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Factors influencing on-resin depsipeptide bond formation: Case studies on daptomycin- and brevicidine-derived sequences

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Abbreviations

6-ClHOBt, 1-hydroxy-6-chlorobenzotriazole; **Ac**, acetyl; **Ala (A)**, alanine; **All**, allyl; **Arg (R)**, arginine; **Asn (N)**, asparagine; **Asp (D)**, aspartic acid; **Boc**, *tert*-butoxycarbonyl; **Cys (C)**, cysteine; **DCM**, dichloromethane; **DIC**, *N*,*N'*-diisopropylcarbodiimide; **DIPEA**, *N*,*N'*-diisopropylethylamine; **DMAP**, 4-dimethylaminopyridine; **DMF**, *N*,*N'*-dimethylformamide; **Fmoc**, 9-fluorenylmethyloxycarbonyl; **Gln (Q),** glutamine; **Glu (E),** glutamic acid; **Gly (G)**, glycine; **h**, hour/s; **HCTU**, O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; **His (H),** histidine; **HMP**, 4-(hydroxymethyl)phenoxyacetic acid; **HRMS**, high resolution mass spectrometry; **Leu (L)**, leucine; **Lys (K)**, lysine; **MeCN**, acetonitrile; **Met (M)**, methionine; **min**, minute/s; **MS**, mass spectrometry; **NMP**, *N*-methyl-2-pyrrolidone; **Pbf**, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; **PG**, protecting group; **Phe (F)**, phenylalanine; **ppm**, parts per million; **Pro (P)**, proline; **PS**, polystyrene; **RP-HPLC**, reverse phase high performance liquid chromatography; **rt**, room temperature; **sec**, second/s; **Ser (S),** serine; ***t*Bu**, *tert*-butyl; **TFA**, trifluoroacetic acid; **Thr (T)**, threonine; **TIPS**, triisopropylsilane; **TMS**, tetramethylsilane; **TritonX100™**, *tert*-octylphenoxypolyethoxyethanol; **Trp (W)**, tryptophan; **Trt**, trityl; **Tyr (Y)**, tyrosine; **UV**, ultra-violet; **Val (V)**, valine.

Experimental

General Information

Chemicals

All reagents were purchased from commercial sources and used without further purification unless otherwise stated from the following sources:

Sigma Aldrich: N,N'-diisopropylethylamine (DIPEA), *N,N*'-diisopropylcarbodiimide (DIC), formic acid,

AK Scientific: 4-(hydroxymethyl)phenoxyacetic acid (HMP) linker, triisopropylsilane (TIPS), Fmoc-L-Asp(OH)-OAll, Fmoc-D-Ser(*t*Bu)-OH

Aapptec: 1-hydroxy-6-chlorobenzotriazole (6-ClHOBt), Fmoc-L-Trp(Boc)-OH, 4-dimethylaminopyridine (DMAP), Boc-L-Trp(H)-OH, O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), Fmoc-D-Asn(Trt)-OH, Fmoc-L-Asp(tBu)-OH, Fmoc-D-Thr(OH)-OH, Fmoc-L-Ser(*t*Bu)-OH, Fmoc-D-Trp(Boc)-OH

Rapp Polymere: Tentagel S resin

Avantor Performance Materials: Acetic anhydride (Ac_2O), diethyl ether (Et_2O)

Oakwood Chemicals: Trifluoroacetic acid (TFA)

Scharlau: N,N'-Dimethylformamide (DMF, AR grade), acetonitrile (MeCN, HPLC grade), acetonitrile (MeCN, MS grade)

ECP Limited: Dichloromethane (DCM), pyridine

AusPep: Fmoc-L-Trp(H)-OH

Polypeptide laboratories: Fmoc-L-Thr(OH)-OH

Chem Supply: piperidine,

ChemPep®: 2-chlorotrityl chloride polystyrene (2-ClTrt PS)resin

Chem Impex Int'l Ltd.: Fmoc-D-Ala-OH

CS Bio (Shanghai) Ltd.: Fmoc-Gly-OH, Fmoc-L-Ile-OH, Fmoc-L-Lys(Boc)-OH

GL Biochem: Ser(*t*Bu)-OMe•HCl

Alloc-L-Ser(*t*Bu)-OH was prepared as described below, according to literature procedures.[1,2]

Analytical equipment

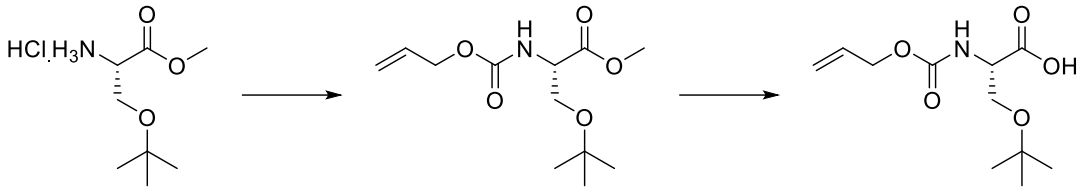
Analytical HPLC was collected on an Agilent 1100 Compact, equipped with an Agilent Zorbax SB300-C8 column (150 x 4.6 mm, 5 µm) using a linear gradient of MeCN with 0.1% (*v/v*) TFA (Buffer B), in H_2O with 0.1% (*v/v*) TFA (Buffer A) at 1 mL/min and the spectra visualised at 214 nm.

LCMS and MS (ESI+) data was collected using an Agilent 1260 Infinity liquid chromatography system equipped with an Agilent Zorbax SB300-C3 column (150 x 3 mm, 3.5 µm), over a linear gradient of MeCN with 0.1% (*v/v*) formic acid, in H_2O with 0.1% (*v/v*) formic acid at 0.3 mL/min and visualised at 214 nm; and attached to an Agilent 6120 Quadrupole electrospray mass spectrometer.

UV readings for the '*Fmoc liberation test'* were collected on a Shimadzu (Kyoto, Japan) UV-1280 system, in quartz cuvettes with a 10 mm pathlength.

Nuclear magnetic resonance (NMR) spectra were recorded at 298 K on a Bruker AVANCE 400 spectrometer. All chemical shifts are reported in parts per million (ppm) from referenced to tetramethylsilane (TMS) at 0 ppm.

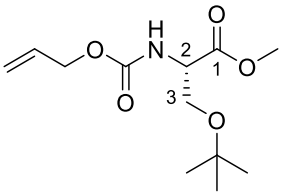
Amino-acid synthesis



H-Ser(*t*Bu)-OMe.HCl **Alloc-Ser(*t*Bu)-OMe** **Alloc-Ser(*t*Bu)-OH**

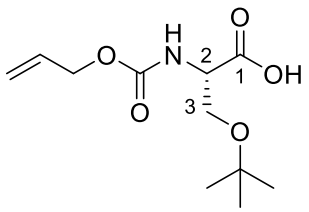
Scheme S1: Alloc-L-Ser(*t*Bu)-OH synthetic scheme

*Methyl-(2S)-2-((allyloxycarbonyl)amino)-3-(tert-butyloxy)propanoate (**Alloc-Ser(tBu)-OMe**)*



A solution of Na_2CO_3 (2.5 g, 23.6 mmol, 5 eq.) in H_2O (7 mL) was added slowly to an ice-water cooled solution of H-Ser(*t*Bu)-OMe.HCl (1.0 g, 4.72 mmol, 1 eq.) in THF (7 mL). The mixture was stirred for 10 min at 0 °C, then allyl chloroformate (1 mL, 9.44 mmol, 2 eq.) was slowly added. The resulting mixture was stirred for 12 h at rt then concentrated *in vacuo.* The reaction was extracted with EtOAc (3 × 20 mL) and the combined organic extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude residue was purified by flash chromatography on silica gel (EtOAc-PET ether 15:85) to afford **Alloc-Ser(*t*Bu)-OMe** as a colourless oil (1.1 g, 95%). $\mathbf{R_f}$ 0.35 (EtOAc-PET ether 15:85); **^{1}H NMR** (400 MHz, $CDCl_3$): *δ* 5.97-5.88 (ddt, *J* = 17.1, 10.9, 5.6 Hz, 1H, Alloc), 5.58 (d, *J* = 8.4 Hz, 1H, NH), 5.32 (dd, *J* = 17.2, 1.3 Hz, 1H, Alloc), 5.22 (dd, *J* = 10.4, 1.1 Hz, 1H, Alloc), 4.59 (ddd, *J* = 5.6, 1.3, 1.3 Hz, 2H, Alloc), 4.44 (ddd, *J* = 8.9, 3.0, 2.9 Hz, 1H, H-2), 3.80 (dd, *J* = 9.0, 2.8 Hz, 1H, H-3), 3.74 (s, 3H, O*CH*$_3$), 3.57 (dd, *J* = 9.0, 3.3 Hz, 1H, H-3), 1.12 (s, 9H, *t*Bu) ppm; **^{13}C NMR** (100 MHz, $CDCl_3$): *δ* 171.1 (*C*$O_2$$CH_3$, C-1), 156.0 (NH*C*$O_2$, Alloc), 132.7 (*CH*=$CH_2$, Alloc), 117.8 (CH=*C*$H_2$, Alloc), 73.4 (*C*$(CH_3)_3$, *t*Bu), 65.8 ($CO_2$*C*$H_2$, Alloc), 62.0 ($CH_2$, C-3), 54.6 (CH, C-2), 52.3 (CH_3, OMe), 27.2 (C(*C*H_3)$_3$, *t*Bu) ppm; **HRMS** (ESI+) *m/z*: 282.1312 (calcd for $[C_{12}H_{21}NO_5+Na]^+$, 282.1312). The spectroscopic data were in agreement with those reported in the literature.[2]

*(2S)-2-((allyloxycarbonyl)amino)-3-(tert-butyloxy)propanoic acid (**Alloc-Ser(tBu)-OH**)*



LiOH (0.23 g, 9.6 mmol, 2 eq.) was dissolved in H_2O (7 mL) and added to a solution of **Alloc-Ser(tBu)-OMe** (1.1 g, 4.8 mmol, 1 eq.) in THF (7 mL). The reaction mixture was allowed to stir at rt for 1 h and concentrated *in vacuo*. The solution was adjusted to pH 2 with 1 M HCl and extracted with EtOAc (3 × 20 mL). The combined organic extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude residue was purified by flash chromatography on silica gel (EtOAc-PET ether 3:7 and 0.1% AcOH) to afford **Alloc-Ser(tBu)-OH** as a colourless oil (0.36 g, 50%). $\mathbf{R_f}$ 0.57 (EtOAc-PET ether 3:7 and 0.1% AcOH);**^{1}H NMR** (400 MHz, $CDCl_3$): *δ* 5.97-5.88 (ddt, *J* = 22.8, 10.9, 5.6 Hz, 1H, Alloc), 5.58 (d, *J* = 8.1 Hz, 1H,

NH), 5.32 (dd, *J* = 17.2, 1.3, 1H, Alloc), 5.22 (dd, *J* = 10.4, 1.3, 1H, Alloc), 4.59 (d, *J* = 5.6, 2H, Alloc), 4.46 (ddd, *J* = 11.6, 7.5, 3.3 Hz, 1H, H-2), 3.90 (dd, *J* = 8.8, 3.0 Hz, 1H, H-3), 3.57 (dd, *J* = 8.9, 4.9 Hz, 1H, H-3), 1.19 (s, 9H, *t*Bu) ppm; **^{13}C NMR** (100 MHz, $CDCl_3$): *δ* 175.1 (CO_2H, C-1), 156.4 ($NHCO_2$, Alloc), 132.7 (*CH*=CH_2, Alloc), 118.2 (CH=*CH_2*, C-8), 74.3 (*C*$(CH_3)_3$, *t*Bu), 66.2 (CO_2*CH_2*, Alloc), 61.9 (CH_2, C-3), 54.4 (CH, C-2), 27.4 (C(*CH_3*)$_3$, *t*Bu) ppm; **HRMS** (ESI+) *m/z*: 268.1155 (calcd for $[C_{11}H_{19}NO_5+Na]^+$, 268.1155). The spectroscopic data were in agreement with those reported in the literature.[1,2]

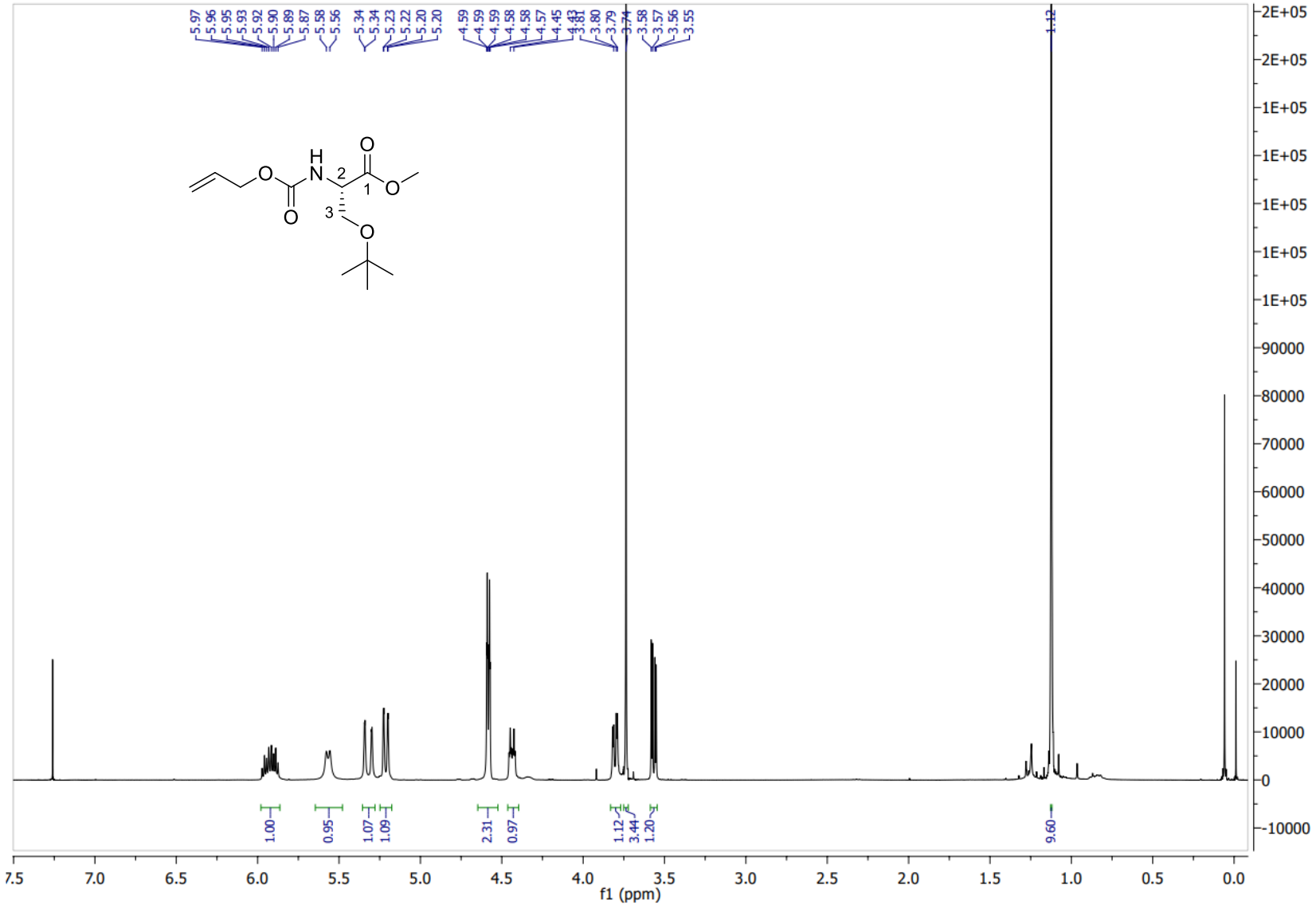


Figure S1. ^{1}H NMR spectrum of methyl (2S)-2-((allyloxycarbonyl)amino)-3-(tert-butyloxy)propanoate (**Alloc-Ser(tBu)-OMe**) ($CDCl_3$, 400 MHz, 298 K).

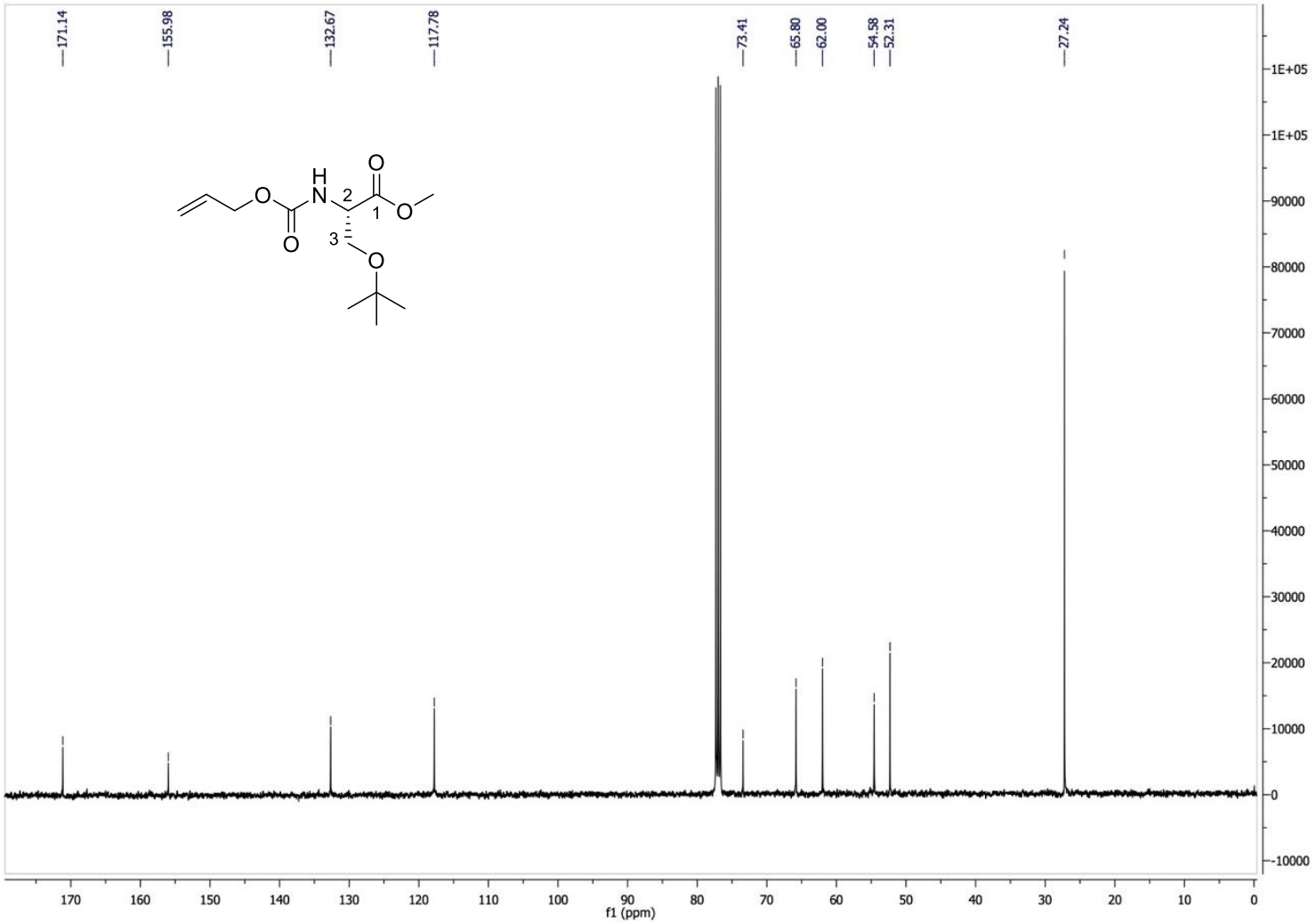


Figure S2. ^{13}C NMR spectrum of methyl (2S)-2-((allyloxycarbonyl)amino)-3- (tert-butyloxy)propanoate (**Alloc-Ser(tBu)-OMe**) ($CDCl_3$, 100 MHz, 298 K)

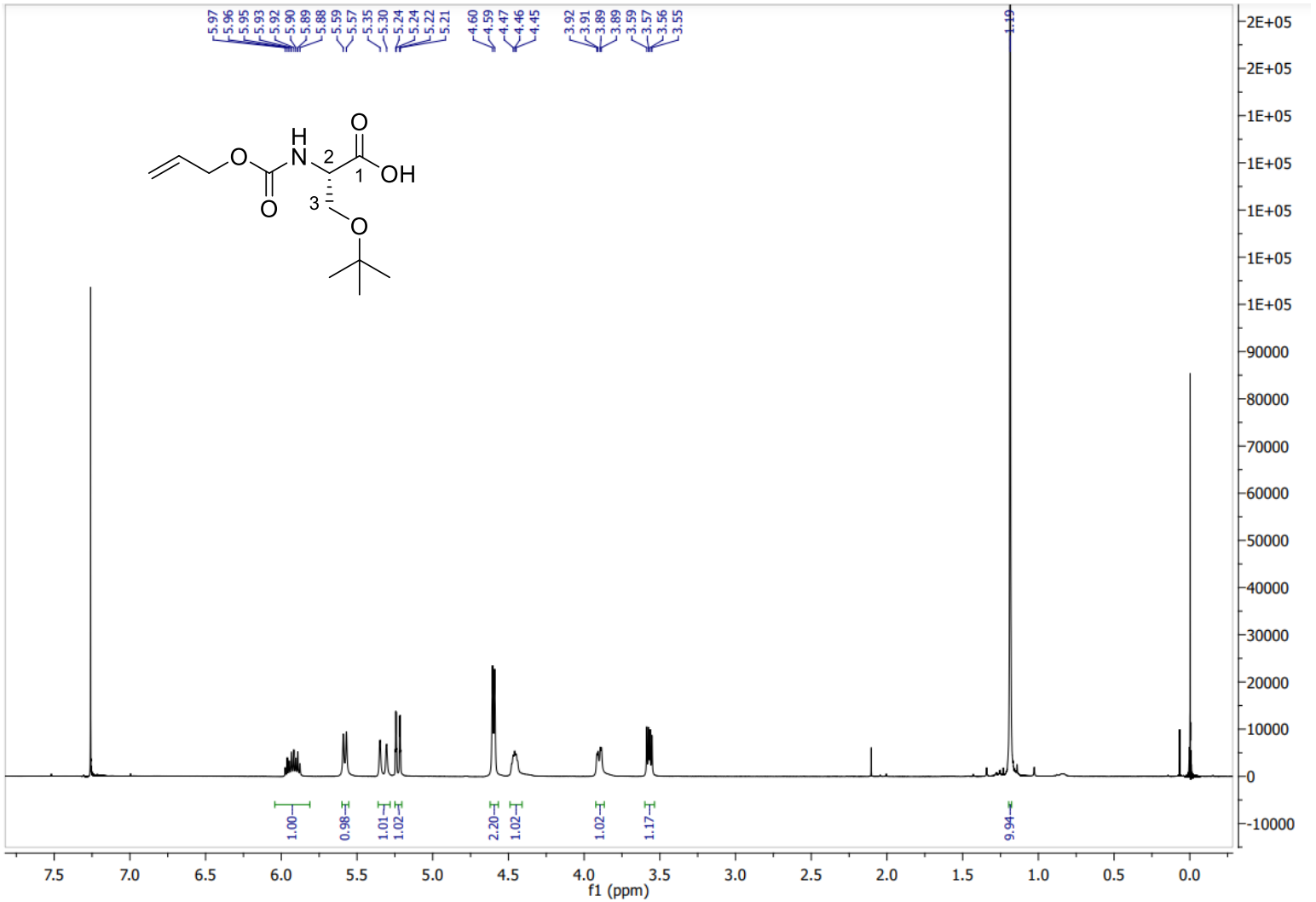


Figure S3. ^{1}H NMR spectrum of (2S)-2-((allyloxycarbonyl)amino)-3-(tert-butyloxy)propanoic acid (**Alloc-Ser(tBu)-OH**) ($CDCl_3$, 400 MHz, 298 K).

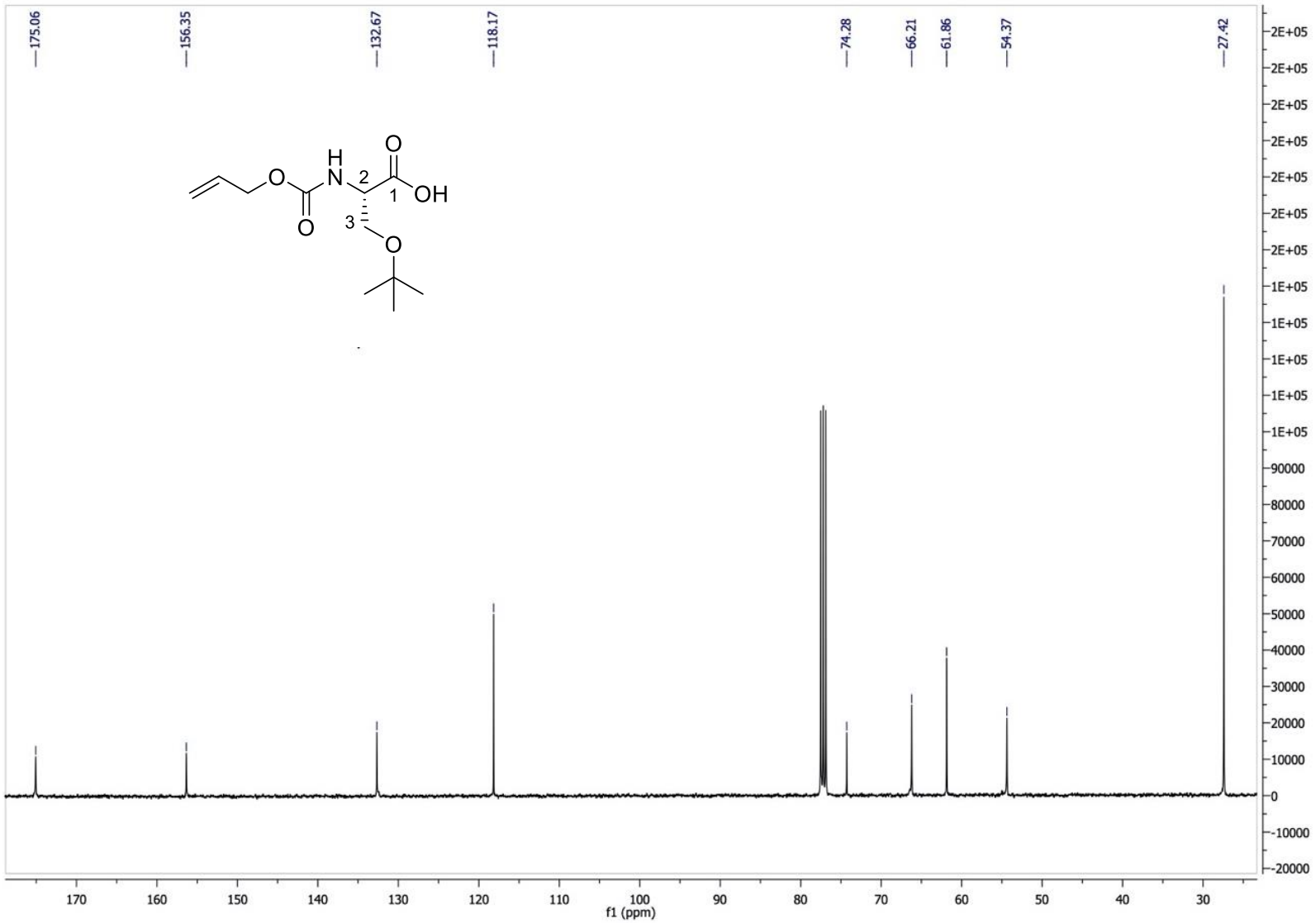


Figure S4. ^{13}C NMR spectrum of (2S)-2-((allyloxycarbonyl)amino)-3-(tert-butyloxy)propanoic acid (**Alloc-Ser(tBu)-OH**) ($CDCl_3$, 100 MHz, 298 K).

Peptide synthesis

All peptides were synthesised, per the general procedures listed below, using the following protected amino-acids, unless otherwise specified: Fmoc-D-Ala-OH, Fmoc-D-Asn(Trt)-OH, Fmoc-L-Asp(OH)-OAll, Fmoc-L-Asp(tBu)-OH, Fmoc-Gly-OH, Fmoc-L-Ile-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Thr(OH)-OH or Fmoc-D-Thr(OH)-OH, Fmoc-L-Trp(Boc)-OH or Fmoc-L-Trp(H)-OH, and Fmoc-L-Ser(*t*Bu)-OH. Identity of the linear peptide sequence, prior to esterification, was confirmed by RP-HPLC and MS (ESI+) by a 'minicleave' of a small portion of resin. Peptidyl resin was then split, and subjected to esterification reaction conditions. Amino-acids building blocks used in esterification reactions included Fmoc-L-Trp(Boc)-OH , Fmoc-L-Trp(H)-OH, Boc-L-Trp(H)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-L-Ser(*t*Bu)-OH, Fmoc-D-Ser(*t*Bu)-OH and Alloc-L-Ser(*t*Bu)-OH.

General procedure for loading of HMP linker and subsequent Fmoc-amino-acid attachment

Tentagel S resin (0.26 mmol/g) was swelled in DMF (7 mL/g resin) for 15 min and then the solution drained from the resin. A solution of 4-(hydroxymethyl)phenoxyacetic acid (HMP linker, 4 equiv), 1-hydroxy-6-chloro-benzotriazole (6-ClHOBt, 4 equiv) and *N*,*N'*-diisopropylcarbodiimide (DIC, 4 equiv) in DMF (4 mL/g resin) was added to the preswelled resin and agitated for 2 h. The solution was drained from the resin, and the resin was washed with DMF (3 × 8 mL/g resin) and DCM (2 × 8 mL/g resin). A '*Kaiser test'* was performed to determine if all amine groups were acylated. The resin was immediately reswelled in DCM, and the solution drained from the resin. A solution of Fmoc-amino-acid (Fmoc-L-Asp(OH)-OAll, Fmoc-Gly-OH or Fmoc-L-Ile-OH) (5 equiv) and DIC (5 equiv) in DCM (4 mL/g resin) was then added to the resin, followed by *N*,*N*-dimethylaminopyridine (DMAP, 0.1 equiv) and the mixture agitated for 4 h, or overnight. Acetic anhydride (250 µL/g resin) was then added to the mixture and agitated for a further 1 h, to ensure any remaining hydroxyl groups were acylated. The solution was then drained from the resin, and the resin washed with DMF (3 × 8 mL/g resin) and DCM (3 × 8 mL/g resin). An '*Fmoc liberation test'* was performed to determine the loading of the Fmoc-amino-acid to the resin. Final resin loadings ranged between 0.10-0.19 mmol/g.

General procedure for loading first amino-acid onto 2-chlorotrityl chloride functionalised resin

2-chlorotrityl chloride functionalised polystyrene resin (0.5 g, 0.89 mmol/g) was swelled in DCM (10 mL) for 15 min. Fmoc-protected amino-acid (0.45 mmol, 1 equiv) was dissolved in DCM (5 mL) and DIPEA (75.6 µL, 2.25 mmol, 5 equiv) added. The amino-acid solution was added to the resin, and the solution shaken (HCl gas evolved) and vented. The mixture was placed on rocker for 2 h and then MeOH (5 mL) added and rocked for a further 30 min to cap any free reactive sites. The solvent was removed and the resin washed with DMF (5 × 8 mL), DCM (2 × 8 mL), DMF (2 × 8 mL) and DCM (5 × 8 mL), then the resin dried by suction. An '*Fmoc liberation test'* was then performed to determine the loading of the Fmoc-amino-acid to the resin. Final resin loadings ranged between 0.077-0.095 mmol/g.

General procedure for peptide synthesis

Resin preloaded with the first Fmoc-amino-acid (0.1 mmol) was preswelled in DMF (5 mL) for 15 min, and the solvent then drained from the resin. Solid-phase peptide chain elongation was carried out on an Activotec Activo-P14 peptide synthesiser. A solution of 20% (*v/v*) piperidine in DMF (3.8 mL) was added to the resin, and the mixture agitated at rt for 3 min. The solution was then drained from the resin, and a fresh portion of 20% (*v/v*) piperidine in DMF (3.8 mL) was added to the resin, and the mixture agitated at rt for a further 9 min. The

solution was then drained from the resin and the resin washed with DMF (5 × 1 min, 4 mL). A solution of Fmoc-amino-acid (5 equiv), HCTU (4.9 equiv, 980 µL, 0.5 M in DMF) and DIPEA (10 equiv, 174 µL) in DMF (made up to 4 mL total) was added to the resin and the mixture agitated at rt for 20 min. The solution was then drained from the resin and the resin washed with DMF (4 × 1 min, 4 mL). The deprotection and coupling cycles were repeated until the desired sequence was achieved. Following the final *N*-terminal Fmoc deprotection, the resin was removed from the synthesiser, and a solution of acetic anhydride (470 µL, 50 equiv) and DIPEA (870 µL, 50 equiv) in DMF (2.5 mL) was added to the resin and agitated for 30 s. The solution was then drained from the resin, and the resin washed with DMF (3 × 5 mL), then DCM (3 × 5 mL) and then airdried.

Kaiser test

A small sample of resin (~2-5 mg) was washed thoroughly with DCM, and then 2 drops of each 80% (w/v) phenol in *n*-butanol (40 g in 20 mL), 5% (w/v) ninhydrin in *n*-butanol (1.0 g in 20 mL) and 200 µM KCN in pyridine (16.5 mg KCN in 25 mL H_2O, 1 mL KCN aq. solution in 49 mL pyridine) added to the resin. The mixture was then heated at 100°C for 5 min and the colour assessed. Blue beads (positive result) indicate the presence of free amines, and colourless/yellow beads (negative result) indicate the absence of free amines.

Fmoc liberation test

Immediately following coupling of the Fmoc-protected building block to the resin, two samples of resin (~1-5 mg) were thoroughly washed with DCM (6 x 300 µL) and airdried with suction. The resin samples were transferred to eppendorfs and the accurate mass of the resin recorded, then freshly prepared 20% (*v/v*) piperidine in DMF (1.0 mL) added to the resin. The mixture was agitated for 20 min at rt. A sample of the supernatant (200 µL) was then taken and diluted 10-fold in 20% (*v/v*) piperidine in DMF (1.80 mL), and the absorbance at 290 nm recorded. Absorbance at 290 nm was first zeroed against a sample of 20% (*v/v*) piperidine in DMF. The loading of the resin was then calculated for each sample, using the following equation:

$$Resin\ loading\ \left(\frac{mmol}{g}\right) = \frac{(A_{290} \times DF \times V_{rxn})}{(\varepsilon_{290} \times m_{resin})}$$

Where, A_{290} is the absorbance at 290 nm as recorded by a UV-Vis spectrophotometer

DF is the dilution factor (here, 10)

V_{rxn} is the volume (in mL) of the deprotection reaction (here, 1 mL)

ε_{290} is the extinction coefficient of the fulvene-piperidine adduct at 290 nm (ε_{290} = 6089)[3]

m_{resin} is the mass of resin in reaction sample (in g)

The two values were averaged (mean) to give the average loading of the resin.

General procedure for peptide cleavage from resin and global deprotection

A solution of 95:2.5:2.5 TFA:TIPS:H_2O (~300 µL) was added to a small portion of the peptidyl resin (~10 mg) and agitated for 1.5 h to cleave the peptides from the resin. The TFA solution was then concentrated under a stream of nitrogen to ~100-200 µL, and cold diethyl ether (1.0 mL) added to precipitate the peptides. The mixture was centrifuged to pellet the precipitate, the solvent was decanted and the pellet dried under a stream of nitrogen. The pellet was resuspended in 50% aq. MeCN with 0.1% TFA (~200 µL), syringe filtered (0.45 µm) analysed by RP-HPLC over a linear gradient at 214 nm, and peptide identity confirmed by MS analysis.

General procedure for esterification of resin bound peptides

Reagents, tryptophan/serine, DIC and DMAP, were freshly made up as stock solutions in the reaction solvent specified (DMF, DCM, NMP, or 1:1:1 DMF:DCM:NMP). A 6-ClHOBt stock, where applicable, was made up in DMF regardless of reaction solvent due to limited solubility in DCM. An *example* of the typical stock solutions prepared is provided,

DIC stock in reaction solvent (5 equiv/reaction, 0.05 mmol/20 µL): 156.3 µL DIC in 243.7 µL solvent

DMAP stock in reaction solvent (0.1 equiv/reaction, 0.001 mmol/20 µL): 36.6 mg DMAP in 6.0 mL solvent

Example of amino-acid stock, Fmoc-L-Trp(Boc)-OH in reaction solvent (5 equiv/reaction, 0.05 mmol/50 µL): 263 mg Fmoc-L-Trp(Boc)-OH in 0.5 mL solvent

Peptidyl resin (0.01 mmol) was preswelled in the reaction solvent (0.5 mL) for 15 min then the solvent pipetted from the resin. Reaction solvent (final reaction volume 0.5 mL) was added to the resin, followed by amino-acid stock (5 equiv, 50 µL), then optional additives as specified (e.g. TritonX100, or 6-ClHOBt as a stock solution in DMF). DIC (5 equiv, 20 µL), and finally, DMAP (0.1 equiv, 20 µL) were added to the mixture, which were then agitated for 1 h, and the solvent mixture then pipetted from the resin and fresh reagents added to the resin. The mixtures were agitated for a further 1 h, the solvent mixture then pipetted from the resin, and the resin was washed with DCM (2 × 0.5 mL). A portion of the resin was taken, and the peptide cleaved from the resin as detailed in '*General procedure for peptide cleavage from resin and global deprotection'.*

Depsipeptide conversion calculation

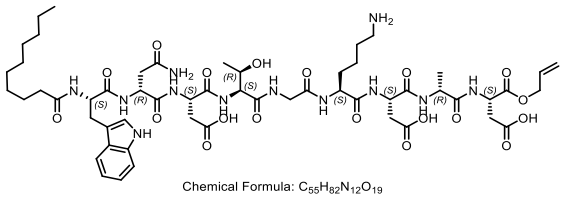
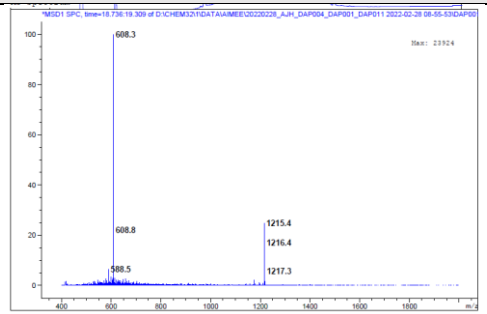
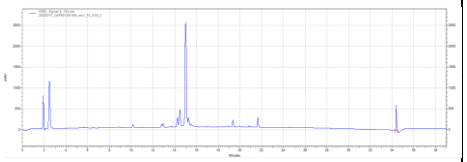
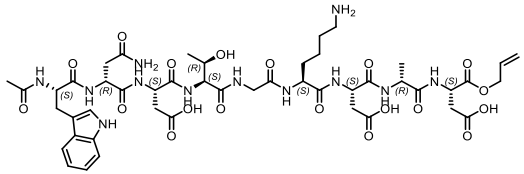
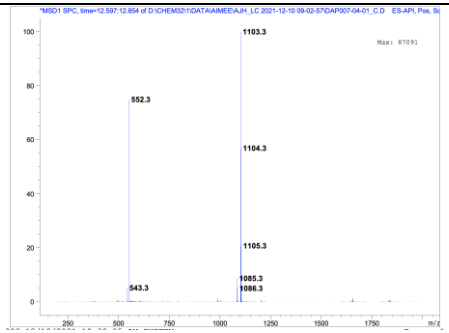
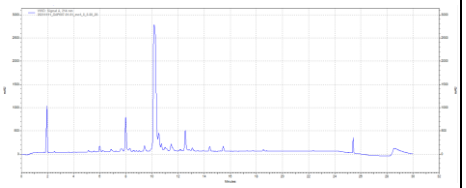
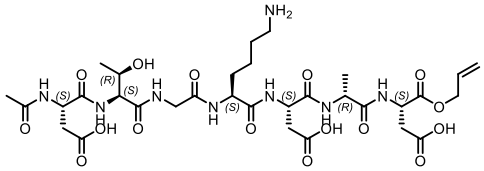
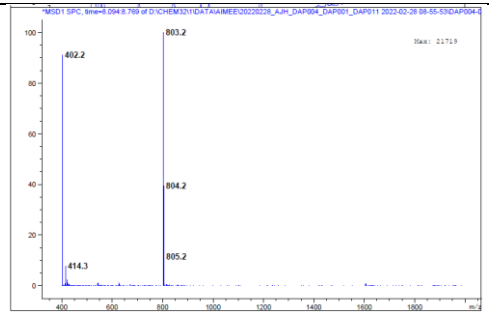
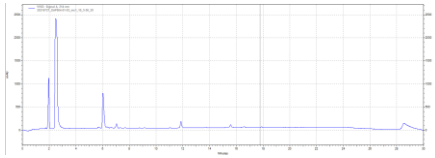
A HPLC spectrum of the reaction mixture was collected and visualised at 214 nm. The resulting spectrum was analysed in the Agilent EZChrom software, and integrated over the relevant gradient time. The percentage (%) area under the curve of the product peak, and any relevant adducts, (e.g. tryptophan CO_2 adducts, as specified in Tables S3, S5, S7, S9, S11, S13, S15, S17, S19, S21, S23, S26), was summed and divided by the sum of the % area of the starting material and product peaks (see generalised equation below). The mean average conversion of at least two experiments is reported.

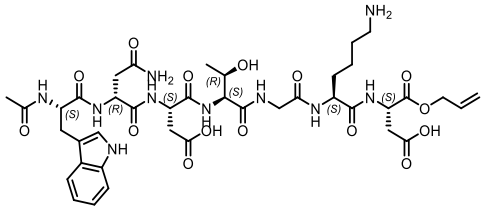
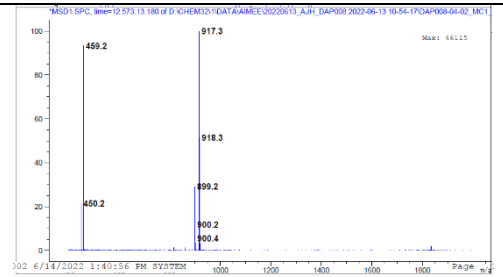
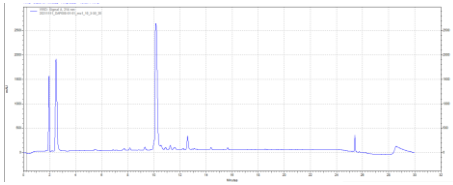
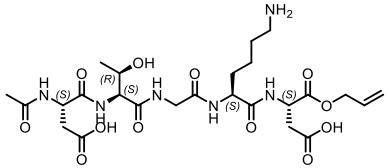
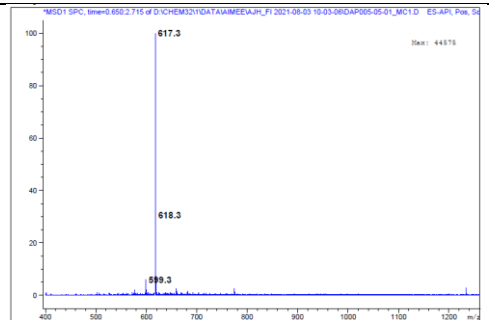
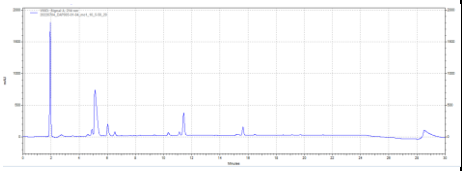
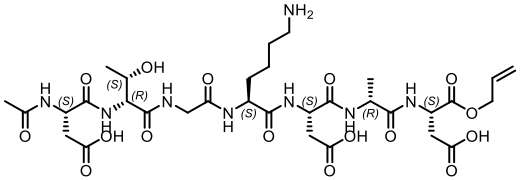
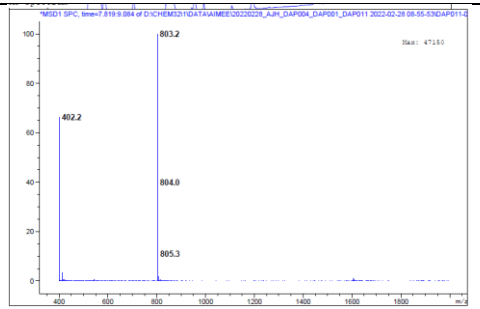
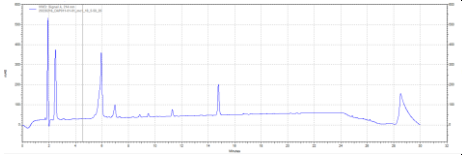
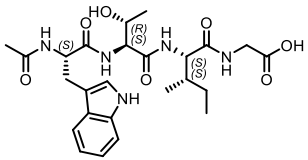
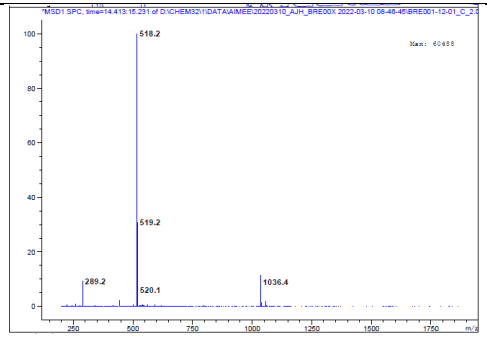
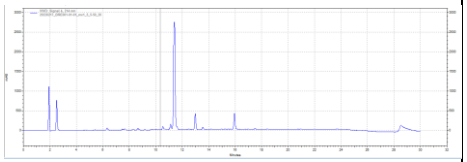
$$\%conversion\ to\ depsipeptide = \frac{\sum Area\ under\ product\ peaks}{\sum area\ under\ product\ and\ starting\ material\ peaks} \times 100$$

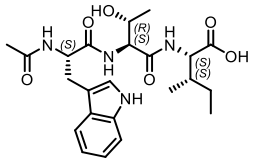
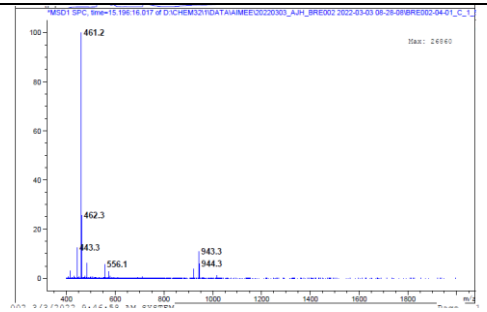
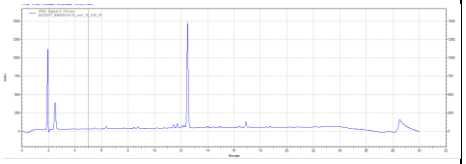
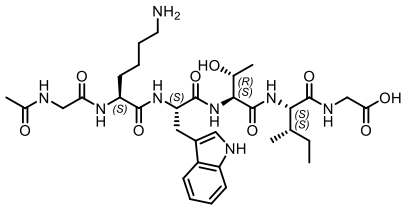
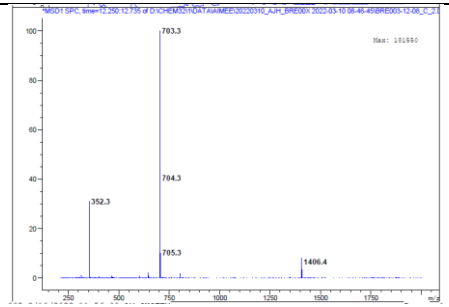
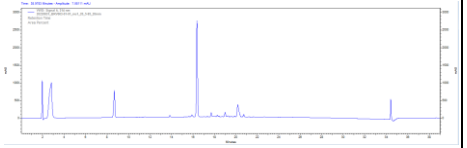
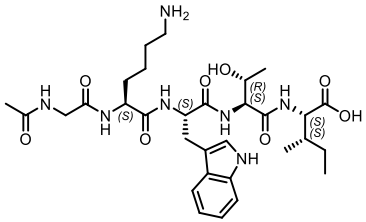
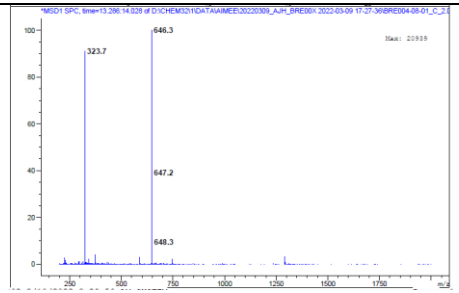
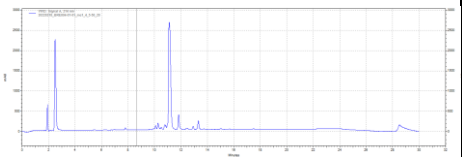
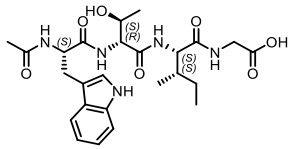
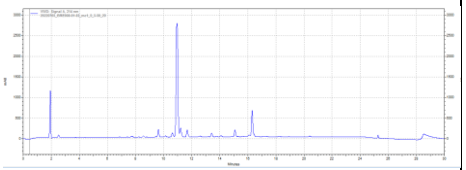
Note: The dried precipitate from the cleavage, was resuspended in 50% aq. MeCN with 0.1% TFA, filtered, and analysed by RP-HPLC within 2 h of redissolution. Extended time in solution, resulted in hydrolysis of the depsipeptide bond for some sequences. Omission of the acid or MeCN resulted in solubility problems for some sequences.

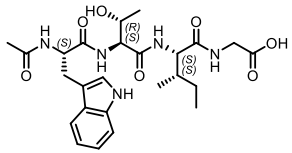
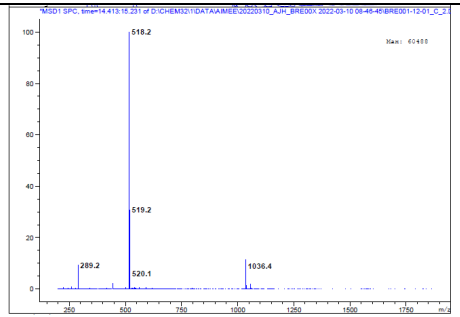
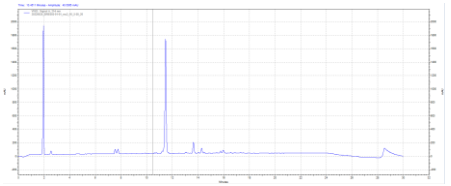
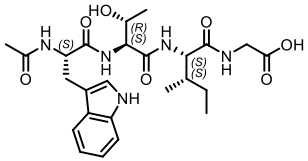
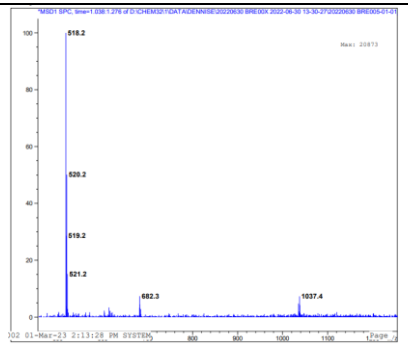
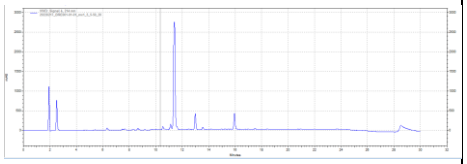
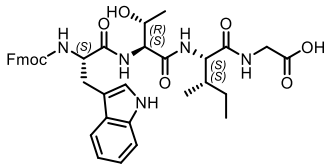
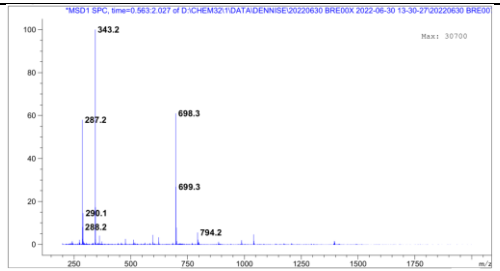
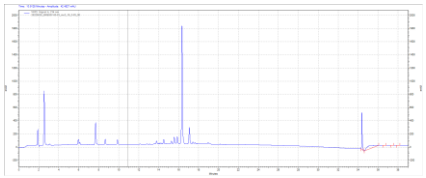
Characterisation of peptides

Table S1: Characterisation of starting material peptides

Peptidyl resin code	Peptide code	Structure	Mass found $[M+H]^+$	MS	R_t	HPLC Representative starting material (crude)
1 (TG)	**S1**	 Chemical Formula: $C_{55}H_{82}N_{12}O_{19}$ Exact Mass: 1214.5819	1215.7		15.1 min	5-95% B over 30min 
3 (TG)	**S3**	 Chemical Formula: $C_{47}H_{66}N_{12}O_{19}$ Exact Mass: 1102.4567	1103.6		10.2 min	5-50% B over 20min 
2 (TG)	**S2**	 Chemical Formula: $C_{32}H_{50}N_8O_{16}$ Exact Mass: 802.3345	803.3		6.0 min	5-50% B over 20min 

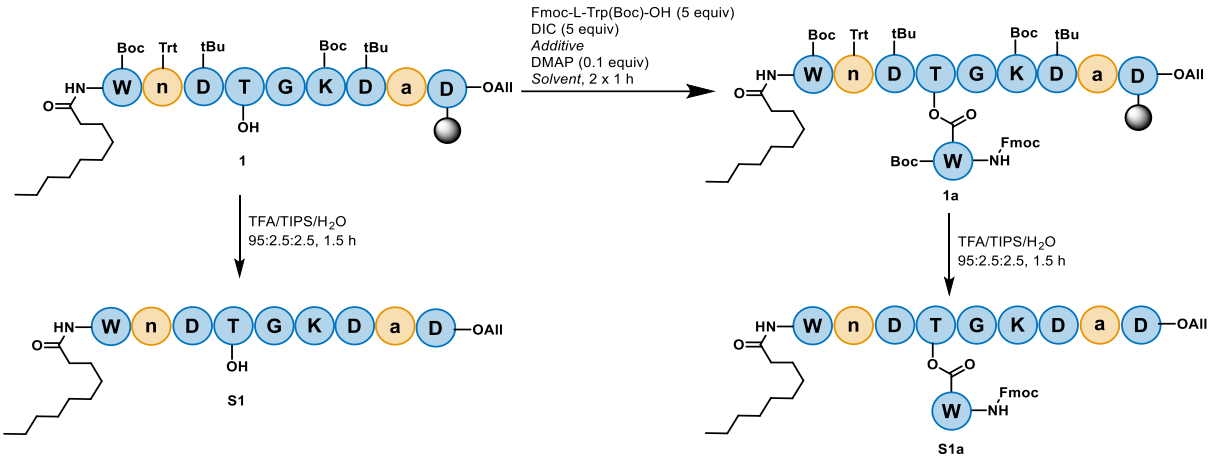
4 (TG)	**S4**	 Chemical Formula: $C_{40}H_{56}N_{10}O_{15}$ Exact Mass: 916.3927	917.5		10.1 min	5-50% B over 20min 
5 (TG)	**S5**	 Chemical Formula: $C_{25}H_{40}N_6O_{12}$ Exact Mass: 616.2704	617.3		5.2 min	5-50% B over 20min 
6 (TG)	**S6**	 Chemical Formula: $C_{32}H_{50}N_8O_{16}$ Exact Mass: 802.3345	803.2		6.0 min	5-50% B over 20min 
7 (TG)	**S7**	 Chemical Formula: $C_{25}H_{35}N_5O_7$ Exact Mass: 517.2536	518.2		11.4 min	5-50% B over 20min 

8 (TG)	**S8**	 Chemical Formula: $C_{23}H_{32}N_4O_6$ Exact Mass: 460.2322	461.2		12.5 min	5-50% B over 20min 
9 (TG)	**S9**	 Chemical Formula: $C_{33}H_{50}N_8O_9$ Exact Mass: 702.3701	703.3		10.1 min	5-50% B over 20min 
10 (TG)	**S10**	 Chemical Formula: $C_{31}H_{47}N_7O_8$ Exact Mass: 645.3486	646.3		11.2 min	5-50% B over 20min 
11 (TG)	**S11**	 Chemical Formula: $C_{25}H_{35}N_5O_7$ Exact Mass: 517.2536	518.3		11.0 min	5-50% B over 20min 

12 (PS)	**S7**	 Chemical Formula: $C_{25}H_{35}N_5O_7$ Exact Mass: 517.2536	518.2		11.4 min	5-50% B over 20min 
13 (PS)	**S7**	 Chemical Formula: $C_{25}H_{35}N_5O_7$ Exact Mass: 517.2536	518.2		11.5 min	5-50% B over 20min 
14 (PS)	**S12**	 Chemical Formula: $C_{38}H_{43}N_5O_8$ Exact Mass: 697.3112	698.3		16.3 min	5-95% B over 30min 

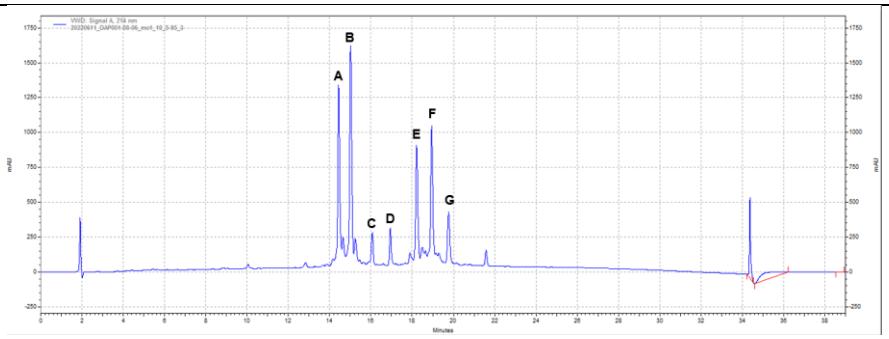
Supplementary Information

Peptidyl resin 1



Scheme S2: General synthetic scheme for reaction of peptidyl resin 1 (HMP-functionalised Tentagel ®) with Fmoc-L-Trp(Boc)-OH for 2 × 1 h, or 20 h (2 × 1 h, + 18 h) as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and products identified in Table S2. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S3.

Table S2: Additional peak characterisation following reaction of peptidyl resin 1. Representative trace following reaction of **1** with Fmoc-L-Trp(Boc)-OH, as shown in Scheme S2, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-95% B)	Observed m/z	Identification (Mw)
A		14.4 min	$[M+H]^+$ 1260.4 $[M+2H]^{2+}$ 630.4	**S1+CO$_2$** (1259.3)

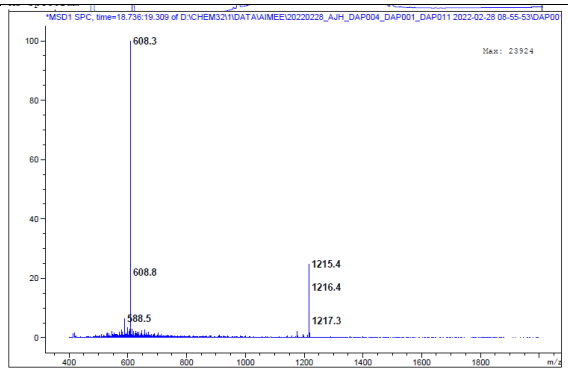
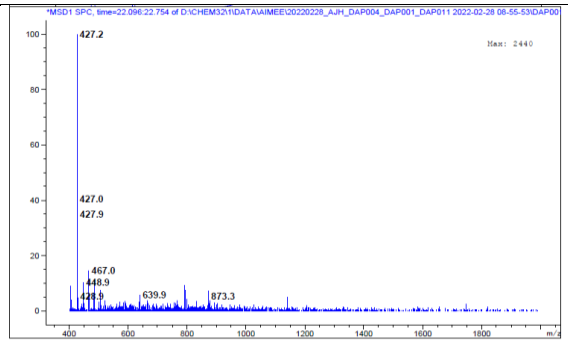
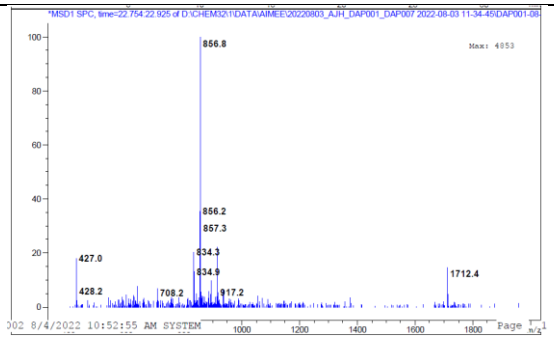
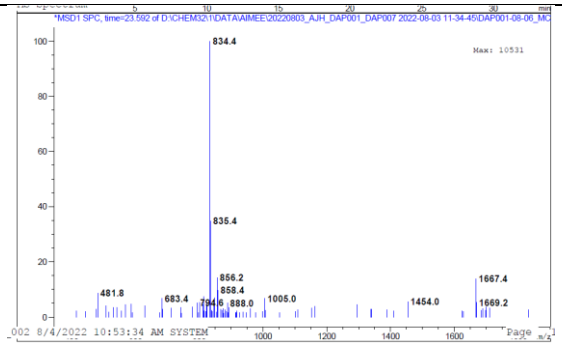
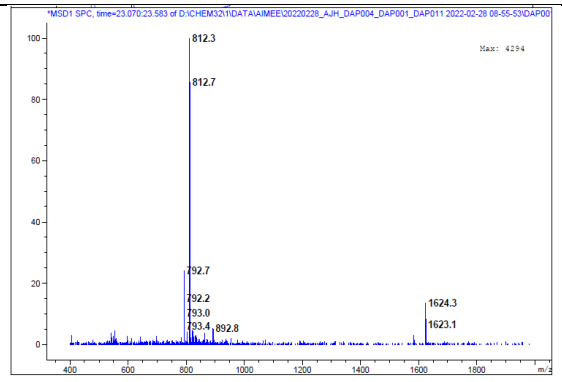
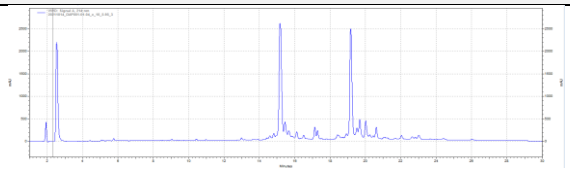
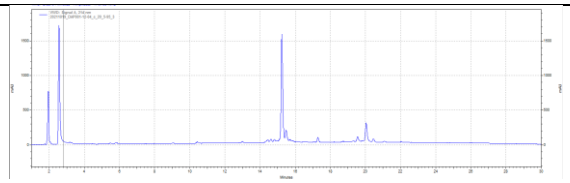
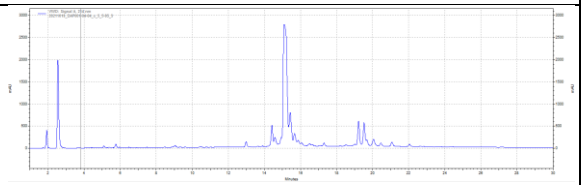
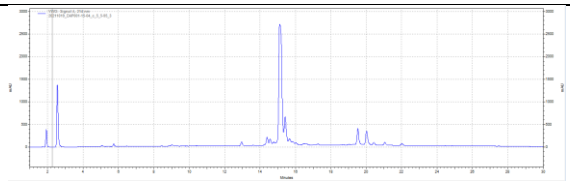
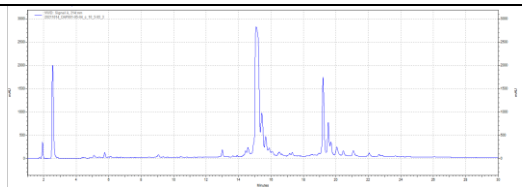
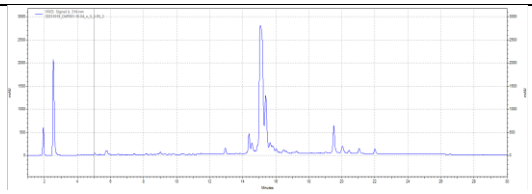
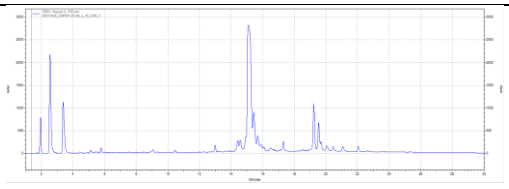
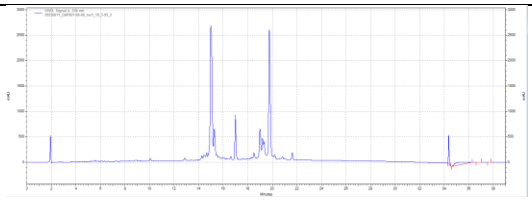
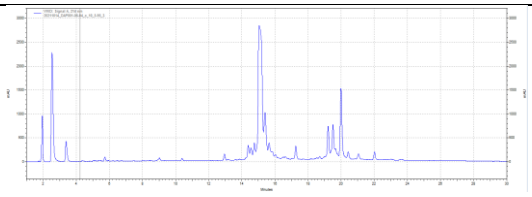
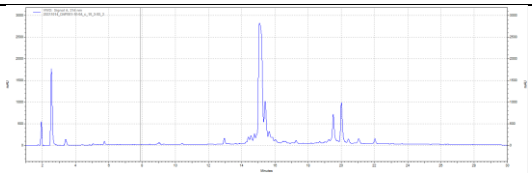
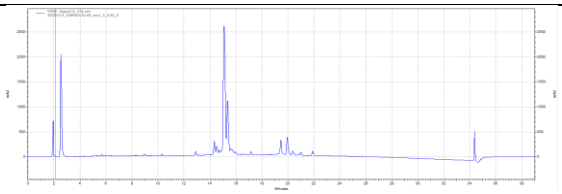
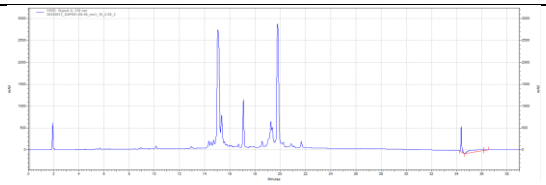
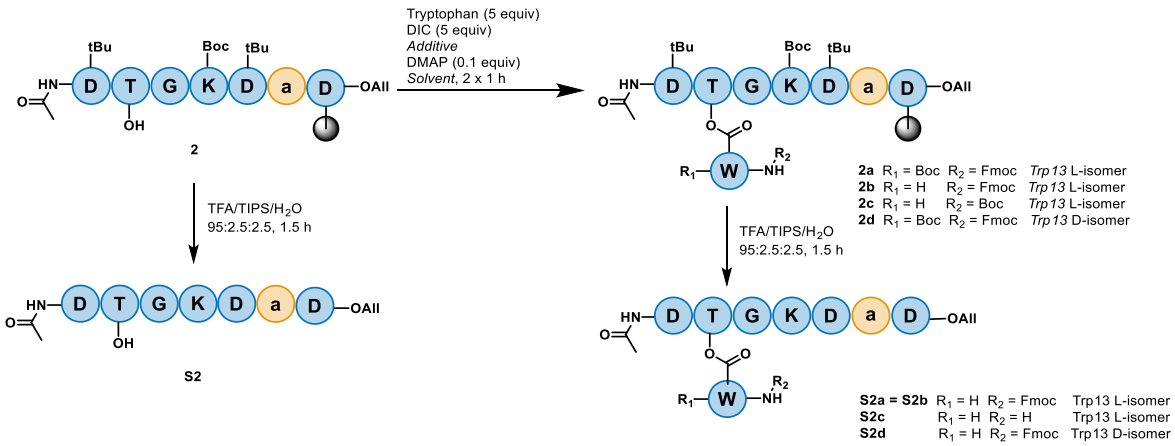
B		15.0 min	$[M+H]^+$ 1215.4 $[M+2H]^{2+}$ 608.2	**S1** (1214.6)
C / D		16.1 min 17.0 min	$[M+H]^+$ 471.0 $[M+H]^+$ 427.2	**C)** Fmoc-Trp-OH + CO_2 (470.2) **D)** Fmoc-Trp-OH (426.2)
E		18.2 min	$[M+H]^{2+}$ 856.8 $[M+H]^+$ 1712.4	**S1a+2CO₂** (1711.8)
F		18.9 min	$[M+H]^+$ 834.4 $[M+2H]^{2+}$ 1667.4	**S1a+CO₂** (1666.7)
G		19.8 min	$[M+H]^+$ 1624.3 $[M+2H]^{2+}$ 812.3	**S1a** (1622.7)

Table S3: RP-HPLC data following reaction of peptidyl resin 1 with Fmoc-L-Trp(Boc)-OH to give depsipeptidyl resin **1a**, as described in the Experimental section and depicted in Scheme S2. Entries 1-7 show data for Table 2 (entries 1-7), and entries 8-9 show data for Table 3 (entry 1). The conversion of peptidyl resin **1** to **1a** was determined as described in the Experimental section using the peaks of the linear (**S1**) and branched depsipeptide (**S1a**), and any corresponding adducts, specified.

Entry	Reaction time	Additive	Solvent	Conversion of 1 to 1a (%, 214 nm)	HPLC (5-95% B, 3%B/min, 214 nm)	R_t of peaks used for conversion calculation
1	2 × 1 h	-	DMF	10.6%		**S1** 15.2 min **S1a** 20.0 min
	2 × 1 h + 18 h	-	DMF	19.0%		**S1** 15.2 min **S1a** 20.0 min
2	2 × 1h	1% TritonX100	DMF	4.8%		**S1** 15.1 min **S1a** 20.0 min
	2 × 1 h + 18 h	1% TritonX100	DMF	8.5%		**S1** 15.1 min **S1a** 20.0 min
3	2 × 1h	10% TritonX100	DMF	4.9%		**S1** 15.1 min **S1a** 20.0 min
	2 × 1 h + 18 h	10% TritonX100	DMF	4.6%		**S1** 15.1 min **S1a** 20.0 min
4	2 × 1 h	-	NMP	3.5%		**S1** 15.1 min **S1a** 20.1 min
5	2 × 1 h	-	DCM	47.1%		**S1+CO_2** 14.4 min **S1** 14.9 min **S1a+CO_2** 19.0 min **S1a** 19.8 min

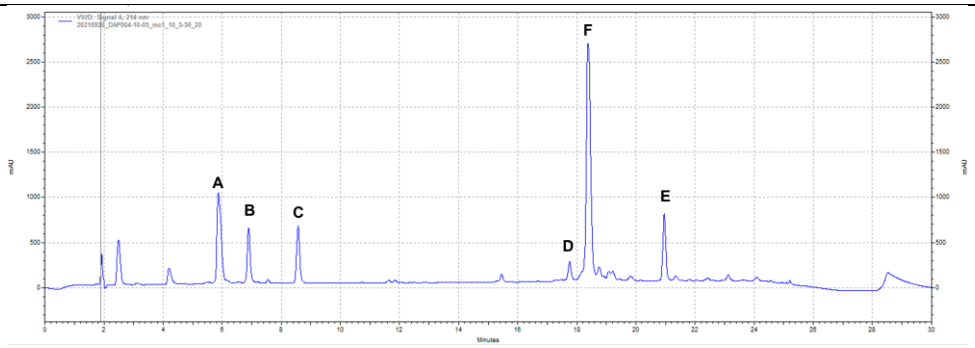
6	2 × 1 h	-	1:1:1 DMF:DCM: NMP	19.5%		**S1** 15.1 min **S1a** 20.0 min
7	2 × 1 h	1% TritonX100	1:1:1 DMF:DCM: NMP	14.3%		**S1** 15.2 min **S1a** 20.0 min
8	2 × 1 h	-	DMF	8.2%		**S1** 15.1 min **S1a** 20.0 min
9	2 × 1 h		DCM	48.2%		**S1** 15.1 min **S1a** 19.8 min

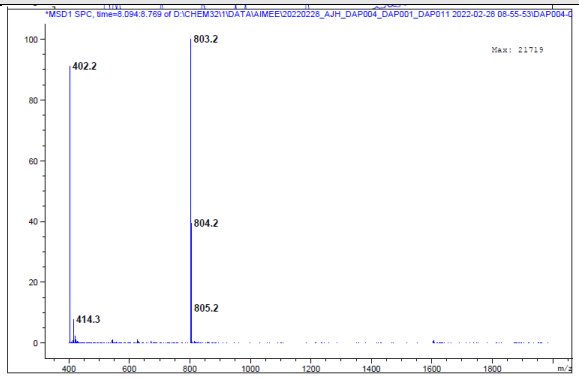
Peptidyl resin 2



Scheme S4: General synthetic scheme for reaction of peptidyl resin 2 (HMP-functionalised Tentagel ®) with appropriately protected tryptophan (specified in Table S5) for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S4. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S5.

Table S4: Additional peak characterisation. Representative trace following reaction of **2** with Fmoc L-Trp(Boc)-OH, as shown in Scheme S4, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification (Mw)
A		5.9 min	$[M+H]^+$ 803.2 $[M+2H]^{2+}$ 462.2	**S2** (802.8)

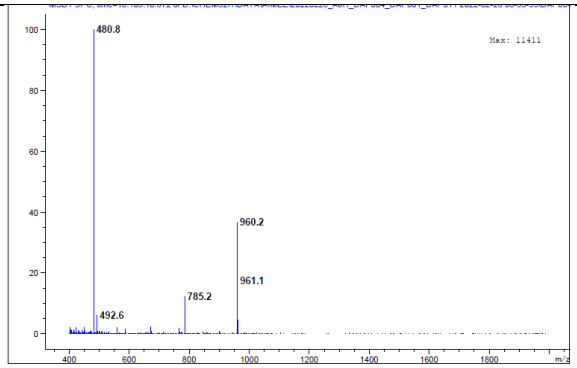
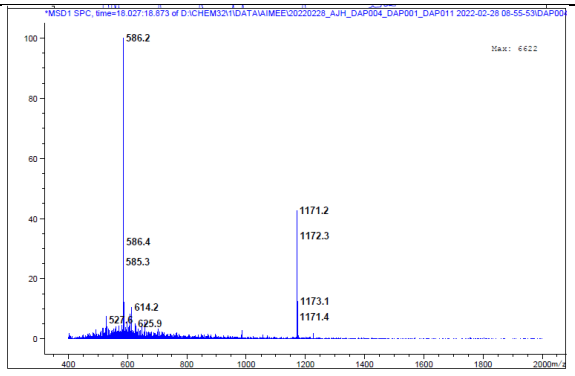
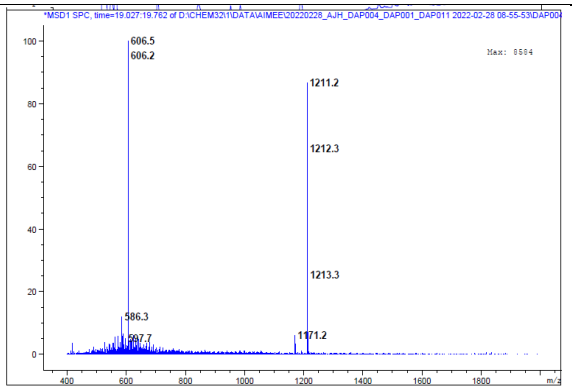
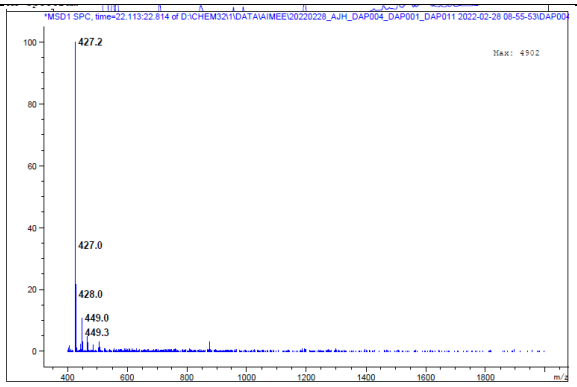
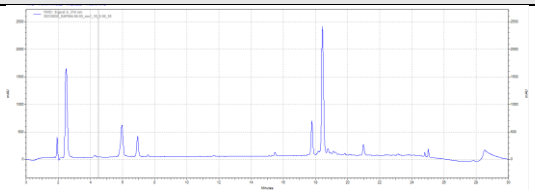
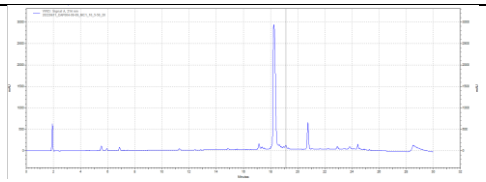
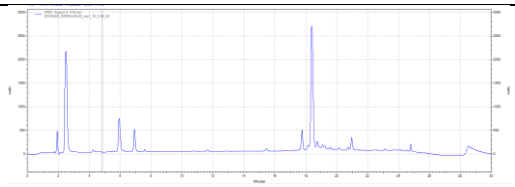
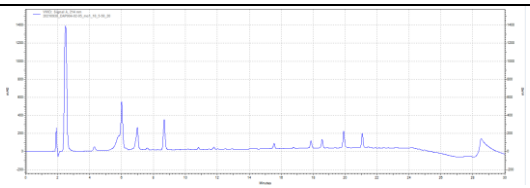
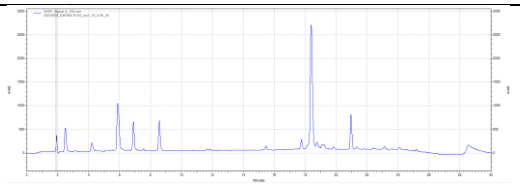
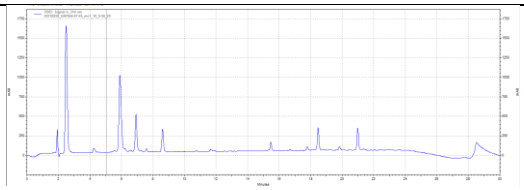
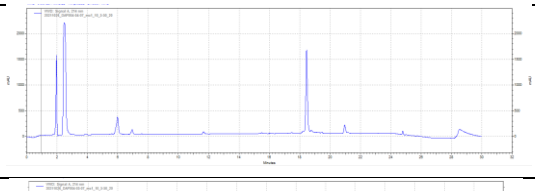
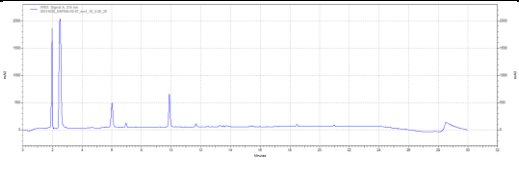
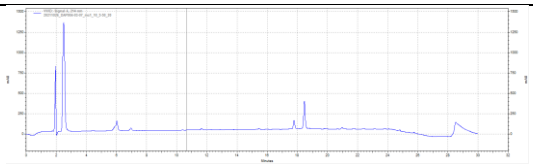
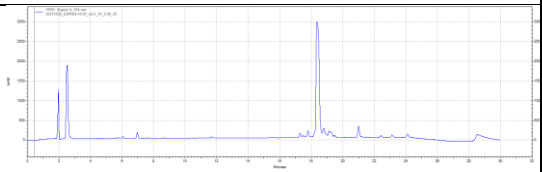
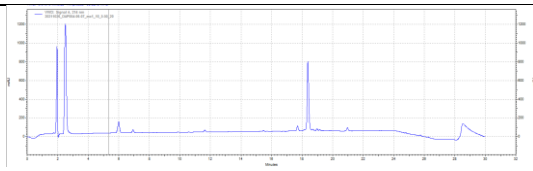
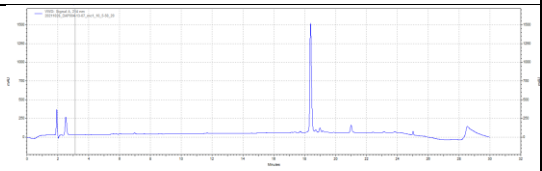
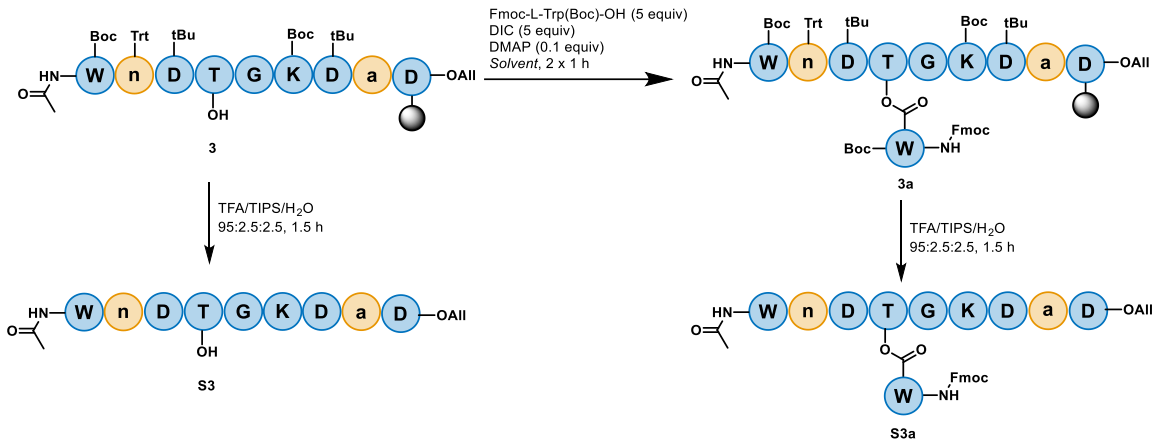
B		6.9 min	$[M+H]^+$ 960.2 $[M+2H]^{2+}$ 480.8	**S2** +156
C	-	8.5 min	*Identified by retention time against standard*	6-ClHOBt (169)
D	Decays readily in aqueous media. Identified in crude MS and by disappearance of HPLC peak over time.	17.7 min		**S2a+CO_2** (1254.2)
E		17.1 min	$[M+H]^+$ 1171.2 $[M+2H]^{2+}$ 586.2	**S2a-OAll** (1171.2)
F		18.4 min	$[M+H]^+$ 1211.2 $[M+2H]^{2+}$ 606.5	**S2a** (1211.2)
G/H		20.8 min	$[M+H]^+$ 427.2	Fmoc-Trp-OH (426.5)

Table S5: HPLC data following reaction of peptidyl resin 2 with an appropriately protected tryptophan to give the corresponding depsipeptidyl resin as described in the Experimental section and depicted in Scheme S4. Entries 1-7 show data for Table 2 (entries 8-15), and entries 8-11 show data for Table 3 (entries 2 & 6).The conversion was determined as described in the Experimental section using the peaks of the linear (**S2**) and branched depsipeptide, and any corresponding adducts, specified below.

Entry	Tryptophan	Additive	Solvent	Depsipeptidyl resin	Conversion of 2 (%, 214 nm)	HPLC (5-50% B over 20 min, 214 nm)	R_t of peaks used for conversion calculation
1	Fmoc-L-Trp(Boc)-OH	-	DMF	**2a**	79.8%		**S2** 5.9 min **S2a+CO_2** 17.8 min **S2a** 18.4 min
2	Fmoc-L-Trp(Boc)-OH	-	DCM	**2a**	98.2%		**S2** 6.0 min **S2a** 18.3 min
3	Fmoc-L-Trp(Boc)-OH	-	Dry DMF	**2a**	82.5%		**S2** 5.9 min **S2a+CO_2** 17.8 min **S2a** 18.4 min
3	Fmoc-L-Trp(Boc)-OH	6ClHOBt	DMF	**2a**	10.1%		**S2** 6.0 min **S2a+CO_2** 17.9 min **S2a** 18.6 min
4	Fmoc-L-Trp(Boc)-OH	6ClHOBt	DCM *	**2a**	57.9%		**S2** 5.9 min **S2a+CO_2** 17.8 min **S2a** 18.4 min
5	Fmoc-L-Trp(Boc)-OH	6ClHOBt	Dry DMF	**2a**	17.1%		**S2** 5.9 min **S2a** 18.5 min
6	Fmoc-L-Trp(H)-OH	-	DMF	**2b**	67.6%		**S2** 6.0 min **S2a** 18.5 min
7	Boc-L-Trp(H)-OH	-	DMF	**2c**	51.2%		**S2** 6.0 min **S2c** 9.9 min

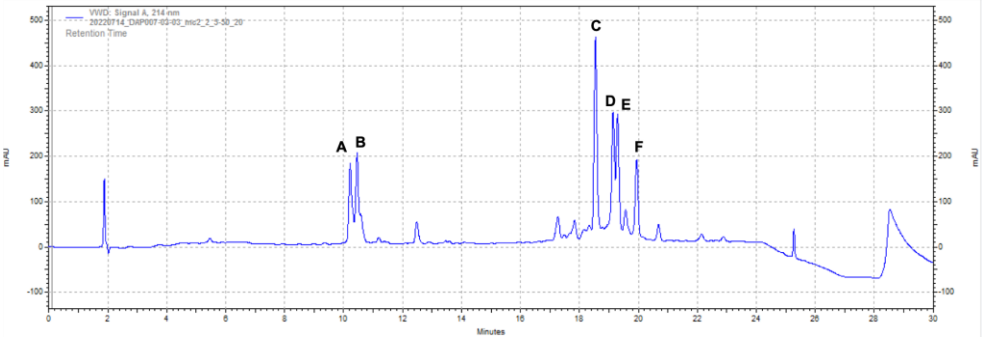
8	Fmoc-L-Trp(Boc)-OH	-	DMF	**2a**	62.5%		**S2** 6.0 min **S2a+CO_2** 17.8 min **S2a** 18.5 min
9	Fmoc-L-Trp(Boc)-OH	-	DCM	**2a**	98.8%		**S2** 6.0 min **S2a** 18.4 min
10	Fmoc-D-Trp(Boc)-OH	-	DMF	**2d**	73.9%		**S2** 6.0 min **S2a+CO_2** 17.7 min **S2d** 18.4 min
11	Fmoc-D-Trp(Boc)-OH	-	DCM	**2d**	99.0%		**S2** 6.0 min **S2d** 18.4 min

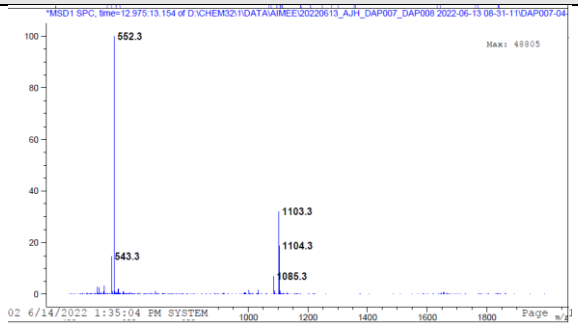
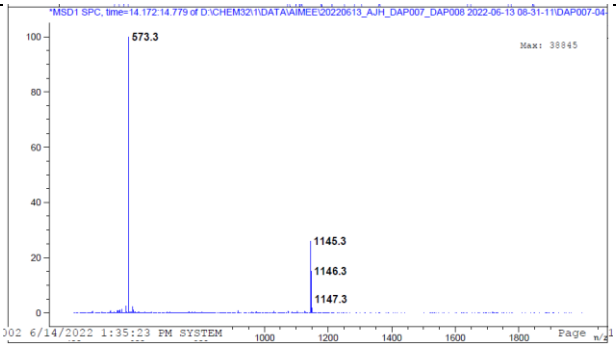
Peptidyl resin 3



Scheme S3: General synthetic scheme for reaction of peptidyl resin 3 (HMP-functionalised Tentagel ®) with Fmoc-L-Trp(Boc)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S6. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S7.

Table S6: Additional peak characterisation. Representative trace following reaction of **3** with Fmoc-L-Trp(Boc)-OH, as shown in Scheme S3, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification (Mw)
A		10.2 min	$[M+H]^+$ 1103.3 $[M+2H]^{2+}$ 552.3	**S3** (1103.1)
B		10.5 min	$[M+H]^+$ 1146.3 $[M+2H]^{2+}$ 573.3	**S3+CO$_2$** (1146.4)

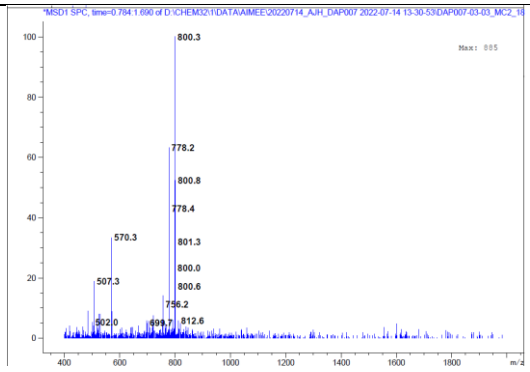
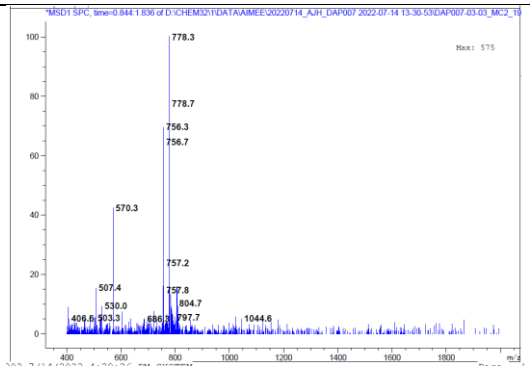
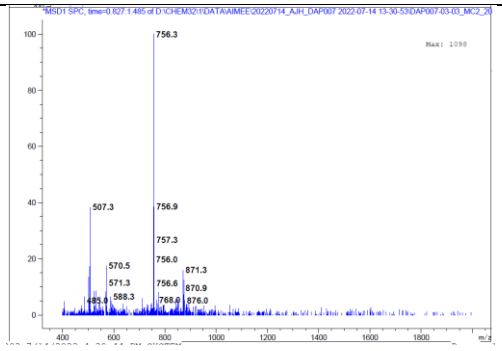
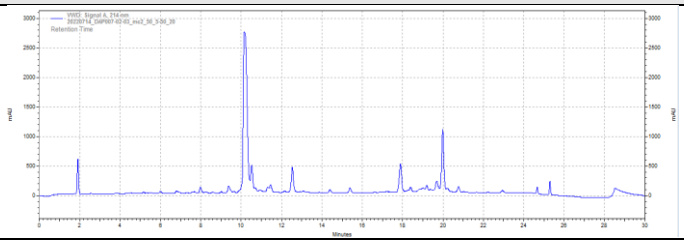
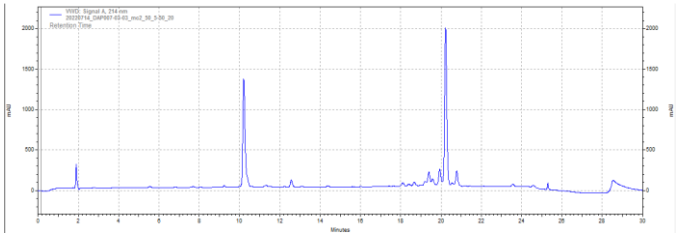
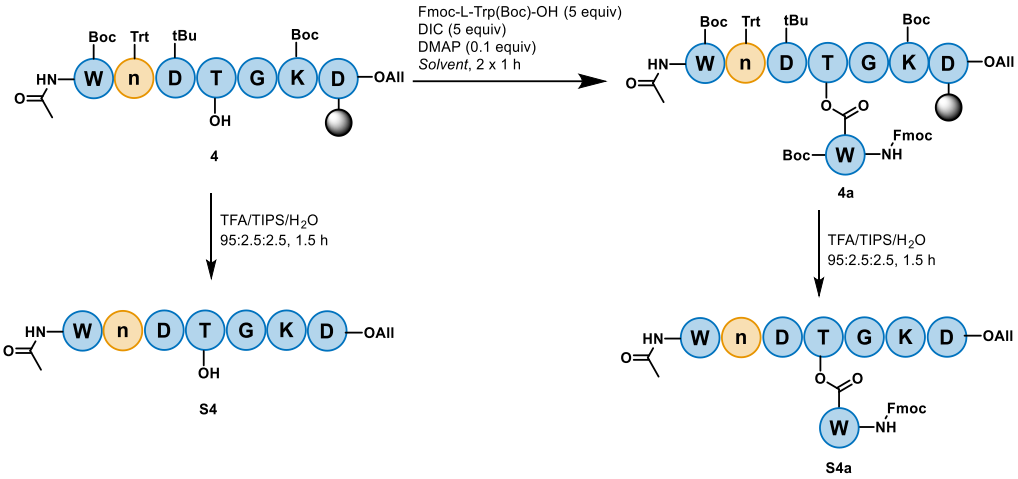
C		18.4 min	$[M+2H]^{2+}$ 800.3 *LCMS overlap/in situ degradation of CO_2 adduct* $[M+2H]^{2+}$ 778.3	**S3a+2CO₂** (1597.6) **S3a+CO₂** (1554.6)
D / E		19.1 min / 19.3 min	$[M+2H]^{2+}$ 778.3 *LCMS overlap/in situ degradation of CO_2 adduct* $[M+2H]^{2+}$ 756.3 $[M+3H]^{3+}$ 507.3	**D/E) S3a+CO₂** (1554.6) Two stereoisomers of CO_2 adduct possible **S3a**
F		19.9 min	$[M+2H]^{2+}$ 756.3 $[M+3H]^{3+}$ 507.3	**S3a** (1511.6)

Table S7: HPLC data following reaction of peptidyl resin 3 with Fmoc-L-Trp(Boc)-OH to give depsipeptidyl resin **3a**, as described in the Experimental section and depicted in Scheme S3. The conversion of peptidyl resin **3** to **3a** was determined as described in the Experimental section using the peaks of the linear (**S3**) and branched depsipeptide (**S3a**), and any corresponding adducts, specified.

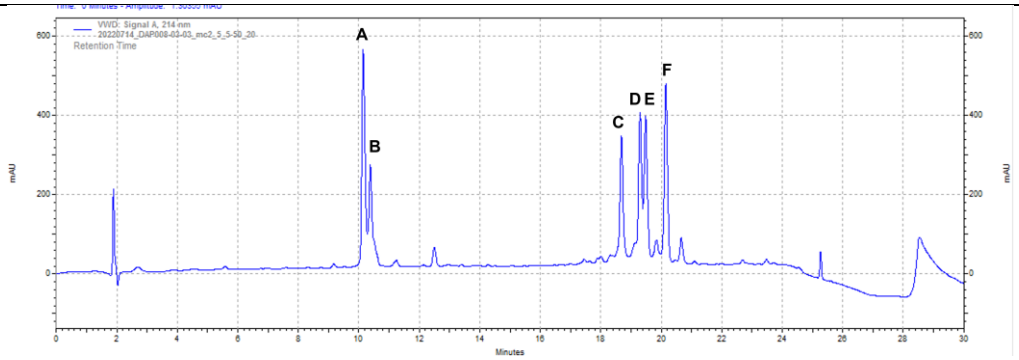
Entry	Solvent	Conversion of 3 to 3a (%, 214 nm)	HPLC (5-50%B over 20 min, 214 nm)	R_t of peaks used for conversion calculation
1	DMF	17.1%		**S3** 10.2 min **S3a** 20.0 min
2	DCM	50.6%		**S3** 10.2 min **S3a** 20.2 min

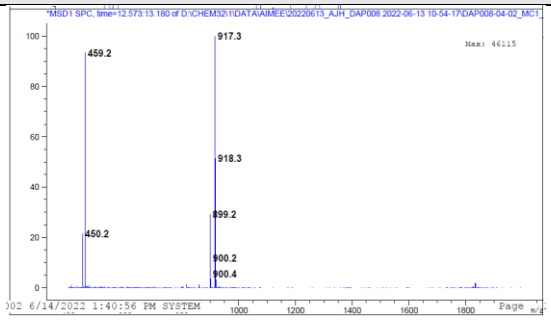
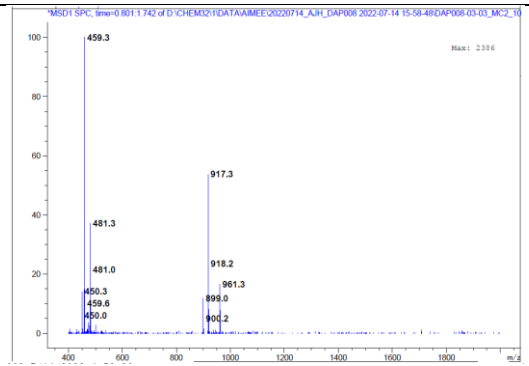
Peptidyl resin 4



Scheme S5: General synthetic scheme for reaction of peptidyl resin 4 (HMP-functionalised Tentagel ®) with Fmoc-L-Trp(Boc)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S8. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S9.

Table S8: Additional peak characterisation. Representative trace following reaction of **2** with Fmoc-L-Trp(Boc)-OH, as shown in Scheme S5, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification (Mw)
A		10.2 min	$[M+H]^+$ 917.3 $[M+2H]^{2+}$ 459.2 $[M+H]^+$ 899.2 $[M+2H]^{2+}$ 450.2	**S4** (916.4) **S4-H_2O** (898.4) Aspartimide
B		10.4 min	$[M+H]^+$ 961.3 $[M+2H]^{2+}$ 481.3 $[M+H]^+$ 917.3 $[M+2H]^{2+}$ 459.2 $[M+H]^+$ 899.2	**S4+CO_2** (960.0) CO_2 adduct on tryptophan *Overlapping signal gives mixed MS spectrum* **S4** (916.4) **S4-H_2O** (898.4)

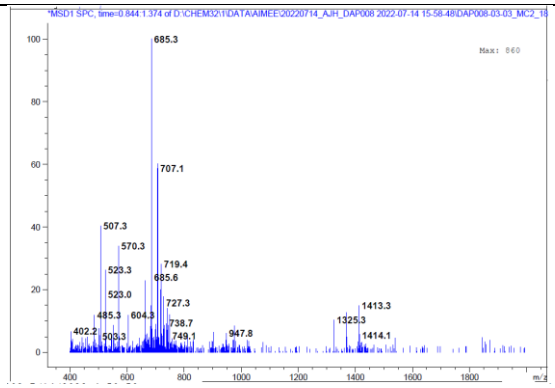
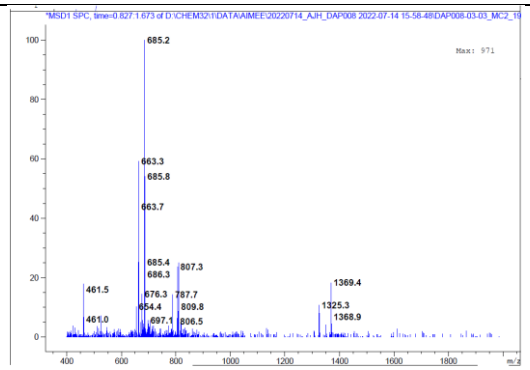
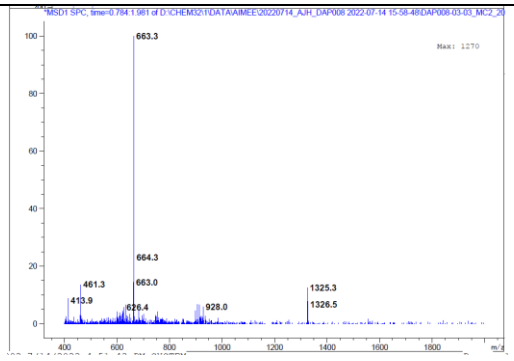
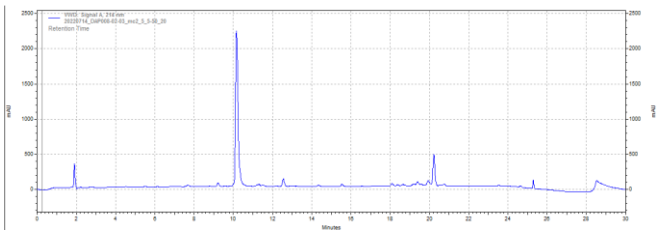
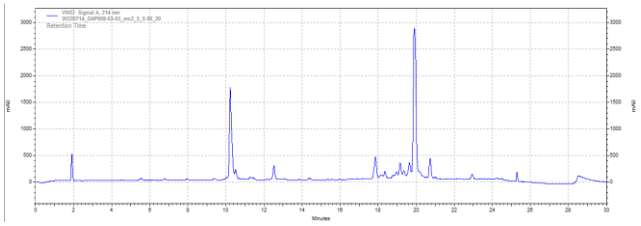
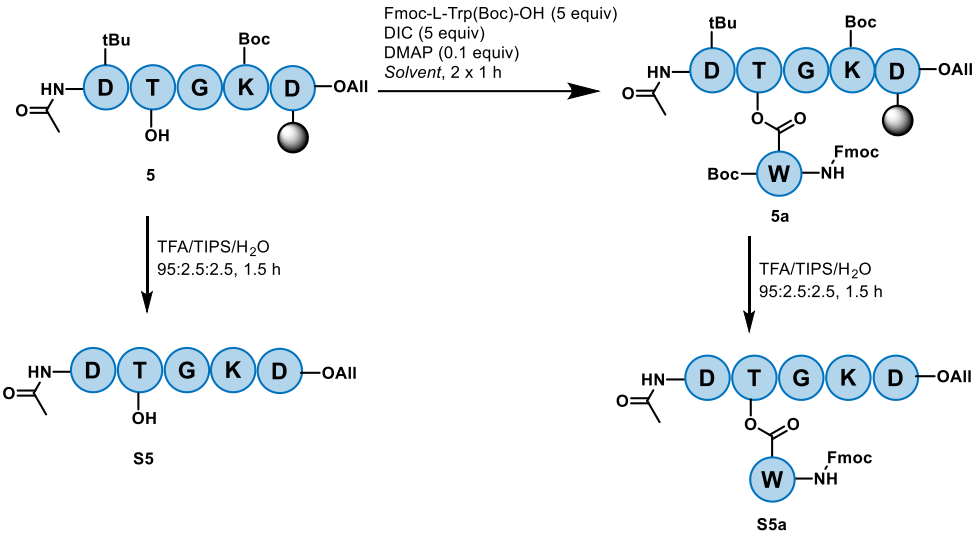
			[M+2H]$^{2+}$ 450.2	Aspartimide
C		18.7 min	[M+H]$^{+}$ 1413.3 [M+2H]$^{2+}$ 707.1 [M+H]$^{+}$ 1369.4 [M+2H]$^{2+}$ 685.3 [M+H]$^{+}$ 1325.3	**S4a+2CO$_2$** (1411.4) CO_2 adduct on both tryptophans *Overlapping MS signals & continual CO_2 adduct decay results in mixed MS spectrum* **S4a+CO$_2$** (1368.4) **S4a** (1325.4)
D/E		19.3 / 19.5 min	[M+H]$^{+}$ 1369.4 [M+2H]$^{2+}$ 685.3 [M+H]$^{+}$ 1325.3 [M+2H]$^{2+}$ 663.3	**D/E) S4a+CO$_2$** (1368.4) *Two stereoisomers of CO_2 adduct possible* *Overlapping MS signals* **S4a** (1325.4)
F		20.1 min	[M+H]$^{+}$ 1325.1 [M+2H]$^{2+}$ 663.3	**S4a** (1325.4)

Table S9: HPLC data following reaction of peptidyl resin 4 with Fmoc-L-Trp(Boc)-OH to give depsipeptidyl resin **4a**, as described in the Experimental section and depicted in Scheme S5. The conversion of peptidyl resin **4** to **4a** was determined as described in the Experimental section using the peaks of the linear (**S4**) and branched depsipeptide (**S4a**), and any corresponding adducts, specified.

Entry	Solvent	Conversion of 4 to 4a (%, 214 nm)	HPLC (5-50% B over 20 min, 214 nm)	R_t of peaks used for conversion calculation
1	DMF	12.2%		**S4** 10.2 min **S4a** 20.2 min

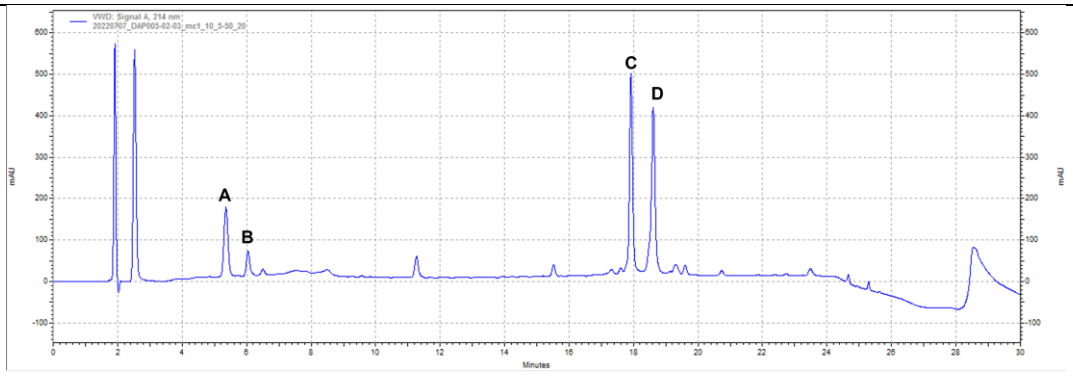
2	DCM	65.9%		**S4** 10.2 min **S4a** 19.9 min **S4a+2CO$_2$** 19.4 min **S4a+CO$_2$** 19.6 min

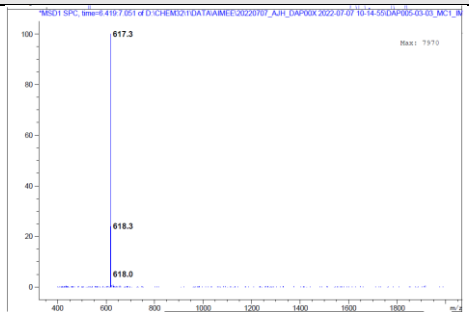
Peptidyl resin 5



Scheme S6: General synthetic scheme for reaction of peptidyl resin 5 (HMP-functionalised Tentagel ®) with Fmoc-L-Trp(Boc)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S10. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S11.

Table S10: Additional peak characterisation. Representative trace following reaction of **5** with Fmoc-L-Trp(Boc)-OH, as shown in Scheme S6, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification (Mw)
A		5.3 min	$[M+H]^+$ 617.3	**S5** (616.6)

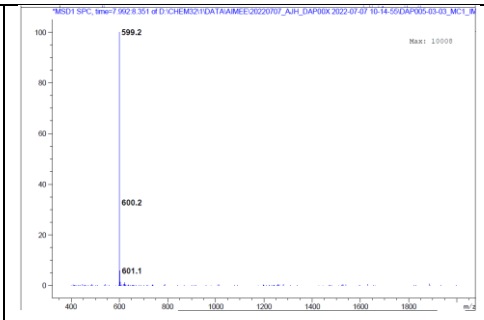
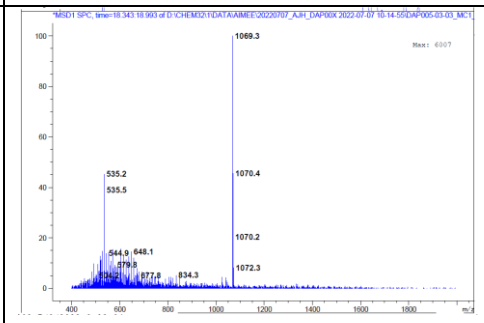
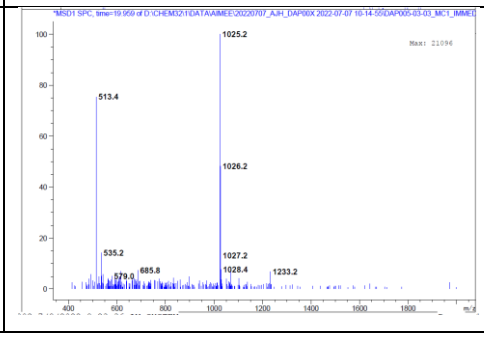
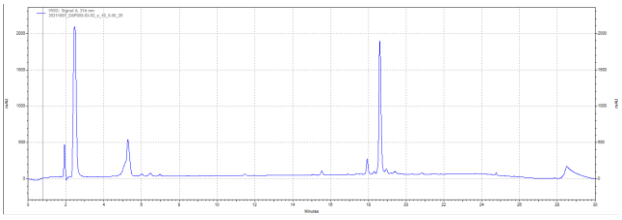
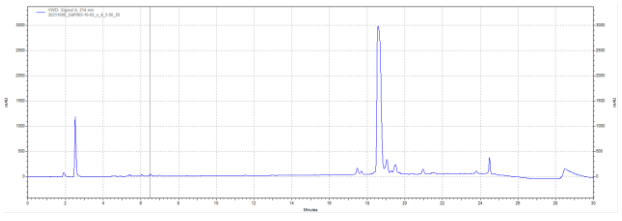
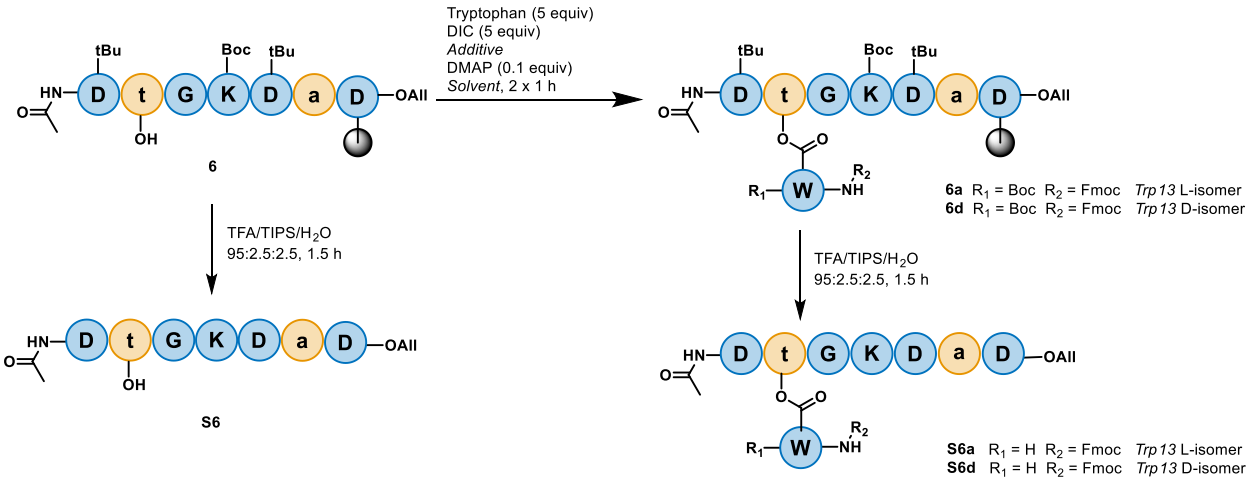
B		6.0 min	$[M+H]^+$ 599.2	**S5-H_2O** (598.6) Aspartimide
C		17.9 min	$[M+H]^+$ 1069.3 $[M+2H]^{2+}$ 535.2	**S5a+CO_2** (1068.1) CO_2 adduct on tryptophan
D		18.5 min	$[M+H]^+$ 1026.2 $[M+2H]^{2+}$ 513.4	**S5a** (1025.1)

Table S11: HPLC data following reaction of peptidyl resin 5 with Fmoc-L-Trp(Boc)-OH to give depsipeptidyl resin **5a**, as described in the Experimental section and depicted in Scheme S6. The conversion of peptidyl resin **5** to **5a** was determined as described in the Experimental section using the peaks of the linear (**S5**) and branched depsipeptide (**S5a**), and any corresponding adducts, specified..

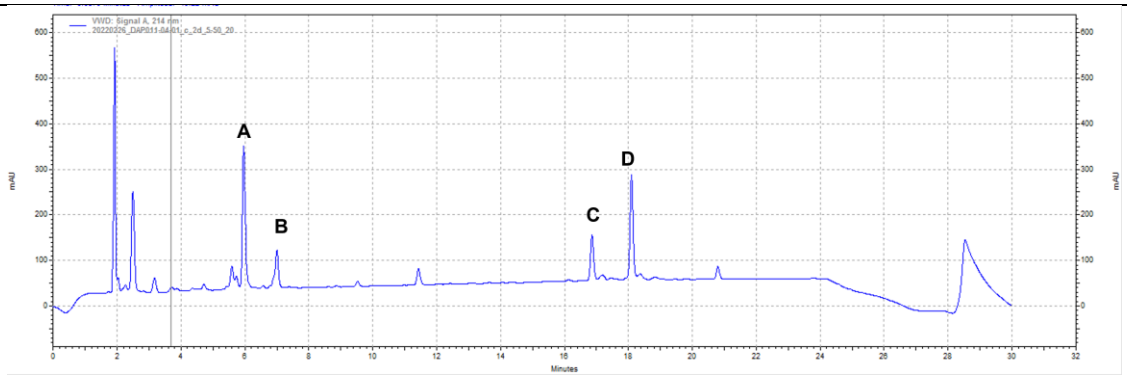
Entry	Solvent	Conversion of 5 to 5a (%, 214 nm)	HPLC (5-50% B, 214 nm)	R_t of peaks used for conversion calculation
1	DMF	74.2%		**S5** 5.3min **S5a+CO_2** 17.8 min **S5a** 18.6 min
2	DCM	99.3%		**S5** 5.1 min **S5a** 18.6 min

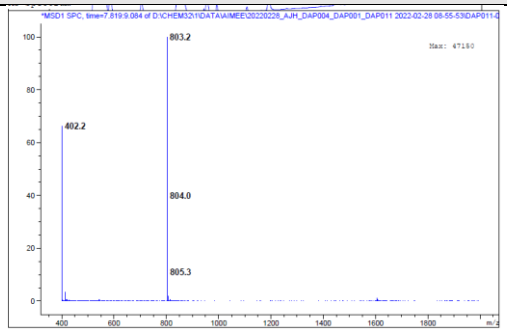
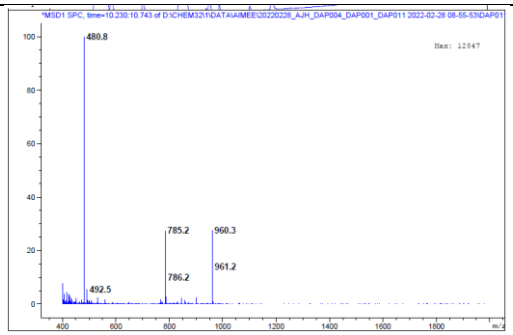
Peptidyl resin 6



Scheme S7: General synthetic scheme for reaction of peptidyl resin 6 (HMP-functionalised Tentagel ®) with Fmoc-Trp(Boc)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S12. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S13.

Table S12: Additional peak characterisation. Representative trace following reaction of **6** with Fmoc-L-Trp(Boc)-OH, as shown in Scheme S7, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification (Mw)
A		5.9 min	$[M+H]^+$ 402.2 $[M+2H]^{2+}$ 803.2	**S6** (802.8)
B		7.0 min	$[M+H]^+$ 960.3 $[M+2H]^{2+}$ 480.8 $[M+H]^+$ 785.2	**S6**+157 **S6-H₂O** (784.8) *Aspartimide*

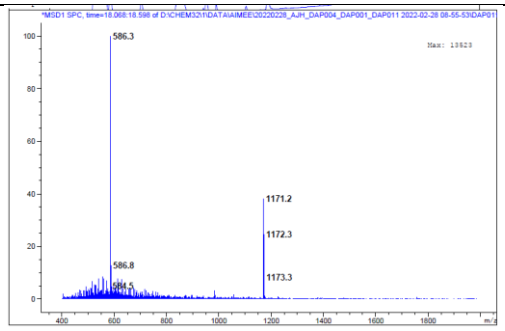
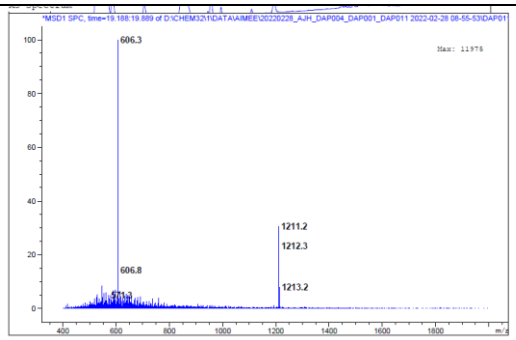
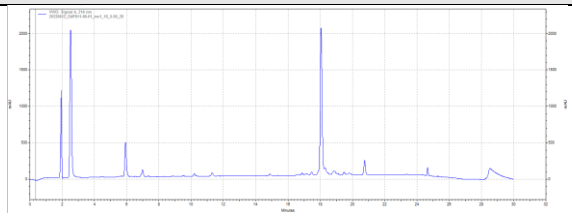
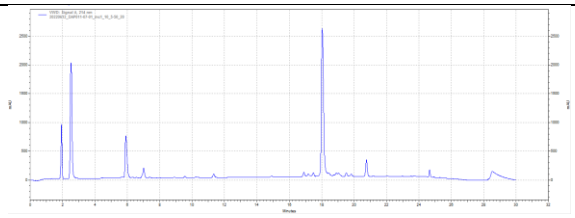
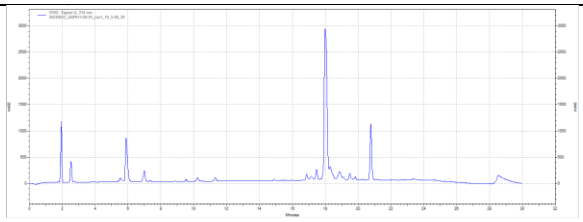
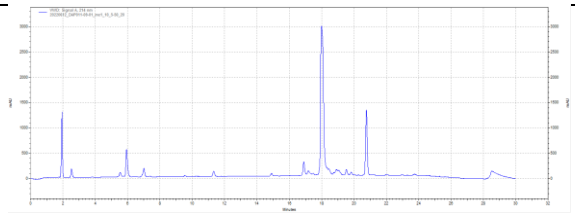
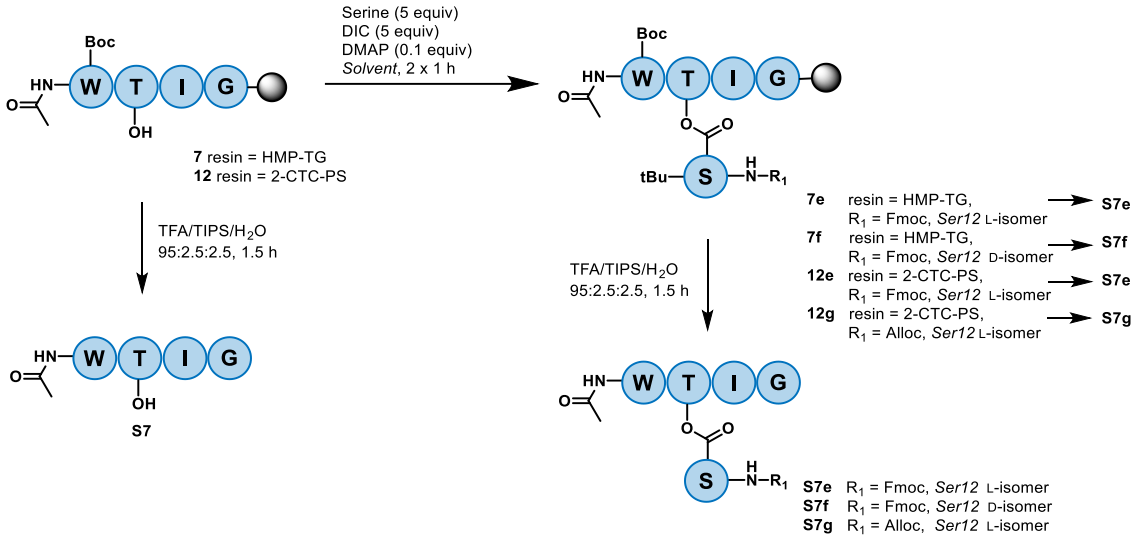
C		16.8 min	$[M+H]^+$ 1171.2 $[M+2H]^{2+}$ 586.3	**S6a-OAll** (1170.5) *Hydrolysis of allyl ester in solution*
D		18.0 min	$[M+H]^+$ 1211.1 $[M+2H]^{2+}$ 606.3	**S6a** (1211.2)

Table S13: HPLC data following reaction of peptidyl resin 6 with Fmoc-L-Trp(Boc)-OH to give depsipeptidyl resin **6a**, or with Fmoc-D-Trp(Boc)-OH to give depsipeptidyl resin **6d**, as described in the Experimental section and depicted in Scheme S7. The conversion of peptidyl resin **6** to **6a** or **6d** was determined as described in the Experimental section using the peaks of the linear (**S6**) and branched depsipeptide (**S6a** or **S6d**, respectively), and any corresponding adducts, specified.

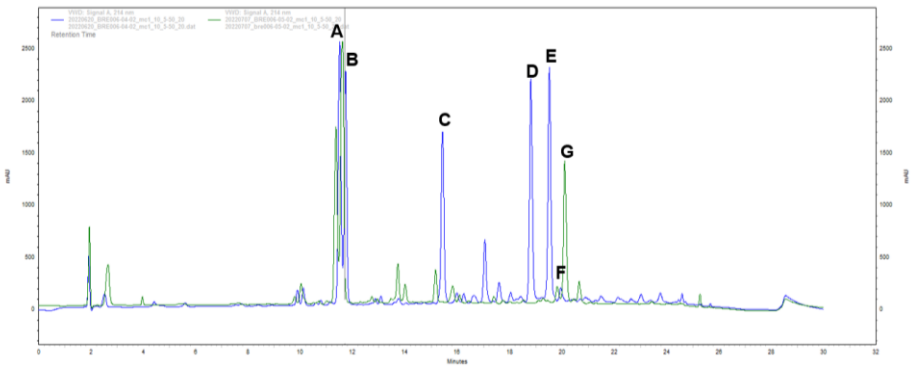
Entry	Fmoc-Trp(Boc)-OH	Solvent	Depsipeptidyl resin	Conversion of 6 (%, 214 nm)	HPLC (5-50% B over 20 min, 214 nm)	R_t of peaks used for conversion calculation
1	Fmoc-L-Trp(Boc)-OH	DMF	**6a**	81.6%		**S6** 5.9 min **S6a** 18.1 min
2	Fmoc-D-Trp(Boc)-OH	DMF	**6d**	78.2%		**S6** 5.9 min **S6d** 18.0 min
3	Fmoc-L-Trp(Boc)-OH	DCM	**6a**	80.9%		**S6** 5.9 min **S6a** 18.0 min
4	Fmoc-D-Trp(Boc)-OH	DCM	**6d**	87.2%		**S6** 5.9 min **S6d** 18.0 min

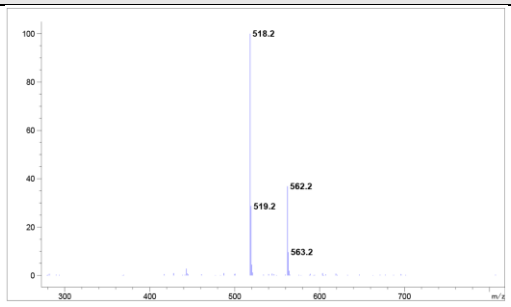
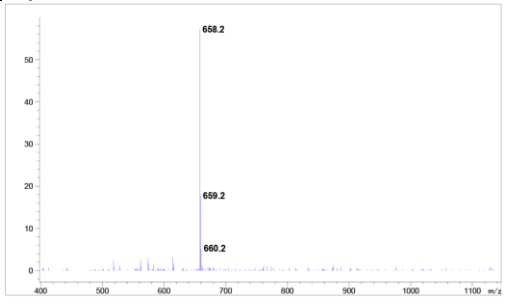
Peptidyl resins 7 & 12



Scheme S8: General synthetic scheme for reaction of peptidyl resins 7 (HMP-functionalised Tentagel ®) **& 12** (2-ClTrt functionalised polystyrene) with appropriately protected serine (specified in Table S16) for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S14. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S15.

Table S14: Additional peak characterisation. Representative trace following reaction of **7**, **12** or **13** with Fmoc-Ser(*t*Bu)-OH (in blue) and Alloc-L-Ser(*t*Bu)-OH (in green) as shown in Scheme S8, or Scheme 12**.** Key products **S7e-g** are identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification (Mw)
A/B		11.5 min / 11.7 min	$[M+H]^+$ 518.2 $[M+H]^+$ 562.2	*Overlapping signals* **A) S7** (517.6) **B) S7+CO_2** (561.6)
C		15.4 min	$[2M+H]^+$ 658.2	Fmoc-Ser-OH (327.1)

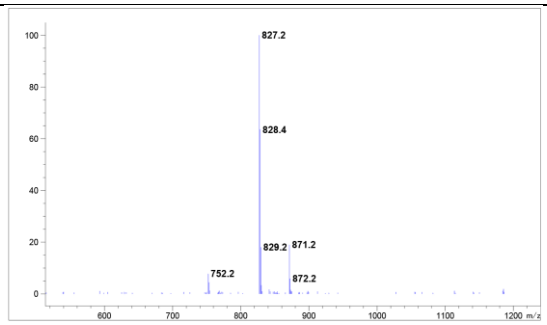
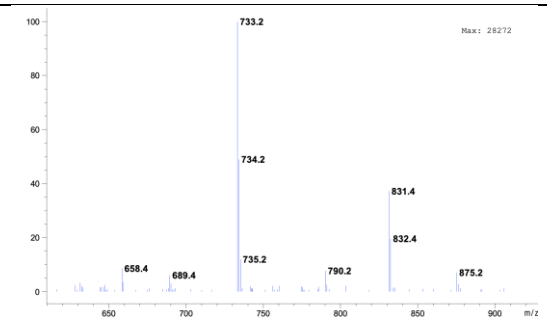
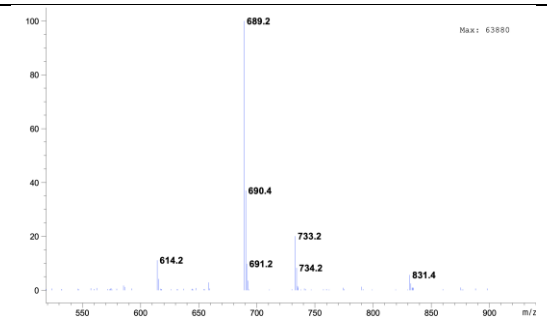
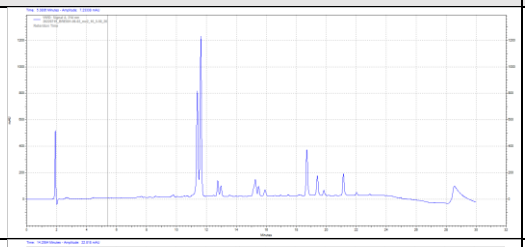
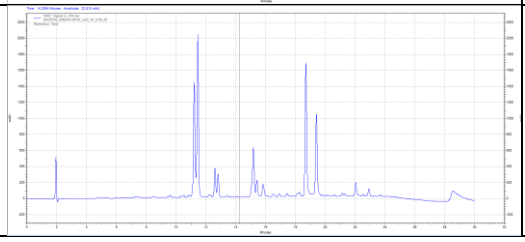
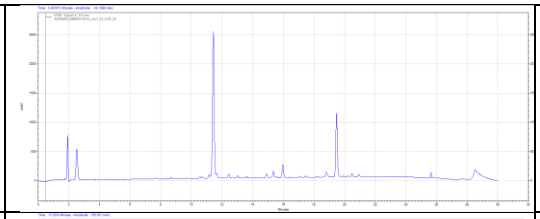
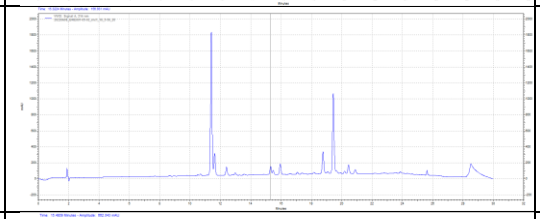
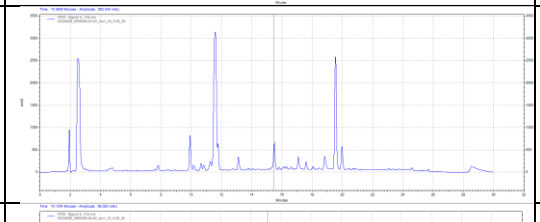
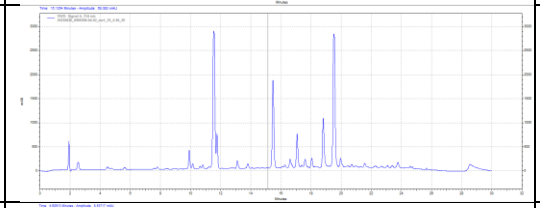
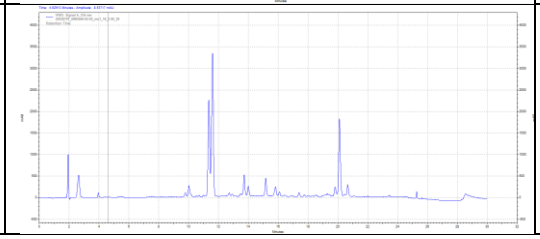
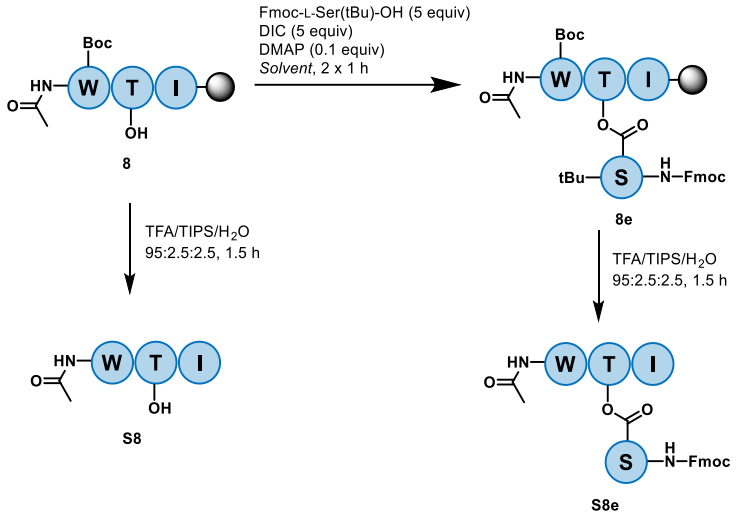
D/E		18.8min/ 19.5 min	$[M+H]^+$ 827.2 $[M+H]^+$ 871.2	*Overlapping signals* **D) S7e** (826.9) **E) S7e+CO_2** (870.9)
F		19.8 min	$[M+H]^+$ 733.2	**S12g+CO_2** (732.7)
G		20.1 min	$[M+H]^+$ 689.2	**S12g** (688.7)

Table S15: HPLC data following reaction of peptidyl resin 7 or 12 with appropriately protected serine to give the corresponding depsipeptidyl resin as described in the Experimental section and depicted in Scheme S8. The conversion was determined as described in the Experimental section using the peaks of the linear (**S7**) and corresponding branched depsipeptide and any corresponding adducts as specified below.

Entry	Peptidyl resin	Solvent	Serine	Depsipeptidyl resin	Conversion of peptidyl resin (%, 214 nm)	HPLC (5-50% B over 20 min, 214 nm)	R_t of peaks used for conversion calculation
1	**7**	DMF	Fmoc-L-Ser(*t*Bu)-OH	**7e**	20.2%		**S7** 11.5 min **S7+CO_2** 11.7 min **S7e+CO_2** 18.8 min **S7e** 19.5 min
2	**7**	DCM	Fmoc-L-Ser(*t*Bu)-OH	**7e**	47.4%		**S7** 11.5 min **S7+CO_2** 11.7 min **S7e+CO_2** 18.8 min **S7e** 19.5 min

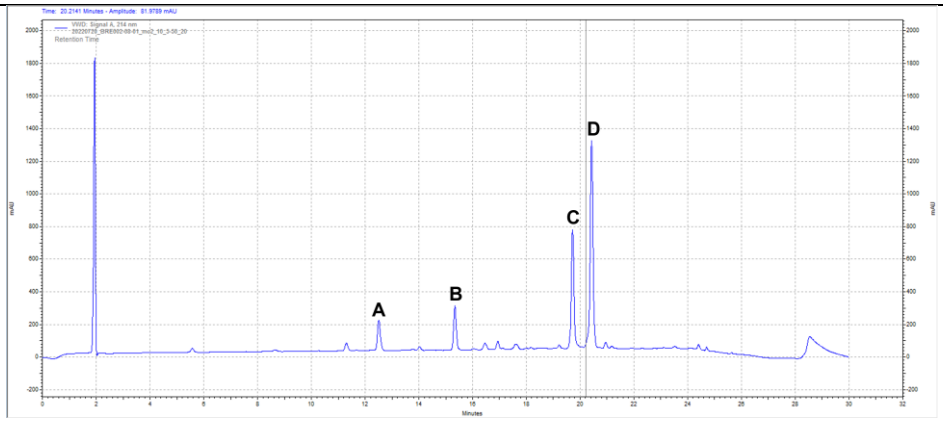
3	**7**	DMF	Fmoc-D-Ser(*t*Bu)-OH	**7f**	29.9%		**S7** 11.4 min **S7+CO_2** 11.6 min **S7f+CO_2** 18.8 min **S7f** 19.5 min
4	**7**	DCM	Fmoc-D-Ser(*t*Bu)-OH	**7f**	28.1%		**S7** 11.4 min **S7+CO_2** 11.6 min **S7f+CO_2** 18.8 min **S7f** 19.5 min
5	**12**	DMF	Fmoc-L-Ser(*t*Bu)-OH	**12e**	26.1%		**S7** 11.6 min **S7+CO_2** 11.8 min **S7e+CO_2** 18.8 min **S7e** 19.5 min
6	**12**	DCM	Fmoc-L-Ser(*t*Bu)-OH	**12e**	51.1%		**S7** 11.6 min **S7+CO_2** 11.8 min **S7e+CO_2** 18.8 min **S7e** 19.5 min
7	**12**	DCM	Alloc-L-Ser(*t*Bu)-OH	**12g**	17.8%		**S7** 11.4 min **S7+CO_2** 11.6 min **S7g+CO_2** 19.8 min **S7g** 20.1 min

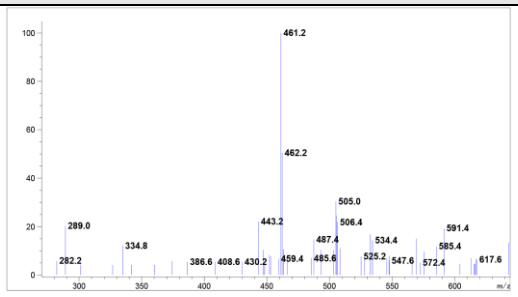
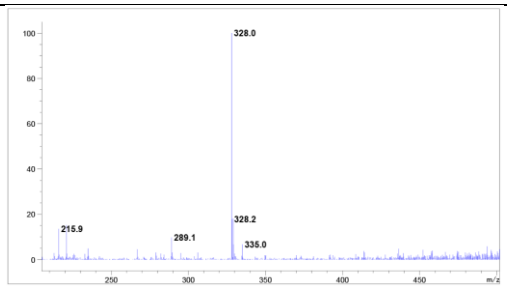
Peptidyl resin 8



Scheme S16: General synthetic scheme for reaction of peptidyl resin 8 with Fmoc-L-Ser(*t*Bu)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S16. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S17.

Table S16: Additional peak characterisation. Representative trace following reaction of **8** with Fmoc-L-Ser(*t*Bu)-OH, as shown in Scheme S9, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification (Mw)
A		12.5 min	$[M+H]^+$ 461.2	**S8** (460.5)
B		15.3 min	$[M+H]^+$ 328.0	Fmoc-Ser-OH (327.3)

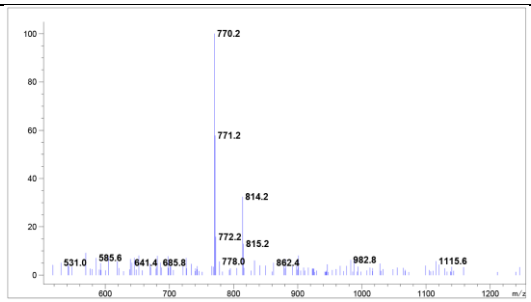
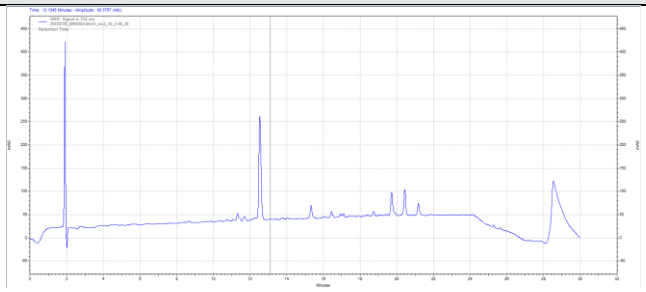
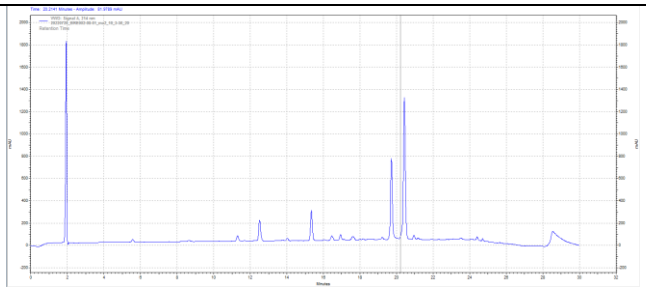
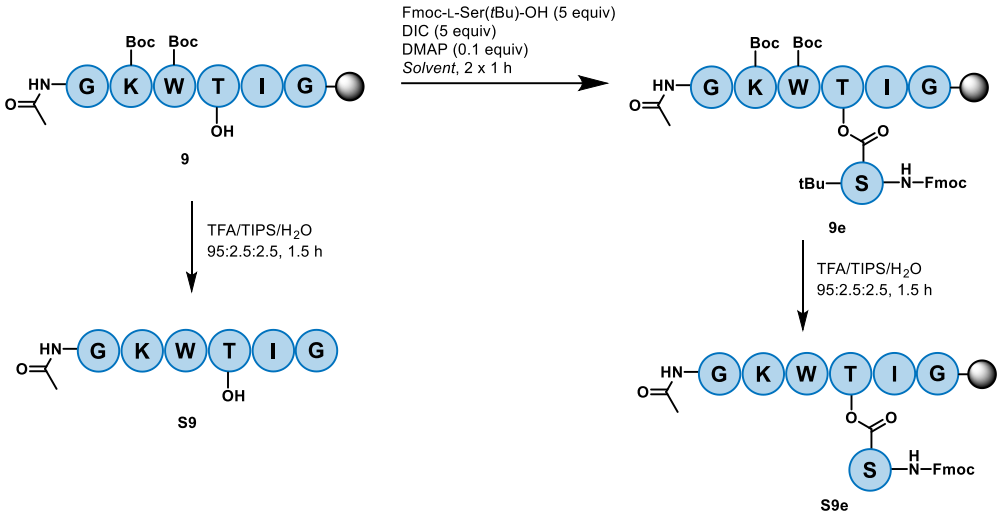
C/D		19.7 min/ 20.4 min	$[M+H]^+$ 814.0 $[M+H]^+$ 770.2	*Overlapping signals* **C) S8e+CO_2** (812.8) **D) S8e** (769.9)

Table S17: HPLC data following reaction of peptidyl resin 8 with Fmoc-L-Ser(*t*Bu)-OH to give depsipeptidyl resin **8e**, as described in the Experimental Section and depicted in Scheme S9. The conversion of peptidyl resin **8** to depsipeptidyl resin **8e** was determined as described in the Experimental Section using the peaks of the linear (**S8**) and branched depsipeptide (**S8e**), and any corresponding adducts, specified.

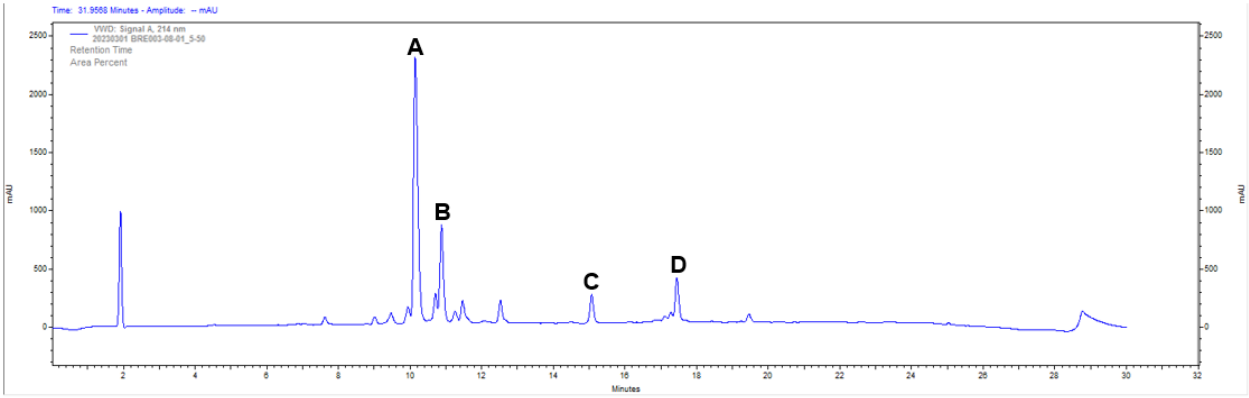
Entry	Solvent	Conversion of 8 to 8e (%, 214 nm)	HPLC (5-50% B over 20 min, 214 nm)	R_t of peaks used for conversion calculation
1	DMF	29.0%		**S8** 12.5 min **S8e+CO_2** 19.7 min **S8e** 20.4 min
2	DCM	90.8%		**S8** 12.5 min **S8e+CO_2** 19.7 min **S8e** 20.4 min

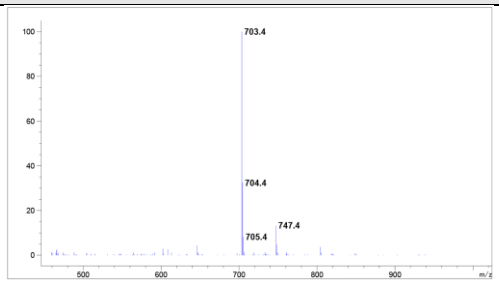
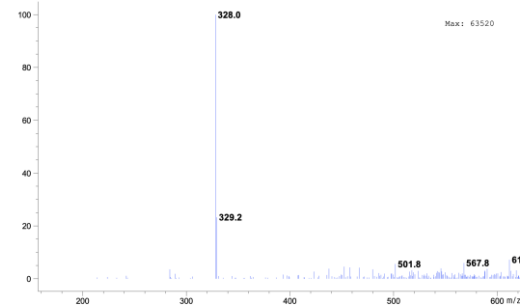
Peptidyl resin 9



Scheme S10: General synthetic scheme for reaction of peptidyl resin 9 with Fmoc-L-Ser(tBu)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the identified in Table S18. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S19.

Table S18: Additional peak characterisation. Representative trace following reaction of **9** with Fmoc-L-Ser(*t*Bu)-OH, as shown in Scheme S10, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification (Mw)
A/B		10.2 min / 10.7 min	[M+H]$^+$ 703.4 [M+H]$^+$ 747.2	*Overlapping signals* **A) S9** (702.4) **B) S9+CO$_2$** (746.4)
C		15.0 min	[M+H]$^+$ 328.0	Fmoc-Ser-OH (327.1)

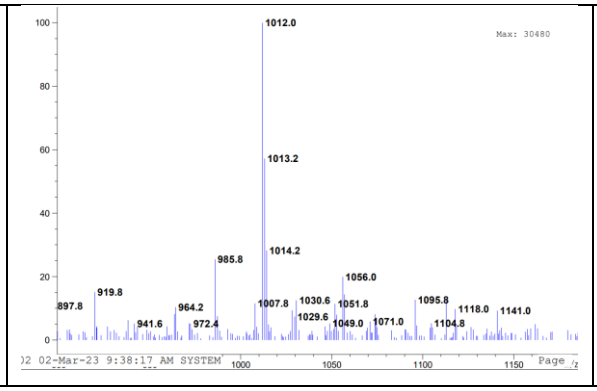
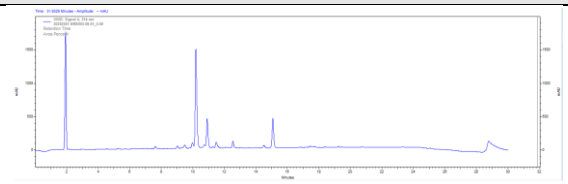
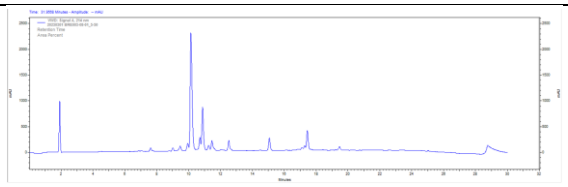
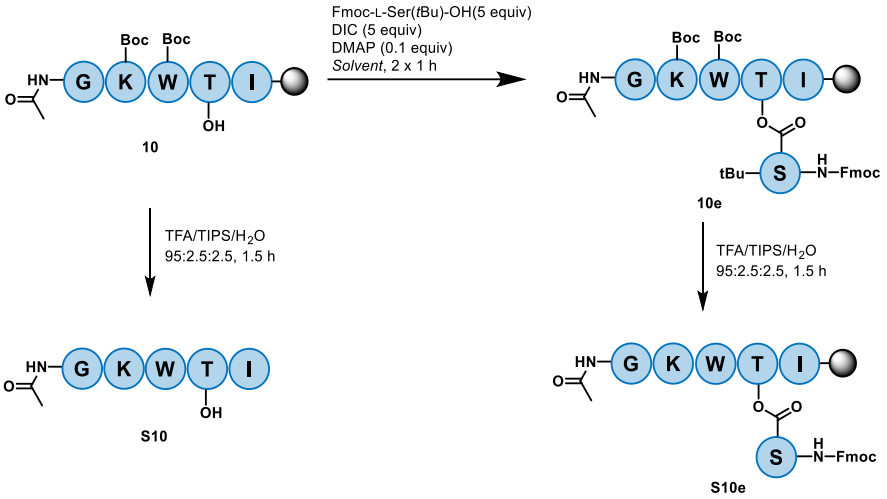
D		17.4 min	$[M+H]^+$ 1012.0	**S9e** (1011.5)

Table S19: HPLC data following reaction of peptidyl resin 9 with Fmoc-L-Ser(*t*Bu)-OH to give depsipeptidyl resin **9e**, as described in the Experimental section and depicted in Scheme S10. The conversion of peptidyl resin **9** to depsipeptidyl resin **9e** was determined as described in the Experimental section using the peaks of the linear (**S9**) and branched depsipeptide (**S9e**), and any corresponding adducts, specified.

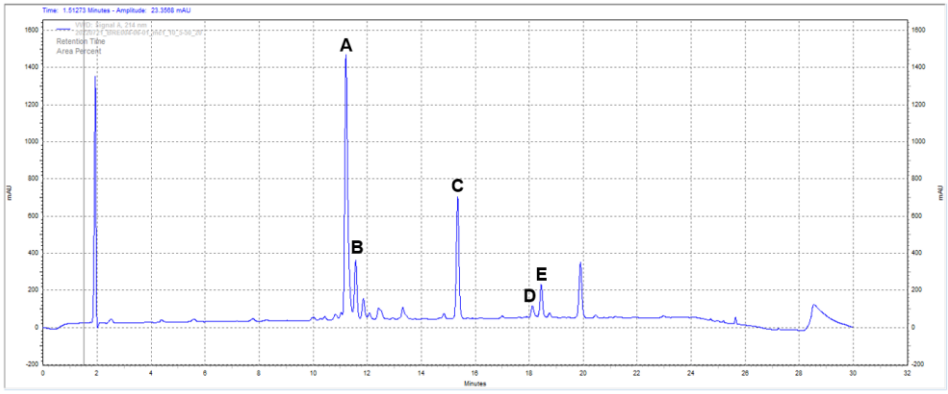
Entry	Solvent	Conversion of 9 to 9e (%, 214 nm)	HPLC (5-50% B over 20 min, 214 nm)	R_t of peaks used for conversion calculation
1	DMF	2.7%		**S9** 10.2 min **S9+CO_2** 10.6 min **S9e** 17.4 min
2	DCM	13.6%		**S9** 10.2 min **S9+CO_2** 10.7 min **S9e** 17.4 min

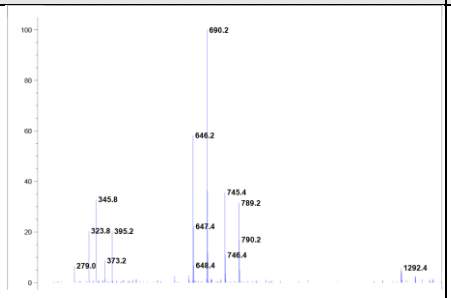
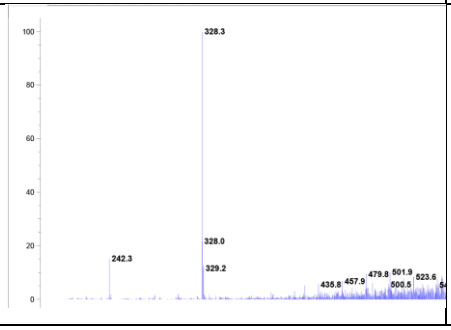
Peptidyl resin 10



Scheme S11: General synthetic scheme for reaction of peptidyl resin 10 with Fmoc-L-Ser(tBu)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S20. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S21.

Table S20: Additional peak characterisation. Representative trace following reaction of **10** with Fmoc-L-Ser(*t*Bu)-OH, as shown in Scheme S11, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification
A/B		11.2 min/ 11.6 min	[M+H]$^+$ 646.4 [M+H]$^+$ 690.2	*Overlapping signals* **A) S10** (645.8) **B) S10+CO$_2$** (689.8)
C		15.3 min	[M+H]$^+$ 328.3	Fmoc-Ser-OH (327.1)

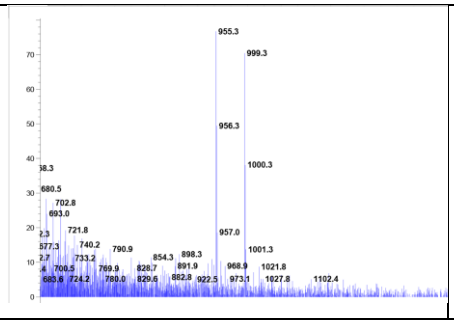
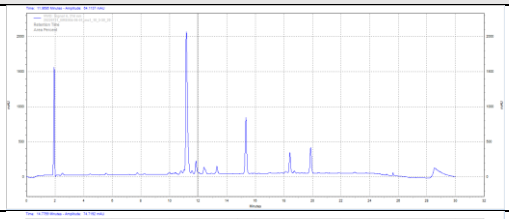
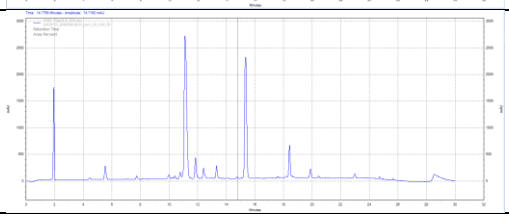
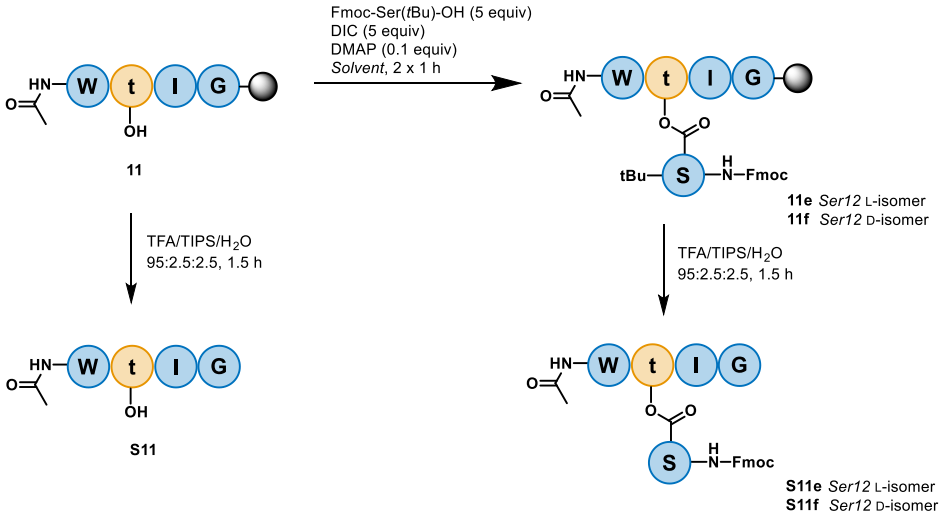
D/E		18.4 min 18.5 min	$[M+H]^+$ 999.3 $[M+H]^+$ 955.3	*Overlapping signals* **D) S10e+CO_2** (999.1) **E) S10e** (955.1)

Table S21: HPLC data following reaction of peptidyl resin 10 with Fmoc-L-Ser(*t*Bu)-OH to give depsipeptidyl resin **10e**, as described in the Experimental section and depicted in Scheme S11. The conversion of peptidyl resin **10** to depsipeptidyl resin **10e** was determined as described in the Experimental Section using the peaks of the linear (**S10**) and branched depsipeptide (**S10e**), and any corresponding adducts, specified.

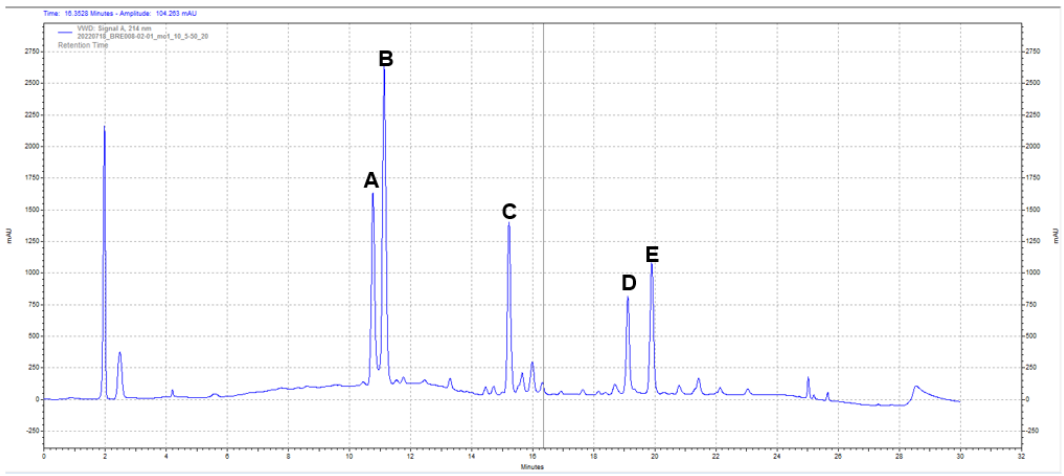
Entry	Solvent	Conversion of 10 to 10e (%, 214 nm)	HPLC (5-50% B over 20 min, 214 nm)	R_t of peaks used for conversion calculation
1	DMF	12.2%		**S10** 11.2 min **S10+CO_2** 11.6 min **S10e+CO_2** 18.1 min **S10e** 18.4 min
2	DCM	12.7%		**S10** 11.1 min **S10e+CO_2** 18.2 min **S10e** 18.4 min

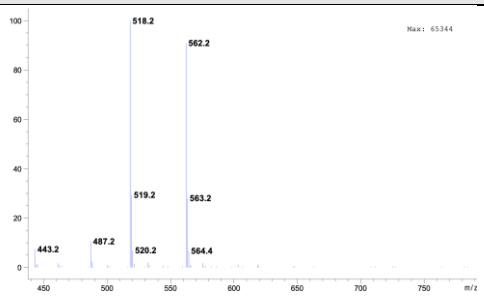
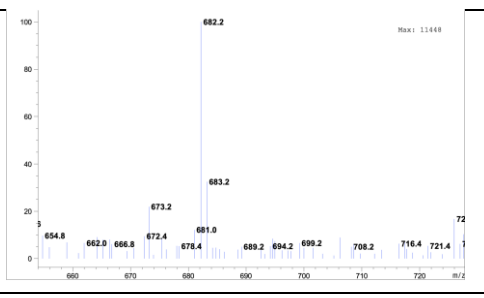
Peptidyl resin 11



Scheme S14: General synthetic scheme for reaction of peptidyl resin 11 with Fmoc-L-Ser(*t*Bu)-OH or Fmoc-D-Ser(*t*Bu)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S22. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S23.

Table S22: Additional peak characterisation. Representative trace following reaction of **11** with Fmoc-Ser(*t*Bu)-OH, as shown in Scheme S14, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-50% B)	Observed m/z	Identification (Mw)
A / B		11.2 min / 11.7 min	$[M+H]^+$ 518.2 $[M+H]^+$ 562.2	*Overlapping signals* **A) S11** (517.6) **B) S11+CO_2** (561.6)
C		15.5 min	$[2M+H]^+$ 682.2	Fmoc-Ser-OH (328.1)

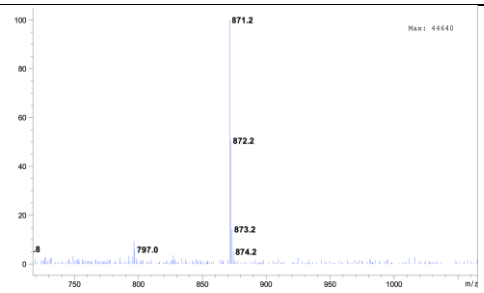
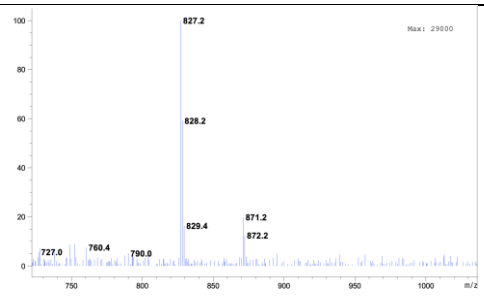
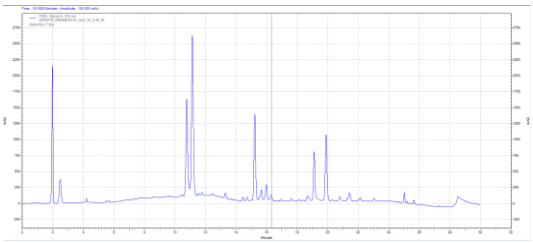
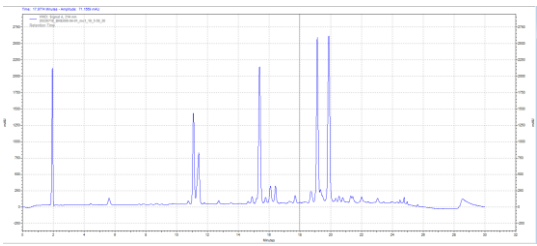
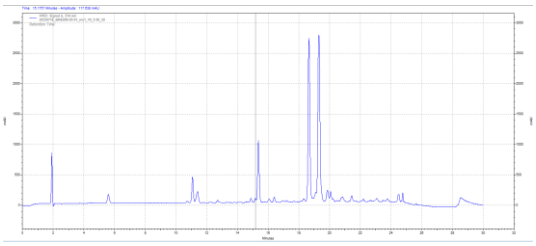
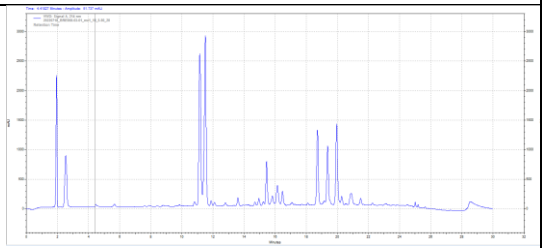
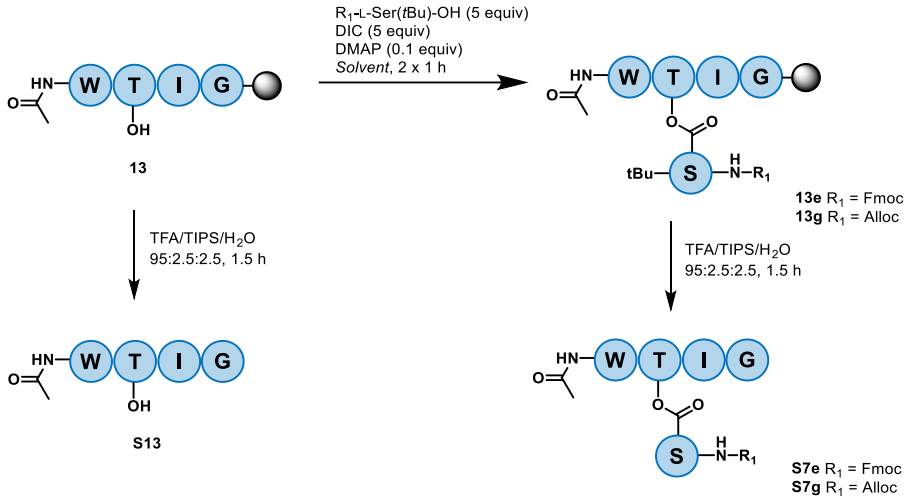
D		19.1 min	$[M+H]^+$ 871.2	**S7e+CO_2** (870.9)
E		19.9 min	$[M+H]^+$ 827.2	**S7e** (826.9)

Table S23: HPLC data following reaction of peptidyl resin 11 with Fmoc-L-Ser(*t*Bu)-OH to give depsipeptidyl resin **11e**; or with Fmoc-D-Ser(*t*Bu)-OH to give depsipeptidyl resin **11f,** as described in the Experimental section and depicted in Scheme S14. The conversion of peptidyl resin **11** to depsipeptidyl resin **11e** or **11f** was determined as described in the Experimental section using the peaks of the linear (**S11**) and branched depsipeptide (**S11e** or **S11f**, respectively), and any corresponding adducts, specified.

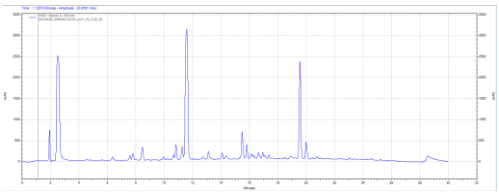
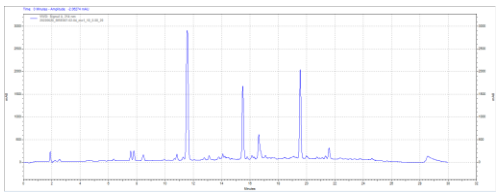
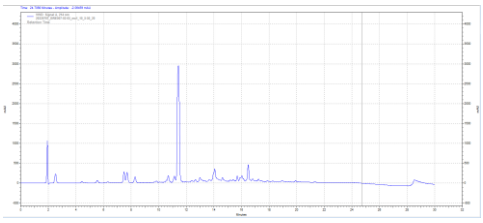
Entry	Serine	Solvent	Conversion of 11 (%, 214 nm)	Depsipeptidyl resin	HPLC (5-50% B over 20 min, 214 nm)	Peaks used for conversion
1	Fmoc-L-Ser(*t*Bu)-OH	DMF	31.1%	**11e**		**S11** 10.8 min **S11+CO_2** 11.1 min **S11e+CO_2** 19.1 min **S11e** 19.9 min
2	Fmoc-L-Ser(*t*Bu)-OH	DCM	62.7%	**11e**		**S11** 11.1 min **S11+CO_2** 11.4 min **S11e+CO_2** 19.1 min **S11e** 19.9 min
3	Fmoc-D-Ser(*t*Bu)-OH	DMF	31.0%	**11f**		**S11** 11.2min **S11+CO_2** 11.5 min **S11f+CO_2** 19.4 min **S11f** 19.9 min
4	Fmoc-D-Ser(*t*Bu)-OH	DCM	90.9%	**11f**		**S11** 11.1 min **S11+CO_2** 11.4 min **S11f+CO_2** 18.7 min **S11f** 19.3 min

Peptidyl resin 13

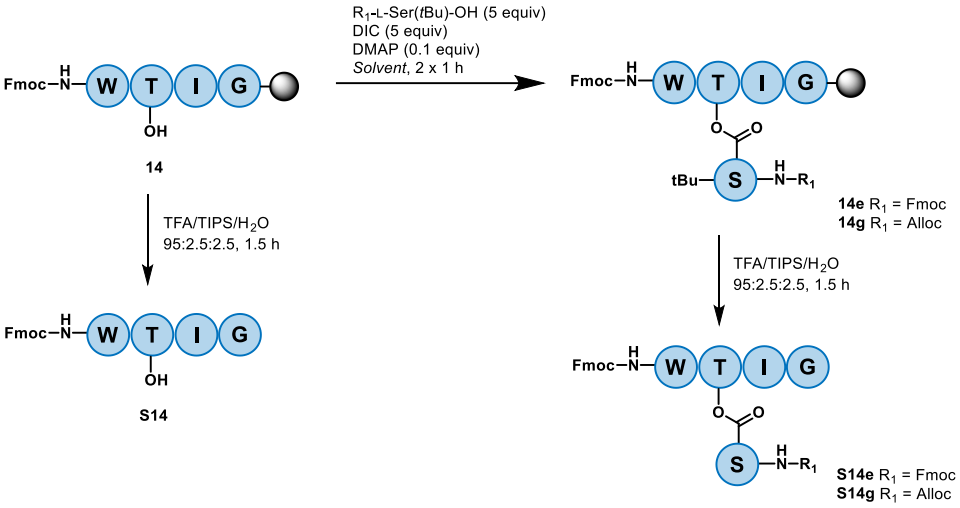


Scheme S12: General synthetic scheme for reaction of peptidyl resin 13 with Fmoc-L-Ser(*t*Bu)-OH or Alloc-L-Ser(*t*Bu)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S14. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S24.

Table S24: HPLC data following reaction of peptidyl resin 13 with Fmoc-L-Ser(tBu)-OH to give depsipeptidyl resin **13e**; or with Alloc-L-Ser(tBu)-OH to give depsipeptidyl resin **13g**, as described in the Experimental section and depicted in Scheme S12. The conversion of peptidyl resin **13** to depsipeptidyl resin **13e** or **13g** was determined as described in the Experimental section using the peaks of the linear (**S7**) and branched depsipeptide (**S7e** or **S7g**, respectively), and any corresponding adducts, specified.

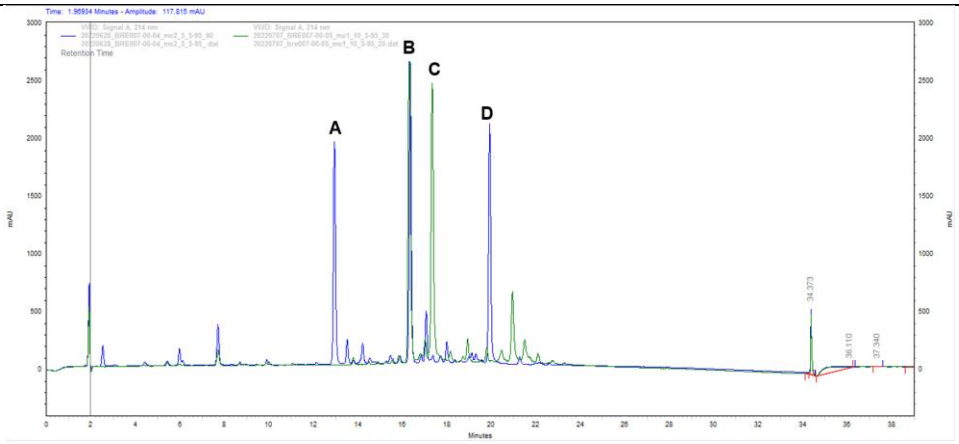
Entry	Solvent	Serine	Depsiptidyl resin	Conversion of 13 (%, 214 nm)	HPLC (5-50% B over 20 min, 214 nm)	R_t of peaks used for conversion calculation
1	DMF	Fmoc-L-Ser(*t*Bu)-OH	**13e**	30.9%		**S7** 11.6 min **S7e** 19.6 min
2	DCM	Fmoc-L-Ser(*t*Bu)-OH	**13e**	33.6%		**S7** 11.6 min **S7e** 19.6 min
3	DCM	Alloc-L-Ser(*t*Bu)-OH	**13g**	11.8%		**S7** 11.4 min **S7g** 16.5 min

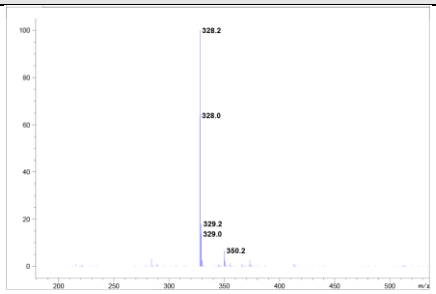
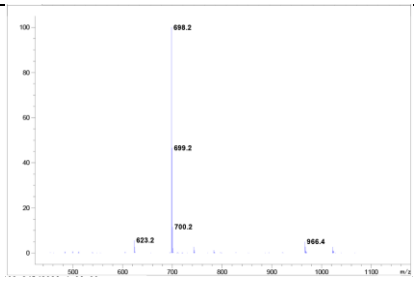
Peptidyl resin 14



Scheme S13: General synthetic scheme for reaction of peptidyl resin 14 with Fmoc-L-Ser(*t*Bu)-OH or Alloc-L-Ser(*t*Bu)-OH for 2 × 1 h as described in the Experimental Section. The resulting reaction mixtures were characterised by RP-HPLC and MS and the products identified in Table S25. The experimental conditions were varied and the resulting RP-HPLC traces are summarised in Table S26.

Table S25: Additional peak characterisation. Representative trace following reaction of **14** with Fmoc-L-Ser(*t*Bu)-OH, as shown in Scheme S13, illustrating key products and potential by-products as identified by ESI+ MS.



Peak	Extracted LCMS ESI+	R_t (5-95% B)	Observed m/z	Identification
A		13.0 min	$[M+H]^+$ 328.2	Fmoc-Ser-OH (327.1)
B		16.4 min	$[M+H]^+$ 698.2	**S14** (697.8)

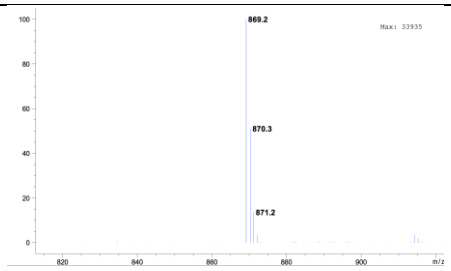
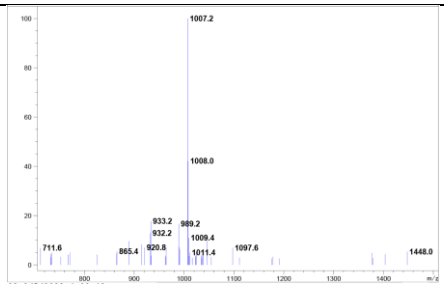
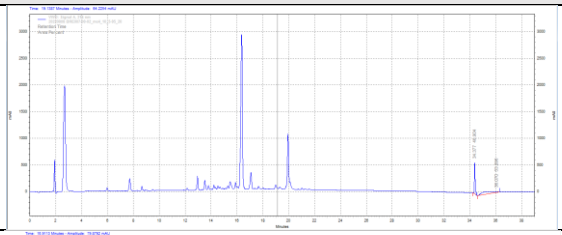
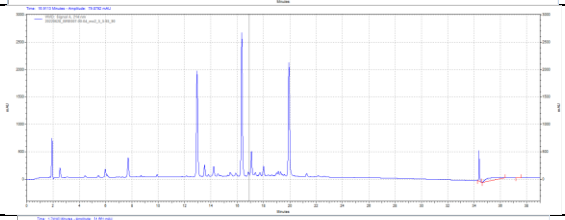
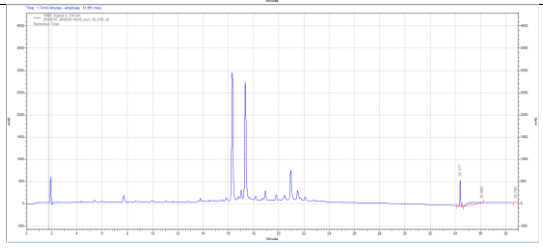
C		17.4 min	$[M+H]^+$ 869.2	**S14g** (868.9)
D		19.9 min	$[M+H]^+$ 1007.2	**S14e** (1007.1)

Table S26: HPLC data following reaction of peptidyl resin 14 with Fmoc-L-Ser(*t*Bu)-OH to give depsipeptidyl resin **14e**; or with Alloc-L-Ser(*t*Bu)-OH to give depsipeptidyl resin **14g**, as described in the Experimental section and depicted in Scheme S13. The conversion of peptidyl resin **14** to depsipeptidyl resin **14e** or **14g** was determined as described in the Experimental section using the peaks of the linear (**S12**) and branched depsipeptide (**S14e** or **S14g**, respectively), and any corresponding adducts, specified.

Entry	Solvent	Serine	Depsipeptidyl resin	Conversion of 14 (%, 214 nm)	HPLC (5-95% B, 3%B/min, 214 nm)	R_t of peaks used for conversion calculation
1	DMF	Fmoc-L-Ser(*t*Bu)-OH	**14e**	20.9%		**S14** 16.4 min **S14e** 19.9 min
2	DCM	Fmoc-L-Ser(*t*Bu)-OH	**14e**	62.3%		**S14** 16.4 min **S14e** 19.9 min
3	DCM	Alloc-L-Ser(tBu)-OH	**14g**	22.4%		**S14** 16.3 min **S14g** 17.4 min

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(3) Eissler, S.; Kley, M.; Bächle, D.; Loidl, G.; Meier, T.; Samson, D. Substitution Determination of Fmoc-Substituted Resins at Different Wavelengths. *J. Pept. Sci.* **2017**, *23* (10), 757–762. https://doi.org/10.1002/psc.3021.