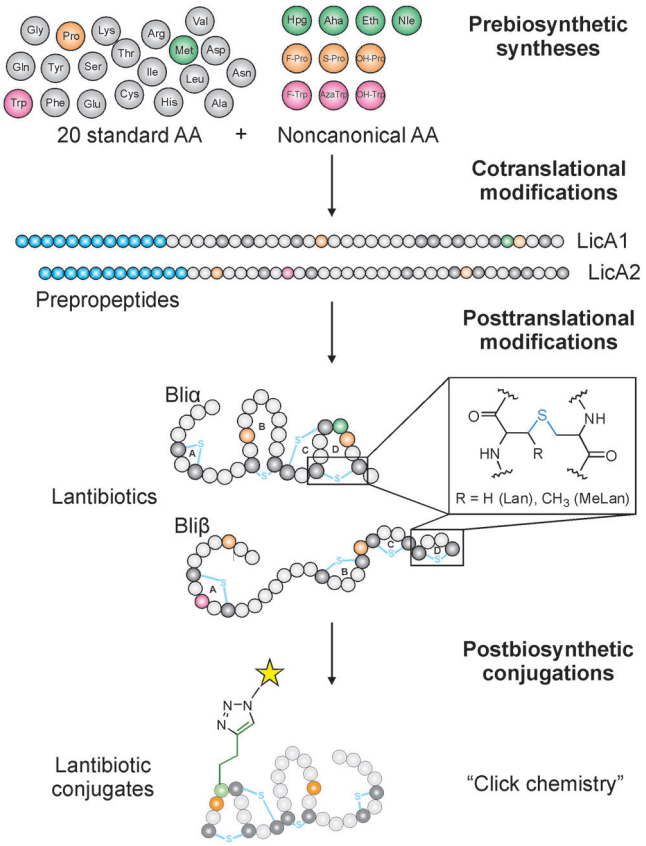
Peptide Synthesis

DOI: 10.1002/anie.201106154

Congeneric Lantibiotics from Ribosomal In Vivo Peptide Synthesis with Noncanonical Amino Acids**

Florian Oldach, Rashed Al Toma, Anja Kuthning, Tânia Caetano, Sónia Mendo, Nediljko Budisa, and Roderich D. Süssmuth**

Ribosomally synthesized peptide antibiotics constitute an important group of secondary metabolites synthesized by bacteria and fungi. Among these are lantibiotics, lanthionine (Lan)-containing bioactive polycyclic peptide antibiotics produced by various Gram-positive bacteria. Lantibiotics exhibit antimicrobial activity against a variety of pathogenic bacteria, for example, *Staphylococcus aureus*.[1] Subsequent to ribosomal peptide synthesis (RPS) peptides are posttranslationally modified. In the case of lantibiotics the amino acids Ser/Cys and Thr/Cys form the thioether-containing amino acids Lan and MeLan, respectively (Scheme 1).[2] Attempts to broaden the structural diversity of such peptides are limited by the restriction of ribosomal peptide synthesis (RPS) to the set of 20 canonical amino acids (cAAs). While the viability of total synthesis approaches to such peptides, albeit artful, is limited,[3] semisynthetic modifications only with easily accessible functional groups have been exploited, and site-directed mutagenesis approaches have also met with restricted success.[4] Expressed protein ligation (EPL) allows the generation of semisynthetic proteins with an almost unlimited number of noncanonical amino acids (ncAAs).[5] Although the intrinsic limitation that ncAAs are delivered only to the peptide part of the target protein could be overcome, as recently shown by Benkovic and Schultz,[6] the product yields of this procedure are extremely low. In contrast, the residue-specific replacement of amino acids,[7] which generally does not suffer from such drawbacks, is a more promising strategy to achieve efficient chemical diversification by directly translating various ncAAs into bioactive peptides.



Scheme 1. Expansion of the synthetic possibilities for the design of ribosomal peptide antibiotics by an expanded amino acid repertoire. Reprogrammed in vivo synthesis of lantibiotics includes three levels of chemical diversification: 1) cotranslational, by insertion of various ncAAs, including some with unique chemical handles, 2) posttranslational, that is, enzymatic processing and assembly of characteristic lanthionines (Lan, MeLan), and 3) postbiosynthetic, enabling the tailoring of the lantibiotics' structures by bioorthogonal conjugations under physiological conditions (e.g. attachment of various ligands such as chromophores and pharmacophores by click chemistry).

Recently, possibilities for lanthionine formation in cell-free translation systems (in vitro) have been reported,[8] whereas we, and others, succeeded in the expression of lantibiotics by RPS in Gram-negative *Escherichia coli* as a heterologous host.[9,10] This bacterial host is, to date, the most efficient platform for genetic code engineering by which one or more types of ncAAs are cotranslationally incorporated into the target polypeptide sequences.[11] Therefore, it should be possible to employ recombinant expression for the

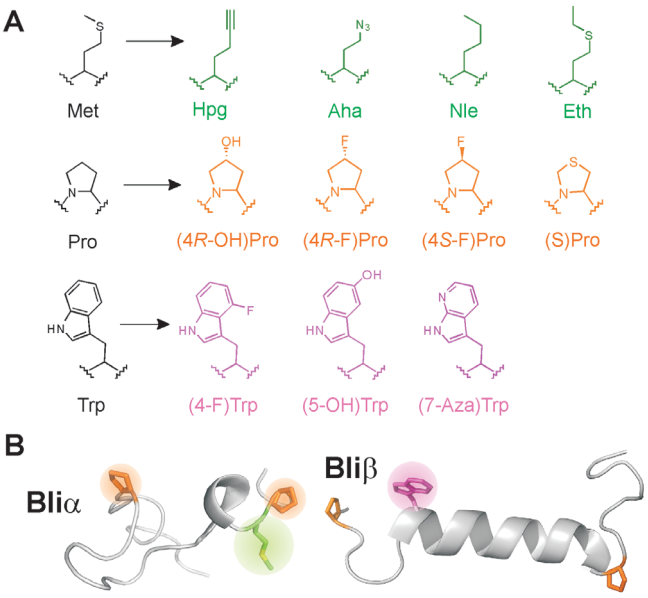
[*] F. Oldach, R. Al Toma, A. Kuthning, Prof. N. Budisa, Prof. R. D. Süssmuth
Institut für Chemie, Technische Universität Berlin
10623 Berlin (Germany)
E-mail: budisa@biocat.tu-berlin.de
suessmuth@chem.tu-berlin.de
Homepage: http://www.biochemie.tu-berlin.de
http://www.biocat.tu-berlin.de
T. Caetano, Prof. S. Mendo
Department of Biology and CESAM, University of Aveiro
3810 Aveiro (Portugal)

[**] This work was supported by the Cluster of Excellence "Unifying Concepts in Catalysis" funded by the DFG and coordinated by the TU Berlin. We are indebted to Waltraud Wenger for technical support at the initial stages of the project. R. Al Toma gratefully acknowledges support from the EMECW (Erasmus Mundus-External Cooperation Window) for a fellowship. Tânia Caetano is supported by "Fundação para a Ciência e Tecnologia". We are grateful to Dr. Lars Merkel for the critical reading and helpful comments and suggestions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201106154.

production of antibiotics with ncAAs. By incorporating different combinations of numbers and functional groups it would be possible to diversify the lantibiotics' structure as well as generate novel and unique sequence combinations. This is of utmost importance for drug development since lantibiotics, aside from possessing well-known antimicrobial activity (e.g. subtilin and nisin[12]), also exhibit other important bioactivities, such as pain-suppression in mice.[13]

In vivo expression of ribosomally synthesized peptide antibiotics has not been examined with a more diverse set of ncAAs. Herein we describe the heterologous expression of the two-component lantibiotic lichenicidin from *Bacillus licheniformis*[14] in *E. coli* (Scheme S2 in the Supporting Information) as a model system for the translation of ncAAs into lantibiotics (Scheme 1). In this system the plasmid-encoded expression of the Bliα and Bliβ prepropeptide is accompanied by expression of the fosmid-encoded posttranslational biosynthesis machinery in the *E. coli* host as described previously.[9] We used a 3D structural model of the two lichenicidin components, termed Bliα and Bliβ, based on NMR data[15] to rationally choose residues to be exchanged during translation (Scheme 2).



Scheme 2. Canonical amino acids and related analogues incorporated into lichenicidin Bliα and Bliβ. A) residue-specific exchange of canonical amino acids (left) with related noncanonical analogues (right) is achieved by a series of single in vivo expression experiments. B) 3D structural model of the two-component lantibiotic lichenicidin, with Met (Bliα), Pro (Bliα and Bliβ), and Trp (Bliβ) side chains; amino acid residues selected for replacement are shaded in color.

The auxotrophic *E. coli* strain B834 (Met$^-$), unable to synthesize Met, was transformed with a plasmid encoding for the synthesis of the Bliα prepropeptide (LicA1, Scheme 1) and a fosmid encoding the posttranslational lantibiotic biosynthesis machinery. Special features of the latter include the lanthionine-cyclases LicM1 and LicM2 for the posttranslational modification, the transporter LicT performing the processing and export from the cytoplasm, and the protease LicP (Scheme S2 in the Supporting Information). Transformed cells were grown in liquid culture to a given cell density (OD_{600} = 0.6–0.8). Then the medium was replaced by new minimal medium (NMM)[7] supplemented with various ncAAs (50 mg L^{-1}): Hpg (homopropargylglycine), Aha (azidohomoalanine), Nle (norleucine), and Eth (ethionine) as replacements for Met. Upon induction of Bliα production with isopropyl β-D-1-thiogalactopyranoside (IPTG), cells were grown for 16 h at 30 °C. Subsequently, peptides were extracted with *n*-butanol and purified by solid-phase extraction.

The purified peptides were analyzed by high-resolution HPLC-ESI-MS, and the incorporation of the amino acid analogues of Met (Scheme 2) was detected based on the characteristic mass shifts of the molecular masses of Bliα (Figure 1 and Figure S1 in the Supporting Information). The incorporation of the amino acid analogues into the B-C-D ring was further confirmed by MS/MS experiments (Figure S3 in the Supporting Information). LC-MS analyses were performed in order to quantify the amounts of produced congeneric Bliα in comparison to the purified wild-type peptide.

Micrococcus luteus was used as an indicator strain in an agar-well diffusion assay to assess the antimicrobial activity of Met congeners of Bliα. To that end Met congeners were quantified by mass spectrometry and applied to the assay in a molar ratio of 1:1 (Bliα:Bliβ). The antibacterial activity displayed by Bliα(Met28Hpg) combined with wt-Bliβ was

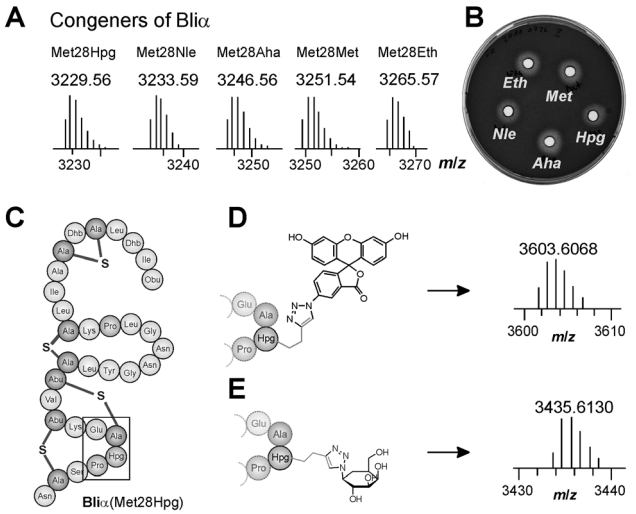


Figure 1. Activity profiles and the conjugation properties of lichenicidin congeners containing Met analogues. A) HR-ESI-MS analysis of lichenicidin peptide Bliα (M_{calc} = 3251.53 Da) and Met congeners: Bliα-(Met28Hpg) (M_{calc} = 3229.53 Da), Bliα(Met28Nle) (M_{calc} = 3233.57 Da), Bliα(Met28Aha) (M_{calc} = 3246.54 Da), Bliα(Met28Eth) (M_{calc} = 3265.54 Da). B) *M. luteus* inhibition assay for Bliα assessing the antibacterial effects of Met congeners. C) Structure of Bliα (Met28Hpg). HR-ESI-MS analysis of Bliα(Met28Hpg) conjugated to D) the dye azido-fluorescein (M_{calc} = 3603.6087 Da) and E) the azido-sugar 1-azido-1-deoxy-β-D-glucopyranoside (M_{calc} = 3435.6087 Da) as examples of postbiosynthetic modifications of ribosomally synthesized lantibiotics.

somewhat less than that of wild-type lichenicidin, nearly equal for congeners containing Eth or Nle analogues, and even fully restored for the Aha congener (Figure 1B and Figure S4 in the Supporting Information).

In a final set of experiments, we sought to apply the principles of structural diversification to lantibiotics by postbiosynthetic modifications using bioorthogonal synthetic chemistry. A Cu^{I}-catalyzed Huisgen 1,3-dipolar cycloaddition (click reaction)[16] was performed with Bliα(Met28Hpg) according to protocols described previously (Table S3 in the Supporting Information).[17] Synthetic coupling to the Bliα peptide was achieved with 1-azido-1-deoxy-β-D-glucopyranoside and azido-fluorescein as shown by HR-ESI-MS (Figure 1).

Finally, we aimed to elucidate the ability of the lichenicidin biosynthetic machinery to tolerate the exchange of further cAAs for ncAAs in Bliα and Bliβ. We chose proline (Pro), represented twice in both Bliα and Bliβ, and tryptophan (Trp), found exclusively in Bliβ, as residues to be replaced (Scheme 2). The incorporation of Pro analogues was performed only in Bliα. All experiments with the related auxotrophic strains JM83 (Pro^{-}) and ATCC 49980 (Trp^{-}) were performed by using the media-shift procedure described above and in the Supporting Information. Thereby, Pro was replaced by (4*R*-OH)Pro (*trans*-4-hydroxyproline), (4*S*-F)/(4*R*-F)Pro (*cis*- and *trans*-4-fluoroproline), and (S)Pro (L-thioproline). Trp analogues (4-F)Trp (4-fluorotryptophan), (5-OH)Trp (5-hydroxytryptophan), and (7-Aza)Trp (7-azatryptophan) were translated into lichenicidin using fermentation and expression procedures described by Lepthien et al.[18] Expectedly, the mass spectrometric analysis clearly showed high levels of incorporation for all these analogues at the defined positions (Figure S2 in the Supporting Information). These experiments demonstrate the general applicability of our approach for the structural and chemical diversification of ribosomally synthesized lantibiotics.

In the natural scenario, lantibiotics (like many other ribosomally synthesized peptides) must first pass the prepropeptide stage with 20 canonical amino acids as standard building blocks before posttranslational modification (PTM), which efficiently transforms the peptides into bioactive lantibiotics (Scheme 1). PTMs constitute an additional level of natural chemical modification and diversification achieved by the battery of enzymes that selectively process various types of amino acid side chains.[19] By the expansion of the amino acid repertoire through the recruiting of other ncAAs for ribosomal peptide syntheses, the diversity of these modifications can be dramatically increased. The synthetic scope of this system has been expanded as we efficiently introduced additional levels of peptide diversity beyond the capacities of living cells, enabling us to generate lantibiotics with chemical properties not developed by natural evolution.

Currently we are expanding our strategy to other ncAAs including fluorinated amino acid analogues. For example, it is well known that fluorinated polymers show enhanced thermodynamic and hydrolytic stability as well as improved bioavailability.[20] Similar effects can be envisioned by systematic fluorination of peptide antibiotics as well. In addition, as we have illustrated with the Hpg-containing lichenicidin, the sequence positions of lantibiotics not affected by PTMs can be occupied by additional bioorthogonal reactive groups making them amenable to other means of bioorthogonal conjugation. On the other hand, sequence positions that are targets for posttranslational processing can be translated by various ncAAs (e.g. with alternative chirality, polarity, molecular volume etc.) in order to harness the catalytic scope of the PTM machinery of lantibiotics.

We anticipate that our findings, in combination with other methods (such as site-directed suppression-mediated ncAA incorporation and the guided evolution of target peptides), will enable us to create toolkits for the generation of antibiotics with novel sequence compositions. These compounds should display greatly enhanced structural and functional diversity while retaining or even improving their structural integrity and bioactivity profile.

Experimental Section

Auxotrophic *E. coli* strains (JM83, B834, and ATCC 49980) were transformed with fosmid pLic5*ΔA1* and plasmid pQE80L-*A2* or with fosmid pLic5*ΔA2* and plasmid pQE80L-*A1*[9] in order to express Bliα and Bliβ separately. Cells were grown in LB media to $OD_{600} = 0.6$–0.8. Then the LB media was replaced by New Minimal Media (NMM)[7] containing the ncAAs (50 mg L^{-1}), and subsequently induced with 1 mM IPTG (final concentration). After growth for 16 h at 30 °C, peptides were extracted with *n*-butanol and purified by solid-phase extraction (SPE-C8). Analytical characterization of the peptides was performed by high-resolution HPLC-ESI-Orbitrap-MS and MS/MS experiments. LC-MS analyses were performed in order to quantify the amounts of produced congeneric Bliα in comparison to the wild-type peptides.

Micrococcus luteus ATCC 9341 was used as an indicator strain in the agar-well diffusion assay to test antimicrobial activity of congeneric Met analogues of Bliα.

A detailed description of the experimental protocols and analytical methods is found in the Supporting Information.

Received: August 30, 2011
Revised: October 20, 2011
Published online: November 30, 2011

Keywords: in vivo peptide synthesis · lantibiotics · lichenicidin · noncanonical amino acid · peptide antibiotics

[1] H.-G. Sahl, G. Bierbaum, *Annu. Rev. Microbiol.* **1998**, *52*, 41–79.
[2] G. Jung, *Angew. Chem.* **1991**, *103*, 1067–1084; *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 1051–1068.
[3] W. Liu, A. S. H. Chan, H. Liu, S. A. Cochrane, J. C. Vederas, *J. Am. Chem. Soc.* **2011**, *133*, 14216–14219.
[4] a) M. Paul, W. A. van der Donk, *Mini-Rev. Org. Chem.* **2005**, *2*, 23–37; b) N. Ghalit, J. F. Reichwein, H. W. Hilbers, E. Breukink, D. T. S. Rijkers, R. M. J. Liskamp, *ChemBioChem* **2007**, *8*, 1540–1554; c) O. P. Kuipers, G. Bierbaum, B. Ottenwälder, H. M. Dodd, N. Horn, J. Metzger, T. Kupke, V. Gnau, R. Bongers, P. Bogaard, H. Kosters, H. S. Rollema, W. M. Vos, R. J. Siezen, G. Jung, F. Götz, H.-G. Sahl, M. J. Gasson, *Antonie Van Leeuwenhoek* **1996**, *69*, 161–169.
[5] K. M. Clark, W. A. van der Donk, Y. Lu, X. Zhang, W. A. van der Donk in *Methods Enzymol.*, *Vol. 462* (Eds.: T. W. Muir, J. N. Abelson), Academic Press, New York, **2009**, pp. 97–115 and 117–134.

[6] T. S. Younga, D. D. Younga, I. Ahmada, J. M. Louis, S. J. Benkovic, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 11052 – 11056.
[7] N. Budisa, B. Steipe, P. Demangel, C. Eckerskorn, J. Kellermann, R. Huber, *Eur. J. Biochem.* **1995**, *230*, 788 – 796.
[8] a) Y. Goto, K. Iwasaki, K. Torikai, H. Murakami, H. Suga, *Chem. Commun.* **2009**, 3419 – 3421; b) F. P. Seebeck, A. Ricardo, J. W. Szostak, *Chem. Commun.* **2011**, *47*, 6141 – 6143.
[9] T. Caetano, J. M. Krawczyk, E. Mösker, R. D. Süssmuth, S. Mendo, *Chem. Biol.* **2011**, *18*, 90 – 100.
[10] Y. Shi, X. Yang, N. Garg, W. A. van der Donk, *J. Am. Chem. Soc.* **2011**, *133*, 2338 – 2341.
[11] a) J. Xie, P. G. Schultz, *Curr. Opin. Chem. Biol.* **2005**, *9*, 548 – 554; b) A. J. Link, M. L. Mock, D. A. Tirrell, *Curr. Opin. Biotechnol.* **2003**, *14*, 603 – 609; c) N. Budisa, *Angew. Chem.* **2004**, *116*, 6586 – 6624; *Angew. Chem. Int. Ed.* **2004**, *43*, 6426 – 6463.
[12] a) C. Klein, C. Kaletta, K. D. Entian, *Appl. Environ. Microbiol.* **1993**, *59*, 296 – 303; b) K. Rayman, N. Malik, A. Hurst, *Appl. Environ. Microbiol.* **1983**, *46*, 1450 – 1452.
[13] K. Meindl, T. Schmiederer, K. Schneider, A. Reicke, D. Butz, S. Keller, H. Gühring, L. Vértesy, J. Wink, H. Hoffmann, M. Brönstrup, G. M. Sheldrick, R. D. Süssmuth, *Angew. Chem.* **2010**, *122*, 1169 – 1173; *Angew. Chem. Int. Ed.* **2010**, *49*, 1151 – 1154.
[14] M. Begley, P. D. Cotter, C. Hill, R. P. Ross, *Appl. Environ. Microbiol.* **2009**, *75*, 5451 – 5460.
[15] Z. O. Shenkarev, E. I. Finkina, E. K. Nurmukhamedova, S. V. Balandin, K. S. Mineev, K. D. Nadezhidin, Z. A. Yakimenko, A. A. Tagaev, Y. V. Temirov, A. S. Arseniev, T. V. Ovchinnikova, *Biochemistry* **2010**, *49*, 6462 – 6472.
[16] R. Huisgen, R. Grashey, J. Sauer, *Chemistry of Alkenes*, Interscience, New York, **1964**, 806 – 877.
[17] V. Hong, S. I. Presolski, C. Ma, M. G. Finn, *Angew. Chem.* **2009**, *121*, 10063 – 10067; *Angew. Chem. Int. Ed.* **2009**, *48*, 9879 – 9883.
[18] S. Lepthien, L. Merkel, N. Budisa, *Angew. Chem.* **2010**, *122*, 5576 – 5581; *Angew. Chem. Int. Ed.* **2010**, *49*, 5446 – 5450.
[19] C. Chatterjee, M. Paul, L. Xie, W. A. Van der Donk, *Chem. Rev.* **2005**, *105*, 633 – 683.
[20] M. Pagliaro, R. Ciriminna, *J. Mater. Chem.* **2005**, *15*, 4981 – 4991.

