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Total Synthesis of Halicylindramide A

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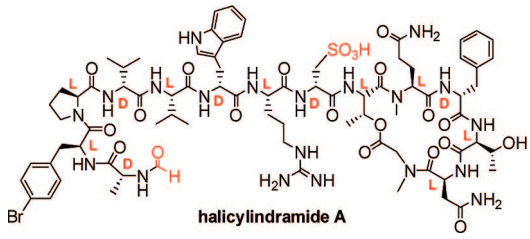

A rapid and efficient Fmoc solid-phase synthesis of halicylindramide A is described. The strategy comprises resin attachment of the first amino acid via the side chain of aspartic acid, stepwise solid-phase synthesis of the linear peptide analog up to the cysteine residue, on-resin head-to-tail cyclization and linear peptide synthesis of the N-terminal region, and finally cysteine oxidation and formylation. The stereochemistry of the halicylindramide A was confirmed by comparison of NMR and RP-HPLC data with the natural molecule. A distinctive conformational change was observed from the CD spectra of the halicylindramide A in sodium dodecyl sulfate.

In 1995, Fusetani and co-workers reported the isolation and structure of halicylindramides A−C (Figure 1), which are cyclic depsipeptides isolated from the Japanese marine sponge *Halichondria cylindrata*.[1] A subsequent paper described the structures of halicylindramide D, a tridecapeptide lactone, and halicylindramide E, a truncated linear peptide.[2] Other sponge peptides, including discodermins A−H,[3] polydiscamide A,[4] and microspinosamide,[5] constitute a family of structurally related trideca- and tetradecapeptides. Recently, corticiamide A[6] and polydiscamides B−D[7] from sponges have also been reported.

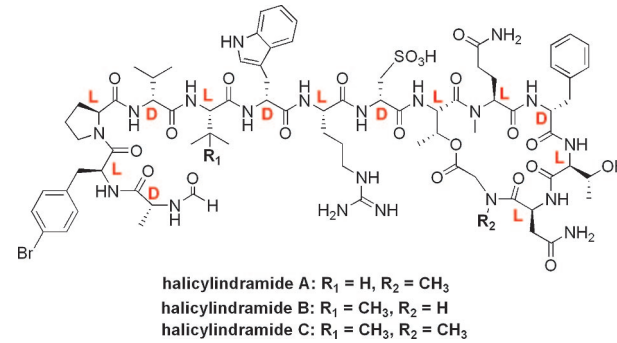

FIGURE 1. Structure of halicylindramide.

These cyclic depsipeptides share many unusual amino acids, such as D-amino acids, *N*-methyl amino acids, and cysteic acid, and contain a formyl group at the N-terminus. These peptides possess various antimicrobial, anticancer, and anti HIV-1 activities and interact with proteins by exhibiting inhibitory activity toward phospholipase A_2 or agonistic activity at a G protein coupled receptor (GPCR). To date, none of these depsipeptides have been synthesized, nor have their conformations been elucidated. Because these peptides are composed of six hydrophobic amino acid residues at the N-terminus and two hydrophilic amino acids in the middle of the sequence, their activities might be related to their association with membranes due to their amphiphilic character. The plasma membrane permeabilization of Ca^{2+} and ATP by discodermin A was reported in 2001,[8] but no other mechanism or structural information was obtained. Therefore, a total synthesis of these products would be valuable to explore their potential therapeutic applications and to understand the mechanism of the membrane interactions of these novel peptides. Here, we describe an efficient solid-phase synthesis of halicylindramide A.

In the retrosynthetic analysis of cyclic depsipeptides, the sites of resin attachment and ring disconnection are crucial for successful synthesis. The selection of the point of cyclization is critical because cyclization often proceeds very slowly, causing side reactions such as dimerization and/or epimerization of the C-terminal residue. In our synthesis, we decided to anchor the side chain of the Asp residue to Rink amide resin and to cyclize the precursor via macrolactamization between the Sar and Asn residues on the resin. In this way, cyclization and coupling of the N-terminal amino acids could be performed while the peptide remained anchored to the resin.

The synthesis of halicylindramide A begins with the attachment of C-terminal Fmoc-D-Asp-OAllyl via the side chain to the Rink amide resin using a PyBOP/HOBt/DIPEA procedure (Scheme 1). Standard Fmoc chemistry was used throughout. To introduce the *N*-methylglutamine residue into the ring, Fmoc-N^{α}-methylglutamine was prepared from commercially available Fmoc-Glu(Trt)-OH according to the published procedure, in 86% yield.[9] To couple the D-Phe and (*N*-Me)Gln residues, 1 h

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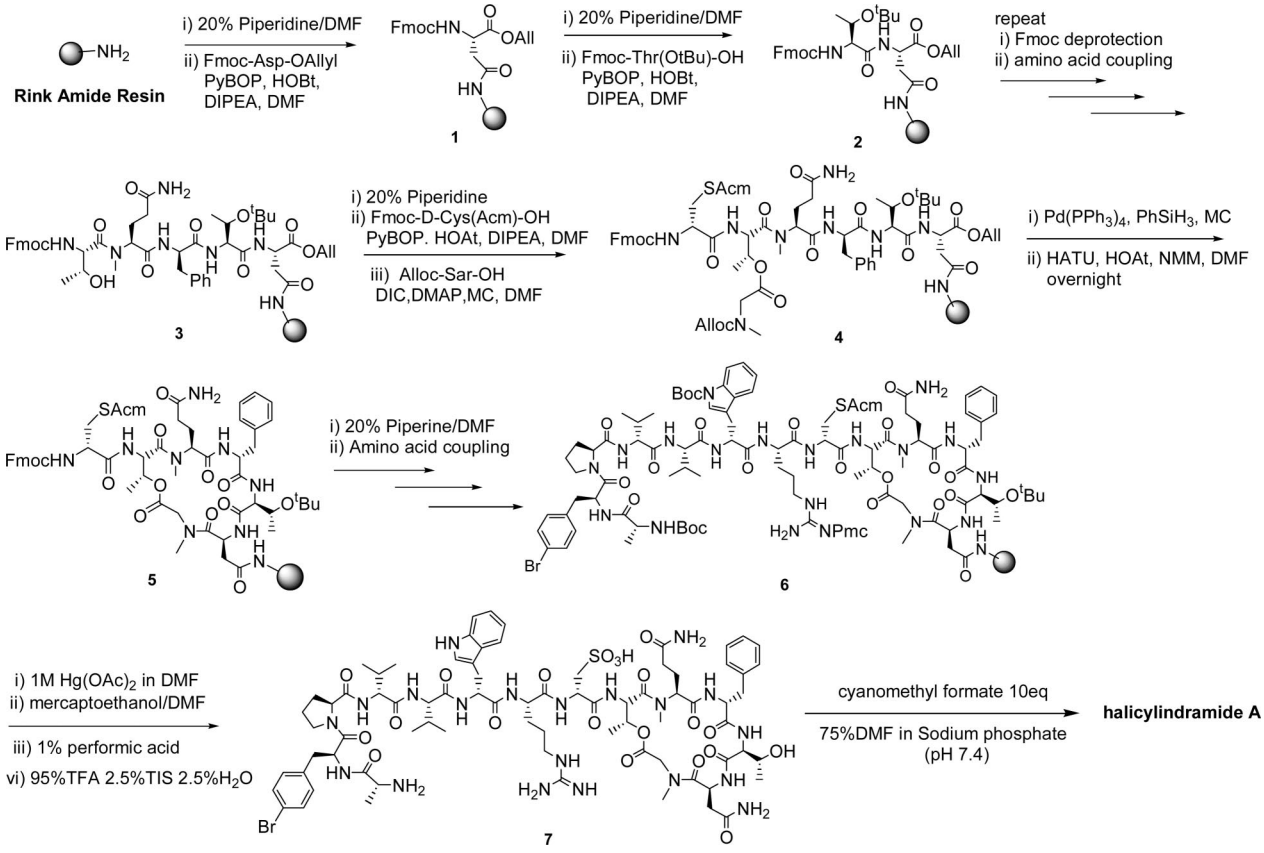
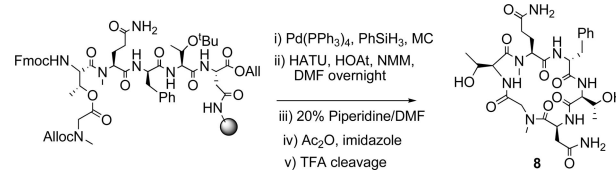
SCHEME 1. Synthesis of Halicylindramide A



of shaking with PyBOP/HOAt/DIPEA was sufficient to complete the reaction. However, repeated coupling (three times) was required for the attachment of Fmoc-Thr to the *N*-Me-Gln residue, providing the resin-bound linear peptide **3.** Coupling of the D-Cys analog was performed while the side chain of the Cys residue was protected with an acetamidomethyl (Acm) group, which can later be removed selectively and oxidized to cysteic acid. To build a cycle on the resin, Alloc-Sar-OH was coupled to the hydroxyl group of the Thr residue via an anhydride method using the DIC/DMAP procedure, and then the Alloc and allyl protecting groups at the Sar and Asn residues were removed selectively by treatment with $Pd(Ph_3P)_4$.[10] The deprotected peptide was cyclized between the Sar and Asn residues with an excess of a HATU/HOAt/NMM mixture, providing the resin-bound depsipeptide **5**. The cyclization reaction was assessed using the chloranil test to detect the absence of the secondary amine of the sarcosine residue.[11] The remaining *N*-terminal amino acids were coupled one by one using PyBOP/HOAt/DIPEA conditions to afford compound **6**.

When we tried to synthesize a cyclic peptide without attaching the Cys analog, we obtained the cyclic peptide **8** as a main product after resin cleavage (Scheme 2). The formation of a byproduct via an O→N acyl shift was anticipated[12] and may be formed during the removal of the Fmoc group from the Thr residue using piperidine. Therefore, the coupling of one more amino acid to the Thr residue prior to depsipeptide cyclization

SCHEME 2. O→N Acyl Shifted Byproduct



was necessary to prevent an undesired O→N acyl shift rearrangement.

The remaining steps of the complete synthesis are the oxidation of Cys and formylation of the N-terminal Ala residue.

Because Trp residues in peptides or proteins can be easily oxidized to form various byproducts during oxidative processes,[13] a protection of the indole ring is important to minimize byproduct formation. Therefore, the oxidation of the side chain of Cys was performed while the Trp residue is protected with a Boc group on the solid phase. The Acm protecting group was removed selectively from the Cys residue using 1 M $Hg(OAc)_2$[14] in DMF, and the resin was washed with 10% mercaptoethanol in DMF to remove Hg^{2+} ions (Scheme 1). The free thiol was treated with freshly prepared 1% performic acid in formic acid,[15] and the resin was treated with dimethylsulfide to remove excess oxidant. Other oxidants, including dimethyldioxirane[16] and

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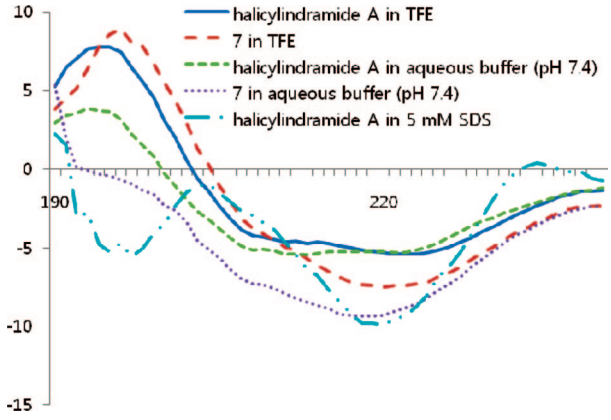

FIGURE 2. Comparison of CD spectra of halicylindramide A and **7**.

chlorite,[17] did not give the desired product. HPLC and mass spectrometry analysis of the byproducts indicated that overoxidized or fragmented peptides were produced. The oxidized depsipeptide from the performic acid oxidation was cleaved from the resin and then purified by RP-HPLC to provide **7** with a 1.5% overall yield.

Finally, formylation of the N-terminal Ala residue was performed using an excess of cyanomethyl formate[18] in a solution of DMF-sodium phosphate buffer (pH 7.4) at room temperature for 48 h. The crude product was purified by semipreparative RP-HPLC to give the desired halicylindramide A in 60% yield. The pure cyclodepsipeptide was analyzed by ^{1}H, HRMS, CD, and analytical RP-HPLC. The ^{1}H and analytical RP-HPLC data of the product were identical to those of the natural halicylindramide A.

CD spectra of the halicylindramide A and deformyl-halicylindramide A (**7**) were examined to compare their conformations under three different conditions (Figure 2). The overall conformations of both compounds were fairly similar, but a significant change was observed in the presence of sodium dodecyl sulfate (SDS), implying that the depsipeptide undergoes a conformational change in the membrane environment.

In summary, we have demonstrated the total synthesis of halicylindramide A via solid-phase peptide synthesis, oxidation, and solution-phase formylation. This strategy may be applicable to the synthesis of other similar depsipeptides containing a cysteic acid residue. In addition, we confirmed the stereochemistry of naturally occurring halicylindramide A. Investigation of the biological activity and conformational studies of halicylindramide A mimetics using NMR and molecular dynamics simulations are underway.

Experimental Section

Peptide Synthesis. Peptide synthesis was accomplished manually via stepwise solid-phase synthesis using Fmoc chemistry. Reaction completion was determined using qualitative Kaiser[19] or chloranil tests. Washings between deprotection and coupling were carried out with DMF (7 × 3 min) and CH_2Cl_2 (3 × 3 min) using 10 mL solvent/g resin for each treatment. After removal of the Fmoc group from the Rink amide resin (500 mg, 0.375 mmol, 100−200 mesh, 1% DVB, 0.75 mmol/g), coupling of the amino acids was achieved by shaking the resin in a solution of Fmoc amino acids, 4 equiv of PyBOP (781 mg), 4 equiv of HOBt (230 mg) or HOAt (204 mg), and 6 equiv of DIPEA (0.37 mL) in DMF (3 mL) for 1 h at room temperature. An excess of amino acids [2 equiv for the coupling of Asp, Thr, D-Phe, (*N*-Me)Gln, and D-Cys derivatives and 3 equiv for Arg, D-Trp, Val, D-Val, Pro, (Br)Phe, and D-Ala derivatives] was used. For the coupling of Fmoc-Thr-OH to the *N*-Me Gln residue of the peptide, three couplings were required to obtain a negative Kaiser test. The Fmoc group was removed using 20% (v/v) piperidine in DMF (3 × 5 min).

Introduction of Alloc-Sar-OH to the side chain of the Thr residue was achieved by mixing the resin with the anhydride solution, which was prepared in a separate flask by combining 6 equiv of Alloc-Sar-OH (390 mg, 2.25 mmol), 5 equiv of diisopropylcarbodiimide (0.29 mL, 1.88 mmol), and 0.1 equiv of DMAP (4.6 mg, 0.0375 mmol) in CH_2Cl_2 (20 mL) at 0 °C for 20 min. A small portion of the resin was cleaved with a solution containing 95% TFA, 2.5% TIS, and 2.5% H_2O, and the crude product was analyzed by RP-HPLC (t_R = 17.2 min, condition A). HPLC conditions are described in Supporting Information.

The Alloc and allyl protecting groups were removed by shaking the resin with 0.1 equiv of $Pd(PPh_4)_4$ (43.3 mg, 0.0375 mmol) and 12 equiv of $PhSiH_3$ (0.55 mL, 4.5 mmol) in CH_2Cl_2 (4 mL) under nitrogen atmosphere for 4 h. The chloranil test was positive, indicating the presence of the secondary amine of the Sar residue. A small portion of the resin was cleaved and the crude product was analyzed by RP-HPLC (t_R = 16.6 min, condition A).

The cyclization step was carried out using 5 equiv of *N*-methyl morpholine (0.21 mL, 1.88 mmol), 5 equiv of HATU (713 mg, 1.88 mmol), and 1 equiv of HOAt (51 mg, 0.375 mmol) in DMF (3 mL). After shaking overnight, a negative chloranil test result was obtained. A small portion of the resin was cleaved with TFA−CH_2Cl_2 (1:1), and the crude product was analyzed by RP-HPLC (t_R = 17.6 min, condition A). LRMS (ESI) calcd 1074.2 for [M + H], found *m/z* 1075.4. Deprotection of *S*-Acm was performed by treatment with 1 M $Hg(OAc)_2$ in DMF (pH 4, adjusted with a few drops of acetic acid) for 3 h in darkness. The resin was washed with DMF (3 × 3 min) and β-mercaptoethanol−DMF (1:9, 3 × 3 min) to remove Hg^{2+} from the resin-bound peptide. A small portion of the resin was cleaved as above and the crude product was analyzed by RP-HPLC (t_R = 11.1 min, condition B).

Oxidation of Cysteine. Performic acid (1%) solution was prepared by mixing 35% hydrogen peroxide and 98% formic acid (3:97, v/v), and the solution was allowed to stand at room temperature for 1 h. The free thiol of the Cys residue was treated with freshly prepared 1% performic acid (5 mL) in a bath at 0 °C for 2 h under a nitrogen atmosphere. Dimethyl sulfide (0.4 mL) was added to the reaction mixture, and the solution was left to stand for 10 min to quench the excess oxidants. A small portion of the resin was cleaved and the crude product was analyzed by RP-HPLC (t_R = 12.7 min, condition B).

Peptide Cleavage and Isolation. The resin-bound peptide was washed thoroughly with DMF (10 × 3 min) and CH_2Cl_2 (5 × 3 min), and then dried *in vacuo* overnight. The peptide was removed from the solid support using TFA containing 2.5% (v/v) *i*Pr_3SiH and 2.5% (v/v) H_2O for 2 h at room temperature. This cleavage step was repeated three times, and the combined solution was concentrated to ~2 mL under reduced pressure. Cold diethyl ether was added to the solution and the precipitated peptide was filtered and dried to obtain 60 mg of the crude **7**. HPLC and mass spectrometry analysis of the crude product indicated that the major peak was the desired peptide. The crude peptide **7** was dissolved in MeOH and allowed to stand for 2 days at room temperature. After purification by semipreparative RP-HPLC, pure product **7** was obtained as a white solid (10 mg, 1.5% overall yield from the starting resin). HRMS (ES^+) calcd for $C_{77}H_{109}BrN_{20}NaO_{21}S$ [M + Na]$^{2+}$ 893.8519, found 893.8511. ^{1}H (DMSO-d_6, 500.1 MHz): δ 10.68 (Trp^{1}NH, brs), 8.79 (Br-Phe-NH, br), 8.62−8.49 (Cys(SO_3)-

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NH, m), 8.35−8.23 (Phe-NH, br), 8.17 (Trp-NH, d, J = 8.5), 8.05 (Arg-NH, d, J = 7.5), 7.98−7.88 (Thr1-NH, Thr2-NH, Ala-NH, br), 7.86−7,79 (D-Val-NH, Val-NH, m), 7.61 (Trp-C_4, d, J = 7.5), 7.52−7.40 (Br-Phe-C_3C_5, Arg-ϵNH, Asn-NH, m), 7.43 (Gln-δNH_2, brs), 7.31 (Trp-C_7, d, J = 8.0), 7.29−7.21 (Phe-ζ, m), 7.19 (Phe-δ1, δ2, d, J = 7.5), 7.17 (Phe-ζ1, ζ2, s), 7.12 (Trp-C_2, s), 7.04−7.01 (Trp-C_6, m), 6.96−6.92 (Trp-C_5, m), 6.74 (Asn-γNH_2, s), 6.67 (Gln-δNH_2, s), 5.14 (Thr1-β, d, J = 7.0), 5.08−5.03 (Thr1-α, m), 5.00−4.94 (Arg-α, Asn-α, m), 4.87−4.85 (Thr2-α, m), 4.83−4.79 (Br-Phe-α, m), 4.70−4.66 (Phe-α, Cys(SO_3)-α, overlap), 4.64−4.59 (Trp-α, m), 4.48−4.42 (Pro-α, Gln-α, m), 4.36−4.32 (D-Val-α, m), 4.20−4.16 (Val-α, m), 3.98−3.93 (Thr2-β, m), 3.76−3.73 (Ala-α, m), 3.66−3.61 (Pro-δ, m), 3.10−3.04 (Arg-δ, Br-Phe-β, m), 3.04−3.01 (Phe-β, Sar-α, m), 3.00−2.96 (Trp-β, m), 2.93 (Gln-NMe, s), 2.82−2.68 (Cys(SO_3)-β, Br-Phe-β', m), 2.73 (Sar-NMe, s), 2.64 (Asn-β, br), 2.14−2.02 (Gln-β, Pro-β, D-Val-β, Gln-γ, m), 1.97−1.78 (Pro-γ, Gln-β', Val-β, m), 1.77−1.72 (Pro-β, m), 1.69−1.59 (Arg-β, m), 1.47−1.36 (Arg-γ, m), 1.16 (Thr1-γ, d, J = 6.5), 1.06 (Ala-β, s), 0.91 (Thr2-γ, d, J = 5.5), 0.84 (D-Val-γ', d, J = 6.5), 0.80 (D-Val-γ, d, J = 6.5), 0.60 (Val-γ', d, J = 6.5), 0.57 (Val-γ, d, J = 6.0).

Halicylindramide A. Formylation of the *N*-terminal Ala residue of **7** using cyanomethyl formate was performed using a minor modification of the method of Deutsch and Niclas.[18] Compound **7** (5 mg, 2.8 μmol) was dissolved in 1.5 mL of 75% DMF in H_2O containing sodium phosphate buffer (20 mM, pH 7.4). To this solution was added cyanomethyl formate (1.3 μL, 10 equiv), and the mixture was allowed to stand at room temperature for 48 h in darkness. The crude product was purified by semipreparative RP-HPLC to give halicylindramide A as a white solid (3 mg, 60% yield). The ^{1}H and HPLC data of the synthetic sample were exactly the same as those of the natural halicylindramide A (see Supporting Material). HRMS (ES^+) calcd for $C_{78}H_{109}BrN_{20}Na_2O_{22}S$ $[M + 2Na]^{2+}$ 919.3443, found 919.3441. Analytical RP-HPLC (condition B): t_R = 14.1 min.

CD Measurement. CD experiments were performed using a Jasco 715 spectropolarimeter with a path length of 1.0 cm at room temperature. Each 100 μM sample was dissolved in TFE or sodium phosphate buffer (20 mM, pH 7.4) in the presence or absence of 5 mM SDS. Data were collected from 260 to 190 nm in 1-nm increments at a scan rate of 20 nm/min and a 3-s signal averaging time.

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Supporting Information Available: General experimental methods; comparison of ^{1}H and RP-HPLC of the synthetic and natural halicylindramide A; ^{1}H and 2D NMR spectra of **7**; UV spectra of halicylindramide A and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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