Enzymatic C–C Bond Formation

DOI: 10.1002/anie.200905909

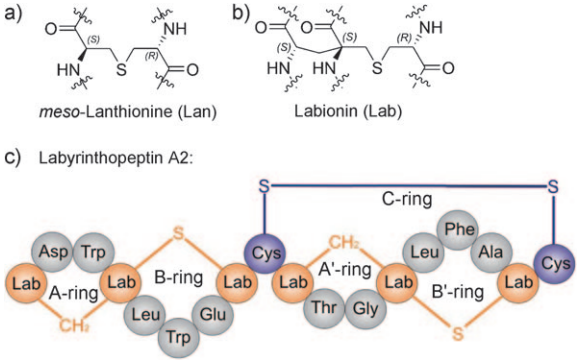
In Vitro Biosynthesis of the Prepeptide of Type-III Lantibiotic Labyrinthopeptin A2 Including Formation of a C–C Bond as a Post-Translational Modification**

*Wolfgang M. Müller, Timo Schmiederer, Paul Ensle, and Roderich D. Süssmuth**

Dedicated to Dr. László Vértesy

Lantibiotics are ribosomally synthesized peptide antibiotics containing the amino acids lanthionine (Lan) and methyllanthione (MeLan) as the most important and characteristic post-translational modification.[1] Work on the in vitro characterization of the enzymatic processing of lantibiotics, in particular for the formation of lanthionine, was described in reports on lacticin 481[2] and haloduracin.[3] Recently, we identified a new family of lantibiotics, named labyrinthopeptins, which are produced by the actinomycete *Actinomadura namibiensis* DSM 6313.[4–6] The structure elucidation of labyrinthopeptin A2 (Scheme 1) mainly performed by X-ray crystallography highlighted a new amino acid, named labionin (Lab) (Scheme 1). Labionin is a triamino acid with a 2*S*,4*S*,8*R* configuration, and consists of a central quarternary carbon atom with a lanthionine motif and an unusual methylene bridge, which establishes a covalent link to a further amino acid moiety. This structure facilitates the formation of two rings in a linear peptide. Bioactivity assays revealed that labyrinthopeptin A2 has an excellent efficacy against neuropathic pain in an in vivo mouse model (ED_{50} = 50 μg kg^{-1}). The additionally found labyrinthopeptins A1 and A3 have been proposed as analogues of the A2 structure containing different amino acid constituents and an alternated ring size of the lanthionine motif.[4]

The overall identification and sequencing of the gene cluster revealed only five genes which could be assigned to the biosynthesis of labyrinthopeptins. These contained two structural genes (*labA1/A2*) as precursors of labyrinthopeptin A1/A3 and A2, two genes with sequence similarity to ATP-dependent ABC transporters for the putative peptide export (*labT1* and *labT2*), and one gene (*labKC*) for a putative modifying enzyme.[4] LabKC (MW = 95 kDa) is a two-domain protein consisting of an N-terminally conserved domain with features of an eukaryotic Ser/Thr protein kinase and a C-terminal domain with low sequence homology to LanC cyclases. In comparison to LanC cyclases, important active site residues, such as the zinc binding motif identified for the nisin cyclase, are missing in LabKC.[7] Moreover, the amino acid sequence of LabKC shows high homology to the SapB modifying enzyme RamC and to sequences from gene clusters of a considerable number of sequenced actinomycete strains (Supporting Information).[4,8] Therefore, we assign labyrinthopeptins as type-III lantibiotics.



Scheme 1. Structures of the amino acids a) lanthionine (Lan) characteristic of lantibiotics and of b) labionin (Lab) characteristic of c) the labyrinthopeptins, a new type-III lantibiotic.

Herein we present the first in vitro reconstitution of the pre-labyrinthopeptin A2 biosynthesis in which a C–C bond formation is catalyzed by LabKC. This reaction involves the unprecedented requirement of guanosine triphosphate (GTP) for the phosphorylation and dehydratation reaction of serines. In other in vitro syntheses of lantibiotics adenosine triphosphate (ATP) and not GTP is used.[2,3] From the obtained data a biosynthetic model has been deduced. To our knowledge, this is the first time that GTP has been shown to act as a co-substrate in the formation of lanthionine-type peptide antibiotics and we assume that the understanding of the function of LabKC as well as of the entire biosynthesis pathway will pave the way for the design of new labyrinthopeptin analogues.

[*] W. M. Müller, Dr. T. Schmiederer, P. Ensle, Prof. Dr. R. D. Süssmuth
Technische Universität Berlin, Fakultät II—Institut für Chemie
Strasse des 17. Juni 124, 10623 Berlin (Germany)
Fax: (+49) 30-314-79651
E-mail: suessmuth@chem.tu-berlin.de
Homepage: http://suessmuth.chem.tu-berlin.de

[**] This research was supported by grants of the Deutsche Forschungsgemeinschaft (DFG SU239/8-1), the Cluster of Excellence "Unifying Concepts in Catalysis" coordinated by the Technische Universität Berlin, and the German Ministry of Education and Research BMBF (no. 0315198). We thank Dr. Mark Brönstrup (Sanofi-Aventis), Anne Hänchen, and Jonny Nachtigall for valuable discussions.

Supporting information for this article (experimental details for the overexpression of labyrinthopeptin kinase–cyclase LabKC, in vitro assays, synthesis of reference compounds, and additional mass spectrometric data) is available on the WWW under http://dx.doi.org/10.1002/anie.200905909.

The DNA sequence of the structural gene *labA2* (coding for 38 amino acids) revealed a Ser-Xxx-Xxx-Ser-Xxx-Xxx-Xxx-Cys motif (Xxx = random amino acid) as precursor amino acids for labionin. For the in vitro synthesis of the labyrinthopeptin A2 prepeptide the kinase-cyclase LabKC was overexpressed in *Escherichia coli* with a C-terminal hexahistidine tag (LabKC-His_6) and purified by immobilized metal affinity chromatography (IMAC; Supporting Information). The LabKC substrate was the 38 amino acid labyrinthopeptin A2 precursor peptide consisting of an N-terminal 20 amino acid leader peptide and of a C-terminal 18 amino acid structural peptide and was synthesized by solid-phase peptide synthesis (SPPS; Supporting Information). Based on the conserved nucleotide binding site of LabKC and the similarity of catalytic domains to other Ser/Thr protein kinases, such as PknB from *Mycobacterium tuberculosis* (Supporting Information),[9] we decided to use ATP as a substrate for the phosphorylation reaction. However, incubation of LabKC-His_6 with the LabA2 precursor in the presence of ATP and Mg^{2+} ions gave no conversion (Figure 1). Subsequently, we systematically examined the other nucleotides guanosine triphosphate (GTP), thymidine triphosphate (TTP), and cytidine triphosphate (CTP) as alternative phosphate donors. Only by incubation in the presence of GTP/dGTP and Mg^{2+} for 12 h, was the LabA2 prepropeptide (M_r = 4272.88 Da) converted into the four-fold dehydrated prepeptide ($[M-72+4H]^{4+}$) as the main product (Figure 1 and the Supporting Information). In addition, signals with mass losses of 54, 36, and 18 Da were observed corresponding to triple, double, and single dehydrations of the 38-mer substrate, as well as three signals corresponding to three monophosphorylated dehydrated intermediates ($M-54+80$ Da, $M-36+80$ Da and $M-18+80$ Da) (Figure 1 and Table 1). These results clearly show that LabKC was obtained as a functional enzyme utilizing GTP rather than ATP for the phosphorylation and subsequent dehydration of serines to α,β-di(dehydro)alanines (Dha).

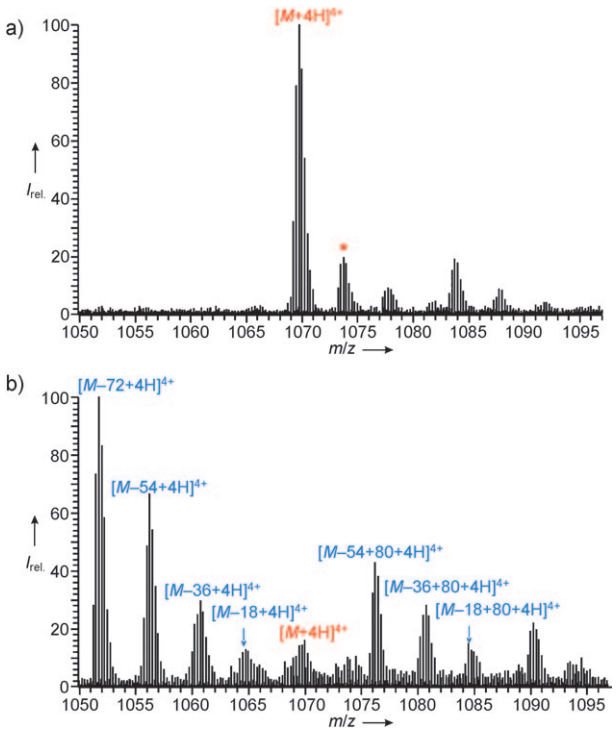


Figure 1. HR-ESI-orbitrap-MS-spectrum of the in vitro assay of kinase–cyclase LabKC-His_6 with 38-mer prepropeptide LabA2. Asterisks indicate the methionine oxidation products of the peptide. a) $[M+4H]^{4+}$ ions of the reaction with ATP (12 h); b) the same assay performed with GTP (1 mM; 12 h).

Table 1: Calculated and experimental molecular masses in the HR-ESI-orbitrap-mass spectra of the in vitro conversion of LabA2 prepropeptide with kinase–cyclase LabKC-His_6 and GTP.

Molecular Mass[a]	Charge state	Exact mass calcd	Mass found	Error [ppm]
M	+4	1069.2277	1069.2316	3.65
M−18	+4	1064.7251	1064.7262	1.03
M−18+80	+4	1084.7166	1084.7182	1.48
M−36	+4	1060.2224	1060.2256	3.02
M−36+80	+4	1080.2140	1080.2174	3.15
M−54	+4	1055.7198	1055.7243	4.26
M−54+80	+4	1075.7114	1075.7163	4.56
M−72	+4	1051.2171	1051.2221	4.76

[a] M = 4272.88 Da unmodified LabA2 prepropeptide, water losses: −18 (−1 H_2O) −36 (−2 H_2O) −54 (−3 H_2O) and −72 (−4 H_2O), +80 = phosphorylation.

Subsequently, ESI-MS/MS experiments were performed to show that LabKC, besides dehydration reactions, also catalyzes the cyclization reaction, that is, the formation of the lanthionine and labionin motives. Analyses were challenging because linear dehydrated (4 × Dha) and cyclic forms (2 × Lan or 2 × Lab) of the prepeptide are isobaric in their molecular masses (M_r = 4200.84 Da) and MS/MS experiments are not insightful because of the high molecular mass. Furthermore, the peptides from the in vitro assay do not show significant differences in retention times on reversed-phase HPLC. Therefore, prior to ESI-MS/MS analyses the assay products of the conversion reaction with LabKC were digested with trypsin to obtain suitable peptide fragment ions amenable to mass spectrometric sequence analysis. The leader peptide contains two cleavage sites at the C-terminal side of Arg-16 and Arg-20 (Figure 2). Tryptic digest of unmodified LabA2 prepropeptide at these sites yielded two C-terminal peptides, a 22-mer and a 18-mer. The linear 22-mer exhibits intensive b- and y-type fragmentations upon collisional activation which could nicely be matched to the C-terminal amino acid sequence of the prepropeptide (Figure 2 and the Supporting Information). In contrast, tryptic digest of the assay mixture gave only a peptide corresponding to the fourfold dehydrated 22-mer suggesting the presence of a fully cyclized peptide, which impedes trypsin cleavage at the C-terminal side of Arg-20. Moreover, ESI-tandem experiments of the fourfold dehydrated 22-mer indicated that this peptide is resistant to fragmentation under identical fragmentation conditions. No fragment ions within the putative lanthionine and labionin motifs could be identified, which indicates that all the ring cyclization reactions have been accomplished in vitro (Figure 2). Finally as a conclusive demonstration, the frag-

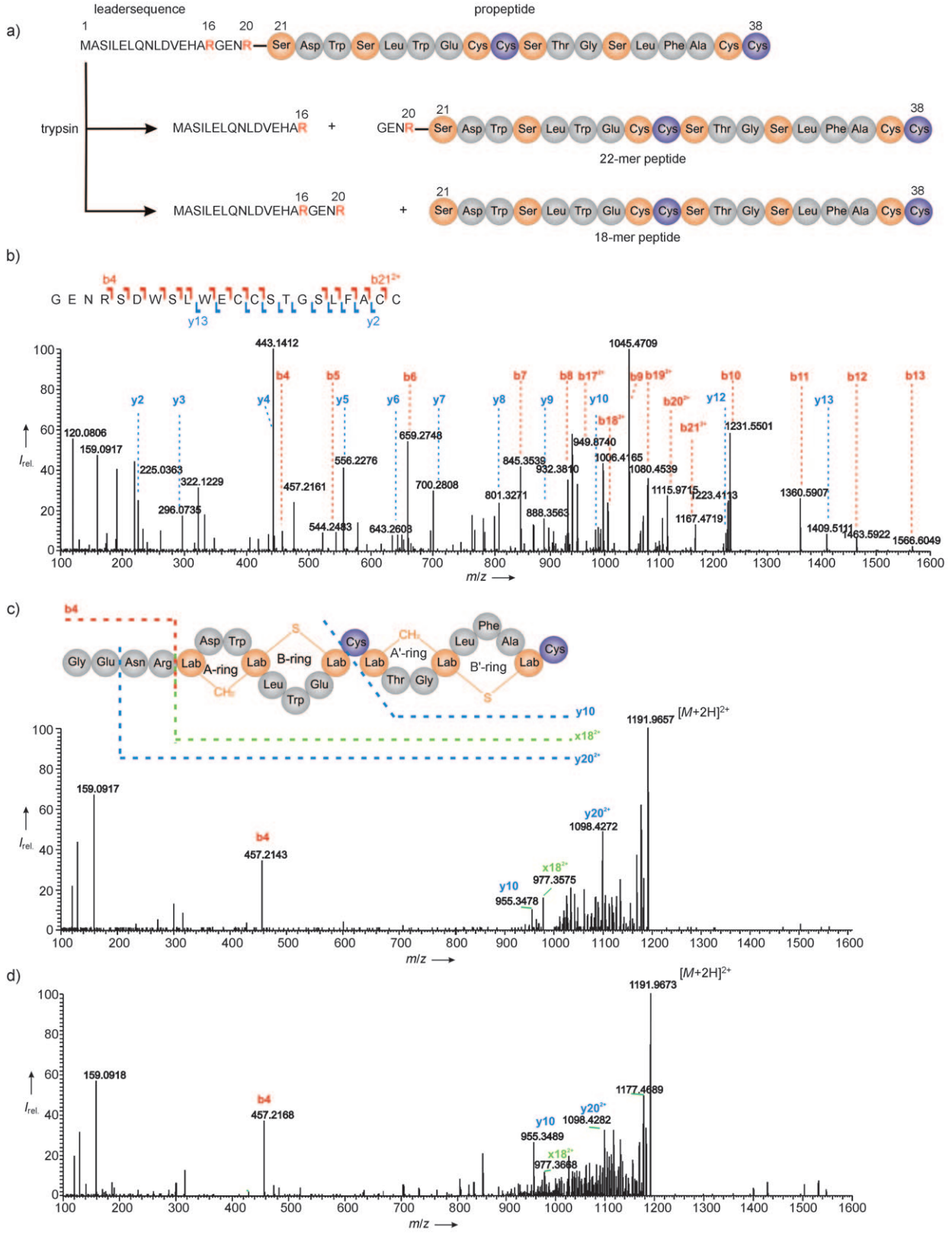


Figure 2. HR-ESI-MS/MS-experiments and fragment assignment of tryptic digests of LabA2 peptides a) Fragments obtained after tryptic digest of a linear LabA2 prepropeptide; b) LC-ESI-MS/MS spectra of the synthetic linear prepropeptide fragment ($[M+2H]^{2+}=1227.4923$) after tryptic digest (2 h) and C-terminal cleavage at Arg-16 (y-fragment and b-fragment series); c) LC-ESI-MS/MS spectra of fourfold dehydrated and cyclized peptide ($[M+2H]^{2+}=1191.4683$) after incubation with LabKC-His_6 and subsequent tryptic digest. No fragment ions were observed in the region corresponding to the fourfold dehydrated propeptide, suggesting a fully cyclized peptide. Assignment of characteristic and diagnostic fragments also found for semisynthetic reference peptide GENR-LabA2, d) LC-ESI-MS/MS of semisynthetic reference peptide GENR-LabA2 ($[M+2H]^{2+}=1191.4686$).

mentation pattern was compared to that of a the corresponding semisynthetic N-terminally modified labyrinthopeptin A2 reference peptide (Figure 2 and Supporting Information) and the same characteristic diagnostic ions have been identified and assigned.

A putative biosynthesis pathway for the assembly of the two labionin amino acids in the backbone of the LabA2 propeptide is shown in Figure 3. According to this mechanism the formation of the amino acid labionin requires two dehydration reactions at the serine residues of the Ser-Xxx-Xxx-Ser-Xxx-Xxx-Xxx-Cys motif to yield two α,β-di-(dehydro)alanines (Dha) which subsequently undergo cyclization by a twofold Michael reaction induced by the nucleophilic attack of a C-terminally activated cysteine side chain. The subsequent biosynthetic steps are proteolytic processing and oxidation of Cys-29 and Cys-38 to cystine.

In summary we succeeded in showing that LabKC functions as a kinase–cyclase acting in a two-step fashion. The first step comprises the phosphorylation and dehydration of the serine residues of LabA2 to yield the corresponding α,β-di-(dehydro)alanines. The second step is the consecutive double Michael addition beginning with the nucleophilic attack of a C-terminal Cys to yield Lan. For the establishment of the carbacyclic ring of labionin we propose the stabilization of a carbanion at the α-C-atom which acts as a C-nucleophile for the reaction at the N-terminal Dha. We suggest, that the function of LabKC is representative of the biosyntheses of type-III lantibiotics of related biosynthetic gene clusters for example, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseus*, and the erythromycin producer *Saccharopolyspora erythraea*. Our future experiments are dedicated to the detailed characterization of LabKC, the characterization of other gene functions of the *lab* gene cluster, and to the identification of the putative protease LabP.

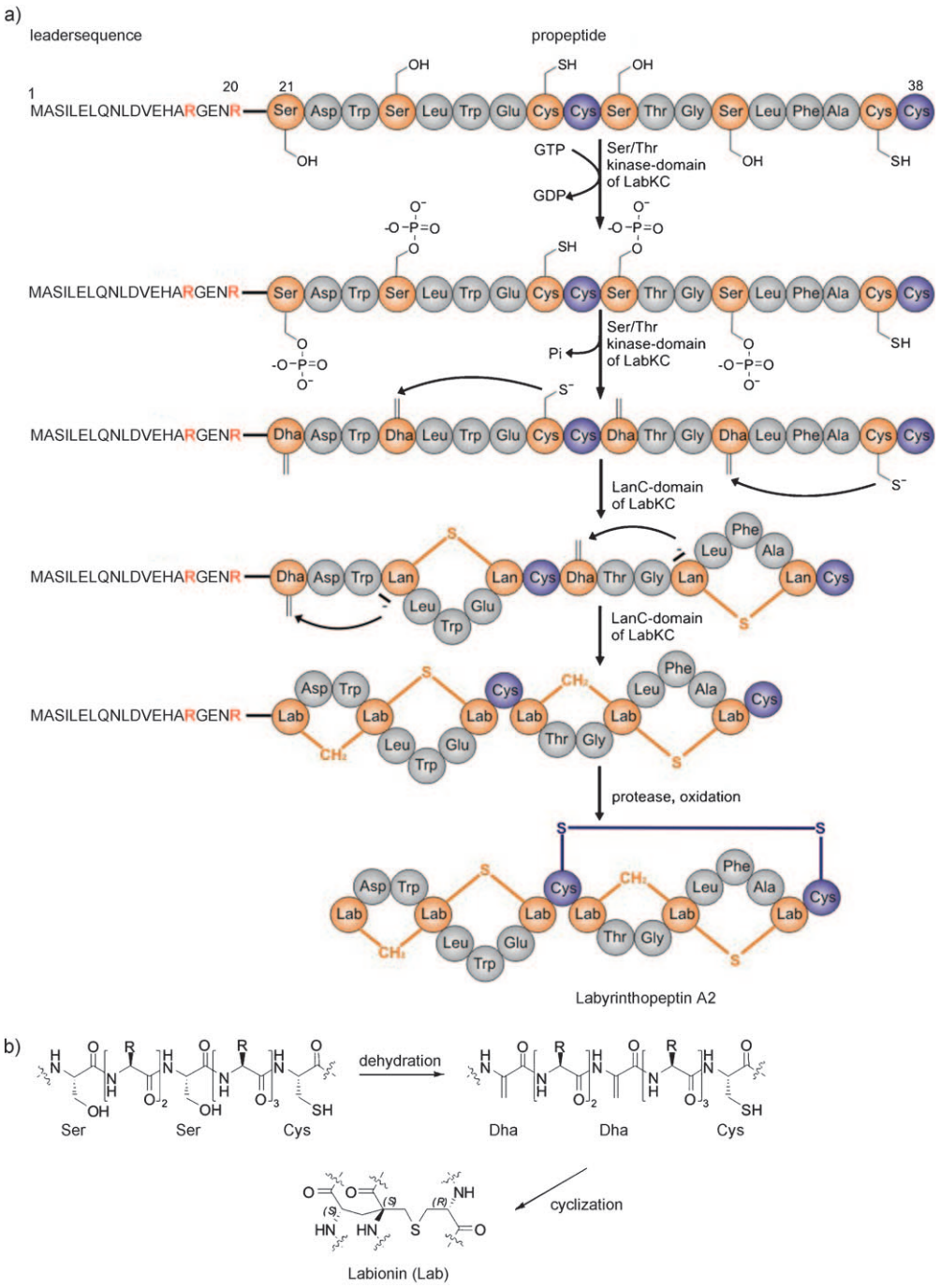


Figure 3. Model of the labyrinthopeptin A2 biosynthesis: a) Upon phosphorylation, dehydration, and cyclization of the prepropeptide by LabKC the labionin rings are assembled by a double Michael addition. Oxidation of the residual cysteine residues and proteolytic processing are the final biosynthetic steps. b) The Ser/Ser/Cys-motif as a biosynthetic precursor of the amino acid labionin.

Received: October 21, 2009
Revised: December 15, 2009
Published online: February 28, 2010

Keywords: C–C coupling · enzymes · lanthionine · lantibiotics · protein modification

[1] J. M. Willey, W. A. van der Donk, *Annu. Rev. Microbiol.* **2007**, *61*, 477 – 501.
[2] L. Xie, L. M. Miller, C. Chatterjee, O. Averin, N. L. Kelleher, W. A. van der Donk, *Science* **2004**, *303*, 679 – 681.
[3] A. L. McClerren, L. E. Cooper, C. Quan, P. M. Thomas, N. L. Kelleher, W. A. van der Donk, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17243 – 17248.
[4] K. Meindl, T. Schmiederer, K. Schneider, A. Reicke, D. Butz, S. Keller, G. Nicholson, H. Gühring, L. Vertesy, 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.
[5] G. Seibert, L. Vértesy, J. Wink, I. Winkler, R. Süssmuth, G. Sheldrick, K. Meindl, M. Broenstrup, H. Hoffmann, H. Guehring, L. Toti WO2008/040469, **2008**.
[6] J. Wink, R. M. Kroppenstedt, G. Seibert, E. Stackebrandt, *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 721 – 724.
[7] B. Li, J. P. J. Yu, J. S. Brunzelle, G. N. Moll, W. A. van der Donk, S. K. Nair, *Science* **2006**, *311*, 1464 – 1467.
[8] S. Kodani, M. E. Hudson, M. C. Durrant, M. J. Buttner, J. R. Nodwell, J. M. Willey, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11448 – 11453.
[9] T. A. Young, B. Delagoutte, J. A. Endrizzi, A. M. Falick, T. Alber, *Nat. Struct. Biol.* **2003**, *10*, 168 – 174.

