Supporting information

Tacrine – Trolox Hybrids: A Novel Class of Centrally Active, Non-Hepatotoxic Multi-Target-Directed Ligands Exerting Anticholinesterase and Antioxidant Activities with Low *In Vivo* Toxicity

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Table S1. Permeability results from PAMPA-BBB assay for tacrine – trolox hybrids **7a-u** and reference compounds **1-4**

Compound	P_e (10^{-6} cm.s^{-1})[a]
7a	2.8
7b	3.4
7c	3.1
7d	3.0
7e	2.9
7f	0.8
7g	3.3
7h	4.4
7i	2.2
7j	1.8
7k	1.5
7l	3.3
7m	3.0
7n	1.3
7o	3.4
7p	2.4
7q	2.1
7r	3.0
7s	3.1
7t	2.3
7u	5.0
Tacrine	4.1
7-Methoxytacrine	4.2
6-Chlorotacrine	5.0
Trolox	0.5
Donepezil (positive control)	5.9
Theophylline (negative control)	0.4

[a] Results are expressed as the mean of at least four experiments.

Microsomal *In Vitro* Metabolism and Stability Analysis

Materials

For LC-MS/MS analyses, acetonitrile (Sigma Aldrich, product No.: 34967), formic acid (Sigma Aldrich, product No.: 34860), both of LC-MS grade purity, and ultrapure water of ASTM I type (resistance 18.2 MΩ.cm at 25°C) prepared by Barnstead Smart2Pure 3 UV/UF apparatus (ThermoFisher Scientific, Bremen, Germany) were used for preparation of mobile phases. A sample stock solution for microsomal metabolism assay was prepared by dissolving of the tested compound in dimethyl sulfoxide (Sigma Aldrich, product No.: D8418). Un-calibrated purity of the target compound was evaluated by LC analysis at λ = 254nm (98%). Calibration series was prepared by

diluting a stock solution of the assayed compound in methanol of LC-MS grade purity (Sigma Aldrich, product No.: 34966).

Pooled human liver microsomes (HLM, from 200 adult donors of mixed gender) in concentration 20.0 mg/mL for the metabolism study were obtained from Tebu Bio Sas (France, 1 mL, product No.: 098H2620). HLM samples contained cytochrome P450 (0.478 nmol/mg protein), cytochrome b_5 (0.369 nmol/mg protein) and NADPH-cytochrome *c* reductase (162 ± 7 nmol/mg protein/min) suspended in 250mM sucrose. After delivery, HLM samples were melted at 4°C, quickly aliqouted to 25 µL and stored at – 80°C. RapidStart NADPH Regenerating System, containing 0.5 mL of 100mM NADP, 0.5 mL of 500mM glucose-6-phosphate, and 0.5 ml of 100 units/mL glucose-6-phosphate dehydrogenase in 20mM potassium phosphate buffer (pH = 7.4) was also purchased from Tebu Bio Sas (France, 1.5 mL, product No.: 098K5000). 1.5 mL of the original RapidStart solution was, according to the supplier instructions, mixed with 3.5 mL ultrapure water, vortexed for 30 s, distributed to 50 µL aliquotes and stored at – 80°C.

Instrumentation

The metabolic study of the tested compound was performed using Dionex UltiMate 3000 analytical LC-MS system coupled with Q Exactive Plus hybrid quadrupole-orbitrap spectrometer (both produced by ThermoFisher Scientific, Bremen, Germany). The LC-MS system consisted of a binary pump HPG-3400RS connected to a vacuum degasser, a heated column compartment TCC-3000, an autosampler WTS-3000 equipped with a 25 µL loop and a VWD-3000 ultraviolet detector. A Waters Atlantis dC18 100Å (1 x 150mm/3µm) column was used as stationary phase. The analytical column was protected against mechanical particles by an in-line filter (Vici Jour) with a frit of 0.5 µm pores. Water (MFA) and acetonitrile (MFB) used for analytical purposes were acidified with 0.1% (v/v) of formic acid. Ions for mass spectrometry (MS) were generated by heated electro-spray ionization source (HESI) working in positive mode with the following settings: sheath gas flow rate 22, aux gas flow rate 2, sweep gas flow rate 0, spray voltage 3.0 kV, capillary temperature 250°C, aux gas temperature 250°C, S-lens RF level 50, microscans 1, maximal injection time 35 ms, normalized collision energy 35, resolution in full-scan 140 000, resolution in all-ions-fragmentation (AIF) 140 000. The wide-range multiple reaction monitoring (MRM) analyses registered ions within m/z range 100 – 1500. To increase the accuracy of HRMS, internal lock-mass calibration was exploited using polysiloxane traces of m/z = 445.12003 ($[M+H]^+$, $[C_2H_6SiO]_6$) present in mobile phases. The chromatograms and mass spectra were processed in Chromeleon 6.80 and Xcalibur 3.0.63 software, respectively. For metabolites and MS/MS fragments predictions Mass Frontier 7.0 and Metworks 1.3 SP3 programs were utilized.

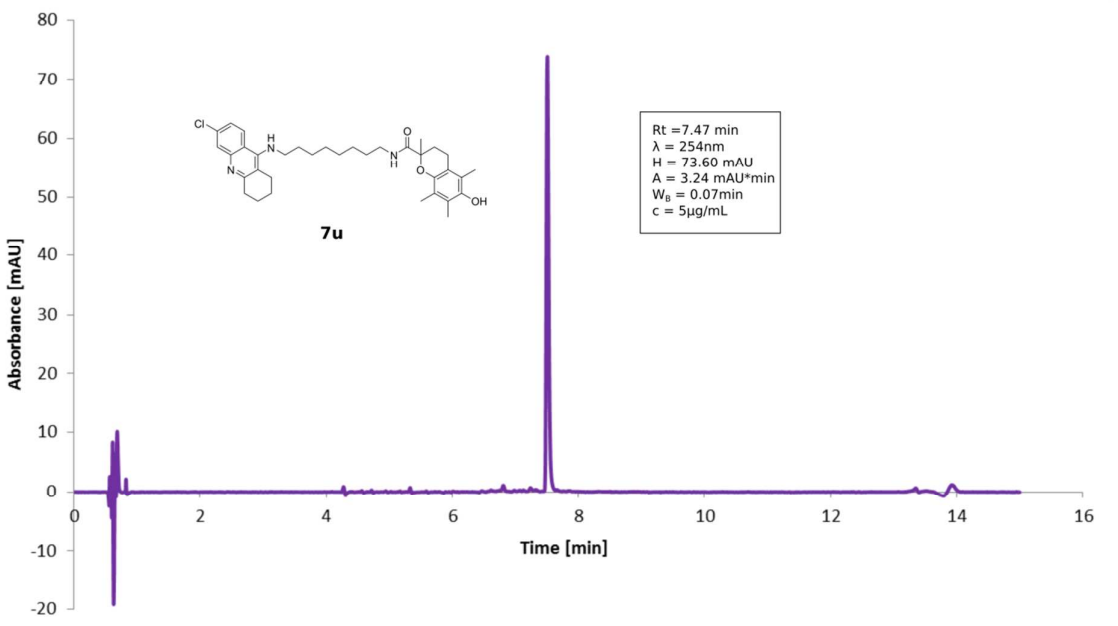

Figure S1. UV-chromatogram of compound **7u** obtained for concentration 5 µg/mL detected at λ = 254nm. Within the LC analysis a linear gradient elution by water and acetonitrile was used.

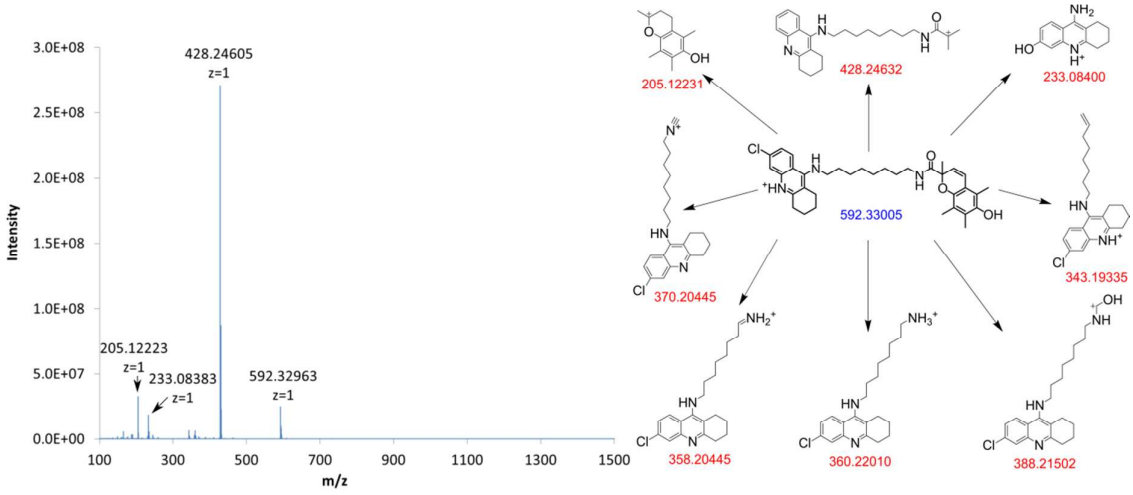

Figure S2. Experimental MS/MS spectrum of the parent compound **7u** and suggested chemical structures of the main fragments. The values in the right-hand part of the Figure are calculated m/z's. All displayed fragments were observed within ±0.001 of m/z in the experimental MS/MS spectra.

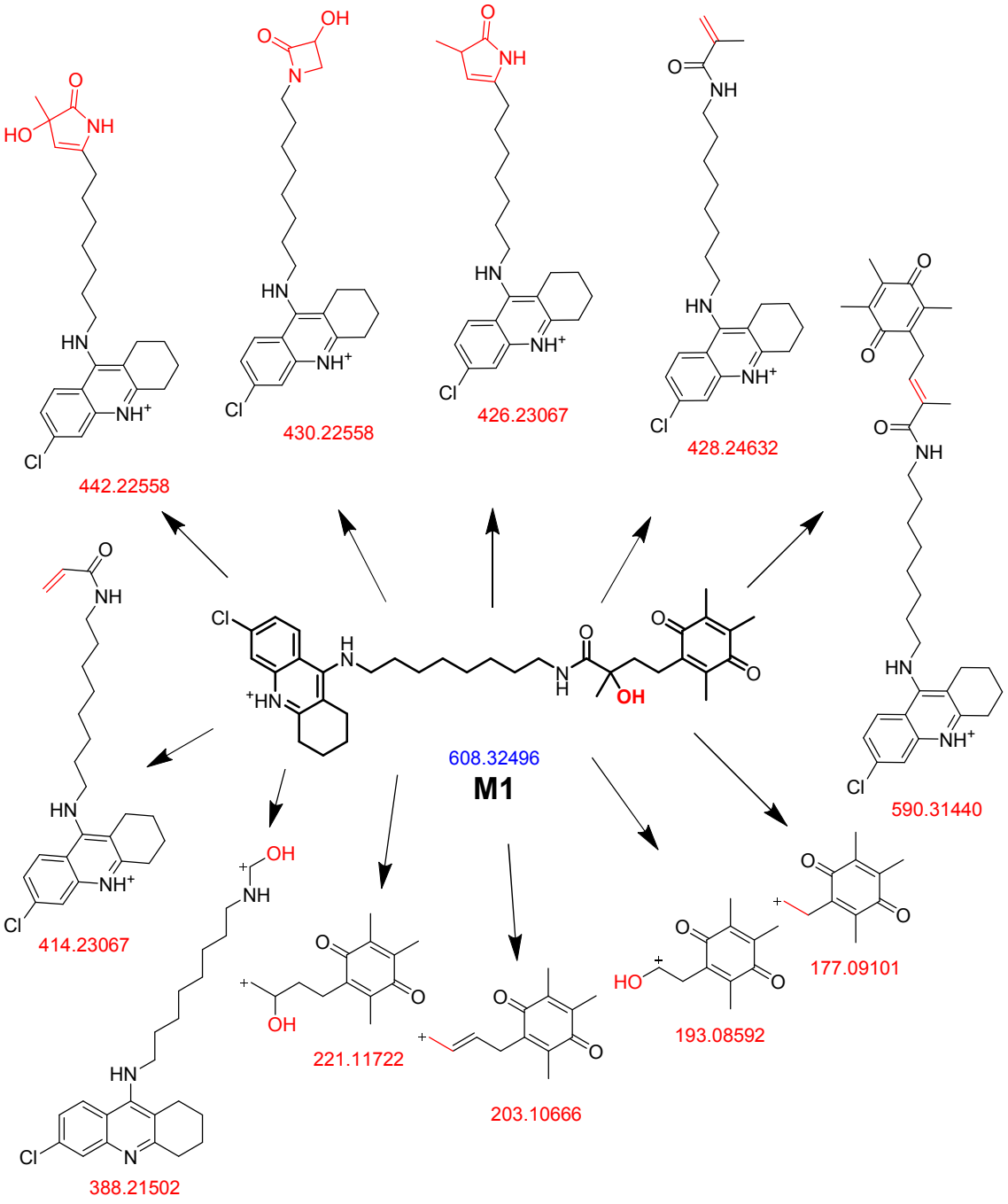

Figure S3. Proposed structure of metabolite **M1** and molecular fragments which were found in MS/MS spectra. The displayed m/z's are expressed as calculated values corresponding to ± 0.001 to m/z's found in the recorded MS/MS spectra.

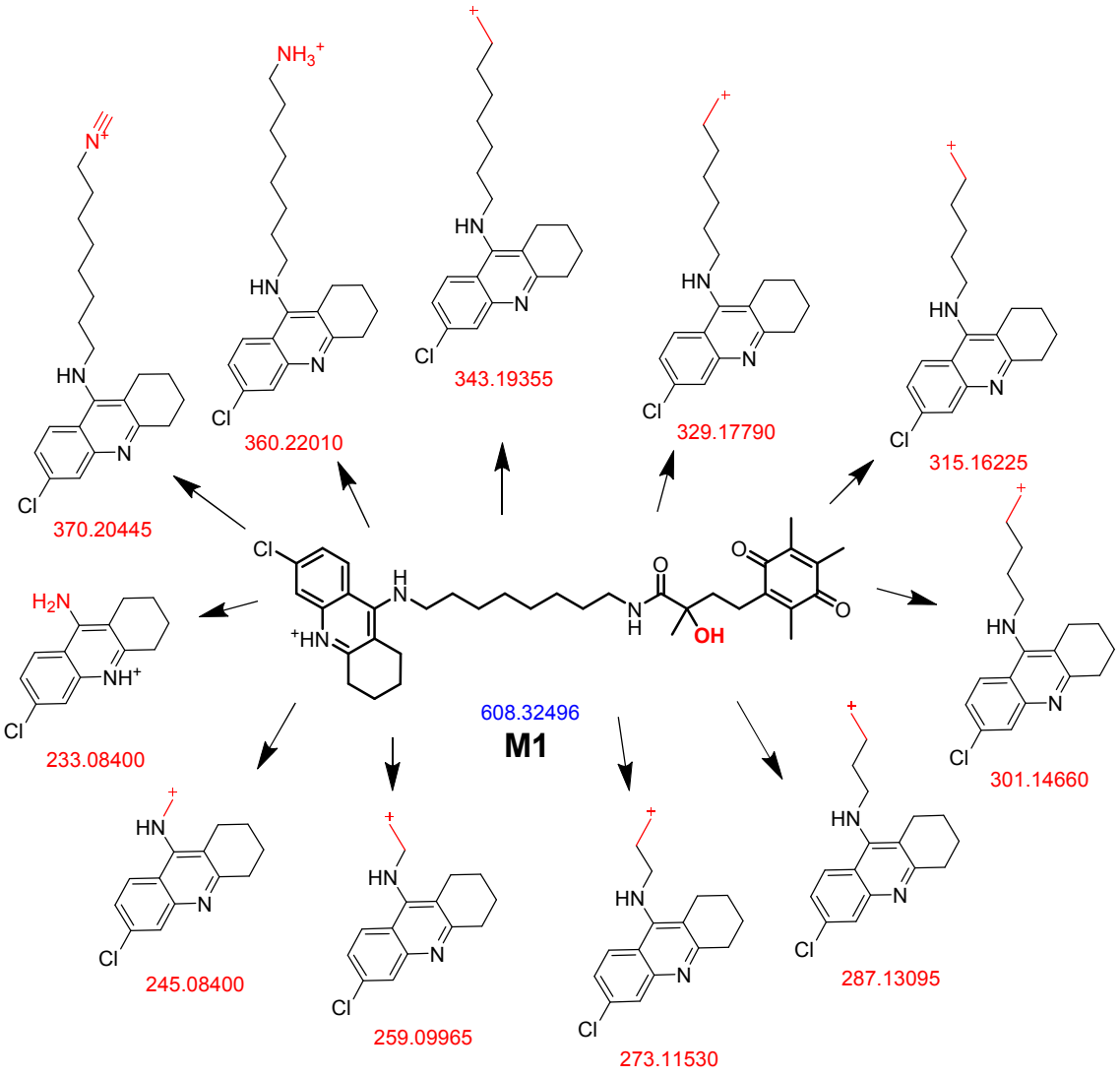

Figure S4. Proposed structure of metabolite **M1** and further molecular fragments which were found in MS/MS spectra. The displayed m/z's are expressed as calculated values corresponding to ± 0.001 to m/z's found in the recorded MS/MS spectra.

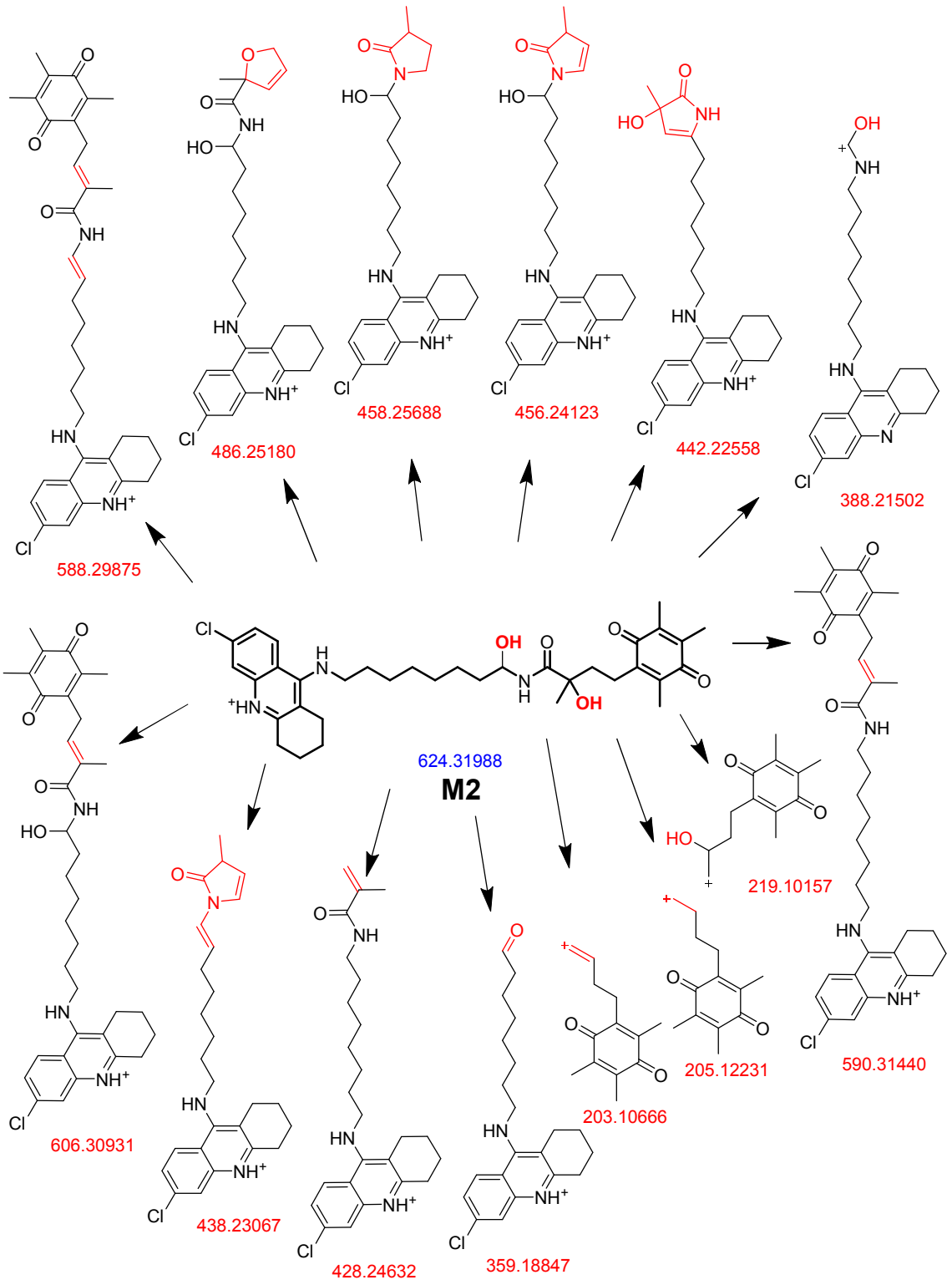

Figure S5. Proposed structure of metabolite **M2** and molecular fragments which were found in MS/MS spectra. The displayed m/z's are expressed as calculated values corresponding to ± 0.001 to m/z's found in the recorded MS/MS spectra.

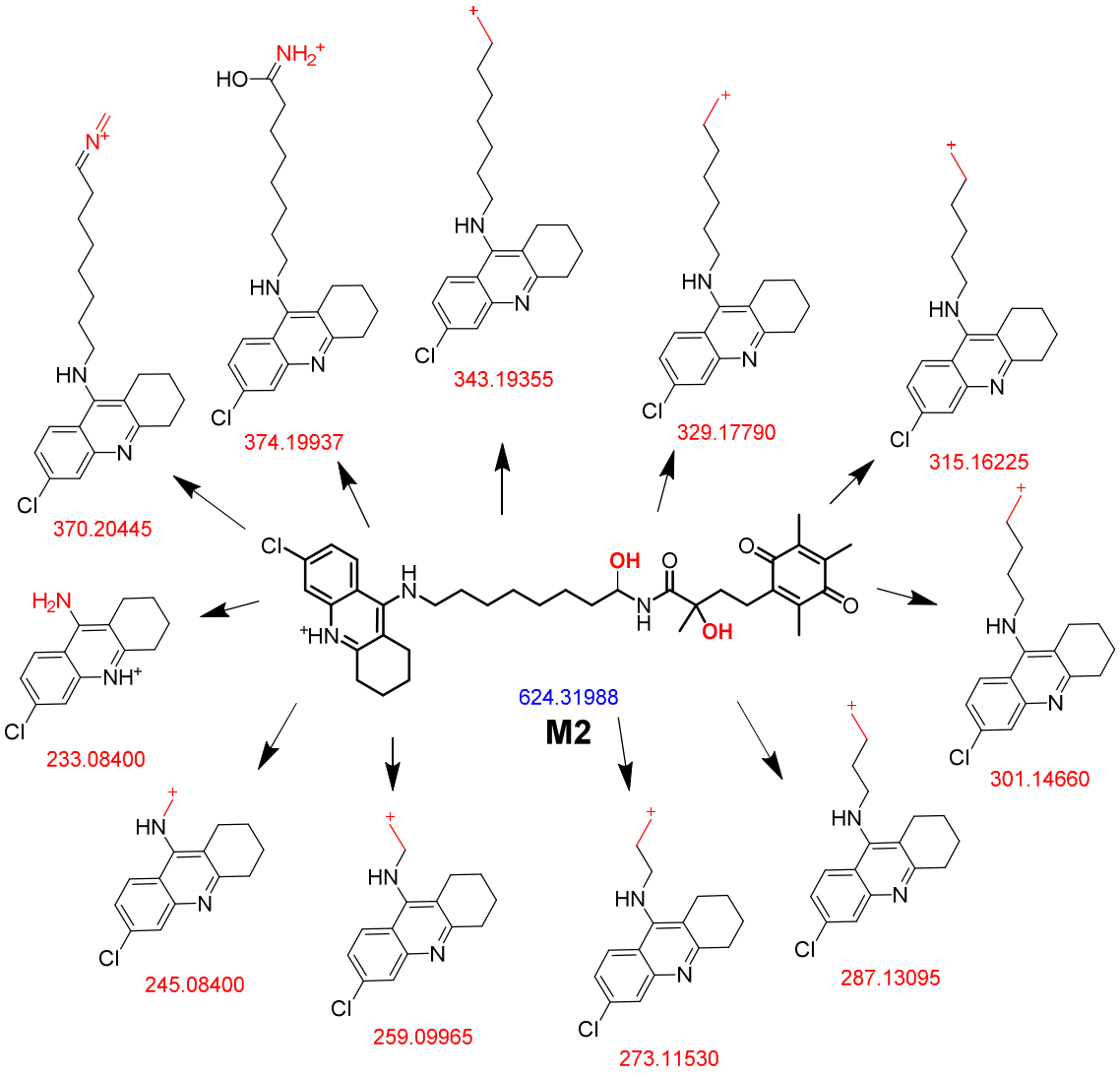

Figure S6. Proposed structure of metabolite **M2** and further molecular fragments which were found in MS/MS spectra. The displayed m/z's are expressed as calculated values corresponding to ± 0.001 to m/z's found in the recorded MS/MS spectra.

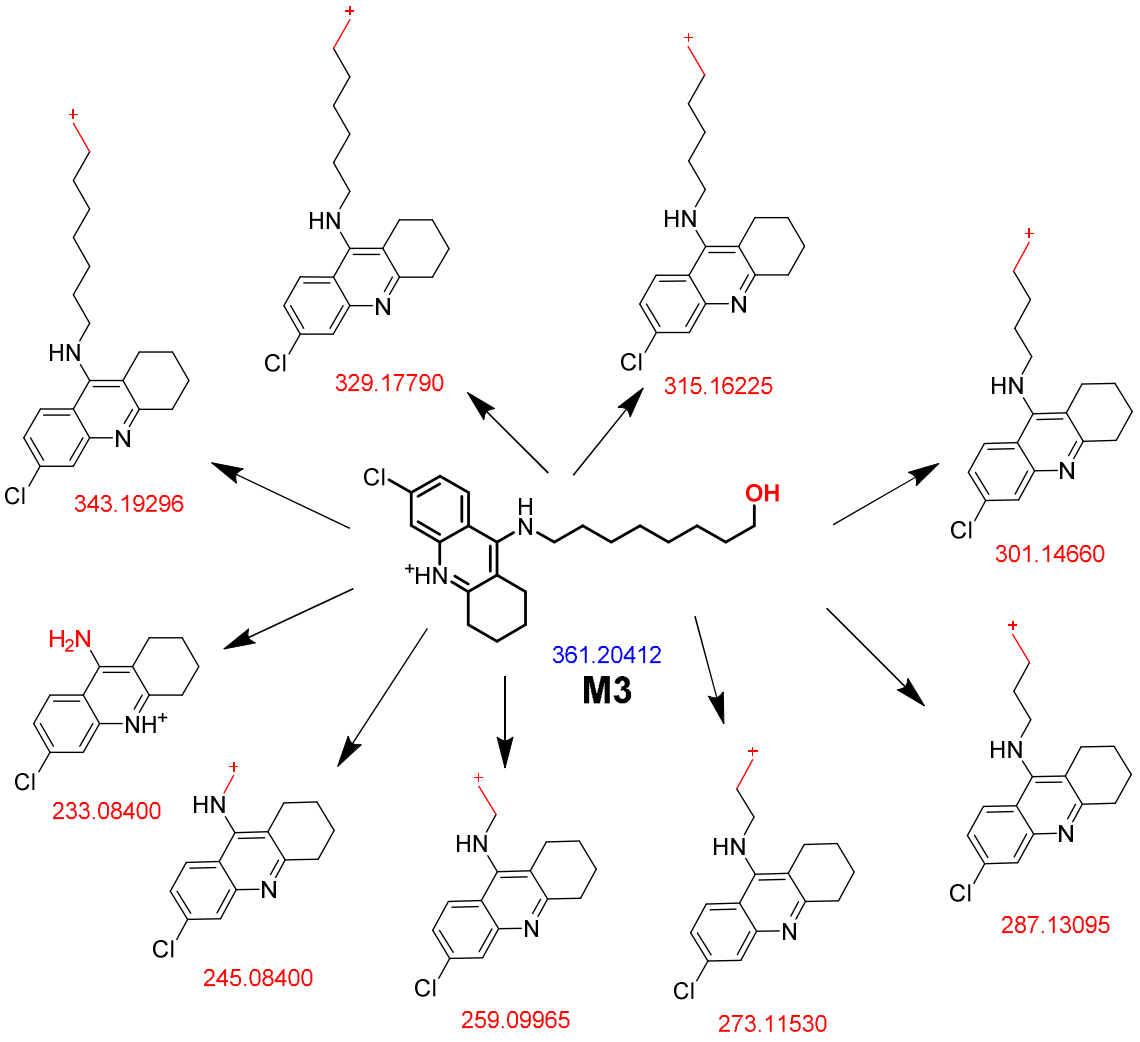

Figure S7. Proposed structure of metabolite **M3** and molecular fragments which were found in MS/MS spectra. The displayed m/z's are expressed as calculated values corresponding to ± 0.001 to m/z's found in the recorded MS/MS spectra.

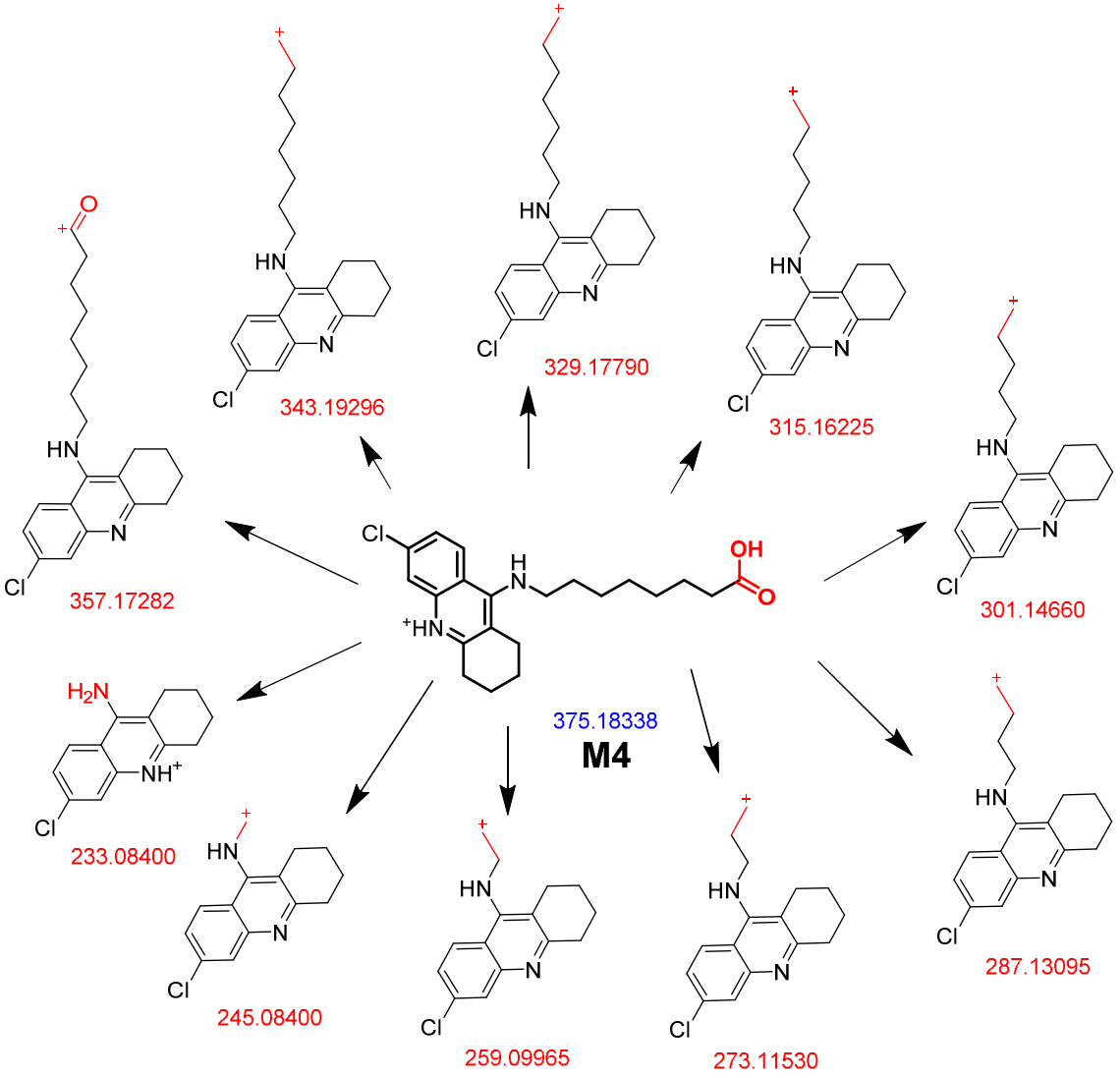

Figure S8. Proposed structure of metabolite **M4** and molecular fragments which were found in MS/MS spectra. The displayed m/z's are expressed as calculated values corresponding to ± 0.001 to m/z's found in the recorded MS/MS spectra.