Supporting Information for:

Stereoisomerism of Stapled Peptide Inhibitors of the p53-Mdm2 Interaction: an Assessment of Synthetic Strategies and Activity Profiles

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1. Peptide Synthesis

1.1 General information

H-Ramage ChemMatrix® resin (0.53 mmol g^{-1}) was obtained from PCAS-Biomatrix (Quebec, Canada) and H-Rink Amide ChemMatrix® resin (100-200 mesh, 0.59 mmol g^{-1}) was obtained from Novabiochem (San Diego, CA). L-amino acids were obtained from Advanced Chemtech (Louisville, KY). Fmoc-threonine, serine, glutamic acid and tyrosine were t-butyl protected. Fmoc-tryptophan was used as both Boc protected and unprotected. Unnatural alkenyl amino acids were purchased from OKeanos (Beijing, China). All other solvents and reagents were obtained from Sigma-Aldrich. 1,2-dichloroethane (DCE) was dried overnight over activated molecular sieves and purged with Argon for 30 min prior to use. All other reagents were used as received.

1.2 Stapled peptide preparation

Solid-Phase peptide synthesis: The peptides were synthesised manually using solid phase and Fmoc chemistry at the 0.1 mmol scale using either H-Ramage-Chemmatrix resin (0.53 mmol g^{-1}) or Rink Amide MBHA resin (0.59 mmol g^{-1}). The dry resin was weighed out into a fritted reaction vessel and swelled with NMP before use. The Fmoc protecting group was removed by treatment with 20% piperidine in DMF and nitrogen gas bubbling (15 min). The Fmoc-protected amino acids (5 equiv.) were coupled using pre-activated (7 min) solutions of HATU (4.9 equiv.) and DIPEA (5 equiv.) in NMP (0.23 M) or DIC (5 equiv.) and HOAt (5 equiv.) in NMP (0.4-0.45 M). The coupling time was 90 min for all amino acids except for (*S*)-*N*-Fmoc-2-(4'-pentenyl)alanine and (*R*)-*N*-Fmoc-2-(7'-octenyl)alanine, which were pre-activated and coupled (4 equiv.) for two hours. The amino acids immediately following the α,α-disubstituted amino acids were double coupled. Following deprotection of the final Fmoc group, the peptides were acetylated using a mixture of acetic anhydride:DIPEA:DMF (2:2:1) for 60 min. After each coupling, deprotection and acetylation reaction, the resin was thoroughly washed with NMP.

On-resin peptide metathesis and cleavage: Ring-closing metathesis of resin-bound, *N*-acetylated peptides was performed manually using a solution of Grubbs I catalyst (20 mol%, 5 mg mL^{-1}) in dry 1,2-dichloroethane (DCE) at room temperature. The reaction was agitated by bubbling with argon gas (3 x 2 h treatments, with the addition of fresh catalyst solution at the start of each cycle). The reaction mixture was drained, the resin washed with DMSO:DMF (1:1, 1 x 2h), DCE (3 x 1 min) and MeOH (3 x 1 min) then dried under vacuum. Cleavage of the peptide from the resin was achieved using a TFA cocktail consisting TFA:triisopropylsilane:water (95:2.5:2.5, 8 mL) for 2 h followed by filtration and precipitation with

diethyl ether. The precipitate was collected by centrifugation, dried under a stream of argon and re-dissolved in a mixture of acetonitrile and water (1:1).

Acetic acid-mediated carboxylate hydrolysis: To a solution of peptide in MeCN:H_2O (3:1, 10 mg mL^{-1}) was added acetic acid (0.2 mL) and the reaction mixture was stirred at room temperature overnight.

Peptide purification and analysis: The success of each synthesis was assessed first by HPLC and ESI-MS analysis of the crude reaction mixture. The peptides were purified by reverse-phase HPLC and the identities and purifies of the peptides were assessed by analytical HPLC (Figure S1-S10), mass spectrometry (Table S1) and NMR spectrometry (Figure S11-S15).

Reverse-phase HPLC was performed using an Agilent 1260 Infinity system fitted with a Phenomenex® analytical column (Jupiter C12, 4 µm, Proteo 90 Å, 150 x 4.6 mm). The eluents were 0.1% aqueous TFA and 0.1% TFA in acetonitrile. Mass spectra were acquired with a Waters 3100 Mass spectrometer. NMR spectra were recorded on a Bruker Ultrashield 600 MHz Plus or on Bruker 400 MHz with CryoProbe. Chemical shifts are reported as δ values in ppm relative to DMSO-d6.

Peptide	analogue	Calculated mass	Observed mass	RP-HPLC gradient elution	Purity (%)
sMTide-02	Early	1461.79	1461.19 [M – H]	40-80% ACN	97.6
sMTide-02	Late	1461.79	1461.16 [M – H]	40-80% ACN	90.7
ATSP-7041	Early	1743.92	1743.40 [M – H]	50-90% ACN	91.0
ATSP-7041	Late	1743.92	1743.38 [M – H]	50-90% ACN	92.1
VIP82	Early	2222.22	1112.19 [M/2 + H]	30-70% ACN	93.2
VIP82	Late	2222.22	1112.14 [M/2 + H]	30-70% ACN	92.0
VIP116	Early	2094.13	1103.12 [M + TFA/2 - H]	30-70% ACN	89.5
VIP116	Late	2094.13	1103.56 [M + TFA/2 - H]	30-70% ACN	94.8
VIP115	Early	2350.32	1176.22 [M/2 + H]	30-70% ACN	96.2
VIP115	Late	2350.32	1176.22 [M/2 + H]	30-70% ACN	93.0

Table S1 Analytical data for unsaturated sMTide-02, ATSP-7041, VIP82, VIP116 and VIP115.

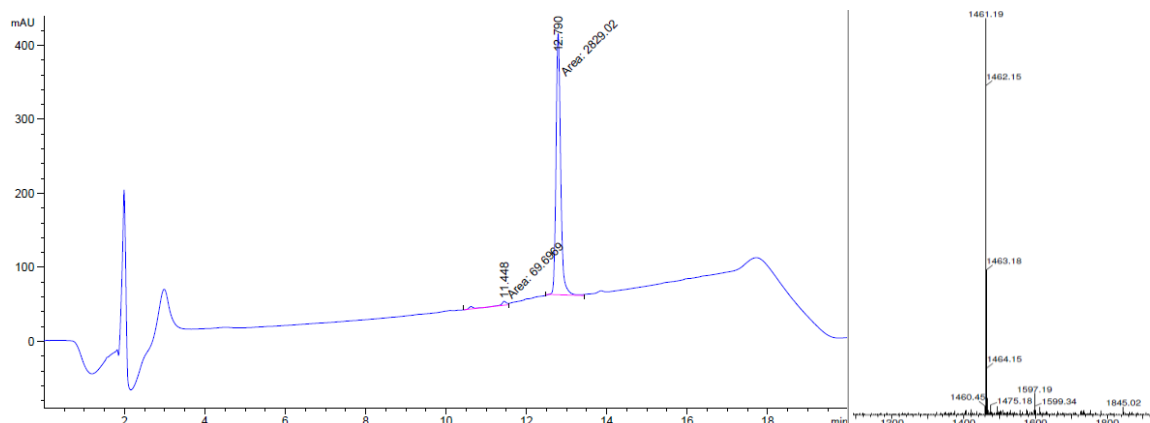**1.3 HPLC/LCMS spectra of unsaturated sMTide-02, ATSP-7041, VIP82, VIP116 and VIP115**

Fig. S1 HPLC/MS of purified sMTide-02, early isomer. RP-HPLC conditions: 40-80% ACN over 20 min.
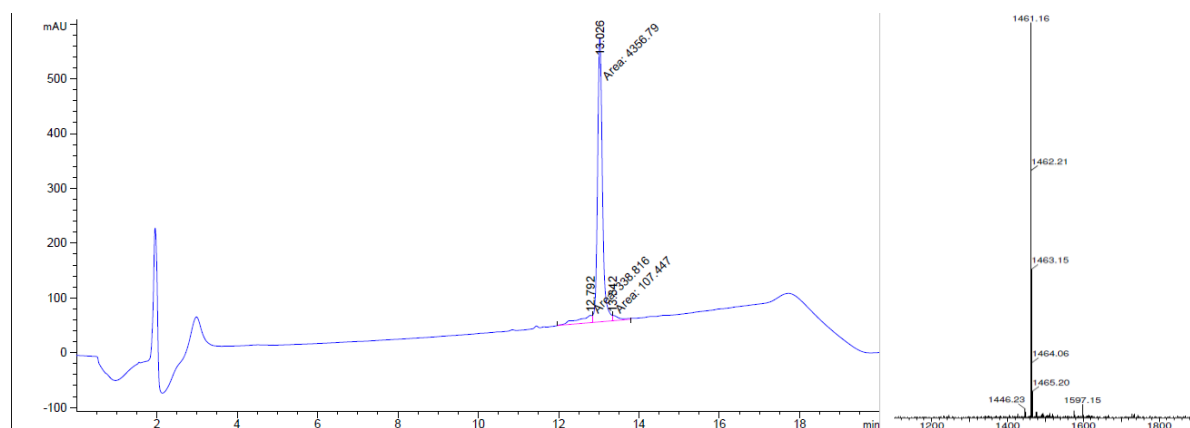

Fig. S2 HPLC/MS of purified sMTide-02, late isomer. RP-HPLC conditions: 40-80% ACN over 20 min.
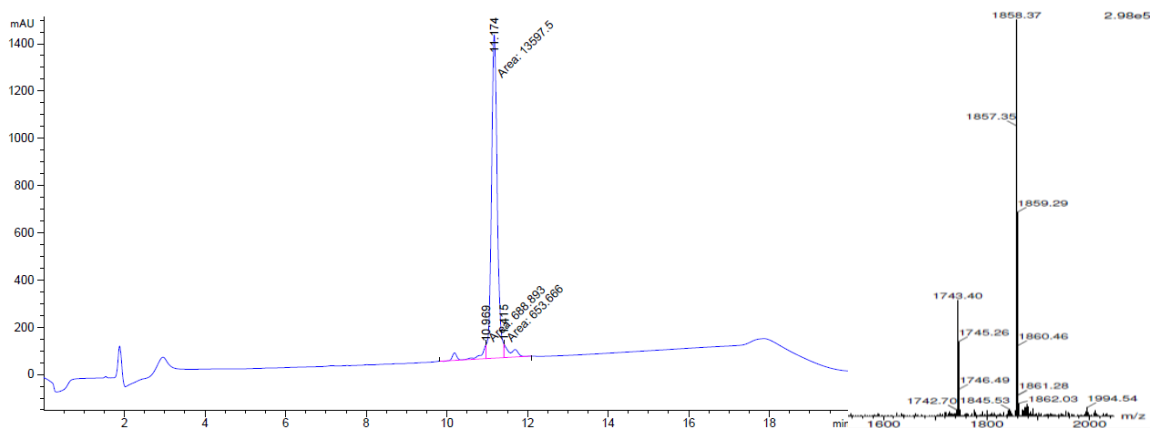

Fig. S3 HPLC/MS of purified ATSP-7041, early isomer. RP-HPLC conditions: 50-90% ACN over 20 min.
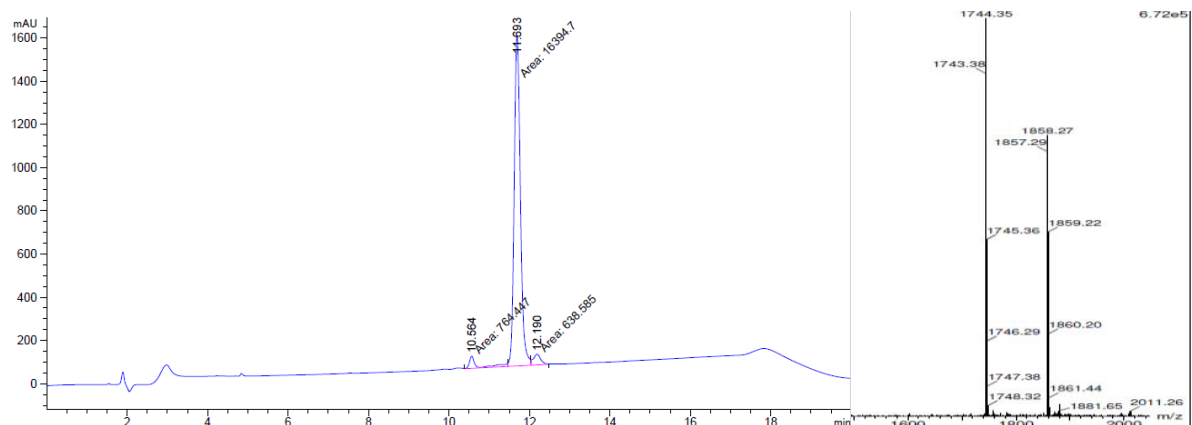
Fig. S4 HPLC/MS of purified ATSP-7041, late isomer. RP-HPLC conditions: 50-90% ACN over 20 min.
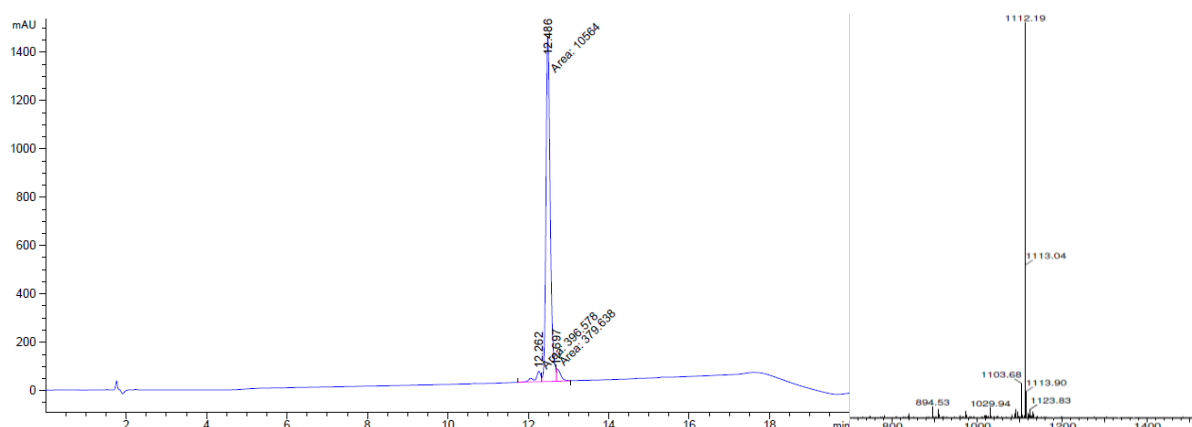

Fig. S5 HPLC/MS of purified VIP82, early isomer. RP-HPLC conditions: 30-70% ACN over 20 min.
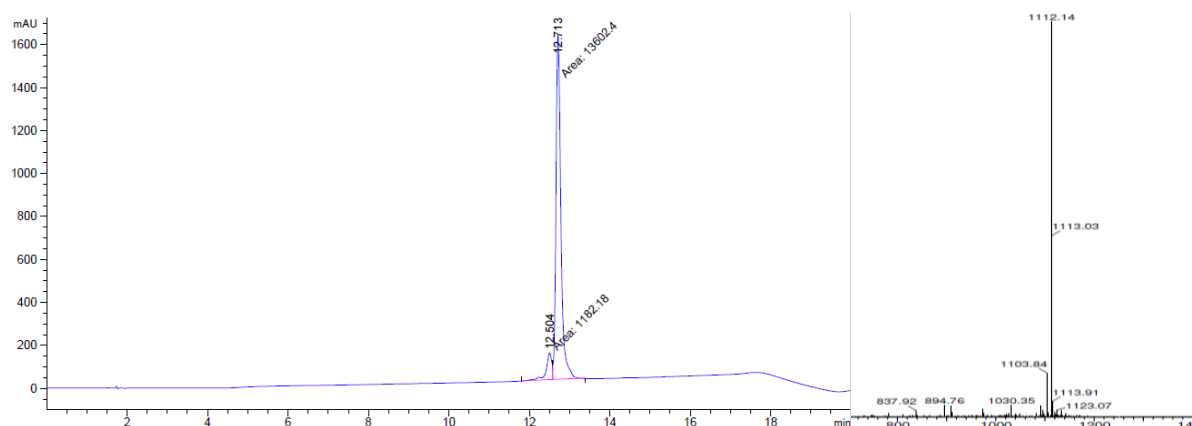

Fig. S6 HPLC/MS of purified VIP82, late isomer. RP-HPLC conditions: 30-70% ACN over 20 min.

Fig. S7 HPLC/MS of purified VIP116, early isomer. RP-HPLC conditions: 30-70% ACN over 20 min.

Fig. S8 HPLC/MS of purified VIP116, late isomer. RP-HPLC conditions: 30-70% ACN over 20 min.
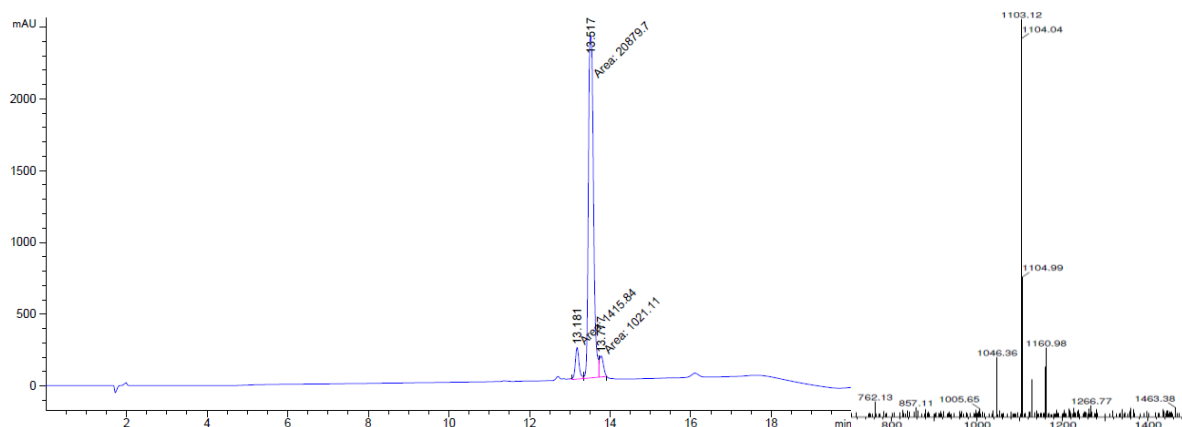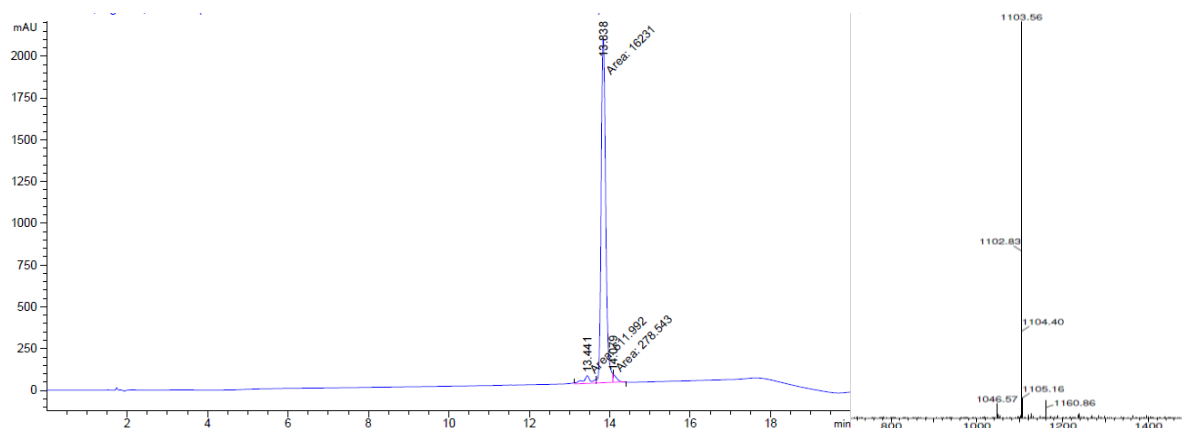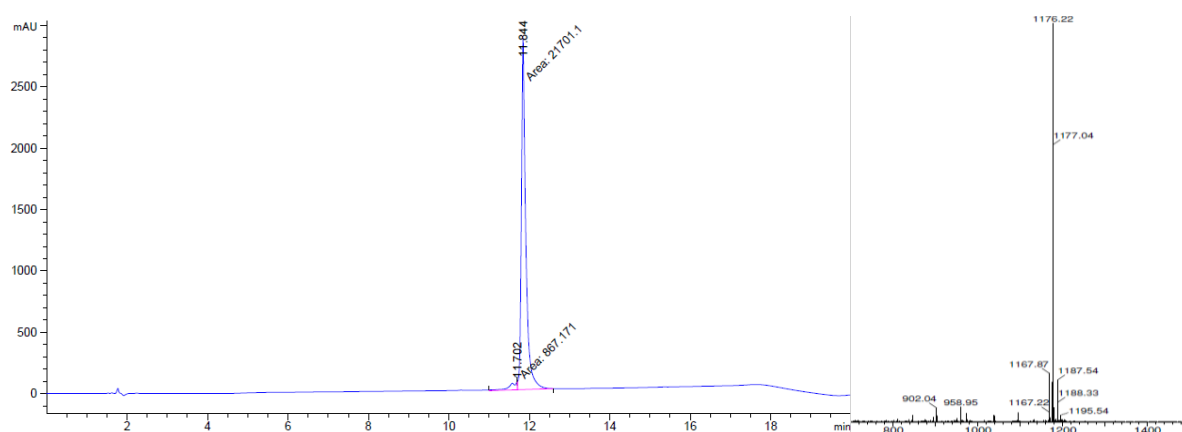

Fig. S9 HPLC/MS of purified VIP115, early isomer. RP-HPLC conditions: 30-70% ACN over 20 min.

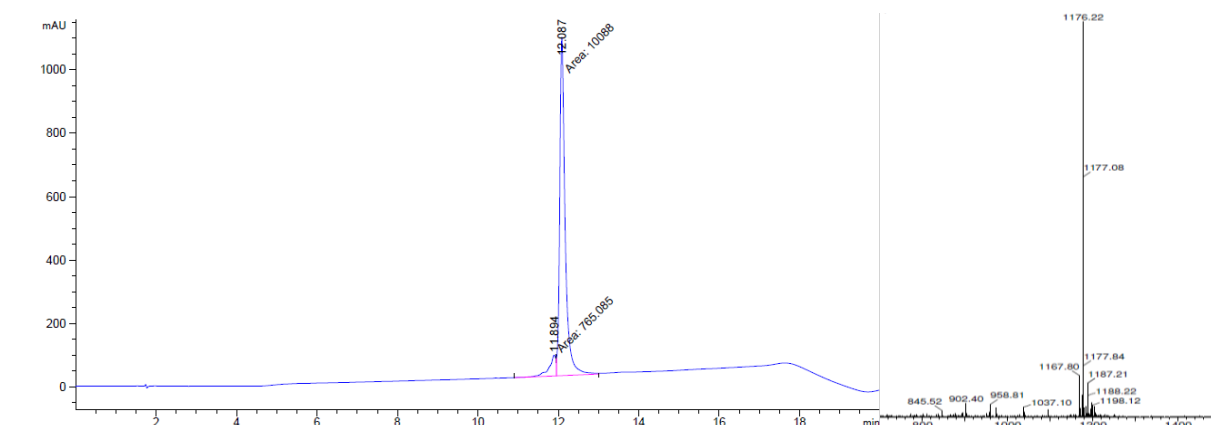Fig. S10 HPLC/MS of purified VIP115, late isomer. RP-HPLC conditions: 30-70% ACN over 20 min**.**

1.4 ^{1}H NMR spectra of unsaturated sMTide-02, ATSP-7041, VIP82, VIP116 and VIP115
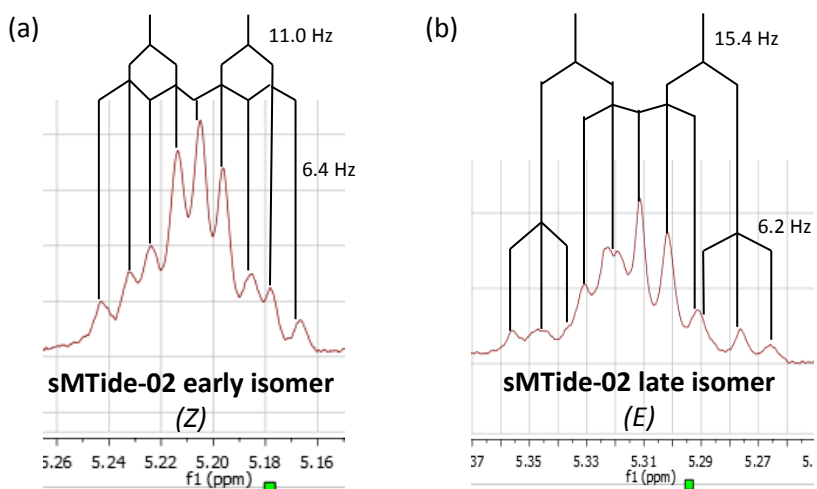

Fig. S11 ^{1}H NMR spectra of the olefinic protons in sMTide-02 early isomer (a) and sMTide-02 late isomer (b).
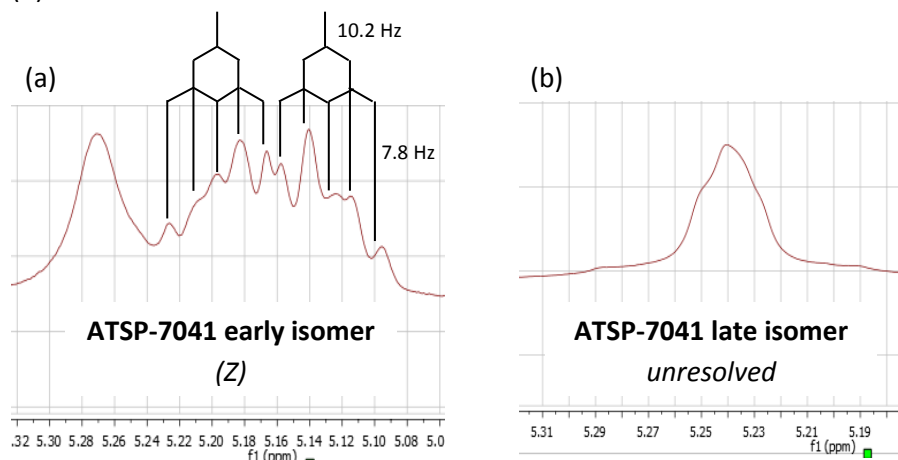
Fig. S12 ^{1}H NMR spectra of the olefinic protons in ATSP-7041 early isomer (a) and ATSP-7041 late isomer (b).

(a) (b)

VIP82 early isomer
unresolved

VIP82 late isomer
unresolved
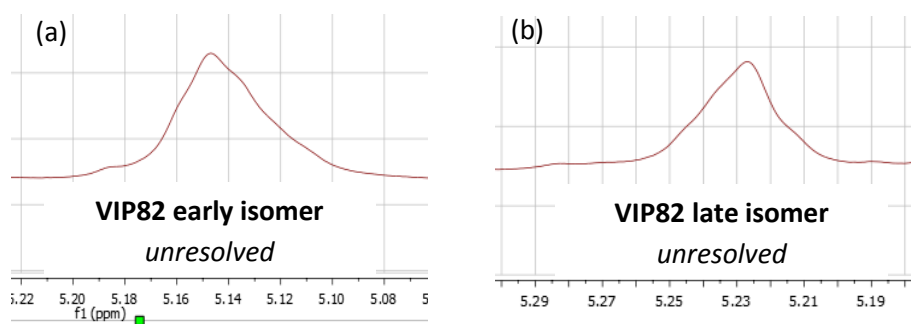
Fig. S13 ^{1}H NMR spectra of the olefinic protons in VIP82 early isomer (a) and VIP82 late isomer (b).

(a) (b)

VIP116 early isomer
unresolved

VIP116 late isomer
unresolved
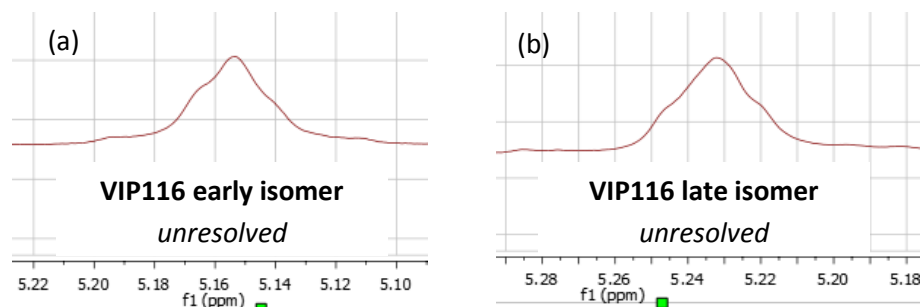
Fig. S14 ^{1}H NMR spectra of the olefinic protons in VIP116 early isomer (a) and VIP116 late isomer (b).

(a) (b)

VIP115 early isomer
unresolved

VIP116 early isomer
unresolved
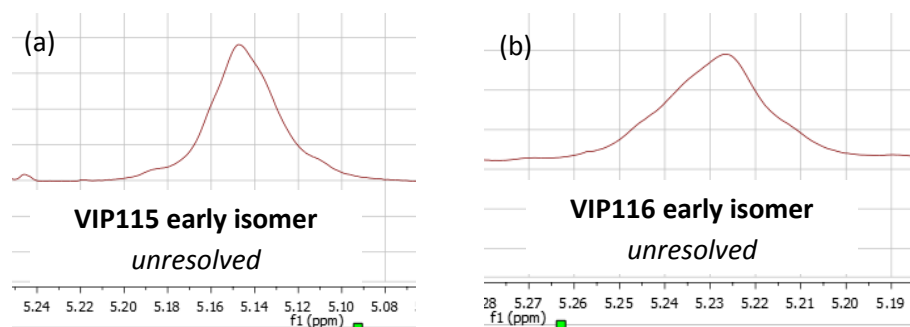
Fig. S15 ^{1}H NMR spectra of the olefinic protons in VIP115 early isomer (a) and VIP115 late isomer (b).

2. Stereoselective metathesis study

2.1 General protocol

Linear, *N*-acetylated sMTide-02 on resin (0.04-0.06 mmol) was weighed out into a fritted reaction vessel and swelled with the appropriate, degassed solvent before use. Ring-closing metathesis was performed by the addition of a solution of the metathesis catalyst (20 mol%, 5 mg mL^{-1}) in the stated solvent at the stated temperature. The reaction vessel was capped at both ends then agitated in a thermomixer (3 x 2 h treatments, with the addition of fresh catalyst solution at the start of each cycle). After each 2 h cycle, an aliquot of the resin suspension was removed, filtered then washed with DCE (2 x 1 min) and MeOH (3 x 1 min). The resin was dried under vacuum and agitated in a suspension of TFA:TIS:H$_2$O (95:2.5:2.5) for 2 h. After filtration and precipitation of the filtrate with diethyl ether, the precipitate was collected by centrifugation and dried under a stream of argon. The pellet was re-dissolved in a mixture of acetonitrile and water (1:1) and analysed by RP-HPLC (Phenomenex® Jupiter C12, 4 µm, Proteo 90 Å, 150 x 4.6 mm, 40-80% ACN over 20 min) for RCM conversion and selectivity.

2.2 Analytical HPLC spectra

Entry	Metathesis conditions	Conversion (%)			Selectivity (*Z*: *E* isomer)		
		1 RCM	2 RCM	3 RCM	1 RCM	2 RCM	3 RCM
1	Grubbs I, DCE, rt	75	92	96	57 : 43	55 : 45	59 : 41
2	Grubbs I, THF, rt	31	57	72	67 : 33	65 : 35	66 : 34
3	Grubbs I, PhMe, rt	45	85	92	67 : 33	64 : 36	65 : 35
4	Grubbs I, DCE, 50 °C	82	96	97	61 : 39	58 : 42	55 : 45
5	Grubbs II, DCE, rt	68	94	98	23 : 77	23 : 77	23 : 77
6	Grubbs II, DCE, 50 °C	98	>98	>98	24 : 76	21 : 79	22 : 78
7	Hoveyda-Grubbs I, DCE, rt	20	33	51	61 : 39	62 : 38	62 : 38
8	Hoveyda-Grubbs I, DCE, 50 °C	57	82	91	63 : 37	61 : 39	59 : 41
9	Hoveyda-Grubbs II, DCE, rt	61	77	85	23 : 77	24 : 76	23 : 77
10	Hoveyda-Grubbs II, DCE, 50 °C	87	>98	>98	27 : 73	25 : 75	25 : 75

Table S2 Catalyst, solvent and temperature effect on *E/Z*-selectivity of sMTide-02. Percent conversion = product/(product + starting material) as determined by reverse phase HPLC (Figures S16-S25).
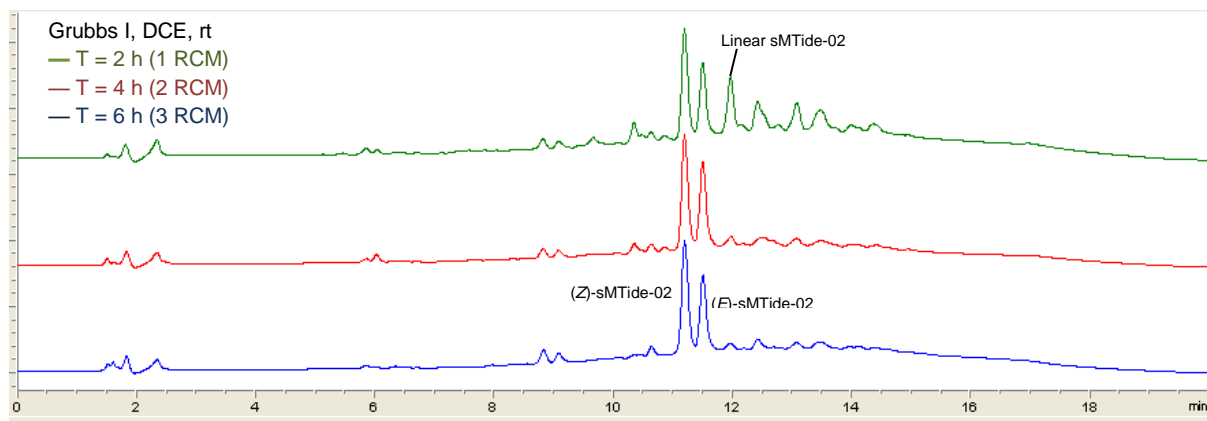
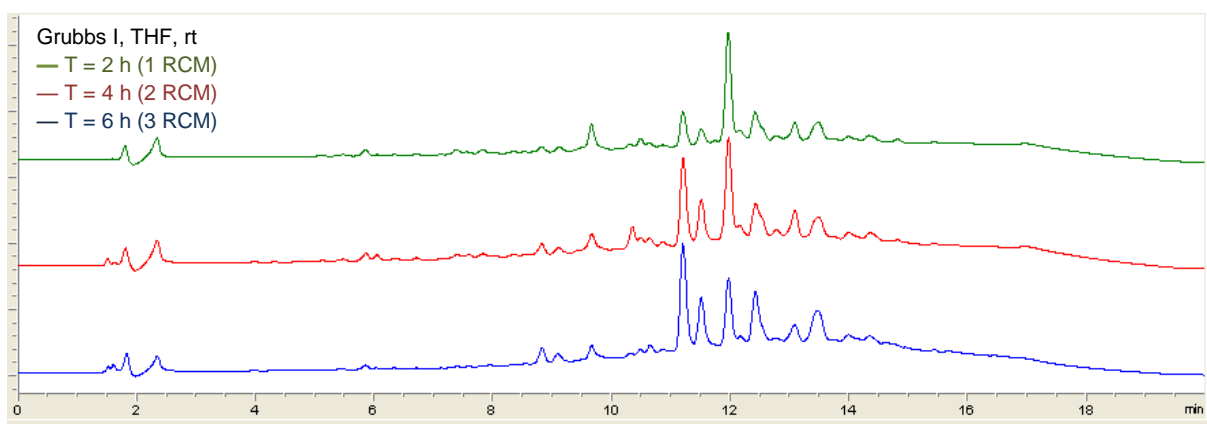Fig. S16 Accompanying analytical HPLC for entry 1, table S2.

Fig. S17 Accompanying analytical HPLC for entry 2, table S2.

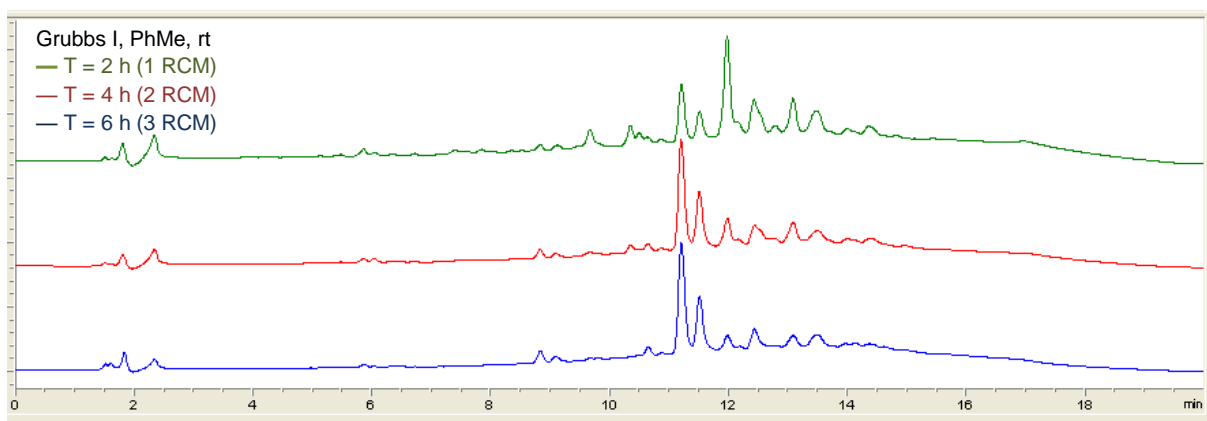Fig. S18 Accompanying analytical HPLC for entry 3, table S2.

Fig. S19 Accompanying analytical HPLC for entry 4, table S2.

Fig. S20 Accompanying analytical HPLC for entry 5, table S2.

Fig. S21 Accompanying analytical HPLC for entry 6, table S2.

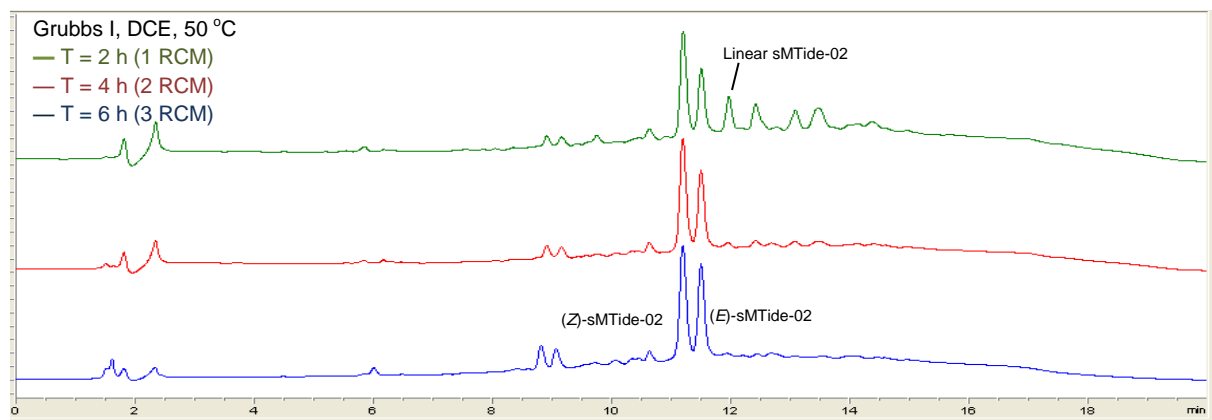Fig. S22 Accompanying analytical HPLC for entry 7, table S2.
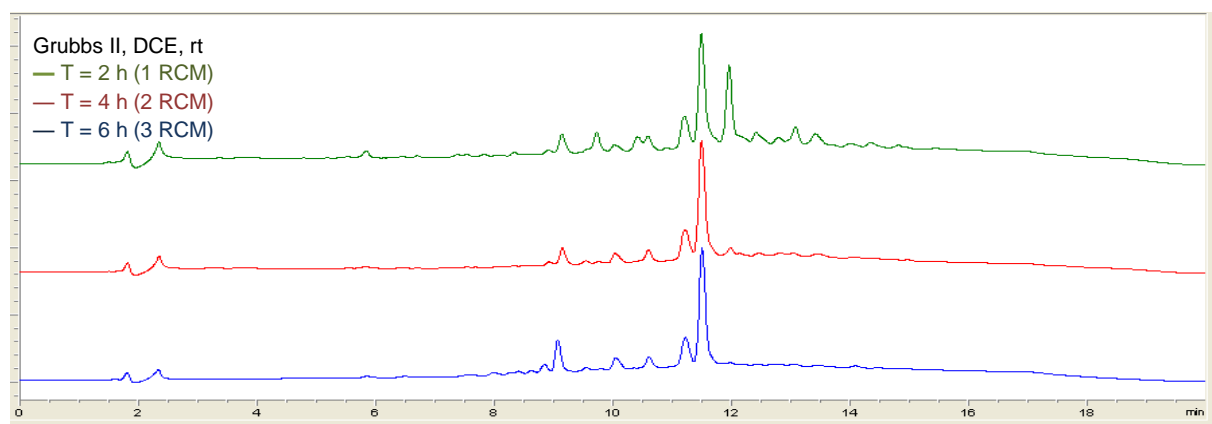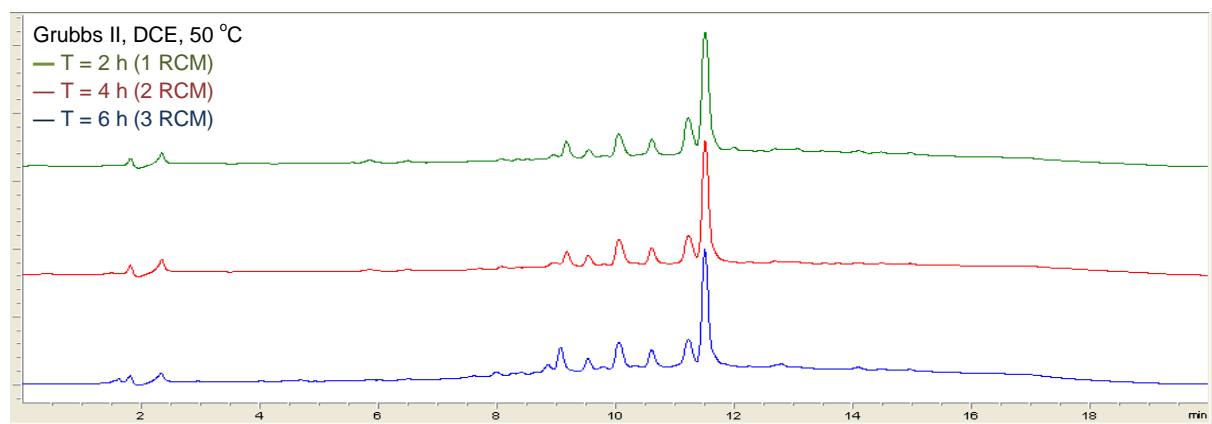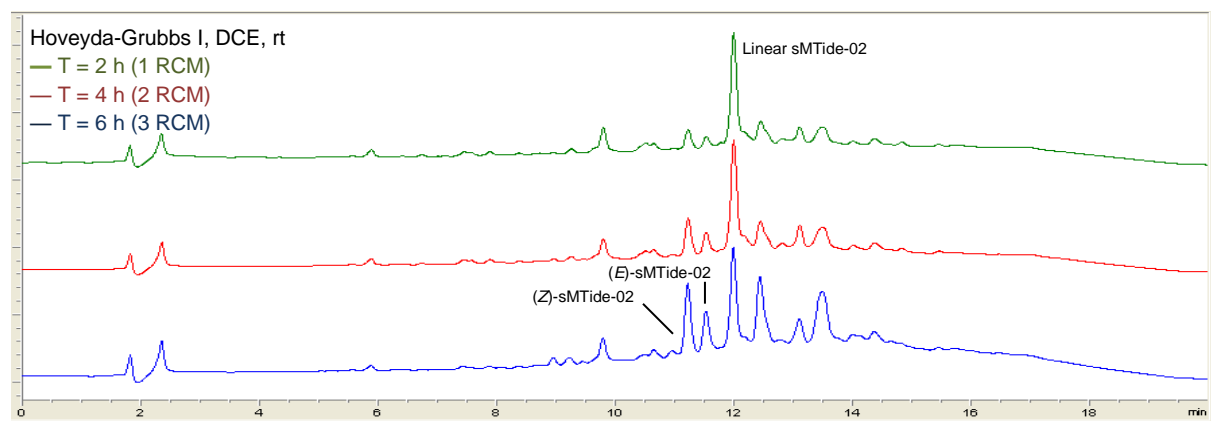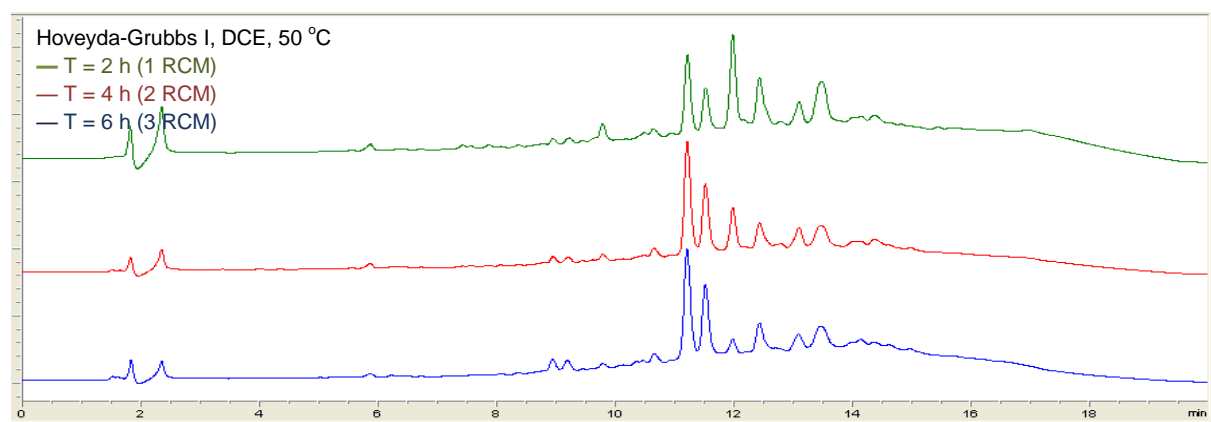

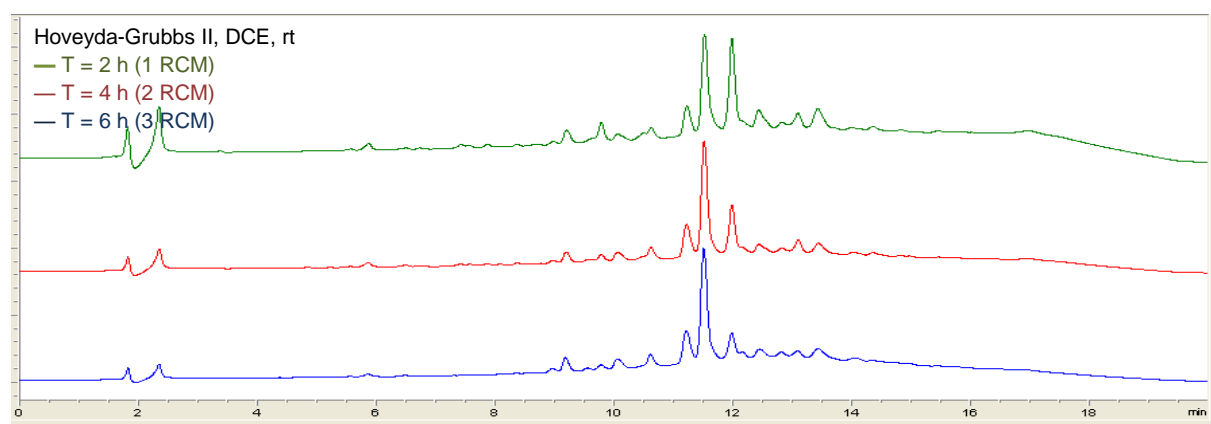Fig. S23 Accompanying analytical HPLC for entry 8, table S2.

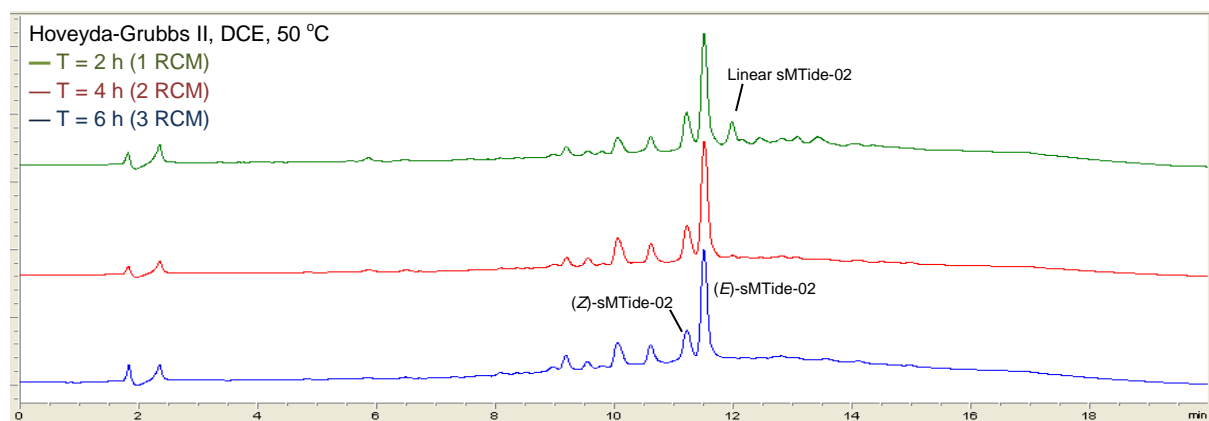Fig. S24 Accompanying analytical HPLC for entry 9, table S2.

Fig. S25 Accompanying analytical HPLC for entry 10, table S2.

2.3 Optimised stereoselective synthesis of the late stapled peptide isomer

Linear, *N*-acetylated peptide on resin (0.1 mmol) was weighed out into a fritted reaction vessel and swelled with degassed DCE before use. Ring-closing metathesis was performed by the addition of a solution of Grubbs II catalyst (20 mol%, 5 mg mL^{-1}) in DCE. The reaction vessel was capped at both ends and agitated in a thermomixer at 50 $^{\circ}$C (2 x 2 h treatments, with the addition of fresh catalyst solution at the start of each cycle). The reaction mixture was drained and the resin washed with DMSO:DMF (1:1, 1 x 2 h), DCE (3 x 1 min) and MeOH (3 x 1 min) then dried under vacuum. Cleavage of the peptide from the resin was achieved using a TFA cocktail consisting TFA:TIS:H$_2$O (95:2.5:2.5, 8 mL) for 2 h followed by filtration and precipitation with diethyl ether. The precipitate was collected by centrifugation and dried under a stream of argon.

3. One-pot metathesis/transfer hydrogenation study

3.1 General borohydride reduction protocol

Linear, *N*-acetylated sMTide-02 on resin (0.04-0.06 mmol) was weighed out into a fritted reaction vessel and swelled with degassed DCE before use. Ring-closing metathesis was performed as stated following the general protocol above. At the end of the final RCM cycle, a solution of the appropriate borohydride (2 equiv.) in MeOH (1/20 volume) was directly added to the metathesis mixture and the hydrogenation carried out at the stated temperature (2 x 2 h treatments and 1 x overnight treatment, with or without an additional aliquot of borohydride at the start of each subsequent cycle). After each hydrogenation cycle, an aliquot of the resin suspension was removed, filtered then washed with DCE (2 x 1 min) and MeOH (3 x 1 min). The resin was dried under vacuum and agitated in a suspension of TFA:TIS:H$_2$O

(95:2.5:2.5) for 2 h. After filtration and precipitation of the filtrate with diethyl ether, the precipitate was collected by centrifugation and dried under a stream of argon. The pellet was re-dissolved in a mixture of acetonitrile and water (1:1) and injected onto an analytical column (Phenomenex® Jupiter C12, 4 μm, Proteo 90 Å, 150 x 4.6 mm) to monitor RCM/hydrogenation conversion.

3.2 General silane reduction protocol:

Linear, *N*-acetylated peptide on resin (0.04-0.06 mmol) was weighed out into a fritted reaction vessel and swelled with degassed DCE before use. Ring-closing metathesis was performed as stated following the general protocol above. At the end of the final RCM cycle, the appropriate silane (5 equiv.) was directly added to the metathesis mixture and the hydrogenation carried out at the stated temperature (2 x 2 h treatments and 1 x overnight treatment, with an additional aliquot of organosilanes at the start of each subsequent cycle). After each hydrogenation cycle, an aliquot of the resin suspension was removed, filtered then washed with DCE (2 x 1 min) and MeOH (3 x 1 min). The resin was dried under vacuum and agitated in a suspension of TFA:TIS:H$_2$O (95:2.5:2.5) for 2 h. After filtration and precipitation of the filtrate with diethyl ether, the precipitate was collected by centrifugation and dried under a stream of argon. The pellet was re-dissolved in a mixture of acetonitrile and water (1:1) and injected onto an analytical column (Phenomenex® Jupiter C12, 4 μm, Proteo 90 Å, 150 x 4.6 mm) to monitor RCM/hydrogenation conversion.

3.3 Analytical HPLC spectra:

Entry	Macrocyclisation conditions	Transfer hydrogenation conditions	Conversion (%)		
			T = 2 h	T = 4 h	T = o/n
1	Grubbs II, DCE, 50 ^{o}C, 2 RCM	TPSH,[a,b] pip., NMP, 60 ^{o}C	17	29	39
2	Grubbs II, DCE, 50 ^{o}C, 2 RCM	NaBH$_4$, MeOH, rt	0	0	6
3	Grubbs II, DCE, 50 ^{o}C, 2 RCM	NaBH$_4$,[b] MeOH, rt	0	0	11
4	Grubbs II, DCE, 50 ^{o}C, 2 RCM	Bu$_4$NBH$_4$,[b]MeOH, rt	1	12	42
5	Grubbs II, DCE, 50 ^{o}C, 2 RCM	Bu$_4$NBH$_4$,[b]MeOH, 50 ^{o}C	16	40	45
6	Grubbs II, DCE, 50 ^{o}C, 2 RCM	Et$_3$SiH,[b] rt	0	0	23
7	Grubbs II, DCE, 50 ^{o}C, 2 RCM	PhSiH$_3$,[b] rt	1	1	1
8	Grubbs II, DCE, 50 ^{o}C, 2 RCM	Ph$_2$SiH$_2$,[b] rt	0	0	0
9	Grubbs II, DCE, 50 ^{o}C, 2 RCM	PMHS,[b] rt	3	6	11
10	Grubbs II, DCE, 50 ^{o}C, 2 RCM	Et$_3$SiH,[b] 50 ^{o}C	13	45	82
11	Grubbs I, DCE, 50 ^{o}C, 3 RCM	Et$_3$SiH,[b] 50 ^{o}C	44	59	84
12	Hoveyda-Grubbs I, DCE, 50 ^{o}C, 3 RCM	Et$_3$SiH,[b] 50 ^{o}C	16	31	75
13	Hoveyda-Grubbs II, DCE, 50 ^{o}C, 2 RCM	Et$_3$SiH,[b] 50 ^{o}C	19	57	78

Table S3 Tandem ring-closing metathesis/hydrogenation study. Percent conversion = product/(product + starting material) as determined by reverse phase HPLC (Figure S26-S38). [a]Solvent exchange required. [b]After each HPLC analysis, an additional aliquot of reagents was added to the reaction mixture.
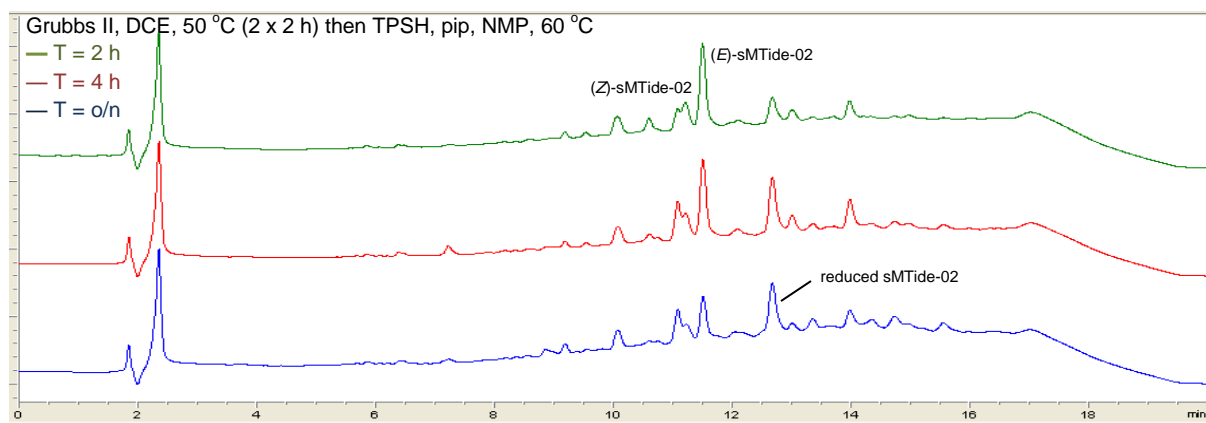

Fig. S26 Accompanying analytical HPLC for entry 1, table S3.
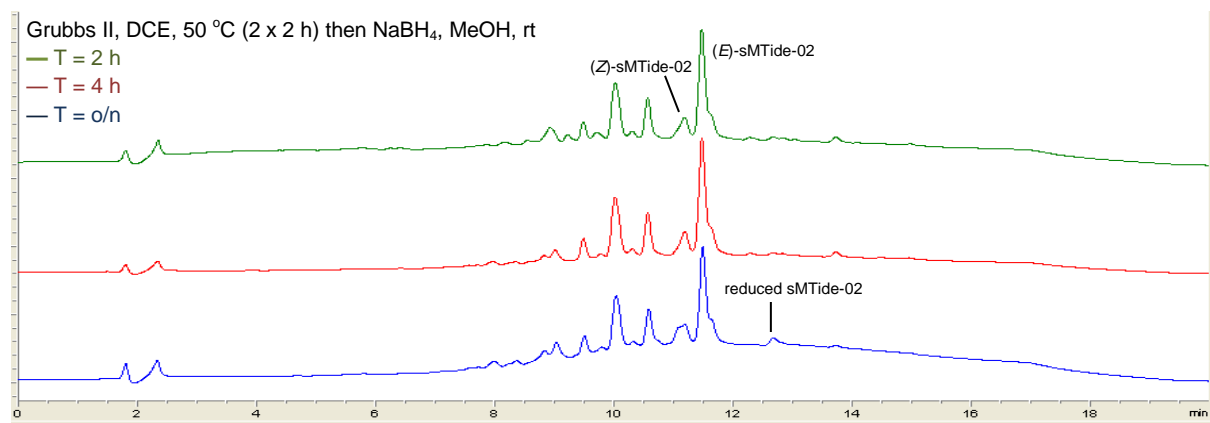
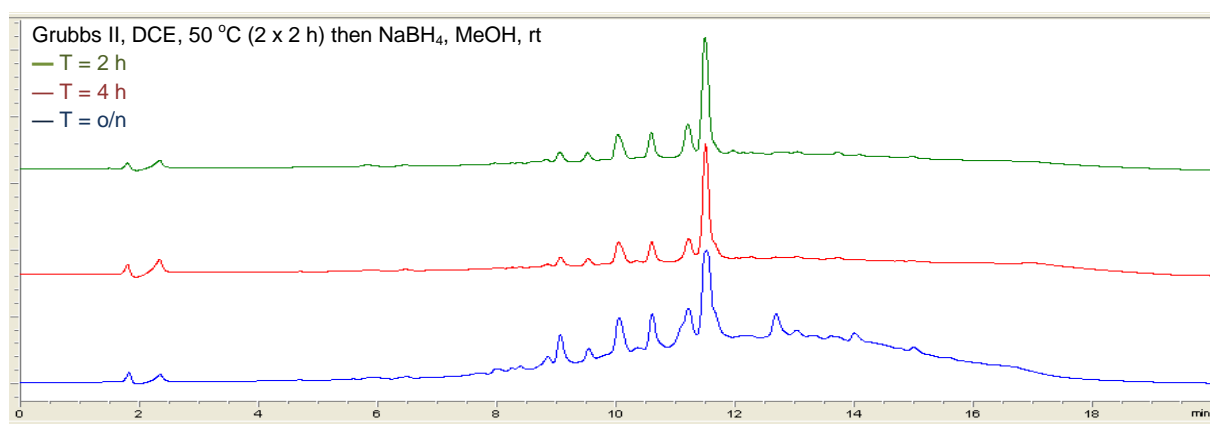Fig. S27 Accompanying analytical HPLC for entry 2, table S3.

Fig. S28 Accompanying analytical HPLC for entry 3, table S3.
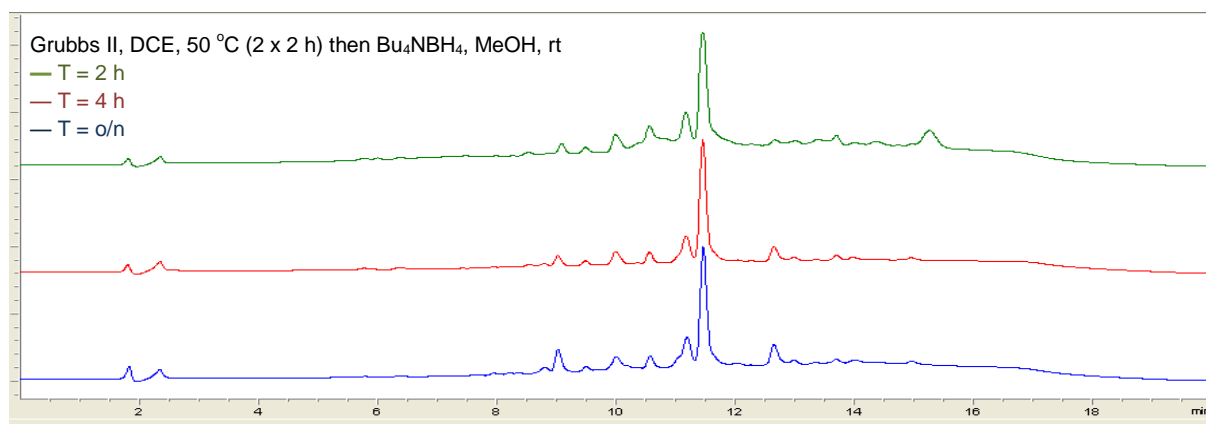

Fig. S29 Accompanying analytical HPLC for entry 4, table S3.
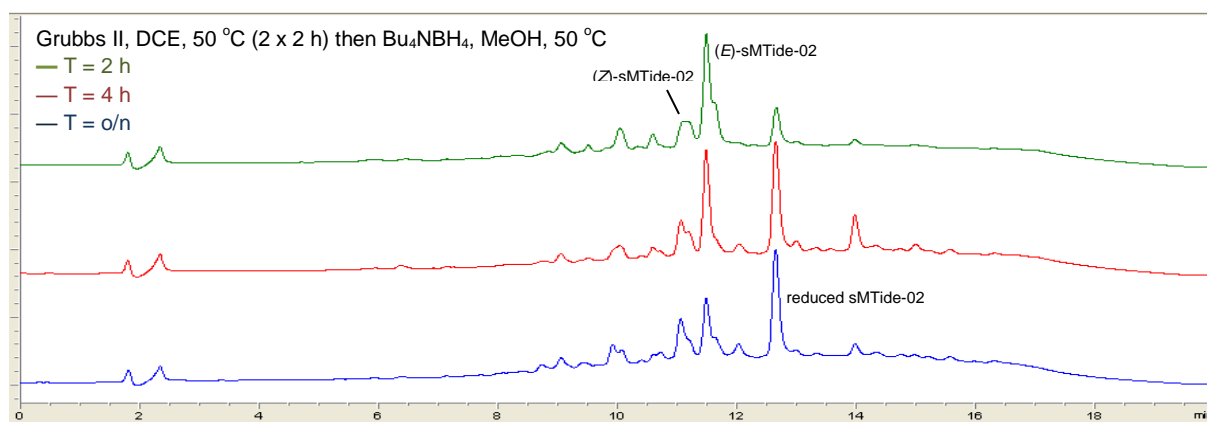
Fig. S30 Accompanying analytical HPLC for entry 5, table S3.

Fig. S31 Accompanying analytical HPLC for entry 6, table S3.

Fig. S32 Accompanying analytical HPLC for entry 7, table S3.

Grubbs II, DCE, 50 °C (2 x 2 h) then Ph$_2$SiH$_2$, rt
— T = 2 h
— T = 4 h
— T = o/n

(E)-sMTide-02

(Z)-sMTide-02

Fig. S33 Accompanying analytical HPLC for entry 8, table S3.

Grubbs II, DCE, 50 °C (2 x 2 h) then PMHS, rt
— T = 2 h
— T = 4 h
— T = o/n

(E)-sMTide-02

(Z)-sMTide-02

reduced sMTide-02

Fig. S34 Accompanying analytical HPLC for entry 9, table S3.

Grubbs II, DCE, 50 °C (2 x 2 h) then Et$_3$SiH, 50 °C
— T = 2 h
— T = 4 h
— T = o/n

(E)-sMTide-02
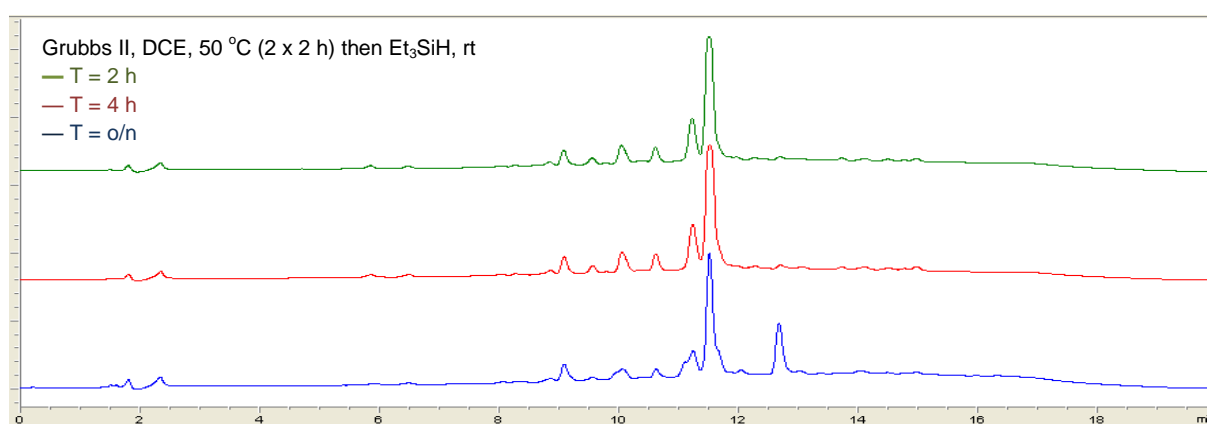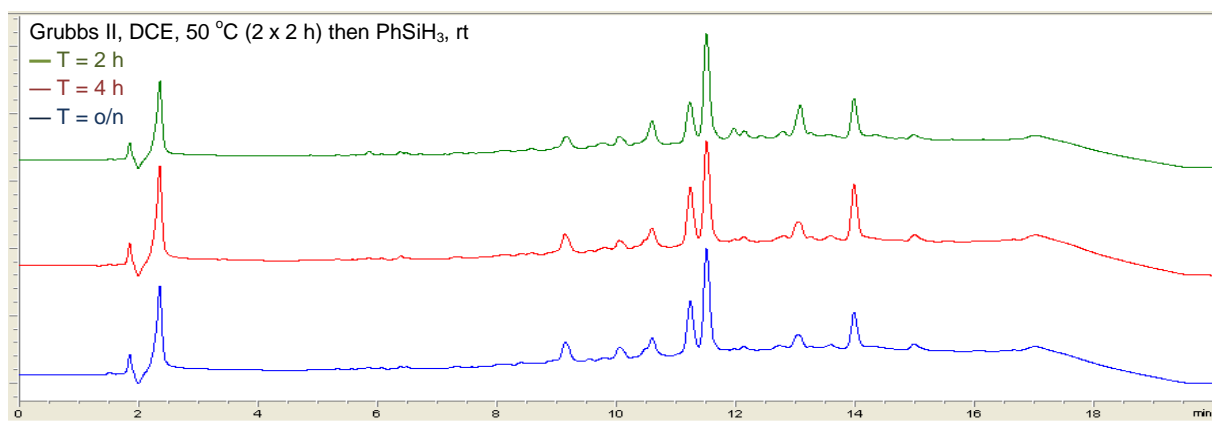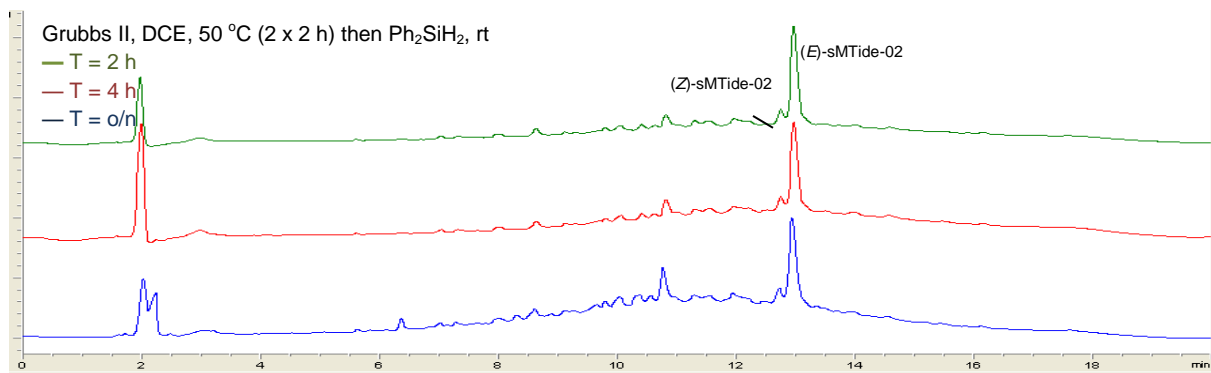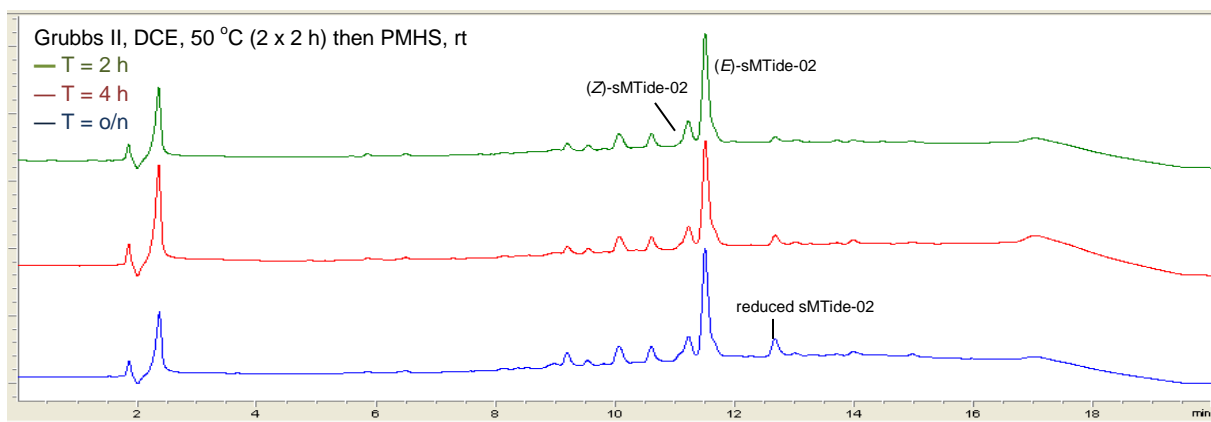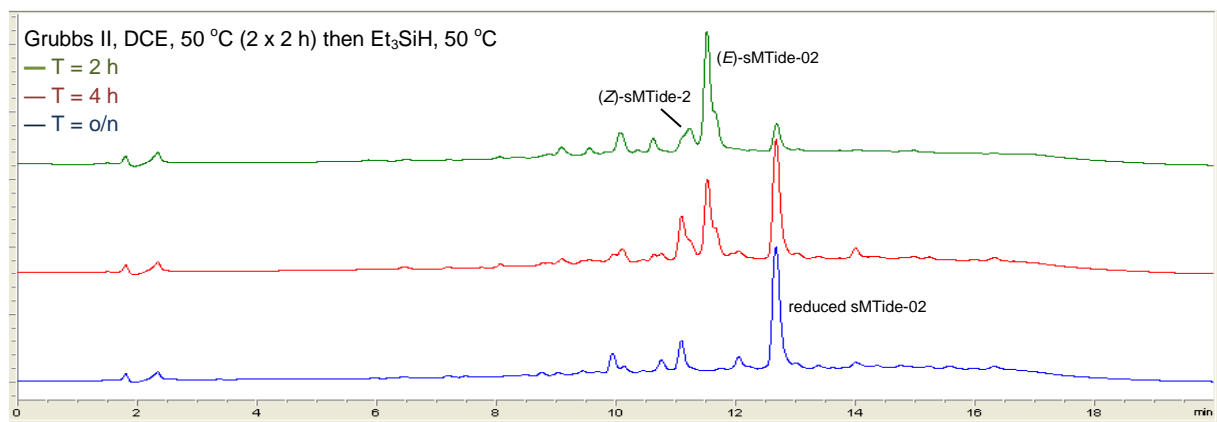
(Z)-sMTide-2

reduced sMTide-02

Fig. S35 Accompanying analytical HPLC for entry 10, table S3.
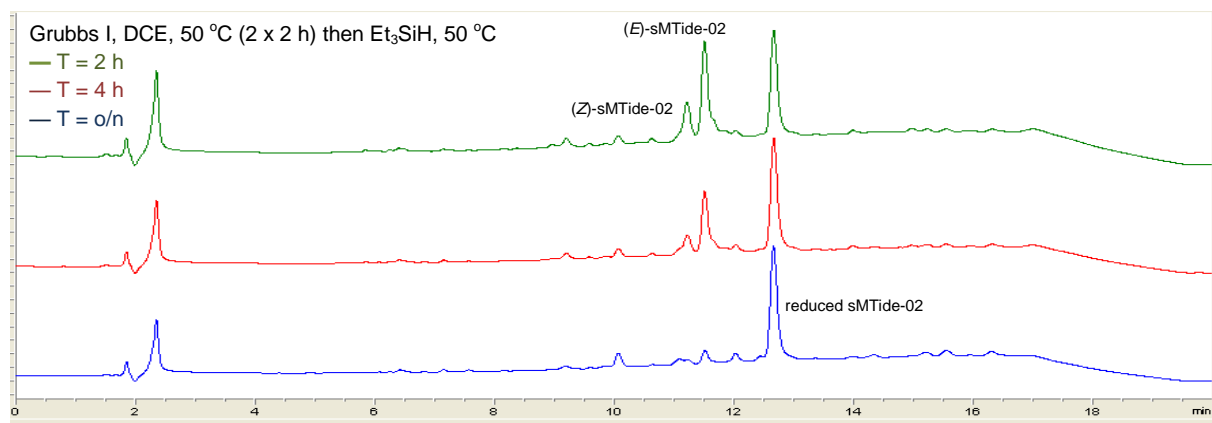
Fig. S36 Accompanying analytical HPLC for entry 11, table S3.

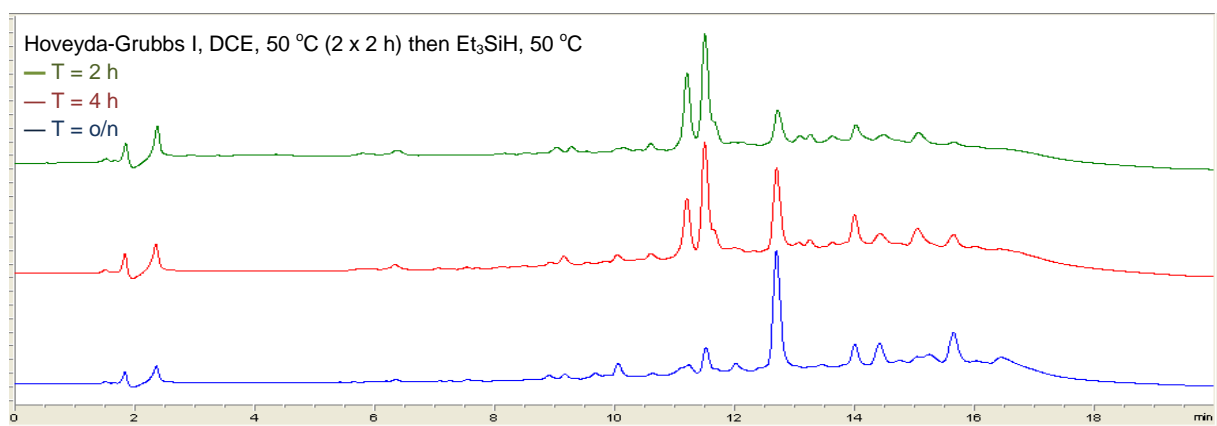
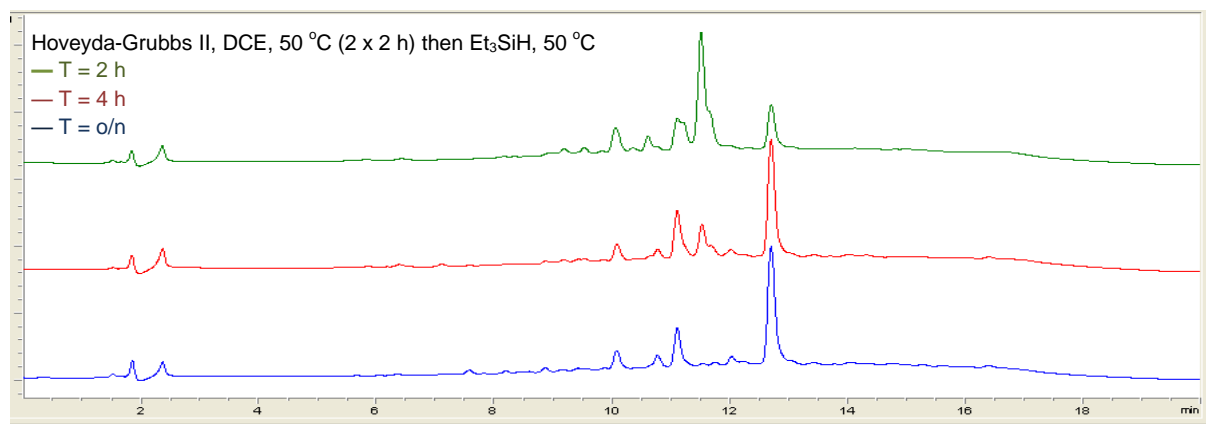Fig. S37 Accompanying analytical HPLC for entry 12, table S3.

Fig. S38 Accompanying analytical HPLC for entry 13, table S3.

3.4 Optimised metathesis/hydrogenation protocol

Linear, *N*-acetylated peptide on resin (0.1 mmol) was weighed out into a fritted reaction vessel and swelled with degassed DCE before use. Ring-closing metathesis was performed by the addition of a solution of Grubbs II catalyst (20 mol%, 5 mg mL^{-1}) in DCE. The fritted reaction vessel was capped at both ends and agitated in a thermomixer at 50 ^{o}C (2 x 2 h treatments, with the addition of fresh catalyst solution at the start of each cycle). At the end of the second RCM cycle, triethylsilane (5 equiv.) was directly added to the metathesis mixture and the hydrogenation carried out at 50 ^{o}C (2 x 2 h treatments and 1 x overnight treatment, with an additional aliquot of Et$_3$SiH at the start of each subsequent cycle). The solution was drained and the resin washed with DCE (2 x 1 min) and MeOH (3 x 1 min). Cleavage of the peptide from the resin was achieved using a TFA cocktail consisting TFA:TIS:H$_2$O (95:2.5:2.5, 8 mL) for 2 h followed by filtration and precipitation with diethyl ether. The precipitate was collected by centrifugation and dried under a stream of argon.

Peptide	analogue	Calculated mass	Observed mass	RP-HPLC gradient elution	Purity (%)
sMTide-02	reduced	1463.81	1463.17 [M − H]	40-80% ACN	89.5
ATSP-7041	reduced	1745.94	1745.36 [M − H]	50-90% ACN	91.7
VIP82	reduced	2223.23	1113.29 [M/2 + H]	30-70% ACN	94.8
VIP116	reduced	2095.14	1104.44 [M + TFA/2 - H]	30-70% ACN	91.0
VIP115	reduced	2351.33	1232.55 [M + TFA/2 − H]	30-70% ACN	95.6

Table S4 Analytical data for reduced sMTide-02, ATSP-7041, VIP82, VIP116 and VIP115.

3.5 HPLC/LCMS spectra of reduced sMTide-02, ATSP-7041, VIP82, VIP116 and VIP115

Fig. S39 HPLC/MS of purified, reduced-sMTide-02. RP-HPLC conditions: 40-80% ACN over 20 min.

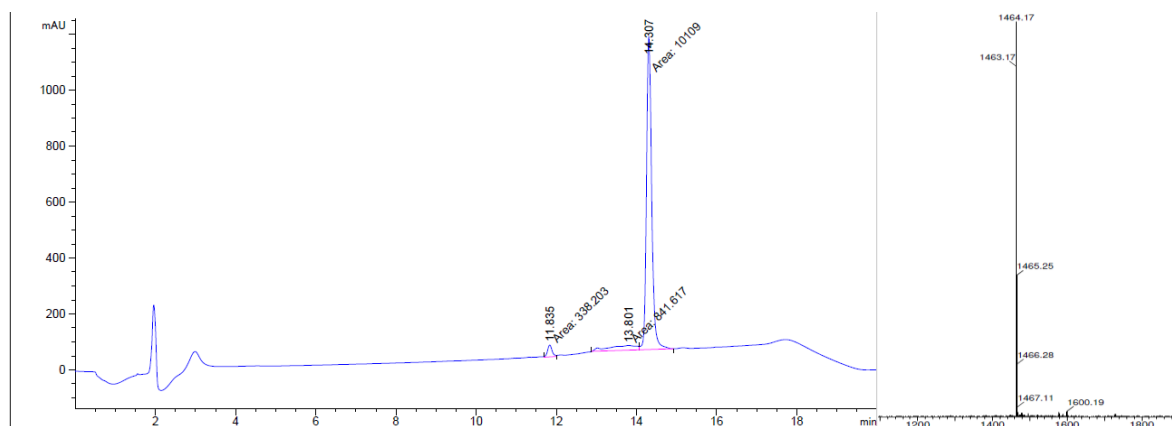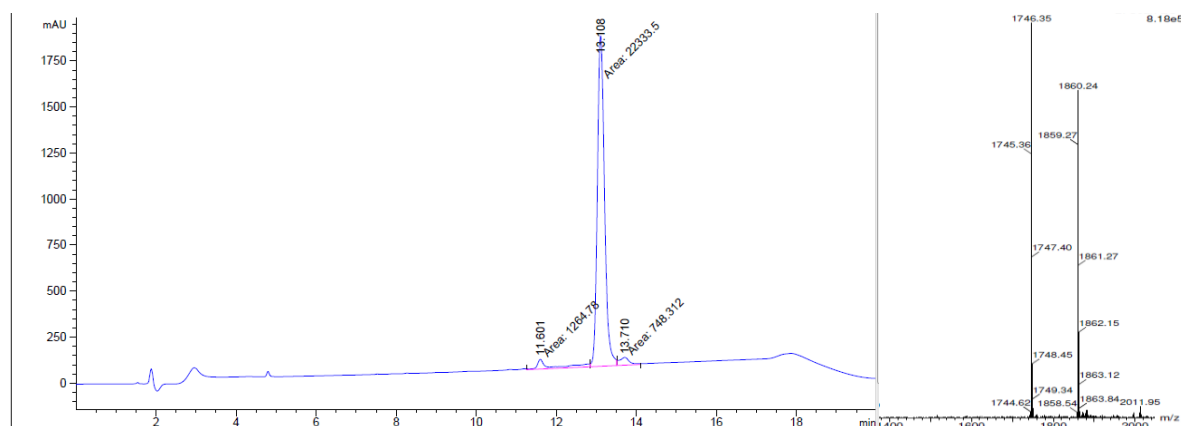Fig. S40 HPLC/MS of purified, reduced-ATSP-7041. RP-HPLC conditions: 50-90% ACN over 20 min.
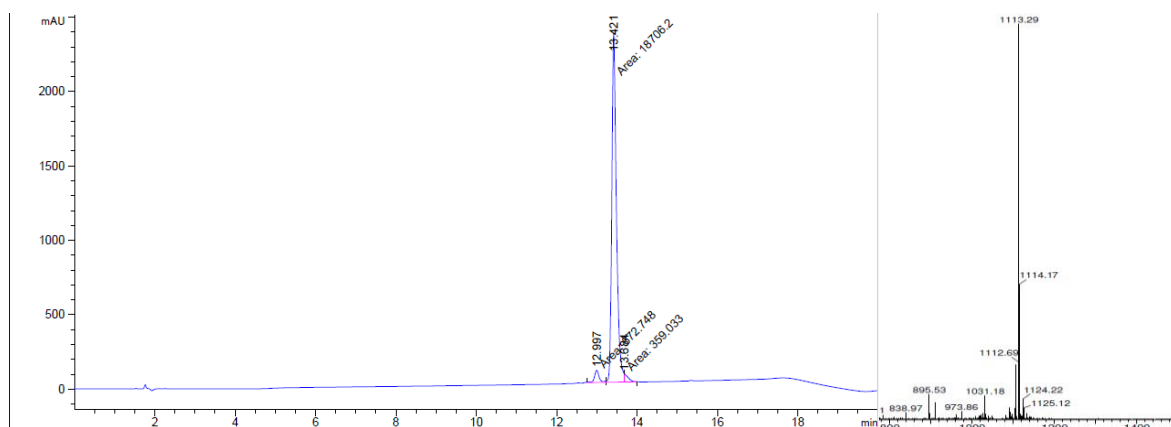

Fig. S41 HPLC/MS of purified, reduced-VIP82. RP-HPLC conditions: 30-70% ACN over 20 min.
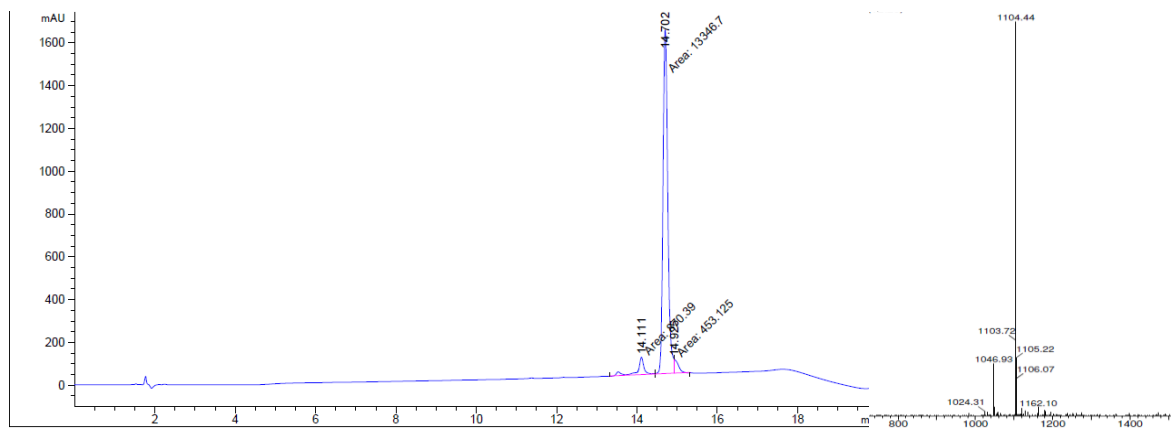
Fig. S42 HPLC/MS of purified, reduced-VIP116. RP-HPLC conditions: 30-70% ACN over 20 min.
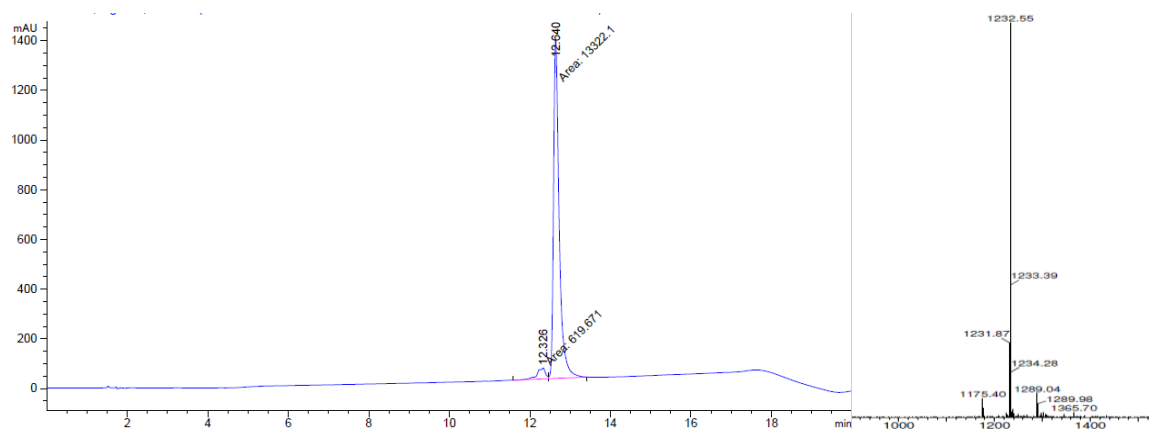

Fig. 43 HPLC/MS of purified, reduced-VIP115. RP-HPLC conditions: 30-70% ACN over 20 min.

4. Adjusted retention time (t_R') calculations

All stapled peptides were re-evaluated using a fixed 35 min linear gradient (0.1 % aqueous TFA and 0.1% TFA in acetonitrile) from 5-95%, at a flow rate of 1 mL/min. The Agilent 1260 Infinity system was fitted with a Phenomenex® analytical column (Jupiter C12, 4 µm, Proteo 90 Å, 150 x 4.6 mm).

Peptide	analogue	t_0 (min)	t_R (min)	t_R' (min)
sMTide-02	Early	2.153	24.276	22.12
sMTide-02	Late	2.152	24.526	22.37
ATSP-7041	Early	2.146	26.109	23.96
ATSP-7041	Late	2.145	26.584	24.44
VIP82	Early	2.167	20.805	18.64
VIP82	Late	2.166	21.046	18.88
VIP116	Early	2.154	21.840	19.69
VIP116	Late	2.155	22.118	19.96
VIP115	Early	2.165	20.252	18.09
VIP115	Late	2.169	20.414	18.25
sMTide-02	Reduced	2.150	25.810	23.66
ATSP-7041	Reduced	2.148	28.085	25.94
VIP82	Reduced	2.167	21.846	19.68
VIP116	Reduced	2.154	23.031	20.88
VIP115	Reduced	2.167	21.193	19.03

Table S5 Adjusted retention times ($t_R' = t_R - t_0$) for sMTide-02, ATSP-7041, VIP82, VIP116 and VIP115 analogues. t_R = the retention time of the product peak, t_0 = the retention time of the unretained peak.

Fig. S44 HPLC of sMTide-02, early isomer. RP-HPLC conditions: 5-95% ACN over 35 min.
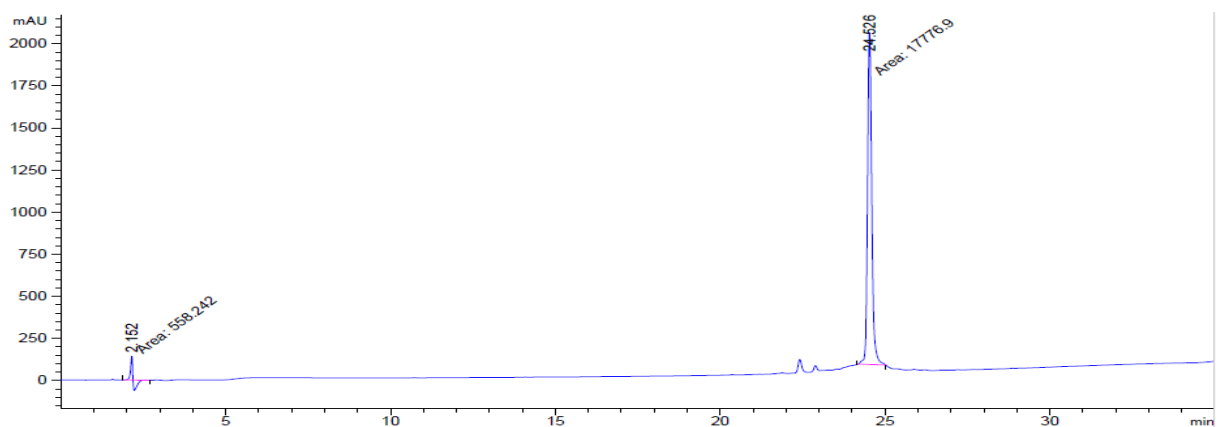
Fig. S45 HPLC of sMTide-02, late isomer. RP-HPLC conditions: 5-95% ACN over 35 min.
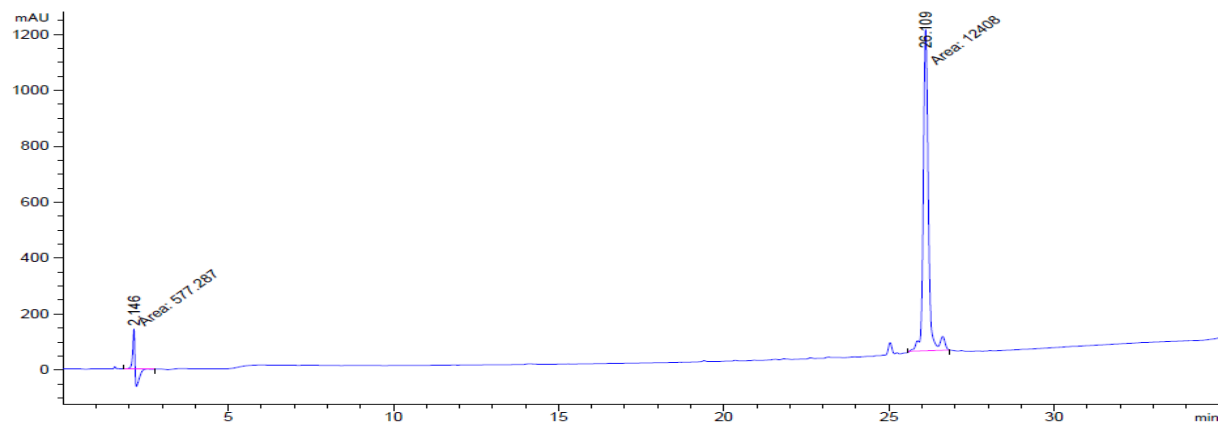
Fig. S46 HPLC of ATSP-7041, early isomer. RP-HPLC conditions: 5-95% ACN over 35 min.

Fig. S47 HPLC of ATSP-7041, late isomer. RP-HPLC conditions: 5-95% ACN over 35 min.
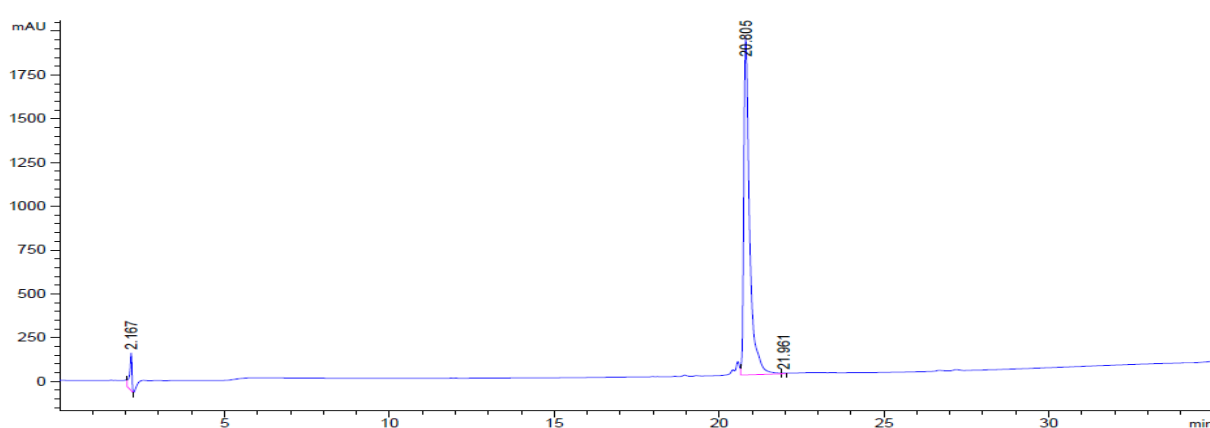
Fig. S48 HPLC of VIP82, early isomer. RP-HPLC conditions: 5-95% ACN over 35 min.
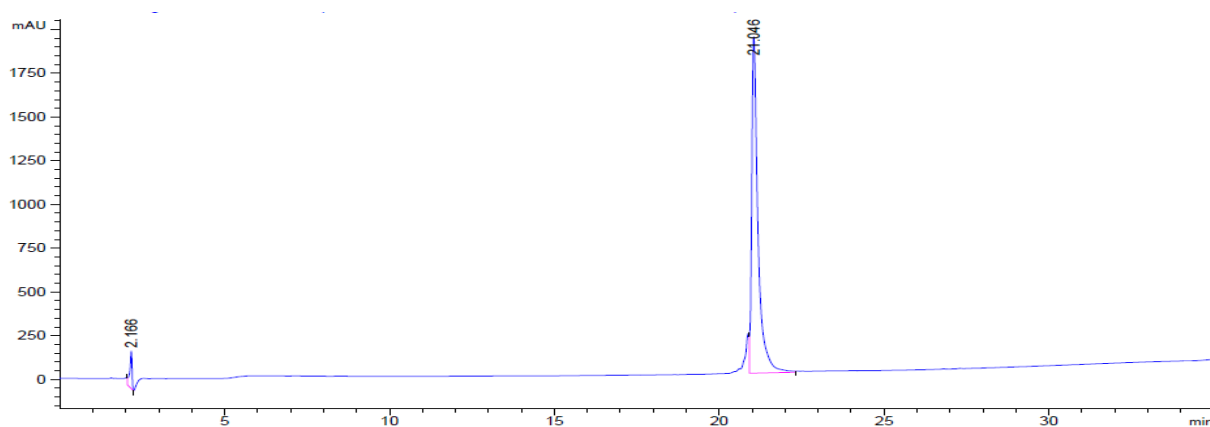
Fig. S49 HPLC of VIP82, late isomer. RP-HPLC conditions: 5-95% ACN over 35 min.
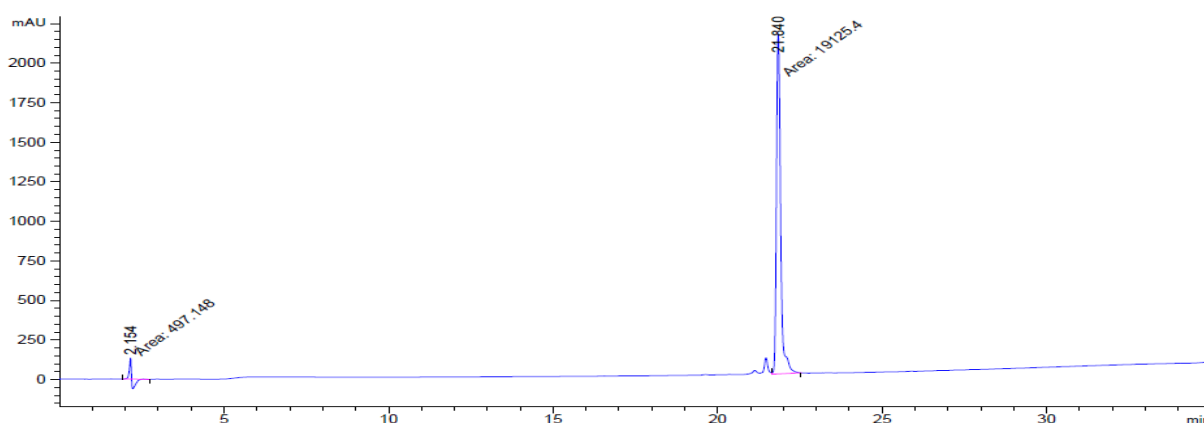
Fig. S50 HPLC of VIP116, early isomer. RP-HPLC conditions: 5-95% ACN over 35 min.
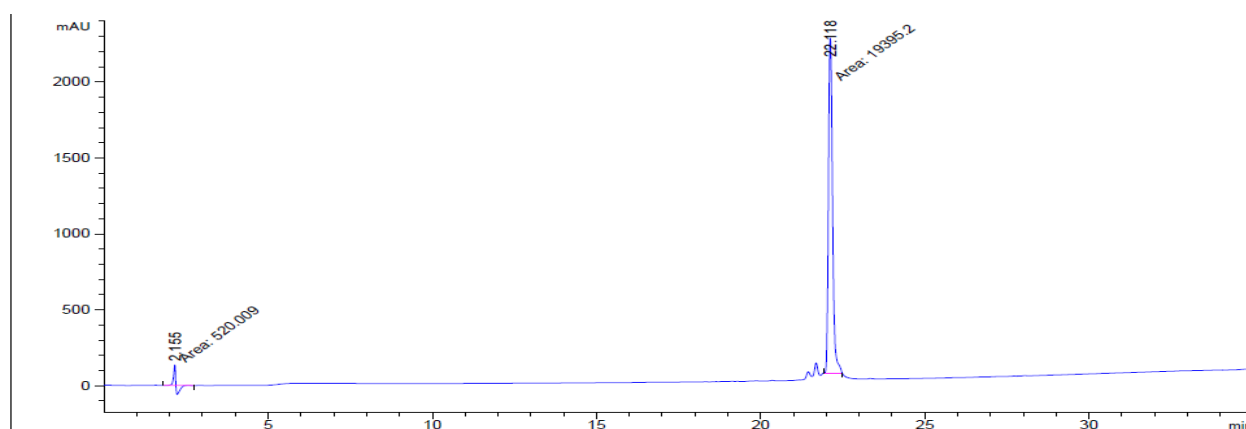
Fig. S51 HPLC of VIP116, late isomer. RP-HPLC conditions: 5-95% ACN over 35 min.
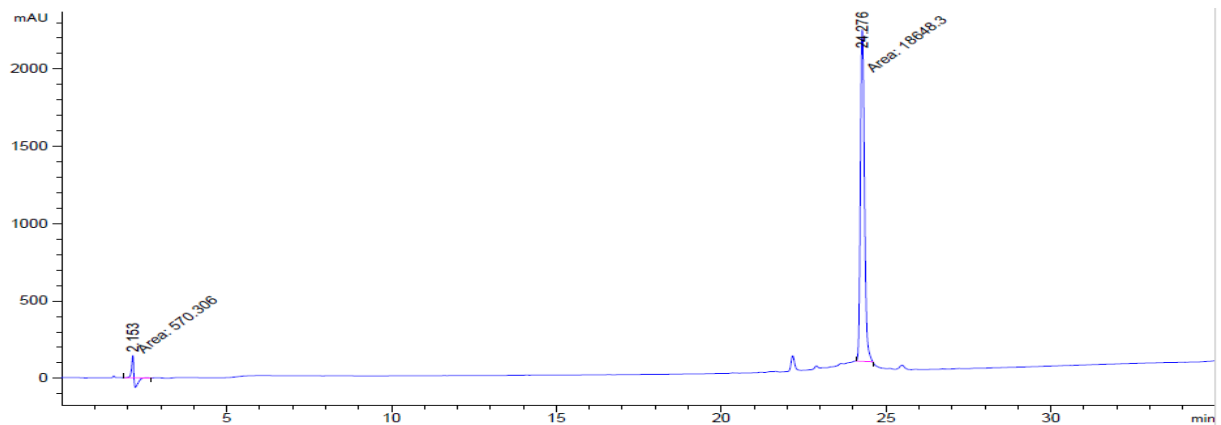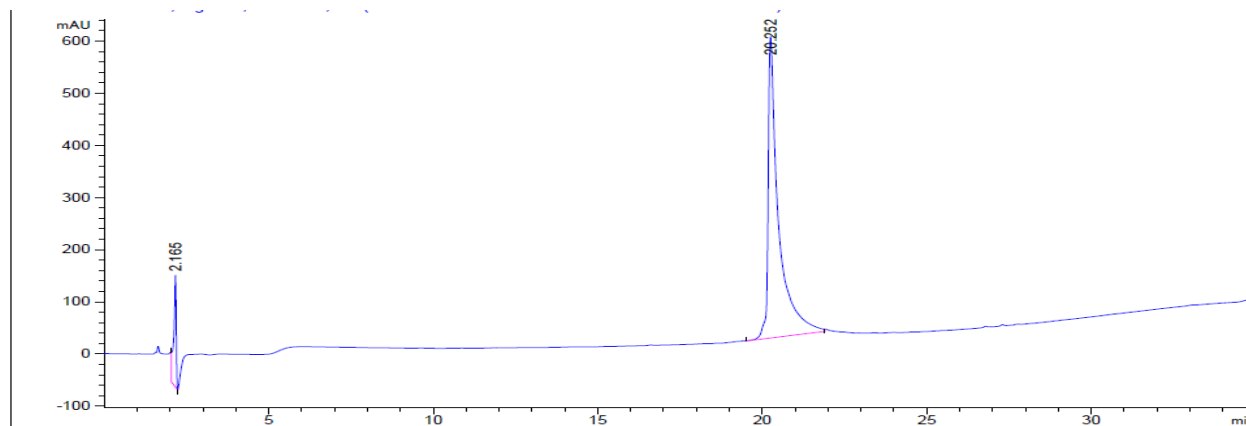

Fig. S52 HPLC of VIP115, early isomer. RP-HPLC conditions: 5-95% ACN over 35 min.

S26

Fig. S53 HPLC of VIP115, late isomer. RP-HPLC conditions: 5-95% ACN over 35 min.
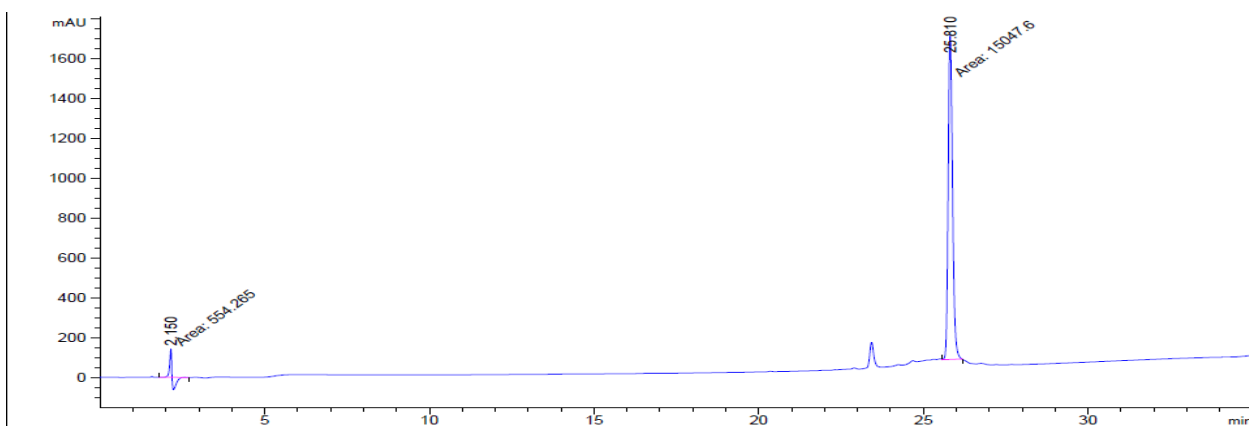
Fig. S54 HPLC of reduced-sMTide-02. RP-HPLC conditions: 5-95% ACN over 35 min.
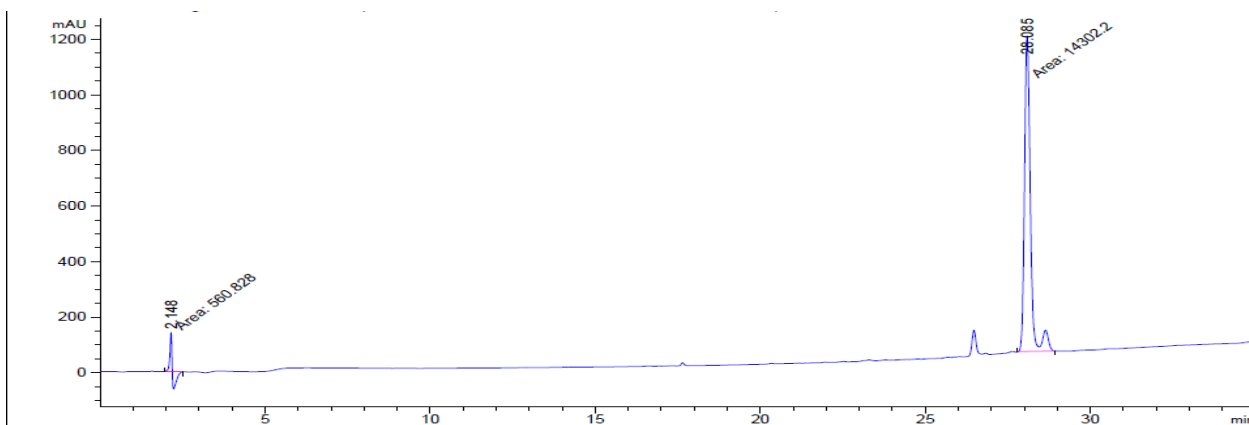
Fig. S55 HPLC of reduced-ATSP-7041. RP-HPLC conditions: 5-95% ACN over 35 min.
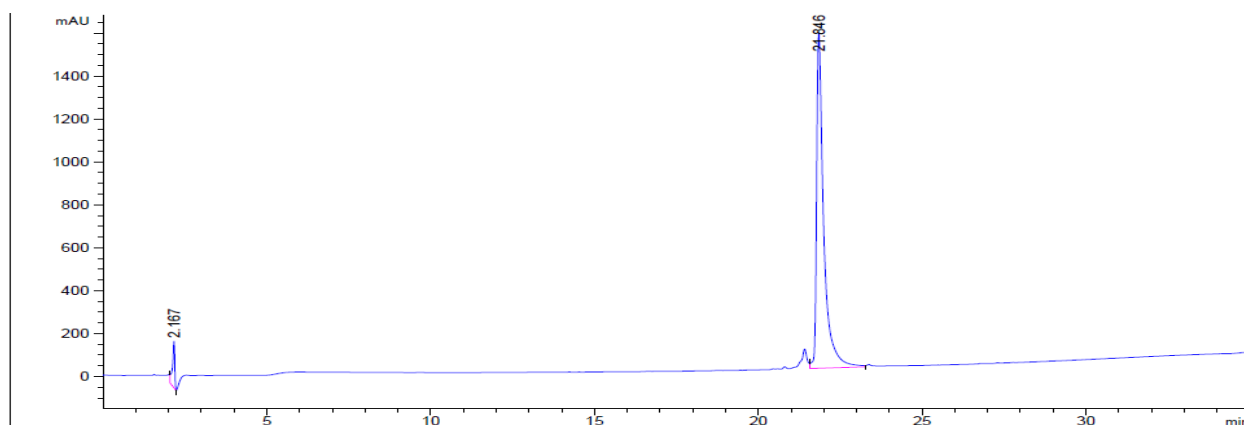
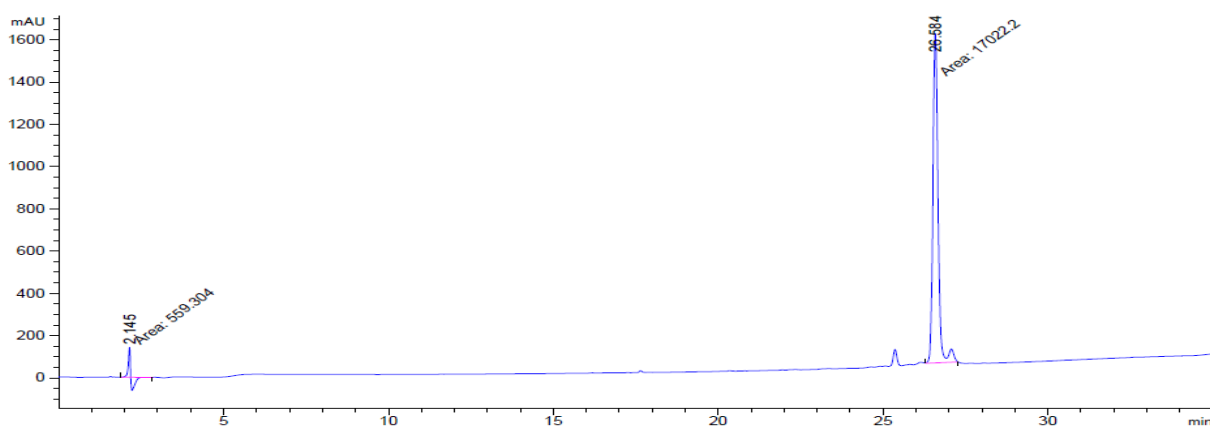Fig. S56 HPLC of reduced-VIP82. RP-HPLC conditions: 5-95% ACN over 35 min.
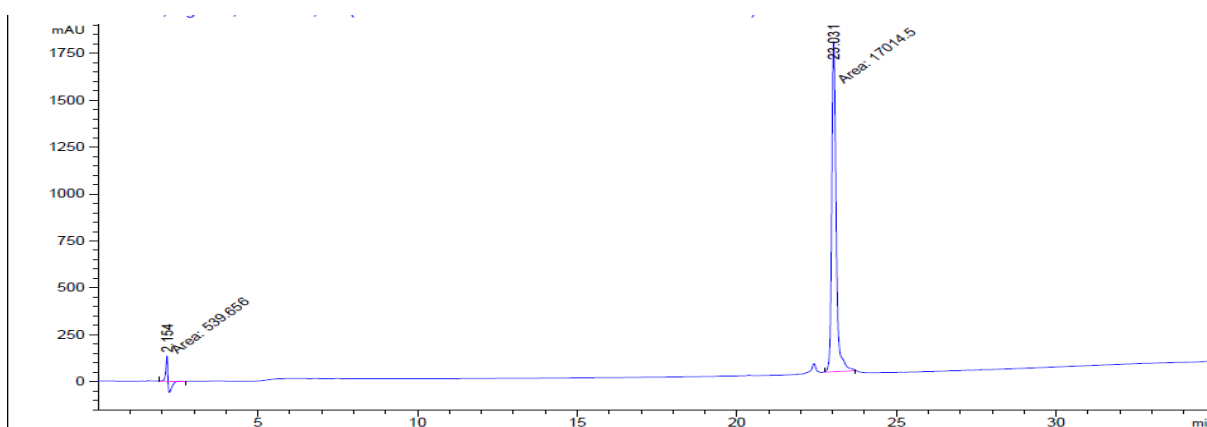
Fig. S57 HPLC of reduced-VIP116. RP-HPLC conditions: 5-95% ACN over 35 min.
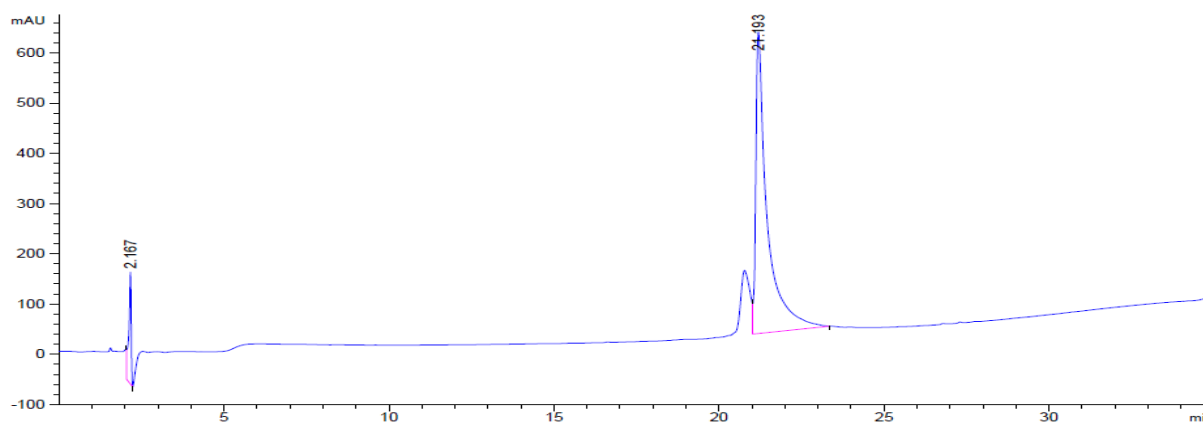
Fig. S58 HPLC of reduced-VIP115. RP-HPLC conditions: 5-95% ACN over 35 min.

5. Circular dichroism (CD)
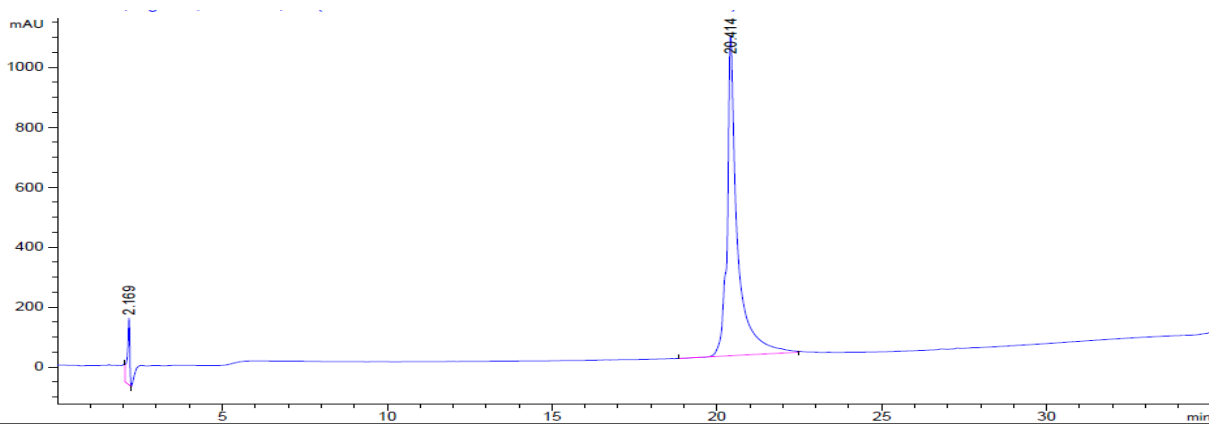
To facilitate buffer exchange, 5 µl of the 10 mM stock peptide, mixed with 45 µl of 100 % methanol, was dried in the SpeedVac concentrator (Thermo Scientific) for 2 hours. Buffer containing 1 mM Hepes pH 7.4 and 5 % methanol was used to reconstitute and dilute the peptide to 1 mM. The CD spectrum of the peptide was recorded from 300 to 190 nm at 25 ˚C, using the Chirascan-plus qCD machine (Applied Photophysics), and a quartz cuvette with a path length of 0.2 cm. Absorbance at 280 nm was used to determine the experimental concentration of the peptide. The CD spectrum was converted to mean residue ellipticity, before deconvoluting using the CDNN software (distributed by Applied Photophysics), to get an estimate of the secondary structure components of the peptide. All experiments were carried out in duplicates.
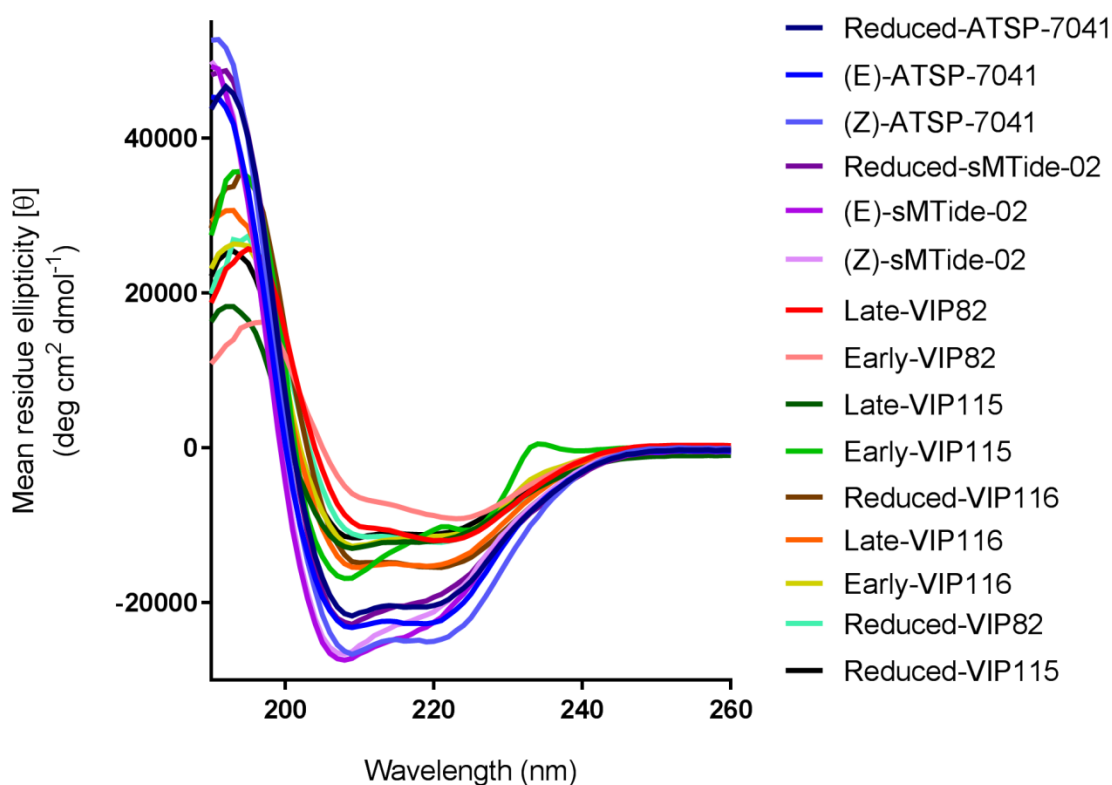
Fig. S59 Circular dichroism spectra of p53-reactivating stapled peptide isomers.

6. Biological assays

T22 p53 reporter assay: T22 p53 β-galactosidase based reporter assay T22 cells, which were stably transfected with a p53 responsive β-galactosidase reporter, were seeded into 96-well plate at a cell density of 8000 cells per well.[1] Cells were also maintained in Dulbecco's Minimal Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin. The cells were incubated for 24 hours and then the media was removed and replaced with 90 µl of DMEM either with 2% FBS. Cells were

treated with compounds/peptide for 18 hours in DMEM with 2% FBS. Final working concentration of DMSO after compound addition was 1% v/v. Corresponding negative control wells with 1% DMSO only were also prepared. β-galactosidase activity was detected using the FluoReporter LacZ/Galactosidase Quantitation kit (Invitrogen) as per manufacturer's instructions. Measurements were carried out using an Envision multiplate reader (Perkin-Elmer). Experiments were carried out independently twice.

Fluorescence Anisotropy Competition Assay: Fluorescence anisotropy assays were performed as previously described.[2] Titrations of purified Mdm2/Mdm4 proteins were incubated with 50 nM of carboxyfluorescein (FAM) labelled 12-1 peptide (FAM-RFMDYWEGL-NH2) to intitially determine the dissociation constants for the peptide-protein interaction. Apparent K_ds of peptides were next determined by competitive fluorescence anisotropy. Titrations of peptides were carried out at constant concentrations of Mdm2/Mdm2-M62A/Mdmx (150 nM), Mdmx-L98V (250 nM) and labelled peptide (50 nM). Anisotropy measurements were carried out using the Envision Multilabel Reader (PerkinElmer). All experiments were carried out in PBS (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4), 3% DMSO v/v and 0.1% Tween-20 v/v buffer. All titrations were carried out in duplicate. Curve fitting was carried out using Prism 5.0 (GraphPad).

Lactate (LD) Dehydrogenase Release Assay: T22 cells cells were seeded into a 96-well plate at a cell density of 5000 cells per well. Cells were maintained in Dulbecco's Minimal Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. The cells were incubated for 24 hours followed by cell media removal and addition of 90 µl of DMEM either with 2% FBS. Cells were then treated with compounds/peptide for 2 hours in DMEM 2% FBS. Final working concentration of DMSO was 1% v/v. Corresponding negative control wells with 1% DMSO only were also prepared. Cytosolic lactate dehydrogenase release was detected using the cytoTox 96® non-radioactive cytotoxicity Assay kit (Promega) as per manufacturer's instructions. Measurements were carried out using an Envision multiplate reader (Perkin-Elmer). Maximum LDH release was defined as the amount of LDH released when cells were lysed in the presence of 0.1% TRITON X-100 and was used to normalize the results. Experiments were carried out independently twice.

Animal Studies: The procedures for all mouse experiments were approved by the A*STAR Institutional Animal Care and Use Committee (IACUC) and performed in compliance with IACUC regulations. Mice were house-breed C57BL/6 strain initially obtained from Jackson Labs. 8-11 week-old mice were used for peptide treatment experiments (n = 2-4). Peptides were dissolved in 100% DMSO as stock and diluted in Hank's Balanced Salt Solution (HBSS) to 2% of DMSO at appropriate concentration 2-4 hours before each injection. The vehicle control consisted of 2% DMSO in HBSS. Mice were injected with 40 mg/kg peptide and vehicle control via the intraperitoneal (i.p.) route and tissues were harvested at various time points post drug administration. Harvested tissues were fixed in cold 10% natural buffered formalin (NFB) for 36-40 hours and sent to histopathology facility (Advanced Molecular Pathology lab, AMPL) for paraffin block embedding.

Immunohistochemistry (IHC): 5 µm tissue sections were used for staining. EDTA based pH 9.0 antigen retrieval solution was used to exposure antigen epitopes. Home-made p53 rabbit serum (1:1500

dilution) was used as primary antibody for IHC. The EnVision HRP Labelled Polymer anti-rabbit IgG (DAKO) was used as secondary antibody (details as previous described).[3] Brightfield images were captured with a Zeiss AxioImager microscope using ZEN software. Immunostaining quantification and statistical analysis were performed using inverted images in ImageJ.[4] The average RGB OD values (0.27980405, 0.57827362, 0.7650414) were entered into the colour deconvolution software to define p53 immunopositivity. Thresholding of images were also carried out and the immunostaining area and intensity were determined.

7. Molecular dynamics

Chains A and C from the crystal structure of M06 peptide complexed with the N-terminal domain of MDM2-M62A (PDB code 4UMN[5]) were used as the initial structures for molecular dynamics (MD) simulations. M06 was converted to PM2 by mutating Trp23 to Ala while MDM2 was reverted to its wild type state by reversing the M62A mutation. Residue protonation states were determined by PDB2PQR.[6] The LEaP program in the AMBER 14 package was then used to solvate each system with TIP3P[7] water molecules in a periodic truncated octahedron box, such that its walls were at least 10 Å away from the MDM2 complex, and for neutralization of charges with chloride ions. The *cis* isomer and reduced form of PM2 were generated using PyMOL.[8]

Energy minimizations and MD simulations were performed with the sander and PMEMD modules of AMBER 14[9] respectively. Four independent explicit-solvent MD simulations using different initial atomic velocities were carried out on each of the complexes of MDM2 with *trans* PM2, *cis* PM2 and reduced PM2 complexes using the ff14SB[10] and generalized AMBER force fields (GAFF).[11] Atomic charges for the stapled residues were derived using the R.E.D. Server,[12] which fits restrained electrostatic potential (RESP) charges[13] to a molecular electrostatic potential (MEP) computed by the Gaussian 09 program[14] at the HF/6-31G* theory level. All bonds involving hydrogen atoms were constrained by the SHAKE algorithm[15], allowing for a time step of 2 fs. Nonbonded interactions were truncated at 9 Å while electrostatic interactions were treated by the particle mesh Ewald method.[16] Energy minimisation was carried out using the steepest descent algorithm for 500 steps, followed by the conjugate gradient algorithm for another 500 steps. The system was then heated gradually to 300 K over 50 ps at constant volume before equilibration at a constant pressure of 1 atm for another 50 ps. Weak harmonic positional restraints with a force constant of 2.0 kcal mol^{-1} Å^{-2} were imposed on the non-hydrogen atoms of the solute during the minimization and the equilibration steps. Subsequent unrestrained equilibration (2 ns) and production (200 ns) runs were carried out at 300 K and 1 atm. The temperature was maintained using a Langevin thermostat[17] with a collision frequency of 2 ps^{-1} while the pressure was maintained by a Berendsen barostat[18] with a pressure relaxation time of 2 ps.

Binding free energy calculations: Binding free energies for the MDM2 complexes were calculated using the molecular mechanics/generalized Born surface area (MM/GBSA) method[19] implemented in AMBER 14.[9] Two hundred equally-spaced snapshot structures were extracted from the last 60-80 ns of each of the trajectories, and their molecular mechanical energies calculated with the sander module. The polar contribution to the solvation free energy was calculated by the pbsa[20] program using the modified generalized Born model described by Onufriev *et al.*[21] while the nonpolar contribution was estimated

from the solvent accessible surface area using the molsurf[22] program with $\gamma = 0.0072$ kcal Å^{-2} and β set to zero.

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