Perfluoroaryl Bicyclic Cell-Penetrating Peptides for Delivery of Antisense Oligonucleotides

Justin M. Wolfe[#a], **Colin M. Fadzen**[#a], **Rebecca L. Holden**[a], **Monica Yao**[b], **Gunnar J. Hanson**[b], and **Bradley L. Pentelute**[*a]

[a]Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 (USA),blp@mit.edu

[b]Research Chemistry, Sarepta Therapeutics, Inc., 215 First Street, Cambridge, MA 02142 (USA)

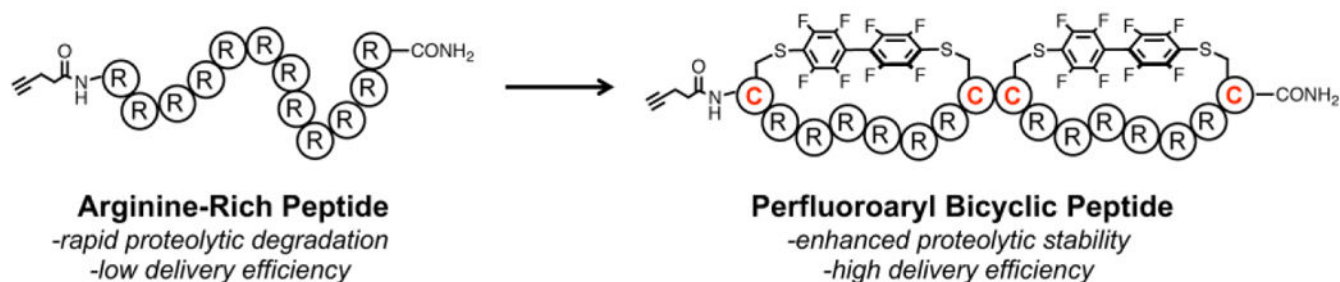[#] These authors contributed equally to this work.

Abstract

Exon-skipping antisense oligonucleotides are effective treatments for genetic diseases, yet exon-skipping activity requires that these macromolecules reach the nucleus. While cell-penetrating peptides can improve delivery, proteolytic instability often limits efficacy. We hypothesized that bicyclization of arginine-rich peptides would improve their stability and their ability to deliver oligonucleotides into the nucleus. Here, we introduce two methods for the synthesis of arginine-rich bicyclic peptides using cysteine perfluoroarylation chemistry. Then, the bicyclic peptides are covalently linked to a phosphorodiamidate morpholino oligonucleotide (PMO) and assayed for exon skipping activity. The perfluoroaryl cyclic and bicyclic peptides improve PMO activity roughly 14-fold over the unconjugated PMO. The bicyclic peptides exhibited increased proteolytic stability relative to the monocycle, demonstrating that perfluoroaryl bicyclic peptides are potent and stable delivery agents. Exon-skipping antisense oligonucleotides are effective treatments for genetic diseases, yet exon-skipping activity requires that these macromolecules reach the nucleus. While cell-penetrating peptides can improve delivery, proteolytic instability often limits efficacy. We hypothesized that bicyclization of arginine-rich peptides would improve their stability and their ability to deliver oligonucleotides into the nucleus. Here, we introduce two methods for the synthesis of arginine-rich bicyclic peptides using cysteine perfluoroarylation chemistry. Then, the bicyclic peptides are covalently linked to a phosphorodiamidate morpholino oligonucleotide (PMO) and assayed for exon skipping activity. The perfluoroaryl cyclic and bicyclic peptides improve PMO activity roughly 14-fold over the unconjugated PMO. The bicyclic peptides exhibited increased proteolytic stability relative to the monocycle, demonstrating that perfluoroaryl bicyclic peptides are potent and stable delivery agents.

Assembling a new bicycle:

Peptide bicycles are synthesized using cysteine perfluoroarylation chemistry. The bicyclic peptides demonstrate enhanced proteolytic stability relative to the monocyclic peptide. Additionally, after conjugation to antisense oligonucleotides, the bicyclic peptide conjugates exhibit a 14-fold increase in cellular activity when compared to the unconjugated oligonucleotide.

Graphical Abstract

Arginine-Rich Peptide
-rapid proteolytic degradation
-low delivery efficiency

Perfluoroaryl Bicyclic Peptide
-enhanced proteolytic stability
-high delivery efficiency

Keywords

antisense agents; bicyclization; cell-penetrating peptides; cysteine arylation; peptides

Exon-skipping antisense oligonucleotides have been developed into successful therapeutic molecules for debilitating genetic diseases, such as Duchenne muscular dystrophy (DMD). [1–3] To promote exon-skipping, these molecules bind to pre-mRNA in the nucleus and sterically block splice junctions or intraexonic ribonucleoprotein binding motifs. The steric blocking alters the spliceosomal processing of pre-mRNA, and results in the exclusion of one or more exons from the mature mRNA transcript. Thus, the oligonucleotide restores the in-frame mRNA and leads to translation of a functional protein.[1]

Phosphorodiamidate morpholino oligonucleotides (PMOs) are one type of exon-skipping oligonucleotide. PMOs are uncharged nucleic acid polymers in which the ribosyl ring is replaced with a morpholino ring and the natural phosphodiester backbone is replaced with phosphorodiamidate.[4] PMOs readily hybridize to DNA and RNA, and their altered backbone structure prevents degradation by serum and intracellular nucleases.[5,6] Eteplirsen, a PMO designed to restore functional dystrophin, recently became the only FDA-approved therapy to address the underlying genetic cause of DMD.

Unfortunately, PMOs, like all antisense oligonucleotides, exhibit limited delivery into the nucleus of cells. To improve PMO delivery, multiple strategies have been explored, such as the covalent attachment of cationic dendrimers or side-chains.[7–9] Arginine-rich cell-penetrating peptides have been widely utilized to improve cargo delivery, and these peptides are one of the most promising strategies for PMO delivery.[10–14] However, arginine-rich sequences suffer from proteolytic instability.[6] Delivery tools with enhanced proteolytic stability and delivery efficiency are therefore widely desired.

Bicyclic peptides are a unique class of peptides characterized by two intramolecular linkages. Excluding disulfide-linked peptides, methods to bicyclize unprotected peptides include bromomethylated aromatics, thiol-ene chemistry, tetrafluoroterephthalonitrile, or multiple orthogonal reactions.[15–19] The constrained conformation of bicyclic peptides can lead to reduced flexibility, improved binding properties, and protease resistance. Additionally, in the context of cellular delivery, bicyclic peptides with certain sequences show robust uptake into cells.[20,21] However, bicyclic peptides have not been explored as tools to promote the intracellular delivery of a large, covalently-attached cargo such as PMOs.

We recently developed a new method for peptide macrocyclization which joins the cysteine side-chains of unprotected peptides with perfluoroaromatic linkers.[22,23] These perfluoroaryl macrocyclic peptides have increased uptake into cells and increased proteolytic stability. Because the synthesis of multiple sizes of macrocycles is facile, we envisioned this perfluoroarylation chemistry could be extended towards the synthesis of bicyclic peptides. We anticipated that these bicyclic peptides would have enhanced stability, and that when covalently attached to oligonucleotides, would promote intracellular delivery.

Our first peptide bicyclization strategy used a trithiol to link together three perfluoroarenes covalently attached to an unprotected peptide (Figure 1). We began with R_{12} peptide **1**. The purified peptide was incubated with an excess of decafluorobiphenyl in dimethylformamide (DMF) with *N,N*-diisopropylethylamine (DIEA) to generate the perfluoroarylated peptide **1a** (35% isolated yield). After purification of **1a**, 1,3,5-benzenetrithiol (1 mM) was added to the peptide (1 mM) in DMF and after 2 hours the bicyclic peptide **1b** was observed. The reaction was quenched and the peptide was purified by high-performance liquid chromatography (HPLC) (62% isolated yield). This strategy also enables the bicyclization of smaller peptides, demonstrated by its applicability to R_6 peptide **2**. This peptide was reacted with excess decafluorobiphenyl and then bicyclized under similar conditions to generate peptide **2b** (35% isolated yield) (SI Fig. 1).

For a second bicyclization strategy, we performed a double macrocyclization of a linear peptide. Initially, we synthesized the R_{12} peptide **3** and utilized orthogonal protection to install two macrocycles sequentially (SI Fig. 2). However, after final purification, the bicyclic peptide **3b** was obtained in an overall yield of 4.5%.

To improve the synthesis of **3b** by eliminating deprotection and purification steps, we developed an alternative method, which we refer to as kinetically controlled bicyclization (Figure 2A). Given the slow rates of *i, i+1* cyclization with decafluorobiphenyl,[23] we anticipated unlinked perfluoroarenes could be pre-installed on resin and peptide cyclization would be favored at specific sites after the addition of base. Peptide **3** was synthesized with two trityl-protected cysteine residues and two *tert*-butylthiol- protected cysteine residues. The *tert*-butylthiol protected cysteines were placed at positions 1 and 9 and deprotected on resin. Then, decafluorobiphenyl was introduced and allowed to react with the free thiol. Cleavage and HPLC purification provided peptide **3a**. Next, the peptide was simply dissolved in DMF with 50 mM DIEA. Complete conversion to the double cycle product **3b** occurred in under 5 minutes (Figure 2B). HPLC purification led to the isolation of **3b** in 81% yield.

Importantly, both methods to synthesize **3b** provided products with identical retention times (Figure 2C), suggesting that a bicycle with two *i, i+7* macrocycles is favored over the bicycle with *i, i+15* and *i, i+1* macrocycles. To confirm our hypothesis that the *i, i+1* reaction is slow, we synthesized the control peptide **4a** containing one cysteine residue adjacent to a cysteine residue linked to decafluorobiphenyl. When treated with 50 mM DIEA in DMF, less than 5% of **4a** cyclized in the first 30 seconds and less than 30% cyclized after 5 minutes (Figure 2D). In comparison, peptide **3a** shows 95% conversion in 30 seconds. The

slow rate for *i, i+1* cyclization indicates that the favored regioisomer for peptide **3b** contains two *i, i+7* cycles.

Next, we tested our bicyclic compounds for their ability to promote the intracellular delivery of an 18-mer, 6 kDa PMO. The PMO-peptide conjugates were synthesized using copper-catalyzed "click" chemistry (Figure 3A). In addition to the bicyclic peptides, the PMO was conjugated to a monocyclic R12 peptide **6c**, linear R12 peptide **5**, a non-fluorinated bicyclic peptide **1nfb**, and a recently-reported lactam-cyclized R10 peptide **7c** (SI Fig. 3).[24]

We measured the activity of the conjugates in the HeLa-654 cell assay.[25] The HeLa-654 cells are stably transfected with an eGFP coding sequence interrupted by a mutant intron from the human β-globin gene (IVS2–654). This intron contains a mutation that alters the normal pre-mRNA splice site to a cryptic splice site, leading to retention of an unnatural exon fragment in the spliced eGFP mRNA. This aberrant mRNA leads to the translation of a *non-fluorescent* form of eGFP. The cargo PMO has a nucleobase sequence complementary to IVS2–654. When the PMO hybridizes to the mutant β-globin exon, it prevents the aberrant gene splicing from occurring and leads to functional eGFP expression. Successful nuclear delivery of PMO leads to the observation of green fluorescence.

To determine if bicyclic peptides improve PMO delivery, the cells were incubated with each PMO-peptide conjugate at 2 and 5 μM in media containing 10% serum. After 22 hours, cellular fluorescence was analyzed by flow cytometry (Figure 3b). At 5 μM treatment, the perfluoroaryl cyclic and bicyclic peptide conjugates all led to an approximately 14-fold increase in eGFP fluorescence relative to PMO. Both the lactam cyclized R10 and the non-fluorinated bicycle exhibited lower fluorescence, demonstrating that the perfluoroarenes contribute to the gains in PMO activity. Additionally, at 2 μM concentration, the bicyclic conjugate PMO-**3b** outperformed all of the other conjugates.

To investigate the role of serum, the PMO conjugates were tested in the eGFP assay using media containing 0, 5, and 10% fetal bovine serum. All conjugates exhibited only a mild reduction in activity at higher serum concentrations, suggesting that the hydrophobic perfluoroarenes are not sequestered by serum proteins (SI Fig. 4). Additionally, a lactate dehydrogenase (LDH) assay was performed to determine if these compounds disrupt the plasma membrane. For PMO-**1b**, LDH release was observed at both 5 μM and 2 μM, indicating early signs of cytotoxicity. At 2 μM, PMO-**3b** exhibited no signs of LDH release while remaining highly active (SI Fig. 5).

We examined if the increase in PMO activity resulted from structural characteristics of the peptides or solely from the combination of arginine residues with a perfluoroarene. PMO-**6c** was tested along with R12 variants cyclized along either the 6 N-terminal residues or the 6 C-terminal residues. The C-terminal cyclized R12 led to less eGFP fluorescence than the fully cyclic peptide **6c**, demonstrating that macrocycle position affects activity (SI Fig. 6). Similarly, for the related arginine-rich peptide Bpep, the fully cyclic peptide led to more activity than the N- and C-terminal cyclic variants (SI Fig. 7). However, investigations with other peptide transporters confirmed the importance of arginine residues (SI Fig. 8).

Lastly, anticipating the peptide bicycles would exhibit enhanced stability, we measured the proteolytic stability of bicyclic *versus* monocyclic peptides (Figure 4). The peptides (100 μM) were incubated with trypsin (0.05 μg/mL) at 37 ℃. After one hour, less than 5% of the cyclic compound **6c** remained, while 45% of bicycle **3b** remained and nearly 70% of trithiol linked bicycle **1b** remained. Ultimately, both bicyclic peptides demonstrated greater stability than the monocyclic peptide, and this stability could be necessary in therapeutic applications.

Here, we demonstrate multiple strategies for peptide bicyclization using cysteine S_NAr chemistry. A trithiol can link three perfluoroarenes on a single peptide together into a bicycle, or two thiol pairs can be selectively crosslinked to synthesize a double macrocyclic peptide. In these studies, our cysteine arylation chemistry is shown to modulate the biological properties of arginine-rich peptides. The bicyclic peptides exhibit substantial protease resistance, and PMO-conjugated **3b** does not display signs of cytotoxicity at 2 μM concentration. The exon-skipping activity of PMOs conjugated to perfluoroaryl cyclic or bicyclic peptides is greater than the activity of PMOs conjugated to other cyclic or bicyclic peptides. Moving towards *in vivo* applications, this control over stability, toxicity, and activity will enable the optimization of our lead compounds. Given these results, we envision that perfluoroaryl CPPs represent next-generation delivery agents for macromolecular cargo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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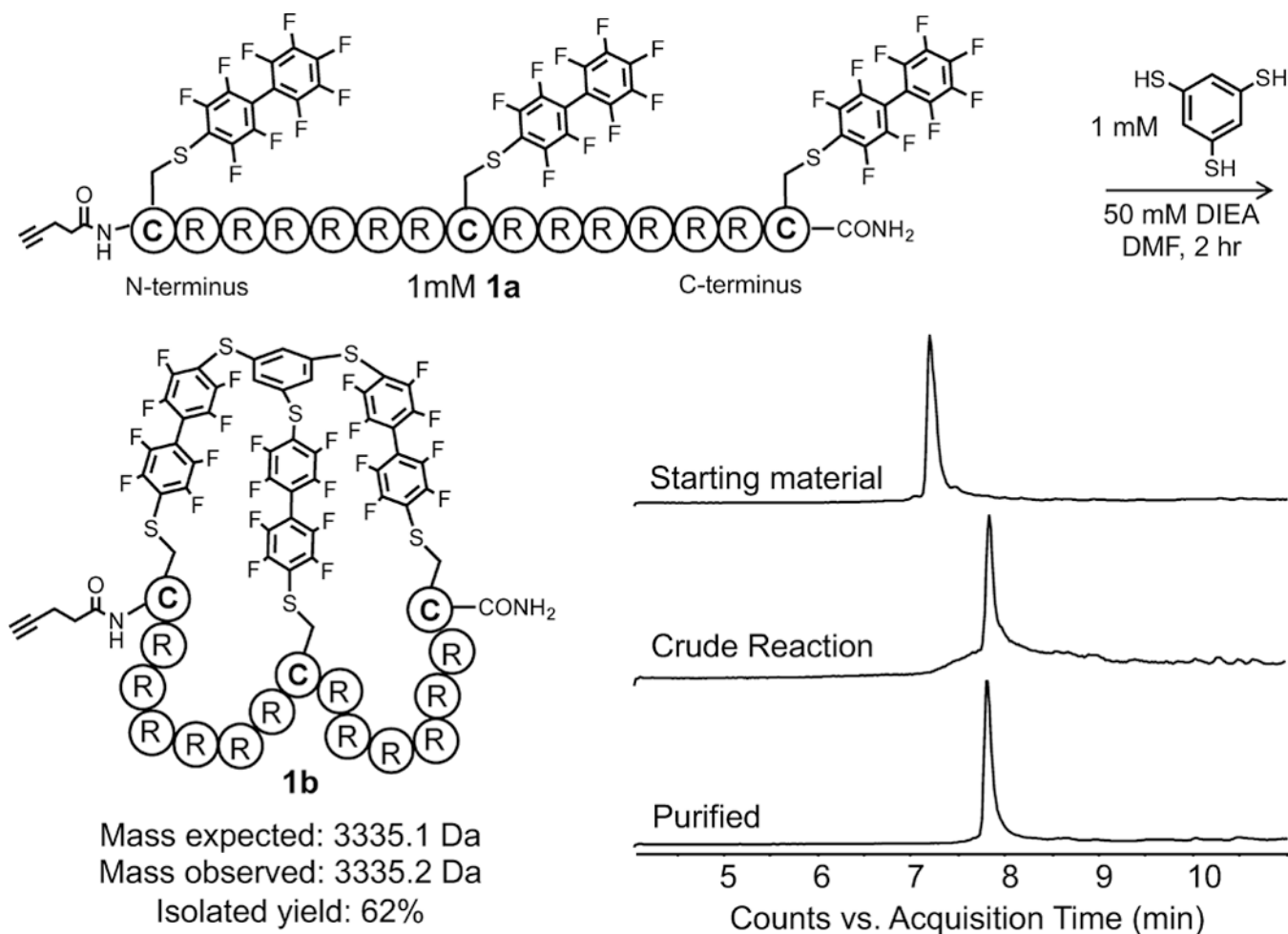
Figure 1: Multiple cysteine residues coupled to a perfluoroarene can be linked with 1,3,5-benzenetrithiol to enable peptide bicyclization.

Peptide 1 is perfluoroarylated to give peptide 1a. Incubating 1a (1 mM) with equimolar 1,3,5-benzenetrithiol for 2 hours generates the bicyclic peptide 1b. LC-MS analysis confirms complete conversion. All chromatograms are total ion currents (TIC). HPLC purification affords 1b in 62% yield.
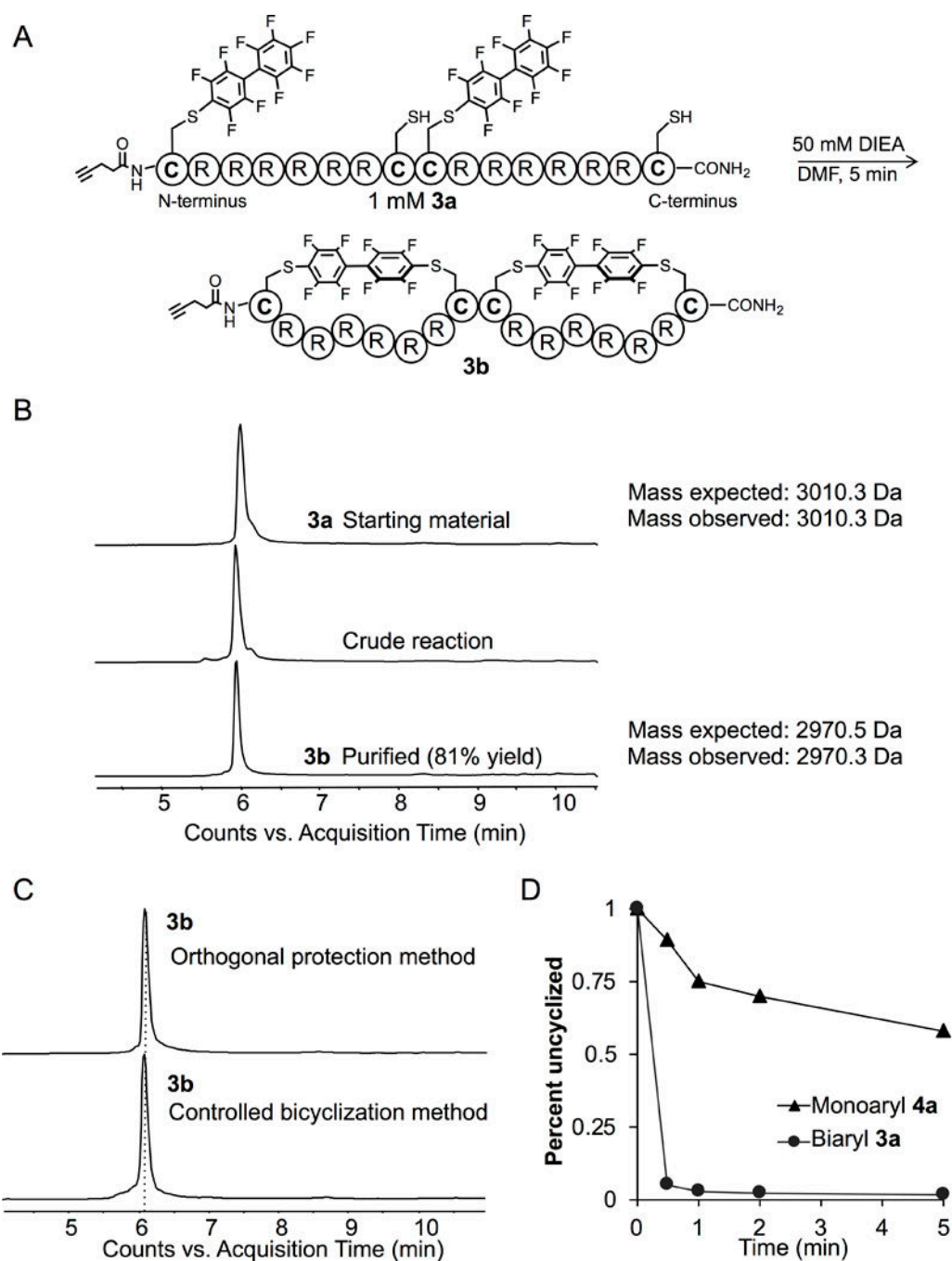
Figure 2: Kinetically controlled bicyclization enables high-yield synthesis of a double perfluoroaryl macrocyclic peptide.

A) Two perfluoroarenes are selectively installed on the peptide on resin. Peptide **3a** undergoes intramolecular $S_N Ar$ after addition of base to preferentially form two *i, i+7* cycles, due to the slow rate of linking adjacent *i, i+1* residues. B) LC-MS analysis and TIC chromatogram demonstrating the conversion of **3a** to **3b** with the appropriate change of mass. C) TIC chromatogram for peptide **3b** synthesized using orthogonal protection is identical to the chromatogram of peptide **3b** synthesized using kinetically controlled

bicyclization. D) The rate of *i, i+1* cyclization of peptide **4a** was compared to cyclization of peptide **3a**. Just 5% of peptide **4a** cyclized in the first 30 seconds, while peptide **3a** shows 95% cyclization in 30 seconds, indicating that the favored regioisomer for peptide **3b** contains two *i, i+7* cycles.
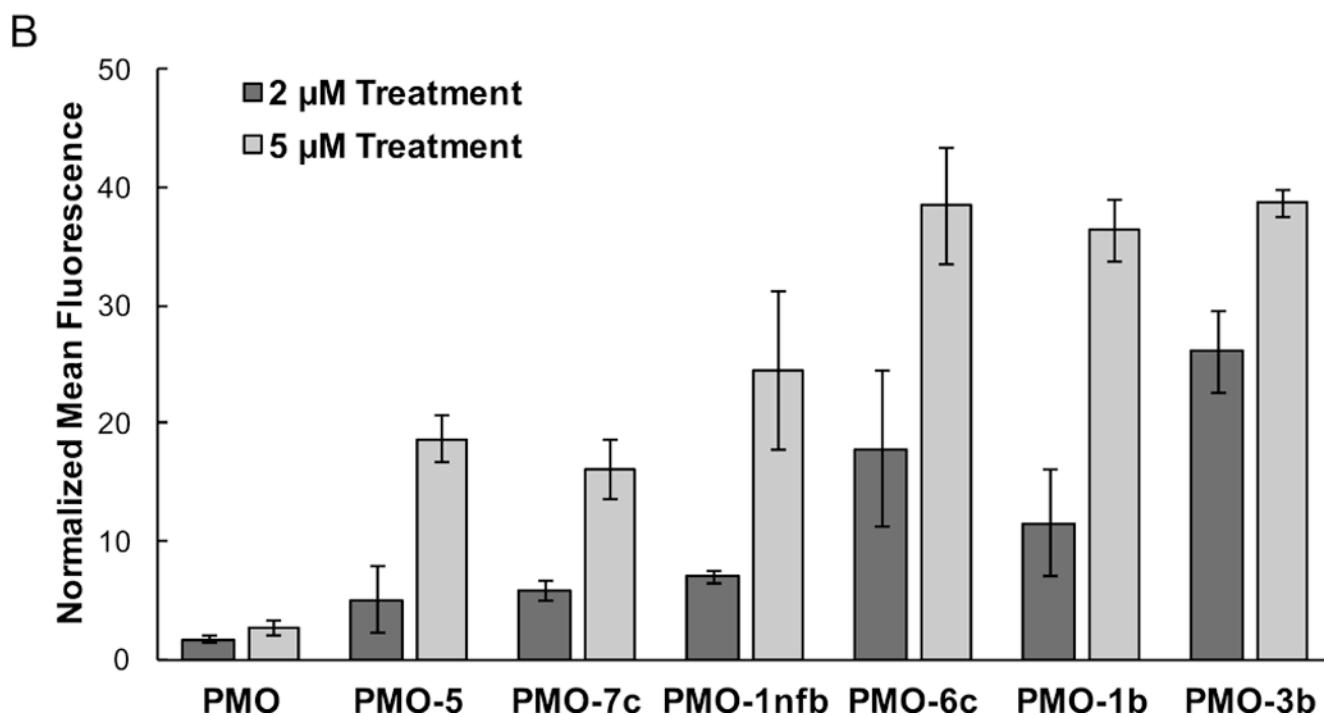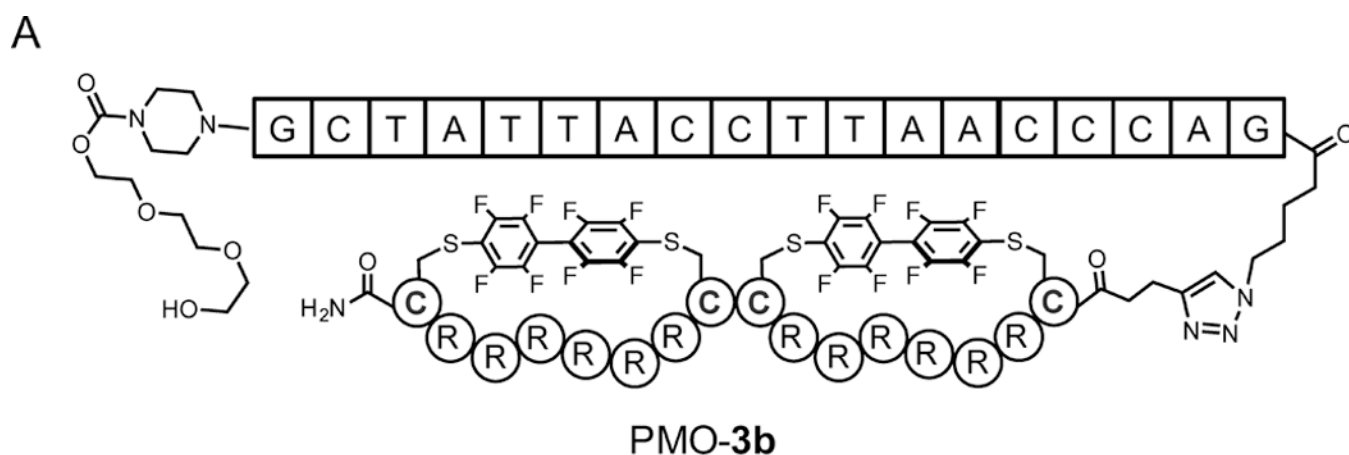
A

PMO-**3b**

B

Figure 3: Bicyclic peptides conjugated to PMO show increased exon-skipping activity.
A) Depiction of bicyclic peptide conjugate PMO-**3b**. B) Conjugates between PMO and perfluoroaryl cyclic or bicyclic peptides (**6c, 1b, and 3b**) lead to more cellular fluorescence than conjugates to linear R12 (**5**), an established cyclic peptide cR10 (**7c**), or a non-fluorinated bicyclic peptide (**1nfb**). The PMO corrects eGFP splicing in a modified HeLa cell line. Cells were incubated with 2 μM or 5 μM of each PMO-peptide conjugate for 22 hours and the mean fluorescence intensity was analyzed by flow cytometry. Error bars are standard deviation (n=3 independent replicates).
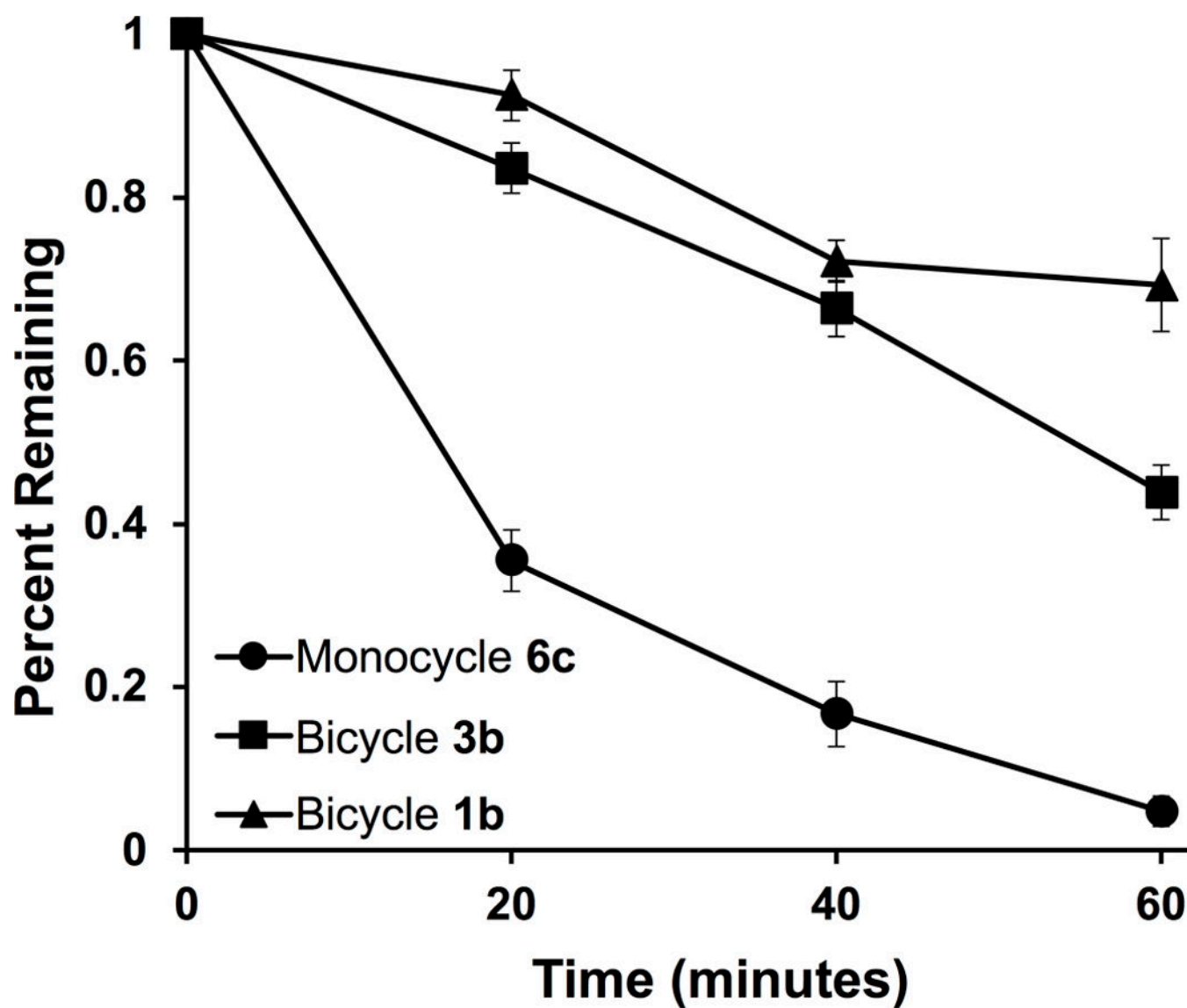
Figure 4: Bicyclic peptides demonstrate enhanced proteolytic stability relative to monocyclic peptides.

The bicyclic peptides **1b** and **3b** and monocyclic peptide **6c** (100 μM) were incubated with trypsin (0.05 μg/mL) at 37 °C. After 1 hour, less than 5% of **6c** remained, while 45% of **3b** and 70% of **1b** remained, demonstrating that bicyclization improves proteolytic stability relative to monocyclization.

Table 1.

Arginine-rich peptides for bicyclization and control sequences

Peptide	Sequence[a]	Cyclic form
1	Z**C**RRRRRR**C**RRRRRR**C**	**1b** - Trithiol bicycle **1nfb** – Non-fluorinated bicycle
2	Z**C**RRR**C**RRR**C**	**2b** - Trithiol bicycle
3	Z**C**RRRRRR**CC**RRRRRR**C**	**3b** - Double macrocycle
4	ZRRRRRR**CC**RRRRRR	**4c** - Monocycle
5	ZRRRRRRRRRRR	None
6	Z**C**RRRRRRRRRRR**C**	**6c** - Monocycle
7	X**K**RrRrRrRrR**E**	**7c** - Lactam monocycle

[a]Z = 4-pentynoyl, X = 5-azidopentyl; all peptides are C-terminal amides