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Synthesis of a Dicarba Analogue of Human β-Defensin-1 Using a Combined Ring Closing Metathesis—Native Chemical Ligation Strategy

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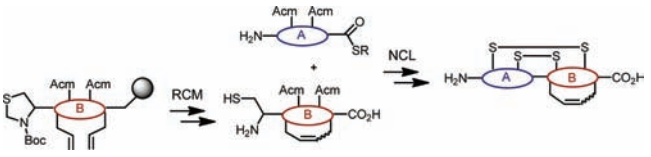
ABSTRACT



We herein describe the first synthesis of the native antimicrobial protein HBD-1 making use of an orthogonal thiol protection strategy and a novel dicarba analogue thereof. The robust hydrocarbon linkage was installed by replacement of one disulfide bond using on-resin ring closing metathesis. The unprecedented 59-membered C-terminal cysteine macrocyclic fragment thus formed then engages in native chemical ligation allowing convergent access to this unique synthetic protein analogue.

The ever-increasing incidence of drug-resistant strains of bacteria demonstrates a continuing imperative for the development of novel classes of effective antibiotics. Naturally occurring antimicrobial peptides (AMPs) form part of the innate immune system and have for some time been recognized as potentially useful therapeutic agents owing to their unique mode of action, which is distinct from and complementary to that of existing drugs.[1] Being cationic and amphipathic in nature they are able to interact in a relatively nonspecific manner with anionic components of the bacterial cell membrane before inserting themselves into the hydrophobic core and inducing disruption of membrane functions. The presence and diversity of AMPs in a multitude of organisms indicate their effectiveness over the evolutionary time frame and suggest their mode of action is not one to which bacteria can easily become resistant.[2] Human β-defensins, first isolated in 1995 from blood filtrate,[3] are a well-known subgroup of AMPs containing three positionally conserved disulfide bonds,[4] ostensibly imparting greater stability to proteolysis and enhancing their effectiveness as antibacterial agents.[5] However, as peptidic drugs have a relatively rapid systemic clearance,[6] it was our intention to investigate the therapeutic potential of stabilized defensin analogues that may possess an extended half-life and an improved dosage profile. The stabilization of peptides by the introduction of a bridging C−C bond, a technique referred to as hydrocarbon stapling, was first described by Grubbs.[7] This process was

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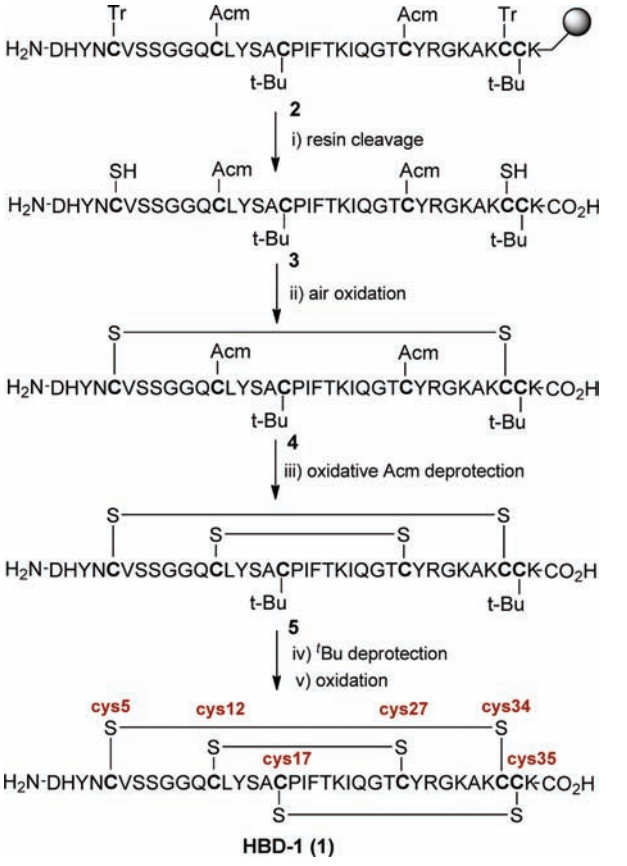
later shown to impart greater resistance to proteolytic degradation and enhance membrane permeability.[8] The process can now be conveniently performed on the solid phase, facilitated by microwave irradiation,[9] and has gained widespread use in the synthesis of bioactive analogues of natural peptides.[10]

Scheme 1. Synthesis of Native HBD-1 (**1**) Using an Orthogonal Protecting Group Strategy for Regioselective Disulfide Bond Formation



Ring closing metathesis (RCM) performed on protected peptide substrates is now a useful addition to the 'toolbox' available to medicinal chemists for α-helix stabilization,[11] the introduction of H-bond surrogates,[12] and substitution of disulfide bonds with chemically inert mimics. In application of this strategy to the present case, it was anticipated that replacing one of the conserved Cys5-Cys34, Cys12-Cys27, or Cys17-Cys35 disulfide bonds of HBD-1 (**1**, Scheme 1) with a carba analogue or 'staple' would impart greater stability while retaining functionality.

All previous chemical syntheses of HBD-1 (**1**) have relied on either a one-step direct oxidation[13] or thermodynamic refolding of the fully reduced linear precursor in the presence of a redox couple.[14] These are capricious methods often characterized by generation of misfolded proteins, presumably with incorrectly formed disulfide linkages. We therefore embarked on the synthesis of HBD-1 (**1**) using an orthogonal cysteine protecting group strategy[15] which enables the installation of these native bridging groups in a controlled, stepwise, and unambiguous manner.

The synthesis, described in Scheme 1, begins with the production of resin-bound peptide (**2**)[16] using MW-assisted Fmoc-SPPS, with HBTU/DIPEA for coupling of amino acid residues and 20% piperidine for Fmoc deprotections. Acid-facilitated cleavage of the peptide from resin with concomitant removal of side-chain protecting groups afforded linear peptide **3**, leaving the Acm groups[17] of Cys12 and Cys27 and the *tert*-butyl thioethers of Cys17 and Cys35 intact. After purification of peptide **3** by HPLC, the Cys5-Cys34 disulfide was generated by aerial oxidation in a tris buffer at pH 8.5 to afford disulfide **4**. The subsequent removal of the Acm groups was challenging in that established oxidative methods using methanolic solutions of iodine afforded complex mixtures, with iodide adducts being detected. However, adjusting the conditions and carrying out the deprotection in 80% AcOH at 0 °C with monitoring by HPLC resulted in satisfactory formation of **5**. The reaction was then quenched with sodium ascorbate, diluted with 10 volumes of water and the product purified by HPLC. An attempted one-pot removal of *tert*-butyl groups and disulfide formation using a standard oxidative cocktail of TFA-DMSO proved unsuccessful. Instead the *tert*-butyl groups were removed from Cys17 and Cys35 by briefly exposing peptide **5** to a 9:1 mixture of trifluoroacetic acid and triflic acid at low temperature.[18] Finally, a solution of the resulting bis-thiol was allowed to oxidize in air at pH 8.2 to afford native HBD-1 (**1**) in 29% isolated yield over four steps.

With the successful synthesis of native HBD-1 (**1**) in hand, attention was turned to synthesizing a carba analogue in which the C-terminal Cys17-Cys35 disulfide

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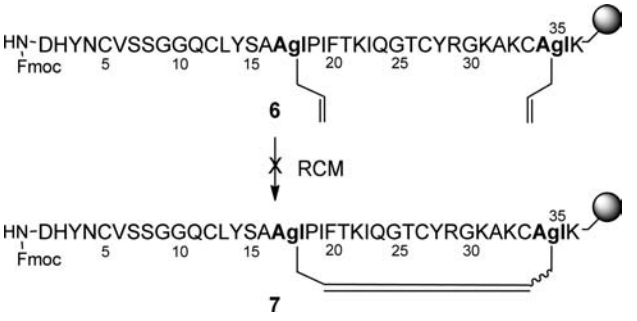
bond was selected for replacement. Thus synthesis of the metathesis precursor **6** (Scheme 2) was performed using Fmoc-SPPS, substituting allylglycine (Agl) for Cys17 and Cys35.

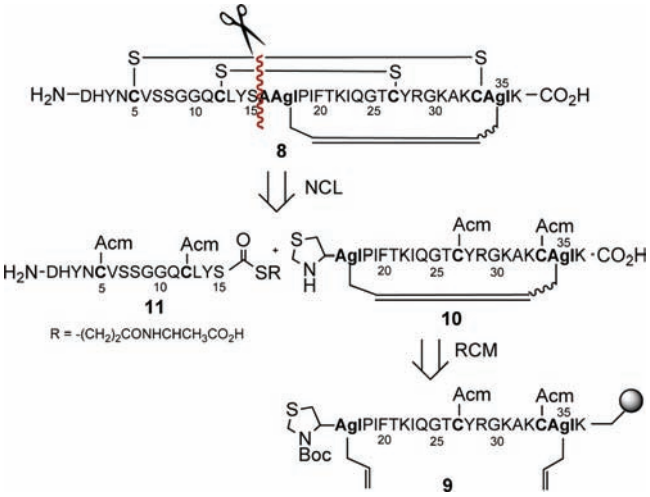
Scheme 2. Initial Attempt to Introduce Dicarba Linkage by RCM on Full Analogue Sequence



To our disappointment, and despite exploration of a variety of conditions, the solid supported metathesis reaction could not be induced to occur, affording none of the desired product **7**. This outcome is consistent with the observation that this reaction in large peptides, especially when forming a large ring, is a capricious and not generally high-yielding process.[19] The approach was accordingly modified as outlined in Scheme 3. It was envisaged that HBD-1 dicarba analogue **8** could be formed in the forward direction by disulfide bond formation following a native chemical ligation (NCL) reaction between protected *N*-terminal cysteine macrocycle **10** and thioester **11** (Scheme 3). Splitting the molecule into two approximately equal sized fragments in this convergent approach would afford the shortest possible metathesis precursor bis-olefin **9** with the most likelihood to undergo successful RCM reaction to form macrocycle **10** after cleavage from the resin. This strategy requires the mutation Ala16 to Cys16 to introduce an appropriately placed ligation site,[20] with the Cys16 being subsequently reductively desulfurized to the native Ala16. The remaining native cysteine thiols were protected with Acm groups, allowing simultaneous deprotection of all thiols following ligation and desulfurization. This strategy both minimizes the number of protecting group manipulations and also enables synthesis of the thioester partner **11** using the more convenient Boc chemistry. It was anticipated that installation of the dicarba bridge would induce partial folding and favor formation of the two disulfide bonds with correct connectivity under thermodynamic conditions.[21]

The alkyl thioester component **11** was synthesized manually on PAM-linked aminomethyl polystyrene

Scheme 3. Revised Retrosynthesis of HBD-1 Dicarba Analogue **8**



resin by *in situ* neutralization Boc-SPPS, using HBTU/DIPEA for coupling activation and 100% TFA for Boc deprotection.[22] Cleavage from the resin using HF afforded thioester **11** with the Cys(Acm) protecting groups intact. NCL partner **10** was obtained by on-resin RCM of bis-olefin **9** which itself was synthesized using Fmoc-SPPS substituting allylglycine for cysteine as necessary and capping with *N*-Boc-1,3-thiazolidine-4-carboxylic acid[23] (a masked cysteine equivalent). Using Fmoc-Cys(Trt) at this position afforded a more complex reaction mixture, presumably caused by greater steric interference of this requisite terminal amine protecting group.

With the bis-olefin RCM precursor **9** in hand, various RCM conditions were investigated and those similar to the conditions reported by Wade[10b] and Robinson[19] were found to be the most efficient. Thus the resin was first swollen in CH_2Cl_2 in the presence of 0.4 M LiCl in DMF (4:1) for 30 min and then degassed with argon. After addition of the Grubbs–Hoveyda second generation catalyst (30 mol %)[24] and sealing of the reaction vessel, metathesis was induced by subjecting the heterogeneous mixture to the optimized 100 °C, 200 W for 5 h. One sharp peak was observed in the LCMS, which exhibited a mass corresponding to the desired ring-closed product **10** (Scheme 4). However, the reaction was low yielding and produced a considerable quantity of material with a broad, poorly defined elution profile consisting of starting peptide and material suggestive of oligomerization. Both decreasing the loading of the resin from

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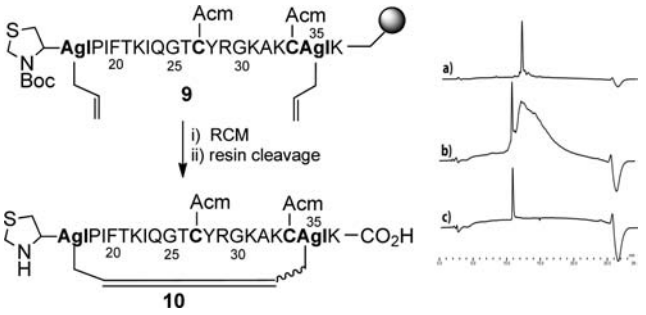
0.8 mmol/g to 0.15 mmol/g and using alternative additives to disrupt unfavorable catalyst interactions such as $Ti(OiPr)_4$[25] and chlorodicyclohexylborane[26] afforded no benefit in conversion.

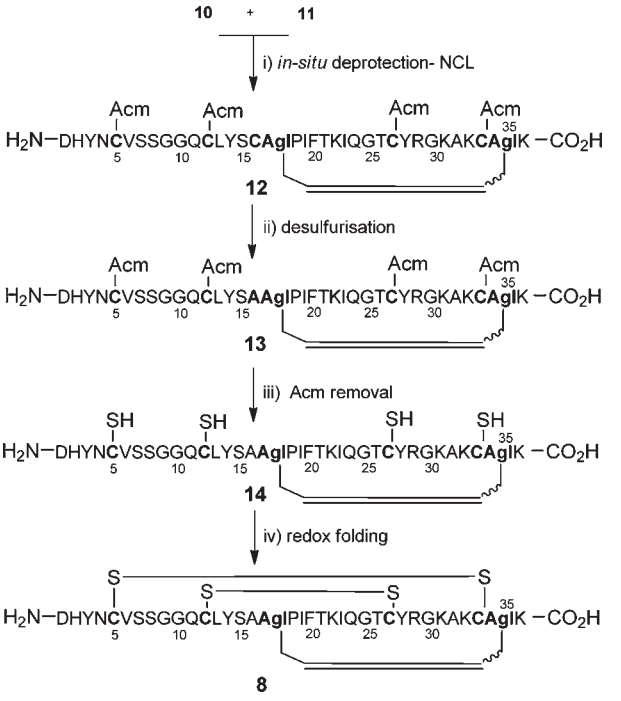
Scheme 4. (Left) RCM Reaction; (R) HPLC Traces[a]



[a] (a) Starting material **9**; (b) crude reaction mixture; (c) purified **10**.

Following metathesis, peptide **10** was cleaved from the resin under standard acidic conditions and purified by RP-HPLC. Ligation between the two fragments was carried out by first subjecting thiazolidine **10** (1.0 equiv, 3 mM) to *in situ* deprotection using $MeONH_2$ to release the terminal cysteine which was then reacted with thioester **11** in the presence of MPAA to afford the intermediary **12**; ligation was complete after 3 h based on consumption of the limiting reagent **10** (Scheme 5). Desulfurization of the resulting cysteine residue[27] was achieved under reducing conditions by using the water-soluble radical initiator VA-044, and the remaining Acm groups were removed using silver acetate in water/acetonitrile. After dilution of the reaction mixture with water and addition of dithiothreitol to sequester the AgOAc, the desired peptide **14**, containing four free cysteine residues, was isolated from the supernatant by SPE. The final step entailed folding this material under well-established thermodynamic conditions to generate the two disulfide bonds. Initially a glutathione redox couple, (GSSG/GSH 10 equiv/100 equiv relative to peptide), 0.1 mM, pH 8.5, was employed to this effect and material with the desired mass was observed to form along with stable glutathione adducts. After further dilution of the reaction mixture the desired material **8**, with an HPLC elution profile similar to that of the native HBD-1 (**1**), was formed as the major product after a period of 5 days.

Scheme 5. NCL Followed by Desulfurization and Protein Folding



In summary, this report describes the first synthesis of the native antimicrobial protein HBD-1 (**1**) using an orthogonal protecting group strategy. Furthermore, we have achieved the synthesis of a novel dicarba-analogue **8** in which one disulfide bond has been replaced with a robust carbon−carbon linkage. To our knowledge, this is the largest macrocycle (59 membered) to be generated by catalytic RCM on a solid support and is also the first example of such a macrocycle being used in the NCL reaction. We are currently investigating the biological properties of this analogue and propose eliminating the occurrence of geometric isomers by reducing the double bond formed upon RCM.

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Supporting Information Available. Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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