Supporting Information

Identification of the significant involvement and mechanistic role of CYP3A4/5 in clopidogrel bioactivation

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Experimental Section

Reagents. (S)-(+)-Clopidogrel hydrogensulfate was purchased from TCI America and its specific rotary power ([α]/D) was measured by a P-1050 Polarimeter (Jasco) to be 52° (t=17.2 °C, MeOH), which is consistent with the specificity provided by the manufacturer and published data.[S1] 2-Oxo-clopidogrel hydrochloride was purchased from Toronto Research Chemicals (North York, Ontario, Canada). (±)-Methyl-alpha-(2-thienylethamino)(2-chlorophenyl)acetate hydrochloride (LC-MS internal standard) was purchased from Higher Biotech (Shanghai, China). β-Nicotinamide adenine dinucleotide 2'-phosphate (reduced, NADPH) was purchased from Roche Applied Sciences (Shanghai, China). Deuterium oxide (99.8% D) was purchased from J&K Scientific (Beijing, China). Pooled HLM, cDNA-expressed supersomes control, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5 were purchased from BD Gentest (Woburn, MA). Other chemicals of the highest available grade were all purchased from Sigma-Aldrich (St. Louis, MO).

Metabolism studies in pooled HLM. Clopidogrel was initially dissolved in DMSO at a concentration of 10 mM. The *in vitro* incubation mixture contains 1.0 mg/mL HLM, 2.0 mM NADPH, 20, 100, or 200 μM clopidogrel, with or without 5 mM glutathione, with or without 100 mM KF, with or without 2 M $CaCl_2$ in a final volume of 100 μL of potassium phosphate (100 mM) buffer (pH = 7.4). Control incubation mixtures were made by replacing NADPH with potassium phosphate buffer. The samples were incubated in a 37 ^{o}C shaking water bath for 30 or 60 min. Each incubation mixture was quenched with 15 μL trichloroacetic acid (10%, w.t.) and kept on ice for 5 min before centrifuged at 12,000 rpm for 10 min on a 5415D Centrifuge (Eppendorf AG, Hamburg, Germany) to fully pelletize the precipitated protein. Supernatant was transferred to sealed vial and placed in an autosampler pending LC-UV-MS/MS analysis.

Reaction phenotyping studies in CYP supersomes. Clopidogrel was initially dissolved in DMSO at a concentration of 50 mM for the phenotyping experiments. Each phenotyping incubation mixture contains 25 pmol/L control or individual CYP supersomes, 1.0 mM NADPH, 50 μM clopidogrel, 5 mM glutathione in a final volume of 100 μL of potassium phosphate (100 mM) buffer (pH = 7.4). The final percentage of DMSO is 0.1% (v/v) that does not denature/inhibit re-constituted P450's as recommended by the reagent provider (no higher than 0.2%, Supersomes Guidelines for Use, available at: www.bdbiosciences.com). The samples were incubated in a 37 ^{o}C shaking water bath for 30 min before quenched with 100 μL of ice-cold CH_3CN/MeOH (4/1, v/v) containing 5 μM internal standard followed by the same process as described above.

2-Oxo-clopidogrel production in HLM with ketoconazole. Incubation mixture contains 1.0 mg/mL human liver microsomes, 20 μM clopidogrel, 5 mM glutathione, 100 mM KF in a final volume of 150 μL of potassium phosphate (100 mM) buffer (pH = 7.4) was evenly divided into three (50 μL each). The first one was added NADPH (final concentration = 1 mM) and CYP3A4 inhibitor ketoconazole (KET, final concentration = 1 μM, which does not lead to cross-inhibition of other CYP enzymes[S2]); the second one was made by replacing the "KET in MeOH" added in the first sample with just MeOH; the

third one was made by replacing the NADPH added in the second sample with KPi buffer. The three samples were incubated in a 37 ^{o}C shaking water bath for 60 min before quenched with equal volumes of ice-cold CH_3CN/MeOH (4/1, v/v) containing 5 μM internal standard followed by the same process as described above.

LC-MS/MS analysis. Chromatographic analysis of the supernatant resulted from the metabolite identification incubation was conducted on a Surveyor HPLC system consisted of an autosampler, a MS pump, and a photo diode array detector (Thermo Fisher Scientific) using a Zorbax SB-Phenyl column (4.6 x 75 mm, 3.5 μm, Agilent Technologies) at room temperature. The volume of each injection was 20 μL. The mobile phase consisted of H_2O (solvent A, containing 0.1% formic acid) and MeOH (solvent B, containing 0.1% formic acid) and was delivered at 750 μL/min. The initial composition of solvent B was maintained at 10% for 2 min and then increased in a linear manner to 50% at 7 min and 90% at 17 min. It was then maintained at 90% solvent B for 1 min and finally decreased to 10% at 19 min. The column was allowed to equilibrate at 10% solvent B for 1 min before the ending of the 20 min gradient elution program for next injection. The scan range of the photo diode array detector was set to be 220-400 nm. Mass spectrometric (MS) analysis was performed on a Thermo LCQ ion trap mass spectrometer (Thermo Fisher Scientific), which was interfaced to the above HPLC system. The HPLC eluate was split after the photo diode array detector and 10% eluate (75 μL/min) was injected onto the mass spectrometer. MS analysis was conducted using a standard electrospray ionization (ESI) source operating in positive ionization mode. Source operating conditions were 4.5 kV spray voltage, 225 °C heated capillary temperature, 20 V capillary voltage, and sheath gas flow at 40 (arbitrary unit), respectively. The MS experiment parameters including the nitrogen gas flow rate, capillary voltage, and the tube lens voltages were all tuned and optimized to give maximum detection sensitivities using a clopidogrel standard solution (10 μM in MeOH/H_2O=1/1, v/v). The MS full scans were monitored over a mass range of m/z 300 to 700. Product ion (MS2) scans were generated via collision-induced dissociation (CID) with helium using normalized collision energy of 60% and a precursor ion isolation width of m/z 2.0. Data was centroid and processed in Qual Browser (Thermo Fisher Scientific). Fragmentations were proposed based on plausible protonation sites, subsequent isomerization and even electron species, as well as bond saturation. For quantitative studies, supernatants of the reaction phenotyping studies were injected onto the same LC-MS/MS system as in metabolite identification experiment with a shorter scan range (m/z=300-350). The chromatographic peaks of metabolites and the internal standards were extracted and integrated in Qual Browser (Thermo Fisher Scientific, Waltham, MA). All the samples were analyzed in triplicates and the averaged metabolite to internal standard ratios were used for quantitative analysis.

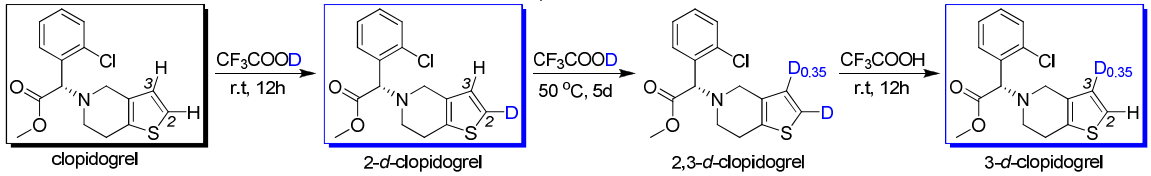
High resolution mass spectrometric (HRMS) analysis was conducted on an Apex IV Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR-MS, Bruker Daltonics, Billerica, MA), which was interfaced to the above HPLC system. The HPLC eluate was split after the diode array detector and 6% eluate (45 μL/min) was injected onto the mass spectrometer. MS analysis was performed using a standard electrospray ionization (ESI) source operating in positive ionization mode. Source operating conditions were: end plate electrode voltage of -3.5 kV, capillary entrance voltage of -4.5 kV,

skimmer voltage of 30 V, dry gas temperature of 250 ^{o}C, and dry gas flow rate of 12 L/min. Data was acquired and processed in DataAnalysis XP (Bruker Daltonics).

Online H-D exchange study. For the purpose of saving deuterated LC solvent, the online hydrogen-deuterium exchange LC-MS/MS study was conducted on another chromatographic system consisted of a 112A oven injector and a 125B binary pump (Applied Biosystem) using a Synergi Max-RP (2.0 x 30 mm, 4 μm, Phenomenex) at room temperature. The volume of each injection was 10 μL. The mobile phase consisted of D_2O (solvent A, containing 0.1% formic acid) and CH_3CN (solvent B, containing 0.1% formic acid) and was delivered at 300 μL/min. The initial composition of solvent B was maintained at 5% for 2 min and then increased in a linear manner to 30% at 12 min and 70% at 20 min. It was then maintained at 70% solvent B for 2 min and finally decreased to 5% at 24 min. The column was allowed to equilibrate at 5% solvent B for 1 min before the ending of the 25 min gradient elution program. The HPLC eluate was split and 25% eluate (75 μL/min) was injected for mass spectrometric analysis using same conditions describe above. This LC system was first tested and optimized with H_2O solvent to provide similar separation results to those obtained on the LC system used for metabolite profiling and identification, then Pump A was purged and flushed with CH_3CN several times to repel the residual H_2O. Finally, D_2O solvent replaced CH_3CN in Pump A and the whole system was flushed and equilibrated.

Preparation of deuterated clopidogrels and metabolism studies. Following the precedents of stepwise thiophene hydrogen-deuterium exchange reaction,[S3] deuterated clopidogrels were prepared as probe analogues to assist pinpointing the fragment of modification for the metabolites. (S)-(+)-Clopidogrel hydrogensulfate (20 mg) was dissolved in 1.0 mL of trifluoroacetic acid-d (CF_3COOD). After placed at room temperature for 12 hours, both MS and HNMR analyses show that the 2-H was completely replaced by deuterium. The reaction mix was placed in a CTO-10A vp column oven ((Shimadzu USA, Columbia, MD), and the temperature was kept at 50 ^{o}C. After 5 days, both MS and HNMR analyses showed that the deuteration of the 3-H reached about 35% and no other carbon was found to be deuterated. Higher temperature and longer reaction time were found to lead to non-specific deuteration. For the purpose of assisting pinpointing sites of metabolism, the 3-$d_{0.35}$ analogue is applicable. After evaporating the CF_3COOD, the deuterium at the 2-position of 2,3-*d*-clopidogrel was changed back to H in 1.0 mL of CF_3COOH. Both MS and HNMR analyses confirmed the structure of the partially deuterated 3-$d_{0.35}$-clopidogrel analogue. 2-*d*-Clopidogrel and 3-*d*-clopidogrel were subjected to the same HLM metabolism studies as described above.

Scheme S1. Preparation of deuterated clopidogrel through stepwise hydrogen-deuterium exchange reactions in CF_3COOH.



Results and Supplementary Discussion

Metabolite profiling of clopidogrel under various incubation conditions. In HLM incubation of clopidogrel, metabolites were detected under various incubation conditions including: different clopidogrel concentration (10 μM, 20 μM, 50 μM, 100 μM, and 200 μM), without KF, without GSH, or incubation for 15 min, 30 min, or 60 min. The following incubation conditions were optimized for metabolite profiling and identification: 100 μM clopidogrel, 100 mM KF, 5 mM GSH, 30 min incubation. Glutathione (5 mM) was included for two purposes: 1. to mimic the *in vivo* hepatic environment where hepatic cell contains 4-5 mM glutathione;[S4] 2. To trap the potentially formed clopidogrel active metabolite,[S5,S6] which is used to monitor the 2-oxo-clopidogrel consumption due to further metabolic activation. Glutathione conjugates of active metabolite ($M+H^+$=661)[S5,S6] were detected only at very low amount, which confirmed that the formed 2-oxo-clopidogrel did not undergo significant further activation.

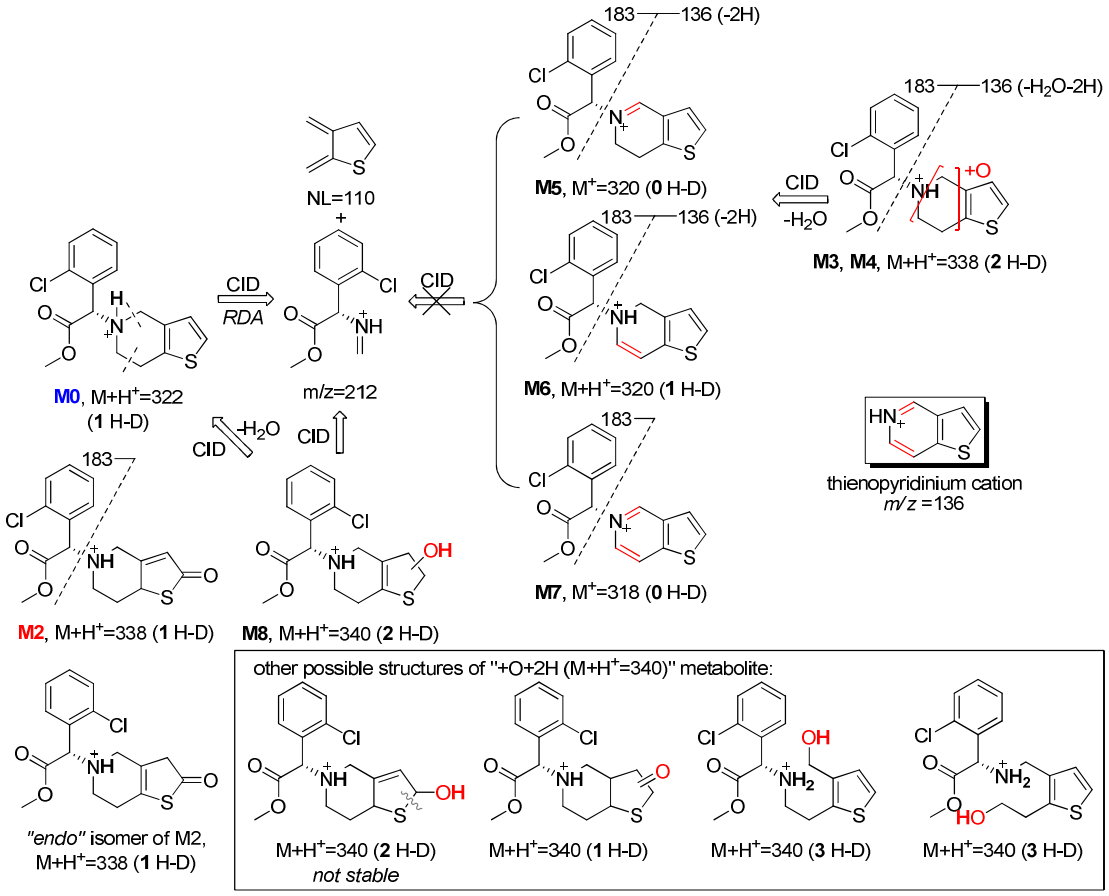


Figure S1. Metabolite structure elucidation based on fragmentation analysis and H-D exchange experiment results. CID: collision-induced dissociation.

Structural elucidation of M5 (-2H), M6 (-2H) and M7 (-4H). The structure elucidation of the dehydrogenation metabolites were aided y fragmentation analyses. The structure of M0 can undergo a "retro-Diels-Alder" (RDA) fragmentation yielding prominent daughter ion of m/z = 212 (Figure S1).[S7] This RDA fragmentation was not

observed with M5, M6, and M7, which supports that the double bonds resulted from dehydrogenation are on the piperidine ring where RDA fragmentation cleaves. M5 and M7 possess much shorter retention times on reversed phase HPLC column than M0 or the other metabolites, which also supports the ionic natures of M5 (iminium) and M7 (pyridinium). Finally, results from online H-D exchange experiment confirmed the structures: M5 and M7 did not show H-D exchange because neither structure needs an extra proton (H^+ or D^+) to be charged due to the existence of the tetravalent *N*-cations of iminium and pyridinium while the protonated M6 showed one H-D exchange like M0. Low level of M5 was also found in HLM incubation sample without NADPH, which is consistent with previously reported results that it is a minor degradation product of clopidogrel.[S7]

M5, M6, M7 of 2-*d*-clopidogrel and 3-$d_{0.35}$-clopidogrel showed no deuterium loss, which supports that dehydrogenation occurs on the piperidine fragment.

Idiosyncratic adverse effects including rash and neutropenia have been associated with clopidogrel therapy.[S8] The CYP3A4/5-catalyzed formation of electrophilic reactive metabolite iminium (M5) and pyridinium (M7) might be responsible for some of these adverse effects based on their well-known toxicological properties.[S9]

Structural elucidation of M3 (+O) and M4 (+O). M3 and M4 are both mono-oxidation metabolites that are isomers of M2. In the online H-D exchange experiment, they both showed two H-D exchanges. The additional H-D exchange suggests that the oxidation confers both metabolites one more exchangeable H-atom (one more O-H or N-H). M3 and M4 both show the major daughter ion of *m/z* 136 (the thienopyridinium cation) and were proposed to be the piperidine hydroxylation metabolites.

M3, M4 of 2-*d*-clopidogrel and 3-$d_{0.35}$-clopidogrel showed no deuterium loss, which supports that hydroxylation occurs on the piperidine fragment.

It was reported that the final active metabolite (M13) with the exocyclic double bond structure exists with minor isomer containing an endocyclic double bond.[S10] Although the "endo" isomer of M2 might not form, it was ruled out to be a possible structure of M3 and M4: first, the "endo" structure shall have only one H-D exchange while M3 and M4 both showed two; second, in the same HLM incubation with inclusion of $CaCl_2$ (activating paraoxonase that can hydrolyze the thio ester of the "endo" isomer[S10]), none of the $M+H^+$=338 peaks showed any change.

Structural elucidation of M8 (+O+2H). M8 has shows a "+18 Da" mass change compared to M0. It is not the hydrolysis metabolite since its formation is NADPH-dependent. One possible biotransformation is "+O+2H", another possible biotransformation, "+3O+2H-S", has been reported for prasugrel (M12), a close analogue of clopidogrel.[S11] LC-HRMS analysis confirmed that the mass shift is "+O+2H" (HRMS calcd for $C_{16}H_{19}ClNO_3S^+$ [$M+H^+$]: 340.0769, found: 340.0778). M8 possesses very similar MS2 spectrum to that of M0. In online H-D exchange experiment, M8 showed two H-D exchanges, which rules out the other possible structures (Figure S1). The tentatively proposed 3- or 2-hydroxy-2,3-dihydrothiophene sub-structure can be found in products of organic synthesis[S12,S13] as well as metabolite of biotransformation[S14,S15,S16,S17,S18] thus is expected to be stable.

Among all the metabolite, M2 and M8 are the only two thiophene oxidation metabolite. M2 of 2-*d*-clopidogrel and 3-$d_{0.35}$-clopidogrel showed loss of deuterium due to 2-hydroxylation and enol-keto tautomerization of thiolactone[S10], respectively, as shown in Figure S2. M8 of the deuterated clopidogrel analogues showed no loss of deuterium, which suggests the "+O+2H" modification on the thiophene motif proceeds as proposed in Figure S2 involving no C-H bond scission.

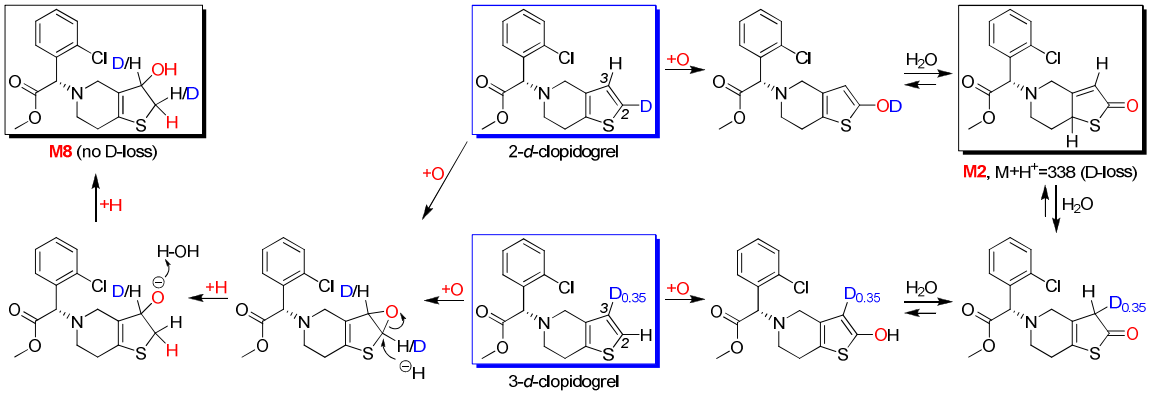


Figure S2. Formation of M2 and M8 from deuterated clopidogrel analogues. One of the two possible M8 structures is shown here.

CYP phenotyping of clopidogrel metabolite formation. It has long been believed that the first step metabolic activation of clopidogrel (M0), thiophene 2-oxidation leading to 2-oxo-clopidogrel (M2) formation, is catalyzed by CYP2C19, CYP1A2 and CYP2B6, as concluded from previous in vitro phenotyping studies in cDNA-expressed cytochrome P450 supersomes.[S19] However, the reported supersome phenotyping experiment contained multiple potential sources of errors that can cause "false negative" results showing only contributions from the three P450 enzymes: 1. the substrate (clopidogrel) concentration was too high (500 μM) and is likely to inhibit the P450 enzymes (clopidogrel has already been shown to be an inhibitor of CYP2C19[S20] and a mechanism-based inhibitor of CYP2B6[S5]) from catalyzing clopidogrel biotransformation; 2. The incubation mixture consisted a high percentage of organic solvent (2% N,N-dimethylacetamide, v/v) which is likely to denature some of the cDNA-expressed recombinant P450 enzymes that is more vulnerable to organic solvents than commonly used sub-cellular fractions like liver microsomes; 3. The metabolite formation was analyzed by UV detection under 220 nm only without more sensitive mass spectrometric detection (clopidogrel has only weak UV absorption and 2-oxo-clopidogrel has even lower UV response after the thiophene motif gets oxidized to thiolactone). The reported quantitative studies of 2-oxo-clopidogrel (M2) formation might also contain significant error as the M2 was eluted under isocratic chromatographic condition, without knowing the existence of its two closely eluted and similarly fragmented isomeric metabolites (M3 and M4), the liquid chromatography tandem mass spectrometry-based quantitative results might contain significant errors affecting the accuracy of the kinetic study results obtained in cDNA-expressed individual P450 enzymes.

The CYP reaction phenotyping experiment was re-tested under conditions eliminating the above potential sources of errors: 1. Final concentration of clopidogrel is 50 μM; 2.

DMSO% is 0.1%; 3. 2-oxo-clopidogrel (M2) was monitored by MS/MS under full chromatographic separation from M3 and M4. The results are shown in Figure S3.

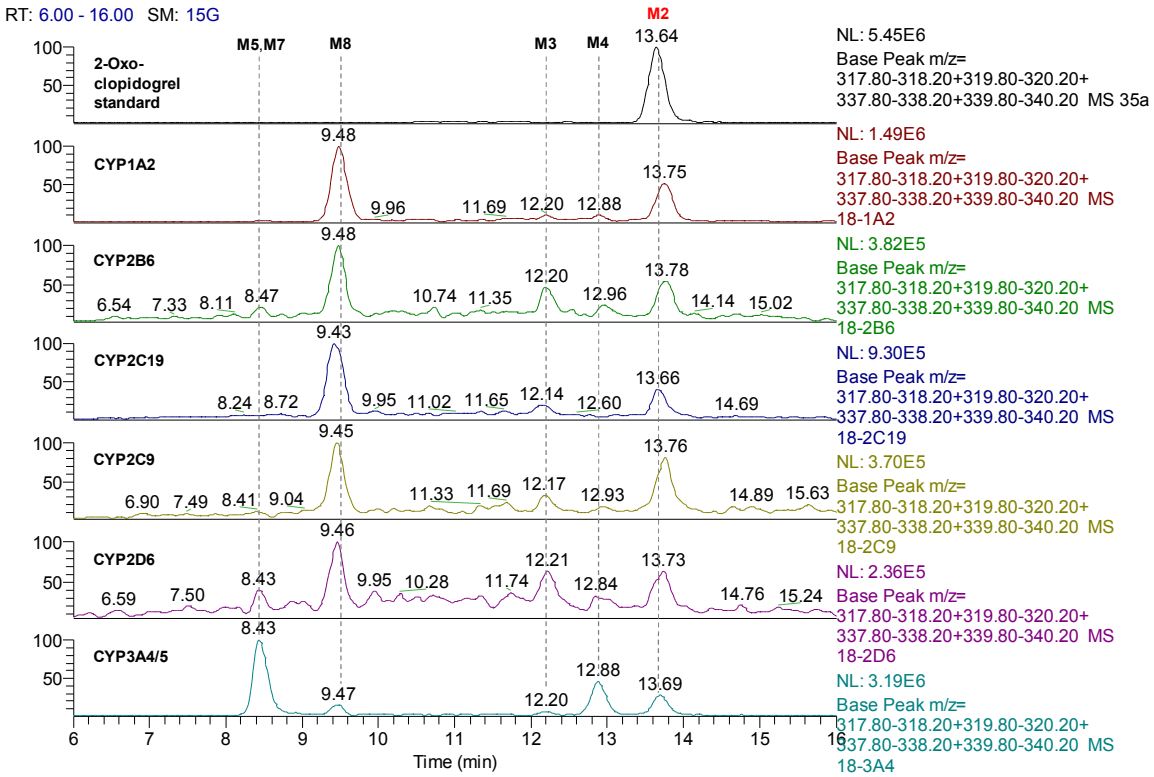


Figure S3. Phenotyping of metabolite formation in cDNA-expressed CYP supersomes. The pharmacologically desired bioactivation metabolite 2-oxo-clopidogrel (M2) was found with all the tested CYP enzymes.

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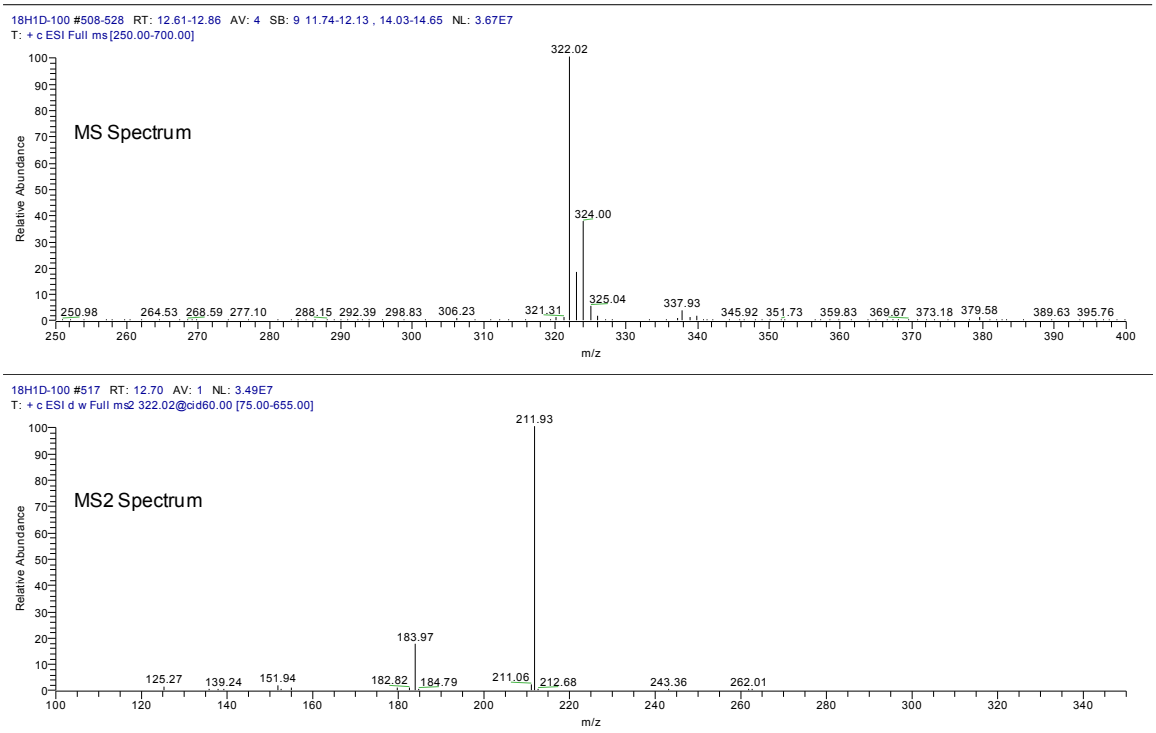

Figure S4. MS and MS2 spectra of M0 ($M+H^+$=322).

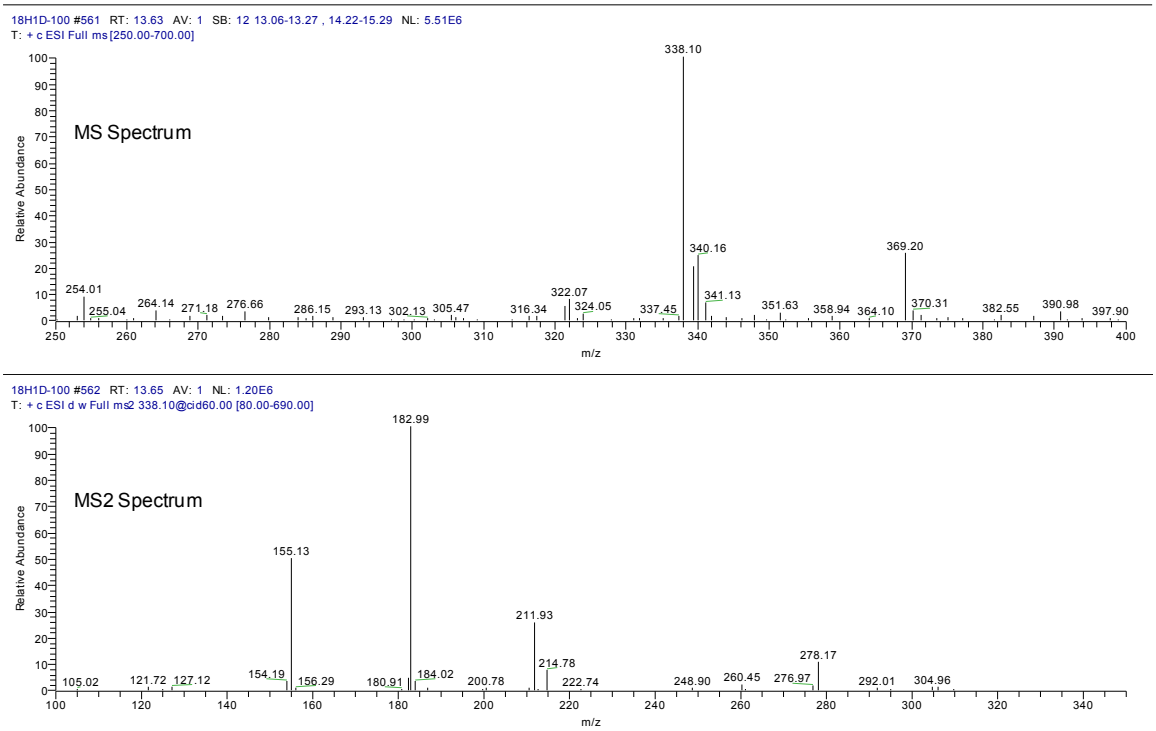

Figure S5. MS and MS2 spectra of M2 ($M+H^+$=338).

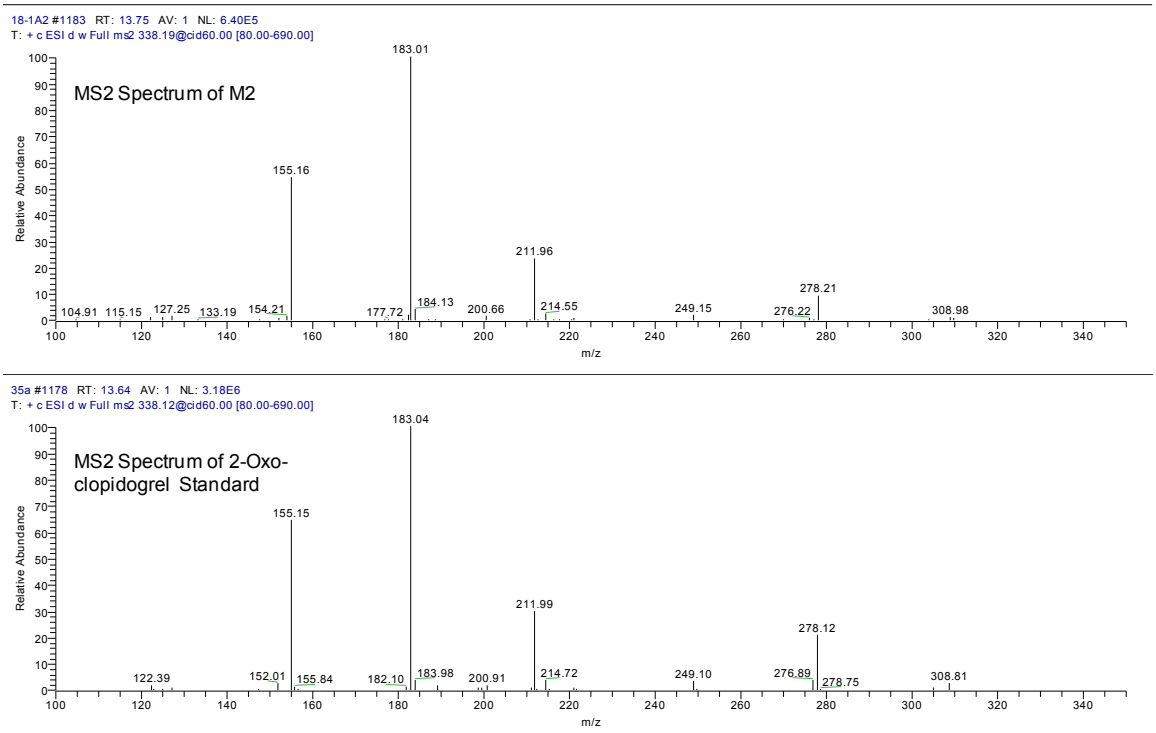

Figure S6. MS2 spectra of M2 ($M+H^+$=338) and 2-oxo-clopidogrel standard.

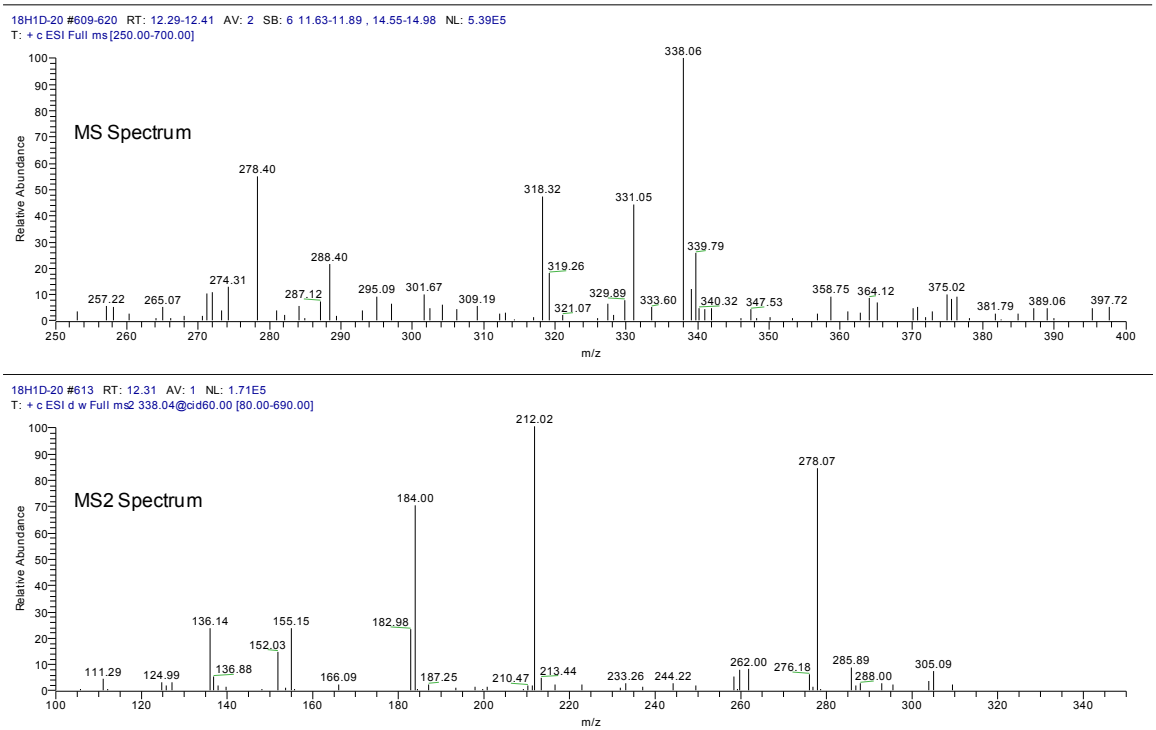

Figure S7. MS and MS2 spectra of M3 ($M+H^+$=338).

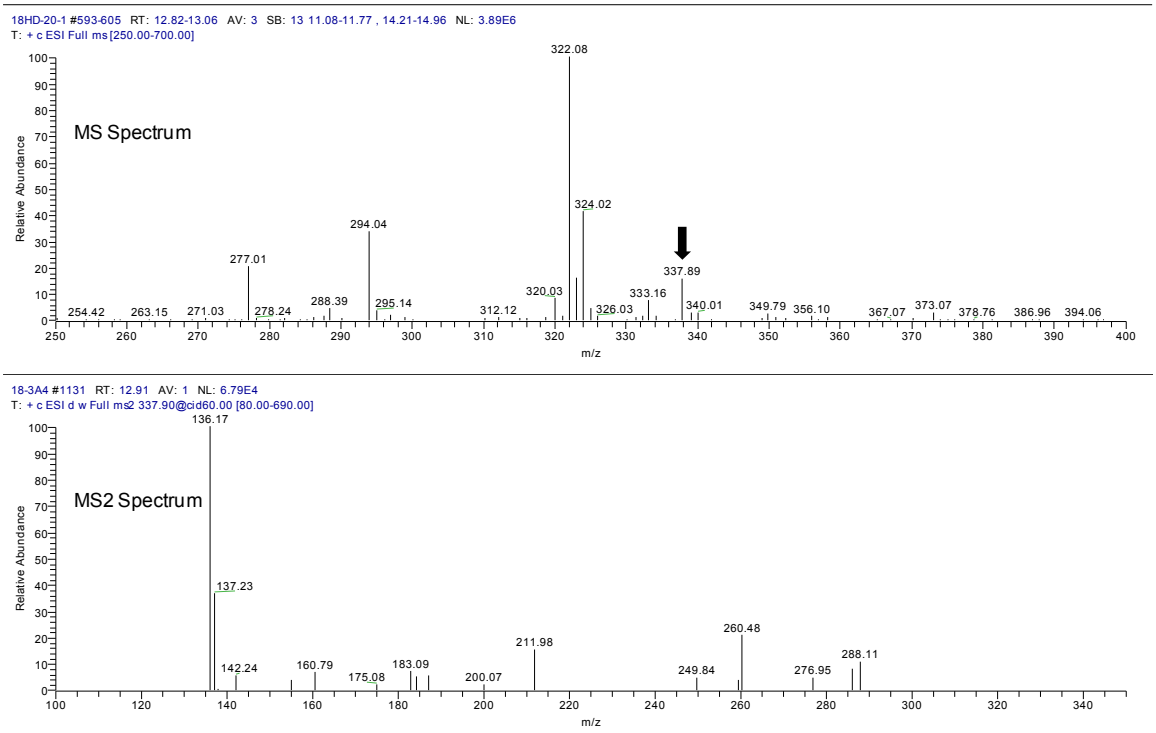

Figure S8. MS and MS2 spectra of M4 ($M+H^+$=338). M4 co-elutes with M0 and M6.

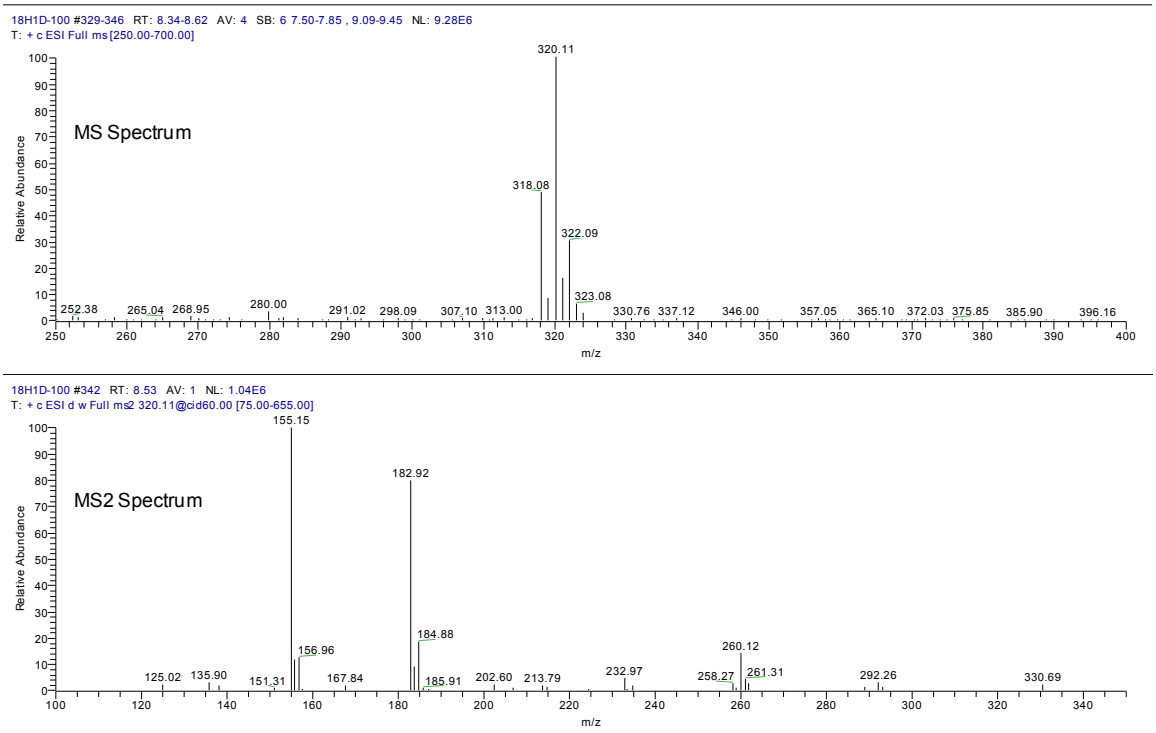

Figure S9. MS and MS2 spectra of M5 ($M+H^+$=320). M5 co-elutes with M7.

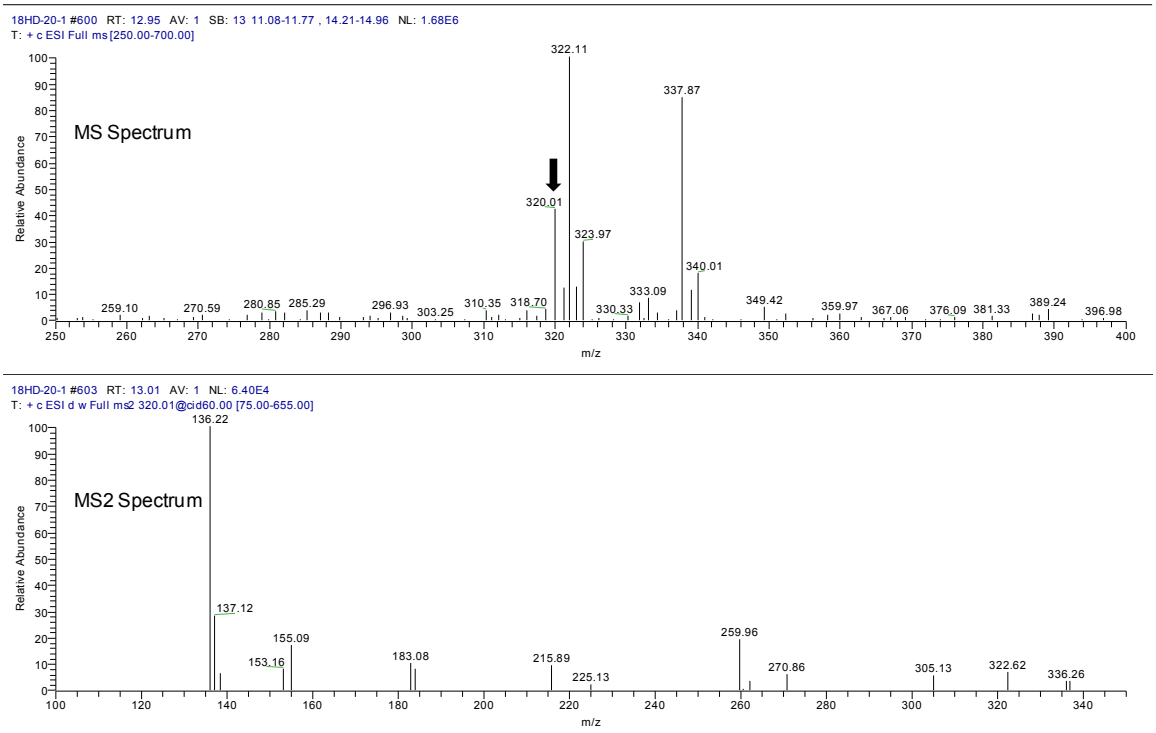

Figure S10. MS and MS2 spectra of M6 ($M+H^+$=320). M6 co-elutes with M0 and M4.

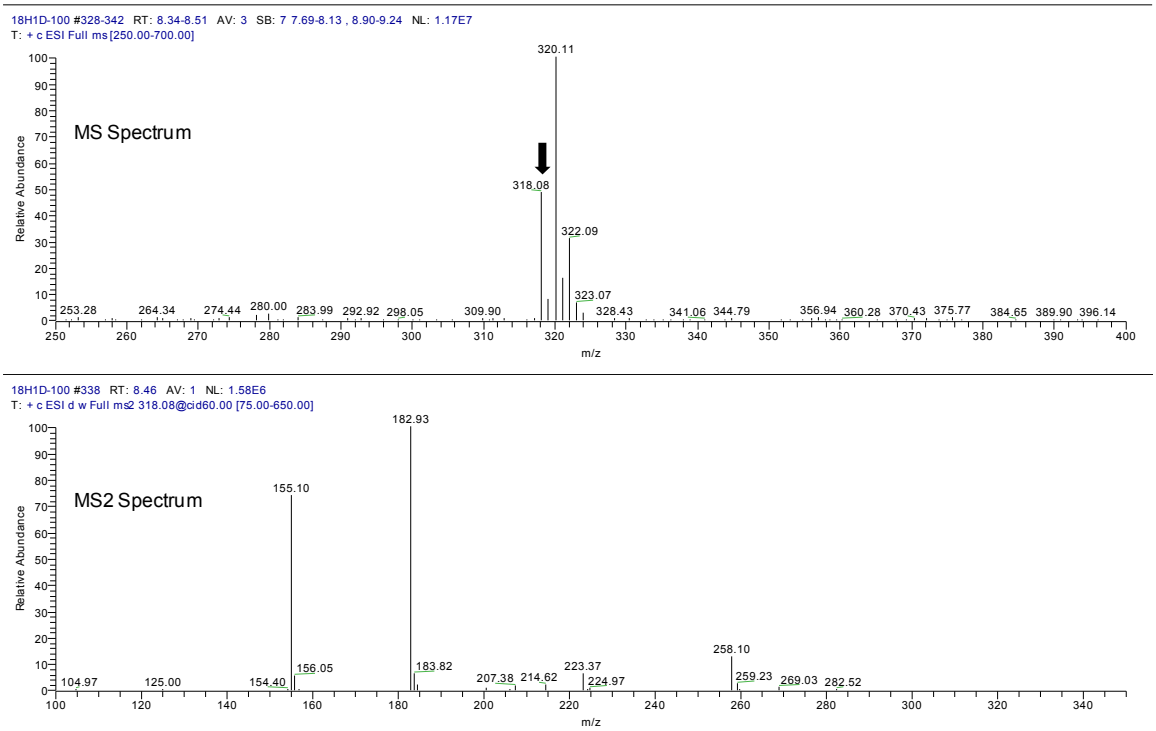

Figure S11. MS and MS2 spectra of M7 ($M+H^+$=318). M7 co-elutes with M5.

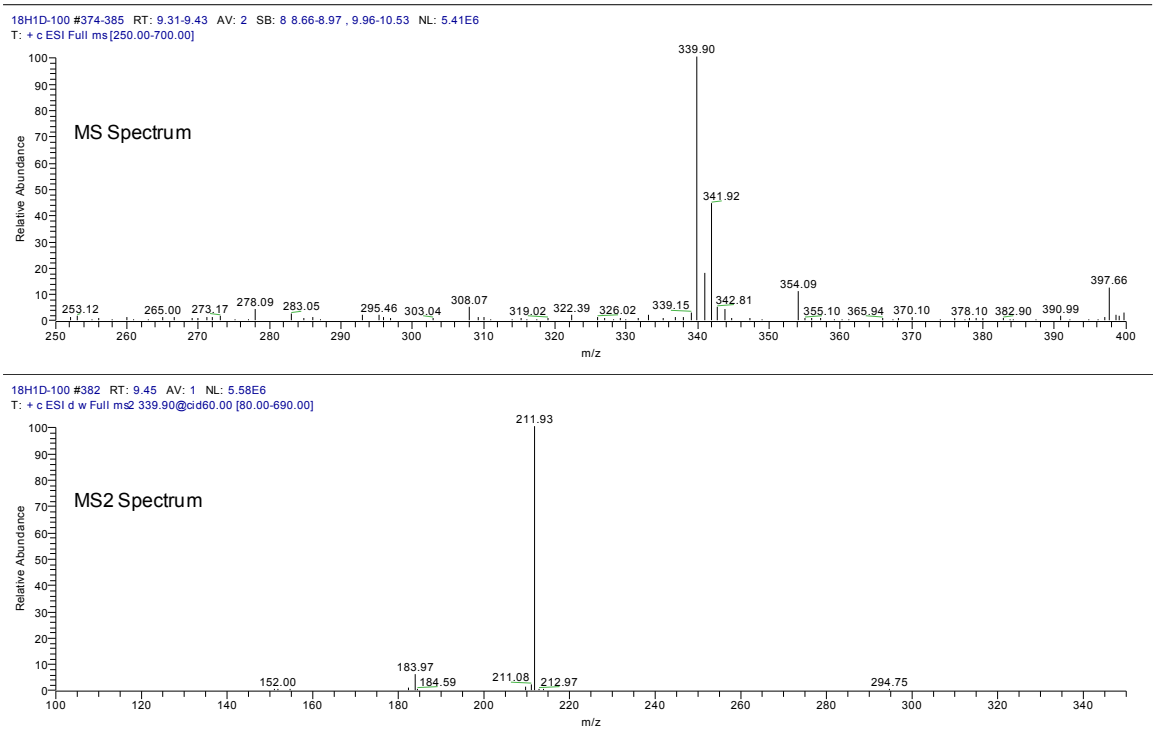

Figure S12. MS and MS2 spectra of M8 ($M+H^+$=340).

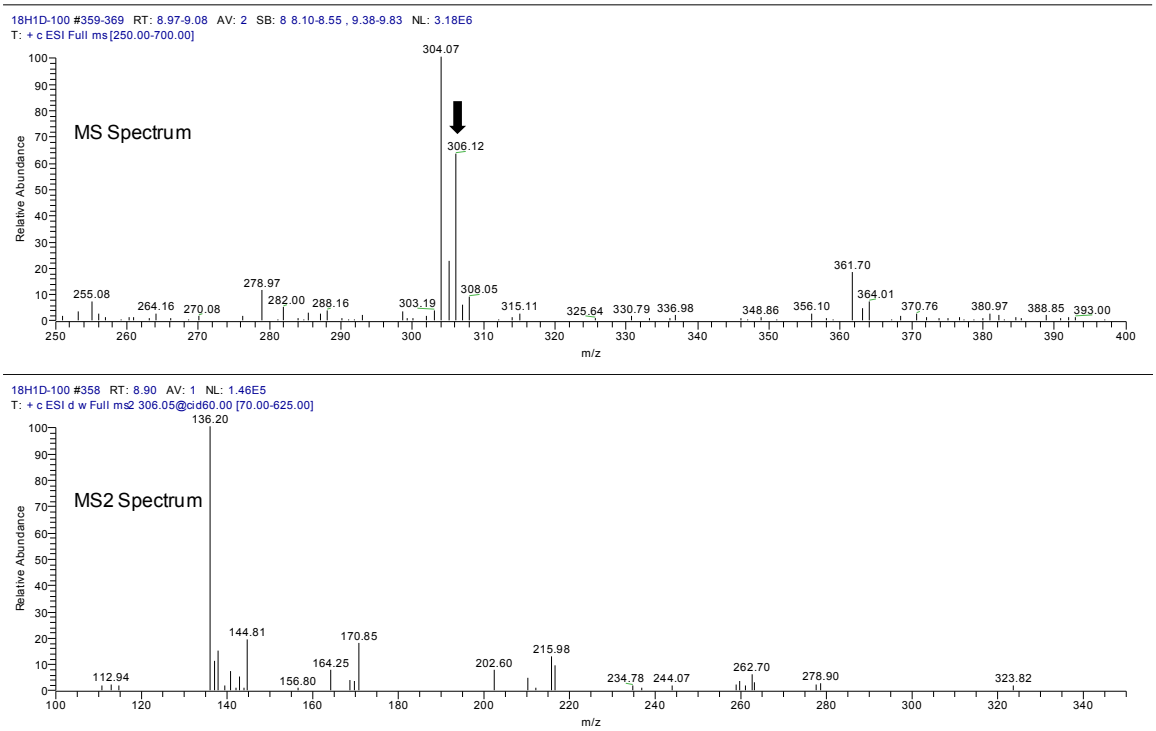

Figure S13. MS and MS2 spectra of M9 ($M+H^+$=306). M9 co-elutes with M10.

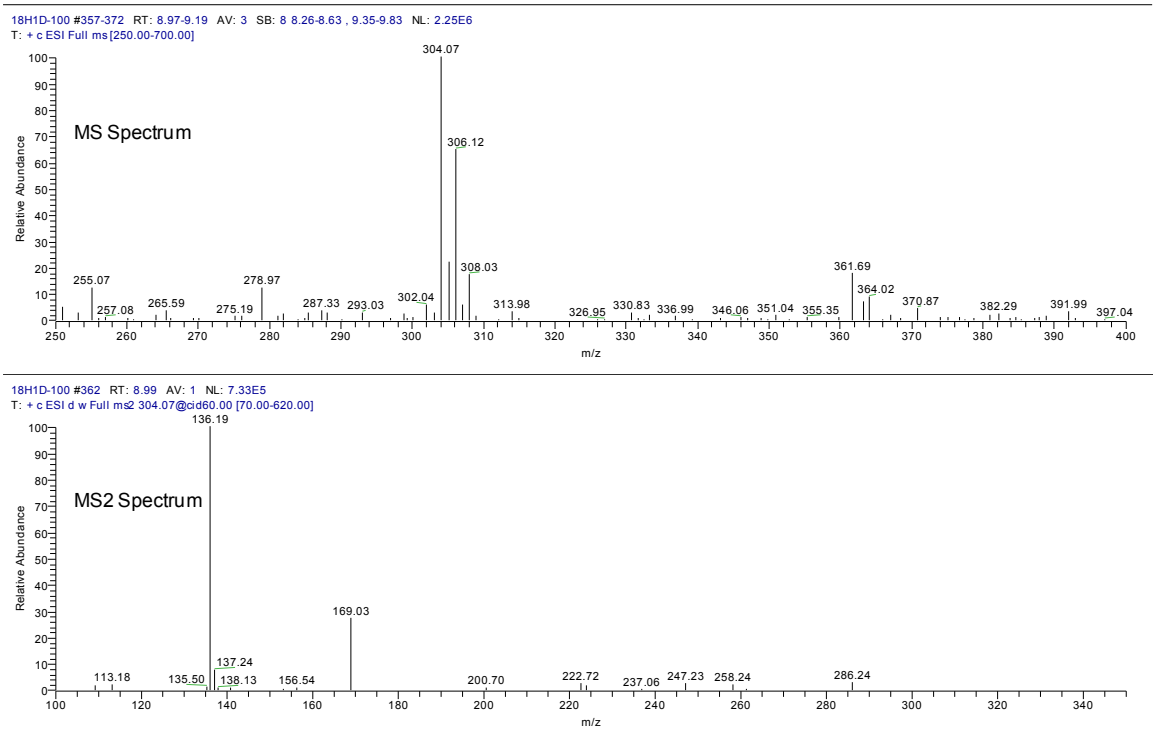

Figure S14. MS and MS2 spectra of M10 ($M+H^+$=306). M10 co-elutes with M9.

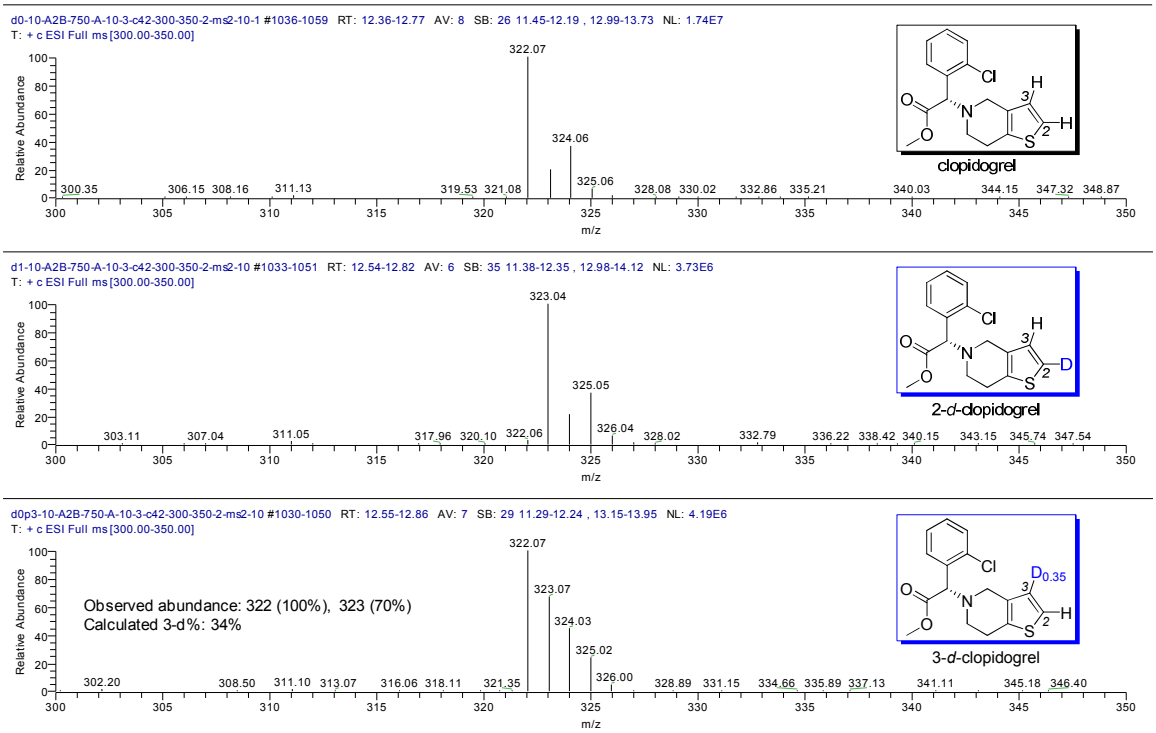

Figure S13. MS spectra of clopidogrel, 2-*d*-clopidogrel, and 3-*d*-clopidogrel.

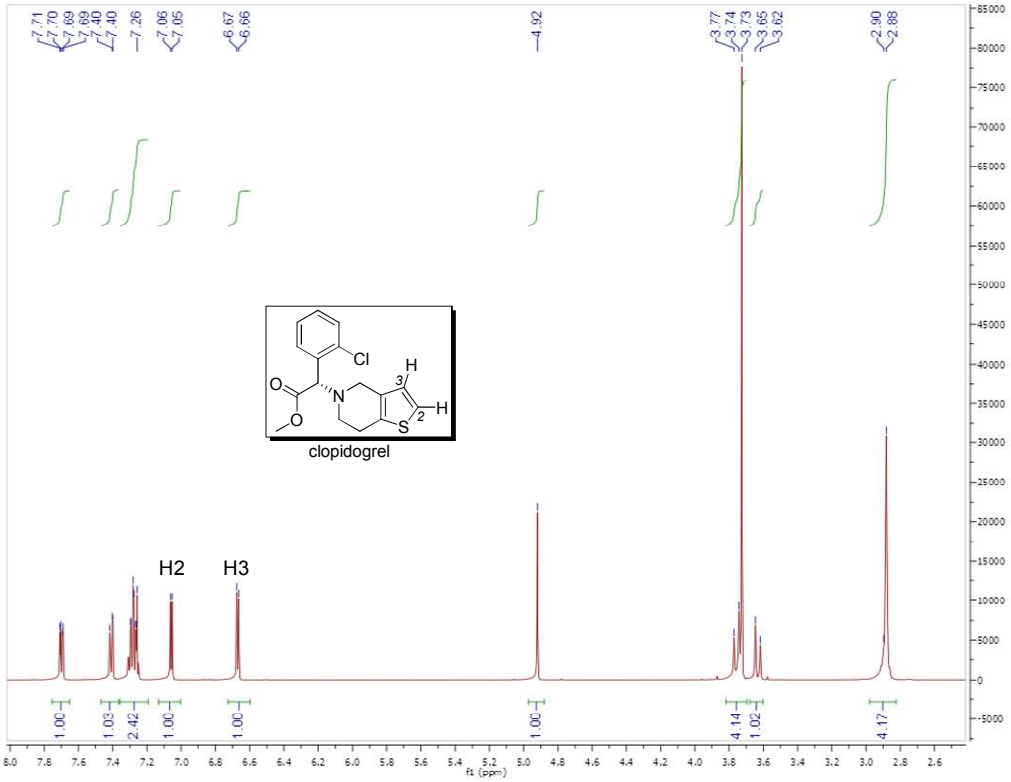

Figure S14. [1]HNMR of clopidogrel.

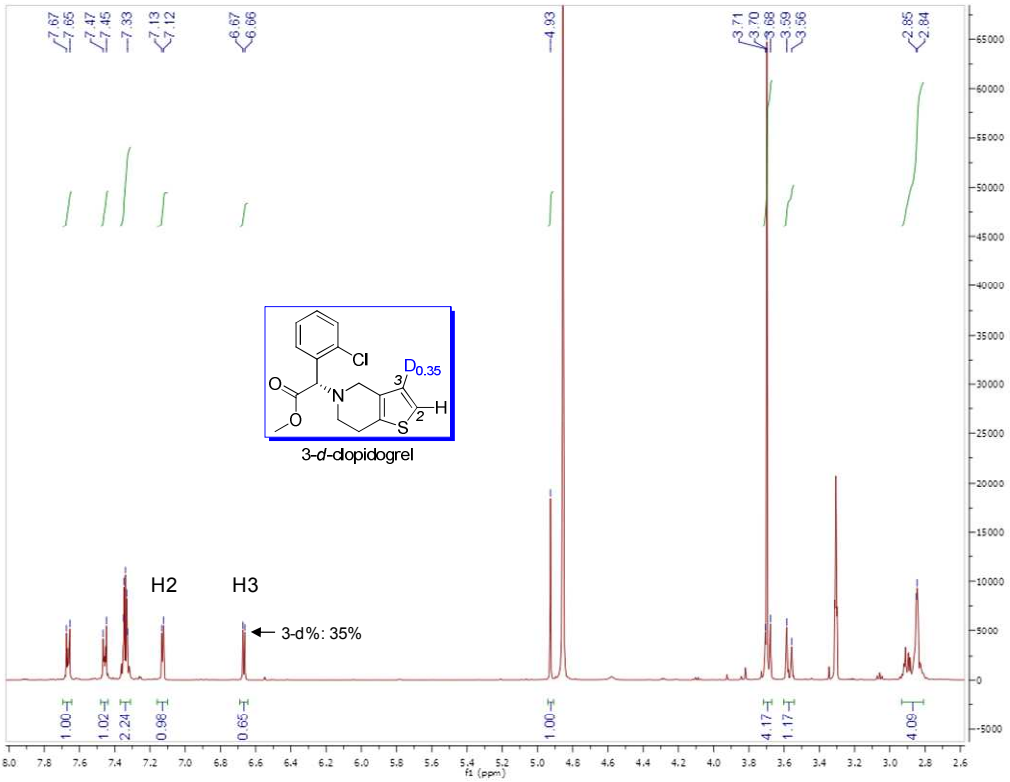

Figure S15. [1]HNMR of 3-*d*-clopidogrel.