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Synthesis of Cell-wall Analogues of Vancomycin-resistant Enterococci using Solid Phase Peptide Synthesis

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Abstract: A convenient method for the direct coupling of lithium D-lactate to 2-chlorotrityl chloride resin enabled the rapid and efficient solid phase synthesis of bacterial cell-wall precursor analogues (depsipeptides) found in vancomycin-resistant enterococci (VRE).

The emergence of antibiotic-resistant strains of bacteria has made it desirable to probe the molecular mode of action of these antibiotics. Recently, resistance to even some of the most potent class of antibiotics, the vancomycin group of antibiotics, has been reported.[1,2] These antibiotics bind to cell-wall peptide precursors terminating in the sequence –Lys-D-Ala-D-Ala.[3,4] In vancomycin-resistant strains of bacteria, the C-terminal residue, D-Ala has been replaced by a D-lactate (D-Lac). This simple change causes a repulsive interaction between the antibiotic and the ligand, such that the antibiotic becomes therapeutically useless.[1,2] Previously, we have developed model cell surfaces in order to study the binding interactions that occur between the vancomycin group antibiotics and cell-wall peptide precursors at the surface of bacteria.[5,6] To this end, we have synthesised suitable cell-wall peptide precursor analogues bearing hydrophobic acyl chains on their N-termini, which can anchor to model membranes.

The solid phase peptide synthesis (SPPS) of D-Ala terminating cell wall peptide analogues is routine, as resins pre-loaded with D-Ala are commercially available. However, resins pre-loaded with D-Lac are not commercially available. Previously, direct attachment of lactic acid to Merrifield resin has been unsuccessful, although Boc-D-Ala-D-Lac was attached using the cesium salt procedure.[7] We report a convenient method for the direct coupling of the commercially available lithium D-lactate (Li D-Lac) to a solid resin support, thereby simplifying the synthesis of depsipeptides terminating in D-lactate by SPPS.

We chose the acid labile 2-chlorotrityl chloride resin[8,9] as the first coupling does not require activation of the first residue, and thus is free from the problems of racemisation and polymerisation. To find suitable conditions for coupling Li D-Lac directly to resin we added the salt to 2-chlorotrityl chloride in various solvents. Li D-Lac proved to be insoluble in a variety of solvents including THF, DCM, and DMF,[10] and coupling could not be detected. However, we found that Li D-Lac was partially soluble in DMSO, and coupling in a solvent mixture DMSO:DCM (3:1) (DCM was required to solubilise 2-chlorotrityl chloride) for one hour gave a new compound by t.l.c..

The analogous experiment was carried out on resin. A four fold excess of Li D-Lac was added directly to 2-chlorotrityl chloride resin (1.3 mmol/gram) in DMSO/DCM (1:1) and the mixture was agitated overnight

(15 hours, under N_2) then washed successively with DMSO, DMF, and DCM. The next residue, Fmoc-D-Ala, was dissolved in DMF and was added to the resin complex and the resulting mixture cooled to 0 °C. DIC and a catalytic amount of DMAP were dissolved in DMF and added dropwise to the resin mixture over 30 minutes (0 °C, under N_2) and agitated at room temperature for 6 hours. DCC can also be used as the coupling reagent, but this generates a large amount of insoluble DCU, whereas DIC generates a small amount of precipitate that is readily removed by successive washes with DMF.

The resin was then washed with DMF, DCM, and dried under vacuum. To determine the loading of the didepsipeptide, a small amount of the resin complex (approx. 1 μmol of original resin loading) was placed in a solution of piperidine in DMF (3 ml, 20 % v/v) and the amount of deprotected Fmoc in free solution was measured by UV at 290 nm. An average corrected absorbance of 0.82 (0.165 = 0.1 μmol Fmoc) gave an estimated loading of 0.5 mmol of D-Lac/gram of resin.

The remaining coupling steps were carried out using standard coupling methods (Table 1). The Fmoc protecting groups were removed using a solution of piperidine in DMF (20 % v/v). Coupling of Fmoc protected amino acids were carried out using PyBOP,[11] HOBt, and DIEA in DMF. Reactions times were typically between 4 - 12 hours depending on the excess of the Fmoc residue and reagents used. The extent of coupling was monitored by the Kaiser ninhydrin test.[12] The Fmoc protected amino acids are commercially available with the exception of the D-glutamic acid residue, where the residue is coupled through the side chain γ-carboxylic acid.

D-Glutamic acid was selectively protected with allyl alcohol, following a procedure analogous to that described for the protection of aspartic acid.[13] Further protection with Fmoc-*N*-hydroxysuccinimide[14,15] and isobutylene[16] resulted in formation of the fully protected *N*-α-Fmoc-D-glutamic acid-γ-allyl ester-α-*t*-butyl ester. The allyl ester was removed using the mild and efficient catalyst tetrakis(triphenylphosphine) palladium (0)[17-19] with HOBt as the accepting nucleophile to produce *N*-α-Fmoc-D-glutamic acid-α-*t*-butyl ester (Fmoc-D-Glu-OtBu) in an overall yield of 33%. Coupling of this protected amino acid to the resin was successfully performed using 1.3 eq. relative to the depsipeptide loading of resin, and with an extended coupling time (48 hours).

After assembling H-Gly-Ala-D-γ-Glu(OtBu)-Lys(Ac)-D-Ala-D-Lac on resin, where the N-terminal Gly acts as a spacer in a sequence otherwise the same as that found in VRE, the resin complex was divided in two equal portions. The two separated portions were used to couple decanoic acid and docosanoic acid respectively, in an analogous manner as the Fmoc amino acid coupling reactions (Table 1). However, due to the poor solubility of docosanoic acid, this coupling was repeated twice using a lower number of equivalents of the acid and warm DCM as the solvent. Cleavage and final deprotection of each product was achieved by treating the resin complex with a solution of TFA:TIPS[20]:H_2O (38:1:1) for 4 hours. The crude $CH_3(CH_2)_8CO$-Gly-Ala-D-γ-Glu-Lys(Ac)-D-Ala-D-Lac product was purified by C_{18} RP-HPLC using a 0% to 80% acetonitrile (0.1 % TFA) gradient over 60 minutes, then lyophilised to give a white solid in a final yield of *ca.* 30%. The crude $CH_3(CH_2)_{20}CO$-Gly-Ala-D-γ-Glu-Lys(Ac)-D-Ala-D-Lac was washed with cold H_2O, then lyophilised to give a white solid in a final yield of *ca.* 40%. The final products gave the expected spectra by FT-ICR electrospray mass spectroscopy (Found: $[M+K]^+$, 781.3751; $C_{34}H_{58}O_{12}N_6K$, and M^+, 911.6069; $C_{46}H_{82}O_{12}N_6$, respectively) and by NMR analysis.

Table 1: Synthetic summary of *N*-Acyl Hexadepsipeptides

Reactants/Conditions	Product
Li D-Lac + 2-Chlorotrityl chloride resin (**P**)	D-Lac-**P**
(i) Fmoc-D-Ala DMF/0 °C; (ii) DIC, DMAP; DMF/0 °C	Fmoc-D-A-D-Lac-**P**
20% piperidine in DMF	D-A-D-Lac-**P**
Fmoc-Lys(Ac), PyBOP, HOBt, DIEA	Fmoc-K(Ac)-D-A-D-Lac-**P**
20% piperidine in DMF	K(Ac)-D-A-D-Lac-**P**
Fmoc-D-Glu-OtBu, PyBOP, HOBt, DIEA	Fmoc-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**
20% piperidine in DMF	D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**
Fmoc-Ala, PyBOP, HOBt, DIEA	Fmoc-A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**
20% piperidine in DMF	A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**
Fmoc-Gly, PyBOP, HOBt, DIEA	Fmoc-G-A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**
20% piperidine in DMF	G-A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**
Addition of Acyl chains	
Decanoic acid, PyBOP, HOBt, DIEA in DCM and G-A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**	$CH_3(CH_2)_8CO$-G-A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**
Docosanoic acid, PyBOP, HOBt, DIEA in DCM/40°C and G-A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-Res	$CH_3(CH_2)_{20}CO$-G-A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**
Deprotection and Cleavage from resin	
TFA 95%; TIPS 2.5%; H_2O 2.5% and $CH_3(CH_2)_8CO$-G-A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**	$CH_3(CH_2)_8CO$-G-A-D-γ-Glu-K(Ac)-D-A-D-Lac
TFA 95%; TIPS 2.5%; H_2O 2.5% $CH_3(CH_2)_{20}CO$-G-A-D-γ-Glu(OtBu)-K(Ac)-D-A-D-Lac-**P**	$CH_3(CH_2)_{20}CO$-G-A-D-γ-Glu-K(Ac)-D-A-D-Lac

In summary, we report a convenient method for the preparation of cell-wall depsipeptide precursors of vancomycin-resistant bacteria. Furthermore, we were able to add acyl chains to these precursors to serve as membrane anchors to our membrane models. These models provide an invaluable tool in understanding the mode of action of the vancomycin group of glycopeptide antibiotics.[5,6]

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10. The following abbreviations were used for solvents, reagents, compound names, etc...
    DCM = dichloromethane; DMF = dimethylformamide; DMSO = dimethylsulfoxide; THF = tetrahydrofuran; TFA = trifluoroacetic acid; TIPS = triisopropylsilane
    DIEA = diisopropylethylamine; DMAP = 4-dimethylaminopyridine; DCC = N,N'-dicyclohexylcarbodiimide; DCU = dicyclohexylurea; DIC = N,N'-diisopropylcarbodiimide; HOBt = N-hydroxybenzotriazole; PyBOP = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; Fmoc = 9-fluorenylmethoxycarbonyl
    RP-HPLC = reverse phase high performance liquid chromatography; NMR = nuclear magnetic resonance; FT-ICR = fourier transform - ion cyclotron resonance; t.l.c. = thin layer chromatography (chloroform:ethyl acetate (9:1);
    Ac = acetyl; A = Ala = alanine; K = Lys = lysine; G = Gly = glycine; Glu = glutamic acid; eq = equivalents; nm = nanometers
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