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Letter

Synthesis of Fluorescent Lanthipeptide Cytolysin S Analogues by Late-Stage Sulfamidate Ring Opening

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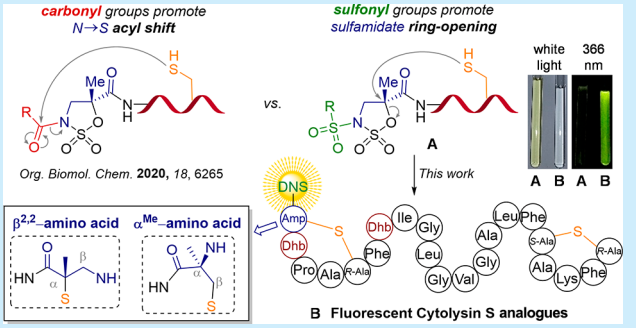
ABSTRACT: Nucleophilic ring opening of cyclic sulfamidates derived from amino acids is a common strategy for the synthesis of lanthionine derivatives. In this work, we report the regio-, chemo-, and stereoselective intramolecular S-alkylation of a cysteine residue with *N*-sulfonyl sulfamidates for the synthesis of cyclic lanthionine-containing peptides. The strategy involves the solid-phase synthesis of sulfamidate-containing peptides followed by late-stage intramolecular cyclization. This protocol allowed for the synthesis of four full-length cytolysin S ($CylL_S''$) analogues, two α-peptides and two hybrid α/β-peptides. Their conformational preferences and biological activities were assessed and compared with those of wild-type $CylL_S''$.



Lanthipeptides are ribosomally synthesized and post-translationally modified peptides (RiPPs) containing sulfur cross-linked lanthionine (Lan) and/or β-methyllanthionine (MeLan),[1,2] formed by a Michael-type addition of a cysteine to a dehydroalanine (Dha) or dehydrobutyrine (Dhb) generated by enzymatic dehydration of Ser or Thr. Many lanthipeptides exhibit antimicrobial properties against Gram-positive[3] and Gram-negative[4] bacteria, including multidrug resistant strains. These cross-linked macrocycles confer lanthipeptides with exceptional properties such as resistance to chemical and enzymatic degradation[5] or conformational rigidity.[6] The number of natural lanthipeptides discovered, as well as engineered analogues, steadily increases in the search for therapeutics to overcome antibiotic resistance.[2,7] However, large-scale production of lanthipeptides still represents a challenge.[8] Solid-phase peptide synthesis (SPPS) is an attractive strategy for the preparation of either natural lanthipeptides or chemically modified variants.[7,8] The use of orthogonally protected bis-amino acids allows for the incorporation of Lan/MeLan residues with defined stereochemistry.[9−11] Biomimetic approaches generating Lan/MeLan by intramolecular Michael addition have been reported.[12,13]

Cyclic sulfamidates have been extensively used for the regio- and stereoselective synthesis of a wide variety of chemicals through regioselective nucleophilic ring-opening reactions.[14−16] In particular, the intermolecular S-alkylation of five-membered ring sulfamidates has been exploited for the synthesis of orthogonally protected Lan and MeLan analogues.[17−19] For instance, the thioether ring B of haloduracin β as well as mimetics has been synthesized by intermolecular ring opening of sulfamidates with short cysteine-containing peptides.[20,21] However, attempts to achieve the intramolecular ring opening of peptides containing *N*-carbonyl sulfamidates and cysteine residues produced an unexpected N → S acyl shift.[22]

Therefore, to avoid this undesired reaction while maintaining the necessary N-activation of the sulfamidate,[23] we envisioned using sulfonamide functional groups, which have been extensively used in the ring opening of aziridines[24,25] but scarcely with sulfamidates.[26] We report the chemo-, regio-, and stereoselective intramolecular ring-opening reactions of peptides incorporating *N*-sulfonyl sulfamidates derived from (*S*)- or (*R*)-α-methylisoserine and (*S*)- or (*R*)-α-methylserine (Figure 1A) as the key step in obtaining four different analogues of enterococcal cytolysin S ($CylL_S''$).

First, we synthesized the linear model peptide that previously led to transacylation (i.e., **1a**-Ala-His-Asn-Cys-Gly)[22] by microwave (MW)-assisted SPPS,[27] and its N-terminus was reacted with dansyl (DNS) chloride (Figure 1B). Upon cleavage and deprotection, peptide **2** was dissolved in a 96:4 mixture of CD_3CN and D_2O and base-promoted cyclization was monitored by ^1H NMR spectroscopy (Figure 1C). After the addition of triethylamine (1.6 equiv), the instantaneous and complete disappearance of the starting

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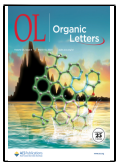


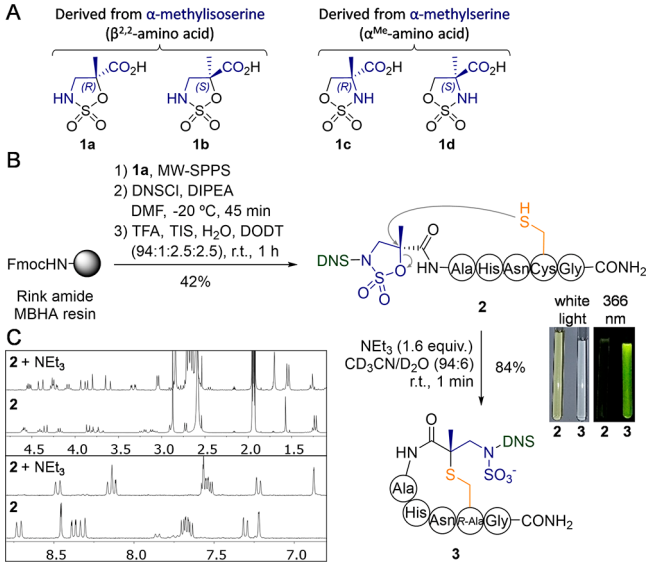
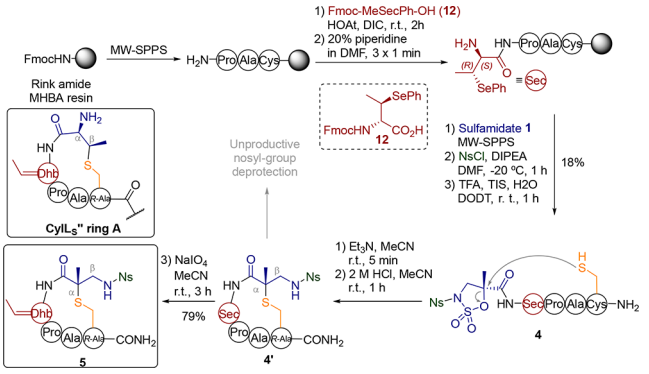


Figure 1. (A) Cyclic sulfamidates used in the synthesis of cytolysin S analogues. (B) MW-SPPS of peptide **2** and subsequent intramolecular sulfamidate ring opening. The inset shows the luminescence of peptides **2** and **3** under white light and ultraviolet light in a CD_3CN/D_2O mixture. (C) Selected regions of the NMR spectra of pure peptide **2** and after reaction for 1 min with triethylamine.

material and the formation of cyclic peptide **3** as a single isomer were observed. The identity of this compound was confirmed by NMR, MS, and RP-HPLC analyses (Figures S1 and S2). The intramolecular sulfamidate ring-opening reaction proceeded with excellent chemo-, regio-, and stereoselectivity and high yield (84%) after purification by HPLC. It is noteworthy that a dramatic increase in fluorescence was observed upon cyclization, likely by dequenching of the dansyl group after sulfamidate ring opening (Figure 1B, inset, and Figure S10). This observation offered the opportunity to prepare fluorescent analogues of cytolysin that may be valuable for investigating the cellular localization of cytolysin, which at present is not known.

Once the ability of *N*-sulfonyl sulfamidates to promote the fast cyclization of small Cys-containing peptides was demonstrated, we focused on the synthesis of an analogue of the A ring of cytolysin S ($CylL_S''$). This lanthipeptide is one of the two components comprising enterococcal cytolysin, which is toxic to Gram-positive bacteria, and is an important virulence factor during infection of eukaryotic cells.[28,29] Recently, cytolysin was directly linked to hepatic cell death and the severity of liver disease in human populations.[30] The A ring contains a Dhb residue, which cannot be directly coupled to the resin using standard SPPS protocols due to the very low reactivity of the enamine−imine tautomers formed upon Fmoc removal, and their hydrolysis to the corresponding ketone, which precludes further couplings.[31] Instead, we used selenocysteine derivative **12** (Fmoc-MeSecPh-OH)[12] as a masked version of Dhb (see the Experimental Section in the Supporting Information). This masking also prevents the *S*-Michael addition to the Dhb residue, which may compete with the desired sulfamidate ring-opening reaction. In this case, the nosyl group was selected as the protecting/activating group of the sulfamidate. This scaffold has been used to promote chemoselective ring opening of aziridines and can be readily removed under mild conditions.[32,33] Linear peptide **4** was synthesized and purified by RP-HPLC in a moderate global yield (18%). The cyclization reaction was then carried out using a slight excess (1.6 equiv) of triethylamine. MS and HPLC analyses confirmed that the reaction took place rapidly and quantitatively after the reagents had been mixed (Figure S3). Further treatment with an aqueous HCl solution to remove the sulfamic moiety followed by oxidation with an excess of sodium periodate (8 equiv) to unmask the Dhb residue afforded cyclic peptide **5** in a high yield (79%) after HPLC purification (Scheme 1). It is noteworthy that no oxidation of the thioether group was detected as judged by MS analysis (Figure S3).

Scheme 1. Synthesis of an Analogue of the $CylL_S''$ A Ring (5)



This $CylL_S''$ ring A analogue (**5**) features (*S*)-3-amino-2-methylpropanoic acid, (*S*)-3-Amp, as the first amino acid in the sequence. (*S*)-3-Amp is a *β*-amino acid in which the thioether linkage is attached to the tertiary *α*-carbon, producing a 15-membered macrocycle, whereas the natural derivative is a 16-membered macrocycle. In addition, the N-terminus is capped with a nosyl group. Attempts to cleave this protecting group were unsuccessful, and intact peptide **4′** was always recovered (Table S1). In view of these results, we decided to proceed with the synthesis of full-length *N*-dansyl analogues of $CylL_S''$, which would confer fluorescent properties to the final peptides.

To synthesize the full-length $CylL_S''$ analogues, ChemMatrix resin was selected due to its effectiveness for the synthesis of highly hydrophobic peptides. First, the orthogonally protected DL-lanthionine building block **6**[34] and the following three natural amino acids (Ala-Lys-Phe) were each manually coupled to the resin. Deprotection and macrolactamization reactions were performed following previously reported conditions[34] to form the C-terminal B ring of $CylL_S''$. The rest of the peptide, including the masked Dhb residues (i.e., Fmoc-MeSecPh-OH), sulfamidate **1a**, and the dansyl group, was successfully coupled to the resin, yielding peptide **7a** upon cleavage. The subsequent late-stage sulfamidate ring-opening cyclization was performed by reacting peptide **7a** with triethylamine in dimethyl sulfoxide (DMSO). We used DMSO instead of acetonitrile in this case due to the better solubility of peptide **7a** in the former solvent. Complete conversion was observed after 5 min, as judged by HPLC and MS analysis, affording peptide **8a** quantitatively. Concentrated aqueous HCl was added to the crude mixture to remove the sulfamic moiety generated upon sulfamidate ring opening. To our delight, concomitant oxidation of the selenides and *β*-elimination of the resulting selenoxides were observed due to the oxidating

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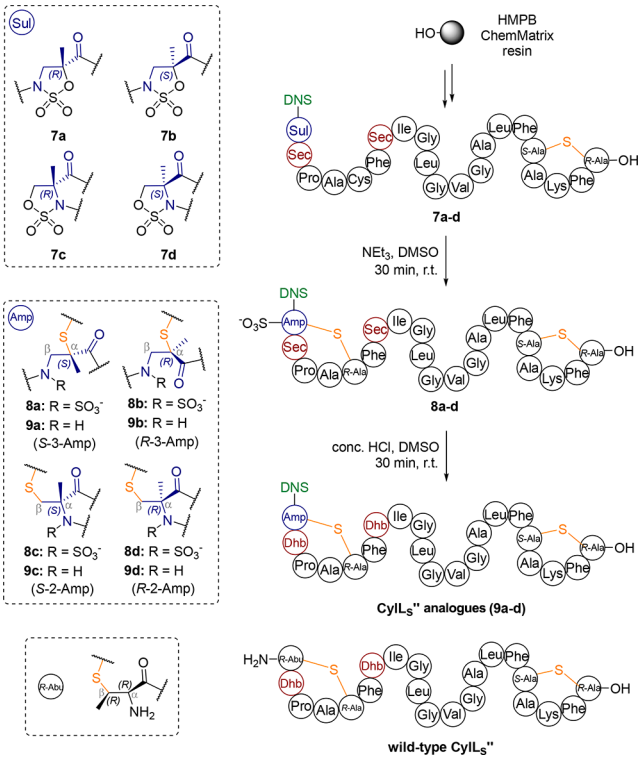
properties of DMSO-HCl mixtures. Importantly, the reaction was mild and selective enough to not oxidize the thioether linkages. Consequently, CylL$_S$″ analogue **9a** was obtained in a 1% global yield (90% per step on average, considering 45 steps from the first deprotection of the Fmoc from the resin to the final oxidation−elimination reaction). Following the same methodology, CylL$_S$″ analogues **9b−d** were synthesized in ∼1% global yields using sulfamidates **1b−d**, where sulfamidate **1b** is the enantiomer of sulfamidate **1a** leading to (*R*)-3-amino-2-methylpropanoic acid, (*R*)-3-Amp, upon cyclization; sulfamidates **1c** and **1d** were derived from (*R*)- and (*S*)-*α*-methylserine and afford (*S*)- and (*R*)-2-amino-2-methylpropanoic acid, (*S*)- and (*R*)-2-Amp, upon cyclization, respectively (Scheme 2).

Scheme 2. Synthesis of the Full-Length Analogues of CylL$_S$″ (9a−d) and Structure of the Natural CylL$_S$″ (DNS = *N*-dansyl)



The conformational preferences and dynamics of natural CylL$_S$″ and analogues **9a−d** in aqueous solution were analyzed by molecular dynamics (MD) simulations (see the Supporting Information). The three-dimensional structure of CylL$_S$″ in methanol was recently determined by NMR[6] and used as a template for the initial geometries of the peptides. Simulations suggested that all peptides are quite flexible and can adopt different conformations (Figure 2 and Figures S11−S15). The native conformation of CylL$_S$″ is frequently observed in all peptides throughout the simulations, particularly at the *α*-helical C-terminal domain (Val12−Phe20). On the contrary, the middle section (Phe6−Gly11) shows a more random coil/bent conformation, likely due to the abundance of glycine residues.

The more significant conformational differences between natural and modified CylL$_S$″ are observed at the N-terminal A ring, where the unnatural amino acids are located. Most

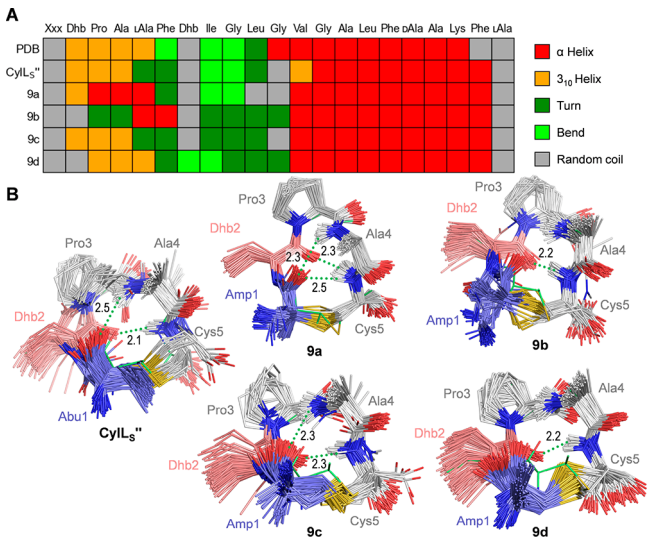


Figure 2. Conformational preferences of CylL$_S$″ and analogues **9a−d**. (A) Most abundant secondary structure for each amino acid along MD simulations. (B) Conformational ensembles of ring A of CylL$_S$″ and analogues **9a−d**. The NMR structure of CylL$_S$″ (Protein Data Bank entry 6VE9) is shown in green as a reference. The C atoms of the first residue and Dhb2 are colored blue and pink, respectively. Only H atoms involved in conserved hydrogen bonds (green dashed lines) are shown. The N-terminal dansyl group and the rest of the peptide (Phe6−Ala21) have been omitted for the sake of clarity.

peptides showed a highly conserved hydrogen bond between Cys5 and the Dhb2 backbone, as well as between Ala4 and Abu1, thus preserving the 3$_{10}$-helix-like conformation observed by NMR (Figures S16−S30 and Table S2). The prevalence of the latter hydrogen bond drops significantly in analogues **9b** and **9d**. An additional hydrogen bond between Cys5 and (*S*)-3-Amp is highly conserved for analogue **9a**, resulting in a more rigid *α*-helix-like motif. These conformational differences exhibited by analogues **9a** and **9b** are due to the incorporation of a *β*-amino acid (3-Amp) at the N-terminus, which also moves the thioether cross-link from the *β*-carbon to the *α*-carbon (Scheme 1). Conversely, the conformations adopted by the A ring of analogues **9c** and **9d** resemble more closely those of natural CylL$_S$″ as only the position of a methyl group is moved from the *β*-carbon to the *α*-carbon (Scheme 2).

The antimicrobial activity of the four analogues (**9a−d**) was tested in combination with native cytolysin L (CylL$_L$″) against *Lactococcus lactis* sp. cremoris. As with natural CylL$_S$″,[35] none of the analogues showed antimicrobial activity in the absence of CylL$_L$″, consistent with the current model in which the two peptides act with a 1:1 stoichiometry.[34] The four synthetic peptides displayed some degree of synergistic activity with CylL$_L$″ in liquid medium antimicrobial assays, suggesting that they still act on the natural target. However, they are significantly less potent than wild-type CylL$_S$″ with minimum inhibitory concentration (MIC) values in liquid culture around 1000-fold higher in the case of *α*/*β*-peptides **9a** and **9b** and 125- and 250-fold higher for **9c** and **9d**, respectively (Figure 3 and Figures S8 and S9). Unfortunately, the high MIC values obtained for all analogues prevented the use of subcellular localization data by fluorescence analysis.[36] These results are in line with previous observations in other synthetic CylL$_S$″ analogues, in which very slight modifications in ring A completely abolished their antimicrobial activity.[34,35,37] Peptides **9a−d** were also assessed in combination with CylL$_L$″ for

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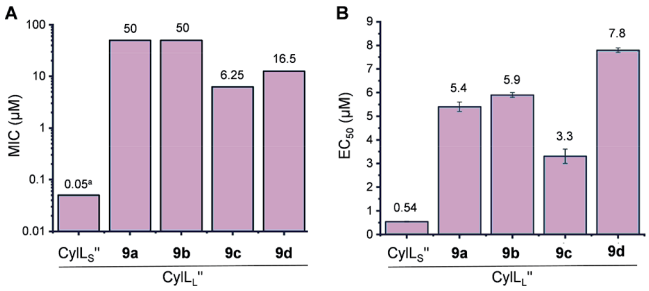

Figure 3. (A) Comparison of MIC values between wild-type ([a]value extracted from ref 37) and analogues of CylL$_S$″ **9a–d** in the presence of equimolar amounts of CylL$_L$″ against *L. lactis* sp. cremoris. (B) Comparison of the EC$_{50}$ values of hemolysis of rabbit red blood cells between the wild type and analogues of CylL$_S$″ **9a–d** in the presence of equimolar amounts of CylL$_L$″.

hemolytic activity against rabbit red blood cells. All analogues showed a decreased activity with half-maximal effective concentration (EC$_{50}$) values 6–14-fold higher than that of natural CylL$_S$″. Analogue **9c** showed the best antimicrobial and hemolytic activities, possibly due to its ability to better mimic the conformational behavior of natural CylL$_S$″ and/or better complementarity with CylL$_L$″.

We have developed a new synthetic methodology for obtaining lanthipeptide analogues with complete control over regio-, chemo-, and stereoselectivity. Our protocol is based on the mild late-stage intramolecular ring opening of *N*-sulfonyl cyclic sulfamidates incorporated in Cys-containing peptides by SPPS. This strategy was validated by synthesizing four full-length analogues of CylL$_S$″ featuring unnatural α- and β-amino acids. The four synthetic peptides showed a dramatic reduction of antimicrobial activity, as well as a decreased hemolytic activity, reinforcing the unique structural properties of CylL$_S$″.[35] Compounds with submicromolar MICs are usually thought to have specific molecular targets,[38] which in the case of cytolysin remains to be identified. Although our analogues of CylL$_S$″ proved to be less active than the wild type, the ability to perform stereocontrolled cyclization reactions on unprotected peptides provides a valuable methodology for the synthesis of conformationally constrained cyclic peptide pools that can be selected for binding to desired targets.[39,40]

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.3c00122.

Experimental procedures, characterization data, and copies of NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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