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Novel Antibiotics: Macrocyclic Peptides Designed to Trap Holliday Junctions

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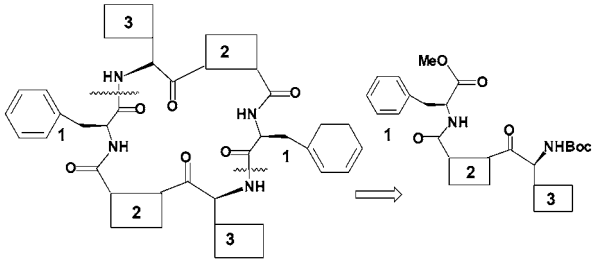
ABSTRACT



Described are the syntheses of eight macrocyclic peptides designed to trap Holliday junctions in bacteria, thereby inhibiting bacterial growth. These macrocycles were designed from linear dimerized hexapeptides that bind to the *C*-2 symmetrical Holliday junction. They were synthesized from three monomers using a combinatorial-like strategy that permits elucidation of the monomer role in accumulation of Holliday junctions and antibiotic activity. These macrocycles are an important step in designing and synthesizing a new class of antibiotics.

Although peptides rarely function well as drugs due to their low bioavailability and rapid degradation within cells,[1] they make convenient initial synthetic targets due to their ease of assembly and modification. Using peptides as initial leads allows rapid identification of the structural requirements of an active biological inhibitor. There are a large number of natural product cyclic peptides that have interesting biological activity.[2,3] Often the conversion of these active peptides into peptidomimetics has been the most successful approach for making new biologically active compounds.[4]

Antibiotic resistance is an extreme public health concern.[5] As more pathogenic bacteria become resistant to first- and second-line antibiotics, easily treatable infectious diseases are becoming life-threatening infections. To keep up with the evolutionary pressure from pathogenic bacteria during the infectious disease process we must continually develop new antibiotics and inhibit new biological targets in bacteria. One such new target is the Holliday junction (HJ) intermediate generated by the XerC/D site-specific recombinase.[6] Site-

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specific recombination can control gene expression, amplify episome copy number, create genetic diversity, and separate chromosomes at bacterial cell division. By trapping the HJ intermediate, we may inhibit site-specific recombination.[7] Blocking this recombination reaction should eventually lead to bacterial death. Thus, compounds that successfully trap the HJ will lead to new antibiotics. Such inhibitors are reminiscent of the quinolone/fluoroquinolone class of antibiotics, which stabilize a normally transient intermediate.

Herein we describe the synthesis of eight potential antibiotics. We based our design on the cocrystal structure[8] of the hexapeptide that dimerizes to give the symmetrical active lead structure[9] and the *C*-2 symmetrical Holliday junction (Figure 1). The previously identified peptide leads

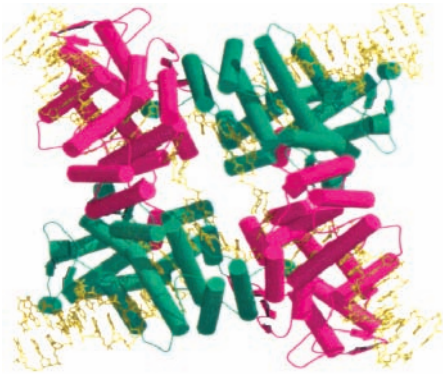

Figure 1. Cocrystal structure was obtained by mixing a mutant Cre protein with loxS DNA substrates. The crystal was dependent on blocking catalysis.[8] A similar cocrystal structure was obtained between wild-type Cre protein and lox substrates, but only in the presence of the linear lead peptides. This latter crystal contains additional electron density in the HJ center, consistent with bound peptide in the central "hole".[8] 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.

are linear dodecapeptides, and their flexibility presumably prevents accurate pinpointing of the specific residues involved in the binding event. However, electron density attributed to the peptides binding to the HJ is detected in the center of the crystal structure.

With the goal of gaining insight into the biological mechanism of action and finding new antibiotics for this unique target, we describe the synthesis of macrocycles that were designed from the *C*-2 symmetric HJ binding site and Segall's discovery of lead compounds.[9] These macrocycles should be more rigid than the linear lead peptides, and they fit the approximate size of the HJ binding site, which is estimated to be ~25 Å by 10 Å.[8] Because symmetrical linear peptides are known to trap the HJ intermediate, we designed *C*-2 symmetric cyclic peptides that mimic the symmetry of the HJ binding site and contain residues found in these lead compounds (Figure 2).[8,9]

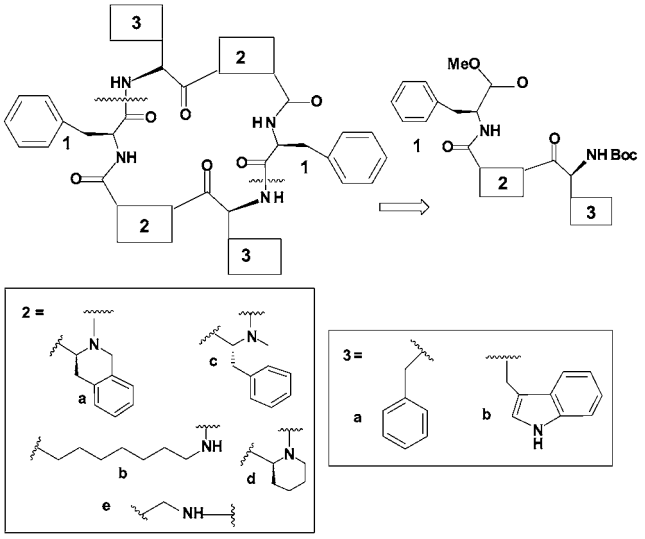

Figure 2. Synthesis strategy.

Our synthetic approach was chosen to simplify the synthesis of *C*-2 symmetrical macrocyclic hexapeptides for rapid biological assessment. By cyclizing the peptides, we aimed to increase their rigidity[10] so that we could identify the exact nature of contacts between the compounds and the HJ. At the same time this should decrease their degradation rate inside cells.

The minimal number of residues involved in binding to the Holliday junction is not known, but several important interactions have been identified.[9] Hydrophobic residues such as tryptophan and phenylalanine are known to be important for binding to the DNA because they appear in all of the lead compounds[9] and are thought to stack with the DNA nucleotides that surround the center of the HJ. Hydrophilic residues such as arginine and lysine may form hydrogen bonds either with the protein that assembles the HJ or with the DNA substrate itself, or both. Starting from commercially available natural and unnatural amino acids, we have synthesized cyclic hexameric peptides. Initially, we chose to use a number of hydrophobic residues in the macrocyclic peptides and sequentially exchange hydrophobic residues with hydrophilic residues.

A great deal of literature has documented the various advantages and disadvantages associated with peptide chemistry.[3] The eight compounds described represent an initial set of potential antibiotics that lay the groundwork for our

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future combinatorial approach. This approach provides the framework from which small, targeted libraries of peptides will be developed, with the eventual goal of converting active peptides into peptidomimetics.

Using *O*-(7-azabenzatrizol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), Hunig's base, and (dimethylamino)pyridine (DMAP) as coupling reagents, L-phenylalanine methyl ester and *N*-Boc-protected residue **2** (Figure 3) were coupled to give the dipeptide **1-2-Boc** (80−94% yield). Deprotection of the amine on residue **2** using 20% TFA and 2 equiv of anisole in methylene chloride gave the free amine **1-2** (∼quantitative yields). Coupling of this dipeptide to monomer **3a** or **3b** gave the desired tripeptide (Fragment 1) in high yields (65−94%).[11]

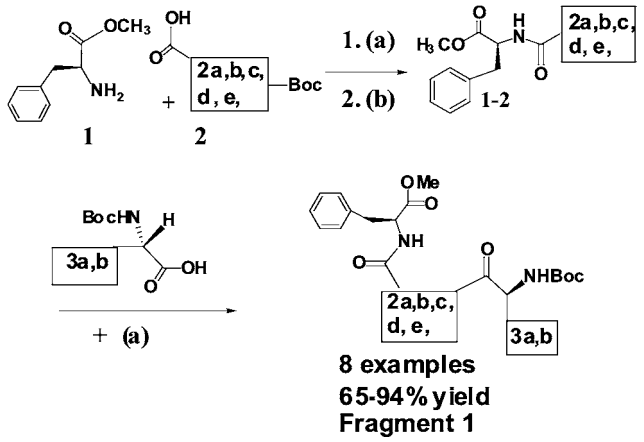

Figure 3. Synthesis of Fragment 1. Reagents and conditions: (a) HATU (1.2 equiv), DMAP (0.2 equiv), Hunig's base (3 equiv), CH_2Cl_2; (b) TFA (20%), CH_2Cl_2, anisole (2 equiv).

Fragment 1 is then separated into two equal aliquots (Figure 4). The acid is deprotected in the first aliquot using 4 equiv of barium hydroxide, while the amine is deprotected in the second aliquot using TFA/anisole. These two trimer peptides are coupled using multiple coupling agents to give the linear hexapeptides.

The linear hexapeptides were cyclized by deprotecting the acid using 4 equiv of barium hydroxide. Upon workup of the free acid, we subjected the compounds to 20% TFA in methylene chloride with 2 equiv of anisole. Following deprotection of the free amine, we subjected the crude, dry product to HATU, TBTU, PyBop, and/or DEPBT coupling reagents (1.2 equiv each), DMAP (0.5 equiv) and Hunig's base (3 equiv) in methylene chloride.[12] The final macrocyclizations took approximately 4 days due to the low concentration (0.005−0.01 M) that was required to maximize the yield. The one-pot ring-closing yields varied from 21 to 48%.[13] The peptides were then purified using reverse-phase HPLC and confirmed using LCMS and high-resolution mass spectroscopy.[13]

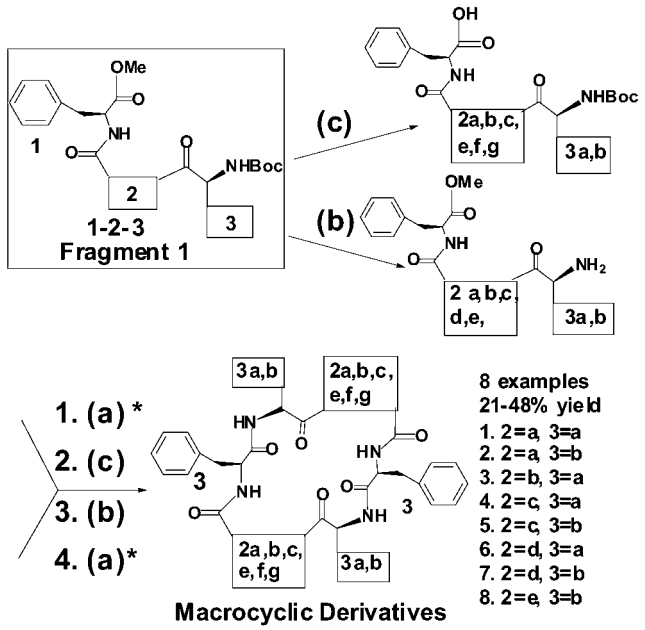

Figure 4. Synthesis of macrocycles. Reagents and conditions: (a) HATU (1.2 equiv; TBTU, PyBOP, and/or DEPBT used as coupling agents), DMAP (0.2 equiv), Hunig's base (3 equiv), CH_2Cl_2; (b) TFA (20%), CH2Cl2, anisole (2 equiv); (c) $Ba(OH)_2$ (4 equiv), MeOH. An asterisk (*) indicates a linear hexamer precursor: 8 examples, yields 31−94%.

These eight macrocyclic compounds have been tested for their ability to bind to the Holliday junction in an in vitro assay (Figure 5). This assay involves a recombination reaction between one radiolabeled double-stranded DNA

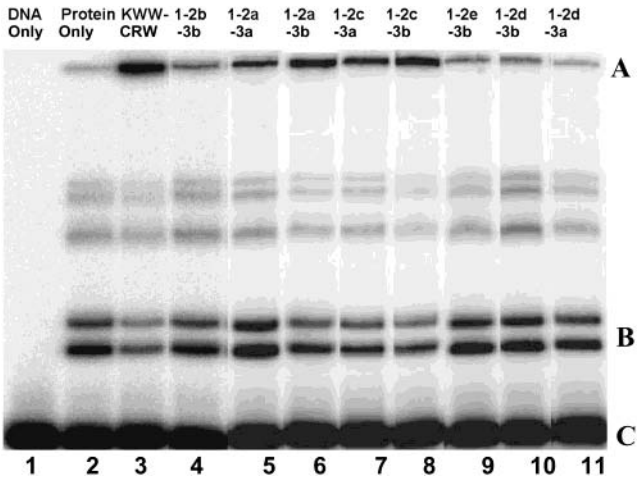

Figure 5. In vitro assay results. (A) Holliday junction intermediate; (B) recombination products; (C) free DNA.

(11) All dipeptide and tripeptide structures were confirmed using ^{1}H NMR. All linear hexameric peptides were confirmed using LCMS and ^{1}H NMR, and cyclized peptides were all confirmed using LCMS, ^{1}H NMR, and high-resolution mass spectrometry. See Supporting Information for spectra.

(12) R. K. Guy (UCSF) communicated his experience where the use of at least three of these coupling reagents facilitates efficient ring-closing reactions by providing a choice of reagents for the specific substrate.

substrate and an unlabeled partner DNA substrate. Successful recombination gives recombination products and very small amounts of HJ intermediate (lane 2). A previously identified lead dimer of Lys-Trp-Trp-Cys-Arg-Trp is used as a positive control because it is known to accumulate HJs[8] (lane 3). During the recombination reaction, two initial DNA cleavage and ligation reactions must take place to form the junction, and two subsequent DNA cleavage and ligation reactions resolve the HJs into double-stranded recombination products.[7] At 0.01 mg/ml concentrations of peptides (Figure 5), a significant amount of HJ intermediates (**A**) accumulate in the presence of four of these macrocyclic hexamers (**1-2a-3a**, **1-2a-3b**, **1-2a-3a**, and **1-2c-3b** seen in lanes 5−8) when compared to a control reaction not treated with peptide (lane 2). These four compounds appear to be as potent as the lead peptide (lane 3) in trapping the HJ intermediate. Tryptophan and phenylalanine are residues within the previously known peptide leads,[8] and it is thought that they stack with the DNA bases in the HJ.[7] The four structures that contain phenylalanine coupled to the 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid moiety (lanes 5−8) appear to stabilize the HJ better than the other four compounds (lanes 4 and 9−11). This binding effect may be the result of the aromatic group in the tetrahydroisoquinoline π-stacking with DNA bases. The relatively low binding of the compounds containing glycine or the piperdine moiety in position 2 (lanes 9−11) may be explained by their inability to π-stack with the DNA bases. The flexibility of the amino octanoic chain may explain the lack of binding for the phenyl and indole moieties present in macrocyclic hexamer **1-2b-3b** (lane 4).

The results from the in vitro assay indicate that our design was an effective initial effort in synthesizing cyclic compounds that trap the Holliday junction. Because the previously known lead peptides contain hydrophilic elements not present in the cyclic hexamers described here, we plan to incorporate these into the next generation of macrocyclic peptides. The syntheses of these new compounds are underway.

In summary, we describe a unique approach to building a potential new class of antibiotics. Our straightforward synthetic route provides novel macrocyclic compounds that stabilize the Holliday junction. The in vitro assays of these compounds suggest that this is a viable approach to designing a new class of antibiotics.

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Supporting Information Available: General experimental procedures, ^{1}H NMR available for dipeptide, tripeptide, and linear hexapeptide intermediates, and high-resolution mass spectra and two wavelength HPLC traces (224 nm and 254 nm; given as evidence of the purity of final macrocyclic peptides). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) R. K. Guy (UCSF) and S. Galicia (UCSF) kindly allowed us to use their preparatory HPLC system for purification of cyclized product.