Supplemental Data: Metabolites of PPI-2458, a Selective, Irreversible Inhibitor of Methionine Aminopeptidase-2. Structure Determination and In Vivo Activity

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Drug Metabolism and Disposition

Chemical synthesis of metabolites.

*(2S)-(–)-2-N-(tert-butoxycarbonyl)amino-3-methyl-1,3 butanediol (**2**, Supplemental Figure 1).* Compound **2** was prepared according to the reported procedure (Dettwiler and Lubell, 2003) starting from N-Boc L-serine methyl ester **1**.

*(R)-(+)-β-Hydroxy-N-(tert-butoxycarbonyl) valine (**3**).* In a 100 mL round bottom flask equipped with a magnetic stirring bar was placed **2** (4.1 g, 18.5 mmol) dissolved in 150 mL of CH_3CN. The flask was cooled to 0°C, and 50% $NaHCO_3$ (25 mL) and TEMPO (0.29 g, 1.85 mmol) were introduced successively into the reaction mixture with stirring. To the resulting red colored solution was added NaOCl (92.6 mmol, 147 mL of 0.63 M commercially available solution) and stirred at 0°C for 1 h, followed by stirring at room temperature for 1-1.5 h. At this stage TLC indicated complete disappearance of starting compound. The reaction mixture was then poured into water (300 mL) and extracted with ether (2 x 50 mL) to remove the neutral impurities. The aqueous layer was acidified with solid citric acid to pH ~3. Sodium thiosulfate (~10g) was then added to the reaction mixture and stirred for 1h. The solution was then extracted with EtOAc (3 x 50 mL) and the combined EtOAc layers were dried over $MgSO_4$. Evaporation of solvent gave Boc-protected hydroxyvaline as a viscous mass in 91% yield, which solidified on storing in refrigerator. Physical characteristics of this compound were similar to those reported (Dettwiler and Lubell, 2003).

*(R)-β-Hydroxy-N-(tert-butoxycarbonyl) valineamide (**4**).* In an oven dried two neck round bottom flask equipped with a magnetic stirring bar, nitrogen inlet and a rubber septa was placed **3** (0.1 g, 0.43 mmol) dissolved in dichloromethane (10 mL). The flask was cooled to 0°C and DCC (0.11 g, 0.54 mmol), HOBt hydrate (0.06 g, 0.43 mmol) were successively introduced into

the reaction mixture. The resulting suspension was allowed to warm to room temperature and stirred at room temperature for 10-20 min. It was then cooled to 0°C, and the nitrogen balloon was replaced with an ammonia balloon. The reaction mixture turned into milky white suspension. It was stirred at room temperature for 2 h under the positive pressure of the ammonia balloon. The balloon was then removed and the reaction mixture was diluted with ether/ hexane (1:1, 50 mL). The white precipitate was filtered off and the filtrate was evaporated to dryness to yield the product contaminated with dicyclohexylurea. The residue was dissolved in minimum amount of ether and the precipitate obtained was filtered. This process repeated until no further precipitation occurred. The viscous mass obtained after evaporation of ether was dried under high vacuum to afford the product in 89% yield. ^{1}H NMR δ ($CDCl_3$) 5.51 (d J = 8.4 Hz, 1H), 3.95 (d, J = 9.2 Hz, 1H), 1.44 (s, 9H), 1.34 (s, 3H), 1.21 (s, 3H).

*(R)-β-Hydroxy valineamide (**5**).* In a 50 mL round bottom flask equipped with a magnetic stirring bar was placed **4** (0.05 g, 0.22 mmol) in dichloromethane (10 mL). It was cooled to 0°C and TFA (2 mL) was introduced. The reaction mixture was stirred at 0°C for 30 min and further allowed to stir at room temperature for 30 min. Most of dichloromethane and TFA were evaporated, and the residue thus obtained was triturated with ether (2 x 20 mL). The resulting viscous mass was dried under high vacuum to yield crude **5** as its TFA salt which was used without further purification in the next step. ^{1}H NMR δ (CD_3OD) 3.67 (s, 1H), 1.38 (s, 3H), 1.25 (s, 3H); ^{13}C NMR 168.68, 69.18, 61.94, 27.21, 24.19.

M1((3R,4S,5S,6R)-5-methoxy-4-((2R,3R)-2-methyl-3-(3-methylbut-2-en-1-yl)oxiran-2-yl)-1-oxaspiro[2.5]octan-6-yl ((R)-1-amino-3-hydroxy-3-methyl-1-oxobutan-2-yl)carbamate). The TFA salt of **5** (2.937g, 11.93 mmole) was dissolved in 300 mL dry CH_3CN. To the solution was added 3.1 mL (17.9 mmole, 1.5 equiv) DIEA. The mixture was stirred at 0ºC, and 10.098 g

(23.86 mmole, 2 equiv) fumagillol-N-hydroxysuccinimidyl carbonate was added while flushing the reaction mixture with dry N_2 gas. An additional 3.1 mL DIEA was added to bring the solution to pH 9. The reaction was stirred at 0ºC under dry N_2 gas for 1.5 h. The reaction mixture was then warmed to room temperature and stirred for an additional 3.5 h. At this time, TLC showed absence of starting material. The reaction mixture was concentrated *in vacuo*, and purified by preparative HPLC with the following conditions: injection quantity ~200mg; column: Phenomenex Luna, C8(2), 5 micron, 100 x 21.2 mm; mobile phase A = H_2O, B = CH_3CN, 1% H_2O; gradient: 25 → 60% B over 20 minutes; flow: 24 mL / min; detection wavelength: 214nm; temperature: ambient. The final yield was 3.4 g (65%).

M2((3R,4S,5S,6R)-4-((2R,3R)-3-(((R)-3,3-dimethyloxiran-2-yl)methyl)-2-methyloxiran-2-yl)-5-methoxy-1-oxaspiro[2.5]octan-6-yl ((R)-1-amino-3-methyl-1-oxobutan-2-yl)carbamate) and M3 ((3R,4S,5S,6R)-4-((2R,3R)-3-(((S)-3,3-dimethyloxiran-2-yl)methyl)-2-methyloxiran-2-yl)-5-methoxy-1-oxaspiro[2.5]octan-6-yl ((R)-1-amino-3-methyl-1-oxobutan-2-yl)carbamate). The nonstereospecific protocol of Curci et al (1980) was used to generate the LC resolvable metabolites as a mixture. In a 250 mL round bottom flask, 800 mg (1.9 mmol) of PPI-2458 was dissolved in 2.3 mL water and 0.9 mL acetone. The solution was cooled to 0ºC, and to it was added, in 1 mL aliquots over an hour, 76 mL of 0.04 M potassium peroxomonosulfate ("Oxone", 2.5 equiv) in 0.04 mM aqueous EDTA. The reaction was maintained at pH 8 - 9 with 0.5 M KOH. The reaction was allowed to stir at 0ºC for 1 hour after addition of Oxone was complete. The reaction mixture was extracted with dichloromethane, and the organic layer then washed with sodium bicarbonate solution, followed by drying over sodium sulfate. The product was the condensed to yield a yellow oil. The diastereomers were resolved by preparative HPLC as follows: injection quantity: ~40 mg; column: Phenomenex Luna, C8, 5 micron, 100 x 21.2 mm;

mobile phases: A = 100% H_2O, B = 85% MeOH in H_2O; gradient: 40 → 65% B over 20 minutes; flow: 24 mL/min; detection wavelength: 214 nm; temperature: ambient. The gradient was adjusted to compensate for any lot to lot variability in the composition of the mobile phases. Aqueous fractions of purified M2 and M3 were found to decompose readily with the addition of water, and required rapid freezing and lyophilization to maintain purity. To generate M2 stereospecifically, the modified protocol of Wang et al (1997) was employed, using the fructose based catalyst 1,2:4,5-di-*O*-isopropylidene-β-D-*erythro*-2,3-hexodiulo-2,6-pyranose.

M4 ((3R,4S,5S,6R)-4-((2R,3R)-3-((E)-4-hydroxy-3-methylbut-2-en-1-yl)-2-methyloxiran-2-yl)-5-methoxy-1-oxaspiro[2.5]octan-6-yl ((R)-1-amino-3-methyl-1-oxobutan-2-yl)carbamate). The procedure described by Bhalero and Rapoport (1971) was used. In a 100 mL round bottom flask, 430 mg (1 mmol) of PPI-2458 and 110 mg (1 mmol) of selenium dioxide were dissolved in 30 mL of t-butanol. The reaction mixture was stirred overnight at 25ºC. The resulting solution was concentrated to an orange solid, taken up in CH_3CN, and shaken until only a red selenium containing precipitate remained. The CH_3CN solution was then decanted, concentrated, and subjected to preparative HPLC using the following conditions: injection quantity: ~40 mg; column: Phenomenex Luna, C8, 5 micron, 100 x 21.2 mm; mobile phases: A = 100% H_2O, B: 99% CH_3CN / water; gradient: 22 → 27% B over 20 minutes; flow: 24 mL/min; detection wavelength: 214 nm; temperature: ambient. Product fractions were combined and lyophilized to yield a white powder, yield = 77 mg (17%).

M5((3R,4S,5S,6R)-4-((2R,3R)-3-(((R)-3,3-dimethyloxiran-2-yl)methyl)-2-methyloxiran-2-yl)-5-methoxy-1-oxaspiro[2.5]octan-6-yl ((R)-1-amino-3-hydroxy-3-methyl-1-oxobutan-2-yl)carbamate) and M6 ((3R,4S,5S,6R)-4-((2R,3R)-3-(((S)-3,3-dimethyloxiran-2-yl)methyl)-2-methyloxiran-2-yl)-5-methoxy-1-oxaspiro[2.5]octan-6-yl ((R)-1-amino-3-hydroxy-3-methyl-1-

oxobutan-2-yl)carbamate). Following a nonstereospecific procedure (Curci et al., 1980) M1 (0.8 g, 1.82 mmole) was dissolved in 0.9 mL acetone and 2.3 mL H_2O. The solution was brought to pH 9 with 0.200 mL 0.5M KOH and cooled to 0ºC. To the reaction mixture was added, in 0.8 mL aliquots over an hour, 114 mL of 0.04 M potassium peroxomonosulfate ("Oxone", 2.5 equiv.) in 0.04 mM aqueous EDTA. The reaction mixture was maintained at pH 9 with 200-400 μL aliquots of 0.5M KOH. When all potassium peroxomonosulfate / EDTA solution had been added the reaction was allowed to stir at pH 9 at 0ºC for 2.5 hours. The reaction mixture was then extracted with 4 x 200 mL DCM. The combined organic layers were washed twice with saturated aqueous $NaHCO_3$, then dried over Na_2SO_4 for 30 minutes. The filtrate was concentrated *in vacuo*, yielding 798 mg crude material. For purification, the crude material was combined with two additional crude lots of product, 1.047 g and 0.085 g weight. The diastereomers were resolved by preparative HPLC with the following conditions: injection quantity ~200mg; column: Phenomenex Luna, C8(2), 5μm, 100 x 21.2 mm; mobile phases A = H_2O pH 8.5 with NH_4HCO_3, B = CH_3CN, 1% H_2O; gradient: first pass: 16 →30% B over 20 minutes, second pass: 18 → 25% B over 20 minutes; flow: 24 mL / min; detection wavelength: 214 nm; temperature: ambient. Yields were 300 mg M5 and 240 mg M6.

PPI-4338 ((3R,5S,6R)-4-((2R,3R)-3-(3-hydroxy-3-methylbutyl)-2-methyloxiran-2-yl)-5-methoxy-1-oxaspiro[2.5]octan-6-yl ((R)-1-amino-3-methyl-1-oxobutan-2-yl)carbamate). To a solution of Hg(II) acetate (319 mg, 1.0 mmole) dissolved in 1:1 water:THF (2 mL) was added PPI-2458 (424 mg, 1.0 mmole, 1 equiv). The clear solution was stirred for 1 h at room temperature, chilled in ice, and then treated with 1 mL of ice cold 3 M NaOH, followed by the addition of a solution of $NaBH_4$ (19 mg, 0.5 mmole, 2 equiv) in 1 mL 3 M NaOH. The reaction mixture was stirred for 1 h at room temperature, and the aqueous layer extracted 3 x with ether.

The combined organic extracts were washed with 5 mL brine, dried over MgSO4, and concentrated to a white foam. The crude material was purified to homogeneity by preparative LC. ^{1}H NMR δ (CD_3OD) 5.42 (s, 1H), 3.94 (d, 1H), 3.68 (dd, 1H), 3.41 (s, 3H), 2.90 (d, 1H), 2.66 (m, 1H), 2.63 (d, 1H), 1.9 – 2.1 (m, 4 H), 1.5 – 1.8 (m, 5 H), 1.21 (s, 3H), 1.20 (s, 3H), 1.19 (s, 3H), 1.08 (m, 1H), 0.99 (d, 3H), 0.96 (d, 3H).

References

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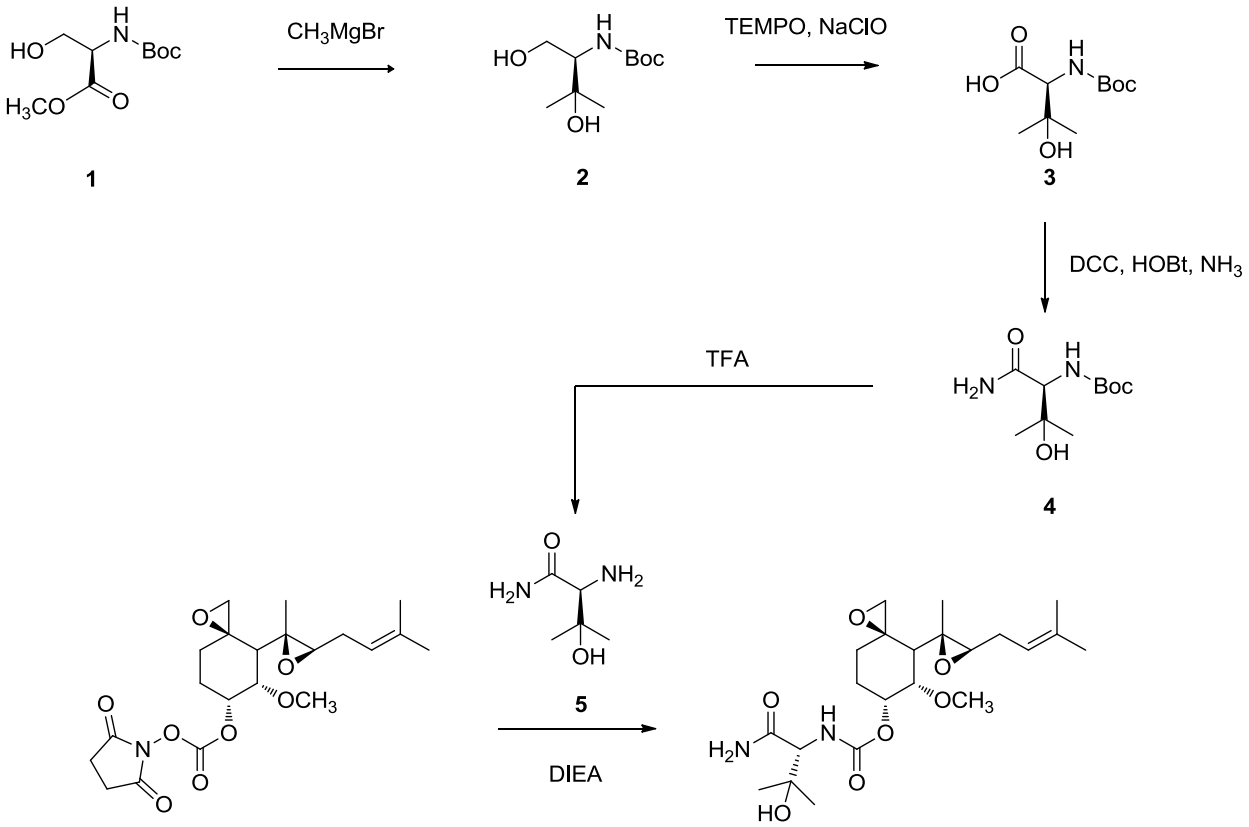
Curci R, Fiorentino M, and Troisi L (1980) Epoxidation of alkenes by dioxirane intermediates generated in the reaction of potassium caroate with ketones. *J Org Chem* **45:** 4758-4760.

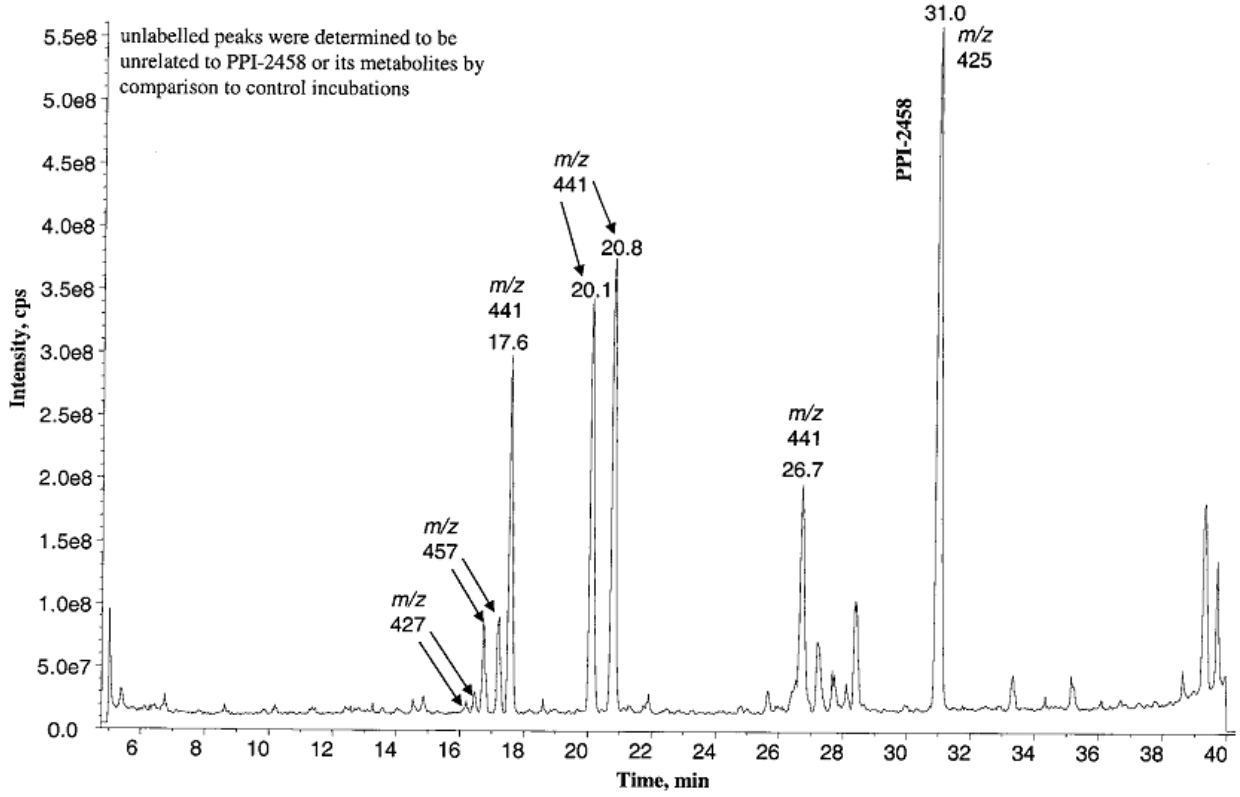
Dettwiler JE and Lubell WD (2003) Serine as chiral educt for the practical synthesis of enantiopure N-protected β-hydroxyvaline. *J Org Chem* **68:** 177-179.

Wang Z-X, Tu Y, Frohn M, Zhang J-R, and Shi Y (1997) An efficient catalytic asymmetric epoxidation method. *J Am Chem Soc* **119:** 11224-11235.

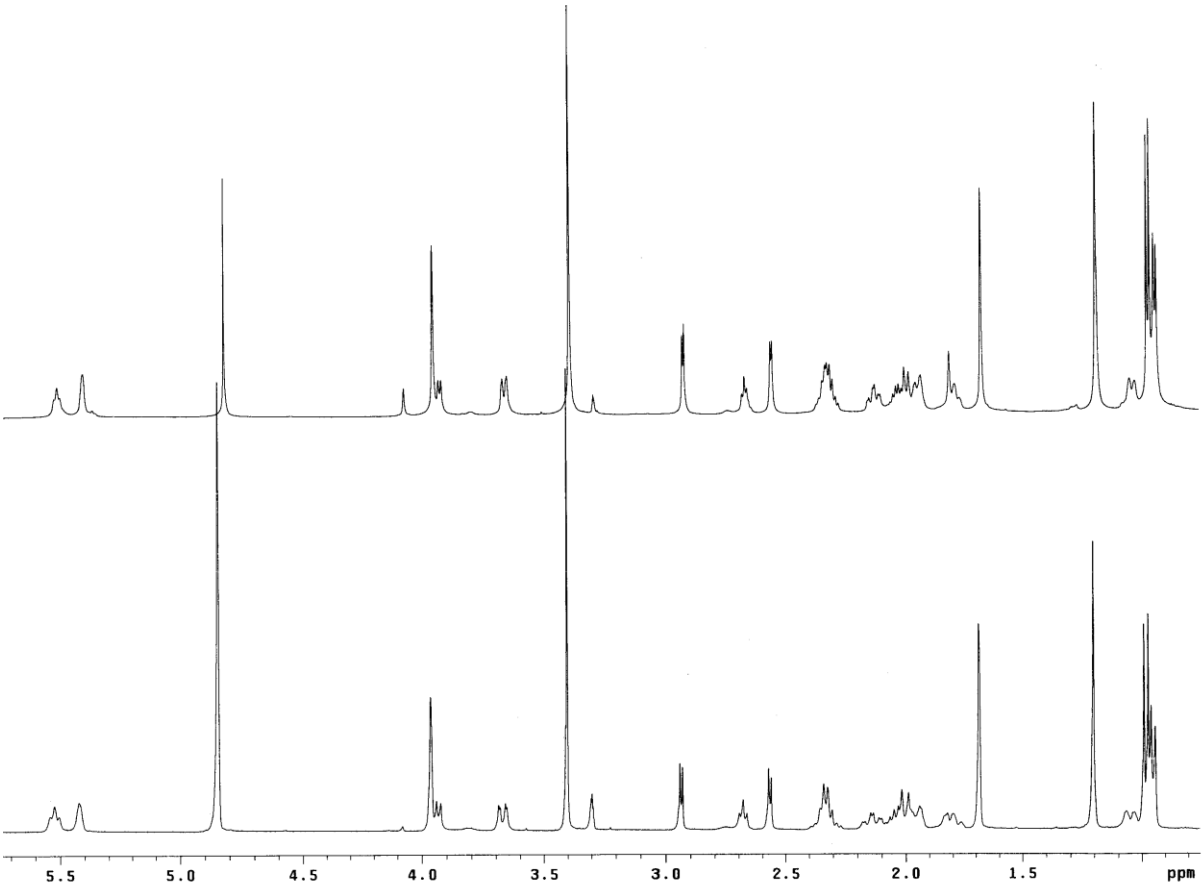
Supplemental Figure 1. Scheme for chemical synthesis of metabolite M1.



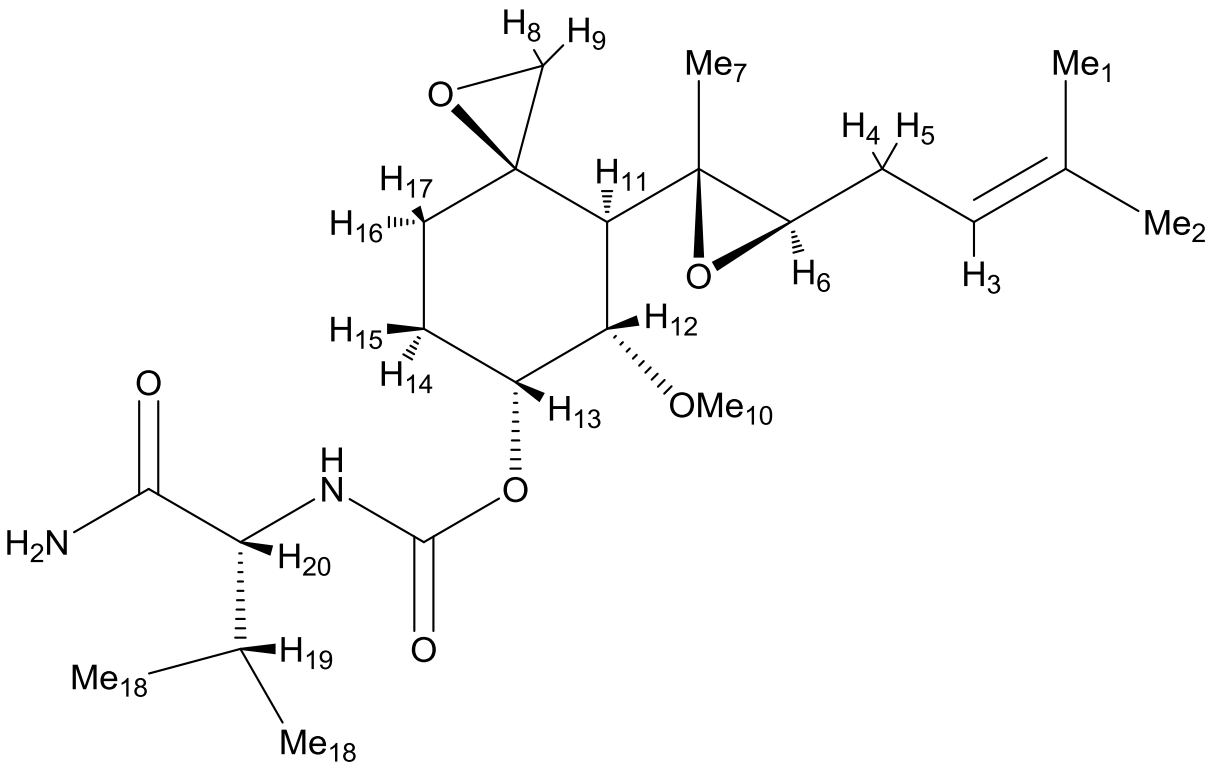
Supplemental Figure 2. Full scan LC-MS of PPI-2458 metabolite formed by incubation with murine liver microsomes.



Supplemental Figure 3. Stacked ^{1}H-NMR spectrum of M4 obtained from liver microsomal preparation (top) and chemical synthesis (bottom).



Supplemental Table 1. ^{1}H chemical shift assignments (ppm) for PPI-2458 and major metabolites in CD_3OD, referenced to TMS at 0.0 ppm. Resonance shifts indicative of the metabolite structure are italicized.



Resonance	**PPI-2458**	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**
Me1	1.67	1.67	*1.29*	*1.31*	1.69	*1.29*	*1.31*
Me2	1.75	1.75	*1.33*	*1.34*	*3.96*	*1.33*	*1.33*
H3	5.24	5.24	*2.97*	*2.94*	*5.53*	*2.97*	*2.94*
H4,5	2.22, 2.31	2.21, 2.32	*1.72, 1.84*	*1.75, 1.91*	2.34, 2.34	*1.72, 1.84*	*1.74, 1.91*
H6	2.64	2.65	*2.84*	*2.81*	2.69	*2.84*	*2.81*
Me7	1.19	1.19	1.16	1.21	1.21	1.16	1.20
H8	2.56	2.57	2.61	2.63	2.58	2.61	2.63
H9	2.96	2.96	3.01	2.93	2.94	3.02	2.93
Me10	3.40	3.40	3.41	3.41	3.41	3.41	3.41
H11	1.97	1.99	2.05	2.05	2.00	2.04	2.04
H12	3.67	3.68	3.69	3.69	3.68	3.70	3.69
H13	5.43	5.46	5.44	5.43	5.43	5.47	5.46
H14	1.96	1.96	1.97	1.96	1.96	1.97	1.97
H15	1.80	1.83	1.83	1.81	1.81	1.84	1.82
H16	2.14	2.15	2.16	2.15	2.15	2.18	2.17
H17	1.04	1.04	1.06	1.09	1.05	1.06	1.09
Me18	0.99, 0.96	*1.28, 1.24*	0.99, 0.96	0.99, 0.96	0.99, 0.96	*1.28, 1.24*	*1.28, 1.24*
H19	2.04	-	2.05	2.04	2.04	-	-
H20	3.94	*4.10*	3.94	3.95	3.94	*4.10*	*4.10*