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A novel strategy for the solid-phase synthesis of cyclic lipodepsipeptides

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Abstract—A rapid and efficient Fmoc solid-phase synthesis of cyclic lipodepsipeptide analogue **1** to antibiotic fusaricidin A is described. Our synthetic approach includes resin attachment of the first amino acid via side chain, successful use of combination of four quasi-orthogonal removable protecting groups, stepwise solid-phase synthesis of linear peptide analogue, lipid tail attachment followed by depsipeptide bond formation and on-resin head-to-tail cyclization. Undesired *O*→*N* acyl shift, which may occur during Fmoc removal, was successfully avoided by the incorporation of the lipid tail into the linear peptide precursor prior to on-resin depsipeptide bond formation and the ring closure.
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Cyclic lipodepsipeptides have been found in many natural organisms such as fungi, bacteria, and marine organisms.[1] Despite their diverse spectrum of biological activities, they have shown the greatest therapeutic potential as antimicrobial agents.[2] However, lack of commercial sources of cyclic lipodepsipeptide producing organisms and unlimited access to their synthetic analogues hampered their utilization as a lead compounds for the development of new antibiotics. Therefore total solid-phase synthesis of these important natural products represents the first step toward complete exploitation of their antibacterial potentials.

Here we described our study on developing a rapid and efficient solid-phase synthesis of cyclic lipodepsipeptides fully compatible with standard Fmoc-chemistry in order to establish a general synthetic route to these natural products and their analogues.

Our strategy for the solid-phase synthesis of cyclic lipodepsipeptides involves side-chain attachment and on-resin head-to-tail cyclization (Scheme 1). The key step in the synthesis of cyclic depsipeptides is the ring closure. Poorly chosen place for ring closure can lead to slow cyclization rates, thus facilitating side reactions such as dimerization, oligomerization, and/or epimerization of the C-terminal residue. Traditional methods to prepare cyclic peptides and therefore depsipeptides involve solid-phase synthesis of the selectively protected linear precursor and cyclization in solution under high dilution conditions.[3,4,7] As an attractive alternative, cyclization could be performed while peptides still remain anchored to the resin. Taking into consideration the greater reactivity of the amino group, and therefore minimal possibility of side reactions, we chose macrolactamization for depsipeptide ring closure. Although this synthetic strategy appears to be a better choice for the solid-phase depsipeptide ring closure, undesired intramolecular *O*→*N* acyl shift may occur if basic conditions were to be used.[5] To test this possibility and to optimize the solid-phase synthesis of cyclic lipodepsipeptides, we have synthesized cyclic fusaricidin A's analogue **1** as a model compound (Scheme 1). Fusaricidin A is a naturally occurring cyclic lipodepsipeptide antibiotic isolated from *Bacillus polymyxa* KT-8 strain.[6] Strong activity against various kinds of fungi and Gram-positive bacteria, including *Staphylococcus aureus*, as well as its short sequence containing mostly standard amino acids, makes this natural product particularly interesting as a lead compound for the development of new antimicrobial agents. Depsipeptide analogue **1** differs from the natural compound in the lipid tail part and D-*allo*-Thr was replaced with D-Thr. Since in fusaricidin A 15-guanidino-3-hydroxypentadecanoic acid is attached via

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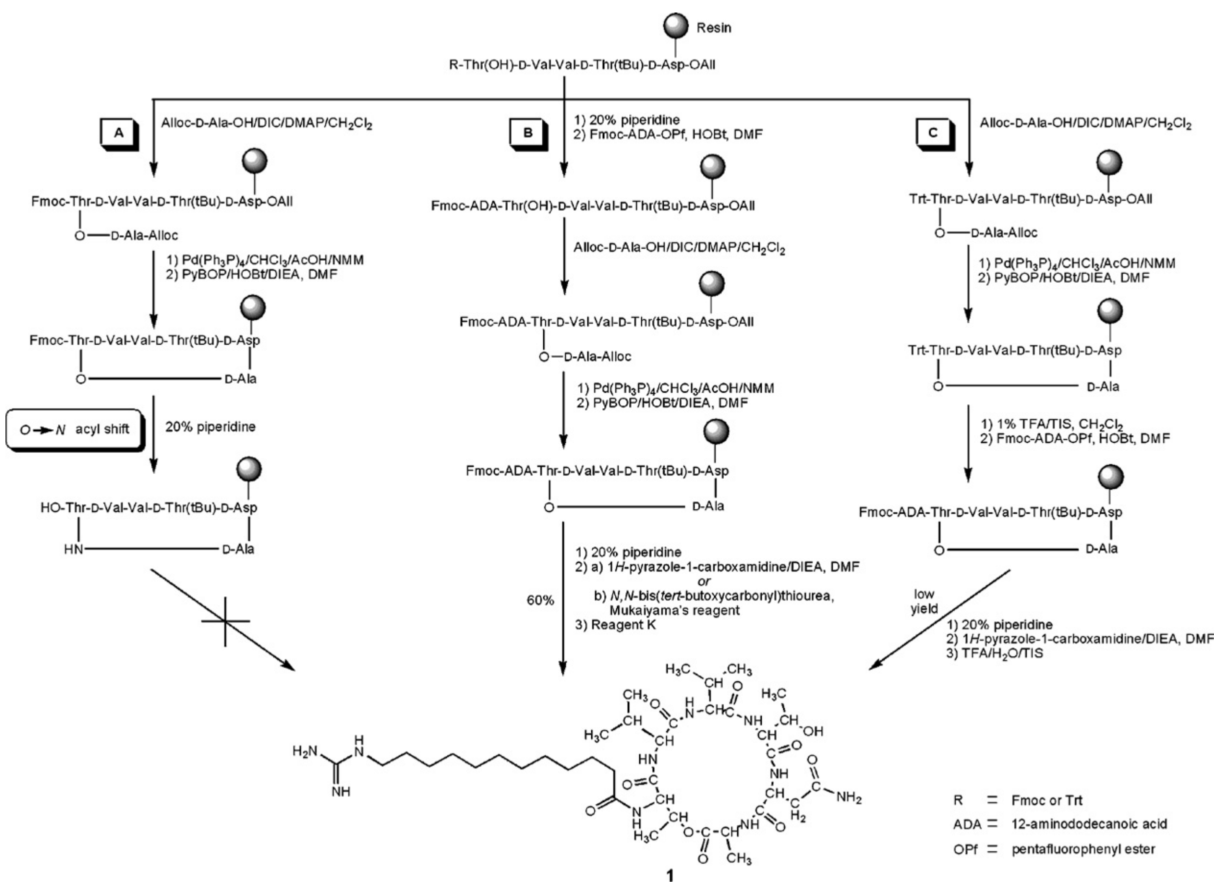

Scheme 1. Solid-phase synthesis of cyclic lipodepsipeptide **1**.

amide bond to the N-terminal L-Thr_1 α-amino group we decided to couple the closest commercially available analogue to this acid instead, 12-aminododecanoic acid, and convert it on the solid support into desirable guanidino form. For depsipeptide's ester bond formation we chose DIC/DMAP method; according to the literature the most efficient method for solid-phase peptide esterification reactions.[7a] Polyethylene glycol (PEG) and polystyrene (PS) based resins were tested for highest esterification yields. The synthesis of **1** is started by attaching C-terminal Fmoc-D-Asp-OAllyl via side chain to amide resins using HBTU/HOBt/DMAP procedure. Standard Fmoc-chemistry was used throughout.[8] The last amino acid in the linear peptide sequence, Alloc-D-Ala_6, was coupled via ester bond to the hydroxyl group of Fmoc-L-Thr_1 using previously mentioned DIC/DMAP method[7] (Scheme 1). Product formation was monitored by MALDI-TOF MS analysis. The best results for this coupling were obtained using PEG based resins such as TentaGel S RAM (0.5 mmol/g, Advanced Chemtech) or PAL-ChemMatrix (0.58 mmol/g, Matrix Innovation) and CH_2Cl_2 as a solvent.[9] The use of polar *N*,*N*-dimethylformamide (DMF), a typical solvent for SPPS, resulted in no ester bond formation. Poor coupling yields were obtained on PS based Rink-MBHA resins (0.66 mmol/g, Novabiochem) regardless of the solvent used. These results could be attributed to a better swelling of PEG-based resins,[10] rapid DIC activation of the carboxylic group,[11] and significant suppression of *N*-acylurea byproduct formation[12] in a non-polar solvent such as CH_2Cl_2. After selective removal of Alloc and Allyl protective groups by treatment with $Pd(Ph_3P)_4$,[13] the linear peptide was cyclized between D-Ala_6 and D-Asn_5 residues with an excess of PyBOP/HOBt/DIEA mixture. An attempt to remove Fmoc protecting group from N^{α}-L-Thr_3 residue using standard piperidine procedure resulted in *O*-acyl ester linkage transfer to *N*-acyl amide linkage to form a peptide bond between Thr_1 and Ala_6 residues (Scheme 1A). Reversible intramolecular $O \rightarrow N$ or $N \rightarrow O$ acyl shifts are well-known side reactions that may occur during peptide synthesis.[5] Peptides containing Ser or Thr residues undergo $N \rightarrow O$ acyl shift under acidic conditions, while the exposure of corresponding depsipeptides to basic conditions leads to opposite $O \rightarrow N$ acyl shift. In our case, the occurrence of $O \rightarrow N$ acyl shift after on-resin Fmoc removal was firstly indicated by a negative Kaiser test, and confirmed by MALDI-TOF MS and RP-HPLC analyses of the crude peptide product. As shown in Figure 1, RP-HPLC analysis of this crude product showed two primary peaks eluting at 13.06 min and 13.62 min.[14] In both cases MALDI-TOF MS gave almost identical mass, $[M+Na]^+$, $m/z = 609.0190$ and $m/z = 609.0219$ (calculated = 608.3014). Since $O \rightarrow N$ acyl shift is a reversible reaction, it is not surprising that both possible products are present in the solution after TFA cleavage from the resins. To avoid this highly undesirable side reaction, we explored the possibility of incorporating into the linear sequence trityl (Trt) protected N^{α}-L-Thr_1 whose deprotection requires acidic conditions; the

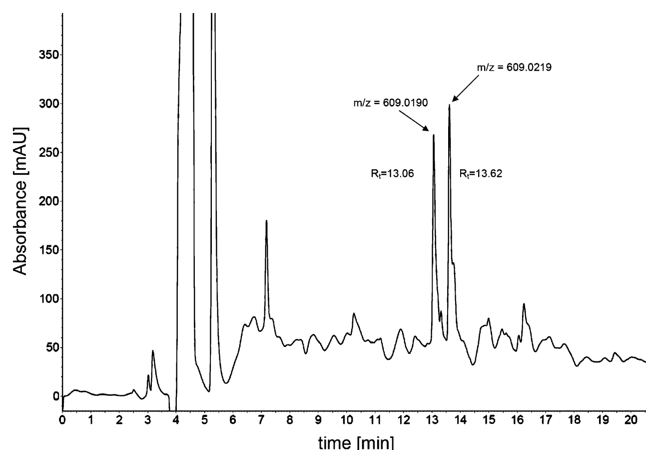

Figure 1. RP-HPLC chromatogram of cyclic peptide products mixture obtained after N-terminal Fmoc deprotection and TFA cleavage indicating occurrence of $O \rightarrow N$ acyl shift.

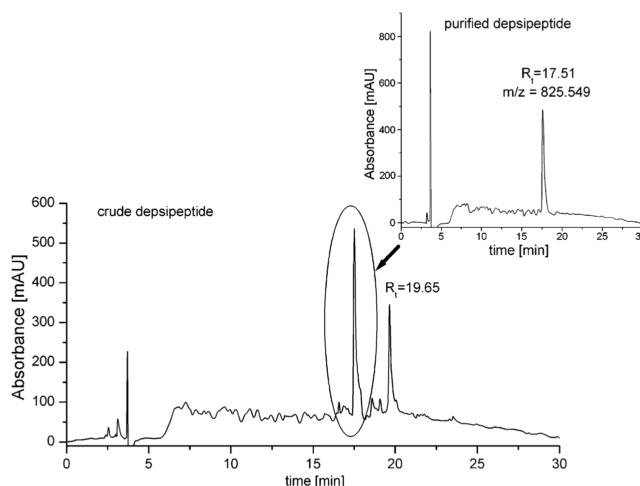

Figure 2. RP-HPLC chromatograms of crude and purified cyclic lipodepsipeptide **1**.

conditions that favor an ester bond formation (Scheme 1C). Although this strategy allowed us to completely suppress undesired $O \rightarrow N$ acyl shift, the desired cyclic lipodepsipeptide **1** was obtained in a poor yield due to observed substantial peptide cleavage from tested PEG based resins and incomplete Trt protecting group removal under generally recommended conditions. Incorporation of the 12-aminododecanoic acid into the linear peptide precursor prior to Ala_6 coupling via ester bond and on-resin cyclization turned out to be a much better choice for avoiding $O \rightarrow N$ acyl shift and obtaining desired cyclic lipodepsipeptide in a high yield (Scheme 1B). Since this strategy utilizes standard peptide deprotection and coupling protocols it is fully compatible with automated solid phase peptide synthesis. After the solid-phase assembly of L-Thr_1-D-Val_2-L-Val_3-D-Thr_4-D-Asn_5 sequence and selective Fmoc removal from terminal N^{α}-L-Thr_1 residue, Fmoc protected 12-aminododecanoic acid pentafluorophenyl ester (2 equiv) was reacted to give the desired linear lipidic peptide precursor. Alloc-D-Ala_6 was then coupled via ester bond followed by simultaneous Alloc and Allyl removal and on-resin cyclization as previously described in the text. The conversion of lipid tail's amino into the desired guanidino group was achieved by the removal of the Fmoc-protecting group using standard piperidine deprotection protocol and the treatment of the peptidyl-resin with either 1*H*-pyrazole-1-carboxamidine/DIEA/DMF mixture or with *N*,*N*-bis(*tert*-butoxycarbonyl)thiourea followed by Mukaiyama's reagent.[15] The best results were obtained using *N*,*N*-bis(*tert*-butoxycarbonyl)thiourea and Mukaiyama's reagent. With these reagents guanylation reaction was completed after 2 h, while the use of 1*H*-pyrazole-1-carboxamidine resulted in incomplete guanylation after 24 h. In both cases the reaction progress was monitored by Kaiser test and MALDI-TOF MS analysis. Final deprotection and cleavage of the cyclic lipodepsipeptide **1** from the resins was carried out with reagent K (Fig. 2, 60% yield based on the HPLC analysis of the crude peptide).[16] Treatment of the crude product **1** with $MeOH/H_2O$/ammonia mixture, that is, known to hydrolyze only depsipeptide's ester bond,[17] followed by RP-HPLC and MALDI-TOF MS analyses of the reaction mixture, confirmed that $O \rightarrow N$ acyl shift did not occur during lipid tail's Fmoc removal and that major product is the desired cyclic lipodepsipeptide **1**.[18]

With the synthesis of fusaricidin A's analogue **1** we demonstrated the feasibility of our synthetic approach that includes resin attachment of the first amino acid via side chain, successful use of combination of four quasi-orthogonal removable protecting groups, stepwise Fmoc solid-phase synthesis of a linear precursor peptide, lipid tail attachment followed by last amino acid coupling via ester bond and on-resin head-to-tail macrolactamization. This strategy allows the complete suppression of undesired $O \rightarrow N$ acyl shift, and an efficient automated solid-phase synthesis of cyclic lipodepsipeptides. The total solid-phase synthesis of a naturally occurring cyclic lipodepsipeptide antibiotic fusaricidin A is in progress and will be reported elsewhere.

Acknowledgments

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9. Alloc-D-Ala-OH (4 equiv), diisopropylcarbodiimide (DIC, 4 equiv) and 4-(dimethylamino)pyridine (DMAP, 0.1 equiv) were dissolved in 5 mL of CH_2Cl_2 or DMF and added to the resin. The mixture was agitated for 1 h in the case of CH_2Cl_2 and overnight in case of DMF at room temperature. The suspension was than washed with DMF (3X) and CH_2Cl_2 (5×). This coupling was performed on a 0.25 mmol scale. The mass spectrometry analyses were performed on MALDI-TOF Voyager-DE STR spectrometer (PerSeptive Biosystems, USA) in reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix. The observed $[M+Na]^+$, $m/z = 973.3043$ (calcd = 972.4331), corresponding to desired depsipeptide product was obtained only when the synthesis was carried out on PEG based resins and CH_2Cl_2 as a solvent.
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14. The crude peptide mixture was analyzed by RP-HPLC (ThermoElectron Corp., USA) on a C-18 column (Grace Vydac Denali, 250 × 4.6 mm, 120 Å, 5 μm) at a flow rate of 1 mL/min using gradient conditions from 5% solution A (water, 0.1% TFA) to 100% solution B (acetonitrile, 0.1% TFA) over 30 min.
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16. The crude peptide was purified by RP-HPLC (ThermoElectron Corp., USA) on a C-18 column (Phenomenex Jupiter, 250 × 20.1 mm, 300 Å, 10 μm) at a flow rate of 7 mL/min using gradient conditions from 20% solution A to 100% solution B over 30 min. The peptide eluted after 17.51 min, and the molecular weight was confirmed by MALDI-TOF MS analysis ($[M+H]^+$ $m/z = 825.5492$, calcd = 824.512).
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18. Cyclic lipodepsipeptide **1** (0.5 mg) was treated with MeOH/H_2O/ammonia mixture (4:1:1, pH 9.0, 0.1 mL) at room temperature for 24 h. RP-HPLC and MALDI-TOF MS analyses of the crude reaction mixture showed existence of only one compound ($[M+H]^+$ $m/z = 843.4928$) corresponding to the product of depsipeptide hydrolysis by methanolic ammonia. Under these experimental conditions the peptidic amide bond is stable, and the presence of corresponding cyclic peptide was not detected.