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Monitoring the Solid Phase Synthesis of Analogues of Lysobactin and the Katanosins using *in situ* MALDI-TOF MS

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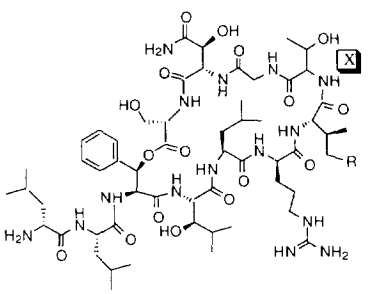
Abstract: *A method of solid phase reaction analysis is described using an in situ cleavage process (TFA vapour) followed by MALDI-TOF MS analysis. The process is demonstrated by the solid phase synthesis of a depsipeptide based on the antibiotic Lysobactin.* © 1997 Elsevier Science Ltd.

Lysobactin **(1)** is a depsipeptide antibiotic (Figure 1) isolated in 1988 from a species of *Lysobacter* (ATCC 53042) by a group from the Squibb Institute.[1-3] It was identified using a screen designed to detect antibiotics that bound to components found in a complex bacterial cell wall preparation, suggesting that it targets bacterial cell wall biosynthesis. It is suspiciously similar to katanosin A **(2)** and B **(3)** isolated from a culture broth of a strain related to the genus *Cytophaga* by a group from the Shionogi research laboratories also in 1988.[4] Interestingly, the katanosins have been reported to have the opposite stereochemistry at the L-*allo*-threonine position (marked X in Figure 1) found in lysobactin yet they still retain potent antibacterial activity.



(1) Lysobactin R = Me, X = L-*allo*-Threonine
(2) Katanosin A, R = H, X = D-*allo*-Threonine
(3) Katanosin B, R = Me, X = D-*allo*-Threonine

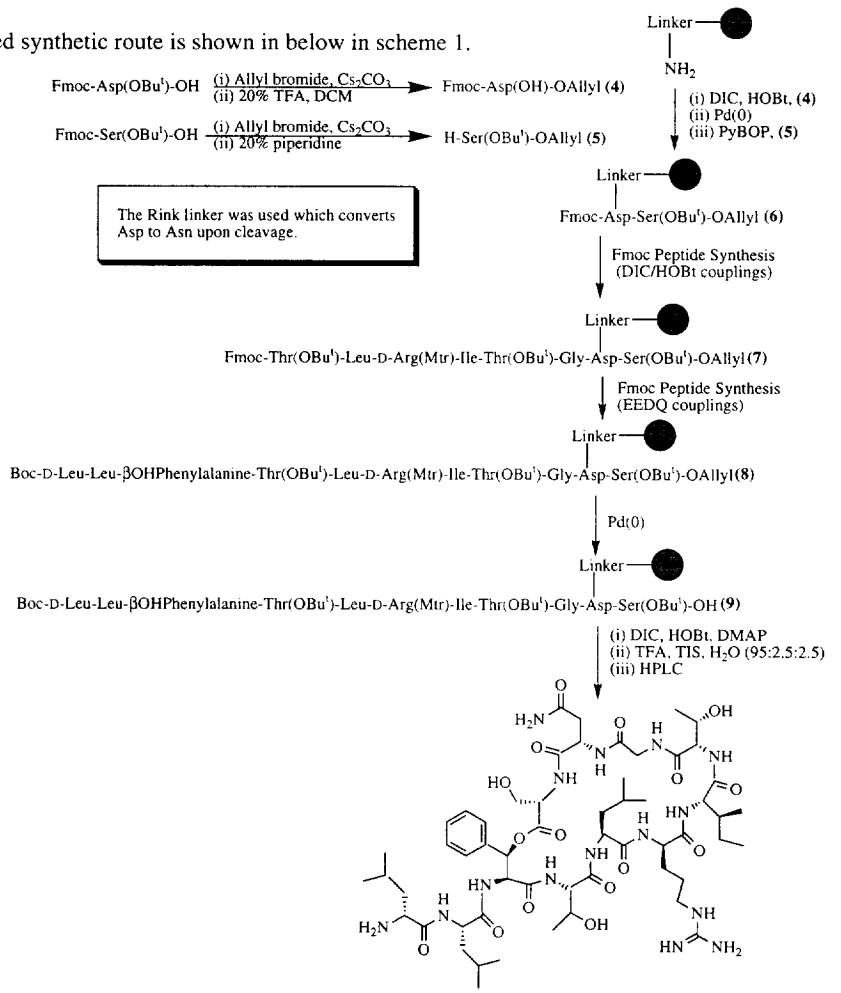
Figure 1

The spectrum of biological activity for lysobactin parallels that of vancomycin[1-3] although lysobactin is four times more potent (MIC mg/ml). The ED_{50} values for lysobactin compare favourably with vancomycin while it has been shown to be several hundred times more efficacious in the treatment of surface wounds. It is however slightly more toxic. Unfortunately, due to the different bacterial strains used in the reports,[1-4] direct comparison of the biological action of katanosin A and lysobactin is impossible. Experiments have also shown that the activity of lysobactin is not due to membrane disruption and that unlike vancomycin its activity is undiminished in the presence of D-Ala-D-Ala containing peptides, with evidence suggesting that lysobactin probably affects a step prior to UDP-*N*-acetylglucosamine formation. Clearly lysobactin and the katanosins are targeting a distinct cell wall precursor and offer an exciting new antibacterial approach.
A solid phase synthesis of lysobactin and the katanosins would allow simple access to peptides of the required size (11 residues), numerous analogues at every point of the synthesis and would be a rare example of a resin based macrolactonisation. Determination of the stereochemistry of the threonine residue would

allow the lysobactin/katanosin structural question to be addressed. The solid phase synthesis is complicated by three factors: (a) the cyclic nature of the peptide; (b) the lactone functionality and (c) the presence of several "unusual" amino acid residues. For our initial studies we decided to simplify the structure by incorporation of L-asparagine, rather than L-hydroxyasparagine (although the synthesis of protected L-*threo*-aspartic acid from maleic acid was readily achieved using literature procedures)[5] and L-threonine or L-leucine rather than L-hydroxyleucine. The crucial L-*threo*-β-hydroxyphenylalanine residue was incorporated, to form part of the lactone, and was obtained in pure form by enzymatic resolution of commercially available DL-*threo*-β-hydroxyphenylalanine in excellent yield.[6] D and L-*allo*-threonines could likewise be prepared in enantiomerically pure form by enzymic (acylase) resolution of commercially available DL-*allo*-threonine but were also commercially available. However due to the uncertainty about this residue it was anticipated all four isomers would eventually be incorporated.

Synthesis:

The proposed synthetic route is shown in below in scheme 1.



Scheme 1. The Solid Phase Synthesis of Analogues of Lysobactin

Four features in the synthesis are of note: (a) the generation of the asparagine residue by cleavage of the aspartate attached to the solid phase *via* the Rink amide linker.[7] (b) The C-terminal allyl deprotection[8] and coupling of H-Ser(OBu^t)-OAllyl carried out at an early stage to reduce the risk of amide mediated epimerisation.[9] (c) The incorporation of the final three residues using EEDQ mediated couplings, a reagent known to be ineffective in esterification chemistry.[10] (d) The serine allyl ester removal and lactonisation on the resin. Fmoc peptide synthesis was carried out in the traditional manner[11] using counterion distribution[12] and ninhydrin tests[13] to monitor the success of the couplings while a new method of solid phase reaction analysis was developed to allow *in situ* cleavage and monitoring using MALDI-TOF MS[14] (figure 2). Thus Fmoc-Asp(OH)-OAllyl (**4**)[15] was attached to the Rink linker,[7] the allyl ester removed[8] using Pd(0) catalysis and the terminal serine residue introduced using H-Ser(OBu^t)-OAllyl (**5**) and PyBOP.[16] Standard Fmoc peptide synthesis gave the peptide (**7**). Incorporation of Fmoc-*threo*-L-β-OH-phenylalanine was accomplished using the coupling reagent EEDQ in DCM, even a slight trace of DMF caused this coupling reaction to fail. The final two residues were likewise coupled using EEDQ and again all reactions monitored using MALDI-TOF MS (figure 2). No evidence of unwanted ester formation was observed under these coupling conditions. The allyl deprotection of (**8**) was slow however and repeated reaction was necessary to give the required product (**9**) (figure 2d,e).

Cyclisation:

The cyclisation of (**9**) was initially attempted using pentafluorophenol ester activation. The activation was successful as shown by MALDI-TOF MS, however, although successful on model reactions in solution the pentafluorophenol active ester of (**9**) was not observed to cyclise under a variety of reaction conditions. In light of this result a range of additional coupling methods were investigated in a variety of solvents (HATU/DMAP, DIC/DMAP/HOBt, PyBOP/DMAP in DCM, DMF, THF or DMSO). The use of DIC/DMAP/HOBt in DCM at 37°C for 10-16h proved to be the ideal coupling mixture, longer time periods leading to decomposition of the lactone. Analysis using MALDI-TOF MS gave the spectrum shown in figure 2f illustrating the cyclisation efficiency. Other peaks in the spectrum, apart from MH^+ were associated with the Arg residue still being side chain protected ($MMtr+H^+$) due to incomplete cleavage in the TFA vapour.

Characterisation:

Cleavage and HPLC purification/analysis (figure 3) gave the lysobactin analogue (**10**) in an overall yield of 15%.

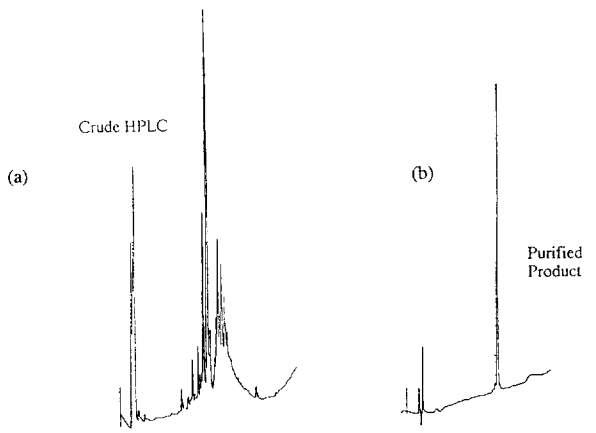


Figure 3. Reverse Phase HPLC Purification of (10). (a) Crude Cleavage; (b) Purified Material.

^{1}H TOCSY NMR, ES MS and Edman sequencing data were fully consistent with the desired product and those published for Lysoabctin although the nmr spectra were found to be broad in a variety of solvents (CD_3CN, CD_3CN/D_2O and D_2O), preventing good n.O.e data, however full spectral assignment was possible. Edman sequencing proceeded as expected. Thus at cycle three two peaks were observed by HPLC which had identical retention times to material produced by PTH derivatisation of βOH-phenylalanine. There was also a 2-3 fold decrease in the yield of PTH amino acid after the second cycle. This was also observed by Tymiak [3] and was suggested to arise by partial transacylation during sequencing.

MALDI-TOF MS Reaction Monitoring:
The success of this solid phase synthesis can be attributed, in part, to the rapid ability to monitor reactions efficiently using a new, powerful and sensitive method of reaction analysis. Several solid phase reactions, such as removal of allyl esters and lactonisation are difficult to monitor on the resin and would normally require large scale cleavage followed by conventional analysis, which is inefficient with respect to material and time. This has led to the development of a method for solid phase reaction analysis using MALDI-TOF and gave the data shown in figure 2. Thus beads (10-100) were removed from the reaction mixture, placed onto the sample plate of the MALDI-TOF mass spectrometer and exposed to TFA vapour for 20mins. Upon removal from the TFA environment, matrix and internal standard (bradykinin) were added and the samples analysed. Figures 2a-f shows some MALDI-TOF MS data for the synthesis of **(10)**. Figure 2a shows the successful C-terminal coupling of H-Ser(OBu^t)-OAllyl **(5)** to Fmoc-Asn(linker-Resin)-OH **(4)** giving the $(M+H)^+$ ion at 481.6 for Fmoc-Asn-Ser-OAllyl as the only major peak. Peptide synthesis continued and gave the peptides shown in figures 2b and 2c. The spectra are complicated here by the incomplete deprotection during the *in situ* cleavage process, especially the Mtr arginine protecting group which is sometimes difficult to cleave but all the peaks are assignable to the expected products. Incorporation of Fmoc-*threo*-L-β-OH-phenylalanine, Fmoc-Leu-OH and Boc-D-Leu-OH gave the spectrum shown in figure 2d. No ester formation was observed. Repeated allyl deprotection of **(8)** was required to give the clean spectrum shown in figure 2e. The final spectrum 2f shows the result of an optimised cyclisation, the loss of 18Da is clearly visible between figures 2e and 2f and the major peak is clearly the desired cyclic compound $(M+H^+)$ along with some cyclic $MMtr+H^+$ material. There was no evidence of any dimeric material.

Conclusion:
We have successfully demonstrated a practical and relatively high yielding route to peptides of the lysobactin and kanatosin type. We have developed a method of solid phase reaction analysis using MALDI-TOF MS to analyse materials cleaved *in situ* from the resin. The use of EEDQ on the solid phase has been demonstrated as has a solid phase lactonisation.

EXPERIMENTAL

General Experimental.
MALDI-TOF mass spectra were recorded on a Laser Tech Bench Top 2, Linear-Laser Desorption Time Of Flight Mass Spectrometer.[16] Reverse phase HPLC was performed on a Techsil5 C18 25 cm x 4.6 mm column (eluting with A = H_2O/0.1%TFA, B = MeCN/0.1%TFA; t = 0, 80% A, 20% B; t = 30, 50% A, 50% B). Normal phase HPLC was performed on a Zorbax Sil 25 cm x 4.6 mm column and Chiral normal phase HPLC was performed on Chiralpak AD 25 cm x 4.6 mm column both eluting with a linear gradient of hexane and isopropyl alcohol.
(a) Standard Fmoc deprotection procedure: The resin, pre-swollen in DMF, was treated with 20% piperidine in DMF (20 ml/g) for 10 mins and filtered. 20% piperidine in DMF (20 ml) was added and the resin shaken for 5 minutes before being filtered and sequentially washed and filtered with DMF (20 ml x2), MeOH (20 ml) and DCM (20 ml x2) and dried.
(b) Standard coupling procedure: The resin (1g) was resuspended in DCM (20 ml) to which were added the Fmoc amino acid (2 eq) and HOBt (2eq) in DCM (5 ml) followed by the addition of DIC (2eq) and the

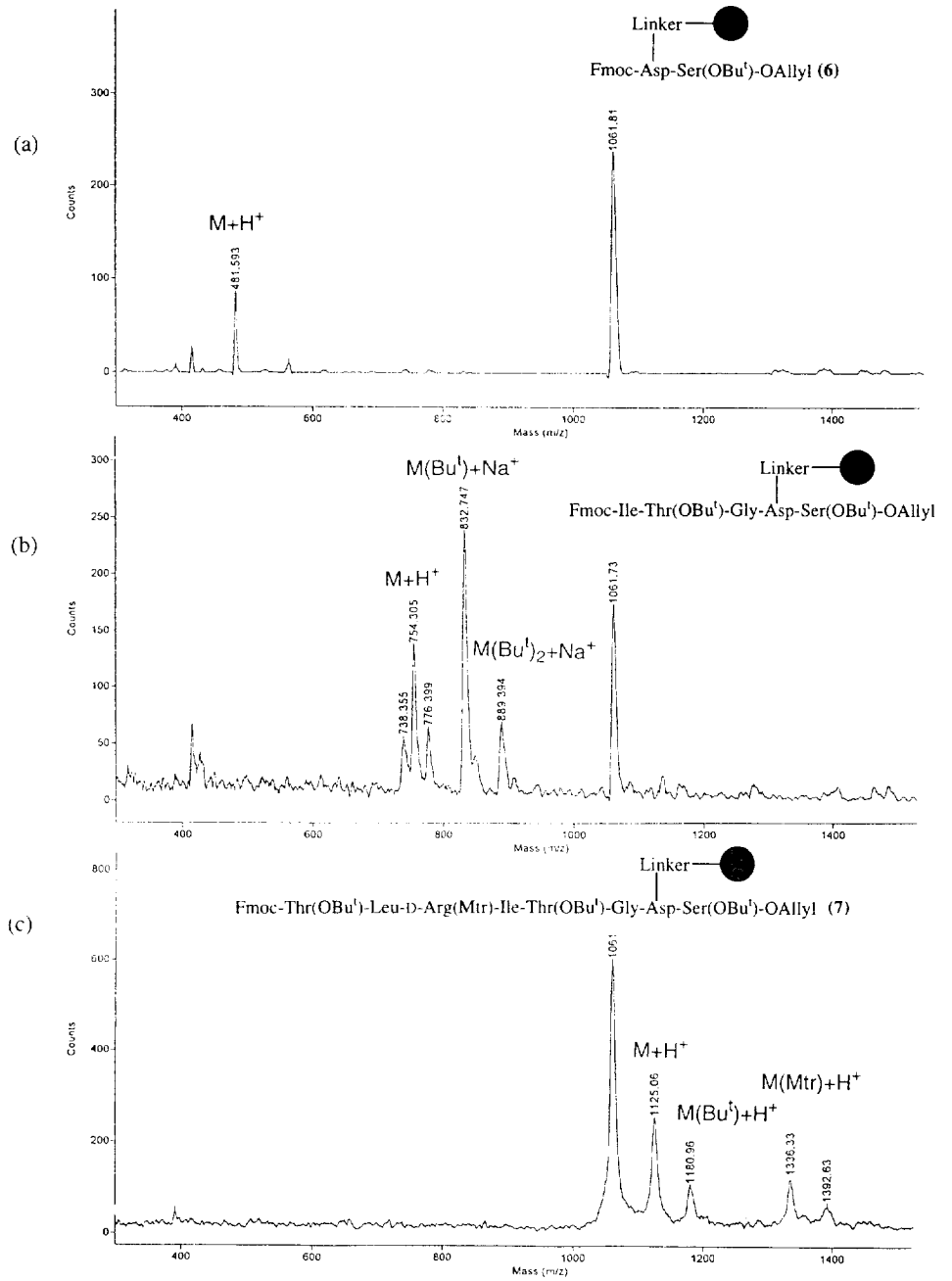


Figure 2. *In Situ* Cleavage and MALDI-TOF MS Analysis of Resin Samples: (a) C-Terminal Coupling of H-Ser(OBu^t)-OAllyl; (b) 5 mer; (c) 8 mer.

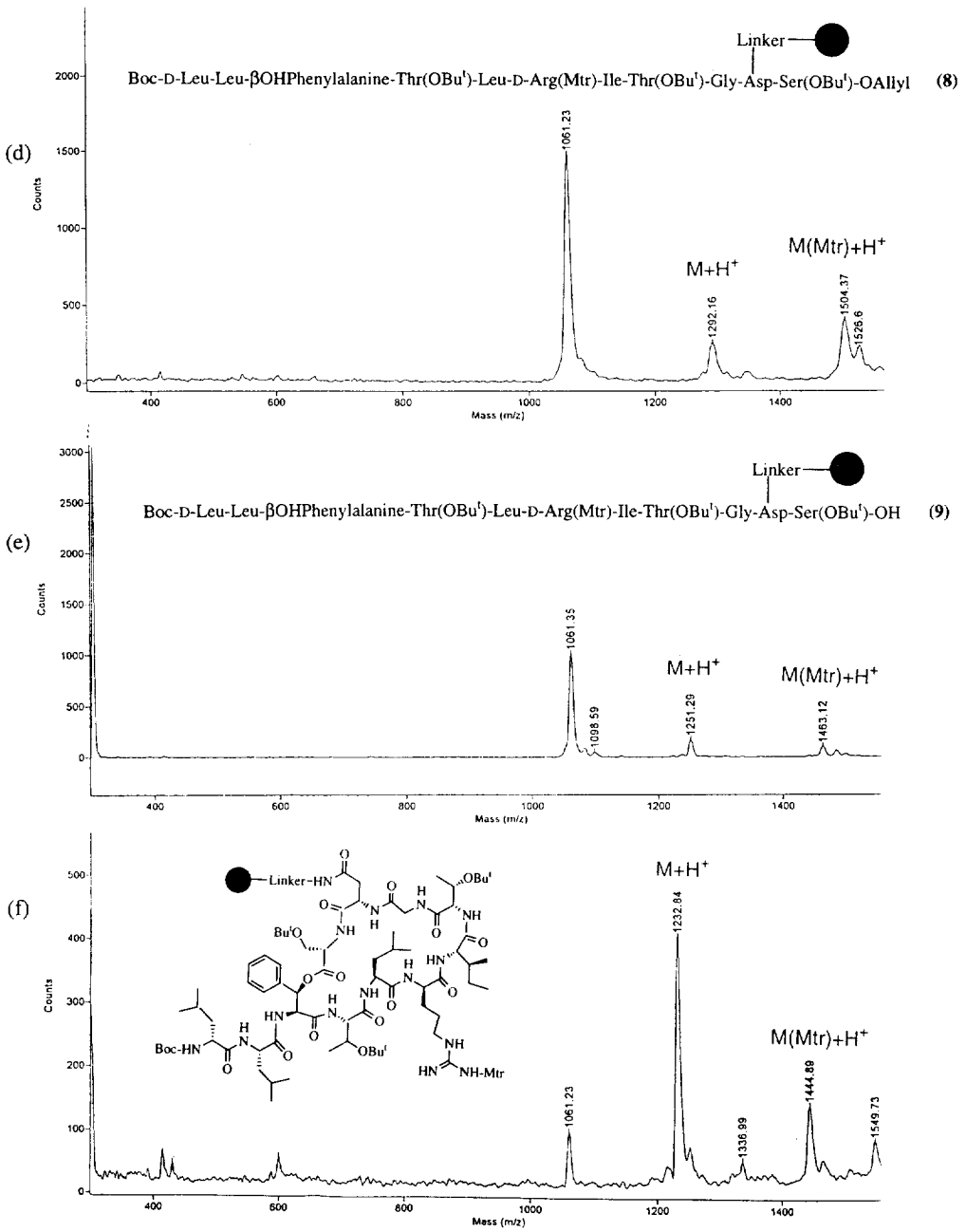


Figure 2. *In Situ* Cleavage and MALDI-TOF MS Analysis of Resin Samples: (d) Full Length Linear, Protected Peptide; (e) Allyl Deprotection of Linear Protected Peptide; (f) Cyclisation Product.

reaction agitated for 2 h before being filtered. The resin was filtered and washed sequentially with DMF (20 ml x2), MeOH (20 ml), and DCM (20 mlx 2).
(c) Standard solid phase allyl deprotection procedure: To a sparged solution of $CHCl_3$/AcOH/NMM (92.5/5/2.5) 20ml/g was added 0.2 molar equivalents of $Pd(PPh_3)_4$, the mixture was excluded from light and shaken for 2 h before being filtered and washed with DCM (20x10 ml).
(d) Standard cleavage procedure: The peptide-resin (pre-swollen in DCM) was treated with TFA:TIS:H_2O, (95: 2.5: 2.5), 10ml/g and shaken for 8 h and filtered. The supernatant was added dropwise to cold ether (10ml of TFA, 40ml of ether) and the precipitate, collected by centrifugation, was washed with ether (40ml) and air dried or redissolved in water and lyophilised.

Fmoc-Asp(OH)-OAllyl (4)
Fmoc-Asp(OH)-OAllyl (4) was prepared according to the literature procedure[11] from Fmoc-Asp(O^{t}Bu)-OH in quantitative yield; mp 87-91°C Lit 93°C; Rf 0.58 (ethyl acetate); δ_H (300MHz, $CDCl_3$): 2.89 - 3.20 (2H,8 line ABX system, J_{AX} 4, J_{BX} 5, J_{AB} 17, β Asp); 4.24 (1H, t, *J* 7, Fmoc-C^{9}H); 4.30-4.77 (3H, m, αCH Asp, Fmoc-CH_2); 4.65 (2H, d, *J* 6, OCH_2 allyl); 5.26 (1H, d, *J* 10, C$\underline{H}_2$=CH *cis*); 5.32 (1H, d, *J* 17, C$\underline{H}_2$=CH *trans*); 5.85 (1H, d, *J* 9, NH); 5.89 (1H, 6 line m *J* 10, 17, 6, C$\underline{H}$=CH_2); 7.34, 7.43 (4H, 2xdd, *J* 7, 7, Ar C^{2,3,6,7}H), 7.60, 7.78 (2H, 2xd, *J* 7, Ar-C^{1,4,5,8}H); 10.29 (1H, br s, COOH); δ_C (75MHz, $CDCl_3$): 36.6 (βC); 47.2 (FmocC^{9}H); 50.5 (αC); 66.7 (FmocCH_2); 67.6 (O$\underline{C}H_2$CHCH_2); 119.2 (OCH_2C$\underline{H}$CH_2); 120.2, 125.3, 127.3, 127.9, (Fmoc Ar-C$^{1-8}$); 131.5 (OC$H_2\underline{C}$HCH_2); 141.5, 143.9 (Fmoc Ar-C^{4a,4b,8a,9a}); 156.3 (urethane); 170.6 (ester); 176.1 (acid); IR υ_{max}/cm^{-1} (NaCl nujol mull): 1730 (s), 1702 (s), 1684 (s), 1535 (m); ES-MS: 418.2 (10%, M+Na$^+$); 813.1 (100%, 2M+Na$^+$); HR-MS; Calculated 396.1369, Found 396.1362.

H-Ser(O^{t}Bu)-OAllyl (5)
To a solution of Fmoc-Ser(O^{t}Bu)-OH (0.50 g, 1.3 mmol) in DMF (20 ml) was added cesium carbonate (0.26 g, 13 mmol) and the suspension stirred for 1h. Allyl bromide (3 ml) was added and stirring continued for 6 h. The solution was extracted with ethyl acetate (3x30 ml), the organic layer washed with 0.1M NaOH (30 ml), 1M HCl (30 ml) and saturated brine (30 ml), dried and the solvent removed *in vacuo* to give 0.54g of a colourless oil. The crude product was dissolved in 20% piperidine in DMF (20 ml) and the solvents immediately evaporated *in vacuo*. This was repeated followed by co-evaporation with toluene (3x20 ml) to give a white solid which was purified by column chromatography (silica gel, eluting with chloroform:ether (6:4)) to give the title compound as a white solid (0.19g, 79%) which was immediately used in the next reaction. Rf 0.2 (ethyl acetate /methanol (4:1)); δ_H (300MHz, d_6-DMSO): 1.11 (9H, s, ^{t}Bu); 3.49-3.62 (3H, m, αCH and βCH_2 Ser); 4.50-4.55, 4.58 (2H, 2xddd, *J* 1, 3, 5, OCH_2 allyl); 5.16 (1H, dt, *J* 12, 1, C$\underline{H}_2$=CH *cis*); 5.26 (1H, dt, *J* 20, 1, olefinic C$\underline{H}_2$=CH *trans*); 5.85 (1H, ddt, *J* 5, 12, 20, CH_2=C$\underline{H}$); δ_C (75MHz, d_6-DMSO): 27.5 (C($\underline{C}H_3$)$_3$); 55.3 (αC); 63.9 (βC); 65.7 (O$\underline{C}H_2$CHCH_2); 73.1 ($\underline{C}$(CH_3)$_3$); 118.3 (OCH_2C$\underline{H}$CH_2); 132.1 (OC$H_2\underline{C}$HCH_2); 174.0 (Ester); IR υ_{max}/cm^{-1} (NaCl nujol mull):1720 (s), 1650 (m); ES-MS: 202.2 (100%, M+H$^+$); HR-MS; Calculated 202.1580, Found 202.1574.

Fmoc-L-*threo*-βOH-Phe
N-Ac-DL-*threo*-β(OAc)-Phe-OH (0.27g, 1.02 mmol), prepared by the treatment of DL-*threo*-β(OH)Phe-OH with Ac_2O, was dissolved in 1 mM $CoCl_2$ (20 ml) at 37°C and the pH adjusted to 8 by the addition of 1M LiOH. *Aspergillus melleus* acylase (1.0 g, 500 units) was added and the solution maintained at pH 8 by the addition of 0.1M LiOH. After 3.5 h the pH was adjusted to pH 5 with 0.1M HCl, the temperature raised to 60°C and acid washed celite (5 g) added, stirred for 30 min and filtered.The filtrate was acidified to pH 1.5 (c.HCl) and extracted with ethyl acetate (30 ml).The aqueous layer was applied to a Dowex 50H$^+$ column (5 g wet, previously washed with 1M NaOH, water, 1M HCl, water). The column was washed with water until the filtrate was neutral then eluted with 1M aqueous pyridine. The eluant was reduced *in vacuo*, taken up in 10% $NaHCO_3$ (10 ml) and dioxane (10 ml) and Fmoc-OSu (0.34 g, 1.05 mmol) in dioxane (2 ml) was added dropwise at 0°C. The reaction was stirred at 0°C for 1h and at room temperature for 4h. The reaction mixture was washed with ether (2x25 ml), acidified to pH 1.5 (c.HCl) and extracted with ethyl acetate (2x25 ml). The extractions were concentrated *in vacuo* to 5 ml and the residue purified by column chromatography (silica gel, eluting with ethyl acetate/petroleum ether/acetic acid (24:74:2)) to give the title compound as a white solid (0.13g, 62%); m.p. 134-138°C; Rf 0.28 (ethyl acetate / petrol / acetic acid (50/50/1)); $[\alpha]_D^{22}$ +31.3° (c. 2.0 acetic acid); δ_H (300MHz, d_6-DMSO): 3.95-4.20 (3H, m, CH_2, Fmoc-C^{9}H$\underline{C}$H$_2$); 4.32 (1H, dd, *J* 3, 8, αH); 5.19 (1H, br m, βH); 5.77 (1H, br s, OH); 7.20 - 7.45 (10H, m, βOHPhe Ar-C^{2,3,4,5,6}H, Fmoc Ar-C^{2,3,6,7}H); 7.62, 7.68 (4H, 2xd, *J* 8, 8, Fmoc Ar-C^{1,4,5,8}H); 12.81 (1H, br s, COOH); δ_C (75MHz, d_6-DMSO): 46.5 (Fmoc C^{9}H); 60.4 (αC); 66.0 (Fmoc CH_2); 72.3 (βC); 120.1, 125.4, 125.6, 126.3, 127.2, 127.7, 127.9 (βOHPhe Ar-C$^{2-6}$, Fmoc Ar-C$^{1-8}$); 140.7, 142.2, 143.8 (Fmoc Ar-C^{4a,4b,8a,9a}, βOHPhe-C^1); 156.3 (Urethane); 172.0 (acid); IR υ_{max}/cm^{-1} (NaCl nujol mull): 3425 (m); 1739 (s), 1700 (s), 1508 (s); ES-MS: 426.0 (100%, M+Na$^+$); 442.1 (35%, M+K$^+$); 829.2 (50%, 2M+Na$^+$).

Fmoc-L-*threo*-βOH-Phe-OMe
Esterification of Fmoc-L-*threo*-βOHPhe-OH (51 mg, 0.13 mmol) was performed using diazomethane in acetone. Recrystallisation from ethyl acetate/hexane gave white needles (48mg, 91%); mp 165-168°C; Rf 0.56 (ethyl acetate / petrol (1:1)); δ_H (300MHz, d_6-DMSO): 3.62 (3H, s, OMe); 4.12 (3H, m, Fmoc-C^9H, FmocCH_2); 4.40 (1H, dd, *J* 3, 10,, αCH); 5.13 (1H, d, *J* 3, 6, βCH); 5.81 (1H, d, *J* 6, OH); 7.55 (1H, d, *J* 10, NH); 7.18-7.49 (9H, m, βOHPhe, Fmoc-$C^{1,4,5,8}$H), 7.65, 7.69, 7.88 (4H, 3xd, *J* 8, Fmoc-$C^{2,3,6,7}$H); δ_C (75MHz, d_6-DMSO): 46.5 (FmocC^9H) 52.1 (OMe); 60.8 (αC); 66.0 (FmocCH_2) 72.4 (βC); 120.1, 125.4, 125.5, 126.3, 127.1, 127.3, 127.7, 127.9, (Fmoc Ar-C^{1-8} and βOHPhe Ar-C^{2-6}); 140.7, 141.7, 143.7 (Fmoc Ar-$C^{4a,4b,8a,9a}$, βOHPhe Ar-C^1); 156.3 (urethane); 171.0 (ester); ES-MS: 418.0 (60%, M+H$^+$); 440.4 (90%, M+Na$^+$); 857.4 (100%, 2M+Na$^+$). Chiral HPLC analysis: Fmoc-DL-*threo*βOHPhe-OMe and Fmoc-L-*threo*βOHPhe-OMe were eluted at 1ml/min over 20 min. Fmoc-D-*threo*βOHPhe-OMe eluted at 5.82 min and the L-isomer at 6.65 min. Integration ratio 1:94.

Boc-DLeu-Leu-β-OHPhe-Thr(O^{t}Bu)-Leu-DArg(Mtr)-Ile-Thr(O^{t}Bu)-Gly-Asn(Linker-Resin)-Ser(O^{t}Bu)-OAllyl (8) and Boc-DLeu-Leu-β-OHPhe-Thr(O^{t}Bu)-Leu-DArg(Mtr)-Ile-Thr(O^{t}Bu)-Gly-Asn(Linker-Resin)-Ser(O^{t}Bu)-OH (9)
Fmoc Rink amide resin (1.20g, 0.44 mmol NH_2/g) was swollen in DCM (20 ml). The resin was filtered and Fmoc deprotected using the standard proceedure (a). Fmoc-Asp-OAllyl was coupled using the general procedure (b), followed by allyl deprotection using general method (c). The resin was resuspended in DCM (20 ml) and H-Ser(O^{t}Bu)-OAllyl (0.22 g, 1.1 mmol), PyBop (0.57g, 1.1 mmol), HOBt (0.18 g, 1.1 mmol) and DIPEA (300 µl, 2.2 mmol) (in DMF/DCM (1:1, 5 ml)) were added and the mixture agitated for 2 h. The resin was washed and filtered sequentially with DMF (20 ml x2), MeOH (20 ml), and DCM (20 mlx2).

Semiautomatic peptide synthesis: Fmoc Peptide synthesis was performed in an Omni column (1x20 cm) using a NovaSyn Gem Peptide Synthesiser. Synthesis was monitored at 360nm using a continuous flow UV spectrophotometer and Nova chrome blue dye counter-ion distribution monitoring.
The amino acids were typically double coupled using 1.5 and 0.3 eq.
aa_3; Fmoc-Gly-OH (0.83 mmol, 0.25 g)
aa_4; Fmoc-Thr(OBu^t)-OH (0.83 mmol, 0.33 g) and (0.22 mmol, 0.087 g)
aa_5; Fmoc-Ile-OH (0.84 mmol, 0.30 g)
aa_6; Fmoc-D-Arg(Mtr)-OH (0.55 mmol, 0.340 g) and (0.22 mmol, 0.134 g)
aa_7; Fmoc-Leu-OH (0.83 mmol, 0.29 g) and 2x (0.22 mmol, 0.078 g)
aa_8; Fmoc-Thr(OBu^t)-OH (0.83 mmol, 0.33 g) and (0.22 mmol, 0.087 g).

The 8mer peptide (**7**) (100 mg) was Fmoc deprotected (method a) and sequentially coupled to: (i) Fmoc-L-*threo*-βOHPhe-OH (18 mg, 44 µmol), (ii) Fmoc-Leu-OH (20 mg, 56 µmol), (iii) Boc-D-Leu-OH (15 mg, 55 µmol), using 4h couplings with EEDQ (1eq) in DCM (10ml), to give the 11-mer linear peptide (**8**). (**8**) was allyl deprotected to give (**9**) using the general allyl deprotection method (c). The 11-mer allyl deprotected peptide (**9**) was cleaved from the resin using the general deprotection method (d) to give a single peak by RP-HPLC, retention time 19.4 min; 625.9 (100%, M+HH^{++}), 1250.4 (10%, M+H$^+$).

Cyclisation to give (10)
Samples of resin bound 11-mer allyl deprotected peptide (**9**) (5 mg, 0.9 µmol) were placed in DCM, THF, DMF or DMSO (1 ml) to which were added HATU/DMAP, DIC/HOBt/DMAP or PyBOP/DMAP. The reactions were shaken at 37°C or 22°C overnight. MALDI TOF MS analysis was performed using a cleavage time of 30 mins in TFA vapour. Cleavage of samples for RP-HPLC (C-18) and MS analysis were performed using standard method (d).

Resin based MALDI-TOF analysis (TFA vapour 30 min)
2-mer (**6**): Fmoc-Asn-Ser-OAllyl; 482.3 (M+H$^+$);
5-mer : Fmoc-Ile-Thr-Gly-Asn-Ser-OAllyl; 753.8 (M+H$^+$), 775.8 (M+Na$^+$), 832.1 (M(Bu^t)+Na$^+$), 888.5 (M(2Bu^t)+Na$^+$);
8-mer (**7**): Fmoc-Thr-Leu-D-Arg-Ile-Thr-Gly-Asn-Ser-OAllyl; 1128.8 (M+H$^+$), 1180.5 (M(Bu^t)+H$^+$), 1336.3 (M(Mtr)+H$^+$), 1392.4 (M(MtrBu^t)+H$^+$)
9-mer: Fmoc-βOHPhe-Thr-Leu-D-Arg-Ile-Thr-Gly-Asn-Ser-OAllyl; 1288.6 (M+H$^+$), 1497.9 (M(Mtr)+H$^+$).
11-mer (**8**): D-Leu-Leu-βOHPhe-Thr-Leu-DArg-Ile-Thr-Gly-Asn-Ser-OAllyl; 1292.0 (M+H$^+$), 1503.9 (M(Mtr)+H$^+$);
11-mer (**9**): D-Leu-Leu-βOHPhe-Thr-Leu-D-Arg-Ile-Thr-Gly-Asn-Ser-OH 1251.9 (M+H$^+$), 1308.0 (M(Bu^t)+H$^+$), 1363.4 (M(2Bu^t)+H$^+$), 1464.1 (M(Mtr)+H$^+$), 1484.6 (M(Mtr)+Na$^+$), 1520.1 (M(Mtr,Bu^t)+H$^+$), 1542.0 (M(Mtr,Bu^t)+Na$^+$), 1598.8 (M(Mtr,2Bu^t)+Na$^+$).

Cyclised 11-mer (**10**): 1291.3 (M(Bu^t)+H$^+$), 1347.5 (M(2Bu^t)+H$^+$), 1448.2 (M(Mtr)+H$^+$), 1479.1 (M(Mtr)+Na$^+$), 1505.6 (M(Mtr,Bu^t)+H$^+$), 1527.7 (M(Mtr,Bu^t)+Na$^+$), 1587.4 (M(Mtr,2Bu^t)+Na

Large Scale Cyclisation and Cleavage of Depsipeptide (10)

Peptide bound resin (**9**) (0.434 g, 0.18 mmol g^{-1}, 0.078 mmol) was treated using the optimised conditions (DIC/HOBt/DCM/37°C) to give the depsipeptide (**10**). The resin (**10**) was treated with TFA:TIS:H_2O, (95: 2.5: 2.5, 15 ml) and shaken for 8 h. The beads were filtered and washed with TFA (10 ml). The filtrates were concentrated *in vacuo* to 3 ml, acetonitrile (10 ml) added and evaporated *in vacuo*. Acetonitrile (5 ml) was added and the resulting solution dripped into an ice cold solution of hexane/diethylether (1:1, 50 ml) and kept on ice for 20 min. The white precipitate was collected by centrifugation (5 min at 4000 rpm). The supernatant was decanted and the pellet resuspended in an ice cold solution of hexane/diethyl ether (1:1, 50 ml) and the process repeated. The pellet was dissolved in water/acetonitrile (90:10, 15 ml) and freeze dried to give 77mg (79%) of the crude cleavage product. Analysis by HPLC and ES-MS showed incomplete Mtr removal. 30 mg of the crude deprotection product was treated with TFA/TIS/H_2O (95/2.5/2.5, 2 ml) for 4 h and the solution evaporated *in vacuo*. Analytical HPLC, with ES-MS analysis, gave 5.9 mg (15.6%) of the desired product. ES-MS; 616.9 (100%, M+HH^{++}); 1232.0 (5%, M+H$^+$); δ_H (500MHz, CD_3CN/H_2O (95:5)): TOCSY analysis:

	-NH	αCHX	βCHX	γCHX	δCHX	other
Leu^2	8.71	4.01	1.72	0.8-1.0	0.8-1.0	
β-OHPhe^3	8.42	5.31	6.26			7.2-7.6[a]
Thr^4	7.26	4.53	4.32	1.18		
Leu^5	7.34	4.32	1.83	0.8-1.0	0.8-1.0	
D-Arg^6	7.04	4.10	1.4-1.7	1.4-1.7	2.84, 3.02	6.35 [b]
Ile^7	7.94	4.01	1.95	1.18	0.8-1.0	0.8-1.0 [c]
Thr^8	8.20	4.18	3.87	1.6		
Gly^9	8.26	3.84, 4.18				
Asn^{10}	8.01	4.77	3.7-2.85			6.53,7.2 [d]
Ser^{11}	7.39	4.38	3.75, 3.85			

^{1}H assignments of depsipeptide 1 from TOCSY analysis (measured in CD_3CN/H_2O 95:5). a; Aromatic H. b; guanidinium NH. c; β-CH_3. d; $CONH_2$, two singlets.

Edman sequencing: The purified cyclic peptide (**10**) was Edman sequenced: amino acid residue (pmoles); leucine(5514); leucine (5981); hydroxyphenylalanine (ND); threonine (1939); leucine (2440); arginine (718); isoleucine (2475); threonine (2154); glycine (1350); asparagine (1144); serine (441).

Abbreviations:

βOH-Phe ; β-hydroxyphenylalanine, βOH-Asp ; β-hydroxyaspartic acid, DCM ; dichloromethane, DIC ; *N,N*-diisopropylcarbodiimide, DIPEA ; diisopropylethylamine, DMAP ; 4-dimethylaminopyridine, DMF ; *N,N*-dimethylformamide, EEDQ ; 2-ethoxy-1-ethoxycarbonyl-1,2-dihydro-quinoline, ES-MS ; electrospray ionisation mass spectrometry, HATU ; *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOAT ; 1-hydroxy-7-azabenzotriazole, HOBt ; 1-hydroxybenzotriazole, MALDI ; matrix assisted laser desorption ionisation, Mtr ; 4-methoxy-2,3,6-trimethylbenzenesulphonyl, NMM ; *N*-methylmorpholine, Pfp ; pentafluorophenol, PyBOP ; benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphoniumhexafluorophosphate, SPIMS ; solid phase *in situ* mass spectrometry, TIS ; triisopropylsilane, TFA ; trifluoroacetic acid, TOF ; time of flight.

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