Chemistry & Biology
Article

Synthesis and Screening of Stereochemically Diverse Combinatorial Libraries of Peptide Tertiary Amides

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http://dx.doi.org/10.1016/j.chembiol.2013.01.013

SUMMARY

Large combinatorial libraries of N-substituted peptides would be an attractive source of protein ligands, because these compounds are known to be conformationally constrained, whereas standard peptides or peptoids are conformationally mobile. Here, we report an efficient submonomer solid-phase synthetic route to these compounds and demonstrate that it can be used to create high quality libraries. A model screening experiment and analysis of the hits indicates that the rigidity afforded by the stereocenters is critical for high affinity binding.

INTRODUCTION

Natural products constructed entirely or partially of N-methyl peptide building blocks display many interesting activities. Some, such as cyclosporine, are in use clinically. N-methylation has the effect of both stabilizing the peptide backbone toward proteolytic degradation and, by removing highly hydrated N-H bonds, generally improving the cell permeability and bioavailability of these compounds. N-methylation can also strongly stabilize one or a few particular conformations of the backbone, particularly in the context of cyclic structures.

N-methylated peptides are a subfamily of peptide tertiary amides (PTAs) (Figure 1) in which the main-chain nitrogen is alkylated. Another PTA subclass that has garnered considerable attention is the peptoid scaffold (Simon et al., 1992) (Figure 1, R_1 = H). Peptoids are oligomers of N-alkylated glycines. Like N-methyl peptides, they are also stable to proteases and exhibit enhanced cell permeability relative to standard peptides (Kwon and Kodadek, 2007; Miller et al., 1994). In contrast to N-methyl peptides, there have been numerous reports of the construction of large synthetic combinatorial peptoid libraries that have been screened successfully for protein ligands (Kodadek, 2010; Kodadek et al., 2004; Sánchez-Pérez et al., 2003; Zuckermann and Kodadek, 2009). This is largely due to the ease of constructing peptoid libraries via the "submonomer" route (Figure 1B, R_1 = H) (Figliozzi et al., 1996; Uno et al., 1999; Zuckermann et al., 1992). In this scheme, an activated ester of 2-bromoacetic acid (or the chloro analog; Burkoth et al., 2003) is added to an amine-displaying resin. The halide is then displaced by a nucleophilic primary amine, providing a peptoid monomer. Because hundreds of suitable primary amines are available commercially or synthesized readily, large one-bead one-compound (OBOC) libraries of peptoids can be made easily using the split and pool strategy (Lam et al., 1991). These libraries can be screened on bead against labeled, soluble proteins (Alluri et al., 2003; Lim et al., 2007; Wrenn et al., 2007; Xiao et al., 2007) or against cells (Aina et al., 2005; Lee et al., 2010a; Udugamasooriya et al., 2008) to identify novel protein ligands. However, these "hits" do not generally display high affinity, with rare exceptions (Zuckermann et al., 1994). This may be due, in part, to the "floppiness" of peptoids. N-substitution and the lack of a chiral center in peptoids results in the loss of the preference for the *trans* amide bond conformation characteristic of simple peptides. Moreover, there is little preference for particular conformations around the carbonyl-Cα or Cα-N bonds. Assuming that the peptoid binds its target protein in a defined conformation, this means a significant entropic penalty must be paid to form a complex, limiting affinity. Peptoid floppiness may also render hit optimization difficult.

Therefore, we and others have been interested in creating peptoid (Chongsiriwatana et al., 2008; Gorske and Blackwell, 2006; Huang et al., 2006; Kwon and Kodadek, 2008; Lee et al., 2010b, 2011; Shah et al., 2008; Shin et al., 2007; Stringer et al., 2011), or peptoid-like (Aquino et al., 2012; Sarma et al., 2011), molecules with greater conformational constraints.

Previous studies of N-methylated peptides have indicated that they are far more constrained conformationally than either peptoids or simple peptides. For example, the CD spectrum of poly-N-methyl-L-alanine indicated a helical structure (Goodman and Fried, 1967), though X-ray crystallography of $(N\text{-Me-Ala})_6$ shows a more extended, β strand-like conformation (Zhang et al., 2006). A striking indication of the much greater conformational constraints in these molecules have been reports of conformational isomers of the same compound that can be separated by high-pressure liquid chromatography (HPLC) (Alfredson et al., 1994; Teixidó et al., 2005). This can be rationalized on the basis of nonbonded steric interactions. For example, as shown in Figure 2A, the *trans* amide bond geometry will be strongly favored in N-methylalanine in order to separate the two branched stereocenters, as is also true in simple peptides. Moreover, an allylic 1,3 ($A_{1,3}$) strain will act to restrict rotation about the carbonyl-Cα and the Cα-nitrogen bonds so as to keep the hydrogen atom at the chiral center in or near the plane of the substituted amide (Figure 2B). These effects are seen clearly in the crystal structure of $(N\text{-Me-Ala})_6$ (Zhang et al., 2006).

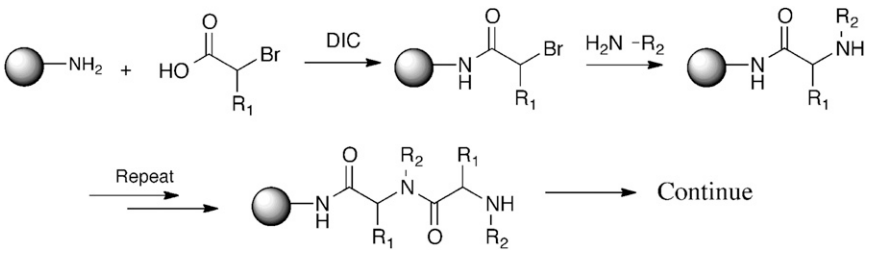

Figure 1. Synthesis of Peptide-like Oligomers
Solid-phase submonomer synthesis of peptoids (when R_1 = H). See also Figures S1 and S2.

Of course, the same arguments would apply to molecules containing other N-substituents besides methyl. Thus, it would be of great interest to construct combinatorial libraries of diverse N-alkylated peptides and investigate them as sources of bioactive molecules, but this has never been done. N-methylated, N-protected amino acids are available (Biron and Kessler, 2005), providing the building blocks required for the synthesis of OBOC libraries of N-methylated peptides by standard peptide bond couplings. But to apply a standard peptide synthesis approach to libraries with different N-substituents would entail the maintenance of a huge number of different N-substituted, Fmoc-protected amino acid building blocks. To address this important goal, we therefore considered a different solution: to create libraries of PTAs via submonomer synthesis in which chiral 2-bromo carboxylic acids are employed in place of 2-bromoacetic acid (Figure 1B, $R_1 \neq H$).

Here, we report a relatively general procedure for the creation of high-quality OBOC libraries of diverse N-substituted alanines and also demonstrate that the same chemistry can be applied to include residues other than methyl at the alpha carbon. Moreover, we demonstrate that these libraries are indeed a source of high-affinity ligands for a protein target. Characterization of one of these PTA-protein complexes provides strong evidence for the importance of conformational constraints in supporting high-affinity binding.

RESULTS AND DISCUSSION

Synthesis of Chiral Submonomers

To construct PTAs via a peptoid-like, submonomer synthetic route (Zuckermann et al., 1992), chiral 2-bromo carboxylic acids must be employed in place of 2-bromoacetic acid (Figure 1, R≠H). These building blocks can be prepared from α-amino acids by treatment with sodium nitrate in the presence of KBr and HBr (see Figure S1A available online) (Izumiya and Nagamatsu, 1952; Tanasova et al., 2009). This reaction proceeds with retention of stereochemistry, presumably because, after conversion of the amine to a diazonium group, a reactive three-membered lactone is formed that is then opened by bromide ion (Izumiya and Nagamatsu, 1952).

To make full use of both enantiomers of a given 2-bromo carboxylic acid in the construction and screening of PTA combinatorial libraries, a method to decode stereochemistry in each monomer is required. Because tandem mass spectrometry is the most convenient method to elucidate the structure of "hits" after screening one-bead one-compound (OBOC) combinatorial libraries (Astle et al., 2010; Paulick et al., 2006; Thakkar et al., 2009), isotope encoding of the absolute stereochemistry in PTAs is required. This meant accessing deuterated amino acids, which can be accomplished through transaminase-catalyzed hydrogen-deuterium exchange using D_2O as the donor. Following the published procedure (Oshima and Tamiya, 1961), more than 90% of the L-alanine was labeled with deuterium on both the α and β carbons. When the procedure was repeated three times, >98% isotopic purity was obtained (see Figures S1 and S2). This simple procedure can be performed on a large scale (>30 g) because L-alanine, D_2O, and transaminase are all inexpensive.

Isotopically labeled L-alanine was then converted to the bromide as described above. After distillation under reduced pressure, the desired acid displayed high stereochemical purity (>95% enantiomeric excess) as determined by HPLC of the corresponding (+)-camphorsultam amide (see Figure S1D).

Submonomer Synthesis of Optically Pure N-Substituted Alanines

The peptide bond-forming step in the submonomer synthesis of peptoids usually employs a carbodiimide, such as diisopropylcarbodiimide (DIC), to activate bromoacetic acid. Although the use of this reagent can be problematic in peptide synthesis because of racemization at the α-carbon, this was not anticipated to be an issue in PTA synthesis via the submonomer route because amino acid racemization involves formation of an intermediate oxazalone that cannot form in this class of molecules (Goodman and Levine, 1964; Yuan et al., 2002). To confirm this, R and S bromopropionic acid were condensed with a chiral amine ((S)-(-)-1-phenylethylamine) under these conditions. No evidence for racemization was observed in the nuclear magnetic resonance spectrum of the final products (see Figure S2).

To determine if oligomers prepared via the submonomer route indeed display the anticipated conformational constraints, we constructed N-methyl-(S)-alanine oligomers of various lengths on Rink amide resin. As shown in Figure 3, an increased circular dichroism signal was observed as the length of the oligomer increased from a dimer to a tetramer to a hexamer, suggesting the buildup of specific conformers as the length of the molecule increases. The CD spectrum of the hexamer shown in Figure 3 is essentially identical with the published spectrum of the same compound made by traditional amino acid couplings (Zhang et al., 2006). Thus, this chemistry provides a route to highly structured oligomers.

Synthesis and Characterization of a Combinatorial Library

With the appropriate conditions in hand, we proceeded to construct a combinatorial library of trimeric PTAs following an invariant linker. The synthesis was conducted on TentaGel beads, which is the preferred resin for subsequent screening (Alluri et al., 2003). 2-bromoacetic acid, (S)-2-bromopropanoic

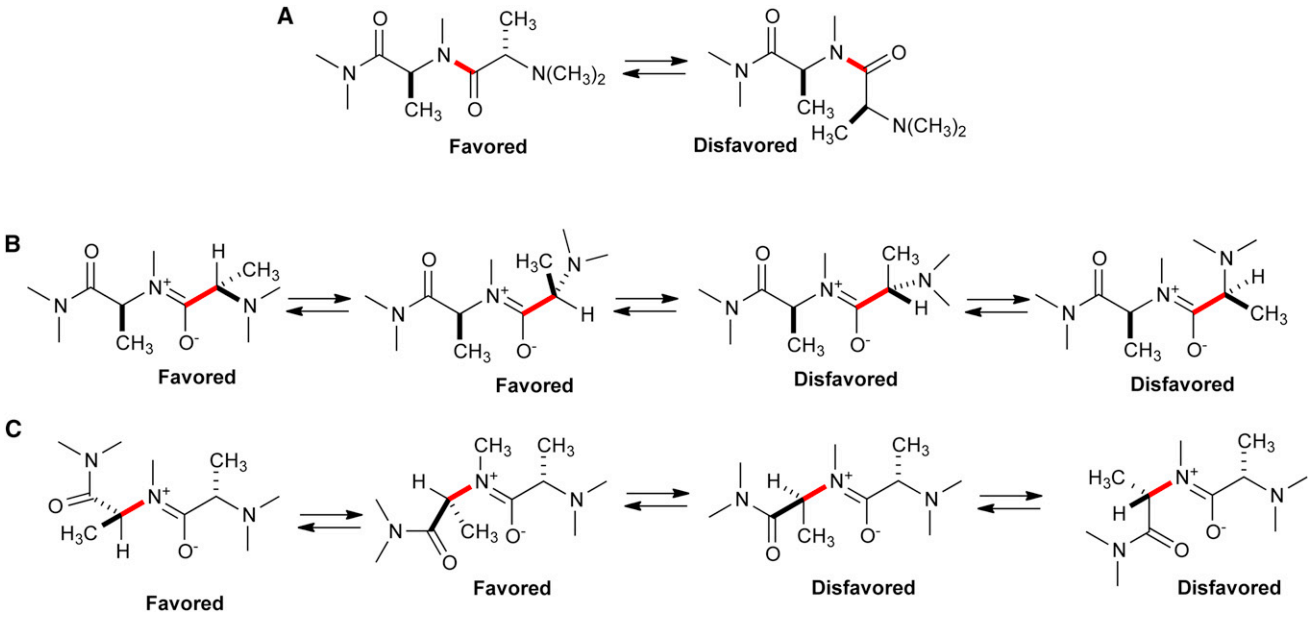

Figure 2. Conformational Analysis of Peptide Tertiary Amides Using a Simple Model Compound in which All of the Substituents Are Methyl Groups

(A–C) The bond whose conformation is considered is highlighted in red.

(A) Amide bond conformations.

(B) Carbonyl-Cα bond conformations.

(C) Cα-N bond conformations. The conformations indicated as favorable are seen in the crystal structure of hexa-N-methylalanine (Zhang et al., 2006).

acid-d_4, and (R)-2-bromopropanoic acid) were employed as submonomers along with 16 amines (see Figure 4). The theoretical diversity of the library was 110,592 compounds. The amines were chosen carefully such that any combination of amine and acid submonomers could be identified uniquely by tandem mass spectrometry upon gas-phase cleavage of the amide bond. Methionine was added to the beads first, using standard peptide coupling conditions (N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate [HBTU], Fmoc-Met-OH, DIEA) to facilitate selective release of the compounds from the beads after the screen by treatment with CNBr. Following the methionine, several invariant peptoid residues were added. The two Nlys residues facilitate display of the molecule in aqueous solution, whereas the alkyne and furan side chains facilitate subsequent labeling of the compound (Hintersteiner et al., 2009) and immobilization on a microarray (Astle et al., 2010) if desired. Moreover, the additional mass serves to move the molecular ion and important fragment peaks out of the range of the matrix peaks, which facilitates compound characterization.

Following linker synthesis, the library was split in three, treated with DIC and (R)-2-bromopropanoic acid, (S)-2-bromopropanoic acid-d_4, or bromoacetic acid, respectively. The beads were then mixed together and then split in 16 equal parts. One of the amines was added to each tube. The same procedures were then repeated two more times in order to form the trimer. The acylation step was monitored by the chloranil test, and the amine substitution was monitored by the silver acetate test (details are described in the Supplemental Experimental Procedures). In addition, individual beads were sampled at each step. After releasing the compound using CNBr, the progress of the reaction was checked by MALDI mass spectrometry. This monitoring is important to create a high-quality library. The acylation step in PTA synthesis, in particular, can be significantly slower than is the case for peptoid synthesis. Thus, although the conditions reported above for DIC-mediated coupling work well much of the time, this depends to some extent on the N-substituent. The blind use of a single set of reaction conditions without monitoring the reaction can result in a significant fraction of incomplete coupling.

In tubes in which the reaction had not proceeded to >90% completion using the standard procedure, a second round of coupling was done by adding a 2:1 mixture of the bromopropanoic acid and DIC (after a preactivation time of 10 min, final concentration of activated bromopropanoic acid at 1 M). Prolonged reaction time (24 hr) provided a satisfactory yield in these difficult reactions. With respect to assessing the progress of the reactions by mass spectrometry, it is noteworthy that the amide bond on the C-terminal side of the PTA fragments easily and is much more acid labile than in standard peptoids (see Figure S5). This results in the production of an abundant b ion on the MALDI mass spectrum even without high-energy tandem mass spectrometry fragmentation. The same effect has been noticed previously in the mass spectrometric analysis of N-methyl peptides. (Vaisar and Urban, 1998).

After the final step, the entire library was washed thoroughly, and 40 beads were picked randomly. The beads were treated with CNBr to release the compounds from the beads, which were then analyzed by tandem MALDI mass spectrometry. We successfully sequenced 37 out of 40 beads for quality control (see Figure S4 for some examples). All 16 of the amines and all

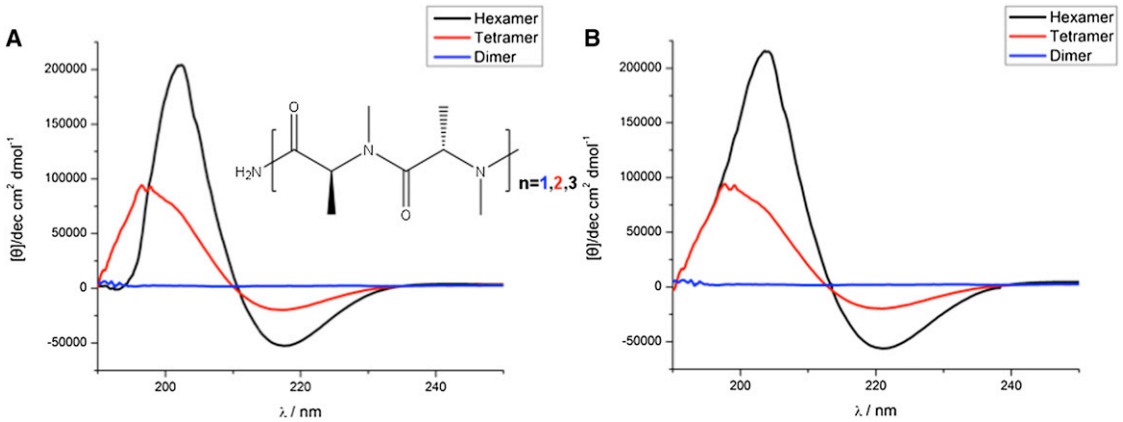

Figure 3. CD Spectrum of the N-Methyl-Alanine Dimer, Tetramer, and Hexamer Synthesized via the Submonomer Route

(A) Spectrum obtained in an aqueous solution (PBS buffer pH = 7).

(B) Spectrum obtained in a trifluoroethanol solution. All spectra were recorded at 0.3 μM compound, path length 10 mm, at 298°K.

See also Figure S3.

three of the acids were found in these spectra, suggesting that all of the submonomers had incorporated in this synthesis. However, we observed no trimers in which a methyl group was present at all three positions, and some dimers were also found. This is clear evidence that in the demanding library synthesis format, the reaction conditions employed resulted in some level of failure when attempting to string together three chiral centers in a row, despite all of our efforts. As will be described below, a modified coupling protocol has solved this problem. Nonetheless, because most compounds in the library could be sequenced, we decided to proceed with a screening experiment to probe the idea that the greater conformational constraints in the PTA relative to peptoids might result in higher affinity ligands.

Isolation and Characterization of Protein Ligands from the PTA Library

The library shown in Figure 4 was screened for ligands to a single-chain variable fragment (scFv) antibody called PX4-4, which was derived from a patient with the skin blistering disease Pemphigus vulgaris (Yamagami et al., 2010). Approximately 250,000 beads were incubated with 10 μM of purified scFv PX4-4 in the presence of 1 mg/ml *Escherichia coli* lysate as a diverse source of competitor proteins. To isolate beads that display ligands that captured significant amounts of the target antibody, magnetic beads displaying protein L were added to the mixture. Protein L will bind tightly to the scFv antibody through the kappa light chains. After a brief incubation, a powerful magnet was employed to segregate the beads that had also affixed to the magnetic protein L-displaying beads (Astle et al., 2010), and 33 beads were isolated as possible hits.

To confirm that these beads were true hits, they were stripped of protein by incubation in a trypsin solution at 37°C for 1 hr and then washed thoroughly. The beads were then incubated with 10 μM of purified scFv PX4-4 again for 30 min and washed gently. A 1:200 dilution of red quantum dots conjugated to protein L was then added. After 30 min of incubation, brightly glowing beads were picked manually using a low-power fluorescence microscope to visualize them. Seventeen of the hits picked up magnetically displayed a red glow (data not shown), consistent with their being PX4-4 ligands. These validated hits were released from the beads with CNBr and sequenced by tandem mass spectrometry.

The most promising hit from the bead screen (as judged by the intensity of the red halo after quantum dot addition), compound **1**, was resynthesized on Rink amide resin with a fluorescein tag, cleaved, and purified by HPLC. As shown in Figure 5A, this compound proved to have chiral centers at the first and third variable positions of the library (highlighted with ovals in Figure 5A), whereas there was no Cα substituent at the second position.

The affinity of compound **1** for PX4-4 was determined by fluorescence anisotropy. As shown in Figure 5B, PTA fluorescein-conjugated **1** exhibited a saturable binding curve when titrated with increasing concentrations of PX4-4, indicating a K_D of approximately 5 μM. In contrast, little binding was observed when fluorescein-tagged **1** was mixed with bulk IgG antibodies, indicating selectivity for PX4-4.

The peptoid analog of **1**, compound **2**, which lacks any chiral centers, was also made. As shown in Figure 5B, this compound had a much lower affinity for PX4-4. The large difference in affinity of PTA **1** and peptoid **2**, which differ only in the presence or absence of the methyl groups at the chiral centers, is striking. Although we cannot rule out the possibility that the Cα methyl groups in PTA **1** contact the scFv antibody directly, these data strongly support the idea that the conformational constraints afforded by the presence of the chiral centers strongly stabilizes binding of the small molecule to the antibody fragment.

To more thoroughly probe the influence of the stereochemistry at each position of PTA **1**, a small library was synthesized in which the amines were held invariant as found in **1**, but all possible combinations of the three acid submonomers were employed at each alpha position. In order to ensure the efficient synthesis of PTA trimers, we performed an extensive optimization of acylation conditions and conditions for amination. Of the eight coupling reagents examined, the triphosgene reagent bis(trichloromethyl) carbonate (BTC) developed by Jung and

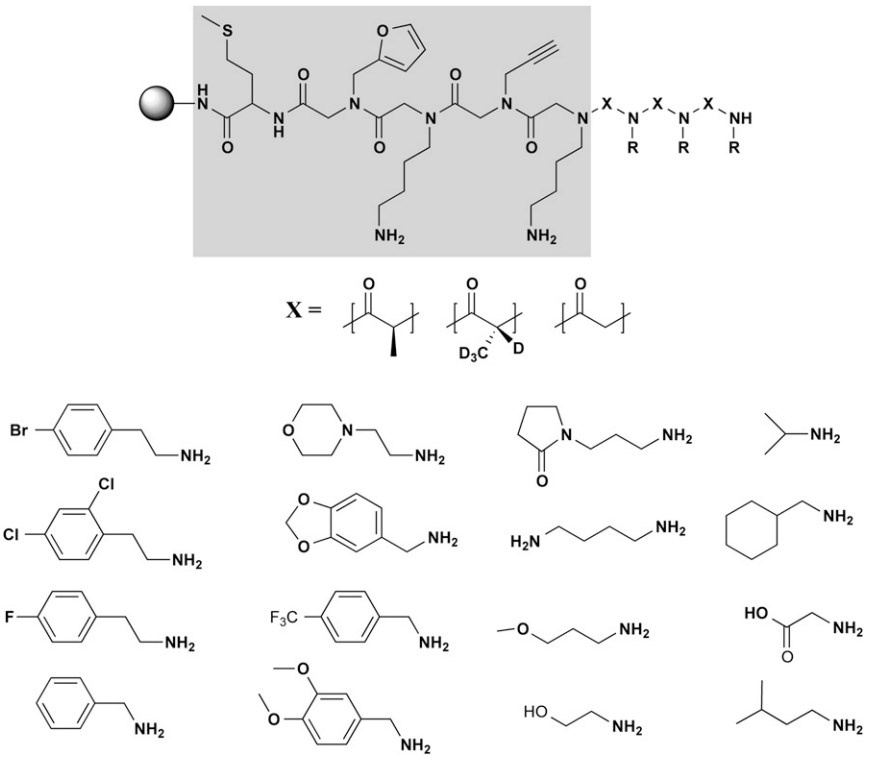

Figure 4. Structure of the Library Constructed: The Invariant Linker Is Boxed
The acid submonomers are indicated as "X". The amine submonomers employed are shown at the bottom of the figure. Protecting groups employed on the acids, alcohols, and diaminobutane are not shown. See also Figures S4 and S5 and Table S1.

colleagues (Thern et al., 2002) was found to be the best by far, providing superior yields with no racemization (see Figure S6).

Several beads were chosen randomly from the library, and the compounds were released and analyzed by mass spectrometry. Gratifyingly, in this case, several of the compounds were trimers with chiral centers at each position. Indeed, close to the expected ratio of compounds was observed (see Figure S6), demonstrating that the use of the BTC-based protocol is appropriate for the synthesis of high-quality PTA libraries.

Ninety-six beads were chosen randomly from the library and placed individually into the wells of a 96-well filter plate. Each well was treated with fluorescein azide and a copper catalyst to attach fluorescein to the alkyne handle (Hintersteiner et al., 2009), and then the compounds were cleaved from beads. The beads were filtered out, and the soluble compounds were used for the fluorescence polarization (FP) assay. All 27 possible structures were found in this collection of molecules by tandem MS sequencing. Semiquantitative binding constants for the PX4-4 antibody for all 27 of the compounds were determined by titrating the compounds with the protein in the microwell format and monitoring the increase in FP (see Table S2). The six highest affinity compounds, which included A48, identical to hit **1** in the variable region, were resynthesized and purified to allow more accurate binding data to be acquired. All six compounds showed an increase in FP when titrated with PX4-4 (Figure 6). Most of the curves approached saturation, allowing for an accurate determination of the K_D. In contrast, compound A41, corresponding to peptoid **2**, was not saturated in the protein concentration range tested (100 nM to 400 μM) (Figure 6).

These data confirmed that compound **1** is the highest affinity PX4-4 ligand of the six and that the chirality of the stereogenic centers can have a profound effect on binding affinity. It is interesting that the compounds with the S,S,R or S,R,R configurations (the same as compound **1** in the first and third positions but with a chiral center at position 2) were not high-affinity ligands for PX4-4 (see Table S2). This indicates that a stereocenter at the second position does not allow the molecule to access the bound conformation.

To address the selectivity of compounds A14, A7, A68, A41, and A24 for PX4-4, the FP experiment was repeated using a mixture of human IgG as we did previously for compound **1**. A14, A7, A68, and A24 showed no binding with whole-human IgG, whereas A41, the analog of peptoid **2**, had a similar affinity for these random antibodies as it did for PX4-4 (Figure 6). This implies that greater conformational flexibility is associated with binding promiscuity, which has been noted previously in studies of N-methylated peptides (Doedens et al., 2010; Fiacco and Roberts, 2008).

Beyond Oligo-N-Substituted Alanines

The synthetic route employed to access the chiral bromides works well with other amino acids besides alanine (Izumiya and Nagamatsu, 1952; Tanasova et al., 2009). Thus, we conducted a small, preliminary study of whether bulkier substituents at the α-carbons in **1** might increase the affinity of the PTA ligand for PX4-4. Four fluorescein-labeled compounds were synthesized in which a benzyl or an isobutyl group was substituted for the chiral methyl groups of **1**. The crude HPLCs of three of these compounds were quite clean, whereas the fourth had some level of impurities (see Figures S7 and S8). Their affinities for PX4-4 were measured by fluorescence polarization spectroscopy. As shown in Figure S7, none of the compounds bound PX4-4 as tightly as the parent compound. The affinities were reduced between 2- to 20-fold for the four compounds. Although improved PX4-4 ligands were not discovered in this small experiment, it shows that PTAs other than oligo-alanines can be accessed via this chemistry.

Conclusions

We report the synthesis of diverse libraries of peptide tertiary amides (PTAs) using the submonomer approach first developed for peptoid synthesis (Figliozzi et al., 1996; Zuckermann et al., 1992). This involves the use of chiral, 2-bromo acids as submonomers in place of 2-bromoacetic acid, several of which can be

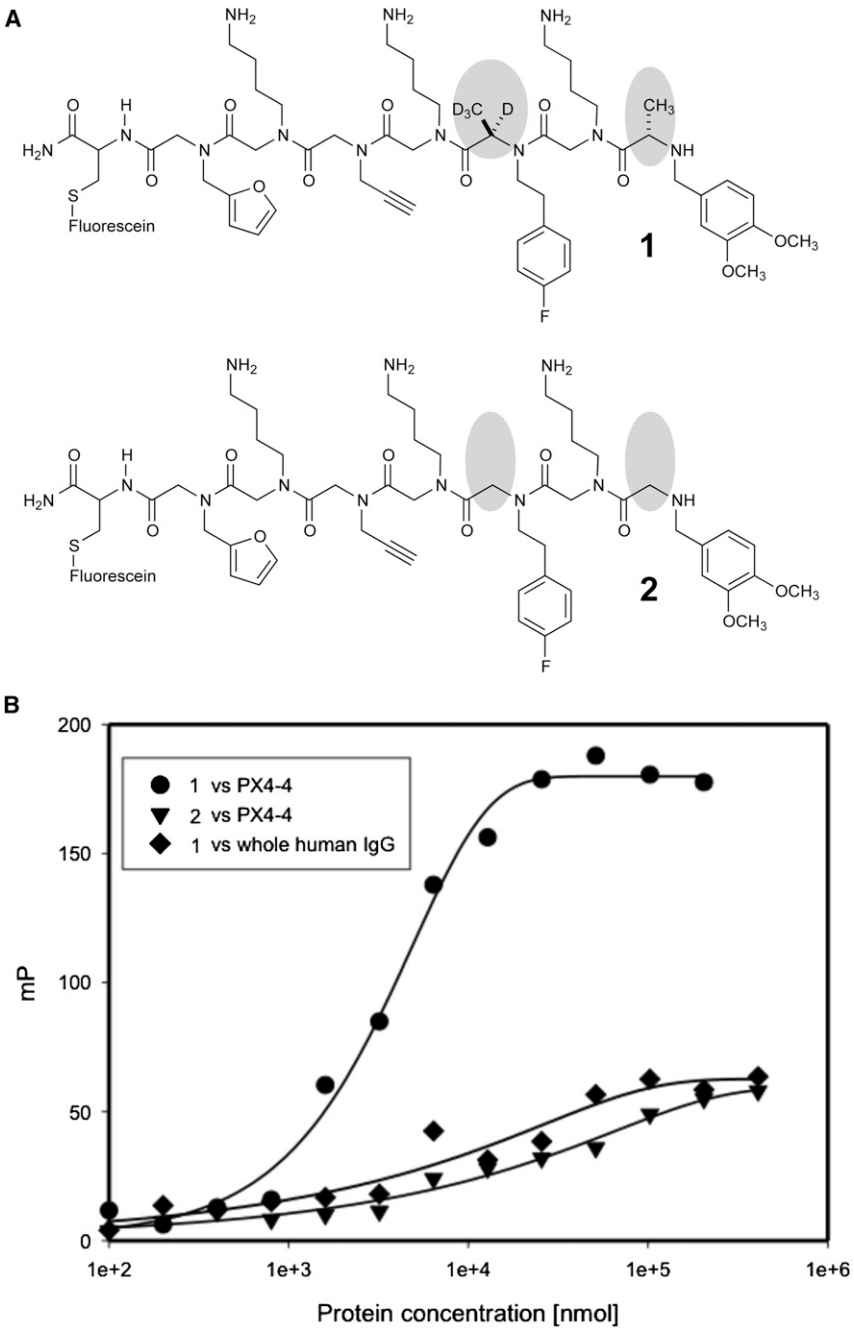

Figure 5. Structure and Characterization of a Ligand for the scFv PX4-4

(A) Structure of a fluorescein-labeled derivative of one of the screening hits, compound **1**, and the peptoid analog **2**, which lacks the methyl groups at the chiral centers. The positions of the chiral centers in **1** are highlighted in the silver ovals, as well as the analogous carbons in **2**.

(B) Fluorescence polarization assay employing the fluorescein-tagged compounds and the indicated proteins. PTA **1** binds the PX4-4 scFv with a K_D of approximately 3 μM but binds much less well to a collection of IgG antibodies, indicating selectivity for PX4-4. The peptoid **2** binds poorly to PX4-4. See also Figure S6.

derived easily from amino acids in high yield and optical purity (Izumiya and Nagamatsu, 1952). Moreover, at least in the case of the alanine-derived 2-bromoacid employed in this study, a simple transaminase-mediated deuteration protocol provides isotopically labeled material to allow the absolute stereochemistry at the chiral center of the PTA to be determined by mass spectrometry. This, in turn, allows both the R and S acids to be used as submonomer diversity elements at any given position in the chain. As anticipated from studies of N-methyl peptide synthesis, the peptide bond couplings are more difficult than is the case for simple peptoids due to greater steric crowding, but the use of highly potent carboxylate-activating agents, such as Jung's BTC reagent (Thern et al., 2002; Videnov et al., 1996), allows the synthesis of high-quality libraries by split and pool synthesis. In this initial report, we have mostly limited the substitution at the α-carbon to methyl (i.e., the synthesis of N-alkylated oligoalanines), but, as shown in Figures S7 and S8, other groups can be accommodated at this position.

The impetus behind the development of this chemistry was the need to create libraries of oligomers with greater conformational constraints than simple peptoids. Peptoid libraries are a useful source of protein ligands, but the primary hits from these libraries tend to be of modest affinity. This is likely due, in part, to the fact that peptoids are quite floppy molecules that must sacrifice a good deal of entropy in order to assume their bound conformation. Although various clever strategies have been reported to conformationally constrain peptoids (Yoo and Kirshenbaum, 2008), none of these have been applied to the synthesis of large combinatorial libraries, probably because of the extremely high efficiency demanded of the chemistry for this application (stepwise yields in excess of 95%).

N-methyl peptides are known to have significant conformational constraints (Chatterjee et al., 2009; Goodman and Fried, 1967; Zhang et al., 2006), resulting from both a strong preference for the trans amide bond geometry as well as potent allylic 1,3 strain effects that bias the conformations of the other two types of bonds in the molecule. Although chemistry exists to access Fmoc-protected, N-methyl amino acid monomers (Biron and Kessler, 2005), only small libraries of N-methyl peptides have been created by parallel synthesis (Ovadia et al., 2011). To the best of our knowledge, large libraries of these molecules have never been created synthetically and screened for protein ligands, though there has been considerable activity in the creation of such libraries using biological approaches (Kawakami et al.,

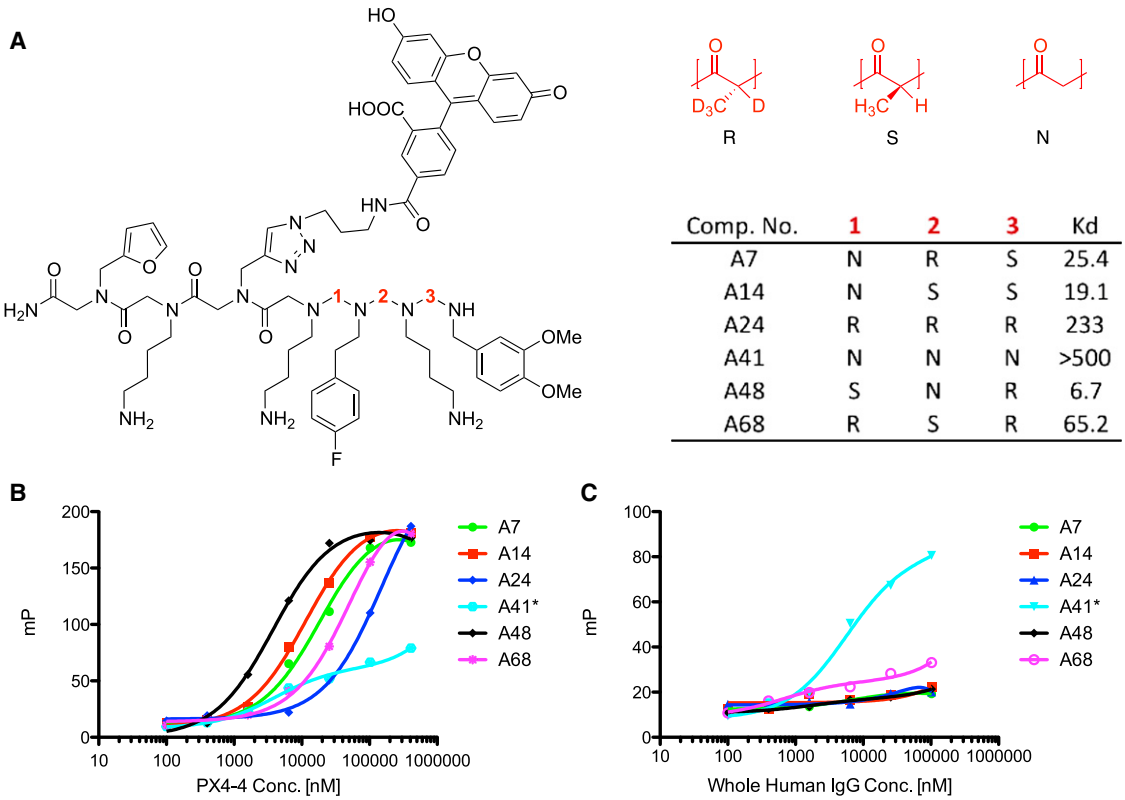

Figure 6. Binding Isotherms for A48 and the Five Other Highest Affinity Members of the Library in which the Stereochemistry at Each Alpha-Carbon of 3 Was Altered: Compound A48 Is Compound 3 with a Fluorescein Label

(A) General structure of the tested compounds and the K_D determined by FP assay after resynthesis as shown in (B). The K_D values are given in micrometers.

(B) Fluorescent polarization assay of a titration of fluorescein-labeled compound with PX4-4.

(C) Titration of fluorescein-labeled compound with a collection of human IgG antibodies (C). Note the different scales of the y axes in (B) and (C). The binding of the peptoid A41 to PX4-4 and whole-human IgG is similar.

See also Table S2 and Figures S7 and S8.

2008; Subtelny et al., 2008). Thus, the PTA library described here is good example of a large, chemically synthesized, and conformationally constrained library of N-alkylated peptides.

To determine if this type of library would indeed be superior to a collection of simple peptoids, we created a library with three variable positions and screened it against an autoantibody of interest. Sixteen amines and three bromoacids (R- and S-2-bromopropionic acid and bromoacetic acid) were employed as submonomers. Thus, the library contained 3,375 peptoids (16^3) among the 110,592 PTAs, yet no simple peptoids were isolated as hits from the screen. All of the hits had at least one chiral center. Moreover, characterization of the best hit, compound **1**, demonstrated that it evinced a high affinity for the target antibody (K_D = 5 μM), whereas the peptoid analog, **2**, had a much lower affinity for the autoantibody and also demonstrated more promiscuous binding behavior. Finally, an analysis of all possible stereoisomers of hit **1** revealed that the compound isolated was indeed the highest affinity ligand among this groups and that the vast majority of stereoisomers evinced much lower affinity for the target. Only two other compounds bound to PX4-4 with an affinity within 10-fold of A48. Although it is impossible to understand exactly what this means in the absence of a structure of the PTA-antibody complexes, it may be that compounds A14 and A7 (Table S2) can place the side chains in something close to the three-dimensional arrangement achieved by A48 in its bound conformation, despite their different backbone stereochemistry. Efforts to obtain crystal structures are ongoing.

In conclusion, this work makes accessible libraries of conformationally constrained N-alkylated peptide oligomers that promise to be exceptional ligands for biological targets of interest.

SIGNIFICANCE

Peptide tertiary amides (PTAs; also called N-alkylated peptides) have long been known to be conformationally constrained. This suggests that libraries of these compounds might be a superior source of protein ligands than the corresponding libraries of relatively "floppy" peptoids or peptides. However, chemistry to create high-quality PTA libraries was lacking. This report demonstrates that large one-bead one-compound libraries of N-alkylated alanines can be constructed by a peptoid-like submonomer synthesis using either enantiomer of 2-bromopropionic acid as a building block. A model screening experiment suggests

that these molecules will indeed provide higher affinity binding to protein targets than unconstrained peptoids.

EXPERIMENTAL PROCEDURES

Synthesis of Chiral Bromoacid

D-alanine (8.9 g, 0.1 mol) and KBr (11.9 g, 0.1 mol) was dissolved in 100 ml, of a 30% HBr water solution and kept in −15°C dry ice/ethylene glycol bath. $NaNO_2$ (10.35 g, 0.15 mmol) were dissolved in 15 ml water and slowly dripped in the above solution under argon atmosphere. The reaction was allowed to proceed for 3 hr and to warm from −15°C to room temperature. It was then put under vacuum for 30 min. Product was extracted by diethyl ether (25 ml × 3). Organic phases were combined and dried over Na_2SO_4. Then the solvent was evaporated under vacuum, and the crude product was further purified by distillation at 115°C, high vacuum. The pure product was obtained as a colorless oil in 82% yield.

Isotopic Labeling of Alanine

L-alanine (300 mg, 3.36 mmol) was dissolved in 10 ml of D_2O, α-ketoglutarate (10 mg, 0.068 mmol) was then added as a cosubstrate, and the whole system was then warmed to 37°C, and the pD was adjusted to 8.5∼8.7 with NaOD. Alanine transaminase (0.1 mg, EC 2.6.1.2 from pig heart, Roche Diagnostics, Indianapolis, IN, USA) was added and incubated at 37°C overnight with mild shaking. Ninety percent of D_2O was recovered by distillation, and L-alanine-d_4 was obtained by lyophilization.

Library Synthesis with DIC

Tentagel beads (1 g, 160 μm, ∼500,000 beads, 0.52 mmol/g, cat# HL 12 162, Rapp-Polymere, Tuebingen, Germany) were swelled in dimethylformamide (DMF) for 2 hr before use. DMF was used as the solvent unless otherwise mentioned. Fmoc-Met-OH (0.77 g, 2.08 mmol) was coupled on the beads using HBTU (0.77 g, 2.08 mmol) and N,N-Diisopropylethylamine (DIPEA) (0.45 ml, 2.6 mmol) for 3 hr. Fmoc was deprotected by 20% piperidine for 30 min. Beads were washed thoroughly with DMF after each step. The beads were split in three portions after deprotection. In a conical tube, 2 ml 1 M DIC solution and 2 ml 1 M corresponding bromoacid (2-Bromoacetic acid, (S)-2-bromopropanoic acid-d4 or (R)-2-bromopropanoic acid) solution were added together for preactivation for 5 min. The 4 ml combined solution was then added to one portion of beads and gently shook till completion. The reaction was monitored by chloranil test, a clear negative result after 5 min indicates the amine was acylated by the corresponding bromoacid. The beads were then thoroughly washed and pooled together, then split in 16 portions. Each portion was incubated with one of the amines listed in Table S1. Amine was used as 2 M DMF solution with an incubation time of 12 hr at 50°C. Silver acetate test and chloranil test were used to monitor the completion of the reaction. When reaction was complete, the beads were washed and pooled together. The acylation and amination steps were then repeated two more times for forming the trimer library.

Peptoid Linker Synthesis with DIC

The peptoid linker was synthesized by standard peptoid synthesis methods using microwave condition and 2 M solutions of DIC, bromoacetic acid, and the corresponding amines in DMF.

Library Synthesis with BTC

Tentagel beads with Rink linker (1 g, ∼100,000 beads, 0.27 mmol/g, cat. # MB 250 230, Rapp-Polymere) were used in order to avoid long exposure to acid during cleavage. Beads were first treated by 20% piperidine for 30 min in DMF in order to remove Fmoc and then the linker part was synthesized as described above. The beads were then split in three equal portions, coupled with 2-Bromoacetic acid, (S)-2-bromopropanoic acid-d_4, and (R)-2-bromopropanoic acid, respectively. For bromoacetic acid, 2 M solution of DIC and 2 M solution of bromoacetic acid were used as described above. For bromopropanoic acids, BTC was used as a coupling reagent. BTC (92.1 mg, 0.31 mmol) was dissolved in 5 ml anhydrous tetrahydrofuran (THF) in a glass vial. Bromopropionic acid (89 μl, 0.95 mmol) was then added to the vial, and the whole vial was kept in a −20°C freezer for 15 min. Beads were washed using DCM, DMF, and then THF, respectively for 5 times each, and then 2:1 THF/DIPEA (750 μl THF, 375 μl DIPEA, 2.2 mmol) was added to the beads, followed by gentle shaking. 2,4,6-Trimethylpyridine (356 μl, 2.7 mmol) was added to the cold solution of bromopropionic acid with BTC, and white precipitation formed following the addition. The white suspension was then applied to the beads, and the reaction vessel was put on a shaker for 2 hr at room temperature. The solution in the vessel should be a pale yellowish suspension during the whole course of the reaction. A darker color is an indication of excessive heat released during the initial addition of the acid chloride solution. It can be solved by further cooling of the acid chloride solution and the beads. The beads were washed with DCM five times when the reaction was done and then with DMF for five times. A chloranil test was used to monitor the completion of the reaction. All three portions of beads were then pooled together, and the beads were incubated with 2 M solution of the corresponding amine in DMF at 60°C overnight. The completion of the reaction was monitored by chloranil and silver acetate test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and two tables, and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.01.013.

ACKNOWLEDGMENTS

This work supported by a contract from the National Institutes of Health Heart, Lung, and Blood Institute (NO1-HV-00242). We thank Prof. John Stanley (University of Pennsylvania) for providing the expression vector for PX4-4.

Received: August 31, 2012
Revised: December 16, 2012
Accepted: January 4, 2013
Published: March 21, 2013

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