Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

amTCO, a new *trans*-cyclooctene derivative to study drug-target interactions in cells

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General

Synthesis: All solvents and reagents were used as supplied without further purification.

Prep HPLC: Preparative high performance liquid chromatography was performed on a Waters autopurification system (2767 Sample manager, 2545 Binary Gradient Module, 2420 ELS Detector, 2996 Photodiode Array Detector, 3100 Mass Detector, Waters SFO, Waters 515 HPLC Pump) fitted with a XBridge BEH C18 OBD prep column (5 µm, 19 mm x 150 mm). The purifications were carried out at a flow rate of 30 mL/min using water and acetonitrile as eluents with 0.2% formic acid as modifier.

UPLC/MS: Ultra performance liquid chromatography coupled to mass spectrometry analyses were carried out on a Waters Acquity system (detectors: Acquity SQD, Acquity ELSD, Acquity PDA) fitted with a Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm x 50 mm). Water + 0.2% formic acid (A) and acetonitrile + 0.2% formic acid (B) were used as eluents. The analyses were performed at a flow rate of 0.5 mL/min with a linear gradient from 5 to 100% over 4 min. Electrospray mass spectra were acquired in positive/negative ion alternate mode. The purity of compounds was determined by detection at 254 nm.

HRMS: High Resolution Mass Spectrometry analyses were performed with a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer at EMBL Metabolomics Core Facility.

NMR: Nuclear magnetic resonance analyses were acquired on a Bruker Advance 400 MHz spectrometer equipped with a 5 mm PABBO BB/19F-1H/D Z-GRD probe at 295 K. The deuterated solvent was used as an internal deuterium lock. Chemical shifts (δ) are reported in parts per million using residual non-deuterated solvents as internal references. Spectra were processed and visualized with MestReNova 14.1.0.

GC: Gas chromatography analyses were performed on an Agilent 7890 B GC chromatograph equipped with a Gerstel MPS autosampler and FID detector using H_2 as carrier gas. Conversion was determined using a Hewlett Packard HP-5 column (30 m x 0.32 mm x 0.25 µm, 1 Bar, 7.557 mL min^{-1} flow). The program is as follows:

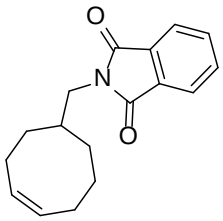
Rate (°C/min)	Temp (°C)	Hold time (min)	Run time (min)
0	50	0	0
10	90	0	4
30	160	0	6.33

FTIR: FT-IR spectra were recorded on a Spectrum 100 FT-IR spectrometer (Perkin Elmer) equipped with a universal ATR accessory.

Log D: Log D values were determined by chromatographic hydrophobicity measurements at pH 7.4.[1,2] The compounds were analyzed by reversed-phase HPLC on a C18 column (50×2 mm 3 µM Gemini NX C18, Phenomenex, Macclesfield, UK) using 50 mM ammonium acetate pH 7.4 and acetonitrile as eluents. The Chromatographic Hydrophobicity Index (CHI) values were derived directly from the retention times by using a calibration curve obtained for standard compounds. ChromlogD was then calculated as follow: $ChromlogD_{7.4} = (0.0857)*CHI_{7.4}-2.00$**.**

cLogP: Calculated Log P values were predicted using ChemDraw® Professional 18.2.0.48. The program uses a proprietary fragment-based method developed by Biobyte.

Phthalimide-protected amCCO



1-(Aminomethyl)-cis-cyclooct-4-ene hydrochloride (supplied by Enamine) (2.00 g, 11.4 mmol, 1 eq) and phthalic anhydride (2.53 g, 17.1 mmol, 1.5 eq) were dissolved in DMF (10 mL) by sonication. The reaction mixture was stirred at 90°C overnight and monitored by UPLC/MS. More phthalic anhydride (3.4 mmol, 506 mg, 0.3 eq) was added and the reaction was stirred at 90°C for another 24 h. A saturated solution of NaHCO3 was added to the reaction mixture and the product was extracted 3 times with DCM. The organic layers were combined, dried over Na2SO4 and concentrated. The product was purified by flash chromatography using 0-40% ethyl acetate in cyclohexane. Phthalimide-protected amCCO was obtained as a transparent oil (2.72 g, 88% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (dd, *J* = 5.5, 3.1 Hz, 2H), 7.71 (dd, *J* = 5.5, 3.1 Hz, 2H), 5.68-5.55 (m, 2H), 3.50 (qd, *J* = 13.4, 7.8 Hz, 2H), 2.36-2.27 (m, 1H), 2.21-1.96 (m, 4H), 1.66-1.18 (m, 6H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 168.88 (C), 134.01 (CH), 132.13 (C), 130.16 (CH), 130.05 (CH), 123.33 (CH), 45.07 (CH_2), 36.83 (CH), 32.86 (CH_2), 30.26 (CH_2), 27.88 (CH_2), 27.04 (CH_2), 25.87 (CH_2), 24.74 (CH_2).
LC/MS (ESI): t_R = 3.22 min, m/z 270.2 $[M+H]^+$.
HRMS (ESI): m/z 270.1491 $[M+H]^+$; calculated for $C_{17}H_{20}NO_2$, 270.1494.

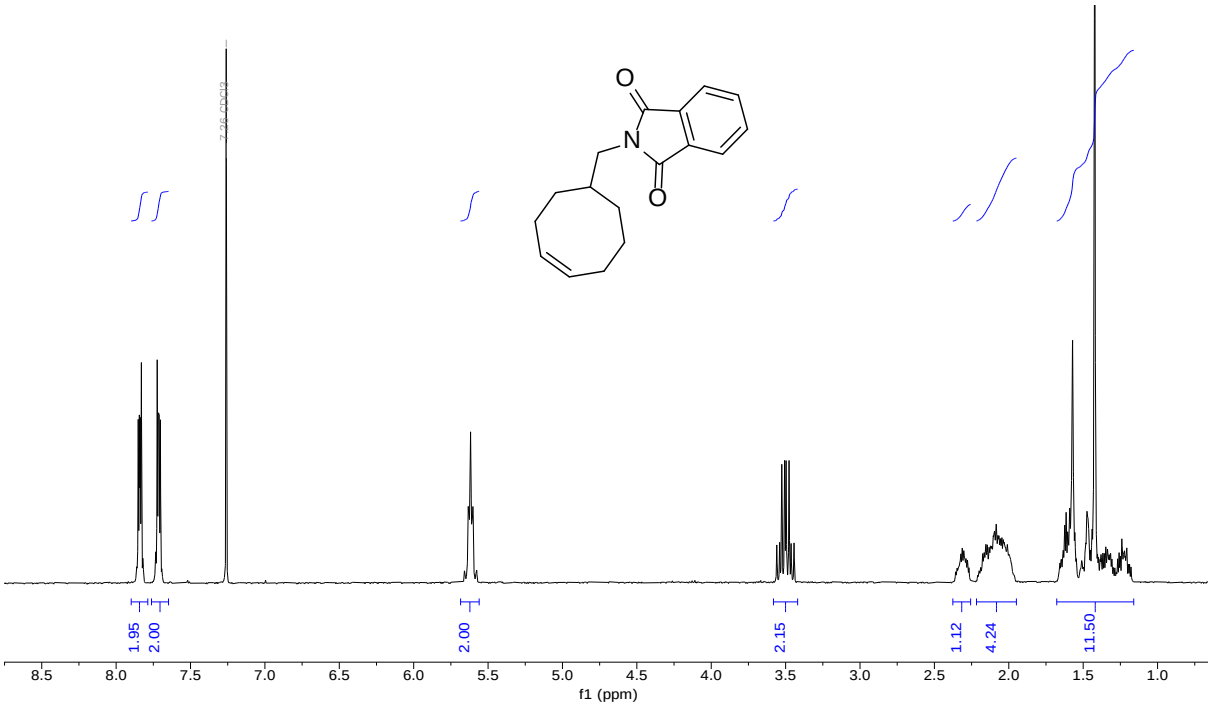


Figure S1. ^{1}H NMR spectrum (400 MHz) of phthalimide-protected amCCO in $CDCl_3$.

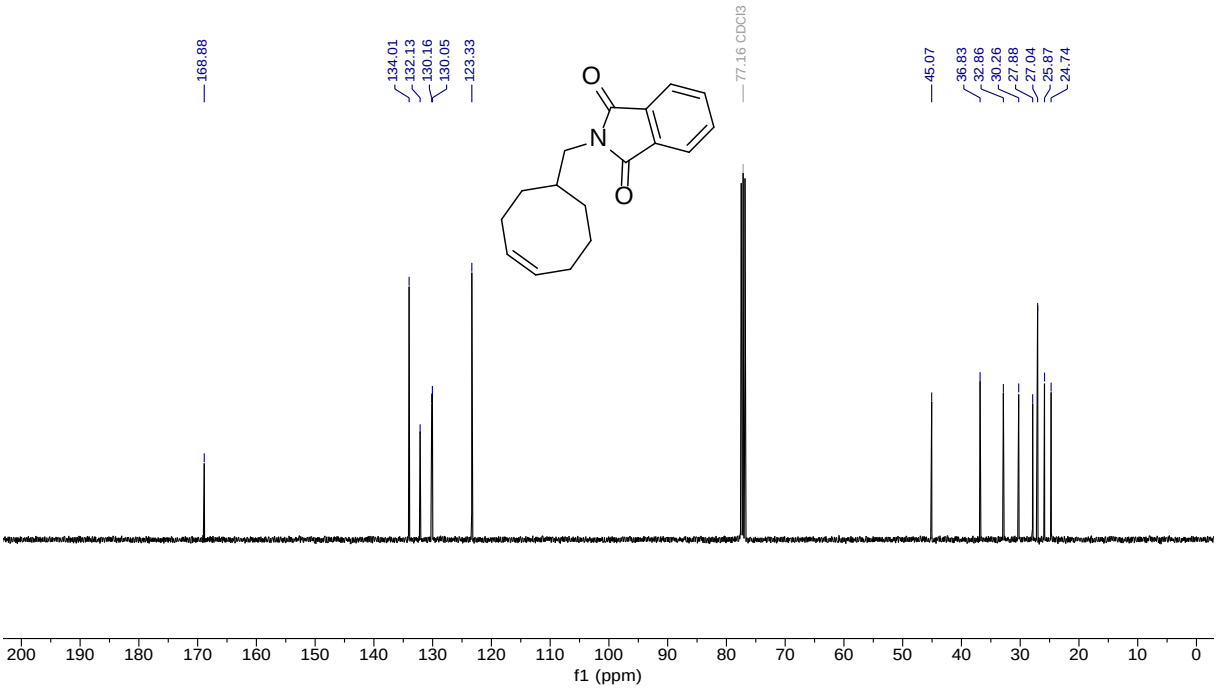
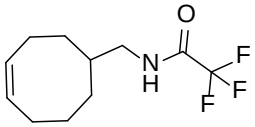


Figure S2. ^{13}C NMR spectrum (101 MHz) of phthalimide-protected amCCO in $CDCl_3$.

TFA-protected amCCO



1-(Aminomethyl)-cis-cyclooct-4-ene hydrochloride (Enamine, 200 mg, 1.14 mmol, 1 eq) was dissolved in a mixture of DCM (1 mL) and DMF (1 mL). DIEA (795 µL, 4.55 mmol, 4 eq) was added. The solution was cooled down to 0°C and placed under nitrogen atmosphere. A solution of 2,2,2-trifluoroacetic anhydride (0.321 mL, 2.277 mmol, 2 eq) in DCM (500 µL) was added dropwise. The reaction mixture was stirred at 0°C for 2 h. Then, water was added and the product was extracted 3 times with DCM. Organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by prep HPLC to give TFA-protected amCCO as a colorless oil (187 mg, 66% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.32 (br s, 1H), 5.72 – 5.57 (m, 2H), 3.21 (t, *J* = 6.6 Hz, 2H), 2.40-2.29 (m, 1H), 2.22-2.05 (m, 3H), 1.78 – 1.54 (m, 3H), 1.49-1.41 (m, 2H), 1.41-1.27 (m, 1H), 1.26-1.15 (m, 1H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 157.68 (C), 157.32 (C), 130.44 (CH), 129.86 (CH), 117.49 (C), 114.62 (C), 47.02 (CH_2), 37.51 (CH), 32.84 (CH_2), 30.72 (CH_2), 27.94 (CH_2), 25.93 (CH_2), 24.52 (CH_2).
LC/MS (ESI): t_R = 2.75 min, m/z 236.2 $[M+H]^+$.
HRMS (ESI): m/z 234.1113 $[M-H]^-$; calculated for $C_{11}H_{15}F_3NO$, 234.1106.

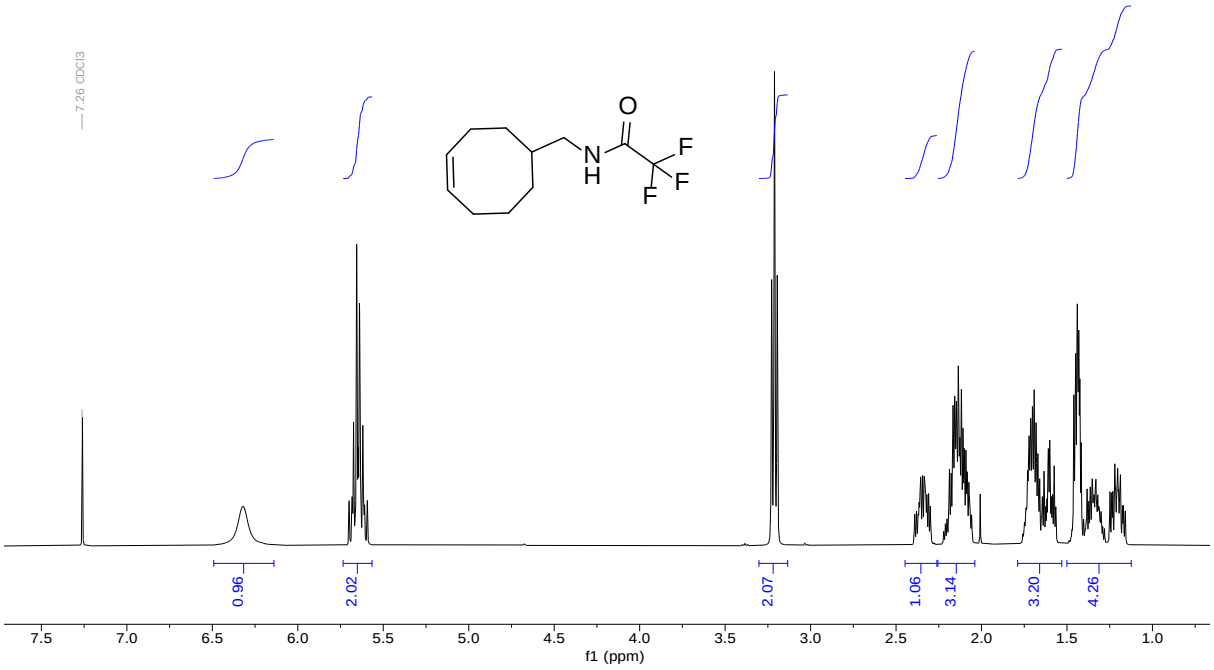


Figure S3. ^{1}H NMR spectrum (400 MHz) of TFA-protected amCCO in $CDCl_3$.

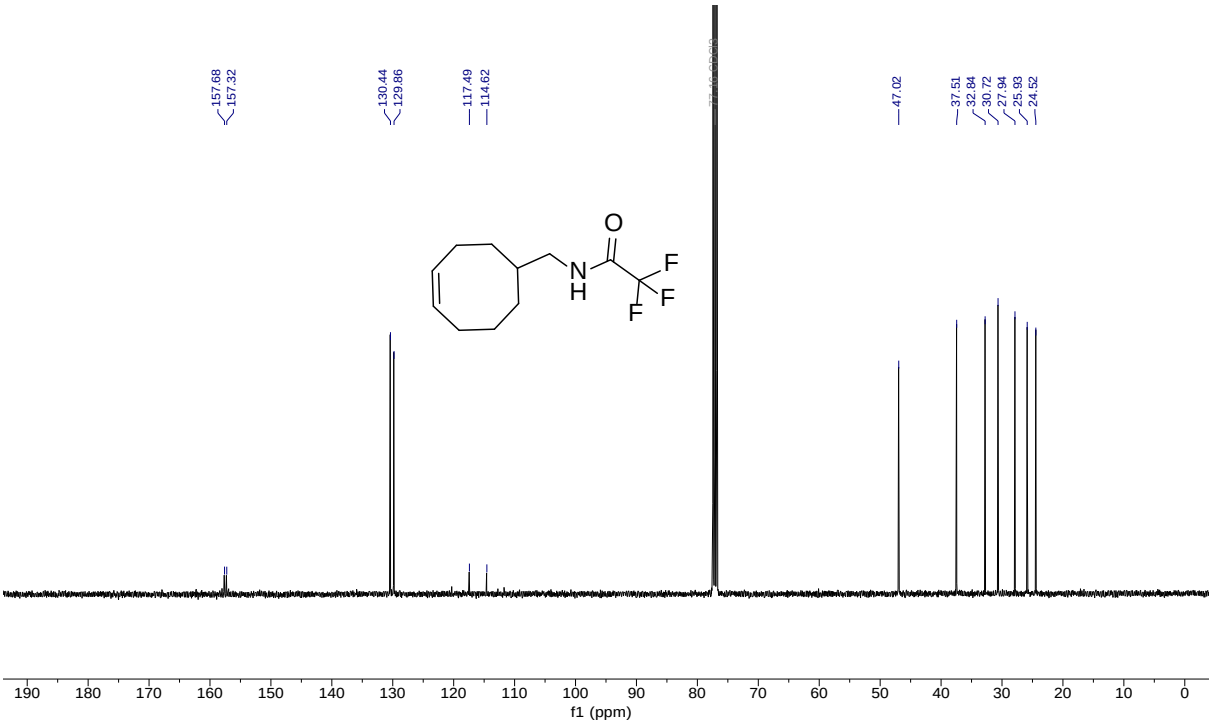
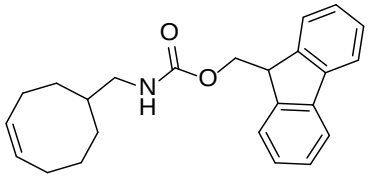


Figure S4. ^{13}C NMR spectrum (101 MHz) of TFA-protected amCCO in $CDCl_3$.

Fmoc-protected amCCO



1-(Aminomethyl)-cis-cyclooct-4-ene hydrochloride (Enamine, 200 mg, 1.14 mmol, 1 eq) was dissolved in a mixture of DCM (1 mL) and DMF (1 mL). DIEA (596 µL, 3.41 mmol, 3 eq) and Fmoc-Cl (353 mg, 1.37 mmol, 1.2 eq) were added. The reaction mixture was stirred at room temperature for 2 h. Then, water was added and the product was extracted 3 times with DCM. Organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography using 0-100% DCM in cyclohexane. Fmoc-amCCO was obtained as a white solid (309 mg, 71% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.5 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 5.71 – 5.58 (m, 2H), 4.78 (br s, 1H), 4.41 (d, *J* = 7.0 Hz, 2H), 4.22 (t, *J* = 6.9 Hz, 1H), 3.04 (t, *J* = 6.5 Hz, 2H), 2.39 – 2.25 (m, 1H), 2.23 – 0.99 (m, 10H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 156.73 (C), 144.14 (C), 141.46 (C), 130.27 (CH), 130.16 (CH), 127.78 (CH), 127.14 (CH), 125.16 (CH), 120.10 (CH), 66.58 (CH), 48.38 (CH_2), 47.47 (CH_2), 38.30 (CH), 32.98 (CH_2), 30.64 (CH_2), 28.12 (CH_2), 25.97 (CH_2), 24.77 (CH_2).
LC/MS (ESI): t_R = 3.61 min, m/z 362.3 $[M+H]^+$, 384.3 $[M+Na]^+$.
HRMS (ESI): m/z 362.2118 $[M+H]^+$; calculated for $C_{25}H_{28}NO_2$, 362.2120.

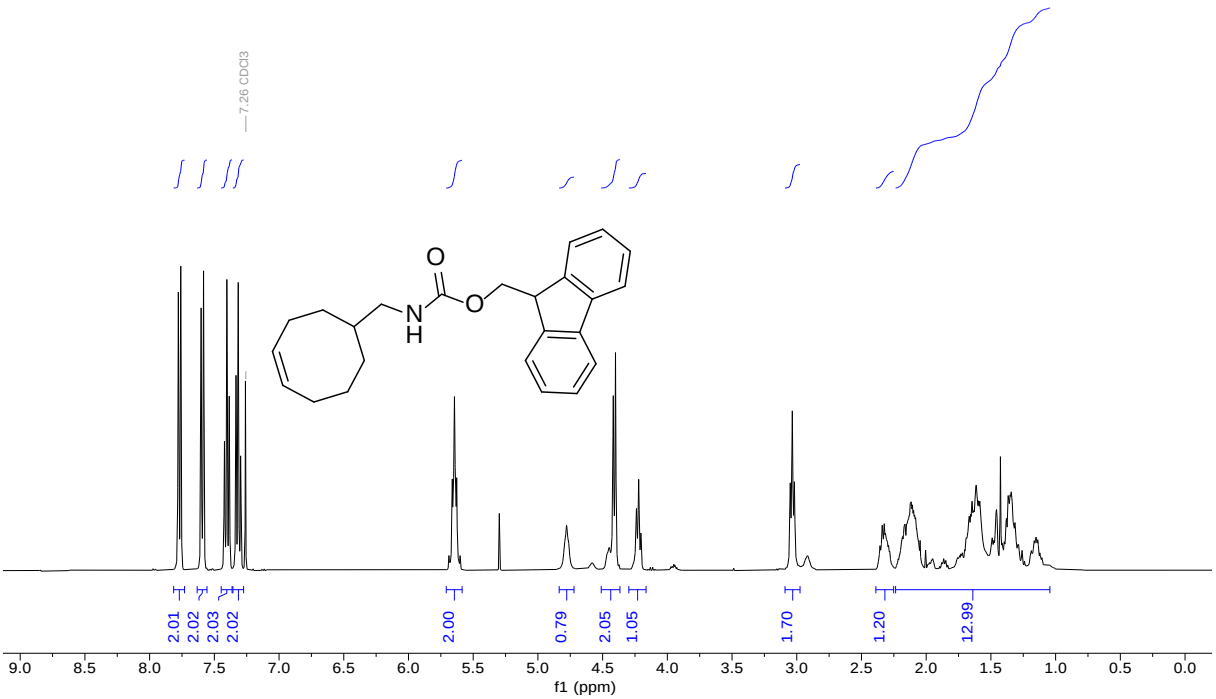


Figure S5. ^{1}H NMR spectrum (400 MHz) of Fmoc-protected amCCO in $CDCl_3$.

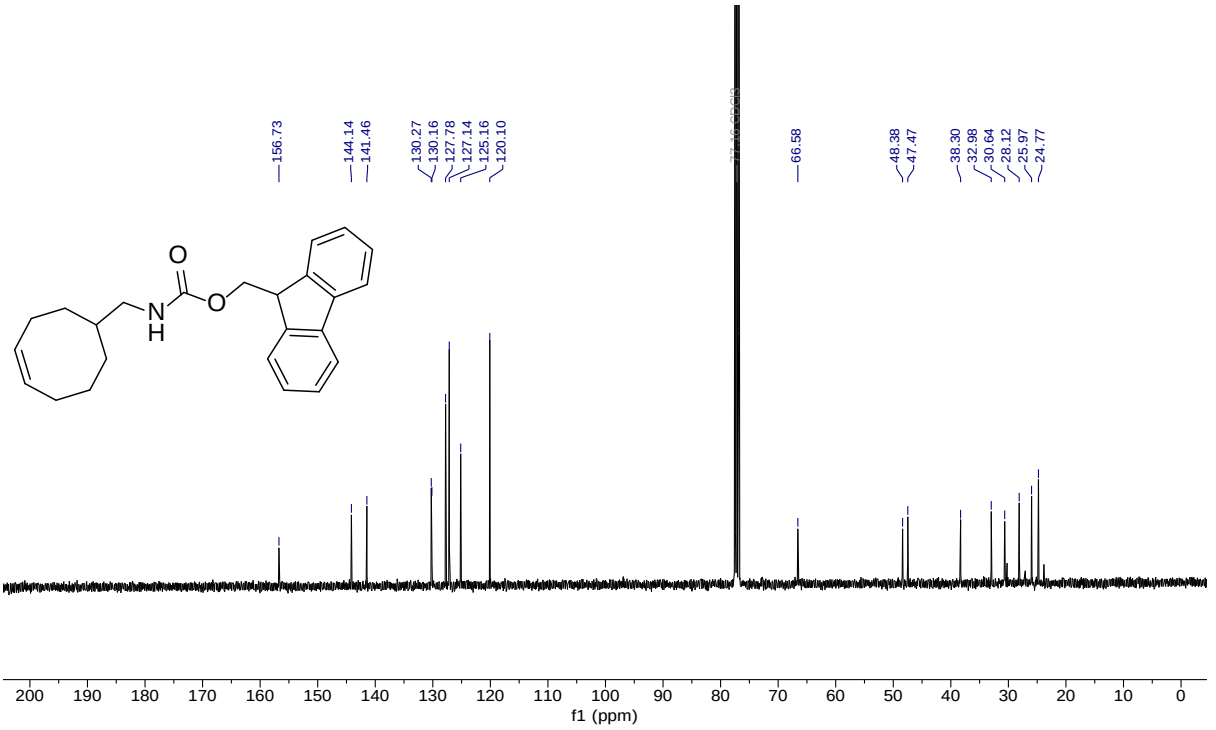


Figure S6. ^{13}C NMR spectrum (101 MHz) of TFA-protected amCCO in $CDCl_3$.

Photoreactor

Photoisomerizations were conducted in an in house-built flow reactor similar to the setup developed by Svatunek *et al*.[3] A 16 W low pressure mercury lamp (Photochemical Reactors Ltd., model 3016) located in a watercooled, double-walled immersion well was used for irradiation. It emits 90% of its radiation at 254 nm. Solutions were pumped into a 25 mL FEP

tubing (Adtech) coiled around the lamp in order to maximise irradiation. Absorption by the irradiated solution was monitored with a CCD spectrometer (Exemplar LS, B&WTek). A trapping column filled with silica topped with 10% $AgNO_3$-coated silica was installed immediately after the coil and cooled down with an ice bag. The eluted solution returned to the reservoir in order to undergo another irradiation cycle. The system was operated within a UV absorbing cabinet equipped with an interlock which ensures the lamp can only be operated when the doors are shut.

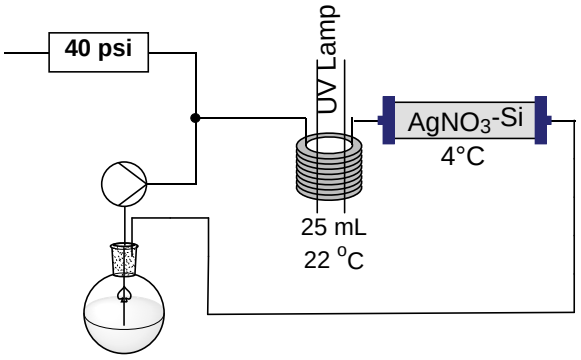


Figure S7. Schematic of the flow setup

Figure S8. FEP extension coil wrapped around the immersion well containing the 16 W low pressure Mercury lamp.

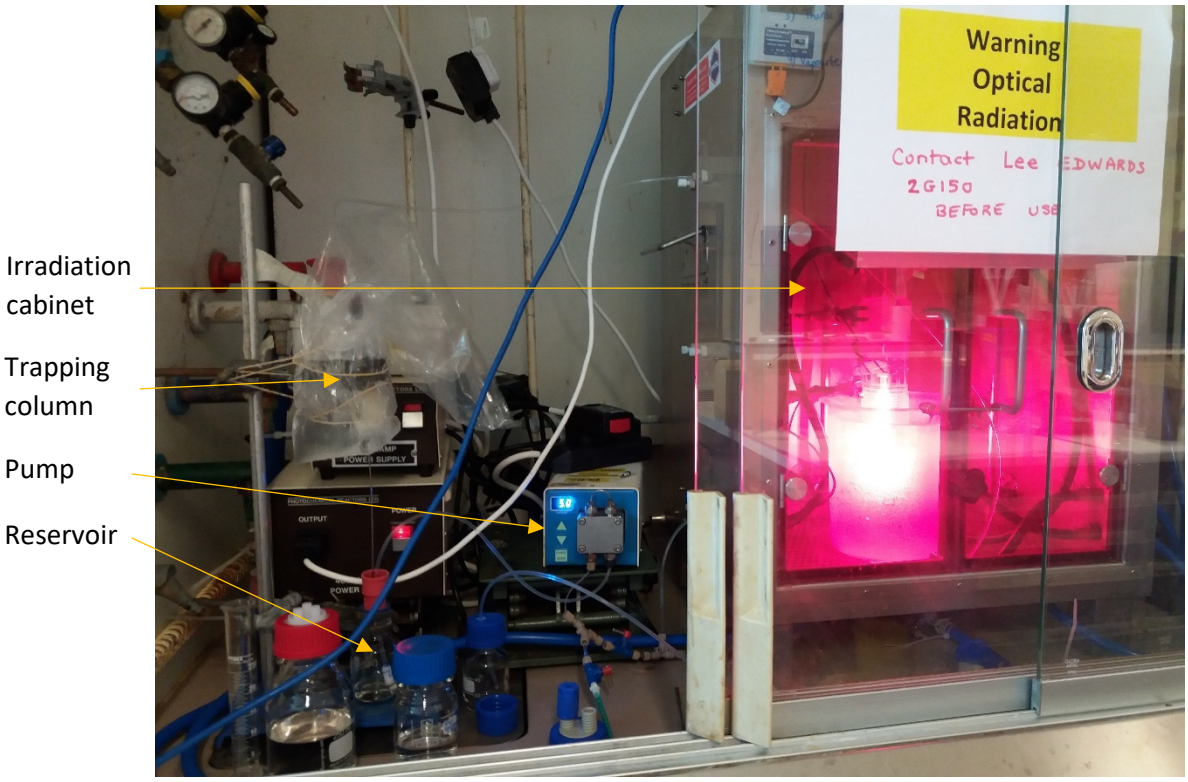


Figure S9. Flow setup

Photoisomerization reactions were monitored by GC. The *cis*-to-*trans* conversion was calculated from remaining *cis*-cyclooctene in the reaction mixture (integration of corresponding GC peak before and after irradiation).

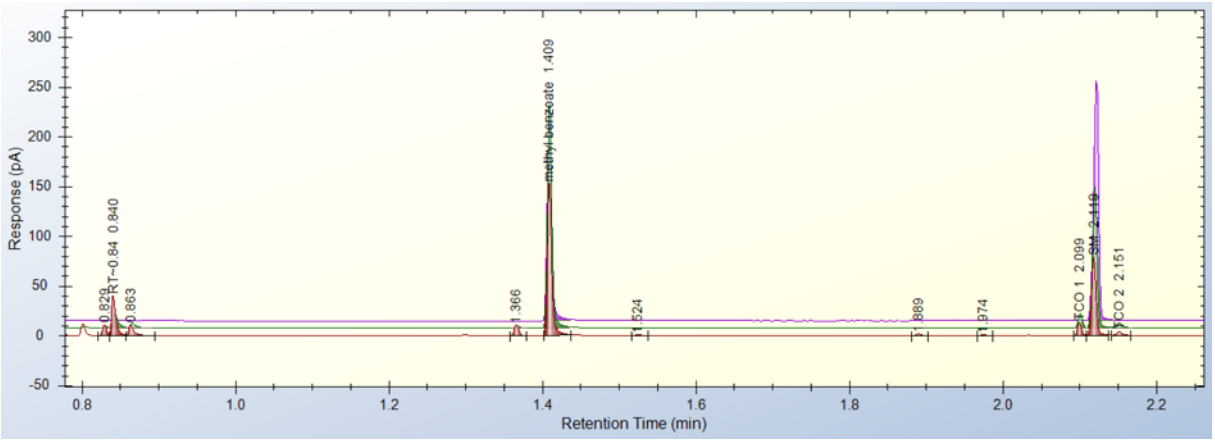


Figure S10. GC chromatograms recorded during the photoisomerization of trifluoroacetamide-amCCO (t_R = 2.12 min) using methyl benzoate (t_R = 1.41 min). Total irradiation time: 0 min in purple, 25 min in green, 1h25min in red.

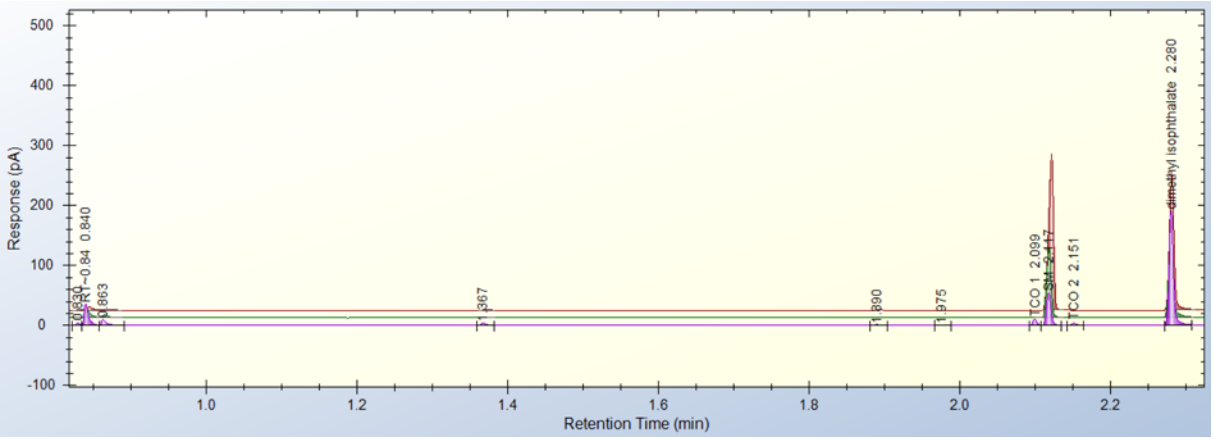


Figure S11. GC chromatograms recorded during the photoisomerization of trifluoroacetamide-amCCO (t_R = 2.12 min) using dimethyl isophthalate (t_R = 2.28 min). Total irradiation time: 0 min in red, 15 min in green, 1h05min in purple.

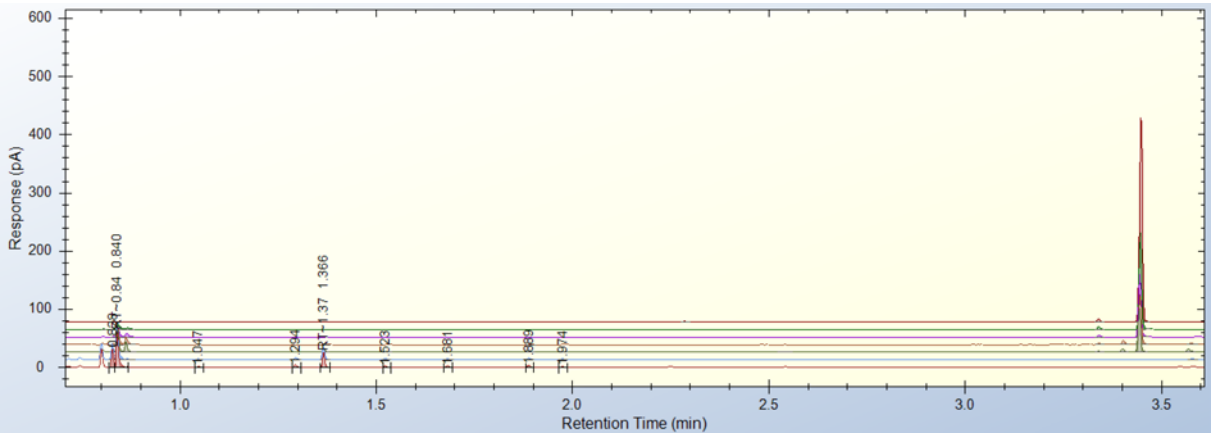


Figure S12. GC chromatograms recorded during the photoisomerization of phthalimide-amCCO (t_R = 3.45 min) in the absence of additional photosensitizer. Total irradiation time (from top to bottom): 0 min in red, 5 min in green, 20 min in purple, 1h05min in orange, 2h05 min in grey. Blue and pink chromatograms were obtained after irradiation with 400 W lamp for 16 and 24 min respectively.

Fmoc-protected amCCO isomerization could not be monitored by GC, LC/MS was used instead (gradient from 3 to 95% ACN 0.1% formic acid in water 0.1% formic acid in 1.5 min).

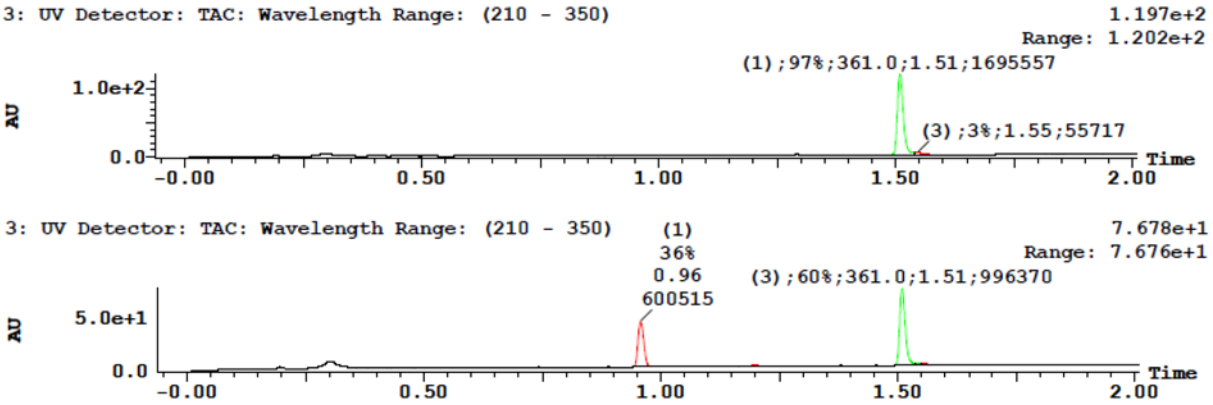
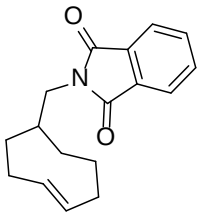


Figure S13. LC/MS analyses recorded during the photoisomerization of Fmoc-amCCO (t_R = 1.51 min) using dimethylisophthalate (t_R = 0.96 min). Top: before irradiation, bottom: after 90 min.

After irradiation, the trans-cyclooctenes were eluted from the trapping column and analysed by LC/MS (gradient from 3 to 95% ACN 0.1% formic acid in water 0.1% formic acid in 1.5 min).

LC/MS (ESI) of trifluoroacetamide-amTCO: t_R = 1.16 min, m/z 236.0 $[M+H]^+$, 234.2 $[M-H]^-$.
LC/MS (ESI) of Fmoc-amTCO: t_R = 1.49 min, m/z 362.0 $[M+H]^+$.
For phthalimide-protected amTCO, see detailed protocol and full characterization below.

Phthalimide-protected amTCO



The photoreactor was flushed with cyclohexane/diethyl ether 50/50 v/v. Phthalimide-protected amCCO (1.972 g, 7.32 mmol, 1 eq) was dissolved in cyclohexane/ diethyl ether 50/50 (14 mL) and the solution was introduced into the reservoir. The system was equilibrated at a flow rate of 10 mL/min for 20 min with the UV lamp on. Then the flow rate was decreased to 5 mL/min and the solution was further irradiated for 7 h 40 min. Phthalimide-protected amTCO was trapped onto an 80 g column filled to half with silica and topped up with 10% $AgNO_3$-coated silica. The amount of light absorbed by the solution in the coil varied with time indicating that the phthalimide-protected amCCO was not evenly distributed along the tubing due to a long residence time in the trapping column. Therefore, the 80 g trapping column was replaced with a 12 g trapping column and the solution was further irradiated for 7 h 15 min. After irradiation, the system was flushed with cyclohexane/diethyl ether 50/50. Phthalimide-protected amTCO - $AgNO_3$ complex was eluted from the trapping columns with methanol (5 column volumes). The eluates were combined and concentrated under reduced pressure. DCM (250 mL), water (175 mL) and 35% aqueous ammonia (175 mL) were added to the residue and mixed. The organic layer was separated, and the aqueous phase was extracted again twice with DCM. Organic layers were combined, dried through a separation phase cartridge and concentrated under reduced pressure. The desired product was purified by flash chromatography using 0 - 40% ethyl acetate in cyclohexane. This afforded phthalimide-protected amTCO as a transparent oil which further crystallized into a white solid (1.341 g, 67% yield). Noteworthy, phthalimide-protected amTCO was obtained as a mixture of diastereomers (axial and equatorial).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86-7.82 (m, 2H), 7.74-7.70 (m, 2H), 5.76-5.45 (m, 2H), 3.84-3.39 (m, 2H), 2.55-1.06 (m, 11H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 168.97 (C), 168.76 (C), 134.76 (CH), 134.52 (CH), 134.08 (CH), 134.00 (CH), 133.86 (CH), 133.75 (CH), 132.24 (C), 132.08 (C), 123.38 (CH), 123.31 (CH), 45.13 (CH_2), 40.91 (CH), 39.57 (CH_2), 39.02 (CH_2), 37.85 (CH_2), 35.44 (CH_2), 34.93 (CH_2), 34.79 (CH_2), 33.75 (CH), 33.63 (CH_2), 31.93 (CH_2), 31.05 (CH_2), 30.38 (CH_2).
LC/MS (ESI): t_R = 3.15 min (major diastereomer) and 3.23 min (minor diastereomer), m/z 270.2 $[M+H]^+$.
HRMS (ESI): m/z 270.1490 $[M+H]^+$; calculated for $C_{17}H_{20}NO_2$, 270.1494.

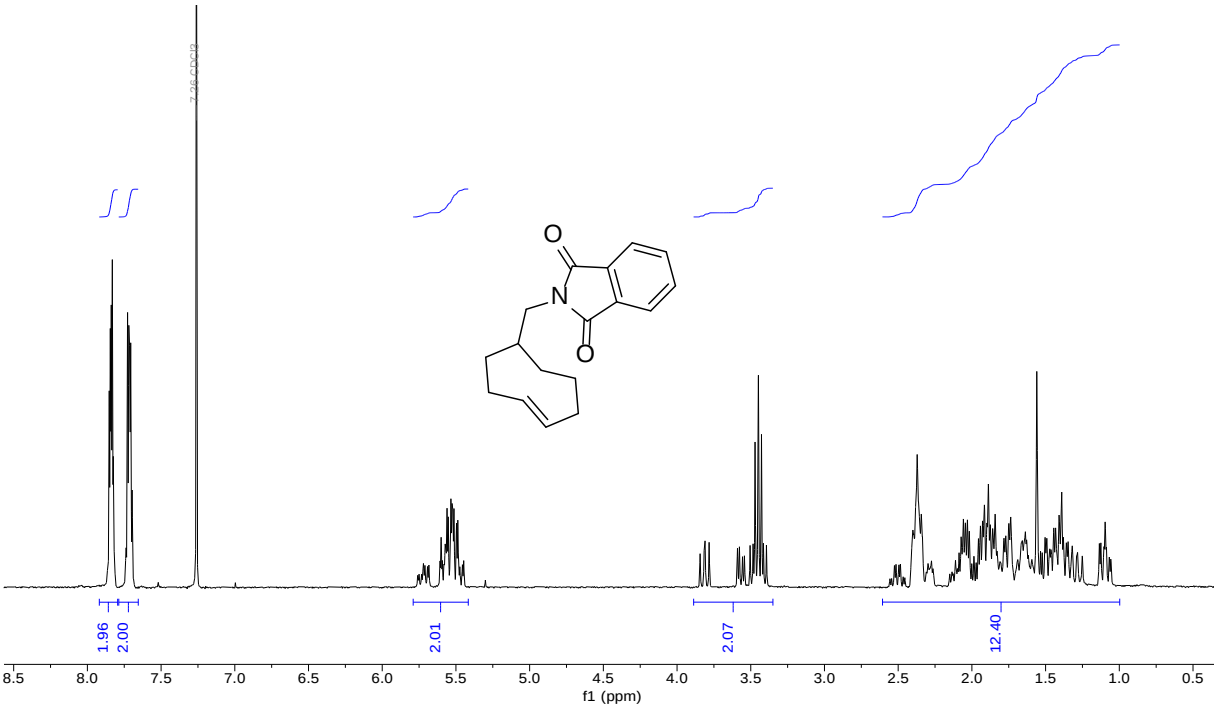


Figure S14. ^{1}H NMR spectrum (400 MHz) of the mixture of diastereomers **(1)** in $CDCl_3$.

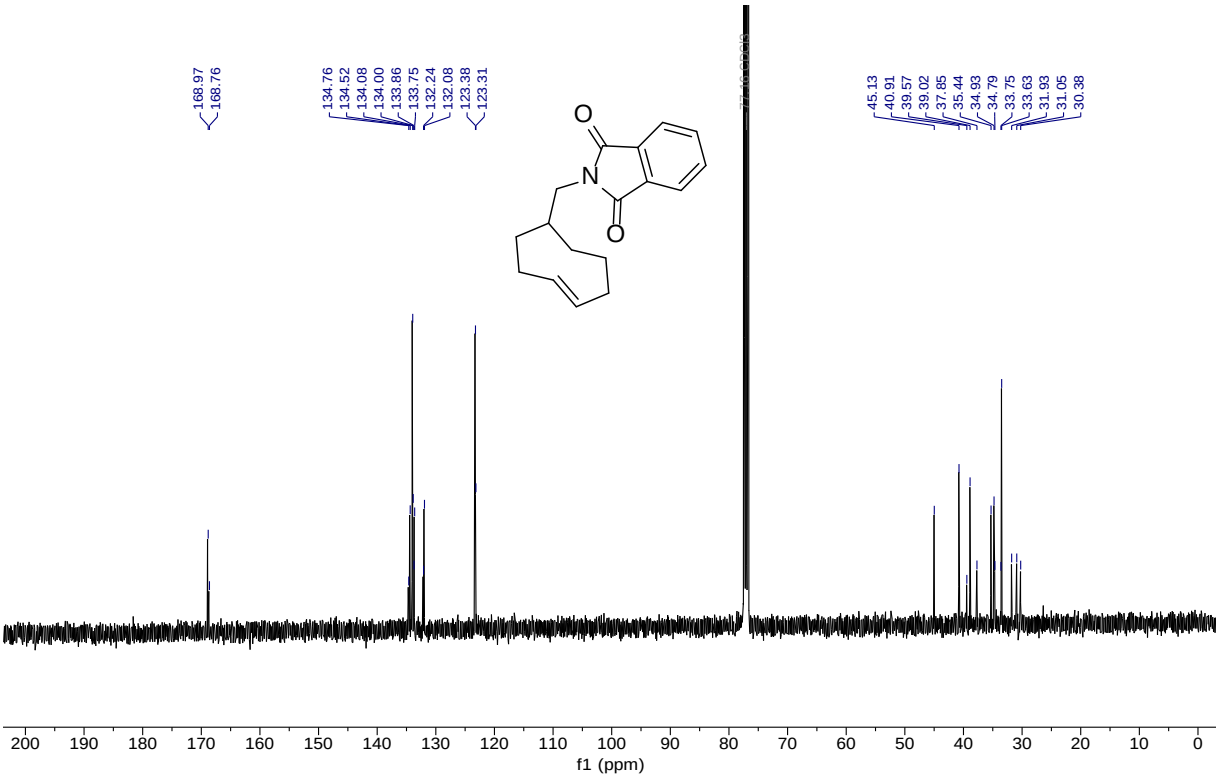
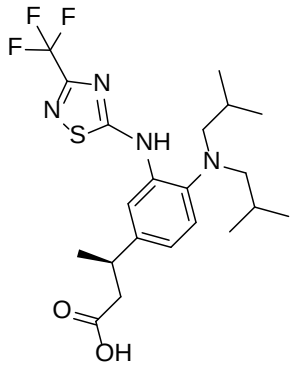


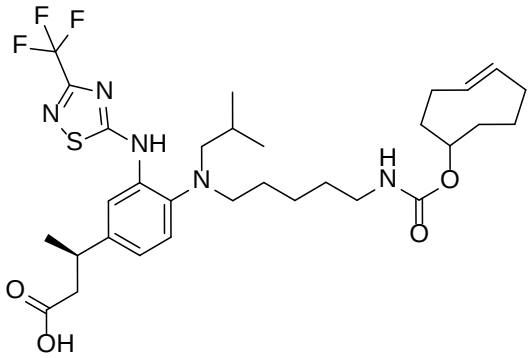
Figure S15. ^{13}C NMR spectrum (101 MHz) of the mixture of diastereomers **(1)** in $CDCl_3$.

GSK5628 (IDO1 inhibitor)



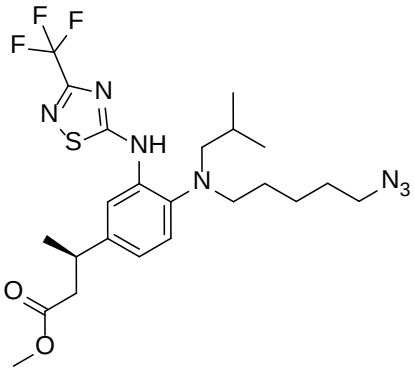
The synthesis of GSK5628 was described in the literature.[4]

TCO-GSK5112



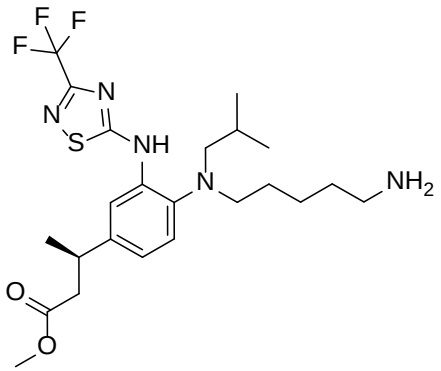
The synthesis of TCO-GSK5112 was described in the literature.[5]

IDO1 inhibitor azide derivative



The azido derivative was prepared following the protocol described in the literature.[6]

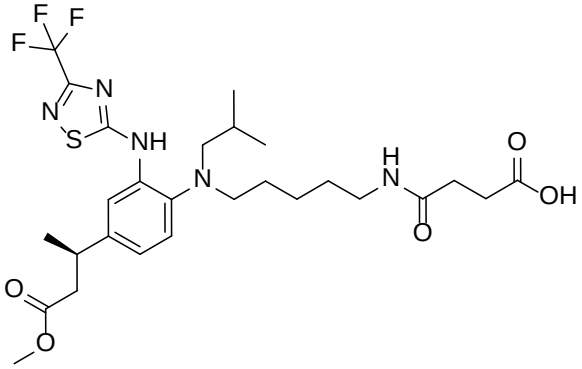
IDO1 inhibitor amine derivative



IDO1 inhibitor azide derivative (500 mg, 0.948 mmol, 1 eq) was dissolved in THF (10 mL). Triphenylphosphine (273 mg, 1.04 mmol, 1.1 eq) was added to this solution. The reaction mixture was stirred at RT overnight and then at 40°C for 4.5 h. Water (500 µL) was added to hydrolyze the iminophosphorane. The reaction was stirred at RT for 1 h. THF was removed under reduced pressure and the product was purified by C18 reverse-phase flash chromatography using 0-50% ACN modified with 0.2% formic acid in water modified with 0.2% formic acid. This afforded IDO1 inhibitor amine derivative as a yellow solid (347 mg, 72% yield).

LC/MS (ESI): t_R = 2.38 min, m/z 502.3 $[M+H]^+$, 500.4 $[M-H]^-$.

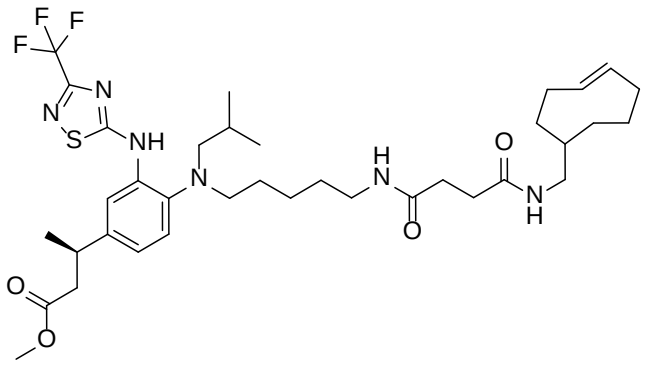
IDO1 inhibitor carboxylic acid derivative



IDO1 inhibitor amine derivative (150 mg, 0.299 mmol, 1 eq) was dissolved in DCM (5 mL). To this solution, DIEA (157 µL, 0.897 mmol, 3 eq) was added followed by succinic anhydride (33 mg, 0.329 mmol, 1.1 eq). The reaction mixture was stirred at RT for 30 min. Water (1 mL) was added and the reaction mixture was stirred again at RT for 30 min. The solution was acidified to pH 2 by addition of concentrated HCl solution. The product was extracted 3 times with DCM. Organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure to yield the desired product as a yellow solid (156 mg, 76% yield). The product was used without further purification.

LC/MS (ESI): t_R = 3.23 min, m/z 600.4 $[M-H]^-$.

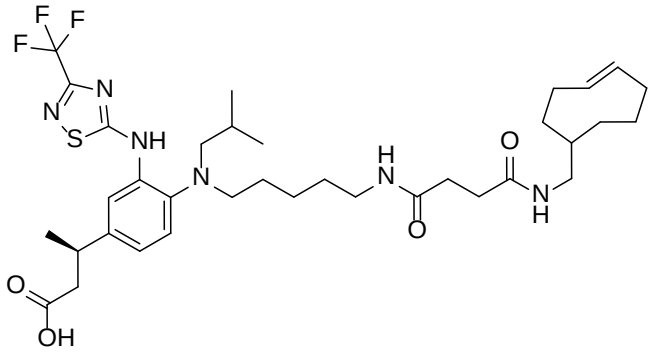
amTCO probe methyl ester



Phthalimide-protected amTCO (10 mg, 37 µmol, 1 eq) was dissolved in a mixture of THF (150 µL) and MeOH (150 µL). Hydrazine (23 µL, 743 µmol, 20 eq) was added. The reaction mixture was stirred at 60°C for 1 h under nitrogen atmosphere. Then, hydrazine was co-evaporated with methanol (3 x 1 mL). Deprotected amTCO was obtained as a white powder. IDO1 inhibitor carboxylic acid derivative (33.5 mg, 56 µmol, 1.5 eq) was dissolved in DMF (500 µL) and activated by addition of DIEA (20 µL, 111 µmol, 3 eq) and HATU (21 mg, 56 µmol, 1.5 eq). The solution contained the activated IDO1 derivative was poured onto the deprotected amTCO. The reaction mixture was stirred at RT for 1 h. The product was purified by C18 reverse-phase flash chromatography using 0-80% ACN in water. This afforded amTCO probe methyl ester as a white solid (10 mg, 33% yield).

LC/MS (ESI): t_R = 3.74 min, m/z 723.4 $[M+H]^+$, 721.5 $[M-H]^-$.

amTCO-GSK9353 probe



amTCO probe methyl ester (10 mg, 13 µmol, 1 eq) was dissolved in MeOH (4 mL). 0.2 M sodium hydroxide in water (1 mL, 200 µmol, 15 eq) was added. The reaction mixture was stirred at RT for 2 days. MeOH was removed under reduced pressure and the product was purified by C18 reverse-phase flash chromatography using 0-80% ACN in water. This afforded amTCO-GSK9353 as a white solid (10 mg, 100% yield).

^{1}H NMR (400 MHz, DMSO) δ 10.43 (br s, 1H), 8.06 – 7.47 (m, 3H), 7.14 (d, *J* = 8.2 Hz, 1H), 7.02 (d, *J* = 8.1 Hz, 1H), 5.56 – 5.31 (m, 2H), 3.14 – 2.99 (m, 2H), 2.93 (q, *J* = 6.6 Hz, 2H), 2.87 – 2.65 (m, 5H), 2.47 – 2.16 (m, 7H), 2.18 – 2.04 (m, 1H), 1.99 – 1.64 (m, 5H), 1.61 – 1.44 (m, 2H), 1.43 – 1.22 (m, 5H), 1.19 (d, *J* = 6.9 Hz, 3H), 1.16 – 1.02 (m, 3H), 0.97 – 0.85 (m, 2H), 0.79 (d, *J* = 6.5 Hz, 6H).
^{13}C NMR (176 MHz, DMSO) δ 171.31 (C), 171.05 (C), 134.35 (CH), 134.10 (CH), 133.10 (CH), 132.96 (CH), 123.60 (CH), 122.60 (CH), 119.38 (CH), 61.39 (CH_2), 55.21 (CH_2), 48.51 (CH), 45.84 (CH_2), 43.43 (CH_2), 41.66 (CH), 38.44 (CH_2), 38.18 (CH_2), 35.61 (CH), 34.87 (CH_2),

34.61 (CH_2), 34.32 (CH_2), 34.17 (CH_2), 33.37 (CH_2), 33.28 (CH_2), 31.03 (CH_2), 30.71 (CH_2), 30.17 (CH_2), 30.12 (CH_2), 29.00 (CH_2), 25.84 (CH), 25.77 (CH_2), 24.16 (CH_2), 21.80 (CH_3), 20.86 (CH_3), 20.65 (CH_3).
LC/MS (ESI): t_R = 3.38 min, m/z 709.5 $[M+H]^+$, 707.6 $[M-H]^-$.
HRMS (ESI): m/z 709.3719 $[M+H]^+$; calculated for $C_{35}H_{52}F_3N_6O_4S$, 709.3723.
FT-IR: ν_{max} 3288, 3094, 2928, 2858, 1645, 1542, 1488, 1437, 1408, 1190, 1171, 1141, 989, 975, 955, 880, 837, 801, 742, 723, 680, 644 cm^{-1}

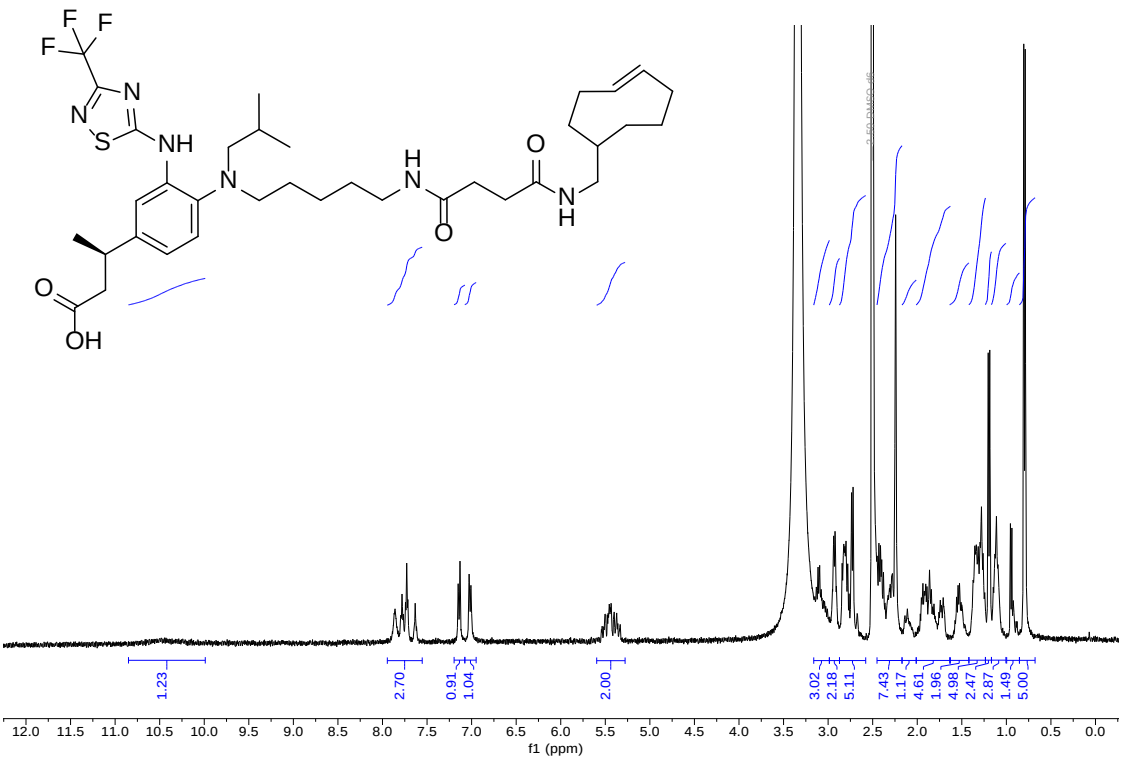


Figure S16. ^1H NMR spectrum (400 MHz) of amTCO-GSK9353 in DMSO-d_6.

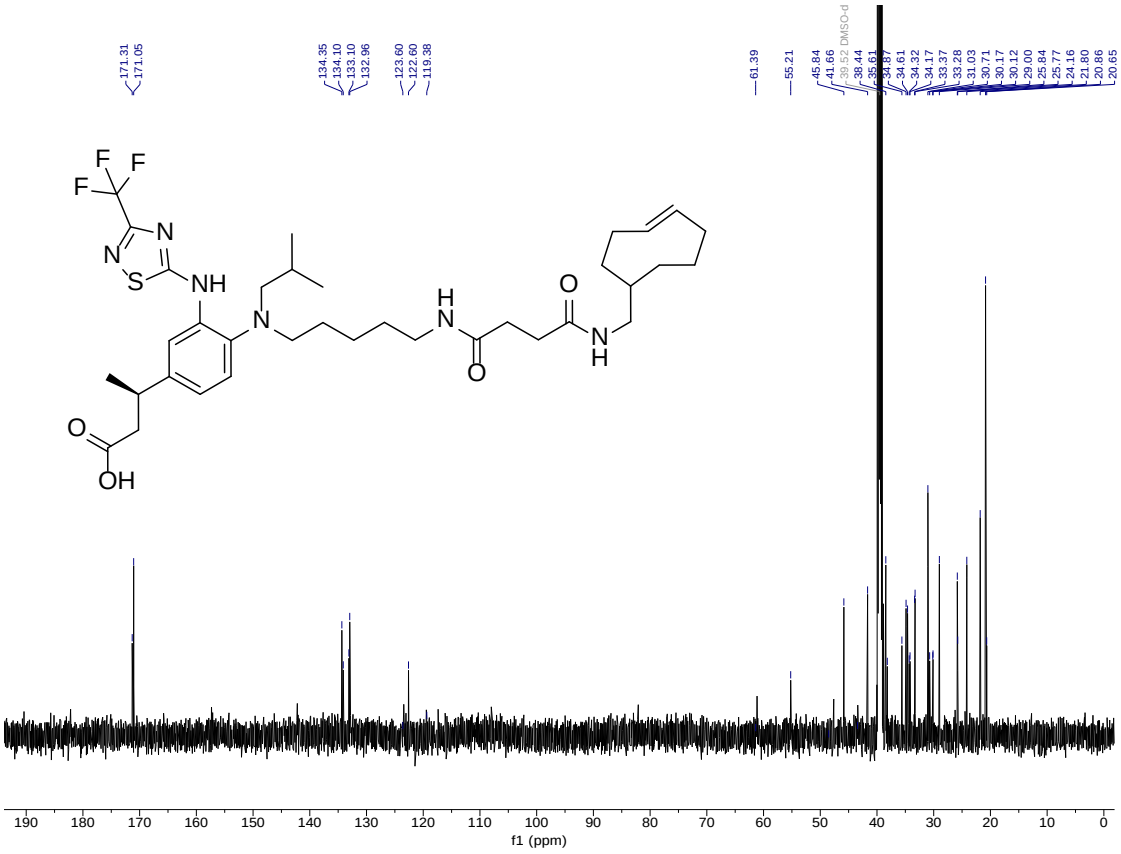


Figure S17. ^{13}C NMR spectrum (176 MHz) of amTCO-GSK9353 in DMSO-d_6.

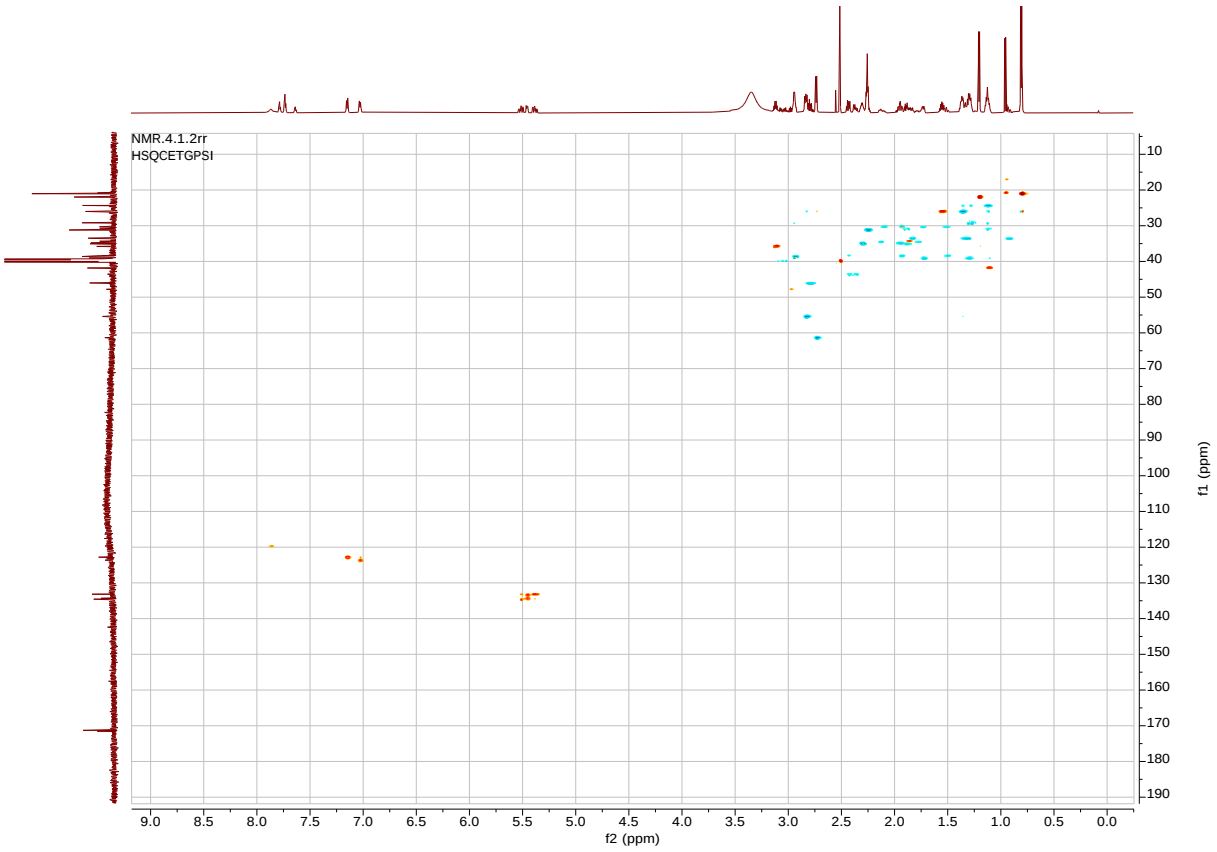


Figure S18. HSQC spectrum of amTCO-GSK9353 in DMSO-d_6 acquired on a Bruker AVIII 700 using the weighted acquisition technique described in *J Biomol NMR*. 2019, 73(3-4):155-165.

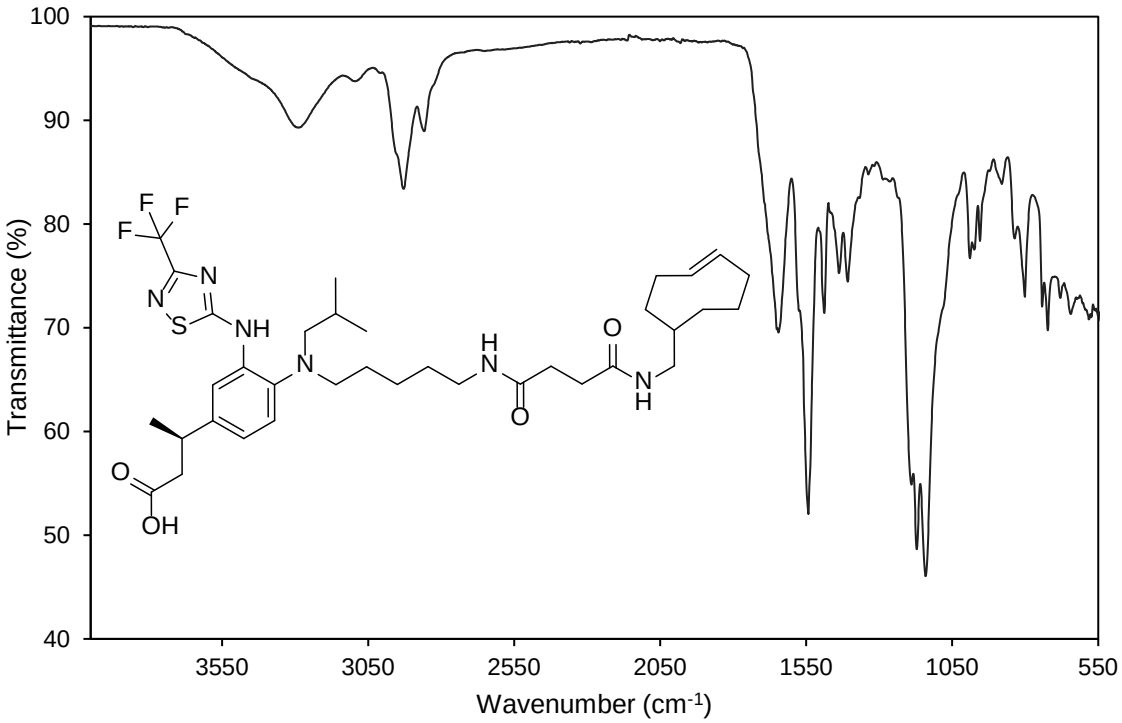


Figure S19. FT-IR spectrum of amTCO-GSK9353.

Cy5-tetrazine

Cy5-tetrazine was obtained from Jena Bioscience.

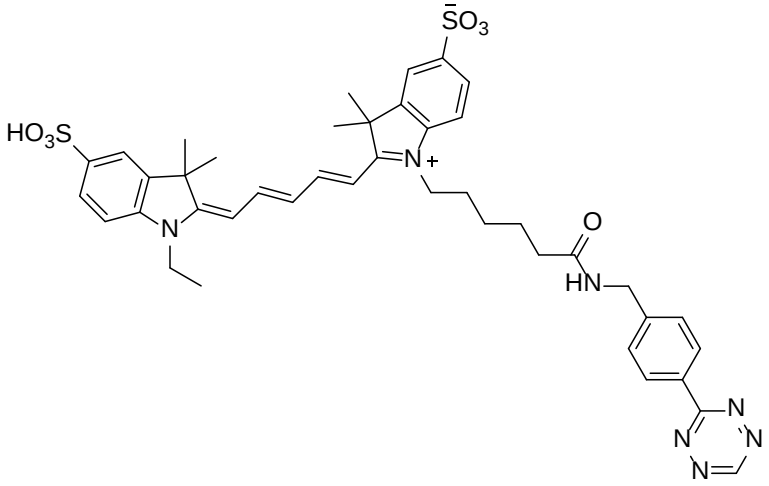


Figure S20. Structure of Cy5-tetrazine.

Absorption and emission measurements

Spectroscopic characteristics of Cy5-tetrazine after click reaction with TCO*-lysine were previously reported.[7]

Absorption and emission spectra of Cy5-tetrazine before and after click reaction with amTCO-GSK9353 probe were recorded on a PerkinElmer Envision plate reader. Three samples were prepared: 1) 2 µM solution of Cy5-tetrazine in PBS, 2) 2 µM Cy5-tetrazine with 2.5 µM amTCO-GSK9353 probe in PBS, 3) amTCO-GSK9353 was pre-incubated with recombinant IDO1 protein (abcam) in PBS for 1h at 37°C and then added to a Cy5-tetrazine solution in PBS (final concentrations: 2.5 µM amTCO-GSK9353, 2.5 µM IDO1 protein, 2 µM Cy5-Tetrazine). Samples (150 µL) were measured in a black, clear-bottom, 96 well plate. Absorbance scans were recorded from 350 nm to 800 nm with a 2 nm step. For fluorescence measurements, the samples were excited at 580 nm and emission was recorded between 600 nm and 800 nm with a 2 nm step. PBS was used as a blank sample.

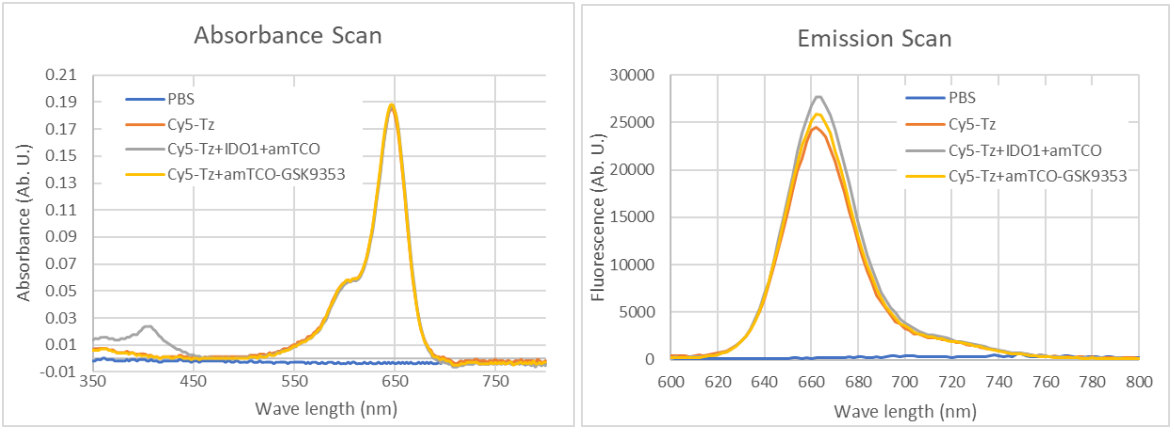


Figure S21. Left: Absorbance spectra. Right: Emission spectra (λ_{exc} = 580 nm). Samples: PBS (blue), Cy5-tetrazine in PBS (orange), Cy5-tetrazine + amTCO-GSK9353 click product in PBS (yellow), Cy5-tetrazine + amTCO-GSK9353 click product in PBS with IDO1 protein (grey).

Affinity enrichment

Affinity enrichment experiment was performed as described in ChemBioChem 2020, DOI: 10.1002/cbic.202000298. In short, amTCO-GSK9353 (1 µM) with and without GSK5628 (10 µM) were spiked into total lysate of INFγ induced Hela cells and incubated for 1.5h at 4 °C. Streptavidin beads pre-incubated with biotin-tetrazine[8] (50 mM stock in DMSO) were equilibrated in lysis buffer, loaded onto a 96-well filter plate (Millipore), and incubated with cell extract to enable the IED-DA bioorthogonal reaction (30 min at 4 °C). Beads were then washed three times with lysis buffer and eluted with 60 µL of 2X-LDS sample buffer with DTT. Eluted proteins were separated on NuPAGE 4–12% (Invitrogen), transferred to a membrane, and immunostained for IDO1 (Abcam, ab76157). Blots were analysed on an Odyssey Scanner (LI-COR).

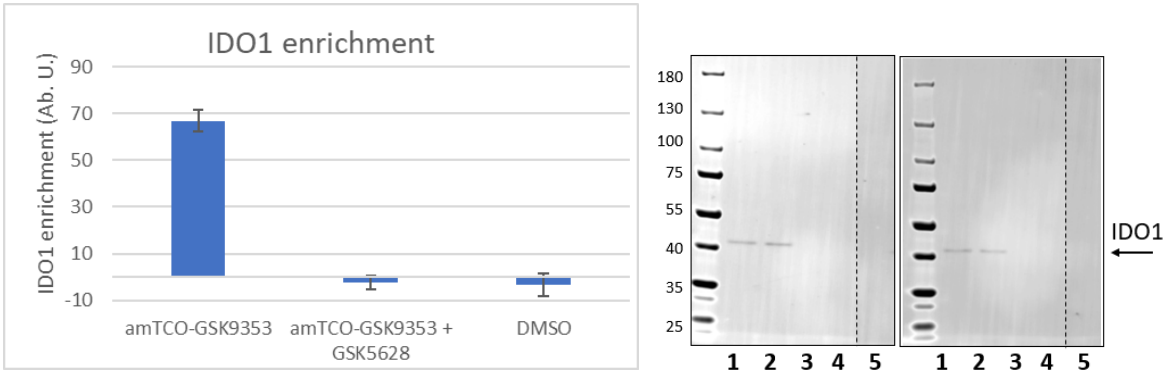


Figure S22. Total lysate of INFγ inducted HeLa cells was incubated with amTCO-GSK9353 in the presence and absence of competitor GSK5628, and subsequently the bioorthogonal partner group (Tetrazine) coupled to biotin and immobilized on streptavidin beads was added. Enrichment of IDO1 was detected in the elution fractions by immunostaining (right) and quantified by densitometry (left). (1)(2) amTCO-GSK9353, (3)(4) amTCO-GSK9353 + GSK5628, (5) DMSO. An average value of two independent experiments, each done in technical replicate, is shown, together with standard deviation.

Imaging assay

HeLa cells were cultured in MEM supplemented with 1% pyruvate, 1% NEAA and 10% FBS at 37°C with a humidified 5% CO_2 atmosphere. Cells (passage 12, 90% confluent) were washed with DPBS and treated with 0.05% trypsin-EDTA for 5 min at 37°C. Trypsin was neutralized by addition of cell culture media. The suspension was adjusted to 200 000 cells/mL and cells were seeded into an 8 well Lab-Tek™ chambered cover glass (200 µL per well). Cells were allowed to adhere for 4 h at 37°C. Then the media was removed completely by aspiration and replaced with media containing 10 nM IFNγ (200 µl/well). Cells were incubated at 37°C for 18 h in order to induce the expression of IDO1. The media was removed and replaced with 200 µL of media containing TCO-GSK5112 (3 µM), amTCO-GSK9353 (3 µM) or DMSO (0.1%) and GSK5628 (10 µM) where applicable. Cells were incubated with the compounds for 1 h at 37°C. After incubation, media was removed and the cells were washed once with media before fixation with 4% PFA in DPBS (10 min, RT, in the dark). Cells were washed twice with DPBS and permeabilized with 0.5% Triton-X100 in DPBS (5 min, RT). Cells were washed twice with PBST (0.1% Tween 20 in DPBS) and stained with 200 nM Cy5-Tetrazine (Jena Bioscience) in DPBS for 10 min at RT in the dark. Cells were washed 5 times with PBST and once with DPBS. Finally, cells were incubated with Hoechst (H3569, Life Technologies, 1:2000 in DPBS) for 10 min at RT in the dark and washed 3 times with DPBS. Cells were imaged in DPBS on a Zeiss NLO 780 microscope using a 63x oil objective. Images were processed and depicted with ImageJ.

As a control, the same imaging assay was also performed with HeLa cells in which IDO1 expression was not induced (Figure S23). After seeding in 8 well Lab-Tek™ chambered cover glass, cells were allowed to adhere overnight. They were not treated with IFNγ. The next day, they were incubated with the probes and competitor, fixed, permeabilized and stained with Cy5-tetrazine and Hoechst as described above.

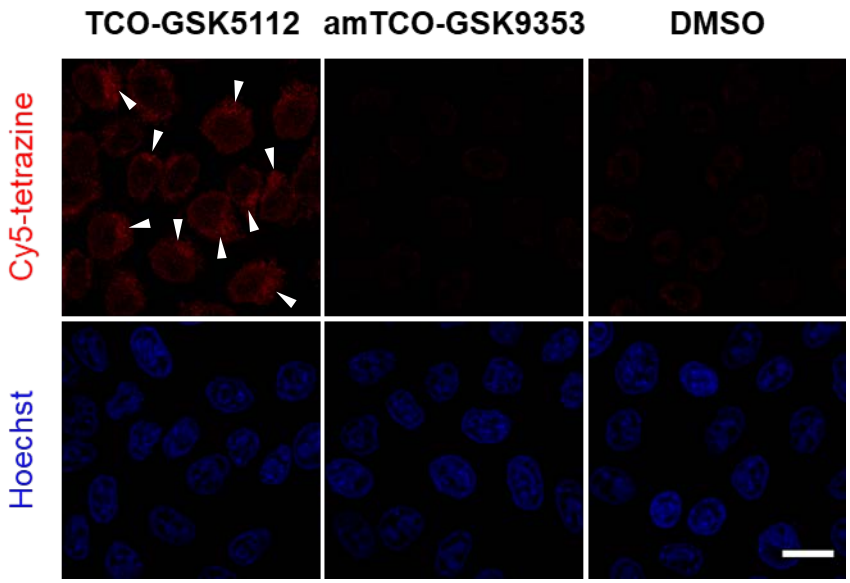


Figure S23. Imaging assay with non-induced HeLa cells (no IDO1 expression) – only unspecific accumulation of GSK5112 could be detected (white arrows). HeLa cells were incubated with 3 µM click probe for 1 h, followed by fixation, permeabilization, click reaction with 200 nM Cy5-Tetrazine and staining with Hoechst. Representative fluorescent images are shown (Cy5: λ_{ex} = 633 nm, λ_{em} = 638-690 nm; Hoechst: λ_{ex} = 405 nm, λ_{em} = 410-468 nm). Scale bar = 20 µm.

For the colocalization study, the protocol above was followed. In addition, cells were stained with:

- either Golgi Cytopainter (Abcam): immediately after fixation, incubation for 30 min at 4°C with 200 µL Golgi Cytopainter solution prepared as recommended by the supplier, followed by a washing with 200 µL 1x Cytopainter assay buffer and a washing with 200 µL PBS,
- or ER Cytopainter (Abcam): after staining with Cy5-tetrazine, incubation for 20 min at 37°C with 200 µL ER Cytopainter solution prepared as recommended by the supplier, followed by washing with 3 x 200 µL PBS.

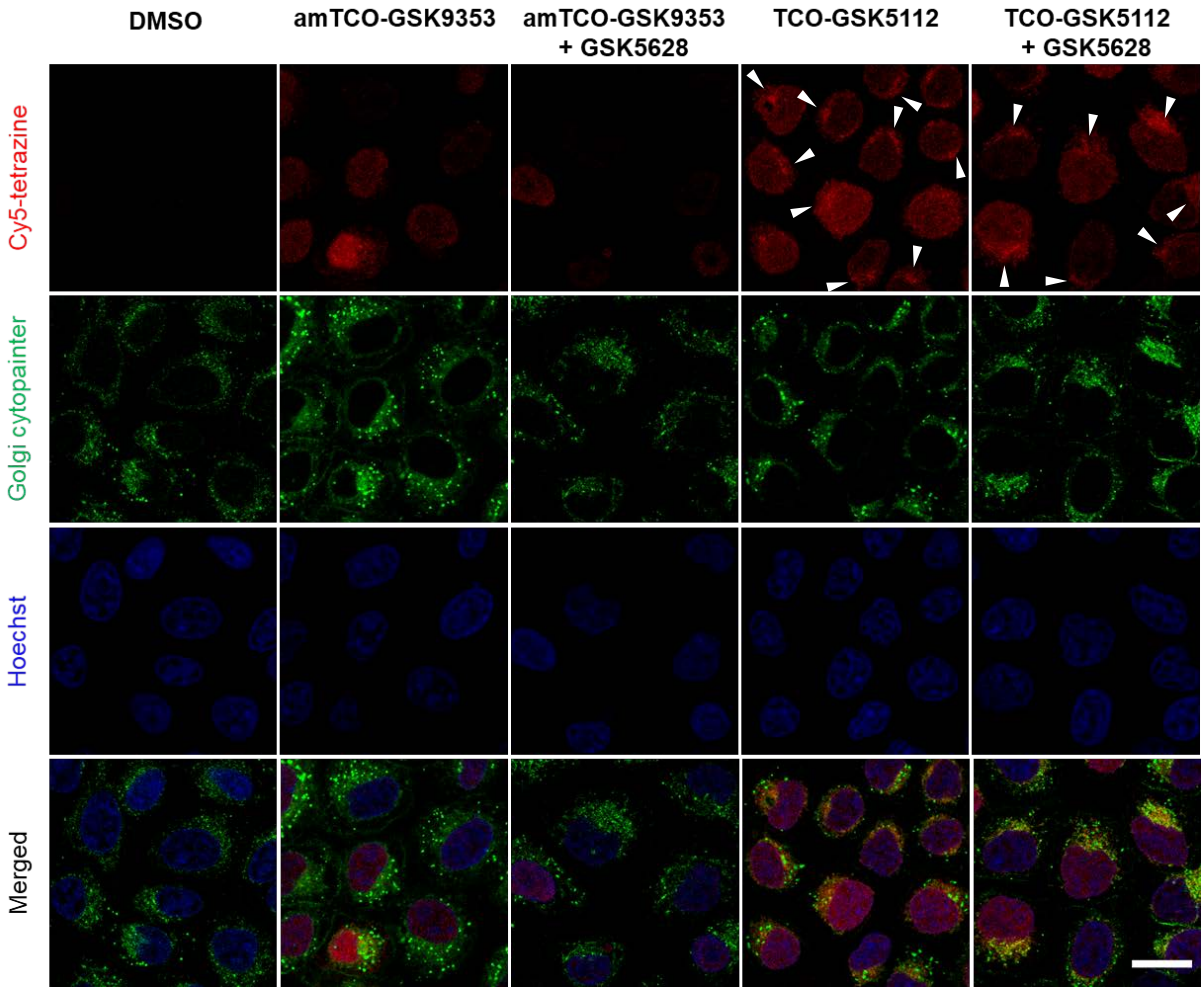


Figure S24. Colocalization study with Golgi Cytopainter. IFNγ-treated HeLa cells were incubated with 3 µM click probe ± GSK5628 for 1 h, followed by fixation, staining with Golgi cytopainter, permeabilization, click reaction with 200 nM Cy5-Tetrazine and staining with Hoechst. Representative fluorescent images recorded are shown (Cy5: λ_{ex} = 633 nm, λ_{em} = 638-690 nm; Golgi cytopainter: λ_{ex} = 488 nm, λ_{em} = 490-611 nm Hoechst: λ_{ex} = 405 nm, λ_{em} = 411-482 nm). Scale bar = 20 µm. Unspecific fluorescence staining of TCO-GSK5112 (white arrows) could be detected with and without competition and partially colocalized with Golgi staining.

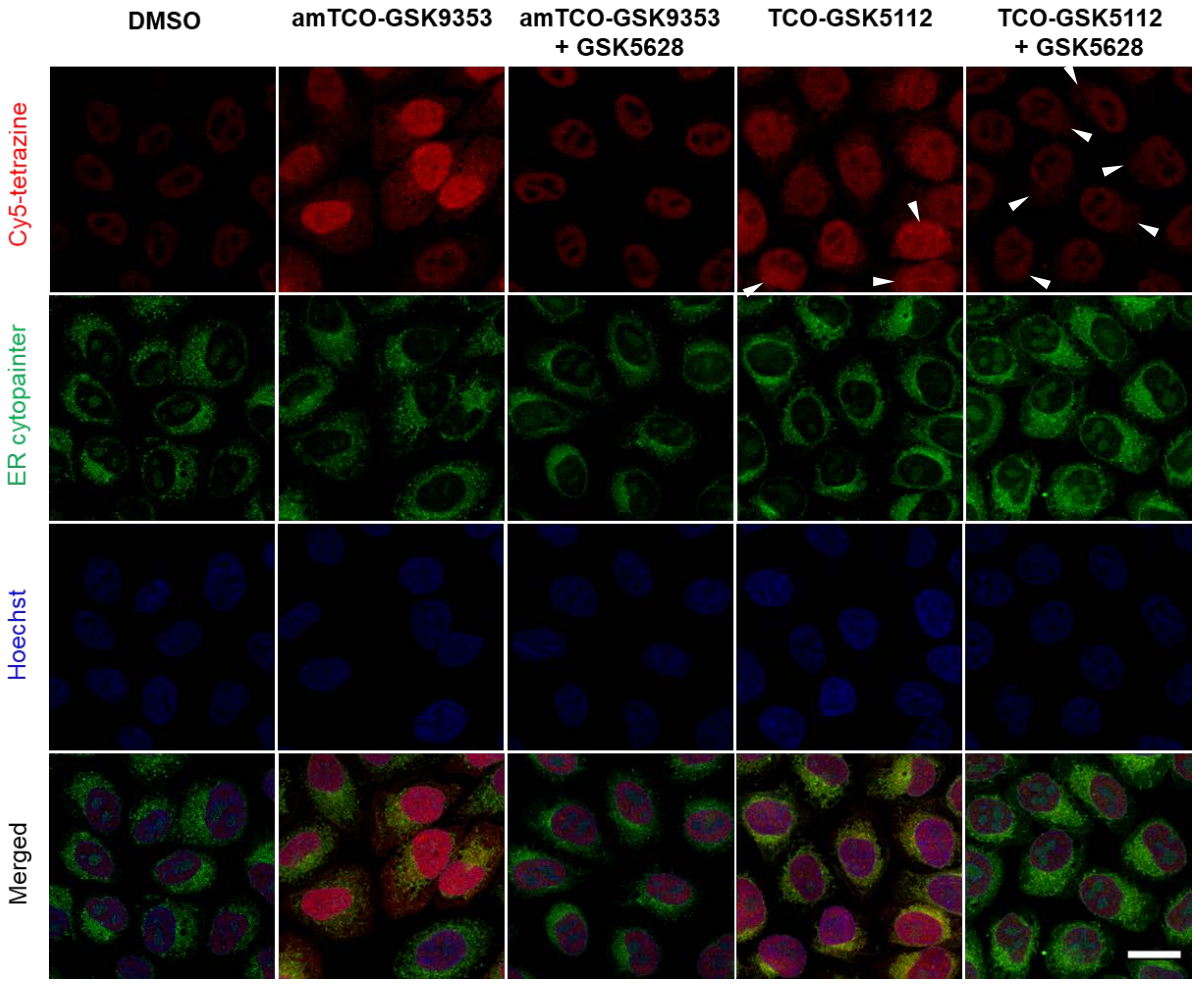


Figure S25. Colocalization study with ER Cytopainter. IFNγ-treated HeLa cells were incubated with 3 µM click probe ± GSK5628 for 1 h, followed by fixation, permeabilization, click reaction with 200 nM Cy5-Tetrazine, staining with ER cytopainter and Hoechst. Representative fluorescent images recorded are shown (Cy5: λ_{ex} = 633 nm, λ_{em} = 638-690 nm; ER cytopainter: λ_{ex} = 488 nm, λ_{em} = 490-611 nm Hoechst: λ_{ex} = 405 nm, λ_{em} = 411-482 nm). Scale bar = 20 µm. Unspecific fluorescence staining of TCO-GSK5112 (white arrows) could be detected with and without competition and partially colocalized with ER staining.

Comparison of TCO-GSK5112 and amTCO-GSK9353 in a competitive assay with quantification of Cy5 fluorescence signal by FACS

HeLa cells were cultured in MEM supplemented with 1% pyruvate, 1% NEAA and 10% FBS at 37°C with a humidified 5% CO_2 atmosphere. Cells (passage 4, 90% confluent) were washed with DPBS and treated with 0.05% trypsin-EDTA for 5 min at 37°C. Trypsin was neutralized by addition of cell culture media. The suspension was adjusted to 500 000 cells/mL and cells were seeded into a 6 cm tissue culture Petri dishes (3 mL per dish, 2 dishes per condition). Cells were allowed to adhere for 4 h at 37°C. Then the media was removed completely by aspiration and replaced with media containing 10 nM IFNγ (3 mL per dish). Cells were incubated at 37°C for 18 h in order to induce the expression of IDO1. The media was removed and replaced with 3 mL of media containing TCO-GSK5112 (3 µM), amTCO-GSK9353 (3 µM) or DMSO (0.1%) and GSK5628 (10 µM) where applicable. Cells were incubated with the compounds for 1 h at 37°C. After incubation, media was removed and the cells were washed once with DPBS. Cells were detached from the plate by treatment with 1 mL 0.05% trypsin-EDTA for 5 min at 37°C. Trypsin was neutralized by addition of 3 mL of media. Cells were transferred into 15 mL Falcon

tubes and centrifuged at 1200 RPM for 5 min. The supernatant was removed by aspiration and cells were resuspended in 4% PFA in DPBS. Cells were incubated with PFA for 10 min at RT in the dark. During fixation, they were distributed into FACS tubes. Each sample was distributed into 3 tubes to obtain technical replicates. Cells were washed twice with DPBS and were permeabilized with 0.5% Triton-X100 in DPBS (5 min, RT). Following on permeabilization, cells were centrifuged for 3 min at 1800 RPM after each washing step. They were washed twice with PBST and then stained with 200 nM Cy5-Tetrazine (Jena Bioscience) in DPBS for 10 min at RT in the dark. Finally, cells were washed 3 times with PBST and once with DPBS. They were resuspended in 200 µL DPBS for FACS acquisition. Flow cytometric analysis was performed on a FACSCalibur instrument (BD Biosciences, San Jose, CA). A total of 50,000 events were acquired for each sample. Cells were excited with a red laser (635 nm) and Cy5 fluorescence was detected using a 660/20 band pass filter (FL4-H channel). Data were analysed using FlowJo software (version 7.6.5). A gate for Cy5-positive cells was defined in a FL1-H/FL4-H scatter plot (FL1-H detecting cell autofluorescence), and the percentage of Cy5-positive cells among all cells was calculated based on the number of events recorded within that gate. Three independent experiments were performed, each of them with 3 technical replicates. A representative experiment is reported in Figure 4B, the other replicates are shown in Figure S27. The error bars represent standard deviations from 3 technical replicates.

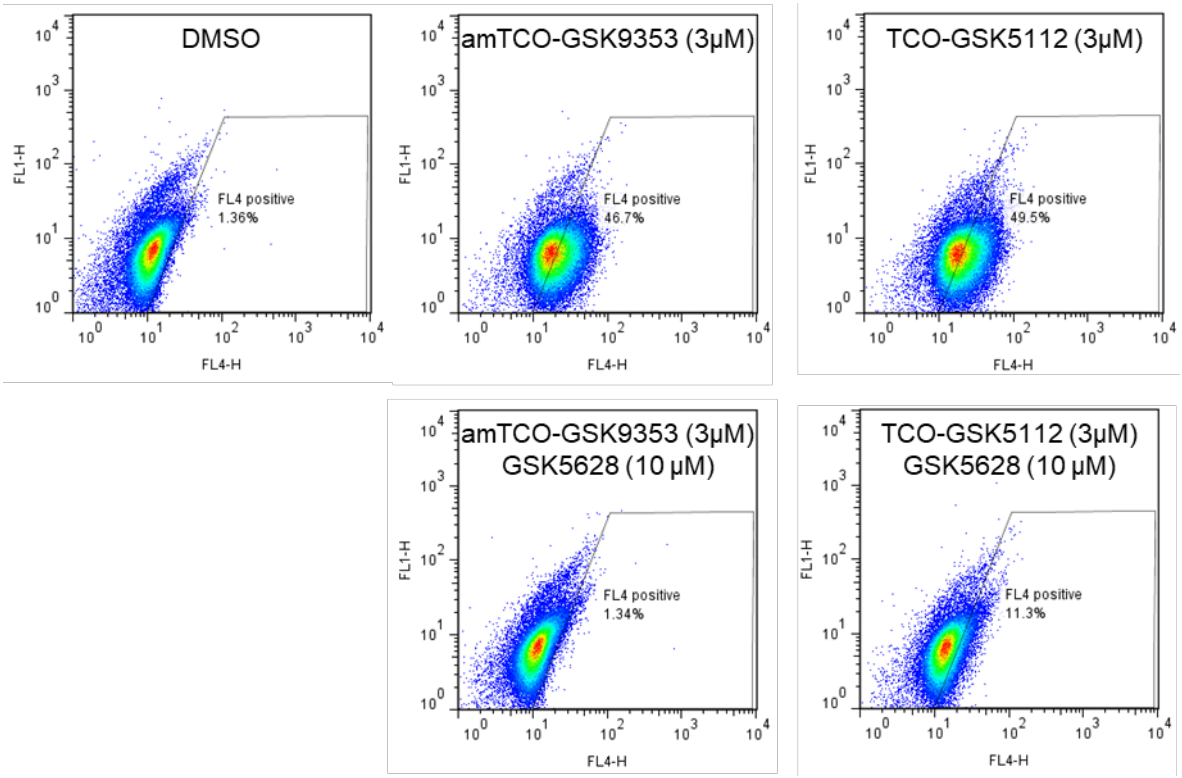


Figure S26. Representative scatter plots for the competitive assay with FACS read-out.

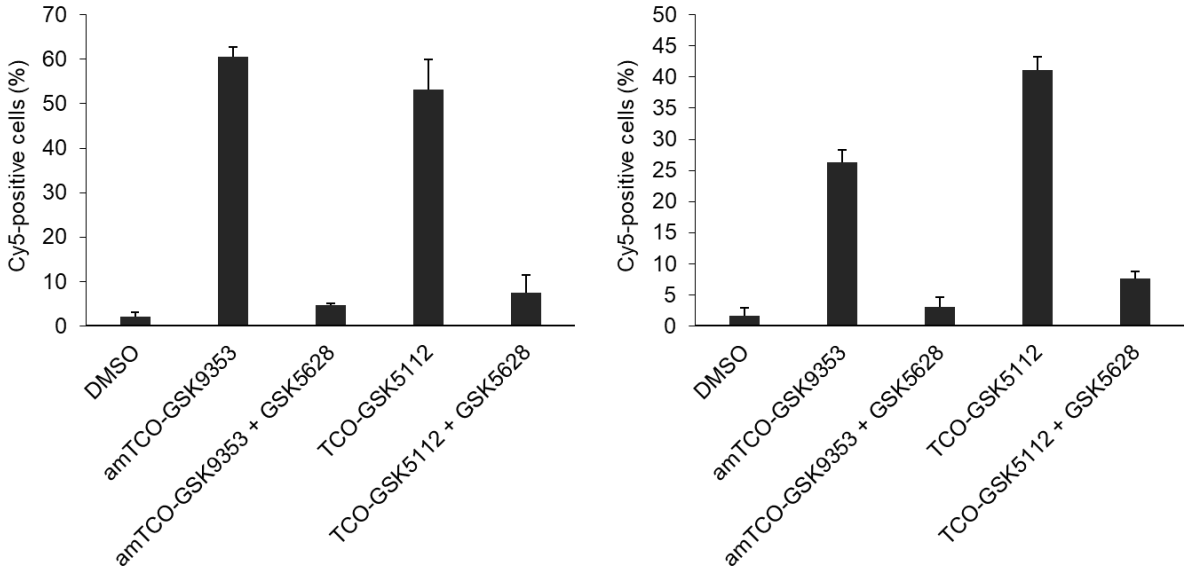


Figure S27. Quantification of Cy5 fluorescence signal in IFNγ-induced HeLa cells treated with 3 µM click probe ± GSK5628 for 1 h, followed by fixation, permeabilization, click reaction with 200 nM Cy5-tetrazine and analysis by flow cytometry. Data from two independent experiments (3rd experiment shown in Figure 4B).

Target engagement measurements

HeLa cells (passage 6, 90% confluent) were treated with 1 µM amTCO-GSK9353 and increasing concentrations of GSK5628 (0-10 µM) following the protocol reported above for the competition assay. Cells were trypsinised, fixed, permeabilised and stained as described above. Flow cytometric analysis was performed as reported in the previous method. Data were analysed using FlowJo software (version 7.6.5). A gate for Cy5-positive cells was defined in a FL1-H/FL4-H scatter plot (FL1-H detecting cell autofluorescence), and the percentage of Cy5-positive cells among all cells was calculated based on the number of events recorded within that gate. Three independent experiments were performed, each of them with two technical replicates. One representative experiment is shown in Figure 4C, the other replicates are shown in Figure S29A. The error bars represent standard deviations from two technical replicates. The data were analyzed using GraphPad Prism software version 6.07. Percentage inhibition was calculated using positive and negative controls as 100% and 0% inhibition, respectively. Concentration-response curves were fitted using a four-parameter non-linear regression fitting module.

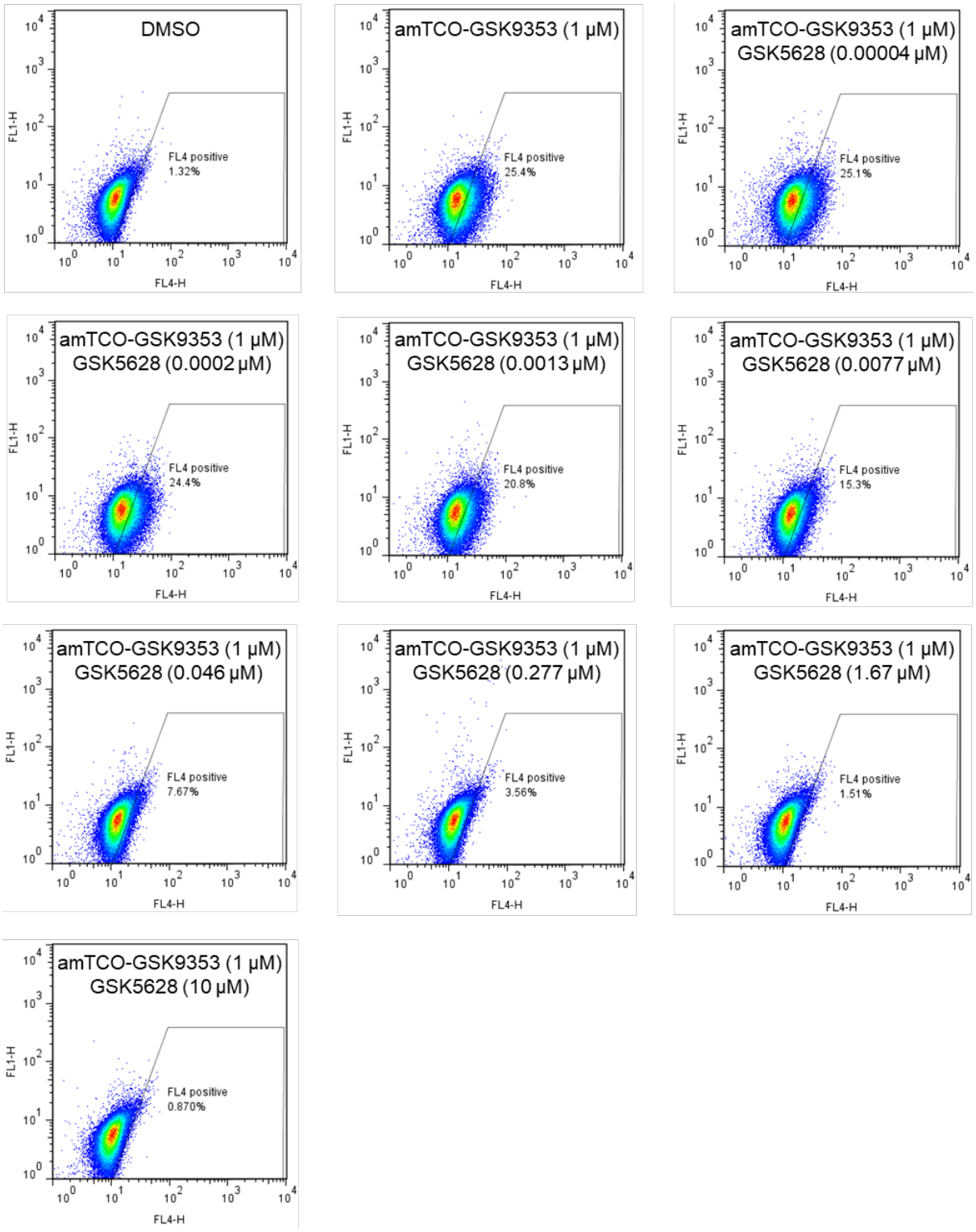


Figure S28. Representative scatter plots for the target occupancy assay with FACS read-out.

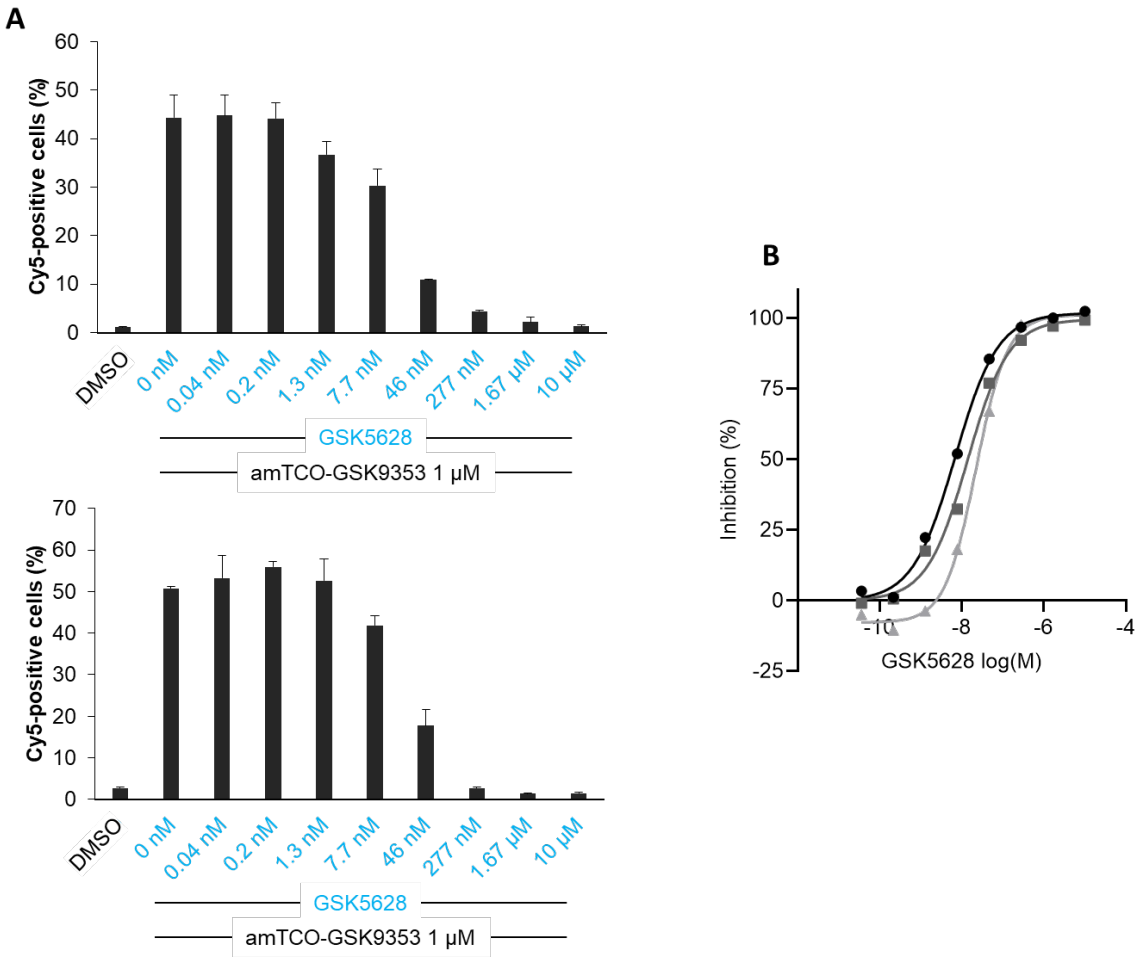


Figure S29. (A) Flow cytometric analysis of IFNγ-induced HeLa cells treated with 1 µM amTCO-GSK9353 and increasing concentrations of GSK5628, followed by trypsinization, fixation, permeabilization and click reaction with Cy5-tetrazine (2nd and 3rd biological replicates, 1st replicate is shown in Figure 4C). (B) Competition binding curves of GSK5628 derived from three independent experiments.

References

1 K. Valkó, C. Bevan and D. Reynolds, *Anal. Chem.*, 1997, **69**, 2022–2029.

2 S. B. Bunally, C. N. Luscombe and R. J. Young, *SLAS Discov. Adv. Sci. Drug Discov.*, 2019, **24**, 791–801.

3 D. Svatunek, C. Denk, V. Rosecker, B. Sohr, C. Hametner, G. Allmaier, J. Fröhlich and H. Mikula, *Monatshefte Chem.*, 2016, **147**, 579–585.

4 World Intellectual Property Organization, WO2017051354A1, 2017.

5 R. F. Ortiz-Meoz, L. Wang, R. Matico, A. Rutkowska, M. De la Rosa, S. Bedard, R. Midgett, K. Strohmer, D. Thomson, C. Zhang, M. Mebrahtu, J. Guss, R. Totoritis, T. Consler, N. Campobasso, D. Taylor, T. Lewis, K. Weaver, M. Mulbaier, J. Seal, R. Dunham, W. Kazmierski, D. Favre, G. Bergamini, L. Shewchuk, A. Rendina and G. Zhang, *ChemBioChem*, , DOI:10.1002/cbic.202000298.

6 World Intellectual Property Organization, WO2018116107A1, 2018.

7 G. Beliu, A. J. Kurz, A. C. Kuhlemann, L. Behringer-Pliess, M. Meub, N. Wolf, J. Seibel, Z.-D. Shi, M. Schnermann, J. B. Grimm, L. D. Lavis, S. Doose and M. Sauer, *Commun. Biol.*, 2019, **2**, 1–13.

8 A. Rutkowska, D. W. Thomson, J. Vappiani, T. Werner, K. M. Mueller, L. Dittus, J. Krause, M. Muelbaier, G. Bergamini and M. Bantscheff, *ACS Chem. Biol.*, 2016, **11**, 2541–2550.