DOI: 10.1002/anie.200905468

Polyketide Biosynthesis

The Final Steps of Bacillaene Biosynthesis in *Bacillus amyloliquefaciens* FZB42: Direct Evidence for β,γ Dehydration by a *trans*-Acyltransferase Polyketide Synthase**

Jana Moldenhauer, Daniel C. G. Götz, Christian R. Albert, Sebastian K. Bischof, Kathrin Schneider, Roderich D. Süssmuth, Marianne Engeser, Harald Gross, Gerhard Bringmann, and Jörn Piel**

The antibiotic bacillaene[1] (**1**; Scheme 1) is the prototype of a growing class of polyketides synthesized by a family of polyketide synthases (PKSs) termed *trans*-acyltransferase (AT) PKSs.[2,3] These poorly studied enzymes are giant multimodular proteins that have evolved independently[4] from the textbook (*cis*-AT) PKSs involved in, for example, erythromycin biosynthesis.[5] Since they are rare in actinomycetes, the first model organisms used for studies on polyketide biosynthesis, *trans*-AT PKSs have long been overlooked. However, they are now known to be widespread among many other rich natural product sources and responsible for the production of several pharmacologically relevant polyketides, including antibiotics of the mupirocin[2c] and streptogramin[2g] groups and the antitumor drug candidate bryostatin 1.[6b] *trans*-AT PKSs have also attracted considerable ecological interest, since they occur in bacteria with unusual lifestyles, such as symbionts, pathogens, and anaerobes.[2,3,6] Another peculiarity that sets them apart from *cis*-AT enzymes and raises fundamental questions about how polyketides are assembled by these proteins is the high frequency of biosynthetic features that do not conform with classical PKS rules.[2,3,7]

During functional studies with **1** as a model, we discovered that deletion of the thioesterase (TE) domain, which releases the assembled polyketide chain from the PKS,[8] results in the production of virtually all intermediates.[9] This unusual phenomenon enabled us to unravel a large part of bacillaene biosynthesis and, in combination with phylogenetic analyses, to establish a set of colinearity rules that can be applied to the prediction and discovery of natural products from *trans*-AT PKS sequences.[3]

Several questions, however, remained unanswered after the studies on **1**. The triene system in the C3–C8 region exhibits rare β,γ-type unsaturation instead of the standard α,β pattern; the resulting enamide moiety is known in few other polyketides.[10] Since **1** and its advanced precursors are highly unstable, our previous structural investigations to characterize the bacillaene assembly had relied on HPLC coupled to high-resolution MS (HRMS).[9] This technique, however, does not distinguish between constitutional isomers and thus leaves unclear when and how *trans*-AT PKSs catalyze olefinic shifts (Scheme 1). We also noticed that in late cultures of the bacillaene producer *Bacillus amyloliquefaciens* FZB42, **1** was almost entirely replaced by a new compound of unknown structure (see Figure S1 in the Supporting Information). This result raised the question of whether **1** is really the true biosynthetic end product. Finally, the bacillaene (*bae*) PKS terminates with two noncanonical modules (modules 16 and 17, Scheme 1), the ketosynthase (KS) domains of which were predicted by bioinformatic analysis to be unable to catalyze chain-elongation reactions.[3] The role of such seemingly superfluous terminal modules, which are ubiquitous in *trans*-AT PKSs, is presently unknown. Another architectural oddity is a dehydratase (DH) domain in module 16 which could not be attributed to any dehydration reaction.

To examine the function of modules 16 and 17, we genetically engineered the PKS mutants JM54-2, with a deletion of module 17 + TE, and JM122, in which module 17 plus the TE domain are fused directly onto module 15. HPLC–HRMS analysis showed that both strains produced a series of prematurely released intermediates, the two most advanced of which had the molecular formulae $C_{34}H_{48}N_2O_6$

[*] J. Moldenhauer, Dr. M. Engeser, Prof. Dr. J. Piel
Kekulé Institut für Organische Chemie und Biochemie
Universität Bonn
Gerhard-Domagk-Strasse 1, 53121 Bonn (Germany)
Fax: (+49) 228-739-712
E-mail: joern.piel@uni-bonn.de
Homepage: http:/www.chemie.uni-bonn.de/oc/ak_piel

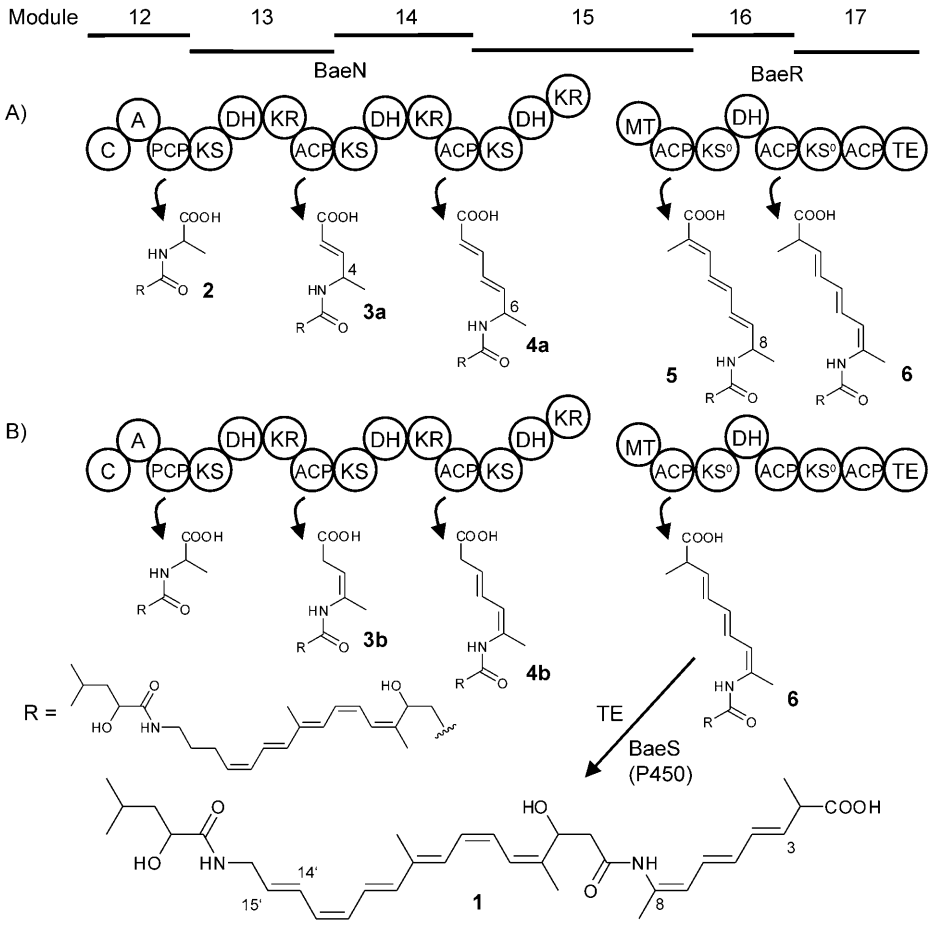
D. C. G. Götz, C. R. Albert, S. K. Bischof, Prof. Dr. Dr. G. Bringmann
Institut für Organische Chemie, Universität Würzburg
Am Hubland, 97074 Würzburg (Germany)
Fax: (+49) 931-888-4755
E-mail: bringman@chemie.uni-wuerzburg.de
Homepage: http://www-organik.chemie.uni-wuerzburg.de/lehr-stuehlearbeitskreise/bringmann

Dr. K. Schneider, Prof. Dr. R. D. Süssmuth
Technische Universität Berlin
Strasse des 17. Juni 124, 10623 Berlin (Germany)

Dr. H. Gross
Institut für Pharmazeutische Biologie, Universität Bonn
Nussallee 6, 53115 Bonn (Germany)

[**] We thank K. Peters-Pflaumbaum, C. Sondag, and M. Grüne for technical assistance, and R. Borriss for the strain FZB42. This project was supported financially by the DFG (SFB 624 to J.P. and M.E., SFB 630 to G.B., and FOR 854 to J.P. and H.G.), by the BMBF (0313805U GenoMik-Plus to R.D.S.), and by fellowships of the Degussa-Stiftung and the Studienstiftung des deutschen Volkes e.V. (to D.C.G.G.)

Supporting information for this article (including experimental details, HPLC chromatograms, mass spectra, NMR spectroscopic data, and a detailed discussion) is available on the WWW under http://dx.doi.org/10.1002/anie.200905468.



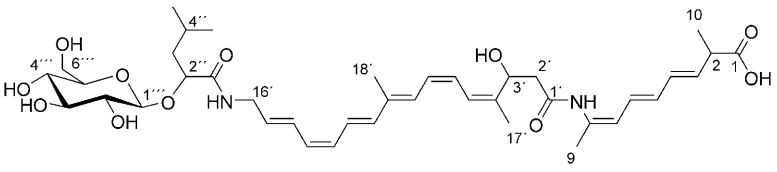
Scheme 1. Two alternative scenarios for the biosynthesis of products with shifted double bonds by the *bae* PKS: A) the double-bond shift occurs after construction of the polyketide chain, or B) the double-bond shift occurs during elongation. The C14′–C15′ bond is olefinic in bacillaene (**1**) and saturated in the precursor dihydrobacillaene (**6**). Abbreviations not defined in the text: C, condensation domain; A, adenylation domain; KR, ketoreductase domain; MT, methyltransferase domain; KS°, nonfunctional KS domain.

and $C_{34}H_{50}N_2O_6$, according to their exact masses. Since these formulae are identical to those of **1** and **6**, the experiment confirmed that modules 16 and 17 are not involved in polyketide elongation. However, in contrast to the previously constructed TE-deletion strain, in which **6** is one of the main polyketides formed,[9] titers of polyketides more advanced than **4a** or **4b** dropped dramatically in JM54-2 and JM122 (see Figure S2 in the Supporting Information). This difference shows that nonelongating modules of *trans*-AT PKSs play a crucial role in maximizing the yields of fully elongated polyketides. These data also revealed that further processing of the trienoic intermediate **5** at module 15 was compromised in the TE-carrying mutant JM122, either because **15** is not a substrate for module 17 (e.g., if scenario A in Scheme 1 applies) or because we had fused an incompatible ACP–KS pair.

We next addressed the question of whether JM122, which lacks module 16, is able to form enamides. Since the polyketides were too unstable for isolation, we carried out NMR spectroscopic analysis of crude polyketide mixtures. To detect the hybridization state of C8 in **5** or **6** by ^{13}C NMR spectroscopy, we fed [2,3-$^{13}C_2$]alanine to cultures of the wild-type producer FZB42 and JM122. When FZB42 extracts were analyzed, two strongly enhanced doublets at $\delta = 133.8$ and 21.6 ppm were observed for the enamide C8 carbon atom and the C9 methyl carbon atom of **1** (see Figure S3 A in the Supporting Information).[1c] This result established alanine as a building block of the pathway. However, in the deletion mutant JM122, late bacillaene intermediates were produced at such low levels that signal overlap caused by other metabolites did not permit an unequivocal interpretation of the NMR spectrum (see Figure S3B in the Supporting Information). We therefore analyzed extracts by liquid chromatography (LC) coupled to NMR spectroscopy in the hope that pure spectra could be recorded before decomposition.

After extensive HPLC optimization, we were able to separate late intermediates of JM122 (see Figure S4 in the Supporting Information). The more prominent compounds were assigned by HRMS as progressively elongated intermediates released from modules 12, 13, and 14.[9] The successful recording of 1D and 2D NMR spectra of **3a/b** and **4a/b** illustrated the potential of the LC–NMR technique for the characterization of highly unstable metabolites (see Figures S5–S15, Data S1 and S2, and Tables S1 and S2 in the Supporting Information). The obtained spectra evidenced a dihydrobacillaene-like[1c] "western" portion (C2′ to C6″). Importantly, however, the compounds were found to contain 9 and 11 olefinic hydrogen atoms, respectively, in agreement with the presence of methyl-branched enamine moieties in the "eastern" part of the molecules. Such moieties are only present in the compounds derived from the postulated scenario B (Scheme 1). Moreover, the spectra revealed three vinylic methyl groups for each of the two intermediates and one methylene group (which is not present in **6**) directly connected to an olefinic carbon atom. For intermediate **4b**, an HMBC interaction between the 2-H methylene hydrogen atoms and the terminal carboxyl group was also observed. For neither compound were the characteristic deshielded β and δ hydrogen atoms of carbonyl-conjugated olefins detected.[11,12] This result proves that at least up to module 14, pathway B (and not A) applies. Thus, the biosynthesis of **1** proceeds via **3b** and **4b**: that is, with the double bonds already shifted into the enamine position (Scheme 1 B).

Thus, deletion experiments in combination with in-depth LC–NMR investigations enabled us to directly observe the



Scheme 2. Structure of bacillaene B (**7**).

installation of two β,γ-olefinic moieties on the same modules that perform the elongation, presumably by the action of a dedicated DH domain (Scheme 1 B). Shifted double bonds are also known for some *cis*-AT PKSs,[13] and a similar biosynthetic scenario was proposed earlier for the ansamitocin PKS on the basis of indirect evidence derived from feeding studies.[13b] The presence of β,γ-dehydrating modules therefore seems to be a general phenomenon that occurs across boundaries of PKS families. The small amounts of fully elongated bacillaenes were not sufficient for us to determine whether the third shifted double bond in **1** is introduced in a similar way. An alternative to β,γ desaturation could be the isomerization of an α,β double bond by module 16 in analogy to rhizoxin biosynthesis.[14]

For the first time, we were also able to detect pure 1D and 2D spectra of the suspected unstable end product of the bacillaene pathway by LC–NMR spectroscopy (see Figures S16–S23 in the Supporting Information). In agreement with the molecular formula $C_{40}H_{58}N_2O_{11}$ (determined by HRMS; see Figure S24 in the Supporting Information), NMR spectroscopic analysis clearly revealed a bacillaene-like structure; additional signals in the carbohydrate region were attributed to a β-hexose unit. On the basis of the NMR spectroscopic data we propose the sugar portion to be a β-glucoside (see Data S3 and Table S3 in the Supporting Information). The position of the sugar moiety was assigned as shown for **7** in Scheme 2 on the basis of the upfield shift of the 2″-H signal in comparison with that observed for **1**, **3b**, **4b**, and **6**[1c] and the occurrence of two distinct signals at $\delta = 0.86$ and 0.84 ppm for the methyl hydrogen atoms of the isopropyl group. Most significantly, a weak HMBC interaction between 1‴-H and C2″ was also observed. Thus, **7** is a novel polyketide designated as bacillaene B.[12] The glucosylated structure was unexpected, since no homologue of known glycosyltransferase genes is present in the *bae* gene cluster.

In conclusion, we have presented direct evidence for the introduction of β,γ double bonds by PKS modules (Scheme 1, path B). In the case of **1**, this noncanonical biochemistry results in the formation of a rare enamide moiety, in yet another example of the remarkable catalytic versatility of *trans*-AT PKSs. Furthermore, advanced NMR spectroscopic techniques identified **7** as the unexpected biosynthetic end product of the *bae* pathway.

Received: September 29, 2009
Published online: January 19, 2010

Keywords: antibiotics · biosynthesis · metabolic engineering · NMR spectroscopy · polyketides

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