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Direct production of ivermectin-like drugs after domain exchange in the avermectin polyketide synthase of *Streptomyces avermitilis* ATCC31272

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Ivermectin™, a mixture of 22,23-dihydroavermectin B1a **9** with minor amounts of 22,23-dihydroavermectin B1b **10**, is one of the most successful veterinary antiparasitic drugs ever produced. In humans, ivermectin has been used for the treatment of African river blindness (onchocerciasis) resulting in an encouraging decrease in the prevalence of skin and eye diseases linked to this infection. The components of ivermectin are currently synthesized by chemical hydrogenation of a specific double bond at C22–C23 in the polyketide macrolides avermectins B1a **5** and B1b **6**, broad-spectrum antiparasitic agents isolated from the soil bacterium *Streptomyces avermitilis*. We describe here the production of such compounds (22,23-dihydroavermectins B1a **9** and A1a **11**) by direct fermentation of a recombinant strain of *S. avermitilis* containing an appropriately-engineered polyketide synthase (PKS). This suggests the feasibility of a direct biological route to this valuable drug.

Introduction

Macrolides are a large and structurally diverse class of natural products which includes many compounds possessing antibiotic or pharmacological properties, such as erythromycin, rapamycin and avermectin. Ivermectin (a mixture of 22,23-dihydroavermectin B1a **9** and 22,23-dihydroavermectin B1b **10** in a molar ratio of at least 80 : 20) is the drug of first choice for the treatment of lymphatic filariasis and river blindness in humans caused by the nematode *Onchocerca volvulus*, and it has enjoyed widespread use as an anthelmintic for livestock and companion animals, and as an agricultural insecticide since 1981.[1–3] The primary target of its antiparasitic action is thought to be a glutamate-gated chloride ion channel.[4] Ivermectin is currently produced by chemical reduction of avermectins, which are biosynthesised by *Streptomyces avermitilis* and consist of a series of pentacyclic polyketides linked to a disaccharide of L-oleandrose.[1] The eight major components in naturally-produced avermectin mixtures result from structural variations at three positions: C5, C22–C23, and C26 (**1**–**8**, Fig. 1). The biosynthesis of avermectin involves the extension of a starter unit, either 2-methylbutyrate (a-components, Fig. 1) or isobutyrate (b-components, Fig. 1), with seven acetate and five propionate derived units. These 12 successive cycles of elongation form the aglycone 6,8a-*seco*-6,8a-deoxy-5-oxoavermectin,[5] (Fig. 2) which is subsequently modified to generate avermectins by oxidative cyclisation, reduction, and optional C5-*O*-methylation (giving either A- or B-components) followed by glycosylation at C13 involving addition of two L-oleandrosyl residues (Fig. 3).[1]

The biosynthetic gene cluster responsible for the production of avermectin has been previously cloned, sequenced and the gene organisation analysed independently by several research groups,[2,6–8] and recently the entire DNA sequence of this 82 kb region has been published.[7] Analysis of the domain organization of the avermectin polyketide synthase (AVES) has revealed the presence of four large polypeptides AVES 1 to 4 containing a total of 12 extension modules (Fig. 2). These 12 modules correspond to the 12 extension cycles required for the synthesis of the 6,8a-*seco*-6,8a-deoxy-5-oxoavermectin aglycone.[2,8] In general, the complement of enzymatic activities within each module is exactly that required to specify the expected degree of reduction of the newly-introduced β-keto group. For complete reduction to methylene[9] these activities comprise: a ketoreductase (KR) domain catalyzing the reduction of the 3-keto group to a hydroxyl group; a dehydratase domain (DH) catalyzing production of an α,β-unsaturated acylthioester; and an enoylreductase (ER) domain for subsequent reduction of this double bond. Enoylreductase domains are not found in the avermectin PKS, which is consistent with the lack of any saturated β-carbon chain in the avermectin aglycone.[3]

Extension module 2, which governs the oxidation-state at the C22–C23 positions of avermectin, is housed within AVES1 and is inferred by sequence comparisons to contain a functional ketoreductase (KR) domain and a possibly inactive dehydratase (DH) domain.[2,6–8,10] Natural avermectin mixtures contain both C22–C23 olefinic compounds ("1" components) and C23-hydroxy compounds ("2" components).[3] The product of the

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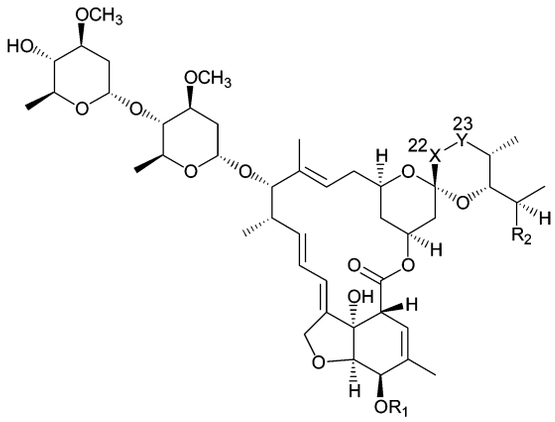

Compound	Avermectin	R_1	R_2	X-Y
1	A1a	CH_3	CH_2CH_3	CH=CH
2	A1b	CH_3	CH_3	CH=CH
3	A2a	CH_3	CH_2CH_3	CH_2-CHOH
4	A2b	CH_3	CH_3	CH_2-CHOH
5	B1a	H	CH_2CH_3	CH=CH
6	B1b	H	CH_3	CH=CH
7	B2a	H	CH_2CH_3	CH_2-CHOH
8	B2b	H	CH_3	CH_2-CHOH
	Ivermectin			
9	B1a	H	CH_2CH_3	CH_2-CH_2
10	B1b	H	CH_3	CH_2-CH_2
11	A1a	CH_3	CH_2CH_3	CH_2-CH_2
12	A1b	CH_3	CH_3	CH_2-CH_2

Fig. 1 Structures of avermectins and 22,23-dihydroavermectins.

aveC gene is apparently required for production of "1" components,[8,11,12] and although the molecular basis for this is not as yet understood, mutations in *aveC* can be used to alter the ratio of 1 : 2 components in the product mixture.[10,12] Other genetic manipulations of the avermectin fermentation have also been used successfully to reduce the complexity of the product mixture in favour of the more bioactive components, and to generate novel analogues with enhanced spectra of biological activity.[13,14]

In the case of the semisynthetic ivermectin derivatives, an important bottleneck still remains: the selective chemical hydrogenation of the double bond at C22–C23 of avermectins B1a and B1b (Fig. 1). Here we present an alternative route to these clinically important compounds by engineering the avermectin PKS. By substituting the DH and KR domains of module 2 of the avermectin PKS with a complete set of β-keto processing enzymes, consisting of the DH, ER and KR domains of module 13 from the rapamycin-producing PKS of *Streptomyces hygroscopicus*,[15] we aimed to promote *in situ* biological reduction of the growing polyketide chain, which would in turn open the way to a direct low-cost fermentation route to 22,23-dihydroavermectins. Such alterations in extent of reduction have been successfully engineered in several other systems (see, for example, ref. 16) but the potential involvement of the *aveC* gene product in direct binding and dehydration at module 2 of the avermectin PKS poses an additional problem. If the integrity of the PKS requires a specific association of AveC with module 2 domains, and the loop swap were to prevent this, the PKS would be inactive; conversely, if AveC were to compete with the swapped domains for PKS-bound intermediates, they would be diverted to the "normal" avermectin products.

Results

To introduce the reductive loop[9] of module 13 from the rapamycin biosynthetic gene cluster into *S. avermitilis*, plasmid pPF137 was isolated and used to transform *S. avermitilis*. The resulting thiostrepton-resistant integrant *S. avermitilis* 1/137/11 (BIOT-1917) was isolated. To verify the location of the chromosomal integration site, Southern blot and PCR analysis were performed; details are described in the Experimental section. BIOT-1917 was then sub-cultured four times in the absence of thiostrepton, followed by protoplasting using standard protocols.[17] The cells were grown on agar plates and colonies were tested for thiostrepton sensitivity. The thiostrepton-sensitive *S. avermitilis* mutant strain SUB388/79 (BIOT-1916) was isolated and the location of the inserted fragment was re-confirmed using Southern blot and PCR analysis methods as described for BIOT-1917.

Analysis of macrocyclic polyketides in cultures of BIOT-1916

Cultures of *S. avermitilis* wild type, BIOT-1917 and BIOT-1916 were extracted and analysis of the extracts by HPLC/UV revealed the presence of at least two new compounds for BIOT-1916 when compared to the pattern of products shown by the *S. avermitilis* wild type (Fig. 4). One of these new products was shown to co-elute with authentic 22,23-dihydroavermectin B1a **9**. No major avermectin or 22,23-dihydroavermectin-related peaks were found in culture extracts of the control strain BIOT-1917. The estimated total amount of avermectins and 22,23-dihydroavermectins produced in BIOT-1916 was decreased approximately 8-fold compared to the amount of avermectins produced in the wild type control. The pattern of avermectins produced by the wild type and by BIOT-1916 was identical with the exception of the new 22,23-dihydroavermectin derivatives that are seen only in BIOT-1916. The extracts of the *S. avermitilis* wild type and BIOT-1916 cultures were also analysed by LCMS. Peaks consistent with the presence of the avermectins A1a **1**, A1b **2**, B1a **5** and A2a **3** were detected in both the wild type samples and also in extracts of BIOT-1916. However, three novel compounds were also present in culture extracts of BIOT-1916 and the masses detected by LCMS corresponded to those expected for 22,23-dihydroavermectins A1a **11**, A1b **12** and B1a **9** respectively. A 4 l fermentation of BIOT-1916 was performed, and the putative 22,23-dihydroavermectins B1a **9** and A1a **11** were purified and analysed by NMR and by high-resolution FT-ICR-MS as described in the Experimental section. These data confirmed that the more polar compound was indeed 22,23-dihydroavermectin B1a **9**. Its molecular mass was measured as 897.4998, within 2.4 ppm of the mass expected for $C_{48}H_{74}O_{14}Na$ (MNa^+). NMR assignments for the less polar compound were likewise fully consistent with 22,23-dihydroavermectin A1a **11**, and its molecular mass was measured as 911.5120, within 1.4 ppm of the mass expected for $C_{49}H_{78}O_{14}Na$ (MNa^+).

Discussion

Swapping of domains between different natural modular polyketide synthases is an emerging technology for the targeted structural alteration of polyketide antibiotics.[9,18,19] In particular, the acyltransferase (AT) domains which govern chemical selection of extender units, and the KR, ER and DH "reductive loop"domains,[9,19,20] which together govern the final oxidation state of each incorporated unit, have been successfully manipulated and the hybrid multienzymes have been shown to produce the anticipated modified compounds, including novel erythronolides and erythromycins.[16,21,22] However, these experiments have also indicated that the catalytic efficiency of such hybrid PKSs may be highly variable, with some of the desired compounds obtained in negligible amounts.[16,22,23] Several factors may potentially contribute to this loss of efficiency: poor expression and/or folding of the hybrid PKS; unfavourable protein–protein interactions reducing the rate of chain transfer between active sites; the inability of one or more individual enzymatic activities to accept the altered substrate; inefficiency of precursor supply; or the rapid removal of stalled intermedi-

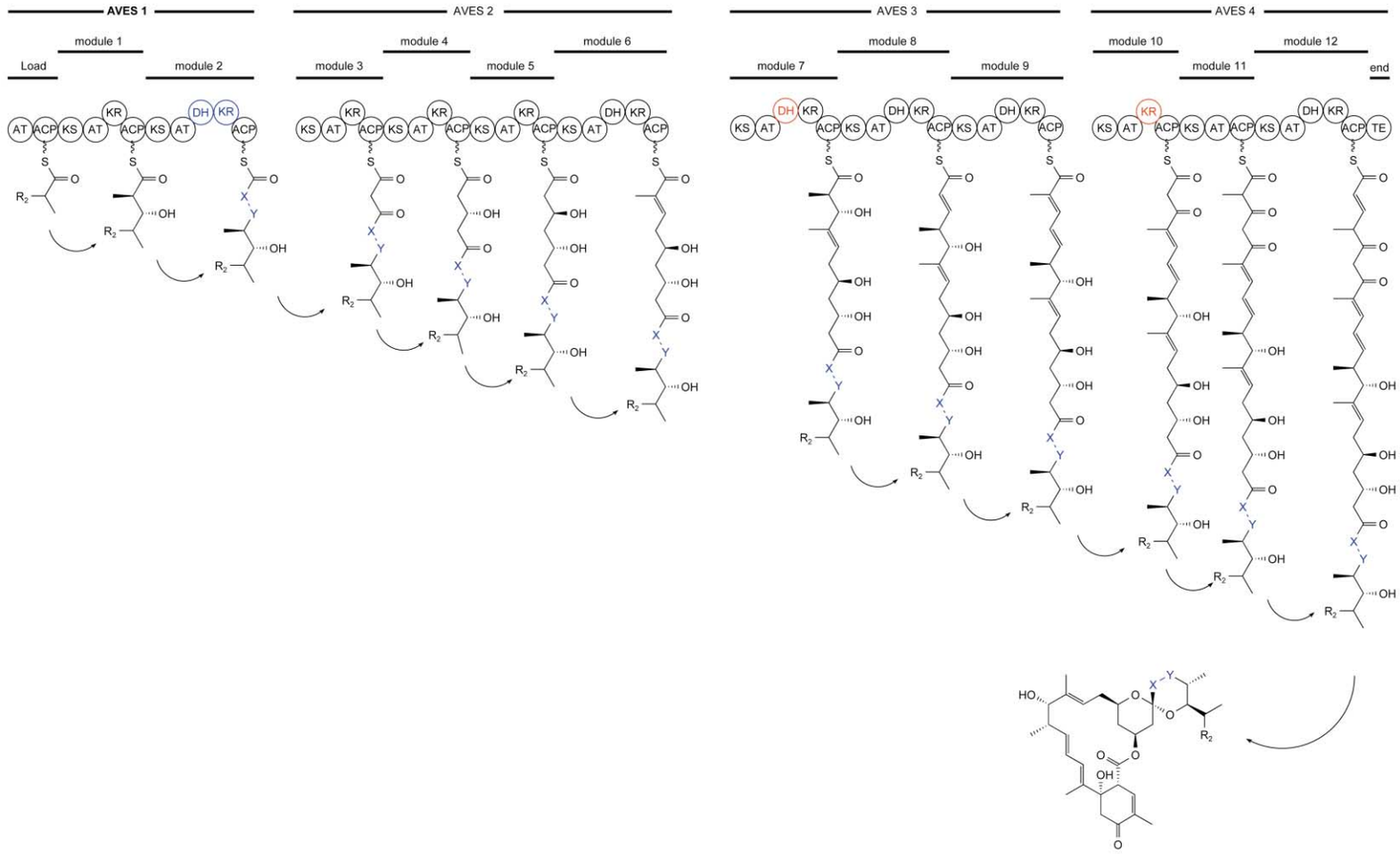

Fig. 2 Organisation of the avermectin PKS and biosynthesis of the avermectin aglycone. Each circle represents an enzyme domain in the PKS multifunctional protein; AT, acyl transferase; KS, β-ketosynthase; DH, dehydratase; KR, β-ketoreductase; ACP, acyl carrier protein; TE, thioesterase. The domains labelled in red are non-functional. The domains and areas of the molecule labelled in blue are those of module 2 and are the topic of this article.

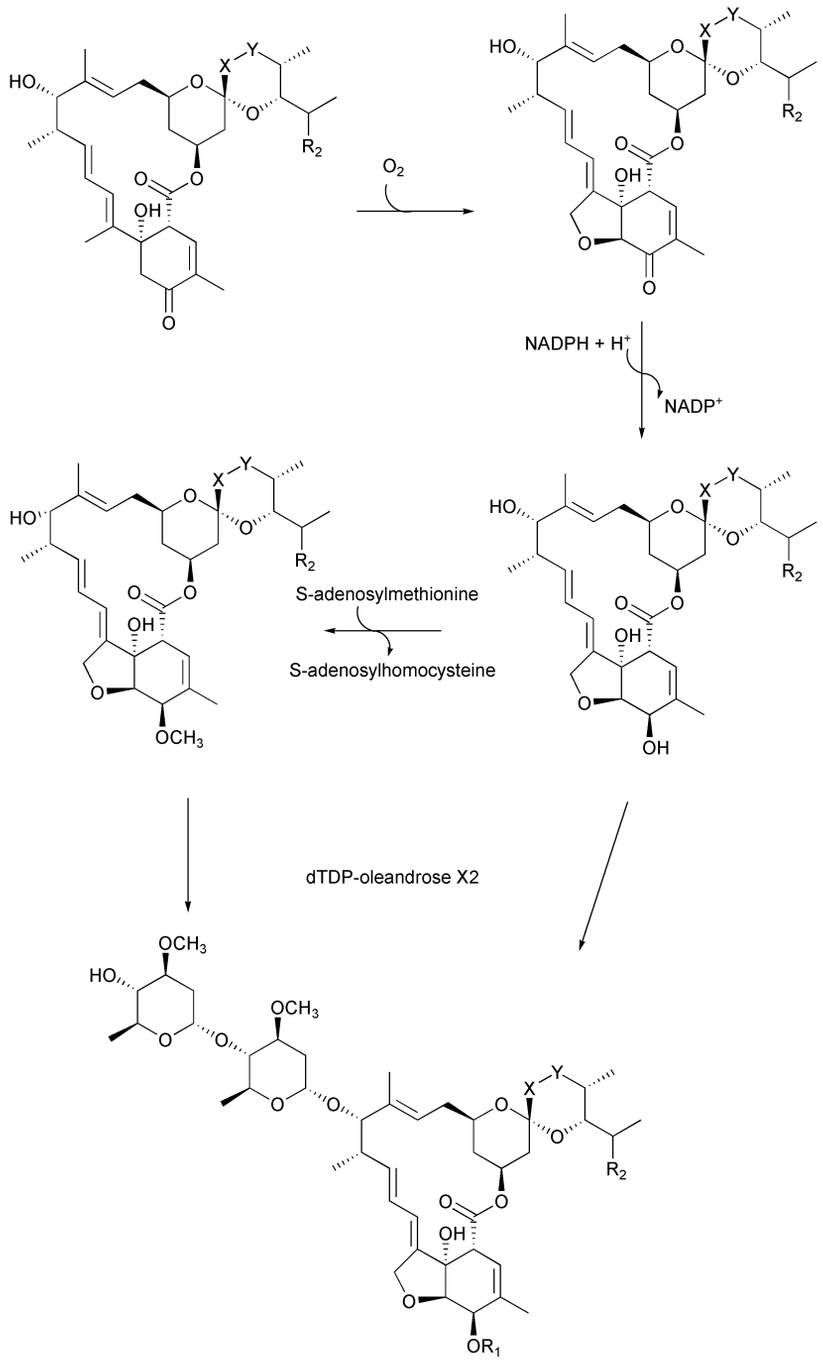

Fig. 3 Biosynthetic sequence from the avermectin aglycone to the final family of avermectin molecules.

ates from the PKS by "proof-reading" thioesterases.[9] While the relative importance of these factors remains unknown, it has repeatedly been demonstrated that in successful hybrids the splice sites for the incoming domain(s) have been chosen to lie either close to those domain boundaries where limited proteolysis experiments have clearly shown the presence of solvent-accessible linker regions,[20,24–26] or in the linker regions themselves. Where disappointing results have been obtained, usually only a single set of splice sites and a single donor domain have been employed.[16,22,23]

In the work presented here, we sought to utilise the domain swap technology to produce a modified avermectin PKS, by exchanging the entire β-processing "reductive loop"[9,18] of module 2 with one derived from module 13 of the rapamycin-producing PKS, providing for complete reduction at C22–C23, and thus creating a potential route for the direct fermentation of 22,23-dihydroavermectins in *S. avermitilis*. The potential complicating factor in this experiment is that the double bond found in C22–C23 olefinic avermectins has a *cis* configuration, very rarely found in polyketide products, and it is not known how this configuration is established. Whereas the KR domain in module 2 carries the conserved active site residues observed in active KR domains of other type I polyketide synthases, the DH domain contains a change in the active site motif.[6,8,10] When the motif was altered by site-specific mutagenesis to match the consensus sequence of active DH domains, the resulting recombinant strain still produced compounds containing both C23-hydroxyl and olefinic functions at C22–C23, with no change in the 2 : 1 ratios, suggesting that module 2 has a limited role in accomplishing dehydration.[8,10,13]

AveC, the product of an additional open reading frame of the avermectin biosynthetic cluster, apparently plays a decisive role in the formation of the functional group at C22–C23. Thus, an *S. avermitilis aveC* mutant has been described which produces only avermectins bearing the C23 hydroxyl, but none of the olefinic compounds,[1] whilst several defined point mutations

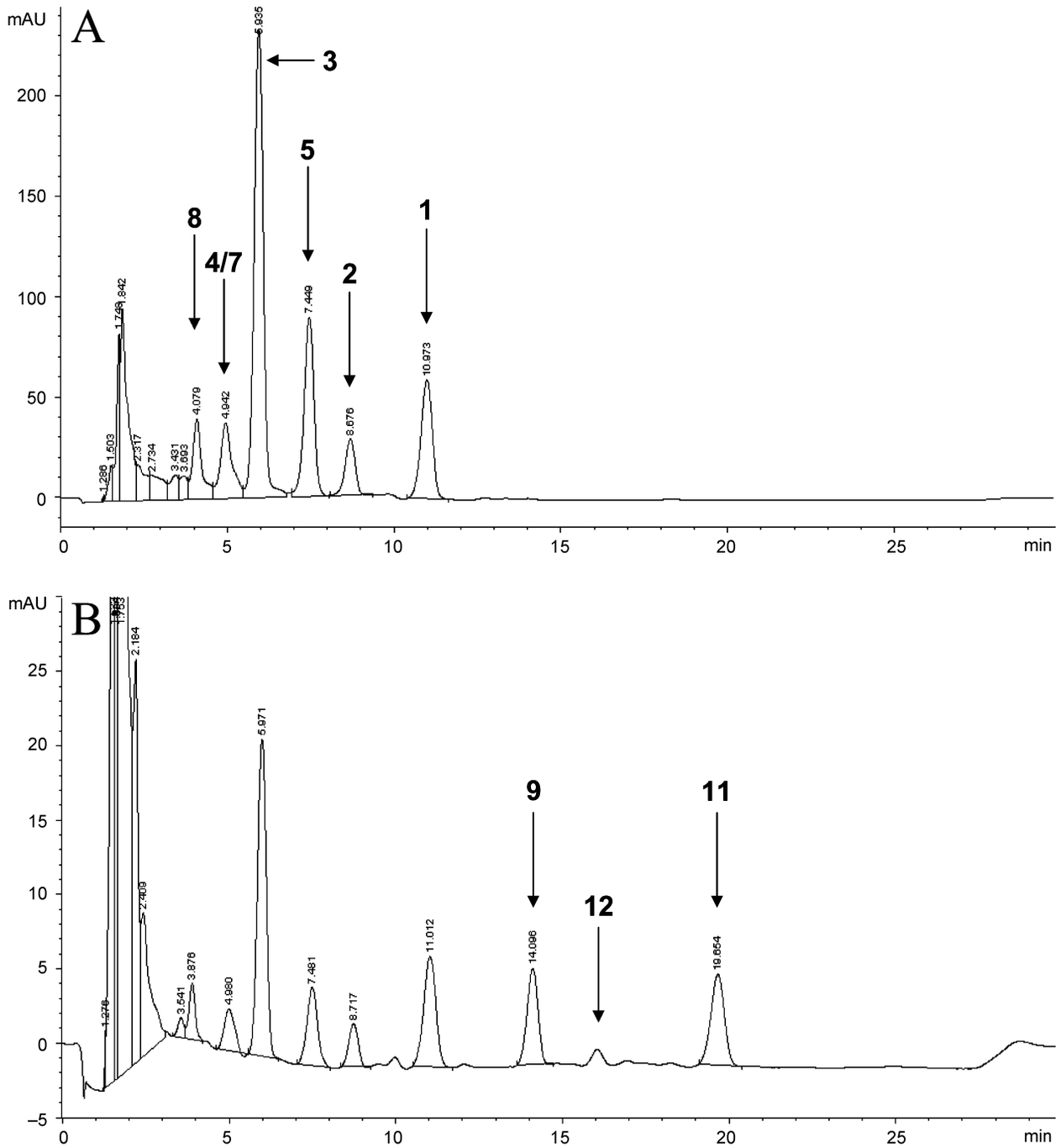

Fig. 4 HPLC analysis of cultures of *S. avermitilis* wild type (A) and BIOT-1916 (B). The locations of the naturally occurring avermectins are shown, as well as novel peaks representing 22,23-dihydroavermectins.

in the *S. avermitilis aveC* gene have been shown to enhance the ratios of the olefinic compounds produced relative to C23 hydroxyl components.[12] Similarly, no olefinic avermectins appear to be produced in mutants from which *aveC* has been deleted.[27] However, there is no amino acid sequence similarity between AveC and authentic PKS- or fatty acid synthase-associated dehydratases. Consequently, it has been postulated that AveC may activate the DH activity of module 2 by associating with the PKS, or may accomplish the dehydration reaction as a post-PKS modification.[7,8,12] Nevertheless, we hoped to over-ride such mechanisms by complete replacement of the reductive loop of module 2.

Conclusions

The results presented here provide clear evidence for the presence of significant amounts of the predicted 22,23-dihydroavermectins in cultures of BIOT-1916. Two of these 22,23-dihydroavermectins were purified and fully characterised by NMR and high-resolution MS analysis. We can conclude that targeted modification of the avermectin PKS is indeed a viable route to the direct production of ivermectins. It is evident that there is considerable scope for improvement of the process, since the overall yield of macrolides is decreased compared to wild type (by approximately eight-fold) and because parent avermectins are still produced in significant amounts, indicating that the β-processing steps at C22–C23 are incomplete. Southern analysis of the recombinant strain of *S. avermitilis* (BIOT-1916) was fully consistent with the expected genetic organisation. It is possible that the heterologous enzymes are less efficient in the context of the avermectin PKS, allowing the ketosynthase of module 3 to recruit the hydroxyacyl chain before it has been reduced.[18] However, our observation that the molar ratios of the avermectin 1 : 2

components are identical in wild type and recombinant strains (Fig. 2) raises the intriguing alternative possibility that AveC competes for the enzyme-bound intermediates at module 2 of the avermectin PKS, and interferes with full reduction. This idea could be tested using *aveC* mutant strains. The use of alternative splice sites and of alternative "donor" domains may well also decisively improve the efficiency of conversion to 22,23-dihydroavermectins,[18] while conventional genetic manipulation can be used to ensure that only desmethyl B-series avermectins are formed.[10] Our results suggest that PKS engineering can indeed provide scaleable and convenient production of a key drug by direct fermentation, avoiding the need for any chemical steps.

Experimental

General

Standard growth media and conditions were used for *Escherichia coli* strains DH10B (Gibco BRL) and ET12567.[2,29] *E. coli* transformants were selected with 100 μg ml^{-1} ampicillin. *Streptomyces avermitilis* ATCC31272 was grown in TSB medium using standard protocols.[17] For production of avermectins or 22,23-dihydroavermectins the strains were grown as described previously.[14] Transformants of *S. avermitilis* were selected with thiostrepton using 20 μg ml^{-1} for solid and 5 μg ml^{-1} for liquid medium. The 22,23-dihydroavermectin B1a standard was purchased from SIGMA. The 4l fermentation of BIOT-1916 was carried out using a 200 ml TSB pre-culture to inoculate the production medium[14] (5% v/v) followed by cultivation at 30 °C with agitation at 450–550 rpm and an air flow of 0.75 v/v/m for 185 h. Transformation of *S. avermitilis* was carried out as described previously.[14]

DNA manipulations, PCR and electroporation procedures were carried out according to standard protocols.[29] Southern hybridizations were carried out with probes labelled with digoxigenin using the DIG DNA labelling kit (Roche Molecular Biochemicals). DNA sequencing was performed using automated DNA sequencing on double stranded DNA templates with an ABM Prism 3700 DNA analyser. Sequence data were analysed using the Genetics Computer Group (GCG, version 10) software package.[28]

Small-scale extractions and mass spectrometry were carried out using methods and procedures described previously.[30] Fermentation of BIOT-1916 was carried out as described above and the harvested culture was centrifuged to separate the supernatant (~2 l) from the cells. The cells were extracted with 1 : 1 (v/v) ethyl acetate–methanol (2 × 250 ml), and the supernatant extracted with ethyl acetate (3 × 2 l). The combined organic extract was dried *in vacuo* and the residue was fractionated (Flash C_{18} cartridge). The products were found only in the first 100% methanol fraction. This was dried *in vacuo* to give a concentrated extract (~180 mg). This residue was semi-purified by reversed-phase (C_{18}) preparative HPLC to isolate fractions **9** and **11**. Fractions containing the putative **9** were purified finally using reversed-phase (C_{18}) semi-preparative HPLC. The solvent was removed *in vacuo* and the aqueous residue was desalted over C_{18} silica cartridges, eluting the product with methanol. Fractions containing the putative **11** were treated in the same manner. NMR spectra were recorded on a Bruker 500 AMX and coupling constants are given in hertz. Samples were run in $CDCl_3$ and referenced to the solvent δ_H = 7.26 ppm and δ_C = 77.0 ppm). The ^1H and ^{13}C NMR spectra obtained for **9** were identical to those found for authentic material. For **11**: δ_H: 3.33 (m, H-2), 5.40 (m, H-3), 1.81 (m, H-4a), 3.97 (m, H-5), 3.51 (s, 3H, 5-OMe), 4.04 (d, *J* 5.7, H-6), 4.63 (dd, *J* 14.4 & 2.5, H-8a), 4.70 (dd, *J* 14.4 & 2.3, H-8a), 5.84 (m, H-9), 5.73 (m, H-10), 5.73 (m, H-11), 2.52 (m, H-12), 1.17 (d, *J* 7.0, H-12a), 3.94 (m, H-13), 1.50 (br s, H-14a), 4.98 (m, H-15), 2.28 (m, H-16), 3.67 (m, H-17), 0.84 (m, H-18), 1.76 (m, H-18), 5.34 (m, H-19), 1.37 (dd, *J* 11.9 & 11.9, H-20), 1.98 (ddd, *J* 11.9, 5.2 & 1.8, H-20), 1.65 (m, H-22), 1.49 (m, H-22 (tentative assignment)), 1.52 (m, H-23), 1.50 (m, H-24), 0.78 (d, *J* 6.8, H-24a), 3.22 (m, H-25), 1.54 (m, H-26), 0.85 (d, *J* 6.8, H-26a), 1.35–1.51 (m, H-27), 0.93 (dd, *J* 7.4 & 7.4, H-28), 4.78 (dd, *J* 4.0 & 1.4, H-1′), 1.55 (m, H-2′), 2.22 (m, H-2′), 3.62 (m, H-3′), 3.43 (s, 3′-OMe), 3.24 (dd, *J* 9.4 & 9.4, H-4′), 3.83 (dq, *J* 9.5 & 6.2, H-5′), 1.26 (d, *J* 6.2, H-6′), 5.40 (m, H-1″), 1.51 (m, H-2″), 2.32 (m, H-2″), 3.47 (m, H-3″), 3.42, (s, 3″-OMe), 3.17 (dd, *J* 9.2 & 9.2, H-4″), 3.77 (dq, *J* 9.5 & 6.2, H-5″), 1.28 (d, *J* 6.2, H-6″). δ_C: 173.9 (C-1), 45.4 (C-2), 118.2 (C-3), 136.2 (C-4), 19.7 (C-4a), 76.9 (C-5), 57.6 (5-OMe), 77.4 (C-6), 80.5 (C-7), 139.8 (C-8), 68.2 (C-8a), 119.7 (C-9), 124.7 (C-10), 137.5 (C-11), 39.6 (C-12), 20.0 (C-12a), 81.8 (C-13), 134.9 (C-14), 15.0 (C-14a), 118.2 (C-15), ~34.0 (C-16), 67.1 (C-17), 36.8 (C-18), 68.5 (C-19), 41.1 (C-20), 97.3 (C-21), 35.6 (C-22), 31.1 (C-23), 27.9 (C-24), 17.3 (C-24a), 76.5 (C-25), 35.2 (C-26), 11.9 (C-26a), 27.1 (C-27), 12.3 (C-28), 94.6 (C-1′), 34.2 (C-2′), 79.3 (C-3′), 56.3 (3′-OMe), 80.3 (C-4′), 67.1 (C-5′), 18.2 (C-6′), 98.3 (C-1″), ~34.2 (C-2″), 78.0 (C-3″), 56.3 (3″-OMe), 75.9 (C-4″), 67.9 (C-5″), 17.5 (C-6″).

Construction and Southern blot analysis of *S. avermitilis* mutant strains

The detailed description is given elsewhere[18] of the isolation of plasmid pJLK137 containing a 2.4 kb DNA fragment of the avermectin PKS gene of *S. avermitilis* encoding the region upstream of the β-processing loop of module 2; a 3.2 kb DNA fragment of the *rapC* gene of *S. hygroscopicus* encoding the β-processing loop of module 13; and a 2.0 kb DNA fragment of the avermectin PKS gene of *S. avermitilis* encoding the downstream region of the β-processing loop of module 2. To create plasmid pPF137, plasmid pJLK137 was digested with *Eco*RI and the insert fragment was isolated and ligated with *Eco*RI digested pCJR24.[31] Plasmid pPF137 was isolated using standard protocols and verified using restriction digests and DNA sequence analysis.[29]

The *S. avermitilis* mutants BIOT-1917 and BIOT-1916 were verified by Southern blot analysis using two different probes. Probe 1 was created by isolating the 2.6 kb *Bam*HI fragment containing parts of module 13 of the rapamycin biosynthetic gene cluster from plasmid pPF137 (indicated in Fig. 5) followed by digoxigenin labelling according to the manufacturers' recommendations (Roche Molecular Biochemicals). Probe 2 was isolated and labelled by PCR amplification using the primers AK7 (5′- TCGTCCTCTACTCCTCCGCCGCCGCC-ACCTTCG–3′) and AK8 (5′-CCGTTTCGGGTGTAGCAG-GTGCCGGGGTGGTC–3′) and genomic DNA of *S. avermitilis* wild type as template. Using probe 1, which is specific for rapamycin module 13, the expected 2.6 kb fragment was detected in *Bam*HI-digested chromosomal DNA of the BIOT-1917, whereas no signal was detected in the control experiment using genomic DNA of the *S. avermitilis* wild type. The expected 5.7 kb fragment was detected when *Not*I/*Bgl*II digested genomic DNA of BIOT-1916 was used for Southern Blot analysis, whereas a 11 kb band was found using BIOT-1917 and no signal was detected in the control with genomic DNA of *S. avermitilis*. Using *Bam*HI digests, the expected 5.1 kb band for BIOT-1917 and the 6.8 kb band for the wild type control were detected with probe 2 in the Southern Blot analysis. The presence of the reductive loop of rapamycin module 13 in BIOT-1917 was also verified using PCR by amplifying DNA fragments with primers framing the exchanged DNA region. The primers AK15 (5′-GTGTCGGGGGTGGTGT-GGCCCAGGACGGTGGCGATGTGGGAGCGGACC-3′) and AK16 (5′-CCCAAACCCACCTCCTCACCAACCTC-GCCAAAACCACCACCACCTGGCAC-3′) were used to amplify the DNA fragment framing the exchanged region applying standard techniques. Using wild type DNA as control, a 2.7 kb DNA fragment was detected, whereas both of the

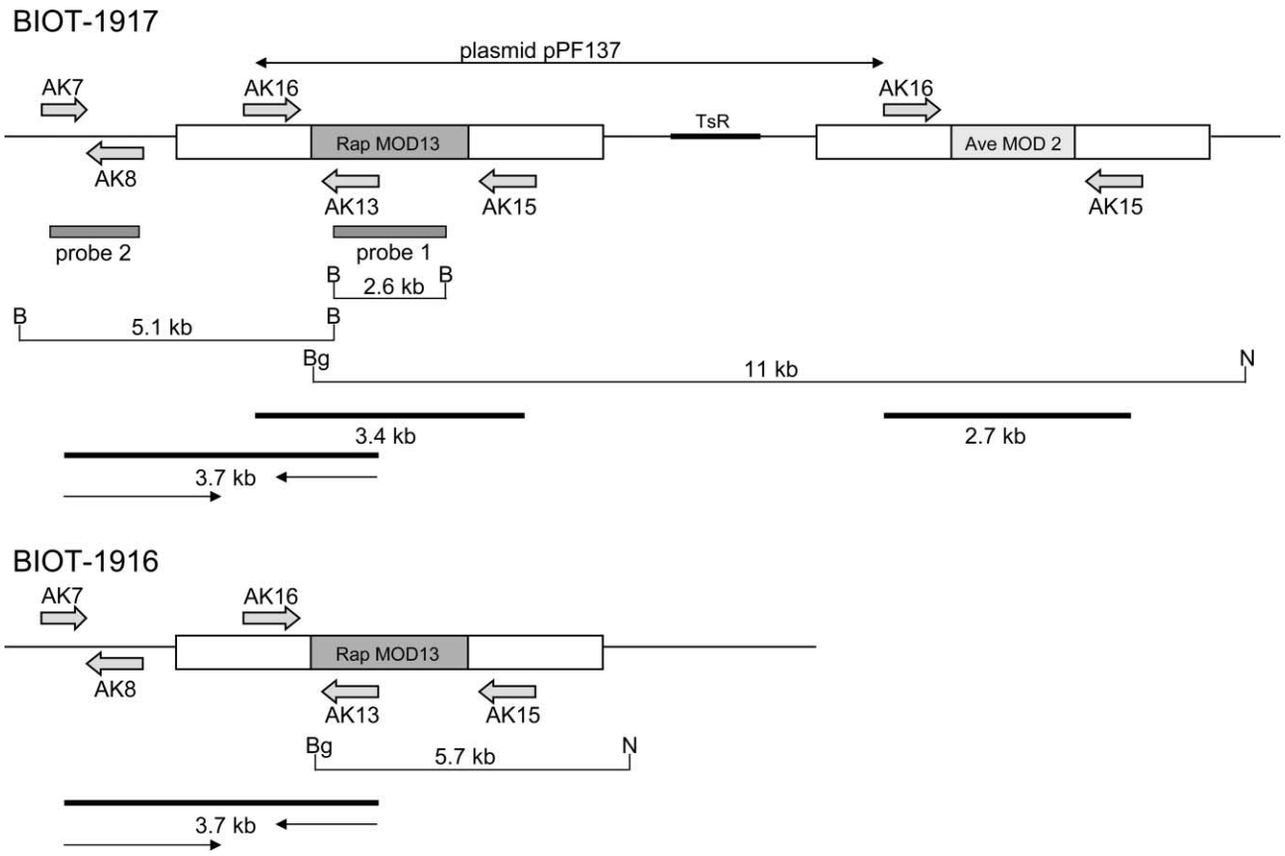

Fig. 5 Southern blot and PCR analysis of the *S. avermitilis* mutants BIOT-1917 and BIOT-1916. The genomic region of the integration site is shown schematically. Grey arrows indicate the primers AK7, 8, 13, 15 and 16. Probes 1 and 2 are shown as grey bars. Relevant restriction fragments are indicated. Black bars denote amplified PCR fragments. Black arrows indicate the sequenced region of the amplified DNA fragment. Abbreviations: B: *Bam*HI, Bg: *Bgl*II, N: *Nco*I.

expected 3.4 kb and 2.7 kb DNA fragments were amplified with chromosomal DNA of BIOT-1917 as template. To verify the genomic integration site, plasmid pALK66 and pALK72 were isolated by amplifying a 3.7 kb DNA fragment with the primers AK7 (see above) and AK13 (5′-CAGGAGCGGGGTT TCGATCACCAACTCGTCAA-3′) and genomic DNA from BIOT-1917 and BIOT-1916 as template respectively, followed by cloning into *Sma*I cut pUC18 using standard protocols. The ends of the cloned fragments were confirmed by sequence analysis. An overview of the Southern blot and PCR analysis is given in Fig. 5.

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