RNA Recognition

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Macrocyclic Helix-Threading Peptides for Targeting RNA**

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The design and synthesis of RNA-binding small molecules with the ability to bind with high affinity and selectivity to specific intracellular targets is an important step in the development of new tools for the study of RNA function and new therapeutics that target RNA.[1–3] Unfortunately, synthetic molecules capable of selective binding to predetermined RNA targets are elusive. Our laboratory has developed helix-threading peptides (HTPs) that target certain duplex RNA structures selectively by threading intercalation.[4–9] These molecules have a heterocyclic core that directs them to sites predisposed to intercalation in RNA.[5] Peptide functional groups attached to the heterocycle extend into the dissimilar RNA duplex grooves.[6] The peptide appendages provide stabilizing interactions in the grooves of the target RNA. However, these short, linear peptides can adopt multiple, energetically similar conformations. Only a subset of these conformations allow for specific amino acid–nucleotide interactions. Thus, entropic losses upon binding can erode the beneficial effects of groove contacts. To overcome this, we are currently exploring different approaches to restrict the conformational flexibility of HTPs. Macrocyclic peptides are an important class of low-molecular-weight ligands for biological receptors and new methods for their synthesis have been reported recently.[10] The cyclic peptide scaffold offers several advantages over the linear counterpart. The limited conformational flexibility of a cyclic scaffold enables orientation of different functional groups on the peptide backbone into distinct positions in space, thereby providing rigid recognition surfaces for receptors. In addition, cyclic peptides often have increased cell permeability compared with their linear counterparts.[11] Herein, we describe the generation of macrocyclic HTPs through ring-closing metathesis and the RNA-binding properties of these new molecules.

Efficiency and functional group tolerance have made ring-closing metathesis (RCM) a popular approach for cyclizing peptides.[12] RCM has been used effectively to generate peptides with medium to large ring sizes.[13–16] To apply this method to HTPs, we chose to append an allyl group to the 2-phenylquinoline core of these molecules.[8,9] If such a structure were introduced at the N terminus of a peptide that also contained an olefin-bearing C-terminal residue, the metathesis reaction would generate a macrocyclic HTP. We anticipated that this would significantly limit conformational flexibility in the peptide backbone given the near planarity of the atoms of the 2-phenylquinoline that are included in the macrocycle. The requisite carboxylic acid was generated through a short, efficient synthesis (Scheme 1). Displacement of a thiomethyl group from the known Meldrum's acid derivative **1**[17,18] by using methyl anthranilate, followed by thermal cyclization, formed the appropriately substituted quinolone **2** in 64% yield (Scheme 1).[19] Chlorination at the C4 atom preceded radical bromination at the benzylic position to give compound **3**.[20] This compound was then subjected to the conditions of a Stille coupling by using vinyl tributyltin to form the *p*-allyl substituent on the 2-phenyl ring of **4**.[21] Amination with Boc-protected 4-aminobenzylamine in the presence of tin tetrachloride[20] followed by hydrolysis of the methyl ester provided acid **5** in good overall yield.

Macrocyclic HTPs were prepared on a Rink amide resin by using standard procedures for assembling peptides with 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids (Scheme 2). Linear peptide synthesis was initiated by coupling commercially available Fmoc-protected allylglycine to the support followed by coupling the additional α-amino acids. The last residue added was 2-(*p*-allylphenyl)quinoline acid **5**. The resin-bound bis-olefin linear peptides were subjected to ring-closing metathesis conditions in dichloroethane at 60 °C for 30 h by using the Hoveyda–Grubbs second-generation ruthenium catalyst (Scheme 2).[13] HPLC purification afforded cyclic HTPs **6** and **7**, which differ in the N→C sequence of the α-amino acids present in the macrocycle. We measured vicinal coupling constants for the major product isolated for each peptide. The high *J* values observed (15.6 Hz for **6** and 15.3 Hz for **7**) are indicative of *trans* stereochemistry for the newly formed double bond. An energy-minimized model for HTP **6** containing the *trans* double bond is shown in Scheme 2 (see the Supporting Information for details).

Ribonuclease footprinting was used to analyze the RNA binding properties for newly synthesized macrocyclic HTPs

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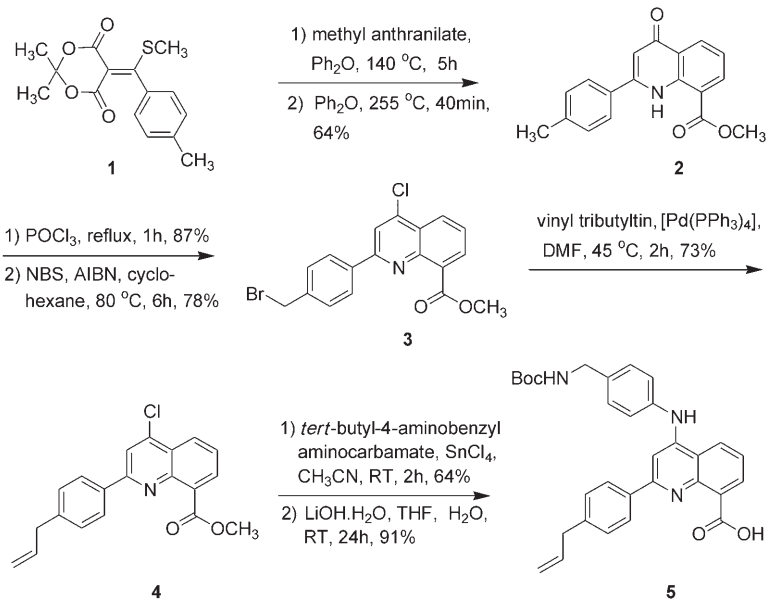
Supporting information for this article (including experimental data) is available on the WWW under http://www.angewandte.org or from the author.

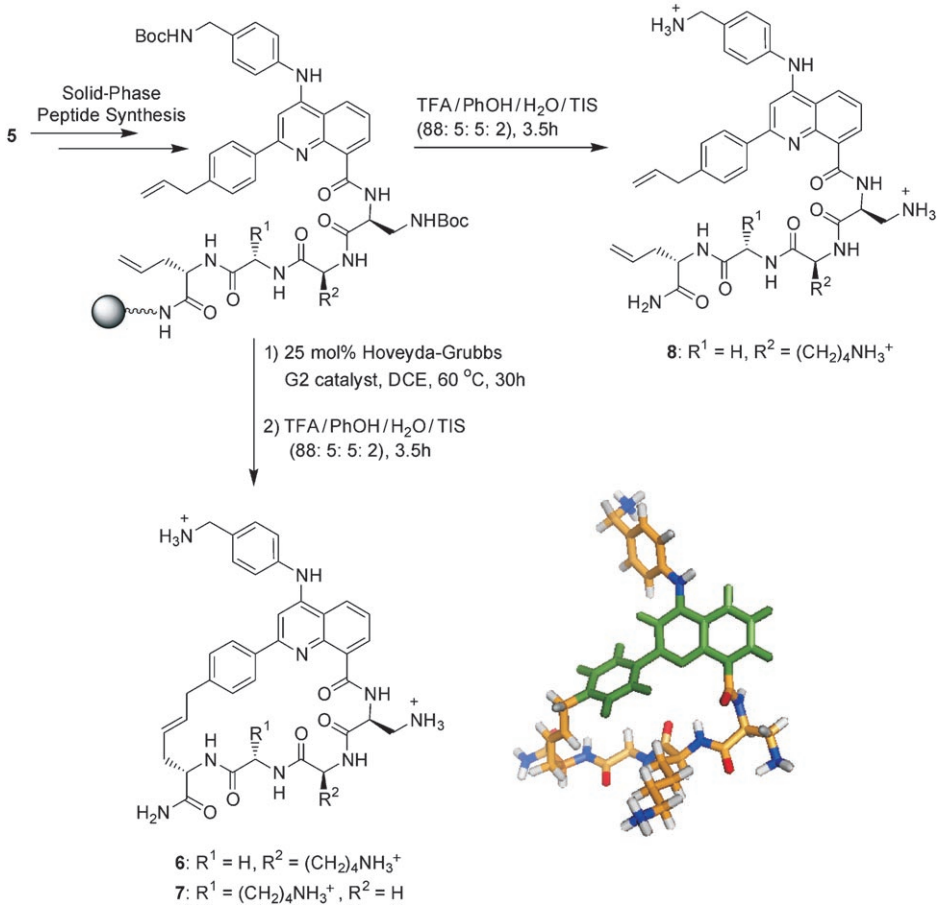


Scheme 1. Synthesis 2-(*p*-allylphenyl)quinoline acid **5**. AIBN = 2,2′-azobisisobutyronitrile, Boc = tert-butoxycarbonyl, DMF = *N*,*N*-dimethylformamide, NBS = *N*-bromosuccinimide.

(Figure 1). RNA **A** selected in vitro, which was previously shown to have a high-affinity HTP binding site, was chosen as the target for our initial studies.[6,7] Both HTPs **6** and **7** bind this RNA selectively, protecting nucleotides near the proposed intercalation site from ribonuclease V1 cleavage (Figure 1). Interestingly, the sequence of α-amino acids in the macrocycle affects affinity for target as **6** binds with a fivefold lower dissociation constant ($K_D = 52$ nM) than does **7** ($K_D = 271$ nM) with RNA **A** (Figure 1, Table 1).

Importantly, cyclization of the HTP increases its affinity for this target RNA as linear peptide **8** binds with a sixfold higher dissociation constant than does the corresponding macrocycle **6** (Figure 1, Table 1). To test the selectivity of the new compounds, we measured the affinity of **6** for RNA molecules **B** and **C**. RNA **B** differs from **A** in the smaller internal loop located adjacent to the intercalation site (one nucleotide versus four nucleotides). RNA **C** is similar to **B** with single nucleotide bulges on each strand on the 5′ side of the intercalation site instead of the 3′ side as in **B** (Figure 1). HTP **6** binds RNA **B** with $K_D =$ 194 nM, whereas no binding could be observed with RNA **C** up to 10 μM HTP. Nonselective protection of this RNA, which is indicative of nonselective binding, is seen at concentrations of **6** greater than 30 μM. Thus, the macrocylic HTP **6** is capable of distinguishing different RNA targets,[9] with binding affinities ranging over two orders of magnitude for the RNA structures described herein. As seen with RNA **A**, cyclization increases affinity for **B** (RNA **B** $K_D =$ 194 nM for **6**, 1.3 μM for **8**).

Selective binding to RNA **B** demonstrated that **6** could target the 5′-PyPu-3′ (Py = pyrimidine, Pu = purine) intercalation site flanked by 3′ bulges preferred by HTPs.[6] This RNA structural motif is present in naturally occurring RNAs. Indeed, a search for this motif in predicted RNA secondary structures identified pre-miRNA23b (pre-miRNA = precursor microRNA), which contains a base paired 5′-UG-3′ step flanked on the 3′ side by a cytidine bulge on one strand and a uridine bulge on the other (Figure 2).[22] Double helical pre-miRNAs are cleaved by the ribonuclease Dicer to generate



Scheme 2. Solid-phase ring-closing metathesis. The model of macrocyclic HTP **6** with the intercalation domain colored in green is also shown. DCE = 1,2-dichloroethane, TFA = trifluoroacetic acid, TIS = triisopropylsilane.

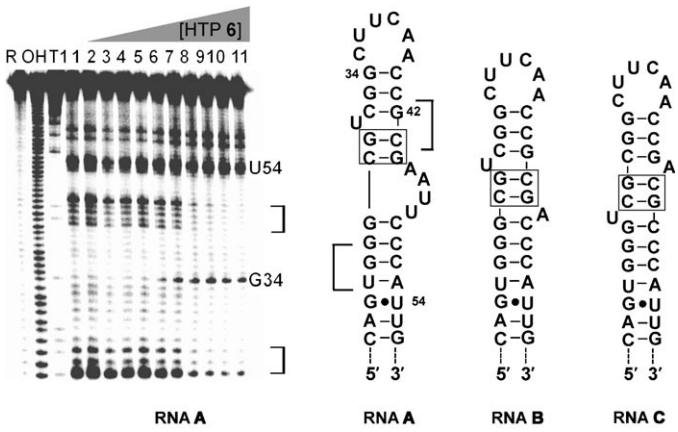


Figure 1. Left: Quantitiative ribonuclease V1 footprinting of HTP **6** binding to RNA **A**. Lane R: RNA alone, lane OH: alkaline hydrolysis, lane T1: ribonuclease T1 products (G lane), lanes 1–11: ribonuclease V1 products. Lane 1: no HTP, lanes 2–11: [HTP **6**] = 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, respectively. Right: Structures of RNA molecules **A**, **B**, and **C** used in this study with the putative intercalation sites indicated with a box.

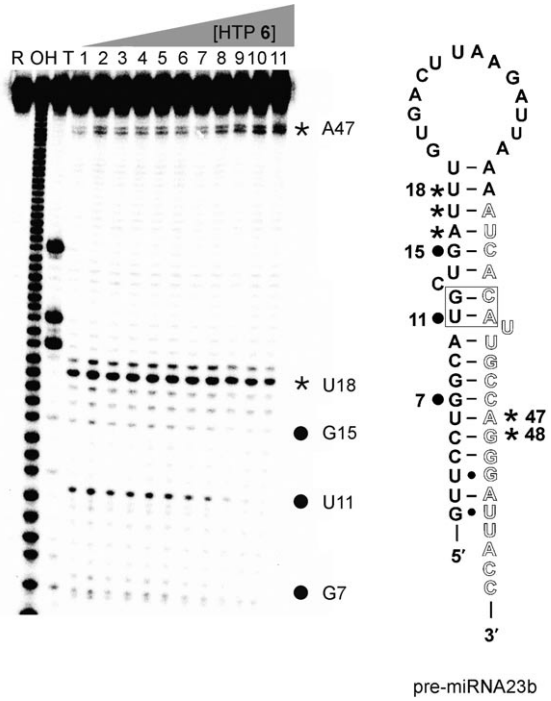


Figure 2. Quantitiative ribonuclease V1 footprinting of HTP **6** binding to pre-miRNA23b. Left: Autoradiogram of polyacrylamide gel used to resolve the footprinting products. Lane R: RNA alone, lane OH: alkaline hydrolysis, lane T1: ribonuclease T1 products (G lane), lanes 1–11: ribonuclease V1 products. Lane 1: no HTP, lanes 2–11: twofold increases in [HTP **6**] starting from 62.5 nM to a maximum of 32 μM. Right: secondary structure of pre-miRNA23b with the putative intercalation site indicated with a box, nucleotides highlighted in outlined text are present in the mature miRNA23b sequence, nucleotides marked with circles are RNAse-V1-dependent bands protected as a result of HTP binding, and nucleotides marked with an asterisk are RNAse-V1-dependent bands not protected by **6**.

Table 1: Dissociation constants for HTP–RNA complexes.

HTP	RNA target	Dissociation constants [nM][a]
6	**A**	52 ± 17
7	**A**	271 ± 108
8	**A**	329 ± 60
6	**B**	194 ± 70
8	**B**	1306 ± 117
6	**C**	> 10000
6	pre-miRNA23b	2333 ± 603

[a] Conditions: 50 mM bis-Tris-HCl (Tris = tris(hydroxymethyl)aminomethane), pH 7.0, 100 mM NaCl, 10 mM $MgCl_2$, and 10 μg mL^{-1} of yeast transfer RNA^{Phe}, 25 °C, 15 min equilibration prior to digestion with ribonuclease V1. Dissociation constants are reported as the average of three independent measurements ± the standard deviation.

miRNAs, which are known mediators of cellular processes including differentiation.[23,24] Indeed, altered expression of certain miRNAs has been linked to human cancers including lung cancer, colon cancer, and leukemia.[25] These observations have stimulated the development of antagonists of miRNA function as potential therapeutic agents (antagomirs).[26,27] A recent report showed that miRNA23b expression is associated with formation of a specific type of pituitary adenoma.[28] Furthermore, blocking miRNA23b function with an antisense RNA was shown to be toxic to lung carcinoma cells, but not a cervical cancer-derived cell line.[29] Small molecules that bind miRNA precursors, like pre-miRNA23b, have the potential to block miRNA function by inhibiting processing steps (e.g. Dicer cleavage) or affecting intracellular localization. Interestingly, we found that macrocyclic HTP **6** binds human pre-miRNA23b, thereby selectively protecting nucleotides near the 5′-UG-3′ site from ribonuclease V1 cleavage (Figure 2). Affinity for this target is reduced relative to RNA **B** ($K_D = 2.3$ μM versus 194 nM; Table 1). This may be due to the differences at the intercalation site (5′-CG-3′ versus 5′-UG-3′) and/or the different bulged nucleotides.

Clearly, HTP binding has RNA sequence preferences in addition to the structural preferences already established (e.g. RNA **B** > RNA **C**).[9] Further structural and structure–activity relationship (SAR) studies with cyclic HTP variants are required to define the basis for this sequence selectivity. In addition, optimization of HTP affinity for pre-miRNA23b may be possible by altering the sequence and/or stereochemistry of the amino acids included in the macrocycle. These studies and others directed at defining the effect HTPs have on miRNA processing steps are currently underway in our laboratories.

In summary, we have developed a high yielding synthesis of a 2-(*p*-allylphenyl)quinoline-containing carboxylic acid that allows for the generation of macrocyclic helix-threading peptides by ring-closing metathesis. A prototypical macrocyclic HTP demonstrated improved affinity for two RNA target sites over its linear bis-olefin precursor and a strong preference for a 5′-CG-3′ step flanked by 3′ bulges. Furthermore, the affinity observed was shown to be dependent on the sequence of amino acids incorporated into the macrocycle. A naturally occurring pre-miRNA is a target for selective

binding, suggesting these compounds may have a potential as antagonists of miRNA function.

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