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STRUCTURAL FEATURES OF AN UNUSUAL SUNFLOWER PROALBUMIN PROTEIN THAT GIVES RISE TO A SMALL CYCLIC PEPTIDE AND A SEED STORAGE PROTEIN

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PawS-Derived Peptides are a novel diverse class of peptides that are found in the sunflower family. They are characterized by an exceptional stability, which makes them ideal candidates for peptide based drug discovery and protein engineering applications. Whereas they are known to be processed from seed storage albumins, their biosynthesis still remains to be fully defined. Here the 116 residue long precursor protein proalbumin PawS1 comprising both the sunflower trypsin inhibitor-1 (SFTI-1) peptide domain and the seed storage albumin domain was produced recombinantly in *E. coli* and enriched with ^{15}N and ^{13}C for NMR studies. Proalbumin PawS1 is post-translationally processed primarily by asparaginyl endopeptidases (AEP) to produce a head-to-tail cyclized SFTI-1 peptide and a mature seed storage albumin. The three-dimensional structure of proalbumin PawS1 reveals two well-defined entities separated by a flexible linker peptide "GLDN". ^{1}H chemical shifts of the SFTI-1 domain are consistent with native SFTI-1 and the albumin in the albumin domain adopts a classic compact helical bundle fold. We also recreate the first *in vitro* biosynthesis steps using the recombinant sunflower precursor protein proalbumin PawS1 and the recombinant sunflower AEP HaAEP1. The two flexible linker peptides "GLDN" and "LRMAVEN" of the proalbumin PawS1 are targeted by HaAEP1 during the albumin maturation to produce the SFTI-1 precursor peptide SFTI-1-GLDN and the cleaved seed storage albumin PawS1. This study provides new structural insights into PawS-Derived Peptides, seed storage albumins and the biological mechanism by which these precursor proteins are processed into their mature forms in the seeds of sunflower.

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DESIGN PRINCIPLES FOR COILED-COIL BASED MATERIALS DERIVED FROM THEIR MOLECULAR MECHANICAL PROPERTIES

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Natural proteins fold into three-dimensional structures that define their functions. One of the most abundant folding motifs, occurring in a variety of natural proteins, is the α-helical coiled-coil. In case of a dimeric coiled-coil motif, two α-helical strands wrap around each other to form a super helical structure. Each α- helical strand is composed of heptad repeats, denoted as ***abcdefg***. Folding and dimerization is driven by this specific heptad pattern where hydrophobic residues at ***a*** and ***d*** positions are internalized and stabilize the dimer structure. Additional stability is contributed by salt bridges between the ***e*** and ***g*** positions. Coiled-coil structures are involved in structural and mechanical tasks and many of the key protein components of the cytoskeleton and the extracellular matrix (ECM) contain coiled-coil building blocks. Considering their crucial mechanical function in Nature and their application potential as ECM mimics, surprisingly little is known about their mechanical properties at the molecular level. With the goal of shedding light on the structure-to-mechanics relationship of coiled-coils, we are applying single molecule force spectroscopy to obtain the force threshold for coiled-coil dissociation. We are currently investigating the effects of sequence, length and pulling geometry on coiled-coil stability via atomic force microscopy (AFM). Using a cysteine-based immobilization method, we are able to immobilize the individual monomers site-specifically at their termini. Depending on the position of the terminal cysteine, we are able to mechanically load the coiled-coils either in shear or unzip geometry. In a first set of experiments, we have analyzed the effect of the sequence length on the rupture forces of heterodimeric coiled-coil motifs consisting of 4 and 3 heptad repeats. We show that the mechanical stability of the coiled-coils differs significantly depending on the sequence length. The most probable rupture forces for these relatively short 4 (CC-A_4B_4) and 3 (CC-A_4B_3) heptad coiled-coils were ~48 pN and ~31 pN, respectively **(Figure 1)**.

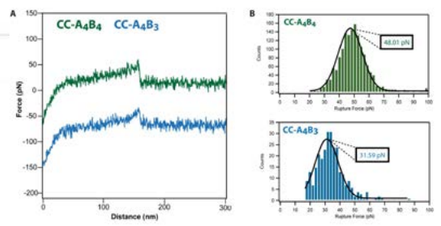

Figure 1. AFM characterization of CC-A_4B_4 and CC-A_4B_3. A) Examples of typical force-extension curves. **B)** Rupture force histograms of CC-A_4B_4 and CC-A_4B_3.

To provide more insights into the coiled-coil mechanics, we are now investigating the effect of site-specific mutations under different force application geometries. This knowledge obtained from single molecule force spectroscopy aids the future design of novel sequences and yields important new input for the assembly of coiled-coil based materials. As cell-generated forces can easily reach to tens of piconewtons, it is absolutely crucial that a newly designed coiled-coil based ECM mimic can withstand these forces. We believe that the coiled-coil length and sequence and, more importantly, the pulling geometry are crucial parameters to ensure a sufficient mechanical stability that is able to support cell growth and differentiation.

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PEPTIDE STAPLING BY SUZUKI-MIYAURA CROSS-COUPLING

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Stapled peptides are potential drugs against diseases such as cancer, infections or neurological disorders in which protein-protein interactions related to signal transduction are involved. In particular, helical secondary structures are predominant in those interactions. This structural motif can be stabilized by means of peptide stapling such as the all-hydrocarbon approach developed by Schafmeister *et al*. Cross-linking based on ring-closure metathesis (RCM) was utilized to generate peptides with increased helicity, membrane permeability and resistance against proteolytic digest.[1] These features gave rise to continuing studies by Bernal *et al*. showing peptides with higher biological activity compared to native ones.[2,3]

C5-, C6- and C7-regioisomers of bromotryptophan are available on a preparative scale using tryptophan halogenases.[4,5] This biocatalytic procedure was used by Frese *et al.* for the one-pot approach of consecutive enzymatic halogenation of tryptophan and derivatization by Suzuki-Miyaura reaction.[5] The intramolecular Suzuki-Miyaura cross-coupling between bromotryptophan and a suitable arylboronic acid provides a novel approach to peptide stapling (Figure 1). A main advantage of this Pd-catalyzed bioorthogonal reaction is the selective derivatization in presence of a broad range of functional groups in biomolecules. Furthermore bromotryptophan and arylboronic acids based on aromatic amino acids such as phenylalanine or tyrosine are derivatives of proteinogenic amino acids which probably leads to a low influence on the biological activity.

Figure 1: Peptide stapling by intramolecular Suzuki-Miyaura cross-coupling of bromotryptophan and an arylboronic acid.

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THE O-CARBAMOYL-TRANSFERASE ALB15 IS RESPONSIBLE FOR THE MODIFICATION OF ALBICIDIN.

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Albicidin is a potent antibiotic and phytotoxin produced by the sugarcane pathogenic bacterium *Xanthomonas albilineans* which targets the plant and bacterial DNA gyrase.[1] The infection occurs through the xylem vessels of the plants where the pathogen induces chlorosis which is characterized by white-pencil streaks.[2] Albicidin is encoded in the alb gene cluster, a polyketide synthase non-ribosomal peptide synthetase hybrid (PKS-NRPS). In addition to the PKS-NRPS megasynthetase machinery their existing several tailoring and post-NRPS proteins, whose function in connection with the processing of albicidin is unclear.[3] We could characterize a new albicidin derivative with a combination of tandem mass spectrometry, in vitro and knockout experiments from cultures of a Xanthomonas overproducer

which is carbamoylated at the N-terminal coumaric acid. The putative ATP-dependent O-carbamoyltransferase Alb15, present in the biosynthesis gene cluster of albicidin, is responsible for the derivatization. Cloning and Expression of Alb15 in Escherichia coli and in vitro reconstitution of the carbamoyltransferase activity confirmed albicidin as the target substrate. The chemical synthesis of carbamoyl-albicidin enabled us to carry out bioactivity assays by means of in vitro gyrase inhibition and antibacterial assays. Carbamoyl-albicidin showed a significantly higher inhibitory efficiency against bacterial gyrase (□8 nM vs 49 nM) then albicidin, which identifies the carbamoyl group as an important structural feature of albicidin maturation.[4]

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A SINGLE-CHAIN DERIVATIVE OF THE RELAXIN HORMONE IS A FUNCTIONALLY SELECTIVE AGONIST OF THE G PROTEIN-COUPLED RECEPTOR, RXFP1

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Human gene-2 relaxin (H2 relaxin) is a pleiotropic hormone with powerful vasodilatory and anti-fibrotic properties which has led to its clinical evaluation and provisional FDA approval as a treatment for acute heart failure. The diverse effects of H2 relaxin are mediated *via* its cognate G protein coupled-receptor (GPCR), Relaxin Family Peptide Receptor (RXFP1), leading to stimulation of a combination of cell signalling pathways that includes cyclic adenosine monophosphate (cAMP) and extracellular-signal-regulated kinases (ERK)1/2. However, its complex two-chain (A and B), disulfide-rich insulin-like structure is a limitation to its facile preparation, availability and affordability. Furthermore, its strong activation of cAMP signaling is likely responsible for reported tumor-promoting actions that may preclude long-term use of this drug for treating human disease. Here we report the design and synthesis of a H2 relaxin B-chain-only analogue, B7-33, which was shown to bind to RXFP1 and preferentially activate the pERK pathway over cAMP in cells that endogenously expressed RXFP1. Thus, B7-33 represents the first functionally selective agonist of the complex GPCR, RXFP1. Importantly, this small peptide agonist prevented or reversed organ fibrosis and dysfunction in three pre-clinical rodent models of heart or lung disease with similar potency to H2 relaxin. The molecular mechanism behind the strong anti-fibrotic actions of B7-33 was shown to involve activation of RXFP1-angiotensin II type 2 receptor heterodimers that induced selective downstream signaling of pERK1/2 and the collagen-degrading enzyme, matrix metalloproteinase (MMP)-2. Furthermore, in contrast to H2 relaxin, B7-33 did not promote prostate tumor growth *in vivo*. Our results represent the first known example of the minimisation of a two-chain cyclic insulin-like peptide to a single-chain linear peptide that retains potent beneficial agonistic effects.[1]

1. Hossain et. al ***Chem. Sci.***, 2016; Advance Article **DOI:** 10.1039/C5SC04754D

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DEVELOPMENT OF HYBRID BIOOLIGOMERS WITH PNA RECOGNITION UNITS TO MIMIC THE ACTION OF SNARE PROTEINS

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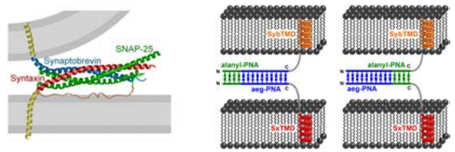
SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins) are regarded as key players in membrane fusion. Located in both sites of opposite membranes, they interact via characteristic recognition motifs bringing membranes into close proximity and eventually leading to fusion.[1] As the exact fusion mechanism is still a matter of debate, the development of model systems with reduced structural complexity is an excellent method to examine distinct steps of the fusion process.

We developed hybrid biooligomers that mimic the SNARE action *in vitro*. They consist of the native transmembrane domains and linker regions of either syntaxin-1A or synaptobrevin-2, two SNAREs of the neuronal exocytosis machinery, and are equipped with an artificial recognition unit. These units are designed as complementary strands of peptide nucleic acid (PNA) which enables precise adjustment of strand orientation, stability, and sequence dependent recognition.[2] In order to mimic the assumed zippering process of the SNARE assembly, the recognition unit consists of two different types of PNA, namely aeg-PNA (N-(2-aminoethyl) glycine-PNA) and alanyl-PNA, which provide contrasting duplex formation rates and thus should set a directionality of complex formation.

Different PNA combinations were tested and melting curve analyses as well as circular dichroism spectroscopy showed that in PNA hybrid oligomers both PNA types clearly contributed to duplex formation. The PNA/peptide hybrid biooligomers were synthesized by means of continuous Fmoc-based solid phase peptide synthesis following an adjusted protocol for the attachment of the PNA monomers. Their fusion capability was examined via FRET (Förster resonance energy transfer) based lipid mixing assays by subsequent incorporation into liposomes. Together with fluorophore dequenching assays, these methods allowed us to distinguish between different stages of the vesicle fusion process.



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DESIGN OF HIGHLY ACTIVE LIGANDS FOR NICOTINIC RECEPTORS ON THE BASIS OF A - CONOTOXIN PNIA

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This study is focused on developing selective and potent α7 nAChR ligands, based on α - conotoxin PnIA.

Applying new Protein Surface Topography (PST) technique to the set of previously produced PnIA analogs with known activities, we were able to identify three positions, where introduction of positive residues could increase ligand's affinity (positions 5, 9, 14). Mutation [Ala10Leu] was also added to experiment, as it is known to improve the target affinity. Three new PnIA analogs were synthesized, containing one (PnIA[R9]), two (PnIA [R9, L10]) and four (PnIA [R5, R9, L10, R14]) substitutions.

Their ability to interact with human α7 nAChR was tested by radioligand binding assay in competition with radioactive α-bungarotoxin ([^{1255}I] - Bgt). This test showed that PnIA [R9,L10] is the most active competitor, with IC_{50} = 270 ± 10 nM. Noteworthy, IC_{50} = 670 ± 10 for PnIA [R5, L10, R14] was our best result obtained in our previous assays.

We also performed radioligand tests to measure the ability of novel α-conotoxin analogs to interact with *A. californica* and *L. stagnalis* acetylcholine-binding proteins (AChBPs), as these proteins are structurally very close to the ligand-binding domain of α7 nAChR. PnIA [R5, R9, L10, R14] was the most active in both cases, with IC_{50} = 24 ± 2 nM for *A. californica* and 1.22 ± 0.04 nM for *L. stagnalis* AChBPs; earlier the best was PnIA[L10,K14], with IC_{50} = 8.2 ± 1.2 nM) for *L. stagnalis* AChBP.

For better quantitative estimation of new PnIA analogs activities, another test was conducted. This time their activities were measured by electrophysiology according to their ability to inhibit the acetylcholine induced current in α7 nAChR heterologously expressed in the *Xenopus laevis* oocytes. All examined analogs efficiently blocked the currents, with the IC_{50} values ranging from 17 to 25 nM. These values were significantly smaller than the IC_{50} = 260 nM for PnIA [L10, K14], earlier the most active analog according to electrophysiology.

Thus, by the PST method we created three highly active analogs of α-conotoxin PnIA. It was enough to add only one positively charged residue to of the 9 positions , suggested by PST to reach maximum activity. While the activities of all analogs are roughly similar, some substitutions resulted in very low dissociation rates making the respective analogs promising for α7 nAChR detection in tissues.

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A-BUNGAROTOXIN AND A-COBRATOXIN, SIMILAR IN BINDING TO NICOTINIC RECEPTORS, DIFFER GREATLY IN THE AFFINITY FOR C-LOOP MODELS

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High affinity (low nM) for nicotinic acetylcholine receptors (nAChRs) of such snake venom α-neurotoxins as α-bungarotoxin (aBgt) or α-cobratoxin (aCtx) has made them invaluable pharmacological tools. Although with a considerably lower affinity, αBgt binds the C-loop fragments of α1 and α7 subunits . Since peptide HAP from a combinatorial library