Antimicrobial Peptides Hot Paper

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Insect-Derived Proline-Rich Antimicrobial Peptides Kill Bacteria by Inhibiting Bacterial Protein Translation at the 70S Ribosome**

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Abstract: *Proline-rich antimicrobial peptides (PrAMPs) have been investigated and optimized by several research groups and companies as promising lead compounds to treat systemic infections caused by Gram-negative bacteria. PrAMPs, such as apidaecins and oncocins, enter the bacteria and kill them apparently through inhibition of specific targets without a lytic effect on the membranes. Both apidaecins and oncocins were shown to bind with nanomolar dissociation constants to the 70S ribosome. In apidaecins, at least the two C-terminal residues (Arg17 and Leu18) interact strongly with the 70S ribosome, whereas residues Lys3, Tyr6, Leu7, and Arg11 are the major interaction sites in oncocins. Oncocins inhibited protein biosynthesis very efficiently in vitro with half maximal inhibitory concentrations (IC_{50} values) of 150 to 240 nmol L^{-1}. The chaperone DnaK is most likely not the main target of PrAMPs but it binds them with lower affinity.*

Antibiotics are a major therapeutic success story, in which pharmacy and chemistry have revolutionized the treatment of bacterial infections and thus prevented bacterial epidemics for almost 70 years. Regrettably, this record of accomplishment is challenged by various resistance mechanisms, which have allowed bacteria to overcome virtually all approved antibiotics.[1] This health threat has recently been partially overcome for Gram-positive pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA),[2] but there are growing concerns relating to multi- or pan-resistant Gram-negative pathogens, especially *Escherichia coli, Klebsiella pneumoniae*, *Enterobacter cloacae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*.[1] There is thus an urgent need to identify novel compound classes, which may need ten to fifteen years to enter clinics. Antimicrobial peptides are a promising class of compounds that has not yet been used pharmaceutically. Proline-rich antimicrobial peptides (PrAMPs), which do not kill bacteria by purely lytic mechanisms but interact with specific bacterial targets, thus preventing adverse effects in humans, appear particularly promising.[3] PrAMPs, which are expressed in mammals and insects, have been evaluated by several research groups, either in their native forms or as chemically optimized or designed lead compounds in different infection models.[4] Collectively, PrAMPs show low sequence homology but are structurally homologous (ca. 30% proline content, one or several Pro-Arg-Pro motifs, disordered structure in solution) and appear to kill bacteria by similar mechanisms.[3] They seem to penetrate the outer membrane freely before being transported by the SbmA transporter into the cytosol.[5] The research group of Laszlo Otvos showed that typical representatives of insect-derived PrAMPs bind in *E. coli* lysates to the 70 kDa bacterial heat shock protein DnaK, with dissociation constants (K_d) in the low micromolar range.[6] We confirmed these interactions and classified PrAMPs into two groups based on their binding mode at the substrate-binding domain of DnaK.[7] Our recent study on the bacterial uptake of fluorescein-labelled apidaecin derivatives, however, challenged DnaK as the major target.[8] We showed that the apidaecin analogue Api88 and two C-terminally truncated versions enter *E. coli* with similar efficiencies and bind equally strongly to DnaK, but only the full-length Api88 is active.[8]

We investigated the sensitivity of the *E. coli* strain BW25113 and the corresponding DnaK null mutant JW0013[9] to PrAMPs (Table 1). The two strains were equally susceptible to the oncocins tested but the JW0013 cells were twofold more vulnerable to apidaecins Api88 and Api137 than the wild-type strain. This result clearly indicates that DnaK is not the major target of these PrAMPs. Further support for this hypothesis comes from a study involving peptides optimized for stronger DnaK binding, which were unexpectedly less active against *E. coli*.[10]

On the basis of these results, we synthesized biotinylated L- and all-D-Api88 analogues, substituting Tyr7 with photo-

Table 1: Sequences and minimal inhibitory concentrations (MIC) of oncocin and apidaecin derivatives.

Peptide	Sequence[a]	MIC [μmol L^{-1}] BW25113	JW0013 (BW25113 Δ*dnaK*)
Api88	gu-ONNRPVYIPRPRPPHPRL-NH_2	0.44	0.27
Api137	gu-ONNRPVYIPRPRPPHPRL-OH	1.75	0.87
Onc72	VDKPPYLPRPRPPROIYNO-NH_2	6.7	6.7
Onc112	VDKPPYLPRPRPPRrIYNr-NH_2	1.7	1.7

[a] gu, O, and r denote *N*,*N*,*N*′,*N*′-tetramethylguanidino, L-ornithine, and D-arginine, respectively.

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reactive *p*-benzoylphenyl-alanine.[20] *E. coli* BL21AI was either incubated with peptide or grown without peptide and then irradiated with UV light.[11] Cross-linked biotin–Api88 proteins were enriched from cell lysates by using streptavidin-coated magnetic beads and separated by SDS-PAGE. The three lanes were cut into 24 equal pieces, digested with trypsin, analyzed by nanoRP-UPLC–ESI-QqTOF-MS, and the relative protein quantities determined. Whereas a few proteins were identified at similar ratios, many were present in the L-Api88 sample in much higher proportions than in the controls.[20] Especially interesting was the 50S ribosomal protein L10, the detection of which indicates that PrAMPs bind to the 70S ribosome (which consists of a 50S and a 30S unit) and might thereby interfere with protein translation.

Based on this data, we studied the in vitro binding of two apidaecin and two oncocin analogues to the *E. coli* 70S ribosome. The 70S ribosome was isolated from *E. coli* strain BL21(DE3)RIL, which is susceptible to Api88, Api137, Onc72, and Onc112 with MIC values ranging from 1 to 7 μmol L^{-1} (Table 2).

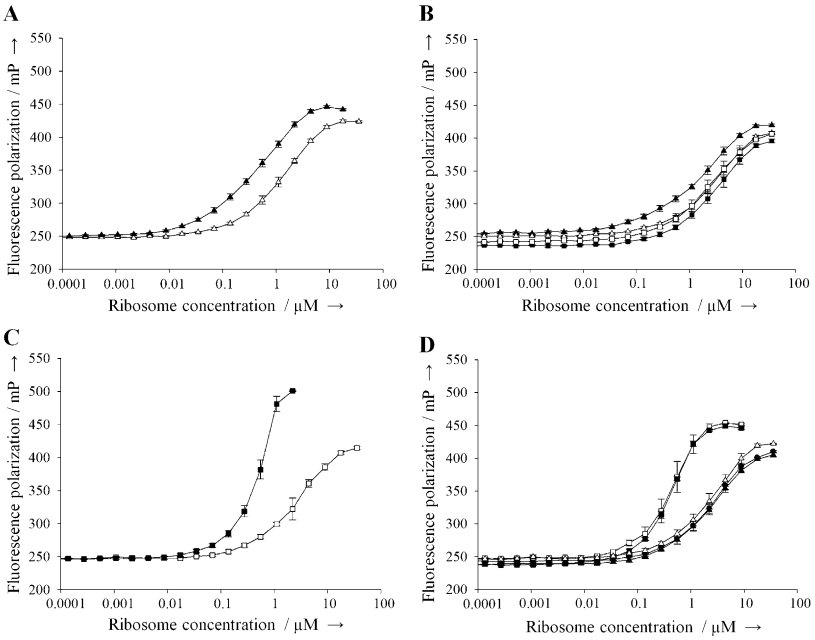

Figure 1. Fluorescence polarization curves recorded for oncocin and apidaecin derivatives with the *E. coli* 70S ribosome after an incubation period of 90 min at 28 °C. A) ▲/△ L/D-Onc72, B) ▲ Onc72 (K3A), ■ Onc72 (Y6A), △ Onc72 (L7A), □ Onc72 (R11A), C) ■/□ L/D-Api137. D) ▲ Api137$^{1-16}$, △ Api137$^{1-17}$, ■ Api137 (R4A), □ Api137 (P14A), ● Api137 (R17A).

Onc72 and Onc112 bound to the 70S ribosome with K_d values of 450 and 90 nmol L^{-1} (Table 2, Figure 1 A), respectively, which are around 10- to 40-fold lower than the corresponding K_d value for Onc72 with DnaK (4 μmol L^{-1}).[12] Api88 and Api137 bound slightly more weakly (K_d = 1.22 and 0.56 μmol L^{-1}, respectively; Figure 1 B) than the oncocins, but they still bound around 4- to 15-fold more strongly to the 70S ribosome than to DnaK (K_d = 5 and 8.7 μmol L^{-1}, respectively).[4d] The corresponding all-D peptides were inactive against *E. coli* and showed higher K_d values, thus indicating stereospecific interactions.

Table 2: Apidaecin and oncocin analogues studied for their activity against *E. coli* BL21(DE3)RIL (MIC values), the binding of the corresponding carboxyfluorescein (Cf)-labelled derivatives towards the *E. coli* 70S ribosome (K_d values), and their inhibition efficiency in cell free GFP expression (IC_{50} values).

Peptide[a]	MIC [μmol L^{-1}]	K_d [μmol L^{-1}]	IC_{50} [μmol L^{-1}]
Api88	0.9	1.22 ± 0.09	> 10[b]
Api137	1.8	0.56 ± 0.06	> 10
Onc72	6.9	0.45 ± 0.03	0.24 ± 0.04
Onc112	3.4	0.09 ± 0.003	0.15 ± 0.03
D-Api88	56	n.d.[c]	> 10
D-Api137	112	2.95 ± 0.15	> 10
D-Onc72	112	1.31 ± 0.06	> 10
D-Onc112	54	n.d.	> 10
Onc72 (K3A)	56	1.95 ± 0.19	4.5 ± 2.7
Onc72 (Y6A)	112	3.31 ± 0.16	> 10
Onc72 (L7A)	112	3.38 ± 0.22	> 10
Onc72 (R11A)	56	2.71 ± 0.10	3.3 ± 1.8
Api137 (R4A)	1.8	0.40 ± 0.01	n.d.
Api137 (P14A)	14	0.42 ± 0.02	n.d.
Api137 (R17A)	112	2.58 ± 0.11	n.d.
Api137$^{1-16}$	253	2.54 ± 0.11	n.d.
Api137$^{1-17}$	29	2.63 ± 0.15	n.d.
Streptomycin	1.7	n.d.	0.08 ± 0.002

[a] D- denotes all-D peptides (see Table S1 in the Supporting Information). [b] > 10 indicates that inhibition occurred but was too low to calculate an IC_{50} value. [c] n.d. = not determined

Two earlier studies had indicated that alanine substitutions of Lys3, Tyr6, Leu7, and Arg11 in Onc72 and Arg17 in Api137 abolish the antibacterial activities.[12,4d] We confirmed this result for *E. coli* BL21(DE3)RIL (Table 2). The corresponding Cf-labelled Onc72 analogues bound to the 70S ribosome around 4.3 to 7.5 times more weakly than Onc72 and even more weakly than the all-D-Onc72 (Table 2, Figure 1 C). Accordingly, Api137 (R17A) and two truncated Api137 peptides missing one (Leu18) or two (Arg17 and Leu18) C-terminal residues bound around 4.6 times less efficiently than Api137, whereas substitutions at Arg4 and Pro14 had no effect (Table 2, Figure 1 D). Interestingly, the important interaction sites contained basic (Arg and Lys) and hydrophobic (Tyr and Leu) residues, with each residue making a similar contribution to binding.

The influence of the two peptide families on protein expression was studied with green fluorescent protein (GFP) in a cell-free system (in vitro translation), in which expression was monitored by fluorescence intensity and SDS-PAGE. T7 RNA polymerase, which was used for plasmid transcription, was not inhibited by any of the apidaecin or oncocin peptides (data not shown). The fluorescence intensity of approximately 7000 recorded for cell-free protein expression represents maximal GFP expression, which was confirmed by SDS-PAGE (Figure 2). Reactions without template DNA or

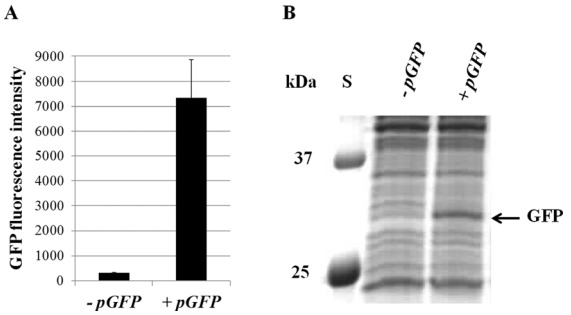

Figure 2. Cell-free expression of GFP. A) Fluorescence intensity and B) SDS-PAGE (Coomassie staining) of a cell-free protein expression assay after 4 h incubation at 37 °C in the absence (−) or presence (+) of p*GFP*. S denotes the Precision Plus Protein Standard (Bio-Rad Laboratories).

with a plasmid not encoding GFP yielded fluorescence intensities of only around 300 and no detectable GFP bands (Figure 2).

Streptomycin, an antibiotic that inhibits protein biosynthesis and is active against *E. coli* BL21(DE3)RIL (MIC $\approx 1.7\ \mu mol\,L^{-1}$), strongly inhibited GFP synthesis with a half maximal inhibitory concentration (IC_{50} value) of 80 $nmol\,L^{-1}$ and full inhibition above 1 $\mu mol\,L^{-1}$ (Table 2, Figure 3). Onc72 and Onc112 showed very similar inhibition curves, with IC_{50} values of 240 and 150 $nmol\,L^{-1}$, respectively (Table 2, Figure 3 A). Interestingly, the IC_{50} values for Onc72 (K3A) and Onc72 (R11A) were 4.5 and 3.3 $\mu mol\,L^{-1}$, respectively, whereas both Onc72 (Y6A) and Onc72 (L7A) had very minor effects on GFP expression (Table 2, Figure 3 C). The IC_{50} values correlated well with the K_d and MIC values; all four Ala-substituted oncocins were only slightly active (MIC $\geq 56\ \mu mol\,L^{-1}$). Api88 and Api137 produced a steady decrease in GFP expression, but only down to approximately 40 % at the highest peptide concentrations (Table 2, Figure 3 B), thus making them less efficient than oncocins. This lower inhibition might be an in vitro effect or it might indicate a different inhibition mechanism that is not so pronounced or easily detectable in vitro.

Api88, Api137, Onc72, and Onc112 bound to DNA and tRNA only at high concentrations, as indicated by gel-shift experiments (Figures S1, S2 in the Supporting Information), with the corresponding all-D peptides showing exactly the same shifts. This result indicates that ribosome binding of the positively charged antimicrobial peptides is unlikely to result from unspecific electrostatic interactions with RNA, although the possibility of specific interactions with folded ribosomal RNA in the ribosome should be investigated.

In conclusion, our data shows for the first time that oncocins, and most likely the related pyrrhocoricin[13] and other PrAMPs, bind to the bacterial 70S ribosome with K_d values in the nanomolar range. This result represents a novel mode of action for gene-encoded antimicrobial peptides. Apidaecins bind strongly to the ribosome but show significantly higher IC_{50} values in vitro, thus indicating that they interfere with the ribosome in a different manner and might bind to a different region as oncocins. PrAMPs most likely also bind to different sites of the 70S ribosome than amino-

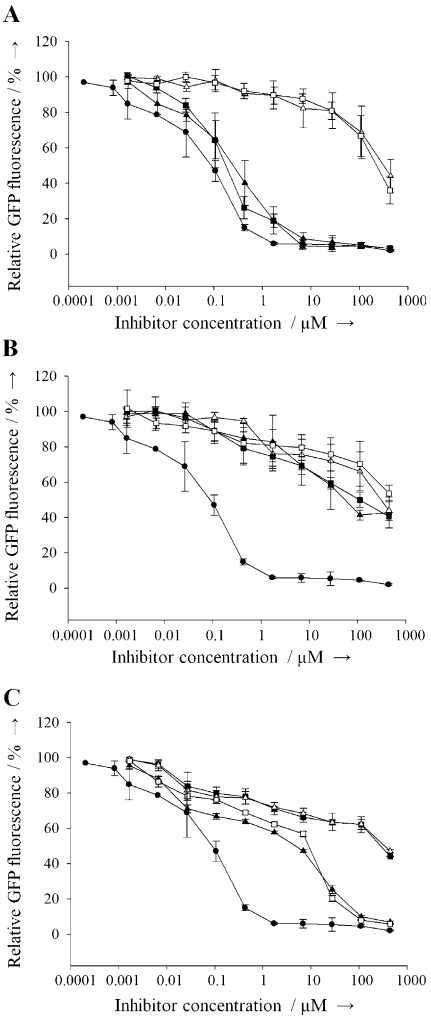

Figure 3. The effect of oncocin and apidaecin derivatives on cell-free GFP expression. GFP expression was monitored by fluorescence, which is displayed relative to the intensities of the control sample (100 %) without inhibiting compounds. A) ▲/△ L/D-Onc72 and ■/□ L/D-Onc112. B) ▲/△ L/D-Api88 and ■/□ L/D-Api137. C) ▲ Onc72 (K3A), ■ Onc72 (Y6A), △ Onc72 (L7A), □ Onc72 (R11A) with increasing concentrations. Streptomycin (●) was used as a control.

glycosides (e.g. streptomycin), tetracyclines, and cyclic peptides.[14] Oncocins bind to the 70S ribosome through at least two basic and two hydrophobic side chains located between positions 3 and 11, which could prevent resistance induced by single mutations to the ribosomal proteins. In apidaecins, Arg17 and Leu18 were identified as major interaction sites, but this doesn't rule out the involvement of other residues. Interestingly, it was noted earlier that even slight structural changes, such as lysine, ornithine, or homoarginine substitutions at Arg17, strongly reduce the antibacterial activity of apidaecins.[4d] This binding site is further confirmed by the above-cited observation that truncated Api88/Api137 sequences are inactive[7] and bind only weakly to the ribosome (Table 2). Inhibition of the 70S ribosome as a mechanism of action explains the activities of different apidaecin and oncocin analogues and would additionally explain why PrAMPs are not toxic to human cell lines.[15] DnaK binds PrAMPs less efficiently but will still capture some of these

peptides. This would explain why DnaK null mutants are equally or slightly more susceptible to apidaecins and oncocins and why better DnaK binders are less efficient. Although further studies are required to identify the exact binding region, a fluorescence polarization assay could be used to screen for more efficient peptide inhibitors or small molecules that bind to the same region.

Experimental Section

Peptides were synthesized on Rink amide or Wang resins by using Fmoc/*t*Bu-chemistry, cleaved with trifluoroacetic acid (TFA), and purified by RP-HPLC on a C_{18} phase by using an aqueous acetonitrile gradient containing 0.1% (v/v) TFA (see the Supporting Information).[4d,16] Peptide masses were confirmed by MALDI-TOF-MS.

Bacterial cultures (see the Supporting Information) were grown in lysogeny broth (LB) Miller (10 g L^{-1} NaCl) or 1% (w/v) tryptic soy broth (TSB; 33% TSB medium) at 37 °C under aerobic conditions. Antibacterial activities were determined in triplicate by a liquid broth microdilution assay in sterile 96-well plates with 33% TSB medium (7.5×10^5 cells per well; 20 ± 2 h, 37 °C).[4d] The MIC was defined as the lowest substance concentration for which the turbidity at 595 nm was equal to that of the medium.

The binding of Cf-labelled peptides to the 70S ribosome was determined by fluorescence polarization (FP) with a two-fold dilution series of the 70S ribosome (starting at 71 μmol L^{-1}) in the presence of Cf-labelled peptide (20 nmol L^{-1}; 28 ± 1 °C). The 70S ribosome was purified from a crude ribosome extract of *E. coli* BL21(DE3)RIL (Stratagene, Cedar Creek, USA) cultivated in LB-media overnight ($OD_{600} \approx 4$; For details see the Supporting Information).[18] FP was recorded after an incubation time of 1.5 h ($\lambda_{Exc.} = 485$ nm, $\lambda_{Em} = 535$ nm) at least twice in triplicate on two different days. Data were fitted to a nonlinear, dose–response logistical transition equation [$y = y_0 + a/(1+(x/x_0)^b)$] by using the Levenberg–Marquardt algorithm with the K_d value being represented by the x_0 coefficients (SigmaPlot, Systat Software Inc., California, U.S.A.).

Cell-free protein expression, including the preparation of S30 and S30-T7 lysates, relied on the protocols reported by Kim et al.[17] Green fluorescent protein (GFP) was expressed from the plasmid p*GFP* (Addgene, Cambridge, MA, USA) and expression was quantified through the increase in fluorescence intensity after 4 h ($\lambda_{Exc.} = 489$ nm, $\lambda_{Em} = 510$ nm). To investigate the effect of apidaecin and oncocin derivatives on protein expression, these peptides or streptomycin[19] were added at different concentrations.

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[1] O. Nolte, *Protein Pept. Lett.* **2014**, *21*, 330 – 335.
[2] K. Kumar, S. Chopra, *J. Antimicrob. Chemother.* **2013**, *68*, 1465 – 1470.
[3] L. Otvos, *Cell. Mol. Life Sci.* **2002**, *59*, 1138 – 1150.
[4] a) E. Ostorhazi, M. C. Holub, F. Rozgonyi, F. Harmos, M. Cassone, J. D. Wade, L. Otvos, Jr., *Int. J. Antimicrob. Agents* **2011**, *37*, 480 – 484; b) D. Szabo, E. Ostorhazi, A. Binas, F. Rozgonyi, B. Kocsis, M. Cassone, J. D. Wade, O. Nolte, L. Otvos, *Int. J. Antimicrob. Agents* **2010**, *35*, 357 – 361; c) M. Benincasa, C. Pelillo, S. Zorzet, C. Garrovo, S. Biffi, R. Gennaro, M. Scocchi, *BMC Microbiol.* **2010**, *10*, 178; d) P. Czihal, D. Knappe, F. Fritsche, M. Zahn, N. Berthold, S. Piantavigna, U. Müller, S. Van Dorpe, N. Herth, A. Binas, G. Köhler, B. De Spiegeleer, L. L. Martin, O. Nolte, N. Strater, G. Alber, R. Hoffmann, *ACS Chem. Biol.* **2012**, *7*, 1281 – 1291; e) N. Berthold, P. Czihal, S. Fritsche, U. Sauer, G. Schiffer, D. Knappe, G. Alber, R. Hoffmann, *Antimicrob. Agents Chemother.* **2013**, *57*, 402 – 409; f) E. Ostorházi, E. Nemes-Nikodém, D. Knappe, R. Hoffmann, *Protein Pept. Lett.* **2014**, *21*, 368 – 373.
[5] a) M. Mattiuzzo, A. Bandiera, R. Gennaro, M. Benincasa, S. Pacor, N. Antcheva, M. Scocchi, *Mol. Microbiol.* **2007**, *66*, 151 – 163; b) G. Runti, C. Lopez Ruiz Mdel, T. Stoilova, R. Hussain, M. Jennions, H. G. Choudhury, M. Benincasa, R. Gennaro, K. Beis, M. Scocchi, *J. Bacteriol.* **2013**, *195*, 5343 – 5351.
[6] L. Otvos, Jr., I. O, M. E. Rogers, P. J. Consolvo, B. A. Condie, S. Lovas, P. Bulet, M. Blaszczyk-Thurin, *Biochemistry* **2000**, *39*, 14150 – 14159.
[7] M. Zahn, N. Berthold, B. Kieslich, D. Knappe, R. Hoffmann, N. Strater, *J. Mol. Biol.* **2013**, *425*, 2463 – 2479.
[8] N. Berthold, R. Hoffmann, *Protein Pept. Lett.* **2014**, *21*, 391 – 398.
[9] T. Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, H. Mori, *Mol. Syst. Biol.* **2006**, *2*, 2006.0008.
[10] M. Liebscher, A. Roujeinikova, *J. Bacteriol.* **2009**, *191*, 1456 – 1462.
[11] G. Dörmán, G. D. Prestwich, *Trends Biotechnol.* **2000**, *18*, 64 – 77.
[12] D. Knappe, M. Zahn, U. Sauer, G. Schiffer, N. Sträter, R. Hoffmann, *ChemBioChem* **2011**, *12*, 874 – 876.
[13] G. Kragol, R. Hoffmann, M. A. Chattergoon, S. Lovas, M. Cudic, P. Bulet, B. A. Condie, K. J. Rosengren, L. J., Montaner, L. Otvos, Jr., *Eur. J. Biochem.* **2002**, *269*, 4226 – 4237.
[14] J. Poehlsgaard, S. Douthwaite, *Nat. Rev. Microbiol.* **2005**, *3*, 870 – 881.
[15] A. Hansen, I. Schäfer, D. Knappe, P. Seibel, R. Hoffmann, *Antimicrob. Agents Chemother.* **2012**, *56*, 5194 – 5201.
[16] F. I. Nollmann, T. Goldbach, N. Berthold, R. Hoffmann, *Angew. Chem. Int. Ed.* **2013**, *52*, 7597 – 7599; *Angew. Chem.* **2013**, *125*, 7747 – 7750.
[17] T. W. Kim, J. W. Keum, I. S. Oh, C. Y. Choi, C. G. Park, D. M. Kim, *J. Biotechnol.* **2006**, *126*, 554 – 561.
[18] U. Bommer, N. Burkhardt, R. Jünemann, C. M. T. Spahn, F. J. Triana-Alonso, K. H. Nierhaus, *Subcellular fractionation. A practical approach* (Eds.: J. Graham, D. Rickwoods), IRL Press, Oxford University Press, Oxford, **1996**, pp. 271 – 301.
[19] L. Luzzatto, D. Apirion, D. Schlessinger, *Proc. Natl. Acad. Sci. USA* **1968**, *60*, 873 – 880.
[20] D. Volke, A. Krizsan, N. Berthold, D. Knappe, R. Hoffmann, Unpublished results; manuscript in preparation..

