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Uncovering the origin of *Z*-configured double bonds in polyketides: intermediate *E*-double bond formation during borrelidin biosynthesis

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Formation of *Z*-configured double bonds in reduced polyketides is uncommon and their origins have not been extensively studied. To investigate the origin of the *Z*-configured double bond in the macrolide borrelidin, the recombinant dehydratase domains BorDH2 and BorDH3 were assayed with a synthetic analogue of the predicted tetraketide substrate. The configuration of the dehydrated products was determined to be *E* in both cases by comparison to synthetic standards. Detailed NMR spectroscopic analysis of the biosynthetic intermediate pre-borrelidin confirmed the *E,E*-configuration of the full-length polyketide synthase product. In contrast to a previously-proposed hypothesis, our results show that in this case the *Z*-configured double bond is not formed *via* dehydration from a 3-L-configured precursor, but rather as the result of a later isomerization process.

Introduction

Reduced polyketides are natural products with an enormous structural diversity and a broad spectrum of biological activities. They are produced by type I polyketide synthases (PKS-I), multimodular mega-enzymes that catalyse the elongation of a growing polyketide chain by decarboxylative *Claisen*-like condensations.[1] In each module, a so-called reductive loop establishes the individual functionalization pattern of the backbone (Scheme 1). A ketoreductase (KR) domain reduces the 3-oxo-thioester **1** to a 3-hydroxy-thioester **2**, the dehydratase (DH) domain removes water and finally the enoylreductase (ER) domain reduces the α,β-unsaturated thioester **3** to the fully saturated chain **4**. During the whole process, the substrate remains tethered to the PKS *via* the phosphopantetheine linker on the acyl carrier protein (ACP) domains.
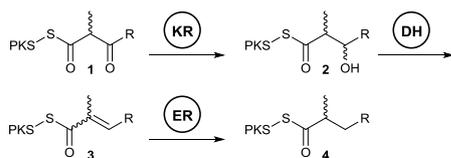
Scheme 1 Processing of PKS intermediates by reductive loop domains.[1]

Most double bonds in polyketides, even those that are immediately reduced by their module-associated ERs, have *E*-configuration. It has been proposed that *E*-double bonds arise from DH-catalysed *syn*-dehydration of 3-D (or 3-*R*) hydroxythioesters and that the rare *Z*-configured double bonds originate from 3-L (or 3-*S*) precursors.[2a] The configuration of this precursor is determined by the preceding KR. Characteristic active site residues in KRs have been identified that directly influence the stereochemical outcome of the reduction on C-3 as well as on C-2.[2] However, there are individual modules (for example in the PKSs for rifamycin, borrelidin halstoctacosanolide, thuggacin and phoslactomycin) where this rule does not seem to apply. In these cases *Z*-configured double bonds are present in the final natural product even though the configuration of the DH precursors in the particular modules is predicted to be 3-D according to the active site residues in the preceding KR.[3]

Although double bonds are important and abundant structural features of reduced polyketides, their DH-catalysed formation has not been intensively studied and only little is known about the stereoselectivity and substrate specificity of these domains. We and others have previously shown that DH domains may retain catalytic competence as isolated domains *in vitro*.[4] X-ray crystal structures have been obtained for *E*-selective DH domains from the curacin and the erythromycin pathway;[5] *E*-selective DH domains from the erythromycin and the nanchangmycin pathway have been studied *in vitro*.[4a,b]

Much less is known about putatively *Z*-selective DH domains. Of the above-mentioned exceptional cases, only the DH domains from the respective modules of the rifamycin and the borrelidin PKS have been studied *in vitro* so far.[4c,d] However, in both cases diketide surrogates have been used which significantly differ from the natural precursors. Additionally, the X-ray crystal structure for RifDH10 from the rifamycin pathway has been reported. Recently, a TE(Fr9-TE2)-catalyzed dehydration reaction leading to the formation of a *Z*-configured double bond was observed in the biosynthesis of the splicosome inhibitor FR901464 and reconstituted *in vitro*.[4e] TE domains could be classified into the same hotdog superfamily as DH domains and therefore they show structural similarity to each other. Further

studies will however have to clarify if mechanistic analogies exist between both domains as well.
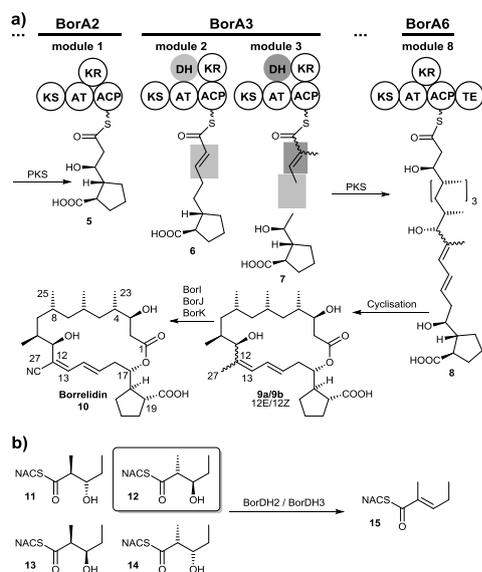
5 **Fig. 1** a) Borrelidin is biosynthesized on a modular PKS-I. The DH domains of modules 2 and 3 are highlighted in light and dark grey. b) Previous study on recombinant BorDH2 and BorDH3. Both DH domains converted only the 2-D,3-D isomer of 3-hydroxy-2-methyl-SNAC-pentanoate, and the product in each case contained an *E*-configured
10 double bond. HSNAC: *N*-acetylcysteamine.[4c,8]

The macrolide borrelidin **(10)** has promising antibacterial, antimalarial and anticancer activities. It acts as an angiogenesis inhibitor by affecting threonyl-tRNA synthetase and the spliceosome-associated protein formin binding protein 21,
15 thereby acting as a modulator of alternative splicing. It contains several unusual structural features, including a cyclopentanecarboxylic acid moiety, which derives from a (1*R*,2*R*)-cyclopentanedicarboxylic acid starter unit, and a carbonitrile substituent at C-12 (Figure 1a).[6] Total synthesis and
20 X-ray diffraction analysis have confirmed the overall structure of borrelidin including the (*Z,E*)-configuration of the conjugated cyanodiene in the southern fragment.[7]

These two double bonds are formed during biosynthesis by the consecutive action of PKS extension modules two and three
25 (Figure 1a).[8] In both modules the KR domains bear the characteristic LDD-motif in their active sites, which predicts a 3-D-configuration in the hydroxyacyl DH-precursors.[2a,b] Interpretation of the BorKR3 sequence according to a recently published algorithm furthermore points towards a D-configuration
30 at the branched 2-position of the BorDH3 precursor.[2c] Based on these analyses the configuration of the borrelidin diene is predicted to be *E,E* and not *Z,E*. This conflict between predicted and confirmed structure makes borrelidin an attractive model system to study the role of PKS-DH domains in the formation of
35 rare *Z*-configured double bonds. Either the BorDH3 represents an exception to the proposed rule, or the *Z*-configured double bond must be established at a later stage in the pathway.[4c] One of the most direct and feasible ways to answer this question would be an *in vitro* experiment with isolated enzymes and a realistic substrate
40 surrogate.

In a previous study we demonstrated the catalytic activity of isolated recombinant BorDH2 and BorDH3 on simple 3-hydroxy-2-methyl-SNAC-pentanoate substrate surrogates *in vitro* (SNAC: *N*-acetylcysteamine).[4c] We found that both domains behave
45 similarly in that they selectively convert only the 2-D,3-D precursor **12** and produce only the *E*-configured product **15** (Figure 1b). While this result is consistent with *syn*-dehydration, assuming that the substrate backbone is similarly positioned as in porcine fatty acid synthase-DH, curacin-DHs and erythromycin-
50 DH, it leaves open the question whether the *Z*-configured double bond in borrelidin is actually installed by a biosynthetic process that occurs after polyketide chain assembly or if the outcome of the *in vitro* experiment with BorDH3 was biased by the use of relatively simple model substrates.

55 It is known from *in vitro* experiments with other isolated PKS domains that the substrate structure and the nature of the thioester exert a crucial influence on the stereochemical course of the reaction.[4d,9] For RifDH10, it was observed that the stereospecificity of the dehydration reaction changes if the
60 hydroxyacyl substrate is attached either to an ACP other than the natural one or to truncated surrogates of the ACP-bound form like SNAC or pantetheine. In the latter cases, the preference switches completely from the native (*2S,3S*) precursor to the enantiomeric (*2R,3R*). Nevertheless, in agreement with a *syn*-dehydration the
65 *E*-selectivity of the domain was not altered by these changes. We now report that further analysis of the BorDH2 and BorDH3 domains with a more realistic substrate has substantiated our previous results, allowed us to study the influence of the substrate structure and better to understand the biosynthetic origin of the
70 (*Z,E*)-diene.

Results and discussion
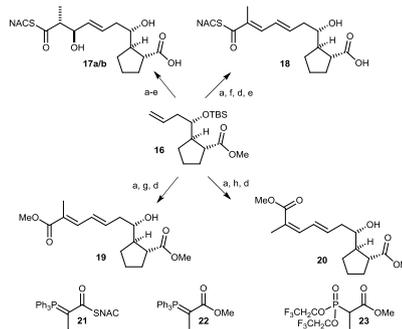
Scheme 2 Synthesis of precursors and reference molecules. a)
75 Crotonaldehyde, Second Generation Grubbs catalyst, CH$_2$Cl$_2$, 40 °C, 2 h; b) PhSCOC$_2$H$_5$, Cy$_2$BCl, NMe$_2$Et, Et$_2$O then aldehyde in Et$_2$O, -78 °C to -30 °C, 16 h, 57% over two steps; c) HSNAC, Et$_3$N, DMF, r.t., 16 h, 70%; d) THF:H$_2$O:FA (6:3:1), r.t., 2 d; e) pig liver esterase, phosphate buffer, r.t., 2-5 d; f) **21**, CH$_2$Cl$_2$, 50 °C, 16 h, 88% over two steps; g) **22**, CH$_2$Cl$_2$,
80 50 °C, 21 h, 64 % over two steps; h) **23**, 18-crown-6, K$_2$CO$_3$, THF, -20 °C to 0 °C, 5 h, 47 % over two steps. Overall yields: 32% over five steps for **17a/b**, 59% over four steps for **18**, 40% over three steps for **19**, 28% over three steps for **20**. Cy: cyclohexyl, FA: formic acid.[10]

We synthesised the tetraketide precursors **17a** and **17b** as a
85 mixture of both 2,3-*anti*-stereoisomers in the form of their SNAC-thioesters, as well as reference molecules **18**, **19** and **20** (Scheme 2).[10] All synthetic routes diverged from a common, α,β-

unsaturated precursor aldehyde, which was obtained by metathesis reaction of olefin **16** with crotonaldehyde and Second Generation Grubbs catalyst. The DH substrates **17a** and **17b** were obtained by an *anti*-selective boron aldol reaction, followed by thiol exchange and ensuing acidic deprotection of the TBS ether and esterase-catalysed cleavage of the methyl ester. The SNAC thioester of the *E,E*-configured product **18** was obtained by *Wittig reaction* with phosphorane **21** and deprotection following the protocol established for **17a** and **17b**. The isomeric dienes **19** and **20** in the form of their *bis*-methyl esters were accessed *via Wittig reaction* with stabilised phosphorane **22** and *Still-Gennari olefination* with phosphonate **23**, respectively, followed by TBS deprotection.
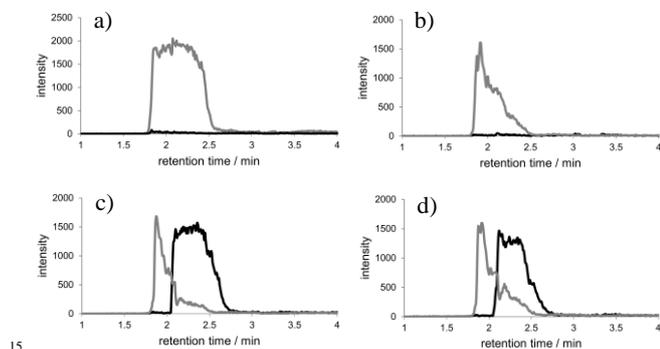

Fig. 2 UPLC-MS analysis of DH assays. The grey line represents the hydrated starting material (**17a/b**: $[M+Na]^+ = 410$), the black line corresponds to the dehydration product (**18**: $[M+Na]^+ = 392$). The starting material is stable towards dehydration in an enzyme-free overnight incubation as well as towards reaction with a deactivated active-site mutant (a) and b)). It shows dehydration only after incubation with the wild-type BorDH domains (c) and d)). a) Enzyme-free overnight incubation of **17a/b**; b) overnight incubation of **17a/b** with BorDH3-H49A; c) overnight incubation of **17a/b** with BorDH2; d) overnight incubation of **17a/b** with BorDH3.

The mixture of the two *anti*-2-methyl-3-hydroxy thioesters **17a** and **17b** was incubated with recombinant BorDH2 and BorDH3, respectively. After 16 h, the reaction was terminated by extraction of the organic materials with ethyl acetate. Direct analysis of this crude assay extract showed conversion of the starting material into a dehydrated compound (Figure 2c/d). Enzyme-free control reactions, which were treated under identical conditions, reproducibly showed no conversion of **17a** and **17b** into the dehydrated product (Figure 2a). To rule out the possibility of catalysis by a contaminant that was purified along with the DH, the experiment was repeated with a purified mutant of BorDH3 in which the active site histidine had been exchanged

to an alanine (Figure 2b). No conversion was obtained in this case, confirming that the recombinant borrelidin DHs were responsible for the observed dehydration.

In order to establish the configuration of the newly formed double bond by comparison to the reference molecules **19** and **20**, we derivatized the crude assay product mixture. Saponification with 1 M NaOH followed by methylation with trimethylsilyl diazomethane gave *bis*-methyl esters, which were analysed by UPLC-MS (Figure 3a, Figure S3 and Figure S4). Comparison to the synthetic reference molecules established the configuration of the products to be *E,E*. A similar result was obtained when BorDH2 was employed instead of BorDH3, showing that this domain also retains activity and its natural stereoselectivity when presented with a substrate of larger size than the natural one.

To exclude the possibility of double bond isomerization during derivatization, we repeated the experiment with BorDH3 on a larger scale and analysed the product by ^{1}H NMR spectroscopy. 10 mg of the mix of **17a** and **17b** were incubated with BorDH3 for 16 h at 37 °C. After extraction into organic solvent, the crude material was directly analyzed by ^{1}H NMR. The resulting ^{1}H NMR spectrum was in full agreement with a partial conversion of **17a/b** into **18**, as judged by comparison to a synthetic sample of **18** (Figure 3b, Figure S5).

Additional NOE experiments were conducted to confirm the (2*E*,4*E*)-configuration of the BorDH3 assay product (Figure 3c). While irradiation at 7.23 ppm (H3) showed a correlation to H5, but not to 2-CH$_3$, irradiation at 6.57 ppm (H4) exclusively showed correlation to 2-CH$_3$. This is in full agreement to a *syn* relationship of the 2-CH$_3$ group and the vinyl fragment along the C2-C3 olefin and therefore a (2*E*,4*E*)-configuration of the diene.

From these results, we conclude that BorDH2 and more importantly BorDH3 each selectively transform the tetraketide precursor into an (2*E*,4*E*)-diene. Accordingly, the isomerization of the double bond must occur at a later stage in biosynthesis.

This result prompted us to examine in detail the configuration of the C12-C13 double bond in 12-desnitrile-12-methylborrelidin (**9a/9b**). This metabolite (pre-borrelidin) was originally isolated as the product of the mutant strain Δ*borI*, which is devoid of BorI.[8b] BorI catalyses the oxidation of the allylic position C27 in one of the potential precursors **9a** or **9b** to activate it for transamination and further oxidation/dehydration to the carbonitrile by BorJ and (probably) BorK (Figure 1a). Previous biotransformation experiments confirmed that pre-borrelidin is indeed a competent intermediate of the pathway and not a shunt product.[8b]
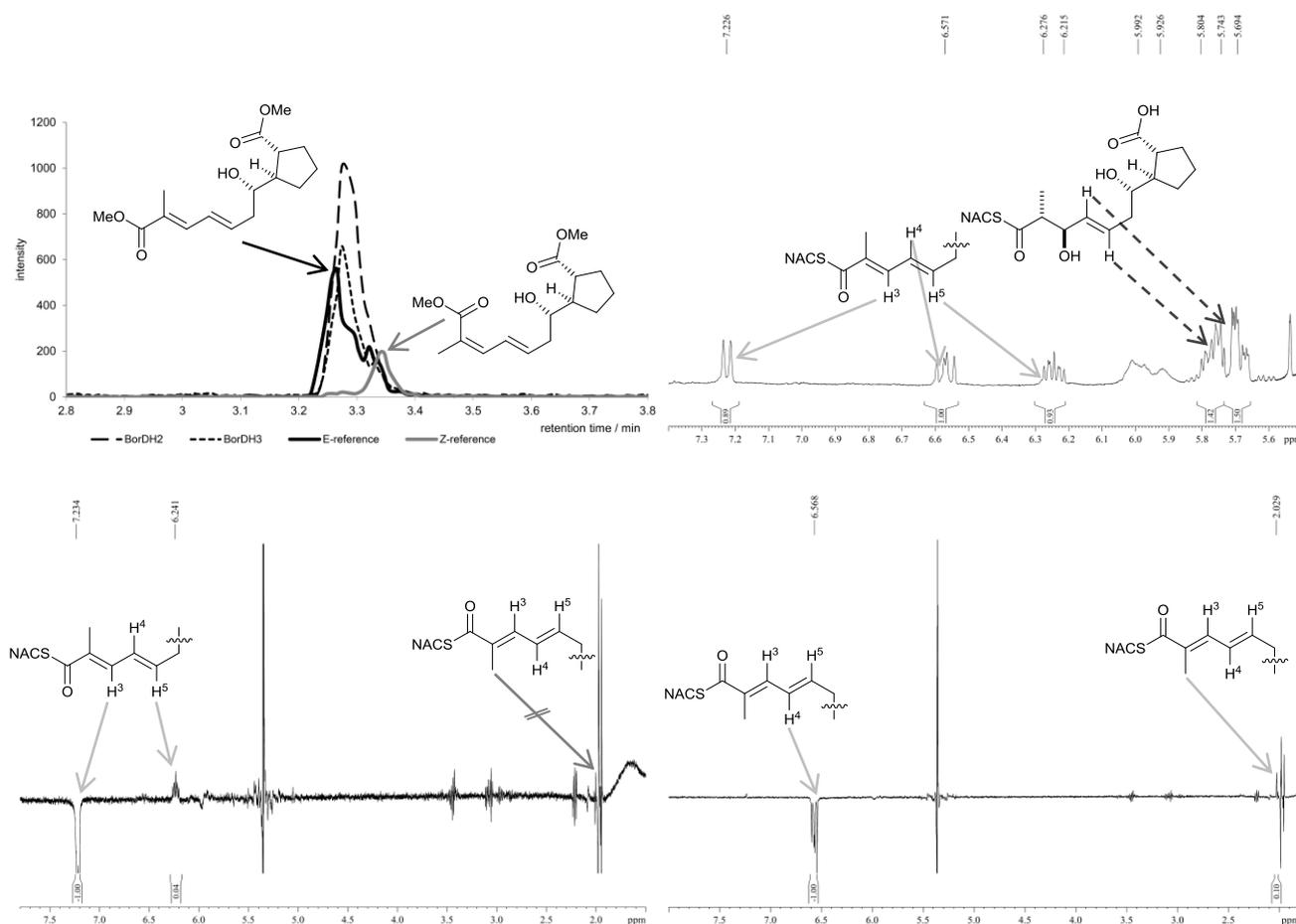
Fig. 3 a) UPLC-MS analysis of derivatized assay products and comparison to reference molecules; grey line: (*Z*)-isomer, reference *bis*-methylester **20**;
black line: (*E*)-isomer, reference *bis*-methylester **19**; dashed line: derivatized dehydration product of BorDH2; dotted line: derivatized dehydration product of BorDH3; b) expansion of the olefinic region in the ^{1}H NMR spectrum of the BorDH3 assay product in d_2-dichloromethane; c) NOE analysis of the BorDH3 assay product. Upon irradiation at 7.23 ppm (H3), correlation to H5 (6.24 ppm), but not to 2-CH$_3$ (2.03 ppm) was observed; d) NOE analysis of the BorDH3 assay product. Irradiation at 6.57 ppm (H4) exclusively showed correlation to 2-CH$_3$.

Initial NMR analysis using ^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC experiments had shown that pre-borrelidin constitutionally differs from borrelidin only in bearing a methyl group instead of a carbonitrile at C27.[8b] We have confirmed these findings and have now carried out additional NOESY and NOE experiments (see Supplementary information, Figure S7 and Figure S8) which firmly establish that the C12-C13 double bond has exclusively the *E*-configuration. Therefore, pre-borrelidin has the (12*E*,14*E*)-configuration shown for **9a** (Figure 1a and Figure 4) and the isomerization to the (12*Z*,14*E*)-configuration found in borrelidin **10** must occur at a later stage of the biosynthesis.
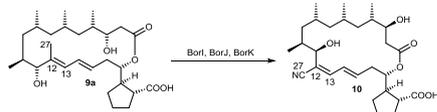

Fig. 4 Proposed final steps of borrelidin biosynthesis. The formation of the carbonitrile is proposed to occur *via* oxidation of pre-borrelidin **9a** at C-27 to an allylic aldehyde (catalysed by BorI), transamination (BorJ) and further oxidation (possibly BorK).[8b]

To exclude the possibility of isomerization having occurred during workup, the fermentation of *ΔborI* was monitored by HPLC-MS, which showed similar retention times for the product formed during fermentation and after final isolation. This result is in accordance with the conclusion from our *in vitro* experiments, that the diene is first formed exclusively in an *E*,*E*-fashion.

Conclusions

This study is the first example in which a potentially *Z*-selective PKS-DH domain was tested *in vitro* with a realistic substrate mimic that resembles the full polyketide portion of the biosynthetic intermediate. Although some *Z*-configured double bonds in reduced polyketides clearly are formed by *syn* dehydration of 3-L alcohols, our results reinforce the view that a subsequent isomerization or other biosynthetic process is likely to be responsible for their formation in many cases.[4e,11]

Our experiments also provide further valuable insight into enzymology of PKS domains. Together with previous reports, our assay results of BorDH2 and BorDH3 with such different substrate surrogates like **11-14**, **17a** and **17b** suggest that PKS-DH domains generally catalyse dehydrations with relaxed

substrate specificity, but with high stereoselectivity with respect to the configuration of the double bond between C-2 and C-3.[4a-d] This result is encouraging for their application in chemoenzymatic synthesis.

5 We can now assign the timing of the *E-Z*-isomerization to the very final steps of borrelidin biosynthesis. Additional *in vitro* experiments with isolated enzymes will be necessary to elucidate the specific mechanism of isomerization and nitrile formation in the terminal steps of the borrelidin pathway.

Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, characterizytion data and spectra of synthesized surrogate molecules. Procedures for enzyme assays and characterization data of 35 assay products and pre-borrelidin. See DOI: 10.1039/b000000x/

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