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

Eduardo Dominguez, Andrea Galmozzi, Jae Won Chang, Ku-Lung Hsu, Joanna Pawlak, Weiwei Li, Cristina Godio, Jason Thomas, David Partida, Sherry Niessen, Paul E. O’Brien, Aaron P. Russell, Matthew J. Watt, Daniel K. Nomura, Benjamin F. Cravatt and Enrique Saez

SUPPLEMENTARY INFORMATION
**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.
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\textsubscript{50} curves are shown (n = 3). IC\textsubscript{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 absorbance. Mock refers to lysate from COS-7 cells transfected with an empty vector control. Error bars represent s.d. (n = 16). (b) IC₅₀ 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

| Protein | 10T1/2 | 3T3-L1 |
|---------|--------|--------|
|         | 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 Et3N (0.58 mL, 4.13 mmol) in dry CH2Cl2 (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 NaHCO₃ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried over MgSO₄, filtered and concentrated. The crude product was used without further purification. To a solution of carbonate intermediate in CH₂Cl₂ (10 mL) we added N-methyl-1-(3-(pyridin-4-yl)phenyl)methanamine (409 mg, 2.06 mmol) and Et₃N (0.58 mL, 4.13 mmol) at 0 °C. After stirring at 0 °C for 6 hours, the reaction mixture was treated with 30.0 mL of saturated aqueous NaHCO₃ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried 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, CDCl₃) 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); ¹³C NMR (100 MHz, CDCl₃) 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 C₂₉H₂₆N₂O₄ [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$_3$N (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$_2$Cl$_2$ (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$_2$Cl$_2$. 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$_2$Cl$_2$ (1.0 mL) we added N-methyl-1-(3-(pyridin-4-yl)phenyl)methanamine (37 mg, 0.186 mmol) and Et$_3$N (0.077 mL, 0.558 mmol) at 0 °C. After stirring at 0 °C 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: $^1$H 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$_3$O$_3$ [M+H]$^+$: 466.2125, found 466.2121.

![Chemical Reaction](image)

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$_3$N (1.99 mL, 14.31 mmol) in dry CH$_2$Cl$_2$ (24.0 mL) we added 4-nitrophenyl chloroformate (961 mg, 4.77 mmol). The reaction mixture was stirred at 0 °C 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: $^1$H NMR (400 MHz, CDCl$_3$) δ 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$_3$) δ 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$_2$O$_5$ [M+H]$^+$: 323.1601, found 323.1604.
**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_{3}N (2.50 mL, 17.61 mmol) in dry CH_{2}Cl_{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}) δ 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); ¹³C NMR (100 MHz, CDCl_{3}) δ 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.

![Image of tert-butyl 2-(3-bromopropyl)piperidine-1-carboxylate (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_{2}O (30.0 mL) we added LiAlH_{4} (326 mg, 8.60 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 hours. After stirring, the reaction mixture was quenched with 5.0 mL of H_{2}O 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
MgSO₄, filtered and concentrated. The crude product was used without further purification. To a solution of alcohol intermediate in CH₂Cl₂ (21.0 mL) we added CBr₄ (2.124 g, 6.40 mmol) and PPh₃ (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 NaHCO₃ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried over MgSO₄, 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): ¹H NMR (400 MHz, CDCl₃) δ 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); ¹³C NMR (100 MHz, CDCl₃) δ 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 C₁₃H₂₆BrNO₂ [M+H]⁺: 306.1063, found 306.1068.

![Structure](image)

**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 °C. The reaction mixture was stirred at 0 °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 NaHCO₃ solution. The organic layer was separated and the aqueous layer extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over MgSO₄, 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
4-nitrophenyl 2-(3-(prop-2-yn-1-yl)oxy)propyl)piperidine-1-carboxylate (JW972)

To a stirring solution of tert-butyl 2-(3-(prop-2-yn-1-yl)oxy)propyl)piperidine-1-carboxylate (141 mg, 0.50 mmol) in dry CH₂Cl₂ (1.0 mL) we added TFA (0.12 mL, 1.50 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 hours. After stirring, the reaction mixture was treated with 5.0 mL of saturated aqueous NaHCO₃ solution. The organic layer was separated and the aqueous layer extracted with CH₂Cl₂. The combined organic extracts were washed with brine, dried over MgSO₄, filtered and concentrated. The crude product was used without further purification. To a solution of 2-(3-(prop-2-yn-1-yl)oxy)propyl)piperidine in CH₂Cl₂ (5.0 mL) we added 4-nitrophenyl chloroformate (111 mg, 0.55 mmol) and Et₃N (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 NaHCO₃ solution. The organic layer was separated and the aqueous layer extracted with CH₂Cl₂. 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 a yellow oil: $^1$H NMR (400 MHz, CDCl$_3$) δ 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$_3$) δ 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$_2$O$_5$ [M+H]$^+$: 347.1601, found 347.1603.
NMR Spectra for WWL113, WWL113U, WWL229, and JW972
