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Cooperation

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Project acronym: SYNPEPTIDE

Project title: **Synthetic Biology for the production of functional peptides**

Figures for Project Final Report
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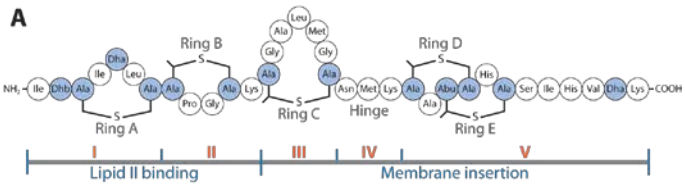


B

Group	Peptide	I	II	III	IV	V
Nisin group	Nisin A	ITSISLC	TPGCK	TGALMGC	NMK	TATCNCSIHVSK
	Subtilin			WKSESLC	FLQ	
	Gallidermin	IASKFLC	TPGCA		KTG	
Epidermin group	Mutacin B-Ny266			FKSWSFC		
Pep5 group	Pep5		SVKQCQ	KTLKAT	RLF	TVSCKGKNGCK
	Epicidin 280		TRQVCP			
	Epilancin K7			SKKYC	KGV	TLTCGCNITGGK
	Paenibacillin		TTIKV	SKAVC		TLTCICTGSCSNCK
Lacticin 481 group	Lacticin 481			KGGSGVIH		
Mersacidin group	Actagardine			SSGWVC		
Cinnamycin group	Lactocin S			SDVAGC	FKY	SAKHHC
Two component	Haloduracin a		TLTVEC			
Synthetic group	Empty			------		
	Synthetic 1				NMKK	
	Synthetic 2				NMKV	
	Synthetic 3				NMS	
	Synthetic 4				G	

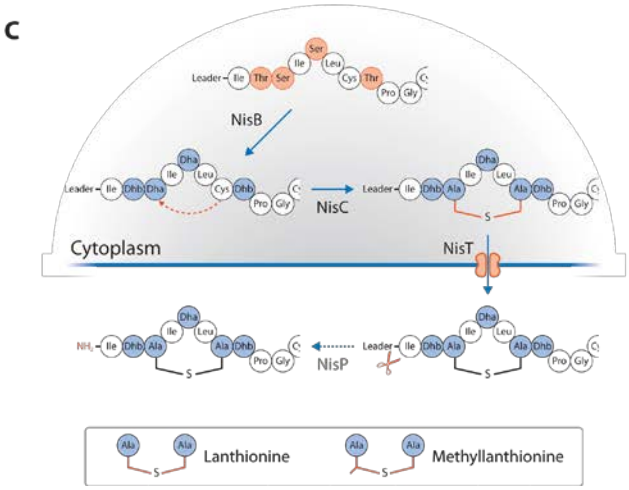


Fig. 1. Modular features and PTMs of lantibiotic peptides. (**A**) Structure of the prototype lantibiotic nisin A with its native ring structure (A to E) and the corresponding module nomenclature scheme (module positions I to V) including functional assignment: Rings A and B (modules I and II) are responsible for lipid II binding and the rest of the molecule for insertion into the cytoplasmic membrane. (**B**) Similar modular structures were identified in 12 natural lantibiotics and used to define a map of 28 natural peptide modules. The set was further supplemented by 4 non-natural modules (to elongate or truncate the hinge-region of the peptides) and an empty ('-----') module at position III to allow for module skipping. Modular recombination at the DNA level then allowed for the synthesis of gene for new-to-nature peptides. Note that the dashed lines on top of the modules indicate the thioether-bridges found in the natural peptide and that the amino acid sequence for each module refers to the genetically encoded amino acid, not the amino acid that is present after modification. Hypothetical rings (expected but not yet experimentally confirmed) are marked with an asterisk. (**C**) For the formation of ring-structures in the combinatorial peptides, three enzymes of the post-translational modification machinery of Nisin were employed. To direct the non-modified prepropeptides synthesized at the ribosome to the modification machinery, they are produced as fusions to the nisin-specific leader peptide. The NisB is used to dehydrate serine and threonine residues to dehydroalanine or dehydrobutyrine, respectively. The NisC catalyzes the ring-formation *via* a cysteine-residue to the dehydroamino acids. NisT secretes the peptide to the extracellular space.

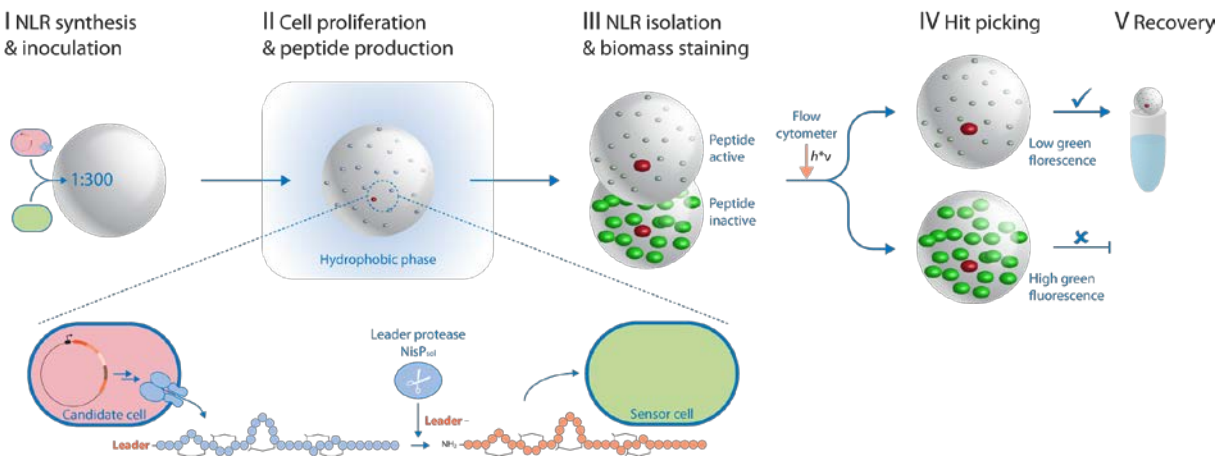


Fig. 2. *Workflow of nanoFleming screening for antimicrobial peptides. (I) Candidate (prenisin-secreting L. lactis NZ9000 [pIL3BTC, pNZE3-library-mcherry]) and sensor (M. flavus) cells are embedded in NLRs such that a single candidate cell (realized as approx. 0.5 cells NLR^{-1} by limiting dilution) is exposed to an excess of sensor cells (approx. 150 cells NLR^{-1}). Note that the candidate strain is additionally equipped with the gene for the red fluorescent protein mCherry to facilitate its identification during later steps. (II) The NLRs are then soaked with growth-medium and incubated in a hydrophobic phase to limit leakage of peptides and crosstalk between reactors. Prior to transfer to the hydrophobic environment, peptide-production is induced and soluble NisP is added to allow for leader-cleavage. During incubation, the prepeptide is secreted within the NLR and can distribute throughout the matrix of the NLR. Upon contact with the leader protease NisP, the prepeptide will be cleaved, thus releasing the activated antimicrobial peptide. Once an activated peptide reaches a sensor cell it can interact with its target and inhibit the growth of the strain to a microcolony. (III) After incubation for 18 h, NLRs are extracted into an aqueous environment and the total biomass within the NLRs is stained by the addition of the DNA-intercalating green fluorescent dye SYTO 9. NLRs that contained a candidate strain able to produce a peptide with antimicrobial activity towards the embedded sensor-strain display lower sensor biomass-levels as compared to NLRs that did not harbor a producer of a bioactive peptide. (IV) To analyze and isolate NLRs, large particle flow-cytometry is employed. NLRs with low biomass levels are isolated and (V) the embedded candidate-cells are recovered by dissolving the NLR matrix and re-growing cells under selective conditions.*

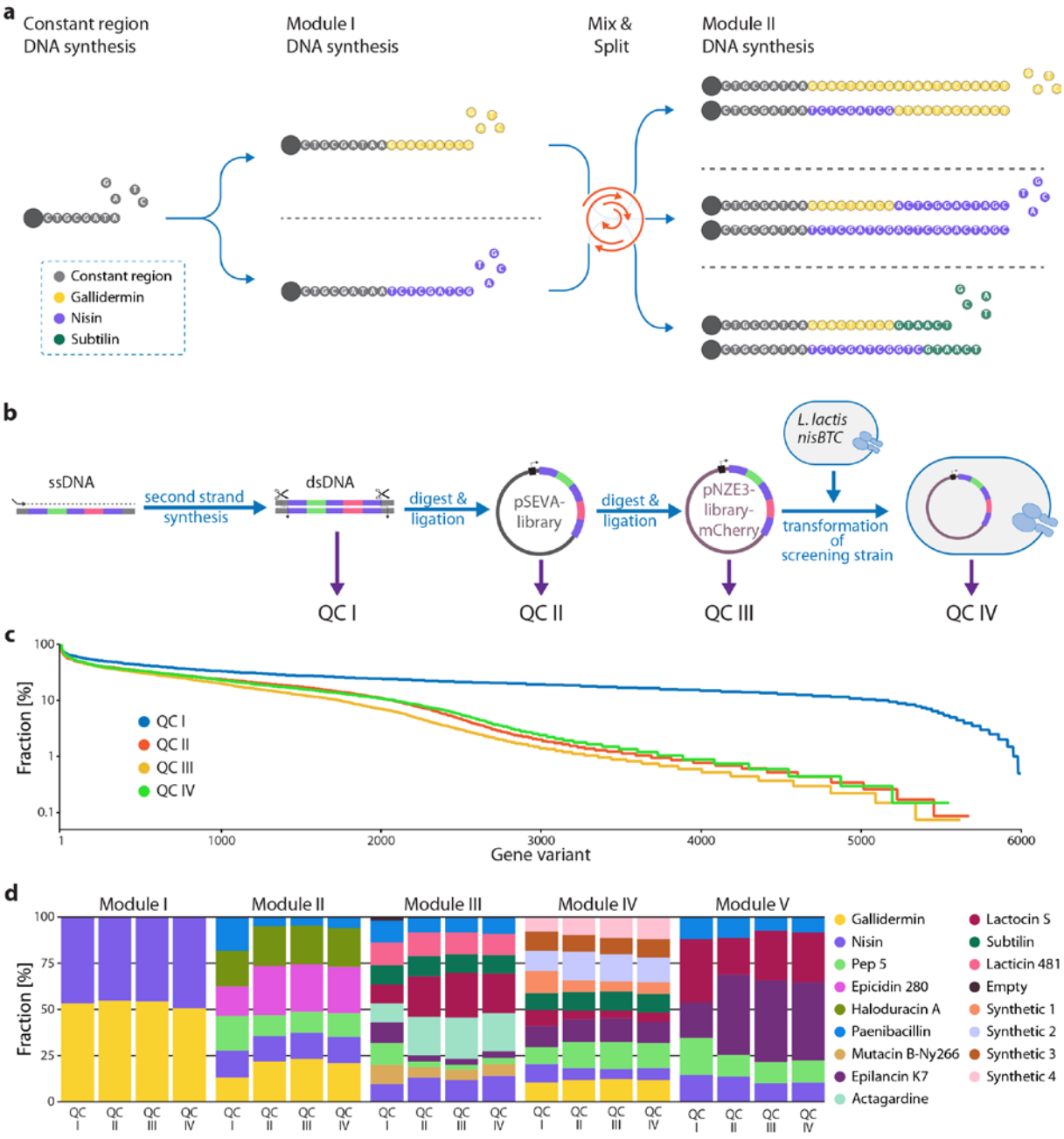


***Figure 3** | Synthesis, cloning and QC of a combinatorial DNA library.*

*(**a**) Synthesis scheme for a combinatorial oligonucleotide library using a mix-and-split approach. The library was generated using chemical solid phase synthesis on controlled pore glass (CPG) combined with a mix-and-split approach. We performed DNA synthesis of the minus strand of the combinatorial genes in on pot. The synthesis starts with the first module, a conserved region among the whole library (grey). This region contains restriction sites later used for cloning. After the synthesis of this constant region, the synthesis batch is, while still bound to the CPG, split up and the oligonucleotide is elongated by the synthesis of the variants of module position I in separate batches (yellow and violet, corresponding to the first modules of the library, gallidermin and nisin A). Next the batches are pooled, mixed and separated again for the synthesis of module position II (yellow, violet, green). The procedure is continued until the last module is reached - which is again a constant region facilitating cloning. (**b**) The cloning of the combinatorial library involves several steps. First, the oligonucleotide is released from the CPG and the second strand (plus strand) of the library is synthesized using an oligonucleotide complementary to the library and binding at its 3' end. After that, the double strand DNA is cleaved and ligated in a subcloning plasmid (see Methods) and proliferated in E. coli. The amplified plasmid is then isolated and the library-fragments are excised via restriction digest, cloned into the expression plasmid (see Methods) and proliferated in E. coli again. In a last step, the plasmid is isolated from E. coli and transferred into the screening host L. lactis NZ9000 [pIL3BTC]. Every step of the*

protocol can result in an introduction of errors in the library (such as a shift in the modular distribution or in accumulation of mutations). We therefore included quality control (QC) of the library-composition at every processing step (indicated as QC I to QC IV). ***(c)*** *The quality of the library is assessed via next generation sequencing with an Illumina MiSeq platform. After sequencing, every possible module-combination is counted, normalized to the variant with the highest abundance and plotted. Whereas for the double stranded oligonucleotide at QC I 5,997 of 6,000 (99.95 %) module combinations were found at least once (at a NGS coverage of 44-fold) for the library in the last cloning step and after the transfer to L. lactis in QC IV yielded in 5,552 of 6,000 (92.5 %) module combinations with counts > 0 (at a NGS coverage of 68-fold). Moreover, the results indicate a shift in the distributions of the module combinations towards the under- and overrepresentation of certain variants. (****d****) Next the distribution for each module was extracted. Whereas in QC I each module position seems to be evenly occupied by every possible module (with except to the 'empty' module position at position III), during the first cloning step and detected at QC II, the modular composition shifts such that certain module-combinations are under- and overrepresented. However, still all modules were detected and are present in the library and therefore qualifies the library for screening.*

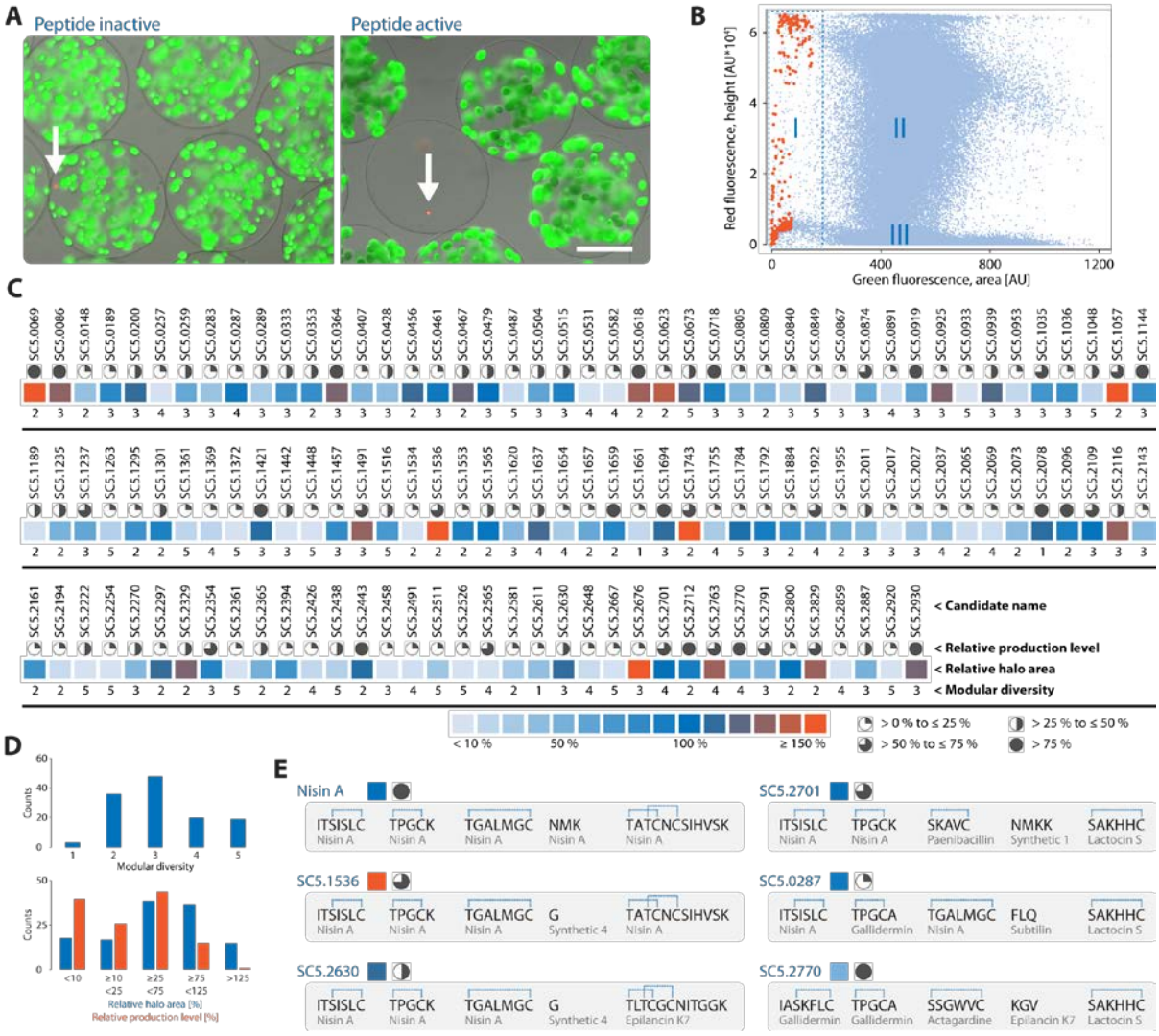


Fig. 4. *Isolation and activity-verification of combinatorial peptides.* ***(A)*** *Microscopic images of NLRs after incubation. Images were generated by the overlay of bright-field and epifluorescence pictures using the GFP and mCherry filter-set. In cases where no or inactive peptides were secreted by the candidate colony (red spot) the sensor cells can grow to microcolonies (green spots, left image). In cases where a potent antimicrobial peptide was secreted no or low microcolony growth of the sensor is observed, yielding in a NLR almost completely cleared from sensor-biomass (right image). Scale bars: 200 µm.* ***(B)*** *The assay was applied to analyze the bioactivity of a combinatorial peptide library consisting of approx. 5,200 variants (as estimated from library quality control, see Supplementary Fig. 1). A total of 3.2 x 10^5 NLRs (blue dots), inoculated with 1.1 x 10^5 (18-fold oversampling) candidate cells were incubated. After incubation, the red fluorescence levels indicated the presence and size of a candidate colony within the NLR (I+II = colony present; III = no colony present) whereas the green fluorescence levels indicated the sensor-biomass levels within the NLR (I = low sensor biomass levels; II+III = high sensor biomass levels). A total of 840 NLRs displaying low green fluorescence levels together with small to large candidate colonies was isolated using large particle flow cytometry. Of those, 617 candidate strains could be regrown (73.5 %). A subset of 326 candidates (0.3 % of the initial candidate population, orange dots) were selected for further analysis.* ***(C)*** *Activity and production profile of combinatorial peptide variants. We identified 126 monoclonal candidates that could be produced and showed halo-formation when tested against the screening strain M. flavus. Modular diversity indicates the number of different natural module origins and thus the degree of module-shuffling for each of the peptides.* ***(D)*** *Candidates isolated were assembled from modules originating from 1 to 5 different natural peptides where most peptides had 3 different module origins. The majority of the peptides showed at least 25 % of relative halo area when compared to nisin A. Production levels were usually lower and many peptides displayed production-levels < 10 % of the prenisin A control.* ***(E)*** *Examples of isolated, bioactive peptide candidates. We observed peptides that where constructed mainly following a building plan of a*

wild-type peptide but with minor changes in one of the module (i.e. SC5.1536) or changes at the C-terminus of the peptide (i.e. SC5.2630). Other peptides were created from more intensive module-shuffling (i.e. SC5.2701, SC5.0287 or SC5.2770).

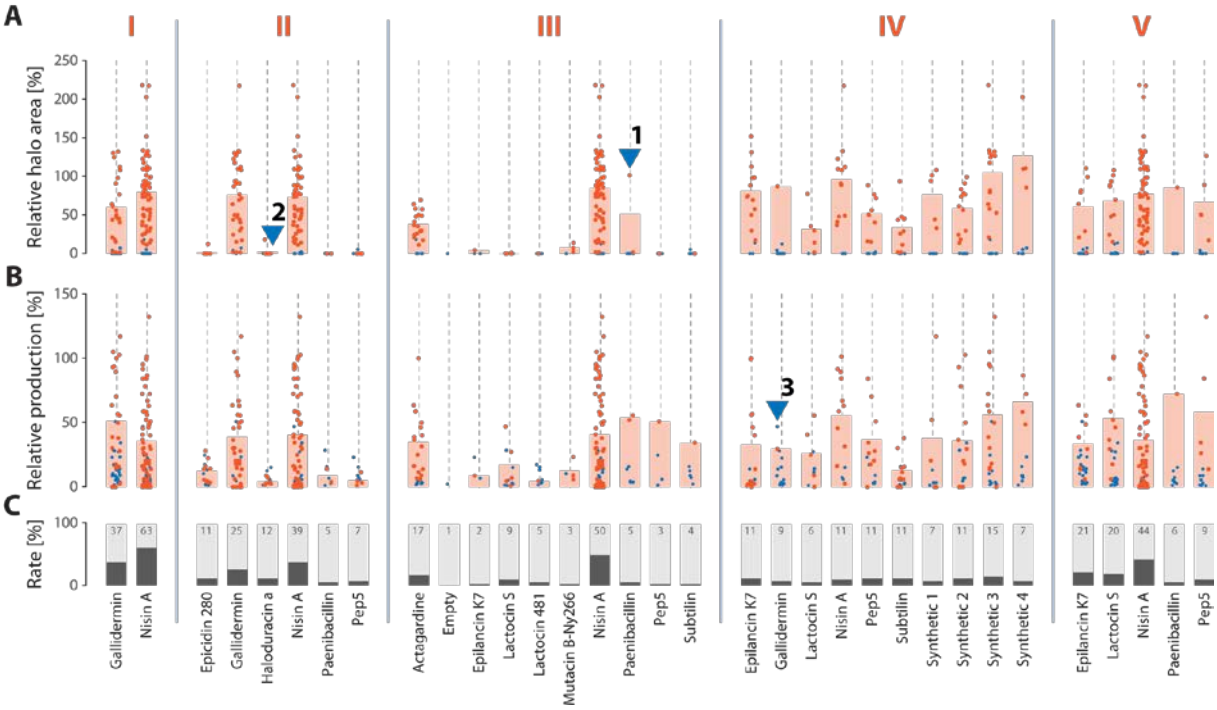
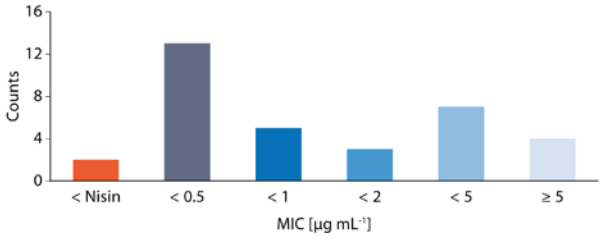


Fig. 5. *Design rules for bioactive combinatorial peptides. Isolated candidates were analyzed for their modular composition to elucidate design rules for a combinatorial assembly that leads to the secretion of bioactive peptides. 204 peptides (including ones that did not show any bioactivity) were imbedded in this analysis. Every dot in the panel corresponds to the relative halo area (**A**) or relative production level (**B**) of one single peptide. Furthermore, and as we are interested in the module-contribution of this data, we split up every peptide into its modular composition, such that each peptide is represented five times with the same halo and leader values, in each of the columns, representing a module position. For example, the data-point labeled with '1' (blue arrow), corresponds to the modular composition of I: Nisin A, II: Nisin A, III: Paenibacillin, IV: Synthetic 1, V: Lactocin S, has a relative halo area of 101 % and a relative leader area of 52 %. In panel (a), the value 101 % of this peptide is therefore present in each of the 5 module columns (I to V). The same is the case for the leader data in panel (b). From this data we define 'allowed' modules that lead to bioactive peptides. In the example from above, the arrow (1) points to module III: Paenibacillin. As Paenibacillin here obviously has led to an active peptide, we defined this module, independent from its context, as 'allowed'. Another example is the data-point labeled with '2', the modular composition is I: Gallidermin, II: Haloduracin a, III: Nisin A, IV: Synthetic 4, V: Nisin A (rel. halo: 0 %, rel. leader: 7 %). The module under investigation is as indicated by the arrow (2) module II: Haloduracin a; as this module did not lead to the secretion of a bioactive peptide, Haloduracin a at module position II is a 'forbidden' module. This analysis is done for all module-positions of a peptide. To highlight the context of a peptide, we introduced a color-coding. If the peptide under investigation has a context (modules outside of the module-position under investigation) that contains 'allowed' modules only, the data-points are colored in orange, whereas data-points from peptides that contain a 'forbidden' module in its context, are colored in blue. For example, the data-point (3) has the composition I: Nisin A, II: Nisin A, III: Lactocin S, IV: Gallidermin, V: Nisin A (rel. halo: 0 %, rel. leader: 47 %) and contains the 'forbidden' module of Lactocin S at position III (no bioactivity detectable) and is therefore presented as a blue dot despites its good production level. Note that the color-code for the relative leader levels and for the relative halo areas are the same as for both the definition of 'allowed' of 'forbidden' is based on the halo-areas. Based on that data, we can define the following modules as 'allowed': module A: Gallidermin, Nisin A; module B: Gallidermin, Nisin A; module C: Actagardine, Nisin A, Paenibacillin; module D: all possible modules; module E: all possible modules. (**C**) The fraction of each module among all peptides used for analysis is given in dark grey bars. The numbers correspond to the percentage. In this case, a large percentage indicates that this module was found more often during nanoFleming screening.*

A

ID	Variants	Module I	Module II	Module III	Module IV	Module V	MIC
SC5.0069	2	Nisin	Nisin	Nisin	Epilancin K7	Nisin	0.27
SC5.0086	2	Gallidermin	Gallidermin	Nisin	Synthetic 3	Nisin	0.59
SC5.0189	1	Nisin	Nisin	Nisin	Synthetic 3	Epilancin K7	3.37
SC5.0333	1	Nisin	Nisin	Nisin	Pep 5	Other	0.49
SC5.0364	2	Nisin	Nisin	Nisin	Synthetic 3	Pep 5	0.44
SC5.0456	1	Nisin	Nisin	Nisin	Epilancin K7	Nisin	0.41
SC5.0479	3	Nisin	Nisin	Nisin	Synthetic 2	Epilancin K7	1.03
SC5.0487	1	Nisin	Gallidermin	Actagardine	Epilancin K7	Pep 5	3.82
SC5.0515	1	Nisin	Gallidermin	Nisin	Gallidermin	Lactocin S	3.03
SC5.0531	3	Gallidermin	Gallidermin	Mutacin B-Ny266	Synthetic 4	Epilancin K7	0.55
SC5.0718	2	Nisin	Nisin	Nisin	Pep 5	Lactocin S	0.45
SC5.0925	2	Nisin	Gallidermin	Nisin	Synthetic 3	Nisin	0.87
SC5.0953	1	Gallidermin	Nisin	Nisin	Synthetic 2	Nisin	0.08
SC5.1048	2	Gallidermin	Nisin	Actagardine	Synthetic 3	Epilancin K7	14.1
SC5.1237	1	Nisin	Nisin	Actagardine	Synthetic 3	Nisin	2.08
SC5.1421	1	Gallidermin	Gallidermin	Nisin	Synthetic 1	Nisin	0.21
SC5.1457	2	Nisin	Nisin	Nisin	Synthetic 3	Epilancin K7	0.06
SC5.1491	2	Nisin	Gallidermin	Nisin	Epilancin K7	Nisin	5.11
SC5.1516	1	Nisin	Gallidermin	Actagardine	EpilancinK7	Lactocin S	2.90
SC5.1536	2	Nisin	Nisin	Nisin	Synthetic 4	Nisin	0.59
SC5.1620	1	Nisin	Gallidermin	Nisin	Synthetic 1	Nisin	0.33
SC5.1659	2	Nisin	Nisin	Nisin	Synthetic 2	Nisin	0.14
SC5.1743	4	Nisin	Nisin	Nisin	Synthetic 3	Nisin	0.14
SC5.1922	2	Gallidermin	Gallidermin	Actagardine	Synthetic 3	Epilancin K7	33.8
SC5.2096	1	Nisin	Nisin	Nisin	Nisin	Pep 5	0.18
SC5.2116	2	Gallidermin	Gallidermin	Nisin	Synthetic 3	Nisin	0.75
SC5.2354	1	Nisin	Nisin	Nisin	Synthetic 4	Paenibacillin	2.95
SC5.2630	2	Nisin	Nisin	Nisin	Synthetic 4	Epilancin K7	2.29
SC5.2701	1	Nisin	Nisin	Paenibacillin	Synthetic 1	Lactocin S	1.49
SC5.2712	1	Gallidermin	Gallidermin	Nisin	Nisin	Nisin	0.13
SC5.2829	1	Nisin	Gallidermin	Nisin	Nisin	Nisin	0.15
SC5.2930	1	Gallidermin	Nisin	Nisin	Synthetic 3	Nisin	0.04
SC5.9487	2	Gallidermin	Epicidin 280	Paenibacillin	Pep 5	Nisin	1.14
SC5.9925	3	Nisin	Haloduracin A	Lactocin S	Synthetic 4	Epilancin K7	73.6



B

ID	*S. pneumoniae* TIGR-4	*S. pneumoniae* D39	*S. aureus* CAL (MRSA)	*S. aureus* MW2 (MRSA)	*E. faecalis* (VRE)	*B. cereus*	*E. faecium* (VSE)
SC5.1421	2.01	2.29	3.73	2.80	1.40	44.80	11.20
SC5.2712	0.53	0.42	7.36	38.90	7.36	37.85	7.36
SC5.2930	0.84	1.13	26.97	17.98	290.0	26.97	6.74
Gallidermin	0.90	1.81	11.30	13.56	27.12	37.29	54.25
Nisin	4.21	4.21	9.86	10.91	6.68	12.19	7.72

C

ID	NZ9000	NZ9803	Immunity
SC5.1536	0.13	3.60	27
SC5.2930	0.12	1.90	16
SC5.0925	0.03	4.00	128
SC5.1659	0.80	12.00	16
SC5.2712	0.05	1.30	27
Nisin	0.03	30.90	1024

D

ID	NZ9000 [pEmpty]	NZ9000 [pNSR]	Resistance
SC5.0515	4.00	4.00	1
SC5.2096	0.50	2.00	4
SC5.1457	0.25	0.50	2
Nisin	0.05	> 50	> 1000

Fig. 6. *Minimal inhibitory concentrations (MICs) of combinatorial peptides. (A) Peptide candidates were HPLC-purified and the activity of 34 peptides was tested against the screening-strain M. flavus. Note that the PTM might results in differently modified peptides (e.g. 7 vs. 8 dehydrations of the peptide). Different modification patterns result in several variants of a peptide with the very same module-combination. Those variants were separated by HPLC, the number of variants observed is given in the corresponding column, and only the MIC value of the most potent variant is listed. (B) MIC values of a selected set of combinatorial peptides against a panel of 7 Gram-positive model pathogens. MIC-values that are improved in comparison to the wild type peptides nisin and gallidermin are highlighted in orange. (C) MIC values of a selected set of combinatorial peptides against a strain carrying the nisin immunity cluster (L. lactis NZ9803) and the comparison to a strain that does not carry the cluster (L. lactis NZ9000). (D) MIC values of a selected set of combinatorial peptides against a strain overexpressing the nisin resistance protein (NZ9000[pNSR]) and the comparison to a strain that carries an empty plasmid only (NZ9000[pEmpty]).*

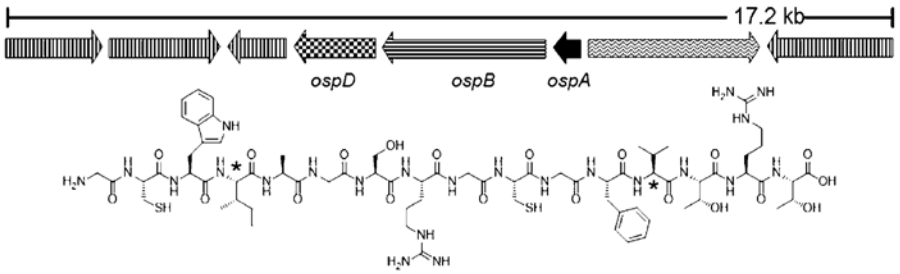


***Fig. 7**, adapted from Morinaka et al, Angew. Chem. Intl Ed. 53 (2014): 8503. Gene cluster containing radical SAM-epimerases. Asterisks denote sites of epimerizations.*

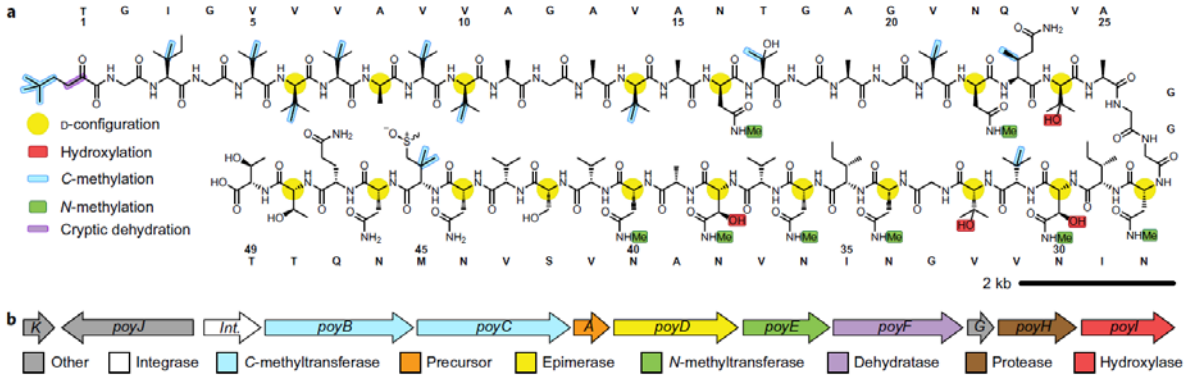


***Fig. 8**, adapted from Freeman et al., Nature Chemistry 9 (2017): 387. a) Shown is the secondary structure of polytheonamides A and B (differing in sulfoxide configuration of Met45) and the different PTMs introduced into the RiPP. b) Structure of the poy gene cluster, color coding in aligment with a).*

Table 1. *Hybrid leaders fused to natural core peptides. Peptides 1, 3, 5, 7, 9 and 11 have nisin core, the rest – OspA core. Leaders are indicated in italics; cleavage sites by peptidases are indicated in red: ASPR – NisP, D – GluC, K – trypsin, respectively. Red background indicates nisin leader conservative motive, green – OspA conservative leader motive.*

1	*MGSSHHHHHHSSGLVPRGSHMSTRKEAEEQLAIKALKDFNLDLVSVSKKDSGASPR*ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
2	*MGSSHHHHHHSSGLVPRGSHMSTRKEAEEQLAIKALKDFNLDLVSVSKKDSGASPR*GCWIAGSRGCGFVTRT
3	*MGHHHHHHSTKDFNLDSTRKEAEEQLAIKALKD*ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
4	*MGHHHHHHSTKDFNLDSTRKEAEEQLAIKALKD*GCWIAGSRGCGFVTRT
5	*MGSSHHHHHHSSGLVPRGSHMSTRKEFNLDLAIKALKDPSFREK*ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
6	*MGSSHHHHHHSSGLVPRGSHMSTRKEFNLDLAIKALKDPSFREK*GCWIAGSRGCGFVTRT
7	*MGHHHHHHSTRKEFNLDLAIKALKDPSFREK*ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
8	*MGHHHHHHSTRKEFNLDLAIKALKDPSFREK*GCWIAGSRGCGFVTRT
9	*MGHHHHHHSTRKEFDLDLAIKALKDPSASPR*ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
10	*MGHHHHHHSTRKEFDLDLAIKALKDPSASPR*GCWIAGSRGCGFVTRT
11	*MGHHHHHHSTRKEFNLDLAIKALKDPSASPR*ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
12	*MGHHHHHHSTRKEFNLDLAIKALKDPSASPR*GCWIAGSRGCGFVTRT

Table 2. *Hybrid leaders (italics) fused to chimeric core peptides. Cores N1O, N2O and N3O are nisin 1, 2 and 3 rings fused to the OspA core peptide(green background), respectively. ASPR – NisP cleavage site.*

Name	Peptide sequence
H1N1O	*MGSSHHHHHHSSGLVPRGSHMSTRKEAEEQLAIKALKDFNLDLVSVSKKDSGASPR*ITSISLCTPGGCWIAGSRGCGFVTRT
H1N2O	*MGSSHHHHHHSSGLVPRGSHMSTRKEAEEQLAIKALKDFNLDLVSVSKKDSGASPR*ITSISLCTPGCKTGGCWIAGSRGCGFVTRT
H1N3O	*MGSSHHHHHHSSGLVPRGSHMSTRKEAEEQLAIKALKDFNLDLVSVSKKDSGASPR*ITSISLCTPGCKTGALMGCNMKGCWIAGSRGCGFVTRT
H11N1O	*MGHHHHHHSTRKEFNLDLAIKALKDPSASPR*ITSISLCTPGGCWIAGSRGCGFVTRT
H11N2O	*MGHHHHHHSTRKEFNLDLAIKALKDPSASPR*ITSISLCTPGCKTGGCWIAGSRGCGFVTRT
H11N3O	*MGHHHHHHSTRKEFNLDLAIKALKDPSASPR*ITSISLCTPGCKTGALMGCNMKGCWIAGSRGCGFVTRT

Table 3. *Modifications made in chimeric core peptides. Cores N1O, N2O and N3O are nisin 1, 2 and 3 rings fused to the OspA core peptide(green background), respectively. Modifications presented here are introduced when single modification module is co-expressed. Possibly epimerized amino acids are indicated in bold; dehydrated – underlined*

Prepeptide	Core peptide sequence	Maximum number of dehydrations	Number of epimerizations
H1N1O	ITSISLCTPGGCW**I**AGSRGCGF**V**TRT	6	2
H1N2O	ITSISLCTPGCKTGGCW**I**AGSRGCGF**V**TRT	6	2
H1N3O	ITSISLCTPGCKTGALMGCNMKGCW**I**AGSRGCGF**V**TRT	8	2

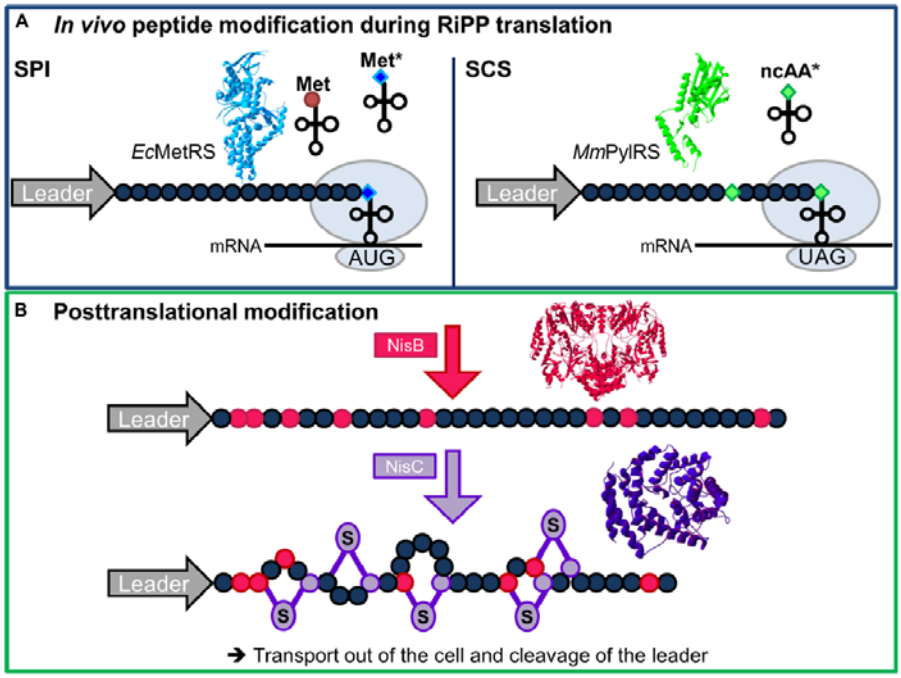


Fig. 9, adapted from Baumann et al, Frontiers in Microbiology 8 (2017):124. Shown are two different methods of ncAA insertion. Upper panel, left: Force feeding (or selective pressure incorporation, SPI): Methionine deprivation allows the insertion of ncAAs similar to methionine into newly synthesized proteins using the standard system of the methionine specific tRNA and the corresponding synthetase. Other proteins receive the Met-line amino acid also instead of Met. Right: Stop-codon suppression (SCS): An orthogonal tRNA is reprogrammed to recognize a STOP-codon, and a stop codon is placed at a specific site of the peptide. A cognate synthetase charges the orthogonal tRNA with tbe ncAA and the tRNA delivers it specifically to suppress the STOP codon. Only other proteins using the same STOP codon to terminate translation receive the ncAA as well. Lower panel: Example for PTMs, specifically thioether bridge formation by enzymes NisB and NisC.

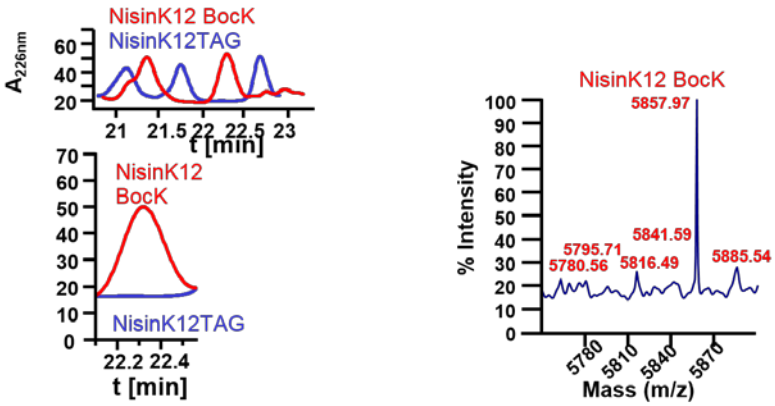


***Fig 10**: Insertion of boc-lysine into nisin produced in* L- lactis *using SCS. Left: HPLC traces (upper panel) and blow up (lower panel) of the peak subsequently submitted to MALDI-TOF analysis (right panel). MS-analysis confirmed correct mass for boc-lysine insertion.*

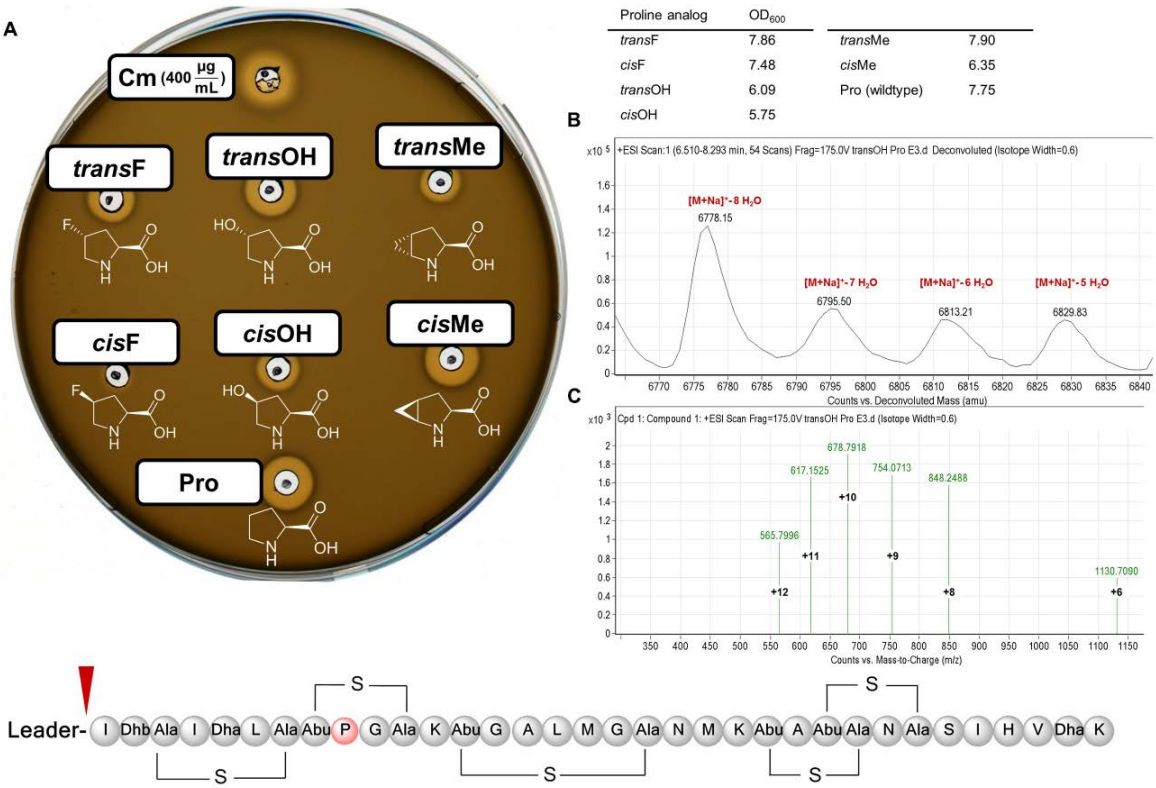


***Fig. 11**, adapted from Baumann et al, Frontiers in Microbiology 8 (2017):124. Modification of nisin with ncAAs. (A) Antimicrobial activity assay using novel nisin variants produced by recombinant expression and SPI using ncAA analogs of proline. E. coli expression samples (harvested cell densities tabulated as OD600) were normalized and tested for inhibition of the Gram-positive indicator strain L. lactis NZ9000 carrying plasmid pNG nisPT for cleavage of the AMP leader (Khusainov and Kuipers, 2013). Cm:400 mg/mL chloramphenicol (antibacterial control); ncAAs used for SPI are abbreviated above and depicted below the corresponding wells: cis/trans-4-fluoroproline((4S/R-F)Pro), cis/trans-4-hydroxyproline ((4S/R-OH)Pro), cis/trans-methanoproline, proline(wild-type control). Nisin structure including (methyl)lanthionine rings and NisP cleavage site (red triangle) depicted at the bottom, highlighting position of proline 9 (red circle) targeted for modification by ncAAs. (B) MS deconvolution chromatogram for recombinant nisin containing trans-4-hydroxyproline.Calculated masses (Da):[MCNa]C – 8H2O D 6779.21, [MCNa]C – 7 H2O D 6795.21, [MCNa]C – 6H2O D 6813.21, [MCNa]C – 5H2O D 6831.21 (C) Compound spectrum for charged species of [MCNa]C – 8H2O*

V) A high throughout assay for GPCR-agonistic activity

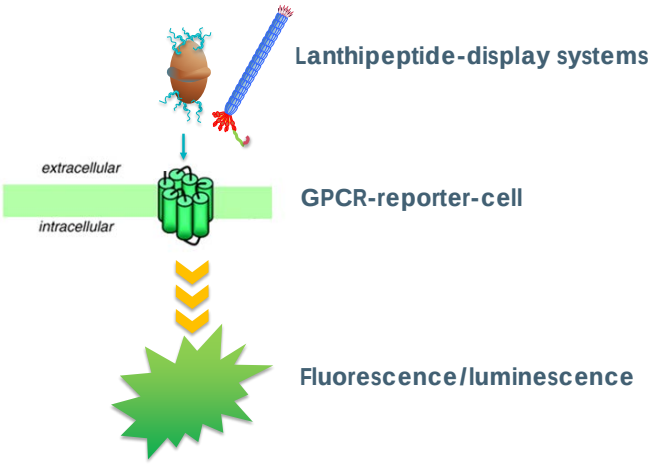


***Fig. 12**. Principle of the envisioned GPCR-screening assay. For details see text.*

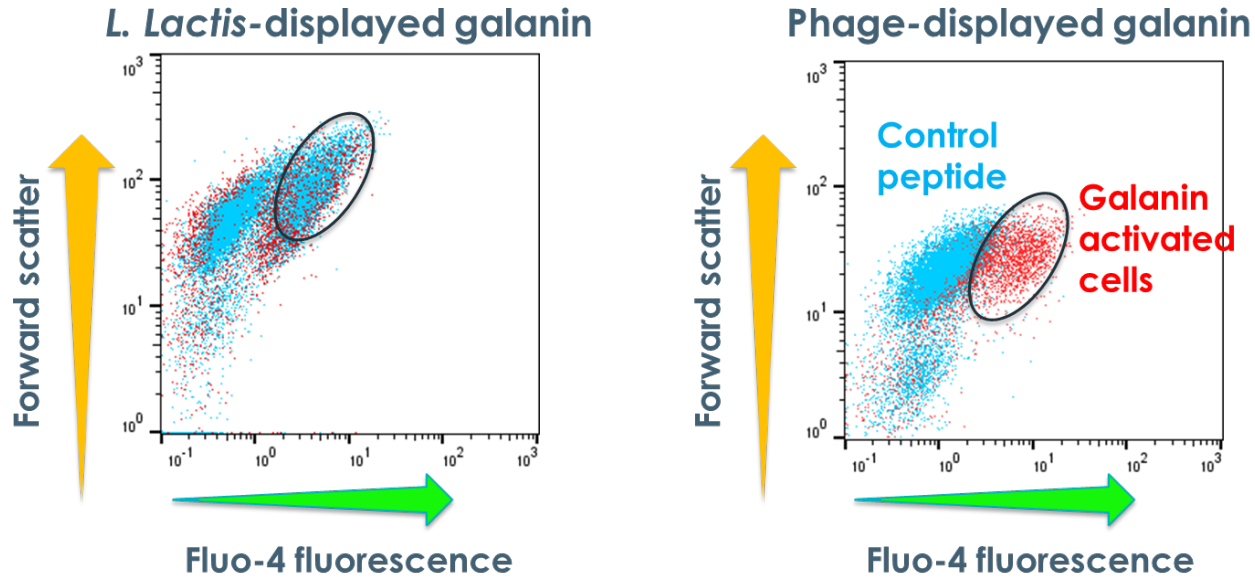


Fig. 13. *N-terminally phage-displayed galanin activates reporter cells eliciting distinct, FACS sortable fluorescence.*

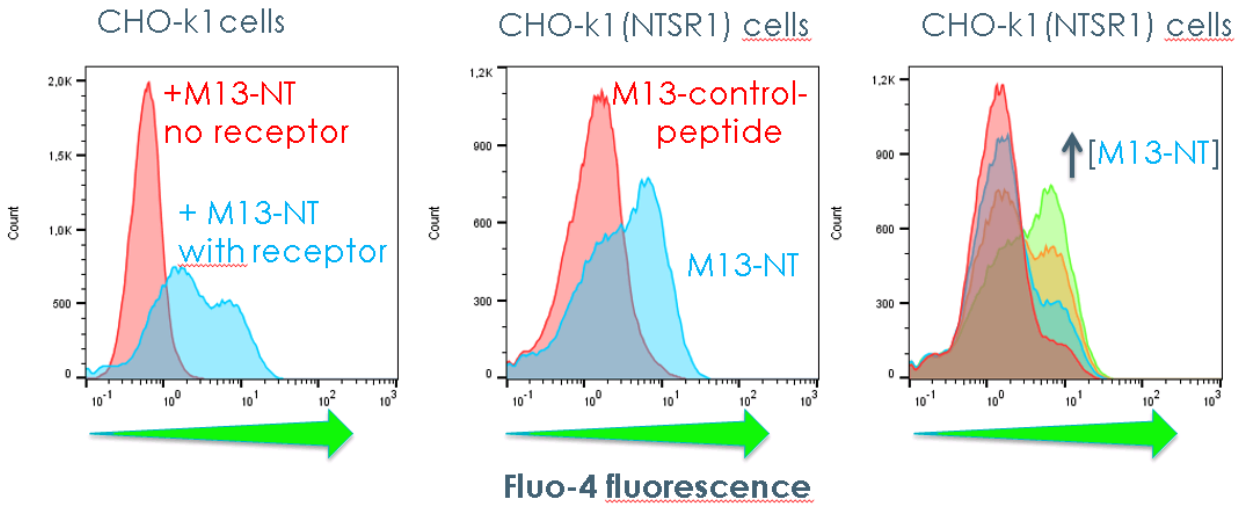


Fig. 14*. C-terminally phage-displayed neurotensin activates neurotensin receptor containing reporter cells inducing FACS sortable fluorescence.*

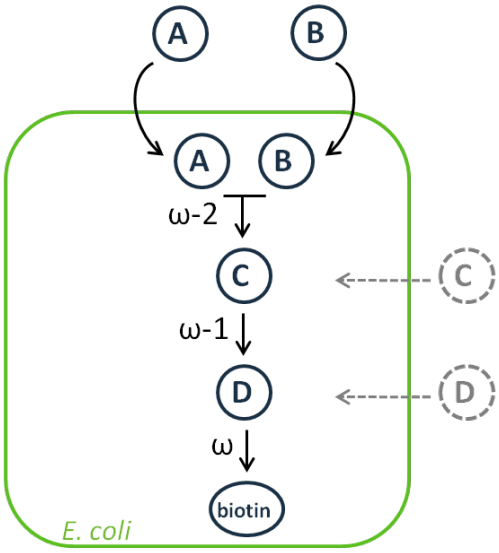


Fig. 15: *A* de novo *designed pathway to biotin. A retrobiochemically designed pathway for synthesis of biotin by E. coli from the two fed precursor substances A and B in three steps (ω-2, ω-1 and ω).*

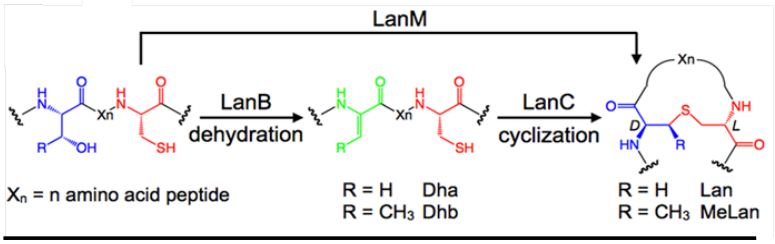


Fig. 16: *Thiophane ring formation and stability of the formed intermediates. Upper panel: Thio-ether ring formation catalysed by the lantibiotic cyclization machinery proceeds via dehydration of alcoholic amino acids threonine or serine to the corresponding enamines follow by enzyme catalysed electrophilic attack of the cysteine sulphur. Lower panel: While the enamines formed as intermediates in the course of the lantibioitic cyclization are rather stable primary amines are readily hydrolysed in water.*

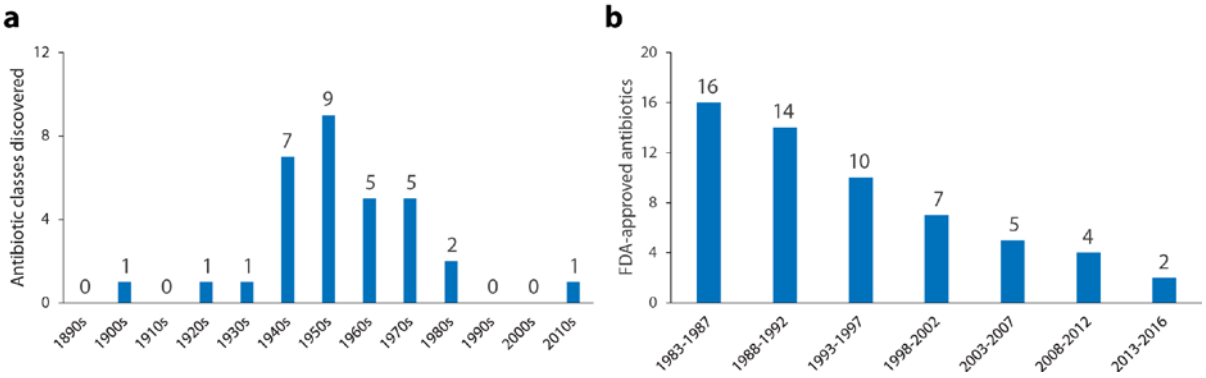


Fig. 17. *Antibiotic drug discovery. (**a**) The number of novel antimicrobial classes discovered (includes data from discoveries and patents) in 10-year time intervals from 1890 to 2016. Note that the last period covers 7 years only. Drug classes include those with new mode of actions or representing novel chemical entities. The latest representatives of novel antibacterial classes, linezolid, daptomycin and the topical agent retapamulin were approved in 2000, 2003, and 2007 respectively. However, the corresponding classes (oxazolidinones, acid lipopeptides, and pleuromutilins) were first reported in 1978, 1987 and 1952, respectively. (**b**) Number of FDA-approved antibiotics in 5-year time intervals from 1983 to 2016. Note that the last period only covers 4 years. Labels on the bars show the exact number of FDA-approvals.*

Table 4 *Antibiotic resistance threats in the United States of America. List of microbial strains, classified as urgent, serious or concerning threats in the United States (as for 2013) according to the Centers for Disease Control and Prevention (CDC)[11]. Note that ranges given for resistant isolates indicate different resistance-levels against several antibiotics or varying fractions of resistant isolates for different species. Definition of threat levels according to CDC are: Urgent: High-consequence antibiotic-resistant threats because of significant risks identified across several criteria. These threats may not be currently widespread but have the potential to become so and require urgent public health attention to identify infections and to limit transmission. Serious: Significant antibiotic-resistant threats. For varying reasons (i.e. low or declining domestic incidence or reasonable availability of therapeutic agents), they are not considered urgent, but these threats will worsen and may become urgent without ongoing public health monitoring and prevention activities. Concerning: Strains for which the threat of antibiotic resistance is currently low, and/or there are multiple therapeutic options for resistant infections. These pathogens cause severe illness. Threats in this category require monitoring and in some cases rapid incident or outbreak response. Note that even though C. difficile is not significantly resistant to antibiotics it is classified as an urgent threat because of its high morbidity and mortality rate as well as its relation to antibiotics use and resistance issues.*

Microorganism	**Threat level**	**Resistant isolates**	**Type**
Clostridium difficile	Urgent	n.d.	Gram-positive
Carbapenem-resistant *Klebsiella* spp.	Urgent	11 %	Gram-negative
Carbapenem-resistant *E. coli*	Urgent	2 %	Gram-negative
Drug-resistant *Neisseria gonorrhoeae*	Urgent	23 - 30 %	Gram-negative
Multidrug-resistant *Acinetobacter* spp.	Serious	63 %	Gram-negative
Drug-resistant *Campylobacter* spp.	Serious	2 - 24 %	Gram-negative
Fluconazole-resistant *Candida* spp.	Serious	7 %	Fungus
Extended spectrum β-lactamase *E. coli*	Serious	14 %	Gram-negative
Extended spectrum β-lactamase *Klebsiella* spp.	Serious	23 %	Gram-negative
Vancomycin-resistant *Enterococcus* spp.	Serious	9 - 77 %	Gram-positive
Multidrug-resistant *Pseudomonas aeruginosa*	Serious	13 %	Gram-negative
Drug-resistant non-typhoidal *Salmonella* spp.	Serious	3 - 8 %	Gram-negative
Drug-resistant *Salmonella Typhi*	Serious	67 %	Gram-negative
Drug-resistant *Shigella* spp.	Serious	2 - 6 %	Gram-negative
Methicillin-resistant *Staphylococcus aureus*	Serious	n.d.	Gram-positive
Drug-resistant *Streptococcus pneumoniae*	Serious	30 %	Gram-positive
Drug-resistant *Mycobacterium tuberculosis*	Serious	10 %	Mycobacterium
Vancomycin-resistant *Staphylococcus aureus*	Concerning	n.d.	Gram-positive
Erythromycin-resistant Group A *Streptococcus* spp.	Concerning	10 %	Gram-positive
Clindamycin-resistant Group B *Streptococcus* spp.	Concerning	49 %	Gram-positive