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Original Research Article

Engineering metabolic pathways in *Amycolatopsis japonicum* for the optimization of the precursor supply for heterologous brasilicardin congeners production

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ABSTRACT

The isoprenoid brasilicardin A is a promising immunosuppressant compound with a unique mode of action, high potency and reduced toxicity compared to today's standard drugs. However, production of brasilicardin has been hampered since the producer strain *Nocardia terpenica* IFM0406 synthesizes brasilicardin in only low amounts and is a biosafety level 2 organism. Previously, we were able to heterologously express the brasilicardin gene cluster in the nocardioform actinomycete *Amycolatopsis japonicum*. Four brasilicardin congeners, intermediates of the BraA biosynthesis, were produced. Since chemical synthesis of the brasilicardin core structure has remained elusive we intended to produce high amounts of the brasilicardin backbone for semi synthesis and derivatization. Therefore, we used a metabolic engineering approach to increase heterologous production of brasilicardin in *A. japonicum*. Simultaneous heterologous expression of genes encoding the MVA pathway and expression of diterpenoid specific prenyltransferases were used to increase the provision of the isoprenoid precursor isopentenyl diphosphate (IPP) and to channel the precursor into the direction of diterpenoid biosynthesis. Both approaches contributed to an elevated heterologous production of the brasilicardin backbone, which can now be used as a starting point for semi synthesis of new brasilicardin congeners with better properties.

1. Introduction

Isoprenoids (also called terpenoids) are small biomolecules representing a large and old family of compounds [1,2]. They are of interest since they are found in almost all living organisms, having a large variety of functions. Isoprenoids are well known as the primary component of the essential oils of plants and for their diverse biological functions including their roles as hormones and components in the respiration and photosynthesis chains. As secondary metabolites, isoprenoids are known to be involved in specialized processes such as communication and defense. Among these secondary metabolites, many pharmaceuticals, biofuels and food additives have been discovered and isolated [3,4]. Despite the structural and functional diversity of isoprenoids, all isoprenoids are biosynthesized from a basic five carbon (C5) precursor unit called isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) [5].

Two unrelated metabolic pathways can synthesize IPP and DMAPP: the mevalonate (MVA) pathway and the methylerythritol

Abbreviations: Aact, acetoacetyl CoA thiolase; BraA, brasilicardin A; BraB, brasilicardin B; BraC, brasilicardin C; BraD, brasilicardin D; BraC-agl, brasilicardin C aglycon; BraD-agl, brasilicardin D aglycon; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; Fpps, farnesyl diphosphate synthase; GlcNAc, *N*-acetylglucosamine; GPP, geranyl diphosphate; Gpps, geranyl diphosphate synthase; GGPP, geranylgeranyl diphosphate; Ggpps, geranylgeranyl diphosphate synthase; Idi, isopentenyl diphosphate synthase; IPP, isopentenyl diphosphate; MEP, Methylerythritol 4-phosphate; MVA, mevalonate; 3HBA, 3-hydroxy-benzoate.

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4-phosphate (MEP) pathway. The MVA pathway is present in most eukaryotes, archea and in a few bacteria, while the MEP pathway can be found in most bacteria and plant chloroplasts [6]. The MVA pathway relies on six enzymes starting with two connective condensation reactions of three acetyl-CoA molecules forming 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). HMG-CoA is then reduced to MVA before two phosphorylation reactions convert MVA to mevalonate diphosphate. Mevalonate diphosphate is subsequently decarboxylated into IPP, forming the endpoint of the MVA pathway (Fig. 1).

The MEP pathway consists of seven enzymatic reactions starting with the condensation of pyruvate and D-glyceraldehyde 3-phosphate. After a reduction and isomerization reaction, 2C-methyl-*D*-erythritol-4-phosphate (MEP) is formed. MEP is successively converted into to 2C-methyl-*D*-erythritol-2,4-cyclodiphosphate (MEcPP) in three reactions. In the last two steps of the MEP pathway, the MEcPP is converted into 4-hydroxy-3-methyl-butenyl 1-diphosphate (HMBPP), which is subsequently transformed into IPP and DMAPP [7].

Thereafter, sequential condensation reactions of the C5 building block IPP with a growing allylic polyisoprenoid diphosphate follows. Thereby in the first step IPP is attached to DMAPP resulting in the formation of geranyl diphosphate (GPP (C10)). Successive addition of IPP forms farnesyl diphosphate (FPP (C15)), geranylgeranyl diphosphate (GGPP (C20)) (Fig. 1) and a series of even longer isoprenoid diphosphate products [8]. These condensation reactions are catalysed by an enzyme family called prenyltransferases or isoprenyl pyrophosphate synthases. Typically, an organism contains several different prenyltransferases (Gpps, Fpps and Ggpps) to ensure the synthesis of a variety of isoprenoid diphosphate molecules [8].

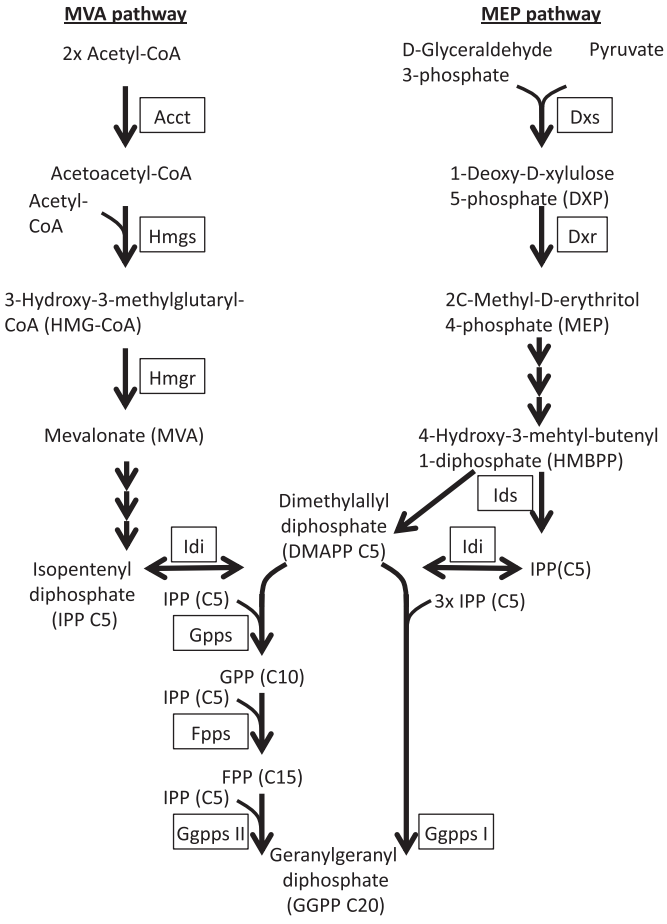

Fig. 1. Characteristic enzymes for the synthesis of isopentenyl diphosphate (IPP)/dimethylallyl diphosphate (DMAPP) and the formation of the isoprenoid diphosphate chain. Mevalonate (MVA) pathway: Aact (acetoacetyl-CoA thiolase), Hmgs (3-hydroxy-3-methylglutaryl CoA synthase), Hmgr (3-hydroxy-3-methylglutaryl coenzyme A reductase). Methylerythritol 4-phosphate (MEP) pathway: Dxs (1-desoxy-D-xylulose-5-phosphate synthase), Dxr (1-desoxy-D-xylulose-5-phosphate reductase), Ids (isopentenyldiphosphate: dimethylallyl diphosphate synthase). Condensation of IPP and other allylic precursors: DMAPP, GPP (geranyl diphosphate), Gpps (geranyl diphosphate synthase), FPP (farnesyl diphosphate), Fpps (farnesyl diphosphate synthase), GGPP (geranylgeranyl diphosphate), Ggpps I and II (geranylgeranyl diphosphate synthase). Carbon sizes of allylic precursors are written in brackets.

Ggpps enzymes, which catalyze the formation of GGPP (C20), can be divided into two classes, characterized by their allylic substrate specificity. The first class represents Ggpps enzymes that use DMAPP (C5) and successively add three molecules of IPP (C5) [9]. The second class of Ggpps enzymes uses FPP (15) and adds one molecule of IPP (C5) to synthesize GGPP (C20) [10,11] (Fig. 1).

Most of the isoprenoid compounds that are used for pharmaceuticals are originally isolated from plants [12]. However, the quantity and efficiency of isoprenoid production in plants is low and prevents a sufficient supply of such products. Heterologous expressions of isoprenoid gene clusters in microorganisms makes the application of metabolic engineering and synthetic biology approaches feasible in order to optimize isoprenoid production [13]. For example, for economical and reliable production of the antimalarial sesquiterpene compound artemisinin, a microbial production of artemisinic acid was developed. In a synthetic biology approach, the artemisinin biosynthetic genes from *Artemisia annua* [14] have been transferred into *Saccharomyces cerevisiae* combined with the endogenous farnesyl pyrophosphate (FPP) pathway. This pathway was further engineered in *S. cerevisiae* to increase artemisinin production [15]. The optimization of isoprenoid production relays on the sufficient provision of the precursor and on its channeling into the direction of the aimed isoprenoid synthesis [16,17].

In this study we focus on the optimization of the production of the isoprenoid brasilicardin A (BraA) (Fig. 2). BraA is a tricyclic diterpenoid consisting of an anti/syn/anti-perhydrophenanthrene skeleton, an amino acid side chain, a 3-hydroxy-benzoate (3HBA), a L-rhamnose and an *N*-acetylglucosamine (GlcNAc) moiety (Fig. 2). It is a promising immunosuppressant, which shows a lower toxicity while having a higher potency compared to one of today's standard immunosuppressants, cyclosporin A [18,19]. Furthermore, BraA was reported to show a new mechanism of immunosuppression. While

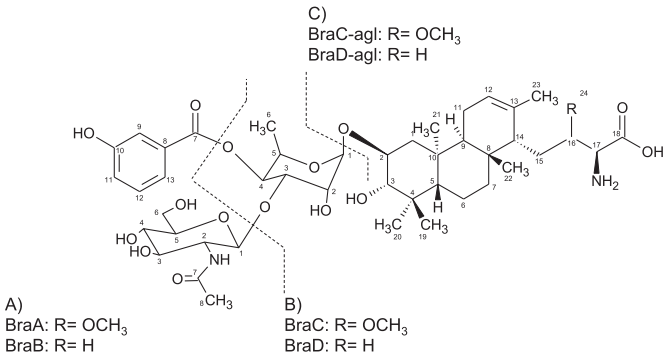

Fig. 2. Chemical structure of brasilicardin congeners produced in *N. terpenica* and/or in *A. japonicum*::pPS1. A) Structure of brasilicardin A (BraA) and brasilicardin B (BraB) produced by *N. terpenica*. B) Structure of brasilicardin C (BraC) and brasilicardin D (BraD) produced by *N. terpenica* and *A. japonicum*::pPS1. C) Structure of brasilicardin C aglycon (BraC-agl) and brasilicardin D aglycon (BraD-agl) produced by *A. japonicum*::pPS1.

commonly used immunosuppressants like cyclosporine A or tacrolimus inhibit interleukin-2 production from T-helper cells [20], BraA interacts with the amino acid transport system L of lymphocytes leading to amino acid deprivation by inhibiting the uptake of large neutral amino acids. Lymphocytes in T cell activation require extracellular nutrients to provide energy for cellular proliferation and effector functions [21].

BraA is produced by the actinobacterium *Nocardia terpenica* IFM0406 [18,22]. However, *N. terpenica* IFM0406 shows a low production titer and is a pathogenic biosafety level 2 organism, which makes metabolic engineering or synthetic biology not feasible. Due to its complex stereochemistry, total synthesis of brasilicardin could not be achieved so far [23–25]. In particular, the difficult synthesis of the anti-syn-anti-perhydrophenanthrene backbone of brasilicardin made the synthesis of brasilicardin and brasilicardin congeners an unsolved task. That is why development of this promising immunosuppressant has been hindered.

However, heterologous expression of the brasilicardin biosynthetic gene cluster proved to be a good alternative for optimizing production. The heterologous production of brasilicardin congeners was achieved by the introduction of a fosmid containing the brasilicardin gene cluster (bcaAB01) into *Amycolatopsis japonicum* MG417-CF17 [26]. Even though *Escherichia coli* and *Saccharomyces cerevisiae* have become the favored choice to optimize the production of non-native terpenoids [27,28] these strains are not suitable for the expression of gene clusters from actinomycetes, which possess a high GC content.

Therefore, the nocardioform actinomycete *A. japonicum* was chosen for heterologous expression. *A. japonicum* is closely related to the native producer *N. terpenica* IFM0406, possesses rhamnose biosynthetic genes for the synthesis of the L-rhamnose moiety of brasilicardin [29] and is genetically manipulable [30]. In *A. japonicum*, the synthesis of the congeners brasilicardin C (BraC) and brasilicardin D (BraD) as well as of BraC-aglycon (BraC-agl), BraD-aglycon (BraD-agl) could be confirmed. All of the structures consist of a tricyclic diterpenoid backbone, an amino acid side chain and an L-rhamnose moiety; but they all lack the 3-hydroxybenzoate (3HBA) and the *N*-acetylglucosamine (GlcNAc) moiety. BraC and BraD differ only in the methoxy group at the C16 position of brasilicardin [31]. The two congeners BraC-agl and BraD-agl lack the L-rhamnose moiety (Fig. 2).

The accomplished production of the brasilicardin diterpene backbone now offers the possibility for the synthesis of novel semi synthetic brasilicardin congeners. Since this chemical derivatization requires high amounts of brasilicardin diterpene backbone, we introduced heterologous genes encoding the MVA pathway to optimize the provision of the isoprenoid precursor IPP in *A. japonicum;* in a second step, we channeled the precursor into the direction of diterpenoid synthesis by expressing diterpenoid specific prenyltransferases. Both approaches contributed to elevated heterologous brasilicardin production resulting in increased production levels compared to the native producer *N. terpenica* IFM0406.

2. Materials and methods

2.1. Bacterial strains and plasmids

All strains and plasmids used in this study are listed in Tables S1 and S2.

2.2. Media and culture conditions

E. coli strains were grown at 37 °C in Luria broth (LB) medium [32] and were supplemented with the respective antibiotics (Table S3) when necessary to maintain plasmids. Liquid cultures of *A. japonicum* were cultivated in an orbital shaker (220 rpm) in 500-ml baffled Erlenmeyer flasks at 27 °C. Liquid/solid media were supplemented with the respective antibiotics (Table S3) to select for strains carrying integrated antibiotic resistance genes. To detect brasilicardin production, the heterologous host *A. japonicum* was inoculated in 50 ml tryptic soy broth medium (TSB) preculture for 48 h, followed by a 50 ml SM17 mainculture ((2 g/l), glycerol (40 g/l), soluble starch (2 g/l), soy flour (5 g/l), peptone (5 g/l), yeast extract (5 g/l), NaCl (5 g/l), $CaCO_3$ (2 g/l)) inoculated with 1% preculture and cultivated for 72 h. 10 ml of the culture were centrifuged for 10 min at 5000 rpm. 500 µl of the supernatant were directly used for HPLC/MS analysis. To calculate production of brasilicardins, HPLC/MS intensities of BraC and BraC-agl were summed up. The summed up intensities were afterwards set into relation with the biomass by dividing the intensities by the dry biomass of 1 ml culture. Cultivation of *N. terpenica* IFM0406 and isolation of brasilicardin congeners were performed as described by Komaki et al. [18].

2.3. Construction of the integrative expression vectors deriving from pIJ10257

Genes cloned into the vector pIJ10257 are under the control of the constitutive promoter *ermEp** [33–36]. For heterologous expression of the *mva* genes, the respective coding region was amplified in two steps, first by using the primer pairs pPSmvapart1.fw/pPSmvapart1.rv and second by using the primer pairs pPSmvap2.fw/pPSmvap2.rv or pPSmvap2-aact.rv when cloning the *mva* pathway genes without *aact.* Primer sequences and included restrictions sites used for cloning into pIJ10257 are listed in Table S4. The fosmid CB23 [37] was used as template DNA.

For heterologous expression of the *mva* pathway genes together with *idi*, the respective coding region was amplified in two steps, first by using the primer pairs pPSmvap1.fw/pPSmvap1/idi.rv and second by using the primer pair pPSmvap2.fw/pPSmvap2.rv. Primer sequences and included restrictions sites used for cloning into pIJ10257 are listed in Table S4. The fosmid CB23 was used as template DNA.

For heterologous expression of the *mva* genes and *idi* in combination with *bra12* and *ggpps-fpps*, the *ermEp** promoter together with the respective coding region was amplified by using the primer pairs pPSmva+bra12.fw/pPSmva+bra12.rv and pPSmva+ggpps_fpps.fw/pPSmva+ggpps_fpps.rv. The plasmids pPSbra12 or pPSggpps+fpps were used as template DNA. First each PCR product was introduced into pPSmva+idi individually downstream of the *mva* pathway genes generating pPSmva+idi+bra12 and pPSmva+idi+ggpps_fpps. Next the *ggpps+fpps* PCR product was introduced into pPSmva+idi+bra12 downstream of *bra12*. Primer sequences and included restrictions sites used for cloning into pIJ10257 are listed in Table S4.

For heterologous expression of idi_{St} and overexpression of idi_{Aja}, the coding regions were amplified by using the primer pairs $pPSidi_{St}$.fw/$pPSidi_{St}$.rv and $pPSidi_{Aja}$.fw/$pPSidi_{Aja}$.rv respectively. Primer sequences and included restrictions sites used for cloning into pIJ10257 are listed in Table S4. Chromosomal DNA of *A. japonicum* to amplify idi_{Aja} and CB23 to amplify idi_{St} was used as template DNA.

For overexpression of *ggpps* and *fpps*, the coding regions were amplified by using the primer pairs pPSggpps.fw/pPSggpps.rv and pPSfpps.fw/pPSfpps.rv respectively. Chromosomal DNA of *A. japonicum* was used as template DNA. For overexpression of *ggpps* together with *fpps*, the *ermEp** promoter together with the coding region of *fpps* was amplified by using the primer pairs pPSfpps.fw/pPSfpps.rv. DNA from the plasmid pPSfpps was used as

template DNA. Primer sequences and included restrictions sites used for cloning into pIJ10257 are listed in Table S4.

2.4. Intergeneric conjugation procedure with A. japonicum spores

For conjugation of *A. japonicum*, the intergeneric conjugation procedure with *A. japonicum* spores and *E. coli* ET12567_pUZ8002 [30] was modified. *E. coli* ET12567_pUZ8002 is a methylation deficient strain carrying the genes required for plasmid transfer (*tra* genes) on the plasmid pUZ8002 [38]. *E. coli* ET12567_pUZ8002 was transformed with the plasmid that was intended to be transferred into *A. japonicum*. 500 µl overnight culture of the transformed *E. coli* ET12567_pUZ8002 was used to inoculate 50 ml LB without antibiotics. The culture was grown to an OD600 of 0.6 and used for conjugation as described by Stegmann et al. [30].

2.5. Detection of brasilicardin congeners by HPLC/MS

HPLC/MS analysis was performed with an Agilent 1200 HPLC series coupled with an Ultra Trap System XCT 6330 (Agilent, Waldbronn, Germany). Samples (2.5 µl) were injected on a 3 µm Nucleosil 100 C18 column (100 × 2 mm, fitted with a precolumn 10 × 2 mm, Dr. Maisch HPLC GmbH, Ammerbuch, Germany) and separated with eluent A 0.1% formic acid in water and eluent B 0.06% formic acid in acetonitrile by gradient elution (20%–50% B over 10 min followed by 50%–100% B over 5 min) at a flow rate of 400 µl/min. Detection was carried out at 220, 240, 300, 360 and 435 nm. Electrospray ionization in ultra scan mode (positive and negative, alternating) was done with a capillary voltage of 3.5 kV and a drying gas temperature of 350 °C [26]. Detection of *m*/*z* values was conducted with Agilent DataAnalysis for 6300 Series Ion Trap LC/MS Version 3.4 (Bruker Daltonik GmbH, Billerica, USA). MS^2 and MS^3 analyses were performed in positive mode under the same conditions. To determine the concentration of brasilicardin congeners produced by *A. japonicum*::pPS1, isolated BraC and BraC-agl ranging from 1 to 1000 mg/l in 10 dilution steps were used to generate a standard curve in HPLC/MS. The concentration of brasilicardin congeners was then determined by analysing the peak height of the MS spectrum in regards to the standard curve.

2.6. Bioinformatics and statistical analysis

Amino acid similarities were analysed with BLASTP [39].

All statistics were performed using GraphPad Prism version 6. The data from production tests were treated statistically, using the Mann-Whitney test [40] to calculate p values ($n = 6$). On each Fig. p values lower than 0.05 and 0.01 are indicated by symbols * and **, respectively. P values higher than 0.05 are indicated with ns (not significant).

2.7. Nucleotide sequence accession number

The GenBank accession number of the genome sequence, of *N. terpenica* IFM0406 is LWGR00000000.1 [41], GenBank accession number of *A. japonicum* is CP008953 [42].

3. Results and discussion

3.1. Quantification of brasilicardin production in A. japoniucum::pPS1

The native brasilicardin producer *Nocardia terpenica* IFM0406 exhibits only low production levels and is a biosafety level 2 organism. Therefore, the brasilicardin gene cluster was heterologously expressed in the closely related actinomycete *Amycolatopsis japonicum*. *A. japonicum* gives the advantage of being easy to cultivate and genetically accessible for further optimization of production.

For heterologous expression of the brasilicardin gene cluster, the fosmid pPS1, a congener of the fosmid bcaAB01, was transferred into *A. japonicum* [26]. pPS1 consists of the brasilicardin biosynthetic gene cluster and the vector pCC1FOS.

A. japonicum::pPS1 produced the congeners BraC, BraD, BraC-agl, and BraD-agl (Fig. 2). In contrast to BraA, produced by *N. terpenica* IFM0406 [18,22] all of them lack the 3-hydroxybenzoate (3HBA) and the *N*-acetylglucosamine (GlcNAc) moiety. Furthermore, BraD and BraD-agl lack the methoxy group at the C16 position of brasilicardin. In the two congeners, BraC-agl and BraD-agl, the L-rhamnose moiety is not attached (Fig. 2). Cytotoxicity assays on the proliferative response of mouse lymphocytes revealed that the presence of a methoxy group at C16 as well as a glucosamine unit and/or a benzoyl group are important for the immunosuppressive and cytotoxic activities [19,31]. BraC congeners exhibit activities 50 times less potent than that of BraA and the unmethoxylated BraD showed no suppressive activity. However, BraC and BraC-agl are suitable precursors for the synthesis of novel congeners using chemical synthesis. In order to find analogs with optimized pharmacological properties regarding potency and toxicity, different sugar moieties or benzoic acid residues can be attached. Therefore, *A. japonicum*::pPS1 was used as a model system for yield optimization via metabolic engineering.

The initial concentration of brasilicardin produced by *A. japonicum*::pPS1 was determined by HPLC/MS (see 2.5). *A. japonicum*::pPS1 was cultivated for 72 h and the culture filtrate was analysed by HPLC/MS. Analysing the cell extracts were omitted since we could show previously that the majority of produced brasilicardins are released into the medium [26]. The concentration of brasilicardin congeners was determined by analysing the peak height of the MS spectrum in regards to a standard curve (see 2.5). The desmethoxy congeners BraD and BraD-agl only contribute to 7% and 3%, respectively, of the brasilicardin production in the heterologous host, *A. japonicum*. Furthermore, the immunosuppressive activity of brasilicardin was shown to be strongly coupled to the methoxylation [31]. Therefore, BraD and BraD-agl were neglected for quantification. *A. japonicum*::pPS1 produced 302 mg/l of BraC and 36 mg/l of BraC-agl.

Even though heterologous expression of the brasilicardin gene cluster itself already increased production of BraC and BraC-agl, optimization of the yield for further chemical synthesis is crucial. Therefore, we aimed to increase heterologous production of the brasilicardins (BraC, BraC-agl) in *A. japonicum*::pPS1 by applying a metabolic engineering approach.

3.2. Optimization of the brasilicardin biosynthesis using the mevalonate (MVA) pathway

It is known that in many cases the limiting step in increasing production is the provision of the precursors [e.g. 43–45]. The diterpenoid backbone of brasilicardin is synthesized from the five carbon precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). IPP can either be synthesized by the mevalonate (MVA) or the methylerythritol 4-phosphate (MEP) pathway. As in most actinomycetes, *N. terpenica* IFM0406 and *A. japonicum* use the MEP pathway to produce IPP [46]. While the MEP pathway undergoes a strong and complex feedback regulation [47,48], the MVA pathway is far more inclined to be manipulated and therefore suitable for a metabolic engineering approach [1]. Therefore, we intended to heterologously express the MVA pathway in *A. japonicum*. However, only a few actinomycetes possess the MVA pathway, which is often located next to an

isoprenoid biosynthetic gene cluster, in addition to the MEP pathway [49].

In *Streptomyces tendae* TÜ1028 the MVA pathway is described to be located upstream to a phenazine gene cluster, where it is putatively responsible for the generation of the prenyl moiety of prenylated phenazines [37]. The genes encoding the MVA pathway (*mva*) from *S. tendae* have been cloned into the cosmid CB23 [37].

The six *mva* genes (*aact*; acetoacetyl-CoA thiolase, *hmgs*; 3-hydroxy-3-methylglutaryl CoA synthase, *hmgr*; 3-hydroxy-3-methylglutaryl coenzyme A reductase, *pmk*; phosphomevalonate kinase, *mdpd*; mevalonate diphosphate decarboxylase, *mk*; mevalonate kinase) were amplified in two steps using the cosmid CB23 as template DNA (primer pairs listed in Table S4). Subsequently, the amplified *mva* genes were introduced into pIJ10257 under the control of the constitutive promoter *ermEp**, generating the plasmid pPSmva. The plasmid was integrated into the genome of *A. japonicum*::pPS1 via site-specific recombination, using the *attP* attachment site of pPSmva. The transfer was mediated via conjugation (see 2.4). A successful introduction was verified by PCR using the primer pair introd.fw/introd.rv amplifying a part of the pIJ10257 plasmid backbone. Transconjugants of the generated *A. japonicum*::pPS1_mva were selected and cultivated for 72 h. 10 ml of the culture were used to obtain the culture filtrates.

Overexpressing the *mva* genes led to a significant increase of brasilicardins (BraC and BraC-agl) production by 27% (Fig. 3), indicating that overexpression of the MVA pathway genes from *S. tendae* constitutes an alternative route to the native MEP pathway of *A. japonicum* for producing IPP.

For further optimization, we searched for effects that possibly negatively influence the flux through the MVA pathway. The first reaction of the MVA pathway is the condensation of two acetyl-CoA molecules forming acetoacetyl-CoA. This reaction is performed by an acetoacetyl-CoA thiolase (Aact), sometimes called acetoacetyl-CoA transferase. Condensation of a third acetyl-CoA molecule then forms HMG-CoA [7]. Aacts are known to play a crucial role in increasing the flux through the MVA pathway. However, it has been described that balanced activity of Aacts in relation to the other enzymes of the MVA pathway is important for the MVA pathway flux [50–52]. For example, overexpression of Aact promotes its thiolysis activity, which is triggered by increased levels of acetoacetyl-CoA. Thiolysis of acetoacetyl-CoA to acetyl-CoA would be detrimental for the MVA pathway flux [52]. Aacts are also involved in fatty acid, carbon and tryptophan metabolism and the synthesis of ketone bodies. Therefore, it is not unusual to find *aact* homologs distributed throughout the genome, since their corresponding enzymes are required in different pathways that incorporate acetyl-CoA and acetoacetyl-CoA.

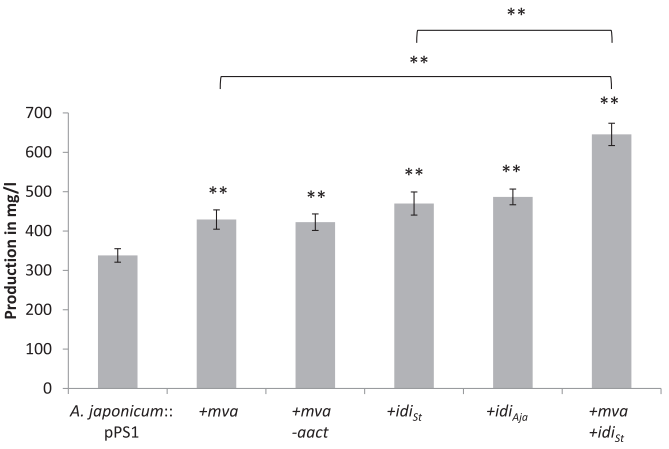

Fig. 3. HPLC/MS analyses of the culture filtrate of different *A. japonicum* strains. Effects of the genes encoding the mevalonate pathway (*+mva),* the *mva* genes without the acetoacetyl-CoA thiolase gene (*+mva -aact*), the IPP isomerase gene of *S. tendae* (*+idi$_{St}$*), the IPP isomerase gene of *A. japonicum* (*idi$_{Aja}$*) and the *mva* genes combined with the IPP isomerase of *S. tendae* (*+mva +idi$_{St}$*), on brasilicardins (BraC, BraC-agl) production of *A. japonicum*::pPS1 are displayed. Production levels of BraC (89%) and BraC-agl (11%) are summed up and given in mg/l. Significance was calculated in comparison to *A. japonicum*::pPS1 production levels or is displayed with brackets. Mann-Whitney test, (**: $P \leq 0.01$). $n = 6$.

However, excessive activity of Aacts in *A. japonicum* could lead to an unbalanced production rate of acetoacetyl-CoA triggering the thiolysis activity of that enzyme, which would negatively influence brasilicardin production. In order to investigate the function of Aact in *A. japonicum*::pPS1, the existence of homologous *aact* genes in *A. japonicum* was determined.

Blast analysis revealed the presence of three genes within the genome of *A. japonicum*, which code for proteins that share between 33% and 36% protein identity to the acetoacetyl-coA-thiolase (Aact) of the MVA pathway from *S. tendae*.

To investigate whether one of the native Aacts of *A. japonicum* can substitute the Aact of the MVA pathway of *S. tendae* (Aact$_{St}$) and bring a better balance to the acetoacetyl-CoA production rate, we introduced the *mva* pathway genes without *aact$_{St}$* into *A. japonicum*::pPS1. The *mva* genes were amplified by using the primer pairs listed in Table S4 and cloned into pIJ10257 generating pPSmva-aact. Conjugation and verification of transconjugants was performed as described for pPSmva. Transconjugants of *A. japonicum*::pPS1_mva-aact were cultivated for 72 h. 10 ml of the culture were used to obtain the culture filtrate, which was analysed via HPLC/MS.

The overexpression of the *mva* pathway genes without the associated *aact$_{St}$* led to an increase of brasilicardins (BraC and BraC-agl) production by 25% compared to *A. japonicum*::pPS1 (Fig. 3).

No extensive difference in production was observed when overexpressing the *mva* pathway genes with or without *aact*. From these results, we concluded that overexpression of the *aact* from *S. tendae* in a background of several native *aacts* of *A. japonicum* does not have any negative effects on increasing production of brasilicardins, but is also not beneficial. Furthermore, in *A. japonicum*::pPS1_mva-aact a native Aact is able to substitute the missing first enzyme of the MVA pathway. In order to establish the MVA pathway in *A. japonicum* only five (*hmgs*; 3-hydroxy-3-methylglutaryl CoA synthase, *hmgr*; 3-hydroxy-3-methylglutaryl coenzyme A reductase, *pmk*; phosphomevalonate kinase, *mdpd*; mevalonate diphosphate decarboxylase, *mk*; mevalonate kinase) out of six MVA pathway genes are required.

3.3. Optimization of the brasilicardin biosynthesis using the isopentenyldiphosphate isomerase (Idi)

A further key point in the biosynthesis of isoprenoids is the availability of the five carbon precursor isopentenyl diphosphate (IPP) (Fig. 1). The availability and isomerization of IPP from its isomer dimethylallyl diphosphate (DMAPP) is regulated by isopentenyldiphosphate isomerases (Idi). These enzymes balance the concentration of IPP and DMAPP in an Mg-dependent, reversible isomerization process [53]. Therefore, they are important for the regulation of the overall biosynthesis of isoprenoids [54,55].

In general Idi, are classified in two distinct subfamilies. While type 1 (Idi1) can be found in a large variety of organisms, from plants to bacteria to humans, type 2 (Idi2) are only present in proteobacteria, cyanobacteria, in gram positive bacteria like actinobacteria, and in some archaea [56]. The Idi of *S. tendae* (Idi$_{St}$), encoded in the *mva* pathway gene cluster, represents a type 2 Idi.

Using the amino acid sequence of Idi$_{St}$ of *S. tendae,* an Idi homolog was identified in *A. japonicum* (Idi$_{Aja}$). In contrast to Idi$_{St}$,

Idi_{Aja} belongs to the type 1 Idi family. Idi1 enzymes are predicted to be more applicable for increasing isoprenoid production than Idi2 enzymes and are therefore used more often for metabolic engineering approaches [1]. A possible explanation for this purpose could be that Idi1 enzymes do not need additional cofactors [56], which could also be a limiting factor.

Since the isomeration of IPP and DMAPP might also be a bottleneck of brasilicardins production in *A. japonicum*::pPS1, we overexpressed *idi* genes from *S. tendae* (idi_{St}) and from *A. japonicum* (idi_{Aja}) in *A. japonicum*::pPS1 and quantified the brasilicardin production. The respective *idi* genes (idi_{St} and idi_{Aja}) were amplified using the primer pair pPSidi$_{Aja}$.fw/pPSidi$_{Aja}$.rv and pPSidi$_{St}$.fw/pPSidi$_{St}$.rv and cloned into the integrative expression vector pIJ10257 under the control of *ermEp**. The generated plasmids pPSidi$_{St}$ and pPSidi$_{Aja}$ were introduced into *A. japonicum*::pPS1 via conjugation. Successful introduction was verified as described before. *A. japonicum*::pPS1_idi$_{St}$ and *A. japonicum*::pPS1_idi$_{Aja}$ were cultivated for 72 h and the culture filtrates were analysed via HPLC/MS. Overexpression of the IPP isomerase genes idi_{St} or idi_{Aja} led to an increase of BraC and BraC-agl production. Moreover, overexpressing the type 1 idi_{Aja} resulted in a slightly higher production of brasilicardins (44%) than overexpressing type 2 idi_{St} (39%) (Fig. 3).

The native MEP pathway of *A. japonicum* is able to produce both isomers (IPP and DMAPP) by using the hydroxymethylbutenyl diphosphate reductase, also called isopentenyldiphosphate:dimethylallyl diphosphate synthase (Ids). Ids uses 4-hydroxy-3-mehtyl-butenyl 1-diphosphate (HMBPP) as a substrate to synthesize IPP and DMAPP in an 85:15 ratio [57]. We could demonstrate that overexpressing *idi* has a significant impact on production. This indicates that Idi is improving the IPP:DMAPP ratio and redirecting the precursors towards brasilicardin synthesis.

Even though Idi1 enzymes seem to be advantageous compared to Idi2 enzymes [1], both classes are most likely equally efficient in isomerization of IPP and DMAPP in *A. japonicum*. This might be because in both classes the range of enzyme activity can vary [58–60]. Therefore, it is of particular interest for biotechnological application to identify IPP isomerases irrespective of their class, which specifically enhance the flux in the aimed terpenoid pathway.

3.4. Optimization of the brasilicardin biosynthesis using the mevalonate (MVA) pathway and isopentenyldiphosphate isomerase (Idi)

Both heterologous expression of the genes encoding the pathway to increase IPP production and overexpression of *idi* to improve balance of IPP and DMAPP resulted in an increase of brasilicardins production in *A. japonicum*::pPS1.

Since the heterologously expressed MVA pathway only produces IPP, which is then subsequently transformed into its isoform DMAPP by Idi [56], we speculated that Idi may play a crucial role for the provision of the precursor DMAPP in *A. japonicum*::pPS1_mva. Therefore, we combined those strategies in the next step.

Given that idi_{St} *and* idi_{Aja} showed no significant difference in increasing production of brasilicardins when overexpressed alone and that idi_{St} is located together with the *mva* genes on the cosmid CB23, we used idi_{St} for the coexpression with the *mva* genes. The entire *mva* gene cluster from *S. tendae* together with idi_{St} was amplified with the primer pairs listed in Table S4. After cloning of the amplified fragment in pIJ10257 the resulting plasmid pPSmva+idi was introduced into *A. japonicum*::pPS1. Successful introduction was confirmed via PCR and *A. japonicum*::pPS1_mva+idi transconjugants were cultivated for 72 h. The culture filtrate was analysed using HPLC/MS.

A. japonicum::pPS1_mva+idi produced 91% more brasilicardins than *A. japonicum*::pPS1 (Fig. 3). In contrast, the separate overexpression of the *mva* pathway genes or idi_{St} contributed only to an increase of brasilicardins production by 27% or 39%, respectively, confirming that Idi is crucial for the MVA pathway to catalyze the isomeration of the precursor IPP to DMAPP.

3.5. Engineering of the (di-)terpenoid biosynthesis

After increasing production of brasilicardins by improving the precursor supply for isoprenoids in general, we intended to specifically improve diterpenoid biosynthesis and to direct the flux of the precursor IPP into the direction of diterpenoids.

For the synthesis of diterpenoids like brasilicardin, GGPP (C20) is needed as a direct precursor. GGPP (C20) as well as mono (C10)-, sesqui (C15)- and all other terpenoids, are synthesized from the C5 building block IPP and its isomer DMAPP [9] via a chain elongation. This elongation is usually a condensation via 10-4 prenyl addition, resulting successively in geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20) (Fig. 1).

An alternative route for the synthesis of GGPP uses IPP and FPP (C15) [10,11] as building blocks (Fig. 1). FPP is provided by an FPP synthase (Fpps) that uses DMAPP and two molecules of IPP. Therefore, not only a GGPP synthase (Ggpps) but also a FPP synthase (Fpps) were taken into consideration in order to increase production of brasilicardins.

Using blast analysis we identified a *ggpps* and a *fpps* gene in *A. japonicum. In silico* analyses do not provide any information concerning the classification of the Ggpps. We amplified the respective genes with the primers listed in Table S4 and cloned *fpps* and *ggpps* alone and in combination in pIJ10257 under the control of the *ermEp** promoter. The resulting plasmids pPSfpps, pPSggpps and pPSfpps_ggpps were introduced via conjugation into *A. japonicum*::pPS1. Transconjugants were analysed via PCR as described before and the successful introduction was confirmed. The strains were cultivated for 72 h and the culture filtrates were analysed using HPLC/MS.

Overexpressing *fpps* alone increased production of brasilicardins by 39% whereas overexpressing *ggpps* alone did not show any effect on production of brasilicardins. However, overexpressing both, *ggpps* and *fpps* significantly increased production of brasilicardins by 67% (Fig. 4).

Ggpps favor different allylic precursors (DMAPP, GPP or FPP) as substrates to synthesize GGPP [9–11]. Our results indicated that the Ggpps in *A. japonicum* is indeed able to use FPP as a substrate since overexpression of *ggpps* (in combination with *fpps*) significantly increased production of brasilicardins. The necessity to use FPP as a substrate is underlined by the fact that there is no increase of brasilicardins production when *ggpps* is overexpressed alone. Hereby we showed that overexpressing an FPP-dependent *ggpps* gene is only beneficial when the FPP supply is assured.

3.6. Combining all positive effects on production of brasilicardins

Previously we were able to identify a positive transcriptional regulator (Bra12) encoded in the brasilicardin gene cluster. Bra12 shows high similarity (protein identity 51%) to a putative SARP family transcriptional regulator of *Nocardia acidivorans*. Introducing a second copy of *bra12* under the control of the *ermEp** promoter into *A. japonicum* carrying the brasilicardin gene cluster increased production of brasilicardins by 63% [26].

In order to yield higher brasilicardin production rates the metabolic engineering approaches of this study were combined

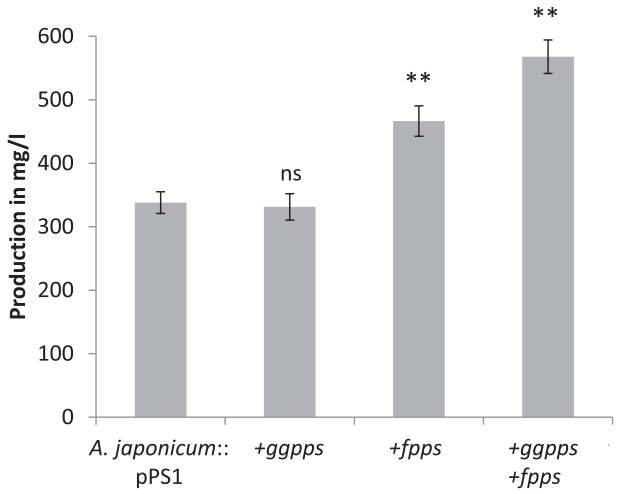

Fig. 4. HPLC/MS analyses of the culture filtrate of different *A. japonicum* strains. Effects of geranylgeranyl diphosphate synthase gene (+*ggpps*), farnesyl diphosphate synthase gene (+*fpps*) alone and in combination (+*ggpps* +*fpps*) on brasilicardins (BraC, BraC-agl) production in *A. japonicum*::pPS1. Production levels of BraC (89%) and BraC-agl (11%) are summed up and given in mg/l. Significance was calculated in comparison to *A. japonicum*::pPS1 production levels. Mann-Whitney test, (**: $P \leq 0.01$). n = 6.

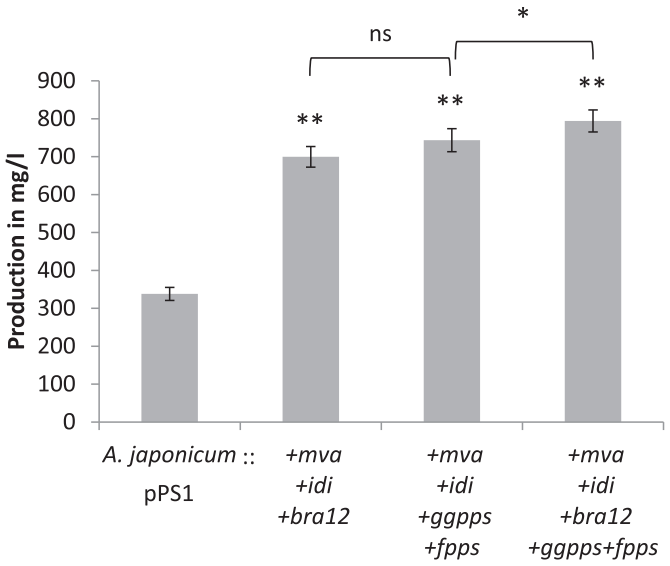

Fig. 5. HPLC/MS analyses of the culture filtrate of different *A. japonicum* strains. Effects of the geranylgeranyl diphosphate synthase gene (+*ggpps*), farnesyl diphosphate synthase gene (+*fpps*) alone and in combination (+*ggpps* +*fpps*) on brasilicardins production (BraC, BraC-agl) in *A. japonicum*::pPS1. Production levels of BraC (89%) and BraC-agl (11%) are summed up and given in mg/l. Significance was calculated in comparison to *A. japonicum*::pPS1 production levels. Mann-Whitney test, (**: $P \leq 0.01$, *: $P \leq 0.05$, ns: not significant). n = 6.

with upregulating the brasilicardin gene cluster expression using Bra12.

For that purpose pPSmva+idi was successively extended, by *ggpps*, *fpps* and *bra12*. The respective genes were amplified with the primer pairs listed in Table S4. First each PCR product was integrated into pPSmva+idi individually downstream of the *mva* pathway genes, generating pPSmva+idi+bra12 and pPSmva+idi+ggpps_fpps. Afterwards, *ggpps* and *fpps* were cloned into pIJmva+idi+bra12 downstream of *bra12* generating pPSmva+idi+bra12+ggpps_fpps. The plasmids were introduced into *A. japonicum*::pPS1 via conjugation. The successful plasmid introduction was confirmed via PCR as described before and a production test was performed.

Overexpression of the *mva* pathway genes and *idi* in combination with *bra12* in *A. japonicum*::pPS1 increased production of brasilicardins by 107%. When overexpressing the *mva* pathway genes and *idi* in combination with *ggpps* and *fpps* production of brasilicardins was increased by 120%. Finally overexpressing all discovered positive effects on production of brasilicardins in *A. japonicum*::pPS1_mva_idi_bra12_ggpps_fpps resulted in increased production levels by 135% when compared with the original, unmodified heterologous production (Fig. 5). Interestingly, throughout our overexpression studies the ratios of produced brasilicardins did not change significantly.

In comparison to *A. japonicum*::pPS1, which produced 338 mg/l of brasilicardins (BraC and BraC-agl), overexpressing the respective genes of the MVA pathway, *idi*, the positive regulator gene *bra12* and *ggpps* and *fpps* increased the yield to 794 mg/l. Producing sufficient amounts of the brasilicardin backbone is now opening the way for brasilicardin semi synthesis and derivatization.

4. Conclusion

Brasilicardin A is a promising immunosuppressant with a novel mode of action and reduced toxicity compared to today's standard drugs [18,19,21]. However, it is labor intensive and time consuming to optimize brasilicardin production in the native biosafety level 2 producer *Nocardia terpenica* IFM0406 or to chemically synthesize brasilicardin A [23–25]. The success of heterologous expression of the brasilicardin biosynthetic gene cluster facilitates brasilicardin related genetic manipulation. Further metabolic engineering approaches of the pathways providing the precursors of brasilicardin can be performed in the heterologous host in order to optimize production. In addition, swapping of genes encoding modification enzymes can be directly carried out on the recombinant fosmid, which harbors the brasilicardin biosynthetic genes, resulting in a brasilicardin derivative library containing hundreds of compounds. The functional elements, addressing the derivatization of brasilicardin, could be planned in the heterologous host with elaborate design directed by synthetic biology studies. This will shorten the fermentation process and reduce costs of brasilicardin production.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.synbio.2017.12.005.

References

[1] Vickers CE, Bongers M, Liu Q, Delatte T, Bouwmeester H. Metabolic engineering of volatile isoprenoids in plants and microbes. Plant Cell Environ

2014;37:1753–75.
[2] Wallach O. Zu Kenntniss der Terpene und ätherischen Öle. Just Lieb Ann Chem 1887;238:78–89.
[3] Heuston S, Begley M, Gahan CG, Hill C. Isoprenoid biosynthesis in bacterial pathogens. Microbiol 2012;158:1389–401.
[4] Bouvier F, Rahier A, Camara B. Biogenesis, molecular regulation and function of plant isoprenoids. Prog Lipid Res 2005;44:357–429.
[5] Oldfield E, Lin FY. Terpene biosynthesis: modularity rules. Angew Chem Int Ed Engl 2012;51:1124–37.
[6] Rohmer M. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat Prod Rep 1999;16:565–74.
[7] Kuzuyama T, Seto H. Two distinct pathways for essential metabolic precursors for isoprenoid biosynthesis. Proc Jpn Acad Ser B Phys Biol Sci 2012;88:41–52.
[8] Kellogg BA, Poulter CD. Chain elongation in the isoprenoid biosynthetic pathway. Curr Opin Chem Biol 1997;1:570–8.
[9] Dogbo O, Camara B. Purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthase from *Capsicum* chromoplasts by affinity-chromatography. Biochim Biophys Acta 1987;920:140–8.
[10] Jiang Y, Proteau P, Poulter D, Ferro-Novick S. BTS1 encodes a geranylgeranyl diphosphate synthase in *Saccharomyces cerevisiae*. J Biol Chem 1995;270:21793–9.
[11] Sagami H, Morita Y, Ogura K. Purification and properties of geranylgeranyldiphosphate synthase from bovine brain. J Biol Chem 1994;269:20561–6.
[12] Kirby J, Keasling JD. Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annu Rev Plant Biol 2009;60:335–55.
[13] Li Y, Wang G. Strategies of isoprenoids production in engineered bacteria. J Appl Microbiol 2016;121:932–40.
[14] Nosten F, White NJ. Artemisinin-based combination treatment of falciparum malaria. Am J Trop Med Hyg 2007;77:181–92.
[15] Hale V, Keasling JD, Renninger N, Diagana TT. Microbially derived artemisinin: a biotechnology solution to the global problem of access to affordable antimalarial drugs. Am J Trop Med Hyg 2007;77:198–202.
[16] Yang J, Zhao G, Sun Y, Zheng Y, Jiang X, Liu W, et al. Bio-isoprene production using exogenous MVA pathway and isoprene synthase in *Escherichia coli*. Bioresour Technol 2012;104:642–7.
[17] Zurbriggen A, Kirst H, Melis A. Isoprene production via the mevalonic acid pathway in *Escherichia coli* (bacteria). BioEnergy Res 2012;5:814–28.
[18] Komaki H, Nemoto A, Tanaka Y, Takagi H, Yazawa K, Mikami Y, et al. Brasilicardin A, a new terpenoid antibiotic from pathogenic *Nocardia brasiliensis*: fermentation, isolation and biological activity. J Antibiot (Tokyo) 1999;52:13–9.
[19] Komatsu K, Tsuda M, Tanaka Y, Mikami Y, Kobayashi J. SAR studies of brasilicardin A for immunosuppressive and cytotoxic activities. Bioorg Med Chem 2005;13:1507–13.
[20] Ho S, Clipstone N, Timmermann L, Northrop J, Graef I, Fiorentino D, et al. The mechanism of action of cyclosporin A and FK506. Clin Immunol Immunopathol 1996;80:S40–5.
[21] Usui T, Nagumo Y, Watanabe A, Kubota T, Komatsu K, Kobayashi J, et al. Brasilicardin A, a natural immunosuppressant, targets amino acid transport system L. Chem Biol 2006;13:1153–60.
[22] Shigemori H, Komaki H, Yazawa K, Mikami Y, Nemoto A, Tanaka Y, et al. Brasilicardin A. A novel tricyclic metabolite with potent immunosuppressive activity from actinomycete *Nocardia brasiliensis*. J Org Chem 1998;63:6900–4.
[23] Coltart DM, Danishefsky SJ. Novel synthetic approach to the 8,10-dimethyl anti-syn-anti-perhydrophenanthrene skeleton. Org Lett 2003;5:1289–92.
[24] Jung ME, Perez F, Regan CF, Yi SW, Perron Q. Se-phenyl prop-2-eneselenoate: an ethylene equivalent for Diels-Alder reactions. Angew Chem Int Ed Engl 2013;52:2060–2.
[25] Jung ME, Chamberlain BT, Koch P, Niazi KR. Synthesis and bioactivity of a brasilicardin a analogue featuring a simplified core. Org Lett 2015;17:3608–11.
[26] Schwarz PN, Buchmann A, Roller L, Kulik A, Gross H, Wohlleben W, et al. The immunosuppressant brasilicardin: determination of the biosynthetic gene cluster in the heterologous host *Amycolatopsis japonicum*. Biotechnol J 2017, 1700527. https://doi.org/10.1002/biot.20170052.
[27] Paramasivan K, Mutturi S. Progress in terpene synthesis strategies through engineering of *Saccharomyces cerevisiae*. Crit Rev Biotechnol 2017;37:974–89.
[28] Kemper K, Hirte M, Reinbold M, Fuchs M, Brück T. Opportunities and challenges for the sustainable production of structurally complex diterpenoids in recombinant microbial systems. Beilstein J Org Chem 2017;13:845–54.
[29] Spohn M, Kirchner N, Kulik A, Jochim A, Wolf F, Muenzer P, et al. Overproduction of Ristomycin A by activation of a silent gene cluster in *Amycolatopsis japonicum* MG417-CF17. Antimicrob Agents Chemother 2014;58:6185–96.
[30] Stegmann E, Pelzer S, Wilken K, Wohlleben W. Development of three different gene cloning systems for genetic investigation of the new species *Amycolatopsis japonicum* MG417-CF17, the ethylenediaminedisuccinic acid producer. J Biotechnol 2001;92:195–204.
[31] Komatsu K, Tsuda M, Shiro M, Tanaka Y, Mikami Y, Kobayashi J, et al. New tricyclic terpenoids [correction of terpernoids] from actinomycete *Nocardia brasiliensis*. Bioorg Med Chem 2004;12:5545–51.
[32] Sambrook JFE, Maniatis T. Molecular cloning: a laboratory manual. second ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.
[33] Kieser TBM, Buttner MJ, Chater KF, Hopwood DA. Practical Streptomyces genetics. Norwich Research Park, Colney, Norwich NR4 7UH, UK: John Innes Foundation; 2000.
[34] Bibb MJ, Janssen GR, Ward JM. Cloning and analysis of the promoter region of the erythromycin resistance gene (ermE) of Streptomyces erythraeus. Gene 1985;38:215–26.
[35] Bibb MJ, White J, Ward JM, Janssen GR. The mRNA for the 23S rRNA methylase encoded by the ermE gene of Saccharopolyspora erythraea is translated in the absence of a conventional ribosome-binding site. Mol Microbiol 1994;14:533–45.
[36] Schmitt-John T, Engels JW. Promoter constructions for efficient secretion expression in Streptomyces lividans. Appl Microbiol Biotechnol 1992;36:493–8.
[37] Saleh O, Bonitz T, Flinspach K, Kulik A, Burkard N, Muhlenweg A, et al. Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines. Medchemcomm 2012;3:1009–19.
[38] Paget MS, Chamberlin L, Atrih A, Foster SJ, Buttner MJ. Evidence that the extracytoplasmic function sigma factor sigmaE is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). J Bacteriol 1999;181:204–11.
[39] (NCBI)[Internet] NCfBI. Bethesda (MD): National library of medicine (US). National Center for Biotechnology Information; 1988.
[40] Mann HB, Whitney DR. On a test of whether one of two random variables is stochastically larger than the other. Ann Math Stat 1947;18:50–60.
[41] Buchmann A, Eitel M, Koch P, Schwarz PN, Stegmann E, Wohlleben W, et al. High-quality draft genome sequence of the actinobacterium *Nocardia terpenica* IFM 0406, producer of the immunosuppressant brasilicardins, using illumina and PacBio technologies. Genome Announc 2016;4.
[42] Stegmann E, Albersmeier A, Spohn M, Gert H, Weber T, Wohlleben W, et al. Complete genome sequence of the actinobacterium *Amycolatopsis japonica* MG417-CF17(T) (=DSM 44213T) producing (S,S)-N,N'-ethylenediaminedisuccinic acid. J Biotechnol 2014;189:46–7.
[43] Liu X, Ding W, Jiang H. Engineering microbial cell factories for the production of plant natural products: from design principles to industrial-scale production. Microbial Cell Factories 2017;16:125.
[44] Backhaus K, Ludwig-Radtke L, Xie X, Li S-M. Manipulation of the precursor supply in yeast significantly enhances the accumulation of prenylated β-carbolines. ACS Synth Biol 2017;6:1056–64.
[45] Liu H, Reynolds KA. Precursor supply for polyketide biosynthesis: the role of crotonyl-CoA reductase. Metab Eng 2001;3:40–8.
[46] Kuzuyama T. Biosynthetic studies on terpenoids produced by *Streptomyces*. J Antibiot (Tokyo) 2017;70:811–8.
[47] Bitok JK, Meyers CF. 2C-Methyl-d-erythritol 4-phosphate enhances and sustains cyclodiphosphate synthase IspF activity. ACS Chem Biol 2012;7:1702–10.
[48] Banerjee A, Wu Y, Banerjee R, Li Y, Yan H, Sharkey TD. Feedback inhibition of deoxy-D-xylulose 5-phosphate synthase regulates the methyl erythritol 4-phosphate pathway. J Biol Chem 2013;288:16926–36.
[49] Kawasaki T, Kuzuyama T, Furihata K, Itoh N, Seto H, Dairi T. A relationship between the mevalonate pathway and isoprenoid production in actinomycetes. J Antibiot (Tokyo) 2003;56:957–66.
[50] Menon N, Pasztor A, Menon BR, Kallio P, Fisher K, Akhtar MK, et al. A microbial platform for renewable propane synthesis based on a fermentative butanol pathway. Biotechnol Biofuels 2015;8:61.
[51] Schadeweg V, Boles E. n-Butanol production in *Saccharomyces cerevisiae* is limited by the availability of coenzyme A and cytosolic acetyl-CoA. Biotechnol Biofuels 2016;9:44.
[52] Tippmann S, Ferreira R, Siewers V, Nielsen J, Chen Y. Effects of acetoacetyl-CoA synthase expression on production of farnesene in *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 2017;44:911–22.
[53] Anderson MS, Muehlbacher M, Street IP, Proffitt J, Poulter CD. Isopentenyl diphosphate:dimethylallyl diphosphate isomerase. An improved purification of the enzyme and isolation of the gene from *Saccharomyces cerevisiae*. J Biol Chem 1989;264:19169–75.
[54] Kajiwara S, Fraser PD, Kondo K, Misawa N. Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. Biochem J 1997;324(Pt 2):421–6.
[55] Albrecht M, Misawa N, Sandmann G. Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin. Biotechnol Lett 1999;21:791–5.
[56] Berthelot K, Estevez Y, Deffieux A, Peruch F. Isopentenyl diphosphate isomerase: a checkpoint to isoprenoid biosynthesis. Biochimie 2012;94:1621–34.
[57] Rohmer M, Knani M, Simonin P, Sutter B, Sahm H. Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem J 1993;295(Pt 2):517–24.
[58] Rothman SC, Helm TR, Poulter CD. Kinetic and spectroscopic characterization of type II isopentenyl diphosphate isomerase from *Thermus thermophilus*: evidence for formation of substrate-induced flavin species. Biochemistry 2007;46:5437–45.
[59] Siddiqui MA, Yamanaka A, Hirooka K, Bamaba T, Kobayashi A, Imanaka T, et al. Enzymatic and structural characterization of type II isopentenyl diphosphate isomerase from hyperthermophilic archaeon *Thermococcus kodakaraensis*. Biochem Biophys Res Commun 2005;331:1127–36.
[60] Hoshino T, Eguchi T. Functional analysis of type 1 isopentenyl diphosphate isomerase from *Halobacterium sp.* NRC-1. Biosci Biotechnol Biochem 2007;71:2588–91.