Published in final edited form as:

Curr Protoc Chem Biol. ; 6(2): 101–116. doi:10.1002/9780470559277.ch130202.

Synthesis of Hydrogen-Bond Surrogate α -helices as Inhibitors of Protein-Protein Interactions

Stephen E. Miller, Paul F. Thomson, and Paramjit S. Arora

Department of Chemistry, New York University, New York, New York

Abstract

The α -helix is a prevalent secondary structure in proteins and critical in mediating protein-protein interactions (PPIs). Peptide mimetics that adopt stable helices have become powerful tools for the modulation of PPIs in vitro and in vivo. Hydrogen-bond surrogate (HBS) α -helices utilize a covalent bond in place of an N-terminal i to i+4 hydrogen bond and have been used to target and disrupt PPIs that become dysregulated in disease states. These compounds have improved conformational stability and cellular uptake as compared to their linear peptide counterparts. The protocol presented here describes current methodology for the synthesis of HBS α -helical mimetics. The solid phase synthesis of HBS helices involves solid phase peptide synthesis with three key steps involving incorporation of *N*-allyl functionality within the backbone of the peptide, coupling of a secondary amine, and a ring-closing metathesis step.

Keywords

α -helix mimetics; hydrogen-bond surrogate; protein-protein interactions

INTRODUCTION

α -helices are the most prevalent secondary structure in proteins, and there has been great interest in mimicking them in order to modulate biomolecular interactions where a helical region dominates the binding energy landscape (Bullock et al., 2011; Jochim and Arora, 2009; Jochim and Arora, 2010; Jones and Thornton, 1995). Because most short peptide fragments are unable to form stable helices outside the context of a protein, strategically placed constraints have been developed to lock peptides in desired conformations (Henchey et al., 2008). The hydrogen bond surrogate (HBS) strategy (Figure 1A) developed in our lab replaces the N-terminal hydrogen bond of a peptide with a covalent linkage, forming a macrocycle within the first turn of an α -helix (Chapman et al., 2004; Patgiri et al., 2008). This constraint forces N-terminal helix nucleation and allows for helix formation to occur more readily in short peptides. Improvement of α -helical character for HBS compounds over linear peptides has been reproducibly shown using circular dichroism spectroscopy (Figure 1B) (Chapman et al., 2004; Henchey et al., 2010a; Henchey et al., 2010b; Wang et al., 2006a; Wang et al., 2006b; Wang et al., 2005; Wang et al., 2008). Further structural analyses by 2D NMR spectroscopy and X-ray crystallography (Figure 1C) confirm the helical conformation adopted by HBS compounds (Liu et al., 2008; Patgiri et al., 2012; Wang et al., 2006b).

HBS helices have been shown to inhibit protein-protein interactions (PPIs) where α -helical segments from one partner contribute significantly to binding (Henchey et al., 2010a; Henchey et al., 2010b; Wang et al., 2005; Wang et al., 2008). Recently, we described disruption of PPIs involved in cancer-related pathways using these compounds. An HBS mimic of the protein Sos was found to inhibit the Ras-Sos complex formation, Ras activation and Erk phosphorylation (Patgiri et al., 2011). Fluorescence microscopy results indicate that the HBS Sos helix is more cell permeable than its linear peptide analog (Figure 2A). An HBS mimic of hypoxia inducible factor-1 α , Hif-1 α , was shown to block the Hif-1 α -p300 interaction in vitro and in cell based assays, while slowing the progression of tumor growth in mouse xenograft models ((Kushal et al., 2013) Figure 2B).

In our efforts to target PPIs, we have synthesized an ever-increasing variety of HBS helices. Here we present an optimized protocol for the synthesis of HBS α -helix mimetics.

STRATEGIC PLANNING

Rational design of protein-protein interaction (PPI) inhibitors centers on the role of protein secondary structures at the protein interfaces (Jochim and Arora, 2009; Jochim and Arora, 2010). We begin helix mimetic design by analyzing and identifying protein complexes that feature helical segments at interfaces using the Protein Data Bank (PDB) for the sequence of interest, as well as computational determination of the energetic contribution of helices important for complex formation (Figure 3). A database that compiles this information from the PDB, HippDB, has been developed, providing a ready starting point for helix design (Bergey et al., 2013). HippDB provides computational alanine scanning and change in solvent-accessible surface area (SASA) values for every interfacial residue. Alanine mutagenesis scanning G value refers to the change in free energy when a residue is mutated to alanine, thus a positive value indicates that mutation to alanine decreases the affinity of PPI and wild type residue contributes to binding. For interfacial residues, the

SASA of a residue is calculated by subtracting the SASA of the residue in the PPI complex from the SASA of the individual residue without any partner protein chains, and a positive value indicates that the residue is buried in the PPI complex and less accessible to solvent. Rosetta(Kortemme and Baker, 2002; Kortemme et al., 2004) and PocketQuery(Koes and Camacho, 2012) offer easily accessible resources for such computational analyses. Once the key interfacial helical domain is identified in the complex of interest, a handful of HBS mimetics are synthesized using the protocol described below.

Basic Protocol 1. SYNTHESIS AND PURIFICATION OF HYDROGEN-BOND SURROGATE α -HELICES

Short α -helices stabilized by a hydrogen-bond surrogate (HBS) are synthesized using solid-phase peptide synthesis approaches. The key synthetic steps are: 1) incorporation of an *N*-allyl group within the backbone of a peptide; 2) a secondary amine coupling for the *N*-allyl peptide of varying difficulty depending on the adjoining residues; 3) a ring-closing metathesis (RCM) reaction to prepare the HBS macrocycle. We have developed three methods in order to obtain *N*-allyl-bearing peptide **2** from peptide **1** (Figure 4). The most general approach, Method **A**, involves a Tsuji-Trost *N*-allylation optimized for HBS (Patgiri

et al., 2010a). Method **B** utilizes an updated variation of Fukayama-Mitsunobu conditions towards the synthesis of the desired olefinic residue (Patgiri et al., 2010b). This approach is comparable to Method A, but is especially efficient for the allylation of alanine. Method **C** involves an efficient, short stepwise synthesis for sequences with glycine as the third amino acid residue (Patgiri et al., 2010b). For all methods, the coupling of Fmoc amino acid to the secondary amine **2** is generally accomplished with DIC/HOAt (*N,N'*-diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole) to obtain peptide **3**. However, for those amino acids found difficult to couple (for example, sterically hindered or β -residues), a triphosgene-mediated amino acid coupling can also be employed (Patgiri et al., 2010b). RCM is the final key step in our synthesis where an intramolecular $i \rightarrow i+4$ hydrogen bond is substituted with a covalent linkage (Figure 4, compound **5**) (Chapman et al., 2004; Patgiri et al., 2008). This is achieved through a microwave-assisted metathesis of two backbone incorporated olefins on the peptide chain using Hoyveda-Grubbs II catalyst (Chapman and Arora, 2006). The strategy outlined below describes the typical synthesis and purification for stabilized HBS α -helices highlighting the key methodologies and steps involved (Figures 4, 5, 6).

Materials

Reagents

Knorr Amide MBHA resin (solid support, capacity 0.4 mmol g⁻¹; Novabiochem)
9-Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids (Novabiochem)
N,N-dimethylformamide (DMF; Sigma-Aldrich)
Dichloromethane (DCM; Sigma-Aldrich)
1-Methyl-2-pyrrolidinone (NMP; Sigma-Aldrich)
Piperidine (Sigma-Aldrich)
2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; Novabiochem).
N,N'-diisopropylethylamine (DIEA; Sigma-Aldrich)
Ninhydrin (Sigma-Aldrich)
Phenol (Sigma-Aldrich)
Potassium cyanide (KCN; Sigma)
Pyridine (Sigma)
Chloranil (Fluka)
Acetaldehyde (Sigma-Aldrich)
2-Nitrobenzenesulfonyl chloride (*o*-NsCl; Aldrich)
2,4,6-Collidine (Sigma-Aldrich)
Triphenylphosphine (Fluka)

Tetrahydrofuran (THF; Sigma-Aldrich)
Tris(dibenzylideneacetone)dipalladium(0) [Pd₂(dba)₃; Aldrich]
Allyl methyl carbonate (Aldrich)
Sodium diethyldithiocarbamate trihydrate (Sigma-Aldrich)
1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU; Aldrich)
2-Mercaptoethanol (Aldrich)
Bis(trichloromethyl) carbonate (Triphosgene; Sigma-Aldrich)
Acetic anhydride (Sigma-Aldrich)
Bromoacetic acid (Aldrich)
N,N'-diisopropylcarbodiimide (DIC; Aldrich)
1-Hydroxy-7-azabenzotriazole (HOAt; Genscript)
Allylamine (Aldrich)
Methanol (Sigma-Aldrich)
Allyl alcohol (Aldrich)
Diisopropyl azodicarboxylate (DIAD; Sigma-Aldrich)
4-Pentenoic acid (Aldrich)
Hoveyda-Grubbs II catalyst (Aldrich)
Anhydrous 1,2-dichloroethane (DCE; Sigma-Aldrich)
Trifluoroacetic acid (TFA; Sigma-Aldrich)
Distilled water
Triisopropylsilane (TIPS; Sigma-Aldrich)
Diethyl ether (Sigma-Aldrich)
Acetonitrile (CAN; Fluka)

Equipment

Fritted solid-phase extraction (SPE) tubes
Vacuum membrane pump
Microwave reaction tubes with caps (10 mL; CEM)
Magnetic stir bars
Centrifuge tubes
HPLC vials
CEM Discover series microwave reactor with fiber-optic temperature probe and magnetic stirrer

Rotary evaporator

Centrifuge

Vacuum dessicator

Preparative and Analytical HPLC systems

Liquid chromatograph–mass spectrometer (LCMS; Agilent 1100 Series LCMSD)

Lyophilizer

CEM Liberty Series Microwave Peptide Synthesizer (CEM)

Nitrogen gas

Automatic peptide shaker

1. Design HBS peptide(s) as previously outlined in Strategic Planning.
2. Prepare peptide synthesis reagents and solutions (Coin et al., 2007).
 - a. For 0.1 mmole-scale synthesis of an HBS helix, weigh 250 mg of Knorr Amide MBHA resin (0.4 mmol g^{-1}) into a fritted SPE tube. Swell resin with 2.0 mL DMF for 15 mins.
 - b. Prepare resin-bound peptides for standard Fmoc solid-phase synthesis protocols. Fmoc removal is achieved by treatment with 20% (vol/vol) piperidine/NMP, twice, for 15 min each. Wash resin sequentially three times with DCM (5 mL) and DMF (5 mL). Each Fmoc amino acid (0.5 mmol) is pre-activated with HBTU (0.45 mmol) and 5% (vol/vol) DIEA/DMF and coupled for 2 h at 25 °C. Alternatively, synthesis of the peptide can be conducted using a CEM Liberty Series microwave peptide synthesizer.

For deprotection of the Fmoc group, it is critical to wash the resin beads thoroughly in order to remove any traces of piperidine before proceeding. The progress of the Fmoc deprotection and peptide synthesis can be monitored as described below using Kaiser and chloranil tests (Kaiser et al., 1970; Vojkovsky, 1995). This provides a qualitative assessment for primary or secondary amines.
 - c. The Kaiser test qualitatively detects primary amines based on the reaction of amines with ninhydrin. A positive test is observed with dark blue-purple color beads, and a negative test is observed with pale-yellow color beads. Preparations for the reagents includes 5 g ninhydrin in 10 mL ethanol (solution A), 80 g phenol in 20 mL ethanol (solution B), and 2 mL of 0.001 M KCN_(aq) in 98 mL pyridine (solution C). Typically, a few resin beads are added to small test tube, 1 drop of solution A, B, and C are added, mixed and heated to 100 °C for 5 min.

- d.** The chloranil test qualitatively detects secondary amines. A positive test is observed with dark blue to green beads, and a negative test is observed with pale-yellow beads. Typically, a few resin beads are added to a small test tube, 1 drop of solution D and E are added and allowed to stand at 25 °C for 5 min. Solution D consists of 20 mg chloranil in 100 mL DMF and solution E contains 2 mL of acetaldehyde in 98 mL DMF.
- e.** The purity and identity of peptide **1** should be confirmed before continuing: Treat a few beads of resin with the micro-cleavage cocktail consisting of 1.0 mL TFA/H₂O/TIPS: 95%/2.5%/2.5% (vol/vol/vol). After 1 h, filter the cleavage mixture, concentrate the filtrate using a rotary evaporator, and precipitate the peptide with cold diethyl ether. Redissolve the solid in 2:1 water:acetonitrile, and analyze the peptide by LCMS.
- 3.** Synthesis of the *N*-allylpeptide **3** can be accomplished using three methods. Method **A (step 4)** is a general approach suitable for any amino acid in the sequence (Figure 5). Method **B (step 5)** is an efficient and high-yielding approach for *N*-allyl alanine (Figure 6, 2, R₃ = CH₃). While Method **C (step 6)** can be employed for sequences with glycine and is generally high yielding (Figure 6, 2, R₃ = H).
- 4.** Method **A**; general approach towards synthesis of *N*-allylpeptide **3**.
- a.** Preparation of nosyl-protected peptide **6** (Figure 5). After peptide elongation and Fmoc deprotection, add 3.0 mL of dry DCM to SPE tube, and allow resin to swell for 5 min.
- b.** Add *o*-NsCl (66 mg, 0.3 mmol) and 2,4,6-collidine (66 µL, 0.5 mmol) to SPE tube with resin and shake for 2 h.
- c.** Filter and wash resin sequentially with DCM (5 mL), DMF (5 mL), MeOH (5 mL), DCM (5 mL), DMF (5 mL). Allow nosyl-protected peptide **6** to dry for at least 1 h by vacuum. Alternatively, the resin can also be dried overnight in a vacuum desiccator.
- Reaction progress can be monitored by the chloranil test. If necessary, a micro-cleavage of a few beads of resin can be performed and analyzed by LCMS.
- d.** Preparation of nosyl-protected *N*-allylpeptide **7** (Figure 5). Transfer dried peptide resin **6** to a 10 mL reaction tube equipped with a septum cap. Add triphenylphosphine (21 mg, 0.08 mmol), then Pd₂(dba)₃ (9 mg, 0.01 mmol). Purge the reaction tube with a continuous flow of argon gas for 20 min.
- e.** Add 3.0 mL of anhydrous THF via syringe and gently shake the reaction tube to dissolve reagents. Continue purging the reaction tube for 10 min.
- f.** Add allylmethylcarbonate (170 µL, 1.5 mmol) and gently agitate. Then allow the mixture to shake at room temperature for 3–6 h.

- g. Filter resin and wash it sequentially with DCM (5 mL), DMF (5 mL), 0.2 M sodium diethyldithiocarbamate trihydrate (5 mL $\times$ 3), DMF (5 mL $\times$ 3), and DCM (5 mL $\times$ 3).

Reaction progress can be monitored by micro-cleavage of a few beads of resin and LCMS analysis.

- h. Deprotection of nosyl group from **7** to obtain *N*-allylpeptide **2**. Add 2.0 mL anhydrous DMF to SPE tube containing dried **7** and swell for 5 min.
- i. Add DBU (75 μ L, 0.5 mmol) to the reaction mixture and then add 2-mercaptoethanol (70 μ L, 1.0 mmol). Shake resin in SPE tube for 30 min, then filter and wash with DMF (5 mL $\times$ 3).
- j. Repeat steps 4 (h and i) two additional times. Filter resin and wash sequentially with DCM (5 mL), DMF (5 mL), MeOH (5 mL), DCM (5 mL), DMF (5 mL).

Deprotection of 2-nitrobenzenesulfonyl group releases a yellow chromophore that can provide visual confirmation of the reaction progress. Progress can also be monitored by the chloranil test, which indicates the presence of secondary amines. If necessary, a micro-cleavage of a few beads of resin can be performed and analyzed by LCMS.

- k. Resin should be dried under vacuum for at least 1 h, then transferred to a microwave tube and purged with N₂ for 1 h. Alternatively, resin can be dried overnight. Once sufficiently dry, continue to step 7.

5. Method B; general synthesis of *N*-allylalanine peptide **2.**

- a. Synthesis of *N*-allylalanine peptide **2** (Figure 6, Method **B**). Prepare a solution of Fmoc-Ala-amino acid (156 mg, 0.5 mmol), HBTU (171 mg, 0.45 mmol) and 2 mL of 5% (vol/vol) DIEA/DMF in a vial and allow the mixture to stir for 15 min at room temperature.
- b. Add the above pre-mix solution to peptide resin **1** in an SPE tube and shake the reaction mixture at room temperature for 2 h.
- c. Filter the resin and wash sequentially with DMF (5 mL $\times$ 3), DCM (5 mL $\times$ 3), DMF (5 mL $\times$ 3).
- d. Deprotect the Fmoc group using 20% (vol/vol) piperidine/DMF, twice, for 15 min. Wash the resin sequentially with DCM (5 mL $\times$ 3) and DMF (5 mL $\times$ 3). Dry the peptide resin **9** in a vacuum dessicator for 2 h.

Reaction progress can be monitored by the Kaiser test. If necessary, a micro-cleavage of a few beads of resin can be performed and analyzed by LCMS.

- e. Nosyl-protection of the free amino alanine resin-bound peptide **9** can be accomplished by performing steps 4 (a–c) as previously described above.

- f.** Add PPh₃ (262 mg, 1.0 mmol) to dried nosyl-peptide resin **9** in SPE tube. Purge with N₂ for 1 h. Swell the resin with 2.0 mL of THF, then add allyl alcohol (68 µL, 1.0 mmol) to the reaction mixture, cap and take reaction tube to a cold room set at 4 °C. After allowing the solution to cool down (15 min), add DIAD (197 µL, 1.0 mmol) and shake at 4 °C for 15 min. Then remove and allow the reaction to shake at room temperature for 12 h.
- g.** Filter resin and wash sequentially with DMF (5 mL × 3), MeOH (5 mL × 3), DMF (5 mL × 3). Dry resin in a vacuum dessicator overnight.
- h.** Nosyl-deprotection of peptide resin **9** can be accomplished by performing steps 4 (h-k) as described above to obtain *N*-allylpeptide resin **2**.

Reaction progress can be monitored by the chloranil test, which indicates the presence of secondary amines. If necessary, a micro-cleavage of a few beads of resin can be performed and analyzed by LCMS.

- 6. Method C; general approach towards synthesis of the glycine derivative *N*-allylpeptide **2**.**
 - a.** Synthesis of *N*-allylglycine peptide **2** (Figure 6, Method C). Prepare a solution of bromoacetic acid (140 mg, 1.0 mmol), DIC (155 µL, 2.0 mmol) and HOBt (153 mg, 1.0 mmol) in 2.0 mL of DMF.
 - b.** Add the above pre-mix solution to peptide resin **1** in SPE tube and shake the reaction mixture at room temperature for 2 h.
 - c.** Filter the resin and wash sequentially with DMF (5 mL × 3), DCM (5 mL × 3), DMF (5 mL × 3).
 - d.** Swell resin in 2.0 mL DMF and add allylamine (150 µL, 2.0 mmol). Shake reaction mixture at room temperature for 20 min.
 - e.** Filter resin and wash sequentially with DMF (5 mL × 3), MeOH (5 mL × 3), DMF (5 mL × 3).

Reaction progress can be monitored by the chloranil test, which indicates the presence of secondary amines. If necessary, a micro-cleavage of a few beads of resin can be performed and analyzed by LCMS.

- f.** Resin should be dried under vacuum for at least 1 h, then transferred to a microwave tube and purged with N₂ for 1 h. Alternatively, resin can be dried overnight.
- 7. Synthesis of *N*-allylpeptide **3**.**
 - a.** Coupling of Fmoc amino acids to *N*-allylpeptide **2** to obtain peptide **3**. Dissolve the desired Fmoc amino acid (1.0 mmol), and HOAt (136 mg, 1.0 mmol) in DMF (3–5 mL), followed by addition of DIC (155 µL, 1.0

mmol) in a vial and allow the mixture to stir for 15 min at room temperature.

Observation of a white precipitate is indicative of diisopropylurea formation, an expected byproduct.

- b. After purging *N*-allylpeptide resin **2** with N₂ in a microwave tube for 1 h, add pre-mixed suspension from step 7a. Allow 5 min for resin to swell, then irradiate the reaction mixture using parameters, 200W power, 2 min ramp time, 45 min hold time, 60 °C on a CEM Discover microwave system. Alternatively, this reaction can be performed by shaking resin **2** with reagents from step 7a in an SPE tube overnight.
- c. Alternative triphosgene-mediated coupling of Fmoc amino acid to **2**. This step is usually employed if the above coupling conditions are ineffective, and/or if β-branched or sterically demanding amino acids are used.

In a microwave tube with a magnetic stir bar and cap, add desired Fmoc amino acid (1.0 mmol) and triphosgene (98 mg, 0.33 mmol). CAUTION: Triphosgene is very toxic. Use of a functional fume hood is required. Purge the reaction tube with nitrogen for 30 min. Add 2.2 mL of anhydrous THF to obtain a final triphosgene concentration of 0.15 M. Stir the reaction mixture for 5 min, and add 2,4,6-collidine (371 μL; 2.8 mmol). A thick yellow-white precipitate of pyridinium salt should be observed after a few minutes. Add the activated Fmoc amino acid to the dried resin, and irradiate using microwave parameters, 150W power, 2 min ramp time, 30 min hold time, 45 °C on a CEM Discover microwave system. Filter the resin with a fritted SPE tube. Wash resin using DCM (5 mL × 3) only. Repeat this triphosgene coupling procedure twice.

- d. Filter the resin with a fritted SPE tube and wash sequentially with DMF (5 mL × 3), DCM (5 mL × 3), and DMF (5 mL × 3).

Reaction progress can be monitored by the chloranil test, which indicates the presence or absence of secondary amines. If necessary, a micro-cleavage of a few beads of resin can be performed and analyzed by LCMS.

8. Synthesis of bis-olefin peptide **4** from peptide **3**.

- a. Standard Fmoc solid-phase peptide chemistry (step 2) is used to couple the next Fmoc amino acid, then 4-pentenoic acid (51 μL, 0.5 mmol).

Reaction progress can be monitored by the Kaiser test. If necessary, a micro-cleavage of a few beads of resin can be performed and analyzed by LCMS.

- b.** Filter the resin and wash sequentially using DCM (5 mL $\times$ 3), MeOH (5 mL $\times$ 3), DMF (5 mL $\times$ 3). Dry the bis-olefin peptide resin **4** in a vacuum dessicator overnight.

The bis-olefin peptide resin must be extensively dried before the start of the olefin metathesis step.

9. Synthesis of HBS α -helix **5 from bis-olefin peptide **4** via metathesis reaction.**

- a. Transfer dried bis-olefin resin **4** to a microwave tube equipped with a magnetic stir bar and cap. Weigh 12.5 mg of Hoveyda-Grubbs II catalyst (0.20 mol %). Add to microwave tube with bis-olefin **4**. Purge with nitrogen for 30 min.
- b. Add 2.0 mL of anhydrous 1,2-dichloroethane per 0.10 mol of resin, and stir the reaction mixture under nitrogen for 15 min.
- c. Irradiate the reaction mixture using the following microwave parameters, 150W power, 2 min ramp time, 15 min hold time, 120 °C on a CEM Discover microwave system.

Reaction progress can be monitored by micro-cleavage of a few beads of resin and LCMS analysis.

- d. Filter resin using a fritted SPE tube. Wash the resin sequentially with DMF (5 mL $\times$ 3), DCM (5 mL $\times$ 3), MeOH (5 mL $\times$ 3), DCM (5 mL $\times$ 3). Allow resin to dry under vacuum for at least 10 minutes before continuing on to step 10.

Dried resin containing **5** can be stored for years in a vacuum dessicator at RT.

10. Cleavage of HBS helix 5 from resin.

- a. Cleavage conditions using a TFA-based cocktail vary depending on the residues in the sequence. A general cocktail contains TFA/H₂O/TIPS with volume ratios of 95/2.5/2.5 respectively. Sequences that contain arginine or cysteine often require the presence of additional protective group scavengers. (Chan and White, 2000; Coin et al., 2007).
- b. Add 4.0 mL of the chosen cleavage cocktail to dried HBS helix resin **5** and stir gently for 2 h.
- c. Filter the cleavage mixture, wash resin with TFA (1.0 mL × 2) and concentrate the combined filtrate using a rotary evaporator.
- d. Carefully add 5.0 mL of cold diethyl ether to the cleavage mixture to precipitate the HBS peptide.
- e. Isolate the precipitate by centrifugation (5,000 g for 5 min). Decant the ether from the tube and repeat the ether wash two more times.

- f.** Dissolve the remaining solid in a mixture of 0.1% (vol/vol) TFA in water and acetonitrile, and lyophilize it.

Lyophilized HBS peptides stored at -80°C can last for years, though sequence may affect longevity.

11. Purification and characterization of HBS α -helix 5.

- a.** HPLC purification of peptides is performed using reversed-phase columns with H_2O and acetonitrile buffers containing 0.1 % TFA. It is important to determine how much peptide is needed before starting the purification process. A typical cleavage of 0.10 mmol resin will yield 100–200 mg of crude peptide, which is more than enough for initial experiments.
- b.** For semipreparative scale purification using a reversed-phase C_{18} column (250 mm $\times$ 9.4 mm, 5 μm), 10 mg of crude peptide can typically be loaded for a single run. After determining the amount to purify, dissolve peptide in acetonitrile/ H_2O (no more than 20 % acetonitrile) to about 2 mg per mL. Inject no more than 5 mL of this solution in to the HPLC system at a time. Peaks associated with compound elution can be monitored at UV detector wavelengths of 220 and 280 nm. Fractions can be collected manually as peaks elute or by using an automated fraction collector. Repeat as necessary.

For the first attempt at purification of a peptide, a typical HPLC run using a gradient of 5–95% acetonitrile in H_2O over 45 min (flow 5 mL min^{-1}). This gradient can be adjusted based on the hydrophilic/hydrophobic nature of the peptide and is typically optimized by trial and error for increased peak separation.

- c.** To assess purity of samples collected, take 20 μL of a fraction and dilute it with 10 μL H_2O . The sample can then be analyzed by LCMS using an analytical HPLC column (C_{18} , 150 mm $\times$ 3 mm, 2.7 μm). Depending on the sensitivity of the machine, an injection volume of 5–10 μL is often sufficient. Peaks associated with compound elution can be monitored at UV detector wavelengths of 220 and 280 nm. A single absorbance peak with a single compound mass indicates a high purity sample.

A typical LCMS run using an analytical column and a gradient of 5–95% acetonitrile in H_2O over 20 min (flow 0.5 mL min^{-1}) will provide acceptable peak separation. This gradient can be adjusted based on the hydrophilic/hydrophobic nature of the peptide and is typically optimized by trial and error for increased peak separation.

COMMENTARY

Background

Design of small molecule inhibitors for PPIs is often difficult (Arkin and Wells, 2004; Raj et al., 2013; Wells and McClendon, 2007). Traditional small molecules (~ 500 MW) are often unable to occupy the large surface area associated with PPIs, forcing researchers to change their approach in targeting these types of interactions. Over the last decade, there have been significant advances in the field of α -helix mimicry leading to potent inhibitors of helical interactions (Azzarito et al., 2013; Henchey et al., 2008; Mahon et al., 2012). These compounds can be classified into three types: 1) surface mimetics – non-peptidic compounds similar to traditional small molecule drugs but designed to display protein-like functionality similar to an α -helix; 2) stabilized peptides – peptides locked into an α -helical structure through strategically placed non-native linkages; 3) foldamers – non-peptidic oligomers that adopt conformations similar to α -helices. (Henchey et al., 2008; Raj et al., 2013)

Analysis of helical PPIs reveals that residues that contribute to binding may be located on a single face, two faces, or all three faces of an interfacial helix ((Bullock et al., 2011) Figure 7, top). A majority of helical interfaces in the HippDB dataset utilize residues on only a single face of the binding helix, allowing for small molecule helical scaffolds to access these interactions. More complicated PPIs involving multiple faces of a substrate helix would require a display of functionality that would be especially arduous for many small molecules to attain. The HBS scaffold is one of a limited number of scaffolds that can access all three faces of a substrate helix, thus maximizing the number of targets for a single scaffold.

Critical Parameters

Fine tuning HBS designs—After the initial design of HBS compounds through alanine scanning (as outlined in Strategic Planning), it is important to acknowledge where minimalistic changes to the peptide sequence may be beneficial. However, one is advised to use caution when making multiple mutations from the native sequence as this could ultimately affect both the binding affinity and specificity. Things to take into consideration:

1. Length of peptide

The desired length of a peptide sequence for an HBS compound is generally based on the positioning of hot spot residues. As many α -helices involved in PPIs contain only 2–4 helical turns, most of the HBS compounds made to date span 8–16 residues. Having hotspot residues within the HBS macrocycle does not appear to be detrimental toward binding efficacy, although this may not be true for every PPI (Henchey et al., 2010b).

2. Amino acid at the bridge

For synthetic ease, our lab often uses *N*-allyl alanine or glycine residues. *N*-allyl alanine is often preferred because it is a better helix-propagating residue. However, for peptides with a sufficient number of helix-promoting amino acids use of *N*-allyl glycine may be acceptable as it is synthetically easier to incorporate. If N-terminal

glycine or alanine is present in the native sequence, these would be ideal locations to install the *N*-allyl functionality as long as all important binding residues are included. Other *N*-allyl amino acids can be used, however, the synthetic efficiency of the secondary amine coupling can be drastically reduced.

3. Solubility of the peptide

Many HBS sequences have enough charged residues for acceptable solubility in aqueous buffer without the addition of solubilizing agents. However, for more hydrophobic peptides or those that exhibit a net charge of zero, addition of DMSO may be necessary for complete solubilization. In order to avoid using DMSO in buffers, design of peptides with additional charges without modification of important binding residues is preferred.

4. Presence of chromophore

In the absence of a chromophore in the native peptide sequence, we will often introduce a tyrosine or tryptophan residue for accurate concentration determination by UV absorbance. The chromophore can be added to the C-terminus of the sequence or by replacing a hydrophobic residue within the sequence not critical for binding.

5. Peptides for initial biological studies

For initial biological studies, our lab typically makes three compounds. One HBS compound based on the native sequence of the protein domain that is being mimicked, and which contains all of the important binding residues. The other two are negative control compounds: an HBS with important binding residues replaced with alanine and a linear peptide of the native sequence without the HBS constraint.

Troubleshooting

General peptide synthesis—Synthesis of peptide **1** (Figure 4; basic protocol, step 2) is the least challenging aspect in making an HBS compound. However, it is critical to confirm the presence and purity (> 90 %) of **1** before continuing on with more difficult steps. A microscale cleavage of resin should be performed and subjected to LC/MS analysis. If crude peptide **1** is <90 % pure, it is suggested to resynthesize **1**. Otherwise, significant problems may arise in subsequent steps.

N-allylation using Method A or B—Excellent allylation can usually be achieved by using either the Tsuji-Trost allylation (Method **A**) or our updated Fukuyama-Mitsunobu conditions (Method **B**). For some amino acids, specifically more bulky residues, the Tsuji-Trost reaction may be a better choice. However, the Fukuyama-Mitsunobu method involves less expensive reagents and provides comparable conversion, especially for alanine. If LC/MS reveals < 90 % conversion to product, dry the resin and repeat the chosen *N*-allylation step. If problems persist with both methods, it is suggested to restart synthesis and choose Method **C**, as the substitution of bromoacetic acid with allyl amine is quantitative.

Secondary amine coupling—Less sterically hindered amino acids typically achieve good conversion using 10 equivalents of Fmoc-AA-OH, HOAt, and DIC. If LC/MS reveals < 90 % conversion, dry the resin and repeat the coupling step. If the coupling to form peptide **3** (Figures 4 and 5) involves hindered amino acids, such as β -branched or ones that contain bulky side chain protecting groups, triphosgene-mediated coupling may be more efficient. However, if the coupling reaction fails to achieve acceptable conversion even when using triphosgene coupling, redesigning the sequence at this region of the peptide chain may be necessary to proceed.

Ring closing metathesis—The efficiency of the ring closing metathesis will depend on the purity of bis-olefin peptide **4** and the sequence used. If LC/MS reveals bis-olefin is < 70% pure, it may be difficult to isolate desired HBS compound from impurities. Sequence-dependent issues may arise, particularly for residues within the desired macrocycle. β -branched residues or ones that contain bulky side chain groups may hinder the ability to close the macrocycle, although acceptable ring closure can usually be achieved through multiple metathesis attempts.

Anticipated results

The overall yield for the synthesis of an HBS compound after purification can be up to 20%, although the yields can vary significantly depending on the sequence used and the efficiency of the more critical steps. We aim for > 95% purity after HPLC purification. The resin scale indicated in this protocol provides more than enough material for all initial structural and biological studies.

Time considerations

An experienced chemist, using an automated synthesizer for the initial peptide sequence, could make and purify a typical HBS compound in about 7–10 days.

For the average 10-mer peptide on Knorr amide resin, automated synthesis using a CEM liberty microwave peptide synthesizer will take < 8 h. Manual synthesis of the same length peptide will take about 2.5 days, using 2 h for each coupling of amino acid under standard conditions and an additional 2 $\times$ 20 min treatment with 20% piperidine for Fmoc removal.

Extended resin drying is necessary for the success of the *N*-allylation, secondary amine coupling, and metathesis steps. Drying times by vacuum and N₂ purging of 1 h each are often sufficient. However, the resin may be left drying overnight under vacuum.

Resin cleavage takes ~2 h per sample. After the TFA is removed, the crude peptide is precipitated in cold ether and subjected to HPLC purification. Purification times will vary depending on the amount of material needed for proposed experiments, the type of HPLC columns used, and the desired purity level. Many semi-preparative columns with an ID of 9.4 mm have a loading capacity of ~ 10 mg of crude peptide. If more material is needed, multiple runs of HPLC would be performed on the crude sample. Lyophilization times for HPLC fractions containing purified product can vary. Smaller volumes of < 2mL per fraction will usually dry in 12 h. Larger volumes of > 5 mL may take up to a day for complete drying. If increased peptide purity is required, a second round of purification using

the semi-pure material can be done using an analytical column. These columns have smaller ID (4.6 mm) and can only handle peptide loading of ~ 1 mg per run.

As most automated peptide synthesizers can make several sequences under 24h, it would be reasonable for an experienced chemist to make 2–3 compounds simultaneously with minimal increase in synthesis time, though an additional day or two may be required for the purification of multiple samples.

Acknowledgments

We thank the NIH (R01GM073943) for financial support. PFT thanks the NIH for a postdoctoral fellowship.

Literature cited

- Arkin MR, Wells JA. Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. *Nat Rev Drug Discov*. 2004; 3:301–317. [PubMed: 15060526]
- Azzarito V, Long K, Murphy NS, Wilson AJ. Inhibition of α -helix-mediated protein-protein interactions using designed molecules. *Nat Chem*. 2013; 5:161–173. [PubMed: 23422557]
- Bergey CM, Watkins AM, Arora PS. HippDB: a database of readily targeted helical protein-protein interactions. *Bioinformatics*. 2013; 29:2806–2807. [PubMed: 23958730]
- Bullock BN, Jochim AL, Arora PS. Assessing Helical Protein Interfaces for Inhibitor Design. *J Am Chem Soc*. 2011; 133:14220–14223. [PubMed: 21846146]
- Chan, WC.; White, PD. *Fmoc solid phase peptide synthesis : a practical approach*. Oxford University Press; New York: 2000.
- Chapman RN, Arora PS. Optimized synthesis of hydrogen-bond surrogate helices: surprising effects of microwave heating on the activity of Grubbs catalysts. *Org Lett*. 2006; 8:5825–5828. [PubMed: 17134282]
- Chapman RN, Dimartino G, Arora PS. A Highly Stable Short α -Helix Constrained by a Main-Chain Hydrogen-Bond Surrogate. *J Am Chem Soc*. 2004; 126:12252–12253. [PubMed: 15453743]
- Coin I, Beyermann M, Bienert M. Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences. *Nat Protoc*. 2007; 2:3247–3256. [PubMed: 18079725]
- Henchey LK, Jochim AL, Arora PS. Contemporary strategies for the stabilization of peptides in the alpha-helical conformation. *Curr Opin Chem Biol*. 2008; 12:692–697. [PubMed: 18793750]
- Henchey LK, Kushal S, Dubey R, Chapman RN, Olenyuk BZ, Arora PS. Inhibition of Hypoxia Inducible Factor 1–Transcription Coactivator Interaction by a Hydrogen Bond Surrogate Alpha-Helix. *J Am Chem Soc*. 2010a; 132:941–943. [PubMed: 20041650]
- Henchey LK, Porter JR, Ghosh I, Arora PS. High Specificity in Protein Recognition by Hydrogen-Bond-Surrogate alpha-Helices: Selective Inhibition of the p53/MDM2 Complex. *ChemBioChem*. 2010b; 11:2104–2107. [PubMed: 20821791]
- Jochim AL, Arora PS. Assessment of helical interfaces in protein-protein interactions. *Mol BioSyst*. 2009; 5:924–926. [PubMed: 19668855]
- Jochim AL, Arora PS. Systematic Analysis of Helical Protein Interfaces Reveals Targets for Synthetic Inhibitors. *ACS Chem Biol*. 2010; 5:919–923. [PubMed: 20712375]
- Jones S, Thornton JM. Protein-protein interactions: A review of protein dimer structures. *Prog Biophys Mol Biol*. 1995; 63:31–65. [PubMed: 7746868]
- Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal Biochem*. 1970; 34:595–598. [PubMed: 5443684]
- Koes DR, Camacho CJ. PocketQuery: protein-protein interaction inhibitor starting points from protein-protein interaction structure. *Nucleic Acids Res*. 2012; 40:W387–392. [PubMed: 22523085]
- Kortemme T, Baker D. A simple physical model for binding energy hot spots in protein-protein complexes. *Proc Natl Acad Sci USA*. 2002; 99:14116–14121. [PubMed: 12381794]

- Kortemme T, Kim DE, Baker D. Computational alanine scanning of protein-protein interfaces. *Science's STKE*. 2004; 2004:pl2.
- Kushal S, Lao BB, Henchey LK, Dubey R, Mesallati H, Traaseth NJ, Olenyuk BZ, Arora PS. Protein domain mimetics as in vivo modulators of hypoxia-inducible factor signaling. *Proc Natl Acad Sci USA*. 2013; 110:15602–15607. [PubMed: 24019500]
- Liu J, Wang D, Zheng Q, Lu M, Arora PS. Atomic Structure of a Short α -Helix Stabilized by a Main Chain Hydrogen-Bond Surrogate. *J Am Chem Soc*. 2008; 130:4334–4337. [PubMed: 18331030]
- Mahon, AB.; Miller, SE.; Joy, ST.; Arora, PS. Rational Design Strategies for Developing Synthetic Inhibitors of Helical Protein Interfaces Protein-Protein Interactions. Wendt, MD., editor. Vol. 8. Springer; Berlin Heidelberg: 2012. p. 197-230.
- Patgiri A, Jochim AL, Arora PS. A hydrogen bond surrogate approach for stabilization of short peptide sequences in alpha-helical conformation. *Acc Chem Res*. 2008; 41:1289–1300. [PubMed: 18630933]
- Patgiri A, Joy ST, Arora PS. Nucleation Effects in Peptide Foldamers. *J Am Chem Soc*. 2012; 134:11495–11502. [PubMed: 22715982]
- Patgiri A, Menzenski MZ, Mahon AB, Arora PS. Solid-phase synthesis of short α -helices stabilized by the hydrogen bond surrogate approach. *Nat Protoc*. 2010a; 5:1857–1865. [PubMed: 21030960]
- Patgiri A, Witten MR, Arora PS. Solid phase synthesis of hydrogen bond surrogate derived alpha-helices: resolving the case of a difficult amide coupling. *Org Biomol Chem*. 2010b; 8:1773–1776. [PubMed: 20449477]
- Patgiri A, Yadav KK, Arora PS, Bar-Sagi D. An orthosteric inhibitor of the Ras-Sos interaction. *Nat Chem Biol*. 2011; 7:585–587. [PubMed: 21765406]
- Raj M, Bullock BN, Arora PS. Plucking the high hanging fruit: A systematic approach for targeting protein-protein interactions. *Bioorg Med Chem*. 2013; 21:4051–4057. [PubMed: 23267671]
- Vojtkovsky T. Detection of Secondary-Amines on Solid-Phase. *Peptide Res*. 1995; 8:236–237. [PubMed: 8527877]
- Wang D, Chen K, Dimartino G, Arora PS. Nucleation and stability of hydrogen-bond surrogate-based alpha-helices. *Org Biomol Chem*. 2006a; 4:4074–4081. [PubMed: 17312961]
- Wang D, Chen K, Kulp JL III, Arora PS. Evaluation of biologically relevant short alpha-helices stabilized by a main-chain hydrogen-bond surrogate. *J Am Chem Soc*. 2006b; 128:9248–9256. [PubMed: 16834399]
- Wang D, Liao W, Arora PS. Enhanced Metabolic Stability and Protein-Binding Properties of Artificial α Helices Derived from a Hydrogen-Bond Surrogate: Application to Bcl-xL. *Angew Chem Int Ed*. 2005; 44:6525–6529.
- Wang D, Lu M, Arora PS. Inhibition of HIV-1 fusion by hydrogen-bond-surrogate- based alpha helices. *Angew Chem Int Ed*. 2008; 47:1879–1882.
- Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. *Nature*. 2007; 450:1001–1009. [PubMed: 18075579]

Figure 1.

The HBS strategy enforces helical conformation in short peptides. A) Comparison of i to $i+4$ hydrogen bond of an α -helix with carbon-carbon bond of HBS. The hydrogen bond surrogate is depicted in orange color. B) Circular dichroism spectra show improved helicity for HBS compounds over analogous linear peptides (Wang et al., 2006b). C) Crystal structure of an HBS helix (Liu et al., 2008).

Figure 2. HBS helices display activity in cellular and animal models. A) Cells incubated with fluorescein-labeled Sos HBS helix shows an intense intracellular fluorescence signal in comparison to cells treated with its linear counterpart; the Sos mimetic inhibits Ras activation and Erk phosphorylation (Patgiri et al., 2011). B) Hif-1 α HBS designed to target hypoxia inducible gene expression reduce tumor burden in mouse xenograft models (Kushal et al., 2013).

Figure 3.

High-resolution structures in the Protein Data Bank have been analyzed to identify helical protein–protein interactions which may be inhibited by HBS helices and other classes of helix mimics (Bergey et al., 2013; Bullock et al., 2011; Jochim and Arora, 2010). The flow chart describes the approach used to construct HippDB, a database of helical interfaces in protein-protein interactions.

Figure 4.

Overview of synthetic protocol for hydrogen-bond surrogate (HBS) α -helical peptides on solid phase. Methods **A**, **B** and **C** are described in detail in the text. R_1 - R_7 are amino acid side chain functionalities.

Figure 5.

Protocol for synthesis of HBS α -helices using Method A. R, R₁–R₇, any amino acid side chain.

Figure 6.

Protocols for synthesis of *N*-allylpeptide **2** using Method **B** (*R*₃ = CH₃) and Method **C** (*R*₃ = H); *R*, *R*₁–*R*₇, any amino acid side chain.

Figure 7.

Interfacial helices may utilize one, two or all three faces for molecular recognition (Bullock et al., 2011). Top: α -helical substrates binding to corresponding targets utilizing different numbers of helical faces to display binding residues (from left to right, PDB: 1XL3, 1XIU, 1OR7). Middle/Bottom: Number of helical faces associated with different types of helix mimetics.