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Solid-Phase Peptide Synthesis of Analogues of the N-Terminus A-ring Fragment of the Lantibiotic Nisin: Replacements for the Dehydroalanine (Dha) Residue at Position 5 and the First Incorporation of a Thioamide Residue

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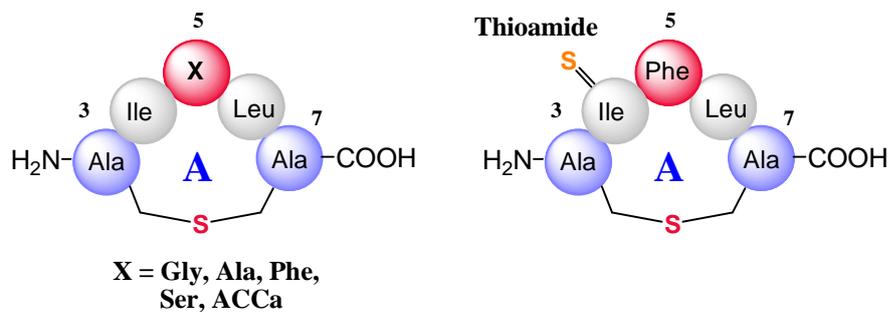
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Graphical abstract:

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X = Gly, Ala, Phe, Ser, ACCa

Solid-phase peptide synthesis of analogues of the N-terminus A-ring fragment of the lantibiotic nisin: Replacements for the dehydroalanine (Dha) residue at position 5 and the first incorporation of a thioamide residue

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Abstract:

A number of A-ring analogues of the lantibiotic nisin, containing replacements for the Dha at position 5, have been successfully prepared by solid-phase peptide synthesis. The Dha replacements include glycine, alanine, phenylalanine, serine and 1-aminocyclopropyl carboxylic acid (ACCa). The incorporation of a thioamide-isoleucine residue at position 4 is also described and is the first report of the preparation of a lantibiotic ring fragment containing a thioamide link.

Keywords: Nisin; thioamide; microwave-enhanced solid-phase peptide synthesis; lantibiotics; dehydroalanine

The lantibiotics are a class of ribosomally synthesised peptides which are subsequently highly post-translationally modified.[1] They are short peptides, with 19-34 amino acid residues, and contain a number of unusual amino acids within their structures. Rather than being linear peptides they contain a number of small cyclic motifs within their overall structures formed by thioether linkages, as compared to the more common disulfide linkages. The best known, and most studied, lantibiotic is nisin, which has been used as a food preservative (E234) for over 50 years without the development of significant antimicrobial resistance. It is produced by a *Lactococcus lactis* species and is highly active against a wide-range of Gram-positive bacterial species including MRSA and the food-

borne pathogen *Listeria monocytogenes*. It is a 34 amino acid residue peptide with five internal rings (**A-E**) within its overall sequence (Figure 1).

Figure 1. The amino acid sequence of nisin A and the structures of its unusual amino acid residues

The unusual amino acids present in nisin are dehydroalanine (Dha), dehydrobutyrine (Dhb), lanthionine and β-methyllanthionine. Of the 34 amino acids of nisin 13 are unusual, which along with the fact that there are no aromatic amino acid residues present makes the overall structure of nisin unique. The A-ring contains a lanthionine bridge (Ala-S-Ala) while the remaining B-E rings contain β-methyllanthionine bridges (Abu-S-Ala). Nisin is both stable and soluble at acidic pH values having its maximum stability at pH 3-4.[2] However, its stability and solubility decrease considerably at physiological pH which precludes its use as a human therapeutic. The A and B rings of the N-terminus of nisin (nisin$_{3-12}$) are critical to its overall biological activity where it binds to the lipid II moiety inhibiting the biosynthesis of the bacterial cell wall.[3] It is known that at physiological pH the two Dha residues at positions 5 and 33 are cleaved by hydrolysis.[4] In particular hydrolysis of Dha5 leads to opening of the A-ring which abolishes its biological activity, while cleavage at Dha33 alone leads to a small drop in activity. A significant number of structure-activity relationship studies on nisin involving mutagenesis have been undertaken which show that it is possible to replace Dha5 while retaining activity.[5] However, little is known about how such amino acid mutations change the overall 3-D conformation of the critical N-terminus region. NMR studies by the Murray group showed that the overall

conformation of the A-ring of nisin changed substantially when the Dha5 residue was replaced by either a L-Ala or D-Ala moiety.[6] For our studies one of the crucial aspects of the endeavour would be the synthesis of useful A-ring analogues of nisin, where Dha5 has been replaced, to study how changes affect both the conformations adopted and the resulting stability of the peptides. Herein, the initial synthetic targets, where the replacement of Dha5 with glycine, L-alanine, L-serine and L-phenylalanine residues is described. The reasons for choosing these initial targets of amino acids were that i) glycine would show the effect of removal of all rigidity at position 5; ii) L-alanine was the direct analogue of Dha where the double bond was removed; iii) serine is a precursor to the Dha moiety by dehydration and iv) phenylalanine is present at position 5 in the closely related lantibiotics epidermin and gallidermin, which also bind to lipid II.[1] A further analogue targeted was the incorporation of a cyclopropyl group, which would require the use of 1-aminocyclopropane carboxylic acid (ACCa). Since the Dha5 moiety is cleaved at physiological pH leading to ring-opening and complete loss of biological activity, it is postulated that the cyclopropyl group would be a more hydrolytically stable mimic of the Dha group, while keeping the overall conformation of the peptide ring. Furthermore, it is known that the group can interact through hyperconjugation with a carbonyl group, in a manner akin to a vinyl group.[7] The N-C_α-$C(=O)$ bond angle of the ACCa is usually close to 116°,[6] which is closer to the theoretical 120° of the sp^2 hybridised system of the Dha than the tetrahedral bond angle of 109.5° (Figure 2).
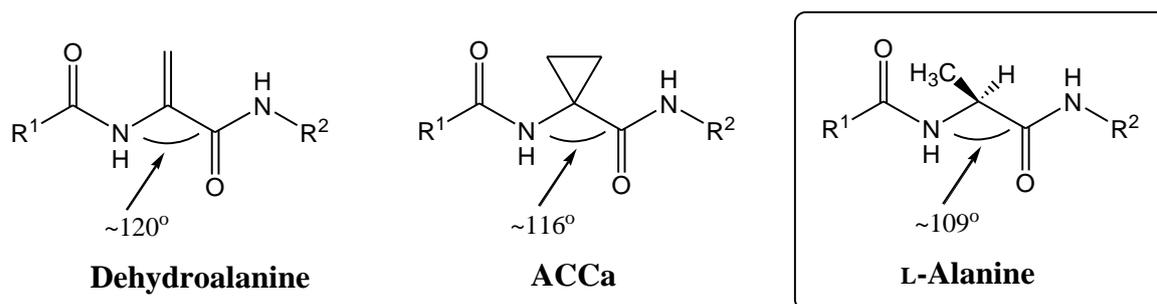

**Figure 2.** N-C_α-$C(=O)$ bond angles for Dha, ACCa and Ala

The proposed Dha5 replacements will ultimately give a greater understanding of how and why structural changes in that region might impact the overall biological activity.

Previously lantibiotics have been prepared by solid-phase peptide synthesis (SPPS), including epilancin X,[8] lacticin 3147a and 3147b,[9] and Cytolysin.[10] These syntheses

required the reliable preparation of significant quantities of orthogonally protected lanthionines and β-methyllanthionines with tailored protecting groups suitable for SPPS.

Lanthionine Synthesis

Although a wide number of methods are published for the synthesis of orthogonally protected lanthionines[11] we found that a variation of the methods described by Schmidt,[12] and van der Donk,[8] using phase-transfer catalysis, to be the most reproducible and high yielding. Thus the two coupling partners, bromoalanine **1** and cysteine **2** were prepared and used immediately due to their instability over time and upon exposure to air. The reaction was conducted in a degassed mixture of ethyl acetate and aqueous sodium hydrogen carbonate in the presence of tetrabutylammonium bromide at pH 8.5. Wavering from these precise conditions, i.e., not sufficiently degassing the reaction mixture nor maintaining it at exactly pH 8.5, resulted in very low isolated yields of **3**, due to oxidation of **2** to the cystine, and/or the formation of significant amounts of a diastereoisomer of **3**. Thus, orthogonally protected lanthionine **3** was successfully prepared in an isolated yield of 55% (based on bromoalanine **1**) as a single diastereoisomer (Scheme 1). Subsequent deprotection of the *t*-butyl ester gave the desired lanthionine **4**, ready for use in SPPS.
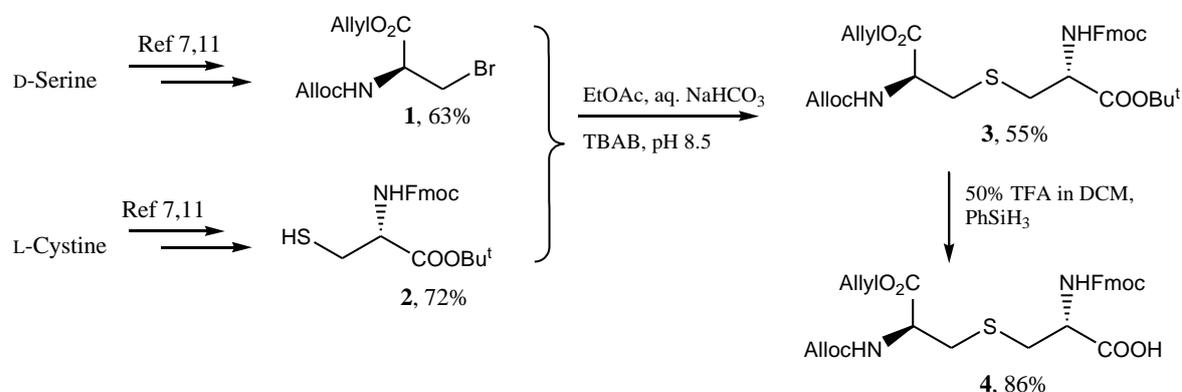

Scheme 1. Synthesis of orthogonally protected lanthionine **4**[7,11]

Preparation of the nisin A-ring analogues by SPPS

Having successfully obtained orthogonally protected lanthionine **4** it was then used for preparation of a number of analogues of the A-ring of nisin by SPPS. The resin chosen for SPPS was a 2-chlorotrityl chloride resin (1.33 mmol/g loading) because of its successful use by Vederas for the synthesis of both components of lacticin 3147.[9] The preparation of the analogue containing a glycine in place of Dha5, i.e. Dha5Gly, is exemplified in Scheme

2. The other analogues were prepared using similar methodology. Lanthionine **4** was first coupled to the resin to give a reduced loading of 0.16 mmol/g,[9] with the remaining reactive sites being capped with acetic acid. A Kaiser test showed the absence of free NH_2 groups.[13] The Fmoc group of the resin-loaded lanthionine **5** was efficiently removed using 20% piperidine in DMF using microwave enhancement at 50 ^{o}C for 10 min. Coupling was undertaken using a pre-activated solution of Fmoc leucine (5 molar equivalents), PyBOP (5 molar equivalents), HOBt (5 molar equivalents) and *N*-methylmorpholine (5.5 molar equivalents), using microwave enhancement at 75 ^{o}C for 10 min, to give peptide **6**.
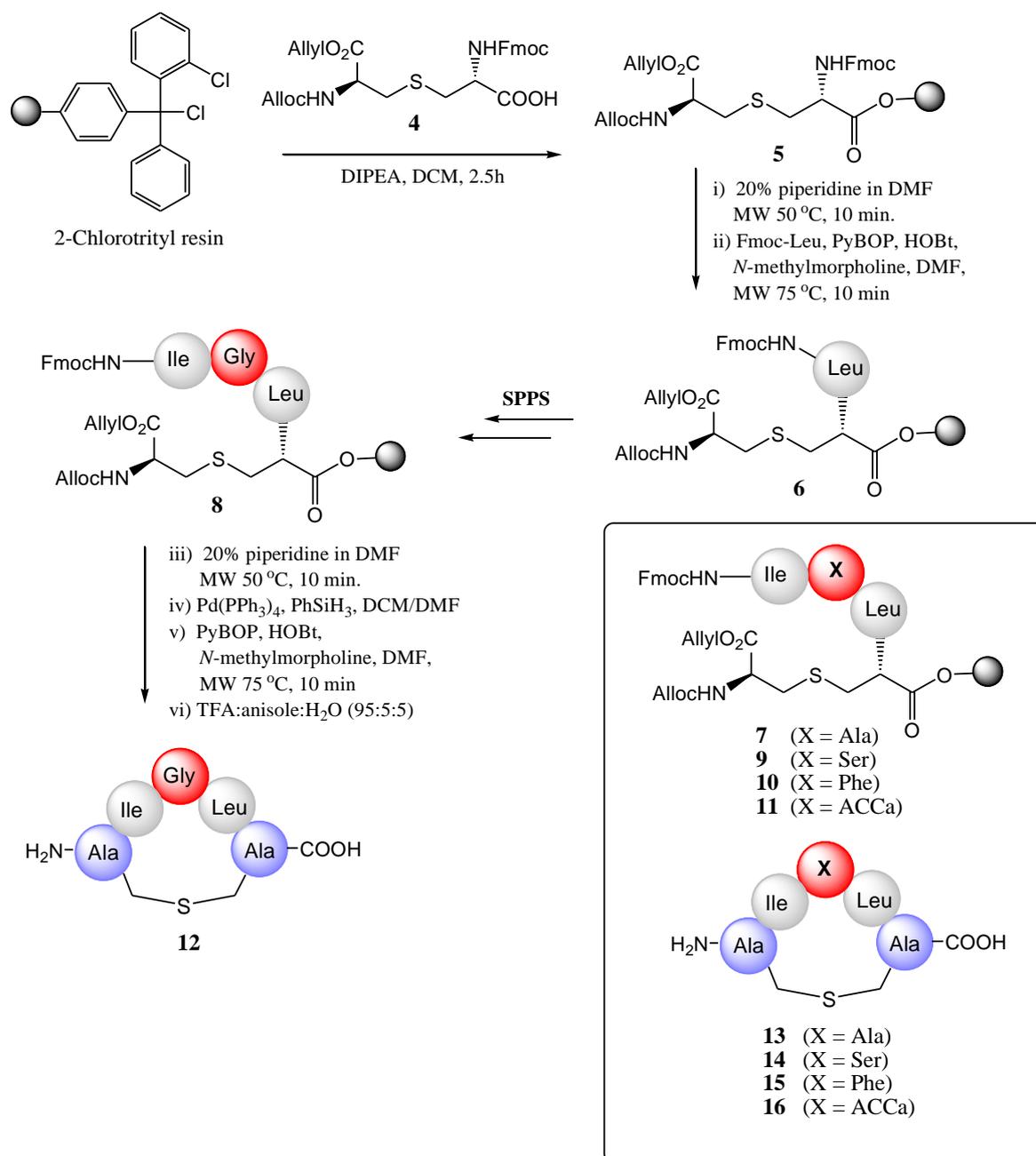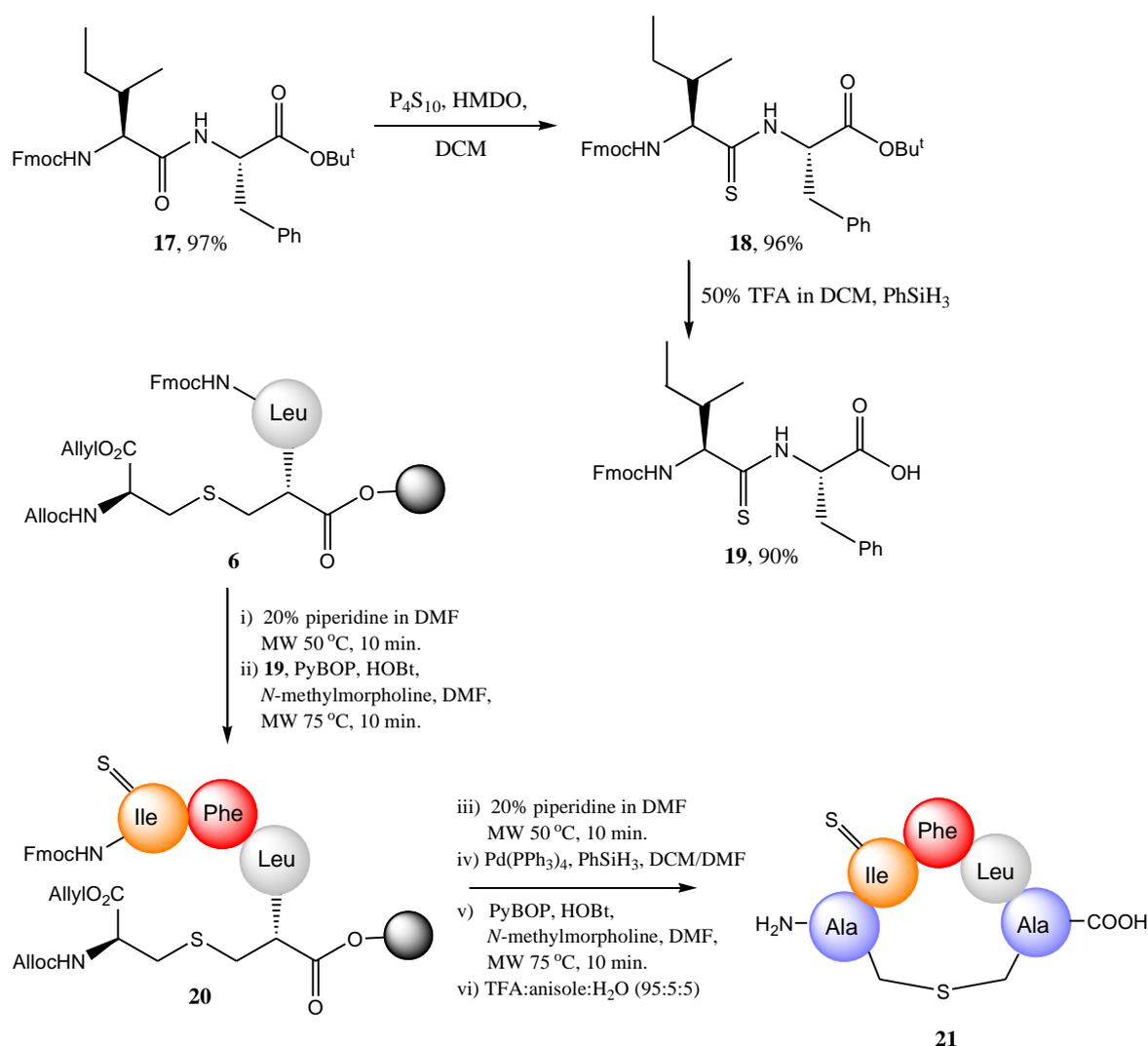

Scheme 2. Synthesis of nisin A-ring analogues (**12-16**), with position Dha5 modified

This loaded resin was then split into five equal portions for the synthesis of the target analogues. Separately, Fmoc L-alanine, Fmoc glycine, Fmoc L-serine, Fmoc L-phenylalanine and Fmoc ACCa were successfully coupled using the same coupling protocol, followed by deprotection and the final coupling of Fmoc L-isoleucine to give peptides **7-11**. Removal of the two allyl based protecting groups was achieved by treatment with Pd(PPh$_3$)$_4$ in DCM/DMF (50:50), in the presence of phenylsilane as a scavenger. Subsequent cyclisation on the resin using PyBop (5 molar equivalents), HOBt (5 molar equivalents) and *N*-methylmorpholine (5.5 molar equivalents), using the method of Vederas,[14] gave the five analogues (**12-16**) of the nisin A-ring, as confirmed by HRMS analysis after cleavage from the resin using TFA (See Table 1 in Supporting Information).

Incorporation of a thioamide residue at position 4 (isoleucine) of the A-ring of nisin

As part of its mechanism of action nisin is known to bind to the pyrophosphate moiety of lipid II, which is present in the bacterial cell wall, through five key H-bonding interactions of the N-H protons of the A and B rings.[15] One of these key interactions involves the N-H of the Dha5 residue of the A-ring.

It is known that thioamides are significantly better H-bond donors than their amide counterparts,[16] therefore, we envisaged that replacement of Ile4 with thioamide-Ile4 would lead to stronger H-bonding to the pyrophosphate moiety. In order to achieve this goal thioamide dipeptide **18** must be synthesised followed by incorporation into the A-ring, using methods employed for the amide analogues **12-16**. We have recently reported the efficient synthesis of such dipeptides via the selective thionation of the amide group of protected dipeptides,[17] using Curphey's method.[18]

Scheme 3. Synthesis of thioamide-Ile4 nisin A-ring analogue **21**

Coupling of the deprotected thioamide dipeptide **19** to resin-loaded peptide **6**, which had been firstly deprotected with 20% piperidine in DMF, gave thioamide peptide **20**. Subsequent deprotection and cyclisation gave the cyclised peptide **21** (Table 1). This is the first report of the successful preparation of a lanthionine-containing ring peptide fragment incorporating a thioamide link.

Peptide isolation and purification

All of the peptides prepared are very hydrophobic in nature and although HRMS analysis of the cleaved product from a small amount of resin showed that the correct cyclic peptides had been formed, there are challenges in the isolation, solubilisation and purification of these peptides. Use of a wide range of published methods for the isolation of hydrophobic peptides, has so far not been fully successful. It is after cyclisation and full cleavage from

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the resin when the solubility problems arise and work is continuing on addressing these issues. However, post cleavage from the resin there is no evidence for any uncyclised peptides remaining by MS analysis, which strongly suggests that cyclisation has successfully occurred, as seen for other related literature syntheses.[8-10,19]

Conclusions

For the purpose of investigating the impact of alteration of the reactive Dha5 residue of the A-ring of nisin has on the conformation of the peptide a series of analogues were synthesised on a small scale using SPPS in order to examine and optimise the synthetic route. Firstly, using phase transfer reaction conditions with a suitably protected bromoalanine (**1**) and cysteine (**2**) the desired lanthionine **4**, a templete for the A-ring, was isolated in 55% yield, as a single diastereoisomer. Using microwave assisted SPPS a collection of useful A-ring analogues, in which the Dha5 was replaced with Gly, Ala, Ser, Phe or ACCa, were successfully prepared. Furthermore, in order to enhance binding to lipid II, Ile4 of the A-ring was successfully replaced with the significantly better H-bond donor thioamide-Ile4 while incorporating Phe at position 5 in place of Dha. Other thioamide-containing A-ring analogues are now being targeted of the key H-bond donors for the binding interaction with the pyrophosphate moiety of lipid II.[15,16] Scale-up of the syntheses is currently being undertaken, along with the optimisation of the purification of all the peptides. Detailed NMR studies of all of these peptides will then be undertaken to examine the effects of replacing Dha5 and incorporation of thioamide moieties on the solution conformations. The results of these studies will be reported in due course.

Acknowledgements

Supplementary Data

Details of the synthesis of compounds **1-4** and **17-19** are described.

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Supporting Information

Solid-phase peptide synthesis of analogues of the N-terminus A-ring fragment of the lantibiotic nisin: Replacements for the dehydroalanine (Dha) residue at position 5 and the first incorporation of a thioamide residue

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Experimental Methods

General Methodology

IR spectra were recorded on a Nicolet Impact 410 FT-IR and characteristic stretches are reported. Solid samples were prepared as dispersions in KBr discs and liquid samples as a film between sodium chloride plates. NMR spectra were obtained on a Bruker Avance III 300 spectrometer operating at 300 MHz for ^{1}H spectra. The typical resolution of this machine is 0.095 Hz or 0.000315 ppm, while coupling constant (*J*) values are accurate to $\pm$ 0.19 Hz. For ^{13}C spectra it operates at 75 MHz, with a typical resolution of 0.28 Hz or

0.0037 ppm. NMR spectra were also obtained on a Bruker Avance III 500 spectrometer operating at 500 MHz for ^{1}H NMR. The typical resolution of this machine is 0.11 Hz or 0.00022 ppm, while coupling constant (J) values are accurate to $\pm$ 0.22 Hz. For ^{13}C spectra it operates at 125 MHz, with a typical resolution of 0.45 Hz or 0.0036 ppm. The 300 MHz spectrometer was used unless otherwise stated. ^{1}H NMR and ^{13}C NMR assignments were achieved with the aid of the following: DEPT (90 and 135), COSY, HSQC, and HMBC. All solvents used were purchased from Sigma Aldrich or Lennox Chemicals. Solvents were dried and stored in accordance with literature procedures.[1] Petroleum ether 40-60 was used for all synthesis and purification methods. High Resolution Mass Spectrometry was performed on an Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS, utilising the Mass-Hunter workstation software. Samples were dissolved in HPLC grade acetonitrile, with volumes injected onto the system of between 0.5–2.0 μL. The mobile phase (all HPLC grade) used was acetonitrile (70%), water (30%), and formic acid (0.1%). A flow-rate of 0.5 mL/min of mobile phase was used, while the column in the LC component was a C18 column. UV analysis was performed using a Hitachi U-2900 double beam spectrophotometer. Thin layer chromatography was performed using Merck aluminium-backed sheets with silica gel 60 F254. Isolated products were purified using flash chromatography with silica gel (0.040-0.0063 mm, 230-400 mesh) obtained from VWR. Polarimetry was carried out at room temperature using a 1 dm optical activity tube and an AA-55 Optical Activity Ltd. Polarimeter. Microwave synthesis was conducted using a CEM Focused Microwave Synthesis System, using a Discover SPS manual microwave peptide synthesiser attachment which included a 25 mL reaction vessel, a reaction vessel holder, a vacuum manifold, and a fiber-optic temperature probe. The manifold consists of two separate fluid paths for both waste and product collection under vacuum.

General Synthetic Methods

1. **General procedure for coupling using Fmoc solid-phase peptide synthesis (SPPS)**

A coupling solution of Fmoc-protected amino acid (5 molar equivalents to resin loading), HOBT (5 molar equivalents), PyBOP (5 molar equivalents) and *N*-methylmorpholine (5.5 molar equivalents) in DMF was prepared and allowed to pre-activate for 5 min. The solution was transferred to the peptide reaction vessel containing pre-swelled resin (DCM for 30 min.). The reaction vessel was placed in the microwave reactor and heated to 75 ^{o}C (50 W) for 10 min. The reaction vessel was then cooled to room temperature and was

shaken for a further 20 min., while nitrogen was gently bubbled through the solution. The resin was washed with DMF (3 x 10 mL) and DCM (3 x 10 mL). A small sample of resin was taken after each coupling step and the peptide was cleaved from the resin (see below) and analysed by LC-MS.

2. General procedure for peptide cleavage from the resin

A small sample of resin was taken and peptide cleavage was achieved by treatment with TFA:anisole:H_2O (95:5:5) and shaking for 1 h, without microwave assistance. The resin was removed by filtration and the filtrate was concentrated *in vacuo*. The resulting residue was dissolved in acetonitrile and analysed by HRMS to determine the content.

3. General procedure for Fmoc deprotection

The *N*-terminal Fmoc protecting group was deprotected by the addition of 20% piperidine in DMF (10 mL) to the peptide reaction vessel. The reaction vessel was placed in the microwave reactor and heated to 50 ^{o}C (25 W) for 10 min. The resin was cooled to room temperature and washed with DMF (3 x 10 mL) and DCM (3 x 10 mL). The deprotection filtrate solution was taken and analysed by UV spectrophotometry, where the presence of the dibenzofulvene-piperidine adduct at 301 nm determined if the deprotection step was successful.

4. General procedure for the removal of a *t*-butyl ester group

To the protected compound (1.0 mmol) was added DCM (5 mL), TFA (5 mL), and phenylsilane (0.13 mL, 0.11 g, 1.1 mmol) and the reaction was stirred for 2 h in an ice-bath, under a nitrogen atmosphere. The reaction was monitored by MS until the molecular ion peak of the starter was no longer present. The reaction mixture was then concentrated *in vacuo* and repeatedly redissolved in DCM and concentrated *in vacuo* to remove residual TFA.

Syntheses

Synthesis of allyl (S)-(1-[(allyloxy)carbonyl)]-N-(2-bromoethyl)carbamate (1). D-Serine (1.00 g, 9.51 mmol) and Na_2CO_3 (1.05 g, 9.90 mmol) were dissolved in water (10 mL) and acetonitrile (5 mL) was added followed by the dropwise addition of allyl chloroformate

(1.05 mL, 1.19 g, 9.88 mmol) over 5 min. The mixture was stirred for 30 h, at room temperature, before being concentrated to dryness *in vacuo*. The residue was then dissolved in dry DMF (20 mL) and $NaHCO_3$ (0.82 g, 9.8 mmol) was added followed by the dropwise addition of allyl bromide (0.85 mL, 1.19 g, 9.8 mmol) over 5 min. The mixture was stirred for 45 h at room temperature and then concentrated *in vacuo*. The residue was dissolved in ethyl acetate (50 mL) and washed with a saturated aqueous $NaHCO_3$ solution (40 mL), water (3 x 40 mL), and brine (2 x 30 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to afford a yellow oil. Purification by flash chromatography on silica in petroleum ether:ethyl acetate (3:2) gave allyl (*R*)-1-[(allyloxy)carbonyl)]-*N*-(2-hydroxyethyl)carbamate as a pale yellow oil (1.52 g, 70%). $[\alpha]^D_{20}$: +2.96 (*c* = 1.35 in $CHCl_3$); R_f 0.56, petroleum ether:ethyl acetate (2:1); ^{1}H NMR (CDCl_3) δ/ppm; 6.21 (d, 1H, *J* = 8.3 Hz), 5.94-5.86 (m, 2H), 5.25-5.19 (m, 4H), 4.67-4.64 (m, 4H), 4.44-4.41 (m, 1H), 3.98 (dd, 1H *J* = 11.5 and 3.7 Hz), 3.86 (dd, 1H *J* = 11.5 and 3.4 Hz); ^{13}C NMR (CDCl_3) δ/ppm; 163.1, 156.2, 132.5, 131.5, 118.4, 117.6, 66.4, 65.8, 62.6, 56.1; IR (Thin Film, NaCl)/cm[-1]; 3404, 2949, 1722, 1689, 1645, 1320; HRMS: ES[+] for $C_{10}H_{15}NO_5$, expected [M+H][+] 230.1028, observed [M+H][+] 230.1024.

To a solution of allyl (*R*)-1-[(allyloxy)carbonyl)]-*N*-(2-hydroxyethyl)carbamate (0.50 g, 2.2 mmol) and CBr_4 (1.24 g, 3.7 mmol) in dry DCM (5 mL) at 0 °C was added PPh_3 (1.17 g, 4.44 mmol) portionwise over a 5 min period. The mixture was then stirred at room temperature for 2.5 hr, after which it was concentrated *in vacuo*. Purification by flash chromatography on silica gel in petroleum ether:ethyl acetate (4:1) gave **1** as a clear oil (0.40 g, 63%). R_f 0.75, petroleum ether:ethyl acetate (2:1); ^{1}H NMR (CDCl_3) δ/ppm; 5.95-5.82 (m, 2H), 5.35-5.17 (m, 4H), 4.81-4.75 (m, 1H), 4.68-4.55 (m, 4H), 3.79 (dd, 1H, *J* = 10.6 and 3.4 Hz), 3.68 (dd, 1H, *J* = 10.6 and 3.5 Hz); ^{13}C NMR (CDCl_3) δ/ppm; 163.5, 152.9, 132.2, 131.3, 118.9, 118.3, 67.0, 66.5, 65.0, 60.8; IR (Thin film, NaCl)/cm[-1]; 3338, 2947, 1724, 1649, 1519, 1333, 665; HRMS: ES[+] for $C_{10}H_{14}NO_4Br$, expected [M+Na][+] 314.0001, observed [M+Na][+] 314.0003.

*Synthesis of N-(9H-fluorenylmethyloxycarbonyl)-(R)-cysteine t-butyl ester (**2**).* Tributylphosphine (0.50 mL, 0.41 g, 2.0 mmol) was added to a solution of *N*-(9H-fluorenylmethyloxycarbonyl)-(*R*)-cystine t-butyl ester[2] (1.50 g, 1.88 mmol) in THF (20 mL) under a nitrogen atmosphere and the solution was stirred for 15 min. Water (2.0 mL) was added and the reaction was stirred for an additional 2.5 h. The reaction was

concentrated *in vacuo* yielding **2** as a clear oil (1.09 g, 72%). The product was used immediately after preparation due to its instability. ^{1}H NMR (CDCl$_3$) δ/ppm; 7.72 (d, 2H, J = 7.5 Hz), 7.53 (d, 2H, J = 7.5 Hz), 7.40 (dd, 2H, J = 7.3 and 7.5 Hz), 7.29 (dd, 2H, J = 6.9 and 7.3 Hz), 5.70 (d, 1H, J = 7.4 Hz), 4.55-4.45 (m, 1H), 4.58-4.33 (m, 2H), 4.20 (dd, 1H, J = 8.9 and 6.5 Hz), 3.03-2.93 (m, 2H), 1.48 (s, 9H); IR (Thin Film, NaCl) cm^{-1}; 3334, 3064, 2978, 2570 (S-H), 1731, 1605.

Synthesis of 3-[2-(R)-allyloxycarbonyl-2-allyloxycarbonylamino-ethylsulfanyl]-2-(S)-(9H-fluorenylmethyloxycarbonylamino)propionic acid t-butyl ester (**3**). Cysteine **2** (1.06 g, 2.7 mmol) and β-bromoalanine **1** (0.82 g, 2.8 mmol) were both dissolved in degassed ethyl acetate (15 mL), under a nitrogen atmosphere. A solution of tetrabutylammonium bromide (3.55 g, 10.0 mmol) dissolved in a saturated aqueous NaHCO$_3$ solution (15 mL, pH 8.5) was then added to the mixture, which was stirred vigorously for 18 h (both the ethyl acetate and NaHCO$_3$ solution were deoxygenated by bubbling nitrogen gas into each solution for 30 min). Ethyl acetate (15 mL) and H$_2$O (15 mL) were added to the mixture and the organic layer was separated, dried over anhydrous MgSO$_4$ and concentrated *in vacuo*. Purification by flash chromatography on silica gel in petroleum ether: ethyl acetate (4:1) gave **3** as a clear oil (0.89 g, 55%). [α]$^D_{20}$: -6.25 (c = 0.32 in CHCl$_3$); R$_f$ 0.28, 3:1 petroleum ether:ethyl acetate; ^{1}H NMR (CDCl$_3$) δ/ppm; 7.72 (d, 2H, J = 7.4 Hz), 7.63-7.54 (m, 2H), 7.39-7.34 (m, 2H), 7.31-7.26 (m, 2H), 5.92-5.79 (m, 2H), 5.77 (d, 1H, J = 7.5 Hz), 5.69 (d, 1H, J = 7.5 Hz), 5.31-5.15 (m, 4H), 4.61-4.55 (m, 4H), 4.52-4.46 (m, 2H), 4.40-4.33 (m, 2H), 4.24-4.19 (m, 1H), 3.07-2.94 (m, 4H), 1.46 (s, 9H); ^{13}C NMR (CDCl$_3$) δ/ppm; 170.1, 169.3, 155.7, 155.6, 143.7, 141.2, 132.2, 131.2, 127.6, 126.9, 125.0, 119.8, 119.0, 117.7, 82.8, 67.1, 66.2, 65.8, 54.6, 53.8, 46.9, 35.7, 35.6, 27.8; IR (Thin Film, NaCl)/cm^{-1}; 3066, 2948, 1724, 1700, 1536; HRMS: ES$^+$ for C$_{32}$H$_{38}$N$_2$O$_8$S, expected [M+Na]$^+$ 633.2266, observed [M+Na]$^+$ 633.2269.

*Synthesis of 3-[2-(R)-allyloxycarbonyl-2-allyloxycarbonylamino-ethylsulfanyl]-2-(S)-(9H-fluorenylmethyloxycarbonylamino)propionic acid (**4**)*. Lanthionine **3** (0.40 g, 0.66 mmol) was deprotected using the general method described. Purification by flash chromatography on silica gel eluting with dichloromethane:methanol (98:2) gave **4** as a pale yellow oil (0.32 g, 86%). [α]$^D_{20}$: +14.47 (c = 0.76 in CHCl$_3$); R$_f$ 0.19, dichloromethane:methanol:acetic acid (25:1:0.1); ^{1}H NMR (CDCl$_3$) δ/ppm; 8.93 (s (br),

1H), 7.75 (d, 2H, J = 7.5 Hz),7.63-7.49 (m, 2H),7.42-7.34 (m, 2H), 7.32-7.27 (m, 2H), 6.03 (d, 1H, J = 7.9 Hz), 5.96-5.78 (m, 2H), 5.35-5.15 (m, 4H), 4.62-4.56 (m, 6H, J = 17.2 and 5.6 Hz), 4.43-4.31 (m, 2H), 4.25-4.19 (m, 1H), 3.13-2.78 (m, 4H); ^{13}C NMR (CDCl$_3$) δ/ppm; 173.2, 170.4, 156.3, 156.1, 143.7, 141.4, 132.3, 131.3, 127.9, 127.2, 125.2, 120.1, 119.5, 118.3, 67.6, 66.7, 66.4, 54.1, 53.7, 47.1, 35.5, 35.4; IR (Thin film, NaCl)/cm^{-1}; 3400, 3318, 3051, 2947, 2935, 1725, 1700; HRMS: ES$^+$ for C$_{28}$H$_{30}$N$_2$O$_8$S, expected [M+H]$^+$ 555.1801, observed [M+H]$^+$ 555.1796.

Preparation of lanthionine-loaded resin **5**. 2-Chlorotrityl chloride resin (2.0 g, 1.3 mmol/g loading) was pre-swelled in DCM (20 mL) with gentle shaking for 30 min. A loading solution of orthogonally protected lanthionine **4** (0.18 g, 0.32 mmol) and DIPEA (0.28 mL, 0.21 g, 1.60 mmol) in DCM (20 mL) was added to the resin in a peptide reaction vessel and the mixture was stirred gently for 2.5 h. The solution was filtered from the resin and the resin was then washed with DCM (3 x 20 mL). The remaining reactive sites on the resin were then capped using a solution of acetic acid (0.11 ml, 0.12 g, 2.0 mmol) and DIPEA (1.39 ml, 1.03 g, 8.00 mmol) in DCM (20 mL) for 2 h with gentle shaking, while N$_2$ was bubbled through the mixture. The resin was washed with DCM (3 x 20 mL). This gave a resin with a reduced loading (0.16 mmol/g) which was ready for SPPS. The Kaiser test[3] was carried out on a few resin beads which confirmed there were no primary amines present. A sample of the resin **5** was taken and cleaved using the general procedure (see above) and analysed by MS. HRMS: ES+ for C$_{28}$H$_{30}$N$_2$O$_8$S, expected [M+H]$^+$ 555.1801, observed [M+H]$^+$ 555.1808.

Preparation of Fmoc-Ile-Phe t-butyl ester **17**. To an ice-cold slurry of Fmoc-L-isoleucine (0.71 g, 2.0 mmol) and and L-phenylalanine *t*-butyl ester (0.44 g, 2.0 mmol) in acetonitrile (10 ml) was added Et$_3$N (0.6 ml, 0.44 g, 4.2 mmol) and HBTU (0.9 g, 2.2 mmol) and the reaction mixture was stirred at room temperature for 2 hr. The mixture was concentrated under reduced pressure and the residue was taken up in EtOAc and washed with HCl (2 M, 10 ml), a saturated aqueous NaHCO3 solution (10 ml), dried over anhydrous MgSO4, and concentrated *in vacuo*. Purification by flash column chromatography on silica gel eluted with petroleum ether:ethyl acetate (4:1) gave **17** as a white solid (1.08 g, 97%). R$_f$ 0.67, petroleum ether:ethyl acetate (2:1); M.pt. 136-137 $^{\circ}$C; [α]$_D^{20}$: -20.00 (c = 0.10 in CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ/ppm; 7.75 (d, 2H, J = 7.4 Hz), 7.59 (d, 2H, J = 7.4 Hz), 7.42-7.35 (m, 2H), 7.33-7.27 (m, 2H), 7.24-7.12 (m, 5H), 6.21 (d, 1H, J = 7.4 Hz), 5.34 (d, 1H, J = 8.8

Hz), 4.74 (dd, 1H, J = 7.3 and 6.2 Hz), 4.46-4.35 (m, 2H), 4.26-4.19 (m, 1H), 3.4.18-4.15 (m, 1H), 3.07 (d, 2H, J = 6.2 Hz), 1.88-1.79 (m, 1H), 1.51-1.45 (m, 1H), 1.39 (s, 9H), 1.18-1.04 (m, 1H), 0.91-0.86 (m, 6H); ^{13}C NMR (CDCl$_3$) δ/ppm; 170.6, 170.2, 156.2, 143.9, 141.4, 135.5, 129.5, 128.4, 127.8, 127.2, 127.1, 125.2, 120.0, 82.6, 67.1, 59.7, 53.6, 47.2, 38.2, 37.6, 27.9, 24.8, 15.4, 11.4; IR (KBr)/cm^{-1}; 3309 (N-H), 3089 (C-H, aromatic), 2965 (C-H, aliphatic), 1735 (C=O, ester), 1700 (C=O, carbamate), 1655 (C=O, amide); HRMS: ES$^+$ for C$_{34}$H$_{40}$N$_2$O$_5$, expected [M+K]$^+$ 595.2574, observed [M+K]$^+$ 595.2571.

Preparation of Fmoc-Ile(C=S)-Phe t-butyl ester **18**. To a solution of **17** (0.60 g, 1.1 mmol)) in dry DCM (20 ml) was added P$_2$S$_5$ (0.60 g, 1.1 mmol) followed by hexamethyldisiloxane (HMDO) (1.2 ml, 0.92 g, 5.5 mmol) and the reaction mixture was stirred at reflux temperature under an atmosphere of nitrogen for 5 hr. A further molar equivalent of P$_2$S$_5$ and 5 molar equivalents of HMDO was then added and the mixture was heated at reflux temperature and monitored by TLC until the reaction went to completion. The solution was concentrated *in vacuo* and the resulting residue was dissolved in EtOAc, washed with water (2 x 15 ml), brine (20 ml) and dried over anhydrous MgSO$_4$. Purification on silica gel eluting with petroleum ether:ethyl acetate (4:1) gave the desired thioamide as a clear oil (0.59 g, 96%). R$_f$ 0.81, petroleum ether:ethyl acetate (2:1); Oil; [α]$_D^{20}$: +47.22 (c = 0.36 in CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ/ppm; 8.35 (d, 1H, J = 6.3 Hz), 7.76-7.71 (m, 2H), 7.60 (d, 2H, J = 7.3 Hz), 7.40-7.34 (m, 2H), 7.32-7.26 (m, 2H), 7.24-7.10 (m, 5H), 5.63 (d, 1H, J = 8.6 Hz),5.30-5.23 (m, 1H), 4.46-4.28 (m, 2H), 4.26-4.18 (m, 2H), 3.21 (d, 2H, J = 6.2Hz), 1.99-1.81 (m, 1H), 1.65-1.53 (m, 1H), 1.37 (s, 9H), 1.18-1.06 (m, 1H), 0.92-0.85 (m, 6H); ^{13}C NMR (CDCl$_3$) δ/ppm; 204.1, 169.5, 156.1, 144.0, 140.4, 135.6, 129.7, 128.6, 127.9, 127.3, 127.3, 125.3, 119.9, 82.2, 67.2, 66.2, 58.9, 47.2, 39.7, 36.6, 28.0, 24.8, 15.8, 11.5; IR (Thin film, NaCl)/cm^{-1}; 3276 (N-H), 3064 (C-H, aromatic), 2967, 2933 (C-H, aliphatic), 1728 (C=O, ester), 1693 (C=O, carbamate), 1031 (C=S, thioamide); HRMS: ES$^+$ for C$_{34}$H$_{40}$N$_2$O$_4$S, expected [M+Na]$^+$ 595.2606, observed [M+Na]$^+$ 595.2572.

Preparation of Fmoc-Ile(C=S)-Phe **19**. To **18** (0.18 g, 0.33 mmol) was added DCM (1.5 ml), TFA (1.5 ml), and phenylsilane (0.04 ml, 0.033 g, 0.33 mmol) and the reaction was stirred for 2 hr. in an ice-bath, under an atmosphere of nitrogen. The reaction was monitored by LC-MS until the molecular ion peak of the starter was no longer present and this indicated the reaction had gone to completion. The reaction mixture was then

concentrated *in vacuo* and repeatedly redissolved in DCM and concentrated *in vacuo* to remove residual TFA. The product was purified by flash chromatography on silica gel eluting with dichloromethane:methanol (98:2) to yield **19** as a pale yellow oil (0.16 g, 90%). R_f 0.07, ethyl acetate:petroleum ether (2:1); ^{1}H NMR (CDCl$_3$) δ/ppm; 8.92 (s, 1H), 7.76 (d, 2H, J = 7.6 Hz), 7.51-7.56 (m, 2H), 7.51-7.35 (m, 2H), 7.34-7.28 (m, 2H), 5.87 (d, 1H, J = 10.0 Hz), 4.89-4.64 (m, 1H), 4.52-4.45 and 4.35-4.29 (m, 2H), 4.35-4.22 (m, 2H), 4.19-4.13 (m, 1H), 3.75 (s (br), 1H), 1.86-1.75 (m, 1H), 1.72-1.46 (m, 1H), 1.19-1.15 (m, 1H), 0.93-0.87 (m, 6H). ^{13}C NMR (CDCl$_3$) δ/ppm; 204.4, 170.9, 156.6, 143.5, 141.3, 127.8, 127.1, 125.1, 120.1, 67.6, 64.4, 47.3, 47.0, 40.7, 25.2, 15.5, 10.7; IR (KBr)/cm^{-1}, 3290 (O-H), 3066 (C-H, aromatic), 2965, 2927 (C-H, aliphatic), 1706 (C=O, acid), 1696 (C=O, carbamate), 908 (C=S, thioamide); HRMS: ES$^+$ for C$_{23}$H$_{26}$N$_2$O$_4$S, expected [M+H]$^+$ 427.1691, observed [M+H]$^+$ 427.1702.

Peptide	Molecular Formula	HRMS (Observed)	HRMS (Expected)
4	C$_{28}$H$_{30}$N$_2$O$_8$S	555.1796 [M+H]$^+$	555.1801[M+H]$^+$
7	C$_{43}$H$_{57}$N$_5$O$_{11}$S	852.3853 [M+H]$^+$	852.3829 [M+H]$^+$
8	C$_{42}$H$_{55}$N$_5$O$_{11}$S	838.3697 [M+H]$^+$	838.3692 [M+H]$^+$
9	C$_{43}$H$_{57}$N$_5$O$_{12}$S	890.3634 [M+Na]$^+$	890.3634 [M+Na]$^+$
10	C$_{49}$H$_{61}$N$_5$O$_{11}$S	928.4166 [M+H]$^+$	928.4161 [M+H]$^+$
11	C$_{44}$H$_{57}$N$_5$O$_{11}$S	864.3853 [M+H]$^+$	864.3850 [M+H]$^+$
12	C$_{20}$H$_{35}$N$_5$O$_6$S	474.2378 [M+H]$^+$	474.2386 [M+H]$^+$
13	C$_{21}$H$_{37}$N$_5$O$_6$S	488.2545 [M+H]$^+$	488.2542 [M+H]$^+$
14	C$_{21}$H$_{37}$N$_5$O$_7$S	504.2491 [M+H]$^+$	504.2508 [M+H]$^+$
15	C$_{27}$H$_{41}$N$_5$O$_6$S	564.2855 [M+H]$^+$	564.2855 [M+H]$^+$
16	C$_{22}$H$_{37}$N$_5$O$_6$S	500.2542 [M+H]$^+$	500.2555 [M+H]$^+$
17	C$_{34}$H$_{40}$N$_2$O$_5$	595.2571 [M+K]$^+$	595.2574 [M+K]$^+$
18	C$_{34}$H$_{40}$N$_2$O$_4$S	595.2606 [M+Na]$^+$	595.2572 [M+Na]$^+$
19	C$_{30}$H$_{32}$N$_2$O$_4$S	517.2161 [M+H]$^+$	517.2169 [M+H]$^+$
20	C$_{49}$H$_{61}$N$_5$O$_{10}$S$_2$	966.3757 [M+Na]$^+$	966.3755 [M+Na]$^+$
21	C$_{27}$4$1_1$N$_5$O$_{15}$S$_2$	597.2892 [M+NH$_4$]$^+$	597.2874 [M+NH$_4$]$^+$

Table 1. High-Resolution MS results for all synthesised peptides.

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