suspensions (6 x 50 mL for HB and DB) were added to 100 mL biotransformation (BT) bottles and unspiked control (UC) reactors. The remaining supernatant (200 mL) was filtered with 0.2 µm filter paper and the filtrate (2 x 50 mL) was added to 100 mL abiotic control (AB) bottles. DB and HB suspensions (2 x 50 mL each) were further added to 100 mL sorption control (SC) bottles. AB bottles were once autoclaved (121 °C and 103 kPa for 20 min), whereas SC bottles were autoclaved twice. All bottle incubations were stirred and air (0.5 bar) was bubbled through. Additionally, CO_2 (1 bar) was bubbled through half of the reactors. All reactors were equilibrated for 1 h prior to incubating with the API mixtures.

Supplementary Table S4. Experimental setup and number of biotransformation and control reactors (HB = high biomass with TSS = 7.1 g/L, DB = dilute biomass with TSS = 0.6 g/L, native sludge with TSS = 2.8 g/L).

	Biotransformation		**Abiotic control**	**Sorption control**		**Unspiked control**	
Treatment	HB	DB		HB	DB	HB	DB
pH-low	2	2	1	1	1	1	1
pH-high	2	2	1	1	1	1	1

The addition of APIs was performed as follows: the spike mixture containing all the APIs (40 µL, 10 mg/L) was added to 16 empty 100 mL amber glass bottle. The solvent was allowed to evaporate over 15 min and then the contents from the equilibrated sludge bottles was transferred to the spiked

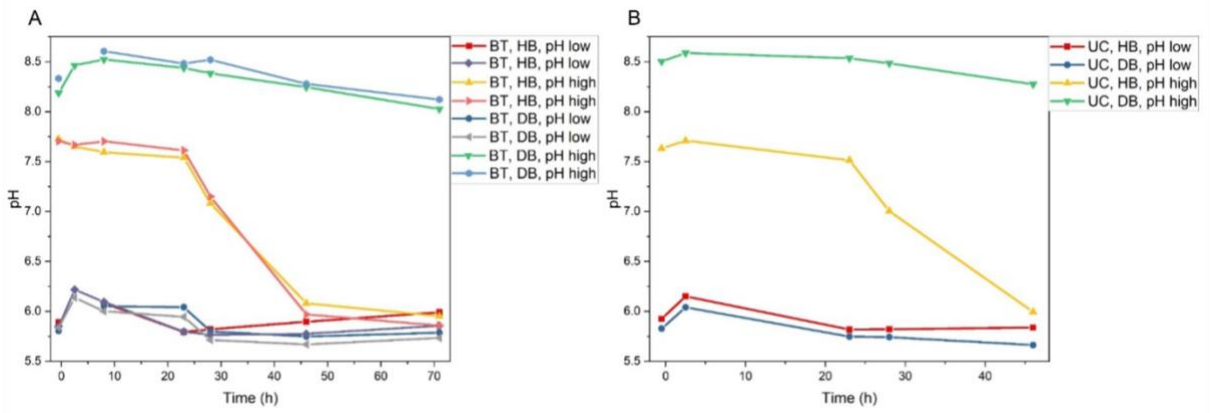
amber bottles. The resulting final concentration was thus 8 µg/L for each parent compound. The remaining four UC reactors were not incubated with APIs.

S3.3 Sampling

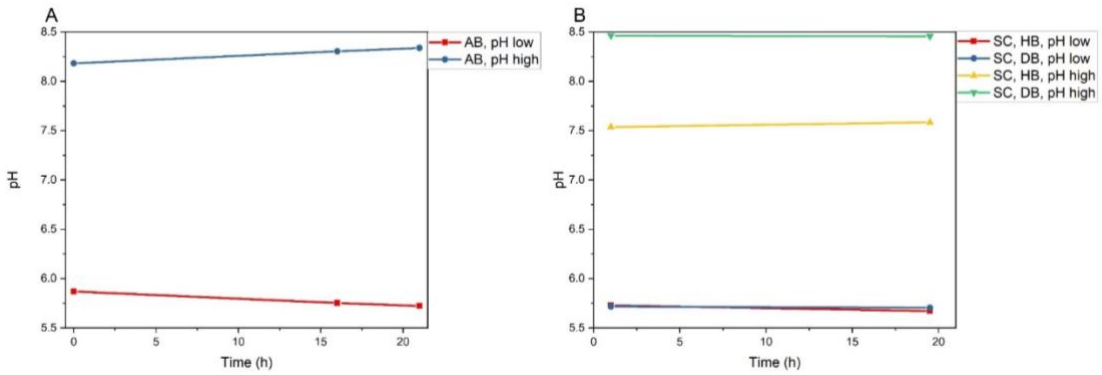
Samples were collected at time points 0 h, 2h, 4 h, 9 h, 24 h, 30 h, 48 h, 54 h and 72 h. Aliquot (1.5 mL) was transferred to 2 mL *Eppendorf* tubes and centrifuged for 10 min at 15'000 rpm. Supernatant (0.8 mL) was added to a 1.5 mL muffled amber vial (500 °C in 2 h, hold for 4.5 h) and stored at 4 °C until 72 h after first sampling. Samples were stored at -20 °C after the experiment. Triplicates were sampled for 0 h from all reactors and 30 h for BT reactors. Weight of reactors were determined before, after taking aliquots, and at the start and end of the day to calculate evaporation losses. Nanopure water was added to compensate for losses.

S3.4 pH measurements in activated sludge experiments

The pH was monitored by measuring it twice a day (*pH Meter 913; Metrohm*, Herisau, Switzerland).



Supplementary Figure S2. Monitoring of pH in biotransformation (BT) reactors with high biomass (HB) or diluted biomass (DB) over the course of the experiment (A) and unspiked control (UC) reactors (B).



Supplementary Figure S3. Monitoring of pH in abiotic (AB) and sorption control (SC) reactors, (A) and (B), respectively, over the first 20 hours of the experiment .

S4. HPLC-HRMS/MS measurements

S4.1 HPLC separation

HPLC-grade (nanopure) water was collected from *Arium pro DI Ultrapure Water System* (*Satorius*, Gottingen, Germany). Samples of 100 µL on-flow with an auto-sampler (mobile phase: H_2O/MeOH + 0.1% HCOOH following flow gradient; flow rate 300 µL min^{-1}, MeOH purchased from Fisher Scientific) were injected into HPLC column (*Atlantis T3* with *VanGuard* pre-column, 3.0 x 150 mm, C18, particle size: 3 µm). For the liquid chromatography, initial conditions for the mobile phase composition were set at 95:5 water/methanol and were maintained at this condition for 1 min. Then, the methanol fraction was increased to 5:95 water/methanol over 16 min and held at this composition for 8 min. Initial conditions were reestablished after 0.1 min and maintained for 4.9 min before starting the next analysis.

S4.2 Scan Methods of HRMS

HPLC was coupled to an electrospray ionization high-resolution mass spectrometer QExactive Plus (*Thermo Fisher Scientific*, Bremen, Germany). Ion source parameters: spray voltage 3.5 kV, capillary temperature 320°C, sheath gas 32 L min^{-1}, s-lens RF level 50.0; full scan MS with data-dependent MS^2; mass calibration <0.5 ppm accuracy in (+/-)-ESI with in-house calibration solution.

S4.2.1 Initial suspect screening

Samples (all timepoints, all treatments and controls) were measured twice (once in positive and once in negative ionizations) first acquiring MS in full scan mode at a resolution of 140,000, scan range of 100 to 1000m/z. Then data-dependent MS^2 spectra were acquired at a resolution of 17,500, maximum ion time of 50 ms, isolation window 1 m/z, dynamic exclusion of 8 s and default NCE of 30 (to be used in any intense ion that is not in the inclusion list). The inclusion list (Section S2.3, Suspect list compilation) contained the calculated mass of $[M+H]^+$ or $[M-H]^-$ ion based on molecular formula of the parents and predicted TPs and a corresponding NCE calculated with Eq 1.

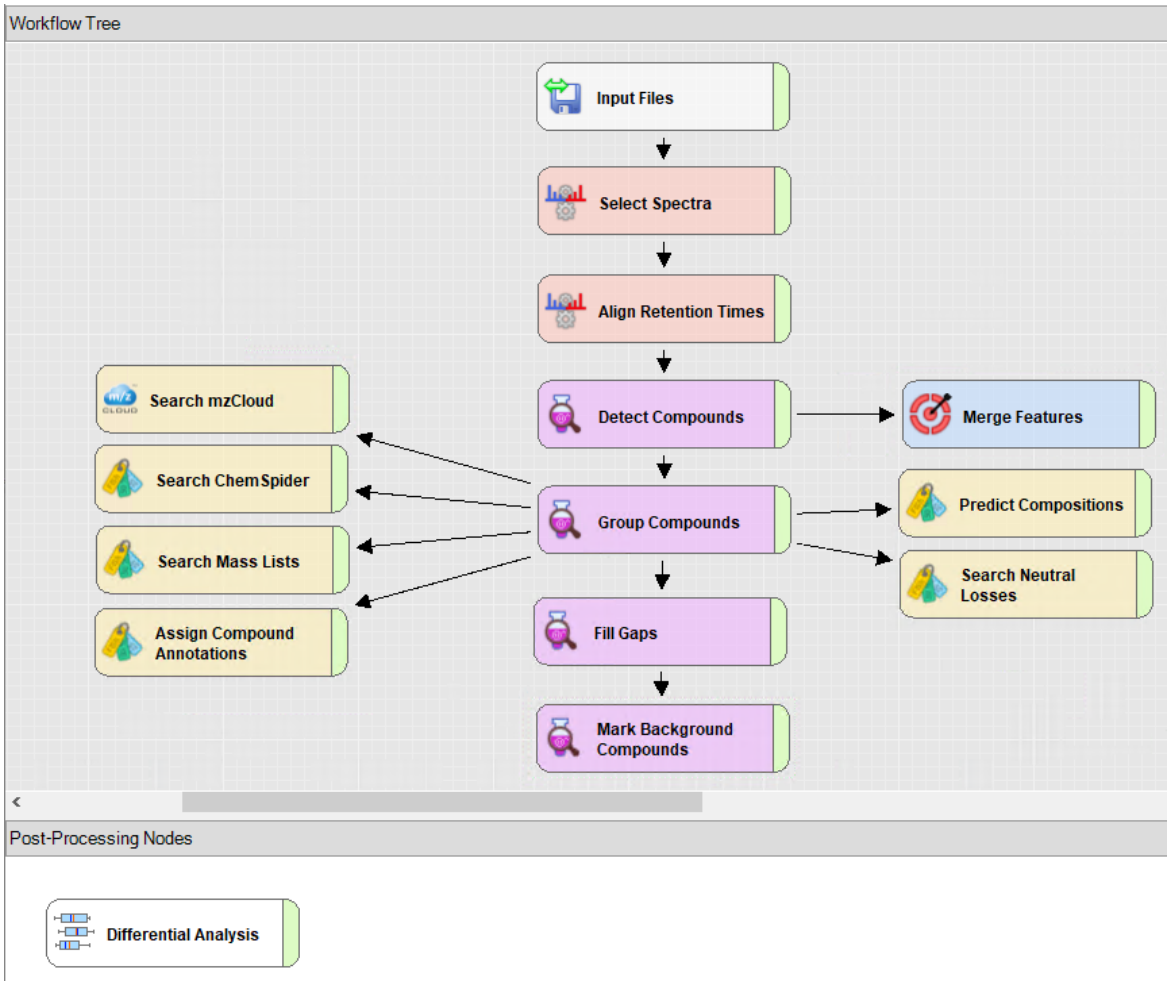
S4.2.2 Stepped Collision energy

Samples (biotransformation bottles at 4 timepoints: 0h, 2h, 24h and 72h) were re-measured once first acquiring MS in full scan in positive mode at a resolution of 70,000, scan range of 100 to 1000 m/z. Then data-dependent MS^2 spectra were acquired at a resolution of 17,500, maximum ion time of 100 ms, isolation window 1 m/z, dynamic exclusion of 6 s and stepped NCE of 15, 35 and 60. The inclusion list contained the calculated mass of $[M+H]^+$ or $[M-H]^-$ ion based on molecular formula of selected predicted TPs where more fragmentation could help in improving structural identification.

S5. Compound Discoverer:

Version 3.2.0.421

S5.1 Workflow and Parameters: Suspect screening



Supplementary Figure S4. Compound Discoverer workflow for the automatic detection of transformation products.

Supplementary Table S5. Parameters used for the used Compound Discoverer workflow.

Compound discoverer nodes	
Select Spectra Node	
Lower RT Limit [min]	2.9
Upper RT Limit [min]	28
Polarity Mode	Any
Align Retention Times Node	
Alignment Model	Adaptive Curve
Maximum shift [min]	2
Mass Tolerance	5 ppm
Detect Compounds Node	
Mass Tolerance	5 ppm
Intensity Tolerance [%]	30
S/N Threshold	3
Min. Peak Intensity	500000
Ions	[M+H]+1; [M+K]+1; [M+Na]+1; [M-H]-1
Min. Element Counts	C H
Max. Element Counts	C60 H100 Br2 Cl5 F10 N10 O20 P5 S5
Merge Features Node	
Mass Tolerance	5 ppm
RT Tolerance [min]	1
Group Compounds Node	
Mass Tolerance	5 ppm
RT Tolerance [min]	0.2
Preferred Ions	[M+H]+1; [M-H]-1
Fill Gaps Node	
Mass Tolerance	5 ppm
S/N Threshold	1.5
Mark Background Compounds Node	
Max. Sample/Blank	5
Max. Blank/Sample	0
Hide Background	True
Search mzCloud Node	
Compound Classes	All
Library	Autoprocessed; Reference
DDA Search	
Identity Search	HighChem HighRes
Match Activation Type	True
Match Activation Energy	Match with Tolerance
Activation Energy Tolerance	20
Apply Intensity Threshold	True
Similarity Search	None
Match Factor Threshold	60
DIA Search	
Use DIA Scans for Search	False

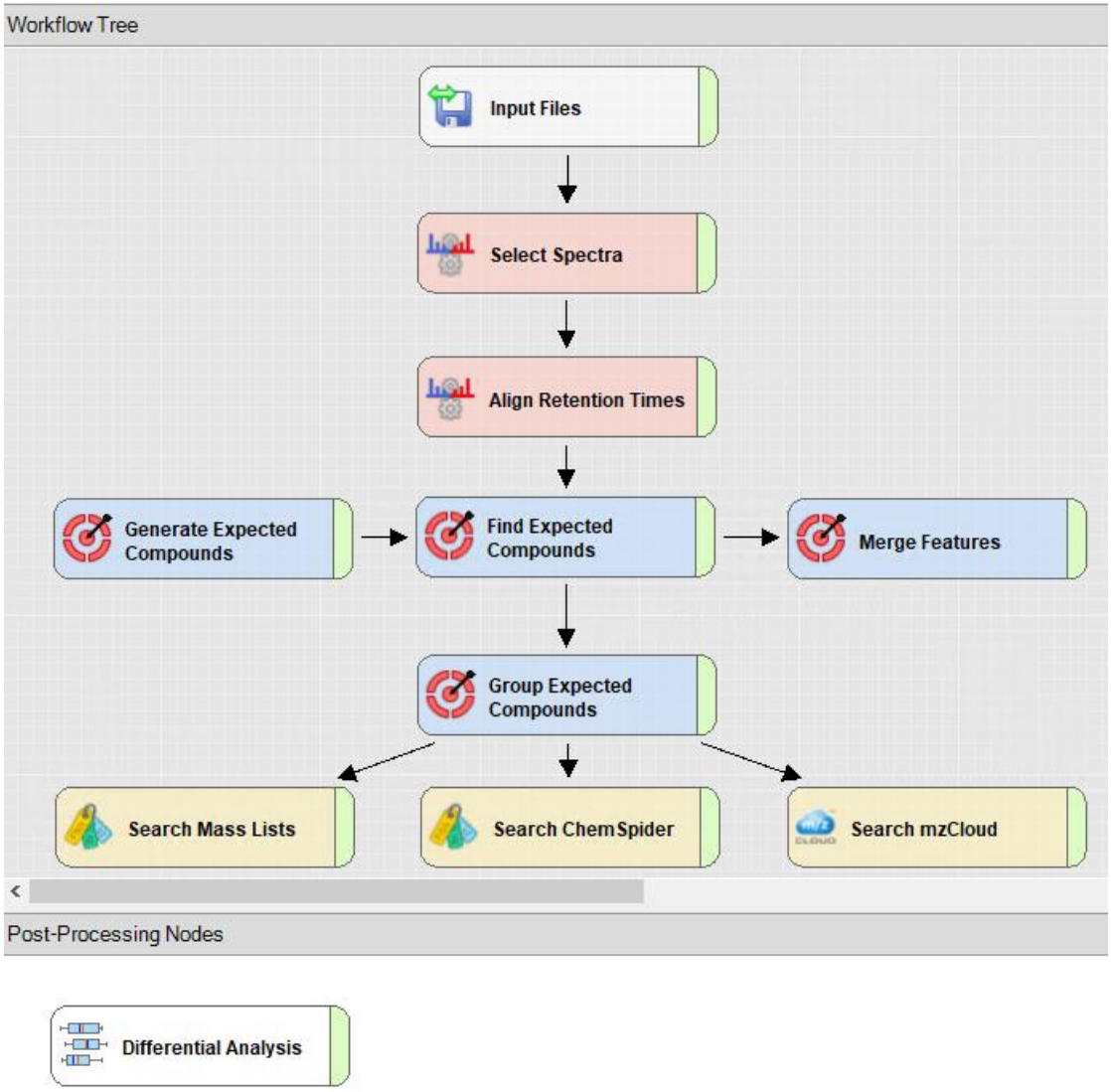
Max. Isolation Width [Da]	500
Match Activation Type	False
Match Activation Energy	100
Apply Intensity Threshold	False
Match Factor Threshold	20
Search ChemSpider Node	
Database(s)	KEGG
Search Mode	By Formula or Mass
Mass Tolerance	5 ppm
Max. # of results per compound	20
Max. # of Predicted Compounds	3
Predict Compositions Node	
Mass Tolerance	5 ppm
Min. Element Counts	C H
Max. Element Counts	C60 H100 Br2 Cl5 F10 N10 O20 P5 S5
Min. RDBE	0
Max. RDBE	40
Min. H/C	0.1
Max. H/C	3.5
Max. # Candidates	10
Intensity Tolerance [%]	30
Intensity Threshold [%]	0.1
S/N Threshold	3
Use Dynamic Recalibration	True
Use Fragments Matching	True
Assign Compound Annotations Node	
Mass Tolerance	5 ppm
Data Source #1	MassList Search
Data Source #2	Predicted Composition
Data Source #3	mzCloud Search
Data Source #4	ChemSpider Search
Use mzLogic	True
Use Spectral Distance	True
Sfit Threshold	20
Sfit Range	20
Search Mass Lists Node	
Mass Lists	Import from csv
Use Retention Time	True
RT Tolerance [min]	2
Mass Tolerance	5 ppm
Search Neutral Losses Node	
High Acc. Mass Tolerance	2.5 mmu
Low Acc. Mass Tolerance	0.5 Da
Neutral Losses	Br, C2 H3, C2 H3 O2, C2 H4 N, C2 H5, C2 H5 O, C2 H6 O, C2 H6, C3 H7, C4 H10, C4 H7, C4 H8, C4 H9, C H2, C2 H2 O, C

	H3, C3 H6, C2 H3 O, C2 H4 O2, C H3 O, C H4 O, C H4, C H5 O, Cl, C O, C O2, C O3, C H2 N O, C6 H10 O4, F, C6 H10 O7, C6 H8 O6, H, H2, C2 H4, C H2 O, H2 O, H2 S, H Br, C2 H2, H Cl, C H N, C6 H10 O5, C12 H20 O10, H F, H I, H S, I, H3 N, N O, N O2, H O, C5 H8 O4, C11 H18 O9, C10 H16 O8, C5 H7 N O3, S, O S, O2 S, C10 H15 N3 O6
S/N Threshold	3
Use DIA Scans for Search	FALSE
Differential Analysis	
Log10 Transform Values	TRUE

Supplementary Table S6. Parameters that were used for the built-in in silico fragmentation tool (FISh Score).

Parameter	Settings
Annotate full spectrum tree	True
Use general rules	True
Use fragmentation libraries	True
Allow aromatic cleavage	True
Max. Depth	5
High accuracy mass tolerance	2.5 mmu
Low accuracy mass tolerance	0.5 Da
S/N threshold	3

S5.2 Workflow and Parameters: Expected Compounds



Supplementary Figure S5. Compound Discoverer workflow for the prediction of TPs resulting from conjugation reactions and their detection.

Supplementary Table S7. Used parameters for the Compound Discoverer workflow for the prediction of TPs resulting from conjugation reactions and their detection.

Compound discoverer nodes	
Select Spectra Node	
Lower RT Limit [min]	2.9
Upper RT Limit [min]	28
Polarity Mode	Any
Generate Expected Compounds Node	
Compounds	All parent imported as .sdf files
Dealkylation	
Apply Dealkylation	FALSE
Apply Dearlyation	FALSE
Max. # of Steps	1
Min. Mass [Da]	100

Transformation	
Max. # of Steps	2
Max. # Phase II	1
Others	
Phase I	
Phase II	Acetylation, Formylation, Fumarylation, Malonylation, Succinylation
Ions	M+H, M+H-H2O, M+K, M+Na, M+NH4, M-H, M-H-H2O
Find Expected Compounds Node	
Avg Peak width [min]	0
Intensity Threshold [%]	0.1
Intensity Tolerance [%]	30
Mass Tolerance	5 ppm
Min. # Isotopes	2
Min. Peak Intensity	500000
S/N Threshold	3
Group Expected Compounds Node	
RT Tolerance [min]	0.2
Preferred Ions	M+H, M-H
Align Retention Times Node	
Alignment Model	Adaptive Curve
Maximum shift [min]	2
Mass Tolerance	5 ppm
Merge Features Node	
Mass Tolerance	5 ppm
RT Tolerance [min]	1
Group Compounds Node	
Mass Tolerance	5 ppm
RT Tolerance [min]	0.2
Preffered Ions	[M+H]+1; [M-H]-1
Search mzCloud Node	
Compound Classes	All
Library	Autoprocessed; Reference
DDA Search	
Identity Search	HighChem HighRes
Match Activation Type	True
Match Activation Energy	Match with Tolerance
Activation Energy Tolerance	20
Apply Intensity Threshold	True
Similarity Search	None
Match Factor Threshold	60
DIA Search	
Use DIA Scans for Search	False
Max. Isolation Width [Da]	500
Match Activation Type	False

Match Activation Energy	100
Apply Intensity Threshold	False
Match Factor Threshold	20
Search ChemSpide Node	
Database(s)	KEGG
Search Mode	By Formula or Mass
Mass Tolerance	5 ppm
Max. # of results per compound	20
Max. # of Predicted Compounds	3
Search Mass Lists Node	
Mass Lists	Import from csv
Use Retention Time	True
RT Tolerance [min]	2
Mass Tolerance	5 ppm
Differential Analysis	
Log10 Transform Values	TRUE