COMMUNICATION

Peptide-Bismuth Bicycles: *In Situ* Access to Stable Constrained Peptides with Superior Bioactivity

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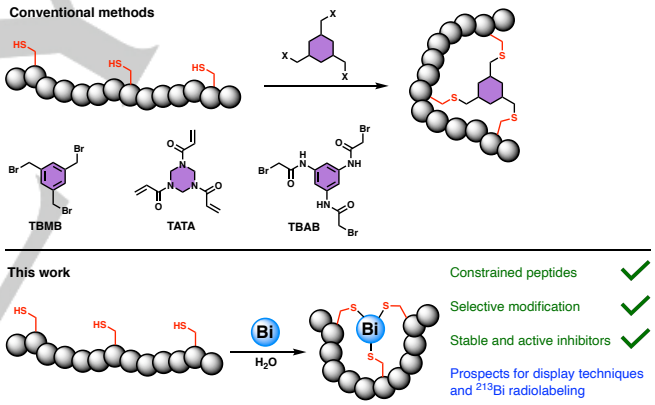
Abstract: Constrained peptides are promising next-generation therapeutics. We report here a fundamentally new strategy for the facile generation of bicyclic peptides using linear precursor peptides with three cysteine residues and non-toxic trivalent bismuth(III) salt. Peptide-bismuth bicycles form instantaneously at physiological pH, are stable in aqueous solution for many weeks, and much more resistant to proteolysis than their linear precursors. The strategy allows the *in-situ* generation of bicyclic ligands for biochemical screening assays. We demonstrate this for two screening campaigns targeting the proteases from Zika and West Nile viruses, revealing a new lead compound that displayed inhibition constants of 23 and 150 nM, respectively. Bicyclic peptides are up to 130 times more active and 19 times more proteolytically stable than their linear analogs without bismuth.

Constrained peptides play an essential role in the discovery of next-generation therapeutics.[1] Their unique properties place them right between small molecules and large antibodies. Bicyclic peptides are an emerging class of constrained peptides that offer even greater conformational rigidity, metabolic stability, and antibody-like affinity and specificity.[2] The modification of three cysteine residues in a peptide allows for bicycle formation even from genetically encoded peptide libraries using alkylating scaffolds such as TBMB, TATA or TBAB (Scheme 1).[3]

Conventional scaffolds like TBMB bind irreversibly and can hence modify other reactive peptide residues, even if only used in slight excess.[4] Nonspecific alkylation and cross-linking of cysteine or lysine residues in proteins may also occur and has, for example, been observed in phage display.[3a] Alternative, reversible linkers that form disulfide bonds between peptide cysteines and scaffolds offer better selectivity but suffer from redox sensitivity.[4] Although scaffolds like TATA and TBAB were specifically developed to stabilize peptide conformations by promoting hydrogen bond networks,[3b] all conventional scaffolds have flexible bonds that ultimately compromise the full potential for rigidification of peptide bicycles.

Here, we present a fundamentally new chemical approach that addresses previous limitations. We introduce bismuth(III) as selective, stable, rigid and green center of bicyclic peptides. With a covalent radius of 1.5 Å,[5] Bi(III) presents the smallest and most rigid 'scaffold' for cysteine modification ever explored, maximizing conformational constraint of bicyclic peptides (Scheme 1). In contrast to related pnictogens like arsenic or antimony and thiophilic transition metals (e.g., mercury, cadmium, lead), bismuth is remarkably harmless and green,[6] as evidenced by the over-the-counter drug Pepto-Bismol (bismuth subsalicylate).[7] A variety of other bismuth-based drugs have been explored and the radioisotope ^{213}Bi is an important α-emitter used in targeted alpha-particle therapy (TAT).[8]



Scheme 1. Conventional methods for the generation of bicyclic peptides are based on cysteine alkylation by reagents with threefold rotational symmetry. The use of trivalent bismuth, presented in this work, offers several advantages. TBMB, 3,5-triacryloyl-1,3,5-triazinane; TATA, 1,3,5-triacryloyl-1,3,5-triazinane; TBAB, *N*,*N*′,*N*″-(benzene-1,3,5-triyl)-tris(2-bromoacetamide).

Our strategy was inspired by the known ability of bismuth(III) to bind to cysteine residues in glutathione, peptides and proteins.[9] Three cysteine residues in a peptide sequence, which are each several residues apart, may form a bicyclic peptide in presence of bismuth(III). In order to assess this initial hypothesis, we designed and synthesized various peptides of the general sequence CX_nCX_nC, with X_n being 3–8 natural and unnatural amino acid residues (**1a**-**18a**, Table 1). To ensure that the approach is biocompatible, experiments were conducted in aqueous solution at physiological pH. Because Bi(III) forms insoluble oxido/hydroxido species in water, we used a highly concentrated stock of $BiBr_3$ in DMSO which was added directly to the aqueous solution containing the peptide at pH 7.5. An excess

of the thiol-free reducing agent tris(2-carboxyethyl)-phosphine (TCEP) was used to ensure full reduction of all cysteine residues.

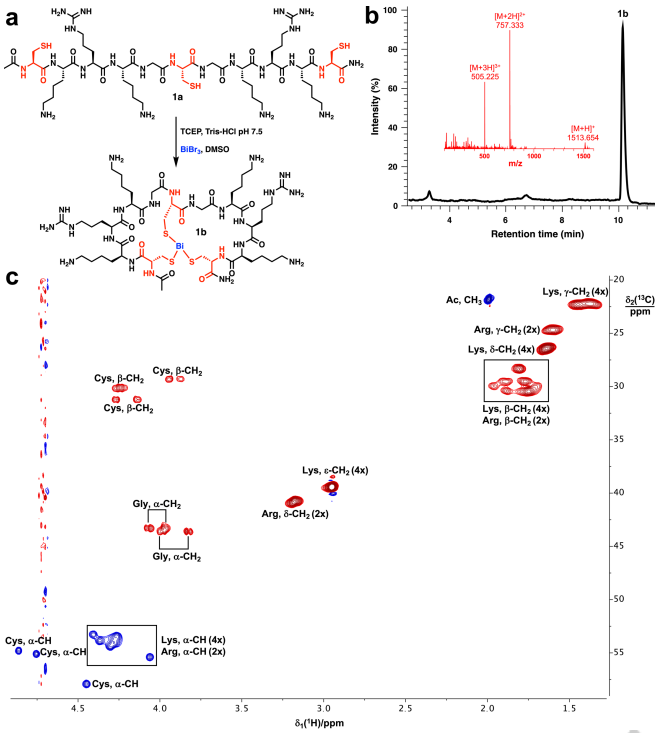


Figure 1. Formation of bicyclic peptide **1b** in aqueous solution in presence of $BiBr_3$. a) 1 mM **1a**, 10 mM Tris-HCl pH 7.5, 10 mM TCEP, 1.2 mM $BiBr_3$ (from 60 mM DMSO stock). b) Crude LC-MS (mass trace) of **1b** after reaction. c) 600 MHz [^{13}C,^{1}H]-HSQC NMR spectrum of purified **1b** (10 mM) in H_2O/D_2O (9:1).

We first evaluated the reaction of $BiBr_3$ with the cationic peptide **1a** (Figure 1a). Slight excess of $BiBr_3$ (1.2 equivalents) resulted in instantaneous and complete transformation of linear peptide **1a** into bicycle **1b**, as confirmed by high-resolution LC-MS (Figure 1b). Insoluble species, resulting from Bi(III) excess, were simply separated by centrifugation. We isolated **1b** using standard peptide purification (HPLC, water, acetonitrile, 0.1% formic acid), indicating superior complex stability even under acidic conditions. Complexes of bismuth with amino acids have previously been described as hydrolytically labile.[10] Owing to the chelate effect, however, bicycle **1b** remains stable in aqueous PBS pH 7.4 at room temperature over several weeks (Figure S1). Partial dissociation of Bi(III) from peptide **1b** was only observed in presence of the hexadentate chelating agent EDTA (Figure S6). Although, even a 2-fold excess of EDTA did not trigger complete Bi(III) dissociation, suggesting an equilibrium between Bi(EDTA) and **1b**. Our stability studies in PBS and competition experiments with EDTA suggested that the bicycle was sufficiently stable to be used in biological applications. Furthermore, LC-MS and NMR measurements of **1b** both indicated a single bicycle species without evidence for Bi(III) dissociation under ESI conditions (Figure 1). The HSQC spectrum of **1b** revealed low-field chemical shifts of the three β-CH_2 cysteine groups in ^{1}H dimension, indicating tight and selective bismuth binding of these residues (Figure 1c). Given the strong symmetry of the peptide sequence of **1b**, the very distinct chemical shifts observed for the diastereotopic cysteine β-CH_2 and glycine α-CH_2 groups suggested a distinct peptide backbone structure that is different from a random coil (Figure 1c).

We analyzed two additional peptides, **2a** and **3a**, for their bismuth-binding properties (Figure 2a). Peptide **2a** is more hydrophobic than **1a** while **3a** contains residues like histidine, aspartate and serine that may interfere with bismuth binding.[11] In presence of Bi(III), both peptides spontaneously form the expected bicycle in quantitative yield, as confirmed by LC-MS (Figure 2b). Like **1b**, both bicycles **2b** and **3b** were isolated by HPLC purification and were stable over several weeks in PBS pH 7.4 at room temperature (Figure S3). Despite the presence of other metal-binding residues, **3b** displayed a single species in LC-MS, indicating exclusive bismuth binding of the three cysteines without any interferences from adjacent histidine, aspartate, or serine residues (Figure 2b).

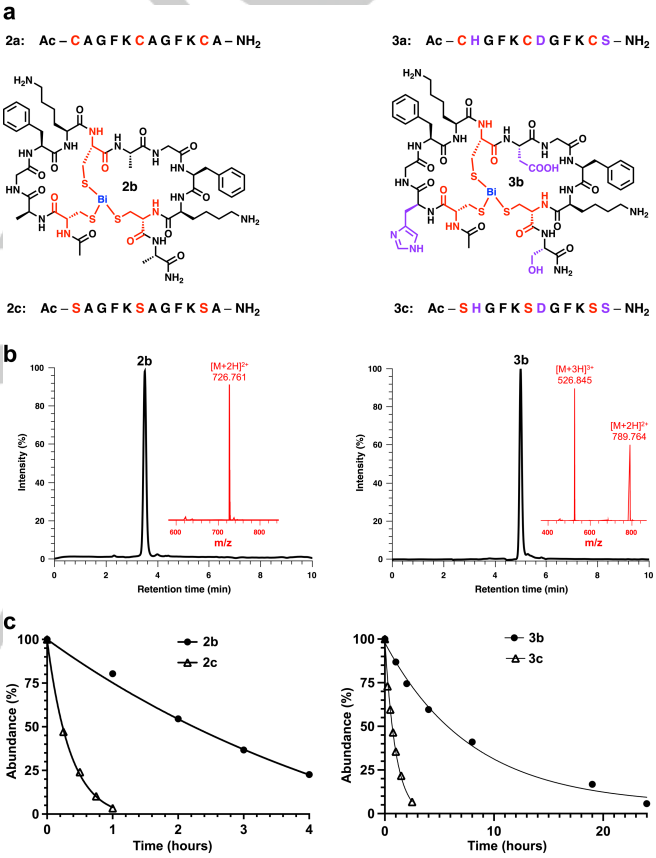


Figure 2. a) Sequences and structures of model peptides **2** and **3**. b) Chromatograms (254 nm) and mass spectra of purified bicycles **2b** and **3b**. c) Tryptic digest of **2b/2c** and **3b/3c**. Peptides (6 mM) were incubated with trypsin (0.13 nM) in 10 mM MOPS pH 7.9 at 37 °C.

Bicycles **2b** and **3b** deliberately contain a lysine residue in each cycle to analyze their digestion by trypsin, which was chosen as a suitable modelling system to assess proteolytic stability. Analogs **2c** and **3c**, in which the three cysteines are replaced by serines, were synthesized to enable a proper comparison of bicyclic and linear analogs over long incubation times. Both bicycles, **2b** and **3b**, show enhanced resistance to tryptic digest compared to their linear analogs **2c** and **3c** (Figure 2c). The half-life of linear **2c** in presence of trypsin is ~15 min, while the half-life of bicycle **2b** is ~2 h. Peptides **3a** and **3b** are less prone to

proteolysis with half-lives of ~45 min and ~6 h for linear peptide **3a** and bicycle **3b**, respectively. These experiments translate into an 8-times slower proteolytic cleavage of the bicycles compared to the linear peptides, clearly demonstrating that bismuth binding can generate constrained peptides that are much better guarded against proteolysis than their linear random-coil analogs.[12]

Table 1. Investigated peptides and their bicycles in presence of bismuth(III).

Cpd.[a]	Sequence[b]	Cpd.[c]	Yield (%)[d]
1a	Ac-CKRKGCGKRKC-NH_2	**1b**	89
2a	Ac-CAGFKCAGFKCA-NH_2	**2b**	98
3a	Ac-CHGFKCDGFKCS-NH_2	**3b**	99
4a	H-CKRKGCGKRKC-NH_2	**4b**	98
5a	H-CKRKGCKRKGC-NH_2	**5b**	97
6a	Ac-CSGSGCGKRKC-NH_2	**6b**	98
7a	Ac-CKRKGCGSGSC-NH_2	**7b**	98
8a	Bz-CSGSGCKOΨΦC-NH_2	**8b**	98
9a	Bz-CSGSGΘKORΦC-NH_2	**9b**	97
10a	Bz-CKORΦCGSGC-NH_2	**10b**	97
11a	Bz-CGSGCKKRC-NH_2	**11b**	98
12a	Bz-CGSGCQKYRC-NH_2	**12b**	76
13a	Bz-CGSGCLRRRRC-NH_2	**13b**	96
14a	Bz-CSIQLACNRKRC-NH_2	**14b**	77
15a	Bz-CROKORCROKORC-NH_2	**15b**	79
16a	Bz-CKRKGCGROKORC-NH_2	**16b**	72
17a	Bz-CKRKGCGROKORGC-NH_2	**17b**	71
18a	Bz-CKRKGCLGFKAFKGC-NH_2	**18b**	91
1c	Ac-SKRKGSGKRKS-NH_2		0
2c	Ac-SAGFKSAGFKSA-NH_2		0
3c	Ac-SHGFKSDGFKSS-NH_2		0

[a] Peptides in absence of Bi(III). [b] Sequences of synthesised and isolated peptides; O, ornithine, Ψ, 4-guanidinophenylalanine; Φ, phenylglycine; Θ, homocysteine. [c] Bicyclic peptides in presence of Bi(III). [d] Yield of bicyclic peptide according to LC-MS (mass trace). Reaction conditions: 1 mM linear peptide, 10 mM Tris-HCl pH 7.5, 10 mM TCEP, 1.2 mM $BiBr_3$.

The ease and reliability of which bicycles can be produced by this methodology, invites the *in-situ* generation of bicyclic peptides for screening campaigns without the need for product purification. To demonstrate this, we conducted screenings against the NS2B-NS3 proteases from Zika and West Nile viruses.[13] While cyclic peptides were previously evaluated as high-affinity inhibitors,[14] for the first time bicyclic peptides are explored in this study. Both proteases contain free cysteine residues, which would likely be modified by an excess of conventional alkylating scaffolds like TBMB. However, as previously demonstrated by us, Bi(III) does not bind to single solvent-exposed cysteine residues in Zika NS2B-NS3 even at very high concentrations.[9e] Consequently, Bi(III) can be used directly in presence of most proteins, rendering it a superior reagent for *in-situ* screening applications.

Subsequently, we designed 15 additional peptides (**4a**-**18a**) as potential inhibitors of NS2B-NS3 protease containing either one or two substrate recognition motifs of NS2B-NS3 flanked with cysteine residues for bicyclization (Table 1). The substrate recognition motif KRKG represents the P_3-P_1' positions of the substrate of NS2B-NS3.[13-14] Various cycle sizes, ranging from 3 to 8 amino acids between two cysteines were explored, and a large variety of canonical as well as non-canonical amino acids were assessed including ornithine and the two unnatural amino acids 4-guanidinophenylalanine and phenylglycine. To further broadening the scope, homocysteine was investigated in place of the central cysteine in **9a**. All peptides (**4a**-**18a**) selectively formed bicycles in presence of Bi(III). None displayed any isomers in LC-MS. Some bicycles (**12b**, **14b**-**17b**) presented lower LC-MS yields, which are a result of challenging purification of the individual linear precursors and low ionizability. The three control peptides **1c**-**3c**, which contain three serines instead of cysteines, did not form any complex with Bi(III) that could be detected by LC-MS, demonstrating the high specificity of this transformation. Even peptide **3c**, which contains histidine and aspartate residues did not display any bismuth complexation.

Compounds **1a**-**18a** were screened in absence and presence of Bi(III) at concentrations of 10 and 1 μM against the proteases from Zika (ZiPro) and West Nile virus (WNVpro) (Figure 3). We applied high substrate concentrations (6K_m for ZiPro and 3K_m for WNVpro) to observe only very strong inhibition. The activities of control peptides **1c**-**3c** were not affected by Bi(III), underpinning the high selectivity and biocompatibility of the transformation. Because constrained peptides can enhance affinity of protease inhibitors by pre-organization,[15] we were interested in examples where the presence of Bi(III) significantly increased inhibition. This effect was pronounced for the pair **1a**/**1b** against both proteases (Figure 3). In case of ZiPro, **1a** indicates an IC_{50} above 1 μM, while **1b** indicates an IC_{50} clearly below 1 μM, even at the high substrate concentration used.

To further validate our screening hits, we determined the inhibition constants (K_i) of purified **1a** and **1b** against ZiPro and WNVpro (Figure 4a-b). In case of ZiPro, we obtained K_i values of 510 and 23 nM for **1a** and **1b**, respectively, translating into a 22-fold affinity increase triggered by bicyclization. The outstanding affinity of **1b** is similar to the best inhibitors reported.[14a, 16] In case of WNVpro, we observed only slightly lower inhibition of **1b** (K_i = 150 nM), but significantly reduced affinity of the linear analog **1a** (K_i = 19,700 nM), translating into a more than 130-fold affinity increase due to bicyclization. We further compared the proteolytic stability of **1a** and the triple-serine analog **1c** in presence of ZiPro and WNVpro over longer time frames (Figure 4c). Bicycle **1b** shows 6-times higher resistance to proteolysis by ZiPro than **1c**. In case of WNVpro, we observed even a 19-fold slower proteolytic digest of **1b** than of **1c**. Hence, **1c** is a high-affinity substrate.

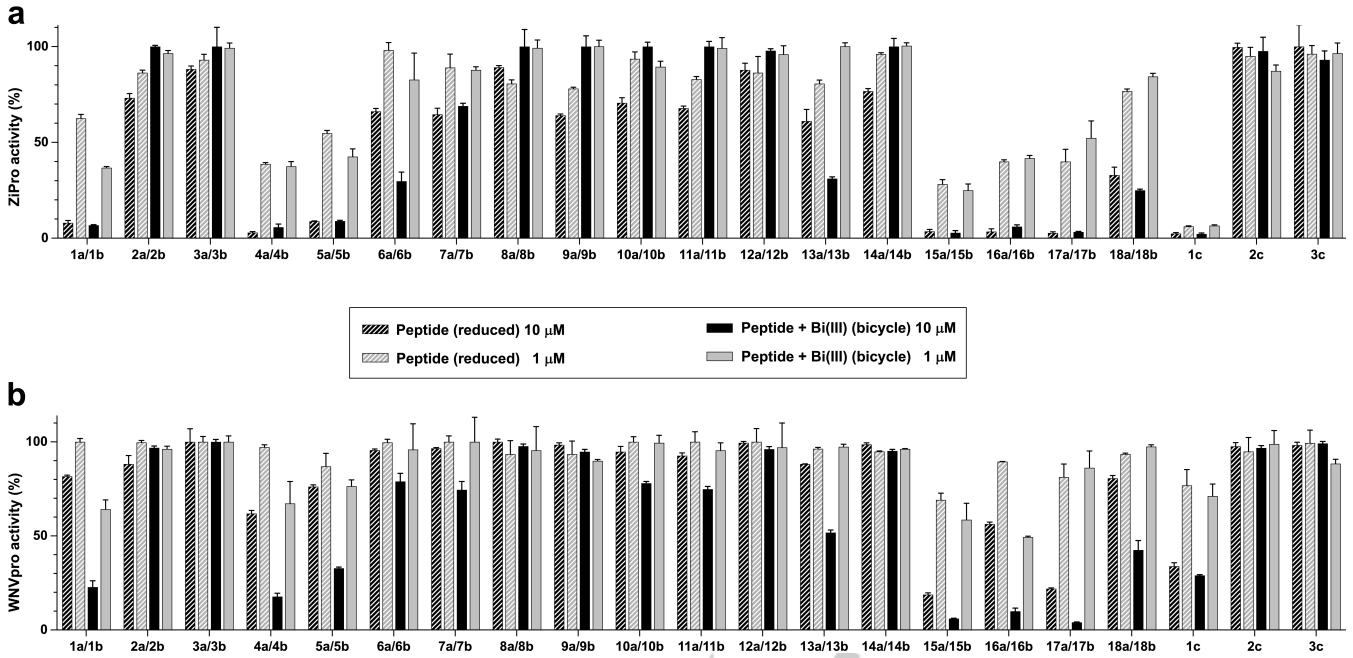


Figure 3. Activity of viral proteases in presence of linear and bicyclic peptides. Peptides were analysed as reduced linear peptides as well as after reaction with 1.2 equivalents of $BiBr_3$. Assays were performed in 10 mM Tris pH 8.5, 0.3 mM TCEP, 1 mM CHAPS, 20% glycerol using the fluorescent substrate Boc-GKR-AMC (100 μM). a) Zika virus NS2B-NS3 protease (ZiPro, 5 nM). b) West Nile virus NS2B-NS3 protease (WNVpro, 7.5 nM).

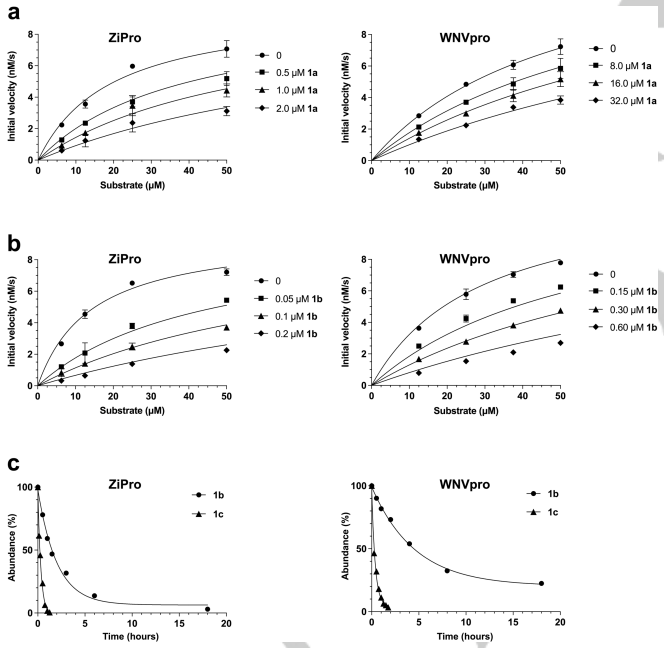


Figure 4. a) Michaelis-Menten kinetics at different concentrations of **1a** (ZiPro, K_i = 510 nM; WNVpro, K_i = 19,700 nM). b) Michaelis-Menten kinetics at different concentrations of **1b** (ZiPro, K_i = 23 nM; WNVpro, K_i = 150 nM). c) Proteolysis of **1b** (4 mM) and **1c** (4 mM) in presence of ZiPro (40 μM) and WNVpro (200 μM) in 16 mM MOPS pH 7.9, 4% glycerol.

In conclusion, we have developed a new concept for the bicyclization of peptides. The thiophilic character of Bi(III) ensures selective cysteine modification with sufficient affinity to generate stable bicycles of high rigidity. The strategy is fully biocompatible and can be used directly in screening campaigns as peptide-bismuth bicycles are formed *in-situ*. We were able to identify a high-affinity bicyclic inhibitor of two viral proteases. Bicyclization not only caused a dramatic increase in target affinity but also enhanced proteolytic stability. Future research will evaluate the potential for peptide display techniques like phage or mRNA display.[17]

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Keywords: peptides • bismuth • inhibitors • macrocyclization • bicycles

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