Synthesis of *Bicycle®* Peptides using Gold-mediated Cysteine Arylation

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Abstract

Bicycles **are a unique class of chemically synthesised peptide-based therapeutics. They are formed by constraining short linear peptides into bi-cyclic structures around a trifunctional, symmetrical scaffold, thus allowing binding to targets of interest with high affinity and specificity.**

New Bicycles are discovered using phage display technology. Key to this strategy is the ability to produce vast, diverse phage libraries which, in turn, increases the probability of finding new hits to targets. Chemical diversity can be derived by varying the peptide sequence, loop size or chemical scaffold used for cyclisation of the peptides. Organometallic complexes, such as Gold(III) complexes, have recently been tested as a new type of scaffold for the cyclisation of cysteine-containing peptides.

Here, we describe the application of gold-based scaffold molecules for the formation of Bicycle® peptides via cysteine arylation. Four novel tris-Gold(III) complexes were synthesised and used as scaffolds for peptide cyclisation with a variety of sequences. We assessed the impact of loop size and the presence of other reactive residues in the peptide sequence on cyclisation efficiency. For all four novel scaffolds, bicyclisation proceeded to completion and no evidence of dimerization was observed. The desired bicyclic products were isolated with yields of 13 - 44 %, illustrating the potential of gold-mediated cysteine arylation for the development of novel *Bicycles*.

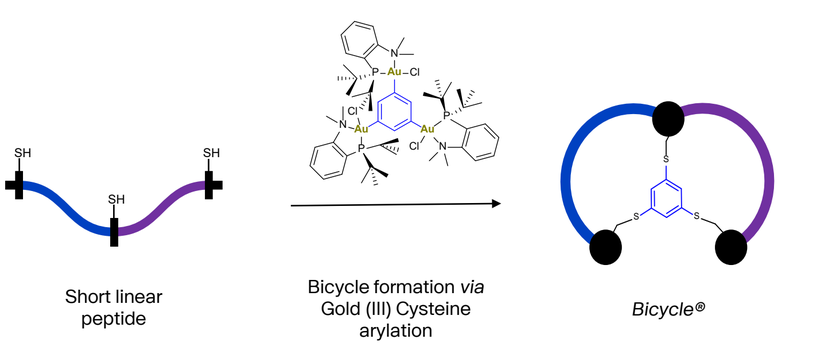

Figure 1: S-arylation of a short linear peptide containing three strategically cysteine residues upon addition of a tris-Gold(III) complex to form a *Bicycle®*.

Introduction

Bicycles are highly constrained bicyclic peptides formed through the reaction of three strategically placed cysteine residues within a short linear sequence (9-20 amino acids in length) and a trivalent scaffold. (Figure **2**).

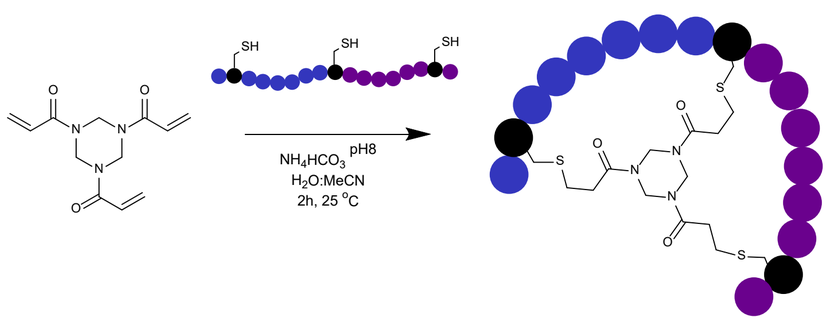

Figure 2: Upon addition of a small molecule scaffold such as 1,3,5-triacryloyl-1,3,5-triazinane (TATA), linear peptides react via Michael addition to form bicyclic peptides.

De novo discovery of new bicyclic peptides against a wide range of targets is achieved using the Bicycle Therapeutics phage screening platform (Figure **3**).[1] This platform provides enormous chemical diversity (>10^{20}) by varying the length of the peptide, the amino acid sequence, the loop size and the small-molecule scaffold used. The development of novel scaffolds for the synthesis of highly constrained peptides has been of great interest to Bicycle Therapeutics.

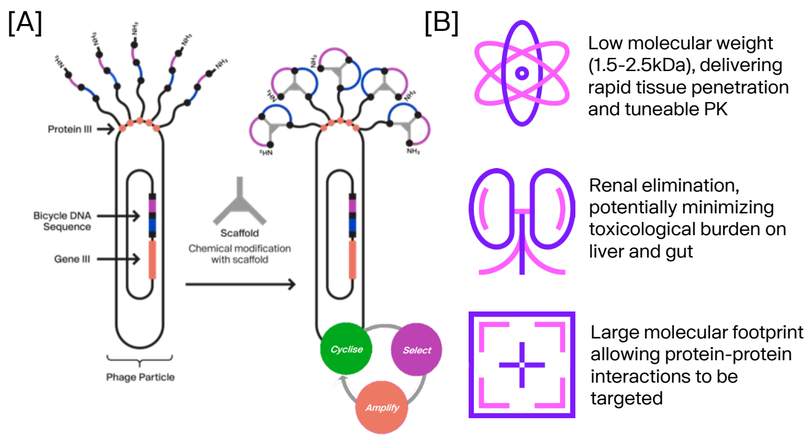

Figure 3: [A] Bicycle® binders to specific targets are identified using phage display. Iterative rounds of affinity maturation creates chemical diversity around a common pharmacophore which can be used to determine structure activity relationships. This directs the tuning of these molecules into potential drug candidates. [B] *Bicycle®* properties.

The Spokoyny group recently demonstrated the use of gold-based complexes to synthesise a bicyclic peptide with a 50% yield via S-arylation.[2,3] Due to the mild reaction conditions required and the high functional-group tolerance of gold-containing complexes, we sought to expand upon the Spokoyny methodology for the synthesis of various Bicycles, with potential to apply this approach to the phage screening platform.[4]

Results

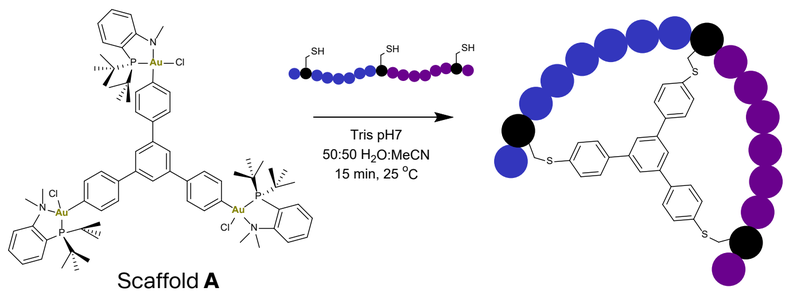

Figure 4: Protocol for the cyclisation of a linear peptide using Scaffold **A.** Scaffold **A** was prepared via oxidative addition of 1,3,5-tris(4-iodophenyl)benzene.

To initiate the study, Scaffold **A** (Figure **4**), designed by the Spokoyny group, was used to modify 8 linear peptides that contained the three cysteine residues required for stapling (Table **1**, Peptide **1 - 8**). To test the functional group tolerance and selectivity of this method, amino acids with reactive side chains (Glu, Lys, Arg, Thr, His, Tyr, Met) were included in the sequence. Peptides of varying lengths and loop sizes were selected for this assessment as well.

Scaffold **A** proved successful in reacting with a range of different peptides to produce *Bicycles* in good yields.

Peptide Entry (sequence and loop size)	Scaffold A	Scaffold B	Scaffold C	Scaffold D
1 (ACEKRVKACTHTYMVCA-NH_2) (6X6)	30 %	26 %	42 %	24 %
2 (ACEKRCVKATHTYMVCA-NH_2) (3X9)	47 %	23 %	34 %	18 %
3 (ACEKRVCKATHTYMVCA-NH_2) (4X8)	34 %	38 %	34 %	34 %
4 (ACEKCRVKATHTYMVCA-NH_2) (2X10)	22 %	13 %	31 %	16 %
5 (ACEKRVKCATHTYMVCA-NH_2) (5X7)	28 %	26 %	39 %	16 %
6 (ACKRVKACTHTYMCA-NH_2) (5X5)	48 %	37 %	33 %	34 %
7 (ACRVKACTHTYCA-NH_2) (4X4)	42 %	18 %	44 %	30 %
8 (ACVKACTHTCA-NH_2) (3X3)	30 %	26 %	39 %	21 %

Table 1: Yields of Bicycle Formation using Scaffolds **A – D** and Peptides **1 – 8**.

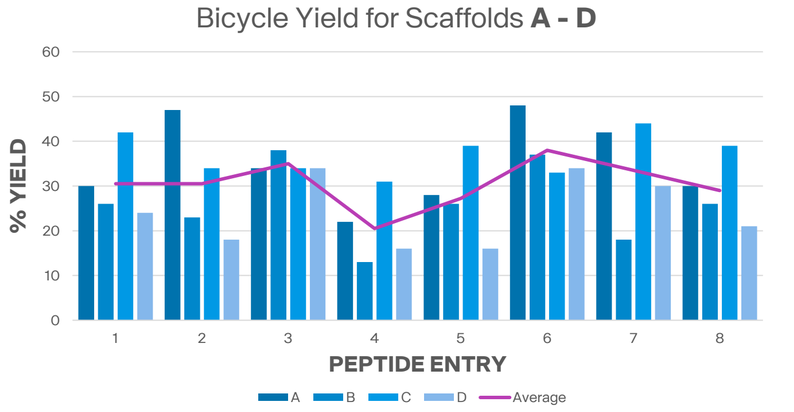

Figure 5: Comparison of the isolated yields of the Bicycles formed using Scaffolds **A – D** and Peptide **1 – 8**. Trendline to show average yield of Bicycle formation for each peptide entry.

Unfortunately, the hydrophobic nature of Scaffold **A** presented an issue. A recent study suggested that hydrophobicity in bicycle toxin conjugates was linked to undesired uptake of the conjugate into the liver, leading to toxicity.[5]

Additional scaffolds (**B – D**) were prepared with structural changes to the central aryl ring to introduce groups that reduced hydrophobicity and that could potentially form positive interactions with the peptides or even the potential protein target (see Figure **6**).

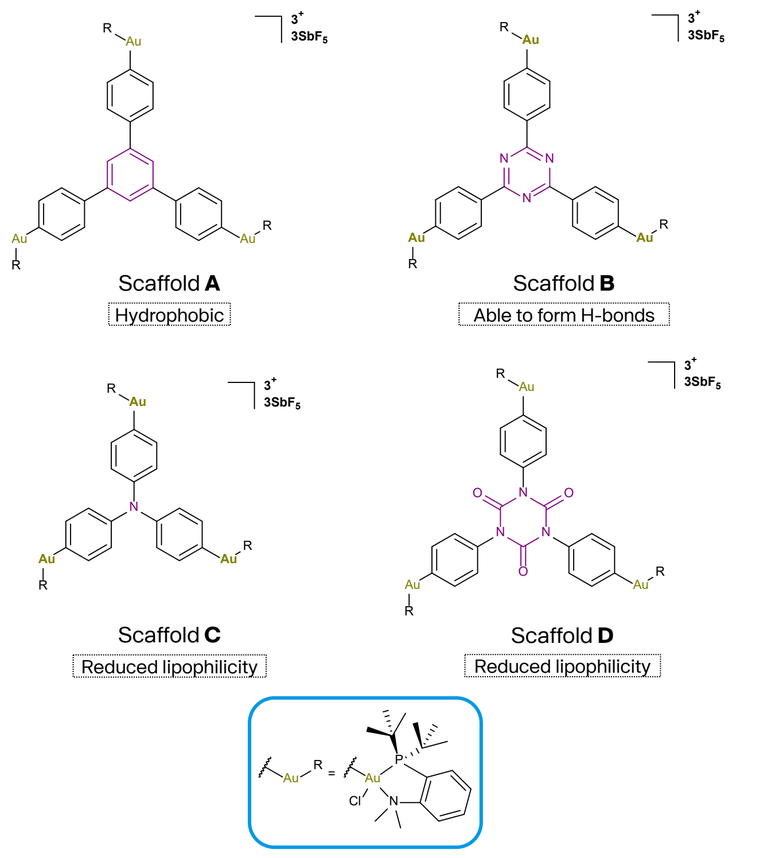

Figure 6: Structures of Gold(III)-based Scaffolds **A – D** prepared for use in Bicycle formation. Scaffold **B – D** were design based on Scaffold **A** but with structural changes to the central aryl ring that were introduced to reduce hydrophobicity.

Scaffolds **B - D** successfully reacted with the full range of linear peptides **1 – 8,** with the more electron rich scaffold, Scaffold **C**, performing the best and giving rise to Bicycles with yields over 30% for all loop formats (Figure **5**).

The final scaffold (Scaffold **E**) was designed to be the smallest possible scaffold. Despite the anticipated steric crowding of three bulky groups around the central ring system, Scaffold **E** was isolated with a 54% yield using an optimized method (Figure **7**).

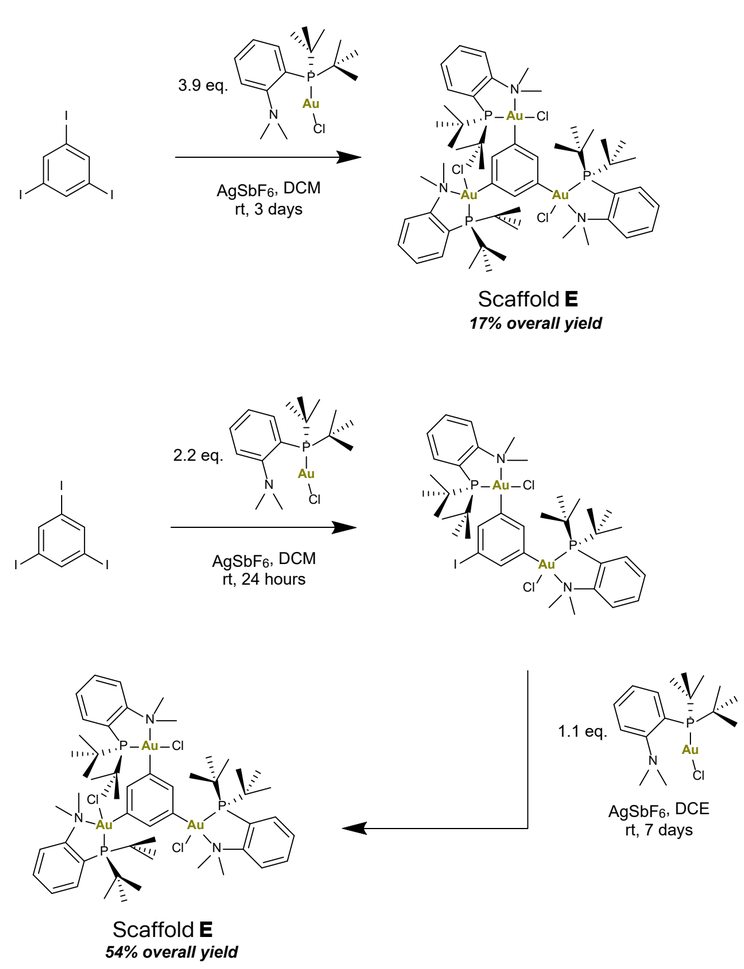

Figure 7: [A] Standard protocol for the synthesis of Scaffold **E**. [B] Optimized protocol for the synthesis of Scaffold **E** consisting of a two-step process.

Upon reaction of Scaffold **E** with Peptides **1 - 8**, the desired bicycles were formed however, the isolated yields were a lot lower compared to all the other scaffolds tested (Table **2**).

Peptide Entry (sequence and loop size)	Scaffold E
1 (ACEKRVKACTHTYMVCA-NH_2) (6X6)	13 %
2 (ACEKRCVKATHTYMVCA-NH_2) (3X9)	24 %
3 (ACEKRVCKATHTYMVCA-NH_2) (4X8)	20 %
4 (ACEKCRVKATHTYMVCA-NH_2) (2X10)	13 %
5 (ACEKRVKCATHTYMVCA-NH_2) (5X7)	15 %
6 (ACKRVKACTHTYMCA-NH_2) (5X5)	21 %
7 (ACRVKACTHTYCA-NH_2) (4X4)	14 %
8 (ACVKACTHTCA-NH_2) (3X3)	18 %

Table 2: Yields of Bicycle Formation using Scaffolds **E** and Peptides **1 – 8**.

Conclusions

- **Scaffolds based on tris-Gold complexes are suitable for directing peptide cyclisation for the formation of novel Bicycles.**
- **Good reactivity was observed with peptides of varying lengths and loop sizes.**
- **The presence of amino acids with reactive groups within the sequence did not hinder the reaction.**
- **Notably, higher yields were obtained when electron-rich scaffolds were employed.**
- **The mild, biologically compatible conditions of this approach allows the possibility for it to be applied to the Bicycle® phage screening platform.**

References:
[1] Heinis et al. (2009) Nature Chemical Biology.
[2] Messina et al. (2018) Journal of the American Chemical Society.
[3] Stauber et al. (2021) Inorganic Chemistry.
[4] Mudd et al. (2022) Bioconjugate Chemistry.
[5] Mudd et al. (2020) Journal of Medicinal Chemistry.

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