Rapamycin-inspired macrocycles with new target specificity

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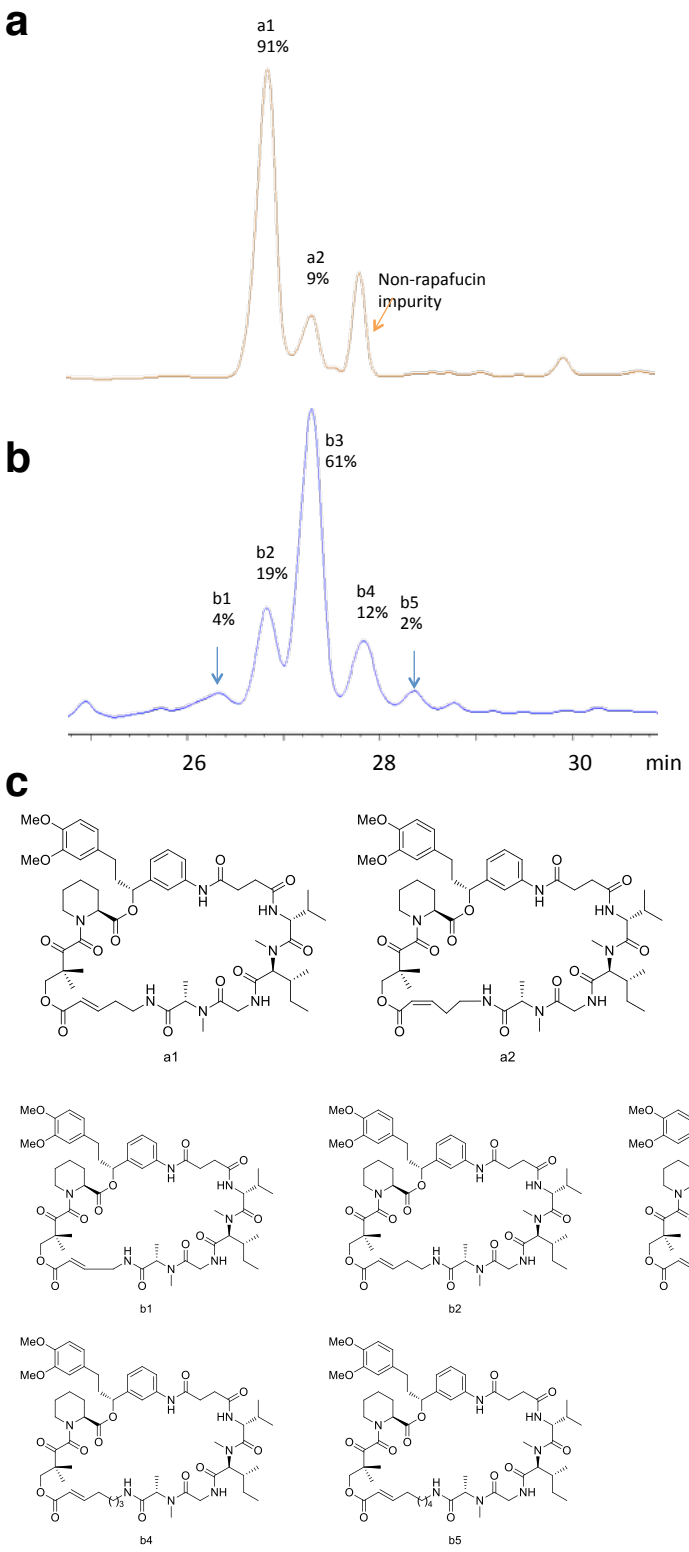
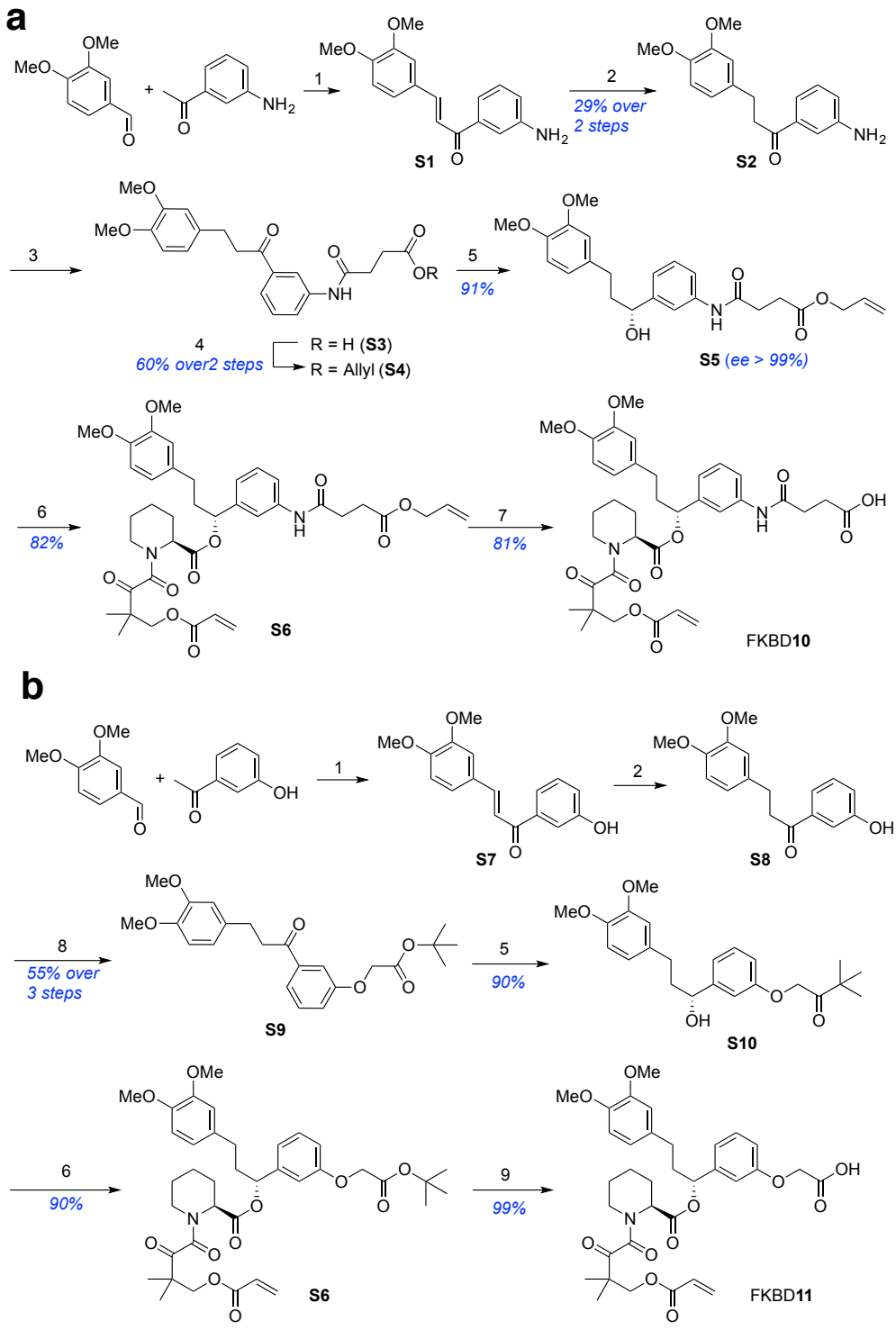
Supplementary Figure 1



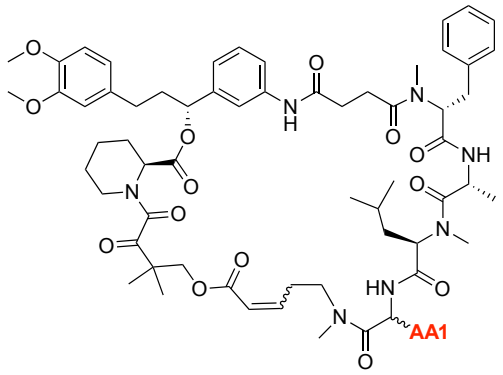
Supplementary Figure 1. Product distribution of ^{D}Val-^{M}Ile-Gly-^{M}Ala with (**a**) *cis*-C6 linker, (**b**) *cis*-C8 linker and FKBD**10** in HPLC analysis. Representative image of $n = 3$ independent experiments with similar results. **c**, Structures for the annotated peaks.

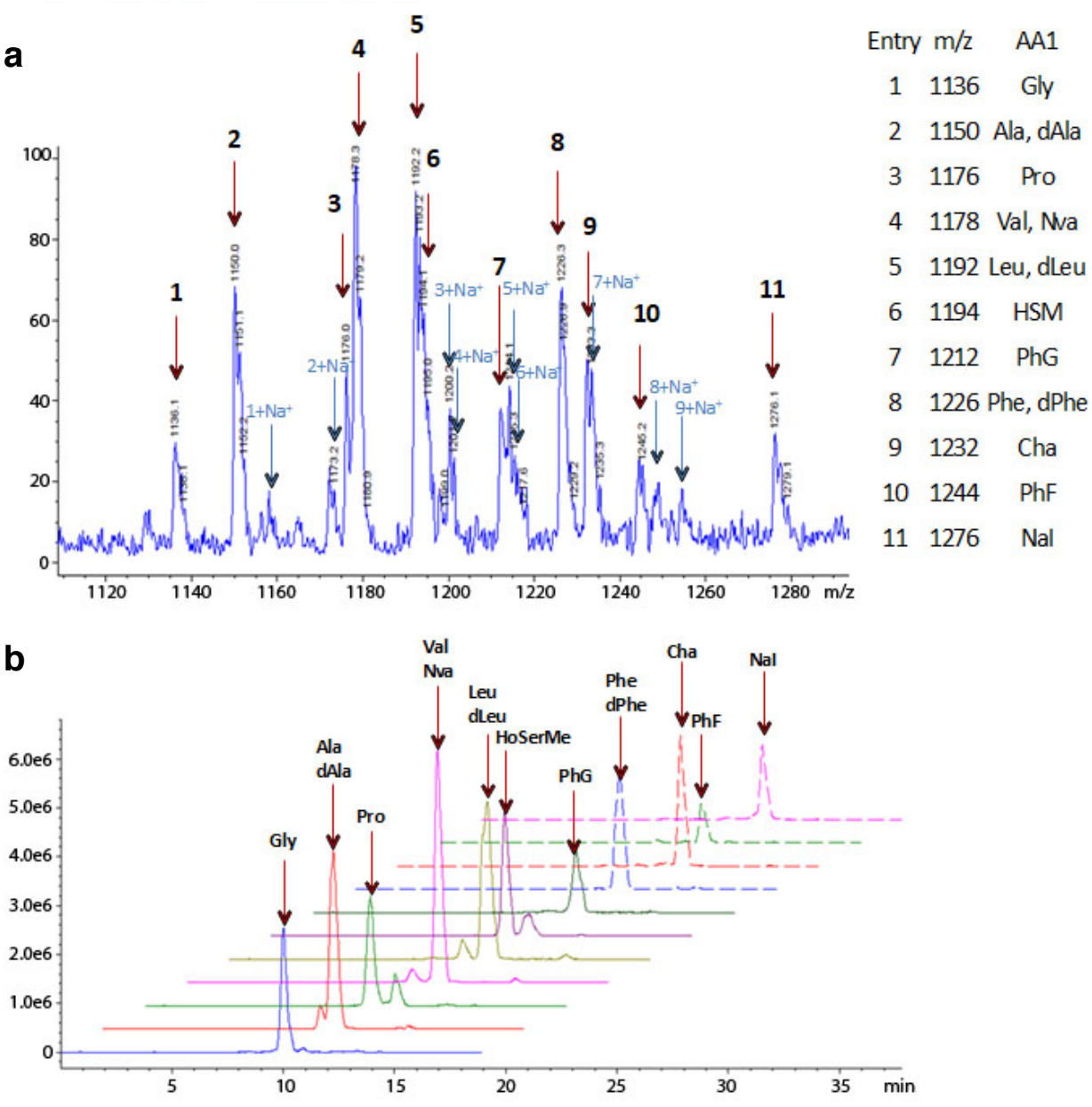
Supplementary Figure 2



Supplementary Figure 2. Synthetic route for (**a**) FKBD**10** and (**b**) FKBD**11**. Reaction conditions: **(1)** KOH, H_2O/EtOH (1/20), RT, 6h; **(2)** Pd/C (10%), H_2, MeOH, RT, 1.5 h; **(3)** succinic anhydride, DMAP (5%), CH_2Cl_2, RT, 3h; **(4)** allyl bromide, Cs_2CO_3, DMF, RT, 2h; **(5)** (+)-DIPCl, THF, -20°C→RT, 5h; **(6) S16** (see synthesis below), benzoyl chloride, DMAP (5%), NEt_3, CH_2Cl_2, RT, 4h; **(7)** $Pd(PPh_3)_4$ (10%), *N*-methylaniline, THF, RT, 6h; **(8)** *tert*-butyl 2-bromoacetate, K_2CO_3, DMF/acetone (1/2), RT, 4h; **(9)** TFA (10%), CH_2Cl_2, RT, 6h.

Supplementary Figure 3

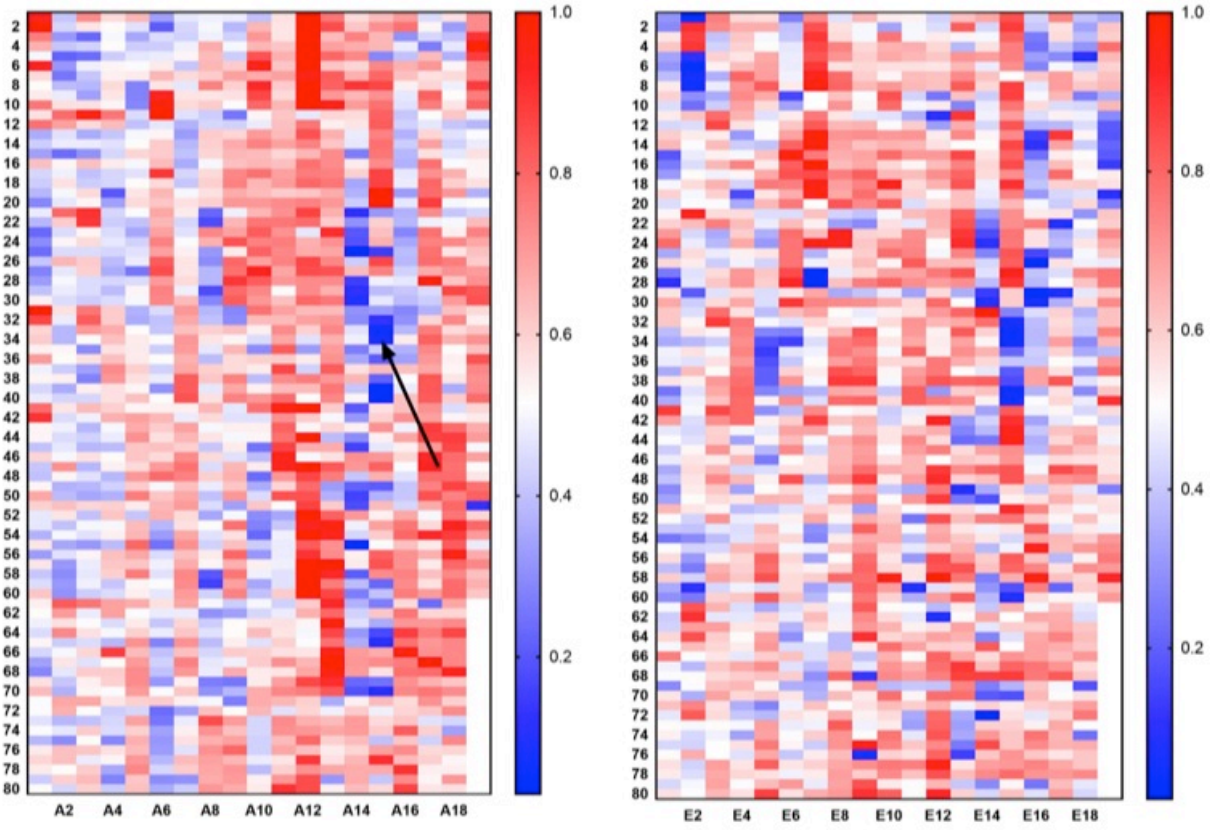




Entry	m/z	AA1
1	1136	Gly
2	1150	Ala, dAla
3	1176	Pro
4	1178	Val, Nva
5	1192	Leu, dLeu
6	1194	HSM
7	1212	PhG
8	1226	Phe, dPhe
9	1232	Cha
10	1244	PhF
11	1276	Nal

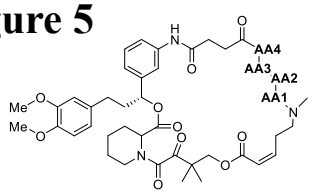
Supplementary Figure 3. a, Mass spectra of pool A8G9 showing all mass peaks as expected according to the structure of rapafucins. No obvious impurities are observed. **b**, Ion extraction of LC-MS chromatogram ensures that all desired rapafucins are obtained. Representative image of $n = 3$ independent experiments with similar results.

Supplementary Figure 4



Supplementary Figure 4. Heatmap of the outcome of the screening of rapamycin libraries using ^{3}H-thymidine uptake assay in HUVEC. FKBD10- (left) and FKBD11-containing libraries (right) were screened at a final concentration of 3 μM. Scale: 1, no inhibition; 0, 100% inhibition. The arrow points to one of the most potent pool of hits at Row 34, Column A15, that was further decoded.

Supplementary Figure 5



Deconvolution

Name	Residue 1	Residue 2	Residue 3	Residue 4	IC_{50} (nM)
A15-34-8	Phe	dmPhe	Pro	mLeu	426 ± 83
A15-34-1	Gly	dmPhe	Pro	mLeu	751 ± 139
A15-34-2	Ala	dmPhe	Pro	mLeu	478 ± 65
A15-34-5	HoSMe	dmPhe	Pro	mLeu	1.1 ± 0.3 μM
A15-34-3	D-Ala	dmPhe	Pro	mLeu	1.5 ± 0.3 μM
A15-34-4	Nva	dmPhe	Pro	mLeu	1.1 ± 0.2 μM
A15-34-6	Leu	dmPhe	Pro	mLeu	>20 μM
A15-34-7	D-Leu	dmPhe	Pro	mLeu	>20 μM
A15-34-9	D-Phe	dmPhe	Pro	mLeu	>20 μM
A15-34-10	PhF	dmPhe	Pro	mLeu	>20 μM
A15-34-11	Pro	dmPhe	Pro	mLeu	5.8 ± 0.9 μM
A15-34-12	Cha	dmPhe	Pro	mLeu	>20 μM
A15-34-13	Nal	dmPhe	Pro	mLeu	>20 μM
A15-34-14	Val	dmPhe	Pro	mLeu	1.2 ± 0.3 μM
A15-34-15	PhG	dmPhe	Pro	mLeu	>20 μM

Orientation Optimization

Name	Residue 1	Residue 2	Residue 3	Residue 4	IC_{50} (nM)
SH-13	mLeu	dPro	mPhe	Phe	366 ± 33
SH-4	Phe	dmPhe	Pro	dmLeu	955 ± 113
SH-7	Phe	dmPhe	dPro	dmLeu	>20 μM
SH-2	Phe	mPhe	Pro	mLeu	>20 μM
SH-3	Phe	dmPhe	dPro	mLeu	>20 μM
SH-5	Phe	mPhe	dPro	mLeu	>20 μM
SH-14	mLeu	dPro	dmPhe	dPhe	3.4 ± 0.8 μM
SH-1	mLeu	Pro	mPhe	dPhe	>20 μM
SH-6	Phe	mPhe	Pro	dmLeu	>20 μM
SH-8	mLeu	Pro	dmPhe	Phe	>20 μM
SH-9	dmLeu	Pro	dmPhe	Phe	>20 μM
SH-10	mLeu	dPro	dmPhe	Phe	>20 μM
SH-11	mLeu	Pro	mPhe	Phe	>20 μM
SH-12	mLeu	Pro	dmPhe	dPhe	>20 μM

Round 1

Name	Residue 1	Residue 2	Residue 3	Residue 4	IC_{50} (nM)
95-15	mLeu	dPro	mPhe	Phe	377 ± 43
95-5	mGly	dPro	mPhe	Phe	440 ± 100
95-1	mPhe	dPro	mAla	Phe	737 ± 130
95-13	mLeu	dPro	mLeu	Phe	787 ± 150
95-14	mLeu	dPro	mSer	Phe	725 ± 120
95-2	mPhe	dPro	mPhe	Phe	>20 μM
95-6	mSer	dPro	mLeu	Phe	>20 μM
95-4	mGly	dPro	mSer	Phe	>20 μM
95-8	mLeu	dPro	mAla	Phe	>20 μM
95-7	mPhe	dPro	mLeu	Phe	>20 μM
95-12	mSer	dPro	mSer	Phe	12.8 ± 3.1
95-10	mAla	dPro	mPhe	Phe	>20 μM
95-11	mPhe	dPro	mSer	Phe	>20 μM
95-9	mAla	dPro	mSer	Phe	>20 μM
95-3	mGly	dPro	mLeu	Phe	>20 μM

Round 2

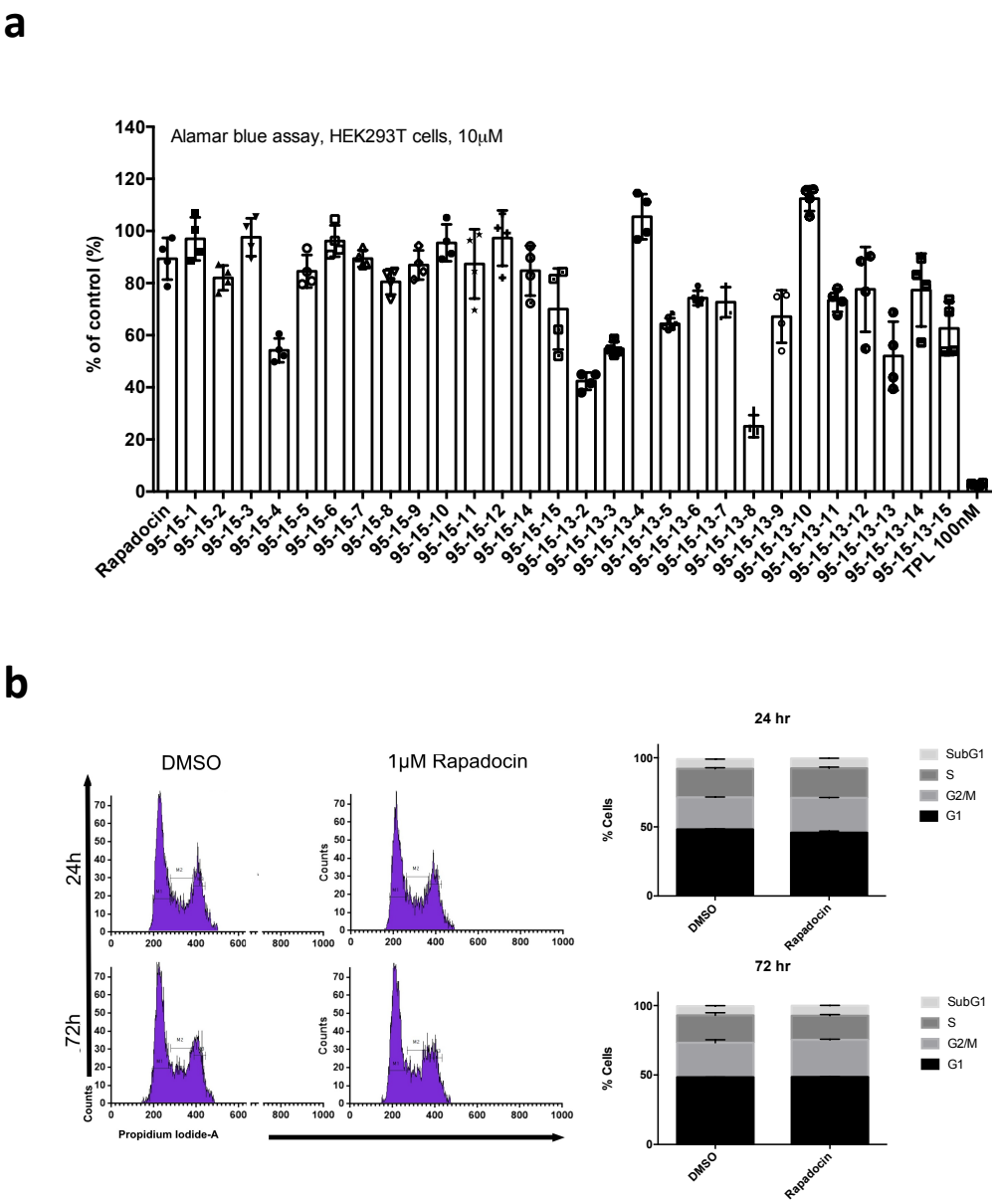
Name	Residue 1	Residue 2	Residue 3	Residue 4	IC_{50} (nM)
95-15-13	mLeu	dPro	mPhe	Phg	5.0 ± 1.2
95-15-9	mLeu	dPro	mPhe	Cha	112 ± 28
95-15-10	mLeu	dPro	Phe	Phe	182 ± 31
95-15-1	mLeu	dPro	mPhe	Phe	377 ± 43
95-15-14	mLeu	dPro	mPhe	PheF	11.1 ± 0.1 μM
95-15-5	mIle	dPro	mPhe	Phe	1.2 ± 0.2 μM
95-15-7	mLeu	Pro	mPhe	Phe	1.9 ± 0.4 μM
95-15-3	mLeu	dPro	mPhe	Tyr	> 20 μM
95-15-6	mNle	dPro	mPhe	Phe	> 20 μM
95-15-15	mLeu	dPro	mPhe	Nal	> 20 μM
95-15-2	mdLeu	dPro	mPhe	Phe	1.3 ± 0.3 μM
95-15-12	mLeu	dPro	mPhe	hoPhe	> 20 μM
95-15-4	mLeu	dPro	mPhe	TyrBu	> 20 μM
95-15-8	mLeu	dPro	mPhe	mPhe	> 20 μM
95-15-11	mLeu	dPro	mPhe	biPhe	> 20 μM

Round 3

Name	Residue 1	Residue 2	Residue 3	Residue 4	IC_{50} (nM)
Rapadocin	mLeu	dPro	mPhe	Phg	6.2 ± 1.3
95-15-13-10	mLeu	dPro	Tyr	Phg	10 ± 2.0
95-15-13-5	mLeu	dPro	hoPhe	Phg	15 ± 2.0
95-15-13-11	mLeu	dPro	TyrOMe	Phg	33 ± 9.0
95-15-13-7	mLeu	dPro	PheF	Phg	57 ± 13
95-15-13-9	mLeu	dPro	PheI	Phg	63 ± 17
95-15-13-3	mLeu	dPro	Phe	Phg	64 ± 15
95-15-13-14	mLeu	dPro	Nal	Phg	88 ± 25
95-15-13-8	mLeu	dPro	PheCl	Phg	123 ± 29
95-15-13-12	mLeu	dPro	PheNO2	Phg	130 ± 25
95-15-13-15	mLeu	dPro	biPhe	Phg	194 ± 51
95-15-13-13	mLeu	dPro	Cha	Phg	199 ± 42
95-15-13-6	mLeu	dPro	Phg	Phg	261 ± 68
95-15-13-4	mLeu	dPro	Pyr	Phg	266 ± 43
95-15-13-2	mLeu	dHoPro	mPhe	Phg	1920 ± 530

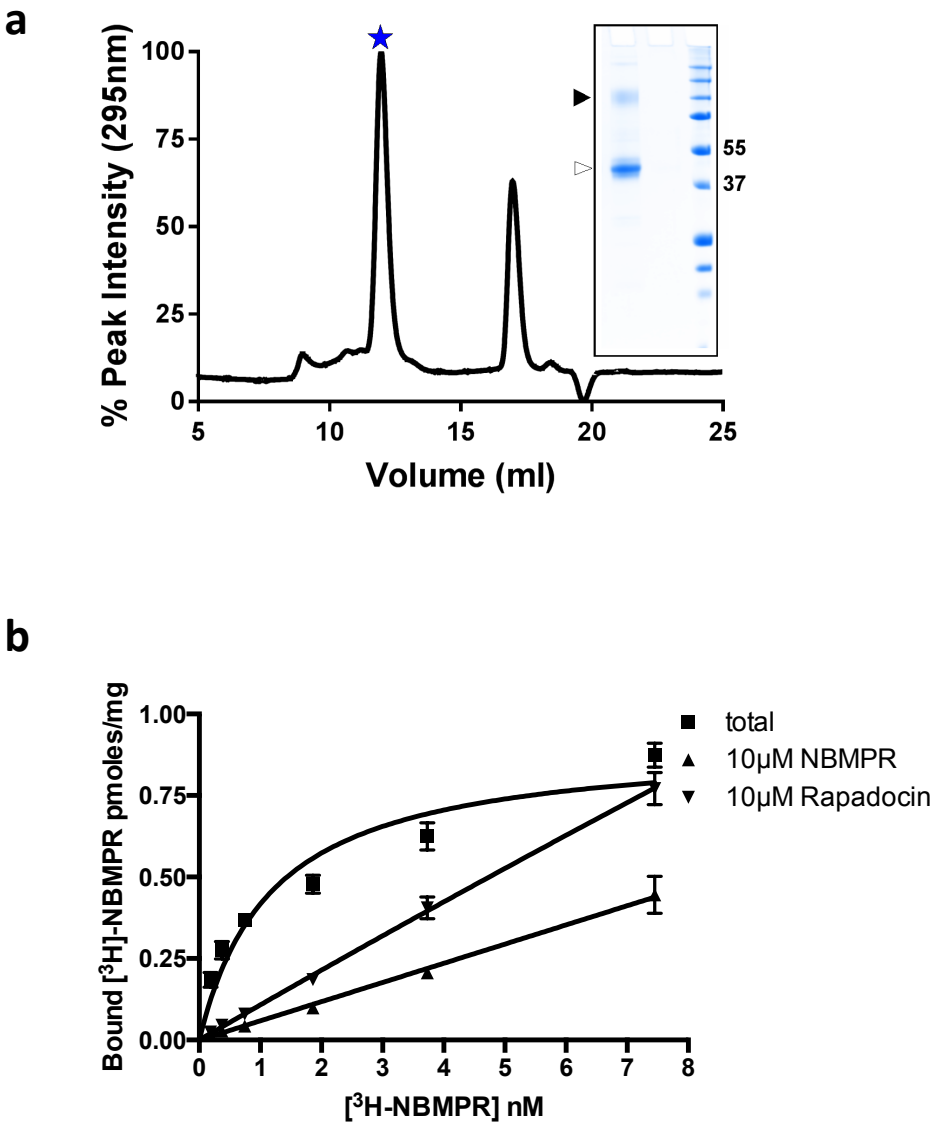
Supplementary Figure 5. Deconvolution and Structure-Activity Relationship Studies (SAR) of Rapadocin. Each of the 15 individual compounds from the hit pool was deconvoluted and their activities assessed to give the most active hit as 95-15. The hit was then subjected to orientation optimization by flipping the tetrapeptide sequence of 95-15 with respect to FKBD to yield an improved lead SH-13. SH-13 was further optimized in three rounds of SAR (Round 1-3) to give the optimally active lead compound 95-15-13, also named "rapadocin". The changed residues from the previous lead sequence are highlighted in yellow. Error bars represent s.d.; data are mean ± s.d; $n = 3$ independent experiments.

Supplementary Figure 6



Supplementary Figure 6. Rapadocin does not inhibit cell proliferation. **a**, Effect of Rapadocin and its analogs on the proliferation of HEK293T cells. Cell Viability was measured using Alamar Blue. Triptolide was included as a positive control. Error bars represent s.d.; data are mean ± s.d; *n* = 3 independent experiments. **b**, HUVEC cells were treated with either DMSO or 1 μM Rapadocin for 24 or 72 h. Cells were stained with propidium iodide and cell cycle analysis was performed. No significant difference observed with Rapadocin treatment compared with DMSO treatment. Representative image of *n* = 2 independent experiments with similar results. Error bars represent s.d.; data are mean ± s.d; *n* = 2 independent experiments. Treated and untreated cells showed no significant variation at both time points, two-sided student *t*-test.

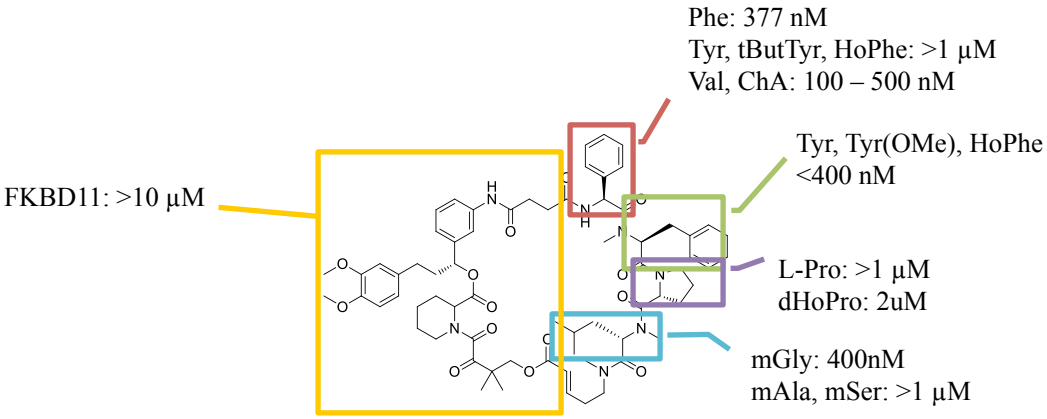
Supplementary Figure 7



Supplementary Figure 7. **Purification and characterization of hENT1 and its inhibition by rapadocin. a,** Size exclusion chromatography analysis of purified hENT1 on Enrich SEC 650 10X300 column (Bio-Rad) with a flow rate of 0.5 ml/min. Inset: SDS-PAGE analysis of purified protein in the monomeric peak fraction(star). The purification is consistent with earlier reports [37]. **b,** Displacement of NBMPR by rapadocin. HEK293 cells were incubated with increasing concentrations of [^{3}H]NBMPR in presence or absence of 10 μM NBMPR or 10 μM rapadocin. There is a decrease in total NBMPR binding in presence of 10 μM rapadocin . The graph presents the pooled data of 3 independent experiments. Error bars represent s.e.m.; data are mean ± s.e.m..

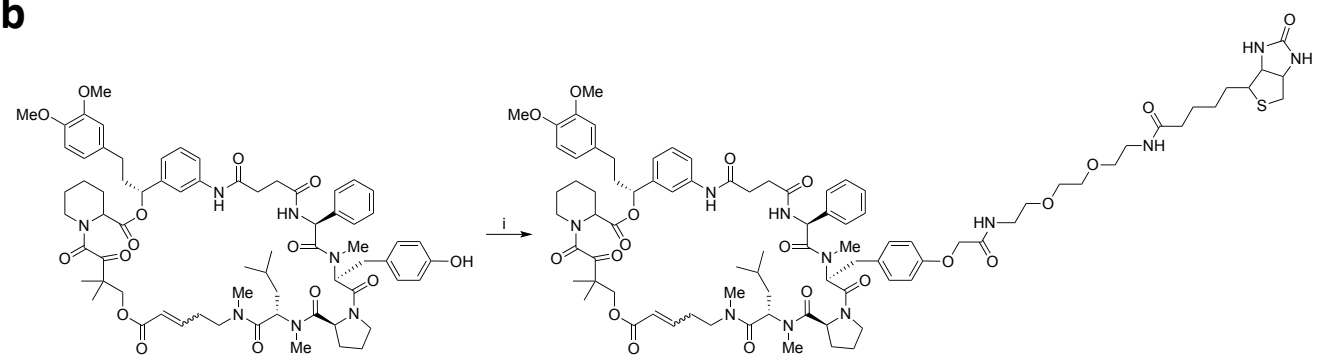
Supplementary Figure 8

a



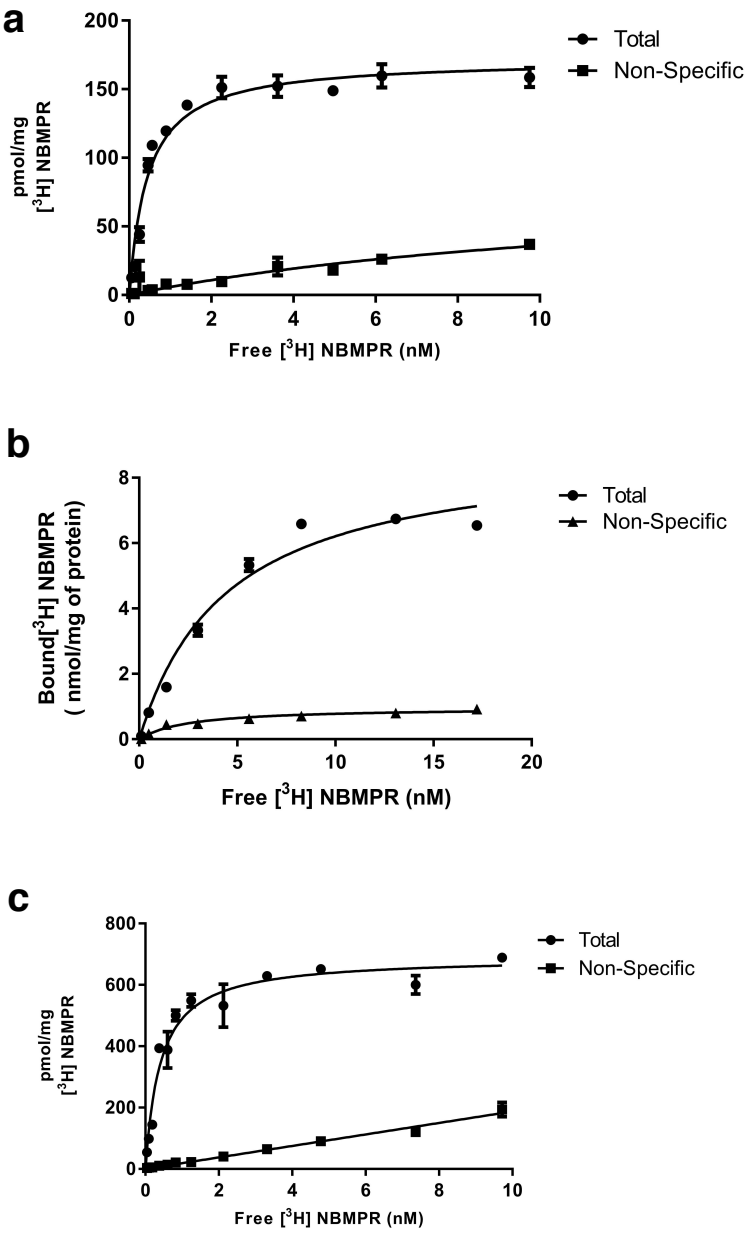
Rapadocin: FKBD**10**-mLeu-dPro-mPhe-PhG;
Norapadocin A: FKBD**10**-mAla-dPro-mPhe-PhG;
Norapadocin B: FKBD**10**-Gly-mLeu-dLeu-mPhe.

b



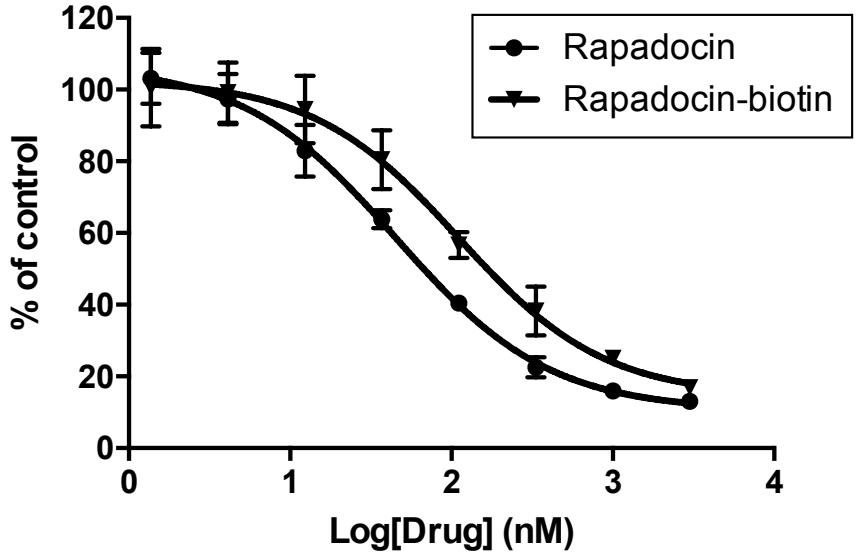
Supplementary Figure 8. **a,** Structure/activity relationship of Rapadocin. The activity of a subset of representative analogs are highlighted. Also shown are the sequences of rapadocin and two inactive analogs, norapadocin A and norapadocin B. **b**, Synthesis of biotinylated rapadocin. Reaction condition: (i) biotin-(PEG)3-iodoacetamide, K_2CO_3, DMF, RT, 24 h, 15%.

Supplementary Figure 9



Supplementary Figure 9. Functional characterization of hENT1 transporter in different lipid or detergent environments. a, [^{3}H]NBMPR saturation binding assay with hENT1 in Sf9 microsomes, **(b)** in LMNG-purified protein and **(c)** liposome reconstituted hENT1 transporter. In all experiments, hENT1 was incubated for 40 min with increasing concentrations of [^{3}H]NBMPR in the absence (total binding) and presence of 10 μM unlabled NBMPR (non-specific binding). The apparent K_d values calculated for conditions in A, B and C were 0.2 ± 0.07 nM, 5.0 ± 2 nM and 0.9 ± 0.05nM, respectively. Error bars represent s.e.m.; data are mean ± s.e.m.; *n* = 3 independent experiments. This data suggests that an apparent decrease in NBMPR affinity for hENT1 in LMNG detergent is restored upon reconstitution into a lipid environment.

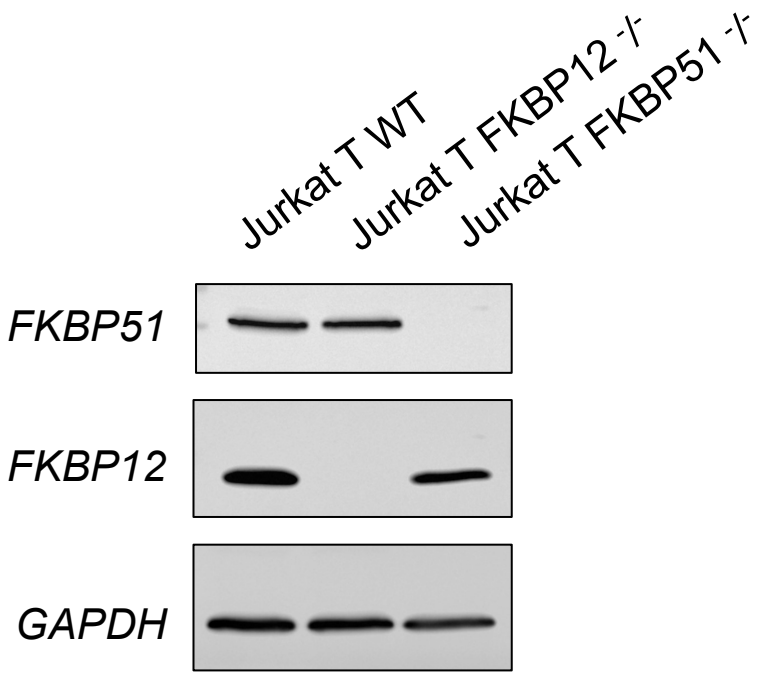
Supplementary Figure 10



	Rapadocin	Rapadocin-biotin
HillSlope	-0.9121	-0.9616
EC50	45.18	110.8

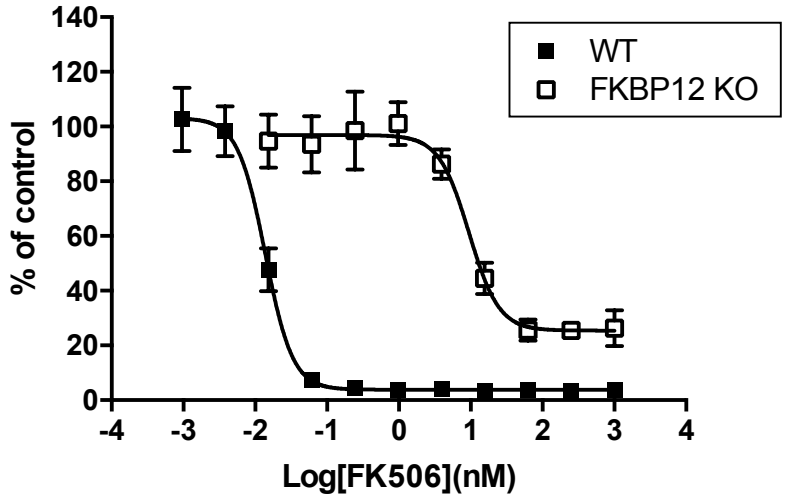
Supplementary Figure 10. Inhibition of [^{3}H]-adenosine uptake in human red blood cells by rapadocin and rapadocin-biotin probe. Error bars represent s.d.; data are mean ± s.d; n = 3 independent experiments.

Supplementary Figure 11



Supplementary Figure 11. FKBP12 and FKBP51 protein levels of Jurkat T wild type and knockout cells analyzed by Western blotting. Representative image of $n = 2$ independent experiments with similar results.

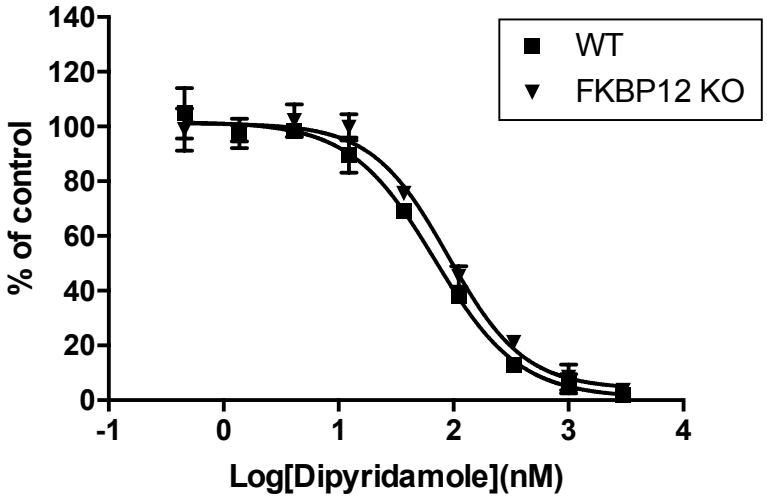
Supplementary Figure 12



	WT	FKBP12 KO
HillSlope	-2.275	-2.081
EC50	0.01379	9.468

Supplementary Figure 12. Validation of FKBP12 knockout Jurkat T cells using NFAT luciferase reporter gene assay. The IC_{50} values of FK506 in the Jurkat T wild type, FKBP12 knockout cells are 0.01 nM and 9.47 nM, respectively (n = 4). Error bars represent s.d.; data are mean ± s.d.

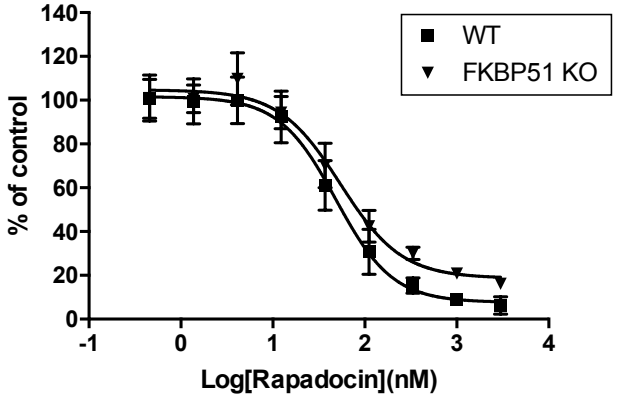
Supplementary Figure 13



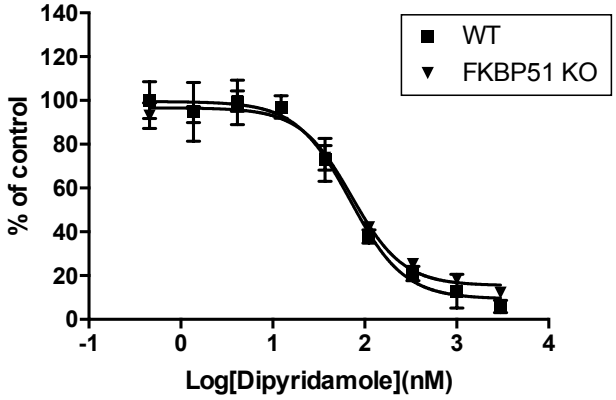
	WT	FKBP12 KO
HillSlope	-1.183	-1.314
EC50	68.73	89.82

Supplementary Figure 13. Inhibition of [^{3}H]-Thymidine uptake in wild type or FKBP12 knock-out Jurkat T cells by dipyridamole. Error bars represent s.d.; data are mean ± s.d; n = 3 independent experiments.

Supplementary Figure 14



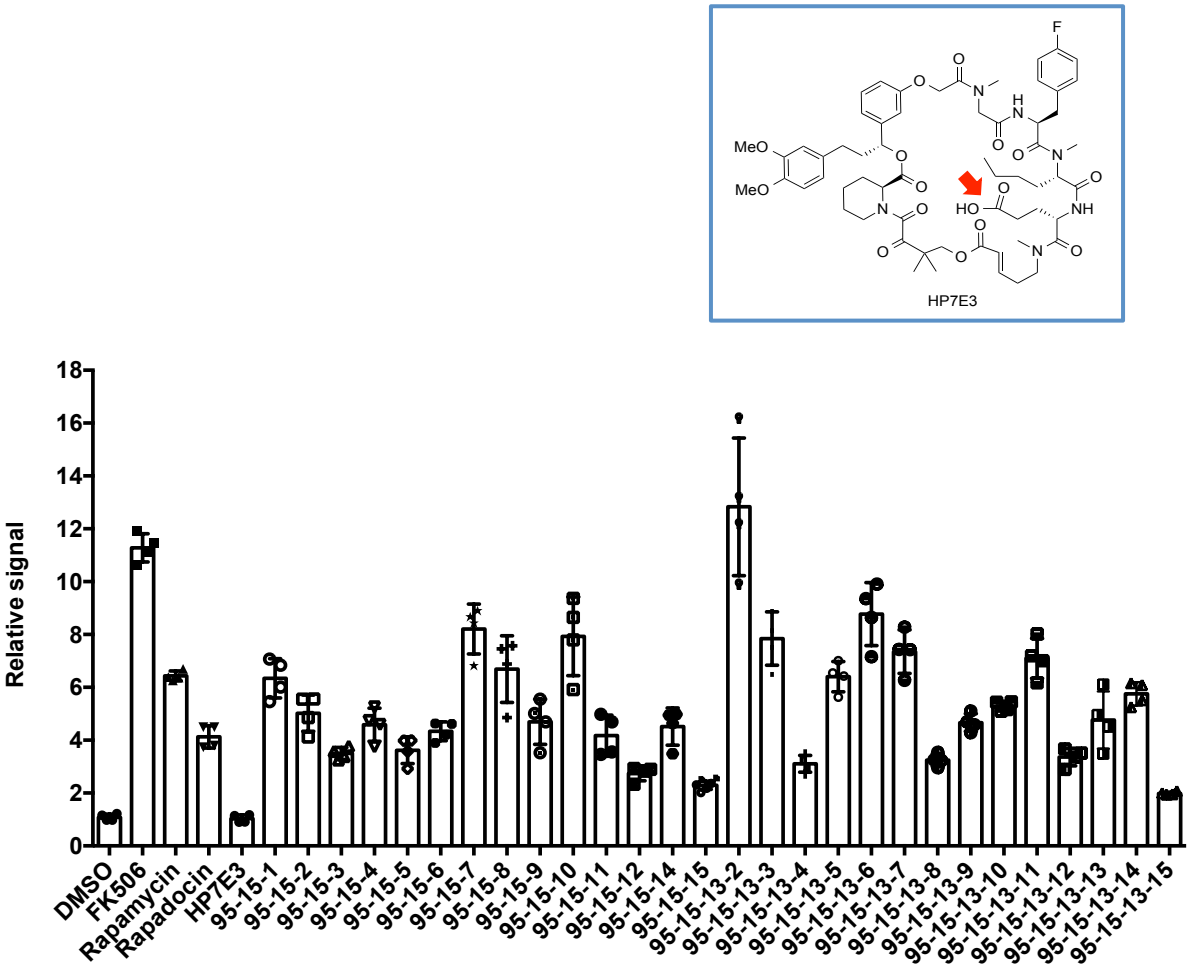
	WT	FKBP51 KO
HillSlope	-1.406	-1.301
EC50	48.45	54.27



	WT	FKBP51 KO
HillSlope	-1.478	-1.554
EC50	69.50	72.27

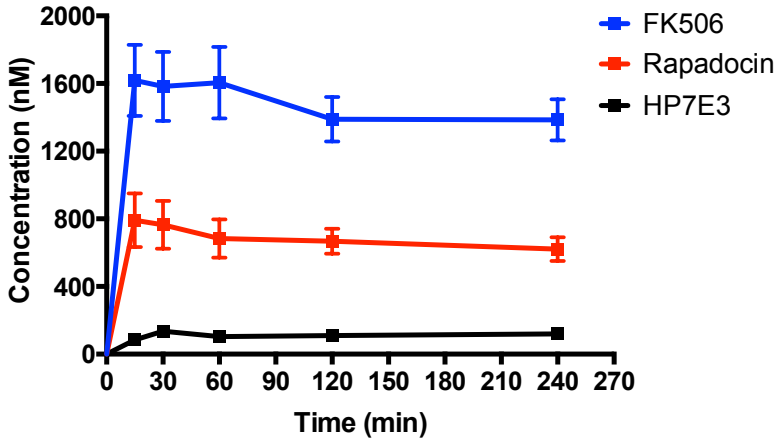
Supplementary Figure 14. Inhibition of [^{3}H]-thymidine uptake in wild type or FKBP51 knock-out Jurkat T cells by rapadocin and dipyridamole. Error bars represent s.d.; data are mean ± s.d; n = 3 independent experiments.

Supplementary Figure 15



Supplementary Figure 15. Cell penetration and activation of a Id1-luciferase reporter gene by rapadocin and analogs as well as FK506 and rapamycin. Jurkat T cells carrying the Id1-luciferase reporter gene was treated with 10 μM of each compound and incubated for 18 h before cells were harvested and luciferase activity was determined. HP7E3 that bears a charged glutamic acid in its effector domain preventing cell penetration was included as a negative control. The structure of HP7E3 is shown in the inset above. The red arrow points to the carboxylic acid side chain in the effector domain. Error bars represent s.d.; data are mean ± s.d; n = 3 independent experiments.

Supplementary Figure 16



Supplementary Figure 16. Permeability of Rapadocin to human cells. Jurkat T cells were treated with 10 μM of each compound and incubated for different times before cells were washed by cold PBS twice and compounds were extracted by saturated $ZnSO_4$ and acetonitrile. The extracted compounds were subjected to LC-MS analysis. FK506 was included as a positive control and rapafucin HP7E3 was included as a negative control. Error bars represent s.d.; data are mean ± s.d; n = 3 independent experiments.

Supplementary Table 1

Supplementary Table 1. Percentage yields for *cis*-C4, -C6 and -C8 linkers with FKBD**10** in the context of random tetrapeptides.

Seq#	Amino acid sequences[a,b]	*cis*-C4	*cis*-C6	*cis*-C8
1	Ala-Val-Phe-Sar	29	39	44
2	^{M}Val-Pro-^{D}Phe-Leu	53	75	77
3	^{M}Ala -Gly-^{M}Ile-^{D}Val	54	77	89
4	^{M}Leu-Val-^{M}Leu-Ala	48	78	69
5	Val-Sar-Gly-^{M}Leu	21	29	46
6	Pro -^{M}Leu-Leu-^{M}Ile	44	42	50
7	Gly-^{M}Val-^{D}Val-^{M}Ser	38	38	61

[a] Sequences are from N- to C-terminus (reversal to synthetic order). [b] Superscript M and D indicates that *N*-Me and D-amino acids are used in the sequences, respectively.

Supplementary Table 2

Supplementary Table 2. Optimized Conditions for Microwave-assisted RCM Reaction

#	T (^{o}C)	catalyst loading (mol%)	time (min)	rapafucin content (%)	isolation yields (%)
a	60	30	30	1/--	--
b	120	30	30	50	34
c	**140**	**30**	**30**	**46**	40
d	160	30	30	42	42
e	140	5	30	14	4
f	140	15	30	57	35
g	140	50	30	31	38

Supplementary Table 3. K_d values for different FKBDs

FKBD	K_d (nM)
3	327
6	62
10	4
11	11

Supplementary Table 4. Binding affinity (K_d) for FKBP12 and rapafucins containing different FKBDs.

Seq#	Amino acid sequences[a,b]	*cis*-C4			*cis*-C6	*cis*-C8				
		3	**7**	**10**	**10**	**3**	**6**	**7**	**10**	**11**
1	Ala-Val-Phe-Sar	175[c]	304	9	133	45	130	1504	16	24
2	^{M}Val-Pro-^{D}Phe-Leu	1473	6130	311	395	140	2131	13270	334	169
3	^{M}Ala-Gly-^{M}Ile-^{D}Val	188	1171	9	24	19	23	2453	57	22
4	^{M}Leu-Val-^{M}Leu-Ala	157	3387	194	126	805	818	2557	161	303
5	Val-Sar-Gly-^{M}Leu	203	ND	ND	38	114	17	ND	4	9
6	Pro-^{M}Leu-Leu-^{M}Ile	1280	ND	ND	112	1196	393	ND	34	224
7	Gly-^{M}Val-^{D}Val-^{M}Ser	191	ND	ND	79	16	306	ND	60	33

[a] Sequences are from N- to C-terminus (reversal to synthetic order). [b] Superscript M and D indicate that *N*-Me and D-amino acids are used in the sequences, respectively. [c] Numbers are K_d values (nM) for FKBP12 and rapafucins.

Supplementary Table 5

Supplementary Table 5. Inhibition constants of rapadocin for the peptidyl prolyl cis-trans isomerase activity of different isoforms of FKBP. Error bars represent s.d.; data are mean ± s.d; $n = 3$ independent experiments.

	FKBP12 K_i (nM)	**FKBP13K_i (nM)**	**FKBP25 K_i (nM)**	**FKBP51 K_i (nM)**	**FKBP52 K_i (nM)**
Rapadocin	3.7 ± 1.6	>5000	>5000	184 ± 54	176 ± 36

Materials and Methods Table of Content

BIOLOGY

CHEMISTRY

BIOLOGY:

Biological Reagents

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Cat#: C2519A). Jurkat T, HEK 293T and A549 cells were purchased from ATCC. Human red blood cells were obtained from Fisher Scientific (Cat#: 50-643-497). EGM-2 medium was purchased from Lonza (Cat#: CC-3162). RPMI-1640 and DMEM media were purchased from Fisher Scientific (Cat#: 11875119 and Cat#: 11885092). [^{3}H]-thymidine was purchased from PekinElmer (Cat#: NET027). [^{3}H]-adenosine was purchased from Moravek (Cat#: MT793). Vectors pET-28a(+) and pDEST15 were purchased from Novagen and Invitrogen, respectively. Ni-NTA agarose beads was purchased from Qiagen (Cat#: 30210). Hi-prep Q anion-exchange column, glutathione beads and sephacryl S-100 beads were purchased from GE life sciences (Cat#: 17115301, 17075601, and 17061210). Streptavidin agarose beads and glutathione magnetic beads were purchased from ThermoFisher Scientific (Cat#: 20359 and 78601). Protein assay kit was purchased from Bio-Rad (Cat#: 5000006). Antibodies anti-FKBP12 and anti-FKBP51 were purchased from Abcam (Cat#: ab92459 and ab126715). Antibodies Ent1 and Ent2 were purchased from Santa Cruz Biotechnology (Cat#: SC377283) and Sigma-Aldrich (Cat#: HPA018168), respectively.

Cell Culture

All cells were grown at 37°C with 5% CO_2 in a humidified environment. HUVEC were grown in EGM-2 bullet kit media and used between passage 2 and 8. Jurkat T cells were grown in RPMI-1640 media with the addition of 10% FBS. HEK 293T and A549 cells were grown in DMEM media with the addition of 10% FBS.

[^{3}H]-thymidine Incorporation Assay

2000 HUVEC/well was seeded in a 96-well plate (Costar) in 180 μL media and other cell lines were seeded at 5000 cells/well. After an overnight recovery, drugs were added. Following a 24 h incubation, cells were pulsed with 1 μCi of [^{3}H]-thymidine for 6 h, washed once with PBS, trypsinized, and transferred to filtermats using a Mach III M Harvester 96 (Tomtec). After drying, [^{3}H]-thymidine retention on the filtermats was determined by scintillation counting using a 1450 Microbeta apparatus

(Wallac). Counts were normalized to vehicle only treated cells. GraphPad Prism (v4.03) software was used to determine IC_{50} values using a four parameter logistic regression.

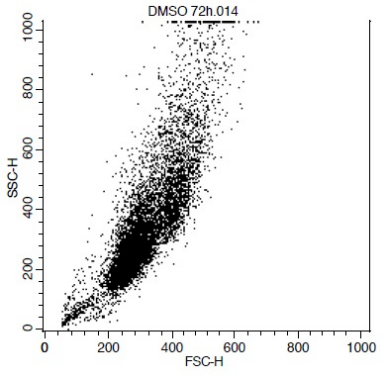
Alamar Blue Cell Viability Assay

500 A549/well was seeded in a 96-well plate (Costar) in 180 µL media. After an overnight recovery, drugs were added. Following a 72 h incubation, cells were added with 20uL of alamar blue reagent and the plates were incubated at 37°C for 6h before reading the fluorescence (544nm Ex / 590nm Em) with a plate reader. GraphPad Prism (v4.03) software was used to determine IC_{50} values using a four parameter logistic regression.

Cell Cycle Analysis

HUVEC were seeded at 5 x 10^5 cells / 15 cm dish, allowed to recover overnight and subsequently treated with drugs or vehicle control for 24 h. Media was then collected and set aside. Cells were washed with PBS, trypsinized, combined with set aside media, pelleted at 500 x *g* and then washed with 10 mL PBS followed by another 500 x *g* spin. The pellet was resuspended in 0.5 mL PBS and added dropwise using a Pastuer pipet to 2 mL 75% ethanol in a 5 mL polystyrene tube being slowly agitated by a vortex. The cells were stored at 4°C until staining. To do so, cells were pelleted at 500 x *g*, resuspended in 5 mL PBS, rested 60 seconds, pelleted again and washed in 5 mL PBS. The cell pellet was then resuspended in 0.5 mL staining solution (0.1% Triton-X-100, 0.2 mg/mL DNase free RNase A, and 0.02 mg/mL propidium iodide). Cells were allowed to stain for 30 min – 1 h prior for analysis. Propidium iodide incorporation was measured using a FACSCalibur. The percentage of cells in each cell cycle stage was determined with FlowJo (v7.5.5) using a Watson analysis (*1*).

A representative gating strategy for singlet discrimination is shown below.



Overexpression and Purification of FKBP12 Protein

The FKBP1A coding cDNA was inserted into either the pET-28a (+) vector between NdeI and XhoI for his-tag fusion protein or pDEST15 using Gateway cloning technology (Invitrogen) for GST fusion protein.

The cloned genes were confirmed not to contain any spurious mutations by sequencing the full length of the cloned inserts. The gene products were then expressed under induction of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4h at 37°C. The 6xHis-FKBP12 protein was purified from the crude extract prepared from the harvested cells, first by metal chelating chromatography using Ni-NTA agarose beads and then by a 5ml Hi-Prep Q anion-exchange column. The GST-FKBP12 protein was purified first by glutathione beads and then by size exclusion chromatography using sephacryl S-100 beads. Purified proteins were quantified by protein assay kit and stored at -20°C until used in PBS buffer (pH 7.4) plus 5 mM DTT and 10% glycerol.

Measurement of K_d Values for Rapafucins

Dissociation constant K_d values for rapafucins were determined with the fluorescence chemical denaturation assay as described previously[3]. Briefly, 1μM of purified FKBP12 protein bearing his-tag was incubated individually with 10μM of each rapafucins in 96-well plates and then equilibrated over a range of chemical denaturant concentrations (GuHCl, 0-4M). After an overnight incubation at RT, the fluorescence (ex 280nm / em 340nm) of FKBP12 or FKBP12-rapafucin-denaturant complexes was measured using a fluorescence plate reader, FLUOstar Optima (BMG Labtech, U.K.). Apparent K_d values for rapafucins were obtained using the data analysis method previously described (*2*).

Expression and Purification of hENT1 and Formation of Proteoliposomes

Human ENT1 (hENT1) was heterologously expressed in Sf9 insect cells and purified as previously described (*3*) with following modifications. Sf9 crude membrane expressing recombinant hENT1 was solubilized with 2% octyl glucoside (βOG) (Anatrace) supplemented with soya lipids (Sigma Aldrich) for 1 hour at 4^{o}C. Solubilized fraction was then allowed to bind Ni-NTA affinity resin for 2 hour. Immediately after elution from the Ni-column protein was mixed with soya lipid mixture solubilized in octyl glucoside at protein to lipid ratio of 1:80 (w/v). Detergent was removed by dialysis overnight at 4^{o}C followed by proteoliposome pelleting at 100,000 g for 30 min. Pelleted proteoliposomes were washed twice with ice cold buffer (10 mM HEPES, 7.4, 10% glycerol, 500 mM NaCl), and resuspended to final hENT1 concentration of approximately 0.5 mg/ml and stored in -70 ^{o}C until further analysis.

[^{3}H]-NBMPR Binding Assays

Whole cell assay: HEK293 cells were grown in DMEM supplemented with 10% FBS at 37^{o}C in a humidified incubator with 5% CO_2. For NBMPR binding experiments cells maintained in 10-cm plates were washed with PBS and collected by scraping in binding buffer (10mM Tris-HCl, 100mM KCl, 0.1mM $MgCl_2$, 0.1mM $CaCl_2$ (pH 7.4)). NBMPR binding was performed by incubating HEK293 cells (approximately 1x10^6 cells) in binding buffer in final volume of 1ml in presence of increasing concentrations (0.186-7.45nM) of [^{3}H]-NBMPR. To measure the displacement by nonradioactive NBMPR or rapadocin, the incubation was done in presence of 10 μM cold NBMPR or 10 μM rapadocin. Cells were incubated for 50 min at room temperature to reach equilibrium binding and the reactions were stop by adding ice-cold binding buffer. The samples were filtered trough Whatman (Kent, UK) GF/B glass microfiber disks using a vacuum manifold Millipore system. The disks were washed twice with the

same buffer before placing them into scintillation vials to count accumulated radioactivity using standard scintillation counting.

Membrane fraction and purified hENT1: The saturation [^{3}H]-NBMPR binding assays were performed as earlier described (*3*). Briefly, crude membranes were diluted to hENT1 concentration of 25 µg/ml in transport buffer (10 mM Tris-HCl, pH 8.0) and incubated with increasing [^{3}H]-NBMPR concentrations, ranging from 0.125-30 nM, for 40 min at 22℃. In order to assess non-specific ligand binding, identical reactions were prepared in the presence or absence of 10 µM unlabeled NBMPR. Unbound ligand was subsequently removed by washing the reaction mixture with ice-cold transport buffer on 0.2 µm 96-well filter plates (Millipore) under vacuum. Subsequently, the plates were allowed to dry at room temperature and the amount of bound radioactivity was determined using a MicroBeta TriLux scintillation counter (Millipore). The amount of specifically bound [^{3}H]-NBMPR was determined by subtracting the signal of the experimental samples with the signal of equivalent control samples treated with excess unlabeled NBMPR.

For [^{3}H]-NBMPR competition binding assays, 5-10 µg of either crude microsomes or hENT1-proteoliposomes were incubated with increasing concentrations of either, rapadocin, or norapadocin-A for 10 min before adding 2.5 nM [^{3}H]-NBMPR. To further characterize the role of FKBP12 in rapadocin inhibition of hENT1 both rapadocin was premixed with 5-10 fold molar excess of purified 6xHis-FKBP12 and [^{3}H]-NBMPR competition binding assay was then performed as described above.

[^{3}H]-thymidine Incorporation Assay in Jurkat T Cells

Jurkat T wild type, FKBP12 knockout, or FKBP51 knockout cells were seeded into 96-well plates at a density of 1.5X10^4 /180µL/well. Cells were added different concentrations of rapadocin or dipyridamole, and then incubated at 37^{o}C for 15min. Cells were then pulsed with 1 µCi [^{3}H]-thymidine for 6 h before they were harvested onto glass fiber filters. The amounts of [^{3}H]-thymidine incorporated into cells were determined using a Perkin-Elmer MicroBeta plate reader. GraphPad Prism (v4.03) software was used to determine IC_{50} values using a four parameter logistic regression.

[^{3}H]-adenosine Incorporation Assay in Human Red Blood Cells

Human red blood cells were washed and re-suspended in uptake buffer (10mM HEPES, 100mM NaCl, 2mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, pH 7.4). Cells were incubated at 37^{o}C for 10min, added different concentrations of rapadocin or rapadocin-biotin conjugate, and then incubated for another 10min. Cells were cooled down to 4^{o}C and transferred to the top layer of an ice-cold 0.7-ml microfuge tube containing 25µL 8% sucrose / 20% perchloric acid (bottom layer), 200µL 1-bromododecane (middle layer), and 50µL uptake buffer containing [^{3}H]-adenosine. Cells were allowed to take up radiolabeled adenosine at 4^{o}C for 2min and then spun through the bromododecane into the acid/sucrose layer. The perchloric acid/sucrose/cell layer was removed and analyzed by liquid scintillation using a MicroBeta scintillation counter, and cell free controls were subtracted. GraphPad Prism (v4.03) software was used to determine IC_{50} values using a four parameter logistic regression.

Extraction of Ent1 Protein from Red Blood Cells

Human red blood cells were washed once in buffer A (10 mM Tris-HCl, 150 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.4), and membranes were prepared by dispersing 1 volume of cells in 24 ml ice cold buffer B (10 mM Tris-HCl, 1 mM EGTA, pH 7.4). After 10 min of incubation on ice, membranes were collected at 17,000 g for 10 min at 4°C. To extract Ent1 protein, membranes were mixed in buffer C (10 mM Tris-HCl, 150 mM KCl, 5 mM $MgCl_2$, 1% OTG, 5% glycerol, 1 mM EGTA, protease inhibitor, pH 7.4) and incubated on ice for 2 h with frequent mixing. The supernatant was collected by centrifugation at 17,000 g for 10min at 4°C, and then diluted four-fold in buffer A plus 5% glycerol to reduce the concentration of OTG. The diluted supernatant was quantified by protein assay kit and placed on ice until use.

Affinity Pulldown with Biotinylated Rapadocin

The supernatant containing extracted Ent1 protein was obtained from previous extraction step and pre-incubated with streptavidin agarose beads at 4°C for 30min to remove the endogenous streptavidin-binding proteins. The supernatant was collected by centrifugation at 2,000 rpm for 1min at 4°C, and diluted to 10mg/ml for pull-down experiment. For a typical biotin pull-down reaction, 300 µL of supernatant was pretreated with 20µM of competitor or equal volume of DMSO (as noted in the text) for 30min, before the addition of 1µM of rapadocin biotin probe or DMSO. After incubation at 4°C for 1h with frequent mixing, 30 µl of streptavidin agarose beads in buffer A plus 5% glycerol was added, and incubation was continued for 2 h. The agarose beads were precipitated by centrifugation and washed three times with 0.8 ml of buffer A plus 5% glycerol and 0.05% OTG. The washed streptavidin agarose beads was then resuspended in 50 µl of 2 x SDS sample buffer, heated at 100°C for 5 min and centrifuged for 2 min. The supernatant was subjected to SDS-PAGE followed by western blot.

GST-FKBP12 Affinity Pull-down

The supernatant containing extracted Ent1 protein was obtained from previous extraction step and pre-incubated with glutathione magnetic beads at 4°C for 30min to remove the endogenous glutathione-binding proteins. The supernatant was collected by precipitation of beads on the magnet and diluted to 10mg/ml for pull-down experiment. For a typical GST-FKBP12 pull-down reaction, 300 µL of supernatant was pretreated with 20 µM of drugs or equal volume of DMSO (as noted in the text) for 30min, before the addition of 2µM of purified GST-FKBP12. After incubation at 4°C for 1h with frequent mixing, 30 µl of glutathione-magnetic beads in buffer A plus 5% glycerol was added, and incubation was continued for 2h. The magnetic beads were precipitated on the magnet and washed three times with 0.8 ml of buffer A plus 5% glycerol and 0.05% OTG. The washed glutathione-magnetic beads was then resuspended in 50 µl of 2 x SDS sample buffer, heated at 100°C for 5 min and centrifuged for 2 min. The supernatant was subjected to SDS-PAGE followed by western blot.

hENT1-proteoliposome Co-pelleting Assay

For liposome co-pelleting assay 1μM FKBP12 was first pre-incubated with 10μM rapadocin for 20 min at room temperature. hENT1-proteoliposomes (at final concentration of 140 nM ENT1) were then added to the reaction mixture and incubated for 30 min at room temperature. Finally, proteoliposomes were pelleted at 100,000 g for 15min in a S100-AT3 rotor (Thermo Scientific). The supernatant and pellet fractions were run on SDS-PAGE and analyzed against FKBP12 and hENT1 specific antibodies.

Western Blot Analysis

For western blot analysis, cells were harvested and lysed by RIPA buffer plus protease inhibitor. Cell lysates or samples from pull-down experiment were subjected to SDS/ PAGE and then transferred to a nitrocellulose membrane. Membranes were first blocked in 5% (wt/vol) BSA in Tris-buffered saline plus 0.1% Tween 20 (TBST) at room temperature for 30min and incubated with primary antibodies at 4 °C for overnight. Membranes were then washed three times with TBST and incubated with secondary antibodies at room temperature for another 1 h. Membranes were washed with TBST three times again and incubated with ECL substrate for 1 min at room temperature. Pictures were captured using a GeneSys Image Station.

CRISPR-Cas9 Knockout Protocol

To create knockout line, cells were transfected with CRISPR all-in-one plasmid (GeneCopoeia) containing transcripts for Cas9, guideRNA, and mCherry fluorescent protein. This was performed by electroporation of 10 million Jurkat T wild type cells in 400μl of FBS/antibiotic free RPMI 1640 and 10μg cDNA (0.4cm gap cuvette) at 250V and 950μF using a Bio-Rad Gene Pulser II. After 48h rest, cells were centrifuged, aspirated, and re-suspended in sorting buffer (1x PBS, 25mM HEPES, 1% FBS, 2% Penn-Strep). Cells were then sorted (single-cell) into three pre-filled 96-well plates (100uL of RPMI 1640 containing 10% FBS and 2% Penn-Strep) for mCherry fluorescence at 561nm. Single cell colonies were allowed to grow 1 week before addition of 100μL of RPMI 1640 containing 10%FBS and 1% Penn-Strep. After 2-3 weeks, visible colonies were transferred to T-25 flasks and grown to 10^5cells/mL before western blot for expression.

Luciferase Reporter Assay

NFAT luciferase reporter assay was performed as described previously (*4*). Briefly, Jurkat T wild type or FKBP12 knockout cells transfected with NFAT reporter construct were seeded into 96-well plates at a density of 5×10^4/180μL/well. Drugs were serially diluted in DMSO as 1000× stocks, which were subsequently diluted to 20× in serum-free RPMI 1640 media before addition to the 96-well plates. After drug incubation at 37^{o}C for 30min, 40nM of phorbol myristate acetate (PMA) and 1μM of ionomycin were added to stimulate cells. After a 6-h incubation, plates were centrifuged and the supernatant was removed. Cells were immediately lysed in situ by adding 100μL/well lysis buffer and kept at room

temperature for 30mins. The luciferase activity was determined with sequential injection of luciferin solution into each well followed by photon emission detection.

BMP Reporter Gene Assay

Jurkat T cells transfected with Id1-Luc reporter construct were seeded into 96-well plates at a density of 5 × 10^4/180μL/well. Drugs were diluted in DMSO as 1000× stocks (10mM), which were subsequently diluted to 20× in serum-free RPMI 1640 media before addition to the 96-well plates. After drug incubation at 37^{o}C for 18h, plates were centrifuged and the supernatant was removed. Cells were immediately lysed in situ by adding 100μL/well lysis buffer and kept at room temperature for 30mins. The luciferase activity was determined with sequential injection of luciferin solution into each well followed by photon emission detection.

Cellular Uptake Permeability Assay

Jurkat T cells were treated with 10 μ M of each compounds and incubated for different times at 37^{o}C before cells were washed by cold PBS twice and compounds were extracted by saturated $ZnSO_4$ and acetonitrile (*5*). The extracted compounds were subjected to LC-MS analysis. Six concentrations of each compound were also subjected to LC-MS analysis to generate calibration curves. Linearity was assessed using the regression analysis implemented in the GraphPad Prism (v4.03) software. FK506 was included as a positive control and rapafucin HP7E3 was included as a negative control. All assays were run in triplicate.

Acute Kidney Ishemia/Reperfusion Assay

All animal studies were conducted in compliance with all relevant ethical regulations set forth by the Johns Hopkins University Animal Care and Use Committee (ACUC).

C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME; 8-10 week of age) were maintained in a pathogen-free facility at Johns Hopkins Medical Institutions and cared for according to National Institutes of Health guidelines and under a protocol approved by the Johns Hopkins University Animal Care Committee.

Compounds were dissolved in a vehicle solution of 80% ethanol/ 20% Cremophor Rh60. This solution was further diluted in normal saline for a final composition of 8% Ethanol, 2% Cremophor Rh60, 90% saline. 100 μL of drug or vehicle solution were injected intravenously through tail vein 15 minutes before surgery. Established model of kidney IRI was used (*6,7*). Animals were anesthetized by intraperitoneal injection with sodium pentobarbital at 75 mg/kg. An abdominal incision at midline was made, and both renal pedicles were bluntly dissected. A nontraumatic microvascular clamp was placed across left renal pedicle for 45 min and the right kidney was excised. During the procedure, animals were kept well hydrated with warm sterile saline, and body temperatures were maintained constantly at 35.5 to 37°C on a

heating pad (40°C) until full recovery from anesthesia. After the allotted ischemia time, the clamp was gently removed and the incision was closed in two layers with 4-0 silver suture, and the mice were allowed to recover with free access to food and water. Animals were sacrificed at 24 hours after IRI and serum levels of blood urea nitrogen (BUN) and creatinine were measured accordingly.

CHEMISTRY

General Experimental for Synthesis

Synthetic Reagents

Piperidine, *N,N*-diisopropylethylamine (DIPEA) were purchased from Alfa Aesar. Anhydrous pyridine was purchased from Acros. Solid support resin with 2-chlorotrityl chloride (Cat#: 03498) was purchased from Chem-Impex. HATU was purchased from ChemImpex. Fmoc protected amino acid building blocks were purchased from ChemImpex, Novabiochem or GL Biochem. Dichloromethane (CH_2Cl_2), methanol (MeOH), hexanes, ethyl acetate (EtOAc), 1,2-dichloroethane (DCE, anhydrous), *N,N'*-dimethylformamide (DMF, anhydrous), Hoveyda-Grubbs catalyst 2nd generation and all the other chemical reagents were purchased from Sigma-Aldrich.

Instruments for Synthesis and Purification

NMR spectra were recorded with Burker-400 and -500. High performance liquid chromatographic analyses were performed with Agilent LC-MS system (Agilent 1260 series, mass detector 6120 quadrupole). Orbital shaking for solid-phase reactions was performed on a Mettler-Toledo Bohdan MiniBlock system for 96 tubes (30-200mg resin in SiliCycle tubes) or a VWR Mini Shaker (0.2-2g resin in a plastic syringe with a fritted disc). Reagents were added with an adjustable Rainin 8-channel pipette for the MiniBlock system. Microwave reactions were performed with a Biotage Initiator Plus or Multiwave Pro with silicon carbide 24-well blocks from Anton Parr. Compound purification at 0.05-50g scale was performed with Teledyne Isco CombiFlash Rf 200 or Biotage Isolera One systems followed by a Heidolph rotary evaporator. Purification at 1-50mg scale was performed with Agilent HPLC system. Mixture of rapafucins in the 45,000-compound library are purified in a high-throughput manner by SPE cartridges (Biotage, 460-0200-C, ISOLUTE, SI 2g/6mL) on vacuum manifold (Sigma-Aldrich, Visiprep™ SPE Vacuum Manifold, Disposable Liner, 12-port) followed by overnight drying with a custom-designed box (50 cm x 50 cm x 15 cm) that allows air flowing rapidly inside to remove the solvent. The high-throughput weighing of the compounds in the library was done by a Mettler-Toledo analytical balance that linked (Sartorious Entris line with RS232 port) to a computer with custom-coded electronic spreadsheet.

General Procedure A: Solid-phase Peptide Synthesis (SPPS)

All orbital shaking was done at 500-600 rpm. Briefly, to prepare the resin, dry resin beads were shaking with 8x (in volume) CH_2Cl_2 for 20min before the solvent was drained and washed with 1x DMF. For coupling reactions, Fmoc-protected amino acid building blocks (3.1 eq.) and HATU (3.0 eq.) were added

in order into the vessel of the prepared resin. The resin and reagent mixture were quickly mixed before DIPEA (3.1 eq.) was added. The reaction usually took 1-3h to completion judged by Kaiser or chloranil test. Reaction with Fmoc-valine or -isoleucine to be coupled to N-methyl amino acid on resin, the reaction mixture was drained after shaking for 3h, briefly washed with DMF, and fresh reaction mixture was added for a second time to ensure high percentage of conversion. For FKBD conjugation, FKBD (1.6 eq.), HATU (1.6 eq.) and DIPEA (1.6 eq.) were used in place. Deprotection of the Fmoc group was achieved by shaking with 8x piperidine/DMF (1/4, v/v) for 20min. Thorough washing was performed in between the SPPS steps by rinsing the beads with CH_2Cl_2 (4x) for 5 times and DMF (1x) for once. To dry the resin, the thoroughly washed beads were soaked in methanol for 5min before the house vacuum was applied to allow the air continuously pass through the fritted reaction vessel. The resin was dried in approx. 20min judged by the unchanged weight. Dried resin with conjugated chemicals can be stored at -20 ^{o}C without observation of lower yield in reactions after 1 year.

General Procedure B: Microwave-assisted RCM Reaction

50-500 mg beads were places in a microwave reactor, followed by the addition of 1,2-dichloroethane (2 mL/100mg) and Hoveyda-Grubbs II (30 mol%). The reactor was then sealed and the reaction was programmed as stirring at 140 ^{o}C for 30 min under the radiation of microwave. Upon the reaction completion, the resulting brown suspension was filtered and the filtrate was concentrated for silica gel purification (MeOH: CH_2Cl_2 1:20→1:5) or HPLC analysis.

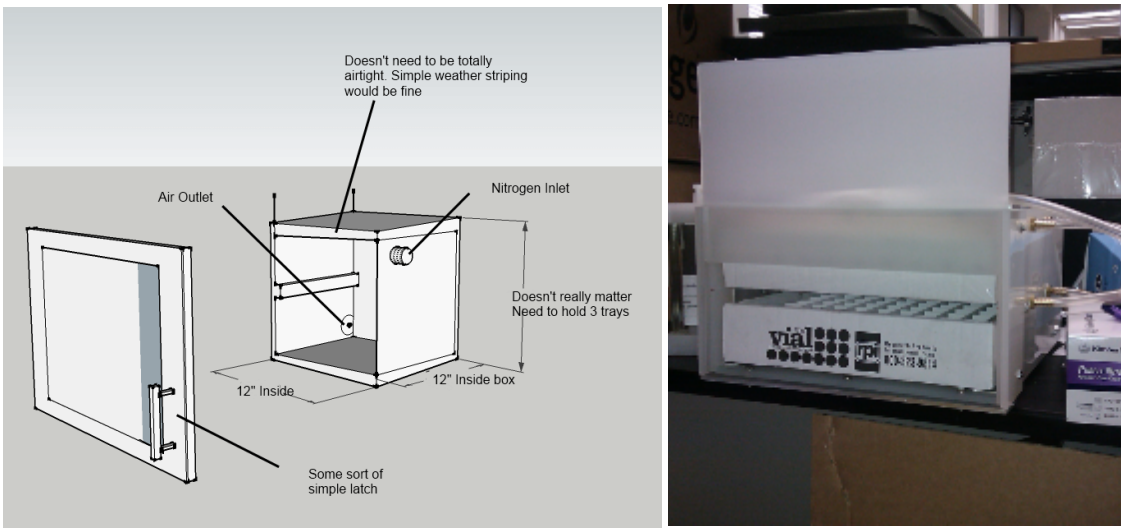
General Procedure C: Macrocycle Purification Protocol

Macrocycles were purified using automated flash chromatography with 4g columns for single compounds or small libraries. The 45,000-compound library was purified on silica loaded SPE cartridges.

For flash chromatography purification, 4g ISCO zip columns were equilibrated in 100% CH_2Cl_2. The samples taken from the microwave reactors were dissolved in minimal DCM and injected using a filtered syringe onto the columns. The flash purification subjected the samples to a linear gradient from 0% methanol in CH_2Cl_2 to 20% methanol in CH_2Cl_2 over 60 column volumes (CV). After 60 CVs, the gradient was set back to 0% methanol so the columns could be equilibrated for the next sample. Each column was used 5 times before discarding.

For SPE purification, 2g silica columns were equilibrated in CH_2Cl_2 until entire the cartridge appeared wet on a 12-channel SPE manifold. Only 6 channels were utilized at one time, the remaining channels were blocked using the protective caps. Samples were dissolved in minimal amount of CH_2Cl_2 and poured directly onto the cartridges. CH_2Cl_2 in a 500 mL squirt bottle was used to wash samples and column until brown color had completely passed through cartridge. After the disappearance of the brown-colored fraction (typically 3 CVs), the manifold cap was removed and preweighed scintillation vials were placed under the outlet tubes. A squirt bottle filled with 20% methanol in CH_2Cl_2 was used to wash the columns until a second colored fraction was eluted (typically 5 CVs). Scintillation vials were removed and returned to their original position in the vial rack. Cartridges were rinsed with 10 CVs of CH_2Cl_2 and reused up to 5 times total.

A polypropylene box was constructed with brass manifolds to hold two stacked scintillation vial racks (see figure below). Scintillation vials with up to 18 mL CH_2Cl_2 was added and air was turned on. Samples were allowed to dry overnight.



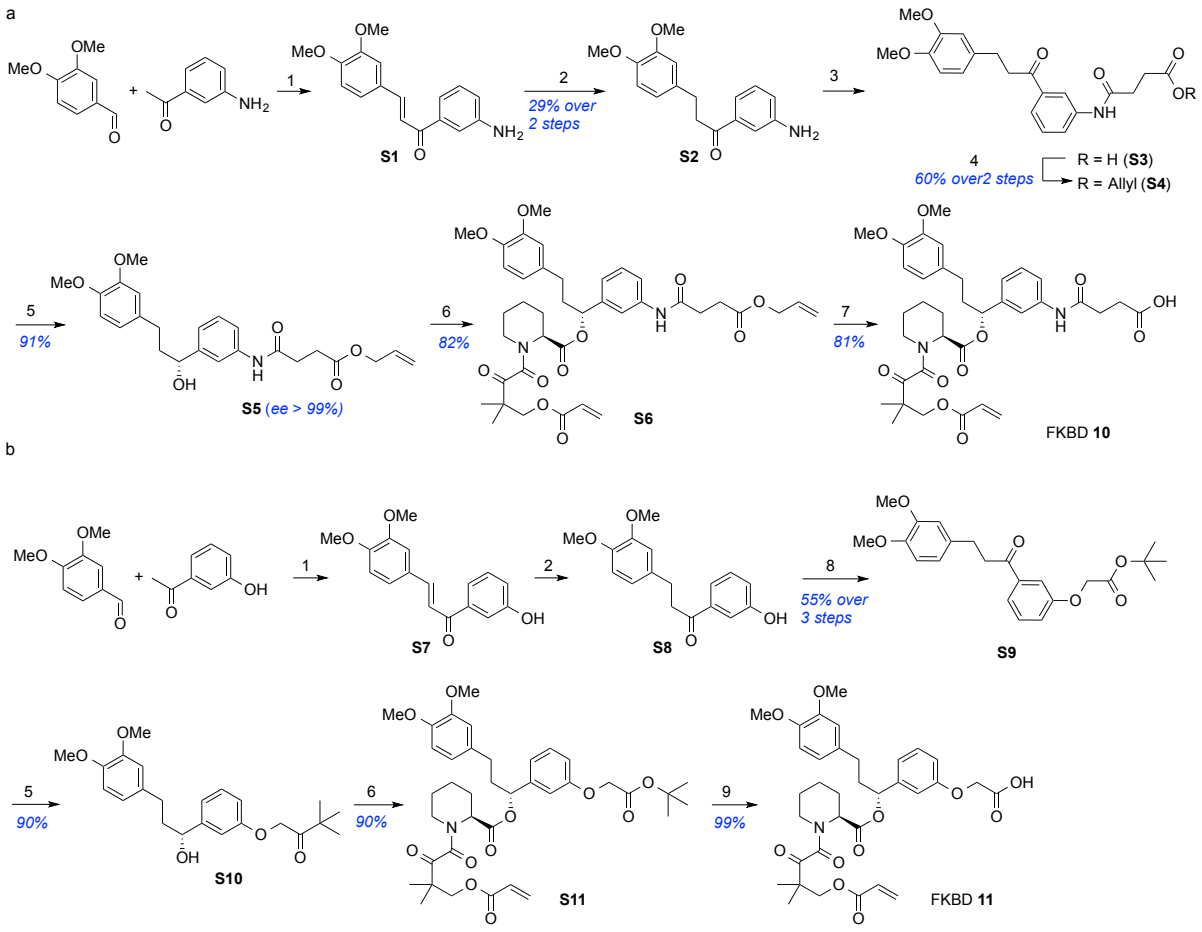
Extended Data Figure 1. Evaporation box. Planned schematic (left) created using Google Sketchup. Actual box (right).

Scintillation vials were preweighed and reweighed after samples were concentrated. Samples were placed onto scale and customized macros were executed to input current scale weight into document. Vials were annotated according to position in the rack.

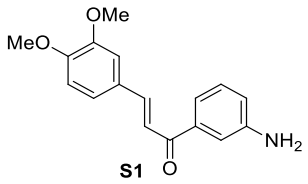
LC-MS Analytical Protocol for Macrocycles

Analytical reversed-phase high-performance liquid chromatography (HPLC) was performed on a C-18 reverse phase HPLC column (5µm Luna, 0.46 cm × 25 cm). Separations were achieved using linear gradients of buffer B in A (A = 0.1% formic acid in H_2O; B = 0.1% formic acid in CH_3CN) at a flow rate of 1 ml/min.

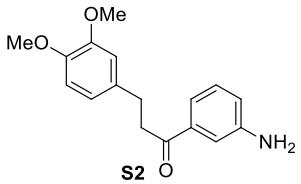
Experimental Procedures and Characterization Data for FKBDs 10 and 11



Extended Data Figure 2. Synthetic Route for a) FKBD **10** and b) FKBD **11**. Reaction conditions: **(1)** KOH, H_2O/EtOH (1/20), RT, 6h; **(2)** Pd/C (10%), H_2, MeOH, RT, 1.5 h; **(3)** succinic anhydride, DMAP (5%), CH_2Cl_2, RT, 3h; **(4)** allyl bromide, Cs_2CO_3, DMF, RT, 2h; **(5)** (+)-DIPCl, THF, -20°C→RT, 5h; **(6) S16** (see synthesis below), benzoyl chloride, DMAP (5%), Et_3N, CH_2Cl_2, RT, 4h; **(7)** $Pd(PPh_3)_4$ (10%), *N*-methylaniline, THF, RT, 6h; **(8)** *tert*-butyl 2-bromoacetate, K_2CO_3, DMF/acetone (1/2), RT, 4h; **(9)** TFA (10%), CH_2Cl_2, RT, 6h.



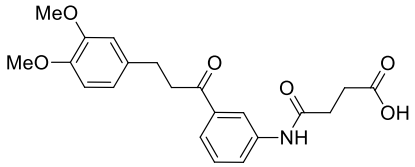
To a solution of 3,4-dimethoxybenzaldehyde (5.10 g) and 3-amino acetophenone (4.15 g) mixture in EtOH (20 mL, 95%), NaOH (0.2 g in 2 mL water) was added. The reaction mixture was stirred at RT for 6h and a slurry of yellow precipitate was formed. The reaction mixture was then diluted with EtOAc (40 mL) and washed with water (30 mL x3). Upon concentrated, the crude product **S1** (9.0 g) is pure enough for the next step.



S2

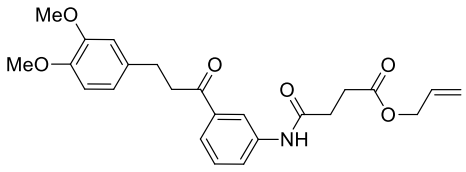
To a solution of α,β-unsaturated ketone **S1** (crude, 9.0 g) in MeOH (20 mL), Pd/C (10%, 1.61 g) was added. The reaction vessel was flushed with hydrogen gas repetitively by using a balloon of hydrogen and high vacuum. The reaction mixture was stirred at RT for 1 h before filtered through a pad of celite. Longer reaction time would render the reaction to generate undesired byproducts. The filtrate was concentrated and subject to column chromatography (50 g silica gel) and eluted with EtOAc/CH_2Cl_2/hexane (1/3/3→1/1/1). **S2** (2.48g) was collected as a yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36 – 7.16 (m, 3H, ar), 6.92 – 6.71 (m, 4H, ar), 3.86 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.81 (s, 2H, NH_2), 3.23 (t, J = 7.5 Hz, 2H, $COCH_2$), 2.99 (t, J = 7.4 Hz, 2H, $ArCH_2$). ^{13}C NMR (126 MHz, $CDCl_3$) δ 199.66 (C=O), 148.90 (ar), 147.38 (ar), 146.82 (ar), 138.03 (ar), 134.03 (ar), 129.49 (ar), 120.19 (ar), 119.61 (ar), 118.44 (ar), 113.91 (ar), 111.87 (ar), 111.35 (ar), 55.98 (OCH_3), 55.87 (OCH_3), 40.80 ($COCH_2$), 29.91 ($ArCH_2$). HRMS for $[M+H]^+$ C17H19NO3, calculated: 286.1443, observed: 286.1436.



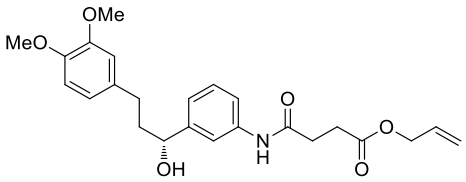
S3

Aniline **S2** (3.50g), succinic anhydride (1.0g) and DMAP (61mg) were mixed in dichloromethane (30mL). After stirring at RT for 3 h, the reaction mixture was washed with HCl (1M, 30mL x 4). Crude product **S3** (3.80g) was collected as a white solid and was used directly in the next step without further purification.



S4

Cs_2CO_3 (1.86g) was added into a solution of carboxylic acid **S3** (crude, 3.80g) in DMF (20mL). The resulting suspension was stirred at RT for 10min before allyl bromide (1.50mL) was added. The reaction mixture was stirred for an extra 2 h. The white precipitate was filtered off with a pad of celite. The filtrate was added with EtOAc (40mL) and H_2O (40mL). Upon stirring for 10min, the product precipitated. Product **S4** (2.11g) was obtained by filtration and air-dried as an off-white solid, and was used in the next step without further purification.



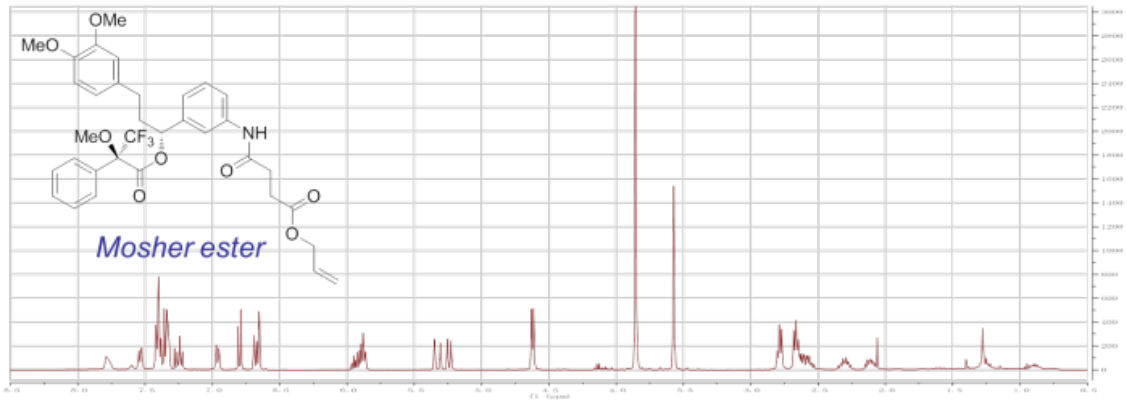
S5 (*ee* > 99%)

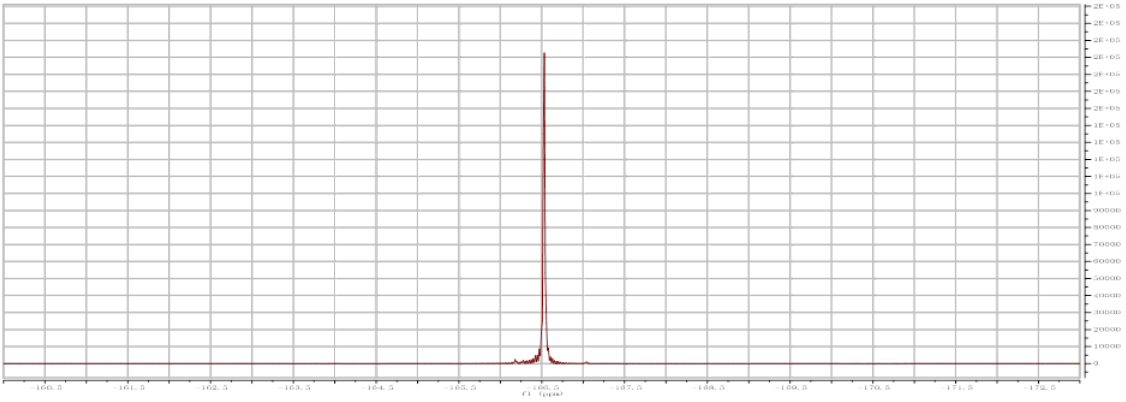
The ketone **S4** (2.11g) was dried under high-vacuum for 1h and the reaction vessel was flushed with argon. THF (anhydrous, 40mL) was added and the solution was cooled down to -20 ^{o}C. (+)-DIPCl (1.6M in hexane, 15mL) was added via a syringe and the resulting yellow solution was stirred at -20^{o}C for 5 hours before being warmed up to RT. Stirring at RT for 30 min, the yellow color disappeared and the reaction mixture was quenched with 2,2'-(ethylenedioxy)diethylamine (1.0 eq to DIPCl) by forming an insoluble complex. After stirring at RT for another 30 min, the suspension was filtered through a pad of celite and concentrated. Column chromatography (80-200 mesh) with EtOAc/hexane (1/1) afforded product **S5** (1.60g) as a white solid.

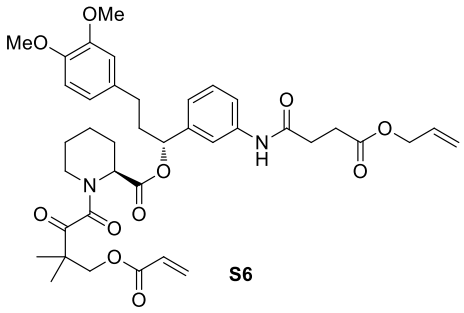
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.72 (s, 1H), 7.50 (s, 1H), 7.40 (d, *J* = 8 Hz, 1H), 7.27 (t, *J* = 8 Hz, 1H), 7.07 (d, *J* = 8 Hz, 1H), 6.78 (d, *J* = 8 Hz, 1H), 6.72 (dd, *J* = 11, 2.0 Hz, 2H), 5.90 (ddt, *J* = 17, 11, 6 Hz, 1H), 5.32 (dd, *J* = 17, 1.5 Hz, 1H), 5.23 (dd, *J* = 10, 1.5 Hz, 1H), 4.65 (dd, *J* = 8, 5 Hz, 1H), 4.60 (dt, *J* = 6, 1.0 Hz, 2H), 3.85 (s, 3H), 3.85 (s, 3H), 2.78 (t, *J* = 6.5 Hz, 2H), 2.73-2.57 (m, 2H), 2.66 (t, *J* = 6.5 Hz, 2H), 2.15 – 1.91 (m, 2H), 2.0 (s, br,1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.9, 169.8, 148.8, 147.2, 145.8, 138.0, 134.4, 131.9, 129.1, 121.8, 120.2, 119.0, 118.6, 117.4, 111.8, 111.3, 73.7, 65.7, 55.9, 55.8, 40.6, 32.0, 31.7, 29.4. HRMS for $[M+H]^+$ C24H30NO6, calculated: 428.2073, observed: 428.2072.

Determination of Enantiomeric Excess (ee) for Compound **S5**

To assess the ee of the asymmetric reduction, compound **S5** was derivatized as a Mosher ester (*8*). No enantiomer was observed in ^{1}H and ^{19}F NMR.



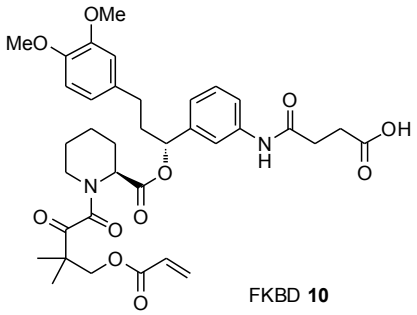




S6

Alcohol **S5** (1.65g) and carboxylic acid **S16** (1.26g, for synthesis see below) were dissolved in a mixture of THF (anhydrous, 5 mL) and dichloromethane (anhydrous, 10 mL). Benzoyl chloride (0.60 mL), Et_3N (1.0 mL) and DMAP (18 mg) were added in order and the resulting suspension was stirred at RT for 2 h. Without further treatment, the mixture was subject to column chromatography (80-200 mesh) with EtOAc/hexane (1/2→1/1). **S6** (2.50 g) was collected as a yellow foam.

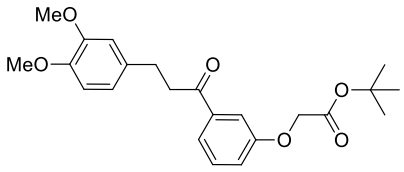
^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.65 (d, *J* = 8 Hz, 1H), 7.46 (s, 1H), 7.28 (t, *J* = 8 Hz, 1H), 7.01 (d, *J* = 8 Hz, 1H), 6.77 (d, *J* = 9 Hz, 1H), 6.69 (d, *J* = 5 Hz, 1H), 6.67 (s, 1H), 6.39 (dd, *J* = 17, 1.5 Hz, 1H), 6.06 (dd, *J* = 17, 10.5 Hz, 1H), 5.90 (ddt, *J* = 17, 10.5, 6 Hz, 1H), 5.83 (dd, *J* = 10.5, 1.5 Hz, 1H), 5.79 (ddd, *J* = 10.5, 8, 3.5 Hz, 1H), 5.31 (dd, *J* = 17, 1.5 Hz, 2H), 5.31 (d, *J* = 6 Hz, 1H), 5.22 (dd, *J* = 10.5, 1.5 Hz, 1H), 4.60 (dt, *J* = 6, 1.5 Hz, 2H), 4.33 (d, *J* = 0.7 Hz, 2H), 3.86 (s, 3H), 3.85 (s, 3H), 3.46 (d, *J* = 14 Hz, 1H), 3.09 (dd, *J* = 18, 8 Hz, 1H), 2.78 (t, *J* = 6 Hz, 2H), 2.70 (t, *J* = 6 Hz, 2H), 2.62 – 2.48 (m, 2H), 2.36 (d, *J* = 14 Hz, 1H), 2.30 – 2.16 (m, 1H), 2.13 – 2.00 (m, 1H), 1.74 (d, *J* = 10.5 Hz, 2H), 1.62 (d, *J* = 12 Hz, 1H), 1.42 (d, *J* = 12.6 Hz, 1H), 1.36 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 205.6, 172.6, 169.8, 169.3, 166.2, 165.6, 148.9, 147.3, 140.7, 138.6, 133.5, 132.0, 131.5, 129.2, 127.8, 122.0, 120.2, 119.3, 118.4, 117.2, 111.7, 111.3, 76.5, 69.2, 65.5, 55.9, 55.9, 51.3, 46.8, 44.1, 38.1, 31.9, 31.1, 29.3, 26.1, 25.1, 22.0, 21.9, 20.9.



FKBD **10**

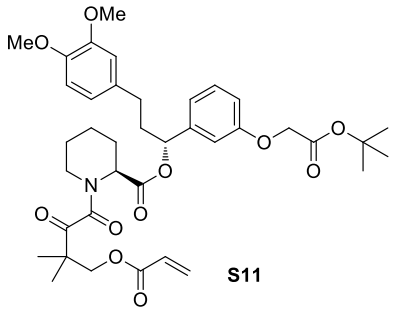
S6 (2.50g), $Pd(PPh_3)_4$ (100 mg), *N*-methylaniline (1.0 mL) were mixed well in THF (20 mL) at RT for 5 h. The reaction mixture was then diluted with EtOAc (50 mL) and washed with HCl (1M, 50 mL x3). The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography (200-400 mesh), where the byproduct can be eluted with 2% MeOH in dichloromethane, followed by the desired product with 3% MeOH and 0.05% AcOH in dichloromethane. FKBD **10** (2.25 g) was collected as an off-white foam.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.40 (s, 1H), 7.62 (s, 1H), 7.48 (s, 1H), 7.26 (t, *J* = 7.5 Hz, 1H), 7.01 (d, *J* = 7.5 Hz, 1H), 6.97 (dd, *J* = 16, 7 Hz, 1H), 6.86 – 6.74 (m, 1H), 6.74 – 6.58 (m, 2H), 5.85 – 5.68 (m, 2H), 5.39 – 5.24 (m, 1H), 4.29 (q, *J* = 11 Hz, 2H), 3.86 (s, 3H), 3.84 (s, 3H), 3.46 (d, *J* = 13 Hz, 1H), 3.13 (t, *J* = 13 Hz, 1H), 2.74 (d, *J* = 5.5 Hz, 2H), 2.69 (d, *J* = 5.5 Hz, 2H), 2.63 – 2.48 (m, 2H), 2.36 (d, *J* = 13 Hz, 1H), 2.30 – 2.15 (m, 1H), 2.15 – 1.99 (m, 1H), 1.85 (d, *J* = 6 Hz, 1H), 1.75 (d, *J* = 12 Hz, 1H), 1.63 (d, *J* = 13 Hz, 1H), 1.55 – 1.38 (m, 2H), 1.34 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 205.6, 176.8, 170.4, 169.4, 166.4, 166.1, 148.9, 147.3, 145.9, 140.7, 138.5, 133.5, 129.2, 122.1, 121.9, 120.2, 119.5, 117.4, 111.8, 111.4, 76.6, 69.0, 55.9, 55.8, 51.4, 46.8, 44.1, 38.1, 31.6, 31.1, 29.3, 26.2, 25.0, 21.8, 20.9, 18.1. HRMS for $[M+H]^+$ C36H44O2N11,calculated: 681.3023, observed: 681.3018.



S9

Compound **S9 was synthesized using the method previously described (*9*).**

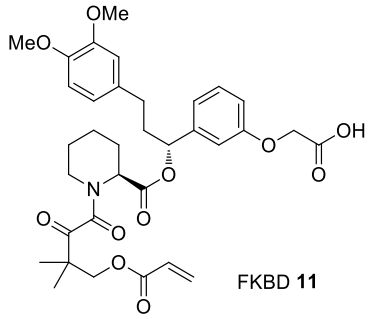


S11

Alcohol **S9** (3.8g, 1.0 eq.), carboxylic acid **S16** (4.1g, 1.2 eq.) and DMAP (134mg, 0.1eq.) were dissolved in a mixture of THF (anhydrous, 35 mL) and dichloromethane (anhydrous, 35 mL) in a round bottom

flask under argon protection. Et_3N (4.7 mL) and benzoyl chloride (2.17 mL, 2.62g, 1.7 eq.) were added dropwise through syringes in order and the resulting suspension was stirred at RT for 2 h. Reaction was monitored through TLC. When full conversion is achieved, the reaction mixture was diluted with 500mL EtOAc, washed with 5% HCl and saturated $NaHCO_3$. Organic phase was washed with brine and dried over Na_2SO_4. Then solvents were removed and product was purified by column chromatography (80-200 mesh) with EtOAc/hexane (1/10→1/3). **S11** (5.3 g, 69%) was collected as a light yellow foam.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26 (d, *J* = 8 Hz, 1H), 6.97 (d, *J* = 8.5 Hz, 1H), 6.93 – 6.89 (m, 1H), 6.86 – 6.81 (m, 1H), 6.78 (d, *J* = 8.5 Hz, 1H), 6.71 – 6.64 (m, 2H), 6.38 (dd, *J* = 17, 1.5 Hz, 1H), 6.06 (dd, *J* = 17, 10.5 Hz, 1H), 5.82 (dd, *J* = 10.5, 1.5 Hz, 1H), 5.78 (dd, *J* = 8, 6 Hz, 1H), 5.29 (d, *J* = 5 Hz, 1H), 4.53 (s, 2H), 4.36 (d, *J* = 11 Hz, 1H), 4.27 (d, *J* = 11 Hz, 1H), 3.86 (s, 3H), 3.84 (s, 3H), 3.48 (d, *J* = 13 Hz, 1H), 3.17 (td, *J* = 13, 3.0 Hz, 1H), 2.67 – 2.44 (m, 2H), 2.37 (d, *J* = 14 Hz, 1H), 2.32 – 2.18 (m, 1H), 2.14 – 1.99 (m, 1H), 1.83 – 1.65 (m, 2H), 1.65 – 1.56 (m, 1H), 1.50-1.43 (m, 2H), 1.48 (s, 9H), 1.35 (s, 3H), 1.35 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 204.8, 169.4, 167.8, 166.4, 165.4, 158.1, 148.9, 147.3, 141.3, 133.4, 131.2, 129.7, 127.9, 120.2, 119.8, 114.2, 113.2, 111.7, 111.3, 82.3, 76.7, 69.2, 65.7, 55.9, 55.8, 51.4, 46.6, 44.0, 37.9, 31.2, 28.0, 26.4, 25.0, 22.1, 21.6, 21.1. HRMS for $[M+H]^+$ C38H49NO11, calculated: 696.3384, observed: 696.3386.

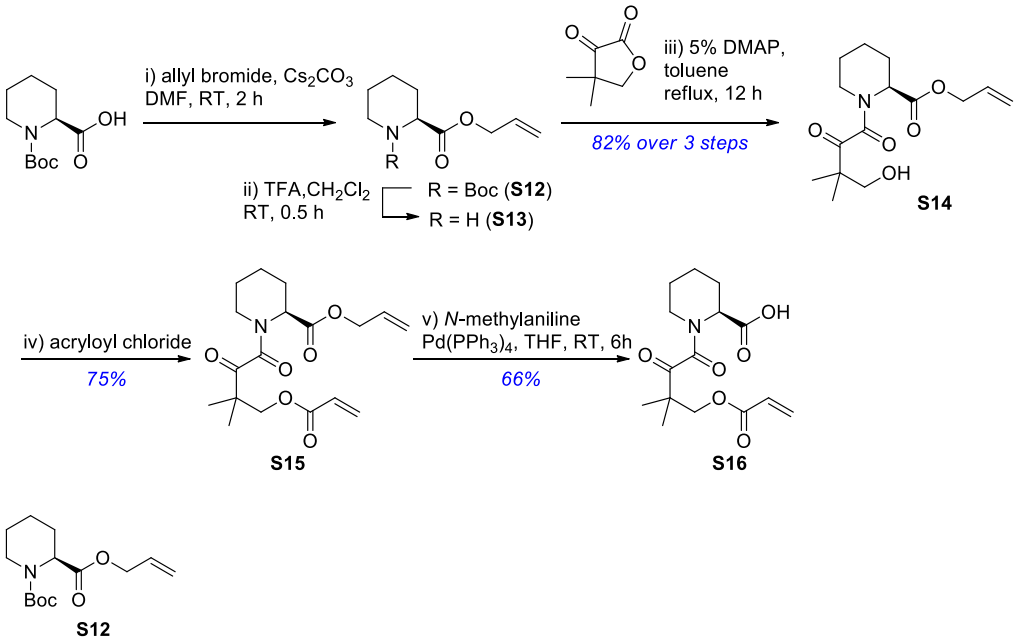


FKBD **11**

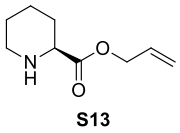
Compound **S11** (5.3g, 1.0 eq.) was dissolved in 60mL of dichloromethane in a round-bottom flask under Ar protection. Then TFA (17mL, 11.4g, 13 eq.) was added through a syringe in 3 portions during 3.5h while stirring at room temperature. The reaction was monitored through TLC. When full conversion was achieved, solvents and TFA were removed under vacuum. Product was purified by column chromatography (80-200 mesh) with EtOAc/hexane (1/5→1/1). FKBD **11** (4.6 g, 96%) was collected as a light yellow foam.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.28 (dd, *J* = 3.5 Hz, 3.5 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 1H), 6.83-6.81 (m, 2H), 6.80-6.78 (m, 1H), 6.69-6.67 (m, 2H), 6.37 (d, *J* = 8.5 Hz, 1H), 6.05-6.02 (m, 1H), 5.83-5.72 (m, 2H), 5.30-5.28 (dd, *J* = 10, 5 Hz, 1H), 4.67 (dd, *J* = 10, 5 Hz, 1H), 4.17 (dd, *J* = 10, 6 Hz, 2H), 3.48-3.45 (m, 1H), 3.24-3.22 (m, 1H), 2.61-2.55 (m, 2H), 2.38 (m, 1H), 2.23 (m, 1H), 2.04 (m, 1H), 1.79 (m, 1H), 1.62 (m, 1H), 1.33 (m, 1H), 1.30 (m, 1H), 1.25 (s, 3H), 1.24 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 204.6, 169.2, 166.7, 165.7, 157.9, 149.0, 147.5, 141.7, 131.4, 129.9, 127.9, 120.0, 115.4, 111.8, 111.4, 111.1, 69.3, 65.2, 60.5, 55.9, 51.7, 44.1, 38.0, 31.4, 22.1, 21.1, 14.2. HRMS for $[M+H]^+$ C34H42NO11, calculated: 640.2758, observed: 640.2761.

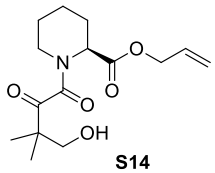
Synthetic Scheme of a Common Synthetic Intermediate **S16** for FKBDs



To a solution of *N*-Boc homoproline (6.30 g) in DMF (40 mL), Cs_2CO_3 (2.90 g) was added. The resulting suspension was stirred at RT for 5 min before the addition of allyl bromide (6.3 g). After stirring at RT for 2 h, the suspension was filtered through a pad of celite, rinsed with EtOAc (50 mL), and washed with HCl (1M, 50 mL x3). The organic layer was dried over Na_2SO_4 and co-evaporated with toluene (30 mL x2). Crude product **S12** (8.10g) was collected as a yellow oil and was pure enough for the next step without further purification.

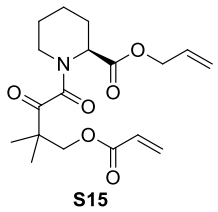


S12 (8.10g) and TFA (4.3g) were mixed well in dichloromethane (20 mL) and stirred at RT for 0.5 h. **S13** (3.00g) was collected as a yellow oil and was pure enough for the next step without further purification.



S13 (3.0 g), dihydro-4,4-dimethyl-2,3-furandione (2.1 g) and DMAP (20 mg) were dissolved in toluene (20 mL) and the reaction was refluxed with an oil bath (120 ^{o}C) for 14 h. After the solvent was removed, the residue was purified by column chromatography (80-200 mesh) with EtOAc/hexane (1/3). **S14** (3.50 g) was collected as a yellow oil.

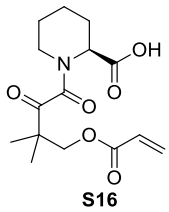
^{1}H NMR (500 MHz, $CDCl_3$) δ 6.04 – 5.80 (m, 1H), 5.36 (d, *J* = 17 Hz, 1H), 5.31 – 5.25 (m, 2H), 4.68 (s, 2H), 3.76 – 3.56 (m, 2H), 3.50 (d, *J* = 13 Hz, 1H), 3.40 (s, 1H), 3.20 (t, *J* = 13 Hz, 1H), 2.37 (d, *J* = 13 Hz, 1H), 1.84 – 1.61 (m, 3H), 1.61 – 1.34 (m, 2H), 1.24 (s, 6H).^{13}C NMR (126 MHz, $CDCl_3$) δ 205.9, 170.1, 168.1, 131.4, 119.2, 69.3, 66.3, 51.6, 49.5, 44.2, 26.3, 24.8, 21.3, 21.2, 21.0.



S15

Acryloyl chloride (0.78g) in dry CH_2Cl_2 (20mL) was added dropwise to a mixture of alcohol **S14** (3.50g) and *N,N*-diisopropylethylamine (2.0mL) in 50mL CH_2Cl_2 with ice-batch over 30min. After addition, the reaction was allowed to stir at RT for 30min before quenched with saturated $NaHCO_3$ solution (20mL). The organic phase was washed with water, dried over Na_2SO_4, concentrated and purified by column (EtOAc : Hexane = 1 : 5) to afford product **S15** (2.21g) as a yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.39 (dd, *J* = 17, 1.5 Hz, 1H), 6.08 (dd, *J* = 17, 11 Hz, 1H), 5.91 (ddt, *J* = 17, 11, 6 Hz, 1H), 5.84 (dd, *J* = 11, 1.5 Hz, 1H), 5.35 (ddd, *J* = 17, 2.5, 1.5 Hz, 1H), 5.28-5.25 (m, 1H), 5.26 (ddd, *J* = 11, 2.5, 1.5 Hz, 1H), 4.66 (ddd, *J* = 6, 4, 2.5 Hz, 2H), 4.37 (d, *J* = 11 Hz, 1H), 4.27 (d, *J* = 11 Hz, 1H), 3.52 (dd, *J* = 13, 1.5 Hz, 1H), 3.23 (td, *J* = 13, 3 Hz, 1H), 2.34 (d, *J* = 14 Hz, 1H), 1.84 – 1.76 (m, 1H), 1.76 – 1.67 (m, 1H), 1.67 – 1.60 (m, 1H), 1.59 – 1.47 (m, 1H), 1.47 – 1.38 (m, 1H), 1.36 (s, 3H), 1.35 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 204.8, 169.8, 166.7, 165.5, 131.5, 131.2, 128.0, 118.9, 69.5, 69.3, 66.0, 51.3, 46.7, 43.9, 26.4, 24.9, 22.2, 21.5, 21.1. HRMS for $[M+H]^+$ C18H25NO6, calculated: 352.1760, observed: 352.1753.



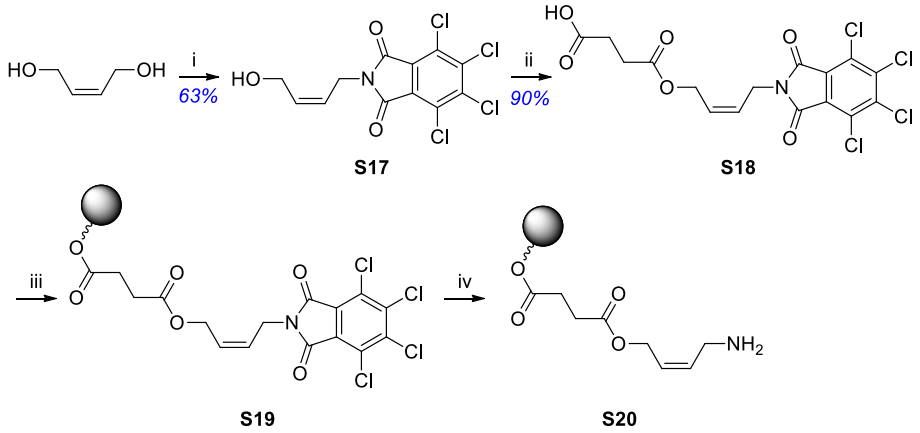
S16

S15 (4.2 g), $Pd(PPh_3)_4$ (230 mg), *N*-methylaniline (2.5 mL) were dissolved in THF (40 mL) and stirred at RT for 6 h. The reaction mixture was then diluted with EtOAc (80 mL) and washed with HCl (1M, 50 mL x3). The organic phase was separated, dried over Na_2SO_4, filtered and concentrated. The crude product was purified using column chromatography (200-400 mesh), where the byproduct can be eluted with 2% MeOH in dichloromethane, followed by the desired product with 3% MeOH and 0.1% AcOH in dichloromethane. **S16** (2.55 g) was collected as a white solid (66%).

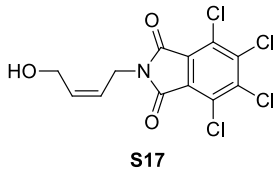
^{1}H NMR (500 MHz, $CDCl_3$) δ 9.96 (s, 1H), 6.39 (d, *J* = 17 Hz, 1H), 6.08 (dd, *J* = 17, 10 Hz, 1H), 5.85 (d, *J* = 10 Hz, 1H), 5.30 (s, 1H), 4.55-4.30 (m, 1H), 4.32 (d, *J* = 6 Hz, 2H), 3.53 (d, *J* = 12 Hz, 1H), 3.24 (t, *J* = 12 Hz, 1H), 2.35 (d, *J* = 13 Hz, 1H), 1.91 – 1.60 (m, 2H), 1.60 – 1.42 (m, 2H), 1.36 (s, 3H), 1.34 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 204.7, 175.3, 166.8, 165.7, 131.4, 127.8, 69.6, 69.5, 51.2, 46.7, 44.0, 26.2, 24.9, 22.1, 21.8, 21.1. HRMS for $[M+H]^+$ C15H21NO6, calculated: 312.1447, observed: 312.1444.

Experimental Procedures and Characterization Data for syntheses of *cis*-C4 linker

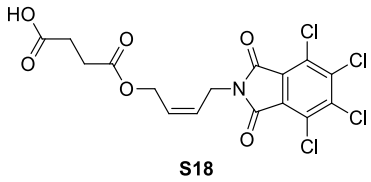
Synthetic Scheme of *cis*-C4 linker



Reaction conditions: i) 3,4,5,6-tetrachlorophthalimide (TCPNH), PPh_3, DIAD, THF, RT, 16h; ii) succinic anhydride, DMAP, CH_2Cl_2; iii) DIPEA, 2-chlorotrityl resin, DMF, RT, 2h; iv) hydrazine, THF, RT, 2h.

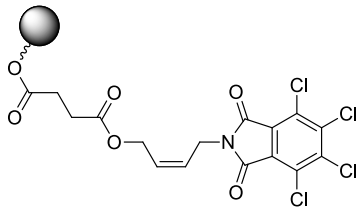


To a mixture of TCPNH (4.0 g, 14 mmol), PPh3 (5.5 g, 20.97 mmol), 2-butene-1,4-diol (1.8 mL, 22 mmol) in anhydrous THF (40 mL) was added DIAD (95%, 4.3 mL, 22 mmol) at RT. The reaction was stirred overnight and concentrated. The residue was added CH_2Cl_2 (40 mL) and filtered to offer the alcohol as a white solid (3.15 g, 63%). The product **S17** was used without further purification.



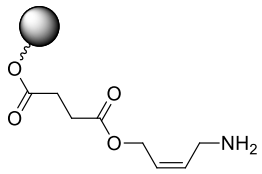
To a solution of the alcohol (3.00 g, 8.45 mmol), DMAP (2.08 g, 17.05 mmol) in anhydrous CH_2Cl_2 was added succinic anhydride (1.32 g, 13.19 mmol). The stirring was continued overnight. The mixture was concentrated and purified by column (CH_2Cl_2 : MeOH = 20 : 1 to CH_2Cl_2 : MeOH : AcOH = 20 : 1 : 0.1) to provide the acid **S18** as a white solid (3.18 g, 83%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.79 (dd, *J* = 17.3, 6.9 Hz, 1H), 5.67 (dd, *J* = 17.9, 7.6 Hz, 1H), 4.88 (d, *J* = 6.7 Hz, 2H), 4.39 (d, *J* = 7.3 Hz, 2H), 2.69 (dd, *J* = 11.7, 5.3 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.4, 171.9, 163.1, 140.2, 129.8, 128.8, 127.6, 126.6, 60.1, 35.5, 28.8.



S19

To a solution of the carboxylic acid (970 mg, 2.13 mmol) and DIPEA (1 mL, 5.73 mmol) in anhydrous DMF (10 mL) was added pre-swollen 2-Cl trityl resin (Rapp Polymere, 1.14 mmol/g, 1.8 g, 2.05 mmol). The resin was shaken for 4 h, and then washed with DMF five times to offer **S19**.

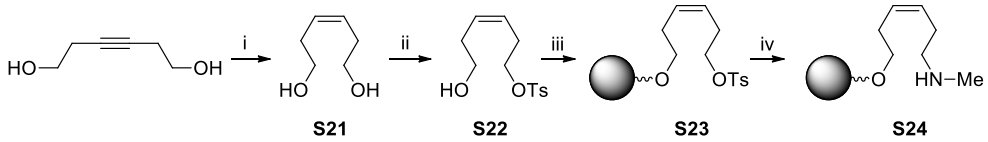


S20

Before couple to the amino acids, the TCP-protected resin **S19** was shaken with ethylenediamine (0.4 mL, 5.99 mmol) in anhydrous DMF at RT for 2h, followed by subsequent washing with CH_2Cl_2 (4x) and DMF (1x). Resin **S20** was obtained with a loading of 0.74 mmol/g.

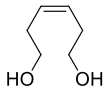
Experimental Procedures and Characterization Data for syntheses of *cis*-C6 linker

Synthetic Scheme of *cis*-C6 linker



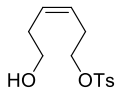
S21 **S22** **S23** **S24**

Reaction conditions: i) H_2, $Pd/CaCO_3$, RT, 12h. ii) TsCl, Ag_2O, KI, CH_2Cl_2, RT, 12h. iii) AgOTf, 2,6-di-*tert*-butylpyridine, 2-chlorotrityl resin, THF, RT, 1h. iv) $MeNH_2$, THF, 40°C, 12h.



S21

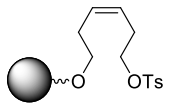
Hex-3-yne-1,6-diol (2.0 g), quinoline (0.12 g) and Lindlar catalyst (0.30 g) were suspended in MeOH (15 mL). Hydrogen was filled in to the flask with a Schlenk line and a positive pressure was maintained with a balloon of hydrogen. The reaction was stirred at RT for 12h before filtered and concentrated. The crude product (2.1 g) was co-evaporated with toluene (20 mL x2) to remove the residue of MeOH. The product **S21** was used without further purification.



S22

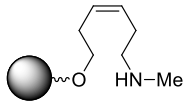
Monotosylation of diol was obtained by a reported Ag_2O-assisted method (*10*). The percentage yield of monotosylation is 90% for *cis*-C6 linker on 2.0 g scale.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.3 Hz, 2H, aromatic), 7.35 (d, *J* = 8.0 Hz, 2H, aromatic), 5.63 – 5.48 (m, 1H, =CH), 5.48 – 5.33 (m, 1H, =CH), 4.04 (t, *J* = 6.7 Hz, 2H, OCH_2), 3.64 (dd, *J* = 12.3, 6.2 Hz, 2H, OCH_2), 2.45 (s, 3H, CH_3), 2.44 (q, *J* = 6.5 Hz, 2H), 2.28 (q, *J* = 6.5 Hz, 2H).^{13}C NMR (126 MHz, $CDCl_3$) δ 129.86 (aromatic), 129.51 (aromatic), 127.93 (=CH), 126.19 (=CH), 69.66 (OCH_2), 61.99 (OCH_2), 30.89, 27.26, 21.69 (CH_3). HRMS for $[M+H]^+$ C13H18O4S, calculated: 271.1004, observed: 271.1004.



S23

To conjugate the Ts-protected alcohol on 2-chlorotrityl chloride solid support, briefly, the resin (9.6 mmol, 1.14 mmol/g), 2,6-di-*tert*-butylpyridine (10.5 mmol) and alcohol (5.9 mmol) was mixed in 100mL CH_2Cl_2. AgOTf (10.0 mmol) was added in two aliquots over 15 min. The red color of the resin persisted and this indicates that the alcohol is depleted in the reaction mixture. MeOH (5 mL) was then added to quench the reaction and the color turned white or pale yellow over 5min. The suspension was stirred at RT for another 1 h before it was filtered and the solid-support was transferred to a separatory funnel with CCl_4. After the mixture standing for 5 min to allow stratification, AgCl precipitation on the bottom was removed by draining the liquid to a level that most floating resin remained. The resin was then collected in a 250 mL solid-support reactor and washed with pyridine (50mL x4) with extensive shaking.

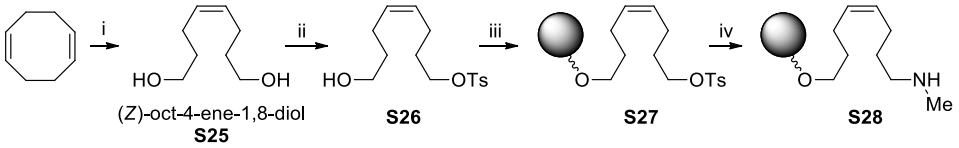


S24

The resin was then transferred into a 250 mL RB-flask with 100 mL THF. Methylamine (33% in MeOH) was added and stirred at 40 ^{o}C for 12h. The resin was filtered and washed with THF (50mL) for twice and CH_2Cl_2 (50mL) for twice. For long time storage at -20^{o}C, the resin was further washed with MeOH and air-dried for 20 min. The molarity of the NH group was determined by UV of the cleaved first coupled Fmoc group (0.40-0.45mmol/g)

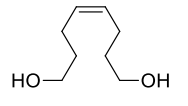
Experimental Procedures and Characterization Data for syntheses of *cis*-C8 linker

Synthetic Scheme of *cis*-C8 linker



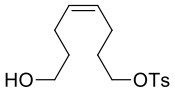
(*Z*)-oct-4-ene-1,8-diol **S25** **S26** **S27** **S28**

Reaction conditions: i) O_3, CH_2Cl_2, -78 ^oC, 1 h, then $NaBH_4$, MeOH, 0^oC, 1h. ii) TsCl, Ag_2O, KI, CH_2Cl_2, RT, 12h. iii) AgOTf, 2,6-di-*tert*-butylpyridine, 2-chlorotrityl resin, THF, RT, 1h. iv) $MeNH_2$, THF, 40^oC, 12h.



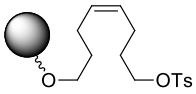
(*Z*)-oct-4-ene-1,8-diol
S25

Cycloocta-1,5-diene (2.0 g) was bubbled with a flow of ozone (ozone generator, 1.5mL/s) at -78 ^oC for 1h in CH_2Cl_2. After concentrated, MeOH (15 mL) was added to dilute the mixture and $NaBH_4$ (2 eq.) was added in 5 aliquots over 10 min at 0 ^oC. The solvent was removed by rotary evaporator until ~10mL remains. Brine (30mL) was added and the mixture was washed with hexane (20mL) twice, and subsequently extracted with EtOAc (20mL) for 5 times. 1.6 g product **S25** was isolated and used in the next step without further purification. Rf = 0.15 (EtOAc/hexane = 1/1, $KMnO_4$ stain).



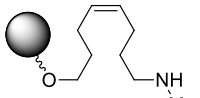
S26

See *cis*-C6 linker synthesis condition (ii). A percentage yield of 83% for *cis*-C8 linker on 2.0 g scale was obtained.



S27

See *cis*-C6 linker synthesis condition (iii).

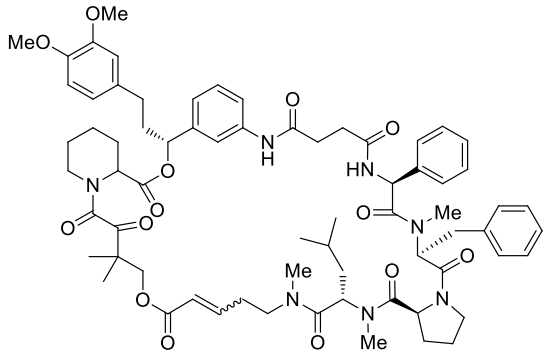


S28

See *cis*-C6 linker synthesis (iv). The molarity of NH group was 0.30-0.40 mmol/g.

Experimental Procedures and Characterization Data for syntheses of Rapadocin and Its Biotinylated Probe

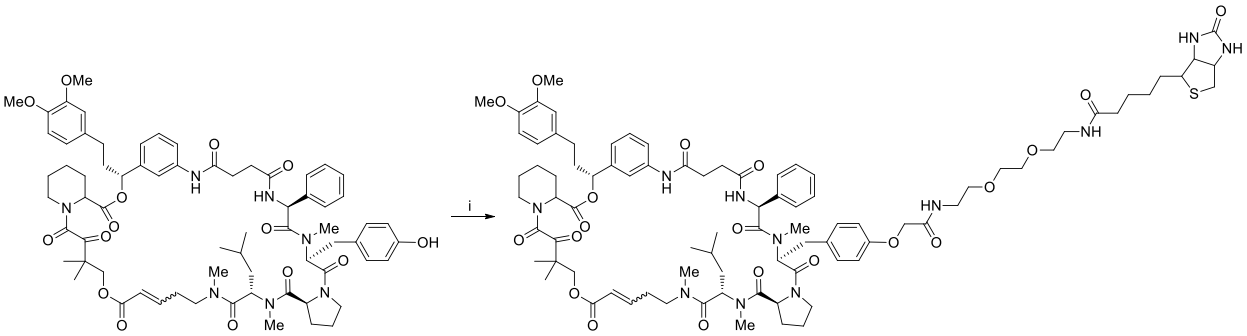
Synthesis of Rapadocin



rapadocin

Fmoc protected *N*-methyl isoleucine, D-proline, phenylalanine, phenylglycine and FKBD **10** were coupled in order on to *cis*-C6 linker conjugated beads (General Procedure A) before microwave-assisted RCM reaction (General Procedure B). The reaction mixture was filtered and purified by silica gel column (MeOH: CH_2Cl_2 1:20→1:5).

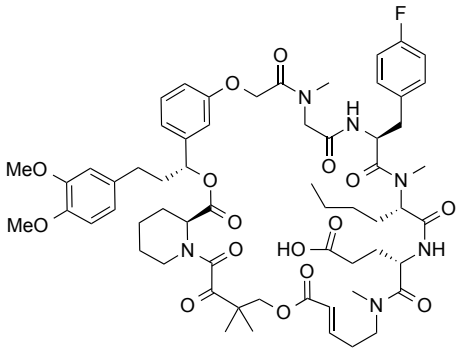
Synthesis of Biotinylated Rapadocin



Extended Data Figure 3. Synthesis of Biotinylated Rapadocin. Reaction condition: i) biotin-$(PEG)_3$-iodoacetamide, K_2CO_3, DMF, RT, 24 h, 15%.

Fmoc protected *N*-methyl isoleucine, D-proline, tyrosine, phenylglycine and FKBD **10** were coupled in order on to *cis*-C6 linker conjugated beads before microwave-assisted RCM reaction. The reaction mixture was filtered and purified by silica gel column (MeOH: CH_2Cl_2 1:20→1:5). 12 mg pure product was stirred vigorously with K_2CO_3 (2mg) and biotin-$(PEG)_3$-iodoacetamide (10mg) in dry DMF (0.2mL) for overnight at RT. The reaction mixture was filtered and purified by silica gel column (MeOH: CH_2Cl_2 1:20→1:5). 2.5mg biotinylated rapadocin was obtained (15%).

Synthesis of HP7E3

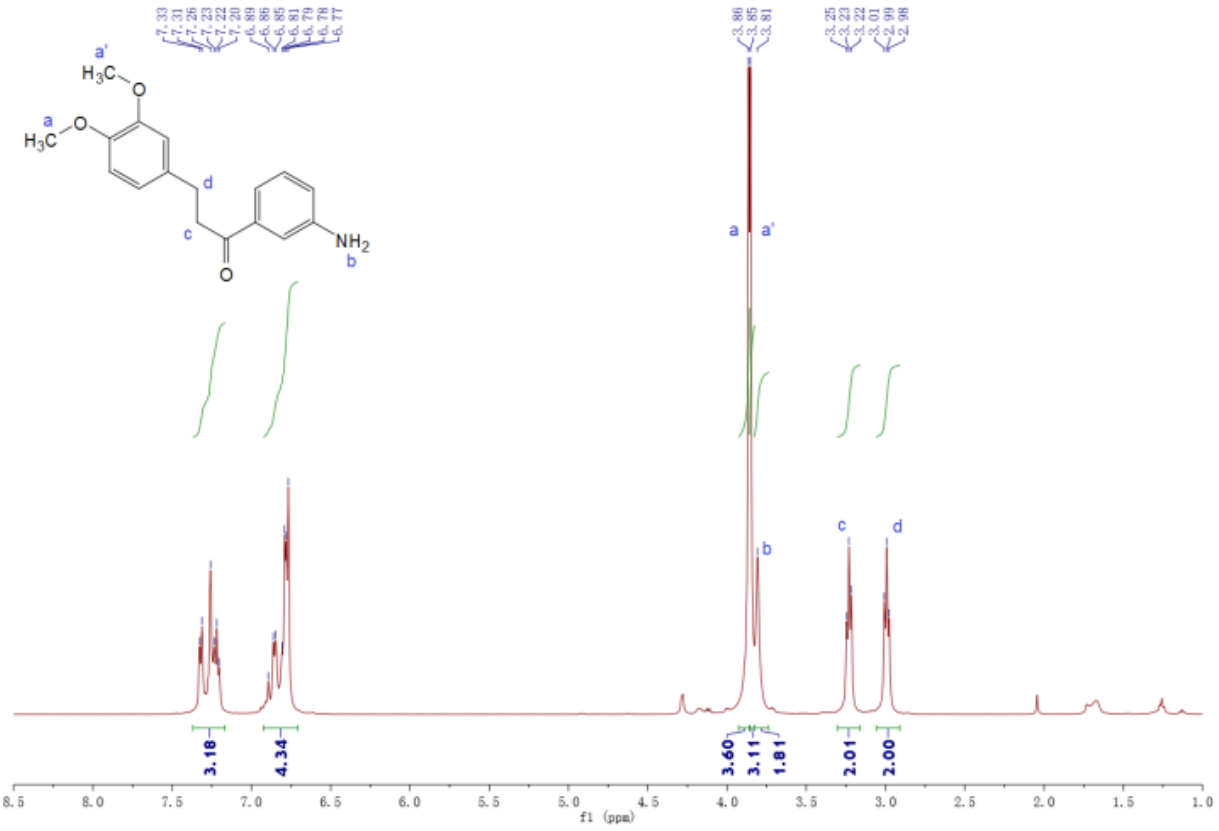


HP7E3

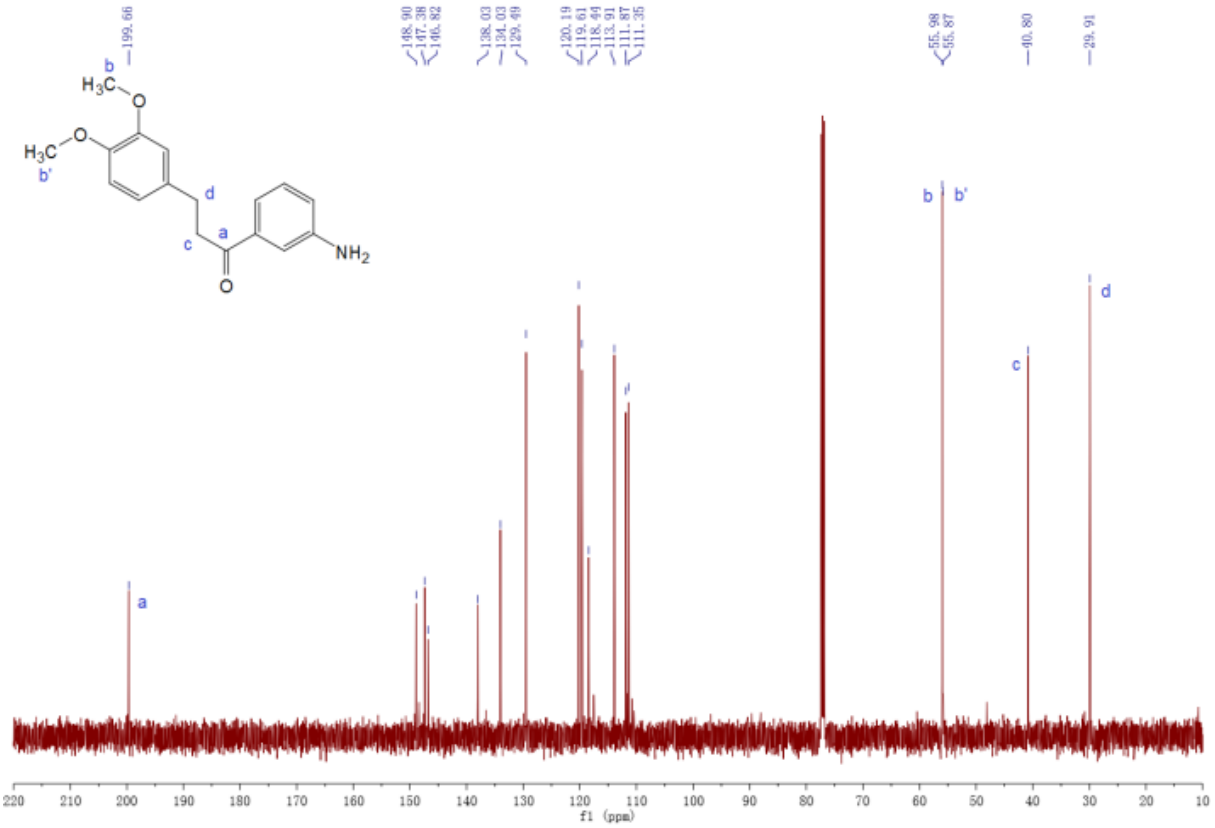
Fmoc protected glutamic acid 5-tert-butyl ester, *N*-methyl norleucine, 4-fluoro-Phenylalanine, *N*-methyl glycine and FKBD **11** were coupled in order on to *cis*-C6 linker conjugated beads (General Procedure A) before microwave-assisted RCM reaction (General Procedure B). The reaction mixture was filtered and purified by silica gel column (MeOH: CH_2Cl_2 1:20→1:5). The purified product was stirred with 10% TFA in DCM at RT for 2h to remove tBu- protection group from glutamic acid. The resulting crude product was dried under vacuum and purified by silica gel column (MeOH: CH_2Cl_2 1:20→1:5).

Spectra for Syntheses of FKBDs 10 and 11

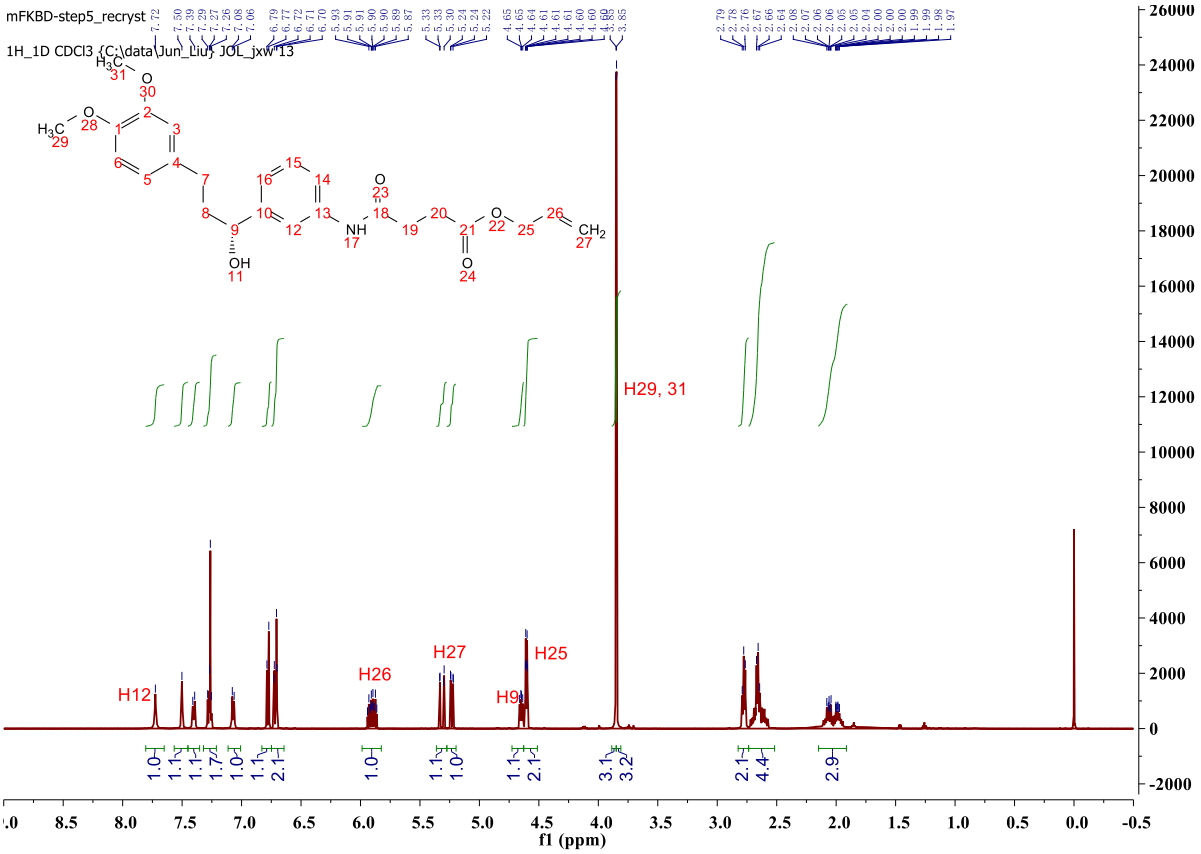
Compound S1: ^{1}H NMR



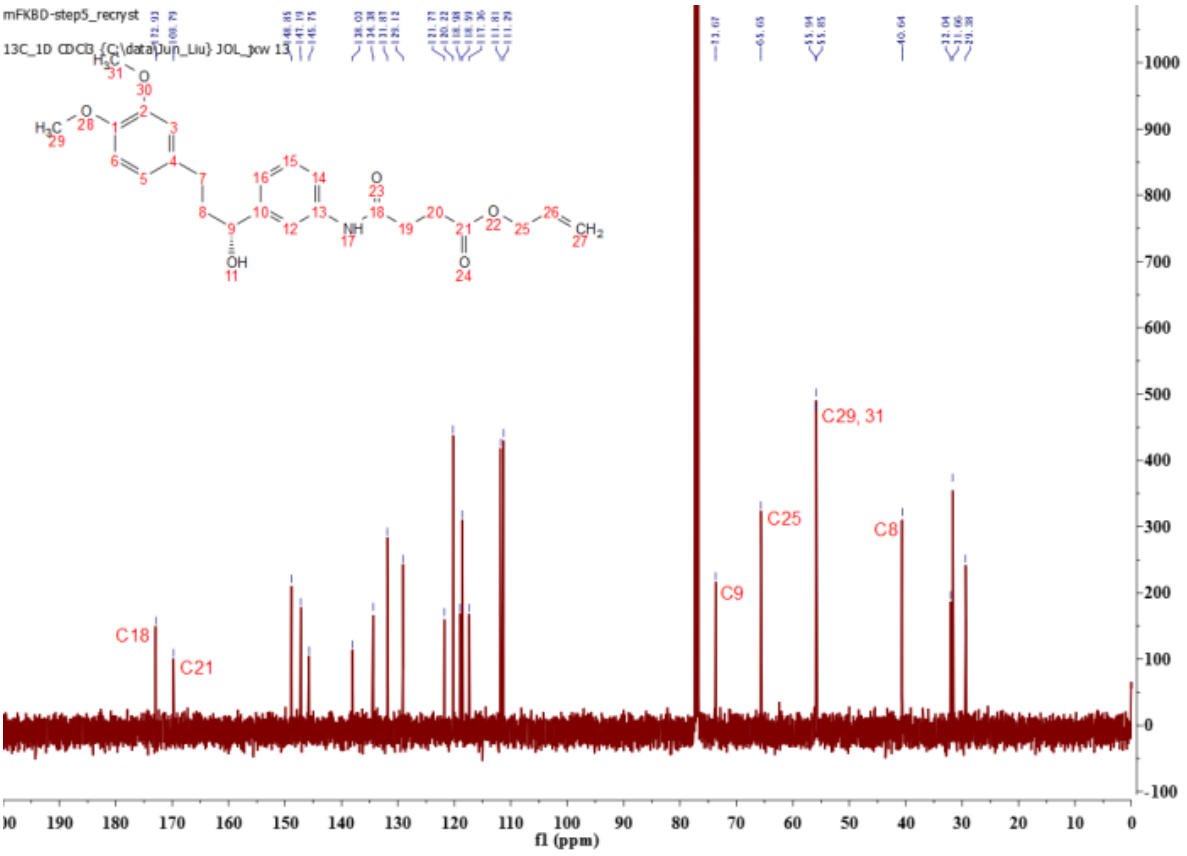
Compound S1: ^{13}C NMR



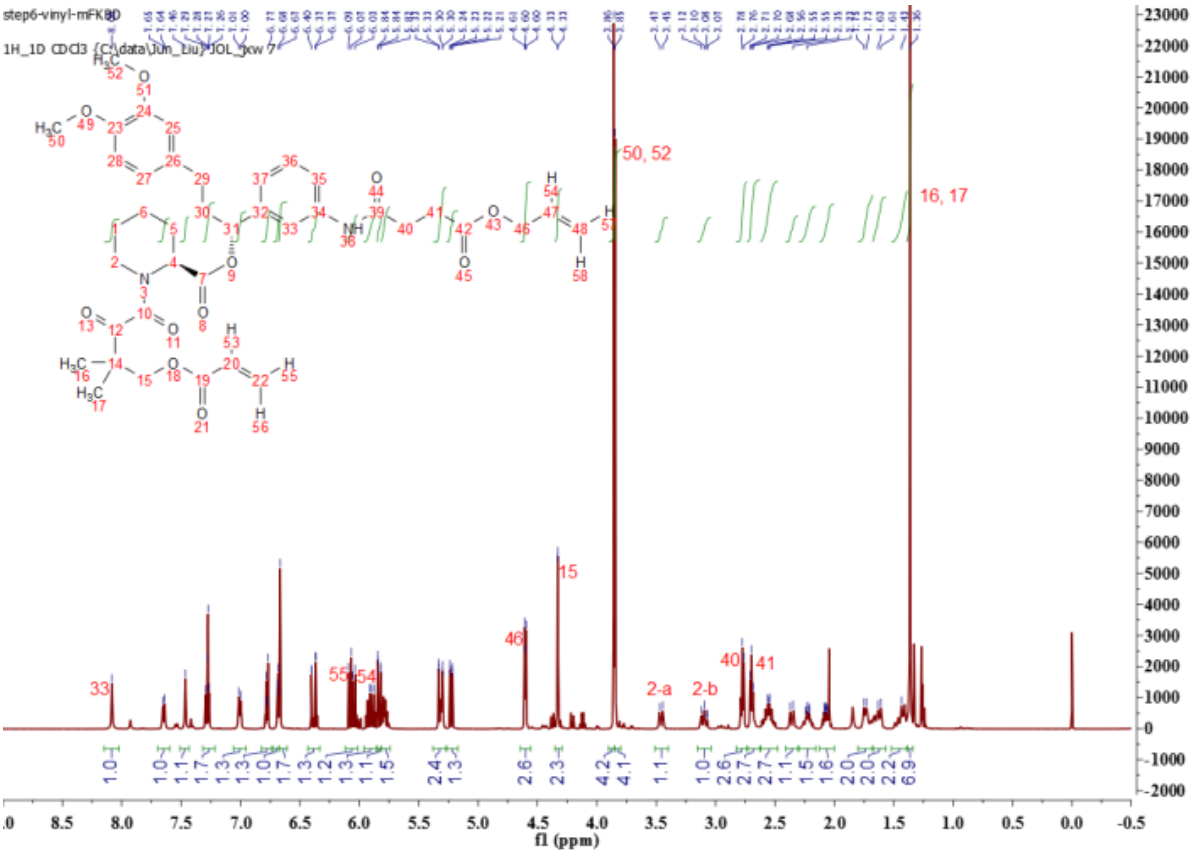
Compound S5: ^{1}H NMR



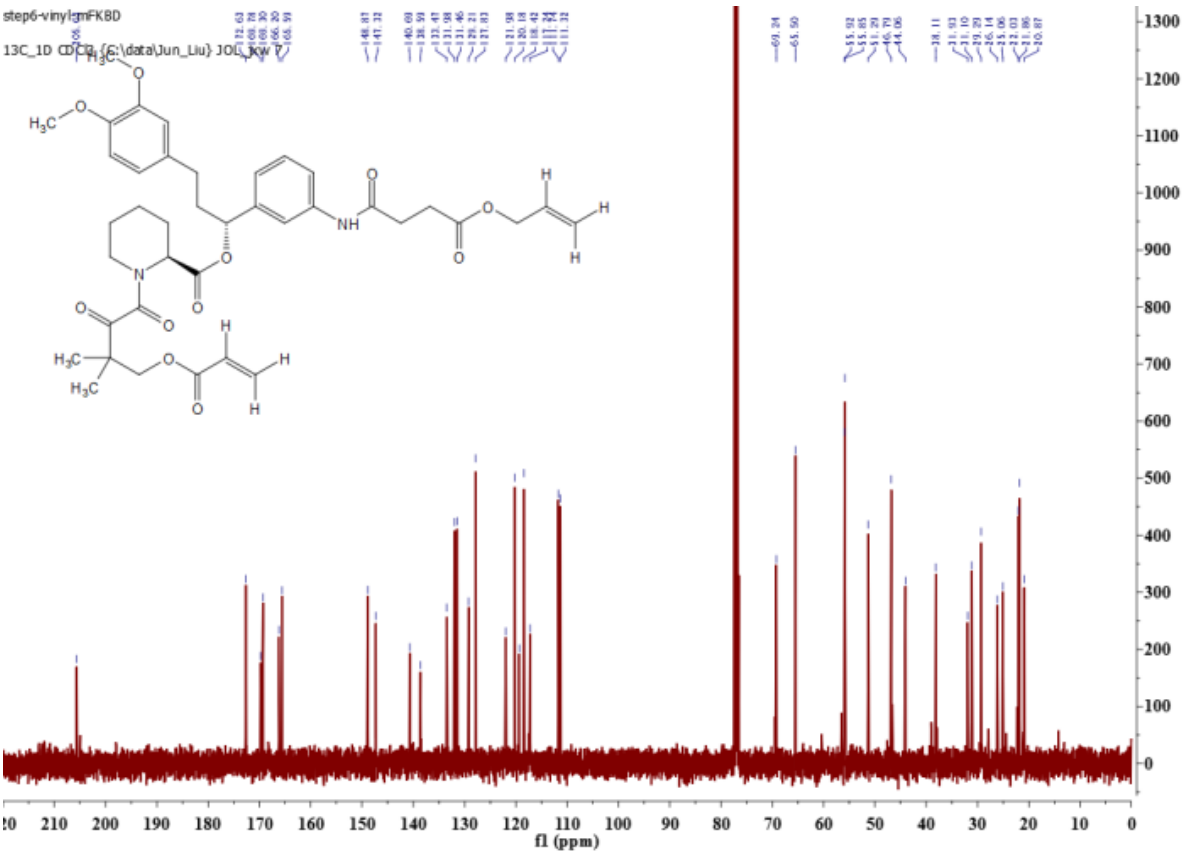
Compound S5: ^{13}C NMR



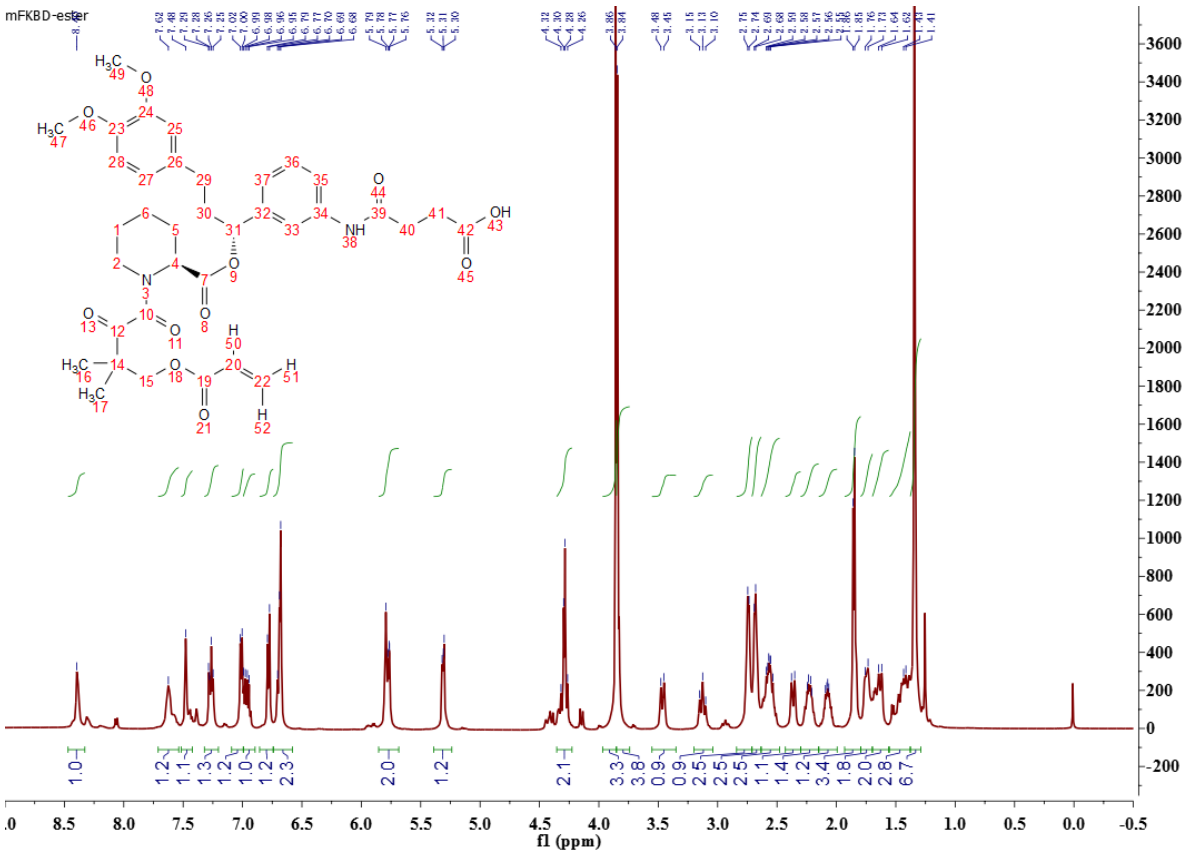
Compound S6: ^{1}H NMR



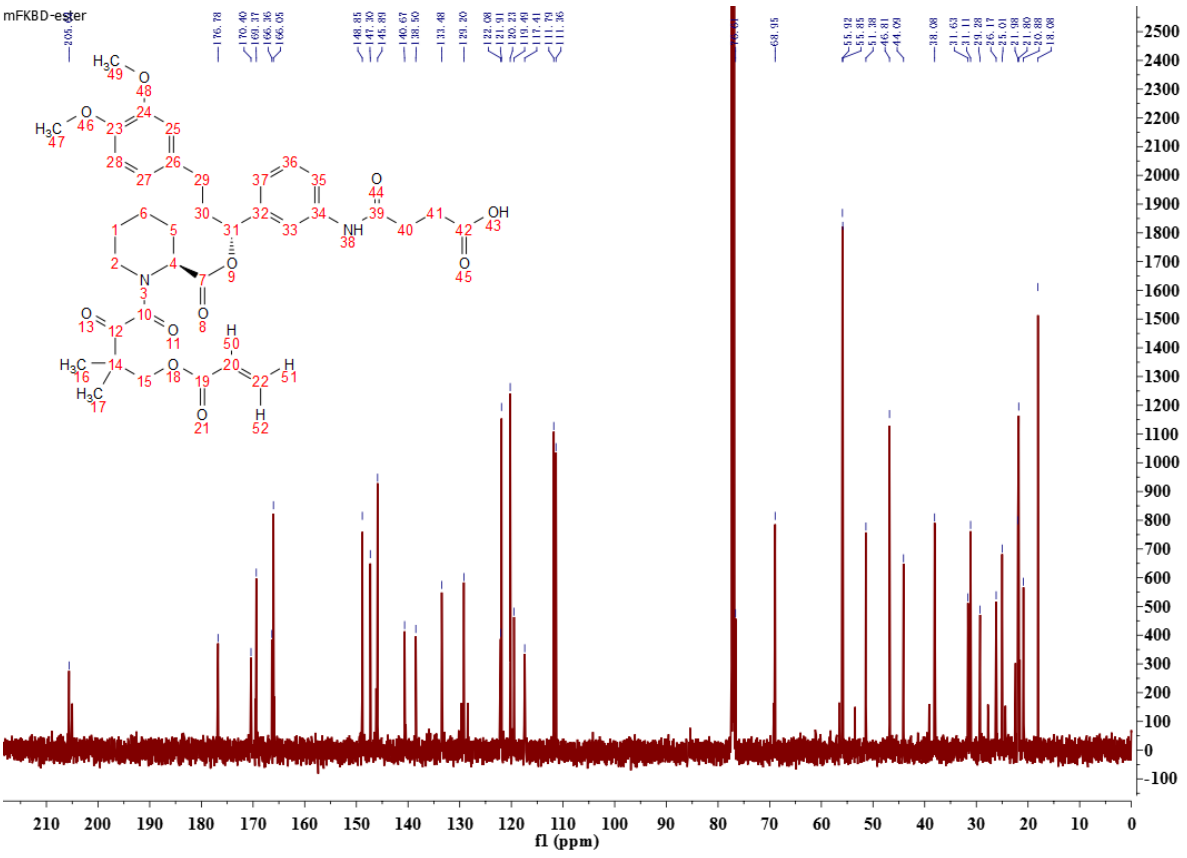
Compound S6: ^{13}C NMR



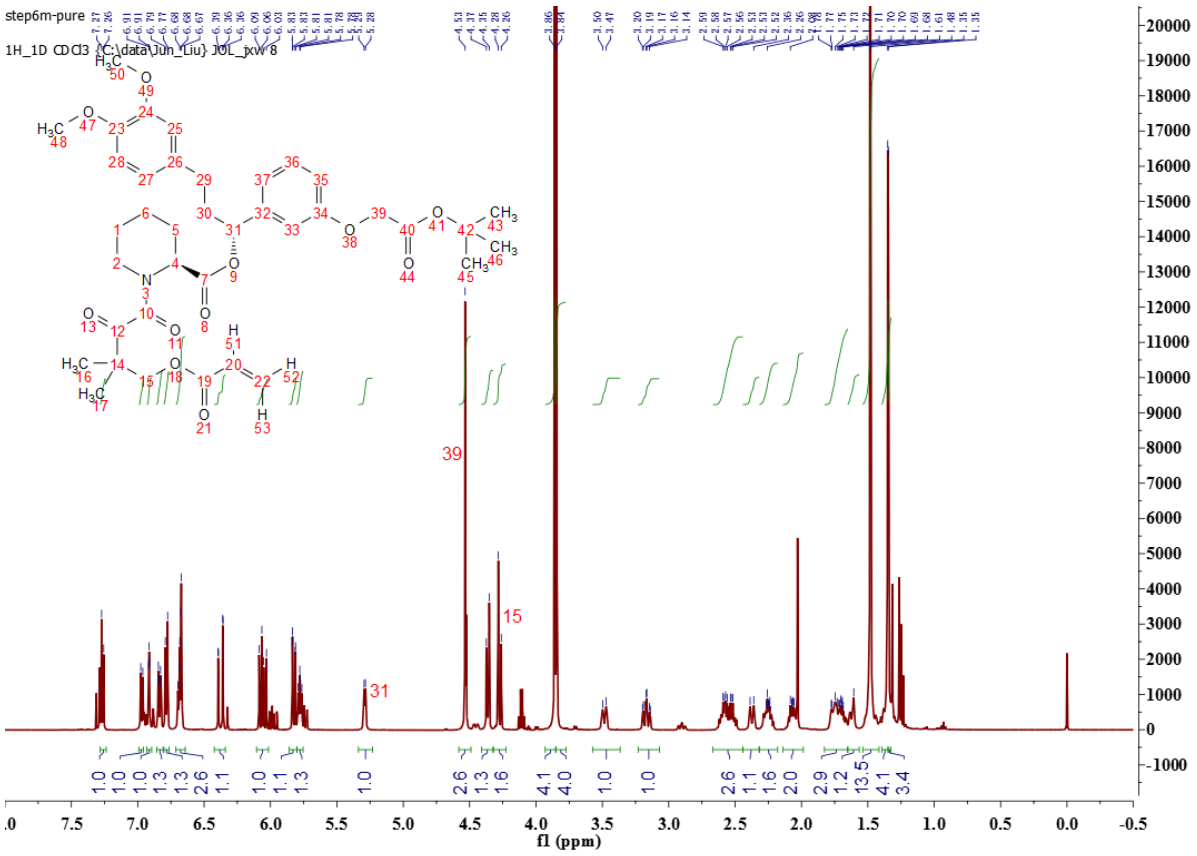
FKBD 10: ^{1}H NMR



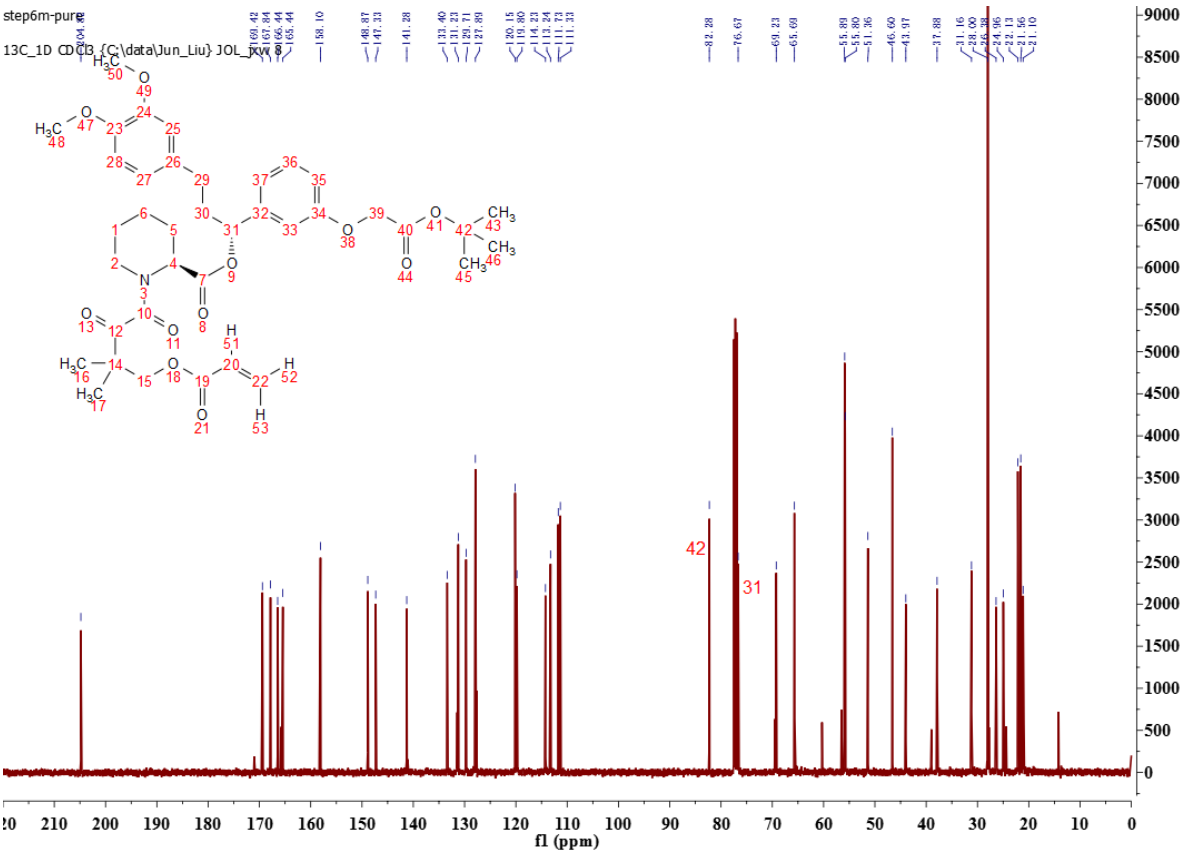
FKBD 10: ^{13}C NMR



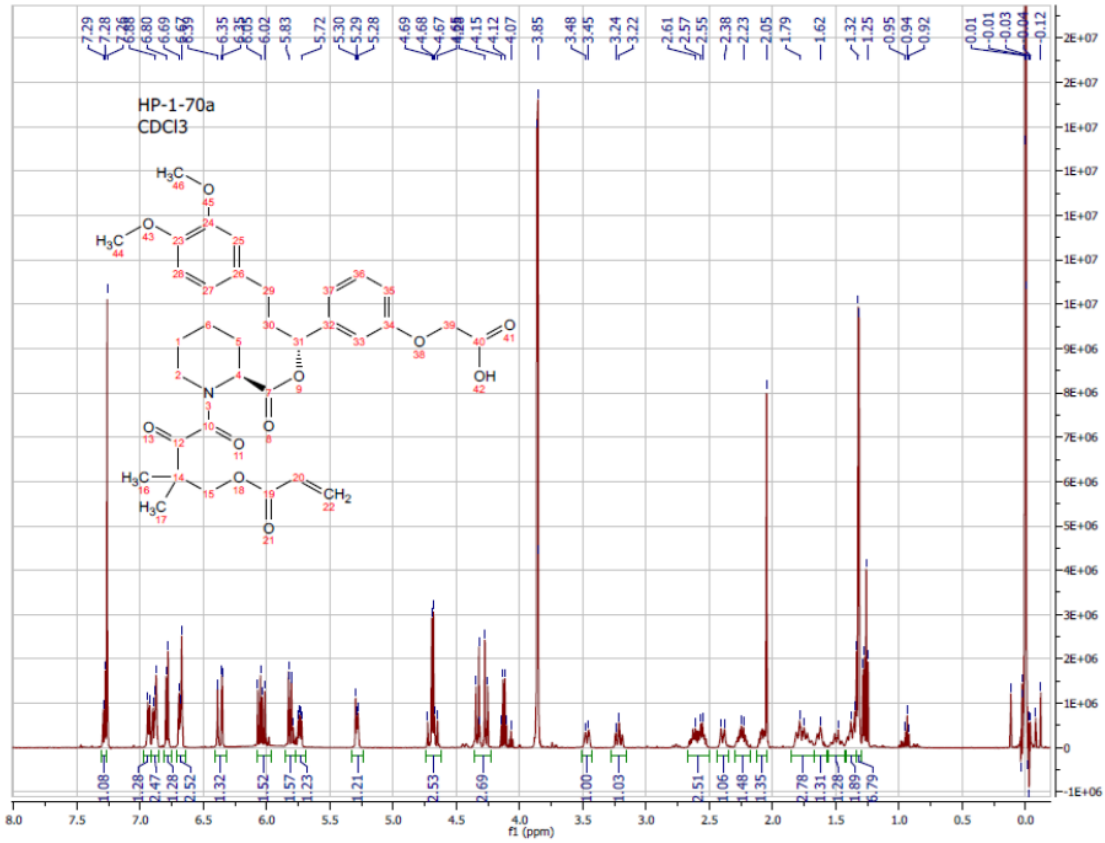
Compound S11: ^{1}H NMR



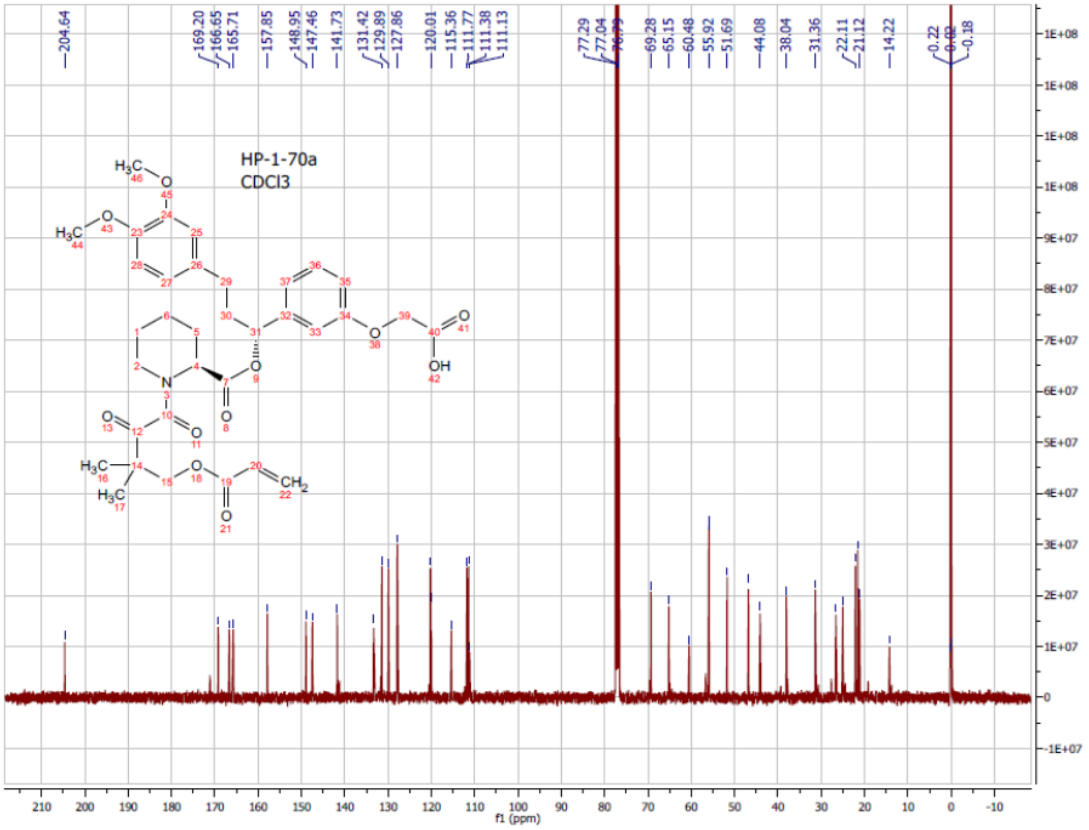
Compound S11: ^{13}C NMR



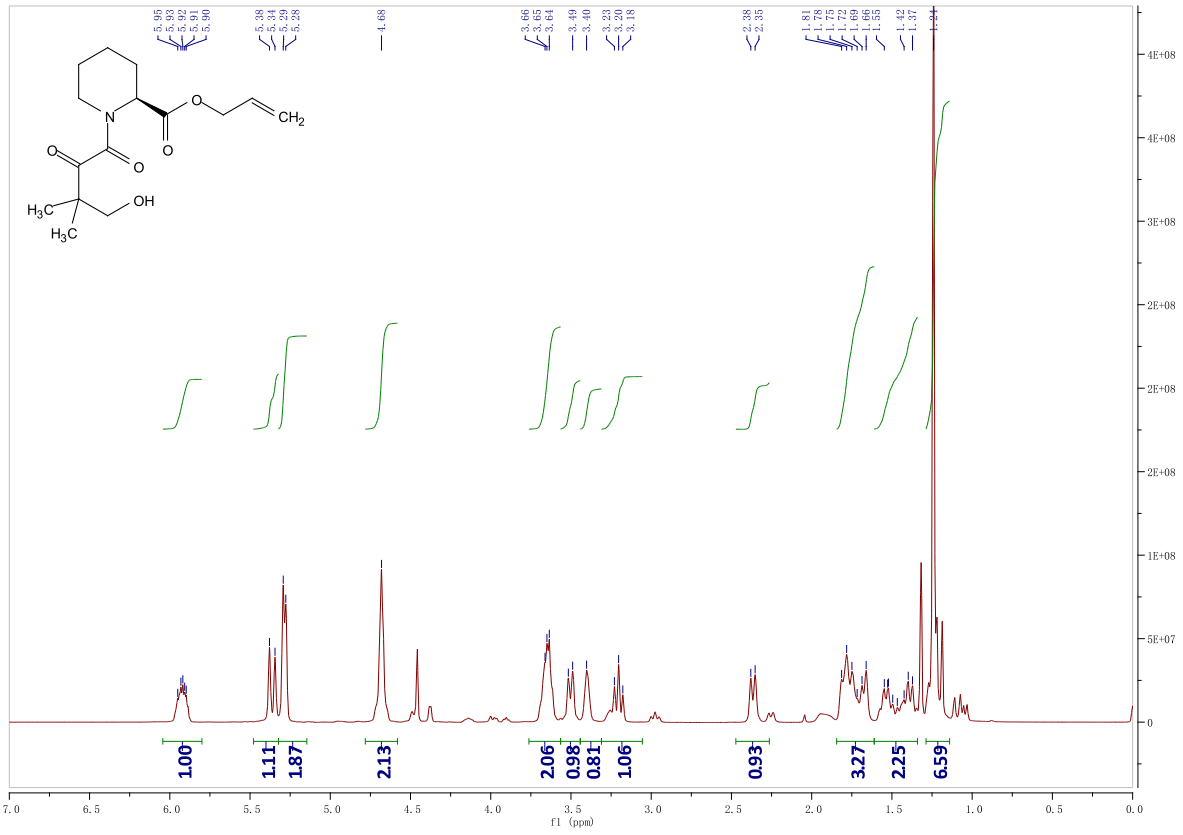
FKBD 11: ^{1}H NMR



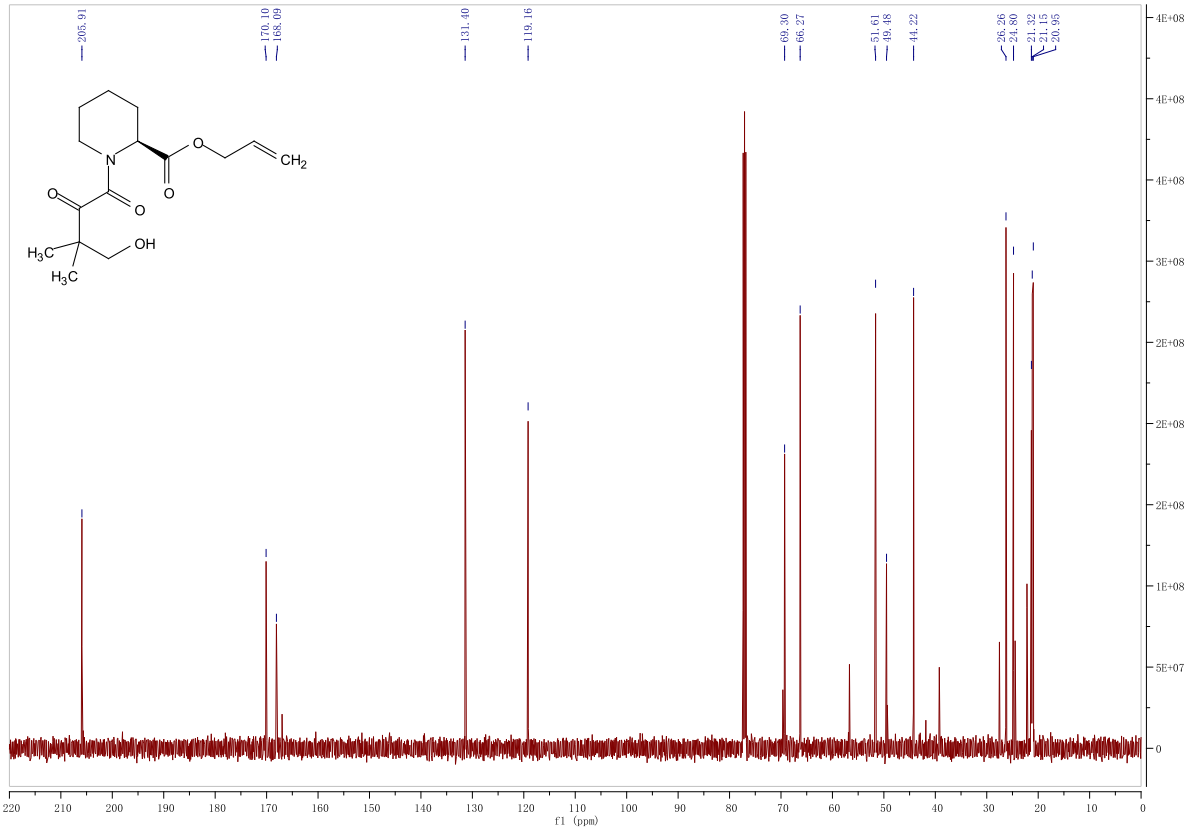
FKBD 11: ^{13}C NMR



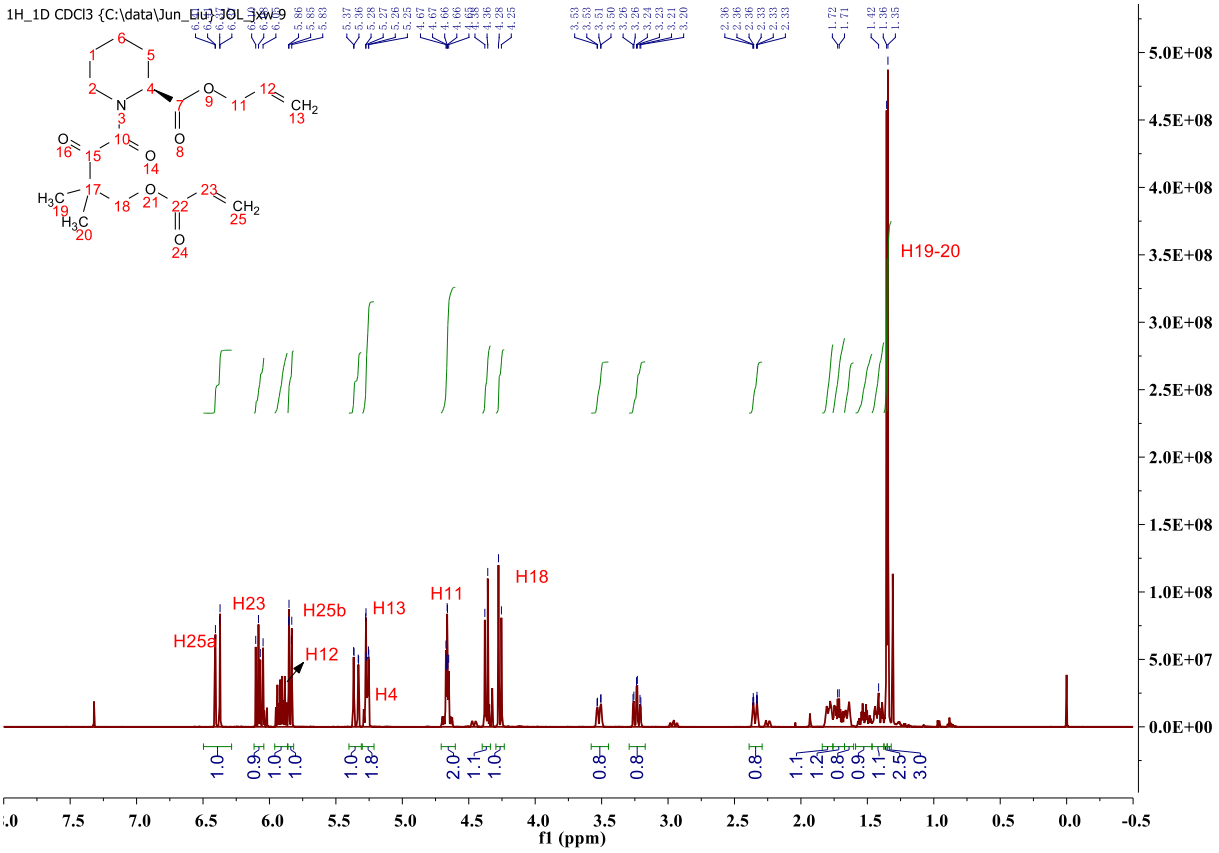
Compound S14: ^{1}H NMR



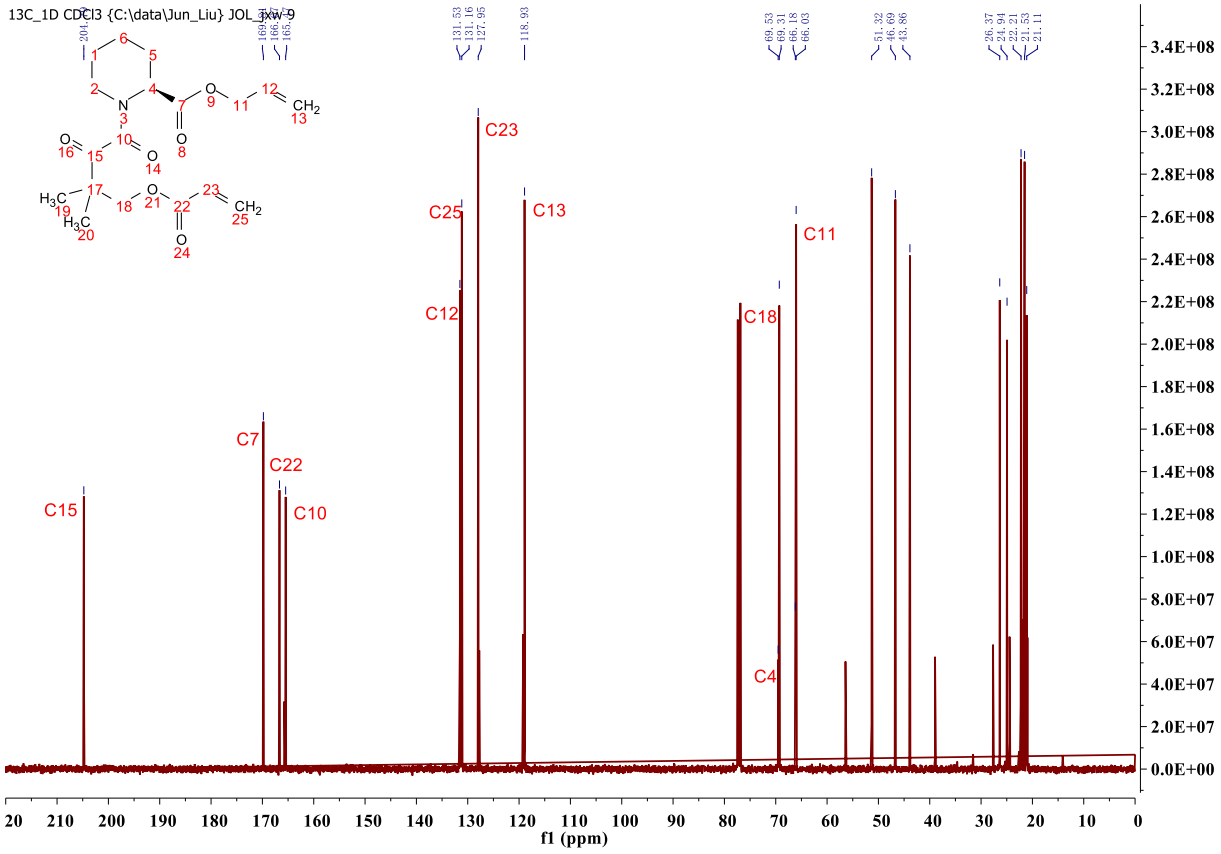
Compound S14: ^{13}C NMR



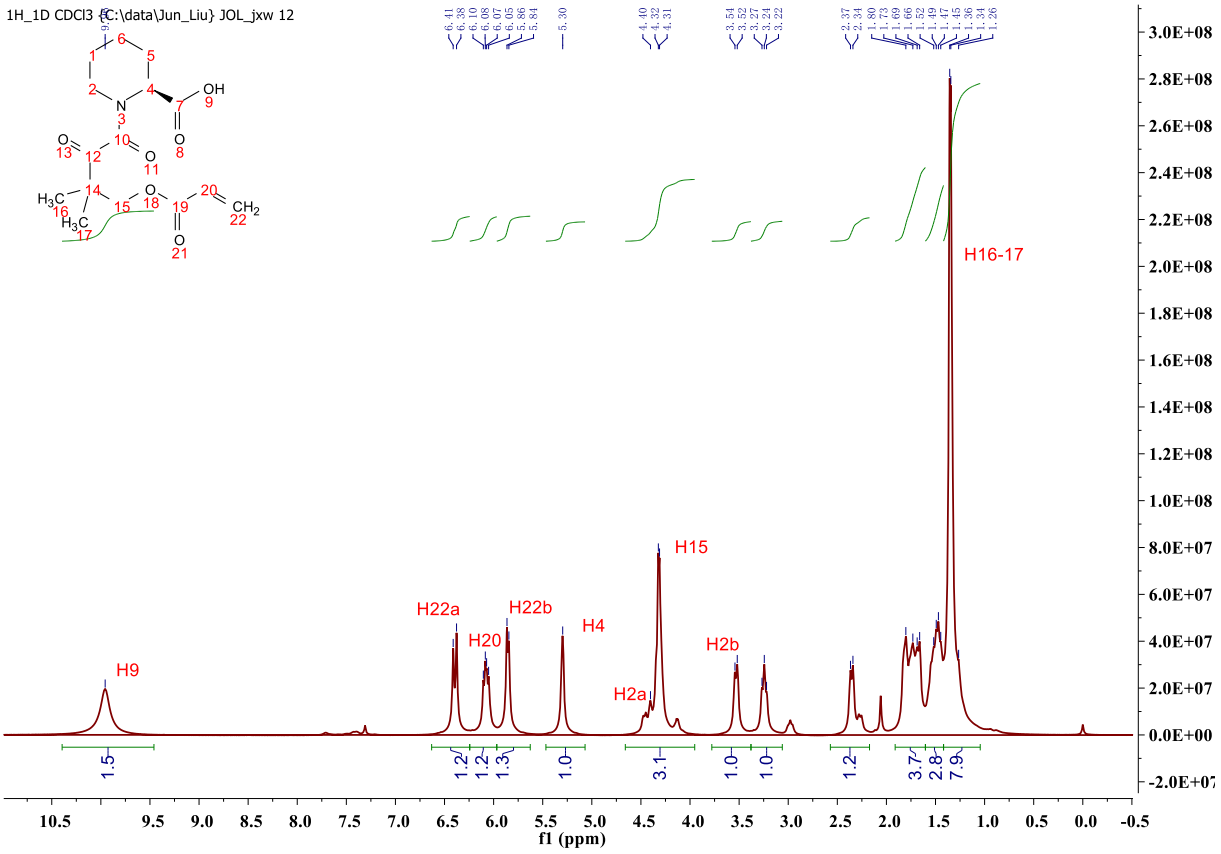
Compound S15: ^{1}H NMR



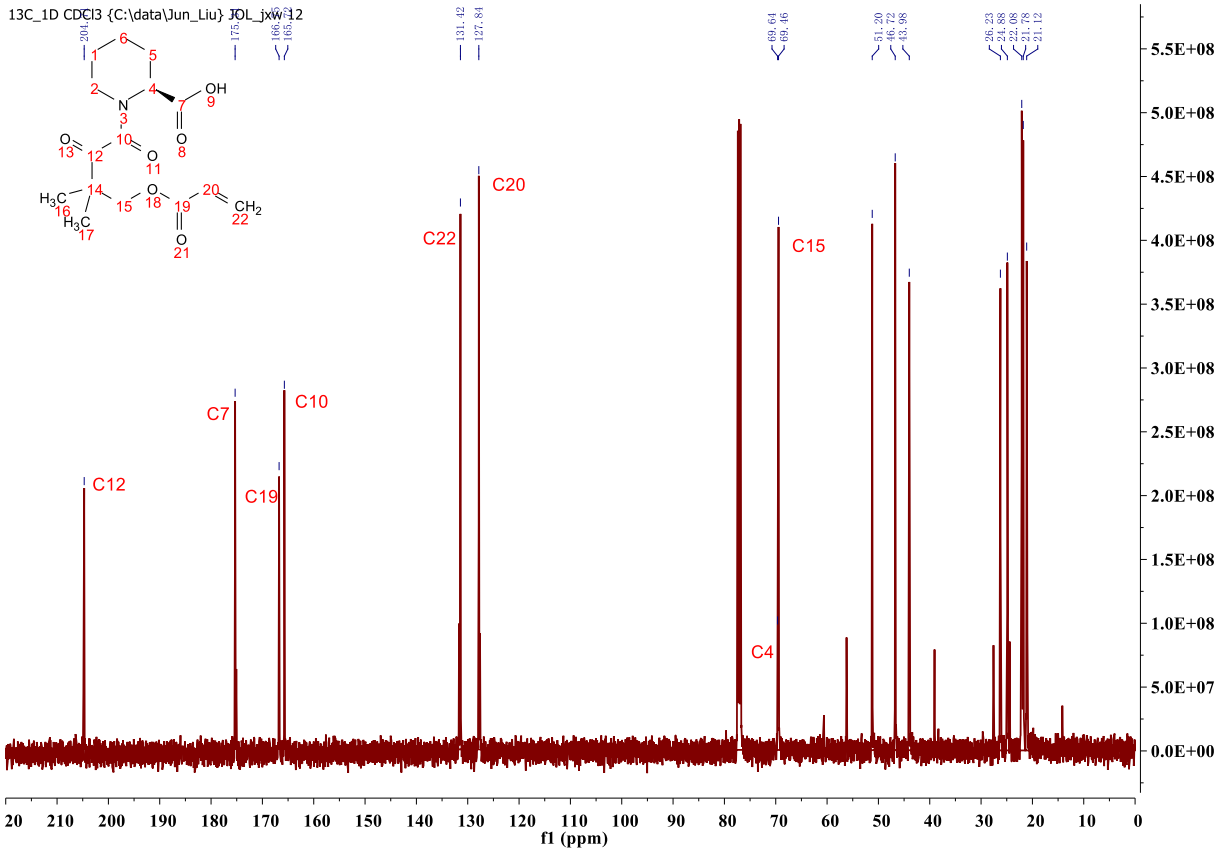
Compound S15: ^{13}C NMR



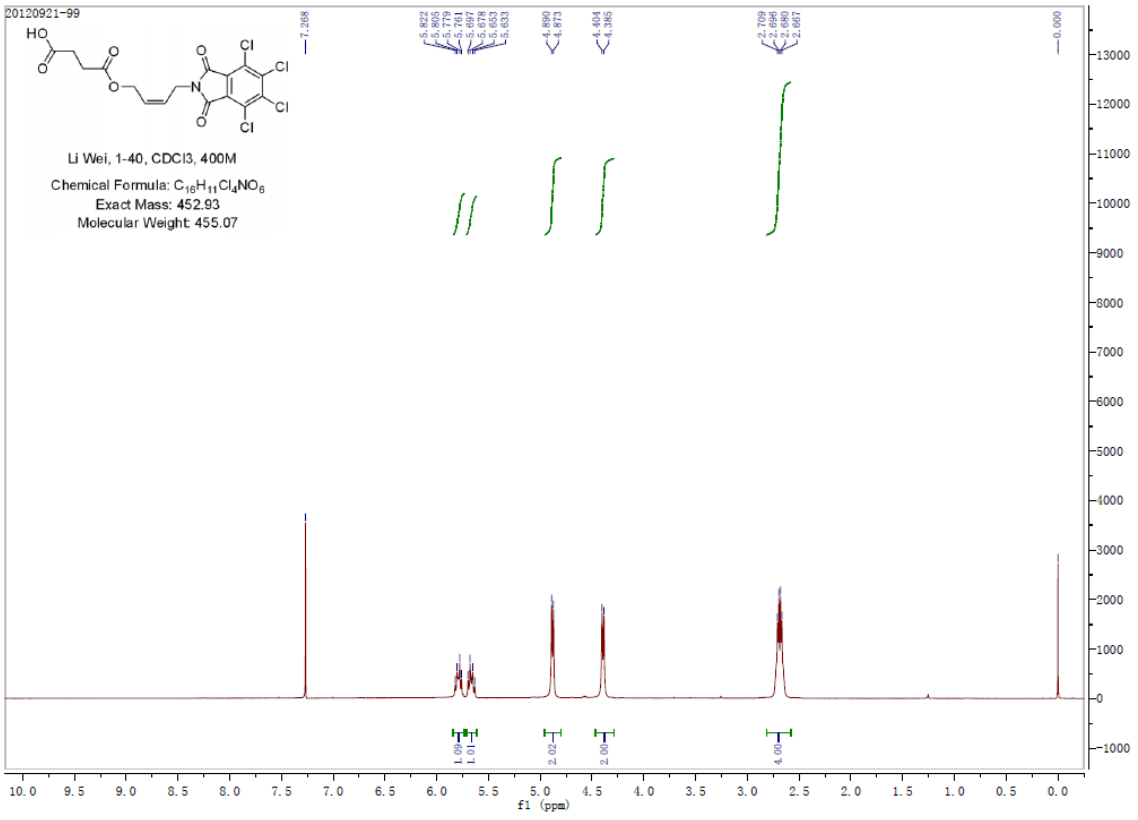
Compound S16: ^{1}H NMR



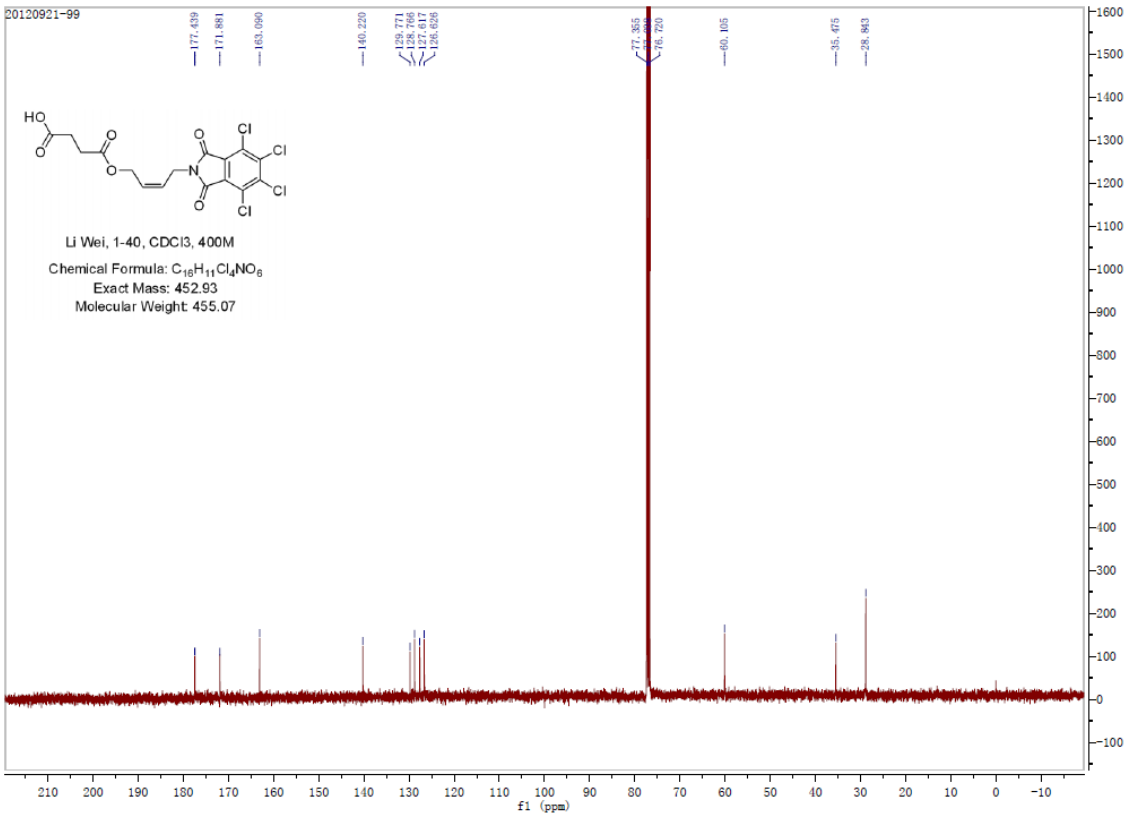
Compound S16: ^{13}C NMR.



Compound S18: ^{1}H NMR

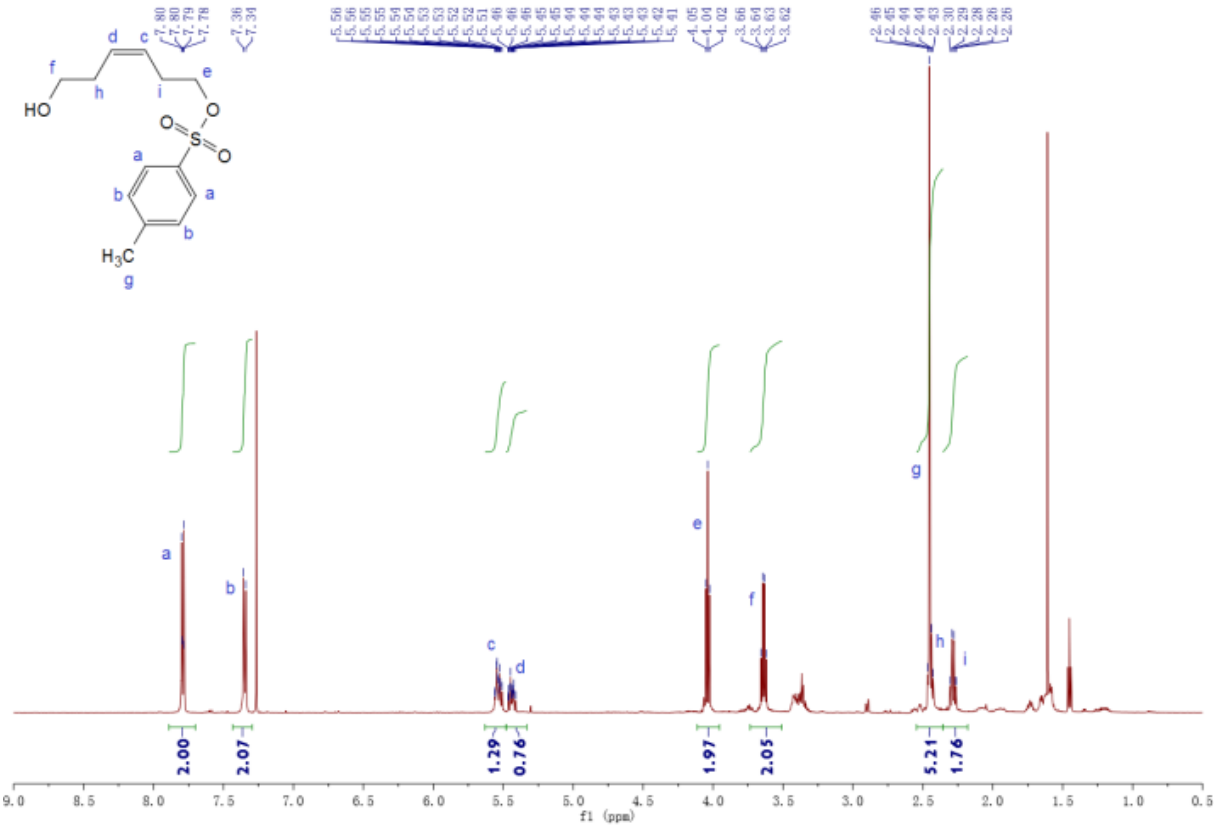


Compound S18: ^{13}C NMR.

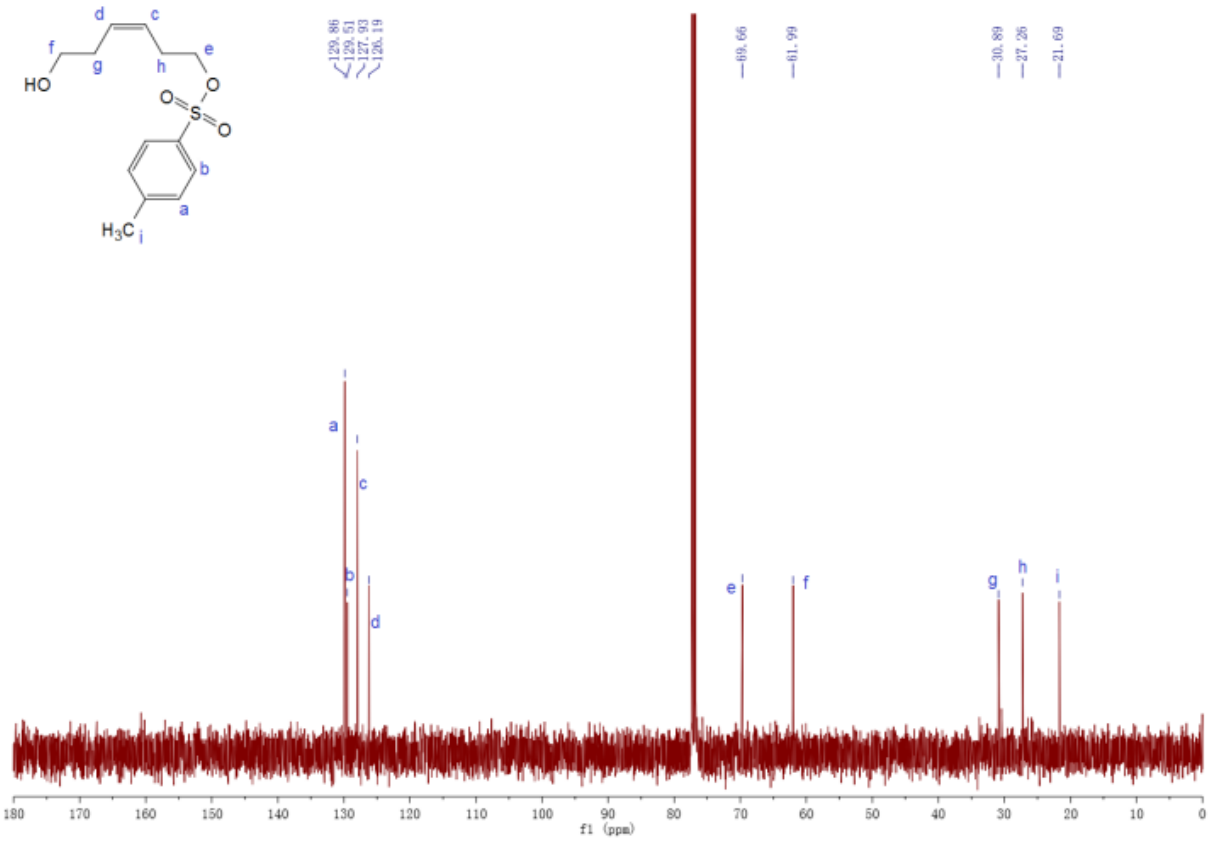


Spectra for Syntheses of *cis*-linkers

Compound S22: ^{1}H NMR

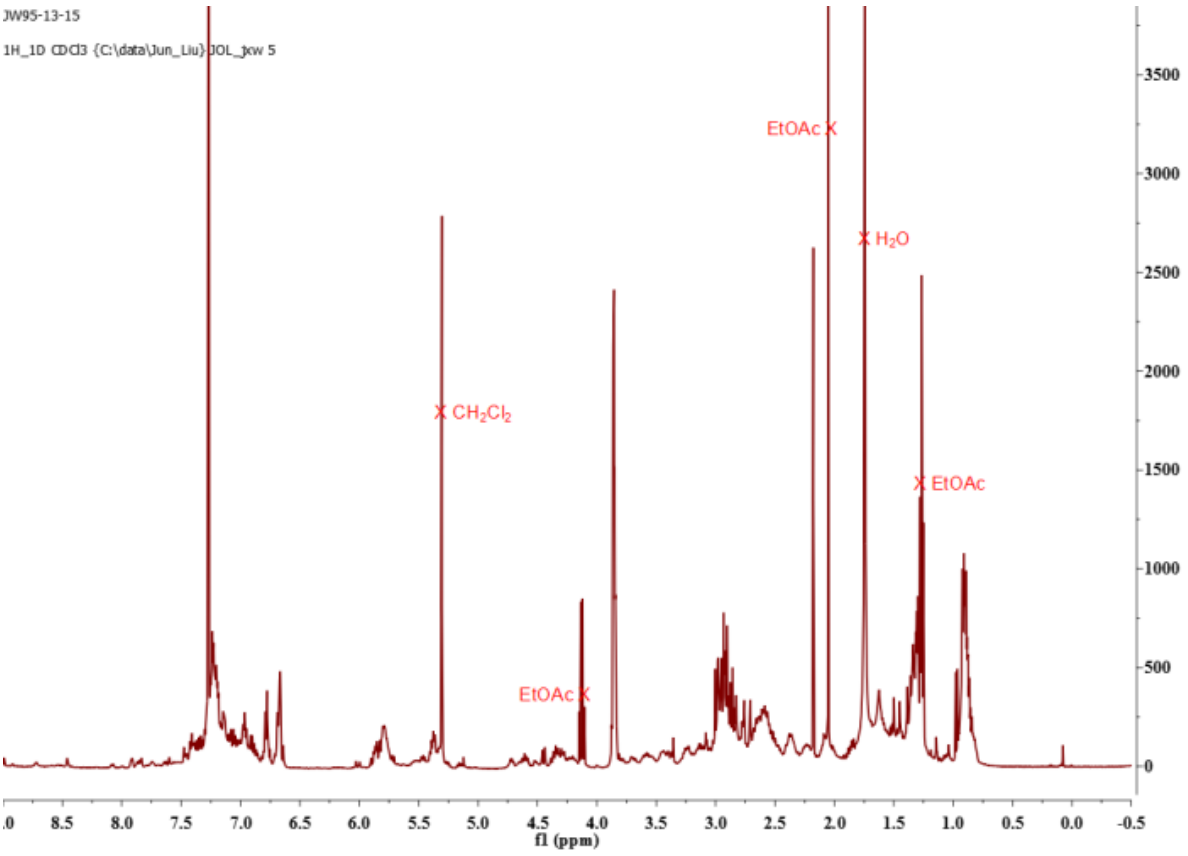


Compound S22: ^{13}C NMR

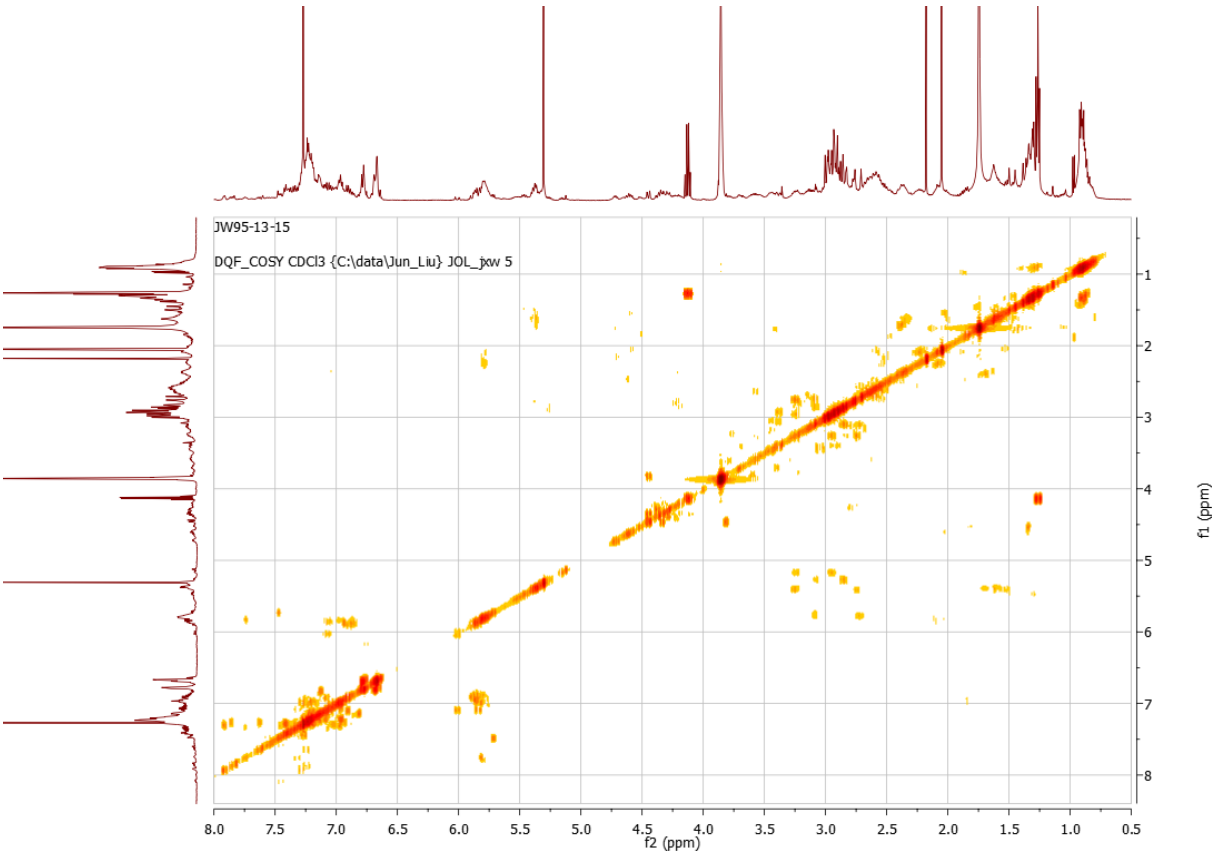


Spectra for Rapadocin, Its Biotinylated Probe and HP7E3

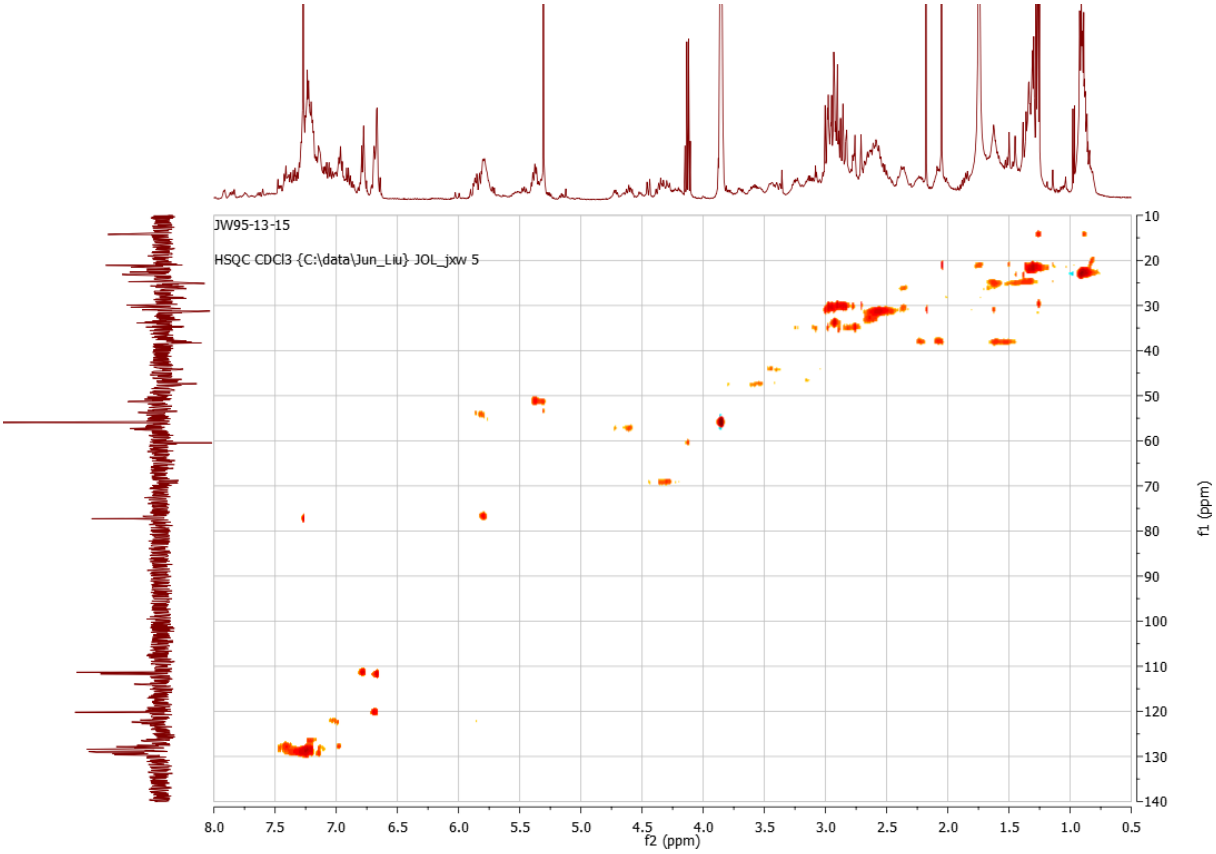
Rapadocin: ^{1}H NMR.



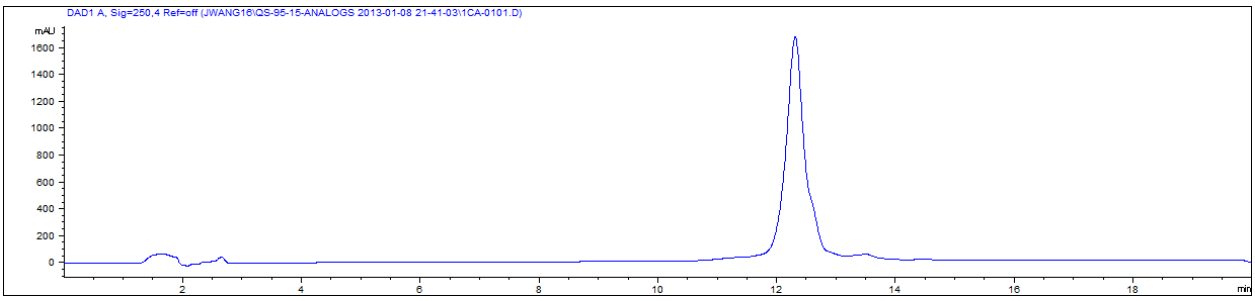
Rapadocin: Double Quantum Filtered COSY



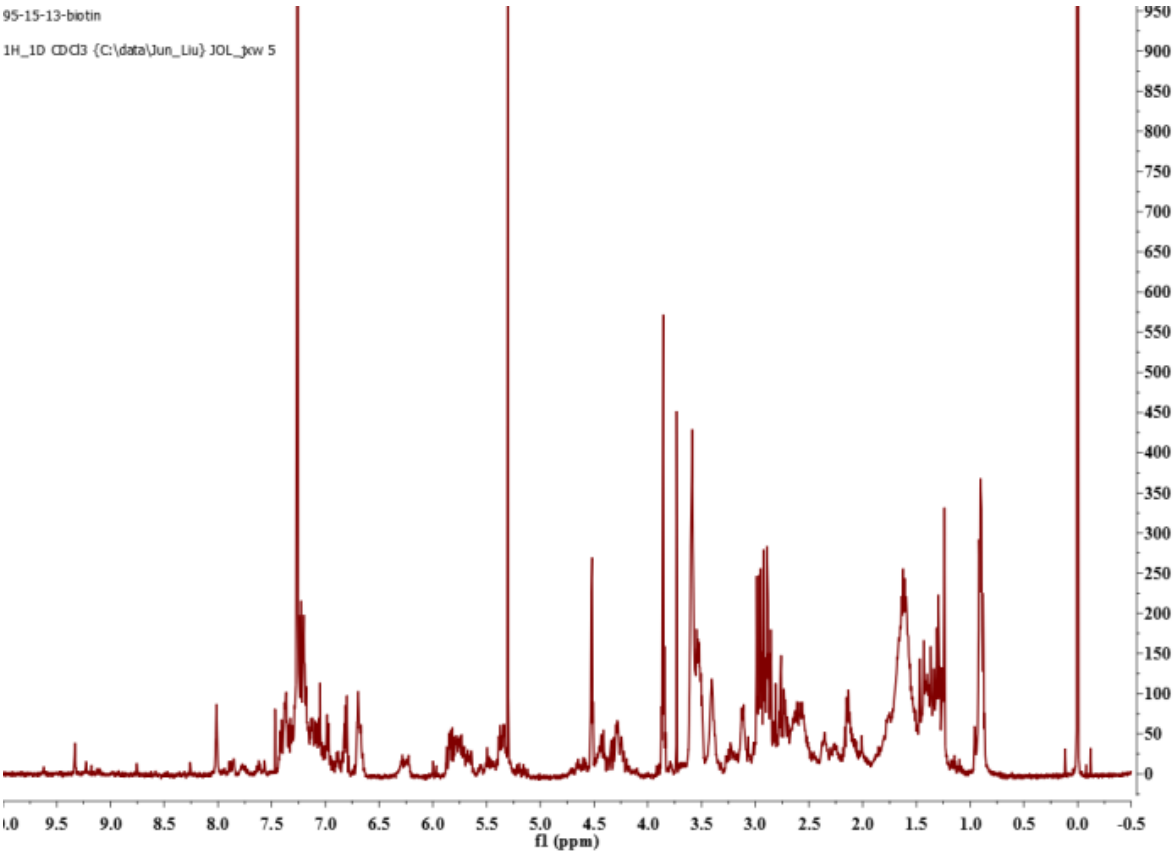
Rapadocin: HSQC (^{1}H-^{13}C DEPT135)



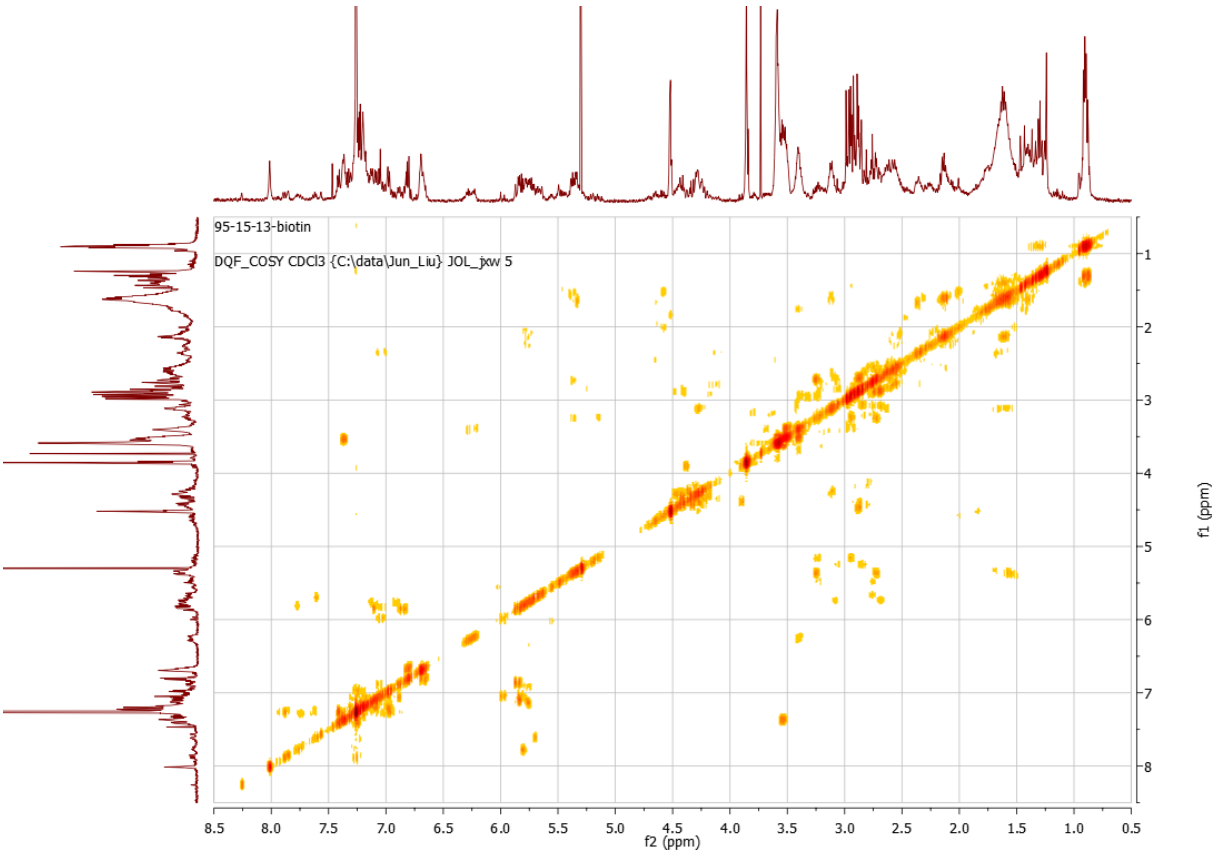
Rapadocin: HPLC Trace



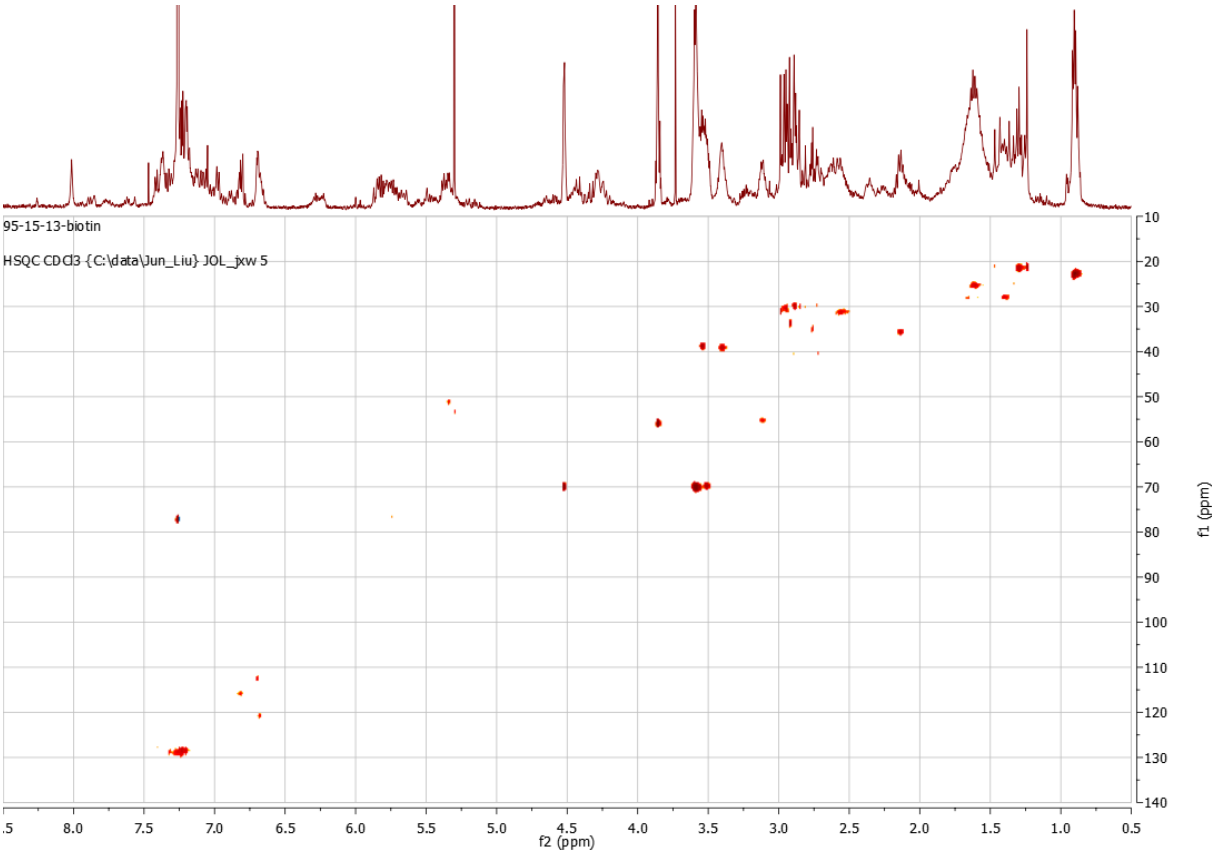
Biotinylated Rapadocin: ^{1}H NMR



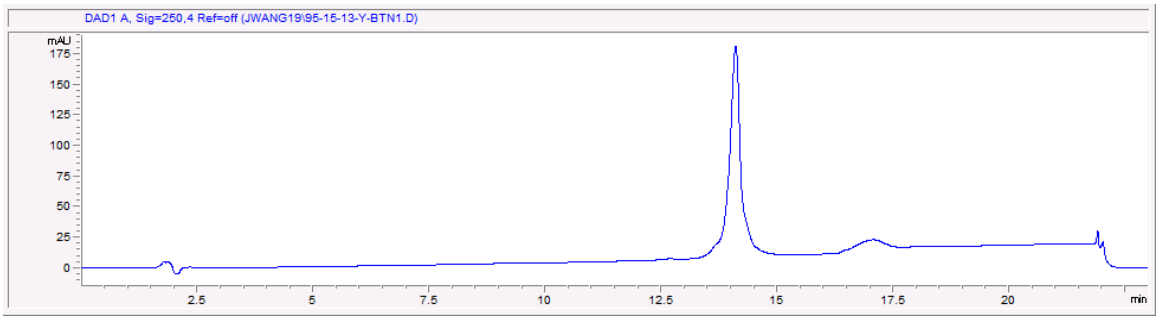
Biotinylated Rapadocin: Double Quantum Filtered COSY

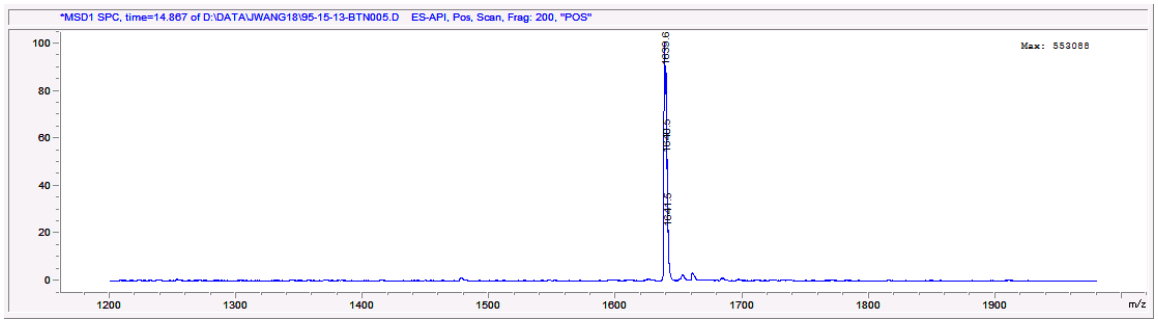


Biotinylated Rapadocin: HSQC (^{1}H-^{13}C DEPT135)

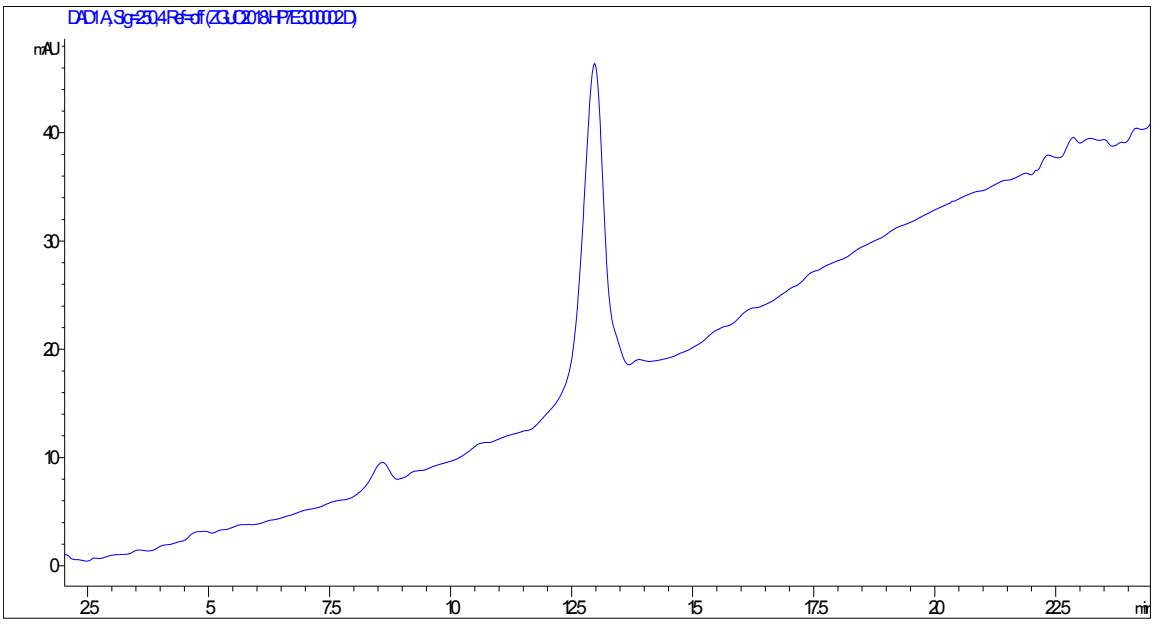


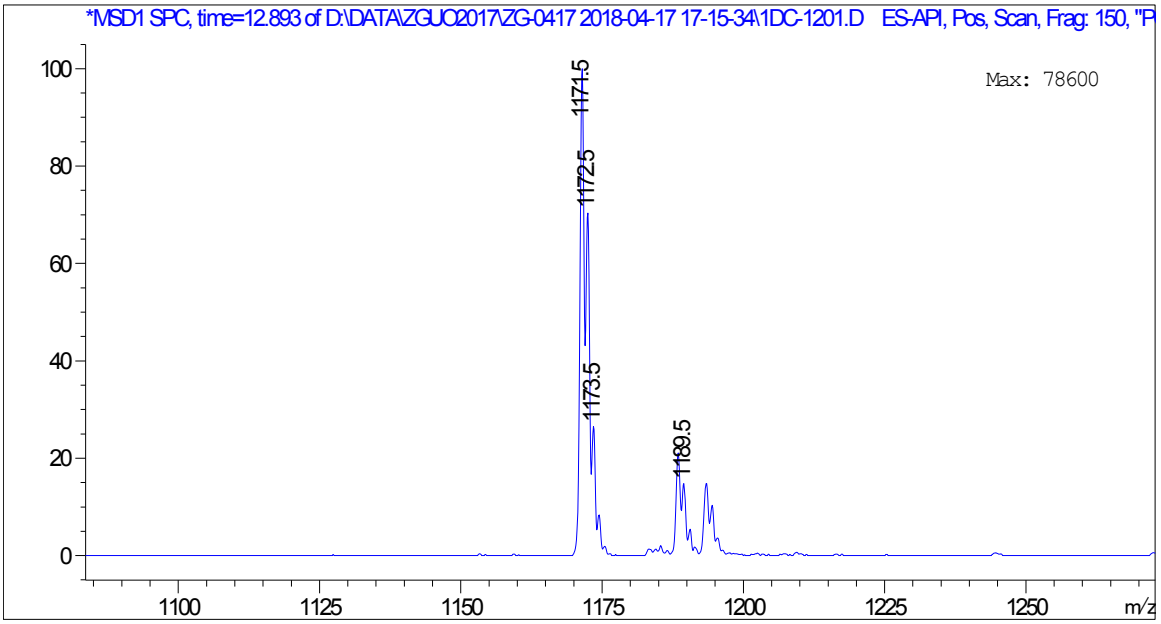
Biotinylated Rapadocin:LC-MS Profile





HP7E3 LC-MS Profile





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