Supplementary Information

Expedited mapping of the ligandable proteome using fully functionalized enantiomeric probe pairs

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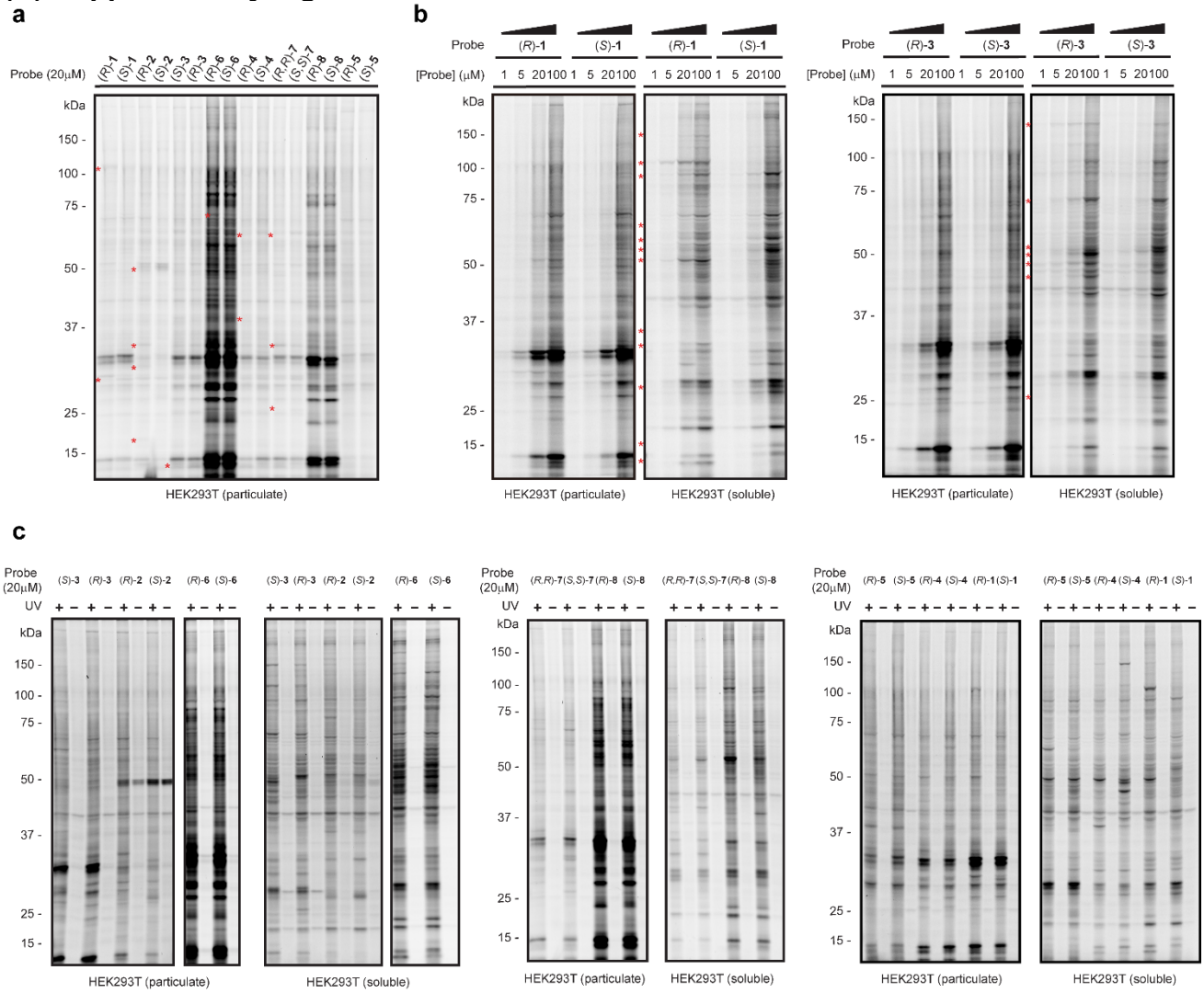
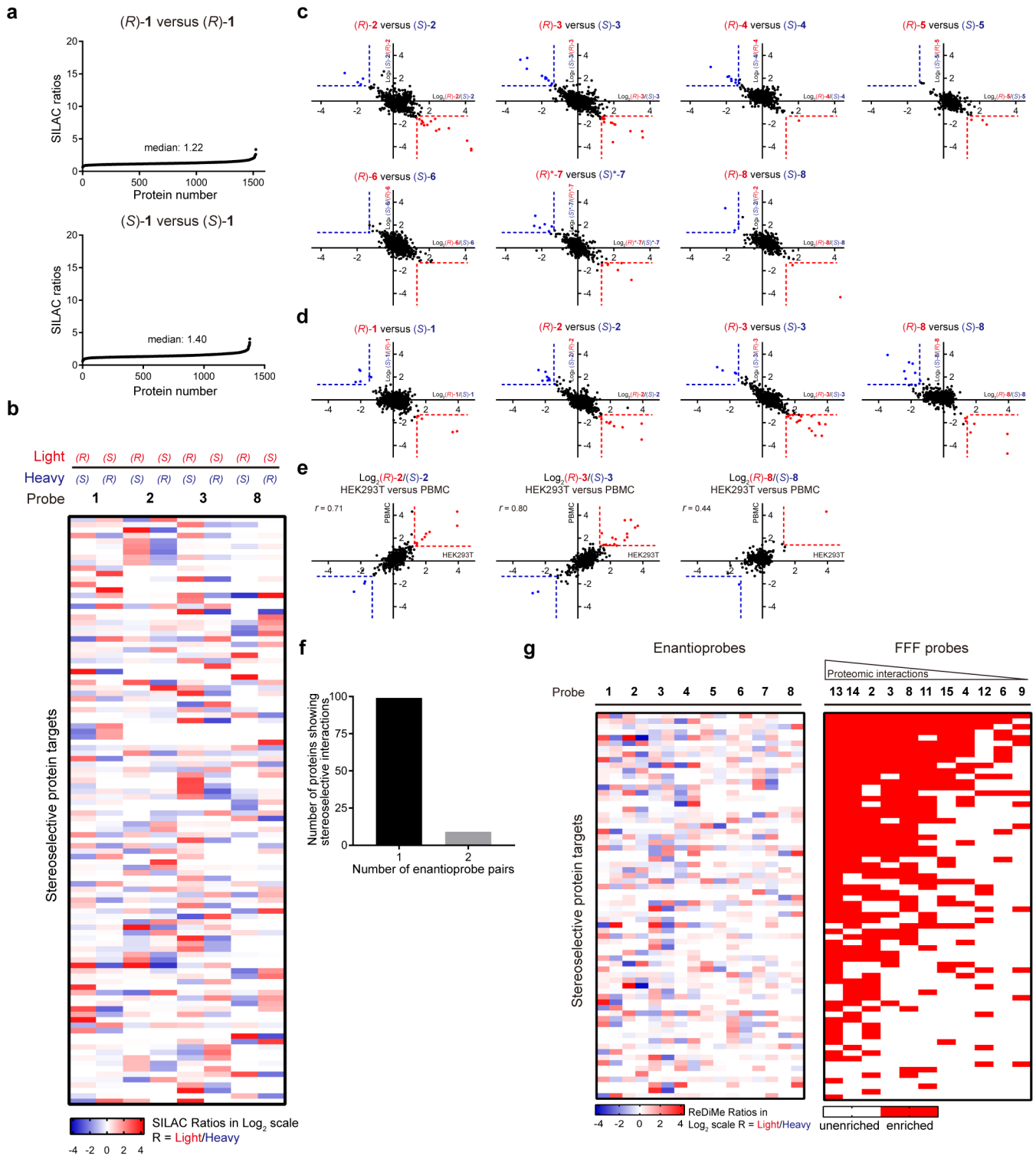
(A) Supplementary Figures

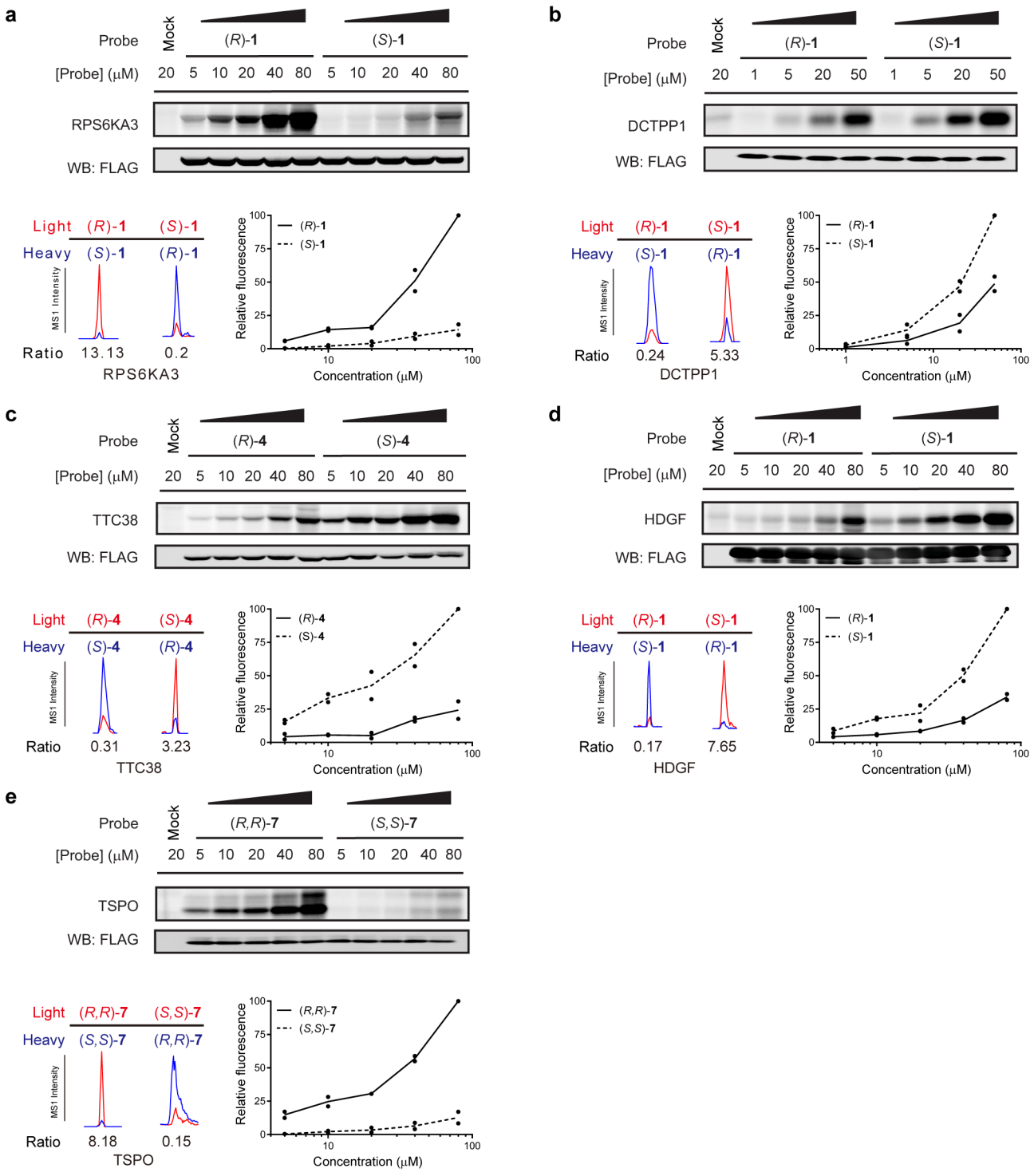


Supplementary Figure 1. Gel-based profiling of enantioprobe-protein interactions in human cells. a, Particulate fraction of gel-based profiles shown in **Fig. 1c.** Red asterisks mark representative stereoselective protein-enantioprobe interactions. **b**, Enantioprobes show concentration-dependent increases in protein labeling in HEK293T cells. **c**, Enantioprobes show UV-dependent protein labeling in HEK293T cells. Gel images reflect representative results from two independent experiments.



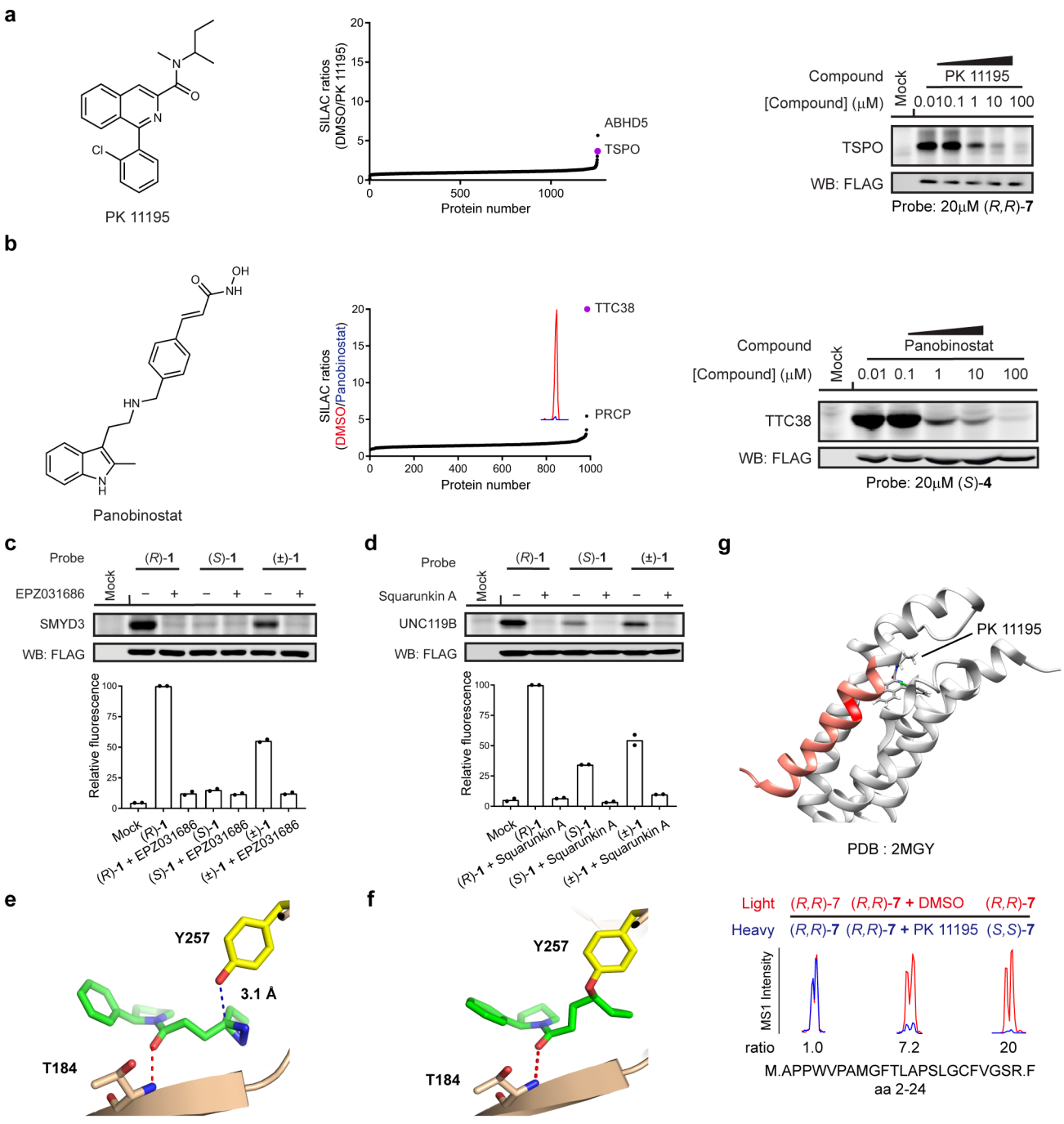
Supplementary Figure 2. MS-based profiling of enantioprobe-protein interactions in human cells. a, Representative SILAC ratio plots for control experiments in which isotopically heavy and light amino acid-labeled HEK293T cells were treated with the same enantioprobe. Results are from an individual experiment representative of two independently performed experiments. **b**, Heatmap showing relative protein enrichment ratios in both isotopic directions for the indicated enantioprobe pairs in HEK293T cells. White signals in the heatmap either correspond to proteins with ratio values of ~ 1 or proteins that were not enriched and quantified with the indicated enantioprobe pair. **c**, **d**, Related to **Fig. 2c.** Scatter plots showing protein enrichment ratios for all enantioprobe pairs in primary human PBMCs (**c**) or HEK293T cells (**d**).

Targets enriched > 2.5-fold by the (*R*) or (*S*)-member of an enantioprobe pair are shown in red and blue, respectively. (*R*)* and (*S*)*- represent (*R,R*) and (*S,S*)- for enantioprobe **7**. Data reflect an average of two independently performed experiments for each isotopic direction that provided similar results. **e**, Related to **Fig. 2d**. Similar stereoselective interactions are observed in different cell types. Plots depict Log_2 values of protein enrichment ratios for indicated enantioprobe pairs in HEK293T cells (x-axis) versus PBMCs (y-axis). *r* is Pearson correlation coefficient. Data reflect an average of two independent experiments that provided similar results. **f**, Related to **Fig. 2f**. Number of proteins showing stereoselective interactions with the indicated number of enantioprobe pairs in HEK293T. **g**, Heatmaps showing relative protein enrichment values for FFF probes (200 μM) versus a methyl control probe (200 μM) for stereoselective protein targets of enantioprobes. FFF profiles were taken from reference 1. White signals in the heatmap either correspond to proteins with ratio values of ~1 or proteins that were not enriched and quantified with the indicated enantioprobe pair or FFF probe.



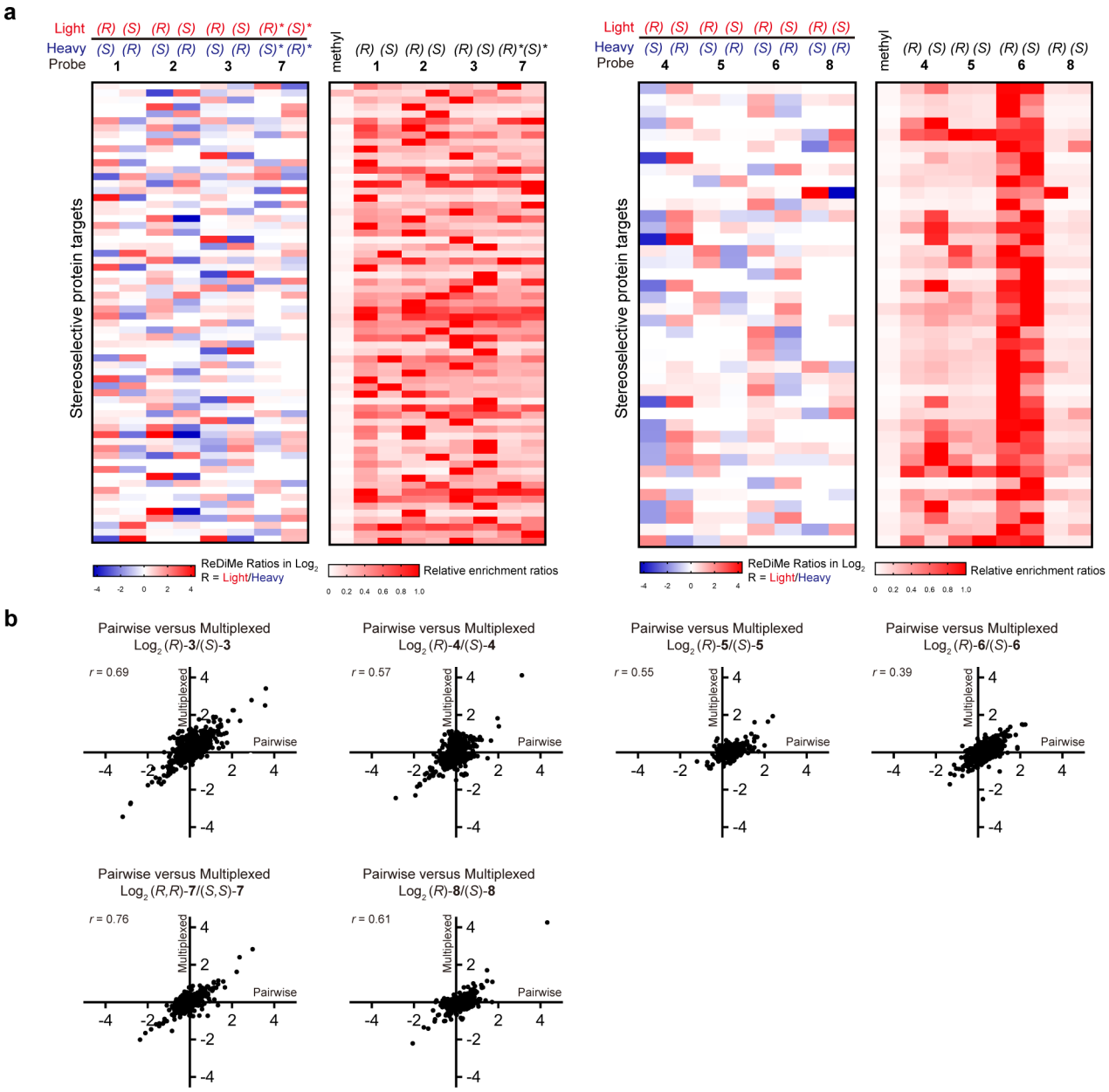
Supplementary Figure 3. Characterization of additional stereoselective protein targets of enantioprobes. a-e, Top: Confirmation of stereoselective enantioprobe-protein interactions with recombinantly expressed proteins. RPS6KA3 (**a**), DCTPP1 (**b**), TTC38 (**c**), HDGF (**d**) and TSPO (**e**) were recombinantly expressed with FLAG epitope tags by transient transfection in HEK293T cells, and transfected cells were then treated with the indicated concentrations of enantioprobes, photocrosslinked, lysed, and proteomes conjugated to an azide-rhodamine tag by CuAAC chemistry and analyze by SDS-PAGE and in-gel fluorescence scanning. Gel images reflect representative results from two independently

performed experiments. Bottom left: Extracted MS1 chromatograms of representative tryptic peptides for endogenous forms of the protein targets in HEK293T cells or PBMCs treated with indicated enantioprobes (200 μM). Bottom right: Quantification of protein labeling by the indicated enantioprobes derived from gel-based profiles show in Top section. Data reflect results from two independently performed experiments that provided similar results.

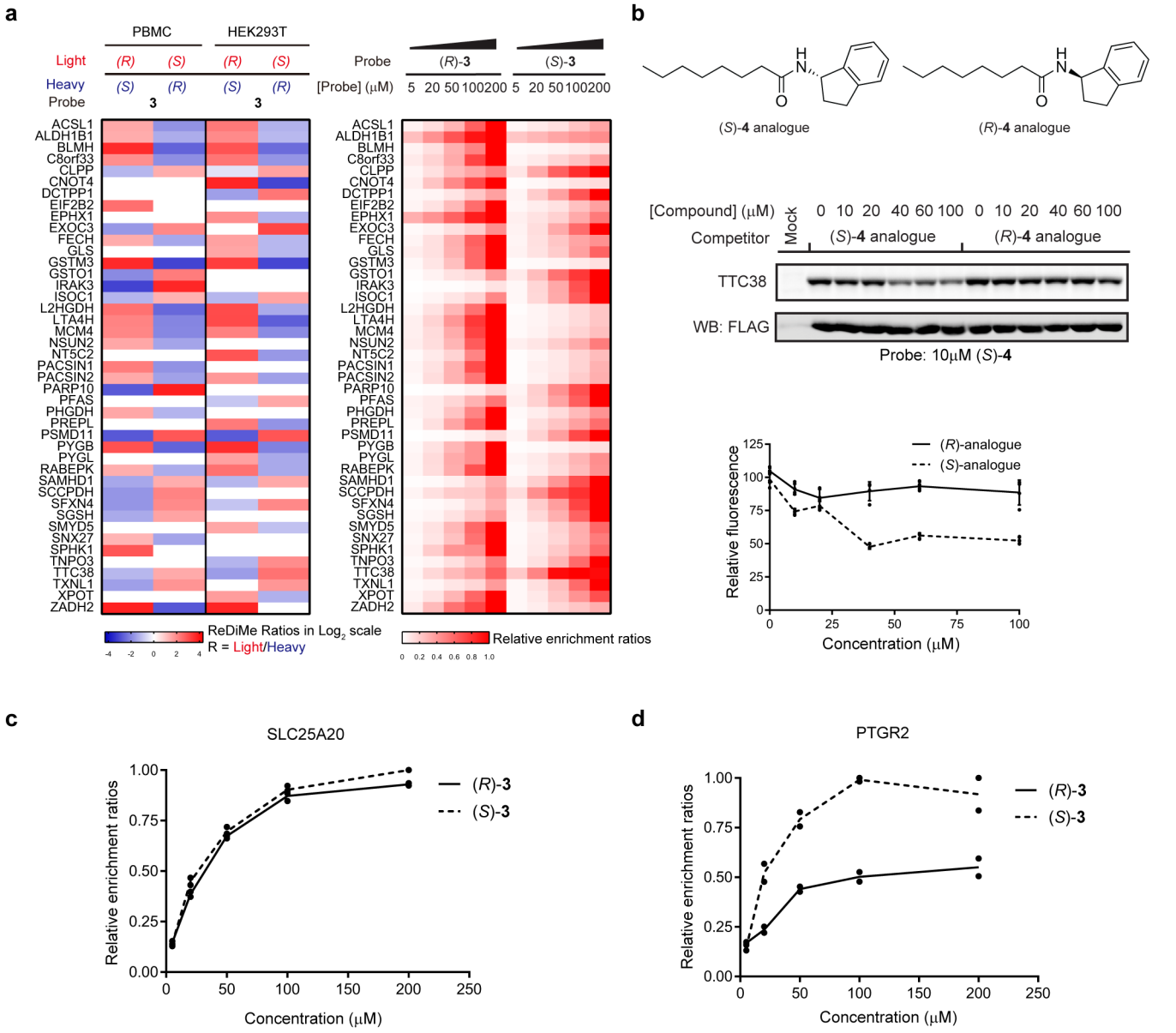


Supplementary Figure 4. Stereoselective interactions occur at functional and druggable sites on protein targets of enantioprobes. a-b, Left: structure of competitor ligands (**a**) PK 11195; (**b**) panobinostat. Middle: Waterfall plots of competitive blockade of enantioprobe (200 μM) interactions with endogenous protein targets for corresponding ligands (20 μM) in HEK293T cells. Data reflect an average of two independent experiments that provided similar results. Inset shows representative MS1 chromatogram for TTC38. Right: Gel-based profiles of competitive blockade of enantioprobe interactions with recombinantly expressed protein targets for corresponding ligands in transfected HEK293T cells. Gel images reflect representative results from two independent experiments. **c-d**, Top: Gel-based profiles of competitive blockade of interactions of enantioprobe (*R*)-**1**, (*S*)-**1**, and a racemic mixture of these enantioprobes with recombinant proteins SMYD3 (**c**) and UNC119B (**d**) expressed with FLAG epitope tags

by transient transfection in HEK293T cells. Transfected cells were treated with the indicated enantioprobes (80 μM, or, for racemic mixture, 40 μM of each enantioprobe) and EPZ031686 (**c**) or squarunkin A (**d**) (20 μM each), photocrosslinked, lysed, and proteomes conjugated to an azide-rhodamine tag by CuAAC chemistry and analyze by SDS-PAGE and in-gel fluorescence scanning. Gel images reflect representative result sfrom two independent experiments. Bottom: Quantification of protein labeling by the indicated enantioprobes derived from gel-based profiles show in Top section. Data reflect two independent experiments. **e**, Binding mode of (*R*)-**1** (green sticks) with SMYD3 (PDB 5CCM, brown cartoons) resulting from conventional docking simulations, with the distance between the diazirine moiety and the Oη of Y257 (yellow sticks) depicted as black dashed line. **f**, Covalent docking pose of (*R*)-**1** bound to Y257 (yellow sticks). **g**, Structure of TSPO in complex with PK 11195 (shown as stick model; PDB 2MGY) highlighting (*R,R*)-**7**-modified tryptic peptide (aa 2-24, light red; predicted probe-modified residue C19, dark red).



Supplementary Figure 5. Multiplexed MS-based quantification for expedited discovery of stereoselective protein-enantioprobe interactions. **a**, Related to **Fig. 5b**. Heatmaps showing enantioprobe enrichment profiles for stereoselective protein targets in multiplexed versus pairwise experiments in PBMCs. White signals in the pairwise heatmap either correspond to proteins with ratio values of ~ 1 or proteins that were not enriched and quantified with the indicated enantioprobe pair. (*R*)* and (*S*)*- represent (*R,R*) and (*S,S*)- for enantioprobe **7**. **b**, Related to **Fig. 5d**. Scatter plots showing the correlation between pairwise and multiplexed experiments performed with the indicated enantioprobe pairs in PBMCs. *r* is Pearson correlation coefficient. Data reflect an average of two independently performed experiments that provided similar results.



Supplementary Figure 6. Concentration-dependent profiles for enantioprobe pair (*R*)-3 and (*S*)-3 in PBMCs. a, Heatmap showing concentration-dependent profiles for stereoselective protein targets of (*R*)-**3** and (*S*)-**3** in PBMCs, at 5, 20, 50, 100, and 200 μM of the enantioprobes (right). The left heatmap shows the profiles for stereoselective protein targets in pairwise experiments performed with the (*R*)-**3** and (*S*)-**3** enantioprobes (200 μM each). White signals in the heatmap either correspond to proteins with ratio values of ~ 1 or proteins that were not enriched and quantified with the indicated enantioprobe pair. **b**, Top: Structure of non-crosslinking (*S*)- and (*R*)-**4** analogues, which consist of the recognition element of enantioprobe **4** and an octanamide group in place of the diazirine-alkyne handle of the enantioprobes. Middle: Representative gel-based profile showing competitive blockade of enantioprobe (*S*)-**4** interactions with recombinant TTC38 by non-crosslinking (*S*)- and (*R*)-**4** analogues in HEK293T cells. Bottom: Quantification of protein labeling by (*S*)-**4** derived from gel-based profiles shown in Middle section. Data represent average values ± SD for four independent experiments. **c**, **d**, Concentration-dependent profiles for representative proteins SLC25A20 (**c**) and PTGR2 (**d**) displaying saturated enrichment signals across the test probe concentration range (5-200 μM). Data reflect two independently performed experiments.

(B) Supplementary Table

Supplementary Table 1. Representative stereoselective protein targets of enantioprobes in human cells.

UniPROT ID	Protein name	Protein type	Cell type	Stereoselective enantioprobe(s)	Highest stereoselective ratio
Q9NUJ1	ABHD10	serine hydrolase	PBMCs	(*R*)-**7**	5.3
Q15018	ABRAXAS2	scaffolding protein	PBMCs, HEK293T	(*R*)-**8**	20
P04083	ANXA1	inflammatory modulator	PBMCs, HEK293T	(*R*)-**2**	3.9
Q13867	BLMH	cysteine endopeptidase	PBMCs, HEK293T	(*R*)-**3**	9.1
P24941	CDK2	protein kinase	PBMCs	(*R*)-**7**	3.9
O95628	CNOT4	E3 ubiquitin ligase	HEK293T	(*R*)-**3**	11
Q9H773	DCTPP1	pyrophosphatase	PBMCs, HEK293T	(*S*)-**1**, (*S*)-**3**, (*S*)-**4**	10
Q13045	FLII	nuclear receptor coactivator	HEK293T	(*R*)-**1**	2.8
P51858	HDGF	transcriptional repressor	PBMCs, HEK293T	(*S*)-**1**	6.3
Q5T447	HECTD3	E3 ubiquitin ligase	HEK293T	(*S*)-**8**	10
O95373	IPO7	nuclear transport receptor	HEK293T	(*R*)-**8**	3.2
Q9Y616	IRAK3	pseudokinase	PBMCs	(*S*)-**3**, (*S*)-**4**	15
Q08J23	NSUN2	RNA methyltransferase	PBMCs	(*R*)-**3**	2.9
Q9UNF0	PACSIN2	membrane-binding protein/adaptor protein	PBMCs, HEK293T	(*R*)-**3**, (*R*)-**6**	4.4
Q53GL7	PARP10	NAD+ ADP-ribosyltransferase	PBMCs	(*S*)-**3**, (*S*)-**4**	20
Q9UBV8	PEF1	calcium-dependent adapter	PBMCs, HEK293T	(*S*)-**1**	4.7
O00329	PIK3CD	phosphoinositide-3-kinase	PBMCs	(*R*)-**2**	3.8
O75475	PSIP1	transcriptional coactivator	PBMCs	(*S*)-**1**	3.7
P63244	RACK1	scaffolding protein	PBMCs, HEK293T	(*R*)-**2**, (*R*)-**7**	3.5
P51812	RPS6KA3	protein kinase	PBMCs, HEK293T	(*R*)-**1**, (*R*)-**2**, (*S*)-**4**	20
Q8WWI5	SLC44A1	choline transporter	PBMCs	(*R*)-**2**	14
Q9H7B4	SMYD3	protein methyltransferase	PBMCs, HEK293T	(*R*)-**1**, (*R*)-**5**	7.0
Q5BJF2	TMEM97	orphan receptor	HEK293T	(*S*)-**8**	7.0
P30536	TSPO	cholesterol transporter	PBMCs	(*R*)-**4,** (*R*)-**7**	7.5
A6NIH7	UNC119B	lipid-binding protein	PBMCs	(*R*)-**1**	2.9
O95619	YEATS4	succinyl-lysine reader	HEK293T	(*R*)-**3**	9.5

(C) Supplementary Dataset Legends

Supplementary Dataset 1. Compiled stereoselective protein targets for enantioprobe pairs **1** – **8** in primary human peripheral blood mononuclear cells (PBMCs) and HEK293T cells. (**Tab 1**) Averaged (3 biological replicates for enantioprobe pair **1**, 4 biological replicates for enantioprobe pair 3, and 2 biological replicates for remainder of enantioprobes) ReDiMe ratios of stereoselective protein targets obtained from pairwise experiments conducted in PBMCs at 200 μM of indicated enantioprobes. (**Tab 2**) Averaged (2 biological replicates) SILAC ratios of stereoselective protein targets obtained from pairwise experiments conducted in HEK293T cells at 200 μM of indicated enantioprobes. (**Tab 3**) Averaged (2 biological replicates) relative enrichment ratios of stereoselective protein targets obtained from multiplexed experiments conducted in PBMCs and HEK293T cells at 200 μM of indicated enantioprobes. Relative enrichment ratios are calculated as a percent of maximum signal per protein. (**Tab 4**) Stereoselective protein targets from pairwise experiments referenced with their relative enrichment ratios from multiplexed experiments. (**Tab 5**) Averaged (2 biological replicates) concentration-dependent relative enrichment values of high-engagement protein targets of (*R*)-**3** and (*S*)-**3** in PBMCs, at 5, 20, 50, 100 and 200 μM of the enantioprobes. High-engagement protein targets are defined as proteins with relative enrichment values greater than 50% at 50 μM and 75% at 100 μM for either (*R*)-**3** or (*S*)-**3**. (**Tab 6**) Averaged (2 biological replicates) concentration-dependent relative enrichment values of stereoselective protein targets of (*R*)-**3** and (*S*)-**3** in PBMCs or HEK293T cells, at 5, 20, 50, 100 and 200 μM of the enantioprobes. (**Tab 7**) Tissue-specific patterns of mRNA expression of stereoselective protein targets discovered solely in PBMCs with enantioprobes **1**, **2**, **3** and **8**. Protein targets with 3 × median mRNA expression amongst lymphoblast, B cells, T cells, dendritic cells, NK cells, monocytes or myeloid cells are considered as enriched in immune-related cells. (**Tab 8**) Averaged (2 biological replicates) SILAC ratios of proteins from HEK293T cells treated with the same indicated enantioprobes in HEK293T cells at 200 μM. The mean of median SILAC ratios was used as the normalization factor to correct incomplete incorporation of heavy amino acids in SILAC experiments.

Supplementary Dataset 2. Data sets from SILAC/ReDiMe experiments. (**Tab 1**) Summary of experimental design for pairwise experiments included in Supplementary Table 2. (**Tab 2-31**) Each tab displays the relative light/heavy ratios for all quantified tryptic peptides per protein from each replicate.

Supplementary Dataset 3. Data sets from TMT experiments. (**Tab 1**) Summary of experimental design for multiplexed experiments included in Supplementary Table 3. (**Tab 2-5**) Averaged (2 biological replicates) relative enrichment ratios of quantified proteins obtained from multiplexed experiments conducted in PBMCs and HEK293T cells at 200 μM of indicated enantioprobes. Relative enrichment ratios are calculated as a percent of maximum signal per protein. (**Tab 6**) Averaged (2 biological replicates) relative enrichment values of quantified protein targets of (*R*)-**3** and (*S*)-**3** in PBMCs, at 5, 20, 50, 100 and 200 μM of the enantioprobes. (**Tab 7-16**) Each tab displays the averaged reporter ions intensity for all quantified proteins from each replicate.

(D) Biological Methods

Cell lines and primary cells
HEK293T cells were maintained in high-glucose DMEM (Corning, 15-013-CV) supplemented with 10% (v/v) fetal bovine serum (FBS, Omega Scientific, FB-01), penicillin (100 U/mL) and streptomycin (100 μg/mL) and L-glutamine (2 mM, Corning, 25-005-CI). Cells were grown at 37 °C in a humidified 5% CO_2 atmosphere. For SILAC experiments, HEK293T cells was passaged at least six times in SILAC DMEM (Thermo), which lacks L-lysine and L-arginine, and supplemented with 10% (v/v) dialyzed FBS (Omega Scientific, FB-03), penicillin, streptomycin (as above), and either [$^{13}C_6$, $^{15}N_2$]- L-lysine (Sigma Aldrich, 608041) and [$^{13}C_6$, $^{15}N_4$]-L-arginine (Sigma Aldrich, 608033) (100 μg/mL each) or L-lysine, HCl and L-arginine, HCl (100 μg/mL each). Heavy and light cells were maintained in parallel and cell aliquots were frozen after six passages in SILAC media and stored in liquid N_2 until needed. Whenever thawed, cells were passaged at least twice before being used in experiments.

All studies using samples from human volunteers follow protocols approved by the TSRI institutional review board. Human blood from healthy donors were obtained after informed consent and peripheral blood mononuclear cells (PBMCs) were purified over Lymphoprep™ (STEMCELL, Catalog # 07861) density gradient medium according to manufacturer's protocol. The purified PBMCs were washed with sterile DPBS (Corning, 21-031-CV) and resuspended in RPMI-1640 (Corning, 15-040-CV) supplemented with penicillin, streptomycin and glutamine (as above) before being used in experiments.

In situ labeling of live cells with enantioprobes
Experiments were performed similarly as previously reported[1]. For gel-based experiments, HEK293T cells were grown in 6-well plates (Olympus, Cat #: 25-105) to near complete confluence before treatment. Cells were washed once with DPBS and replenished with 1mL fresh serum-free media before treated with enantioprobes, and, if applicable, competitors or DMSO vehicle. The treated cells were incubated at 37 °C for 30 min, exposed to 365 nm UV light (Stratagene, UV Stratalinker 1800) for 10 min in a cold room. For no-UV control experiments, cells were placed at 4 °C in the cold room for 10 min under ambient light. Following photocrosslinking, cells were harvested by scraping, centrifuged (1,400 g, 4 min, 4 °C), and washed with cold DPBS (2X). Pellets were either directly used for analysis or kept frozen at - 80 °C until use. For MS-based experiments, HEK293T cells were treated similarly as above with minor modifications, including cells were grown in 6 cm plates (Olympus, Cat # 25-260) in 'heavy' or 'light' SILAC DMEM media, and treated in 1.5 mL fresh serum-free media. Human PBMCs were obtained as described above, resuspended in serum-free RPMI-1640 media and seeded in 6-well plates immediately before treated similarly as HEK293T cells.

Preparation of proteome for gel- and MS-based analysis
Cell pellets were lysed in cold DPBS (100 – 400 μL) using a Branson Ultrasonics Sonifier S-250A cell disruptor (~6 pluses, 35% duty cycle, output setting =3.5). For experiments with soluble and particulate fraction of proteomes, cell lysates were centrifuged (100,000 g, 20 min) to provide soluble (supernatant) and particulate (pellet) fractions. Particulate fractions were resuspended in cold DPBS with sonication. Protein concentration was determined with the DC Protein Assay (Bio-Rad) and the absorbance was measured using a CLARIOstar microplate reader following manufacturer's instructions. Protein concentration was diluted to 1.5 mg/mL with cold DPBS for further use.

Gel-based analysis of probe labelled proteins in cells
To each sample (1.5 mg/mL, 50 μL), 6 μL of freshly prepared 'click' mixture (3 μL of 1.7 mM TBTA in 4:1 *t*-BuOH:DMSO, 1 μL of 50 mM $CuSO_4$ in H_2O, 1 μL of 1.25 mM Tetramethylrhodamine (TAMRA) azide in DMSO, 1 μL of freshly prepared 50 mM TCEP in DPBS) was added. The reaction mixture was mixed by pipetting and incubated at room temperature for 1 hour before quenching with 4X SDS gel loading buffer (17 μL). Proteins (30 μg total protein loaded per gel lane) were resolved by SDS-PAGE (10% acrylamide) made in-house and visualized by in-gel fluorescence on a Bio-Rad ChemiDoc MP Imaging System. The images were processed using Image Lab (version 5.2.1) software.

Preparation of isotopically labeled samples for pairwise MS-based analysis

For isotope dimethyl labeled experiments, samples were prepared as previous reported[2]. To 0.5 mL cell lysates in a 1.5 mL Eppendorf tube, 55 μL freshly prepared 'click' mixture (30 μL of 1.7 mM TBTA in 4:1 *t*-BuOH:DMSO, 10 μL of 50 mM $CuSO_4$ in H_2O, 5 μL of 10 mM Biotin-PEG4-azide (ChemPep, cat # 271606) in DMSO, 10 μL of freshly prepared 50 mM TCEP in DPBS) was added and the samples were rotated at room temperature for 1 hour. The mixtures were transferred to 15 mL falcon tubes and 3 mL cold methanol was added. The resulting cloudy mixtures were centrifuged (5,000 g, 10 min, 4 °C) to obtain protein pellets. After removing the supernatant, the protein pellets were washed with cold 1:1 MeOH:$CHCl_3$ (2 × 1 mL) and were resuspended in cold 4:1 MeOH:$CHCl_3$ (3 mL) by sonication. The cloudy mixtures were centrifuged (5,000 g, 10 min, 4 °C) to pellet the proteins. The pellets were solubilized in proteomics-grade urea (500 μL, 6 M in DPBS) containing 10 μL of 10% SDS by sonication. 50 μL 1:1 mixture of TCEP (200 mM in DPBS) and K_2CO_3 (600 mM in DPBS) was added to each sample and the mixture was incubated at 37 °C for 30 min to reduce the disulfides. Reduced thiols were then alkylated by adding 70 μL iodoacetamide (400 mM in DPBS) at room temperature protected from light for 30 min. To each solution, 130 μL of 10% SDS (in DPBS) was added and diluted to ~0.2% SDS with DPBS (5.5 mL) and incubated with pre-equilibrated streptavidin agarose beads (Thermo Fisher Scientific, Cat # 20347, 100 μL 1:1 slurry) for 1.5 hours at room temperature on a rotator. The beads were centrifuged (1,400 g, 2 min) and washed sequentially with 0.2% SDS in DPBS (1 × 5 mL), DPBS (2 × 5 mL) and H_2O (1 × 5 mL) to remove unbound proteins, excess detergent and reagents. The beads were transferred to a low-binding 1.5 mL microfuge tube (Axygen, CNT-1.5FL) and the enriched proteins were on-beads digested overnight at 37 °C in ~200 μL of 2 M urea in DPBS containing 2 μg sequencing grade porcine trypsin (Promega, V5111) in the presence of $CaCl_2$ (1 mM). To each digested sample, 8 μL of 4% 'light' formaldehyde (Sigma Aldrich, 252549) or 8 μL of 4% 'heavy' formaldehyde-^{13}C, d_2 (Sigma Aldrich, 596388) and 8 μL of sodium cyanoborohydride (0.6 M in H_2O) were added and the reaction was incubated at room temperature for 1 hour before quenching with 32 μL of 1% NH_4OH (in H_2O) followed by 16 μL of formic acid. The corresponding 'light' and 'heavy' sample were combined and centrifuged (1,400 g, 2 min). The supernatant was transferred to a low-binding microfuge tube and stored at -20 °C until analysis. For Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) samples, a procedure adapted from methods previously reported[1] was followed.

Liquid chromatography-mass spectrometry (LC-MS) analysis of probe labeled proteins

Protein digests were pressure loaded onto a 250 μm (inner diameter) fused silica capillary column packed with 4 cm C18 resin (Phenomenex, Aqua 5 μm). Samples were analyzed on an LTQ-Orbitrap Elite mass spectrometer (Thermo Scientific) coupled to a UltiMate 3000 Series Nano/Cap Pump NCP-3200RS and a WPS-3000PL (RS) autosampler (Thermo Scientific Dionex). Peptides were eluted by two-dimensional separation on a column with a 5 μm tip [100 μm fused silica, packed with 10 cm C18 (Phenomenex, Aqua 5 μm) and 3 cm strong cation exchange resin (SCX, Phenomenex)] using a 5-step 'MudPIT' protocol that injects 5 μL 0%, 25%, 50%, 80%, 100% salt bumps of ammonium acetate (500mM) in Buffer A (95% H_2O, 5% acetonitrile, 0.1% formic acid) followed by an increasing gradient of buffer B (20% H_2O, 80% acetonitrile, 0.1% formic acid) in Buffer A in each step. The flow rate was 0.5 μL/min and the voltage applied to the nano-LC electrospray ionization source was 2.5 kV. Spectra were collected in a data-dependent acquisition mode with dynamic exclusion enabled (repeat count 2, exclusion duration 20 s). One full MS1 scan (400 -1800 m/z) was followed by 30 MS2 scans (CID-ITMS) of the nth most abundant ions. Parent ions with unassigned or +1 charge state were excluded from fragmentation.

Peptide and protein identification and quantification

The MS2 spectra were extracted from the raw file using RAWconverter (available at http://fields.scripps.edu/rawconv/) with 'select monoisotopic m/z in DDA' enabled. MS2 spectra data were searched using ProLuCID algorithm against a reverse-concatenated, nonredundant variant of the Human UniProt database (2016-07) and filtered using DTASelect 2.0 within the Integrated Proteomics Pipeline (IP2). The precursor ion mass tolerance for a minimum envelop of three isotopic peaks was set to 50 ppm. All cysteines were specified with a static modification for carbamidomethylation (+57.02146) and up to one differential modification was allowed per peptide for either methionine oxidation (+15.994915) or glutamine/asparagine deamidation (+ 0.984016). For stable isotope dimethyl labeled samples, lysine and N-terminus were also specified with a static modification for demethylation (+28.0313). In addition, peptides were required to have at least one tryptic terminus. Each dataset was simultaneously searched for both 'light' and 'heavy' isotopic labeling by specifying the mass shifts on selected labeled amino acids, specifically, lysine (+8.0142) and arginine (+10.0083) for SILAC samples or lysine (+6.03182) and N-terminus (+6.03182) for

dimethyl labeled samples. The minimum peptide length was set to six residues, at least 2 peptides per protein was required and the false-positive rate was set at 1% at spectrum level. Light/Heavy peptide ratios were quantified based on peak areas on MS1 chromatogram with the in-house software (CIMAGE) as previously described[3]. The co-elution correlation score filter was set to $R^2 > 0.5$ for stable isotope dimethyl labeled samples and $R^2 > 0.8$ for SILAC experiments, default parameters were used for all other experiments. In certain cases, CIMAGE did not quantify a close-to-zero peptide ratio in light-over-heavy due to 2.5-fold signal to noise cutoff for peak detection, in these instances, the multiplicative inverse of heavy-over-light ratio was used. Finally, MS1 chromatograms were manually reviewed for all stereoselective protein targets to ensure correct ratio assignments.

Proteomics data analysis of stereoselectively labeled proteins in pairwise comparisons

To minimize false stereoselective protein targets introduced by, for instance, altered protein expression levels in 'light' versus 'heavy' SILAC medium, probe treatment was performed in both 'forward' and 'reverse' experimental design. In 'forward' experiment, the isotopically 'light' and 'heavy' proteomes were treated with (*R*)- and (*S*)-probe, respectively; then the isotopically 'light' and 'heavy' proteomes were treated with (*S*)- and (*R*)-probe in 'reverse' experiment. Both isotopic directions were performed in biological duplicate to furnish four independent experiments for each enantioprobe pair. Within individual experiment dataset, unique peptides from the same protein were grouped together, and the median ratio from at least two unique peptides was calculated as the protein ratio if its standard deviation was less than 10; if the standard deviation was greater than 10, the peptide ratio closest to 1 was assigned to the protein ratio. Protein ratios processed as above were then averaged across replicate experiments if their standard deviation is less than 60% of the mean; otherwise the ratio closest to 1 was taken as the final ratio for a protein in certain experiment condition.

In addition to the above criteria, to qualify as stereoselective protein targets, proteins must fulfill the following additional criteria: (1) quantified in at least two datasets for any enantioprobe pair. Proteins quantified in only one dataset were considered for stereoselective protein target designation if the protein was stereoselectively labeled by other probes (**Supplementary Table 1**); (2) For proteins quantified in both 'forward' and 'reverse' isotopic directions, average stereoselectivity ratios greater than 2.5 were required; for proteins only quantified in one isotopic direction, stereoselectivity ratios greater than 4 were required; (3) have at least 2 unique quantified peptides (unique sequences, ignoring MudPIT salt pump steps, charge states, modifications, etc.) in individual dataset. But if a protein had evidences of being stereoselectively labeled by other probes or in the other cell type, single unique quantified peptide was also reported.

Meta-analysis of stereoselective protein targets

Stereoselective protein targets were queried against the DrugBank database[4] (v. 5.1.1 released on 2018-7-3; group "All") and fractionated into DrugBank and non-DrugBank proteins. Protein targets were queried against the KEGG and UniProtKB/Swiss-Prot Protein Knowledge database for protein classification. Top level terms from KEGG BRITE and Gene Ontology terms were parsed to place each protein into a category (Transporter, Channel and Receptors; Enzymes; Gene Expression and Nucleic Acid Binding; Scaffolding, Modulators and Adaptors). If a protein can be classified into different categories, the abovementioned order of categories was used to prioritize the protein class. If no Gene Ontology term was available to assign protein class, the protein was sorted into the category 'Uncategorized'. Stereoselective protein targets only discovered in human PBMCs are queried against GeneAtlas U133A, gcrma dataset[5] on BioGPS[6] for tissue-specific patterns of mRNA expression. Protein targets with 3 × median mRNA expression in any of lymphoblast, B cells, T cells, dendritic cells, NK cells, monocytes or myeloid cells are defined as enriched in immune-related cells.

Cloning and transient overexpression of proteins in HEK293T cells

Open reading frame (ORF) of genes of interest were cloned from the Human ORFome V8.1 Library (Dharmacon) into a pRK5-derived plasmid generated using the gateway vector conversion system (Invitrogen, cat # 11828029). Final plasmids contain a CMV promoter followed by a gateway cloning linker, a start methionine, the respective ORF without stop codon, a second gateway cloning linker and a C-terminal DDK tag with its own stop codon. All gene constructs were verified by DNA sequencing. To transiently overexpress proteins for *in situ* treatment, HEK293T cells were grown to ~60% confluency in 6-well plates (gel-based experiments) or 6-cm plates (MS-based experiments) in complete growth DMEM. 2 – 4 μg of desired plasmid and 6-12 μg of PEI (polyethyleneimine, MW 40,000; Polysciences) were mixed in serum-free DMEM and incubated at room temperature for 30 min and added dropwise to the cells. Cells were grown for 48 – 72 hours before treatment. The pRK5 vector was a gift from David Sabatini lab (MIT).

Western blot analysis
After scanning for fluorescence, proteins were transferred to a nitrocellulose membrane (Amersham Protran, cat # 10600011) in Towbin buffer, the membrane was blocked for 1 hour at room temperature with 5% nonfat dry milk (w/v) in Tris-buffered saline with Tween 20 (TBST) and incubated with primary antibodies in the same solution overnight at 4 °C or 1 hour at room temperature. Blots were washed (3 × 5 min, TBST), incubated with secondary antibodies (IRDye 800CW) in milk for 1 hour at room temperature, washed (3 × 5 min, TBST), rinsed in water and scanned with a LI-COR Odyssey Scanner.

Preparation of tandem mass tag (TMT) labeled samples for multiplexed MS-based analysis
The procedure to prepare stable isotope dimethyl labeled samples as reported above was modified with the following modifications. After streptavidin enrichment, the beads were centrifuged (1,400 g, 2 min) and washed sequentially with 0.2% SDS in DPBS (1 × 5 mL), DPBS (2 × 5 mL) and 200mM EPPS (Sigma Aldrich, E9502, pH 8, 1 × 5 mL). The beads were transferred to a low-binding 1.5 mL microfuge tube (Axygen, CNT-1.5FL) and the enriched proteins were on-beads digested overnight at 37 °C in ~200 μL of 2 M urea in 200mM EPPS buffer containing 2 μg sequencing grade porcine trypsin (Promega, V5111) in the presence of $CaCl_2$ (1 mM). The samples were centrifuged to separate the beads and supernatant, then anhydrous acetonitrile was added to the supernatant to 30% final volume. 6 μL (20 μg/μL) of respective 10-plex TMT tag (Thermo Scientific, cat # 90110) was added and the reaction was incubated at room temperature for 1 hour with occasional vortex before quenching with 6 μL of 5% hydroxylamine for 15 min followed by 4 μL formic acid. All 10-plex samples were vacuum-centrifuged to near dryness, reconstituted in Buffer A (95% H_2O, 5% acetonitrile, 0.1% formic acid), combined and stored at -80°C until analysis.

Mass spectrometry analysis of tandem mass tag (TMT) labeled peptides
Labeled peptides were pressure loaded onto a 250 μm (inner diameter) fused silica capillary column packed with 4 cm C18 resin (Phenomenex, Aqua 5 μm). Samples were analyzed on an Orbitrap Fusion mass spectrometer (Thermo Scientific) coupled to an UltiMate 3000 Series Rapid Separation LC system and autosampler (Thermo Scientific Dionex). Peptides were separated on a 100 μm inner diameter capillary column with a 5 μm tip packed with 10 cm C18 (Phenomenex, Aqua 5 μm) and 3 cm strong cation exchange resin (SCX, Phenomenex)] using a 10-step 'MudPIT' protocol that injects 5 μL 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 80%, 100% salt bumps of ammonium acetate (500mM) in buffer A (100% H_2O, 0.1% formic acid) followed by an increasing gradient of buffer B (100% acetonitrile, 0.1% formic acid) in Buffer A in each step. The flow rate was 0.6 μL/min and the voltage applied to the nano-LC electrospray ionization source was 1.9 kV. A MS3-based TMT method was used for data acquisition. The scan sequence began with a MS1 master scan (Orbitrap analysis, resolution 120, 000, 400−1700 m/z, RF lens 60%, automatic gain control [AGC] target 2E5, maximum injection time 50 ms, centroid mode) with dynamic exclusion enabled (repeat count 1, duration 15s). The top ten precursors were then selected for MS2/MS3 analysis. MS2 analysis consisted of: collision‐induced dissociation (CID), quadrupole ion trap analysis, AGC 1.8E4, CID collision energy 35%, Activation Q 0.25, maximum injection time 120 ms, and isolation window at 0.7. Following acquisition of each MS2 spectrum, Synchronous Precursor Selection (SPS) was enabled to include up to 10 MS2 fragment ions for the MS3 spectrum. MS3 precursors were fragmented by HCD and analyzed using the Orbitrap (collision energy 55%, AGC 1.5E5, maximum injection time 120 ms, resolution was 50, 000). For MS3 analysis, we used charge state–dependent isolation windows. For charge state z = 2, the MS isolation window was set at 1.2; for z = 3-6, the MS isolation window was set at 0.7.

The spectra files were uploaded to Integrated Proteomics Pipeline (IP2) and searched using ProLuCID algorithm against a reverse-concatenated, nonredundant variant of the Human UniProt database (2012-11). The precursor ion mass tolerance for a minimum envelop of three isotopic peaks was set to 50 ppm. All cysteines were specified with a static modification for carbamidomethylation (+57.02146) and up to two differential modification was allowed per peptide for methionine oxidation (+15.994915). Lysine and N-terminus were also specified with a static modification for TMT tag (+229.1629). The minimum peptide length was set to six residues, at least 1 peptide per protein was required and the false-positive rate was set at 1% at spectrum level. MS3-based peptide quantification was performed with reporter ion mass tolerance set to 20 ppm and intensity threshold set to 5000 for sum of all reporter ions. Proteins were required to have at least three unique peptides (unique sequences, ignoring salt pump steps, charge states, modifications, etc.) and quantified by summing reporter ion intensities across all matching PSMs and normalized to the highest signal channel per protein. All TMT-based experiments were performed in duplicate, and the averaged values were reported.

Preparation for MS-based analysis of probe-modified peptides

A procedure previously reported[1] was followed. In brief, HEK293T cells were grown in 6 cm plates and *in situ* labeled with probes (200 – 250 μM) as described above. The harvested proteomes were adjusted to 1.8 mg/mL (500 μL), to which a freshly prepared 'click' mixture (30 μL of 1.7 mM TBTA in 4:1 *t*-BuOH:DMSO, 10 μL of 50 mM $CuSO_4$ in H_2O, 10 μL of 5 mM either 'light' or 'heavy' isotopically labeled Biotin-TEV-azide[7] in DMSO, 10 μL of freshly prepared 50 mM TCEP in DPBS) was added. The samples were rotated at room temperature for 1 hour and centrifuged (16,000 g, 5 min, 4°C). The resulting protein pellets were sonicated in ice-cold methanol (500 μL) and the corresponding 'light' and 'heavy' samples were then combined and centrifuged. The pellets were sonicated in 1mL of 1.2% SDS in DPBS and heated to 95 °C for 5 min. Then the samples were transferred to 15 mL falcon tubes and diluted with 5 mL DPBS, to which 100 μL of pre-equilibrated streptavidin-agarose beads (Thermo Fisher Scientific, Cat # 20347, 1:1 slurry) was added. After 3 hours incubated on a rotator, the beads were centrifuged (1,400 g, 2 min) and washed sequentially with DPBS (2 × 10 mL) and H_2O (2 × 10 mL). The beads were transferred to Eppendorf tubes and resuspended in 500 μL DPBS containing 6 M proteomics-grade urea. DTT (25 μL of 200 mM stock in DPBS) was added and the samples were incubated at 65 °C for 15 min. Then iodoacetamide (25 μL of 400 mM stock in DPBS) was added and allowed to react at 37 °C for 30 min. The beads mixture was diluted with 900 μL DPBS, centrifuged and resuspended in 200 μL of 2 M urea in DPBS containing 2 μg sequencing grade porcine trypsin (Promega, V5111) in the presence of $CaCl_2$ (1 mM). In the next day, the beads were centrifuged, washed with DPBS (2 × 1 mL), H_2O (2 × 1 mL), transferred to low-binding Eppendorf tubes, then washed with 150 μL of TEV buffer (50 mM Tris, pH 8, 0.5 mM EDTA, 1 mM DTT). To the washed beads, 150 μL of fresh TEV buffer with 80 μM TEV protease was added and the samples were rotated overnight at 29 °C. The TEV digests were separated from the beads by centrifugation (1,400 g, 3 min) and the beads were washed once with water (100 μL). The samples were then acidified with formic acid (~5% final v/v) and stored at -80°C until analysis. The MS data were collected on a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) as described above with differences in the salt bumps applied in the LC gradients, which in this case were 0%, 30%, 60%, 90% and 100% NH_4OAc (500 mM). The spectra searches were performed with the following changes applied to identify the peptides modified with the corresponding probe and the cleaved TEV tag. All amino acids were considered as possible residues for differential modification. The mass of the modification used to search was +651.38567 m/z for (*R*)-**1** and (*S*)-**1**, +695.41188 m/z for (*R,R*)-**7** and (*S,S*)-**7**, which are the monoisotopic masses for the corresponding probe plus the light TEV-tag and an additional +6.01381 m/z for the heavy counterpart. To account for potential diazirine insertion at cysteine thiols, an additional search only considering differential modification on cysteine without static carbamidomethylation (+57.02146) was also performed. The isoTOP ratios for probe labeled peptides were quantified using the in-house software CIMAGE. All MS1 chromatograms and MS2 fragment spectra were manually reviewed to ensure correct assignments.

Molecular modeling of enantioprobe docking into SMYD3

The crystal structure of the protein SMYD3 was retrieved from the Protein Data Bank (PDB: 5CCM), the hydrogens were added with Reduce[8], then was prepared using AutoDockTools[9] following the standard AutoDock protocol[10]. For the covalent docking, flexible side chain method was applied: the ligand structure was created by removing the diazirine moiety, then adding the two receptor atoms of the residue to the ligand coordinates, then processed following the covalent docking protocol (available at http://autodock.scripps.edu/resources/covalentdocking). Grid box was defined as following: center (x: 20.770, y: 9.873, z: 14.853); size 60x60x60 points. All dockings were performed using AutoDock 4.2.6[9], generating 100 poses using the default LGA parameters. Poses with the best energy score were selected.

Enantiopurity analysis of enantioprobes in cells

HEK293T cells were grown in 10-cm plates (Olympus, Cat #: 25-105) to near complete confluence before treatment. Cells were washed once with DPBS and replenished with 2mL fresh serum-free media before treated with 200μM (R)- or (S)-enantioprobe, or 100μM (R)- and 100μM (S)-enantioprobe, or DMSO as control. The treated cells were incubated at 37 °C for 30 min, washed with cold DPBS (2X), and harvested by scraping with cold DPBS (1mL). Cells were transferred to 2-dram vials before adding 1:2 MeOH:$CHCl_3$ (3 mL) to extract small molecule metabolites. The vials were centrifuged (1,400 g, 3 min, 4°C) to separate the organic and aqueous phases. The aqueous phase was extracted again with $CHCl_3$ (1 mL) and the organic layers were combined, blown down by N_2, and resuspended in acetonitrile (100μL) for enantiopurity analysis by heart-cutting 2D LC-SFC. LC dimension: The sample was separated on a BEH C18 column (1.7 μm, 2.1x100 mm) under gradient conditions [15-99% ACN / (0.1% aq. NH_4OH in H2O) over 1.1 minutes, followed by an isocratic hold] at 0.6

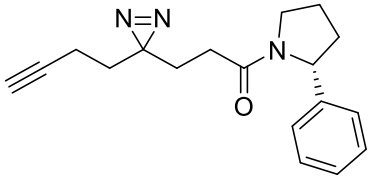
mL/min and 55 °C. SFC dimension: for (*R*)-**3** and (*S*)-**3**, the heart cut was analyzed on a Daicel IA column (3 μm, 4.6x250 mm) under gradient conditions [1 minute isocratic hold, then 3-30% (0.5% 7N methanolic NH_3 in MeOH) / CO_2 over 3 minutes, followed by an isocratic hold] at 3.6 mL/min, 1600 psi backpressure, and 30 °C; for (*R,R*)-**7** and (*S,S*)-**7**, the heart cut was analyzed on a Daicel IG column (3 μm, 4.6x250 mm) under isocratic conditions [20% (0.5% 7N methanolic NH_3 in MeOH) / CO_2] at 4 mL/min, 1600 psi backpressure, and 30 °C. The enantiomers were detected by UV light (214 nm). The heart cut was triggered at a fixed time (1.22 minutes for (*R*)-**3** and (*S*)-**3**; 1.24 minutes for (*R,R*)- **7** and (*S,S*)-**7**) using a 6-port 2-position valve equipped with a 10 μL transfer loop.

(E) Synthetic Methods

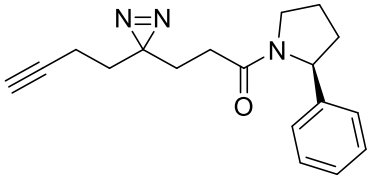
Chemicals and reagents were purchased from commercial vendors, including Sigma-Aldrich, Fisher Scientific, CombiBlocks, Alfa Aesar and AstaTech, and were used as received without further purification, unless otherwise noted. Anhydrous solvents were purchased from Sigma-Aldrich in Sure/Seal™ formulations. All reactions were monitored by thin-layer chromatography (TLC, Merck silica gel 60 F-254 plates). The plates were stained either with potassium permanganate, anisaldehyde, iodine or directly visualized with UV light. Reaction purification was carried out using Flash chromatography (230 – 400 mesh silica gel) or preparative thin layer chromatography (pTLC, Analtech, 500-2000 μm thickness). NMR spectra were recorded on Bruker DPX-400 or Bruker AV-500 spectrometers in the indicated solvent. Multiplicities are reported with the following abbreviations: s singlet; d doublet; t triplet; q quartet; p pentet; m multiplet; br broad; dd doublet of doublets; dt doublet of triplets; td triplet of doublets; Chemical shifts are reported in ppm relative to the residual solvent peak and J values are reported in Hz. Mass spectrometry data were collected on an Agilent 6120 single-quadrupole LC/MS instrument (ESI, low resolution) or an Agilent ESI-TOF instrument (ESI-TOF, HRMS).

3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propanoic acid was prepared according to literature procedures[1]. Analytical data are in agreement with previously reported data.

General Procedure: coupling procedure for the synthesis of chiral fragment probes. 3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propanoic acid (0.1 mmol, 1 equiv.) and the commercially-available chiral amine (0.11 mmol, 1.1 equiv.) were dissolved in anhydrous dichloromethane (1.5 mL) in a 4 mL vial. DIPEA (2.2 equiv.), EDCI (1.1 equiv.) and HOAt (1.1 equiv.) were added. The reaction mixture was stirred overnight at room temperature or when TLC indicated reaction completed. The crude mixture was concentrated by blowing a constant stream of nitrogen over it to remove the excess solvent. The resulting mixture was diluted with ethyl acetate (5 mL) and washed with saturated aqueous NH_4Cl (5 mL), saturated aqueous $NaHCO_3$ (5 mL) and brine (5 mL). The organic phase was dried over anhydrous Na_2SO_4, concentrated by removing solvent under reduced pressure and purified by flash chromatography or preparative TLC. The chromatography was run with the indicated solvent and the product was eluted from the silica. Evaporation of the solvent resulted in the desired product.



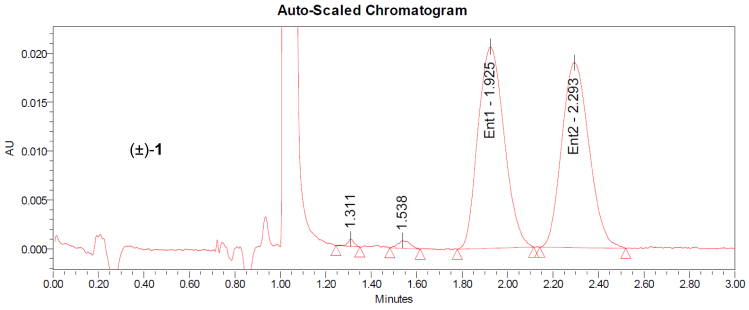
(R)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-1-(2-phenylpyrrolidin-1-yl)propan-1-one ((*R*)-1). General procedure. The flash chromatography was run with n-hexane/ethyl acetate 3:1. 21.4 mg (72%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) (70:30 mixture of rotamers, peaks corresponding to minor rotamer starred) δ 7.35 (m, 1.35H), 7.27 (m, 1.35H), 7.21 – 7.16* (m, 0.3H), 7.16 – 7.10 (m, 2H), 5.19* (dd, *J* = 8.1, 2.7 Hz, 0.3H), 4.88 (dd, *J* = 8.0, 2.4 Hz, 0.7H), 3.79 – 3.64 (m, 1.7H), 3.54* (ddd, *J* = 9.9, 8.6, 7.2 Hz, 0.3H), 2.45 – 2.32 (m, 0.7H), 2.29 – 2.18* (m, 0.3H), 2.09 (t, *J* = 7.6 Hz, 0.6H), 2.04 – 1.95 (m, 1.4H), 1.95 – 1.82 (m, 5.7H), 1.80 – 1.62 (m, 2.3H), 1.62 – 1.55 (m, 0.7H), 1.55 – 1.41 (m, 1.3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.10, 169.95, 143.53, 143.34, 129.28, 128.77, 127.82, 127.09, 125.80, 125.77, 83.22, 83.16, 77.69, 77.43, 77.18, 69.46, 61.99, 60.97, 47.92, 47.58, 36.76, 34.35, 33.03, 32.78, 28.85, 28.79, 28.23, 27.88, 24.07, 22.16, 13.73, 13.61. HRMS (m/z) calculated for $C_{18}H_{22}N_3O$ $[M+H]^+$: 296.1757; found: 296.1760.

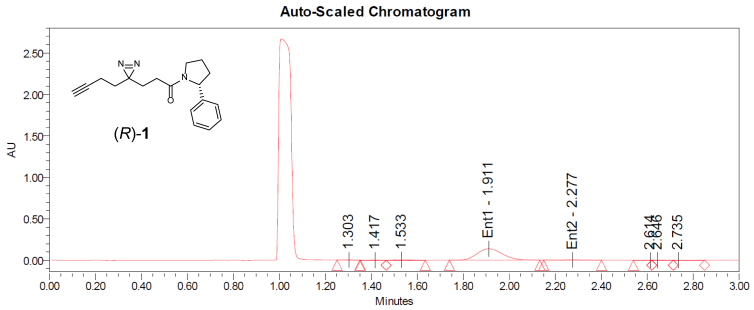


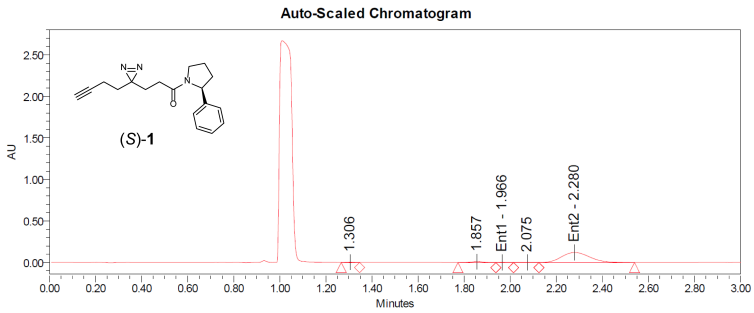
(S)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-1-(2-phenylpyrrolidin-1-yl)propan-1-one ((*S*)-1). General procedure. The flash chromatography was run with n-hexane/ethyl acetate 3:1. 22.3 mg (75%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) (70:30 mixture of rotamers, peaks corresponding to minor rotamer starred) δ 7.38 – 7.32 (m, 1.35H), 7.31 – 7.25 (m, 1.35H), 7.21 – 7.16* (m, 0.3H), 7.16 – 7.10 (m, 2H), 5.19* (dd, *J* = 8.1, 2.7 Hz, 0.3H), 4.88 (dd, *J* = 8.0, 2.4 Hz, 0.7H), 3.79 – 3.64 (m, 1.7H), 3.54* (ddd, *J* = 9.9, 8.6, 7.3 Hz, 0.3H), 2.44 – 2.34 (m, 0.7H), 2.29 – 2.19* (m, 0.3H), 2.09 (t, *J* = 7.6 Hz, 0.6H), 2.04 – 1.95 (m, 1.4H), 1.94 – 1.84 (m, 5.7H), 1.79 – 1.62 (m, 2.3H), 1.61 – 1.55 (m, 0.7H), 1.55 – 1.41 (m, 1.3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.76, 169.61, 143.12, 142.94, 128.90, 128.39, 127.44, 126.71, 125.42, 125.39, 125.37, 82.84, 82.78, 77.29, 77.04, 76.78, 69.08, 68.98, 61.61, 61.59, 60.59, 60.57, 47.54, 47.20, 47.18, 36.36, 33.96, 32.62, 32.38, 29.71, 28.46, 28.40, 28.38, 28.03, 27.83, 27.81, 27.48, 23.66, 21.76, 21.74, 13.34, 13.22. HRMS (m/z) calculated for $C_{18}H_{22}N_3O$ $[M+H]^+$: 296.1757; found: 296.1761.

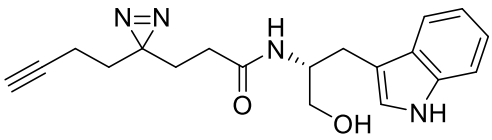
Enantiopurity was determined by SFC (see below). T_{rac} = 1.925 and 2.293 min.

	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.*
(*R*)-**1**	1.911	2.277	1049882	12758	97.6
(*S*)-**1**	2.280	1.966	1015497	4775	99.1

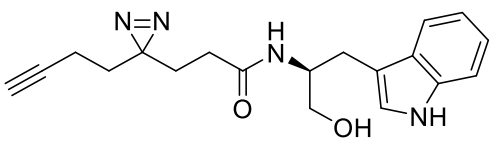








(R)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(1-hydroxy-3-(1H-indol-3-yl)propan-2-yl)propenamide ((*R*)-2). General procedure. The flash chromatography was run with n-hexane/ethyl acetate 2:3. 24.7 mg (73%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.25 (s, 1H), 7.64 (d, *J* = 7.9 Hz, 1H), 7.36 (d, *J* = 8.1 Hz, 1H), 7.24 – 7.17 (m, 1H), 7.17 – 7.09 (m, 1H), 7.04 (d, *J* = 2.4 Hz, 1H), 5.83 (d, *J* = 7.6 Hz, 1H), 4.35 – 4.21 (m, 1H), 3.78 – 3.55 (m, 2H), 3.01 (d, *J* = 6.9 Hz, 2H), 2.90 (d, *J* = 5.1 Hz, 1H), 2.01 – 1.93 (m, 3H), 1.87 (t, *J* = 7.1 Hz, 2H), 1.81 – 1.73 (m, 2H), 1.57 (t, *J* = 7.4 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.91, 136.30, 127.62, 122.76, 122.32, 119.74, 118.68, 111.47, 111.34, 82.77, 77.31, 77.05, 76.80, 69.31, 64.47, 60.45, 52.26, 32.27, 30.52, 28.30, 27.91, 26.51, 14.21, 13.26. HRMS (m/z) calculated for $C_{19}H_{23}N_4O_2$ $[M+H]^+$: 339.1815; found: 339.1817.

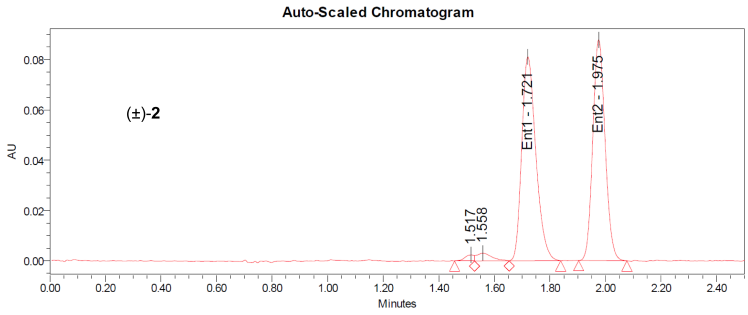


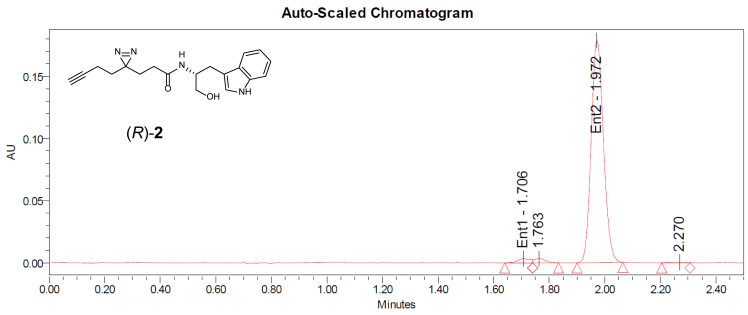
(S)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(1-hydroxy-3-(1H-indol-3-yl)propan-2-yl)propenamide ((*S*)-2). General procedure. The flash chromatography was run with n-hexane/ethyl acetate 2:3. 27.8 mg (82%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.17 (s, 1H), 7.65 (d, *J* = 7.9 Hz, 1H), 7.37 (d, *J* = 8.1 Hz, 1H), 7.24 – 7.18 (m, 1H), 7.14 (t, *J* = 7.5 Hz, 1H), 7.06 (d, *J* = 2.4 Hz, 1H), 5.77 (d, *J* = 7.5 Hz, 1H), 4.35 – 4.22 (m, 1H), 3.77 – 3.61 (m, 2H), 3.02 (d, *J* = 6.9 Hz, 2H), 2.72 (s, 1H), 2.03 – 1.92 (m, 3H), 1.88 (t, *J* = 7.2 Hz, 2H), 1.83 – 1.72 (m, 2H), 1.58 (t, *J* = 7.2 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.85, 136.30, 127.60, 122.70, 122.37, 119.79, 118.69, 111.53, 111.32, 82.75, 77.29, 77.03, 76.78, 69.27, 64.60, 52.27, 32.30, 30.95, 30.52, 28.29, 27.89, 26.52, 13.26. HRMS (m/z) calculated for $C_{19}H_{23}N_4O_2$ $[M+H]^+$: 339.1815; found: 339.1813.

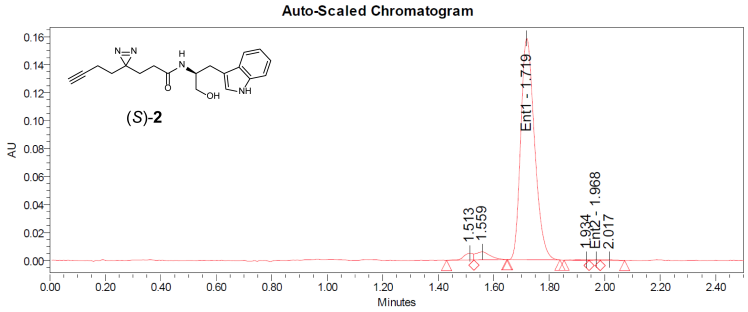
Enantiopurity was determined by SFC (see below). T_{rac} = 1.721 and 1.975 min.

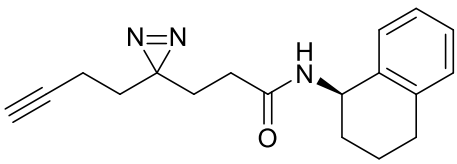
	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)

(*R*)-2	1.972	1.706	547954	10462	96.3
(*S*)-2	1.719	1.968	547686	1357	99.5

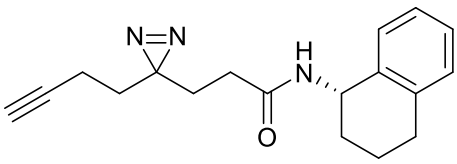








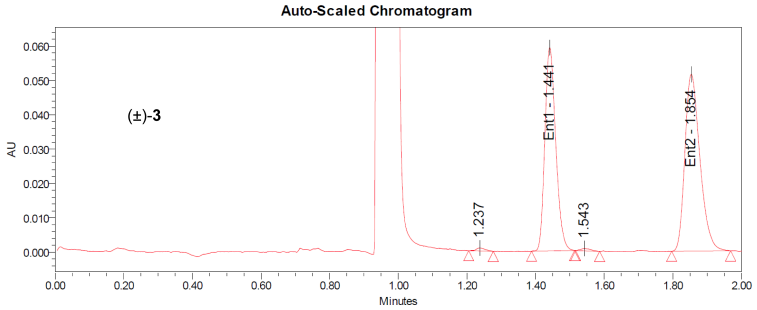
(R)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(1,2,3,4-tetrahydronaphthalen-1-yl)propenamide ((*R*)-3). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 3:1. 19.6 mg (66%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.25 (d, *J* = 2.5 Hz, 1H), 7.18 (td, *J* = 6.8, 6.0, 3.9 Hz, 2H), 7.13 – 7.07 (m, 1H), 5.67 (d, *J* = 8.5 Hz, 1H), 5.17 (dd, *J* = 8.9, 5.2 Hz, 1H), 2.78 (qt, *J* = 17.0, 5.9 Hz, 2H), 2.03 (td, *J* = 7.6, 2.9 Hz, 3H), 1.98 – 1.92 (m, 3H), 1.89 (m, 2H), 1.83 (m, 3H), 1.67 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.20, 137.63, 136.45, 129.23, 128.76, 127.39, 126.31, 82.72, 77.29, 77.04, 76.78, 69.22, 47.53, 32.46, 30.61, 30.04, 29.71, 29.21, 28.40, 27.88, 19.85, 13.32. HRMS (m/z) calculated for $C_{18}H_{22}N_3O$ $[M+H]^+$: 296.1757; found: 296.1757.

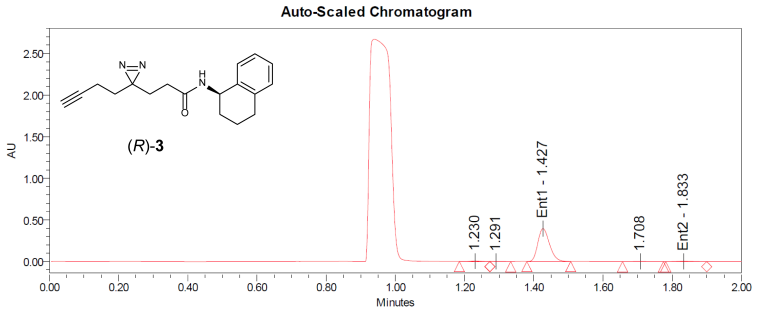


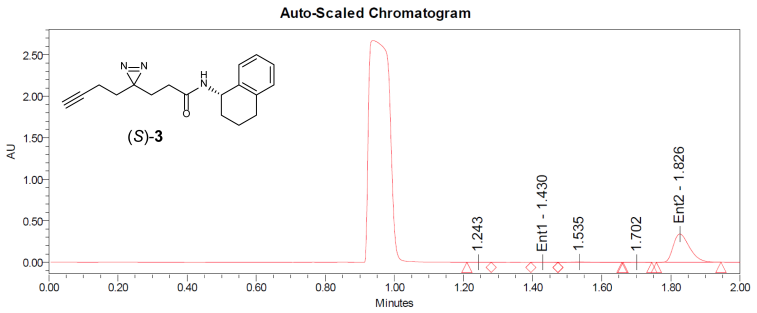
(S)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(1,2,3,4-tetrahydronaphthalen-1-yl)propenamide ((*S*)-3). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 3:1. 22.6 mg (77%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.25 (d, *J* = 2.6 Hz, 1H), 7.18 (td, *J* = 6.8, 5.9, 3.8 Hz, 2H), 7.12 – 7.06 (m, 1H), 5.67 (d, *J* = 8.5 Hz, 1H), 5.17 (dt, *J* = 9.4, 5.4 Hz, 1H), 2.78 (qt, *J* = 17.0, 6.0 Hz, 2H), 2.03 (ddd, *J* = 10.4, 8.3, 4.5 Hz, 3H), 1.95 (dt, *J* = 12.2, 3.3 Hz, 3H), 1.89 (m, 2H), 1.83 (m, 3H), 1.66 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.20, 137.63, 136.45, 129.23, 128.76, 127.39, 126.31, 82.72, 77.29, 77.04, 76.78, 69.22, 47.53, 32.46, 30.61, 30.04, 29.71, 29.21, 28.40, 27.88, 19.85, 13.32. HRMS (m/z) calculated for $C_{18}H_{22}N_3O$ $[M+H]^+$: 296.1757; found: 296.1756.

Enantiopurity was determined by SFC (see below). T_{rac} = 1.441 and 1.854 min.

	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)
(*R*)-**3**	1.427	1.833	915936	11204	97.6
(*S*)-**3**	1.826	1.430	1113421	2175	99.6



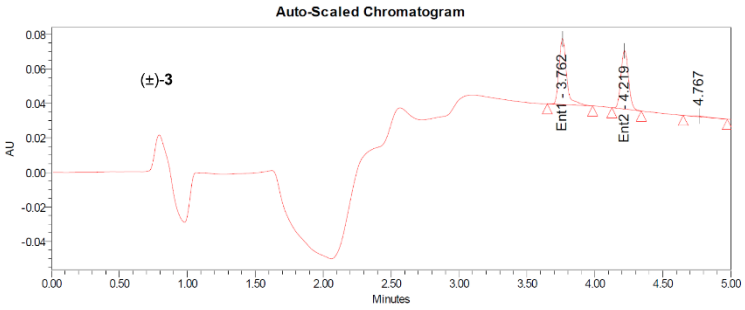


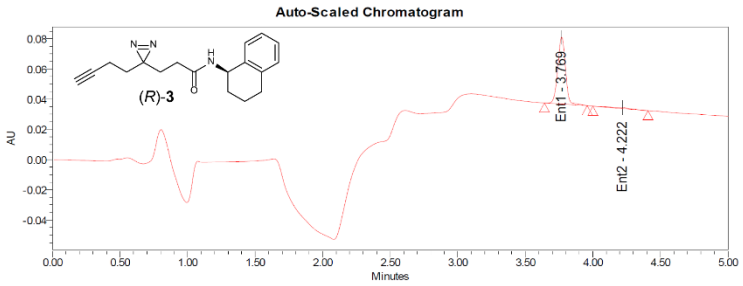


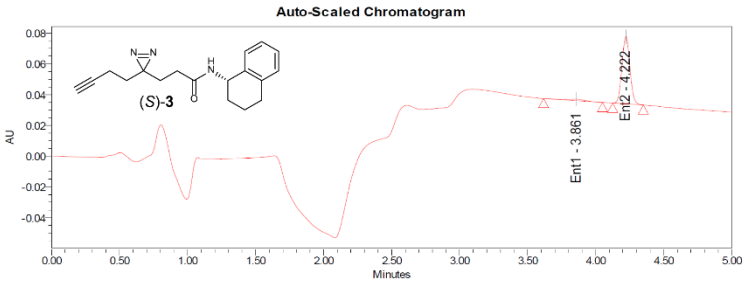
2-D LC-SFC enantiopurity analysis for enantioprobe **3** in cells (see Biological Methods for details)

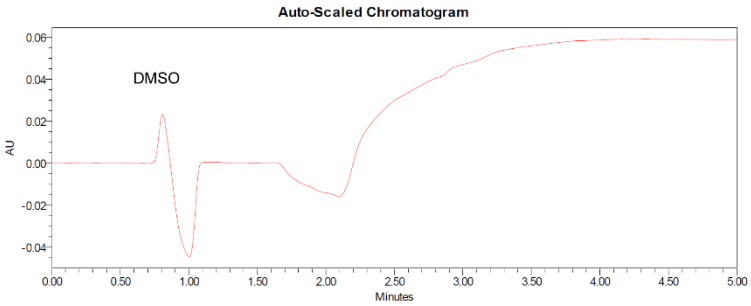
Enantiopurity was determined by SFC (see below). T_{rac} = 3.762 and 4.219 min.

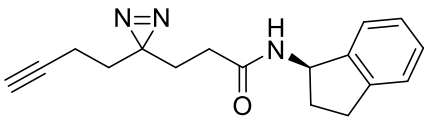
	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)
(±)-**3**	3.762	4.219	146681	130443	5.9
(*R*)-**3**	3.769	4.222	175122	6739	92.6
(*S*)-**3**	4.222	3.861	177246	8661	90.7
DMSO	n.d.	n.d.	n/a	n/a	n/a



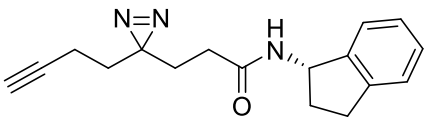








(R)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(2,3-dihydro-1H-inden-1-yl)propenamide ((*R*)-4). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 3:1. 21.0 mg (75%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.30 – 7.27 (m, 1H), 7.25 – 7.18 (m, 3H), 5.69 (d, *J* = 8.5 Hz, 1H), 5.46 (q, *J* = 7.7 Hz, 1H), 2.98 (m, 1H), 2.87 (dt, *J* = 16.0, 8.1 Hz, 1H), 2.58 (m, 1H), 2.03 (td, *J* = 7.5, 2.6 Hz, 2H), 1.98 – 1.93 (m, 3H), 1.89 (m, 2H), 1.80 (m, 1H), 1.66 (t, *J* = 7.5 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.81, 143.47, 142.96, 128.09, 126.82, 124.86, 124.03, 82.72, 77.30, 77.04, 76.79, 69.24, 54.77, 33.99, 32.45, 30.48, 30.23, 28.40, 27.89, 13.32. HRMS (m/z) calculated for $C_{17}H_{20}N_3O$ $[M+H]^+$: 282.1601; found: 282.1598.

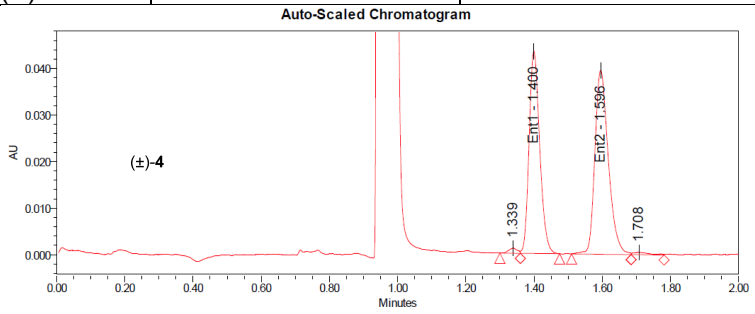


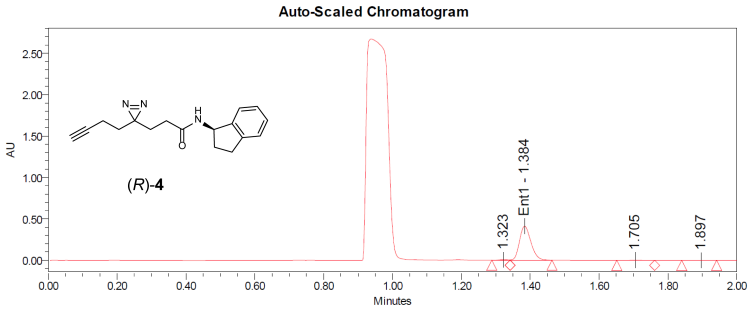
(S)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(2,3-dihydro-1H-inden-1-yl)propenamide ((*S*)-4). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 3:1. 25.4 mg (90%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.30 – 7.27 (m, 1H), 7.26 – 7.18 (m, 3H), 5.68 (d, *J* = 8.5 Hz, 1H), 5.46 (q, *J* = 7.7 Hz, 1H), 2.98 (m, 1H), 2.87 (dt, *J* = 16.1, 8.1 Hz, 1H), 2.59 (m, 1H), 2.03 (td, *J* = 7.5, 2.7 Hz, 2H), 1.99 – 1.93 (m, 3H), 1.92 – 1.87 (m, 2H), 1.81 (m, 1H), 1.67 (t, *J* = 7.5 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.81, 143.47, 142.96, 128.09,

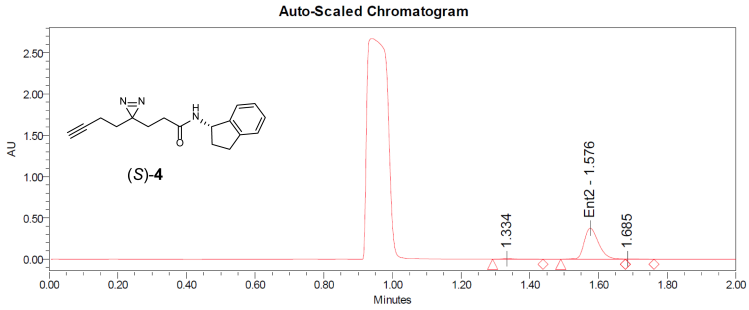
126.82, 124.86, 124.03, 82.72, 77.30, 77.04, 76.79, 69.24, 54.78, 33.99, 32.46, 30.49, 30.23, 28.40, 27.89, 13.32. HRMS (m/z) calculated for $C_{17}H_{20}N_3O$ $[M+H]^+$: 282.1601; found: 282.1601.

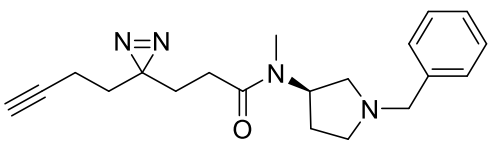
Enantiopurity was determined by SFC (see below). T_{rac} = 1.400 and 1.596 min.

	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)
(*R*)-**4**	1.384	n.d.	940583	n/a	100
(*S*)-**4**	1.576	n.d.	1082102	n/a	100

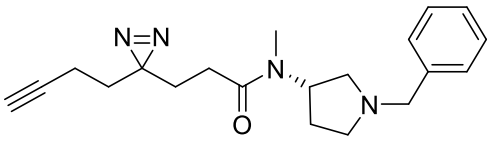








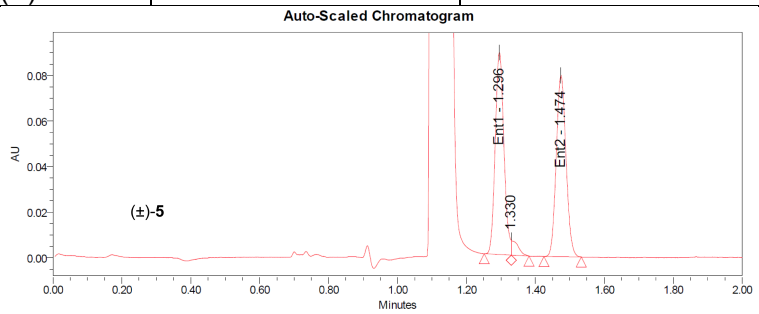
(R)-N-(1-benzylpyrrolidin-3-yl)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-methylpropanamide ((*R*)-5). General procedure. The flash chromatography was run with n-hexane/ethyl acetate 3:2 to 2:3. 27.8 mg (82%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) (60:40 mixture of rotamers, peaks corresponding to minor rotamer starred) δ 7.30 (m, 4H), 7.27 – 7.21 (m, 1H), 5.22 (m, 0.6H), 4.37* (m, 0.4H), 3.64 (d, *J* = 12.9 Hz, 1H), 3.50 (dd, *J* = 18.2, 12.9 Hz, 1H), 2.92 (m, 3.6H), 2.79* (td, *J* = 8.7, 4.1 Hz, 0.4H), 2.62 – 2.57 (m, 1H), 2.54* (m, 0.4H), 2.39 (q, *J* = 8.6, 7.9 Hz, 1H), 2.27 – 2.10 (m, 2H), 2.10 – 1.94 (m, 5H), 1.85 – 1.80 (m, 2H), 1.65 (t, *J* = 7.5 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.05, 170.50, 139.07, 138.65, 128.56, 128.53, 128.37, 128.29, 127.17, 127.01, 82.81, 77.31, 77.05, 76.80, 69.13, 69.10, 60.18, 60.11, 57.19, 57.11, 55.54, 53.83, 53.58, 51.82, 32.58, 32.55, 30.05, 29.71, 29.06, 28.96, 28.30, 28.17, 27.98, 27.91, 27.62, 27.24, 13.33. HRMS (m/z) calculated for $C_{20}H_{27}N_4O$ $[M+H]^+$: 339.2179; found: 339.2185.

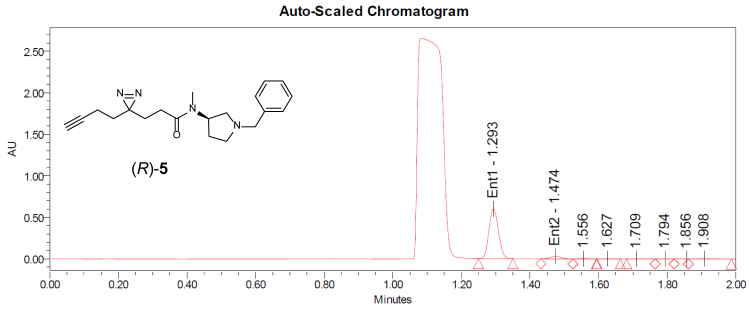


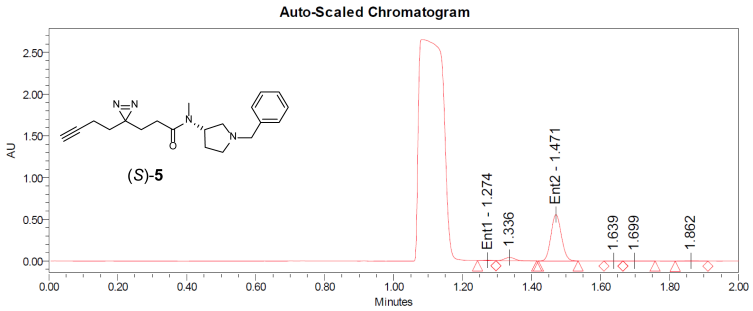
(S)-N-(1-benzylpyrrolidin-3-yl)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-methylpropanamide ((*S*)-5). General procedure. The flash chromatography was run with n-hexane/ethyl acetate 3:2 to 2:3. 25.7 mg (76%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) (60:40 mixture of rotamers, peaks corresponding to minor rotamer starred) δ 7.36 – 7.30 (m, 4H), 7.26 (m, 1H), 5.25 (m, 0.6H), 4.40* (m, 0.4H), 3.67 (d, *J* = 12.9 Hz, 1H), 3.53 (dd, *J* = 17.8, 12.9 Hz, 1H), 2.95 (m, 3.6H), 2.82* (td, *J* = 8.7, 4.3 Hz, 0.4H), 2.62 (dd, *J* = 10.1, 3.8 Hz, 1H), 2.56* (dd, *J* = 10.1, 8.0 Hz, 0.4H), 2.46 – 2.38 (m, 1H), 2.29 – 2.12 (m, 2H), 2.12 – 1.96 (m, 5H), 1.89 – 1.82 (m, 2H), 1.68 (t, *J* = 7.6 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.05, 170.50, 138.64, 128.56, 128.53, 128.37, 128.29, 127.17, 127.01, 82.80, 77.30, 77.04, 76.79, 69.12, 69.10, 60.17, 60.11, 57.18, 57.11, 55.54, 53.83, 53.57, 51.82, 32.58, 32.55, 30.05, 29.71, 29.06, 28.95, 28.30, 28.17, 27.98, 27.91, 27.63, 27.24, 13.33. HRMS (m/z) calculated for $C_{20}H_{27}N_4O$ $[M+H]^+$: 339.2179; found: 339.2184.

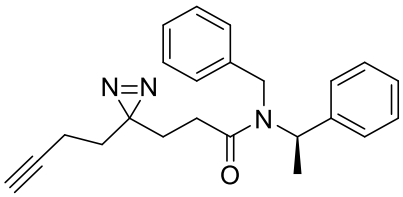
Enantiopurity was determined by SFC (see below). T_{rac} = 1.296 and 1.474 min.

	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)
(*R*)-**5**	1.293	1.474	1152767	64955	89.3
(*S*)-**5**	1.274	1.471	1164051	10419	98.2



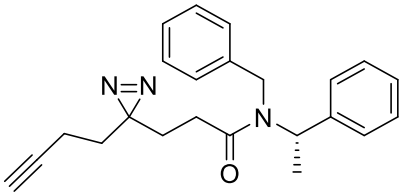






(R)-N-benzyl-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(1-phenylethyl)propenamide ((*R*)-6). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 13:7. 26.0 mg (72%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) (70:30 mixture of rotamers, peaks corresponding to minor rotamer starred) δ 7.33 – 7.16 (m, 8H), 7.12 (m, 0.7H), 7.05 (m, 1.3H), 6.17 (q, *J* = 7.2 Hz, 0.7H), 5.13 (q, *J* = 7.2 Hz, 0.3H), 4.94 (d, *J* = 15.5 Hz, 0.3H), 4.35 (d, *J* = 17.9 Hz, 0.7H), 4.18 (d, *J* = 17.9 Hz, 0.7H), 3.98 (d, *J* = 15.5 Hz, 0.3H), 2.23 (m, 0.7H), 2.06 – 1.77 (m, 6.3H), 1.66 (m, 0.7H), 1.58 (m,

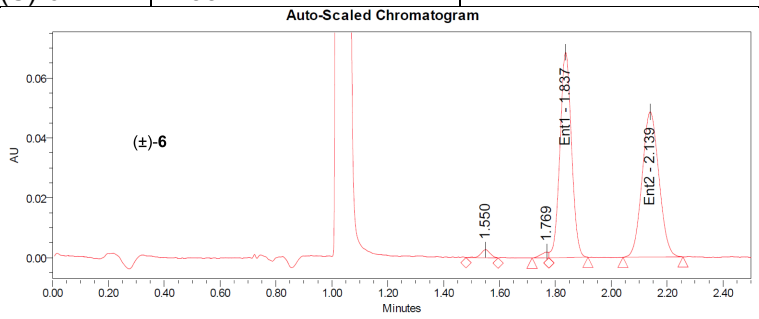
1.3H), 1.46 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.42, 140.93, 138.09, 128.83, 128.72, 128.53, 128.28, 127.55, 127.51, 127.27, 127.20, 126.58, 125.83, 82.80, 77.29, 77.04, 76.78, 69.06, 51.80, 47.19, 46.55, 32.55, 28.11, 27.97, 16.96, 13.29. HRMS (m/z) calculated for $C_{23}H_{26}N_3O$ $[M+H]^+$: 360.2070; found: 360.2070.

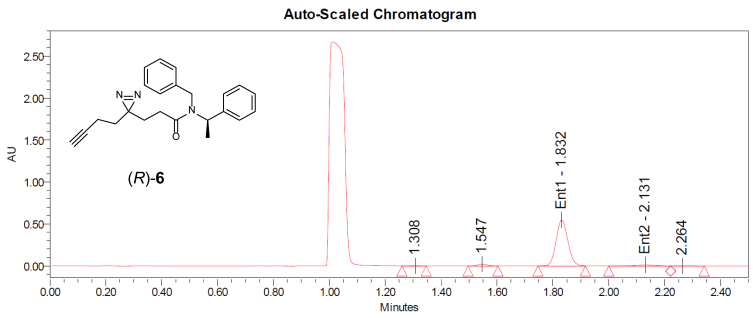


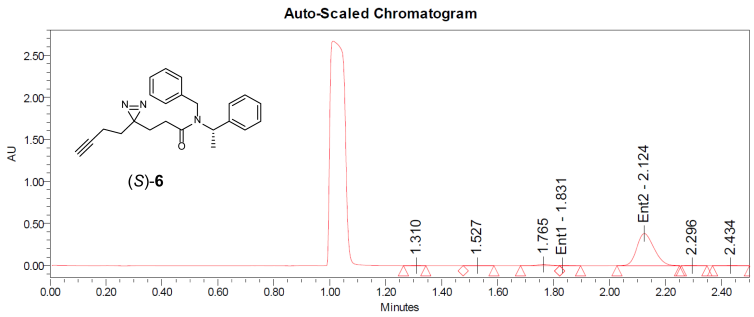
(S)-N-benzyl-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(1-phenylethyl)propenamide ((*S*)-6). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 13:7. 29.2 mg (81%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) (70:30 mixture of rotamers, peaks corresponding to minor rotamer starred) δ 7.39 – 7.17 (m, 8H), 7.14 (m, 0.7H), 7.07 (m, 1.3H), 6.20 (q, *J* = 7.2 Hz, 0.7H), 5.16* (q, *J* = 7.0 Hz, 0.3H), 4.96* (d, *J* = 15.5 Hz, 0.3H), 4.37 (d, *J* = 17.9 Hz, 0.7H), 4.21 (d, *J* = 17.9 Hz, 0.7H), 4.00* (d, *J* = 15.5 Hz, 0.3H), 2.25 (m, 0.7H), 2.08 – 1.79 (m, 6.3H), 1.68 (m, 0.7H), 1.60 (m, 1.3H), 1.48 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.42, 140.93, 138.09, 128.83, 128.72, 128.53, 128.28, 127.55, 127.51, 127.28, 127.20, 126.58, 125.83, 82.79, 77.29, 77.04, 76.79, 69.06, 51.80, 47.19, 32.55, 28.11, 27.97, 16.96, 13.29. HRMS (m/z) calculated for $C_{23}H_{26}N_3O$ $[M+H]^+$: 360.2070; found: 360.2070.

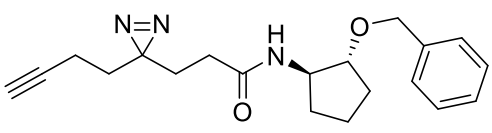
Enantiopurity was determined by SFC (see below). T_{rac} = 1.837 and 2.139 min.

	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)
(*R*)-**6**	1.832	2.131	1581571	50450	93.8
(*S*)-**6**	1.831	2.124	1624433	5316	99.4



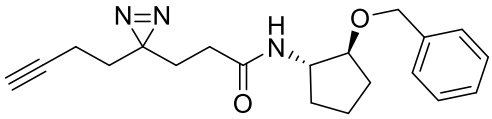






N-((1R,2R)-2-(benzyloxy)cyclopentyl)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propenamide ((*R,R*)-7). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 13:7. 24.1 mg (71%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*)

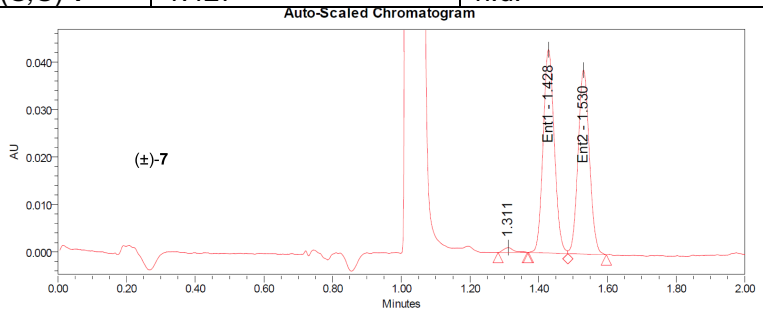
δ 7.36 – 7.30 (m, 4H), 7.29 – 7.26 (m, 1H), 5.30 (d, *J* = 7.3 Hz, 1H), 4.60 (q, *J* = 12.0 Hz, 2H), 4.20 (m, 1H), 3.77 (dt, *J* = 6.3, 3.9 Hz, 1H), 2.18 (m, 1H), 2.04 – 1.94 (m, 3H), 1.91 – 1.66 (m, 7H), 1.66 – 1.60 (m, 3H), 1.39 (m, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.60, 138.67, 128.36, 127.69, 127.51, 84.59, 82.72, 77.29, 77.03, 76.78, 71.09, 69.22, 55.93, 32.45, 30.57, 30.48, 30.46, 28.31, 27.88, 21.68, 13.29. HRMS (m/z) calculated for $C_{20}H_{26}N_3O_2$ $[M+H]^+$: 340.2019; found: 340.2027.

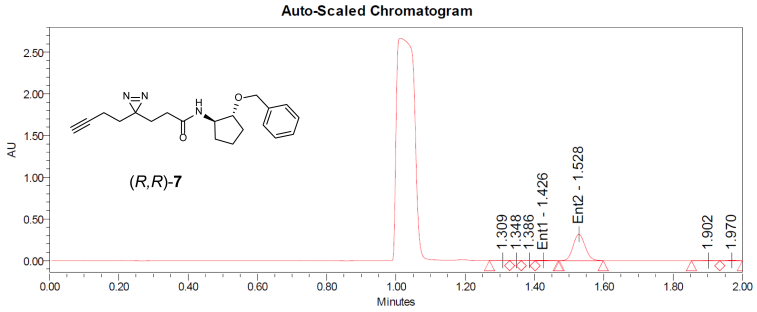


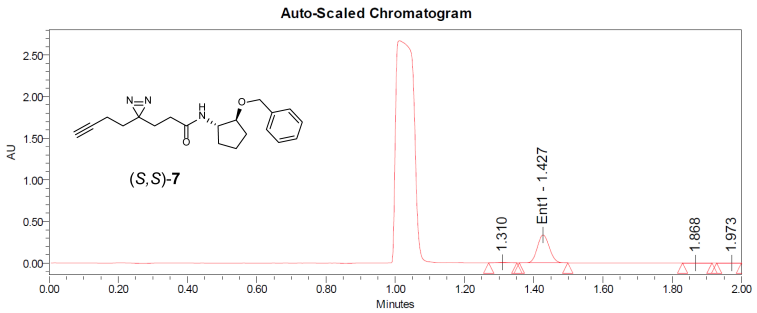
N-((1S,2S)-2-(benzyloxy)cyclopentyl)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propenamide ((*S,S*)-7). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 13:7. 21.5 mg (63%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.35 – 7.31 (m, 4H), 7.29 – 7.26 (m, 1H), 5.37 – 5.20 (m, 1H), 4.60 (q, *J* = 12.1 Hz, 2H), 4.20 (m, 1H), 3.77 (dt, *J* = 6.3, 3.9 Hz, 1H), 2.18 (m, 1H), 2.04 – 1.94 (m, 3H), 1.93 – 1.76 (m, 6H), 1.76 – 1.69 (m, 1H), 1.68 – 1.57 (m, 4H), 1.39 (m, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.59, 138.67, 128.36, 127.69, 127.51, 84.59, 82.72, 77.28, 77.03, 76.78, 71.09, 69.22, 55.93, 32.46, 30.57, 30.48, 30.46, 28.31, 27.88, 21.68, 13.29. HRMS (m/z) calculated for $C_{20}H_{26}N_3O_2$ $[M+H]^+$: 340.2019; found: 340.2025.

Enantiopurity was determined by SFC (see below). T_{rac} = 1.428 and 1.530 min.

	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)
(*R,R*)-**7**	1.528	1.426	753576	4809	98.7
(*S,S*)-**7**	1.427	n.d.	805126	n/a	100



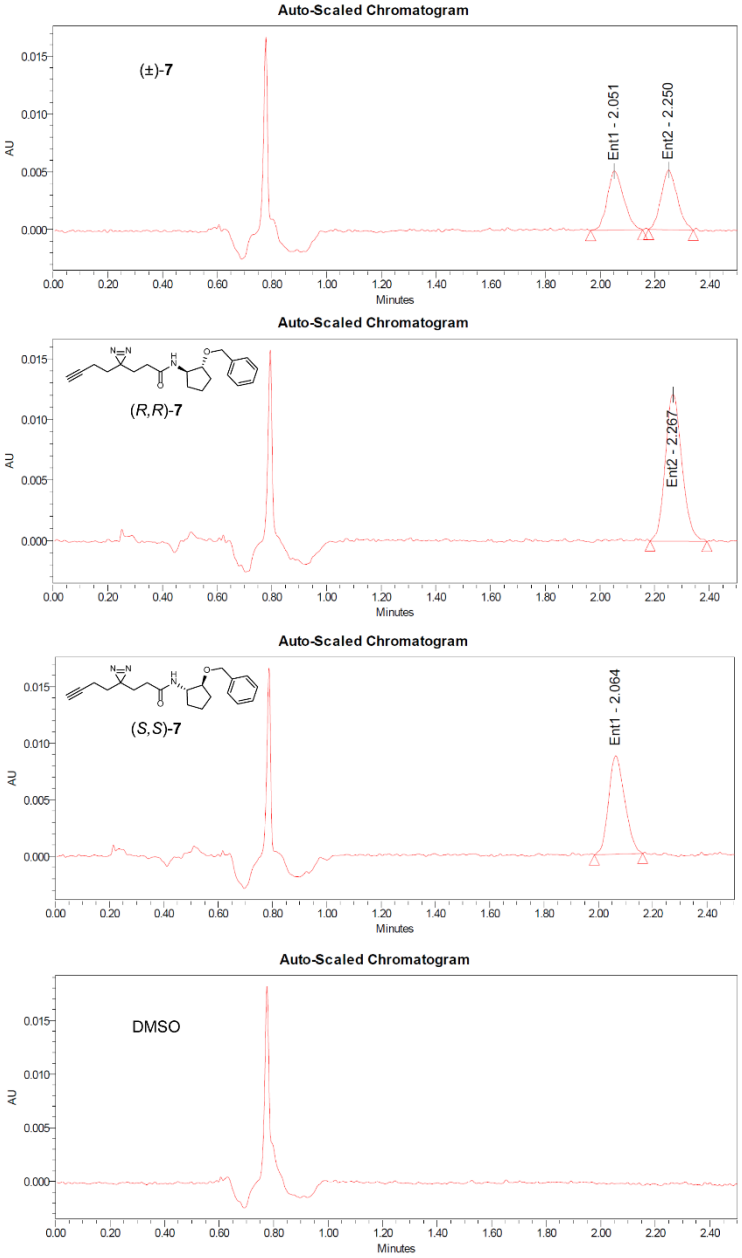


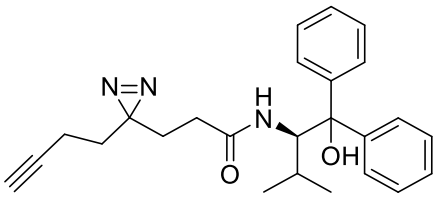


2-D LC-SFC enantiopurity analysis for enantioprobe **7** in cells (see Biological Methods for details)

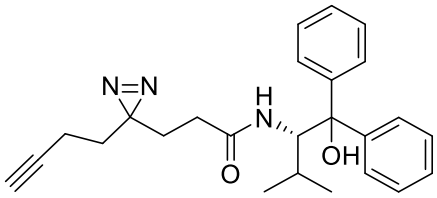
Enantiopurity was determined by SFC (see below). T_{rac} = 2.051 and 2.250 min.

	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)
(±)-**7**	2.051	2.250	21348	21184	0.39
(*R*,*R*)-**7**	2.267	n.d.	51483	n/a	100
(*S*,*S*)-**7**	2.064	n.d.	36052	n/a	100
DMSO	n.d.	n.d.	n/a	n/a	n/a





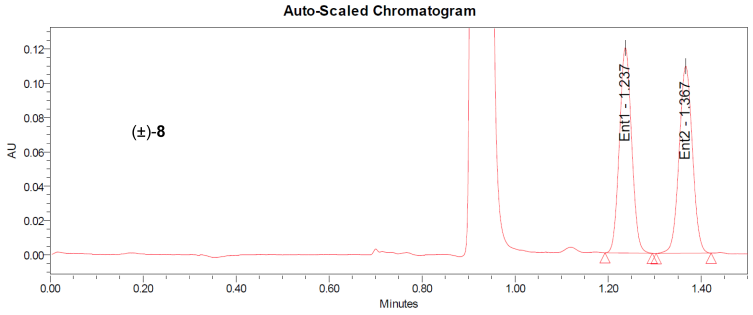
(R)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(1-hydroxy-3-methyl-1,1-diphenylbutan-2-yl)propenamide ((*R*)-8). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 5:3. 38.7 mg (96%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.46 (m, 4H), 7.31 (dd, *J* = 8.5, 7.1 Hz, 2H), 7.28 – 7.24 (m, 2H), 7.23 – 7.14 (m, 2H), 5.97 (d, *J* = 9.9 Hz, 1H), 4.95 (dd, *J* = 9.9, 2.3 Hz, 1H), 2.87 (s, 1H), 1.96 – 1.92 (m, 3H), 1.90 – 1.78 (m, 2H), 1.76 – 1.62 (m, 2H), 1.56 – 1.46 (m, 2H), 0.90 (dd, *J* = 7.7, 6.8 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.25, 146.02, 145.32, 128.49, 128.41, 126.99, 126.97, 125.33, 125.19, 82.73, 82.31, 77.29, 77.04, 76.78, 69.19, 57.85, 32.23, 30.59, 28.81, 28.39, 27.79, 22.88, 17.81, 13.30. HRMS (m/z) calculated for $C_{25}H_{29}N_3O_2Na$ [M+Na]$^+$: 426.2152; found: 426.2159.

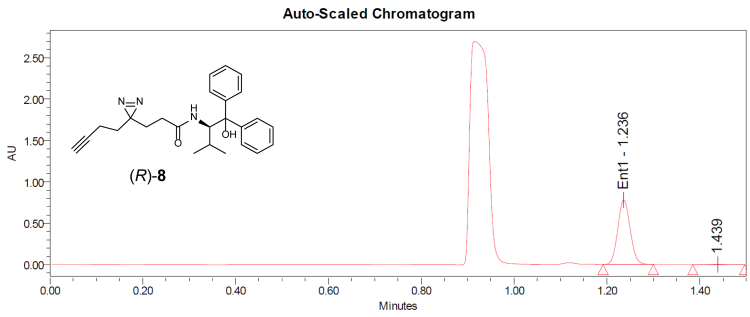


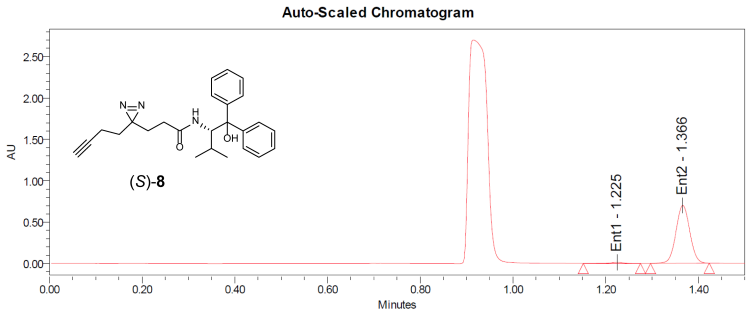
(S)-3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(1-hydroxy-3-methyl-1,1-diphenylbutan-2-yl)propenamide ((*S*)-8). General procedure. The preparative TLC was run with n-hexane/ethyl acetate 5:3. 38.4 mg (95%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.46 (m, 4H), 7.31 (dd, *J* = 8.5, 7.1 Hz, 2H), 7.26 (t, *J* = 7.8 Hz, 2H), 7.23 – 7.14 (m, 2H), 5.98 (d, *J* = 9.9 Hz, 1H), 4.95 (dd, *J* = 10.0, 2.3 Hz, 1H), 2.88 (s, 1H), 1.97 – 1.91 (m, 3H), 1.90 – 1.77 (m, 2H), 1.77 – 1.62 (m, 2H), 1.56 – 1.49 (m, 2H), 0.90 (dd, *J* = 8.5, 6.8 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.26, 146.03, 145.32, 128.49, 128.41, 126.99, 126.97, 125.33, 125.20, 82.73, 82.31, 77.29, 77.04, 76.78, 69.19, 57.86, 32.22, 30.60, 28.82, 28.39, 27.79, 22.88, 17.81, 13.30. HRMS (m/z) calculated for $C_{25}H_{29}N_3O_2Na$ [M+H]$^+$: 426.2152; found: 426.2158.

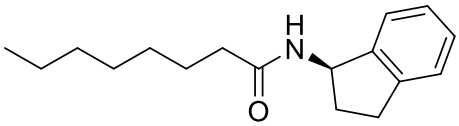
Enantiopurity was determined by SFC (see below). T_{rac} = 1.237 and 1.367 min.

	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)
(*R*)-**8**	1.236	n.d.	1447134	n/a	100
(*S*)-**8**	1.366	1.225	1446498	26237	96.4

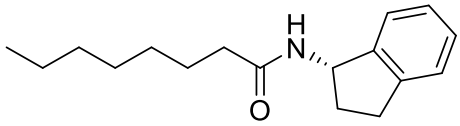






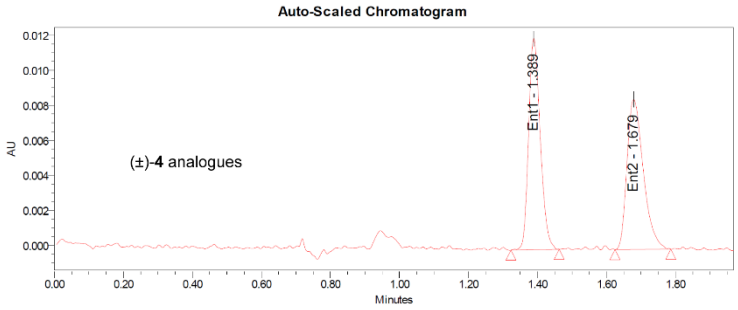


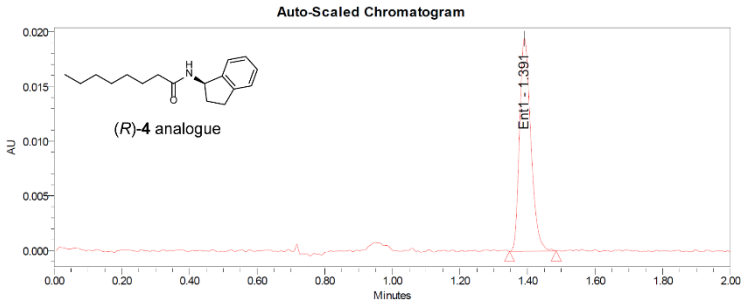
(R)-N-(2,3-dihydro-1H-inden-1-yl)octanamide ((*R*)-4 analogue). General procedure with octanoic acid (0.1 mmol). The preparative TLC was run with n-hexane/ethyl acetate 3:1. 22.6 mg (87%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-d) δ 7.28 – 7.20 (m, 4H), 5.66 (d, J = 8.6 Hz, 1H), 5.50 (q, J = 7.7 Hz, 1H), 2.98 (ddd, J = 16.0, 8.7, 3.9 Hz, 1H), 2.87 (dt, J = 16.1, 8.1 Hz, 1H), 2.60 (m, 1H), 2.25 – 2.16 (m, 2H), 1.78 (dtd, J = 12.9, 8.5, 7.3 Hz, 1H), 1.72 – 1.62 (m, 2H), 1.35 – 1.25 (m, 8H), 0.91 – 0.85 (m, 3H). ^{13}C NMR (126 MHz, CDCl3) δ 172.91, 143.46, 143.34, 143.32, 127.96, 126.77, 124.82, 123.99, 77.29, 77.04, 76.78, 54.55, 36.99, 34.21, 34.20, 34.18, 31.71, 30.24, 29.71, 29.27, 29.02, 25.91, 25.88, 22.67, 22.62, 14.08, 14.06. HRMS (m/z) calculated for $C_{17}H_{26}NO$ $[M+H]^+$: 260.2009; found: 260.2010.

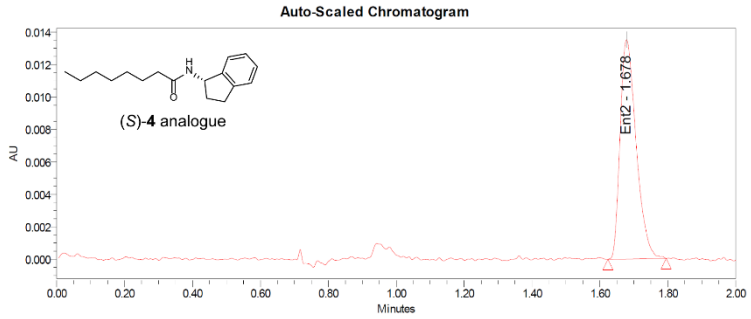


(S)-N-(2,3-dihydro-1H-inden-1-yl)octanamide **((*S*)-4 analogue).** General procedure with octanoic acid (0.1 mmol). The preparative TLC was run with n-hexane/ethyl acetate 3:1. 21.9 mg (85%) of the product were obtained. ^{1}H NMR (500 MHz, Chloroform-d) δ 7.29 – 7.18 (m, 4H), 5.66 (d, J = 8.5 Hz, 1H), 5.50 (q, J = 7.8 Hz, 1H), 2.98 (ddd, J = 16.0, 8.8, 3.9 Hz, 1H), 2.87 (dt, J = 16.1, 8.2 Hz, 1H), 2.60 (m, 1H), 2.25 – 2.14 (m, 2H), 1.78 (dtd, J = 12.9, 8.5, 7.3 Hz, 1H), 1.72 – 1.61 (m, 2H), 1.35 – 1.24 (m, 8H), 0.90 – 0.86 (m, 3H). ^{13}C NMR (126 MHz, CDCl3) δ 172.93, 172.91, 143.46, 143.34, 127.96, 126.80, 126.77, 124.82, 123.99, 77.29, 77.04, 76.78, 54.55, 54.53, 36.99, 34.24, 34.18, 34.16, 31.71, 31.69, 30.24, 29.71, 29.27, 29.07, 29.02, 25.88, 25.86, 22.66, 22.63, 22.62, 22.60, 14.13, 14.08, 14.05, 14.03. HRMS (m/z) calculated for $C_{17}H_{26}NO$ $[M+H]^+$: 260.2009; found: 260.2011. Enantiopurity was determined by SFC (see below). T_{rac} = 1.389 and 1.679 min.

	Retention (major)	Retention (minor)	Area (major)	Area (minor)	*e.e.* (%)
(*R*)-**4** analogue	1.391	n.d.	45183	n/a	100
(*S*)-**4** analogue	1.678	n.d.	44261	n/a	100

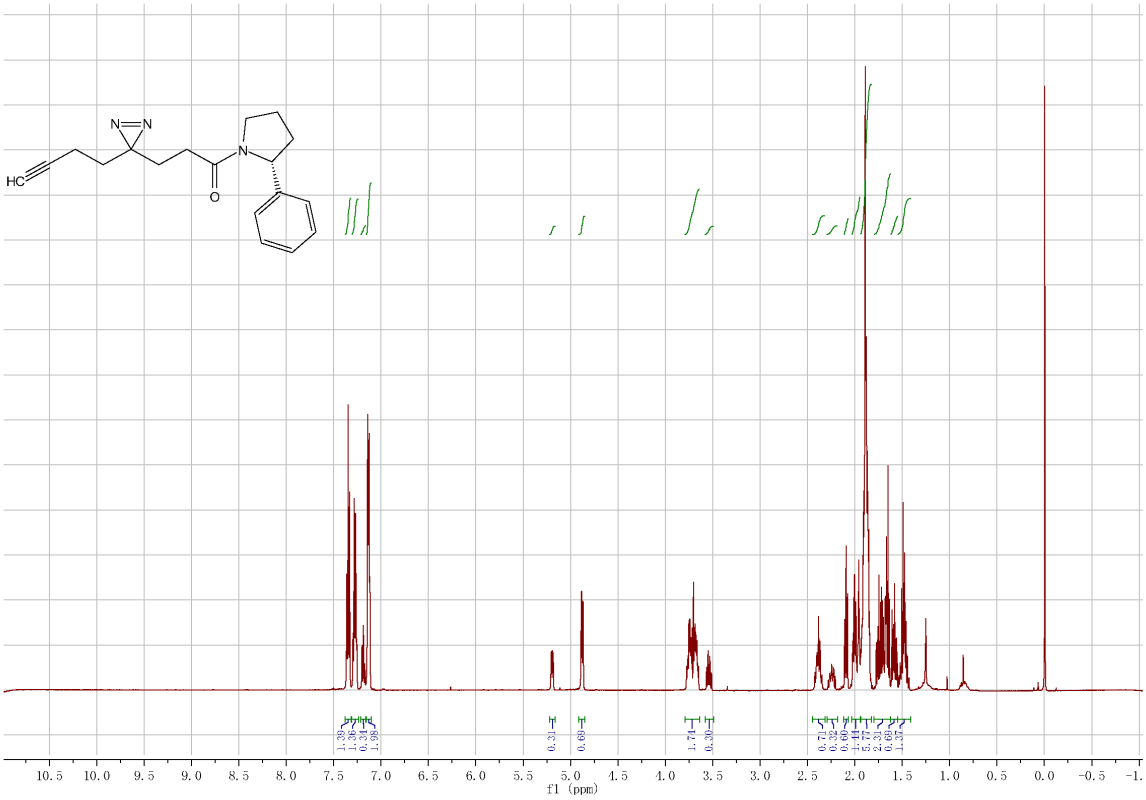

Auto-Scaled Chromatogram
(±)-4 analogues
Ent1 - 1.389
Ent2 - 1.679
AU
Minutes


Auto-Scaled Chromatogram
(R)-4 analogue
Ent1 - 1.391
AU
Minutes

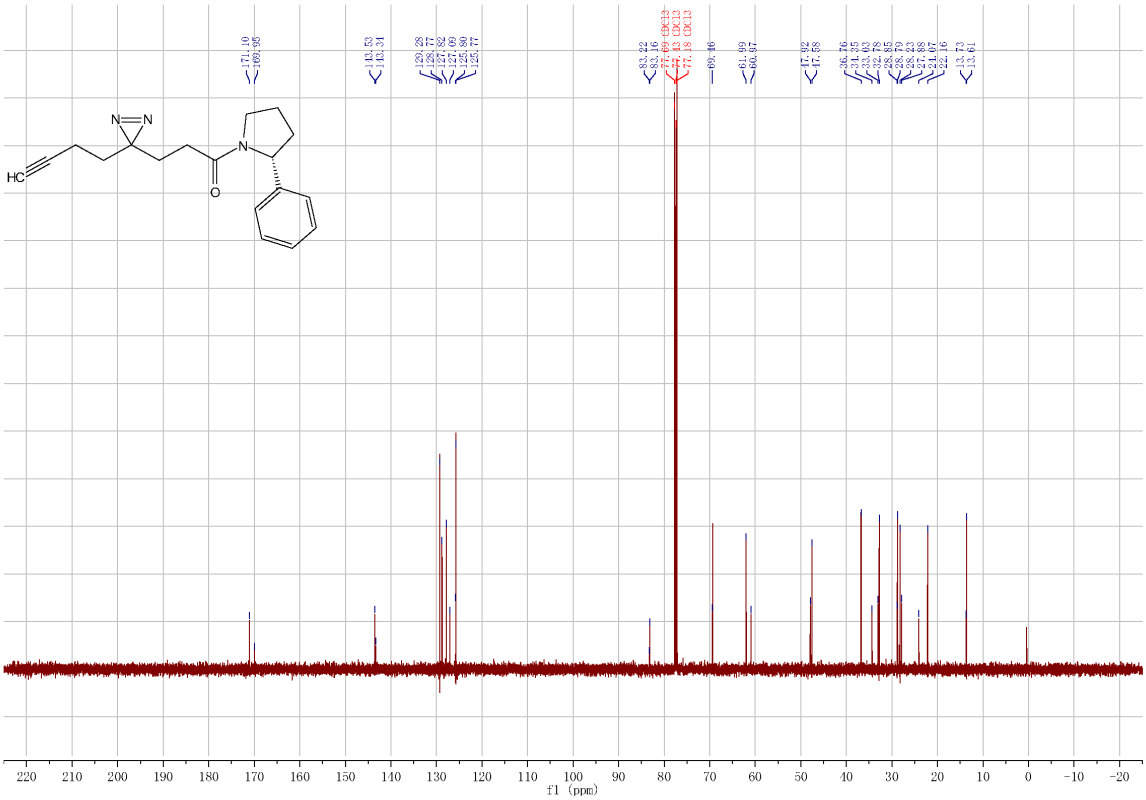

Auto-Scaled Chromatogram
(S)-4 analogue
Ent2 - 1.678
AU
Minutes

(F) NMR spectra

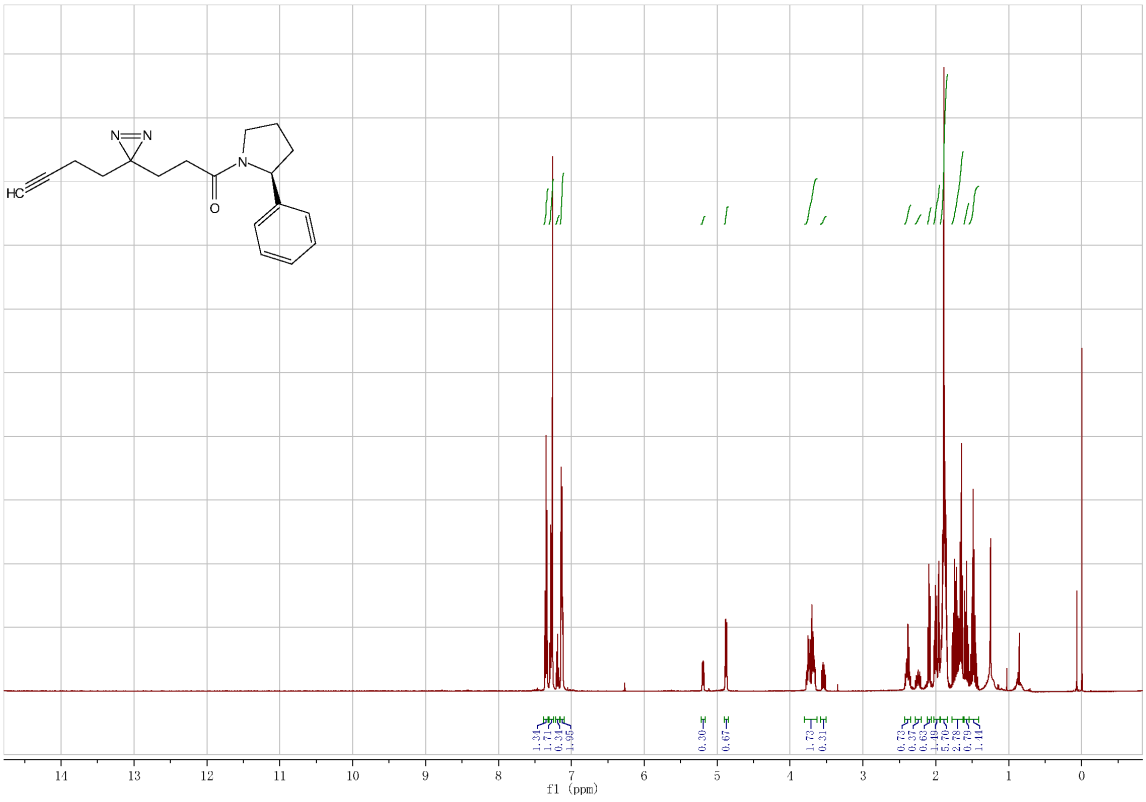
^{1}H-NMR for (*R*)-**1**



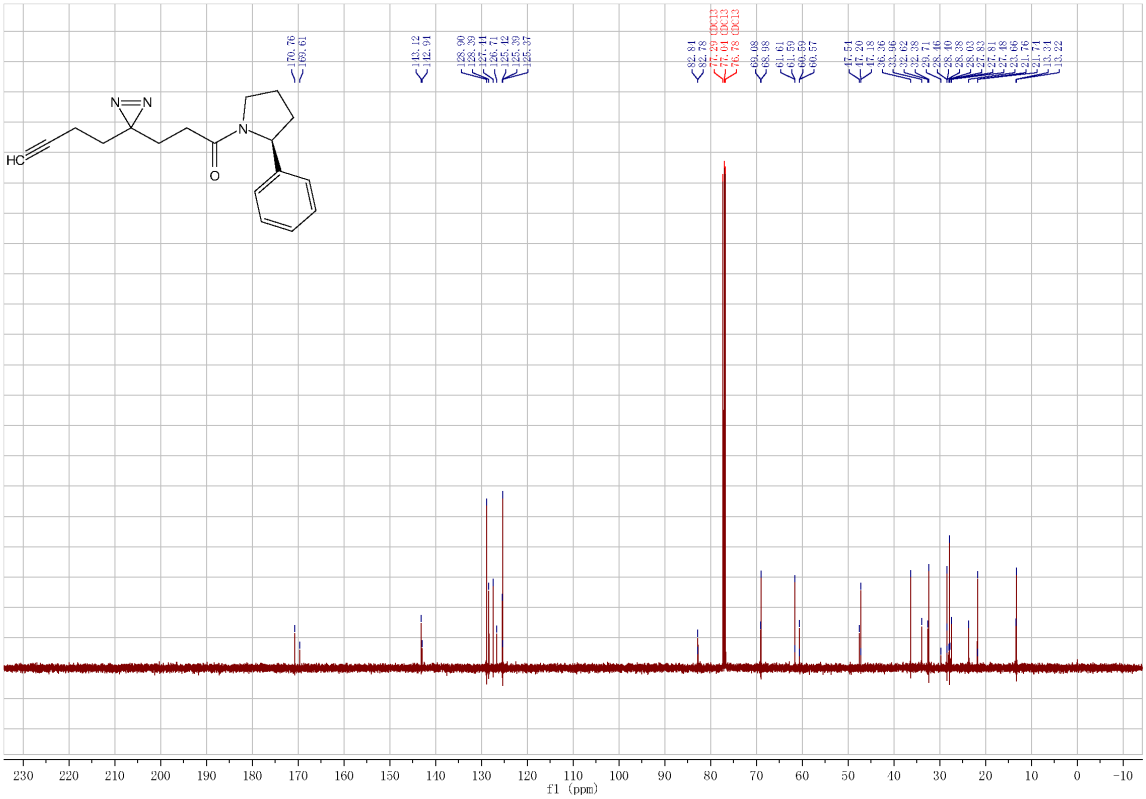
^{13}C-NMR for (*R*)-**1**



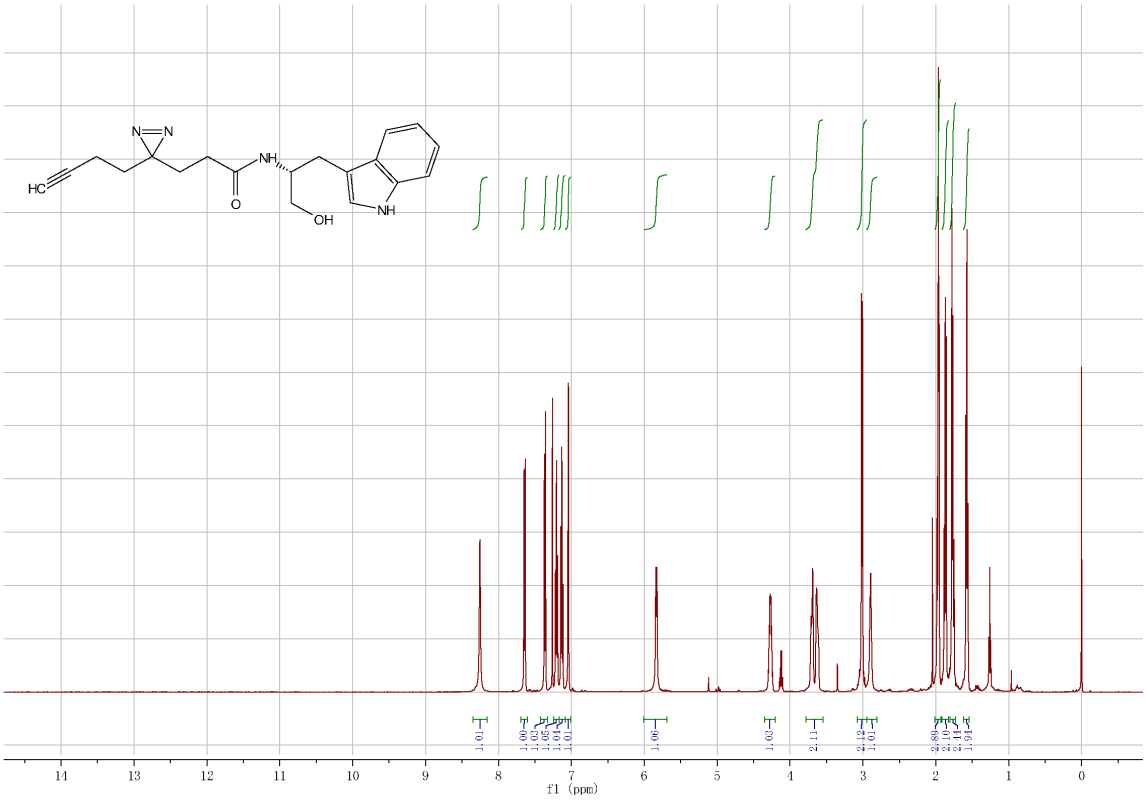
^{1}H-NMR for (*S*)-**1**



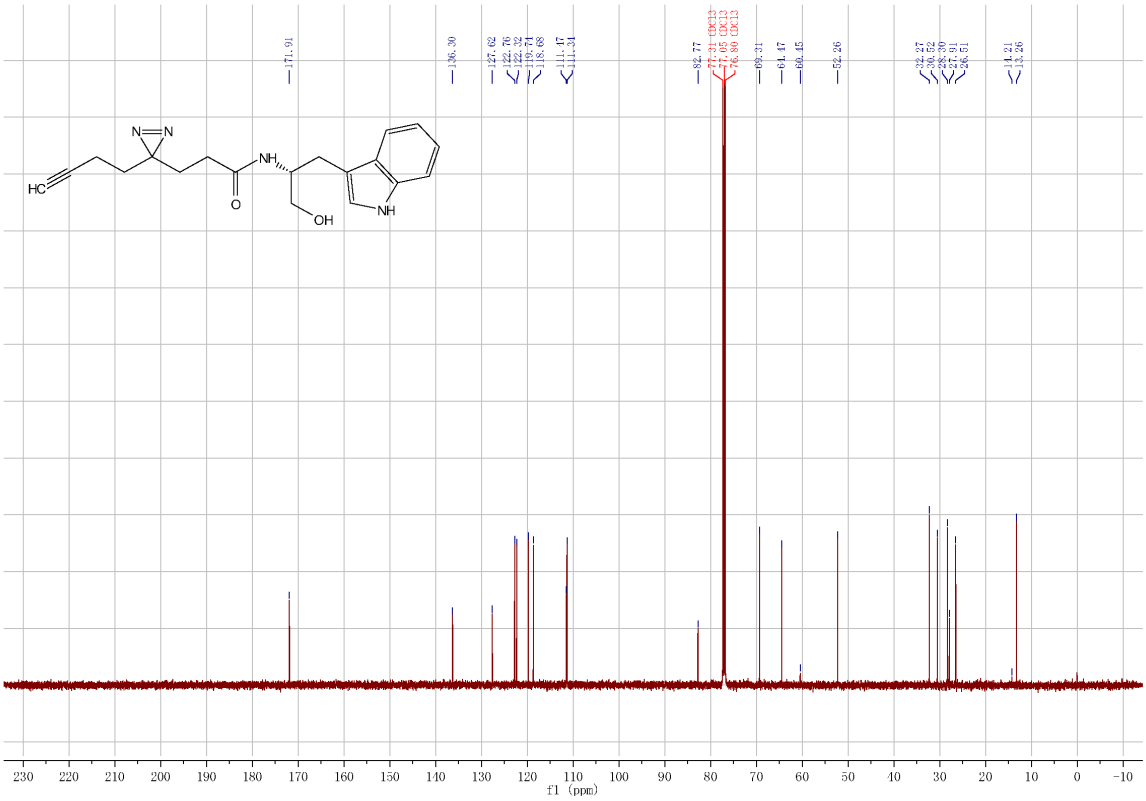
^{13}C-NMR for (*S*)-**1**



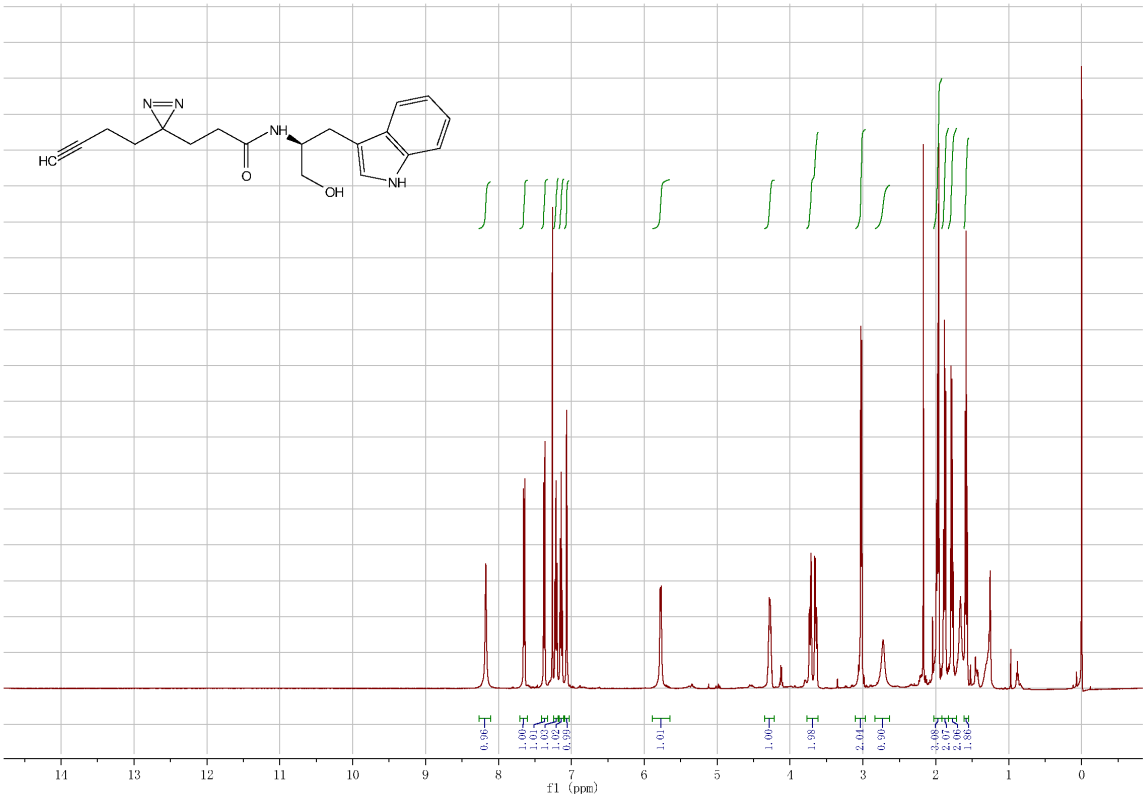
^{1}H-NMR for (*R*)-**2**



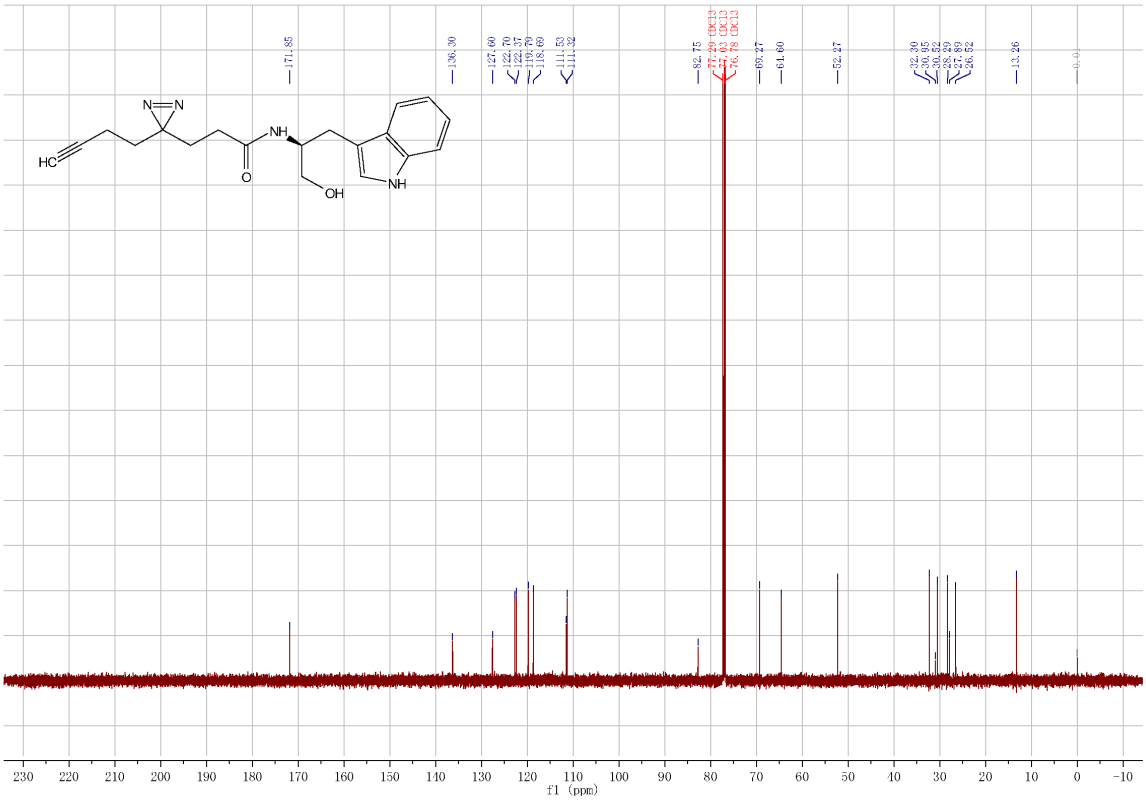
^{13}C-NMR for (*R*)-**2**



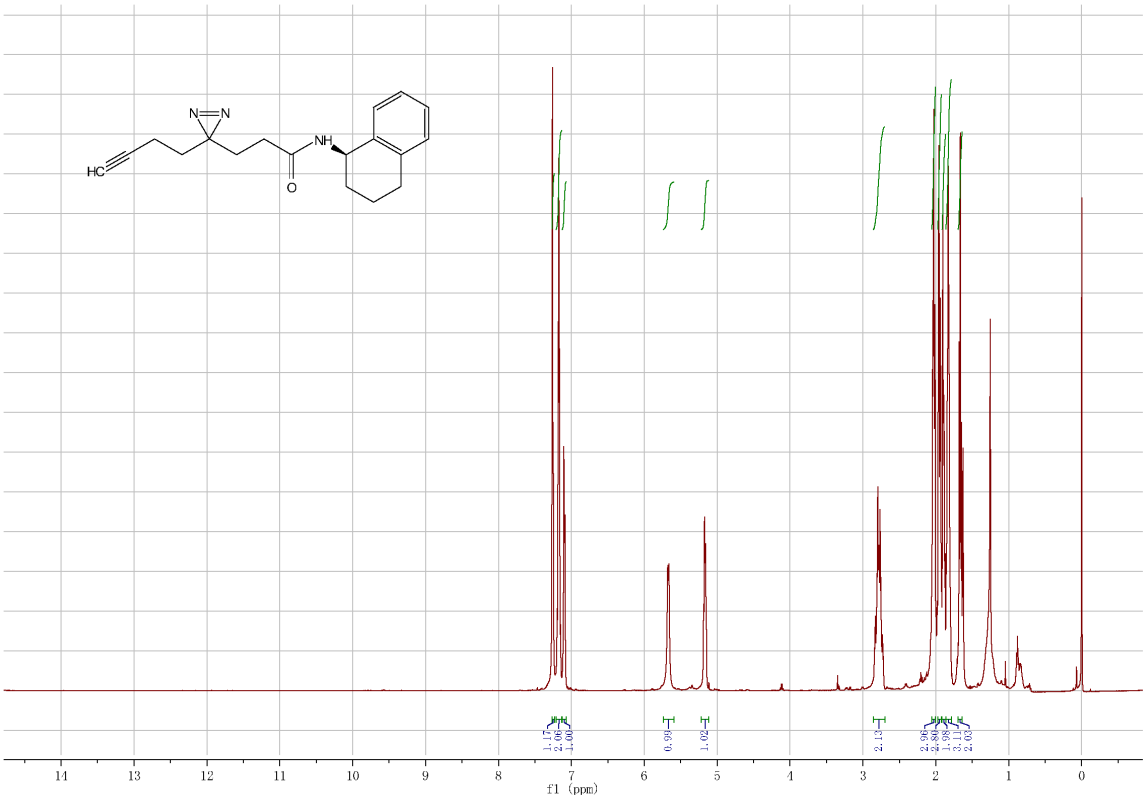
^{1}H-NMR for (*S*)-**2**



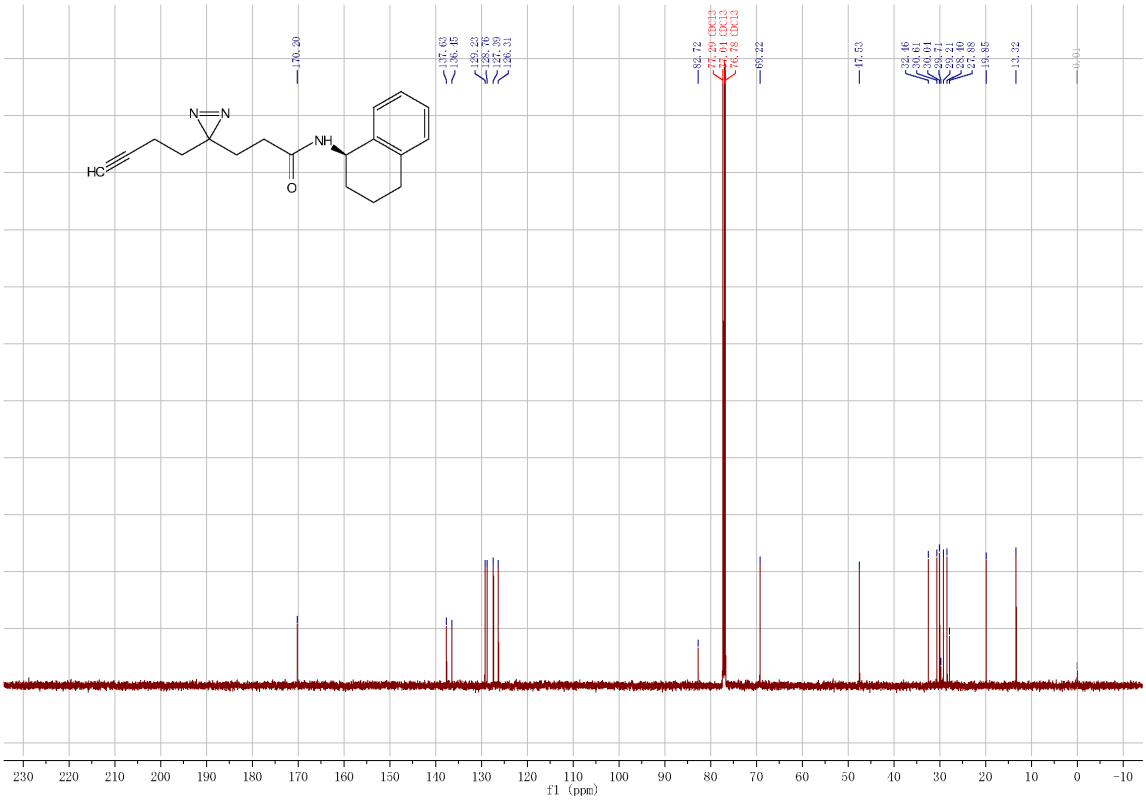
^{13}C-NMR for (*S*)-**2**



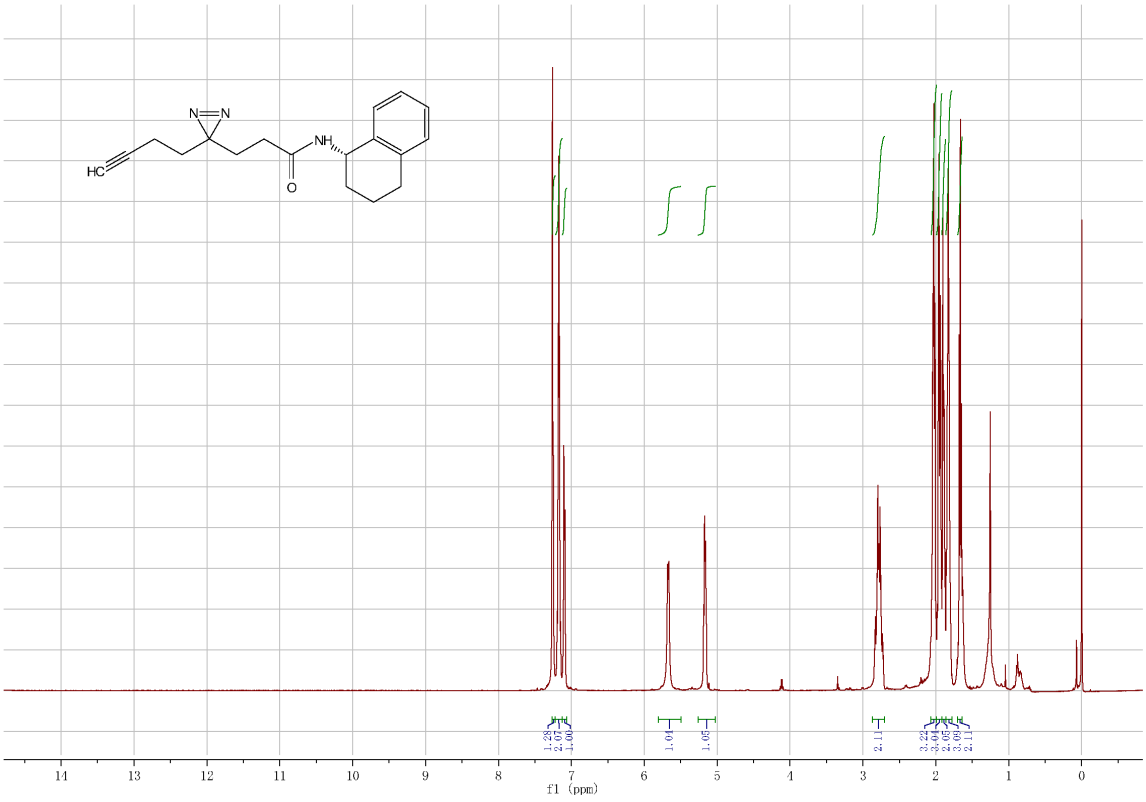
^{1}H-NMR for (*R*)-**3**



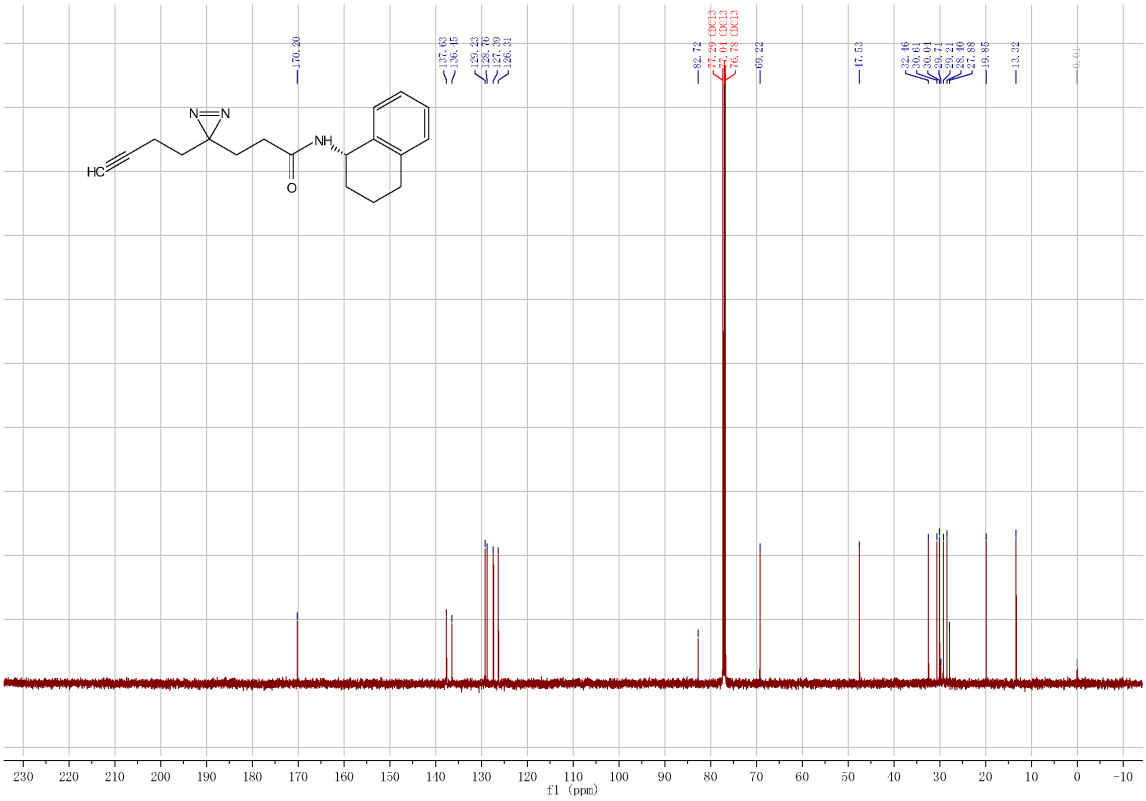
^{13}C-NMR for (*R*)-**3**



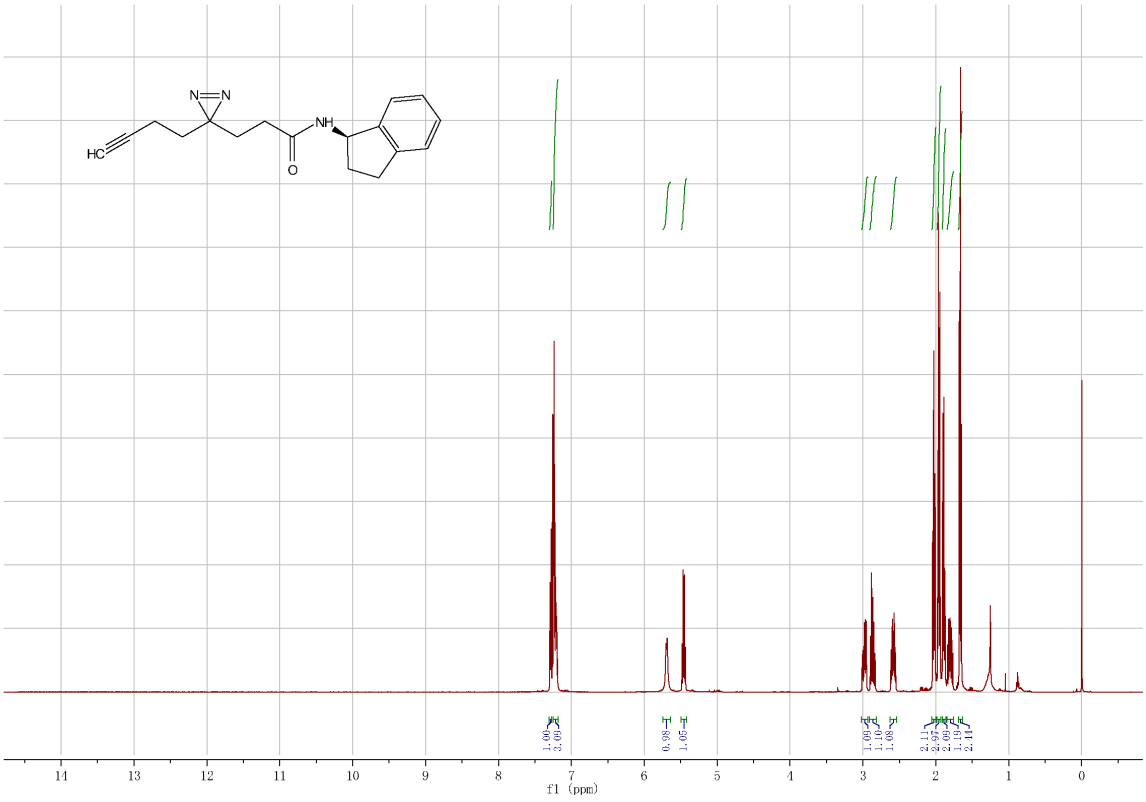
^{1}H-NMR for (*S*)-**3**



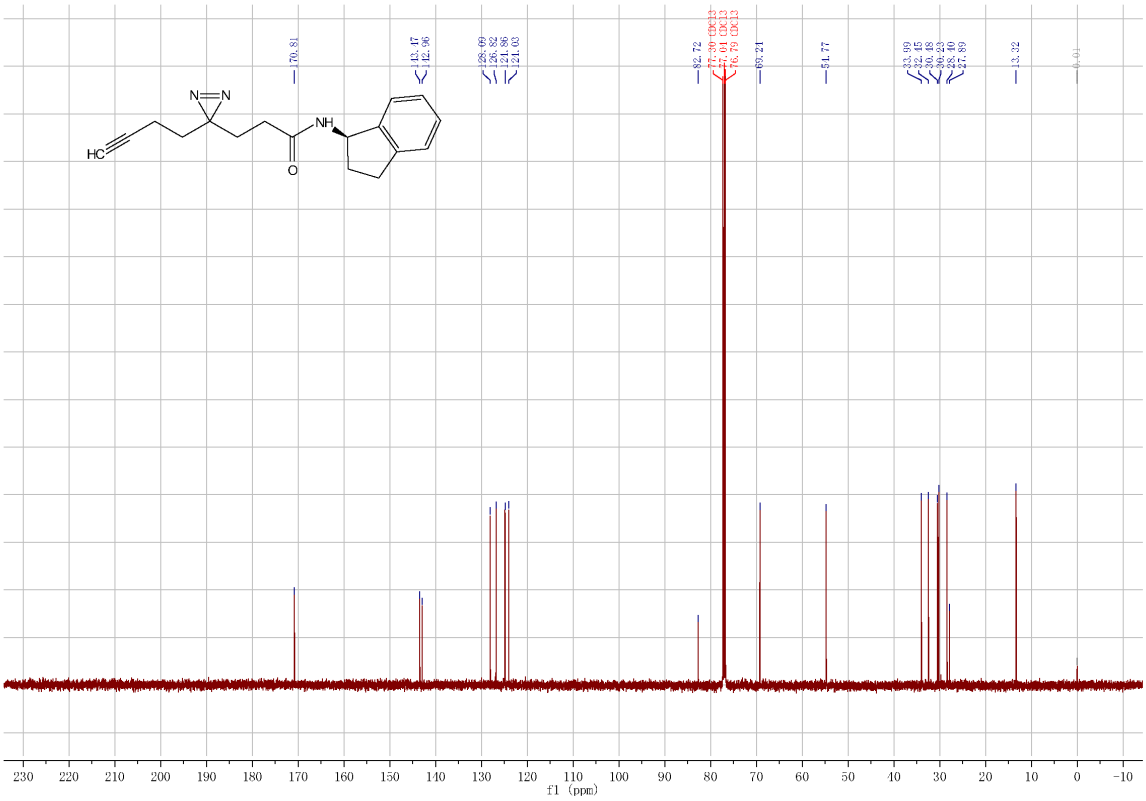
^{13}C-NMR for (*S*)-**3**



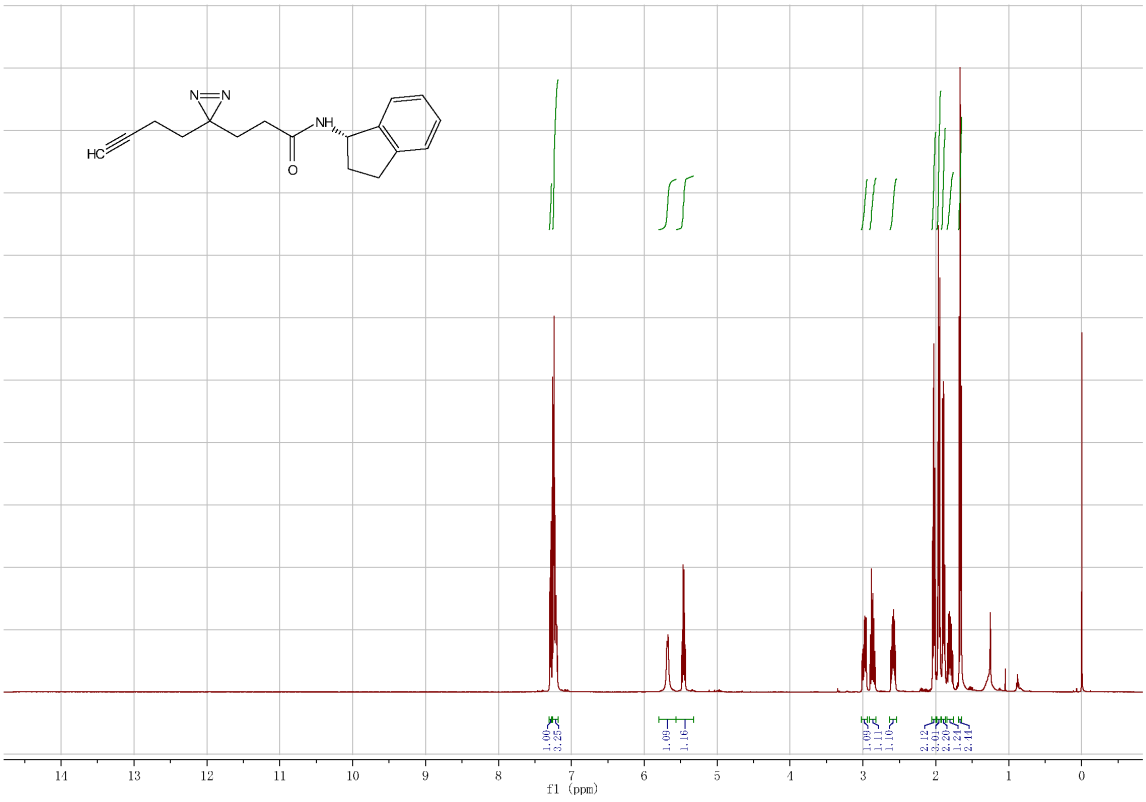
^{1}H-NMR for (*R*)-**4**



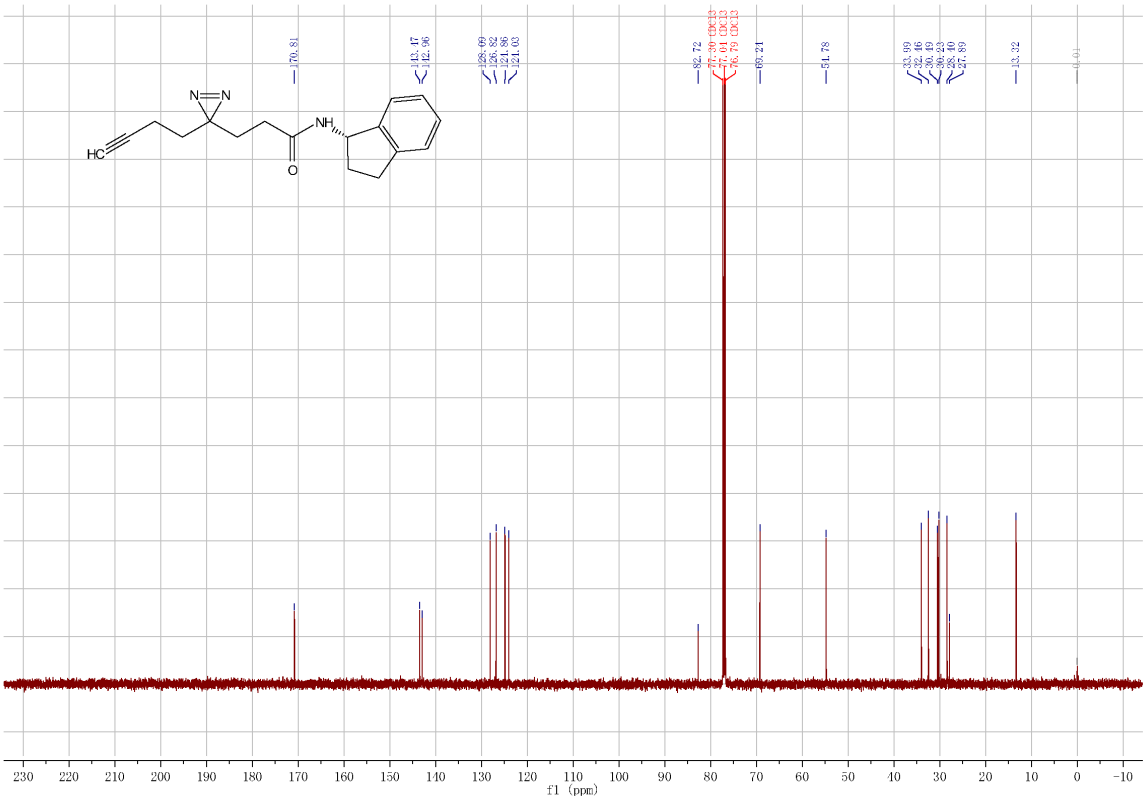
^{13}C-NMR for (*R*)-**4**



^{1}H-NMR for (*S*)-**4**



^{13}C-NMR for (*S*)-**4**



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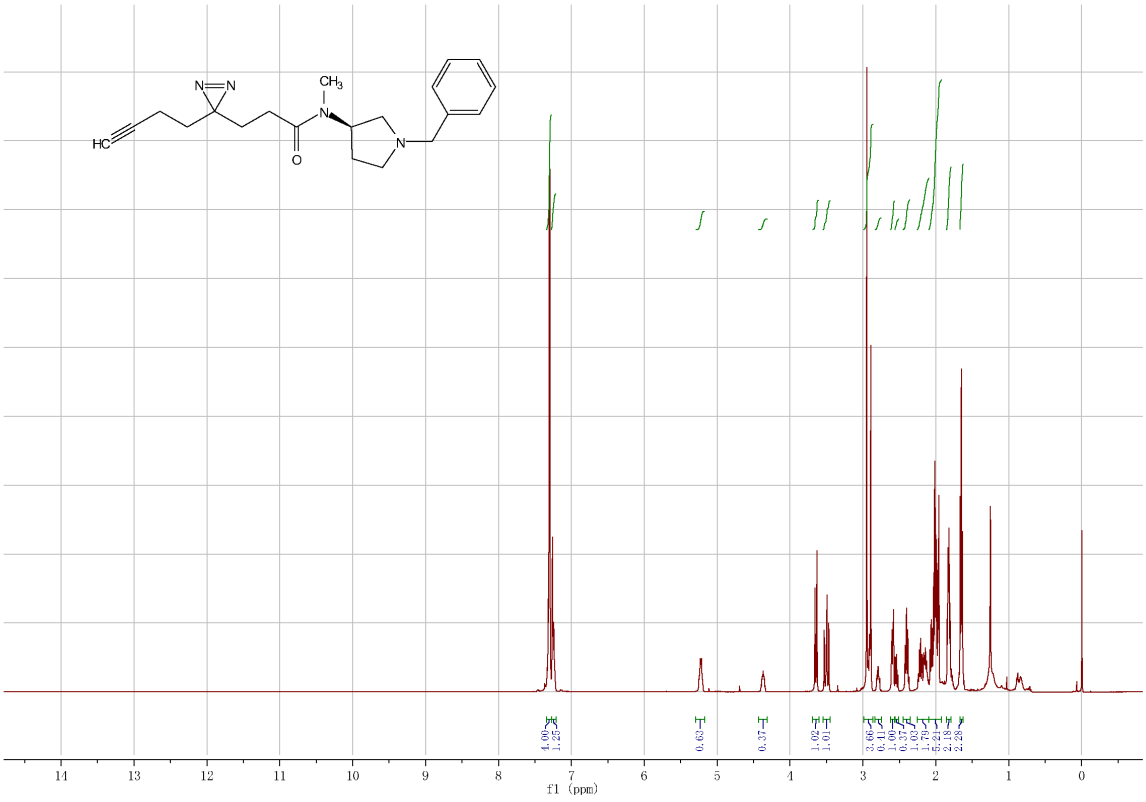
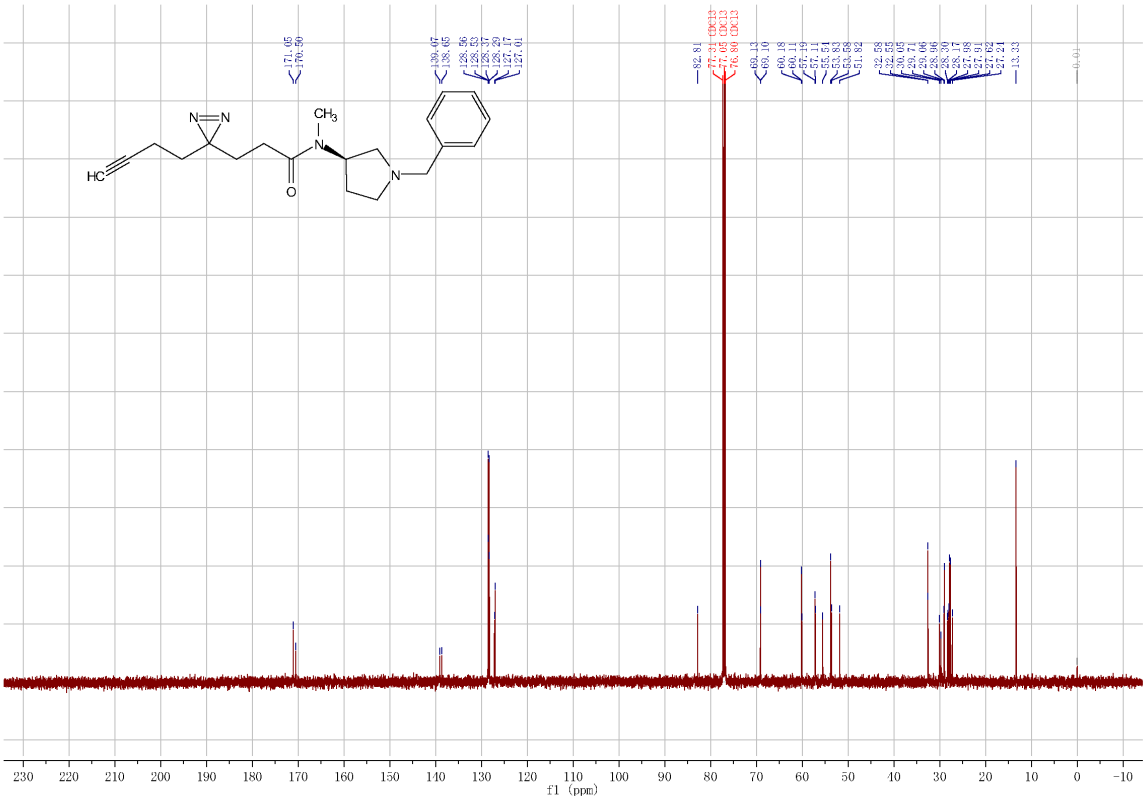
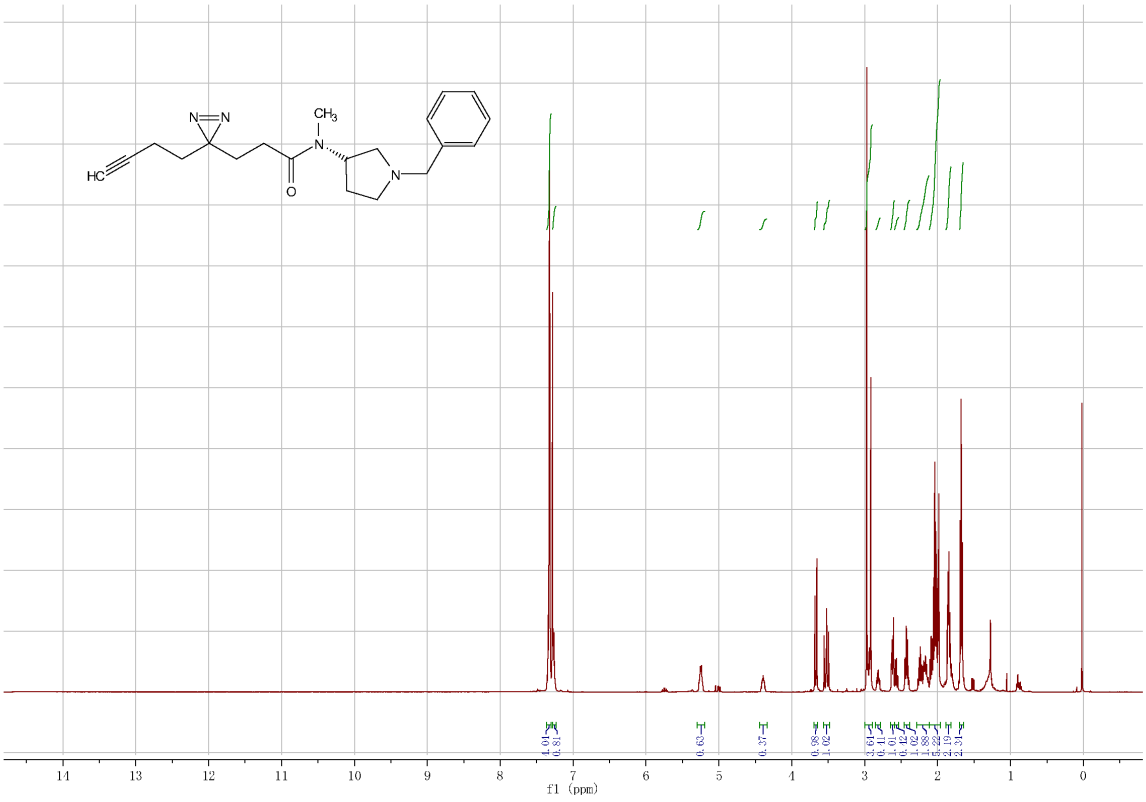


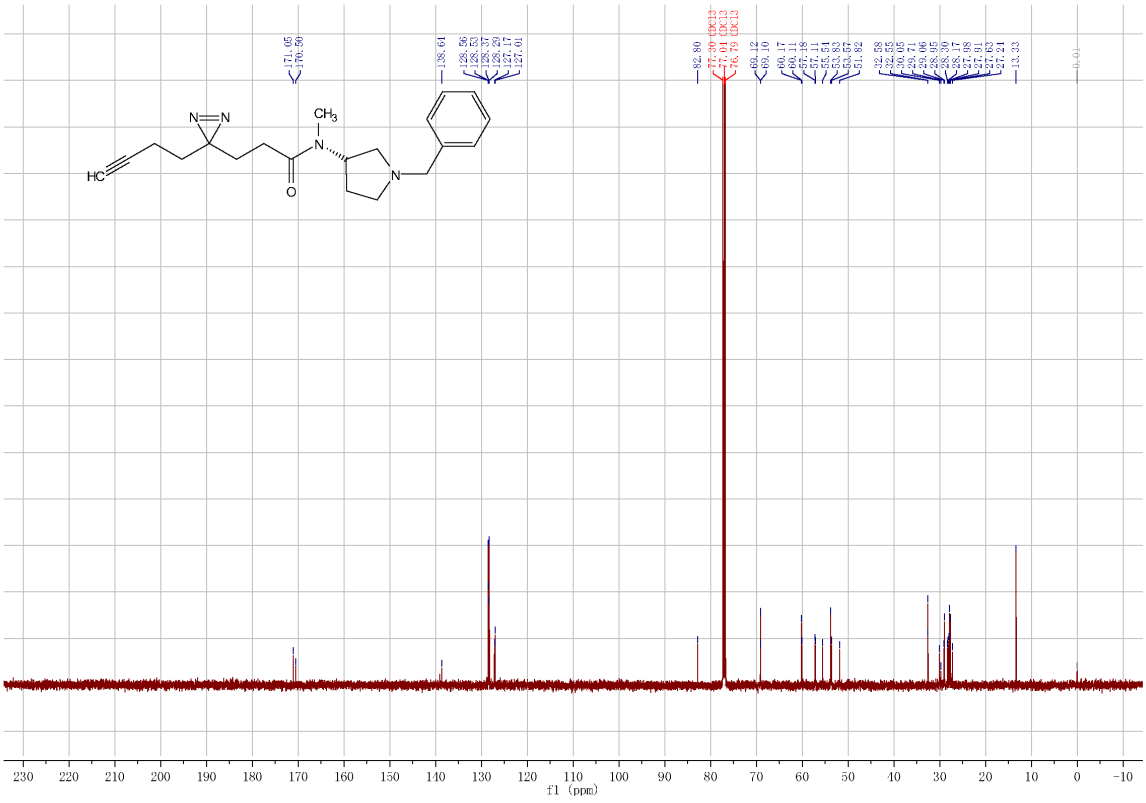
^{13}C-NMR for (*R*)-**5**



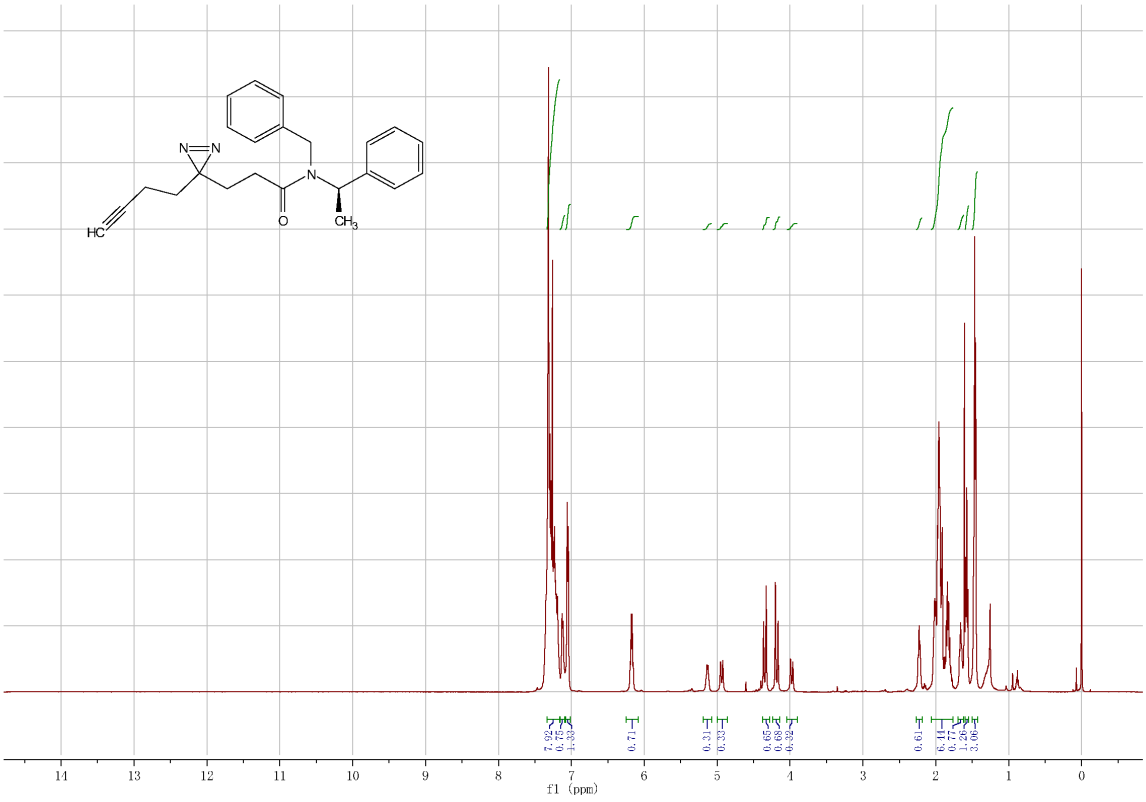
^{1}H-NMR for (*S*)-**5**



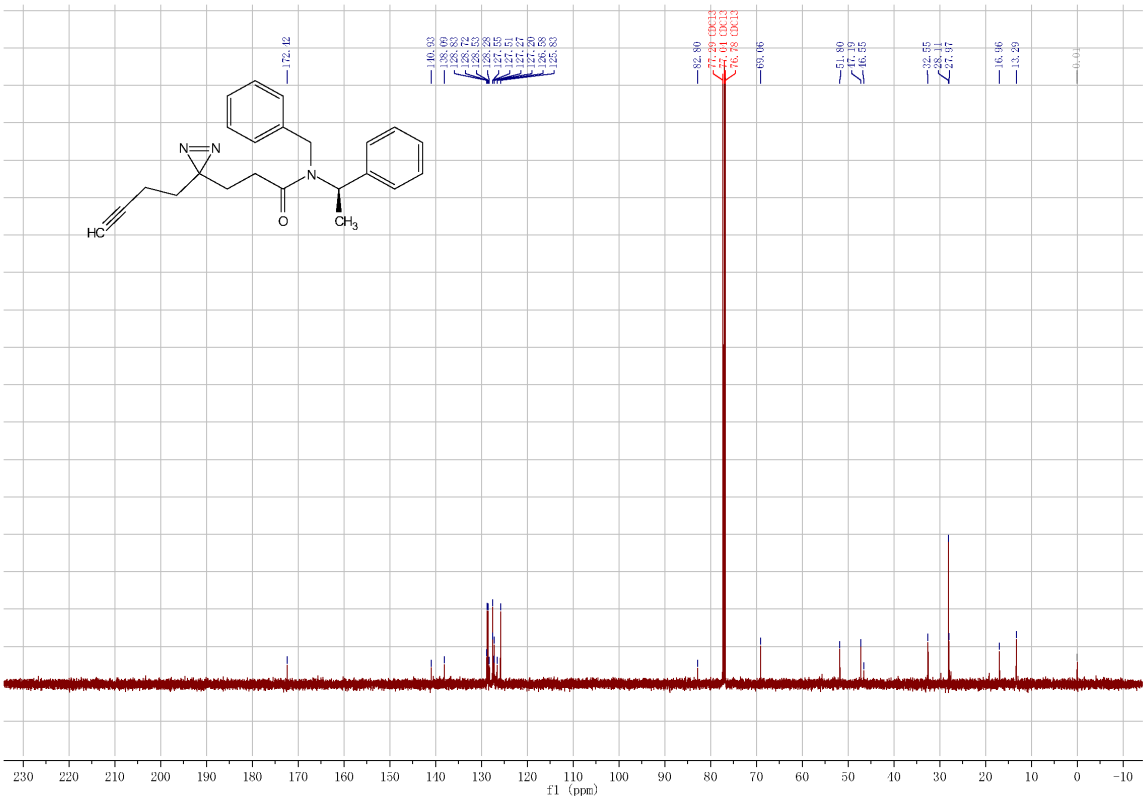
^{13}C-NMR for (*S*)-**5**



^{1}H-NMR for (*R*)-**6**



^{13}C-NMR for (*R*)-**6**



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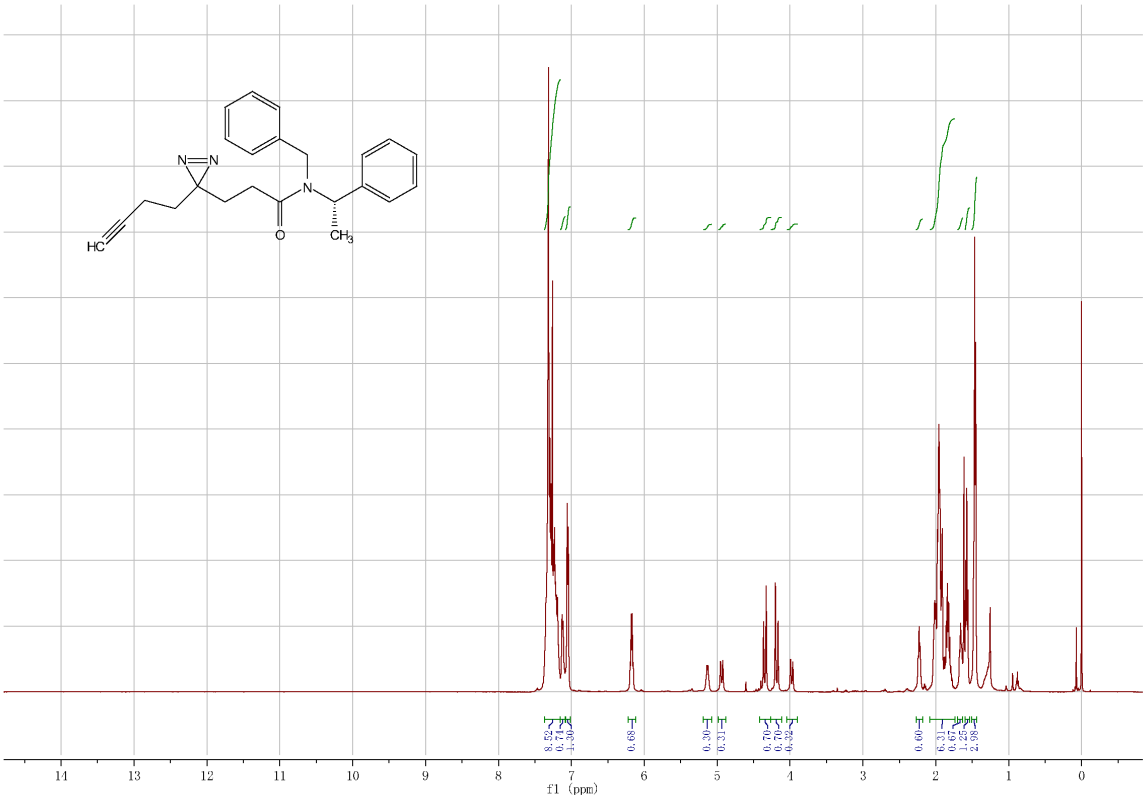

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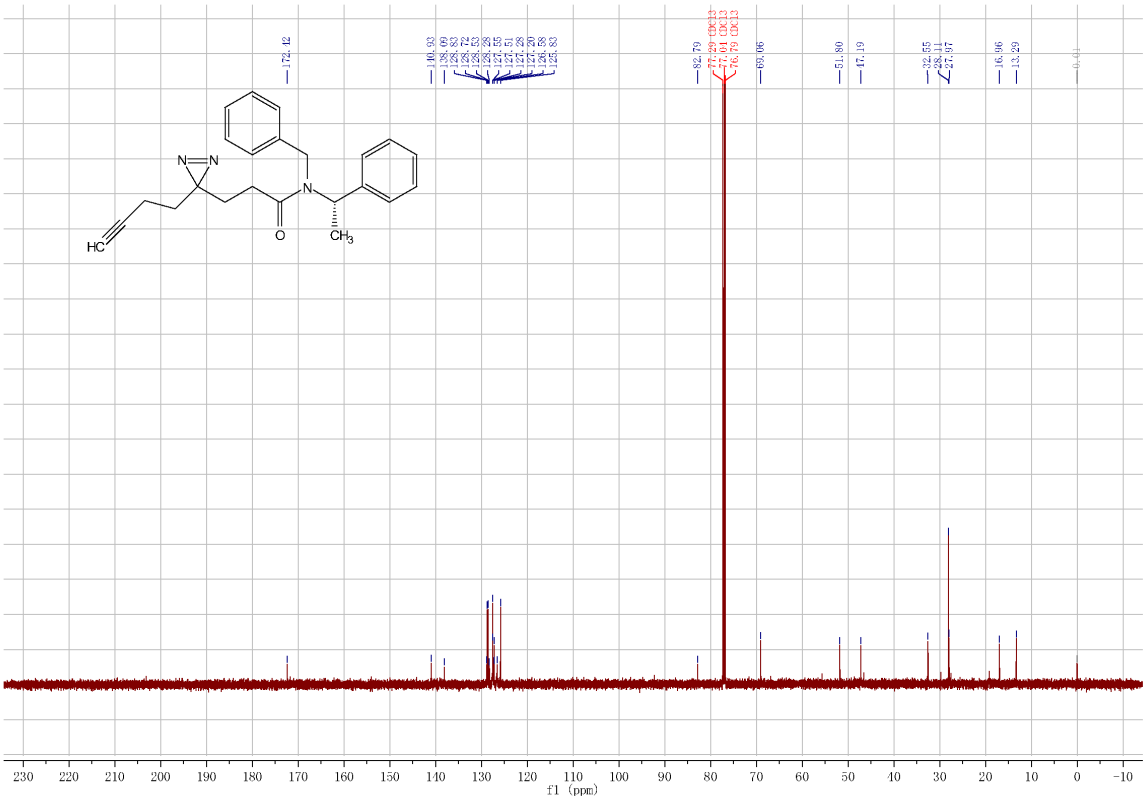
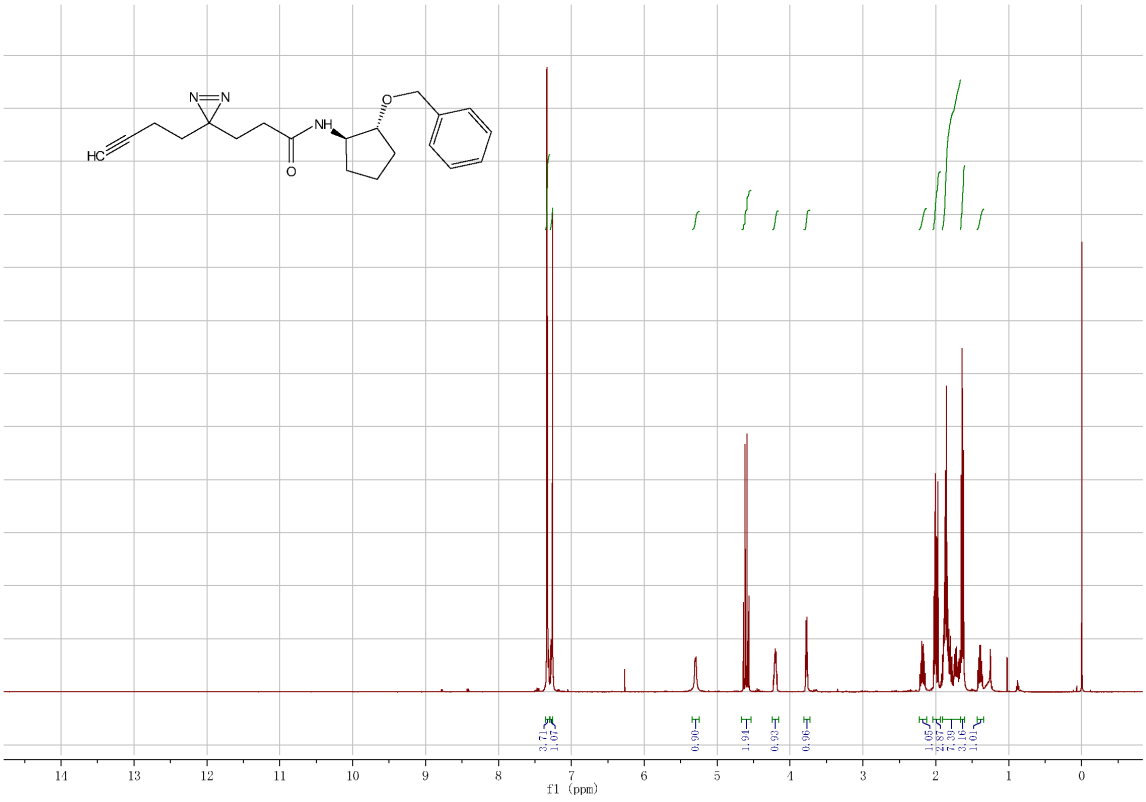


^{1}H-NMR for (*R*)-**7**



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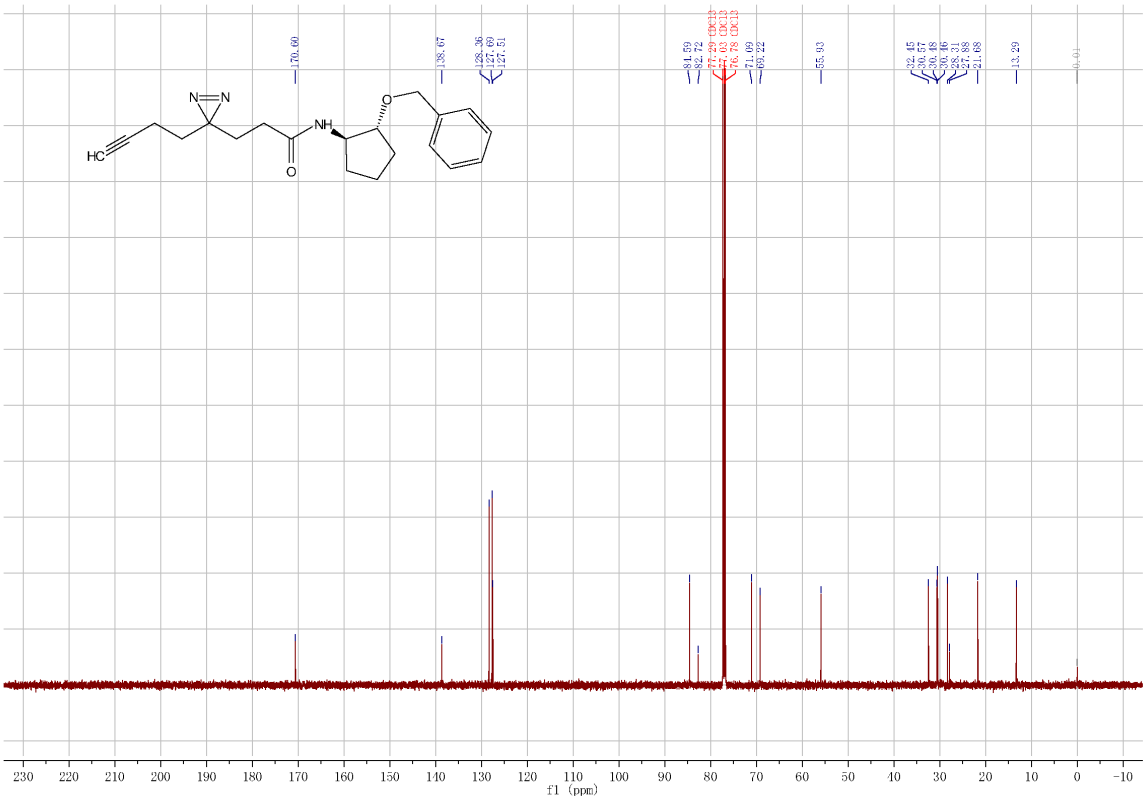
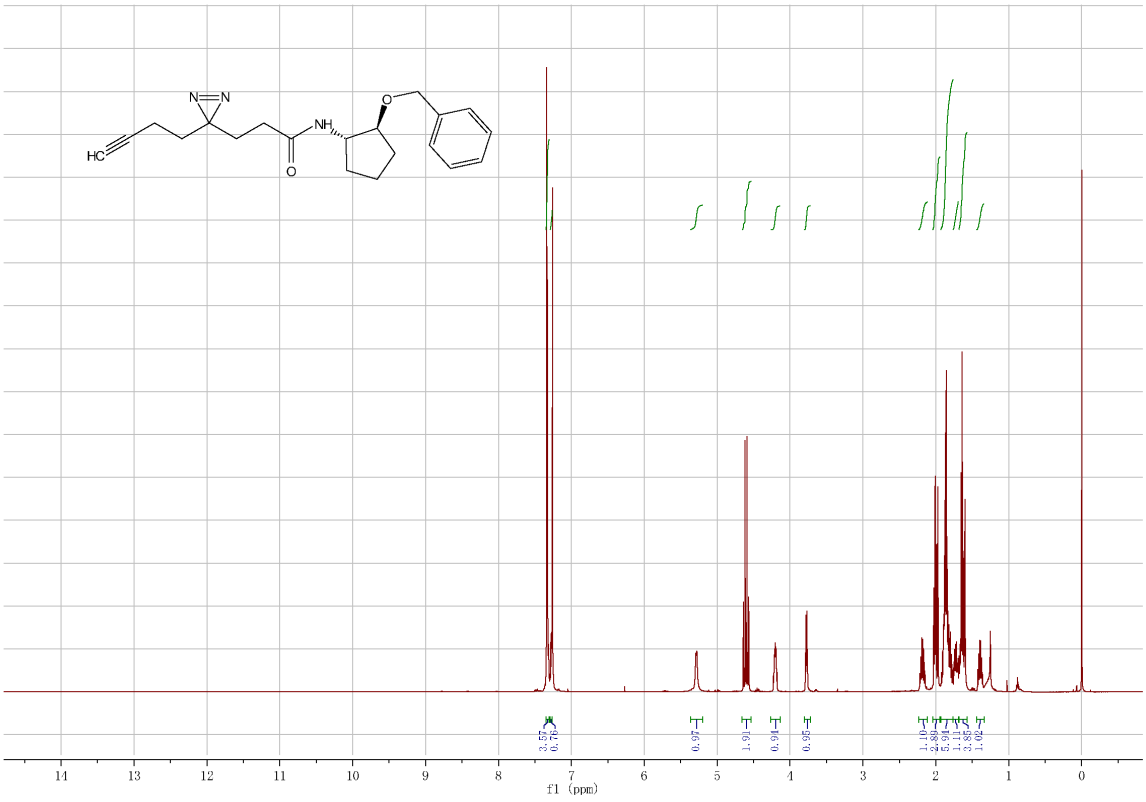
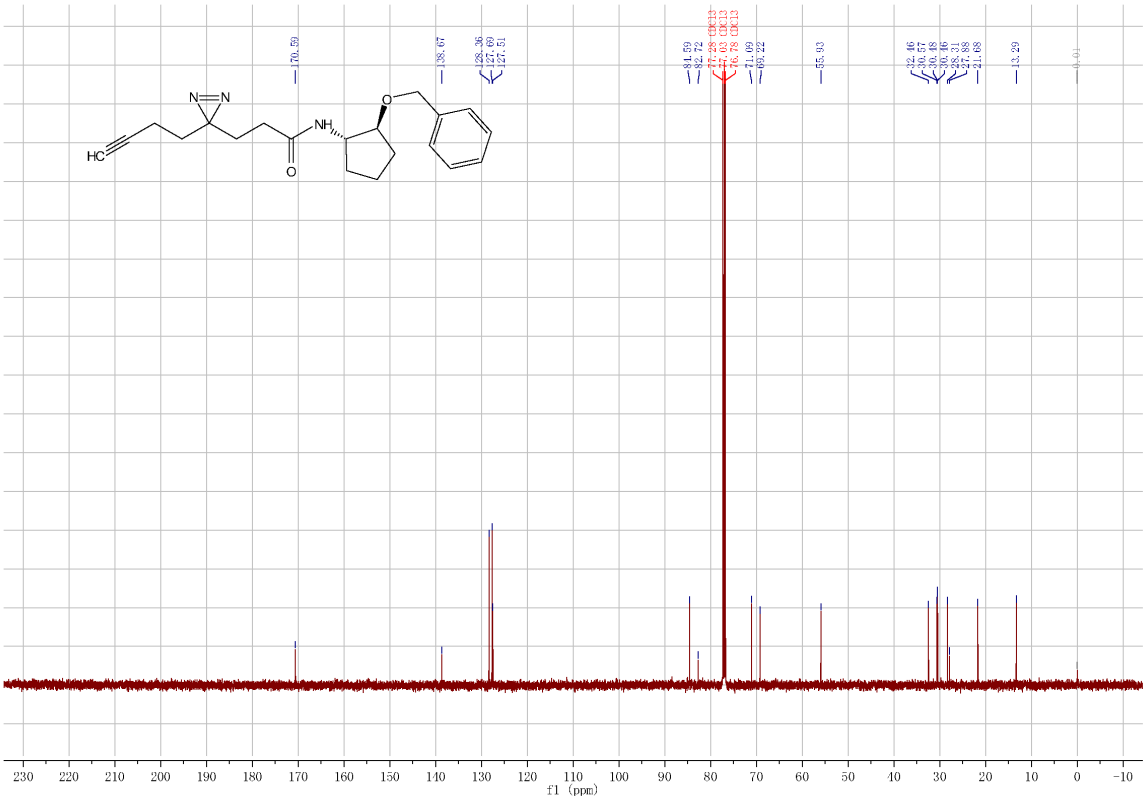


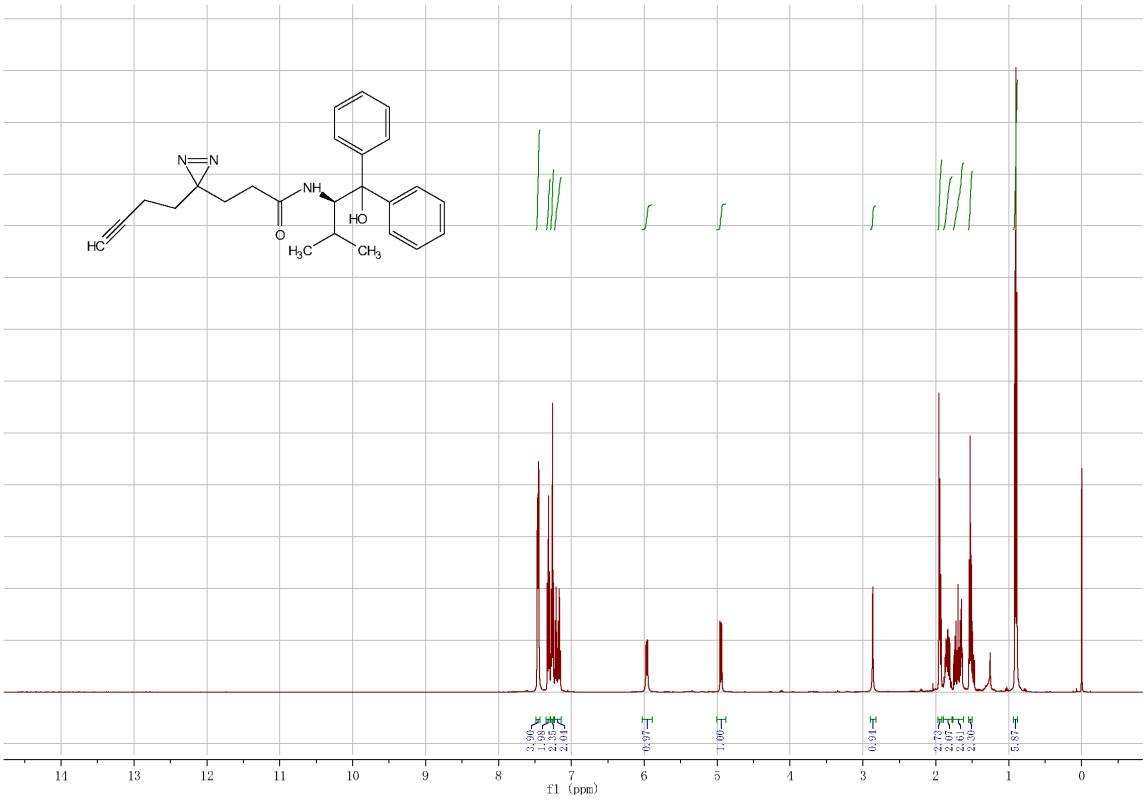
^{1}H-NMR for (*S*)-**7**



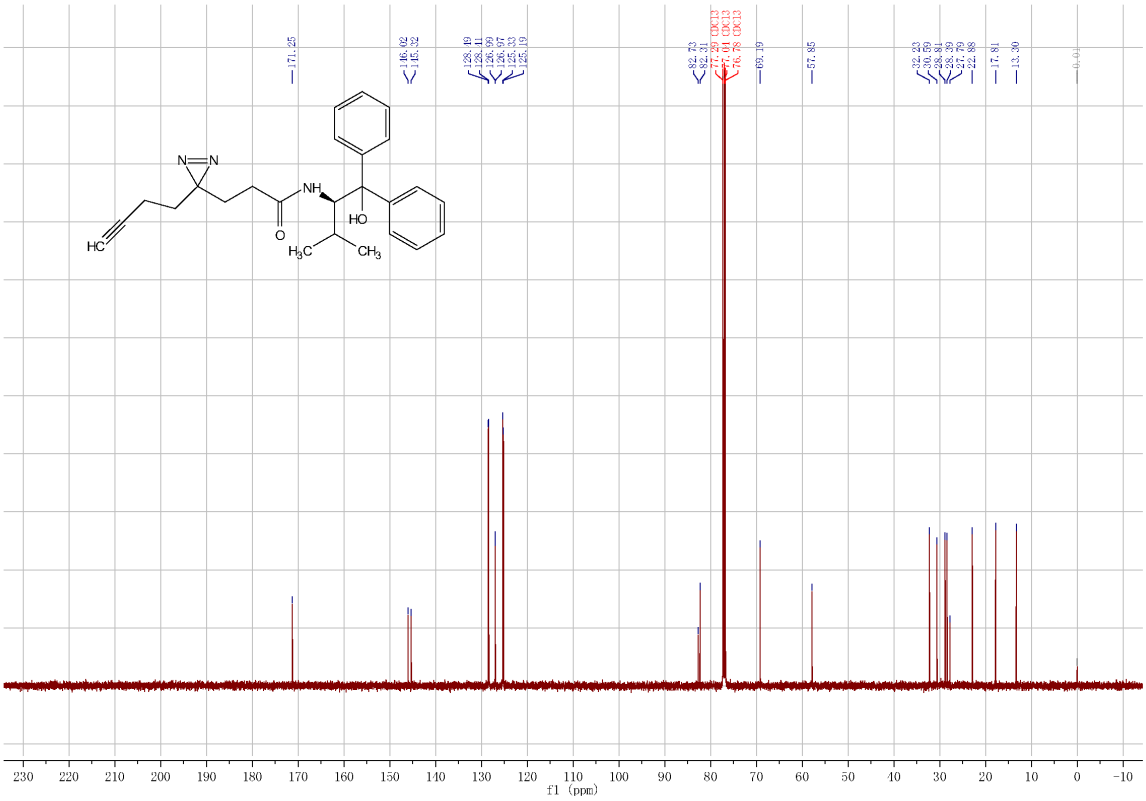
^{13}C-NMR for (*S*)-**7**



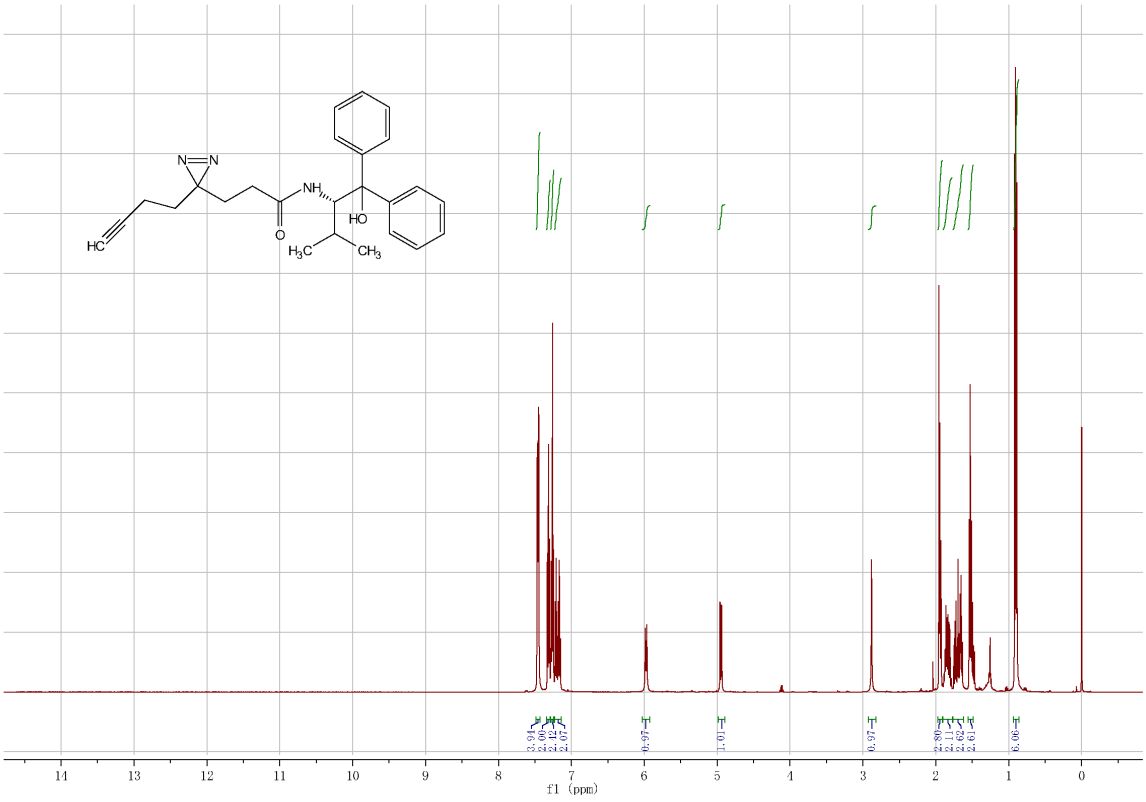
^{1}H-NMR for (*R*)-**8**



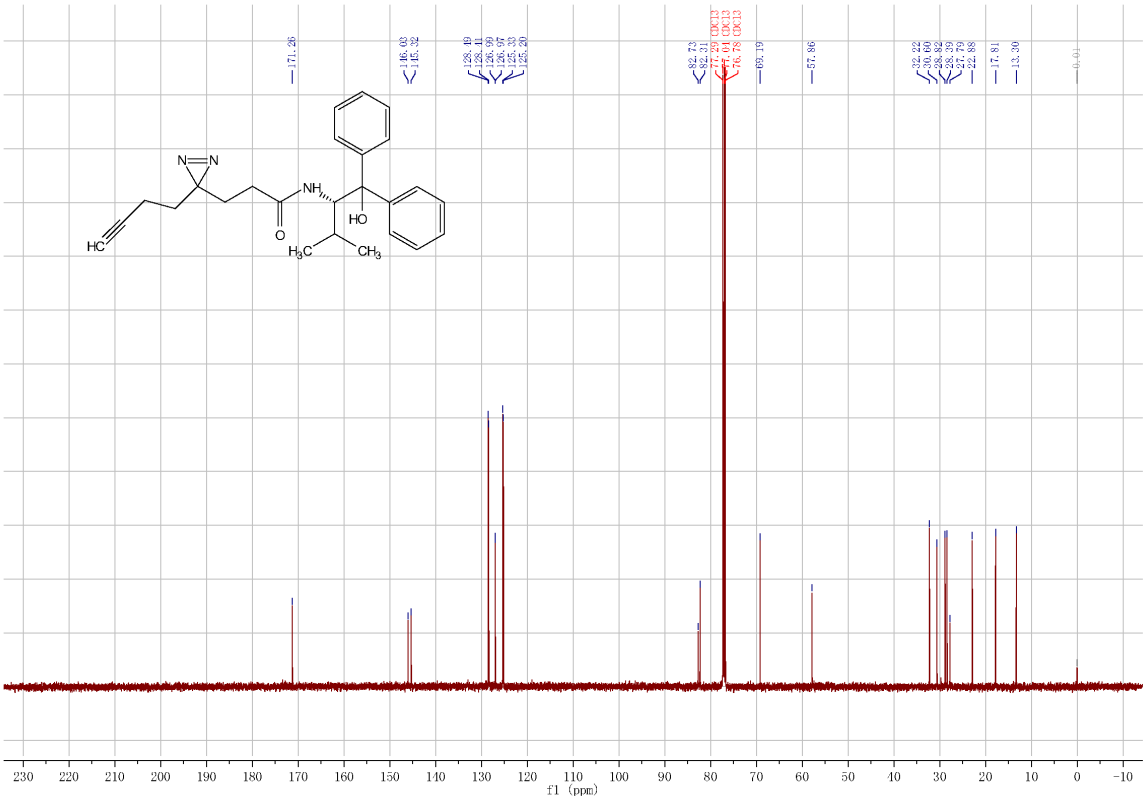
^{13}C-NMR for (*R*)-**8**



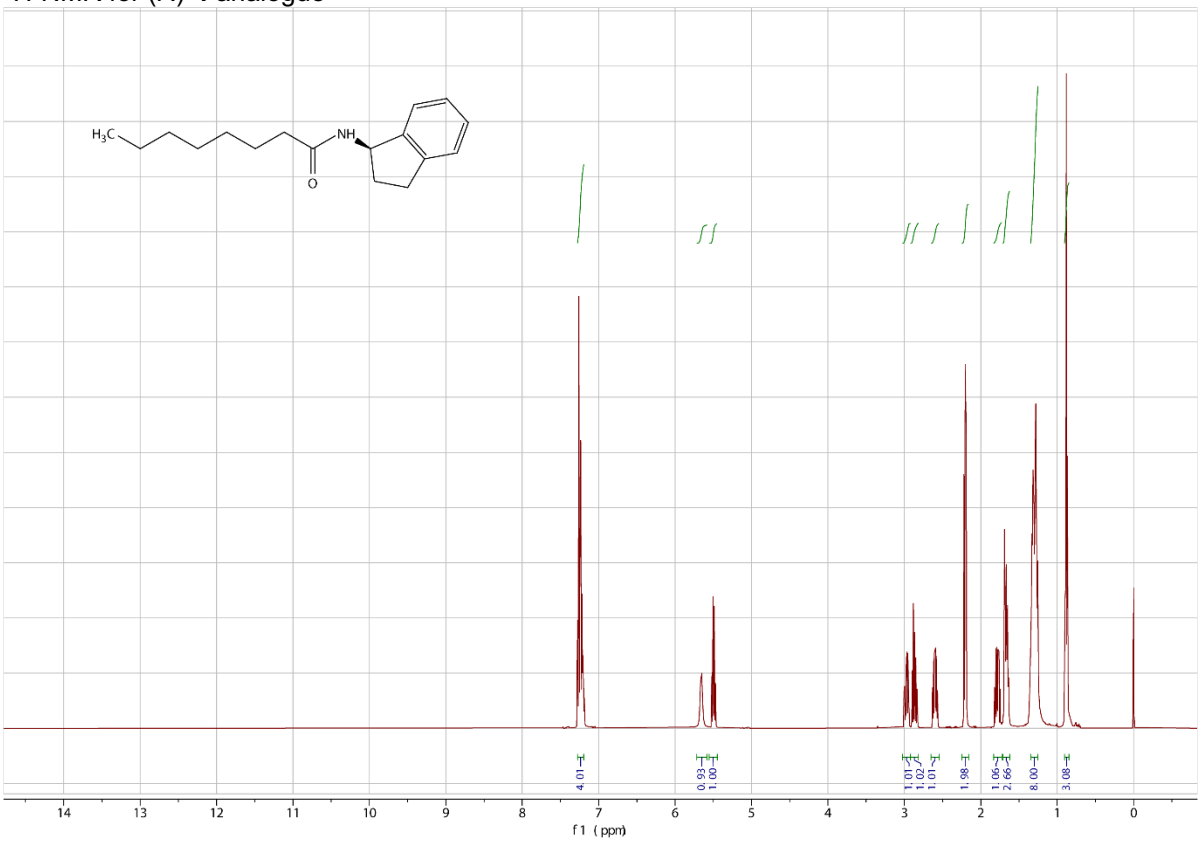
^{1}H-NMR for (*S*)-**8**



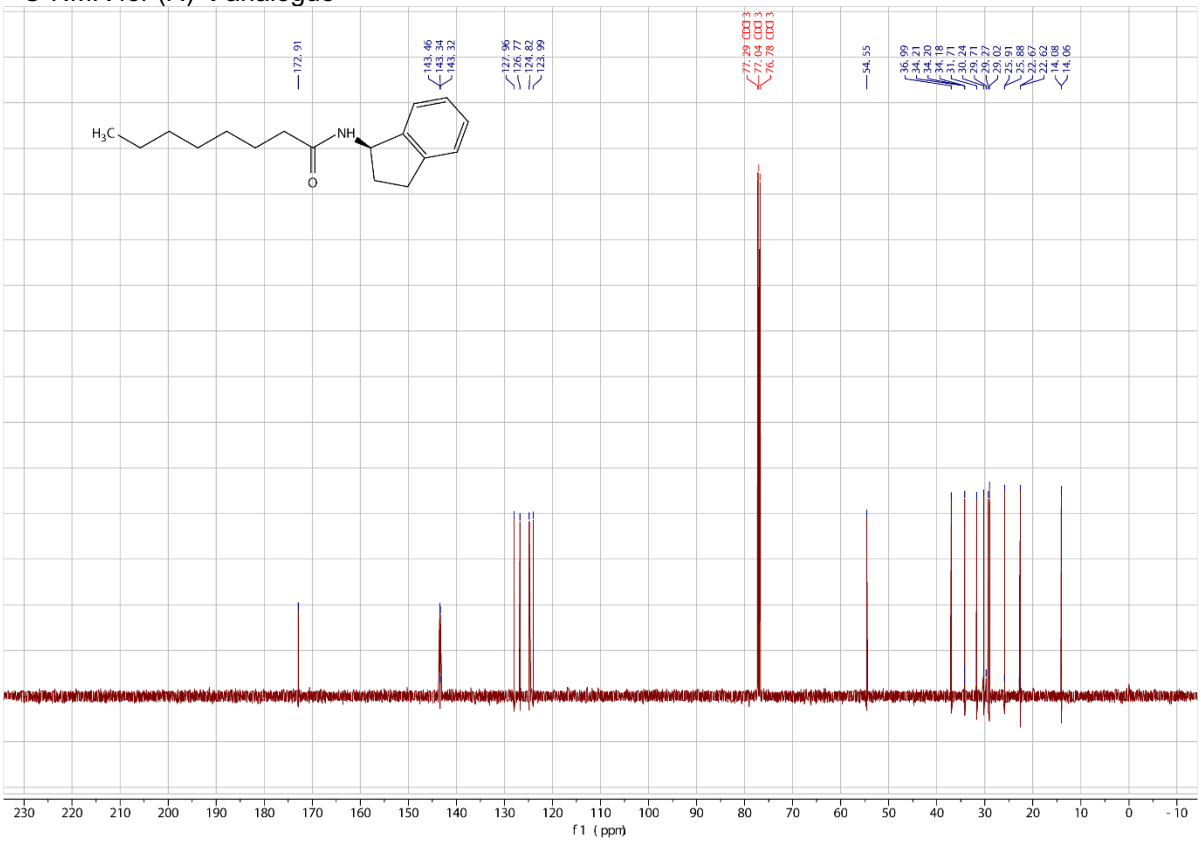
^{13}C-NMR for (*S*)-**8**



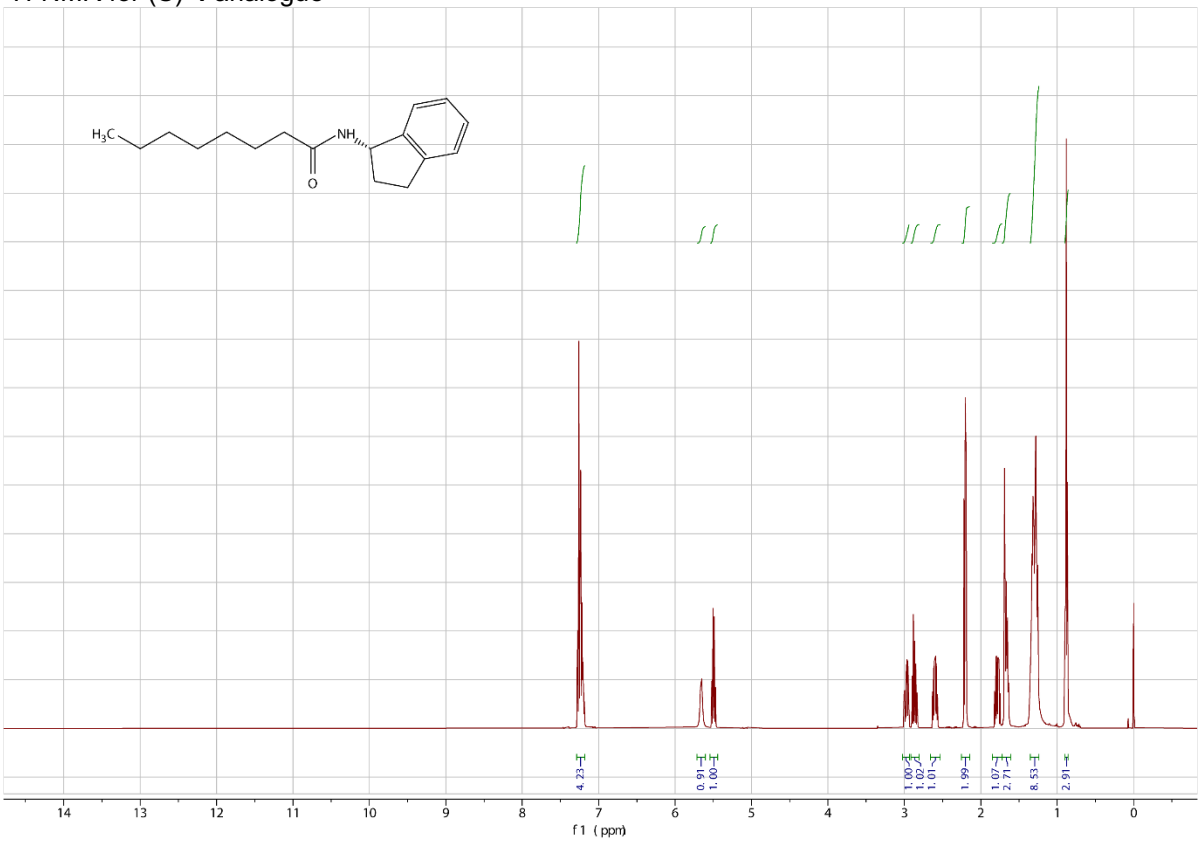
^{1}H-NMR for (*R*)-**4** analogue



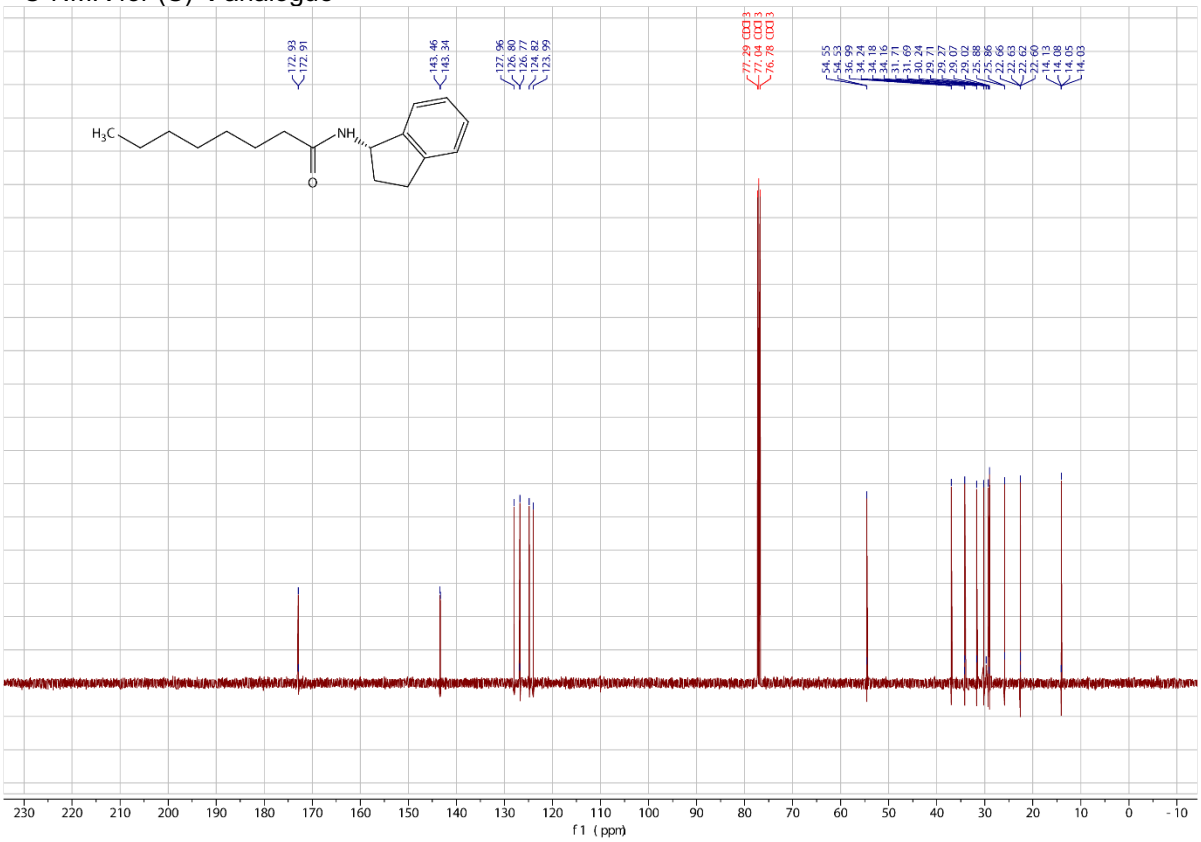
^{13}C-NMR for (*R*)-**4** analogue



^{1}H-NMR for (*S*)-**4** analogue



^{13}C-NMR for (*S*)-**4** analogue



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