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Muramyl Peptide Analogs : Synthesis of a Depsipeptide using Orthogonal SPPS

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Abstract: *A depsipeptide mimic of the Gram-positive muramyl peptide was synthesized on resin using both Boc and Fmoc protection strategies. The depsipeptide unit is chemically and stereochemically compatible with both Boc and Fmoc chemistries and with HF cleavage conditions.*

High molecular mass penicillin binding proteins (HMM-PBP's) catalyze peptidoglycan transpeptidation and transglycosylation, which are necessary for the synthesis of bacterial cell walls, and are targets of the β-lactam antibiotics. An *in vitro* functional assay for inhibition of transpeptidase activity would be a useful tool for antibiotic discovery.[1] Recently there have been reports which show that ester and thioester mimics of *D*Ala-*D*Ala[11] are substrates of HMM-PBP's.[2] Although Ac-Lys(Ac)-*D*Ala-*D*Ala and Ac-Lys(Ac)-*D*Ala-*D*Lac appear to be a poor substrates of HMM-PBP's,[1,3] we were interested in examining the value of a substrate which more closely resembles the natural muramyl peptide. It would contain a pentaglycine chain branching from the ε-amine of the Lys (which characterizes Gram-positive bacterial peptidoglycan) and a marker for transpeptidase activity (*D*-lactate release). We chose to target peptide (**1**).

If we were to install the lactate unit of (**1**) in the early stages of a resin linked construction we would need to assess the structural and stereochemical integrity of this unit in the depsipeptide to repeated bouts with 20% piperidine, 50% TFA and HF that would be necessary for orthogonal synthesis. Initial attempts to circumvent such potential problems centered on synthesizing a protected peptide fragment (Ac-A-*D*IsoGln-K(Fmoc-G_5)-*D*A-OH) and esterifing it with benzyl *D*-lactate or methyl *D*-lactate. Unfortunately this approach failed due to solubility problems. We were therefore compelled to investigate the resin bound lactic acid strategy. We had no success in attaching lactic acid to Merrifield Resin, presumably again due to solubility problems. However the protected depsipeptide Boc-*D*Ala-*D*lactic acid[4] was readily attached to Merrifield Resin using the cesium salt procedure.[5]

Boc-*D*Ala was coupled with benzyl *D*-lactate, in solution, to yield Boc-*D*Ala-*D*Lac-OBn which was then hydrogenated in ethyl acetate with Pd/charcoal to remove the benzyl protection (see Scheme). The Boc-*D*Ala-*D*lactic acid was converted to its cesium salt. This material was only partially soluble in dry DMF. Nonetheless Merrifield resin was added and the mixture sonicated at 48°C for 72 hours. After Boc removal, the resin was subjected to a quantitative ninhydrin[6] test which indicated that 99% of the potential reactive groups on the resin were occupied with *D*Ala-*D*lactic acid.

Scheme : Summary of Depsipeptide Synthesis

Step	Reactants/Conditions	Product
1)	Boc-*D*A + *D*Lac-OBn ; CDI / CH_2Cl_2	Boc-*D*A-*D*Lac-OBn
2)	H_2 / Pd / EtOAc	Boc-*D*A-*D*Lac-OH
3)	Cs_2CO_3 / EtOH / H_2O	Boc-*D*A-*D*Lac-O$^-$ Cs$^+$
4)	Merrifield resin ; DMF / 50°C	Boc-*D*A-*D*Lac-Resin
5)	(i) TFA (ii) DIEA (iii) N-α-Boc-N-ε-Fmoc-K-Obt	Fmoc Boc-K-*D*A-*D*Lac-Resin
6)	piperidine	Boc-K-*D*A-*D*Lac-Resin
7)	Fmoc-G-Obt	Fmoc-G Boc-K-*D*A-*D*Lac-Resin
8-15)	repeat steps 6 & 7 - x4	Fmoc-G-G-G-G-G Boc-K-*D*A-*D*Lac-Resin
16)	(i) TFA (ii) DIEA (iii) Boc-*D*Isogln-Obt	Fmoc-G-G-G-G-G Boc-*D*Isogln-K-*D*A-*D*Lac-Resin
17)	(i) TFA (ii) DIEA (iii) Boc-A-Obt	Fmoc-G-G-G-G-G Boc-A-*D*Isogln-K-*D*A-*D*Lac-Resin
18)	(i) TFA (ii) DIEA (iii) Ac_2O	Fmoc-G-G-G-G-G Ac-A-*D*Isogln-K-*D*A-*D*Lac-Resin
19)	piperidine	G-G-G-G-G Ac-A-*D*Isogln-K-*D*A-*D*Lac-Resin
20)	HF	G-G-G-G-G Ac-A-*D*Isogln-K-*D*A-*D*Lac-OH (1)

At this point the rest of the molecule was synthesized using standard solid phase peptide synthesis[7] with both Boc and Fmoc amine protection (see Scheme). All acylations were performed with BOP activation[8] and monitored with ninhydrin testing. Boc deprotection was achieved with 50% TFA/CH_2Cl_2 for a total of 25 minutes followed by neutralization by 5% DIEA in CH_2Cl_2. Fmoc was removed with 20% piperidine/80% NMP for 20 minutes. N-α-Boc-N-ε-Fmoc-Lys was used to acylate *D*Ala-*D*Lac-Merrifield Resin. The segment extending out from the ε-amine of the Lys was successively deprotected and acylated with Fmoc-Gly five times leaving the last Gly protected with Fmoc. The α-amine of the Lys was deprotected and acylated with Boc-*D*Isoglutamine which was then deprotected and acylated with Boc-Ala. The Boc group was removed and the N-terminus acylated with acetic anhydride/pyridine. The Fmoc on the Gly was then removed. The resin peptide was then washed, dried, and cleaved in 100% HF at 0°C for one hour. The crude peptide was purified by C18 RP-HPLC with a 0% to 40% acetonitrile(0.1% TFA) gradient over 60 minutes. The product had a mass of 815 by electrospray mass spectroscopy and, using isocratic HPLC, it was chromatographed to yield a single peak at 7.36 minutes using 6% acetonitrile on a C-18 analytical column. The overall yield was 32% of theoretical.

NMR analyses have previously been performed on the products of Boc protected depsipeptide fragment couplings to rule out epimerization during these processes.[9] However, to our knowledge, retention of stereochemical integrity of lactate units under the basic conditions of Fmoc removal and the rigours of HF cleavage has not been established. To satisfy our concerns about racemization, the Boc group was removed from a portion of the Boc-*D*Ala-*D*Lac-Resin and the resultant terminal amine was then acetylated. This resin was treated with 20% piperidine/NMP for 1 hour, 50% TFA/CH_2Cl_2 for one hour and HF cleavage conditions for one hour to simulate actual synthesis conditions. Ac-*D*Ala-*D*lactic acid and Ac-*D*Ala-*L*lactic acid were synthesized in solution as controls. The 500MHz ^{1}H-NMR spectra of these two compounds were compared and it was clear that the C-α-H signals of Ala, both individually and in mixtures, were distinguishable. The ^{1}H-NMR of the material retrieved from the resin gave no indication of racemization both when compared to, and mixed with, Ac-*D*Ala-*L*lactic acid.[10]

In summary we have been able to prepare a lactate containing analog of a Gram-positive muramyl peptide as a potential substrate for a peptidoglycan transpeptidation assay. This synthesis has allowed us to demonstrate the applicability of Fmoc solid phase peptide synthesis and of the HF resin cleavage process to the construction of depsipeptides.

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10. Lack of racemization was also confirmed by mild alkaline hydrolysis of (1). The liberated lactate was identified upon exposure to *L*- and *D*- lactate dehydrogenase. There was no evidence of *L*- lactate dehydrogenase activity above background levels. These hydrolysis conditions do not racemize *D*-Lactate. We would like to thank Dr. Paul Mazur for this determination.
11. Ac = acetyl; Ala = A = alanine; Bn = benzyl; Boc = t-butyloxycarbonyl;
    BOP = benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate;
    CDI=1,1'carbonyldiimidazole; DIEA = diisopropylethylamine;
    Fmoc = 9-fluorenylmethyloxycarbonyl; Gly = G = glycine; HF = hydrogen fluoride;
    Lac = lactic acid; Lys = K = lysine; NMP = N-methylpyrrolidinone; Obt = oxybenzotriazole;
    RP-HPLC = reverse phase high performance liquid chromatography;
    TFA = trifluoroacetic acid; SPPS = solid phase peptide synthesis.

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