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Evidence from engineered gene fusions for the repeated use of a module in a modular polyketide synthase

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Functional evidence for programmed loss of co-linearity on the borrelidin modular polyketide synthase (PKS) is presented.

Introduction

Macrocyclic polyketides include some of the most important pharmaceutical agents in current clinical use. They are biosynthesised by modular (type-I) polyketide synthases (PKSs) that contain multiple modules of enzyme activities. Each module catalyses a single cycle of chain extension of the polyketide backbone and concomitant reductive processing of the β-keto groups generated *in situ*.[1] Engineering this modular assembly process should considerably enhance the utility of natural products in drug discovery,[2–4] particularly if we can understand and harness the fundamental process of chain extension. In the course of our sequencing and analysis of the biosynthetic gene cluster for the angiogenesis inhibitor borrelidin (*bor*) from *Streptomyces parvulus* Tü4055, we found that the *bor* PKS contains two fewer extension modules than required by the strict rule of "one extension, one module". It seems that module 5 (BorA5) must catalyse (with perfect fidelity) three rounds of chain elongation and processing to a methylene moiety. Engineered versions of the *bor* PKS in which module 5 was translationally fused to either or both of its flanking modules remained able to synthesize borrelidin. Here we show that the paradigm for these multienzymes must be expanded, since one copy of a single PKS module is necessary and sufficient to catalyse three successive extension cycles.

Borrelidin (Fig. 1) is an 18-membered macrolide polyketide produced by several streptomycete species. It was first discovered due to its antibacterial activity,[5] which involves selective inhibition of threonyl-tRNA synthetase,[6] but current interest centres on its anti-angiogenesic activity and its potential as an antitumor agent.[7–9] In a rat aorta matrix culture model, borrelidin exhibited a potent angiogenesis-inhibiting effect causing disruption of formed capillary tubes by inducing apoptosis of the capillary-forming cells. The IC_{50} value for anti-angiogenesis activity is 50-fold lower than that for inhibition of protein synthesis through threonyl-tRNA synthetase inhibition, indicating different activities of the compound. Borrelidin also displays potent inhibition of angiogenesis *in vivo* using a mouse dorsal air sac model, and was very effective in the inhibition of the formation of spontaneous lung metastases of B16-BL6 melanoma cells at the same dosage that inhibited angiogenesis.[8]

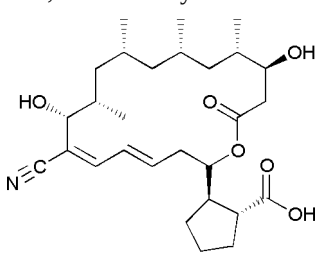

Fig. 1 Chemical structure of the angiogenesis inhibitor borrelidin.

The chemical structure of borrelidin contains several unique features, including an intriguing nitrile moiety at C12 and a *trans*-1,2 disubstituted cyclopentanecarboxylic acid moiety at C17, which corresponds to the start of the polyketide chain. For a better understanding of the biosynthetic pathway for the generation of novel derivatives through genetic engineering, we cloned, sequenced and analysed the genes encoding the biosynthetic gene cluster for borrelidin from *Streptomyces parvulus* Tü4055.[10] The *bor* biosynthetic gene cluster contains six genes (*borA1-A6*) that encode the PKS (Fig. 2), and several plausibly involved in either the production of the putative *trans*-cyclopentane-1,2-dicarboxylic acid starter unit or the nitrile function at C12. Surprisingly the borrelidin PKS contains only six extension modules instead of the anticipated eight. This observation led to the hypothesis that one extension module of the *bor* PKS is capable, with remarkable fidelity, of catalysing three successive condensations, rather than one, before passing the intermediate to the next module.

Based on the structure of borrelidin three rounds of chain elongation must occur consecutively, in which methylmalonyl-CoA extension units are incorporated and reduced to a methylene moiety (Fig. 2). Only BorA5 contains all of the catalytic activities capable of performing this operation. The remaining modules contain precisely the catalytic activities required to synthesize the rest of the polyketide chain in the established manner. Clearly BorA5 is the only module that can be used repeatedly (three times) during polyketide chain assembly.

To test the repeated use of BorA5, several alternative mechanisms were considered for the observed deficiency of two extension modules. The possibility that during construction of the cosmid library a DNA rearrangement had occurred in this region was ruled out on the basis of detailed and extensive Southern analysis. Three further explanations presented themselves.

Firstly, that the extra PKS modules responsible for chain elongation are located elsewhere on the chromosome. This would be unprecedented, but to test this an experiment was designed whereby BorA5 was translationally fused, both separately and together, to its immediate neighbours BorA4 and BorA6 (see below).

A second scenario involves non-stoichiometric amounts of the various PKS proteins. Extra copies of BorA5 could be thus created, and three copies of BorA5 could then assemble between BorA4 and BorA6 of the resultant multienzyme complex. Biosynthesis of the macrolactone by this system would effectively follow the established paradigm of modular PKS biosynthesis with the three copies of BorA5 each used for one round of chain elongation. However, such a situation is unlikely, as it is known that the docking regions between polyketide synthases are crucial for recognition and transfer of

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the extending acyl chain.[11,12] This means that three copies of the same protein should not be able to assemble in the manner described above. However, we chose once again to eliminate this possible scenario by translational fusion of BorA5 to its neighbours; in this system BorA4-A6 would exist as a single protein, and multiple copies of BorA5 could not assemble and dock together.

The third explanation is that one or more of the *bor* genes undergo differential transcription–translation to produce the required complement of extension modules, not as complimentary copies as described above, but as iso-forms of BorA5. Precedent for this exists in the pikromycin gene cluster of *Streptomyces venezuelae*, which is responsible for the biosynthesis of both the heptaketide pikromycin and the hexaketide methymycin.[13] These macrolides derive from alternative expression of the final PKS protein PikA4[14] giving iso-forms that allow the polyketide chain to "skip" through without condensation.[15] It therefore seemed possible that alternative forms of BorA5 differing in their *N*-termini might be capable of assembly and then utilized to catalyse successive condensations. This hypothesis could be tested by translational fusions of BorA5.

To delineate between these various mechanistic possibilities we generated translational fusions, head-to-tail, between *bor* modules 4, 5 and 6 (Fig. 3). None of the above mechanisms, with the exception of BorA5 reusing its active sites for three successive extensions of the polyketide chain, would permit borrelidin synthesis by any of these mutants. A mutant of the *bor* PKS (A4-A5) was constructed in which the unimodular multienzyme BorA5 was fused in-frame at its *N*-terminus to the *C*-terminus of BorA4. Analogously, a second mutant (A5-A6) was constructed by in-frame translational fusion, so that BorA5 was tethered *via* its *C*-terminus to the *N*-terminus of BorA6. Finally, a double mutant (A4-A5-A6) was constructed in which BorA4, A5 and A6 are housed in the same multienzyme protein. The mutants were compared with wild type *S. parvulus* Tü4055 for their ability to produce borrelidin. HPLC analysis revealed that the mutants produced the following levels of borrelidin relative to the wild type: mutant A4-A5, 21 ± 4%; mutant A5-A6, 27 ± 4%; and mutant A4-A5-A6, 18 ± 5%. This demonstrates, unambiguously, that the full-length polyketide backbone of the nonaketide borrelidin is produced using only the starter and six extension modules. These results allow us to conclude that the repeated use of a module during chain extension is indeed occurring on the *bor* PKS.

These data represent the first functional evidence for the repeated use of a module in a type-I modular PKS. Previously this possibility had been proposed by comparing the structure of polyketide metabolites with the sequence analysis of their respective biosynthetic gene clusters. It was shown that the gene cluster responsible for stigmatellin biosynthesis in *Stigmatella aurantiaca* contains a PKS comprising only nine extension modules instead of the predicted ten required for the undecaketide product.[16] Recently, the gene cluster responsible for lankacidin biosynthesis was isolated and characterised from a large linear plasmid in *Streptomyces rochei*.[17] This type-I modular PKS contains only five modules, where eight condensation cycles are required. These conjunctures represent the only other genetic (sequence), but not functional, evidence reported so far to indicate that certain modular type-I PKSs reuse modules during polyketide chain assembly through a defined number of cycles as their natural function. In addition, through the careful analysis of metabolite profiles it has been suggested that modular PKSs reuse an extension module more than once, although not as their natural function. The first example of this was observed for module 4 of the erythromycin PKS.[18] It was discovered that octaketide macrolides related to 6-deoxyerythronolide B (6dEB), the product of the erythromycin PKS, were also present, albeit at low levels, in *Saccharopolyspora erythraea* strains in which the PKS was fully functional. An *S. erythraea* mutant deleted in the PKS was incapable of producing either 6dEB or the new octaketide products. These compounds appeared to be derived from a process in which module 4 of the erythromycin PKS had operated twice, incorporating two extension units. This aberrant process was termed stuttering. Stuttering also presumably occurs in the epothilone producer *Sorangium cellulosum* from which minor epothilone-related metabolites extended by one

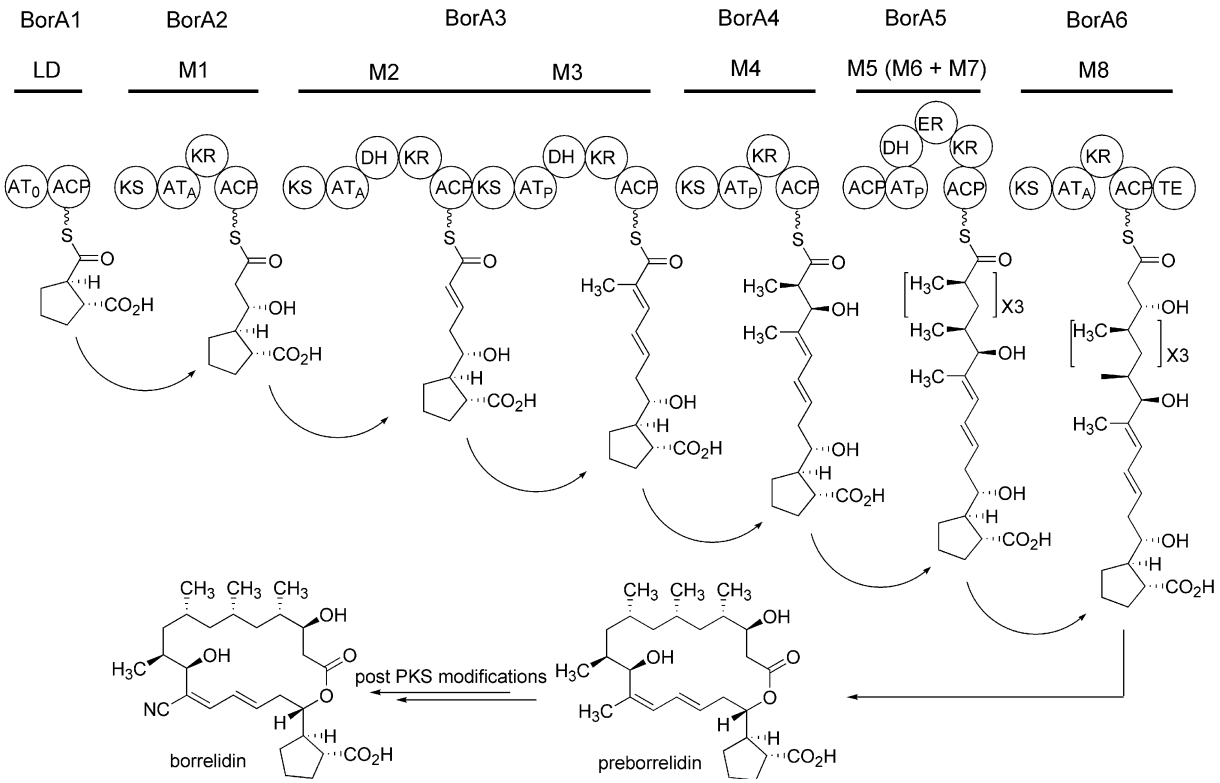

Fig. 2 Modular organization of the borrelidin PKS: LD, loading domain; M, module; ACP, acyl carrier protein; AT, acyltransferase; AT_a, malonyl-specific acyltransferase; AT_p, methylmalonyl-specific acyltransferase; ER, enoyl reductase; KR, β-keto reductase; DH, dehydratase; KS, β-ketoacyl-ACP synthase; TE, thioesterase.

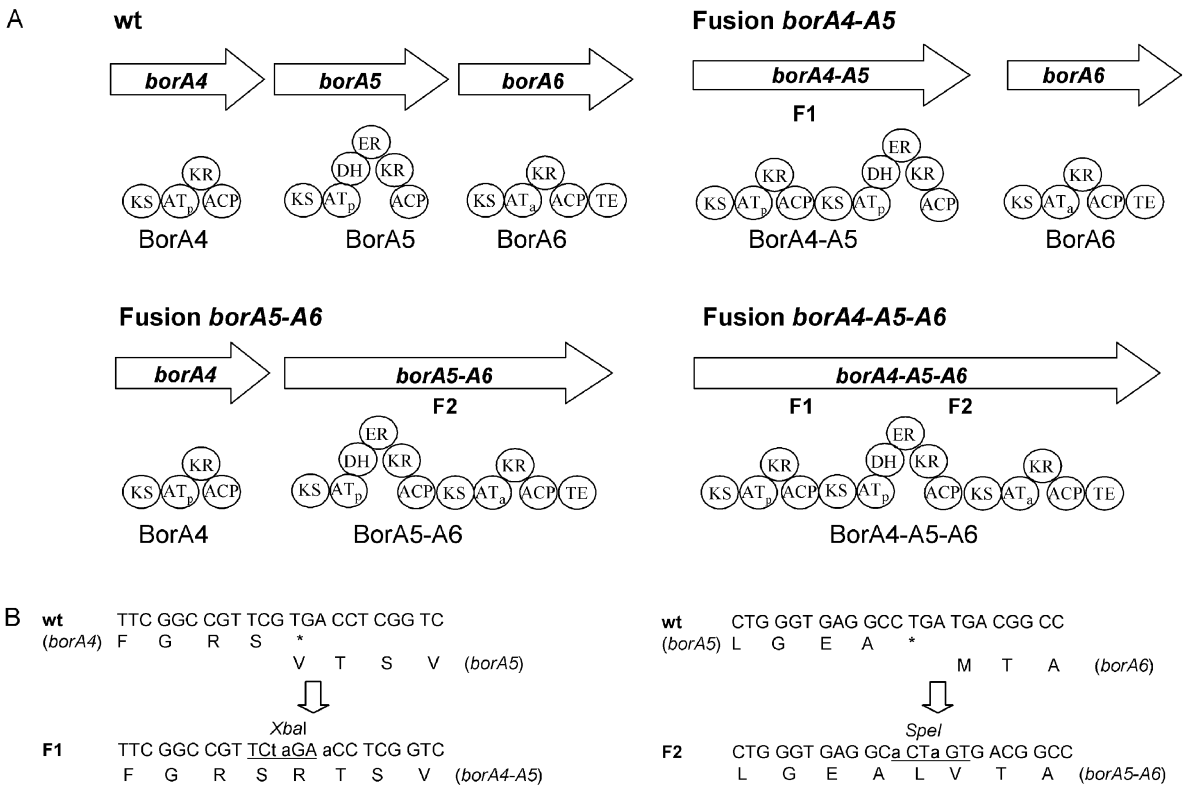

Fig. 3 A. Schematic representation of the gene fusions between *borA4*, *borA5* and *borA6* and the resultant protein products. B. Detail of the fusions at the nucleotide level indicating the formation of the new restriction sites. Lower case indicates the two new nucleotides introduced by PCR amplification to generate the gene fusions and the new restriction sites. F1 and F2 indicate the two fusions generated.

extra unit have been isolated.[19] A distinction should be drawn between stuttering on a modular PKS to produce minor metabolites and the repeated use of a module to synthesize a parent compound. In the first case 'stuttering' is an aberrant process, whereas in borrelidin, and presumably stigmatellin biosynthesis, the PKS has evolved to produce these compounds *specifically* through the repeated use of a module during polyketide chain extension.

The ability of BorA5 to operate in a repeated manner and with perfect fidelity (no borrelidin related hepta-, octa- or decaketide analogues could be identified even after exhaustive HPLC and LCMS analysis) is remarkable, indicating that under specific circumstances modular PKSs are able to either measure the size of the growing chain or to count the number of extension cycles, while also recognising the functionality incorporated. Analysis of BorA5 through sequence alignments with other type-I PKSs, both bacterial modular and fungal iterative ones, and to type-I fatty acid synthase (FAS) provides no evidence for any unusual function of the enzyme. Recent studies with bacterial type-II PKSs have provided evidence for a measuring mechanism,[20] which is also the case for FAS systems.[21] Whether the mode of operation of BorA5 is related to the organisation of domains, *i.e.* that it contains a full set of reductive domains and as such is similar, at least in a functional sense, to type-I FAS remains to be seen. The specific mechanisms that govern the repeated use of BorA5 in this way remain to be uncovered; in the first instance the roles of the ketosynthase from BorA5 and its downstream counterpart BorA6 in controlling the number and fidelity of chain extensions will be particularly worthy of study.

Now the role of BorA5 in this process has been defined it can be applied along with other strategies for the combinatorial biosynthesis of complex polyketides. This work lays the foundation for future studies aimed at engineering more potent analogues of borrelidin as angiogenesis inhibitors and antitumor agents.

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