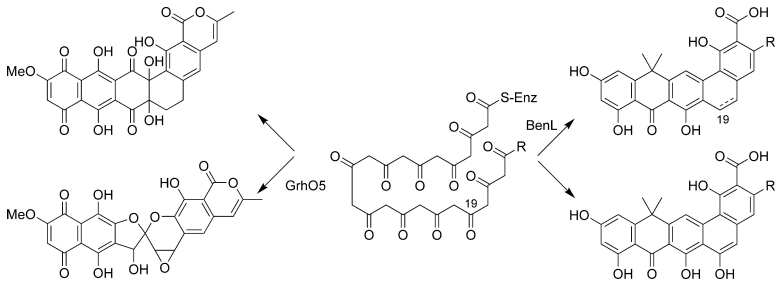
Article

Biosynthesis of Pentangular Polyphenols: Deductions from the Benastatin and Griseorhodin Pathways

Gerald Lackner, Angla Schenk, Zhongli Xu, Kathrin Reinhardt, Zeynep S. Yunt, Jrn Piel, and Christian Hertweck

J. Am. Chem. Soc., **2007**, 129 (30), 9306-9312• DOI: 10.1021/ja0718624 • Publication Date (Web): 11 July 2007



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 11 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

J|A|C|S
ARTICLES
Published on Web 07/11/2007

Biosynthesis of Pentangular Polyphenols: Deductions from the Benastatin and Griseorhodin Pathways

Gerald Lackner,[†] Angéla Schenk,[†] Zhongli Xu,[†] Kathrin Reinhardt,[‡] Zeynep S. Yunt,[‡] Jörn Piel,*[,‡] and Christian Hertweck*[,†,§]

Contribution from the Department of Biomolecular Chemistry, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knöll-Institute, Beutenbergstrasse 11a, 07745 Jena, Germany, Kekulé-Institute for Organic Chemistry and Biochemistry, University of Bonn, Gerhard-Domagk-Strasse 1, 53121 Bonn, Germany, and Friedrich-Schiller-University, Jena, Germany

Received March 16, 2007; E-mail: christian.hertweck@hki-jena.de; joern-piel@uni-bonn.de

Abstract: The benastatins, pradimicins, fredericamycins, and members of the griseorhodin/rubromycin family represent a structurally and functionally diverse group of long-chain polyphenols from actinomycetes. Comparison of their biosynthetic gene clusters (*ben*, *prm*, *fdm*, *grh*, *rub*) revealed that all loci harbor genes coding for a similar, yet uncharacterized, type of ketoreductases. In a phylogenetic survey of representative KRs involved in type II PKS systems, we found that it is generally possible to deduce the KR regiospecificity (C-9, C-15, C17) from the amino acid sequence and thus to predict the nature of the aromatic polyketide (e.g., angucycline, anthracycline, benzoisochromanequinones). We hypothesized that the new clade of KRs is characteristic for biosynthesis of polyphenols with an extended angular architecture we termed "pentangular". To test this hypothesis, we demonstrated the biogenetic relationship between benastatin and the structurally unrelated spiro ketal griseorhodin by generating a mutant producing collinone, a pentangular pathway intermediate. The benastatin pathway served as a model to characterize the KR. Gene inactivation of *benL* resulted in the formation of a series of 19-hydroxy benastatin and bequinostatin derivatives (e.g., benastatin K and benastatin L). These results clearly showed that BenL functions as a C-19 KR in pentangular pathways.

Introduction

Aromatic polyketides comprise an important group of polyphenols, many of which have emerged as leads for drug development.[1] Their biosynthesis, which involves repetitive Claisen condensations of an acyl starter with malonyl extender units, has been the subject of intense research.[2] In bacteria, aromatic polyketides are mainly produced by dissociable enzyme complexes named type II polyketide synthases (type II PKSs).[1,3] A hallmark of type II PKSs is the presence of a ketosynthase (KS_α) chain length factor (CLF/KS_β) heterodimer and an acyl carrier protein (ACP). The proper cyclization of the nascent polyketide chain is governed by cyclases and aromatases, which give rise to various ring topologies.[4] In many cases, ketoreductases (KR) that catalyze the stereospecific hydrogen transfer from NAD(P)H onto a keto group have a marked effect on the overall structure. Ketoreduction usually occurs at a position where bends or kinks are found in the final polyketide structure, suggesting that the reduced open-chain intermediate may take up a favored orientation for regiospecific aldol reaction. Therefore, ketoreductases may at least indirectly contribute to the polyketide cyclization process.[1,3] It has been demonstrated that incomplete, KR-deficient sets of type II PKS enzymes either are nonfunctional[5] or may result in incorrectly folded polyketides.[6] Notably, most known KRs reduce the C-9 keto group, as in tetracyclines, benzoisochromane (BIQ) quinones, and anthracyclines. In the latter, an additional C-17 reduction takes place, albeit after formation of the ring system. In contrast to these examples, the role of ketoreduction is thus far unknown in a structurally diverse group of aromatic polyketides that stand out because of their extended ring systems. Examples of compounds belonging to this type are the antifungal pradimicin (**1**), benastatin A (**2**), which inhibits glutathione *S*-transferases and induces apoptosis,[7] the telomerase inhibitor griseorhodin A (**3**) of the rubromycin family, and the topoisomerase inhibitor fredericamycin A (**4**) (Figure 1). The isolation of collinone (**5**) from a bacterium expressing an uncharacterized DNA segment from the rubromycin producer[8] provides circumstantial evidence that rubromycin-type compounds are biosynthetically related to

[†] Hans-Knoll-Institute.
[‡] University of Bonn.
[§] Friedrich-Schiller-University.

(1) Hertweck, C.; Luzhetskyy, A.; Rebets, Y.; Bechthold, A. *Nat. Prod. Rep.* **2007**, *24*, 162−190.
(2) Shen, B. *Top. Curr. Chem.* **2000**, *209*, 1−51.
(3) Rawlings, B. J. *Nat. Prod. Rep.* **1999**, *16*, 425−484.
(4) Jakobi, K.; Hertweck, C. *J. Am. Chem. Soc.* **2004**, *126*, 2298−2299.
(5) Hertweck, C.; Xiang, L.; Kalaitzis, J. A.; Cheng, Q.; Palzer, M.; Moore, B. S. *Chem. Biol.* **2004**, *11*, 461−468.
(6) Fu, H.; Hopwood, D. A.; Khosla, C. *Chem. Biol.* **1994**, *1*, 205−210.
(7) Xu, Z.; Schenk, A.; Hertweck, C. *J. Am. Chem. Soc.* **2007**, *129*, 6022−6030.
(8) Martin, R.; Sterner, O.; Alvarez, M. A.; De Clercq, E.; Bailey, J. E.; Minas, W. *J. Antibiot.* **2001**, *54*, 239−249.

10.1021/ja0718624 CCC: $37.00

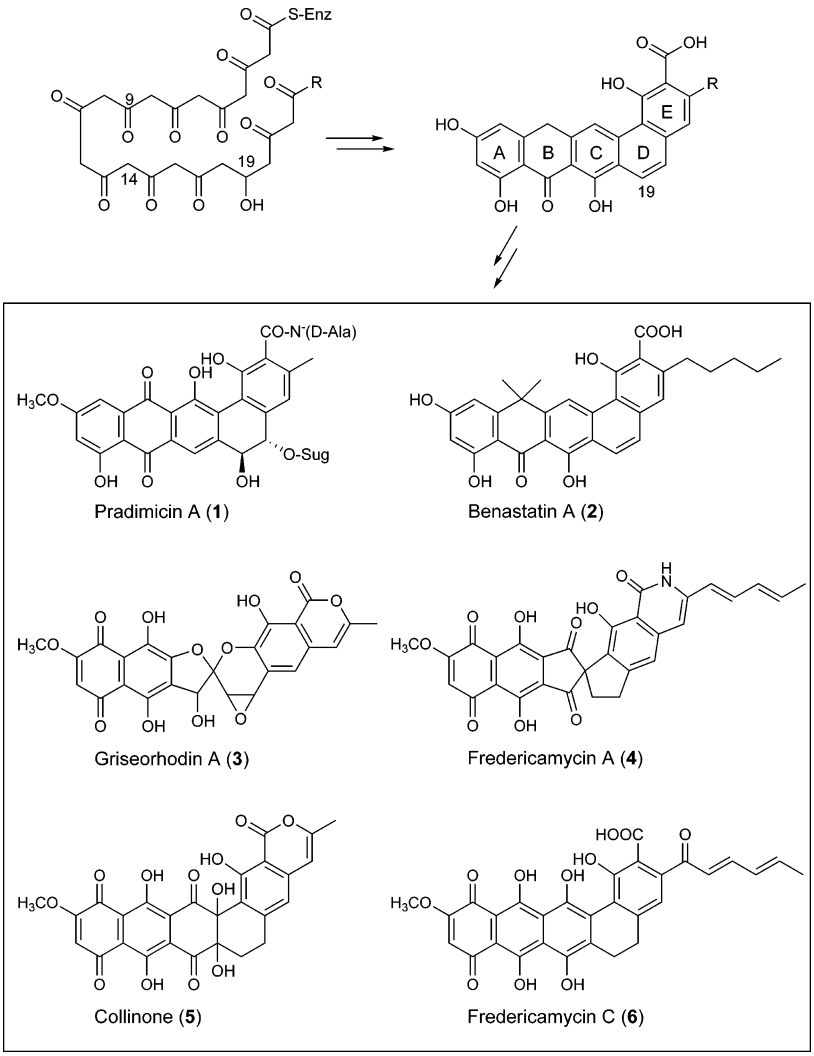


Figure 1. Structures of pentangular polyketides and their proposed formal biosynthesis involving a C-19 ketoreduction.

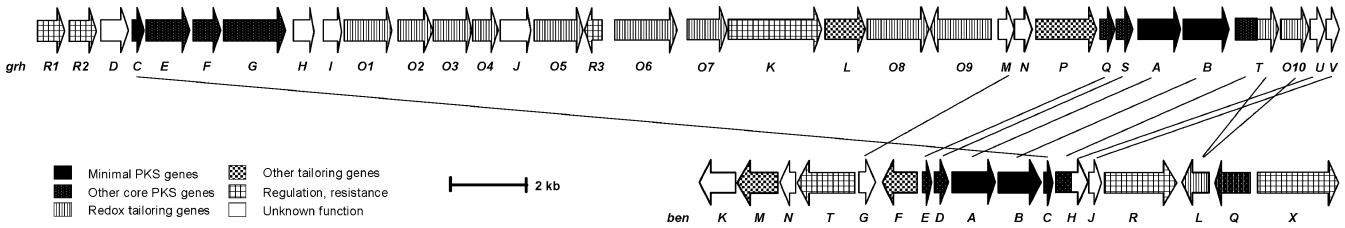


Figure 2. Organization of the *grh* and *ben* gene loci and their functionally related biosynthesis genes.

the pradimicins and benastatins. Fredericamycin C (**6**) isolated from a fredericamycin A producer[9] exhibits a similar extended angucyclic structure, suggesting that fredericamycin A also belongs to the same biosynthetic family,[10] which we propose to term pentangular polyketides. Here we show that the polyketide cyclization pattern as well as the regiochemistry of reduction can be predicted by sequence analysis of KRs. For the new group of KRs from pentangular polyketide pathways, functional analysis of a member from benastatin biosynthesis was conducted, yielding five novel polyketides.

Results and Discussion

Our groups have recently cloned and sequenced the gene clusters encoding the biosyntheses of the spiroketal griseorhodin A (*grh*)[11] and the fatty acid−polyketide hybrid benastatin (*ben*)[7] (Figure 2). Most of the deduced *grh* and *ben* proteins share high sequence similarities, suggesting a close relationship of both pathways that was not apparent from the polyketide structures. Particularly striking was that both gene clusters harbor genes (*grhO10*, *grhT*, and *benL*) that code for a similar, unusual type of ketoreductase of the short-chain dehydrogenase/reductase family.[12]

To gain insights into the relationship of the diverse KRs participating in aromatic polyketide biosynthesis, multiple

(9) Sontag, B.; Muller, J. G.; Hansske, F. G. *J. Antibiot.* **2004**, *57*, 823−828.
(10) Wendt-Pienkowski, E.; Huang, Y.; Zhang, J.; Li, B.; Jiang, H.; Kwon, H.; Hutchinson, C. R.; Shen, B. *J. Am. Chem. Soc.* **2005**, *127*, 16442−16452.
(11) Li, A.; Piel, J. *Chem. Biol.* **2002**, *9*, 1017−1026.
(12) Oppermann, U.; Filling, C.; Hult, M.; Shafqat, N.; Wu, X.; Lindh, M.; Shafqat, J.; Nordling, E.; Kallberg, Y.; Persson, B.; Jornvall, H. *Chem.-Biol. Interact.* **2003**, *143−144*, 247−253.

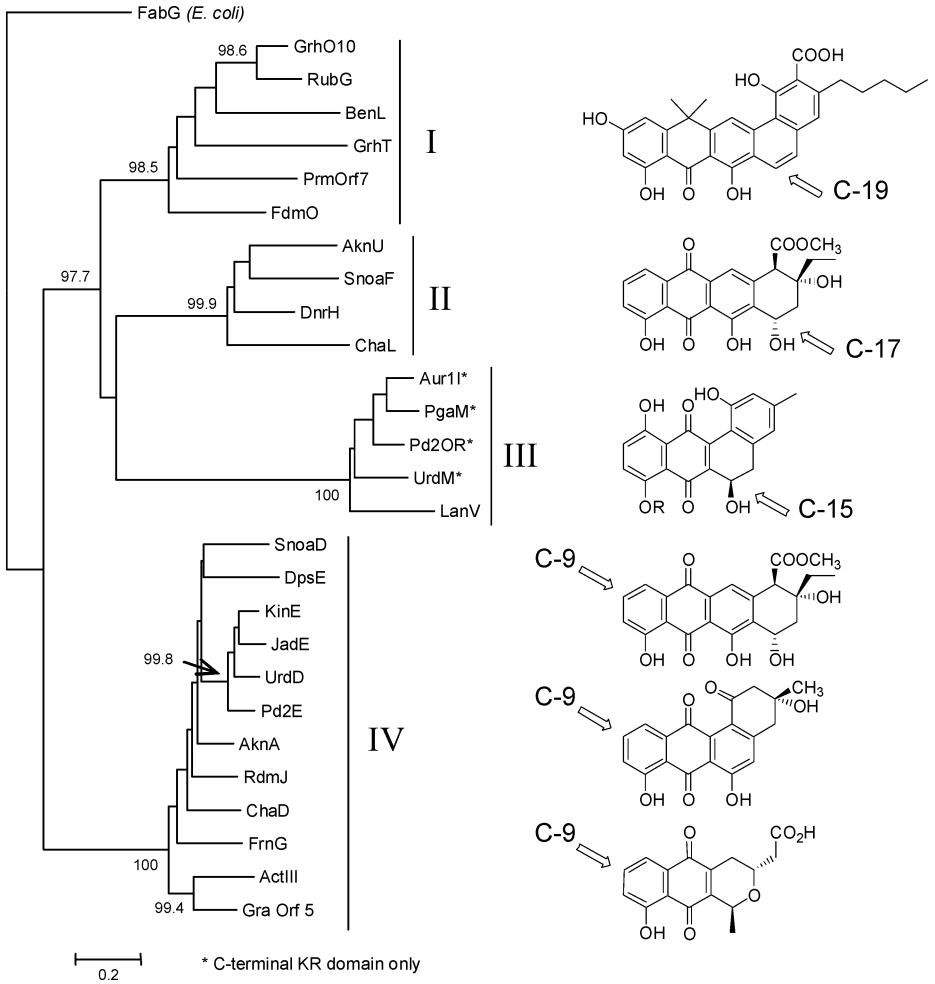


Figure 3. NJ phylogenetic tree of representative KRs involved in aromatic polyketide biosynthesis. FabG was used as an outgroup. Structures on the right exemplify KR regiospecifity. * = part of a bifunctional protein.

sequence alignment and phylogenetic analysis using the fast and robust neighbor joining (NJ) algorithm were performed. FabG KR from the *Escherichia coli* fatty acid biosynthesis pathway was included as an outgroup.[13] In the inferred phylogenetic tree,[14] KRs with known function generally grouped according to their regioselectivity and to the cyclization topology of the polyketide (groups II−IV, Figure 3). The presence of a further well-supported clade (group I) containing the *ben* and *grh* KRs along with homologues from the rubromycin (unpublished; accession No. AF293355), pradimicin,[15] and fredericamycin[10] clusters indicated that the structurally diverse compounds are biosynthetically related and that the KRs introduce reductions at identical positions of pradimicin-type, pentangular polyketides. Since thus far there had been no direct proof that the highly modified griseorhodin/rubromycin-type compounds are indeed derived from a pentangular pathway, we first sought to establish the biosynthetic origin of griseorhodin A in the marine-derived strain *Streptomyces* sp. JP95 by in-frame gene inactivations.

Knock-out experiments were conducted on the *grh* cluster present on the cosmid pMP31[11] by PCR targeted recombination in *E. coli*.[16,17] Twelve individual genes were deleted using long primers designed from flanking *grh* gene regions and plasmid pIJ778 as template, which harbors the spectinomycin resistance marker *aadA*. After FLP-mediated excision of the resistance gene in the resulting constructs, each cluster variant carrying an in-frame deletion in a different gene was transferred by conjugation into the expression host *Streptomyces albus* J1074 for heterologous production of polyketides. These strains carried the genes stably integrated into the genomic *attB* site. To investigate which polyketides resulted from the knock-out experiments, crude extracts of the engineered bacteria grown on MS plates were compared by HPLC with the extract of *S. albus* MP31 harboring the entire griseorhodin cluster. While many mutant strains produced new, but unstable, compounds (data not shown), *S. albus* KR8 containing a deletion in the FAD-dependent oxygenase gene *grhO5* generated a stable red pigment as main product (Figure 4). For structural characterization, the engineered strain was fermented in 6 L of liquid TSB medium and extracted with acidified ethyl acetate. The high-resolution mass of the compound obtained by ESI-HRMS was determined as *m*/*z* 557.069, which suggested an identical molecular formula as the previously reported collinone.[8] ^{1}H NMR, ^{13}C NMR, HMQC, and HMBC spectra of the compound

(13) Price, A. C.; Zhang, Y.-M.; Rock, C. O.; White, S. W. *Biochemistry* **2004**, *40*, 12772−12781.
(14) Saitou, N.; Nei, M. *Mol. Biol. Evol.* **1987**, *4*, 406−425.
(15) Dairi, T.; Hamano, Y.; Yasuhiro, I.; Furumai, T.; Oki, T. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1445−1453.
(16) Murphy, K. C.; Campellone, K. G.; Poteete, A. R. *Gene* **2000**, *246*, 321−330.
(17) Datsenko, K. A.; Wanner, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6640−6645.

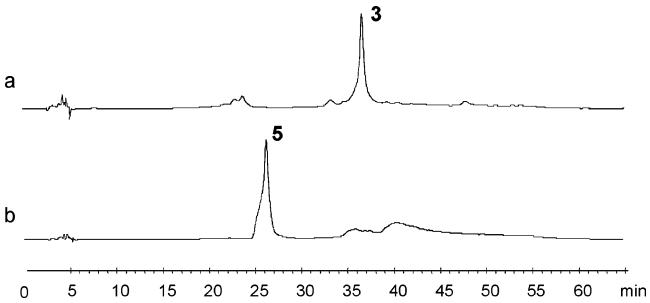


Figure 4. Chromatographic profile of extracts from (a) *S. albus* MP31 (griseorhodin (**3**) producer) and (b) *S. albus* KR8 (mutant lacking the *grhO5* gene, collinone (**5**) producer).

were recorded for structure determination, and the compound was identified as collinone by comparison with NMR data by Martin et al.[8] Collinone is highly oxygenated but still contains two carbon atoms more than griseorhodin. Our results thus suggest that the oxygenase GrhO5 is a key enzyme in the elaboration of the unique epoxyspiroketal moiety and might catalyze one of the subsequent carbon−carbon bond cleavage steps.

The identification of collinone also established that all KRs of clade I indeed act on pentangular polyketide precursors. Therefore, we next intended to gain functional information about the regiochemistry of ketoreduction by further mutational analyses. Because the *grh* cluster encodes two KR representatives of clade I that might complement each other, we focused on the single putative KR BenL from the benastatin cluster. It appeared most suitable to replace *benL* in cosmid clone p5H09, which contains the entire *ben* gene cluster, by a resistance cassette bearing an *oriT* sequence for conjugative gene transfer. This goal was achieved by a similar PCR targeting strategy as above, using primers designed from *benL* flanking regions. The manipulated gene cluster was introduced into *S. albus* by conjugation, and the metabolic profiles of selected transconjugants were examined. Five clones were selected that were cultured on agar plates and liquid media and soon showed heavy dark brown to black pigmentation. Monitoring of the crude extract by HPLC revealed that essentially none of the known benastatins (**1**, **7**−**10**, Figures 5 and 6) were formed by the mutant. Instead, several other compounds (**11**−**15**) were detected that showed strong UV absorption at 375 nm. HR-ESIMS data from HPLC fractions suggested that **11**−**15** represent novel metabolites.

A liquid culture of strain *S. albus* p5H09 Δ*benL* was scaled up (14 L) to obtain the new compounds in sufficient amounts for full structural elucidation. The crude extract was treated with hexanes to remove lipids and subjected to flash chromatography on silica impregnated with aqueous 0.1 M KH_2PO_4. Isolation of the major product was achieved by repeated preparative reverse-phase HPLC. Since the metabolites proved to be highly unstable, all HPLC fractions were immediately lyophilized and stored under argon. In this way, 2 mg of pure compound **14** was obtained for full characterization. HRMS and ^{13}C NMR data indicated the molecular formula of $C_{29}H_{26}O_9$. In light of biosynthetic considerations, this result already suggested that **14** represents a 19-hydroxy-substituted benastatin derivative. Comparison of the NMR spectra of **14** and **2** and 2D NMR data of **14** enabled the full correlation of all C and H signals (Table 1). While signals from the A and E ring atoms and the side chain remained unaltered, differences in C-13, ring C, and ring D were observed. Indeed, in comparison to benastatin A, signals for the dimethyl moiety (C-22 at 35.1 ppm and C-21 at 39.5 ppm) were missing. As in the benastatin derivative BE43767A produced by the wild-type strain, the C-21 methyl carbon signal can be detected at 39.5 ppm. Because of the additional OH group, the C-13 signal is shifted from 40.4 to 71.5 ppm. It was possible to observe an HMBC coupling of 13-OH proton (5.36 ppm) with C-21. The signal of C-6 provided evidence for D ring substitution. While this carbon resonates at 123.5 ppm in benastatin A, it is shifted downfield (158.2 ppm) in **14** and shows no coupling to any directly bound proton. This finding underscores the presence of an electronegative substituent, such as the expected OH group. C-5 was attributed to the signal at 108.5 ppm. The corresponding ^1H signal at 6.88 ppm does not form a doublet as in benastatin A, but appears as a singlet because of the missing proton at C-6. HMBC couplings with C-6 and C-6a, C-14b and C-4 confirm the position of the H-atom in the ring system. The assignment of C-6 and C-14 was hampered by the small difference in chemical shifts of C-6a (114.0 ppm) and C-14b (113.8 ppm) but could be solved by means of HMBC correlations. All NMR data provide strong support for the structure of a 6-hydroxy derivative of BE43767A (**8**), named benastatin L (**14**). Compound **12**, which showed very similar NMR data, proved to be the 6-hydroxy derivative of benastatin A (**1**) and was named benastatin K (**12**). Low production and instability of further side products hampered their full characterization. However, HRMS, MS^n, and limited NMR data indicated the presence of a 6-hydroxy bequinostatin derivative (**11**) and suggested that **15** and **13** represent the decarboxylated analogues of **14** and **11**, respectively. All metabolites formed by the mutant feature enolized D-ring structures and thus clearly indicate that BenL functions as a KR. However, to exclude any secondary effect resulting from gene deletion, the Δ*benL* mutant was complemented. The gene region containing *benL* and its genuine ribosome binding site was amplified by PCR, sequenced, and cloned downstream of the constitutive *ermE* promoter on the integrative shuttle vector pKJ55, yielding pGL11. This construct was transferred into *S. albus* p5H09 Δ*benL* by conjugation. Five clones investigated showed the typical yellow pigmentation of benastatin producer strains. According to HPLC, the complemented mutant produces benastatins **1** and **7**−**10**, but the ratio differs from the wild type. Benastatins with a single bond between C-19 and C-20 (i.e., **7** and **9**) were only formed in barely detectable trace amounts. To exclude the influence of altered titers of enzyme components, an additional copy of the KR gene was introduced into the benastatin wild-type producer. However, this manipulation did not alter the metabolic profile. It is possible that rather low expression is the reason, considering that *benL* is inserted into the genome as a singular copy, while the mutated *ben* gene cluster is located on a multicopy vector. This is also supported by the observation that traces of **9** could be detected in the complemented mutant. Nonetheless, future studies will be needed to understand double bond reduction in the D-ring and the cause for the occurrence of different product ratios.

According to the results of the inactivation experiments, BenL represents the prototype of a novel group of ketoreductases involved in the biosynthesis of pentangular polyphenols. A prediction of the BenL secondary structure on the basis of a

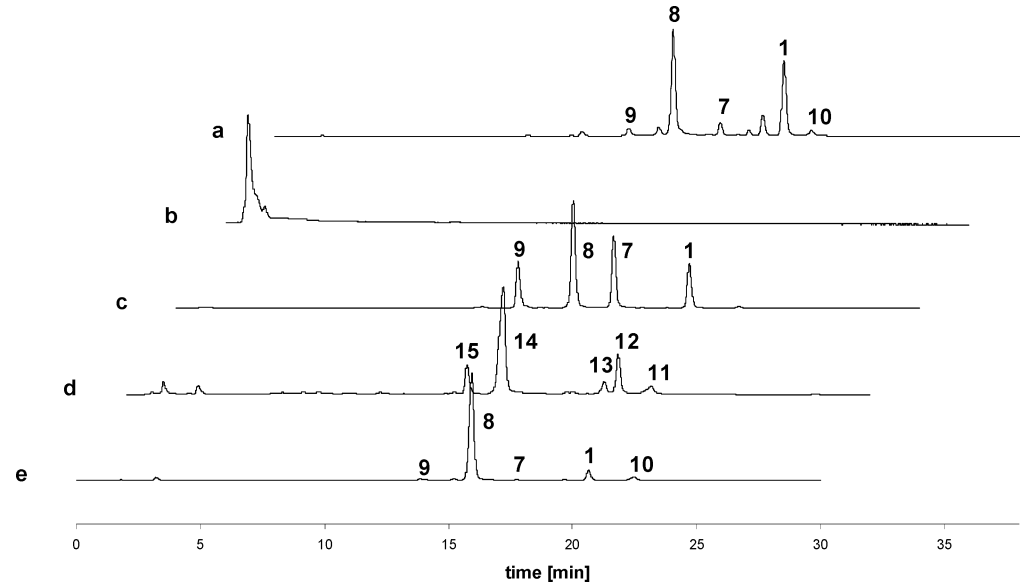


Figure 5. HPLC profiles of extracts of (a) benastatin producer, (b) *S. albus* expression host, (c) *S. albus* p5H09 bearing the entire *ben* gene cluster, (d) *S. albus* p5H09 Δ*benL*, mutant lacking the *benL* gene, and (e) *S. albus* p5H09 Δ*benL*::pGL11 (complemented mutant).

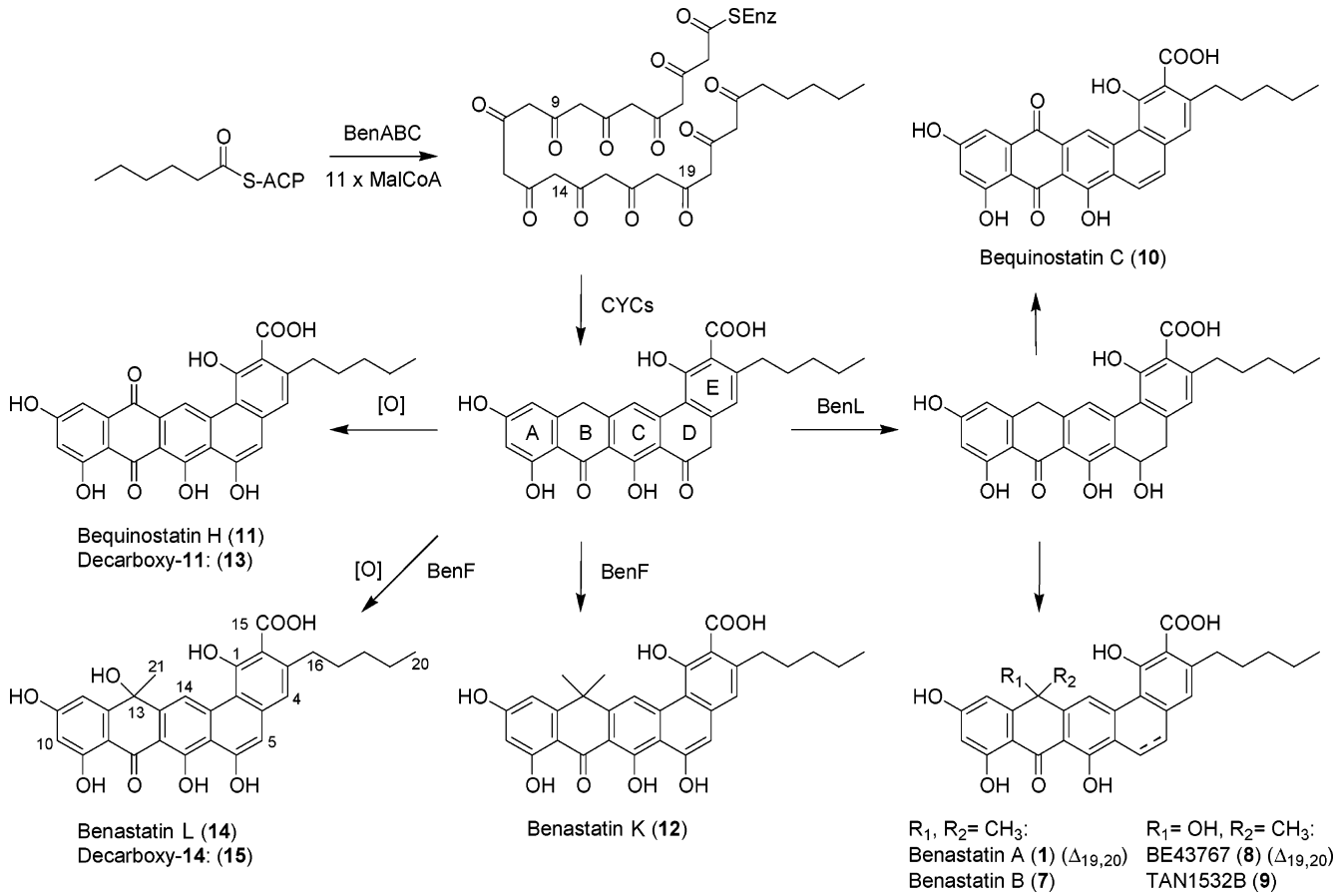


Figure 6. Model for the biosynthesis of pentangular benastatin derivatives produced by the wild type and Δ*benL* mutant.

Hidden−Markov−Model[18−21] was performed (Supporting Information). There is a Rossmann fold in BenL, which enables binding of NAD(P)H. The fold is highly conserved and consists of two $\beta-\alpha-\beta-\alpha-\beta$ motifs that are linked through helix α-3.[13,22] Typically, the catalytic tetrad consists of asparagine (N), serine (S), tyrosine (Y), and lysine (K).[23] However, it should be highlighted that in BenL the conserved N is substituted by

(18) Karplus, K.; Barrett, C.; Hughey, R. *Bioinformatics* **1998**, *14*, 846−856.
(19) Park, J.; Karplus, K.; Barrett, C.; Hughey, R.; Haussler, D.; Hubbard, T.; Chothia, C. *J. Mol. Biol.* **1998**, *284*, 1201−1210.
(20) Karplus, K.; Karchin, R.; Barrett, C.; Tu, S.; Cline, M.; Diekhans, M.; Grate, L.; Casper, J.; Hughey, R. *Proteins: Struct., Funct., Genet.* **2001**, *45*, 86−91.
(21) Karplus, K.; Karchin, R.; Draper, J.; Casper, J.; Mandel-Gutfreund, Y.; Diekhans, M.; Hughey, R. *Proteins: Struct., Funct., Genet.* **2003**, *51*, 504−514.
(22) Hadfield, A. T.; Limpkin, C.; Teartasin, W.; Simpson, T. J.; Crosby, J.; Crump, M. P. *Structure* **2004**, *12*, 1865−1875.

Table 1. ^{1}H NMR Data of Novel Benastatin Derivatives

	benastatin K (**12**)		benastatin L (**14**)	
position	^{13}C	^{1}H (J [Hz])	^{13}C	^{1}H (J [Hz])
1	167.6		167.0	
2	106.3		106.2	
3	145.8		145.8	
4	120.7	7.03 (s, 1H)	120.6	7.04 (s, 1H)
4a	142.5		142.5	
5	108.1	6.85 (s, 1H)	108.5	6.88 (s, 1H)
6	158.1		158.2	
6a	113.5		114.0	
7	162.5		162.3	
7a	109.6		109.4	
8	191.2		190.7	
8a	108.1		108.0	
9	167.4	12.51 (s, 1H, OH)	167.7	12.05 (s, 1H, OH)
10	102.2	6.28 (d, 1.8, 1H)	102.5	6.26 (d, 2.1, 1H)
11	167.4		167.6	
12	108.0	6.75 (d, 1.8, 1H)	107.1	7.02 (d, 2.1, 1H)
12a	156.3		156.9	
13	40.2		71.5	5.36 (br s, 1H, OH)
13a	148.5		148.4	
14	119.1	9.86 (s, 1H)	118.4	10.04 (s, 1H)
14a	140.0		140.0	
14b	113.4		113.8	
15	176.3		176.1	
16	37.9	3.11 (m, 2H)	37.9	3.11 (t, 7.7, 2H)
17	32.6	1.69 (m, 2H)	32.6	1.72 (m, 2H)
18	33.1	1.40 (m, 4H)	33.1	1.42 (m, 4H)
19	23.4		23.4	
20	14.4	0.92 (t, 7.0, 3H)	14.4	0.95 (t, 7.4, 3H)
21	34.7	1.80 (s, 6H)	39.5	1.59 (s, 3H)
22	34.7	1.80 (s, 6H)		

S. It is conceivable that both amino acid residues bind water and are involved in proton transfer to the substrate (proton relay system).[23]

The most surprising observation results from the general correlation between KR regioselectivity and sequence similarity in aromatic polyketide pathways. The NJ approach appeared suitable for the detection of groupings, each of which contained enzymes with similar regiospecificities (Figure 3). On the basis of known or putative functions, at least four types of KRs involved in polyketide biosynthesis were distinguished: group IV comprises C-9 KRs from BIQ (ActIII,[24] Gra ORF5[25]), angucycline (JadE,[26] UrdD[27]), and anthracycline (AknA,[28] SnoaD[29]) pathways. C-17 KRs from anthracycline biosynthesis pathways (e.g., such as DnrH[30] or ChaL[31]) and angucycline C-15 KRs, such as LanV,[32] represent clades II and III, respectively. Finally, the well-supported clade I consists of KRs that reduce the keto group at C-19 in pentangular polyketides. Interestingly, these KRs form a superfamily with group II (anthracycline C-17 KRs) and group III (angucycline C-15 KRs), which all reduce a keto group at either angular or terminal ring positions.

(23) Korman, T. P.; Hill, J. A.; Vu, T. N.; Tsai, S.-C. *Biochemistry* **2004**, *43*, 14529−14538.
(24) Fernandez-Moreno, M. A.; Martinez, E.; Boto, L.; Hopwood, D. A.; Malpartida, F. *J. Biol. Chem.* **1992**, *267*, 19278−19290.
(25) Ichinose, K.; Bedford, D. J.; Tornus, D.; Bechthold, A.; Bibb, M. J.; Revill, W. P.; Floss, H. G.; Hopwood, D. A. *Chem. Biol.* **1998**, *5*, 647−659.
(26) Han, L.; Yang, K.; Ramalingam, E.; Mosher, R. H.; Vining, L. C. *Microbiology* **1994**, *140*, 3379−3389.
(27) Decker, H.; Haag, S. *J. Bacteriol.* **1995**, *177*, 6126−6136.
(28) Tsukamoto, N.; Fujii, I.; Ebizuka, Y.; Sankawa, U. *J. Bacteriol.* **1994**, *176*, 2473−2475.
(29) Ylihonko, K.; Tuikkanen, J.; Jussila, S.; Cong, L.; Mantsala, P. *Mol. Gen. Genet.* **1996**, *251*, 113−120.
(30) Grimm, A.; Madduri, K.; Ali, A.; Hutchinson, C. R. *Gene* **1994**, *151*, 1−10.
(31) Xu, Z.; Jakobi, K.; Welzel, K.; Hertweck, C. *Chem. Biol.* **2005**, *12*, 579−588.
(32) Mayer, A.; Taguchi, T.; Linnenbrink, A.; Hofmann, C.; Luzhetskyy, A.; Bechthold, A. *ChemBioChem* **2005**, *6*, 2312−2315.

In this work, we have demonstrated the function of a representative of a new type of C-19 specific KRs from pentangular polyketide biosynthesis, which remained cryptic in the biosynthetic pathways of the spiro metabolites. The bioinformatic studies in conjunction with the mutational analyses of the *ben* and *grh* pathways and the structural elucidation of novel benastatin derivatives now clearly demonstrate the biogenetic relationship of the structurally diverse benastatins and griseorhodins. Our studies show that it is possible to gain valuable information on the general type of biosynthetic pathway as well as the reduction regiochemistry from KR phylogeny even in heavily modified aromatic polyketides. The occurrence of two clade I KR genes in the *grh* gene cluster implies the formation of an intermediate that is related to pradimicin or benastatin. This assumption was confirmed by identifying the pentangular compound collinone formed by a mutant lacking the FAD-dependent oxygenase GrhO5. The fact that the fredericamycin cluster also contains a copy of a clade I KR gene is in perfect agreement with the isolation of the pentangular fredericamycin C from the fredericamycin A producer[9] and corroborates earlier predictions that both compounds are biosynthetically related.[10]

Finally, it appears that in the biosynthesis of pentangular polyketides ketoreduction is not crucial for proper polyketide assembly. Thus, inactivation of the C-19 KRs may be a promising approach to yield hydroxyl-functionalized D-rings. Providing the labile compounds can be readily trapped, this approach could be exploited to generate further functionalized polyketides by synthesis or biotransformation.

Experimental Section

General. Mass spectra were obtained from an LCQ electrospray MS (Thermo Electron). High-resolution MS spectra were recorded on a MAT 95 XL (Thermo Electron). One- and two-dimensional ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE dpx 300 and drx 500, respectively, in THF-d_8 (if not indicated otherwise) using the solvent signals as reference. IR spectra were obtained on a Satellite FTIR manufactured by Mattson. Thin layer chromatography was performed on silica gel 60 F_{254} plates (layer thickness 0.2 mm, Merck). For column chromatography, silica gel 60, 40−63 μm, treated with aqueous 0.1 M KH_2PO_4, was used. Preparative HPLC separation was performed on a Macherey-Nagel Nucleosil 100-7, C-18 (20 × 250 mm) column, the analytic HPLC on a Chrom Nucleosil 100-5, C-18 (4.6 × 250 mm) column. Flow rate: 15 mL min^{-1} (preparative HPLC), 1 mL min^{-1} (analytical HPLC).

Bacterial Strains and Culture Conditions. Benastatin producer *Streptomyces* sp. A2991200 was kindly provided by Dr. P. Krastel (Novartis). *S. albus* J1074 (kindly provided by Prof. J. Salas) served as host strain for heterologous expression experiments. For polyketide production, wild-type and mutant strains were cultivated in a tryptic soy broth (TSB) and mannitol soya (MS) flour[33] medium for 6 days at 30 °C with shaking. *S. albus* transconjugants were selected with thiostreptone at 5 μg mL^{-1}, apramycin at 50 μg mL^{-1}, and/or spectinomycin at 400 μg mL^{-1} in solid or liquid medium depending on the specific requirements. *E. coli* strain XL1 blue served as host for routine subcloning. For intergeneric conjugation, *E. coli* ET12567 containing the RP4 derivative pUZ8002 was used.[33] *E. coli* strains were grown in LB medium supplemented with ampicillin (100 μg mL^{-1}), apramycin (50 μg mL^{-1}), or spectinomycin (50 μg mL^{-1}) for selection.

Plasmids and General DNA Procedures. DNA isolation, plasmid preparation, restriction digests, gel electrophoresis, and ligation reactions

(33) Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics;* The John Innes Foundation: Norwich, U.K., 2000.

were conducted according to standard methods.[33,34] pBluescript II SK-(−), pGEM T-easy (Promega), and p*MOS*Blue (Amersham) were the routine vectors for subcloning and preparation of DNA templates for sequencing. Restriction enzyme digested DNA fragments were recovered from agarose gel by the GFX PCR DNA and Gel Band Purification Kit (Amersham).

Inactivation of *grhO5*. The *grhO5* in-frame gene deletion mutant was constructed by PCR-mediated gene replacement based on the λRed technology.[16,17] The streptomycin and spectinomycin resistance cassette (*aadA*) flanked by FRT sites (FLP recognition target) was amplified with two long primers, each comprising 39 nt homology from sense/antisense strands ending in start/stop codons. Primers for the *grhO5* gene were grhO5_F: 5′-CCC GCG GAG AGC GAC CAA CCG CGA GAG GGG TTC TTT GTG ATT CCG GGG ATC CGT CGA CC-3′ (59 nt) and grhO5_R: 5′-CGC CCG TGC CCG GCG CGC CGG GGC GGT GGT CCG TGC TCA TGT AGG CTG GAG CTG CTT C-3′ (58 nt). The extended resistance cassette was introduced into *E. coli* BW 25113/pIJ790 containing the cosmid pMP31, which carries the entire *grh* biosynthesis gene cluster, with concurrent substitution of the *grhO5* gene. Subsequently, the antibiotic resistance cassette was eliminated by FLP-mediated excision in *E. coli* DH5α/BT340 with a remaining 81 bp "scar" sequence. The resulting *grhO5* knock-out cosmid pKR8 was introduced into *S. albus* J1074 by conjugation using *E. coli* ET12567/pUZ8002. The ex-conjugants were selected for their apramycin resistance, and the integration was verified by PCR on the genomic DNA. The obtained mutant strain *S. albus* KR8 was further used for heterologous expression.

Production, Isolation, and Analysis of Collinone (5). *S. albus* KR8 spores were inoculated into 100 mL of TSB medium and grown overnight. The resultant seed culture was used to inoculate 6 L of TSB medium, which was transferred to 500-mL Erlenmeyer flasks with a stainless steel spring, so that each flask contained 200 mL of culture for production. Flasks were incubated for 4 days at 30 °C with shaking at 200 rpm. For polyketide isolation, the mycelia were harvested by centrifugation and adjusted to pH 2 with 2 N HCl solution and extracted with ethyl acetate. The combined extracts were fractionated by flash chromatography on a NaH_2PO_4-impregnated silica gel column (9:1, $CHCl_3$/MeOH). Fractions containing the compound were detected by TLC and HPLC, combined, and dried. Further purification was carried out by preparative HPLC using a PerfectChrom 100 C18 reverse-phase semipreparative column (250 mm × 8 mm, 5μm) with UV detection at 254 and 488 nm using a Jasco MD-2015 PDA detector. A 25−75% to 40−60% CH_3CN in H_2O (0.1% TFA) gradient was used over 22 min at a flow rate of 6 mL min^{-1}. The isolated yield of collinone was 8 mg. The identity of the compound was confirmed by ESI-MS analysis (*m*/*z*: 557.067 ($M + Na^+$)) and NMR analysis (in $CDCl_3$). All spectral data were identical to the data in the literature.[8]

Inactivation of *benL*. The *benL* disruption mutant was constructed by PCR targeting based on the λRed method as described above. The primers used were: benL_F: 5′-CCG TGG ACA CCA GCC GAC ACG ACA CGG CCG CGG ACA TCA TGT AGG CTG GAG CTG CTT C-3′ (58 nt) and benL_R: 5′-GCC ATC CCT TCC CCA CAT ACG AAC TCG GAG CGC CCC GTG ATT CCG GGG ATC CGT CGA CC-3′ (59 nt). The amplicons were introduced into *E. coli* BW25113/pIJ790 containing cosmid p5H09 with concomitant substitution of *benL* by the extended antibiotic resistance cassette. The inserted cassette was maintained in the cosmid for the sake of facilitating conjugation. The resulting construct, p5H09 Δ*benL*, was introduced into *S. albus* J1074 by intergeneric conjugation using *E. coli* ET12567 containing the RP4 derivative pUZ8002. The transconjugants were selected for their spectinomycin resistance.

Cloning and Heterologous Expression of *benL* for Complementation. *benL* was amplified from cosmid p5H09 by PCR using primers benL_for: ACA TCA GCC GAC ACG ACA C and benL_rev: CTG ACC AGC AAG GTC CGA AAG. The 875 bp amplicon was first subcloned into pGEM-T (Promega) and then ligated into the *Eco*RI site downstream of the *ermE* promoter of pKJ55. The resulting plasmid, pGL11, was introduced into *S. albus* p5H09 Δ*benL* by intergeneric conjugation.

Production, Isolation, and Analysis of Benastatin Derivatives. One hundred microliters of a spore suspension of strain *S. albus* p5H09 Δ*benL* was used to inoculate 100 mL of a seed medium (15 g L^{-1} glucose, 15 g L^{-1} soy bean meal, 5 g L^{-1} NaCl, 1 g L^{-1} $CaCO_3$, 0.3 g L^{-1} KH_2PO_4) in 250-mL Erlenmeyer flasks, supplemented with thiostreptone (5 mg L^{-1}) for antibiotic selection, and cultivated at 28 °C for 2 days on a rotary shaker (180 rpm). Fifteen milliliter aliquots of the seed culture were transferred into 250-mL portions of 14-L MS production medium (20 g L^{-1} mannitol, 20 g L^{-1} soy bean meal) in 1-L Erlenmeyer flasks and cultivated at 28 °C for 4 days on a rotary shaker (180 rpm). To isolate the secondary metabolites, the culture broth was separated into the mycelial cake and the culture filtrate. The mycelial cake was extracted with methanol (4 L). The extract was concentrated under reduced pressure to an aqueous solution. It was re-extracted with ethyl acetate, and the organic phase was dried over *anh* and Na_2SO_4 and concentrated under reduced pressure, yielding 135 mg of mycelial crude extract. The culture filtrate was exhaustively extracted with ethyl acetate, and the organic phase was dried and concentrated under reduced pressure. The combined extracts (brown oil) were subjected to open column chromatography on silica gel (impregnated with aqueous 0.1 M KH_2PO_4) using a chloroform/methanol (100:0 to 50:50) gradient for elution. Benastatin-containing fractions were subjected to further purification by preparative RP-HPLC using acetonitrile/0.1% AcOH in water (60:40 to 100:0). The purity of the obtained fractions was tested by analytical HPLC using acetonitrile/0.1% AcOH in water (60:40 to 100:0) gradient.

Benastatin L (14): For NMR data, see Table 1. IR (powder) ν 3356, 2952, 2924, 2853, 1627, 1598, 1457, 1375, 1268, 1035, 873 cm^{-1}. ESI-MS (*m*/*z*, %): 517 ($[M - H]^-$, 100). HRMS calcd for $C_{29}H_{25}O_9$: 517.1493, observed: 517.1457.

Benastatin K (12): For NMR data, see Table 1. IR (powder) ν 3384, 3068, 2952, 2923, 2854, 1631, 1592, 1431, 1363, 1336, 1256, 1155, 1047, 847, 768 cm^{-1}. ESI-MS (*m*/*z*, %): 515 ($[M - H]^-$, 100). HRMS calcd for $C_{30}H_{27}O_8$: 515.1711, observed: 515.1706.

Phylogenetic Analyses. As a basis for the phylogenetic tree construction, a multiple sequence alignment was created using ClustalX (version 1.83).[35] To obtain an optimal amino acid sequence alignment, overhanging sequence ends were trimmed and gap opening penalty was set to seven. The resulting alignment of 28 sequences with an overall length of 260 positions was chosen to construct an NJ bootstrap tree (10 000 replicates) using the tree-building function of ClustalX. The sequence of *E. coli* FabG was used as an outgroup to root the tree. To validate the tree topology, a maximum likelihood analysis was performed using Tree-Puzzle 5.2.[36]

Acknowledgment. This work has been financially supported by the BMBF GenoMik program (C.H.), a Humboldt postdoctoral fellowship to A.S., and the DFG (PI 430/4-1, PI 430/5-1, GRK 804 to J.P., and HE 3469-2 to C.H.). We are grateful to M.-G. Schwinger for fermentation, and A. Perner, Dr. F. A. Gollmick, Dr. H. Gross, and Dr. M. Engeser are acknowledged for performing MS and NMR measurements. We thank Dr. P. Krastel (Novartis) for providing the benastatin producer *Streptomyces* sp. A2991200. We thank Prof. J. Salas for the kind gift of the *S. albus* host strain.

JA0718624

(34) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.
(35) Thompson, J. D.; Gibson, T. J.; Plewniak, F.; Jeanmougin, F.; Higgins, D. G. *Nucleic Acids Res.* **1997**, *24*, 4876−4882.
(36) Schmidt, H.; Strimmer, K.; Vingron, M.; von Haeseler, A. *Bioinformatics* **2002**, *18*, 502−504.