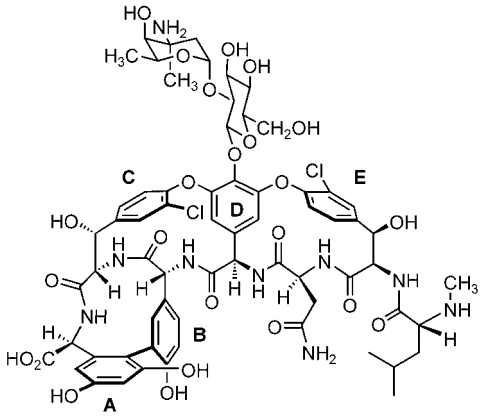
The Biosynthesis of Vancomycin-Type Glycopeptide Antibiotics—A Model for Oxidative Side-Chain Cross-Linking by Oxygenases Coupled to the Action of Peptide Synthetases

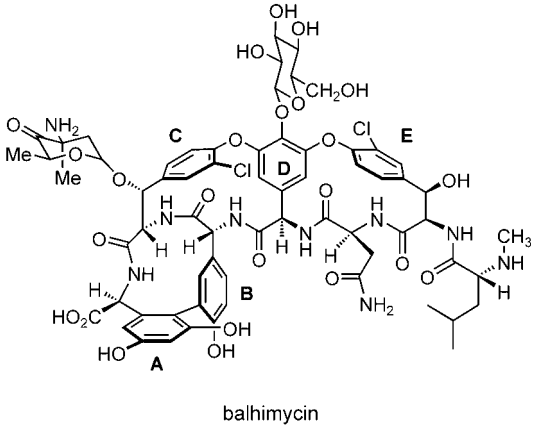
Daniel Bischoff,[a] Bojan Bister,[a] Marcelo Bertazzo,[a] Volker Pfeifer,[b] Efthimia Stegmann,[b] Graeme J. Nicholson,[a] Simone Keller,[a] Stefan Pelzer,[b] Wolfgang Wohlleben,*[b] and Roderich D. Süssmuth*[a, c]

Vancomycin (Scheme 1) and teicoplanin are "last resort" antibiotics for the treatment of severe infections with enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA) strains. However, over the past 15 years, vancomycin-resistant enterococci (VRE) and intermediate resistant staphylococci (VISA) have emerged. One approach to counter such resistance is the generation of novel glycopeptides with altered antibiotic activity by combinatorial biosynthesis, that is, the reprogramming of glycopeptide biosynthesis, a basic requirement for which is the understanding of the process.[1]

The recent sequencing of glycopeptide biosynthesis gene clusters has provided deeper insights into glycopeptide antibiotic biosynthesis.[2] Subsequent biosynthesis investigations have been performed by heterologous expression and characterization of enzymes, as well as gene inactivation combined with the characterization of accumulated peptide intermediates.[1] The latter approach has mainly been performed with balhimycin (Scheme 1) produced by *Amycolatopsis balhimycina*,[3] formerly referred to as *A. mediterranei*.[4]

Glycopeptides are assembled from amino acid precursors by the action of nonribosomal peptide synthetases (NRPS),[1] and modified by the action of so-called "tailoring enzymes". The tailoring enzymes include three P450-dependent oxygenases responsible for the cross linking of the aromatic side chains,[5–7]





Scheme 1. *Structural formulae of glycopeptide antibiotics balhimycin and vancomycin.*

glycosyl transferases for the attachment of carbohydrate residues and an N-methyl transferase[1] that introduces a methyl group at the amino group of leucine. The three oxidative side-chain cyclizations were assigned to three oxygenase genes (*oxyA/B/C*). A sequence for the assembly of the glycopeptide aglycon from linear peptide precursors was deduced as: 1) CD-ring (OxyB), 2) DE-ring (OxyA) and 3) AB-ring (OxyC) coupling.[5]

Whether oxidative formation of AB, CD and DE rings occurs before or after cleavage of the linear peptide from the NRPS complex has not been determined.[1] Our previous observation of considerable amounts of various linear and cyclized hexa- and heptapeptides isolated from oxygenase mutants (*oxyA/B/C*) cast doubt on whether side-chain-cyclized hexapeptides were degradation products or were rather related to true biosynthesis intemediates.[5,7] Here we report on the characterization of metabolites accumulated from balhimycin biosynthesis mutants inactivated in the central step of heptapeptide formation. These studies lead to the important conclusion that peptide assembly on the NRPS appears to be intimately coupled to the action of the oxygenases (OxyA/B/C).

Two *A. balhimycina* in-frame deletion mutants, described in earlier work and both inactivated in different stages of hepta-

[a] *Dr. D. Bischoff, Dr. B. Bister, Dr. M. Bertazzo, G. J. Nicholson, S. Keller, Prof. Dr. R. D. Süssmuth*
Institut für Organische Chemie der Universität Tübingen
Auf der Morgenstelle 18, 72076 Tübingen (Germany)
Fax: (+49) 7071-29-5560
E-mail: suessmuth@chem.tu-berlin.de

[b] *Dr. V. Pfeifer, Dr. E. Stegmann, S. Pelzer, Prof. Dr. W. Wohlleben*
Lehrstuhl für Mikrobiologie/Biotechnologie der Universität
Auf der Morgenstelle 28, 72076 Tübingen (Germany)
Fax: (+49) 7071-29-5979
E-mail: wolfgang.wohlleben@biotech.uni-tuebingen.de

[c] *Prof. Dr. R. D. Süssmuth*
Present address: Technische Universität Berlin
Institut für Chemie, FG Organische Chemie
Straße des 17. Juni 124, 10623 Berlin (Germany)
Fax: (+49) 30-314-324783

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

 DOI: 10.1002/cbic.200400328

peptide formation, have been investigated.[9,10] In the *dpgA* mutant, a gene involved in the biosynthesis of 3,5-dihydroxyphenylglycine (^{7}Dpg)[9] is inactivated and thus lacks the ability to produce Dpg for delivery to the peptide synthetase, BpsC (Figure 1a). The *bpsC* mutant[10] has a deletion in the first condensation domain of BpsC that is responsible for condensation of a hexapeptide with Dpg to yield the heptapeptide precursor (Figure 1b). In both cases, the antibiotically inactive culture filtrates were analyzed by HPLC-ESI-MS. This revealed chlorinated peptide metabolites closely related to the putative balhimycin biosynthesis intermediates. However, compared to the wild-type strain, peptide production rates of these mutants were significantly decreased (~100-fold). In order to obtain representative metabolite-production profiles, fermentations were scaled up to 80 (*dpgA* mutant) and 60 litres (*bpsC* mutant).

A previously described linear hexapeptide SP-969 (**4**)[7] from culture filtrates of the *dpgA* mutant was found to be the main metabolite. C-terminally truncated penta-, tetra-, tri- and dipeptides **5**, **6**, **7** and **8** were detected in significantly lower amounts, along with two bicyclic hexapeptides (DB-979/DB-993; **2a**/**b**). In addition, two isobaric peptides, DB-1126^{A} (**1**) and DB-1126^{B} (ESI-FTICR-MS: $[M+H]^+ = 1127.2960$ and $[M+H]^+ = 1127.2959$) with the molecular formula $C_{53}H_{52}O_{16}N_8Cl_2$, were isolated. The preparative HPLC yielded: **1** (~500 μg), DB-1126^{B} (~70 μg), **2a**/**b** (~50 μg) and **4** (~5 mg). Peptide sequences were deduced from ESI-MS-MS experiments, in which structures of **4**–**8** could be assigned (Figure 2a and Scheme 2). 2D NMR experiments for DB-1126^{A} (**1**) revealed a tricyclic glycopeptide aglycon with ^{7}Dpg replaced by 4-hydroxyphenylglycine (^{7}Hpg; Scheme 2). This remarkable finding that a heptapeptide can be produced by this mutant is in contrast to the unavailability of the amino acid ^{7}Dpg in the mutant. The configuration of ^{7}Hpg (D or L) in DB-1126^{A} could not be assigned. Surprisingly, the newly formed AB-ring of compound **1** is expanded to a 13-membered ring; this is a possible reason for the lack of antibiotic activity against the indicator strain *Bacillus subtilis*. The second derivative, DB-1126^{B}, is assumed to have a similar structure, but insufficient quantities were available for detailed NMR studies.

Other important results came from investigation of culture filtrates of the *bpsC* mutant, which were also found to contain chlorinated metabolites. The linear hexapeptide **4** (SP-969; ~0.7 mg), the monocyclic hexapeptide **3** (DB-967; ~0.8 mg; Scheme 2) and di- to pentapeptides **5**–**8** were detected by HPLC-ESI-MS from the culture filtrates. In contrast to the *dpgA* mutant, no bicyclic hexapeptide and no heptapeptide derivatives were detected in extracts of the *bpsC* mutant by HPLC-ESI-MS. As a consequence, except for compounds **4**–**8**, both

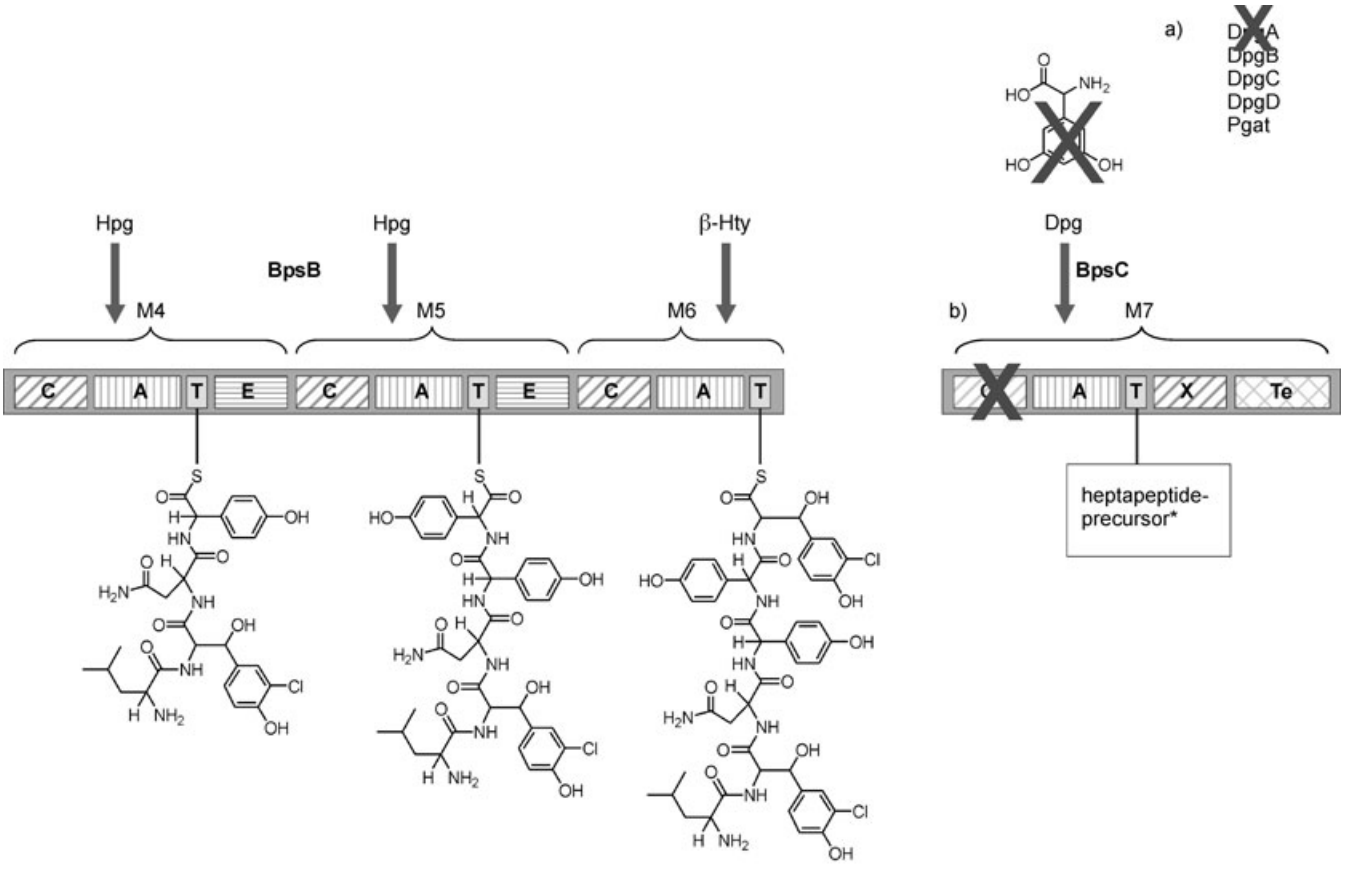


Figure 1. *Scheme of a part of the nonribosomal peptide synthesis of balhimycin (BpsB/C, modules M4–7): gene inactivations of a) the Dpg assembly (*dpgA *mutant) and b) the heptapeptide condensation (*bpsC *mutant) are assigned. The heptapeptide precursor * is only present in the wild-type; Hpg, 4-hydroxyphenylglycine, β-Hty, β-hydroxytyrosine, Dpg, 3,5-dihydroxyphenylglycine, DpgA-D, Pgat proteins of the Dpg biosynthesis pathway;[9] Domains: C, condensation; A, adenylation; T, thiolation; E, epimerization; Te, thioesterase; X, condensation domain of unknown function).*

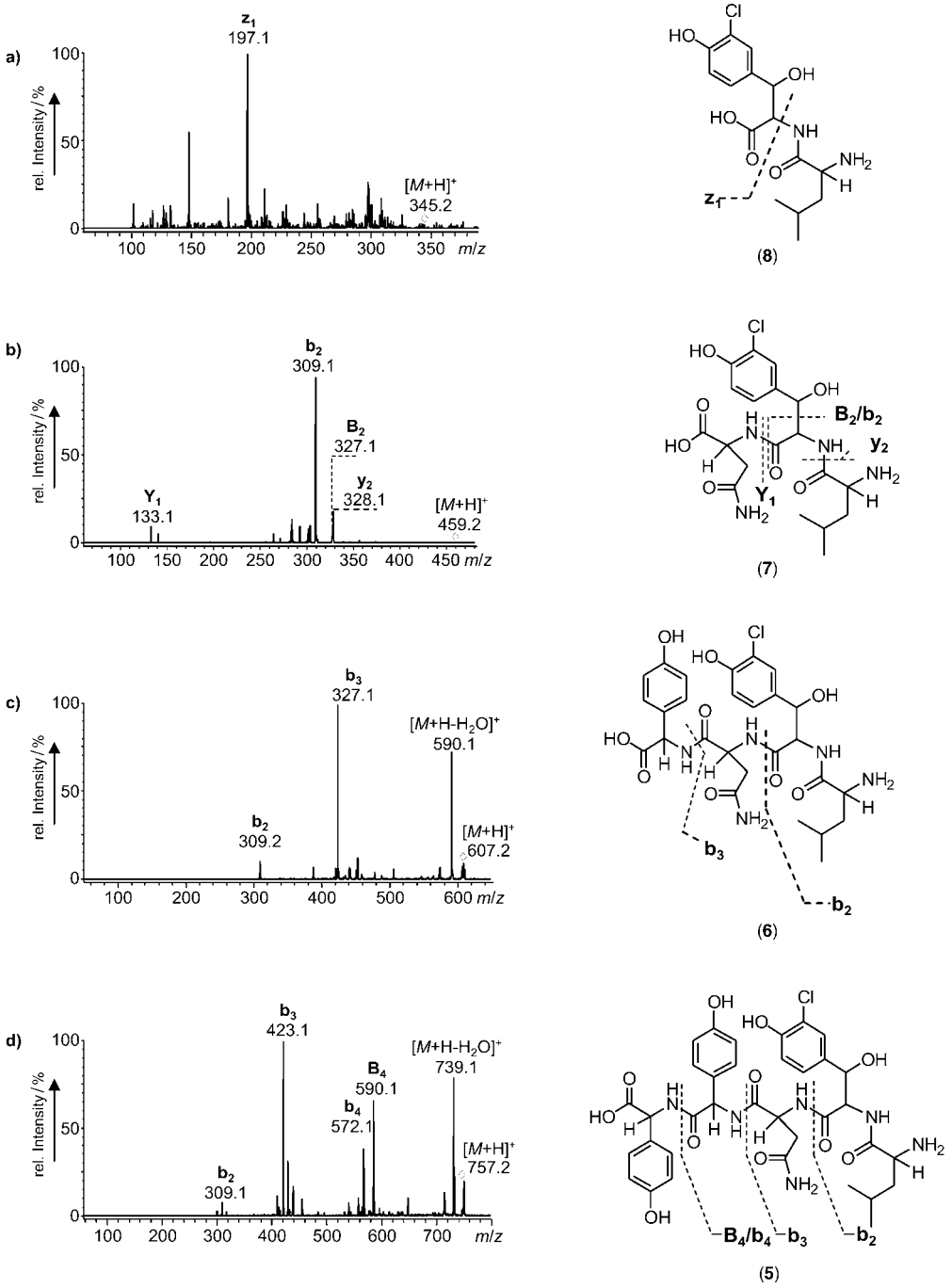
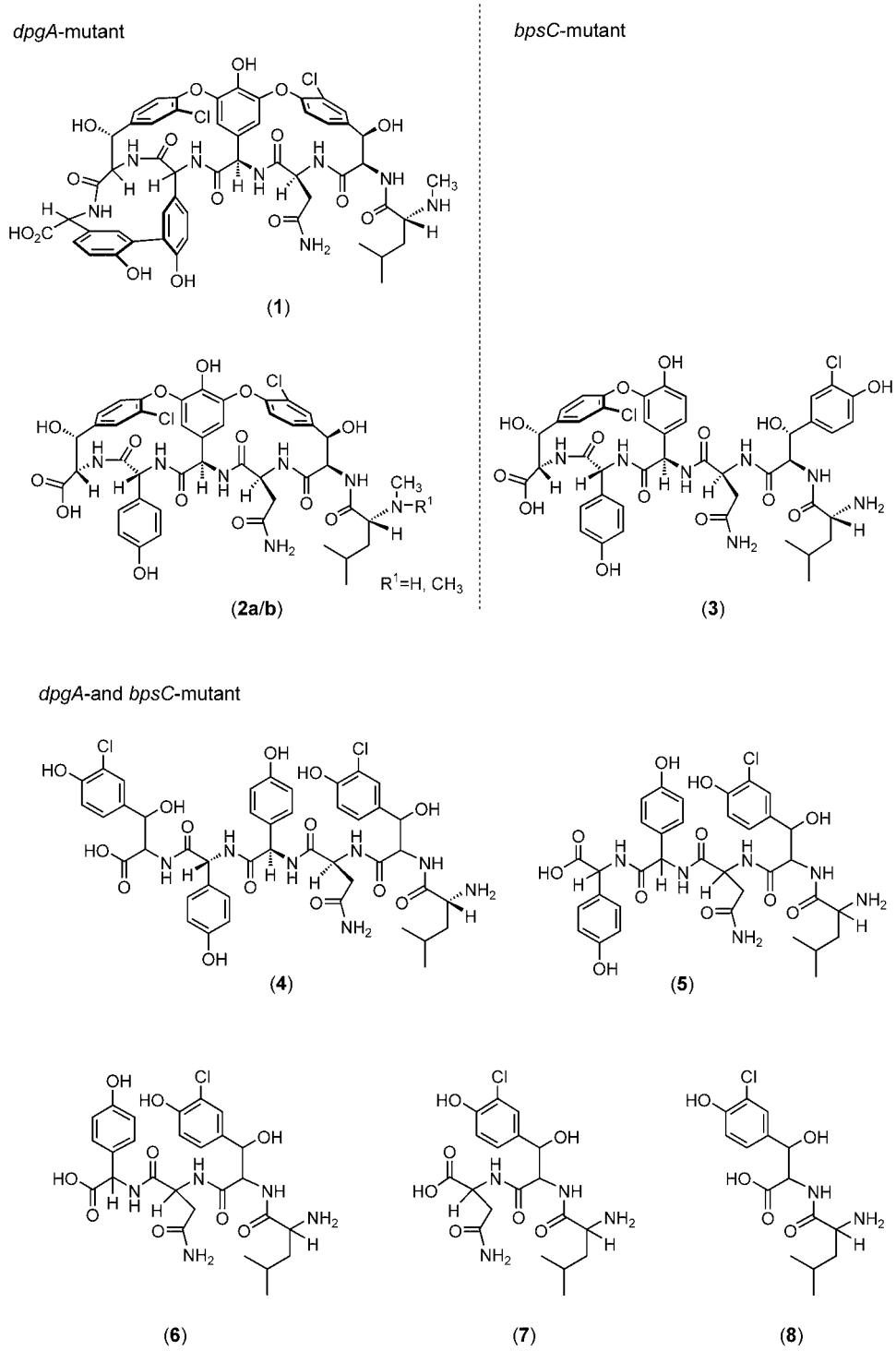


Figure 2. *ESI-MS-MS spectra of peptides* ***5****–****8*** *from the* A. balhimycina bpsC *mutant. The same spectra were obtained for peptides* ***5****–****8*** *from the* dpgA *mutant. Peptide sequences have been deduced as indicated from characteristic fragments (lower-case letters indicate fragment ions* $[F-H_2O]^+$*).*

mutants show markedly different peptide metabolite and side-chain cyclization profiles. However, it was not clear whether the truncated di- to pentapeptides **5**–**8** found in both of these mutant strains were derived from proteolytic degradation of a hexapeptide precursor. When the linear hexapeptide **4** and heptapeptide SP-1134[7] were incubated with cell lysates from the *A. balhimycina bhp* mutant (OP-696),[11] which is incapable of producing balhimycin peptide metabolites, C-terminal degradation of the heptapeptide to the hexapeptide **4** was observed. However, the hexapeptide was not significantly degraded. Vancomycin aglycon was also stable in these cell extracts.

Semiquantitative HPLC-ESI-MS analyses of both *dpgA* and *bpsC* mutants showed significantly higher amounts of tripeptide **7** and hexapeptides **4** compared to di-, tetra- and pentapeptides **5**, **6** and **8** (Figure 3). Interestingly, the tripeptide **7** and hexapeptide **4** correspond to the enzyme-free forms of the products of NRPS BpsA and BpsB, respectively. The tripeptide **7,** bound as a thioester to BpsA, should be transferred to BpsB. Similarly, the thioester-bound form of hexapeptide **4**



Scheme 2. *Structural formulae of peptide metabolites from* A. balhimycina dpgA *(**1**, **2a/2b**, **4**–**8**) and* bpsC *mutants (**3**, **4**–**8**).*

should be transferred to the third NRPS, BpsC. According to our previous results,[5,7] the occurrence of significant amounts of hexapeptide could only be explained by cellular degradation from heptapeptide precursors; this is confirmed by the presence of a suitable protease. However, the degradation characteristics of hexa- and heptapeptides with cell lysates clearly differs from the peptidic metabolite profiles found for the *dpgA* and *bpsC* mutants. In this context, N-terminally truncated peptides or nonchlorinated precursor peptides were not detected in extracts of the *dpgA* and *bpsC* mutants. Apparently, hydrolysis of the intermediate thioesters could compete with transfer between peptide synthetases BpsA/B/C to preferentially generate free **4** and **7**.

A current model[1b] for the biosynthesis of glycopeptide antibiotics is based on the assembly of a linear heptapeptide on the NRPS (BpsA/B/C) followed by its cleavage from the NRPS complex by a thioesterase (Te) domain located at the C terminus of BpsC. Subsequent tailoring and decorating steps occur, including side-chain bridging by oxygenases (OxyA/B/C), N-methylation and glycosylation (Figure 4a). However, the exceptional detection of monocyclic hexapeptide **3** (Scheme 2) from the *bpsC* mutant demands a reconsideration of this model because CD ring cyclization could occur even when the hexapeptide is linked as a thioester to BpsB. This suggests that the hexapeptide linked as a thioester to BpsB can act as a substrate for OxyB.

Particularly for the *bpsC* mutant, peptide intermediates must have been released by premature hydrolysis from peptide synthetases BpsA/B. The same is likely to be the case for the *dpgA* mutant since Hpg is usually not accepted as a natural substrate by BpsC.[8] Thus a proper heptapeptide assembly is slowed down, resulting in premature hydrolysis of peptide intermediates. Similar observations of intermediates in the field of polyketide biosynthesis support our findings.[12] Even more significantly, the complete absence of monocyclic hexapeptide **3** in the *dpgA* mutant clearly argues against a possible cytoplasmic cyclization of a free peptide precursor.

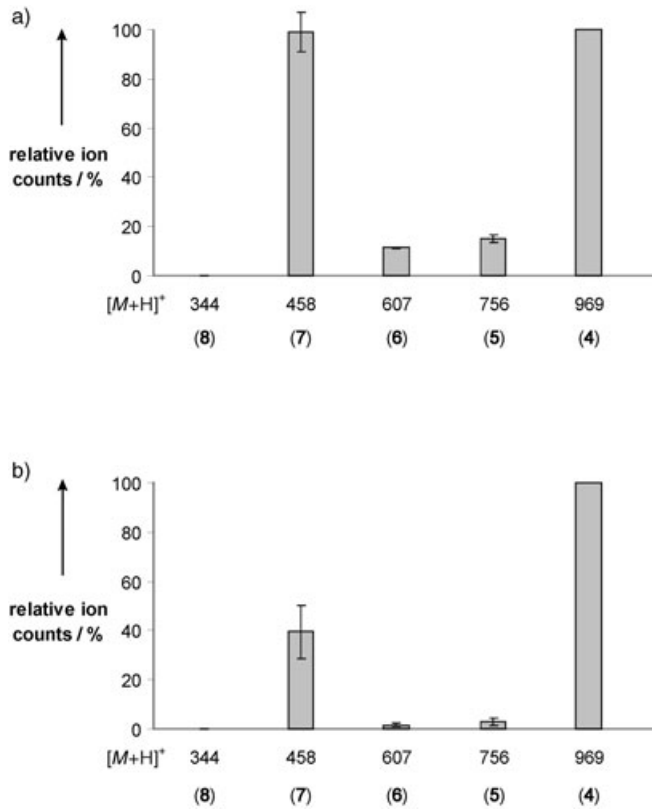


Figure 3. *Comparison of relative peptide amounts of hexa- (**4**), penta- (**5**), tetra- (**6**), tri- (**7**) and dipeptide (**8**) from* A. balhimycina *balhimycin biosynthesis mutants a)* bpsC, *and b)* dpgA, *determined by HPLC-ESI-MS.*

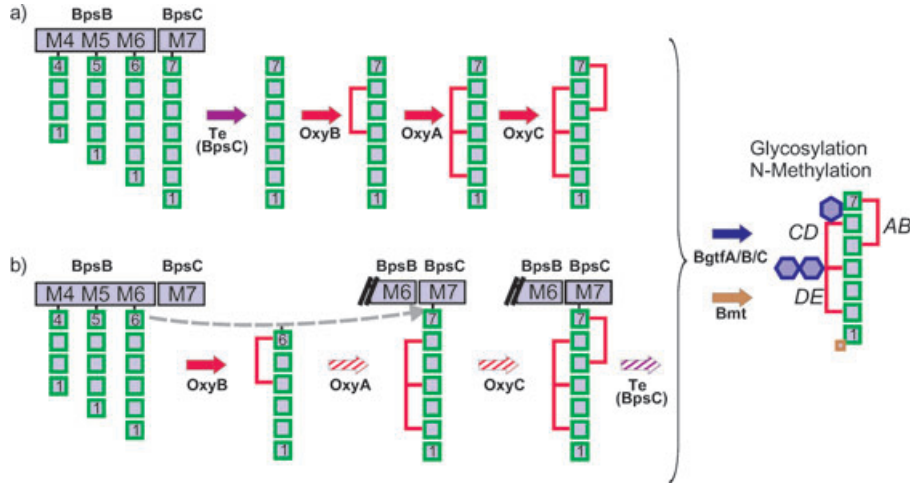


Figure 4. *Models for the biosynthesis of vancomycin-type glycopeptide antibiotic balhimycin: a) NRPS independent cyclizations by oxygenases OxyA/B/C. b) Proposed model: formation of the CD-ring on module 6 before or during transfer to module 7. Dashed arrow: the formation of DE- and AB-rings is possibly also coupled to the NRPS. Green squares symbolize the peptide backbone, red lines the AB, CD and DE rings, and blue hexagons the carbohydrate residues of balhimycin.*

The experimental results suggest an alternative model for vancomycin biosynthesis in which the first ring-closure reaction of OxyB and possibly even all oxygenase reactions occur on NRPS thiolester-bound peptides (Figure 4b). Thus, formation of the CD ring (OxyB) should occur shortly before or during the transfer of the hexapeptide from module 6 on BpsB to module 7 on BpsC. Then the occurrence of only linear and monocyclic hexapeptide in *bpsC*-mutant cultures can be explained by the absence of an essential interaction site between OxyA and the NRPS complex due to the deletion in BpsC.

This model provides an explanation for previously unsuccessful attempts to convert linear peptide precursors to vancomycin aglycon with over-expressed oxygenases,[13] and shares similarities with the proposed biosynthesis of β-hydroxytyrosine from a NRPS-bound tyrosine.[14] Furthermore, the presented model stimulated experiments in which peptide carrier domain-bound hexapeptide was successfully cyclized with over-expressed oxygenase, OxyB.[15] The observation of the unusual substitution pattern in glycopeptide derivatives DB-1126^{A} (**1**) and DB-1126^{B} indicates a certain substrate tolerance of intact peptide synthetase, BpsC, and of the oxygenases towards altered peptidic substrates. This will certainly stimulate future attempts to vary glycopeptide structures by mutasynthesis[8] or combinatorial biosynthesis or by a combination of both.

Experimental Section

LC-MS experiments were performed on a Bruker Esquire 3000+ (Bruker Daltonics, Bremen, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). In order to obtain semiquantitative data of peptide distribution patterns (**4**–**8**), ES-ionisation yields were assumed to be approximately equal. FTICR-ESI-MS spectra were recorded on an APEX II FTICR mass spectrometer (4.7 T, Bruker Daltonics). NMR experiments were recorded on an AMX 600 NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5 mm triple-resonance probehead with z-gradients. Further data on the isolation and characterization of compounds (**1**, **2a**/**b**, **3**, **4**, **5**–**8**) as well as peptide degradation experiments are given in the Supporting Information.

Acknowledgements

The work of R.D.S. has been supported by an Emmy-Noether Fellowship for young investigators from the Deutsche Forschungsgemeinschaft (DFG; SU 239/2-2). Further support was granted from the EU (MEGA-TOP, QLK3-CT-1990-00650 to W.W. and COMBIGTOP, LSHB-CT-2003-503491 to R.D.S. and W.W.) and from the DFG (Wo 485/3-3, SU 239/2-3).

Keywords: antibiotics · biosynthesis · glycopeptides · nonribosomal peptide synthetases · vancomycin

[1] a) R. D. Süssmuth, W. Wohlleben, *Appl. Microbiol. Biotechnol.* **2004**, *63*, 344 – 350; b) B. Hubbard, C. T. Walsh, *Angew. Chem.* **2003**, *115*, 752 – 789; *Angew. Chem. Int. Ed.* **2003**, *42*, 730 – 765.
[2] a) A. M. A. van Wageningen, P. N. Kirkpatrick, D. H. Williams, B. R. Harris, J. K. Kershaw, N. J. Lennard, M. Jones, S. J. M. Jones, P. J. Solenberg, *Chem. Biol.* **1998**, *5*, 155 – 162; b) S. Pelzer, R. Süssmuth, D. Heckmann, J. Recktenwald, P. Huber, G. Jung, W. Wohlleben, *Antimicrob. Agents Chemother.* **1999**, 1565 – 1573; c) H. T. Chin, B. K. Hubbard, A. N. Shah, J. Eide, R. A. Fredenburg, C. T. Walsh, C. Khosla, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 8548 – 8553; d) J. Pootoolal, M. G. Thomas, C. G. Marshall, J. M. Neu, B. K. Hubbard, C. T. Walsh, G. D. Wright, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 8962 – 8967; e) M. Sosio, M. Kloosterman, A. Bianchi, P. de Vreugd, L. Dijkhuizen, S. Donadio, *Microbiology* **2004**, *150*, 95 – 102; f) M. Sosio, S. Stinchi, F. Beltrametti, A. Lazzarini, S. Donadio, *Chem. Biol.* **2003**, *10*, 541 – 549; g) T. L. Li, F. Huang, S. F. Haydock, T. Mironenko, P. F. Leadlay, J. B. Spencer, *Chem. Biol.* **2004**, *11*, 107 – 119.
[3] J. Wink, R. M. Kroppenstedt, B. N. Ganguli, S. R. Nadkarni, P. Schumann, G. Seibert, E. Stackebrandt, *Syst. Appl. Microbiol.* **2003**, *26*, 38 – 46.
[4] S. Chatterjee, E. K. S. Vijayakumar, S. R. Nadakarni, M. V. Paterl, J. Blumbach, B. N. Ganguli, H.-W. Fehlhaber, H. Kogler, L. Vértesy, *J. Org. Chem.* **1994**, *59*, 3480 – 3484.
[5] D. Bischoff, S. Pelzer, B. Bister, G. J. Nicholson, S. Stockert, W. Wohlleben, G. Jung, R. D. Süssmuth, *Angew. Chem.* **2001**, *113*, 4825 – 4827; *Angew. Chem. Int. Ed.* **2001**, *40*, 4688 – 4691.
[6] D. Bischoff, S. Pelzer, A. Höltzel, G. J. Nicholson, S. Stockert, W. Wohlleben, G. Jung, R. D. Süssmuth, *Angew. Chem.* **2001**, *113*, 1736 – 1739; *Angew. Chem. Int. Ed.* **2001**, *40*, 1693 – 1696.
[7] R. D. Süssmuth, S. Pelzer, G. Nicholson, T. Walk, W. Wohlleben, G. Jung, *Angew. Chem.* **1999**, *111*, 2096 – 2099; *Angew. Chem. Int. Ed.* **1999**, *38*, 1976 – 1979.
[8] S. Weist, C. Kittel, D. Bischoff, B. Bister, V. Pfeifer, G. J. Nicholson, W. Wohlleben, R. D. Süssmuth, *J. Am. Chem. Soc.* **2004**, *126*, 5942 – 5943.
[9] V. Pfeifer, G. J. Nicholson, J. Ries, J. Recktenwald, A. B. Schefer, R. M. Shawky, J. Schröder, W. Wohlleben, S. Pelzer, *J. Biol. Chem.* **2001**, *276*, 38 370 – 38 377.
[10] J. Recktenwald, R. Shawky, O. Puk, F. Pfennig, U. Keller, W. Wohlleben, S. Pelzer, *J. Microbiol.* **2002**, *148*, 1105 – 1118.
[11] O. Puk, P. Huber, D. Bischoff, J. Recktenwald, G. Jung, R. D. Süssmuth, K.-H. van Pée, W. Wohlleben, S. Pelzer, *Chem. Biol.* **2002**, *9*, 225 – 235.
[12] A. Stratmann, C. Toupet, W. Schilling, R. Traber, L. Oberer, T. Schupp, *J. Microbiol.* **1999**, *145*, 3365 – 3375.
[13] K. Zerbe, O. Pylypenko, F. Vitali, W. Zhang, S. Rousett, M. Heck, J. W. Vrijbloed, D. Bischoff, B. Bister, R. D. Süssmuth, S. Pelzer, W. Wohlleben, J. A. Robinson, I. Schlichting, *J. Biol. Chem.* **2002**, *277*, 47 476 – 47 485.
[14] H. Chen, C. T. Walsh, *Chem. Biol.* **2001**, *8*, 301 – 312.
[15] K. Zerbe, K. Woithe, D. B. Li, F. Vitali, L. Bigler, J. A. Robinson, *Angew. Chem.* **2004**, *116*, 6877 – 6881; *Angew. Chem.* **2004**, *43*, 6709 – 6713.

Received: September 28, 2004
Published online: January 13, 2005

