Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Activation of Protein Splicing by Protease or Light Triggered O-N Acyl Migration

Miquel Vila-Perelló, Yuichiro Hori, Marc Ribó and Tom W. Muir

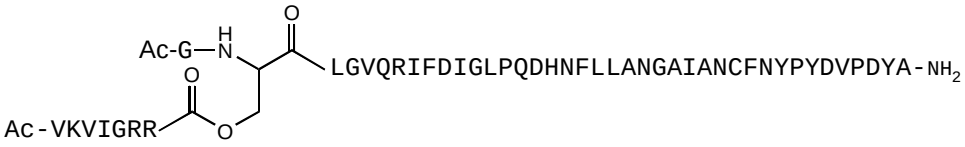
General Methods

All Fmoc amino acid derivatives and HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) were obtained from Novabiochem (San Diego, CA). All other reagents were from Sigma-Aldrich (St. Louis, MO). Nvoc-Ser-OH was synthesized as previously described.[1] Analytical RP-HPLC was performed on a Hewlett-Packard 1100 series instrument equipped with a C18 Vydac column (5 μm, 4.6 x 150 mm) at a flow rate of 1 mL/min. Semi-preparative RP-HPLC was performed on a Waters DeltaPrep 4000 system with a Waters 486 tunable detector. All runs used linear gradients of 0.1 % TFA (trifluoroacetic acid) in water (solvent A) and 0.1% TFA 90 % acetonitrile in water (solvent B). Mass spectrometric analysis for all peptides and proteins was performed by ESI-MS on a Sciex-API-100 single quadrupole spectrometer using positive ionization. Protein purifications were carried out on an ÄKTA FPLC system (Amersham Biosciences, Uppsala, Sweden).

Peptide Synthesis

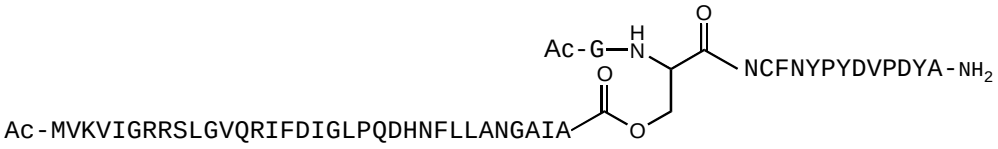
All peptides were synthesized using standard Fmoc protocols on a Rink-amide ChemMatrix resin (0.5 mmol/g, Matrix Innovations, Canada). Linear peptides were assembled using a Liberty peptide synthesizer equipped with a Discovery microwave module (CEM, Matthews, NC). Amino acids were activated with HBTU, HOBt (N-hydroxybenzotriazole) and DIEA (N,N-diisopropylethylamine) in DMF (dimethylformamide) and Fmoc deprotections were carried out using 20% piperidine in DMF. Cleavage from the resin was performed by treatment with 95 % TFA, 2.5 % water, 2.5 % tri-isopropyl-silane for 2 h at r.t. Peptides were isolated by precipitation with cold Et_2O and centrifugation and taken up in 50 % solvent B. *O*-acyl peptides were synthesized using optimized Fmoc protocols (short deprotections and base-free coupling methods for the incorporation of the 3^{rd} residue after the ester bond) to minimize diketopiperazine formation.[2] RP-HPLC chromatograms of all purified peptides are shown in Figure S1.

Synthesis of Dna$E_C$dep9-HA (**1**).



Residues 10 to 48 (residue numbers referenced to linear Dna*$E_C$wt-HA*, **7**) were assembled using microwave assisted automated peptide synthesis methods as described above. Fmoc-Ser-OH and Ac-Gly-OH were next manually coupled using 5 eq. of amino acid activated using 5 eq. of DIC (diisopropyl-carbodiimide) and HOBt in DMF (60 min). Any undesired acylation of the Ser side chain was cleaved by treatment with ethanolamine in 5% water/DMF (2 x 30 min). Next, Fmoc-Arg(Pmc)-OH was coupled to the β-hydroxyl of the serine side chain using the symmetric anhydride method with DIC and 0.1 eq. DMAP (dimethylaminopyridine) (1 x 5 h and 1 x O/N). The following Fmoc-Arg(Pmc)-OH was coupled using HBTU/DIEA and deprotected with 20% piperidine (1 x 10 min). Finally, the last five amino acids were manually coupled using 5 eq. of amino acid and 5 eq. of DIC and HOBt (1 x 1 h) and deprotections were carried out with 20% piperidine/DMF (1 x 10 min). Following cleavage, the crude peptide (synthesis yield of 33%) was purified by RP-HPLC and the identity confirmed by ESI-MS (found 5363.3 ± 0.2, expected 5363.1 Da).

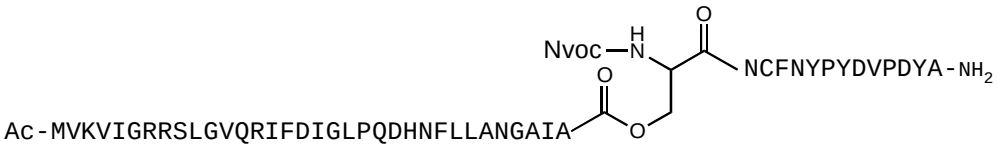
Synthesis of Dna$E_C$dep35-HA (**2**).



Residues 34-48 were assembled using microwave assisted automated peptide synthesis methods as described above. Fmoc-Ser-OH and Ac-Gly-OH were next manually coupled using 5 eq. of amino acid activated using 5 eq. of DIC and HOBt in DMF (60 min). Any undesired acylation product at the Ser side chain was cleaved by treatment with ethanolamine in 5% water/DMF (2 x 30 min). Next, Fmoc-Ala-OH was coupled to the β-hydroxyl of the serine side chain using the symmetric

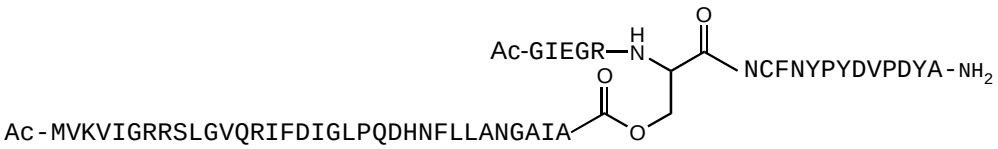
anhydride method with DIC and 0.1 eq. DMAP (1 x 5 h and 1 x O/N). Fmoc-Ile-OH was coupled using HBTU/DIEA and deprotected with 20% piperidine (1 x 10 min). The following five amino acids were manually coupled using 5 eq. of amino acid and 5 eq. of DIC and HOBt (1 x 1 h) and deprotections were carried out using 20% piperidine/DMF (1 x 10 min). Chain elongation was completed in the Liberty using non-microwave assisted methods to minimize ester hydrolysis. Following cleavage, the crude peptide (synthesis yield of 32%) was purified by RP-HPLC and the identity confirmed by ESI-MS (found 5510.5 ± 0.9, expected 5510.3 Da).

Synthesis of Nvoc-DnaE$_C$-dep35-HA (**3**).



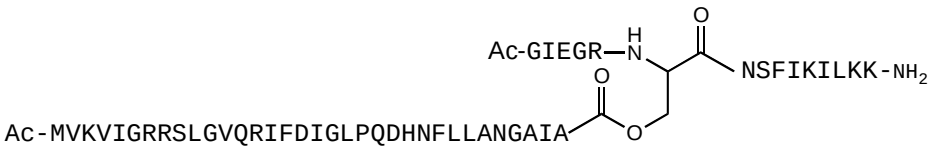
Residues 35 to 48 were assembled as in peptide **2**. Nvoc-Ser-OH was then coupled using 2 eq. of amino acid, 2 eq. of DIC and 2 eq HOBt (1 x 2 h and 1 x O/N). After β-hydroxyl acylation with Fmoc-Ala-OH synthesis was completed as for **2**. Following cleavage, the crude peptide (synthesis yield of 30%) was purified by RP-HPLC and the identity confirmed by ESI-MS (found 5651.3 ± 0.2, expected 5650.3 Da).

Synthesis of Xa-DnaE$_C$-dep35-HA (**4**).



Residues 35 to 48 were assembled as for peptide **2**. The Factor Xa recognition site (GIEGR) was then introduced by coupling each of the Fmoc-amino acid derivatives with DIC and HOBt. The synthesis was then completed as for **2** and **3**. Following cleavage, the crude peptide (synthesis yield of 37%) was purified by RP-HPLC and the identity confirmed by ESI-MS (found 5966.0 ± 1.4, expected 5964.0 Da).

Synthesis of Xa-DnaE$_C$-dep35-MA (**5**).



The peptide was synthesized using the same procedure employed for peptide **4**. Following cleavage, the crude (synthesis yield of 10%) peptide was purified by RP-HPLC and identity confirmed by ESI-MS (found 5475.8 ± 1.7, expected 5475.4 Da).

Synthesis of DnaE$_C$wt-HA (**7**), *DnaE$_C$A35S-HA* (**8**) *and DnaE$_C$MA$_C$* (**9**).

Ac-MVKVIGRRSLGVQRIFDIGLPQDHNFLLANGAIAANCFNYPYDVPDYA-NH_2 (**7**)

Ac-MVKVIGRRSLGVQRIFDIGLPQDHNFLLANGAIASNCFNYPYDVPDYA-NH_2 (**8**)

H-MVKVIGRRSLGVQRIFDIGLPQDHNFLLANGAIASNSFIKILKK-NH_2 (**9**)

Following chain assembly and cleavage (synthesis yields of **7**, **8** and **9** were 36, 30 and 48% respectively), peptides were purified by RP-HPLC. The identities of the three peptides, **7**, **8** and **9** were confirmed by ESI-MS; 5395.3 ± 1.1 (expected 5395.2 Da), 5413.4 ± 1.7 (expected 5411.2 Da) and 4879.2 ± 1.4 (expected 4878.9 Da), respectively..

Magainin analogues synthesis: Antimicrobial peptides were synthesized using the Liberty peptide synthesizer as described above. Crude peptides were purified by RP-HPLC and identify confirmed by ESI-MS, results are summarized in Table S1.

Carbamidomethylation of peptides **2**, **7** *and* **8**

The catalytic Cys of peptides **2**, **7** and **8** was blocked by reaction with iodoacetamide prior to performing fluorescence and circular dichroism experiments. Crude peptides were dissolved in a buffer containing 6 M Gn·HCl, 50 mM Tris-HCl, pH 7.5 and reacted with 250 eq. of iodoacetamide

in the dark for 1 h. Reaction products were negative on an Ellman test for thiols. Modified peptides **2b**, **7b** and **8b** were purified by HPLC and their identities confirmed by ESI-MS (**2b** 5567.3 ± 0.2, expected 5566.4 Da; **7b** 5454.3 ± 1.6, expected 5452.3 Da and **8b** 5467.8 ± 0.5, expected 5468.3 Da).

Protein Expression

Expression and purification of His_6-MBP-Ub(R42E)-$DnaE_N$ (**10**)

The plasmid pMUEn encoding His_6-MBP-Ub(R42E)-$DnaE_N$ in a pMAL vector was constructed from pHis6-GFP-DnaEn[3], pTBX-Ub and pEB4[4] using standard molecular cloning techniques. The plasmid was characterized by DNA sequencing. *E. coli* BL21 cells transformed with pMUEn were grown in 6 L of Luria-Bertani (LB) media supplemented with ampicillin (100 μg/mL) at 37 ºC until OD_{600} = 0.8, then cells were cooled down to 22-24 ºC and expression was induced by addition of 0.1 mM IPTG for 5 h at 22 ºC. After harvesting the cells by centrifugation, the cell pellet was resuspended in cold lysis buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, 1mM DTT, pH 7.5) supplemented with Complete protein inhibitor tablets from Roche Diagnostics (Mannheim, Germany). Cells were lysed by passage through a French press, the soluble fraction was recovered by centrifugation and further cleared by filtration through a 5 μm filter. The soluble fraction was then loaded onto a column containing 15 mL of amylose resin (New England Biolabs, MA), previously equilibrated with 8 column volumes (CVs) of 50 mM Tris, 300 mM NaCl, 1 mM DTT at pH 7.5. After washing, the protein was eluted with the same buffer supplemented with 10 mM maltose. Fractions containing **10** were pooled and dialyzed into a buffer containing 50 mM sodium phosphate, 300 mM NaCl, 1 mM β-mercaptoethanol, 10 mM imidazole, pH 8.0. The dialyzed protein was then loaded onto a second column containing 6 mL of Ni-NTA (Novagen, Madison, WI) beads previously equilibrated in dialysis buffer. After overnight incubation at 4 ºC, beads were washed with 5 CV of dialysis buffer, followed by 5 CV of a washing buffer containing 50 mM sodium phosphate, 300 mM NaCl, 1 mM β-mercaptoethanol, 20 mM imidazole, pH 8.0 and finally

the protein was eluted with a buffer containing 50 mM sodium phosphate, 300 mM NaCl, 1 mM β-mercaptoethanol, 250 mM imidazole, pH 8.0. Fractions containing the purified protein **10** were pooled, dialyzed into 100 mM Tris, 500 mM NaCl, 1 mM EDTA, 10 mM DTT pH 7.5, aliquoted and frozen.

Expression and purification of His_6-Ub(R42E)-$DnaE_N$ (**11**)

The plasmid encoding His_6-Ub(R42E)-$DnaE_N$ (pMR-MVP) was obtained by digesting pUEn, generated during the preparation of pMUEn, with NdeI and HindIII and ligating the excised Ub-$DnaE_N$ gene into a pTYB1 vector from New England Biolabs. The plasmid was characterized by DNA sequencing. *E. coli* BL21 cells transformed with pMR-MVP were grown in 2 L of LB supplemented with ampicillin (100 μg/mL) at 37 ºC until OD_{600} = 0.6. Expression was then induced by addition of 0.5 mM IPTG and cells grown for 3.5 h at 37 ºC. After harvesting the cells by centrifugation, the cell pellet was resuspended in lysis buffer (50 mM Tris, 100 mM NaCl, pH 7.5) supplemented with Complete protein inhibitor tablets. Cells were lysed by passage through a French press, the insoluble fraction was recovered by centrifugation and washed two times with isolation buffer (50 mM Tris, 100 mM NaCl, 1% triton-X, pH 7.5). The inclusion bodies were solubilized in re-suspension buffer (6 M urea, 50 mM sodium phosphate, 300 mM NaCl, 2 mM β-mercaptoethanol, pH 8.0) and separated from the cellular debris by centrifugation. Solubilized inclusion bodies were then loaded onto a column containing 5 mL of Ni-NTA beads pre-equilibrated in re-suspension buffer and incubated for 1 h at 4 ºC. After discarding the flow-through, the loaded Ni-NTA beads were washed with 5 CV of re-suspension buffer and 5 CV of wash buffer (6 M urea, 50 mM sodium phosphate, 300 mM NaCl, 2 mM β-mercaptoethanol, 2 mM imidazole, pH 8.0) and the protein was eluted with 5 CV of elution buffer (6 M urea, 50 mM sodium phosphate, 300 mM NaCl, 2 mM β-mercaptoethanol, 500 mM imidazole, pH 8.0). Fractions containing pure protein **11** were pooled and diluted to 1 mg/mL with re-suspension buffer. Protein **11** was refolded by stepwise dialysis from re-suspension buffer to a final buffer containing 100 mM

phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0 and then purified by gel filtration chromatography using an S200 column (Amersham Biosciences, Uppsala, Sweden). Fractions containing the monomeric protein were pooled, aliquoted and frozen.

Expression and purification of MA_N-$DnaE_N$ (**6**)

GIGKFLKKAKKFGKCLSFGTEILTVEYGPLPIGKIVSEEINCSVYSVDPEGRVYTQAIAQWHDRGE
QEVLEYELEDGSVIRATSDHRFLTTDYQLLAIEEIFARQLDLLTLENIKQTEEALDNHRLPFPLLD
AGTIK

The plasmid pMVP23 encoding His_6-SUMO-$MA_N$$DnaE_N$ was constructed by PCR amplification of the $DnaE_N$ sequence from pMR-MVP using primers NotI-MA1-En (5'-AGCGGCCGC<u>AA-AAAATTTGGCAAA</u>TGCCTCAGTTTTGGCACCG-3') and XhoI-En (5'-TCTCGAGTTATTT-AATTGTCCCAGCGTCAAGTAATGG-3'), which also resulted in the incorporation of 15 nucleotides (underlined) from the magainin sequence. After digestion with NotI and XhoI, the PCR product was ligated into a pET-SUMO vector previously digested with the same enzymes. The 27 nucleotides encoding the remainder of the N-terminus of MA were introduced by a restriction enzyme free cloning strategy[5] using the QuickChange kit from Stratagene (La Jolla, CA) and the primers fwrd-MA2 (5'-GAGAACAGATTGGTGGTGGCATTGGCAAATTTCTGAAAAAAGCG-AAAAAATTTGGCAAATG-3') and rev-MA2 (5'-CATTTGCCAAATTTTTTCGCTTTTTTCAG-AAATTTGCCAATGCCACCACCAATCTGTTCTC-3'). This plasmid, pMVP23, encoding for His_6-SUMO-$MA_N$$DnaE_N$, was characterized by DNA sequencing. *E. coli* BL21 cells transformed with pMVP23 were grown in 1 L of LB media supplemented with kanamycin (50 μg/mL) at 37 ºC until OD_{600} = 0.6, protein expression was induced by addition of 1 mM IPTG and the cells grown for 3.5 h at 37 ºC. After harvesting the cells by centrifugation, cell pellets were resuspended in lysis buffer (50 mM Tris, 100 mM NaCl, pH 7.5) supplemented with Complete protein inhibitor tablets. Cells were lysed by passage through a French press and the insoluble fraction was recovered by centrifugation and washed two times with isolation buffer (50 mM Tris, 100 mM NaCl, 1% triton-X, pH 7.5). Inclusion bodies were dissolved in re-suspension buffer (6 M urea, 50 mM sodium

phosphate, 300 mM NaCl, 2 mM β-mercaptoethanol, pH 8.0) and separated from the cellular debris by centrifugation. Solubilized inclusion bodies were loaded onto a column containing 2.5 mL of Ni-NTA beads pre-equilibrated in re-suspension buffer and incubated for 1 h at 4 ºC. After discarding the flow-through Ni-NTA beads were washed with 5 CV of re-suspension buffer and 5 CV of wash buffer (6 M urea, 50 mM sodium phosphate, 300 mM NaCl, 2 mM β-mercaptoethanol, 2 mM imidazole, pH 8.0) and the protein was eluted with 5 CV of elution buffer (6 M urea, 50 mM sodium phosphate, 300 mM NaCl, 2 mM β-mercaptoethanol, 500 mM imidazole, pH 8.0). Fractions containing the pure protein were pooled together and diluted down to 0.75 mg/mL with re-suspension buffer. Protein was refolded by stepwise dialysis from re-suspension buffer to a buffer containing 50 mM phosphate, 150 mM NaCl, 1 mM DTT, pH 7.5. The SUMO domain was removed by cleavage with SUMO protease (1 U / 50 μg of protein, 2 h at r.t.), cleaved His_6-SUMO and un-cleaved precursor were depleted from **6** by Ni-NTA purification: the cleavage mixture was diluted two times in 50 mM phosphate, 300 mM NaCl, 20 mM imidazole, 2 mM β-mercaptopethanol, pH 8.0 and loaded into the Ni beads. The flow-through fraction containing the desired protein was then dialized into PBS or TBS buffer and immediately used in splicing or antimicrobial activity assays.

Fluorescence Spectroscopy

Fluorescence spectra were recorded at 25 ºC using a Jobin Yvon/Spex Fluorolog-3 spectrofluorometer (Instrument S.A., Inc., Edison, NJ). Measurement parameters were as follows: excitation wavelength, 280 nm; emission wavelength, 310 – 405 nm; averaging time, 0.2 sec; increment, 1.0 nm; scans, 2; excitation slit width, 2.5 nm; emission slit width, 10 nm. For equilibrium binding measurements, a solution of **11** (200 nM) in 100 mM sodium phosphate, 500 mM NaCl, 1 mM EDTA, pH 7.0 was titrated with carbamidomethylated-$DnaE_C$ peptides (from stocks in the same buffer) to give final concentrations of between 0 and 800 nM. Note, that protein **11** contains a single Trp residue within the $DnaE_N$ segment that can be used to monitor association

with the $DnaE_C$ fragment. The fluorescence emission of this Trp was monitored at 337 nm as a function of added carbamidomethylated-$DnaE_C$ peptides, corrected for dilution effects and plotted vs. the concentration of $DnaE_C$. Titration curves (Figure S3) were fit to a quadratic equation (Equation 1) to extract the dissociation constant (K_D) for the interaction. K_D's obtained from at least 3 independent experiments were averaged to obtain the reported values and their SD (Table S2).

Equation 1:

$$F_{obs} = F_o + \left[\frac{F_\infty - F_o}{2[UE_N]} \times \left([UE_N] + [E_c] + K_D - \left(([UE_N] + [E_C] + K_D)^2 - 4[UE_N][E_C] \right)^{1/2} \right) \right]$$

Were F_{obs}, F_o and F_8 are the observed, initial and saturation fluorescence signal, $[UE_N]$ and $[E_C]$ the total concentration of the N and C terminal halves of the intein at each titration point and K_D is the dissociation constant.

Stopped-flow Fluorescence Spectroscopy

Stopped-flow fluorescence measurements were conducted using an Applied Photophysics SX.18MV instrument. The change in fluorescence of Trp47 in $DnaE_N$ was followed above 325 nm with excitation at 280 nm. Experiments were conducted in 100 mM sodium phosphate, 500 mM NaCl, 1 mM EDTA, pH 7.0 at 25°C. The final $DnaE_N$ concentration after mixing was around 0.77 μM and those of $DnaE_C$ between 0.5 and 2 μM. See Figure S4.

Circular Dichroism (CD) Spectroscopy

CD spectra were collected on an Aviv 62DS spectropolarimeter (Aviv Biomedical, Inc., Lakewood, NJ). Quartz cuvettes of 0.1 and 1 cm were used for the far and near UV CD measurements, respectively, and temperature was controlled at 25 ºC by circulating water. Protein samples were prepared in 100 mM sodium phosphate, 150 mM NaCl, 0.4 mM DTT, pH 7.0. Far UV CD spectra were recorded at 10 μM protein concentration by accumulating five 190-260 nm scans using 1 nm steps and 3 s of averaging time. Near UV CD spectra were obtained using the same acquisition

parameters but with a 250-350 nm spectral window and at 20 μM protein concentration. The spectra of free ubiquitin was collected in both the far and the near UV CD and subtracted from that of Ub-$DnaE_N$ in order to obtain the intein signal. Theoretical spectra of the intein complexes were calculated by adding the signal from $DnaE_C$ and $DnaE_N$ by themselves and transforming it to Molar Ellipticiy per residue ($[\Theta]_{MR}$). See Figure S5.

Peptide antimicrobial activity assays

The bioactivity of antimicrobial magainin analogues against *S. aureus* was assayed using the broth micro-dilution method[6]. Mueller-Hinton broth (Fluka, St. Louis, MO) was used as growth medium; an initial inoculum of *S. aureus* (RN6911, ca 5 x 10^5 CFU/mL) at exponential growth phase was used. The MIC was defined as the lowest concentration of peptide that completely inhibits growth of the organism in the micro-dilution wells measured by turbidimetry (OD_{650}) after 18 h at 37 ºC in a THERMOmax microplate reader (Molecular Devices). Concentrations of antimicrobial peptides ranged from 250 to 7.5 μg/mL. See Table S3 for results. The analogue MA-SFI (also abbreviated as MA) was almost as active as the wild type peptide and contained the SFI sequence, known to be tolerated by the *Ssp* DnaE split intein at the splicing junction. MA-SFI was spliced between Lys14 and Ser15, its N- and C-terminus were fused to $DnaE_N$ and $DnaE_C$, respectively to generate constructs **6** and **9**. Fusion of the C-terminus of MA to a peptide containing the Factor Xa trigger gave peptide **5**.

Splicing mediated antimicrobial activity assays

Antimicrobial activity against *E. coli* (BL21) and *S. aureus* (RN6911) of DnaE mediated magainin splicing reactions was assayed as above, but at 25 ºC so that the splicing reaction could proceed during the whole duration of the experiment. Bacterial growth was measured as the ratio between the OD at 650 nm for each sample and a control in culture media after 18 h incubation at 25 ºC. Reactions with wild type inteins (**6** and **9**) in buffer were incubated for 2 h prior addition to the

bacterial inoculum. For Factor Xa activated reactions 0.1 U of Factor Xa were added per 50 μg of **5** and the splicing reaction was allowed to proceed at r.t. for 8-10 h before adding it to the bacteria. See Figure S12 and S13.

Trans-splicing assays

Trans splicing assays were performed in splicing buffer (100 mM phosphate, 150 mM NaCl, 1mM EDTA, 1 mM DTT, pH 7.5) or in Factor Xa buffer (20 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT, pH 8.0) in the case of experiments carried out in the presence of the protease. *MBP-Ub-E_N* (**10**) at 20 μM and the indicated *DnaE_C* analogue at 12.5 μM were incubated at room temperature and aliquots were taken after 0, 2, 4, 6 and 20 h for analysis. A control sample with wild-type intein was allowed to react for 24 h and used as a standard for the maximum level of splicing to which other samples were normalized. For Factor Xa triggered reactions 0.1 U of Factor Xa (New England Biolabs) per 50 μg of peptide was added 1 h prior the addition of **10**. For the light activated trans-splicing reaction, samples containing both **10** and **3** were irradiated 3 times for 15 seconds with a He-Cd laser (325 nm, 4.74 W/cm^2, Kimmon Electric Co., Englewood, CO). See Figures S2, S6, S7 and S8.

DnaE mediated trans-splicing of MA.

Splicing of the magainin analogue `GIGKFLKKAKKFGKSFIKILKK` was carried out by mixing **6** and **9** (Figure S9 and S10) or **6** and **5** (Figure S11) at 20 μM in splicing buffer and allowing the reaction to proceed at room temperature. For the Factor Xa activated splicing 0.1 U of protease per 50 μg of peptide were added to a mixture of **6** and **5** at 20 μM.

Western blot analysis and quantification

Protein samples were diluted with 2 X SDS-PAGE loading buffer and resolved by SDS-PAGE before being transferred to Immobilon-P membrane (Millipore) using standard Western blotting

procedures. Immuno-blotting was performed using anti-HA mouse monoclonal antibody and a fluorescent goat anti-mouse IRDye 800 from Odyssey (Lincoln, NE) as the secondary. Western blot quantification was performed using the Odyssey Infrared Imaging System and the Odyssey v 2.1.10 analysis software from Li-COR.

Table S1: ESI-MS of Magainin analogues.

Peptide	**Sequence**[a]	observed (Da)	expected (Da)
MA-78 (wt)	H-GIGKFLKKAKKFGKAFVKILKK-NH_2	2477.96	2477.24
MA-SFN	H-GIGKFLKKAKKFGKSFNKILKK-NH_2	2508.3	2508.6
MA-SFI	H-GIGKFLKKAKKFGKSFIKILKK-NH_2	2507.4	2507.27

[a] Sequences were designed to incorporate either the SFN or SFI residues which are known to be tolerated at the splicing junction by the DnaE intein (S.W. Lockless and T.W. Muir, unpublished results).

Table S2: Dissociation constants (±SD) measured for the $DnaE_C$ analogues for His-Ub-$DnaE_N$ (**11**)

Peptide	K_D (nM)
7b	35 ± 20
8b	37 ± 12
2b	32 ± 22

Table S3: Antimicrobial activity of Magainin-78 analogues against *S. aureus*.

Peptide	**Sequence**	**MIC (μg/mL)** [a]	**MIC (μM)** [a]
MA-78 (wt)	H-GIGKFLKKAKKFGK**AFV**KILKK-NH_2	31.25	12.6
MA-SFN	H-GIGKFLKKAKKFGK**SFN**KILKK-NH_2	250	99
MA-SFI	H-GIGKFLKKAKKFGK**SFI**KILKK-NH_2	52.1	20.8

[a] MICs were determined as an average from 3 independent experiments.

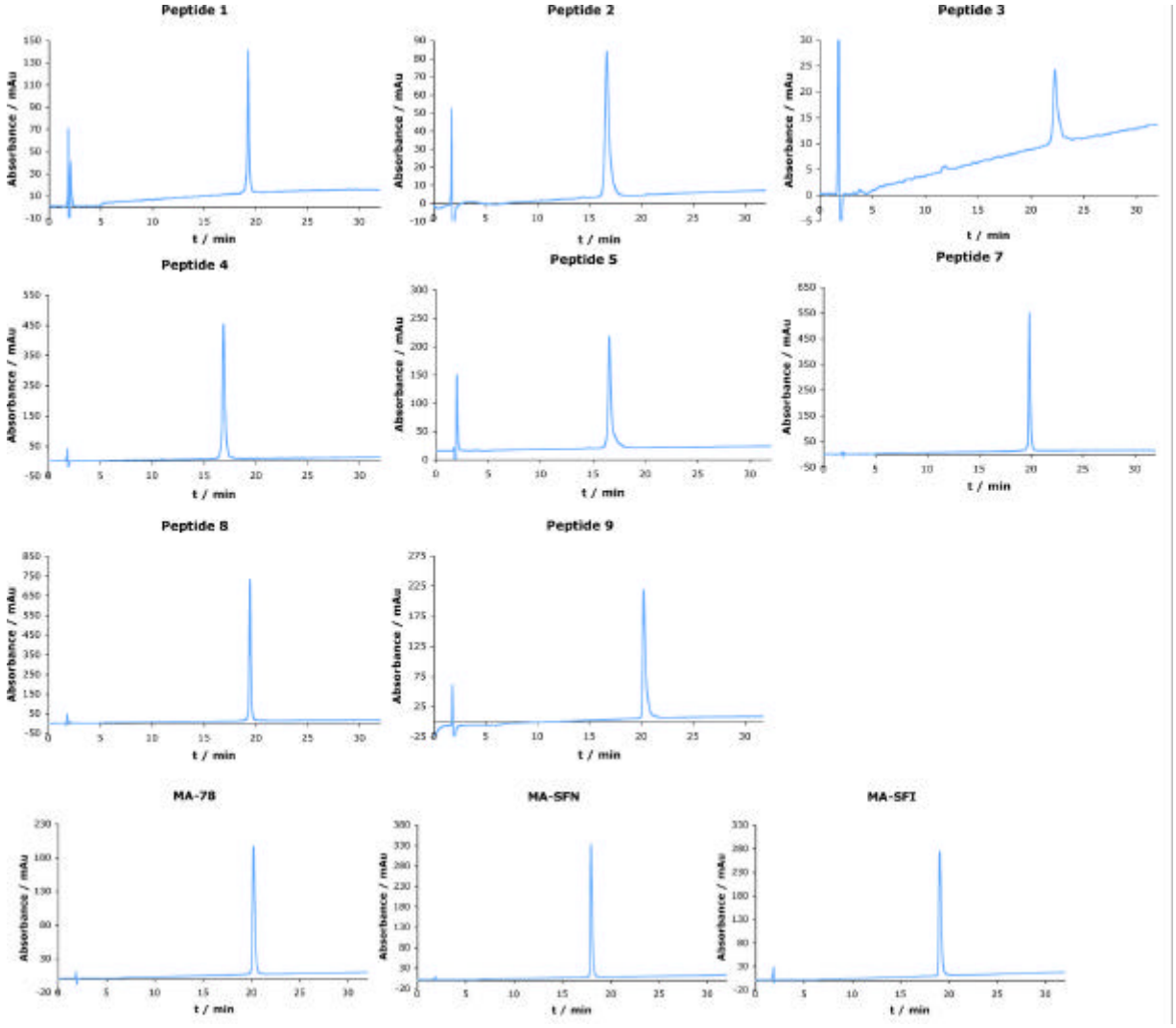


Figure S1: HPLC analysis of purified peptides **1-5** (C18 column, 30-50 % B over 30 min, 214 nm detection), **7-9** (C18 column, 20-60 % B over 30 min, 214 nm detection) and magainin analogues (C18 column, 25-45 % B over 30 min, 214 nm detection).

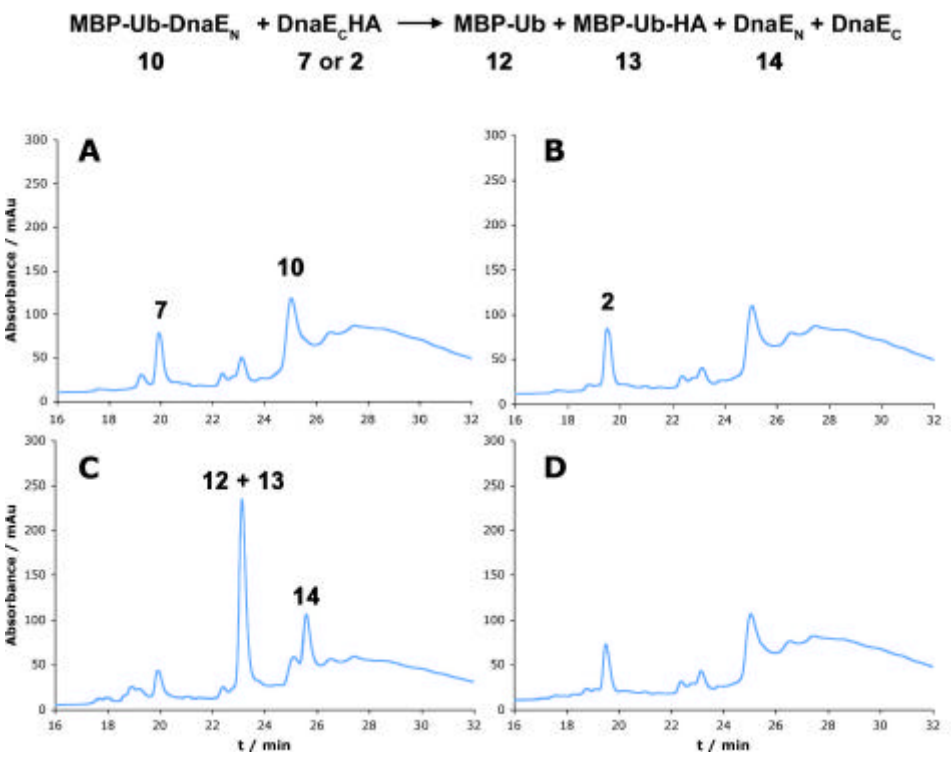


Figure S2: Analysis of N-terminal cleavage and trans-splicing. Top) Scheme of the trans-splicing reaction. Bottom) RP-HPLC analysis (C18 column, 10-73 % B over 30 min, 214 nm detection) of splicing reaction between 20 µM of **10** and 12.5 µM of **7** (**A**, t = 0 h and **C**, t = 20 h) or *O*-acyl peptide **2** (**B**, t = 0 h and **D**, t = 20 h). The peak at 23 min contained a mixture of splicing product (**13**, *m/z* 55000 Da, expected 54989 Da) as well as N-terminal cleavage side-product (**12**, *m/z* 53705 Da, expected 53696 Da). A 45 % yield of the splicing reaction was measured by HPLC based on the consumption of **7** after 24 h.

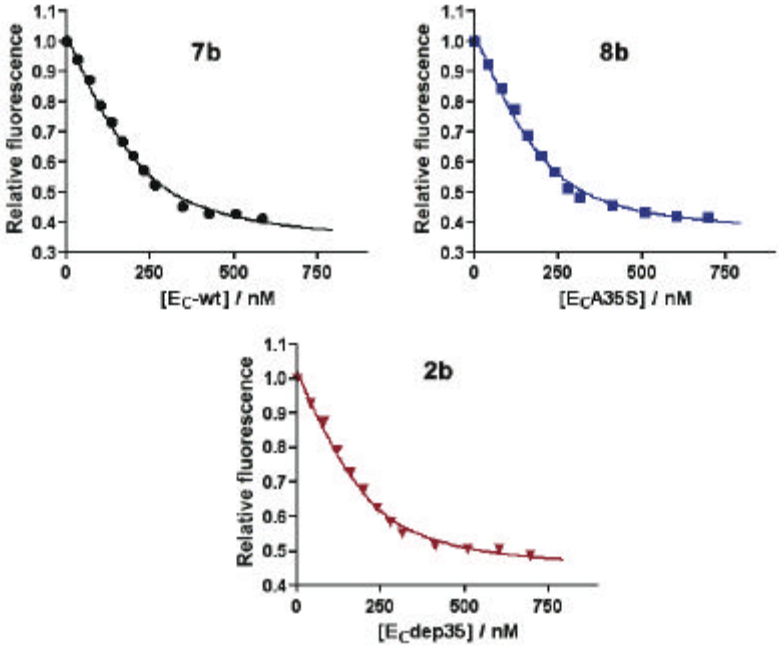


Figure S3: Representative binding isotherms of *Ub-DnaE$_N$* (**11**) with carbamidomethilated *DnaE$_C$wt* (**7b**), *DnaE$_C$A35S* (**8b**) and *DnaE$_C$dep35* (**2b**) in 100 mM sodium phosphate, 500 mM NaCl, 1 mM EDTA, pH 7.0 at 25°C. The change of the relative fluorescence at 337 nm *vs* peptide concentration was plotted and fitted to a quadratic equation to obtain the dissociation constants.

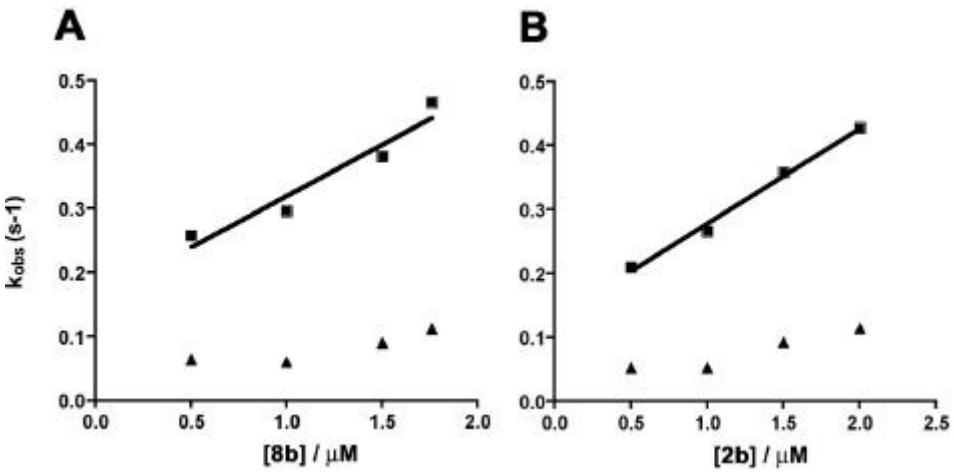


Figure S4: Stopped-flow kinetic analysis of $DnaE_N$ and $DnaE_C$ association. Stopped-flow fluorescence decay curves were fitted to a double exponential equation and two observed rate constants: k_{obs1} (■) and k_{obs2} (π) were obtained for each of the four concentrations of $DnaE_C$ used. The observed rate constants at different concentrations for **8b** (**A**) and **2b** (**B**) are almost identical. k_{obs1} clearly depends linearly on $DnaE_C$ concentration allowing k_{on} (1.61 ± 0.31 and 1.50 ± 0.09 x 10^5 $s^{-1}M^{-1}$ for **8b** and **2b**, respectively) and k_{off} (0.16 ± 0.04 and 0.13 ± 0.01 s^{-1} for **8b** and **2b**, respectively) to be determined from the slope and intercept, respectively .

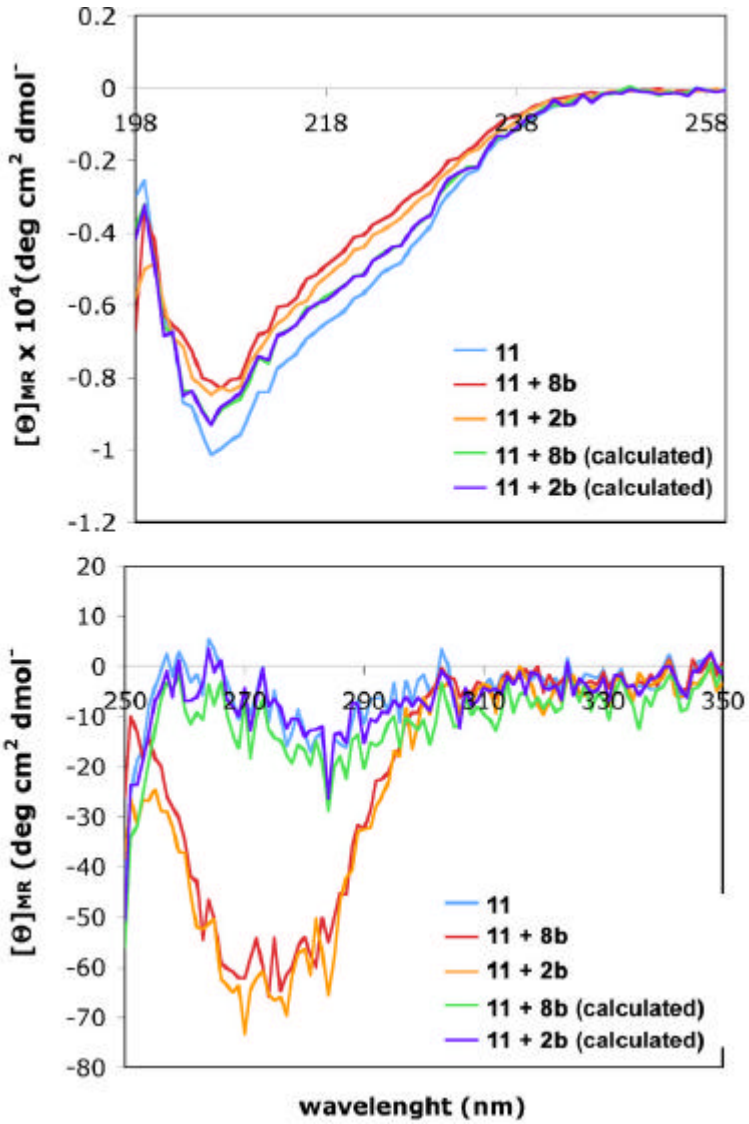


Figure S5: Circular Dichroism (CD) analysis of DnaE complexes. Far (top) and near (bottom) UV CD spectra of indicated DnaE complexes (10-20 µM) were recorded at 25 ºC in 100 mM phosphate, 150 mM NaCl, 0.4 mM DTT, pH 7.0. The spectra of Ub-DnaE$_N$ (**11**) alone (Ub signal subtracted) and of the various DnaE complexes are shown. Calculated spectra correspond to the addition of the signal from each of the halves of the intein complex obtained separately to illustrate that the change observed in the near UV is due to complex formation.

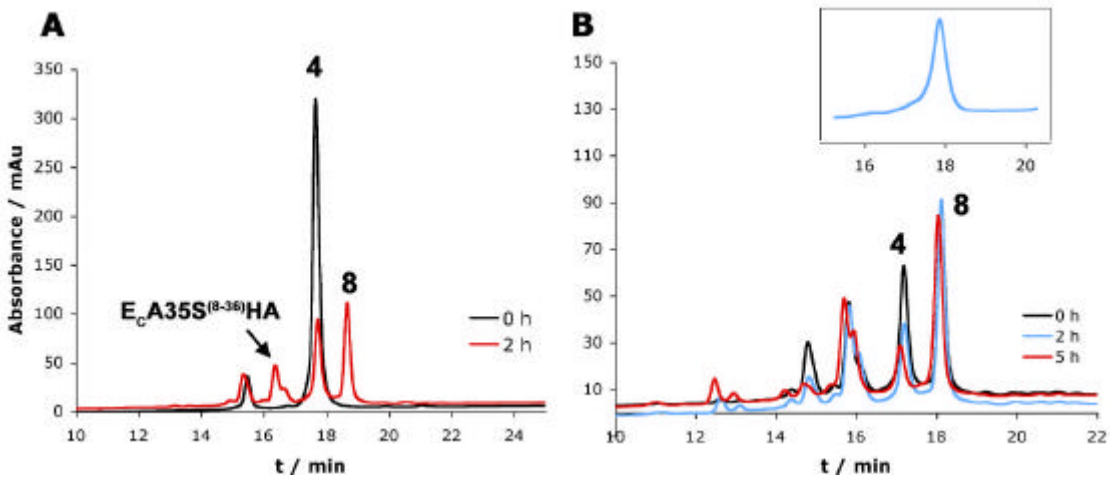


Figure S6: Factor Xa digestion of *XaDnaE$_C$dep35-HA* (**4**). **A**) RP-HPLC analysis (C18 column, 30-50 % B over 30 min, 214 nm detection) of 50 µg of peptide **4** incubated with 0.1 U of Factor Xa in 20 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT, pH 8.0. After 2 h, a new peak with a molecular weight corresponding to **8** was identified (*m/z* 5413.5 ± 1.7 Da, expected 5411.2 Da), as well as a product of non-specific cleavage at Arg8 (*$E_CA35S^{(8-36)}HA$*, *m/z* 4587.1 ± 0.79 Da, expected 4585.2 Da). **B**) RP-HPLC analysis of proteolysis reaction mixtures incubated at pH 11 for 5 h. *O*-acyl peptides are consumed due to ester hydrolysis while all-amide peptides are stable. Factor Xa activity was quenched by addition of EDTA. Inset, shows co-elution of synthetic **8** with the product of proteolysis of **4**, which further demonstrates that the *O*-to-*N* acyl migration has occurred.

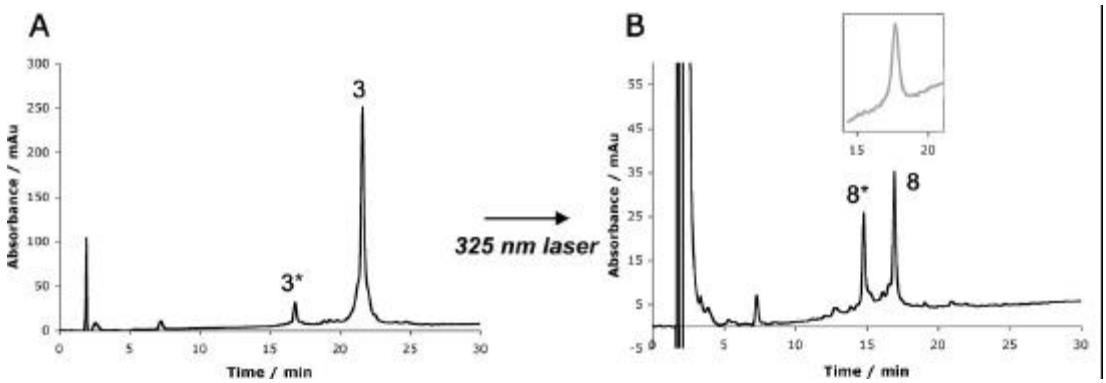


Figure S7: Photo-deprotection of *Nvoc-$E_C$dep35-HA* (**3**). Peptide dissolved in splicing buffer (100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5) was irradiated 3 x 15 s with a 325 nm He-Cd laser. RP-HPLC traces (C18 column, 30-50 % B over 30 min, 214 nm detection) of the reaction mixture before (left) and after (right) irradiation are shown. After photo-deprotection two main peaks are observed: one corresponds to **8** (observed 5411.24 ± 0.1 Da, expected 5411.2 Da) and the other (**8***) was assigned to a product of N-terminal Met oxidation (observed 5426.74 ± 0.7 Da, expected 5427.2 Da). Inset shows co-elution of synthetic **8** with the product of **3** photo-deprotection.

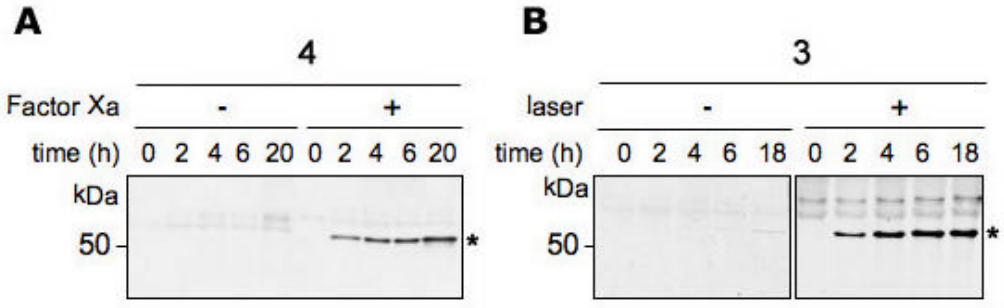


Figure S8: Representative anti-HA Western blot analysis of Factor Xa (**A**) and laser (**B**) activated protein trans-splicing. See text for experimental details. Spliced product, MBP-Ub-HA, is indicated by *

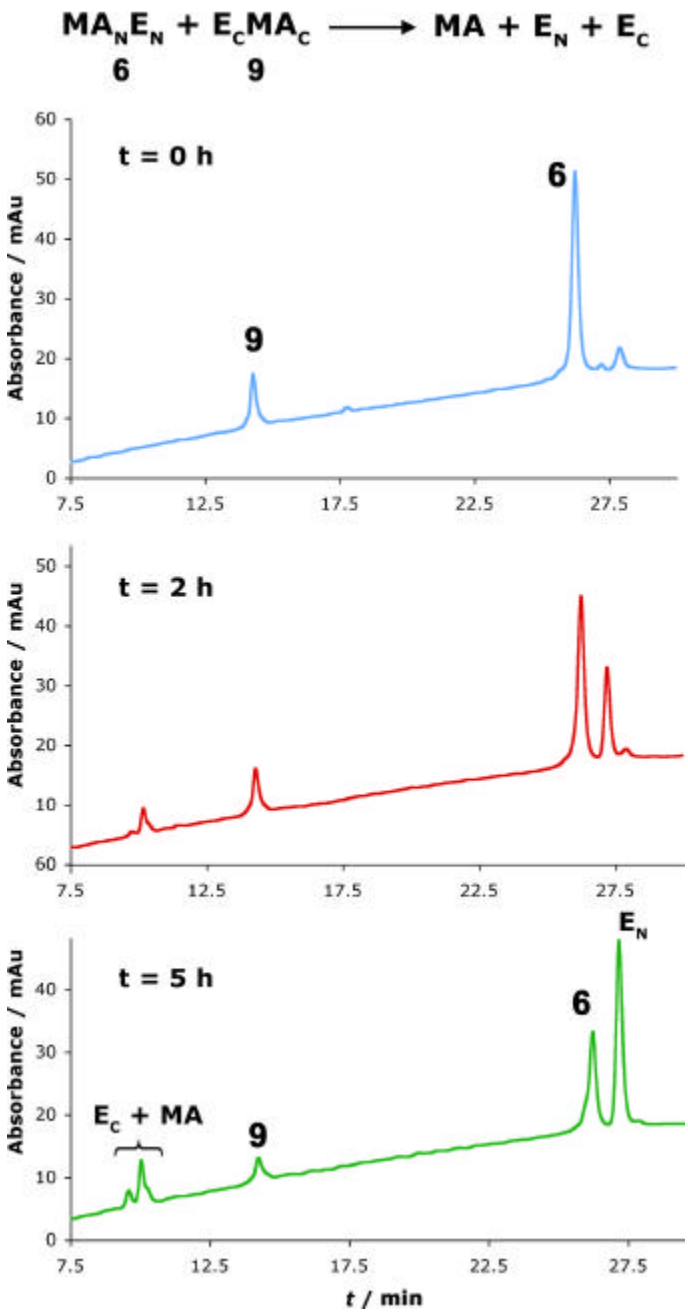


Figure S9: Trans-splicing reaction between *MA_NDnaE_N* (**6**) and *$DnaE_CMA_C$* (**9**) at 20 µM in splicing buffer. Top) scheme of the splicing reaction. Bottom) RP-HPLC analysis (C18 column, 30-50 % B over 30 min, 214 nm detection) of the reaction at different time-points. Consumption of both **6** and **9** and generation of MA was observed. See also Figure S10.

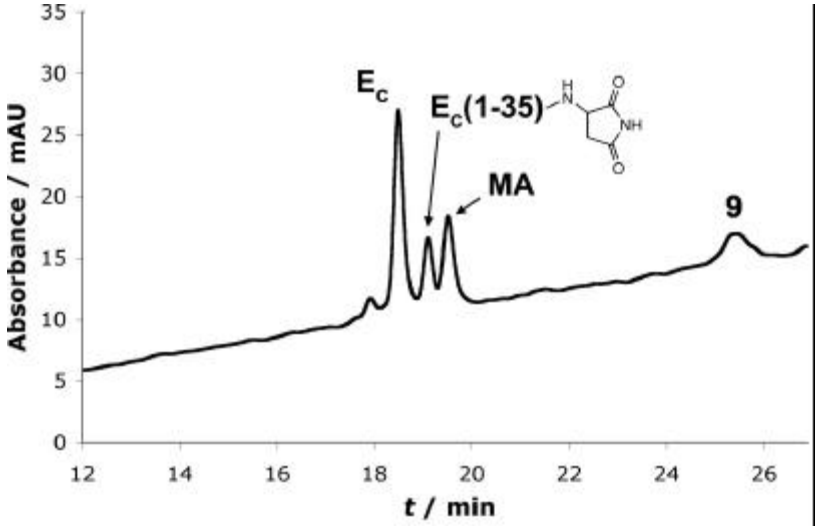


Figure S10: Trans-splicing reaction between **6** and **9** at 20 μM in splicing buffer. RP-HPLC analysis (C18 column, 25-45 % B over 30 min, 214 nm detection) of the splicing reaction after 18 h. Peaks corresponding to $DnaE_C$ (observed 3920.60 ± 0.8 Da, expected 3919.1 Da), $DnaE_C$-succinimide (observed 3903.20 ± 0.8 Da, expected 3901.1 Da) and the product of splicing magainin (observed 2507.8 ± 0.1 Da, expected 2507.3 Da) could be detected as well as low amounts of unreacted **9**. The yield of magainin trans-splicing was determined to be 38 % based on initial amount of **9**.

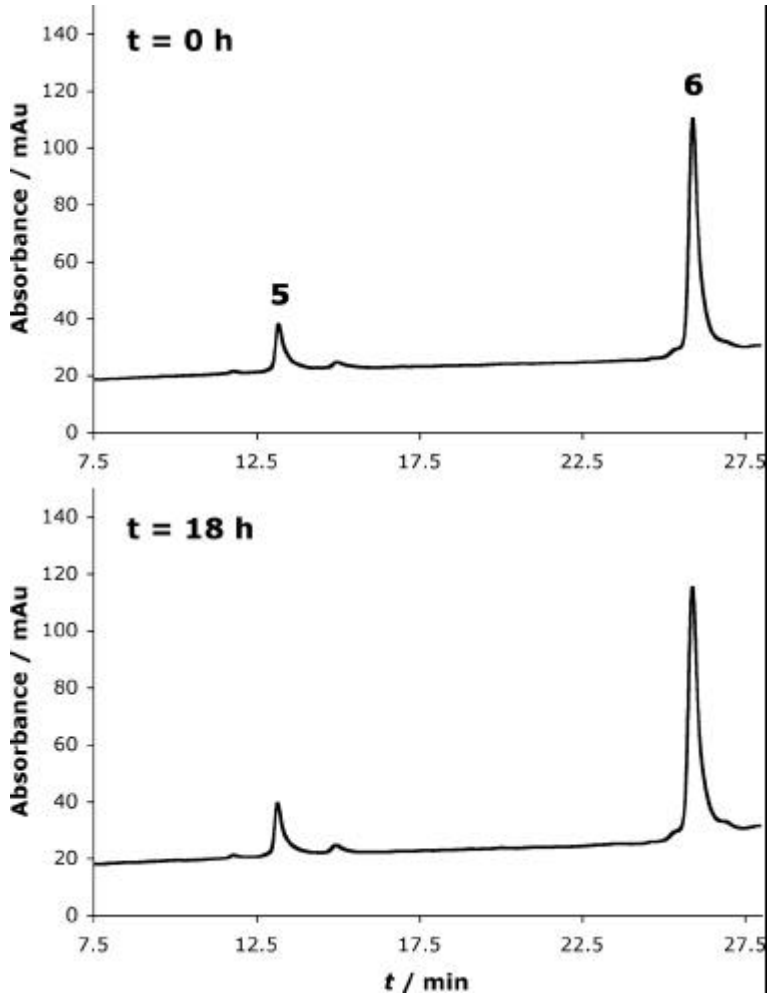


Figure S11: Splicing reaction between **6** and *O*-acyl peptide **5** at 20 μM in splicing buffer. RP-HPLC analysis (C18 column, 30-50 % B over 30 min, 214 nm detection) of the splicing mixture at 0 h and 18 h. In the absence of protease, neither splicing nor N-terminal cleavage products could be detected after 18 h incubation.

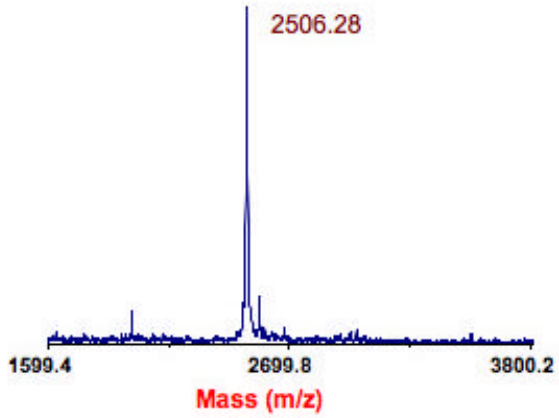


Figure S12: MALDI-TOF analysis of the product of the trans-splicing reaction between **6** and **5** at 20 μM in splicing buffer in the presence of Factor Xa protease (expected 2506.64 $M+H^+$, monoisotopic).

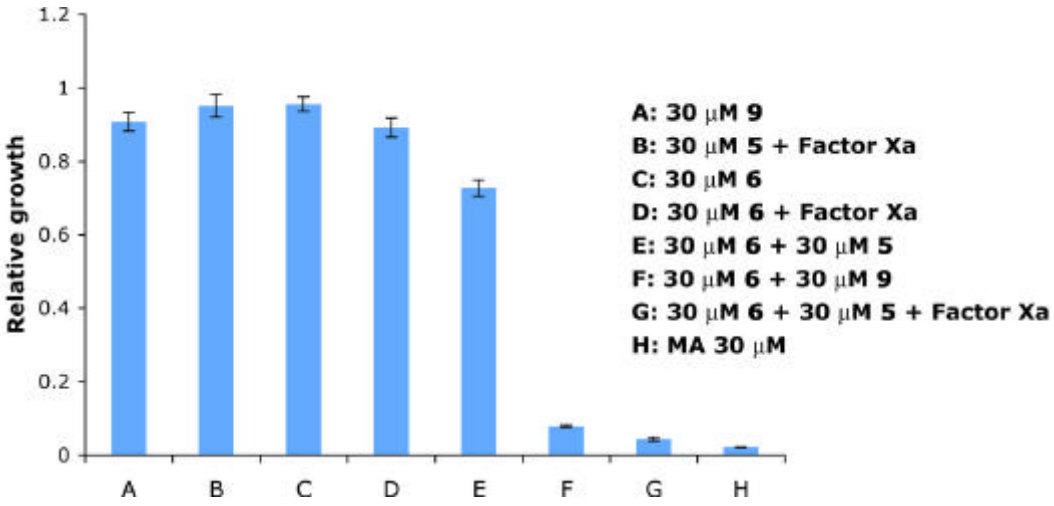


Figure S13: Antimicrobial activity against *E. coli*. Bacterial growth (measured as the OD_{650} after 18 h of incubation) relative to control in culture media for each protein sample is shown, errors = s.d. (n=3). See text for experimental details. We presume that the small amount of anti-microbial activity observed when **5** and **6** are incubated without the protease may reflect some residual activity within pre-splicing complex.

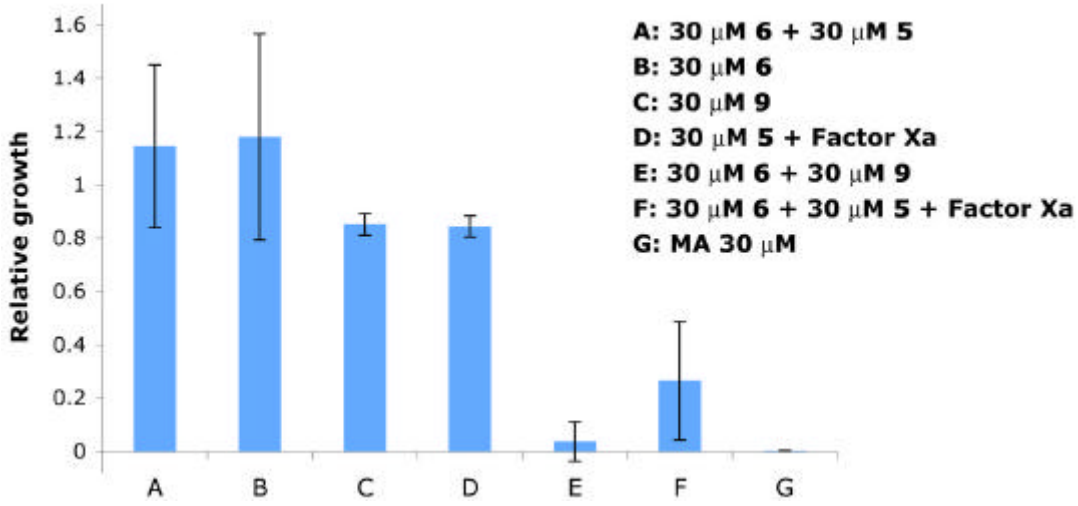


Figure S14: Antimicrobial activity against *S. aureus*. Bacterial growth (measured as the OD_{650} after 18 h of incubation) relative to control in culture media for each protein sample is shown. Data represents the average of two independent experiments each of them performed by triplicate, errors = s.d. (n=6). Experiments were done as for *E. coli*.

References

[1] A. Taniguchi, Y. Sohma, M. Kimura, T. Okada, K. Ikeda, Y. Hayashi, T. Kimura, S. Hirota, K. Matsuzaki, Y. Kiso, *J Am Chem Soc* **2006**, *128*, 696.

[2] I. Coin, R. Dolling, E. Krause, M. Bienert, M. Beyermann, C. D. Sferdean, L. A. Carpino, *J Org Chem* **2006**, *71*, 6171.

[3] J. Shi, T. W. Muir, *J Am Chem Soc* **2005**, *127*, 6198.

[4] H. D. Mootz, E. S. Blum, A. B. Tyszkiewicz, T. W. Muir, *J Am Chem Soc* **2003**, *125*, 10561.

[5] F. van den Ent, J. Lowe, *J Biochem Biophys Methods* **2006**, *67*, 67.

[6] J. M. Saugar, M. J. Rodriguez-Hernandez, B. G. de la Torre, M. E. Pachon-Ibanez, M. Fernandez-Reyes, D. Andreu, J. Pachon, L. Rivas, *Antimicrob Agents Chemother* **2006**, *50*, 1251.