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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

Paul N. Schwarz [a], Luisa Roller [a], Andreas Kulik [a], Wolfgang Wohlleben [a, b], Evi Stegmann [a, b, *]

[a] *Microbiology/Biotechnology, Interfaculty Institute of Microbiology and Infection Medicine Tübingen (IMIT), Eberhard Karls University Tübingen, Tübingen, Germany*

[b] *German Centre for Infection Research (DZIF), Partner Site Tübingen, Tübingen, Germany*

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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 4-phosphate (MEP) pathway. The MVA pathway is present in most eukaryotes, archea and in a few bacteria, while the MEP pathway

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.

* Corresponding author. Microbiology/Biotechnology, Interfaculty Institute of Microbiology and Infection Medicine Tübingen (IMIT), Eberhard Karls University Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany.
E-mail address: evi.stegmann@biotech.uni-tuebingen.de (E. Stegmann).

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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].

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 commonly used immunosuppressants like cyclosporine A or tacrolimus inhibit interleukin-2 production from T-helper cells

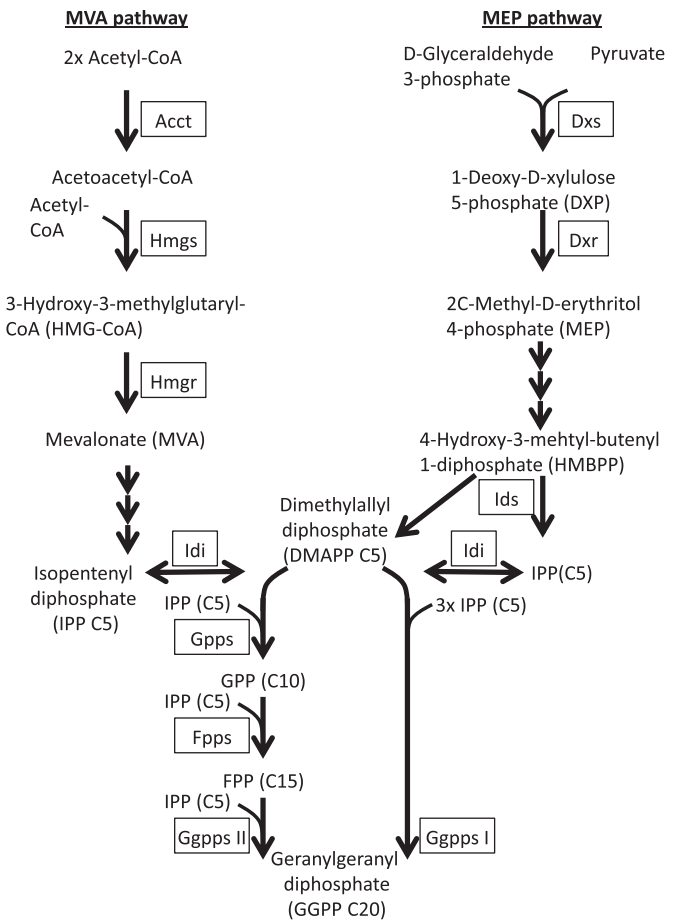

Fig. 1. Characteristic enzymes for the synthesis of isopentenyl diphosphate (IPP)/dimethylallyl diphosphate (DMAPP) and the formation of the isoprenoid diphosphate chain. Mevalonate (MVA) pathway: Aact (acetoacetyl-CoA thiolase), Hmgs (3-hydroxy-3-methylglutaryl CoA synthase), Hmgr (3-hydroxy-3-methylglutaryl 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.

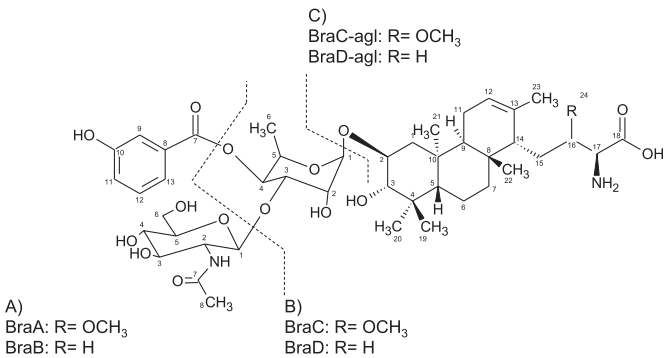

Fig. 2. Chemical structure of brasilicardin congeners produced in *N. terpenica* and/or in *A. japonicum*::pPS1. A) Structure of brasilicardin A (BraA) and brasilicardin B (BraB) produced by *N. terpenica*. B) Structure of brasilicardin C (BraC) and brasilicardin D (BraD) produced by *N. terpenica* and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

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