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Total Synthesis of Malacidin A by β-Hydroxyaspartic Acid Ligation Mediated Cyclization and Absolute Structure Establishment

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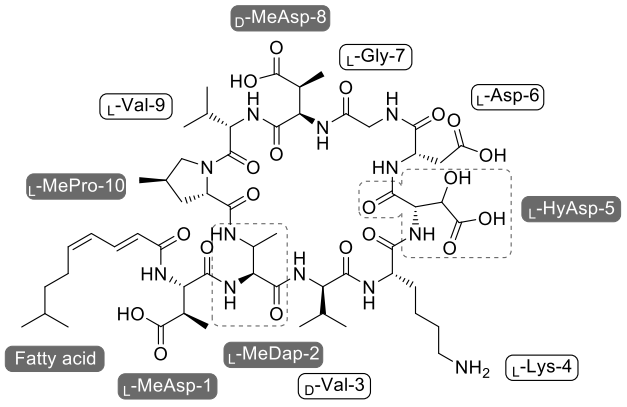
Abstract: The development of novel antibiotics is critical to combating the growing emergence of drug-resistant pathogens. Malacidin A is a new member of the calcium-dependent antibiotic (CDAs) family with activity against antibiotic-resistant pathogens. Its mode of action is distinct from classical CDAs. However, the absolute structure of malacidin A has not been established. Herein, the total syntheses of malacidin A and its analogues are reported by a combination of Fmoc-based Solid-Phase Peptide Synthesis (SPPS) and β-hydroxyaspartic acid ligation mediated peptide cyclization. The total synthesis enabled us to establish the absolute configuration of malacidin A, which is in agreement with those for natural malacidin A confirmed by advanced Marfey's analysis in our study.

More than ninety years since the discovery of penicillin, developing new classes of antibiotics remains an urgent need with the increasing emergence of bacterial pathogens with multi-drug resistance.[1] Among the different antibiotic skeletons, (e.g., β-lactam, tetracycline, aminoglycoside, etc.), cyclic peptides represent an important class of antibiotics. This includes bacitracin, vancomycin, daptomycin and polymyxins which are all used in the clinic. Known cyclic peptide antibiotics often work through non-protein targets. With emerging technologies, such as environment-mimic cultivation method[2] and genome mining of Biosynthetic Gene Clusters (BGCs)[3], several cyclic peptides with new structural motifs have been recently discovered and exhibited promising potency. Through genome mining of BGCs from environmental DNA, malacidins A and B were identified as a new class of calcium dependent antibiotics (CDAs).[4] Malacidin A exhibits broad activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE), and has potent MIC values (0.2-2 μg/mL) in the presence of the divalent cation calcium.

Interestingly, the malacidin family contains a calcium-binding motif (HyAsp-Asp-Gly) that differs from the canonical sequence (Asp-Xaa-Asp-Gly) observed in other known CDAs[5]. In addition to the lack of the variable spacer amino acid (Xaa), a non-proteinogenic β-hydroxyaspartic acid (β-HyAsp) replaces the first Asp residue in the calcium-binding motif of malacidin. Unlike the described modes of action (MOAs) of CDAs, which typically depolarize the bacterial cell membrane[6] or bind to the undecaprenyl phosphate (C_{55}-P)[7], malacidin A exerts bactericidal activity by binding to lipid II. As no cross resistance with vancomycin was observed, the interaction between malacidin A and lipid II should be different and comprehensively studied in the future. Moreover, bacterial resistance to malacidin A has not been observed under the laboratory resistance-inducing conditions.



Malacidin A

1a: L-(3S)-MeDap, L-(3R)-HyAsp **1b**: L-(3R)-MeDap, L-(3R)-HyAsp
1c: L-(3S)-MeDap, L-(3S)-HyAsp **1d**: L-(3R)-MeDap, L-(3S)-HyAsp

Figure 1. Structure of malacidin A. Residues with asterisk have uncertain stereochemistry.

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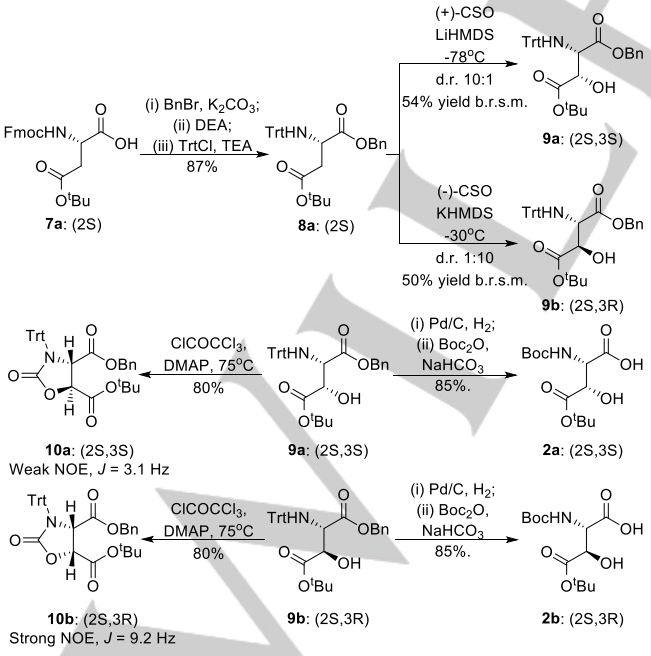
Malacidin A consists of a cyclic nonapeptide anchored with an amino-acid-linked unsaturated C9-fatty acid (Figure 1), as well as one D-Val and five non-proteinogenic amino acids, including β-methylaspartic acid at position 1 and 8 (MeAsp-1 and -8), β-methyldiaminopropionic acid-2 (MeDap-2), β-hydroxyaspartic acid-5 (HyAsp-5) and γ-methylproline-10 (MePro-10). Based on NMR characterization, the configuration of the lipid tail was readily determined as 2*E*,4*Z*-methyl-nonadienoic acid. Combining NMR technique with the Marfey's analysis, configurations of MePro-10 and HyAsp-5 were suggested as L-(4R)-MePro and L-HyAsp-5. Bioinformatically, MeDap-2, MeAsp-1, and MeAsp-8 were predicted as L-MeDap-2, L-(3S)-MeAsp-1 and D-(3S)-MeAsp-8 respectively.[4] However, the β-stereochemistry of L-MeDap-2 and L-HyAsp-5 remained uncertain and needed further investigation. This left four possible diastereomeric structures: **1a**, **1b**, **1c** and **1d**. If a chemical synthetic route could be established, malacidin A's structure could be confirmed by NMR comparison to **1a**-**1d**. The final route would also provide opportunities for structure-activity relationship (SAR) studies and the development of the next generation of CDAs. To this end, we initiated a program on the total synthesis of malacidin A.

Prior to the total synthesis attempt, building blocks of non-proteinogenic amino acids with proper protecting groups were required. Although there are several strategies reported to construct HyAsp, few of them could be conveniently utilized to prepare suitable building blocks for this synthetic route. This includes the tedious route starting from tartaric acid[8], non-suitable side chain protection in the masked acyl cyanide reaction[9] and difficult observation of *trans* product in the Sharpless aminohydroxylation reaction[10]. Inspired by the Sardina's work[11], herein we developed a concise synthetic route for selectively preparing **2a** or **2b** in five steps with 37~40% overall yield (Scheme 1.).



Scheme 1. Concise Syntheses of **2a** and **2b**. Abbreviations: DEA, diethylamine; TEA, triethylamine; LiHMDS, lithium bis(trimethylsilyl)amide; KHMDS, Potassium bis(trimethylsilyl)amide; (+)/(-)-CSO, (+)/(-)-(camphorylsulfonyl) oxaziridine; DMAP, 4-Dimethylaminopyridine.

Starting from a commercially available building block of L-aspartic acid **7a**, its carboxylic acid was protected by benzylation. After removal of Fmoc group, **8a** was prepared by tritylation of the amine to shield the α-proton from epimerization in later steps. Then with LiHMDS at -78°C, stereo-selective hydroxylation of the enolate from **8a** was performed by (+)-CSO to afford **9a**. The d.r. was up to 10:1 in this oxidation and both diastereomers could be well separated by column chromatography. Likewise, using the oxaziridine with the opposite chirality, i.e., (-)-CSO, the stereochemistry of **8a** was successfully reverted to produce **9b** at -30°C with 1: 10 d.r.. KHMDS was used instead of LiHMDS due to different chelation preference for better setereoselectivity.[11] The configurations of the β-position from **9a** and **9b** were confirmed through determining the coupling constants and nuclear overhauser effect (NOE) of the α and β-protons in their cyclic carbamate derivatives **10a** and **10b**. While **10a** exhibited a weak NOE signal (*J* = 3.1 Hz), an apparent NOE and larger *J* value (9.2 Hz) was observed from **10b**, indicative of a *trans*-proton pair in **10a** (i.e. 2S, 3S) and *cis*-proton pair in **10b** (i.e. 2S, 3R). Next, **9a** or **b** was hydrogenated by catalytic palladium to remove the trityl and benzyl groups at the same time, followed by Boc installation in one pot to afford **2a** or **2b**, which were ready for Fmoc-based SPPS.

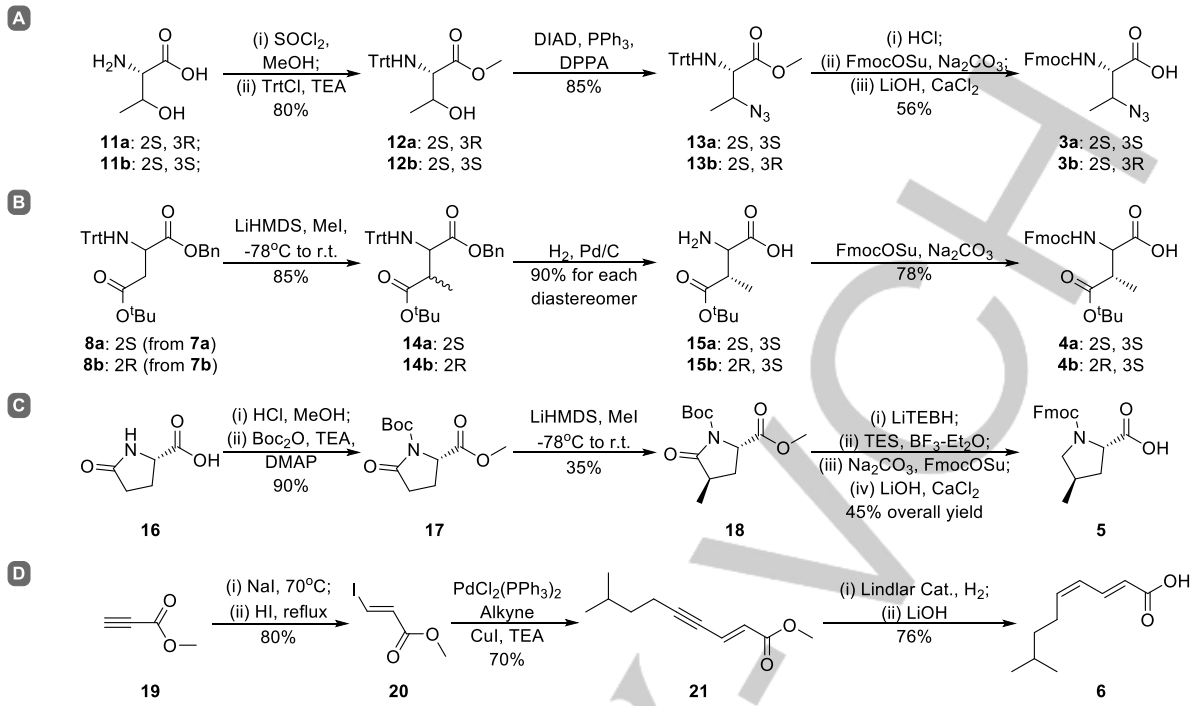
This synthetic route provided the following two advantages. Firstly, the two carboxylic groups were differentiated early on. Secondly, the protecting groups of **9** could be easily modified because trityl, benzyl and tert-butyl group could be removed under mild conditions with orthogonality. Through this facile and stereoselective introduction of the β-hydroxy group to aspartic acid, various protection strategies could be adapted to this key intermediate **9** to fulfill the requirements of SPPS.

As for the MeDap building block (Scheme 2A), **13** was prepared from L-Thr or L-*allo*-Thr (**11a** or **11b**) by the Mitsunobu reaction of **12a** or **12b** to introduce an azido group[12]. After conversion of the trityl to an Fmoc group, followed by hydrolysis of the methyl ester, **3a** or **3b** was produced in high yield, respectively.

The scaffold of MeAsp **14** was generated from trityl protected **8a** or **8b** by methylation (Scheme 2B). Although there is no stereo-selectivity at this step, each diastereomer of **15** could be separated by column chromatography after hydrogenolysis of methylated mixtures[13]. Reinstallation of the Fmoc group gave high yield of **4a** and **4b**, which could serve directly as the building blocks for MeAsp-1 and -8.

Next, the synthetic route of the (2*S,* 4*R*)-methyl proline, building block **5,** was developed using a combination of Li's[14] and Pedregal's method[15] (Scheme 2C). Briefly, the pyroglutamic acid **16** was protected as **17** and then methylated at its γ-position to generate the (2*S,* 4*R*) intermediate **18**. After that, it underwent a chemoselective amide reduction and manipulation of protecting groups to afford compound **5**.

For the fatty acid building block (Scheme 2D), the *trans*-iodoalkene **20** generated from **19** was coupled with the terminal alkyne of 5-methyl-1-hexyne through the Sonogashira cross coupling conditions to obtain **21**. This *trans*-configuration maintaining intermediate was selectively hydrogenated using the Lindlar catalyst, followed by hydrolysis of methyl ester to give rise to the 2*E*, 4*Z*-fatty acid **6** (see SI for more details of building blocks syntheses).



Scheme 2. Synthesis of **3a**, **b**; **4a**, **b**; **5** and **6**. Abbreviations: DIAD, diisopropyl azodicarboxylate; DPPA, diphenylphosphoryl azide; LiTEBH, Lithium triethylborohydride; TES, triethylsilane; Alkyne, 5-methyl-1-hexyne.

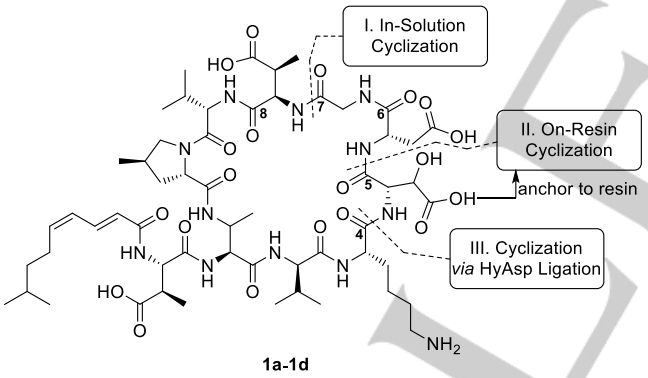


Figure 2. Three cyclization strategies of malacidin A.

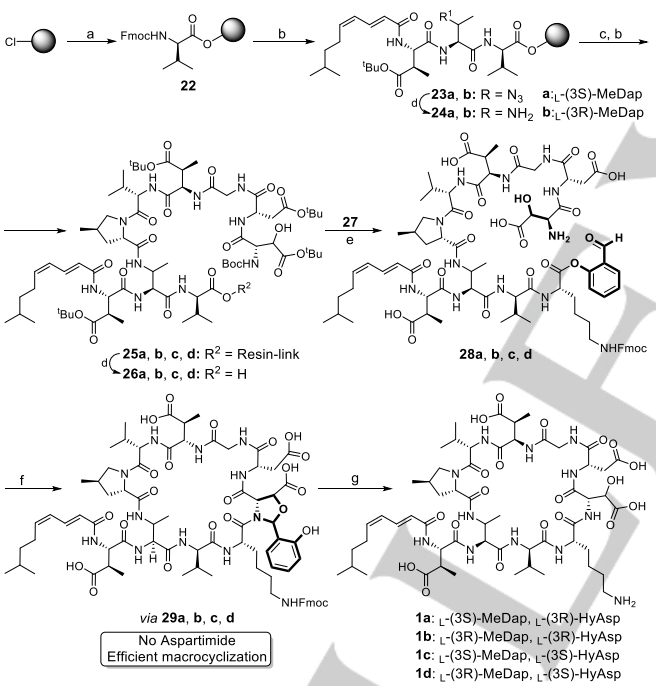
With all the building blocks in hand, we proceeded towards the total synthesis of malacidin A. Our first attempt used solid phase peptide synthesis followed by in-solution peptide cyclization. In this route, the Gly7-MeAsp8 peptide bond (Figure 2, Scheme S6) was disconnected as the cyclization site of the linear peptide, as cyclizing the C-terminal glycine should avoid epimerization at the C-terminus due to the achirality of glycine. Unfortunately, during Fmoc-SPPS we observed significant aspartimide formation after the coupling of HyAsp as the third residue. All attempts to vary building blocks, deprotection methods or coupling conditions failed to resolve this issue. Furthermore, no cyclized product was found when the linear peptide (**S7**) was activated by benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop) with N,N-diisopropylethylamine (DIEA) under highly dilute concentrations. Although changing coupling reagents offered trace amounts of product **1,** by UPLC it eluted as a broad peak with overlapping aspartimide and related epimerization peaks, making it extremely difficult to purify this cyclized product.

Considering the facile formation of aspartimide at the junction of HyAsp5-Asp6 and suspected hindrance in cyclization, an on-resin cyclization synthetic plan was attempted in which the side chain of the HyAsp building block was directly anchored to the resin (Figure 2, Scheme S7). In theory, anchoring the side chain of HyAsp to resin would avoid aspartimide formation as no other residue would be linked to the C-terminus until the macrocyclization step. As outlined, peptide elongation proceeded smoothly without any trace of aspartimide. Unfortunately, the cyclization precursor **S12** was found to fall off the resin under the C-terminus carboxylic acid allyl deprotection conditions. A similar problem has been reported previously when using Asp and 2-Cl-trityl resin[16]. More importantly, the cyclization of prematurely released peptide would be non-regioselective due to the presence of both the C-terminal and side-chain carboxylic acids and it would lose the pseudo-high-dilution advantage of on-resin cyclization.

In the end, we turned to an unconventional peptide cyclization synthetic route that relied on chemical ligation[17]. We envisioned that linear peptide **28** with a C-terminal salicylaldehyde ester and an N-terminal β-hydroxyaspartic acid might undergo a similar reaction as Ser/Thr ligation[18–20]. The advantages of this route, if successful, include avoiding epimerization during the peptide cyclization and using an unprotected linear peptide precursor. However, it remains to explore whether the hydroxylated amino acid could effectively proceed under the Ser/Thr ligation

conditions. To this end, the Lys4-HyAsp5 linkage (Figure 2) was chosen as the cyclization site.

As shown in Scheme 3, the crude peptide **26** with side-chain protection was prepared from Fmoc-D-Val-COOH linked trityl resin **22** *via* on-resin reduction of **23** to **24** and cleavage of **25** from resin. To enable the HyAsp ligation, the linear peptide with C-terminal salicylaldehyde ester **28** was prepared.[21] Under Sakakibara conditions (EDC, HOOBt, TFE/$CHCl_3$), the side-chain-protected Lys salicylaldehyde semicarbazone **27** was coupled to peptide **26**, followed by global deprotection in the presence of pyruvic acid. HPLC purification afforded the side chain unprotected peptide salicylaldehyde ester **28**. With this ligation precursor in hand, different conditions for cyclization were screened. Gladly, the linear salicylaldehyde ester cyclized smoothly within a few hours in pyridine/acetic acid buffer at a concentration of 3 mM. Detection of the oxazolidine **29** by UPLC before acid addition to the ligation mixture suggests that the linear peptide underwent HyAsp ligation mediated cyclization by a mechanism similar to Ser/Thr ligation. After acidolysis and deprotection of the Fmoc group in one pot, HPLC purification gave malacidin A candidate **1a**. Ultimately, this work established a robust synthetic route to the total synthesis of malacidin A.



Scheme 3. Assembly of **1a**-**1d**. Conditions: (a) Fmoc-D-Val-OH, DIEA, DCM, 2 h; (b) (i) 20% Piperidine in DMF, 10 min; (ii) Fmoc-AA-OH, HATU, DIEA, DMF, 1-2 h; (c) $SnCl_2$/PhSH/DIEA, DMF, 1 h; (d) HOAc/TFE/DCM, 2 h; (e) (i) EDC, HOOBt, $CHCl_3$/TFE, 6 h; (ii) Pyruvic acid, H_2O/TFA, 3 h; (iii) HPLC purification, 10% yield; (f) Acetic acid/pyridine, 3-6 h; (g) (i) TFA/TIPS/H_2O, 15min; (ii) 10% DEA in ACN, 1 h; (iii) HPLC purification, 50-60% yield. Abbreviations: DCM, dichloromethane; DMF, N,N-dimethylformamide; HATU, (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide; TFE, trifluoroethane; EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; TFA, trifluoroacetic acid; ACN, acetonitrile.

The other three possible diastereomeric structures, **1b**-**1d**, were synthesized following the same route. After careful comparison and analysis of spectral data from all four synthetic compounds, fortunately, **1b** was found to be nearly identical to the reported NMR spectra for malacidin A (Table S1). Based on this analysis, the absolute stereocenters of its five non-proteinogenic amino acid residues are suggested as (2*S,* 3*S*)-MeAsp1, (2*S,* 3*R*)-MeDap2, (2*S,* 3*R*)-HyAsp5, (2*R,* 3*S*)-MeAsp8 and (2*S,* 4*R*)-MePro10. To confirm these results, we carried out an advanced Marfey's analysis on the natural malacidin A (Figures S10-S12). This analysis validated the stereochemical predictions suggested by our total synthesis studies. Our synthetic malacidin **1b** showed the same calcium dependent antibacterial activity against MRSA as the natural product[4].

In summary, the total syntheses of malacidin A and its diastereomeric analogues was completed, from which we established its absolute configuration. Our synthetic route involved the HyAsp-mediated ligation as a key step for peptide cyclization. Compared with classical strategies that might be used to construct this natural product, the HyAsp ligation enables chemoselective and efficient macrolactamization free of epimerization and aspartimide formation during the precursor preparation. It is worth noting that HyAsp-mediated peptide cyclization was conducted at 3 mM concentration without observed dimerization and polymerization. Indeed, this outcome opens up new possibilities for the synthesis of cyclic peptides with HyAsp or similarly hydroxylated amino acids. Through such a practical protocol, analogues of malacidin A can be obtained routinely, providing the foundation for its SAR study.

Acknowledgements

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Keywords: Cyclic peptide antibiotics • Malacidin • Total Synthesis • β-Hydroxyaspartic Acid • Chemical ligation

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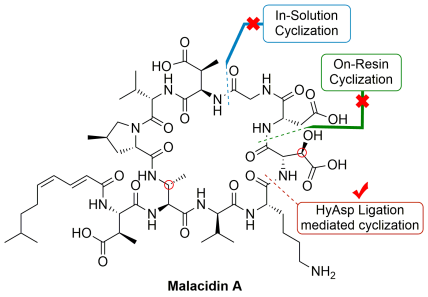

Malacidin A

A novel cyclization strategy mediated by hydroxy aspartic acid ligation was developed to construct malacidin A, a new calcium-binding antibiotic discovered recently without comprehensive stereochemistry elucidation. Such technique allows facile syntheses of malacidin A and its analogues for establishment of its absolute structure.