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Aza-amino acid scanning of chromobox homolog 7 (CBX7) ligands

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An aza-amino acid scan of peptide inhibitors of the chromobox homolog 7 (CBX7) was performed to study the conformational requirements for affinity to the methyllysine reader protein. Twelve azapeptide analogues were prepared using three different approaches employing respectively *N*-(Fmoc)aza-amino acid chlorides and submonomer azapeptide synthesis to install systematically aza-residues at the first four residues of the peptide, as well as to provide aza-lysine residues possessing saturated and unsaturated side chains. The aza-peptide ligands were evaluated in a chromobox homolog 7 binding assay, providing useful insight into structural requirements for affinity. Copyright © 2017 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: aza-peptides; chromobox homolog 7 ligands; submonomer solid-phase synthesis; trimethyllysine peptides mimics

Introduction

Enzymes involved in protein post-translational modification are promising targets for therapeutic intervention. The chromobox homolog 7 (CBX7) is a member of the family of histone reader proteins and binds *N*-trimethyllysine-27 of histone-3 (H3K27me3) in a recognition event that silences tumor suppressors [1–3]. Several lines of evidence suggest targeting CBX7 for cancer therapy [3–5]. For example, overexpression of CBX7 in hematopoietic stem cells drives proliferation and genesis of T-cell leukemia [5]. On the other hand, diminished expression of CBX7 induces a senescent phenotype with reduced cell proliferation in prostate cancer cell lines [1].

A series of short *N*-trimethyllysine containing peptides were recently reported that inhibited formation of the CBX7-H3K27me3 complex [6]. For example, pentapeptide **1** (Figure 1) inhibited the association of CBX7 and FITC-labeled H3K27me3 with IC_{50} 11 ± 0.4 μM and exhibited almost twofold selectivity for CBX7 over CBX4. Peptide **1** and analogues represent the first examples of inhibitors of any chromodomain [6] and form an initial set of research tools for studying therapeutic hypotheses related to chromodomain-containing proteins [7–12].

Employing **1** as lead peptide, we have performed an aza-amino acid scan in which each amino acid residue was replaced by its corresponding semicarbazide counterpart in order to gain insight into the conformational preferences of the parent peptide. A set of constrained aza-lysine analogues was specifically made to study the influence of side chain geometry and ε-amine methylation on affinity. Two strategies were employed to make aza-lysine analogues possessing saturated and unsaturated side chains. Alkylation of aza-glycine semicarbazone **12** (Scheme 1) with α,ω-dihaloalkanes gave the corresponding aza-ω-haloalkylglycine peptides that were reacted with various amines or sodium azide to give lysine residues with both saturated and double bond containing side chains [13–15]. Alternatively, copper-catalyzed Mannich reaction on aza-propargylglycine provided the corresponding lysine analogues possessing a triple bond in the side chain [16]. Finally, aza-phenylalanine and aza-leucine residues were introduced through the application of *N*-(Fmoc)aza-amino acid chlorides [17]. This combination of different azapeptide synthetic approaches has provided 12 analogues (**2**–**7**, Figure 1) that were subsequently evaluated in a competitive binding assay, which has demonstrated the importance of backbone conformation for receptor affinity.

Chemistry

Aza-amino amides are semicarbazides. On introduction into so-called azapeptides, the semicarbazide induces conformational constraint about the peptide backbone due to its planar urea and the repelling nitrogen lone pairs of the *N*,*N*′-diacylhydrazine component [18]. Substitution of aza-residues into peptides has been shown to induce turn conformation, enhance molecular recognition and prevent protease degradation [18–20].

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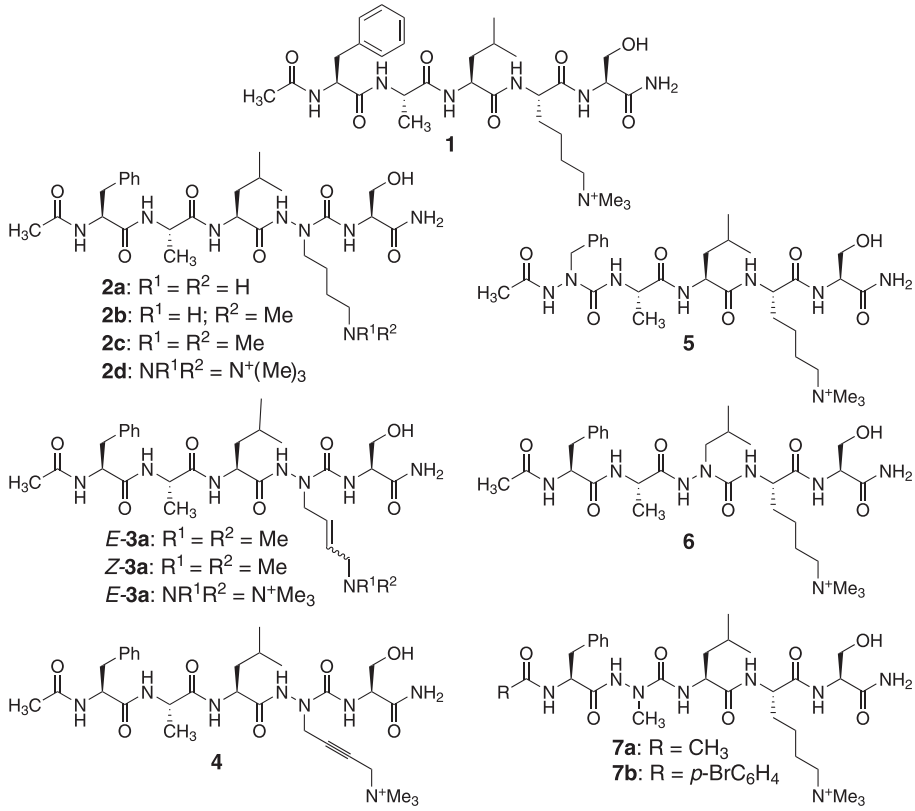

Figure 1. CBX7 ligand parent pentapeptide **1** and aza-anologues **2**–**7**.

To study peptide **1** with semicarbazides, a variety of methods were employed for the synthesis of azapeptides on solid-phase. Considering the importance of the geometry and amine substituents of the lysine residue side chain, a submomomer strategy for the solid-phase incorporation of aza-lysine residues was employed featuring alkylation of azaglycine semicarbazone **12** with 1-bromo-4-chlorobutane, as well as *E*- and *Z*-1,4-dichlorobutene to install, respectively, saturated and unsaturated ω-haloalkyl side chains that were subsequently displaced by various amines (Scheme 1) [13–15].

On Rink amide resin, supported benzhydrylidenyl-azaGly-Ser(OtBu)-amide **12** was synthesized by acylation of serine resin **11** with activated carbazate **10** generated from mixing benzophenone hydrazone (**8**) with *p*-nitrophenyl chloroformate (**9**, Scheme 1). Alkylation of resin **12** was, respectively, performed with 1-bromo-4-chlorobutane and 1,4-dichlorobut-2-ene at room temperature using tetraethylammonium hydroxide (300 mol % of a 35% aqueous solution) in tetrahydrofuran (THF) to provide the corresponding aza-chloroalkylglycine resins **13**, and *E*- and *Z*-**14**. Conversion was assessed to be complete by liquid chromatography–mass spectrometry examination of the residue obtained on treatment of resin aliquots with trifluoroacetic acid/water/triethylsilane [TFA/H_2O/TES (95:2.5:2.5)] and resin filtration. Subsequently, the resin **13** was treated with sodium azide in dimethylformamide (DMF) to displace the chloride and provide the corresponding azide **15**. Access to other degrees of ω-amine methylation was gained by displacement of chlorides **13**, and *E*- and *Z*-**14** with methylamine, dimethylamine and trimethylamine. Secondary ε-methylamine **17b** was protected using Boc_2O and diisopropylethylamine (DIEA) in DCM to give carbamate **18b**. The semicarbazone was removed using a 1.5 M solution of hydroxylamine hydrochloride in pyridine, and the resulting semicarbazides were coupled to Fmoc-Leu-OH by way of its symmetric anhydride, which was prepared using diisopropylcarbodiimide (DIC). The resulting azatripeptides were elongated using standard Fmoc-based solid-phase peptide synthesis protocols [21]. Acetylation of the *N*-terminal phenylalanine residue was performed with acetic anhydride and DIEA in DCM. Azide **19** was reduced chemoselectively using tris(2-carboxy)ethylphosphine (TCEP) in THF/H_2O (9:1) to provide lysine analogue **20** [13]. Resin cleavage was performed using a cocktail of TFA/H_2O/TES (95:2.5:2.5). Peptides **2a**–**d**, *E*- and *Z*-**3c**, and *E*-**3d** were, respectively, shown to be of 25–80% crude purity, purified on a preparative column (250 × 21.2 mm, 5 μm, Gemini™ C18) using gradients of 10–80% distilled water containing 0.1% formic acid in methanol (0.1% formic acid) at a flow rate of 10.0 ml/min, and isolated in 1–9% yields (see Supporting Information).

To introduce a triple bond into the aza-lysine side chain, a copper-catalyzed Mannich addition was performed on aza-propargylglycine **21** (Scheme 2) [16]. Propargylation was performed on semicarbazone **12** using tetrabutylammonium hydroxide and propargyl bromide to give aza-dipeptide **21** [22], which was treated with CuI (20 mol %), dimethylamine (600 mol %) and 37% aqueous formaldehyde (600 mol %) in dimethylsulfoxide at rt for 3 h, to furnish the azalysine dipeptide **22** [16]. Peptide elongation using solid-phase synthesis protocols provided azapeptide resin **23** [21], which was treated with methyl iodide (1 equiv) in DMF for 1 h to give the tetra-alkylamonium salt **24**. Azapeptide **4** was cleaved from the resin using a solution of TFA/H_2O/TES (95/2.5/2.5), characterized to be of 75% crude purity by analytical high performance liquid chromatography, and isolated as described above in 9% yield.

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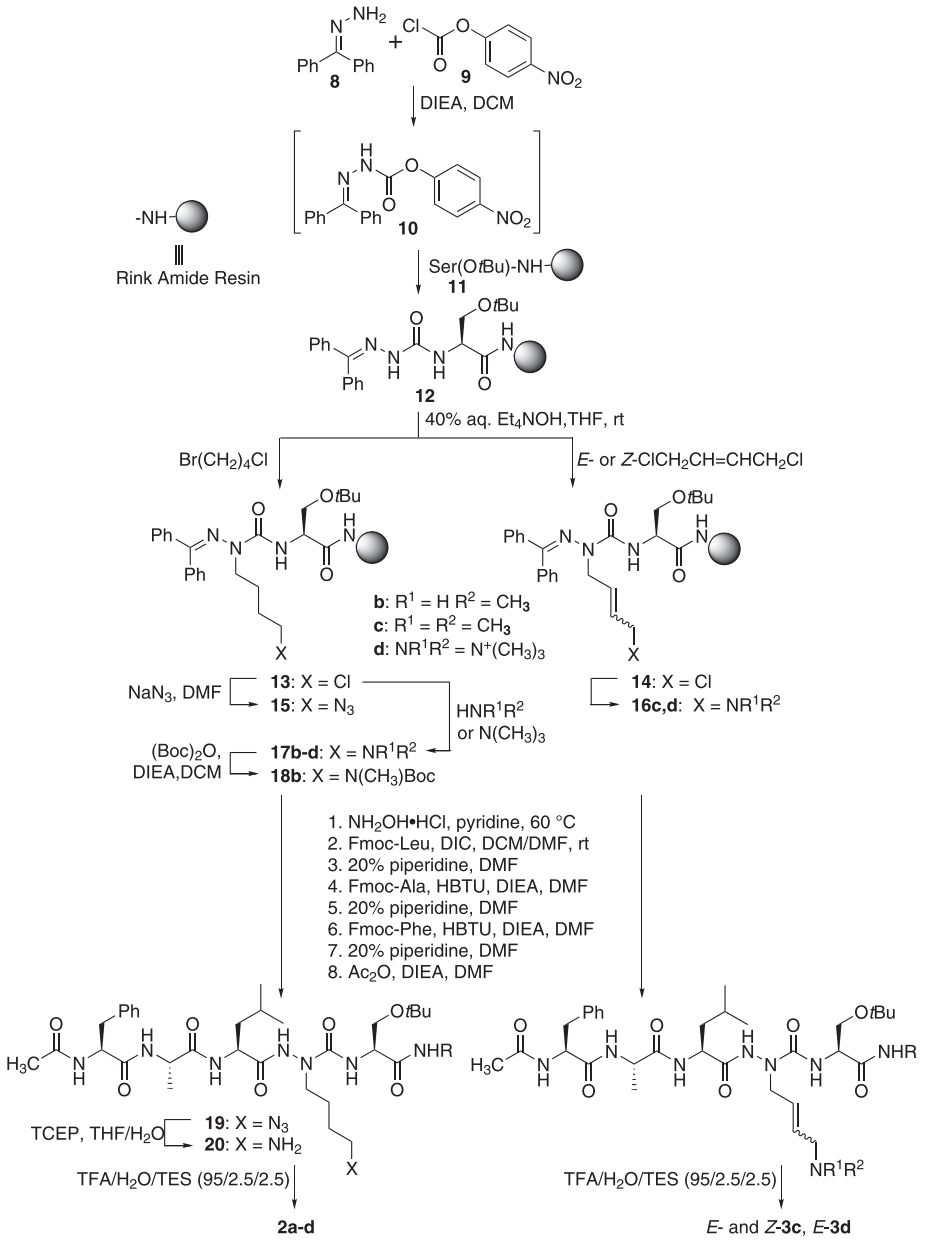
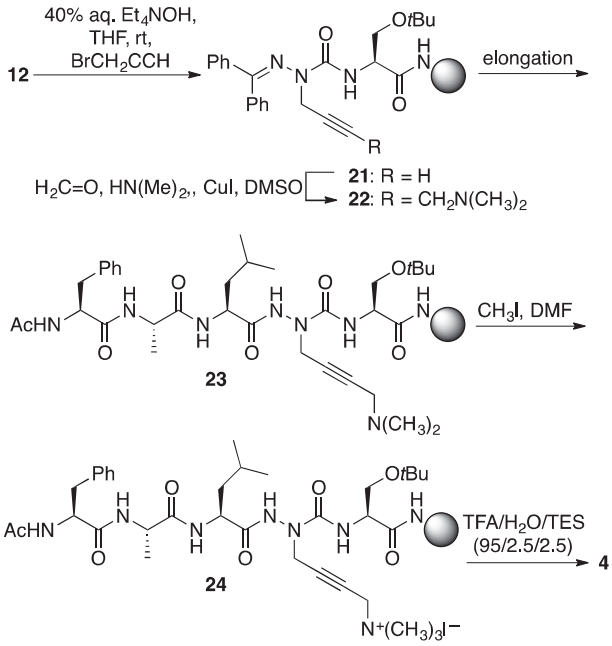


Scheme 1. Solid-phase synthesis of aza-Lys derivatives **2** and **3**.

The *N*-terminal portion of the peptide was scanned with aza-residues by employing *N*-(Fmoc)aza-amino acid chlorides to install the aza-phenylalanine [17], and aza-leucine residues, and by using a submonomer approach to add the aza-alanine residue (Schemes 3 and 4) [22].

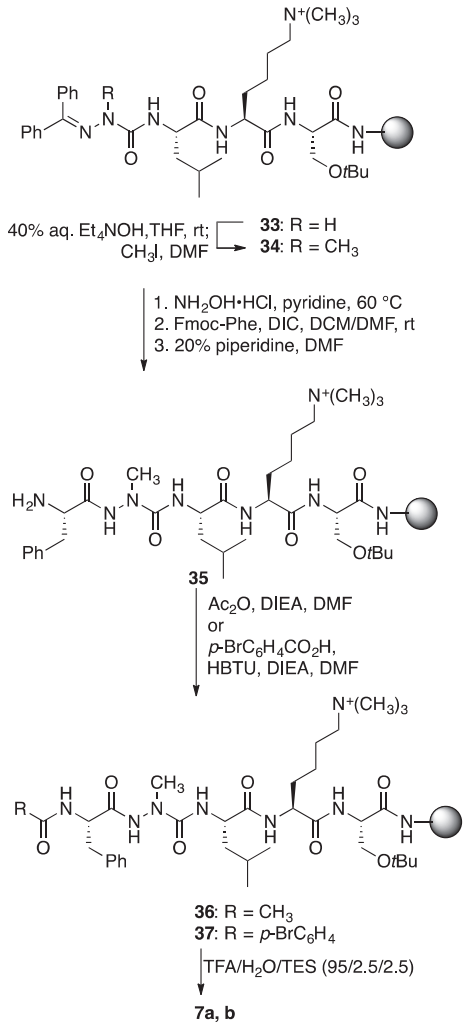
The aza-amino acid chlorides were, respectively, prepared from fluorenylmethylcarbazate **28** [17]. Reduction of the corresponding semicarbazones from condensation of **28** with *iso*-butanal and benzaldehyde using sodium cyanoborohydride gave *N'*-alkyl carbazates **29**, which were treated with phosgene in toluene to give the corresponding *N*-(Fmoc)aza-amino acid chlorides **30** after evaporation of the volatiles [17]. Aza-amino acid chlorides **30** were used without further purification, dissolved in dichloromethane (DCM) and added to resin-bound peptides **26** and **27**. The latter were made from resin **25**, which was obtained by coupling of Fmoc-Lys[*N*,*N*-$(CH_3)_2$] [23] to H-Ser(tBu)-resin, treatment with methyl iodide and removal of the Fmoc group to give corresponding trimethyllysine resin **26** that was elongated to give peptide **27**. Acylation of peptide **26** with Fmoc-aza-leucine amino acid chloride **30a** gave aza-tripeptide resin **31**. Similarly, treatment of the resin **27** with Fmoc-aza-phenylalanine amino acid chloride **30b** furnished aza-pentapeptide **32**. Azapeptide **5** was prepared from **31** by Fmoc group removals, acylation with the symmetric anhydride from treatment of Fmoc-Ala-OH with DIC, coupling with Fmoc-Phe-OH using HBTU, acetylation with acetic anhydide and DIEA, resin cleavage and purification as described for the synthesis of peptide **2** above. Similarly, azapeptide **6** was prepared, respectively, from **32** by Fmoc group removal, acetylation with acetic anhydide and DIEA, resin cleavage and purification.

The aza-alanine residue was introduced at the peptide 2-position by alkylation of aza-glycine semicarbazone **33** [22], which was prepared from Leu-Lys(N^+Me_3)-H-Ser(tBu)-resin using similar protocols as described for the synthesis of semicarbazone **12**



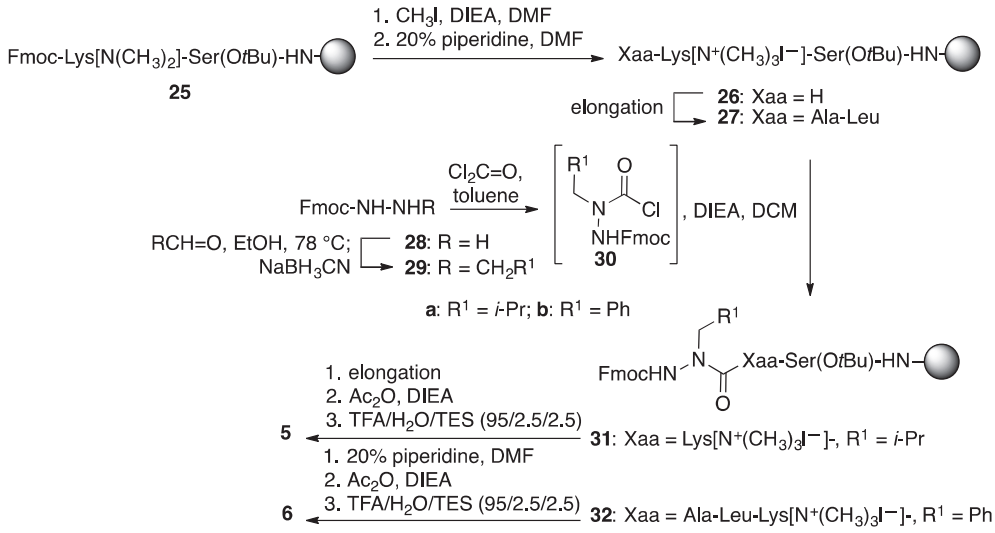
Scheme 2. Synthesis of azalysine **4** possessing a triple bond.

above. Exposure of **33** to tetraethylammonium hydroxide followed by iodomethane in THF provided aza-alanine resin **34** (Scheme 4). The semicarbazone was removed using $NH_2OH{\cdot}HCl$ in pyridine, and the resulting semicarbazide was coupled to Fmoc-phenylalanine using DIC as described above. After Fmoc removal using 20% piperidine in DMF, resin **35** was, respectively, acylated with acetic anhydride and DIEA in DMF to give acetamide **36**, and *p*-bromobenzoic acid using O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) and DIEA in DMF to provide the benzamide **37**. Resins **36** and **37** were, respectively, cleaved and the peptides purified by high performance liquid chromatography as described above to provide aza-alanine peptides **7a** and **7b**.



Scheme 4. Submonomer synthesis of aza-alanine peptide **7**.

Different methods were employed to synthesize azapeptides **2**–**7** to best introduce specific functional groups effectively. For example, submonomer solid-phase synthesis enabled access to aza-lysine peptides **2** and **3** possessing saturated and olefin aza-lysine side chains having diverse ω-amino substituents, because alkylation of a common aza-glycine intermediate with



Scheme 3. Solid phase synthesis of aza-Phe, and aza-Leu analogues **5** and **6.**

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α,ω-dihaloalkane and alkene residues installed effectively an ω-chloroalkyl side chain for diversification of the terminal amine by nucleophilic displacements. Although a triple bond may in principle be installed using a similar alkylation strategy, attempts to alkylate the aza-glycine residue with 1,4-dichlorobutyne were unsuccessful; instead, the copper-catalyzed Mannich reaction on an aza-propargylglycine residue proved an effective alternative for introducing the acetylene into the aza-lysine side chain in azapeptide **4**. Application of submonomer chemistry to install the aza-phenylalanine and aza-leucine residues in the presence of the trimethyl-lysine reside was however complicated likely by the presence of the tetra-alkyl ammonium residue, such that the application of *N*-(Fmoc)aza-amino acid chlorides was used to provide azapeptides **5** and **6** (Scheme 3) with better crude purity and isolated yield. On the other hand, alkylation of the aza-glycine residue with iodomethane in the presence of the trimethyl-lysine was successful and provided access to aza-alanine peptide **7**.

The 12 new azapeptide analogues of ligand **1** were evaluated for their affinity for CBX7 in a competitive fluorescence polarization assay that measured the displacement of a dye-labeled peptide ligand, as previously reported [6,7]. Most of the new analogues were not measurably active in the assay up to the limits of their most concentrated solutions (0.8–1.8 mM). At 1 mM, azapeptide **2d** showed 10% of the response of the positive control pentapeptide. Azapeptides **6** and **7a** showed 50 and 90% responses, respectively, at 1.5 mM, but their respective binding curves did not saturate and could not be fitted to provide IC_{50} values due to limits in their solubility. The potency of **7a** suggests that CBX7 is more tolerant of backbone conformational changes in the area of the leucine residue, which may be explained because unlike along the rest of the binding interface, the protein does not make a hydrogen bond to the peptide's backbone carbonyl at this position [6, 24].

Although the azapeptides maintain all side-chain structural components present in parent ligand **1** (IC_{50} 11 μM), a loss of affinity was generally seen across the series. Considering the backbone conformational preferences of azapeptides [18], as well as the flatter nature of the semicarbazide residue [25], such constraints on peptide **1** were not tolerated in the protein binding site, likely because they disturb the preferred β-strand conformation [24].

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.

Table S1. Azapeptide yields and purities.