Biosynthesis of the angiogenesis inhibitor borrelidin: directed biosynthesis of novel analogues†

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We report the directed biosynthesis of borrelidin analogues and their selective anti-proliferative activity against human cancer cell lines.

The polyketide macrolide borrelidin **1** is a potent inhibitor of angiogenesis. This activity was discovered using an *in vitro* rat thoracic aorta tube formation assay where **1** was found to inhibit capillary tube formation and to disrupt newly-formed capillary tubes, both in a dose-dependent manner.[1] Borrelidin has been shown previously to inhibit threonyl-tRNA synthetase[2] but the IC_{50} value for inhibition of capillary formation was 0.8 nM, 50-fold lower than that for the inhibition of protein synthesis.[1] It also inhibits angiogenesis *in vivo*, and is effective in the inhibition of spontaneous lung metastases of B16-BL6 melanoma cells at the same dosage that inhibits angiogenesis.[3]

As part of our anti-cancer drug discovery programmes **1** was selected as a lead molecule. We previously cloned and sequenced the gene cluster from *Streptomyces parvulus* Tü4055 that governs the biosynthesis of **1**.[4] This allowed us to propose a biosynthetic pathway in which a modular polyketide synthase (PKS) generates the macrolactone structure using a molecule of cyclopentane-*trans*-1,2-dicarboxylic acid **2** as a starter unit to initiate polyketide chain synthesis (see Figs. 1 and 2 for structures). The only post-PKS modification involves the unusual conversion of the methyl group at C12 of the first PKS-free intermediate (pre-borrelidin, **3**) into a nitrile moiety.[5] The absolute configuration of **2** as the 1*R*,2*R* isomer follows from the known absolute structure of **1**.[6] We now report the rational biosynthetic engineering of the *bor* gene cluster of *S. parvulus* Tü4055 in order to produce novel analogues of **1**.

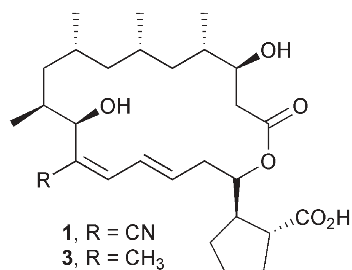

Fig. 1 Chemical structures of borrelidin **1** and pre-borrelidin **3**.

In previous work we identified *S. parvulus* Tü4055 mutants which did not produce **1**, or any analogues of **1**, unless **2** was added (Fig. 2).[4] These mutants therefore allow analogues of **2** to be tested as starter units in order to produce novel borrelidin analogues by mutasynthesis. This approach has previously been used with success to produce analogues of several therapeutically important polyketides[7] including the mTOR inhibitor rapamycin[8] and the antihelmintic avermectin.[9] For the experiments described here we utilised a *S. parvulus* mutant disrupted in the gene *borG* (BIOT-1302).[4] Mutants deleted in other *bor* genes could also be restored to borrelidin production by added **2**, however when supplemented with **2** BIOT-1302 unexpectedly produced **1** at levels between 15- and 20-fold higher than the parent strain under identical conditions, and around 5-fold higher titre than the parent strain supplemented with equivalent levels of **2**. Although we cannot yet assign a definite function to the *borG* product BorG, we speculate it may be involved in starter unit biosynthesis or in the regulation of borrelidin biosynthesis.

More than 40 mono- and dicarboxylic acids were fed to BIOT-1302 cultures (see supplementary information for full details). All of the mono- and most of the dicarboxylic acids examined failed to initiate the biosynthesis of novel analogues of **1**. Indeed, of the several cyclic dicarboxylic acids examined, only cyclobutane-*trans*-1,2-dicarboxylic acid produced a novel compound when fed to BIOT-1302; this was produced at ~ 50% of the titre of **1** obtained when **2** was fed. Fermentation was then scaled up and after purification the new compound was shown to have the expected

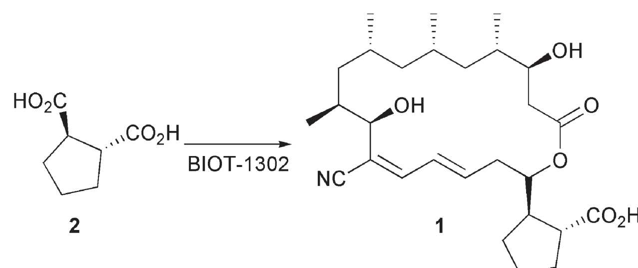

Fig. 2 Precursor directed biosynthesis of **1** from **2** using *S. parvulus* mutant BIOT-1302.

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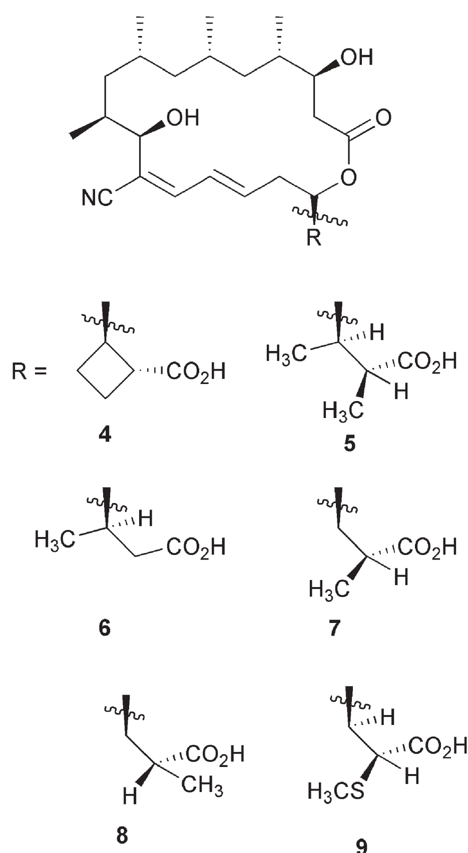

Fig. 3 Novel borrelidin analogues generated by precursor directed biosynthesis.

structure, **4** (Fig. 3; see supplementary information for NMR and MS details). The *cis*-isomer (*meso* form) of cyclobutane-1,2-dicarboxylic acid failed to act as a PKS starter unit, as did the *cis*-cyclohexane and *trans*-cyclohexane analogues, the *cis*-cyclopentane and cyclopent-1-ene-1,2-dicarboxylic acids, and the *trans*-cyclopropane analogue. Assuming that all of these analogues are converted by a CoA ligase to activated CoA monoesters inside the cell, these results suggest that the PKS loading module recognises a rather specific geometrical arrangement of the adjacent carboxylate groups. It is consistent with this idea that, although succinic acid itself was not a substrate, the 2-methyl- and 2,3-dimethylsuccinic acids were accepted and novel compounds were produced. 2,3-Dimethylsuccinic acid is commercially available either in the *meso* form, or as a mixture of racemic and *meso* forms. When fed the mixture BIOT-1302 produced only a (single) novel compound. This was expected based on the stereochemistry of **2**. The new compound was produced at $\sim$15% of the titre of **1** obtained when **2** was fed. The compound was isolated from a larger-scale fermentation and its structure was confirmed as **5**. When fed the *meso* form only no products were produced. When racemic 2-methylsuccinic acid was fed three new compounds were produced, with the major compound **6** produced at approximately half of the titre for **5** above. After scale-up of fermentation and isolation, the three compounds were identified as **6–8** respectively. The major product **6** has a methyl branch adjacent to the carboxylic acid moiety utilised for polyketide biosynthesis. No other α-methyl regioisomer was obtained and the absolute structure of **6** is based on that of **1** and on the results of further feeding experiments described below. **7** and **8** derive from the incorporation of 2-methylsuccinic acid with the alternative regiochemistry, *i.e.* where incorporation by the PKS gives a β-methyl substitution relative to the carboxylic acid moiety used for polyketide chain extension. Apparently, both isomers of the fed substrate are regiospecifically incorporated. The stereochemistry at the β-methyl for each of these was assigned based on the experiments described below.

To verify that **6** was indeed derived from the (2*R*)-isomer of 2-methylsuccinic acid, the (2*R*)- and (2*S*)-2-methylsuccinic acids were fed separately to BIOT-1302. As expected, feeding the (2*R*)-isomer produced a major compound **6** and a minor compound **7**, but no **8**. When the (2*S*)-isomer was fed only **8** was produced.

In addition to feeding analogues of the PKS starter unit as their free acids, we examined the effect of adding substituted succinates as their anhydrides (readily available and cheap) to the fermentation. In fact, similar titres were obtained as for the parent acids. We also fed itaconic anhydride, which gave the same product profile as (2*R*)-2-methylsuccinic acid, demonstrating that itaconic anhydride is reduced to (2*R*)-2-methylsuccinic anhydride, or its free acid, prior to incorporation by the loading module of the PKS. We then fed racemic 2-(mercaptoacetyl)succinic acid anhydride to cultures of BIOT-1302 and observed the production of a new compound, albeit at significantly lower titre than in our other experiments. Larger-scale fermentation and isolation allowed its identity to be established as the 2-thiomethyl substituted compound **9**. It appears that *in vivo* 2-(mercaptoacetyl)succinic acid anhydride or a macrocyclic precursor of **9** undergoes an *S*-deacetylation followed by *S*-methylation; we predict that the timing of this will prove to be prior to utilisation by the PKS.

We examined the cytotoxicity of these novel borrelidin analogues against a number of cancer cell lines *in vitro* (MCF-7, MDA-MB-231, MDA-MB-468, NCI-H460, SF-268, OVCAR-3, A498, GXF 251L, MEXF 394NL, UXF 1138L, LNCAP and DU145;[10] see supplementary information for experimental details). None of **4–9** were as potent as **1** in inhibiting the mean proliferation of these cancer cell lines as judged by mean growth inhibition of treated cells *versus* untreated (%T/C) (Table 1). However, the compounds showed interesting selectivity against certain cell lines. For example, **4** and **6** are equipotent to **1** against mammary MCF-7 and uterus 1138L although both are less active than **1** against other cell lines. Further analysis of these and anti-angiogenic evaluations are ongoing.

Table 1 Results of growth inhibition assays with borrelidin analogues (dosed at 1 and 10 μM) against twelve selected cancer cell lines. Full details of the cell lines assayed and experimental detail are provided in the supplementary information. Mean %T/C, mean percentage of viable cells in the treated group relative to the control group; *n*, the number of times the monolayer assay was carried out

Compound	Mean %T/C		
	at 1 μM	at 10 μM	*n*
Borrelidin, **1**	7	6	2
4	33	8	2
5	21	7	3
6	73	22	3
7	83	28	3
8	96	74	3
9	94	62	3

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We have demonstrated that structural changes can be readily introduced into the portion of borrelidin derived from the starter unit by using mutasynthesis and, notably, that a mutant strain (BIOT-1302) of *S. parvulus* Tü4055 which carries a disrupted *borG* gene can be used to generate greatly improved titres of both **1** and these novel compounds.

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