Integrated phenotypic and activity-based profiling links Ces3 to obesity and diabetes

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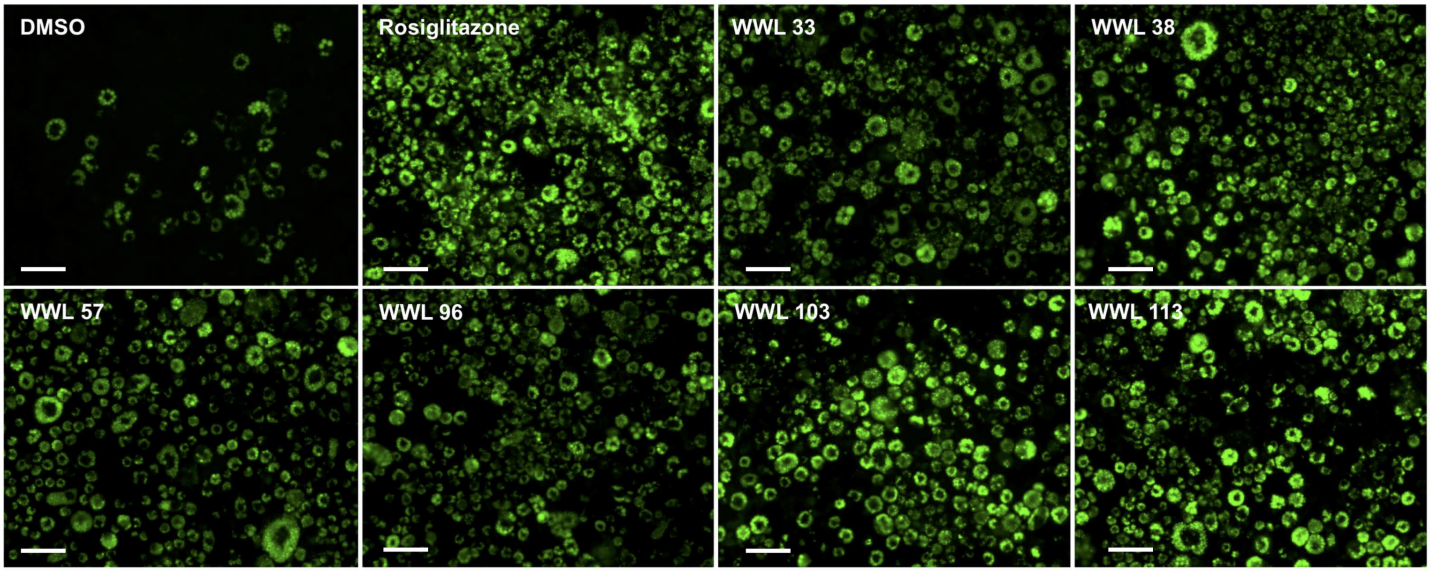
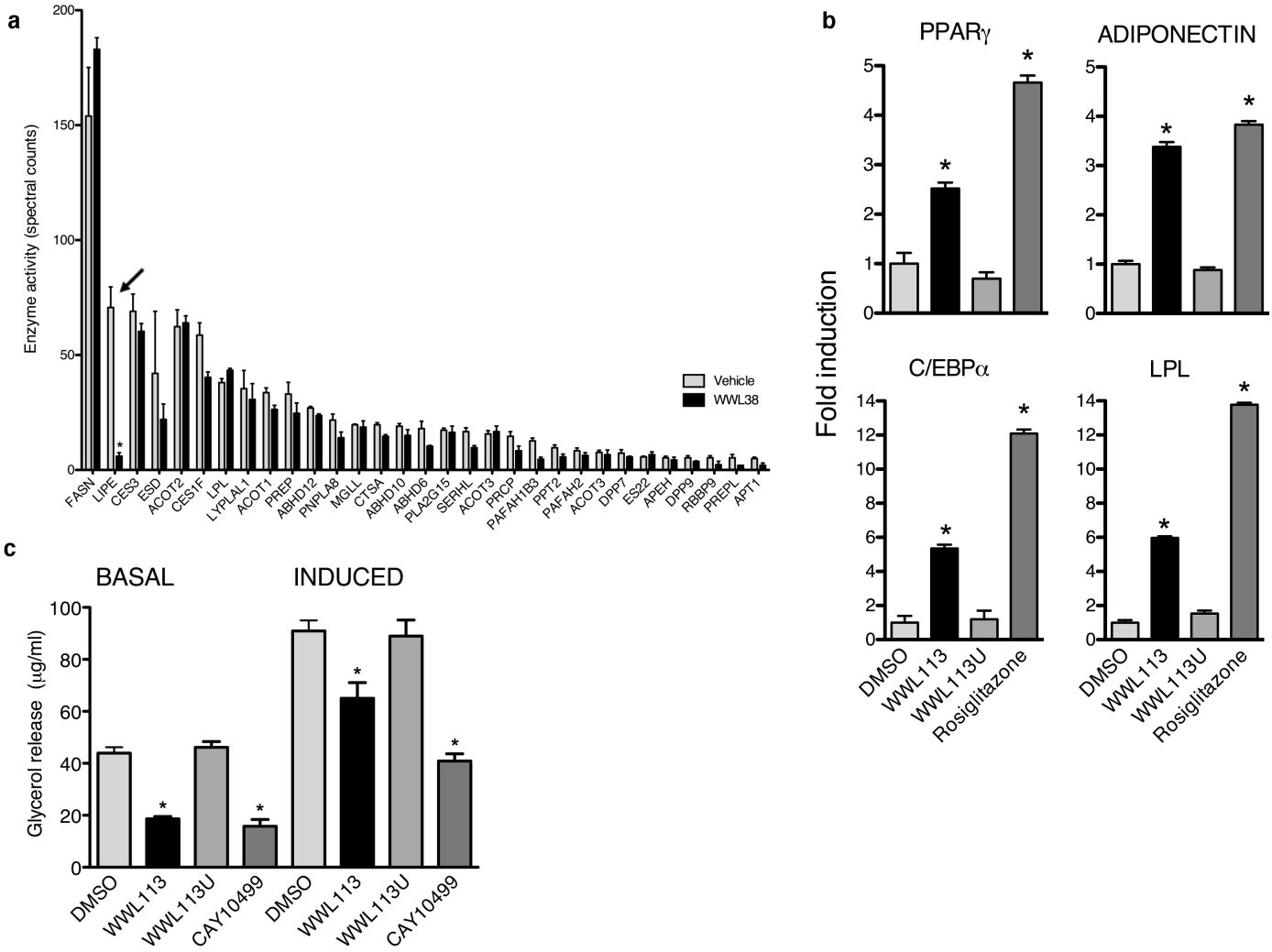
SUPPLEMENTARY INFORMATION

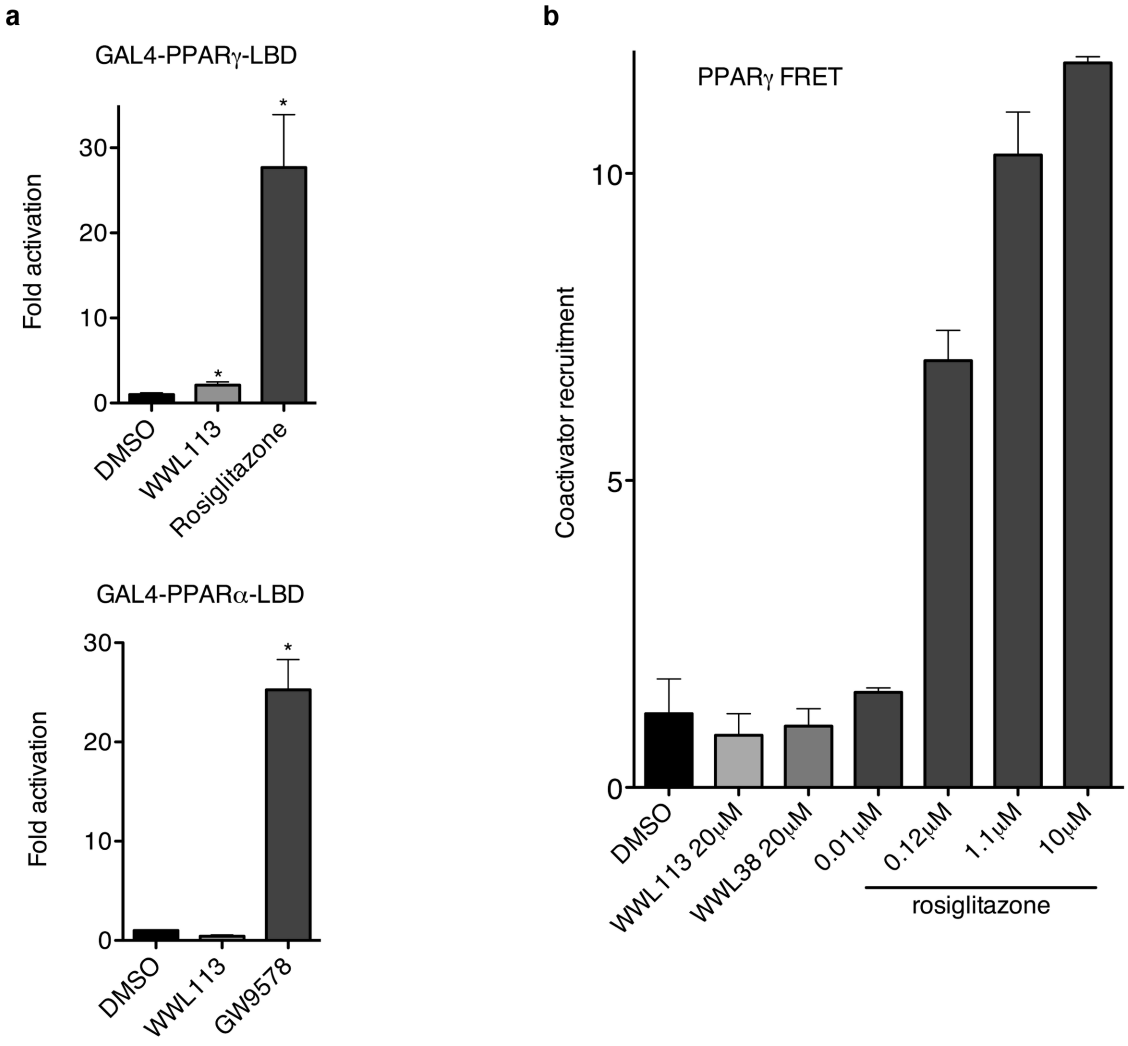
SUPPLEMENTARY RESULTS



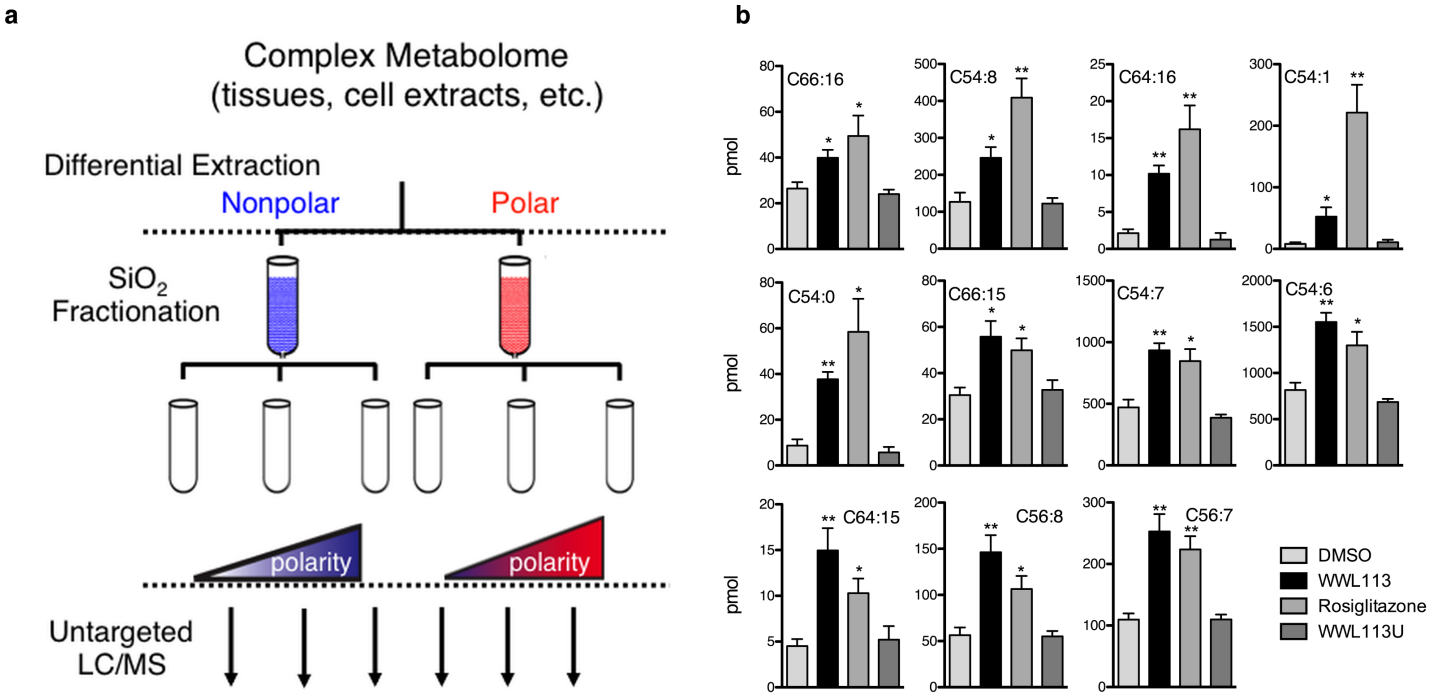
Supplementary Figure 1. Phenotypic screening of carbamates in 3T3-L1 cells. 3T3-L1 preadipocytes were induced to differentiate into adipocytes two days post-confluence in the presence of 10 μM individual carbamates. Two days later, induction media was replaced with maintenance media and compounds refreshed. The extent of lipid accumulation and adipocyte differentiation was evaluated on day 7 using the fluorescent lipid Nile red (shown in green). Representative hits are shown. Scale bar indicates 100 μm.



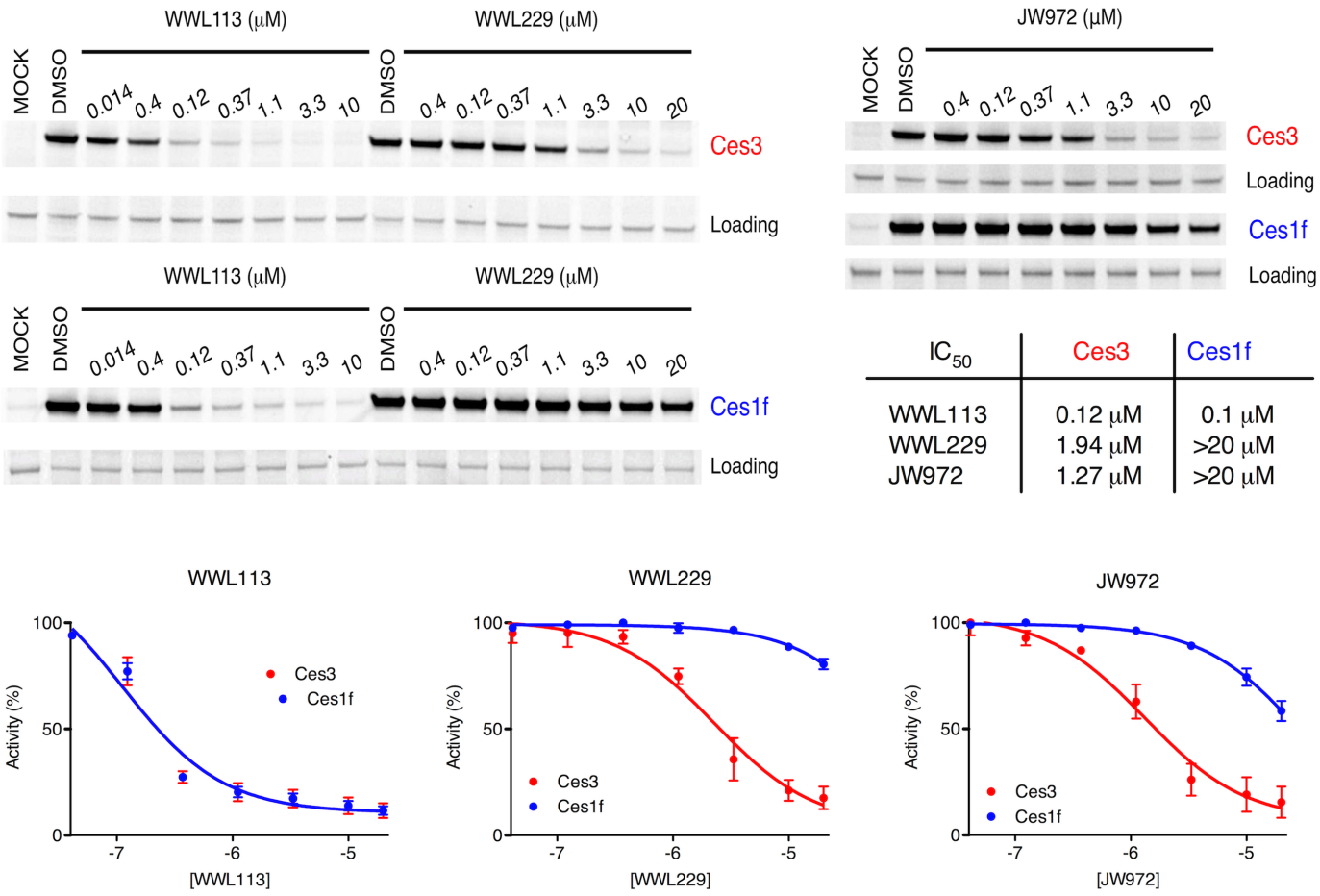
Supplementary Figure 2. WWL113 blocks basal lipolysis in adipocytes. (**a**) Competitive ABPP-MudPIT using 3T3-L1 adipocytes shows that WWL38 is an inhibitor of HSL (Lipe, arrow). Error bars indicate s.d. (n = 3), *p <0.05 vehicle vs. WWL38-treated cells. (**b**) Expression of adipocyte markers is increased in 10T1/2 cells differentiated for 8 days in the presence of WWL113. Error bars represent s.d. (n = 3), *p < 0.05 vs. vehicle-treated cells. (**c**) WWL113 blocks basal lipolysis and, to a lesser extent, hormone-induced lipolysis in 10T1/2 adipocytes exposed to compounds for 24 hr. Data are presented as mean ± s.d. (n = 3), *p <0.05 vehicle vs. treated cells.



Supplementary Figure 3. WWL113 is not a PPARα or PPARγ ligand. (**a**) Transient transfections using heterologous GAL4 DNA binding domain::PPAR ligand binding domain (LBD) chimeric proteins show that WWL113 (20 μM) does not directly activate PPARγ or PPARα. 10T1/2 preadipocytes (for PPARγ) or HepG2 hepatoma cells (for PPARα) were exposed to compounds for 48 hr. Rosiglitazone (1 μM) and GW9578 (10 μM) are synthetic PPARγ and PPARα ligands used as controls. Data are presented as mean ± s.d. (n = 3), **p* <0.05 vehicle vs. treated cells. (**b**) A Fluorescence Resonance Energy Transfer assay using a His-tagged PPARγ LBD and a coactivator peptide from SRC-1 shows that neither WWL113 nor WWL38 directly activate PPARγ. Error bars represent s.d. (n = 3).

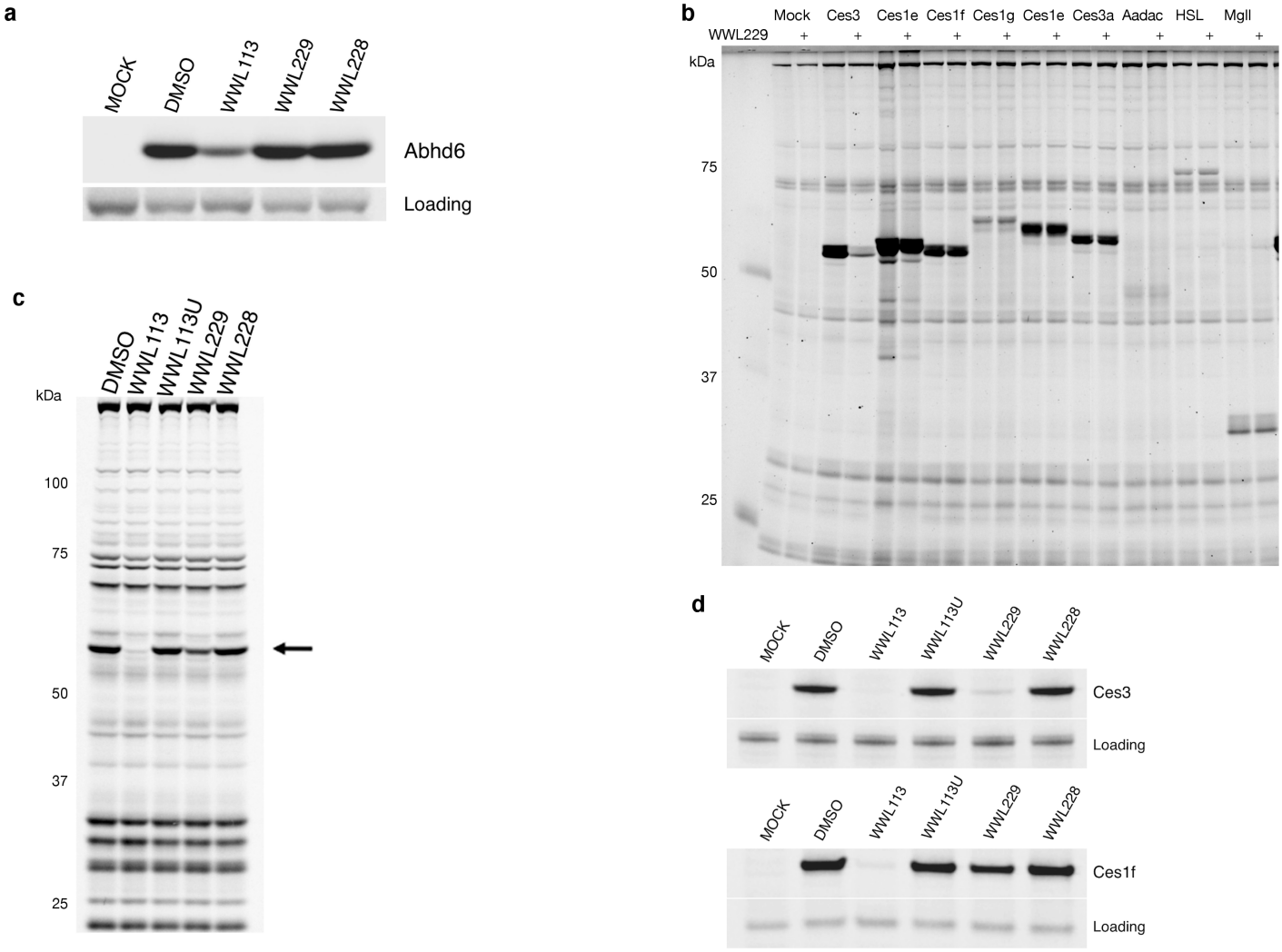


Supplementary Figure 4. Lipidomic analysis of WWL113-treated adipocytes. (**a**) Workflow of multidimensional Discovery Metabolite Profiling. (**b**) WWL113 treatment increases accumulation of long chain polyunsaturated TAG species in 10T1/2 cells differentiated for 8 days in the presence of the indicated compounds (10 μM). Error bars represent s.e.m. (n = 4), **p* <0.05, ***p* <0.01 vehicle vs. treated cells.

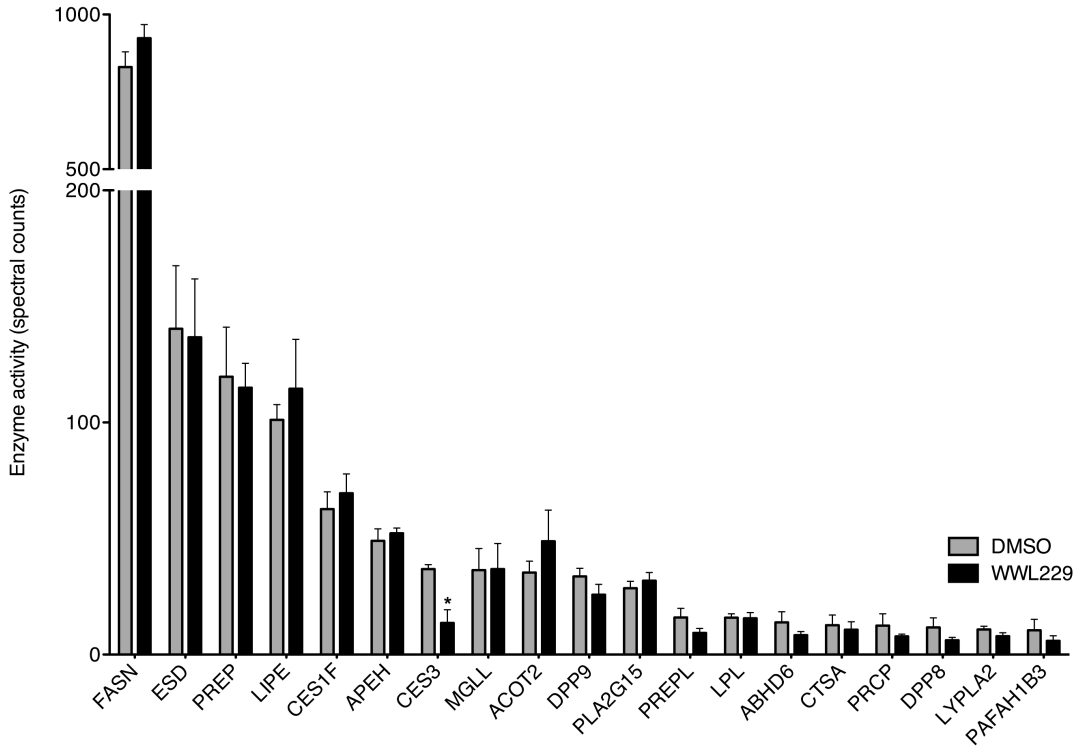


IC_{50}	Ces3	Ces1f
WWL113	0.12 μM	0.1 μM
WWL229	1.94 μM	>20 μM
JW972	1.27 μM	>20 μM

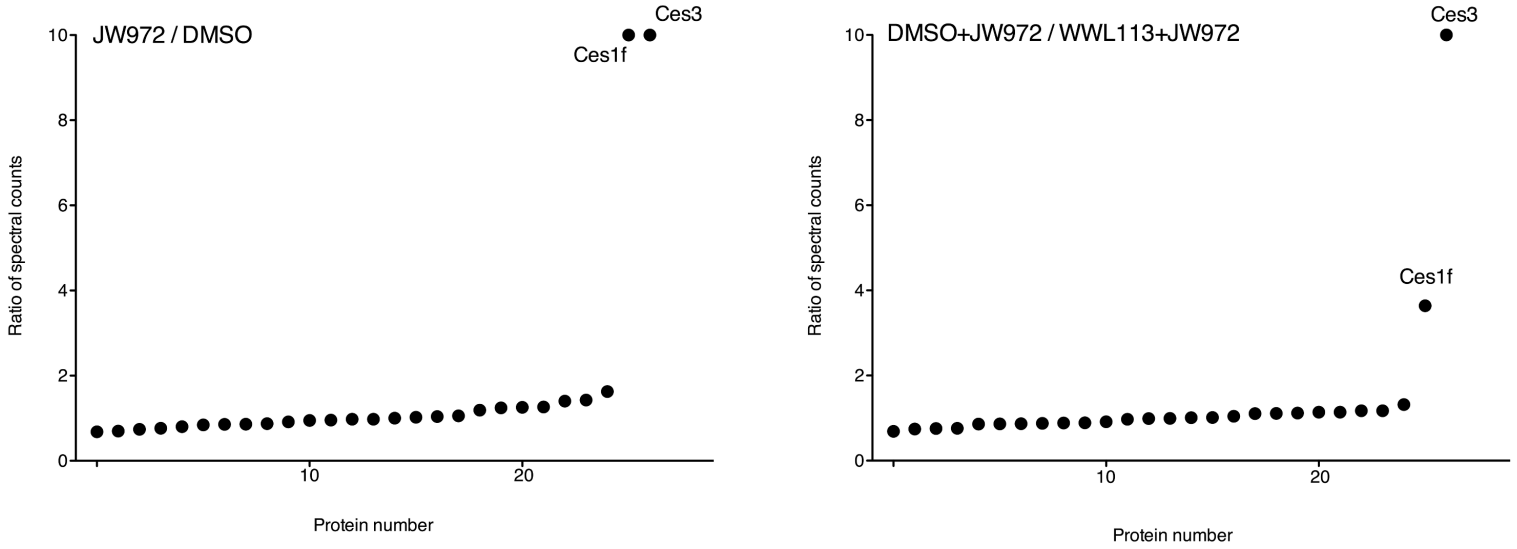
Supplementary Figure 5. WWL113 inhibits Ces3 and Ces1f with similar potency, but WWL229 selectively inhibits Ces3. Gel-based competitive ABPP data for WWL113, WWL229, and JW972 tested against Ces3 and Ces1f recombinantly expressed in HEK293T cells. Proteomes were incubated with the indicated concentration of each compound prior to incubation with the FP-rhodamine probe. Note that the top dose of WWL229 and JW972 (20 μM) is greater than that of WWL113 (10 μM). Scans of representative gels used to fit IC_{50} curves are shown(n = 3). IC_{50} values were calculated by normalizing the intensity of scanned enzyme bands to the abundance of a common Comassie-stained protein band. DMSO lanes were set as 100% enzyme activity. Error bars represent s.d. (n = 3).



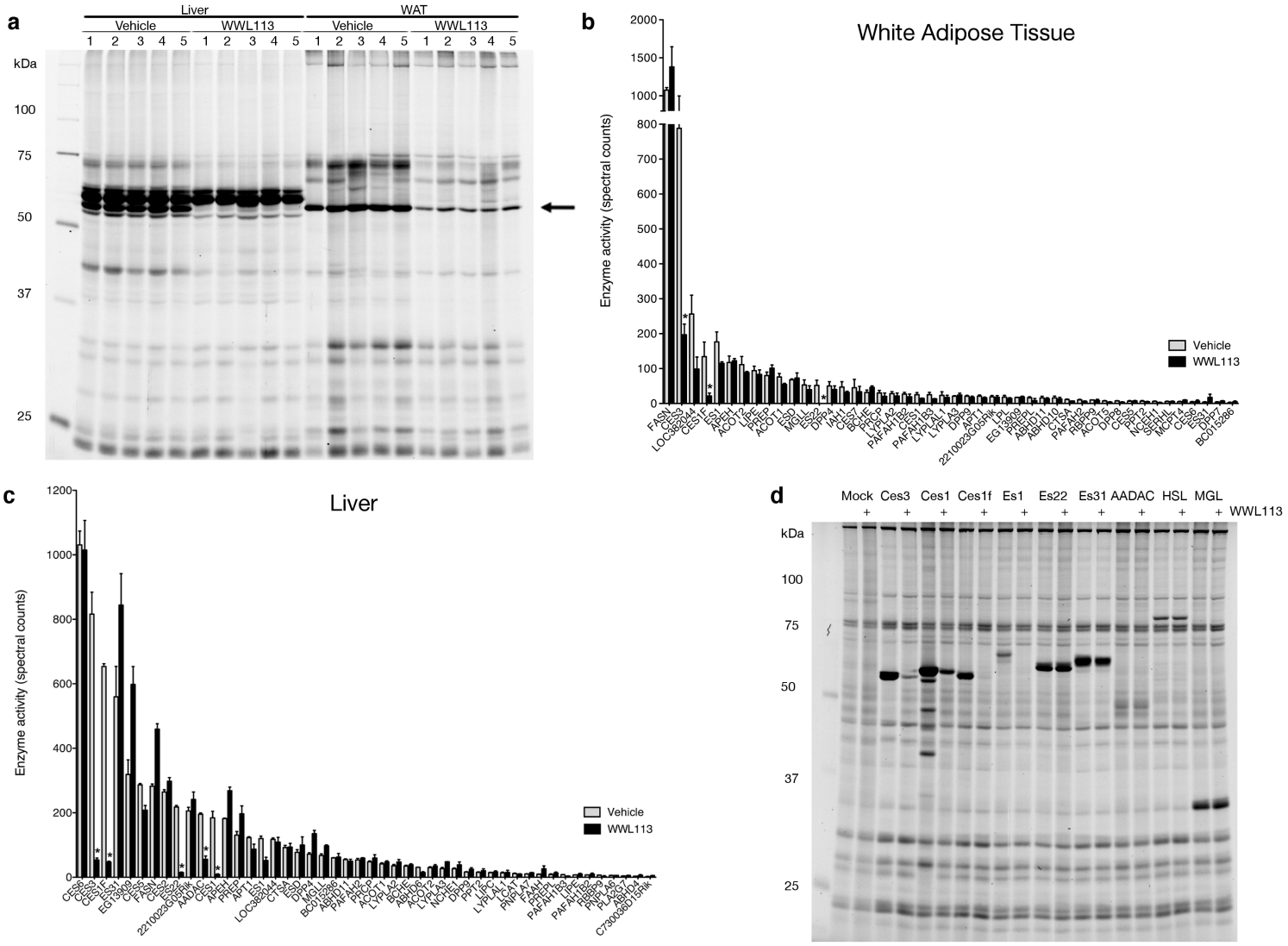
Supplementary Figure 6. WWL229 is a selective Ces3 inhibitor. (**a**) WWL113, but not WWL229, inhibits recombinant ABHD6 expressed in HEK293T cells. Proteomes were incubated with 10 μM of each compound prior to gel-based competitive ABPP. (**b**) Gel-based competitive ABPP was used to establish the selectivity of WWL229 (20 μM) on a set of serine hydrolases related to Ces3, using COS-7 lysates from cells overexpressing each enzyme individually. WWL229 inhibits Ces3, but not Ces1, Ces1f, or Ces1c. Gel-based competitive ABPP was also used to profile WWL113 (10 μM), WWL113U (10 μM), WWL228 (20 μM), and WWL229 (20 μM) in (**c**) 10T1/2 adipocyte proteomes, and (**d**) lysates from COS-7 cells overexpressing Ces3 or Ces1f. Arrow points to Ces3/Ces1f band. WWL229 inhibits Ces3, but not Ces1f. WWL228 does not inhibit Ces3 or any other serine hydrolase in the adipocyte proteome.



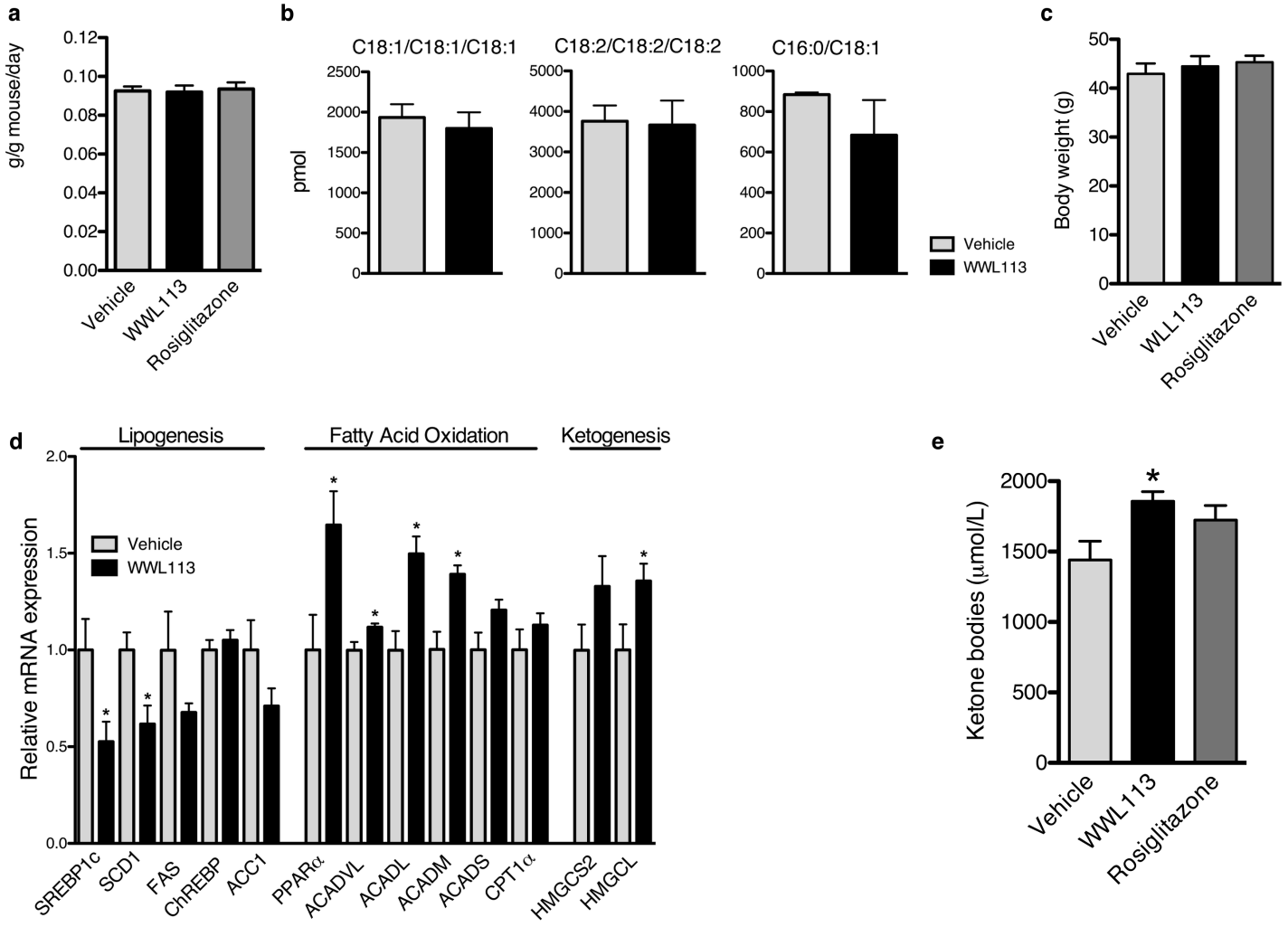
Supplementary Fig. 7. Competitive ABPP-MudPIT confirms that WWL229 is a selective Ces3 inhibitor in mouse adipocyte proteomes. Proteomes from differentiated 10T1/2 adipocytes were incubated with 10 μM WWL229 prior to treatment with the FP-biotin probe and MudPIT analysis. Error bars indicate s.e.m., **p* <0.05 vehicle (n = 3) vs. WW229 (n = 4).



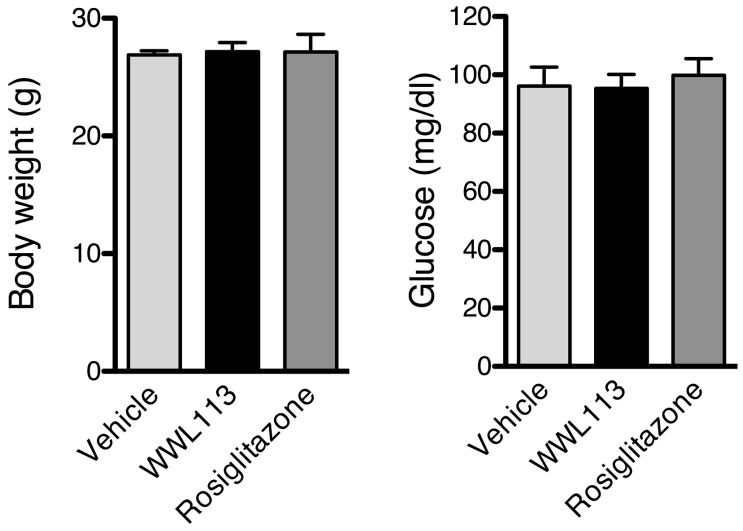
Supplementary Figure 8. The click probe JW972 selectively labels and enriches Ces3 and Ces1f from adipocyte proteomes. Adipocyte proteomes were incubated with either DMSO or JW972 (1 μM) for 45 min prior to conjugation with a biotin azide tag by copper-catalyzed azide-alkyne cycloaddition and ABPP-MudPIT analysis. Left graph shows that Ces3 and Ces1f are the only two proteins enriched by JW972 as determined by comparing the average spectral count values for proteins in DMSO- vs. JW972-treated proteomes. Pre-treatment with 10 μM WWL113 prior to JW972 labeling, click chemistry, and MudPIT analysis (right graph) blocked the enrichment of Ces3 and Ces1f, confirming that they are specific targets of the JW972 probe. Spectral count values were averaged for three replicates per group and these average values were used to calculate the ratio values shown in the graphs.



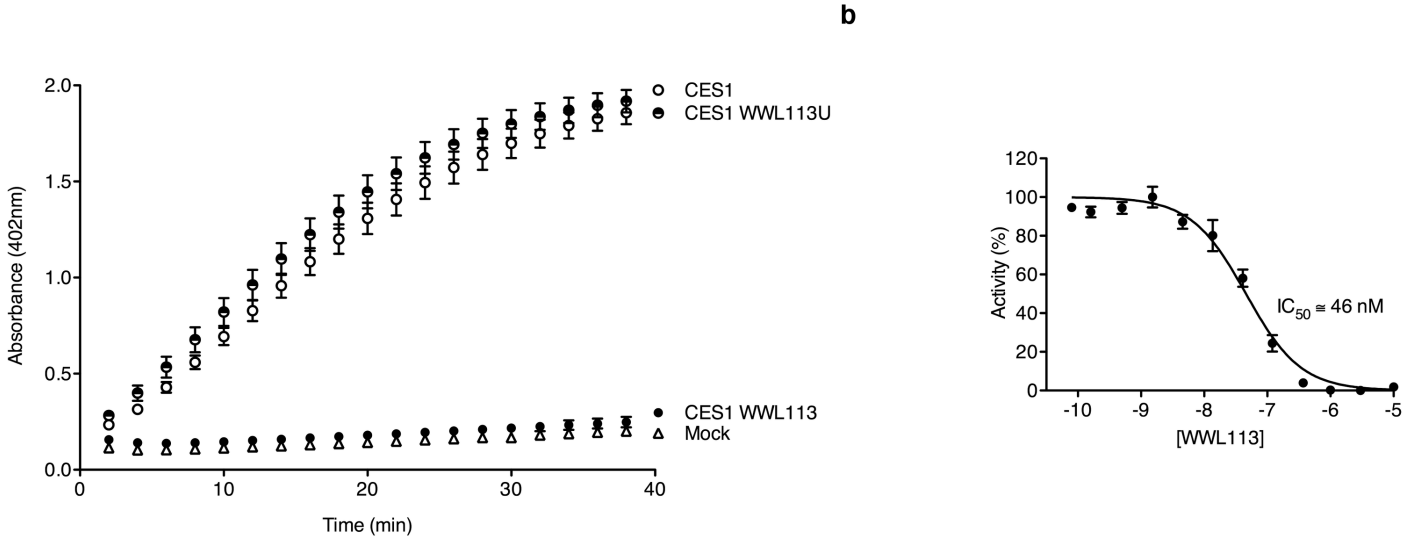
Supplementary Figure 9. Inhibition profile of WWL113 *in vivo*. (**a**) ABPP of white adipose tissue and liver from mice treated orally with a single dose of 30 mg/kg of WWL113 4 hr prior to analysis. This dose of WWL113 is sufficient to block Ces3 (arrow). Lanes represent individual mice (n = 5 per group). (**b,c**) Competitive ABPP-MudPIT confirms that WWL113 inhibits Ces3 in fat (75%) and liver (93%). Several additional serine hydrolases appear blocked by WWL113. Error bars represent s.e.m. (n = 5 per group), *$p < 0.05$ vehicle vs. treated mice. (**d**) To differentiate direct and indirect WWL113 targets, putative targets identified by MudPIT were overexpressed in 293T cells and proteomes incubated with 10 μM WWL113 prior to ABPP analysis. This carbamate directly blocks Ces3, Ces1f, and two other enzymes that are considerably less active in liver (Ces1 and Ces1c).



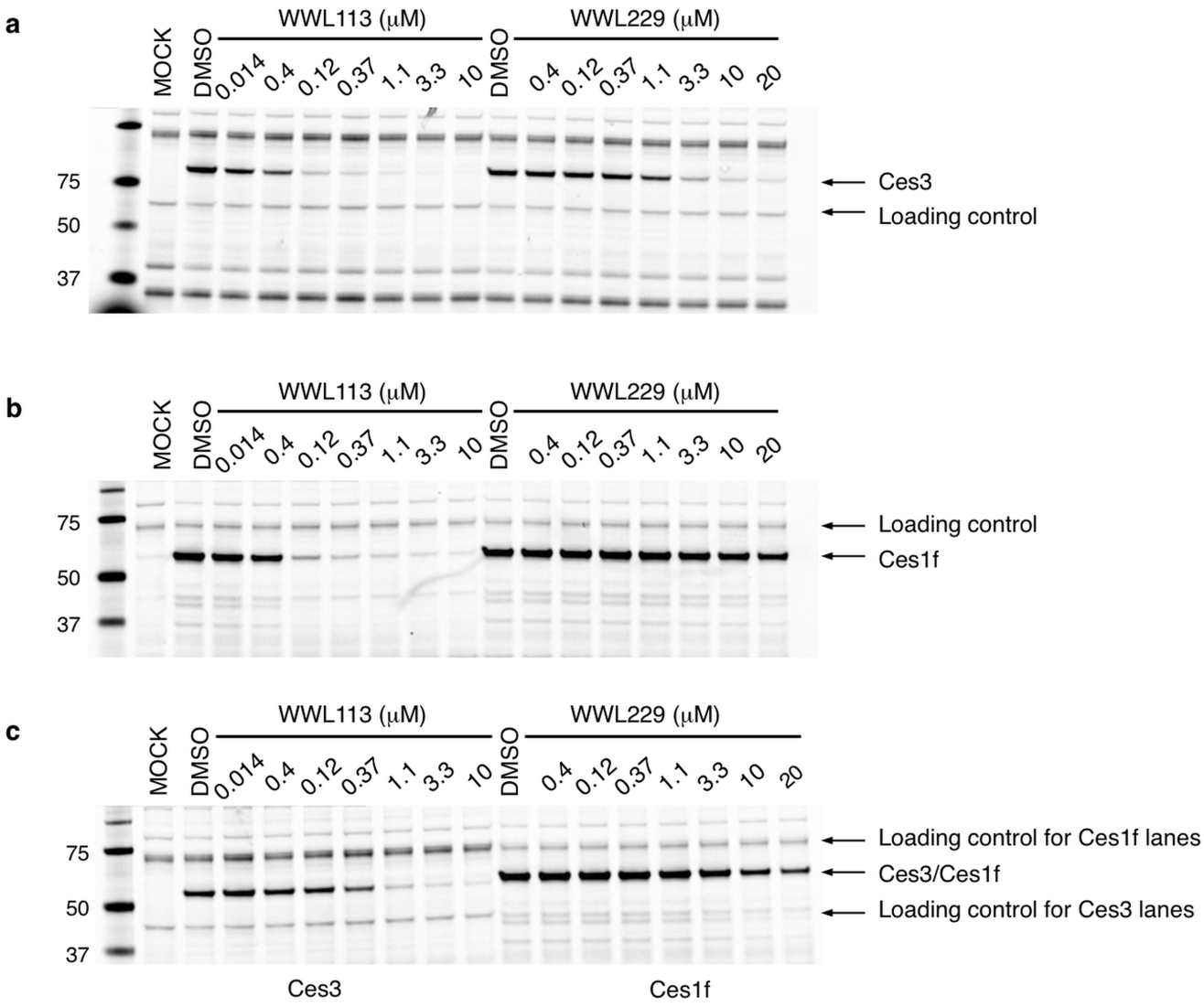
Supplementary Figure 10. Effects of WWL113 in *db/db* mice. (**a**) Food intake and (**b**) intestinal lipid absorption are not affected by WWL113 treatment. Analysis of the major lipid species present in feces is shown. *Db/db* mice were dosed orally once a day with vehicle, 30 mg/kg WWL113, or 4 mg/kg rosiglitazone. Error bars represent s.e.m. (n = 8 per group). (**c**) No difference in weight is seen in *db/db* mice treated with WWL113 for 8 days. Error bars represent s.e.m. (n = 10). (**d**) Gene expression analysis shows decreased expression of genes involved in lipogenesis and increased expression of genes implicated in fatty acid oxidation in livers of *db/db* mice treated chronically (3 months) with WWL113. RT-qPCR data is presented relative to levels in vehicle-treated animals. Error bars represent s.e.m. (n = 8 per group). (**e**) Plasma ketone bodies are increased in *db/db* mice treated with WWL113 for 14 days. Error bars represent s.e.m. (n = 10).



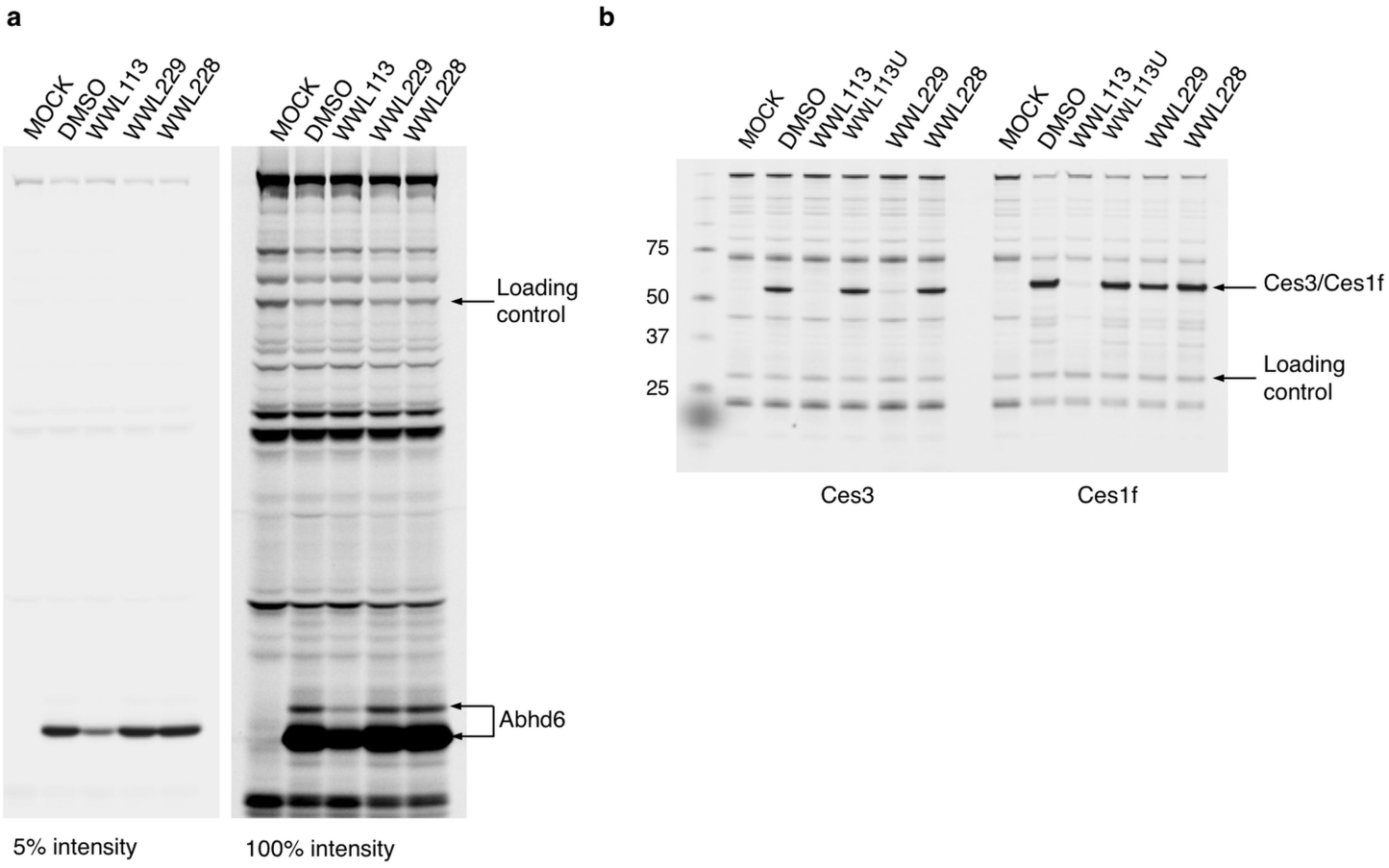
Supplementary Figure 11. Wild-type mice treated chronically with WWL113 show no side effects. C57BL/6 mice were dosed orally once a day for 30 days with vehicle, 30 mg/kg WWL113, or 4 mg/kg rosiglitazone. At the end of treatment, no differences in weight or fasting glycemia were evident. Error bars represent s.e.m. (n = 8 per group).



Supplementary Figure 12. WWL113 inhibits CES1, the human orthologue of Ces3, in a substrate hydrolysis assay. (**a**) WWL113, but not WWL113U, inhibits CES1. Lysates of COS-7 cells overexpressing human CES1 were pre-incubated with 10 μM WWL113 or WWL113U for 20 min prior to addition of the substrate 4-nitrophenyl butyrate (4-NPB). Hydrolysis of 4-NPB generates 4-nitrophenol, which is detected at 402 nm^{-1} absorbance. Mock refers to lysate from COS-7 cells transfected with an empty vector control. Error bars represent s.d. (n = 16). (**b**) IC_{50} of WWL113 on CES1 calculated using the 4-NPB hydrolysis assay. Error bars represent s.d. (n = 4).



Supplementary Figure 13. Full images of gels shown in Supplementary Figure 5**.**



Supplementary Figure 14. Full images of gels shown in Supplementary Figure 6**.**

Supplementary Table 1 – Profile of Serine Hydrolase Activity During Adipocyte Differentiation

	10T1/2		3T3-L1	
Protein	**Preads**	**Adipocytes**	**Preads**	**Adipocytes**
IPI00387289 - CES3	0	185±34**	0	69±13**
IPI00321386 - ABHD6	0	8±2**	nd	nd
IPI00115871 - ACOT1	3±2	24±4**	5±3	34±4**
IPI00131216 - CES22	0	6±1**	nd	nd
IPI00113223 - FASN	82±44	251±25**	104±23	154±37
IPI00228826 - LIPE	1±1	50±13**	0	71±16**
IPI00653566 - ACOT2	31±5	154±35**	10±4	62±13**
IPI00318006 - SERHL	0	11±3**	1±1	17±3**
IPI00761930 - PREP	16±3	5±3**	74±13	33±9**
IPI00118821 - PAFAH1B beta	10±2	2±2**	7±2	2±2
IPI00403586 - NCHE1	6±3	0**	5±2	1±1*
IPI00128399 - CES1F	0	70±29**	0	59±9**
IPI00132874 - MGLL	0	22±9**	nd	nd
IPI00319188 - LPL	0	20±8**	0	38±3**
IPI00387245 - APEH	15±2	8±3	9±5	5±1
IPI00120080 - PNPLA8	1±2	6±2	0	22±5**
IPI00462461 - DPP9	13±4	7±2	10±7	5±2
IPI00170213 - ABHD11	0	13±8*	1±1	20±2**
IPI00130369 - ACOT5	1±1	5±3	0	10±1**
IPI00406375 - ACOT6	1±1	5±3	nd	nd
IPI00308769 - ACOT4	1±1	9±6	nd	nd
IPI00118819 - PAFAH1B gamma	7±4	3±2	22±14	13±2
IPI00658539 - CTSA	6±4	4±2	47±19	20±1
IPI00165731 - ABHD12	5±4	7±1	6±4	27±1**
IPI00131216 - CES22	nd	nd	0	6±1**
IPI00226414 - ABHD10	nd	nd	5±1	19±2**
IPI00136676 - ACOT3	nd	nd	0	16±3**
IPI00130764 - PPT2	nd	nd	3±1	10±2**
IPI00321386 - ABHD6	nd	nd	1±1	18±6**
IPI00153133 - LYPLAL1	nd	nd	8±3	35±14*
IPI00130018 - APT1	nd	nd	3±1	5±1
IPI00330837 - DPP8	nd	nd	7±5	0
IPI00649456 - PAFAH2	nd	nd	3±3	8±2
IPI00331550 - DPP2	nd	nd	10±2	7±3
IPI00132020 - PRCP	nd	nd	12±3	15±4
IPI00135277 - RBBP9	nd	nd	6±1	5±2
IPI00224078 - PREPL	nd	nd	7±6	5±3

Data represent mean ± s.d. spectral counts of 3-4 replicates; *p<0.05, **p<0.01; nd = not detected. IPI, International Protein Index identifiers.

Supplementary Data Set 1: Quantification of protein activities in predifferentiated and mature 10T1/2 adipocytes.

Supplementary Data Set 2: Quantification of protein activities in predifferentiated and mature 3T3-L1 adipocytes; quantification of protein activities in competitive ABPP-MudPIT analysis of 3T3-L1 adipocyte proteome pre-incubated with WWL38 (10 μM).

Supplementary Data Set 3: Quantification of protein activities in competitive ABPP-MudPIT analysis of 10T1/2 adipocyte proteome pre-incubated with WWL113 (10 μM).

Supplementary Data Set 4: Quantification of protein activities in competitive ABPP-MudPIT analysis of 10T1/2 adipocyte proteome pre-incubated with WWL229 (10 μM).

Supplementary Data Set 5: Quantification of protein activities labeled by JW972 (1 μM) in 10T1/2 adipocyte proteome.

Supplementary Data Set 6: Quantification of protein activities labeled by JW972 (1 μM) in 10T1/2 adipocyte proteome pre-incubated with WWL113 (10 μM).

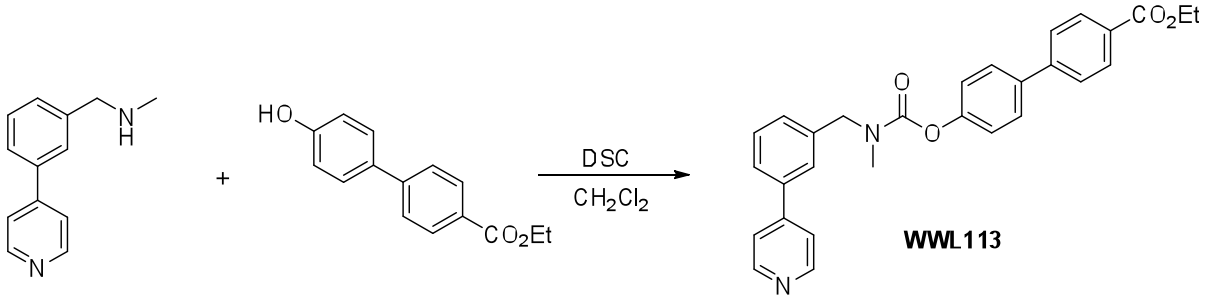
Supplementary Data Set 7: Quantification of protein activities in competitive ABPP-MudPIT analysis of white adipose tissue of mice treated with 30 mg/kg WWL113 4 hr prior to analysis.

Supplementary Data Set 8: Quantification of protein activities in competitive ABPP-MudPIT analysis of liver of mice treated with 30 mg/kg WWL113 4 hr prior to analysis.

SUPPLEMENTARY NOTE

Compound synthesis. All commercially available chemicals were obtained from Aldrich, Acros, Fisher, Fluka, or Maybridge and were used without further purification, except where noted. Dry solvents were obtained by passing these through activated alumina columns. All reactions were carried out under an inert nitrogen atmosphere using oven-baked glassware unless otherwise noted. Flash chromatography was performed using 230-400 mesh silica gel 60. NMR spectra were generated on Varian 400 MHz instrument. Chemical shifts were recorded in ppm relative to tetramethylsilane (TMS) with multiplicities given as s (singlet), bs (broad singlet), d (doublet), t (triplet), dt (double of triplets), q (quadruplet), qd (quadruplet of doublets), m (multiplet). Full synthesis details of the carbamate library, including that of WWL228 are provided in reference 21. Synthesis of FP-rhodamine is described in reference 51, and FP-biotin is described in reference 52. Azide-rhodamine and azide-biotin synthesized and characterized as described in reference 29.

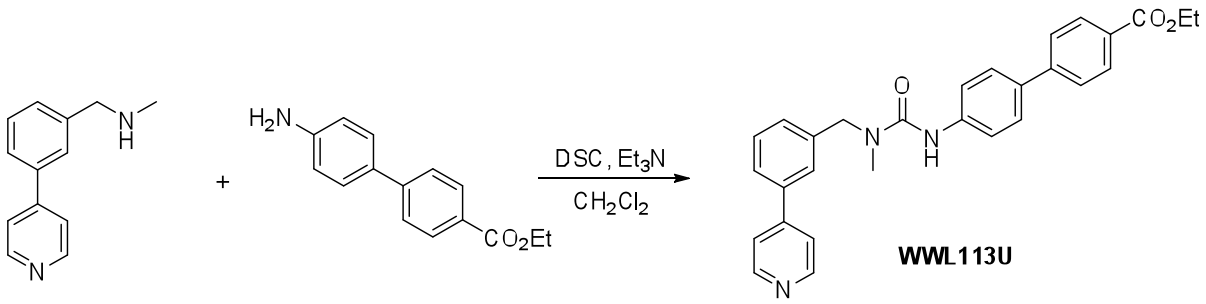
Synthesis of WWL113, WWL113U, WWL229 and JW972



Ethyl 4'-((methyl(3-(pyridin-4-yl)benzyl)carbamoyl)oxy)-[1,1'-biphenyl]-4-carboxylate (WWL113)

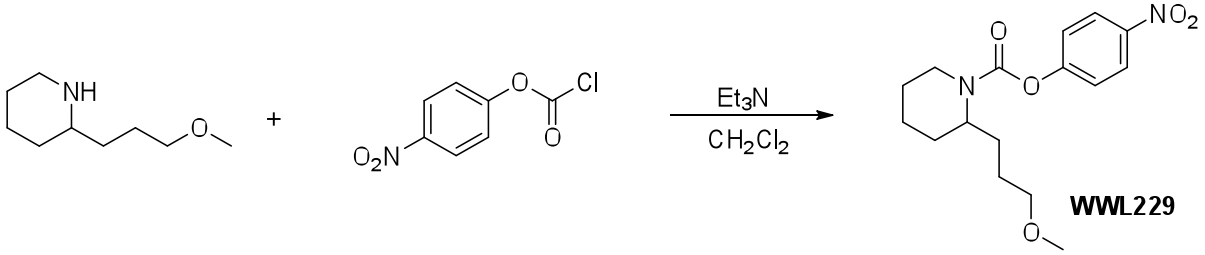
To a stirring solution of *N*,*N*'-disuccinimidyl carbonate (2.643 g, 10.32 mmol) and Et_3N (0.58 mL, 4.13 mmol) in dry CH_2Cl_2 (10.0 mL) was added ethyl 4'-hydroxy-[1,1'-biphenyl]-4-carboxylate (500 mg, 2.06 mmol). The reaction mixture was stirred at room temperature for 2 hours and then treated with 10.0

mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was used without further purification. To a solution of carbonate intermediate in CH_2Cl_2 (10 mL) we added N-methyl-1-(3-(pyridin-4-yl)phenyl)methanamine (409 mg, 2.06 mmol) and Et_3N (0.58 mL, 4.13 mmol) at 0 ^oC. After stirring at 0 ^oC for 6 hours, the reaction mixture was treated with 30.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography using 98:2 v/v dichloromethane:methyl alcohol as solvent to afford title compound (850 mg, 89 % yield) as a colorless oil: ^1H NMR (400 MHz, $CDCl_3$) d 8.74 (bs, 1H), 8.12 - 8.10 (d, J = 8 Hz, 2H), 7.64 – 7.43 (m, 10H), 7.28 – 7.20 (m, 3H), 4.76 - 4.66 (d, 2H), 4.43 - 4.38 (q, J = 8 Hz, 2H), 3.11 - 3.07 (d, 3H), 1.43 - 1.40 (t, J = 4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) d 166.68, 144.93, 144.88, 138.33, 138.27, 137.49, 130.28, 129.91, 129.77, 129.45, 128.97, 128.64, 128.48, 128.44, 128.23, 127.13, 126.92, 126.59, 126.12, 61.20, 53.10, 35.17, 34.55; HRMS (ESI-TOF+) *m/z* calc'd for $C_{29}H_{26}N_2O_4$ $[M+H]^+$: 467.1966, found 467.1962.



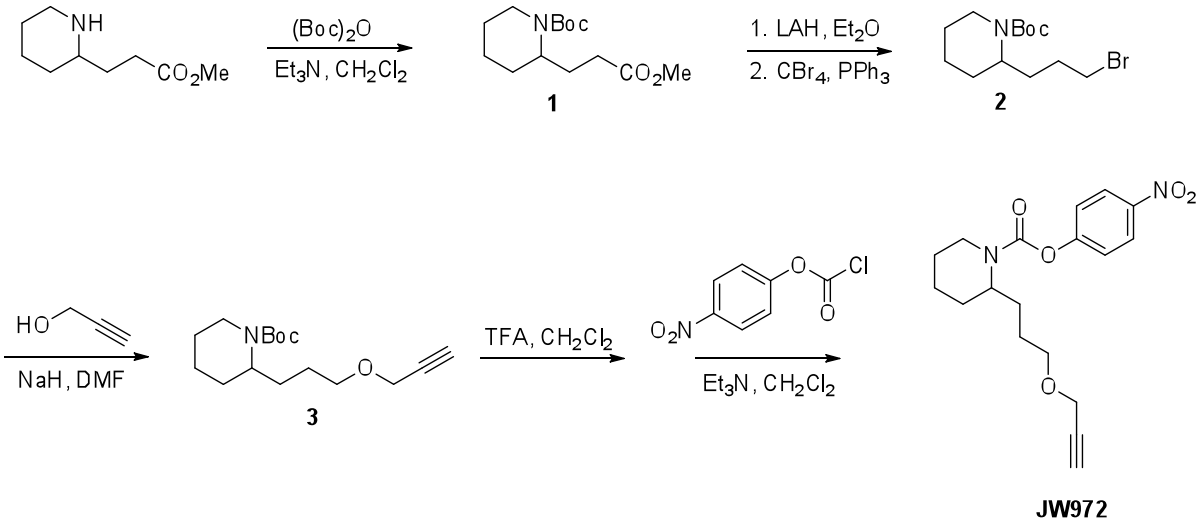
Ethyl 4'-(3-methyl-3-(3-(pyridin-4-yl)benzyl)ureido)-[1,1'-biphenyl]-4-carboxylate (WWL113U).

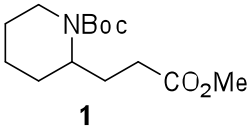
N,*N*'-disuccinimidyl carbonate (296 mg, 1.116 mmol) and Et_3N (0.077 mL, 0.558 mmol) were added to a solution of ethyl 4'-amino-[1,1'-biphenyl]-4-carboxylate (45 mg, 0.186 mmol) in CH_2Cl_2 (1.0 mL) at room temperature and the reaction mixture was stirred for 1 hour and then treated with 2.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was used without further purification. To a solution of carbonate intermediate in CH_2Cl_2 (1.0 mL) we added N-methyl-1-(3-(pyridin-4-yl)phenyl)methanamine (37 mg, 0.186 mmol) and Et_3N (0.077 mL, 0.558 mmol) at 0 ^oC. After stirring at 0 ^oC for 4 hours, the reaction mixture treated with 2.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by Preparative TLC using 50:50 v/v hexane:ethyl acetate as solvent to afford title compound (43 mg, 50 % yield) as a white solid: ^1H NMR (400 MHz, $CDCl_3$) 8.64 - 8.63 (d, J = 4 Hz, 2H), 8.08 - 8.06 (d, J = 8 Hz, 2H), 7.61 - 7.59 (d, J = 8 Hz, 2H), 7.56 – 7.45 (m, 8H), 7.39 – 7.37 (m, 1H), 6.80 (s, 1H), 4.69 (s, 2H), 4.41 - 4.35 (q, J = 8 Hz, 2H), 3.06 (s, 3H), 1.41 - 1.38 (t, J = 8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 166.71, 155.75, 150.34, 148.11, 145.04, 139.42, 138.86, 138.81, 134.66, 130.20, 129.78, 128.89, 128.27, 127.79, 126.62, 126.40, 126.15, 121.84, 120.33, 61.08, 52.33, 34.87, 14.50; HRMS (ESI-TOF+) *m/z* calc'd for $C_{29}H_{27}N_3O_3$ $[M+H]^+$: 466.2125, found 466.2121.



4-nitrophenyl 2-(3-methoxypropyl)piperidine-1-carboxylate (WWL229)

To a stirring solution of 2-(3-methoxypropyl)piperidine (750 mg, 4.77 mmol) and Et_3N (1.99 mL, 14.31 mmol) in dry CH_2Cl_2 (24.0 mL) we added 4-nitrophenyl chloroformate (961 mg, 4.77 mmol). The reaction mixture was stirred at 0 ^oC for 4 hours. The reaction mixture was then treated with 20.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography using 9:1 v/v hexane:ethyl acetate as solvent to afford title compound (1.350 g, 86 % yield) as a colorless oil: ^1H NMR (400 MHz, $CDCl_3$) d 8.25 - 8.22 (d, J = 12 Hz, 2H), 7.29 - 7.26 (d, J = 12 Hz, 2H), 4.39 (m, 1H), 4.14 - 4.10 (m, 1H), 3.44 - 3.41 (m, 2H), 3.33 (s, 3H), 3.10 - 3.03 (m, 1H), 1.73 - 1.54 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) d 156.80, 152.75, 144.86, 125.26, 122.48, 72.54, 58.87, 52.22, 40.34, 29.16, 26.73, 26.00, 25.57, 18.96; HRMS (ESI-TOF+) *m/z* calc'd for $C_{16}H_{22}N_2O_5$ $[M+H]^+$: 323.1601, found 323.1604.

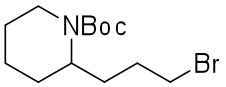




1

***tert*-butyl 2-(3-methoxy-3-oxopropyl)piperidine-1-carboxylate (1)**

To a stirring solution of methyl 3-(piperidin-2-yl)propanoate (1.005 g, 5.87 mmol) and Et_3N (2.50 mL, 17.61 mmol) in dry CH_2Cl_2 (30.0 mL) we added di-tert-butyl dicarbonate (2.562 g, 11.74 mmol). The reaction mixture was stirred at room temperature for 8 hours. After stirring at room temperature 8 hours, the reaction mixture treated with 20.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography using 9:1 v/v hexane:ethyl acetate as solvent to afford title compound (1.30 g, 83 % yield) as a colorless oil: ^1H NMR (400 MHz, $CDCl_3$) d 4.26 - 4.24 (bs, 1H), 3.98 (bs, 1H), 3.67 (s, 3H), 2.78 - 2.71 (t, J = 12 Hz, 1H), 2.36 - 2.23 (m, 2H), 2.15 - 2.05 (m, 1H), 1.73 - 1.51 (m, 7H), 1.45 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) d 174.09, 155.19, 79.44, 51.74, 50.06, 38.62, 31.10, 29.09, 28.60, 25.73, 25.16, 19.24; HRMS (ESI-TOF+) *m/z* calc'd for $C_{14}H_{25}NO_4$ $[M+H]^+$: 272.1856, found 272.1866.

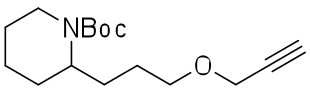


2

***tert*-butyl 2-(3-bromopropyl)piperidine-1-carboxylate (2)**

To a stirring solution of tert-butyl 2-(3-methoxy-3-oxopropyl)piperidine-1-carboxylate (1.167 g, 4.40 mmol) in dry Et_2O (30.0 mL) we added $LiAlH_4$ (326 mg, 8.60 mmol) at 0 ^oC. The reaction mixture was stirred at 0 ^oC for 2 hours. After stirring, the reaction mixture was quenched with 5.0 mL of H_2O and 30.0 mL Ethyl acetate were then added. The organic layer was separated and the aqueous layer was extracted with Ethyl acetate. The combined organic extracts were washed with brine, dried over

$MgSO_4$, filtered and concentrated. The crude product was used without further purification. To a solution of alcohol intermediate in CH_2Cl_2 (21.0 mL) we added CBr_4 (2.124 g, 6.40 mmol) and PPh_3 (1.343 g, 5.12 mmol) at room temperature. After stirring at room temperature for 12 hours, the reaction mixture was treated with 20.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography using 9:1 v/v hexane:ethyl acetate as solvent to afford title compound (1.104 g, 84 % yield): ^{1}H NMR (400 MHz, $CDCl_3$) d 4.25 (bs, 1H), 3.98 (bs, 1H), 3.47 - 3.44 (t, J = 8 Hz, 2H), 2.79 - 2.71 (m, 1H), 1.94 - 1.81 (m, 3H), 1.69 - 1.58 (m, 7H), 1.46 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) d 154.92, 79.12, 49.40, 38.58, 33.79, 29.56, 28.85, 28.38, 28.23, 25.61, 19.06; HRMS (ESI-TOF+) *m/z* calc'd for $C_{13}H_{24}BrNO_2$ $[M+H]^+$: 306.1063, found 306.1068.

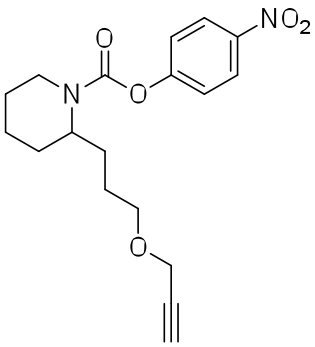


3

***tert*-butyl 2-(3-(prop-2-yn-1-yloxy)propyl)piperidine-1-carboxylate (3)**

To a stirring solution of prop-2-yn-1-ol (145 mg, 2.58 mmol) in dry DMF (2.6 mL) we slowly added NaH (93 mg, 3.88 mmol) at 0 ^{o}C. The reaction mixture was stirred at 0 ^{o}C for 1 hour and tert-butyl 2-(3-bromopropyl)piperidine-1-carboxylate (396 mg, 1.29 mmol) was then added at room temperature. After stirring at room temperature for 12 hours, the reaction mixture was treated with 5.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography using 85:15 v/v hexane:ethyl acetate as solvent to afford title compound (301 mg, 82 % yield) as a

colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) d 4.20 (bs, 1H), 4.14 - 4.13 (d, J = 4 Hz, 2H), 3.95 (bs, 1H), 3.58 - 3.48 (m, 2H), 2.74 - 2.71 (m, 1H), 2.43 - 2.41 (m, 1H), 1.81 - 1.76 (m, 1H), 1.61 - 1.54 (m, 9H), 1.45 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) d 155.29, 80.06, 79.32, 74.41, 70.08, 62.61, 58.15, 51.20, 38.71, 28.63, 26.45, 26.24, 25.76, 19.14; HRMS (ESI-TOF+) *m/z* calc'd for $C_{16}H_{27}NO_3$ $[M+H]^+$: 282.2064, found 282.2068.



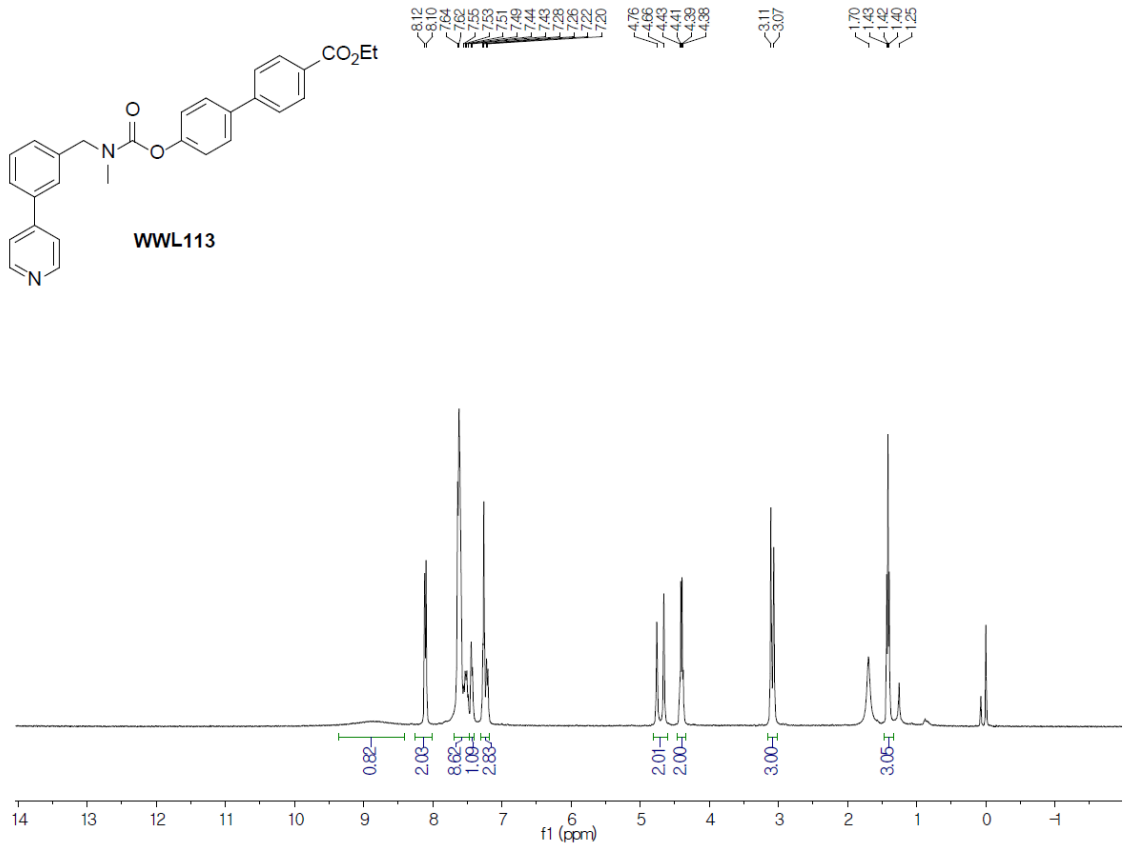
JW972

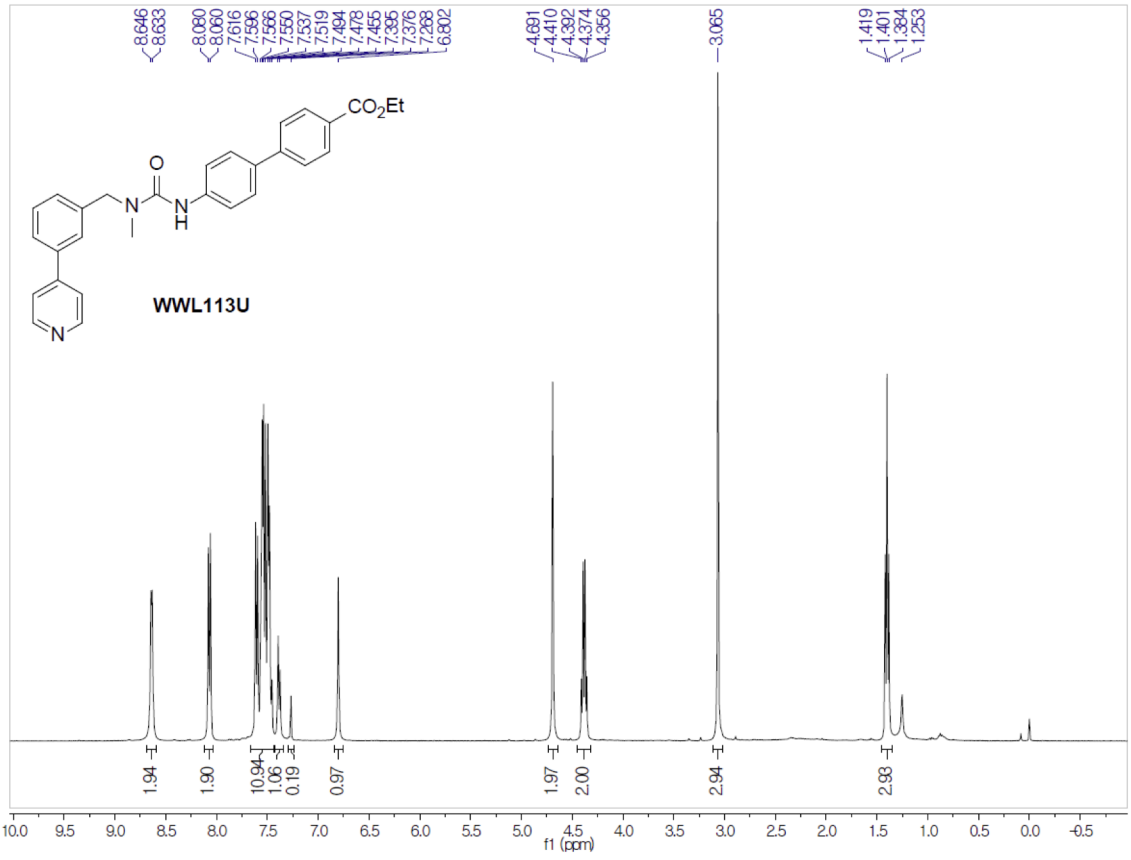
4-nitrophenyl 2-(3-(prop-2-yn-1-yloxy)propyl)piperidine-1-carboxylate (JW972)

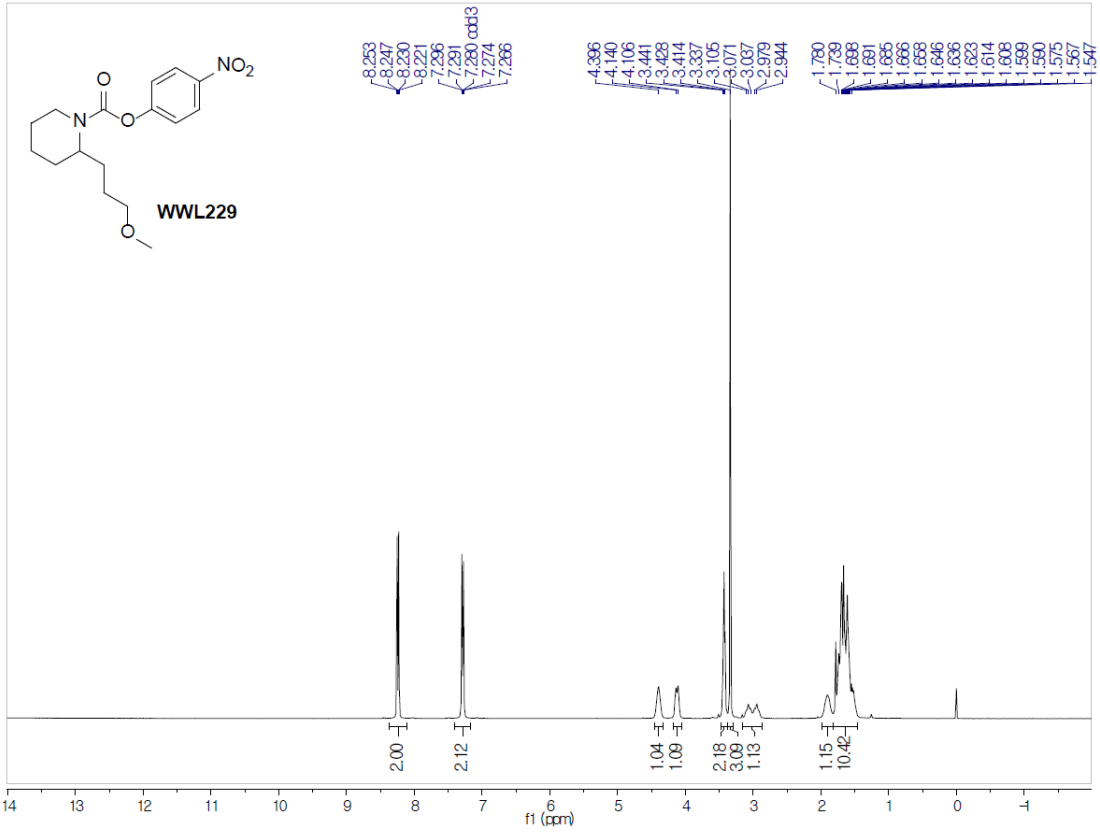
To a stirring solution of tert-butyl 2-(3-(prop-2-yn-1-yloxy)propyl)piperidine-1-carboxylate (141 mg, 0.50 mmol) in dry CH_2Cl_2 (1.0 mL) we added TFA (0.12 mL, 1.50 mmol) at 0 ^{o}C. The reaction mixture was stirred at 0 ^{o}C for 2 hours. After stirring, the reaction mixture was treated with 5.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was used without further purification. To a solution of 2-(3-(prop-2-yn-1-yloxy)propyl)piperidine in CH_2Cl_2 (5.0 mL) we added 4-nitrophenyl chloroformate (111 mg, 0.55 mmol) and Et_3N (0.26 mL, 1.50 mmol) at room temperature. After stirring at room temperature for 12 hours, the reaction mixture was treated with 5.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer extracted with CH_2Cl_2. The combined organic

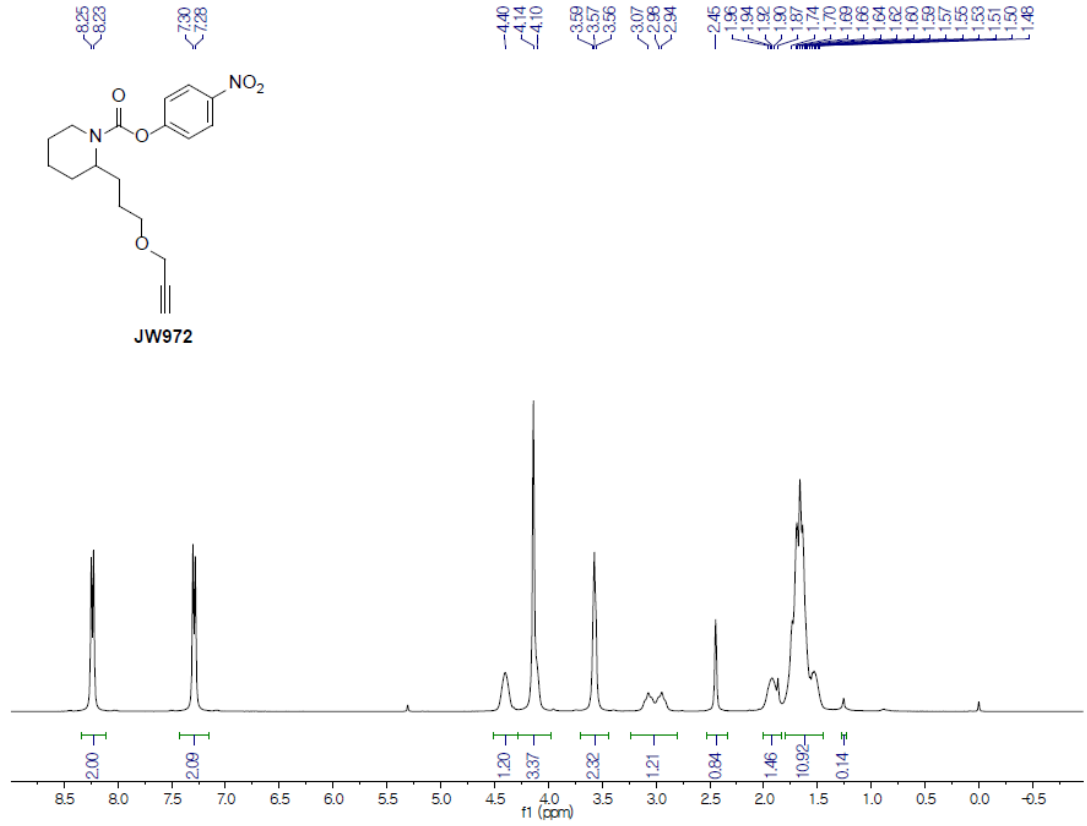
extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography using 85:15 v/v hexane:ethyl acetate as solvent to afford title compound (132 mg, 79 % yield) as an yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) d 8.25 - 8.23 (d, J = 8.0 Hz, 2H), 7.30 - 7.28 (d, J = 8.0 Hz, 2H), 4.40 (bs, 1H), 4.14 - 4.10 (m, 3H), 3.59 - 3.56 (m, 2H), 3.07 - 2.94 (m, 1H), 2.45 (s, 1H), 1.74 - 1.57 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) d 156.75, 152.75, 144.79, 125.19, 122.46, 80.05, 74.55, 69.84, 58.30, 52.10, 39.67, 29.10, 26.69, 26.38, 25.94, 18.91; HRMS (ESI-TOF+) *m/z* calc'd for $C_{18}H_{22}N_2O_5$ $[M+H]^+$: 347.1601, found 347.1603.

NMR Spectra for WWL113, WWL113U, WWL229, and JW972






WWL229
f1 (ppm)


JW972
f1 (ppm)