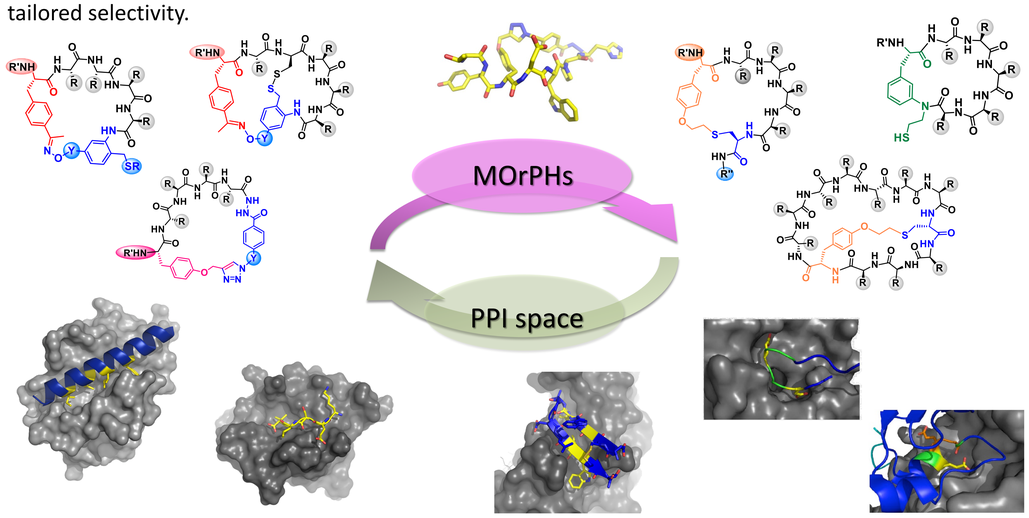
Expanded toolbox for directing the biosynthesis of macrocyclic peptides in bacterial cells

Jacob A. Iannuzzelli and Rudi Fasan*

Department of Chemistry, University of Rochester, Rochester, NY 14627. Email: jiannuz2@ur.rochester.edu

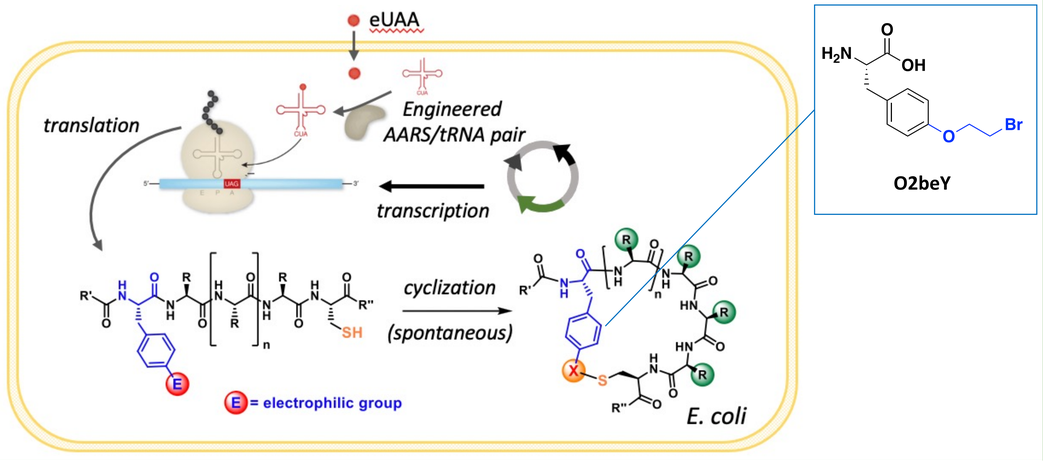
Macrocyclic Organo-Peptide Hybrids (MOrPHs)

Our group is interested in the development of new strategies to direct the synthesis, diversification, and evolution of Macrocyclic Organo-Peptide Hybrids (MOrPHs) as potent and selective modulators of protein-protein interactions (PPIs). MOrPHs are generated through the cyclization of ribosomally produced precursor peptides by means of genetically encoded non-canonical amino acids (ncAAs) and/or in combination with non-peptidic linkers.[1] These strategies couple the versatility of chemical synthesis with the power of genetic encoding and combinatorial mutagenesis, providing unique opportunities for the creation and functional exploration of large and structurally diverse libraries of peptide macrocycles. Our group is exploring the potential of this new class of peptide macrocycles for generating chemical agents capable of targeting and disrupting different types of protein-mediated interactions with high potency and tailored selectivity.



Thioether-bridged MOrPHs

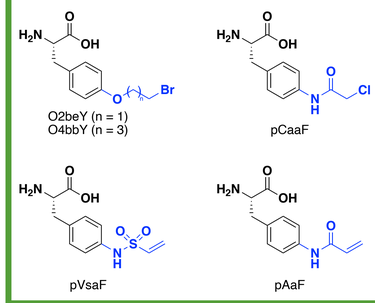
We have reported a versatile MOrPH synthesis method that relies on a spontaneous, chemoselective cross-linking reaction between an electrophilic unnatural amino acid (eUAA) and a proximal Cys residue, resulting in the formation of peptide macrocycles constrained by an inter-side-chain-to-side-chain thioether bridge.[2] We have primarily utilized the eUAA, *O*-(2-bromoethyl)-tyrosine (O2beY), as the peptide crosslinking agent. So far, however, only a few eUAAs are available for these applications, thus limiting opportunities for structural diversification of the resulting macrocyclic peptides through variation of the eUAA module.



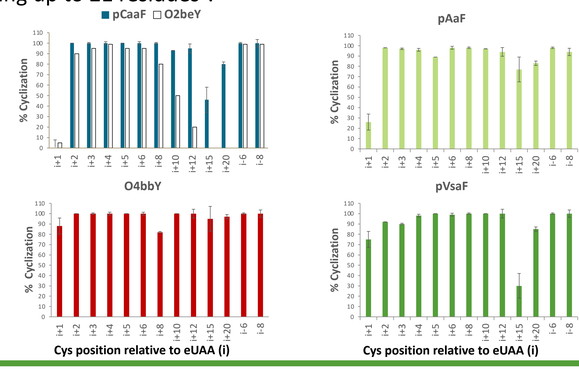
Expanded Toolbox of eUAAs

With the goal of expanding the range of macrocyclic peptide scaffolds accessible, we selected four target eUAAs to use with this methodology. Namely, *p*-(vinyl-sulfonamido) phenylalanine (pVsaF), *p*-(acrylamido) phenylalanine (pAaF), *p*-(2-chloroacetamido) phenylalanine (pCaaF), and *O*-(4-bromobutyl) tyrosine (O4bbY). We prepared a series of model polypeptides encompassing a macrocycle precursor sequence in which the eUAA and Cys residues are spaced from each other by an increasing number of intervening residues, leading to peptides ranging from an i/i+1 to i/i+ 20 macrocycle, where i and n correspond to the eUAA and Cys residue, respectively, along with two additional constructs with an inverted eUAA/Cys orientation (i.e. i/i-6 and i/i-8). These eUAAs are able to mediate long-range macrocyclizations as well as medium- and short-range ones, resulting in the efficient formation of peptide macrocycles spanning up to 21 residues[3].

electrophillic Unnatural Amino Acids (eUAAs)



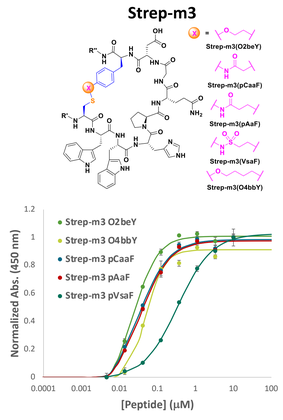
Name	Target Sequence	Cys
Z1C	(eUAA)CGSKLAEY	i + 1
Z2C	(eUAA)TCSKLAEY	i + 2
Z3C	(eUAA)TGCKLAEY	i + 3
Z4C	(eUAA)TGSCLAEY	i + 4
Z5C	(eUAA)TGSKCAEY	i + 5
Z6C	(eUAA)TGSKLCEY	i + 6
Z8C	(eUAA)TGSKLAEC	i + 8
Z10C	(eUAA)TGSHYLNAEC	i + 10
Z12C	(eUAA)TGSHIYLTNAEC	i + 12
Z15C	(eUAA)TGSHIYLTNAEGSAC	i + 15
Z20C	(eUAA)TGSHIYLTNAEGSAHITLTC	i + 20
C6Z	CTGSKL(eUAA)EY	i - 6
C8Z	CTGSKLAE(eUAA)	i - 8



Scaffold-dependent Effect on Bioactive Cyclic Peptides

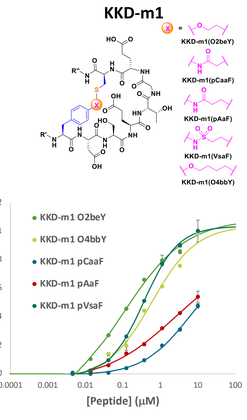
We envisioned the different eUAAs could also provide a means to tune the functional properties of the corresponding peptide macrocycles. To investigate this aspect, we selected and tested two bioactive cyclopeptides we have recently isolated from the screening of phage displayed libraries of O2beY-based macrocycles[4]. These correspond to Strep-m3, a (i/i-8)-linked macrocycle with nanomolar affinity for streptavidin (KD: 20 nM), and KDD-m1, a (i/i+ 7)-linked macrocyclic peptide capable of targeting Kelch-like ECH-associated protein 1 (Keap1) (KD: 107 nM). The different eUAAs had a profound and scaffold-dependent effect on the target binding properties, highlighting the value of this strategy toward modulating the protein recognition properties of a functional macrocyclic peptide[3].

Strep-m3



eUAA	Streptavidin K_D (nM)	Keap1 K_D (nM)	X Linker
O2beY	20 ± 1.3	107 ± 24	-O$(CH_2)_2$-
pCaaF	31 ± 3	>10,000	-NHCOCH_2-
pAaF	35 ± 3	>10,000	-NHCO$(CH_2)_2$-
pVsaF	343 ± 20	327 ± 23	-NHS$O_2(CH_2)_2$-
O4bbY	50 ± 3	1,200 ± 150	-O$(CH_2)_4$-

KKD-m1



Summary

We have developed an expanded methodology for directing the biosynthesis of thioether-linked macrocyclic peptides via a cysteine cross-linking reaction by means of electrophilic non-canonical amino acids. The peptide cyclization strategies reported here are expected to expand opportunities for the creation of structurally and functionally diverse libraries of peptide macrocycles which can be produced in bacterial cells and can be explored through functional assay or in combination with high-throughput display platforms. This methodology will facilitate the discovery of chemical agents capable of targeting and modulating biomolecular interactions.

References:
[1] Smith *et al.*, *ACIE* **2011** 50(22):5075; Satyanarayana *et al.*, *Chem. Comm.* **2012** 48(10):1461; Frost *et al.*, *Chembiochem* **2013** 14(1):147; Smith *et al.*, *Org. Biomol. Chem.* **2014** 12(7):1135; Frost *et al.*, *ACS Chem. Biol.* **2015** 10(8):1805. [2] Bionda *et al.*, *ACS Chem. Biol.* **2014** 9(9):2008; Bionda *et al.*, *Chembiochem* **2015** 16(14):2011; Owens *et al.*, *JACS* **2017** 139 (36): 12559; Fasan *et al*, US Patent No.10,544,191. [3] Iannuzzelli *et al.*, *Chem. Sci.* **2020**, 11, 6202-6208 [4] Owens *et al.*, *ACS Cent. Sci.* **2020**, 6, 368-381.

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