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Novel antibiotics: second generation macrocyclic peptides designed to trap Holliday junctions

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Abstract—Described are the syntheses of 15 macrocyclic peptides designed to trap Holliday junctions (HJs) in bacteria during site-specific and homologous recombination. This leads to inhibiting bacterial growth. These second generation macrocycles were based on the *C*-2 symmetrical HJ. They were synthesized using a strategy that permits elucidation of the amino acid role in binding HJs. The syntheses of these macrocycles are an important step in the development of a new class of antibiotics.

Peptides rarely function well as drugs due to their low bioavailability and rapid degradation within cells.[1] However, they make convenient initial synthetic targets due to ease of assembly. Moreover, a large number of natural product cyclic peptides have interesting biological activity.[2] The conversion of these active peptides into peptidomimetics has been a successful approach for making new biologically active compounds.[3]

Antibiotic resistance has become a major public health concern.[4] As pathogenic bacteria become resistant to first and second line antibiotics, easily treatable infectious diseases are becoming life threatening. In order to keep up with the evolutionary pressure from pathogenic bacteria during the infectious disease process, we must continually develop new antibiotics and aim at new biological targets within bacteria. One such new target is the Holliday junction (HJ) generated as an intermediate in XerCD site-specific recombination, in RecA-dependent genetic exchange and by replication fork regression.[5] Site-specific recombination can control gene expression, amplify episome copy number, separate chromosomes during bacterial cell division and create genetic diversity. Homologous recombination also promotes diversity, although its main function is to maintain genomic integrity by facilitating DNA repair and replication restart.[5] By trapping the HJ, we may inhibit either or both of these pathways.[6] Blocking these recombination reactions in vivo has led to bacterial death.[7–9] Segall and co-workers have shown that linear dodecapeptides successfully trap the HJ in nanomolar concentrations and cause bacteria cell death via this mechanism of action.[9] Compounds that successfully trap the HJ and lead to bacterial cell death represent a new class of antibiotics. Such inhibitors are reminiscent of the quinolone/fluoroquinolone class of antibiotics, which stabilize a normally transient intermediate.

The initial peptide leads were linear hexapeptides, which dimerize via a disulfide bridge giving the active dodecapeptide structure. These leads were problematic given their size, solubility, and flexibility, making it difficult to identify specific residues involved in the binding event. To elucidate the biological mechanism of action, and find soluble, effective compounds that trap this unique target, we synthesized a first generation of macrocycles. These were based on the *C*-2 symmetrical HJ binding site[7,10] and Segall's lead linear dodecapeptides (Fig. 1).[8,9] The first generation was cyclized to maximize

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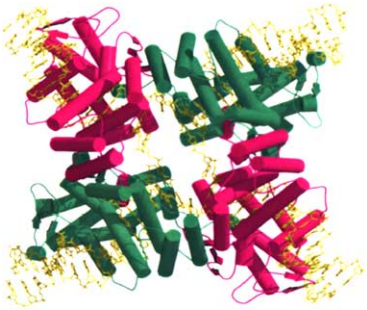

Figure 1. A co-crystal structure of the Holliday junction. The co-crystal structure was obtained by mixing a mutant Cre protein with *loxS* DNA substrates. The crystal was dependent on blocking catalysis. A similar co-crystal structure was obtained between wild type Cre protein and *lox* substrates, but only in the presence of the linear lead peptides. This second crystal contains additional electron density in the HJ center, consistent with bound peptide in the central 'hole'. Note the *C*-2 symmetry of the structure. One lead linear hexapeptide structure is **Lys-Trp-Trp-Cys-Arg-Trp** where the active peptide is a dimer of this linear peptide. Bacterial RuvA, RuvC, and RecG target similar DNA structures.

inherent rigidity of the compounds,[11] identify the contacts between the compounds and HJ, and decrease degradation inside cells.

This first generation of macrocycles contained six amino acid residues, estimated to fit the approximate size of the HJ binding site, which is approximately 25 Å by 10 Å.[10,12] These compounds also mimicked the symmetry of the HJ and contained residues found in the lead compounds. Like the linear lead dodecapeptides, a number of first generation macrocycles successfully trapped the HJ.[12] However, unlike the linear lead peptides, they were not bactericidal. This led us to design and synthesize a second generation of macrocycles that we anticipate will be more effective antibiotics.

Hydrophobic residues may play an important role in intercalating into the DNA involved in the HJ intermediate, and/or the proteins involved in the HJ formation. They are known to be important for binding to the DNA because they appear in all lead compounds[8,9] and are thought to stack with DNA nucleotides that surround the center of the HJ. The first generation peptides contained only hydrophobic residues, making them relatively insoluble. Since they contained no hydrophilic residues, the side chains were unable to hydrogen bond with HJs. As a result, this second generation of macrocycles has incorporated hydrophilic residues to increase the solubility and hydrogen bonding properties. In addition, the minimal number of residues involved in binding to the HJ is not known. It is estimated that six to eight amino acid residues will fit into the HJ binding site.[10] Therefore, our addition of macrocyclic octapeptides in this synthesis will examine an ideal 'fit' in the HJ.

Herein we describe the synthesis of 13, second generation, macrocyclic peptides. Our synthetic approach for the second generation was chosen to simplify the synthesis of the macrocycles, while providing flexibility in amino acid placement (Fig. 2). Starting from commercially available natural and unnatural amino acids, this generation incorporates hydrophobic residues shown to trap the HJ, while including hydrophilic residues not present in our first generation.

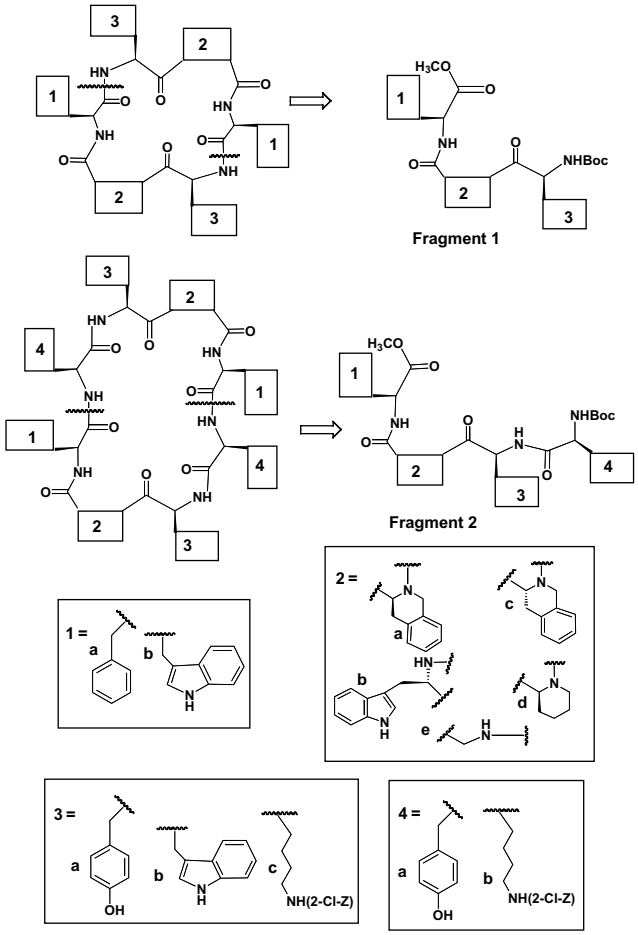

Figure 2. Synthesis strategy.

Using 2(1-H-benzotriazole-1-yl)-1,1,3-tetramethyl-uronium tetrafluoroborate (TBTU) as a coupling reagent, and diisopropylethylamine (DIPEA), acid protected residue **1**(**a**,**b**) and *N*-Boc protected residue **2**(**a**–**e**) (Fig. 3) were coupled to give the dipeptide 1-2-Boc (80–94% yield). Deprotection of the amine on residue **2** using TFA gave the free amine **1**–**2** (~quantitative yields). Coupling of this dipeptide to monomer **3**(**a**–**c**) gave the desired tripeptide (**Fragment 1**) in good yields (65–94%).[13]

Fragment 1 was separated into two equal aliquots (Fig. 4). The acid was deprotected in the first aliquot using 4 equiv of sodium hydroxide, while the amine was deprotected in the second aliquot using TFA. These two trimer peptides were coupled using multiple coupling agents[12,14] yielding 11 examples of linear hexapeptides (36–94% yield). The synthesis of **Fragment 2** was completed by deprotecting the tripeptide using TFA and coupling the free amine to residue **4**(**a**,**b**). In a similar fashion to **Fragment 1**, **Fragment 2** was separated into two equal aliquots (Fig. 4), where upon the first

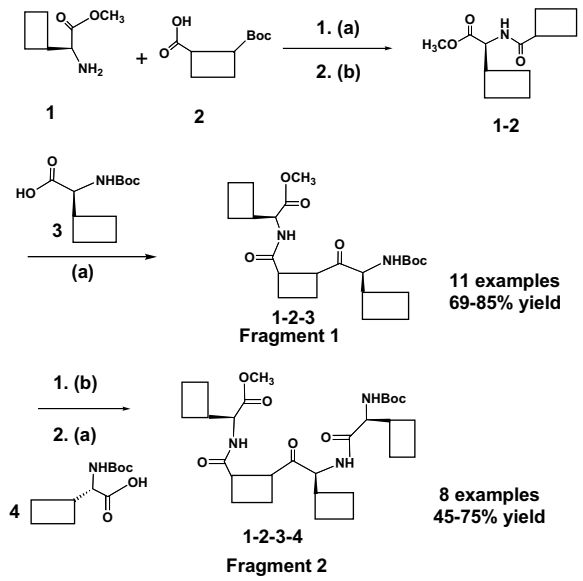

Figure 3. Synthesis of **Fragment 1** and **Fragment 2**. Reagents and conditions: (a) TBTU (1.2 equiv), DIPEA (3 equiv), CH_2Cl_2; (b) TFA (20%), CH_2Cl_2, anisole (2 equiv).

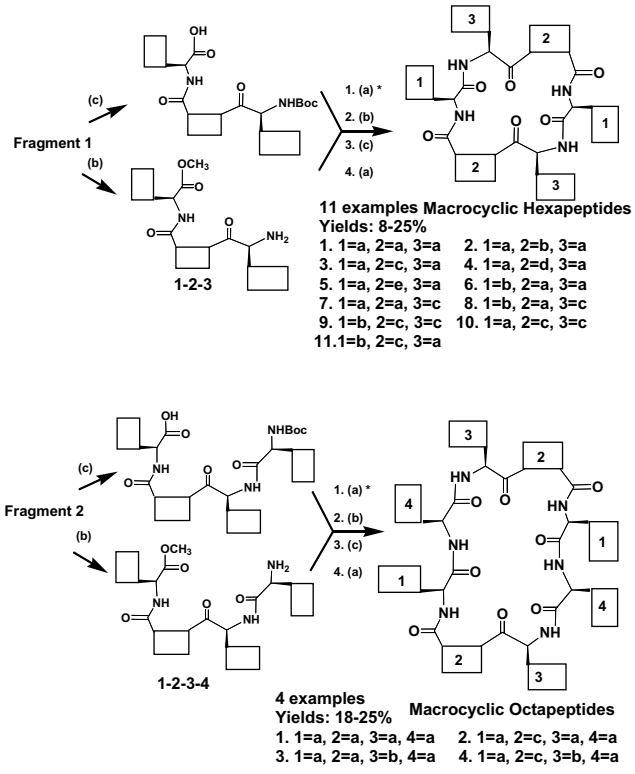

Figure 4. Synthesis of macrocycles. Reagents and conditions: (a) coupling agent‡, DIPEA (3 equiv), CH_3CN; (b) HCl, MeOH, anisole (2 equiv); (c) NaOH (4 equiv), MeOH. *Linear hexapeptide precursor: 11 examples, yields 31–94%, linear octapeptide precursor: 8 examples, yields 36–68%.

‡For the formation of linear peptides, typically two coupling reagents were used: HATU and TBTU (~1 equiv each). For cyclizations HATU, DEPBT, and/or TBTU were used as coupling agents (0.6 equiv each).

aliquot was acid deprotected and the second was amine deprotected. The subsequent coupling of the tetrapeptide free acid and free amine using multiple coupling agents gave eight examples of linear octapeptides (36–68% yield).

The linear hexapeptides and octapeptides were amine deprotected using HCl (pH < 3). Upon completion, the reaction was concentrated in vacuo, and the acid was deprotected by neutralizing the reaction with sodium hydroxide, and then adding four additional equivalents of sodium hydroxide in methanol to give pH = 11. Following acid deprotection, the reaction was concentrated in vacuo and subjected to HATU, TBTU, and DEPBT coupling reagents (~1.0 equiv each), and DIPEA (~6 equiv).[12,14] The final macrocyclizations took approximately 4 days due to the low concentration (0.005–0.01 M) required to maximize the yield.[15] The one-pot ring-closing yields varied from 8% to 25%. The final compounds were then purified using reverse phase HPLC and confirmed via LC–MS.[16] These 15 macrocyclic compounds are currently being tested for their ability to bind the HJ in the in vitro assay used to test the first generation of compounds.[12]

To establish that these second generation macrocycles can function in blocking HJs we investigated their impact on DNA binding by *E. coli* RuvC. RuvC is a Holliday junction-specific endonuclease important in eliminating joint molecules during homologous recombination. All six of the compounds tested significantly reduced the ability of RuvC to form complexes with a radioactively labeled HJ substrate (Fig. 5). Most of these were at least as effective as the first generation cyclic peptides. The results confirm that these macrocycles can bind to HJs and restrict access of a bacterial junction processing enzyme in vitro. All final macrocycles are currently being tested in growth inhibition assays and in vitro assays.

In summary, we describe the synthesis of a second generation of compounds for a unique target in bacteria, the HJ. This new generation of compounds should help elucidate structure–activity relationships of substrates binding to HJs. In addition, these compounds will allow isolation and study of this transient HJ intermediate via co-crystal structure. Furthermore, this class of macrocyclic compounds may be viable leads for a novel class of

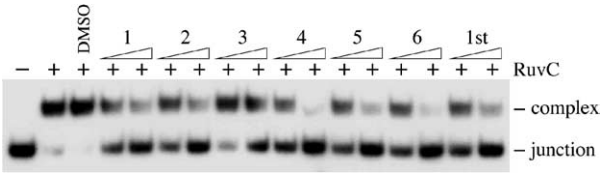

Figure 5. Macrocycle inhibition of *E. coli* RuvC binding to HJ. Reagents and conditions: Junction DNA was prepared and binding assays performed as described.[17] RuvC (50 nM) was mixed with 0.4 nM ^{32}P-labeled synthetic Holliday junction (J11) in the presence or absence of second (1–6) or first (1st) generation macrocycles at 100 and 1000 nM. Protein–DNA complexes were separated on 4% polyacrylamide gels.

antibiotics. Additional assays are currently being run, and will be published in due course.

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13. All dipeptide and tripeptide structures were confirmed using ^{1}H NMR. All linear hexameric peptides were confirmed using LC–MS and ^{1}H NMR and cyclized peptides were all confirmed using LC–MS, ^{1}H NMR and High Resolution Mass Spectrometry.
14. (a) Robinson, J. L.; Taylor, R. E.; Liotta, L. A.; Bolla, M. L.; Azevedo, E. V.; Medina, I.; McAlpine, S. R. *Tetrahedron Lett.* **2004**, *45*, 2147–2150; (b) Ring closing reactions are slow and typically low yielding. Unpublished results from the Guy lab at UCSF, and recently our lab, have found that the use of several coupling reagents facilitates ring-closing reactions by providing a choice of reagents for the specific substrate. This is performed in lieu of optimizing each individual reaction for each individual coupling agent.
15. Macrocycles containing 2-Cl-Z protected lysine residues (**3c**, **4b**) are subjected to palladium catalyzed hydrogenation for the final amine deprotection.
16. One representative example of a macrocyclic hexapeptide and macrocyclic octapeptide in Figure 4 are as follows (note: MS data is given as major peaks with +23[Na^+], and +1 being those peaks):

    Macrocyclic Hexapeptides
    1a-2b-3a (MW=992.5) MS: 1015.5, 993.4,
    |_____| Yield: 15%
    Macrocyclic Octapeptides
    1a-2c-3a-4a (MW=1264) MS:1265.4
    |_____| Yield: 10%.

17. Sharples, G. J.; Curtis, F. A.; McGlynn, P.; Bolt, E. L. *J. Mol. Biol.* **2004**, *340*, 739–751, Compounds 1–6 are: Compound 1 = **1a**–**2c**–**3a**–**4a**, Compound 2 = **1a**–**2b**–**3a**, Compound 3 = **1a**–**2a**–**3a**, Compound 4 = **1a**–**2a**–**3a**–**4a**, Compound 5 = **1a**–**2c-3a**, Compound 6 = **1a**–**2d**–**3a**.