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MINI-REVIEW

# Echinocandins: production and applications

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**Abstract** The first echinocandin-type antimycotic (echinocandin B) was discovered in the 1970s. It was followed by the isolation of more than 20 natural echinocandins. These cyclic lipo-hexapeptides are biosynthesized on non-ribosomal peptide synthase complexes by different ascomycota fungi. They have a unique mechanism of action; as non-competitive inhibitors of β-1,3-glucan synthase complex they target the fungal cell wall. Results of the structure–activity relationship experiments let us develop semisynthetic derivatives with improved properties. Three cyclic lipohiexapeptides (caspofungin, micafungin and anidulafungin) are currently approved for use in clinics. As they show good fungicidal (*Candida* spp.) or fungistatic (*Aspergillus* spp.) activity against the most important human pathogenic fungi including azole-resistant strains, they are an important addition to the antifungal armamentarium. Some evidence of acquired resistance against echinocandins has been detected among *Candida glabrata* strains in recent years, which enhanced the importance of data collected on the mechanism of acquired resistance developing against the echinocandins. In this review, we show the structural diversity of natural echinocandins, and we summarize the emerging data on their mode of action, biosynthesis and industrial production. Their clinical significance as well as the mechanism of natural and acquired resistance is also discussed.

**Keywords** Natural echinocandins · Semisynthetic echinocandins · Non-ribosomal peptide synthase · β-1,3-Glucan synthase · Echinocandin resistance · Paradoxical growth

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## Introduction

*Candida* and *Aspergillus* strains are responsible for the majority of increasing invasive fungal infections; however, some other species, including *Cryptococcus neoformans*, *Pneumocystis carinii* and *Histoplasma capsulatum*, are also of great biomedical importance. The increasing threat to human health, by fungal infections, observed in the past decades is explained by the aging patient population, the increasing number of immunocompromised individuals as well as the wide-spread use of central venous catheters and broad-spectrum antibiotics (De Rosa et al. 2009; Rüping et al. 2008). Secondary metabolites of microbes represent an inexhaustible source of chemical structures leading to novel (antifungal) drugs. The discovery of echinocandins was the result of the systematic screening of microorganisms for novel antimycotics. Despite their relatively narrow spectra and weak solubility, echinocandins proved to be promising antifungal agents owing to their strong anti-*Candida* and anti-*Aspergillus* activities, their unique mode of action and their remarkable efficiency against azole-resistant strains. In the last few years, several reviews have been published on echinocandins; most of them summarize the clinical significance and limitations of the FDA-approved echinocandins anidulafungin, caspofungin and micafungin (Chen et al. 2011; Howard and Arendrup 2011; Kofla and Ruhnke 2011; Mukherjee et al. 2011; Perlin 2011; Pfaller 2012; Scott 2012). In the present review, we draw attention to the properties of semisynthetic echinocandins which influences their clinical applications and to the mechanisms of acquired resistance currently developing against them. In addition, we also emphasize the rich diversity of natural echinocandins and summarize the available data on their biosynthesis and industrial production.

## Natural echinocandins

The first echinocandin-type antimycotic, echinocandin B, was isolated independently by the researchers of Ciba-

Geigy, Sandoz and Eli Lilli from the fermentation broth of "*Aspergillus nidulans* var. *echinolatus*", "*Aspergillus nidulans* var. *roseus*" and *Aspergillus rugulosus* in the 1970s in random screening of the available strain collections (Benz et al. 1974; Keller-Juslén et al. 1976; Nyfeler and Keller 1974). Echinocandin B was followed by the isolation and characterization of more than 20 natural echinocandins. All these secondary metabolites are produced by ascomycota fungi, they have a cyclic lipo-hexapeptide structure and they all act as β-1,3-glucan synthase inhibitors. Representative members of the natural echinocandins as well as the most important producers are summarized in Table 1.

The detailed chemical structure of several of these compounds has been determined (Hensens et al. 1992; Iwamoto et al. 1994; Kanasaki et al. 2006c; Keller-Juslén et al. 1976; Mukhopadhyay et al. 1987, 1992; Satoi et al. 1977; Strobel et al. 1999; Traber et al. 1979; Tscherter and Dreyfuss 1982). The hexapeptide scaffold of echinocandin B consists of the following six amino acids: 4*R*,5*R*-dihydroxyl-L-Orn, L-Thr, 4*R*-hydroxyl-L-Pro, 3*S*,4*S*-dihydroxyl-L-homoTyr, L-Thr and 3*S*-hydroxyl,4*S*-methyl-L-Pro (Fig. 1a). The macrocycle is formed by a peptide bond between the δ-amino-group of dihydroxyl-Orn and the carboxyl-group of methyl-Pro. The linoleoyl moiety binds to the α-amino group of dihydroxyl-Orn. The presence of nonproteinogenic amino acids (Orn, homoTyr and methyl-Pro) is a typical feature of many non-ribosomal peptides. The long-chain fatty acyl amide as well as the multiple hydroxyl groups, including the vicinal diols on the Orn and homoTyr residues, are characteristic properties of echinocandin B and several other echinocandins. The presence of the hydroxyl group on the Cδ of Orn is particularly interesting because it forms a hydrolytically labile hemiaminal in the hexapeptide scaffold (Hensens et al. 1992; Leonard et al. 2007). Due to the internal hydrogen bonds between the two Thr residues and the β-turn conformation of the methyl-Pro and Pro residues, echinocandin B has a rigid conformation (Keller-Juslén et al. 1976, Traber et al. 1979). The chemical structures of other echinocandins are presented in Fig. 1b, c. Aculeacins are similar to echinocandin B but their acyl-moiety is either the myristoyl (aculacin Aα–Dα) or palmytoyl (aculacin Aγ–Dγ) group (Satoi et al. 1977). Mulundocandins have 10,12-dimethyl-myristoyl acyl-moiety and they contain L-Ser (instead of L-Thr) at position 5 from the N-terminus Orn (Mukhopadhyay et al. 1987, 1992). Pneumocandins differ from mulundocandins by the 3*R*-hydroxyl-L-Gln at position 5. The "Ser analogues" of pneumocandins (e.g. those of pneumocandin $B_0$ and $B_5$) contain L-Ser instead of L-Thr at position 2 (Connors and Pollard 2004). Sporiofungins do not contain Thr but they have 3*R*-hydroxyl-L-Gln, and L-Ser residues at position 5 and 2, respectively. Besides the 3*R*-hydroxyl-L-Gln moiety, they also have a 10,12-dimethylmyristoyl acyl-group, similar to pneumocandins (Bryskier 2005; Tscherter and Dreyfuss 1982). "Catechol-sulfate" echinocandins represent a diverse group of metabolites. All of them have a catechol-sulfate core in the homoTyr residue, they contain 3*R*-hydroxyl-L-Gln at position 5 and they are all acylated by palmitic acid. In some "catechol-sulfate" echinocandins, the second amino acid is L-Thr whilst in others it is L-Ser (Iwamoto et al. 1994; Kanasaki et al. 2006c) and they also show some heterogeneity in the position of the sulfate-group (both *meta* and *para* positions may occur). Cryptocandin differs from echinocandin B in its palmitoyl–moiety and the Gln residue at position 5 (Strobel et al. 1999).

Echinocandins show a high heterogeneity in the number and position of hydroxyl-groups on L-Orn, L-homoTyr, L-Gln and (mehyl)-L-Pro and/or the presence of the methyl-group on L-Pro even within the main groups. Instead of 4*R*,5*R*-dihydroxyl-L-Orn, they may contain L-Orn (e.g. pneumocandin $A_4$; Hensens et al. 1992), 5*R*-hydroxyl-L-Orn (e.g. FR901382; Iwamoto et al. 1994) or even 4*R*-hydroxyl-5*R*-methoxyl-L-Orn (e.g. sporiofungin C; Bryskier 2005; Tscherter and Dreyfuss 1982). The 3*S*,4*S*-dihydroxy-L-homoTyr residue can be replaced by 3*S*-hydroxyl-L-homoTyr (e.g. in deoxymulundocandin or in sporiofungins; Mukhopadhyay et al. 1992; Tscherter and Dreyfuss 1982) or L-homoTyr (e.g. in pneumocandin $A_4$; Hensens et al. 1992). Instead of the 3*S*-hydroxyl,4*S*-methyl-L-Pro residue, they may contain 3*S*-hydroxyl-L-Pro, 4*S*-hydroxyl-L-Pro and 3*S*,4*S*-dihydroxyl-L-Pro (pneumocandin $B_0$, $C_0$ and $D_0$ respectively; Hensens et al. 1992, Morris et al. 1994), or even 3-hydroxyl,4-hydroxymethyl-L-Pro residue (cryptocandin; Strobel et al. 1999). In many cases these analogues are produced by the same strain (e.g. echinocandin B-D by *A. rugulosus*; von Traber et al. 1979, pneumocandins by *Glarea lozoyensis*; Hensens et al. 1992, Morris et al. 1994).

## Mode of action

Several experimental data have demonstrated that the primary mode of action of echinocandins is the obstruction of the biosynthesis of the fungal cell wall by inhibiting the β-1,3-glucan synthase complex (Debono et al. 1994; Douglas et al. 1997; Mizoguchi et al. 1977; Perez et al. 1981; Radding et al. 1998; Sawistowska-Schröder et al. 1984; Yamaguchi et al. 1985). It is worth mentioning that besides being an indispensable component of the fungal cell wall, β-1,3-glucans are also present in certain fungi as stored compounds (Stone and Clark 1992). In addition, these important polysaccharides may also occur in prokaryotes, protozoa, chromista and plants, but not in vertebrates (Bacic et al. 2009). The majority of the echinocandins have only been tested on fungal β-1,3-glucan synthases with few exceptions. Klaus and Jeblick (1986) found that—depending on the experimental conditions, echinocandin B can either

**Table 1** Representative members of natural echinocandins and their producers

| Echinocandins | producer | Reference |
|---|---|---|
| Echinocandin B-D | "*Aspergillus nidulans* var. *echinulatus*" [a] | Nyfeler and Keller (1974) |
| | "*A. nidulans* var. *roseus*"[b] | Keller-Juslén et al. (1976) |
| | *A. rugulosus* | von Traber et al. (1979) |
| Aculeacin A-G | *A. aculeatus* | Mizuno et al. 1977 |
| | "*A. japonicus* var. *aculeatus*" | Satoi et al. (1977) |
| | | Hino et al. (2001) |
| Mulundocandin deoxymulundocandin | *Aspergillus sydowi* | Roy et al. (1987), Mukhopadhyay et al. (1992) |
| Pneumocandin A-E | *Glarea lozoyensis*[c] *Pezicula carpinea* | Schwartz et al. (1989, 1992) Nobel et al. (1991) |
| | *Cryptosporiopsis* sp. | Morris et al. (1994) |
| | | Bills et al. (1999) |
| Sporiofungin A-C | *Cryptosporiopsis* sp. | Tscherter and Dreyfuss (1982) |
| "Catechol-sulfate" echinocandins (FR901379, FR901381-82, FR190293, FR209602-4, FR220897, FR220899, FR227673) | *Coleophoma empetri* *Coleophoma crateriformis* | Iwamoto et al. (1994); Kanasaki et al. (2006a, b, c) |
| | *Chalara* sp. | |
| | *Tolypocladium parasiticum* | |
| Cryptocandin | *Cryptosporiopsis quercina* | Strobel et al. (1999) |

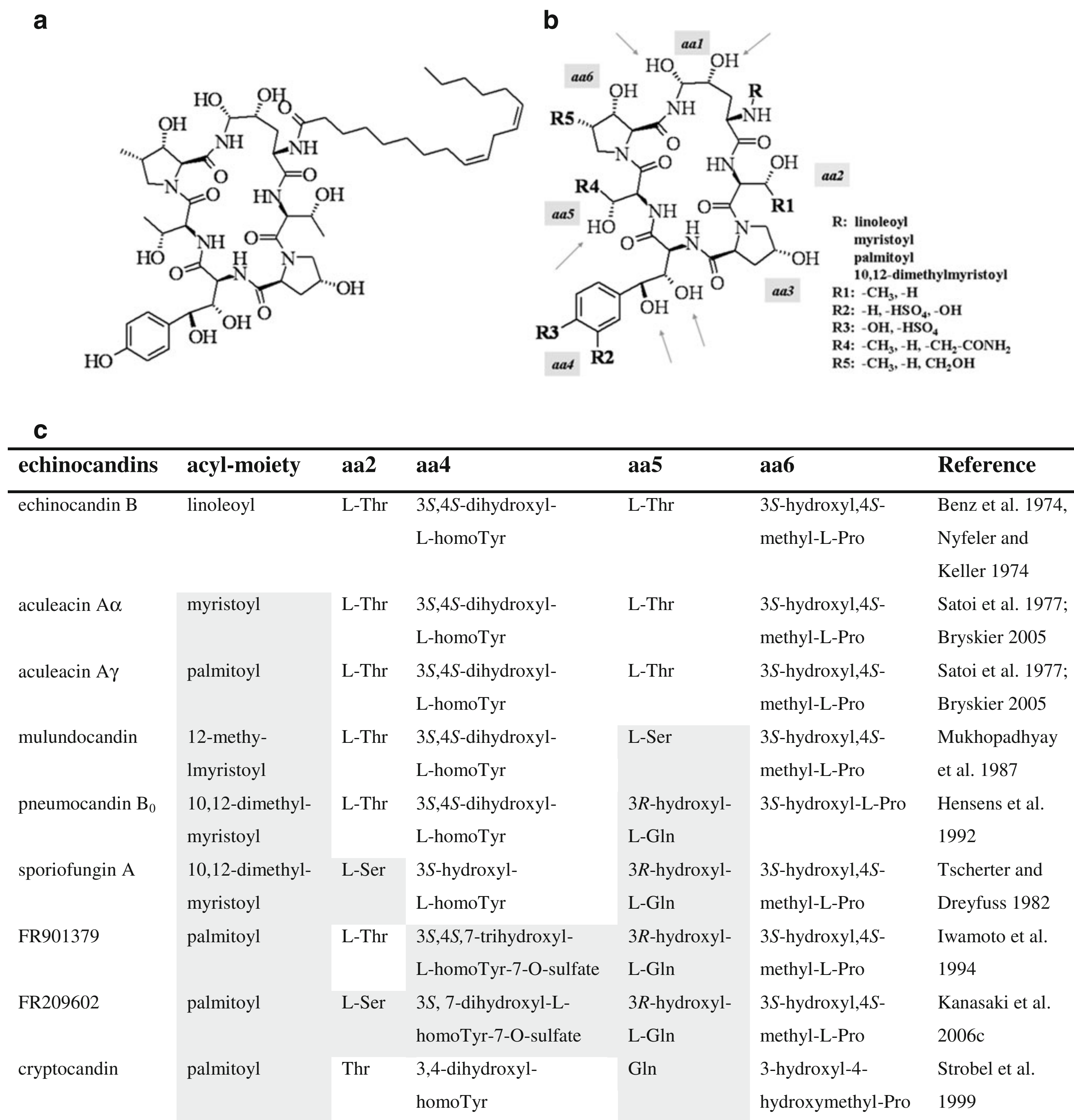
[a]The taxonomical position of *Aspergillus* subspecies is questionable, and therefore their name is not a validly published name (Geiser et al. 2007; Peterson 2008).

[b]This strain was reclassified as *A. rugulosus* (Tóth et al. 2011)

[c]Originally described as *Zalerion arboricola* but later reclassified (Bills et al. 1999)

inhibit or increase, most likely indirectly—the β-1,3-glucan synthase activity of the legume, *Glycine max*.

In the case of fungi, echinocandins bind non-competitively to the catalytic subunit of β-1,3-glucan synthases, which are membrane protein complexes and are responsible for the synthesis of cell wall β-1,3-glucans (Perez et al. 1981; Radding et al. 1998; Sawistowska-Schröder et al. 1984; Taft et al. 1988; Tang et al. 1991). A study by Chen et al. (2011), suggested that the acyl side chain of the echinocandins may interact with the cell membrane. In ascomycetous yeasts, there are three paralog genes encoding β-1,3-glucan synthase catalytic subunits. In the baker's yeast *Saccharomyces cerevisiae*—and in many other yeasts—Fks1 is the most active, whilst Fks2 is expressed during the sexual cycle, sporulation starvation conditions (Mazur et al. 1995), and Fks3 is involved in spore wall assembly (Ishihara et al. 2007). In certain yeasts—such as *Candida glabrata*—Fks1 and Fks2 are functionally redundant (Katiyar et al. 2012). The genomes of *Aspergillus* species typically contain only one β-1,3-glucan synthase catalytic subunit gene (*fks1*). Indirect evidence demonstrates that in addition to Fks1, echinocandins can also inhibit Fks2 β-1,3-glucan synthase catalytic subunits (Katiyar et al. 2012). The inhibition of β-1,3-glucan synthesis requires the uptake of echinocandins by sensitive cells (Paderu et al. 2004). Paderu et al. (2004) demonstrated that at low (<1 mg/ml) concentrations, a high-affinity facilitated-diffusion transporter mediates the uptake of caspofungin, a semisynthetic pneumocandin $B_0$ derivative (see below) in *Candida albicans*. At higher concentrations, nonspecific uptake can also progress (Paderu et al. 2004). The specificity of this group of antifungals for β-1,3-glucan synthesis was demonstrated by the observation that echinocandins were ineffective in inhibiting chitin or mannan synthesis (Perez et al. 1981). In baker's yeast, echinocandins increase the osmotic sensitivity of the cells and cause lysis even in biofilms as a consequence of the inhibition of β-1,3-glucan synthesis (Bachmann et al. 2002a, b; Cassone et al. 1981; DiDone et al. 2011; Drouhet et al. 1990; Yamaguchi et al. 1982). Cell lysis is restricted mainly to the growing tip of the daughter cell (Cassone et al. 1981; DiDone et al. 2011; Drouhet et al. 1990; Yamaguchi et al. 1982). Among the secondary effects, reductions in the membrane sterol content as well as increased cell wall chitin contents are notable (Pfaller et al. 1989; Walker et al. 2012). The inhibition of cell wall synthesis also causes morphological alterations. After echinocandin treatment, cells form aggregates because the separation of daughter and mother cells fails (Drouhet et al. 1990), and the formation of hyphae is also inhibited (Gil et al. 1994; Petraitiene et al. 1999). More recently, Hao et al. (2012) demonstrated that besides necrotic cell lysis, caspofungin also induced a metacaspase-1 dependent apoptosis in *C. albicans*. In *Aspergillus* spp., the effects of echinocandins are fungistatic rather than fungicidal (Bowman et al. 2002; Douglas 2006), which can be explained by differences in cell wall composition and in the regulation of cell wall stress responses in yeasts and




| echinocandins | acyl-moiety | aa2 | aa4 | aa5 | aa6 | Reference |
|---|---|---|---|---|---|---|
| echinocandin B | linoleoyl | L-Thr | 3*S*,4*S*-dihydroxyl-L-homoTyr | L-Thr | 3*S*-hydroxyl,4*S*-methyl-L-Pro | Benz et al. 1974, Nyfeler and Keller 1974 |
| aculeacin Aα | myristoyl | L-Thr | 3*S*,4*S*-dihydroxyl-L-homoTyr | L-Thr | 3*S*-hydroxyl,4*S*-methyl-L-Pro | Satoi et al. 1977; Bryskier 2005 |
| aculeacin Aγ | palmitoyl | L-Thr | 3*S*,4*S*-dihydroxyl-L-homoTyr | L-Thr | 3*S*-hydroxyl,4*S*-methyl-L-Pro | Satoi et al. 1977; Bryskier 2005 |
| mulundocandin | 12-methy-lmyristoyl | L-Thr | 3*S*,4*S*-dihydroxyl-L-homoTyr | L-Ser | 3*S*-hydroxyl,4*S*-methyl-L-Pro | Mukhopadhyay et al. 1987 |
| pneumocandin $B_0$ | 10,12-dimethyl-myristoyl | L-Thr | 3*S*,4*S*-dihydroxyl-L-homoTyr | 3*R*-hydroxyl-L-Gln | 3*S*-hydroxyl-L-Pro | Hensens et al. 1992 |
| sporiofungin A | 10,12-dimethyl-myristoyl | L-Ser | 3*S*-hydroxyl-L-homoTyr | 3*R*-hydroxyl-L-Gln | 3*S*-hydroxyl,4*S*-methyl-L-Pro | Tscherter and Dreyfuss 1982 |
| FR901379 | palmitoyl | L-Thr | 3*S*,4*S*,7-trihydroxyl-L-homoTyr-7-O-sulfate | 3*R*-hydroxyl-L-Gln | 3*S*-hydroxyl,4*S*-methyl-L-Pro | Iwamoto et al. 1994 |
| FR209602 | palmitoyl | L-Ser | 3*S*, 7-dihydroxyl-L-homoTyr-7-O-sulfate | 3*R*-hydroxyl-L-Gln | 3*S*-hydroxyl,4*S*-methyl-L-Pro | Kanasaki et al. 2006c |
| cryptocandin | palmitoyl | Thr | 3,4-dihydroxyl-homoTyr | Gln | 3-hydroxyl-4-hydroxymethyl-Pro | Strobel et al. 1999 |

**Fig. 1** Natural echinocandins. **a** Chemical structure of echinocandin B. **b** Generalized chemical structure of natural echinocandins. **c** Composition of some natural echinocandins aa1 and aa3 are 4R,5R-dihydroxy-L-Orn and 4*R*-hydroxyl-L-Pro, respectively, in the echinocandins presented in this table

moulds (Dichtl et al. 2012). The inhibition of the biosynthesis of β-1,3-glucans in moulds decreases the growth rate, increases the susceptibility of hyphal tips to osmotic lysis, elevates the chitin content of the cell wall and causes severe morphological changes (Bowman et al. 2002; Kurtz et al. 1994a, b; Verwer et al. 2012). However, echinocandin treatment fails to completely inhibit hyphal growth and only has a minor effect on the sub-apical regions (Bowman et al. 2002). Morphological alterations include the formation of highly branched hyphal tips, swollen germ tubes and balloon-like cells (Bowman et al. 2002; Kurtz et al. 1994a, b).

Structure–activity relationship (SAR) experiments demonstrated that the free phenolic group in the homoTyr

residue as well as the presence of the fatty acyl chain are essential for antifungal activity (Balkovec et al. 1993; Debono et al. 1988, 1989; Klein et al. 2000; Zambias et al. 1992). The acyl chain should be C-12 or longer (C-18 is the optimal length) and should possess a sufficient lipophilicity for optimal antifungal activity (Debono et al. 1988, 1989). The rigidity of the hexapeptide ring also proved to be important, and changes in the peptide scaffold disrupting the internal hydrogen bonds reduced the antifungal activity (Zambias et al. 1992). The presence of the 3-hydroxy-4-methylproline residue enhanced the activity, whilst the vicinal diols were not essential (Lal et al. 2003; Zambias et al. 1992). More recently, Yao et al. (2012) suggested that it is beneficial when the first three amino acid residues (aa1–aa3) represent a hydrophobic structural motif whilst the last three amino acid residues (aa4–aa6) form a hydrophilic core. They also demonstrated that the structure of the hexapeptide scaffold could affect the SAR of the acyl side chain (Yao et al. 2012).

## Biosynthesis of echinocandins

Only limited information is available on the biosynthesis of echinocandins. Given their chemical structure, it can be assumed that the cyclic hexapeptide scaffold is synthesized by a non-ribosomal peptide synthase (NRPS) consisting of six modules. Early studies, based on stable isotope tracer techniques, demonstrated that labels from DL-[2-$^{13}$C]-Tyr and [2-$^{13}$C]-acetate enriched the L-homoTyr residues of pneumocandin $A_0$ at positions C-3 and C-2, respectively, in *G. lozoyensis* (Adefarati et al. 1991). This observation suggests that L-homoTyr should be synthesized from Tyr (phenyl-piruvate) via chain elongation with acetyl-CoA. The same study demonstrated that hydroxyl-L-Gln, and dihydroxyl-L-Orn originate from L-Gln whilst 10,12-dimethylmyristyl acid is synthesized from acetyl-groups, except for the methyl-moieties which are supplied by *S*-adenosyl-Met (Adefarati et al. 1991, 1992). 3-Hydroxyl-4-methyl-Pro is formed from L-Leu and not from L-Pro (Adefarati et al. 1991), as has also been demonstrated in several other microorganisms. For example in the case of nostopeptolide produced by *Nostoc* sp. GSV224, (2S,4S)-methyl-Pro is synthesized from L-Leu via (2S, 4S)-5-hydroxy-Leu, (2S, 4S)-$\gamma$-methyl-glutamic acid–$\gamma$-semialdehyde and (3S, 5S)-3-methyl-$\Delta^1$-pyrroline-5-carboxylic acid as intermediers (Hoffmann et al. 2003).

The involvement of non-heme iron, $\alpha$-ketoglutarate dependent oxygenases in pneumocandin biosynthesis (formation of hydroxyl-L-Pro) was demonstrated by Petersen et al. (2003). The addition of 3*S*- or 4*S*-hydroyl-L-Pro to the fermentation medium efficiently altered the ratio of pneumocandin $B_0$ and $C_0$ (containing 3*S*- and 4*S*-hydroyl-L-Pro residues, respectively) suggesting that at least the hydroxylation of L-Pro precedes the synthesis of the hexapeptide scaffold (Connors and Pollard 2004). Besides the diversity of pneumocandins at position 6 (e.g. 3*S*-, 4*S*-hydroyl-L-Pro, 3*S*-hydroxyl,4*S*-methyl-L-Pro), the relatively broad substrate specificity of the NRPS active sites can be responsible for the formation of “Ser analogue” pneumocandins (Connors and Pollard 2004). Whole genome sequencing of *G. lozoyensis* has been completed (Youssar et al. 2012), and preliminary data predict the presence of three PKS-NRPS hybrid clusters and six NRPS clusters in this species, among which two putative NRPS have six modules, as expected for the biosynthesis of pneumocandin (Youssar et al. 2012).

The recent discovery of the *ecd* gene cluster responsible for the synthesis of echinocandin B in *A. rugulosus* brought a breakthrough in this field (Cacho et al. 2012). The *ecd* gene cluster involves 12 genes (*ecdA-L*), where *ecdA* encodes a NRPS consisting of 6 modules, which all contain a condensation (C), adenylation (A) and thiolation (T) domain, start with an extra $T_0$ domain at N-terminus and end with a terminal condensation domain ($C_T$) (Fig. 2). Genes *ecdI* and *ecdH* encode a fatty-acyl-AMP ligase and a cytochrome P450 heme-iron-dependent oxygenase, respectively. Meanwhile, *ecdK* and *ecdG* code for non-heme iron, $\alpha$-ketoglutarate dependent oxygenases, *ecdC*, *ecdD* and *ecdL* are transporter genes, and *ecdB*, *ecdE*, *ecdF* and *ecdJ* encode a transcription factor, a glycosyl hydrolase, a glycosidase and a hypothetical protein, respectively. In their work, Cacho et al. (2012) proposed the following biosynthetic steps (Fig. 2): lipo-initiation (1), extension (2), termination (3) and hydroxylation (4).

The biosynthetic steps in detail:

Step (1): Lipo-initiation. Due to the importance of the acyl-chain in the biological activity (Debono et al. 1995), lipo-initiation is a critical step in the biosynthetic process of lipopeptides. In certain NRPSs (e.g. in the synthesis of mycosubtilin; Hansen et al. 2007), a fatty acid synthetase-like module at the N-terminus of NRPS is responsible for lipo-initiation. In the case of other lipopeptides, a fatty acyl ligase activates and transfers the lipid chain either to coenzyme A (e.g. in surfactin formation; Kraas et al. 2010) or to a free thiolation domain (e.g. in the synthesis of daptomycin; Wittmann et al. 2008). After that, the fatty acid condensates with an amino acid in a reaction catalyzed by the first condensation domain of the NRPS. A variation of the latter case occurs during ECB biosynthesis. Namely, EcdI forms linoleoyl-AMP, transfers it to the $T_0$ domain of EcdA, and the first condensation domain responsible for the formation of

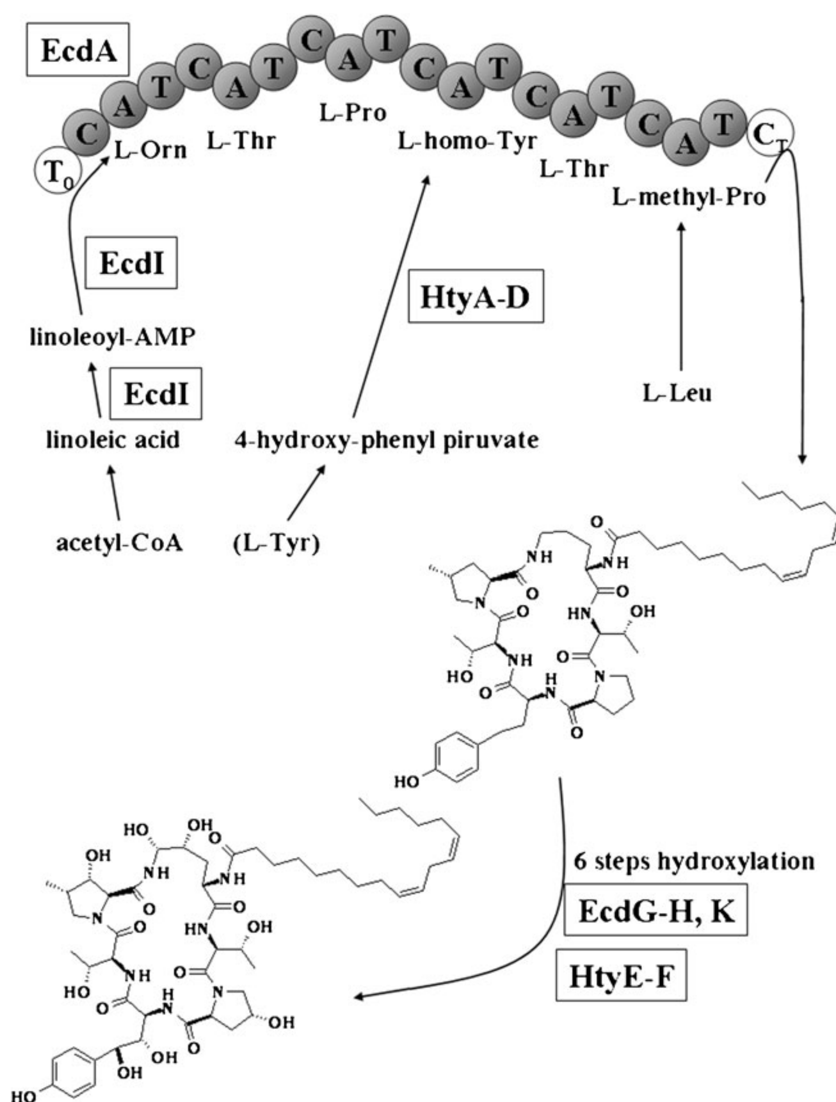



**Fig. 2** Schematic representation of the echinocandin B biosynthetic pathway according to Cacho et al. (2012). C, A, T, $C_T$ and $T_0$ are domains of the NRPS encoded by the *ecdA* gene. C – condensation domain, A – adenylation domain, T- thiolation domain, $T_0$ – an extra thiolation domain involved in lipo-initiation, $C_T$ - a terminal condensation domain responsible for macrocyclization. The gene *ecdI* encodes a fatty-acyl-AMP ligase responsible for lipo-initiation. The *ecdH* and *htyF* encode cytochrome P450 heme-iron-dependent oxygenases; *ecdK*, *ecdG* and htyE code for non-heme iron, α-ketoglutarate dependent oxygenases. The *htyA*-D genes encode enzymes responsible for the 4-hydroxy-phenyl-piruvate → L-homoTyr conversion. See text for further details

*N*-linoleoyl-Orn intermediate. The only essential difference between lipo-initiation of ECB and daptomycin biosynthesis is that the fatty acyl acceptor thiolation domain is part of the NRPS in the case of ECB biosynthesis (Cacho et al. 2012).

Step (2): Extension. *N*-linoleoyl-Orn is sequentially extended with L-Thr, L-Pro, L-homo-Tyr, L-Thr and *4S*-methyl-L-Pro to form a linear lipohexapeptide.

Step (3): Termination. The $C_T$ domain of EcdA, which is a typical domain of fungal NRPSs, catalyses the formation of cyclic compounds (Gao et al. 2012) and, hence, is responsible for the macrocyclization of the hexapeptide chain. The cyclic lipopeptide scaffold is released from the NRPS thereafter.

Step (4): Hydroxylation. The released unhydroxylated intermediate undergoes six rounds of hydroxylation catalyzed by non-heme iron, α-ketoglutarate-dependent oxygenases and cytochrome P450 heme-iron-dependent oxygenases. Vicinal diols of ECB are assumed to be formed by two enzymes (Cacho et al. 2012).

An alternative pathway suggests that amino acids should be hydroxylated prior to, and not following, hexapeptide formation (Cacho et al. 2012). Considering the lability of hemiaminals (Hensens et al. 1992), at least the formation of dihydoxyl-L-Orn occurs most likely after the synthesis of the hexapeptide scaffold (Cacho et al. 2012). Since the genome of *A. rugulosus* contains only one echinocandin gene cluster (Cacho et al. 2012), *ecd* should also be responsible for the formation of echinocandins C and D, which only differ from echinocandin B in the hydroxylation of the amino-acid scaffold.

Among the substrates of echinocandin B synthesis, linoleic acid, L-Orn, L-Thr and L-Pro, but not L-homoTyr and L-

methyl-Pro, are intermediates of the primary metabolism. Enzymes catalyzing the synthesis of L-homoTyr are encoded by the *hty* gene cluster, which is thought to be located in the same chromosome as the *ecd* cluster. Among the six genes belonging to *hty*, four genes (*htyA-D*, encoding a synthase, isomerase, dehydrogenase and a transaminase) are responsible for the 4-hydroxy-phenyl-piruvate → L-homoTyr conversion, which involves a chain elongation step by acetate. The *htyE-F* genes, which code for a non-heme iron, α-ketoglutarate-dependent oxygenase and a cytochrome P450 heme-iron-dependent oxygenase, respectively, are most likely involved in the hydroxylation of L-homoTyr prior to or after the synthesis of the hexapeptide (Cacho et al. 2012). The origin of L-methyl-Pro has not yet been elucidated but L-methyl-Pro may be synthesized from L-Leu. If this were the case, only the enzyme responsible for hydroxyl-Leu formation (possibly a non-heme iron, α-ketoglutarate dependent oxygenase) would be encoded by the *ecd* or the *hty* gene cluster (Cacho et al. 2012). All other genes involved in methyl-Pro biosynthesis cannot belong to these clusters, suggesting that ECB is a product of the cooperative and concerted action of several gene clusters located in the genome of *A. rugulosus*.

## Fermentation of echinocandins

Because echinocandin-type lipohexapeptides possess a high-complexity chemical structure, their industrial-scale production is based on fermentation. Among the natural echinocandins, echinocandin B, pneumocandin $B_0$ and FR901379 are produced for commercial purposes. Since the fermentation and purification costs of natural echinocandins are the primary reason for the high costs of semisynthetic derivatives, the optimization of the fermentation process is crucial to make a competitive product. Not surprisingly, little information has been published in this field so far.

Pneumocandin $B_0$ is fermented by *G. lozoyensis*, where the wild-type strain produces predominantly pneumocandin $A_0$ with the ratio of pneumocandin $A_0$/pneumocandin $B_0$= 7:1 in basal medium. Classic microbial biotechnological techniques including mutation-selection and medium optimization were employed to obtain suitable producer strains and to achieve satisfactory fermentation conditions for the industrial production of pneumocandin $B_0$, an antimycotic, which is structurally less similar to echinocandin B than pneumocandin $A_0$ (Connors et al. 2000; Masurekar et al. 1992; Petersen et al. 2001; Tkacz et al. 1993). The main challenge of any cost-effective production technology is the reduction of the formation of minor side-products (e.g. pneumocandin $C_0$, $D_0$, $E_0$ and the "Ser analogue pneumocandins"), which may cause severe problems during the isolation and purification of pneumocandin $B_0$ (Connors and Pollard 2004). Among the minor pneumocandins, pneumocandin $C_0$ (containing 4*R*-hidroxyl-L-Pro residue instead of 3*R*-hydroxyl-L-Pro of pneumocandin $B_0$) is the most important contaminant. Several factors have been identified, which increase both the pneumocandin $B_0$ titer and the pneumocandin $B_0$/pneumocandin $C_0$ ratio. High osmolality, which is achieved by high residual fructose concentrations or by midcycle additions of NaCl or $Na_2SO_4$, slightly decreases pneumocandin $B_0$ formation but markedly decreases the quantity of pneumocandin $C_0$ (Connors et al. 2000). The addition of L-Pro to the medium at a concentration of 5–10 g/l increases the yield of pneumocandin $B_0$ and also hinders the intracellular formation of 4*R*-hidroxyl-L-Pro and, therefore, the production of pneumocandin $C_0$. However, at high L-Pro levels, this amino acid can also be incorporated into the hexapeptide scaffold instead of 4*R*-hidroxyl-L-Pro, which results in elevated pneumocandin $E_0$ titers (Connors and Pollard 2004). The addition of either L-Thr or L-Ser at a high concentration (5 g/l) reduces pneumocandin $B_0$ titers but L-Ser increases the ration of "Ser analogues", whilst L-Thr decreases it (Connors and Pollard 2004).

A highly dissolved oxygen concentration is needed for the efficient production of pneumocandin. The critical dissolved oxygen tension for antimycotic production is 20 % air saturation although for growth this value is only 2 % (Connors and Pollard 2004; Pollard et al. 2002). Similarly to other secondary metabolites, high oxygen tensions are critical in the scale-up of the fermentation processes (Pollard et al. 2002, 2007). High oxygen tensions facilitate the hydroxylations of amino acids and, consequently, increase the ratio of highly hydroxylated derivatives such as pneumocandin $D_0$ with 10 hydroxyl-groups whilst decreasing the formations of pneumocandin $B_1$ and $B_5$ with "only" 8 hydroxyl-groups (Connors and Pollard 2004). A narrow temperature range between 23.5 and 25 °C can be regarded as optimal for both pneumocandin production and the growth of *G. lozoyensis*. Elevations in the cultivation temperature decrease the antimycotic yields and above 28 °C both the production and the growth is blocked (Connors and Pollard 2004). Among other factors, such as magnesium limitation, optimization of phosphate concentration and C/N ratio, and the addition of L-Glu to the medium, which may increase pneumocandin production under certain fermentation conditions (Connors and Pollard 2004; Masurekar et al. 1992, Tkacz et al. 1993), the use of soybean oil and/or the application of complex organic nitrogen sources such as Pharmamedia enhance pneumocandin formation (Masurekar et al. 1992). This is remarkable because plant oils (e.g. rice oil, sunflower oil and soybean oil) and complex nitrogen sources are also clearly beneficial for the formation of other echinocandins including echinocandin

B and FR901379 (Boeck and Kastner 1981; Kanda et al. 2009).

FR901379 is fermented by overproducing mutants of *Coleophoma empetri* at the optimal growth temperature of the strains (23–27 °C) (Kanda et al. 2009). Besides the supplementation of rice oil and complex organic nitrogen sources, the addition of appropriate amounts of $MgSO_4$ as a sulfate source (sulfate is needed to support the biosynthesis of the catechol-sulfate moiety) together with the maintenance of a low phosphate concentration, a high osmolality and a sufficient oxygen supply in the culture medium were beneficial for the production of the antimycotic (Kanda et al. 2009, 2010). Culture viscosity is reported as a serious but solvable problem during the scale-up of the fermentation (Kanda et al. 2010).

Echinocandin B is produced by industrial *A. rugulosus* (*Emericella rugulosa*) strains. In addition to increasing echinocandin B titers and reducing the formation of echinocandin B analogues, it is important to achieve a competitive technology to eliminate other contaminants (Klich et al. 2001; Tóth et al. 2011). Among these contaminants, sterigmatocystin, an aflatoxin-related, carcinogenic mycotoxin is the most important (Rank et al. 2011). Since the elevated echinocandin B formation is generally accompanied by enhanced sterigmatocystin production in industrial strains, the high sterigmatocystin contents of the fermentation broths increase the costs of downstream processing and wastewater treatments considerably during echinocandin B fermentations. Hodges et al. (1994, 2000) isolated and characterized a mutant defective in sterigmatocystin production and the authors found that a chromosomal translocation occurred within the *stcW* gene encoding the enzyme catalyzing the conversion of averufin to 1-hidroxyl-versicolorone. Since this mutation blocks the formation of any product harboring a carcinogenic *bis*-furan ring, this strain is significantly safer than the wild-type strain (Hodges et al. 2000). Importantly, the mutation did not affect echinocandin B production (Hodges et al. 1994).

The temperature optimum for echinocandin B production is below 30 °C (generally between 24 and 28 °C) similarly to those observed with pneumocandin $B_0$ and FR901379. However, the growth optimum of *A. rugulosus*, in contrast to *G. lozoyensis* and *C. empetri*, is well above 30 °C (around 37 °C) and *A. rugulosus* grows quite poorly at echinocandin B-producing temperatures (Klich et al. 2001, Tóth et al. 2011). Complex organic nitrogen sources such as yeast extract, soybean peptone, casein peptone and soybean powder are suitable nitrogen sources for echinocandin B production. In the absence of complex nitrogen sources, both echinocandin B and sterigmatocystin productions decreased and the addition of certain amino acids to this simplified medium enhanced only echinocandin B synthesis without increasing the sterigmatocystin titers (Tóth 2012). Interestingly, the best results were obtained with L-Tyr and L-Phe (15 g/l) (Tóth 2012), which are good sources of 4-hydroxyl-phenyl-pyruvate, the precursor of homoTyr synthesis (Cacho et al. 2012). Importantly, plant oils also enhance echinocandin B production (Boeck and Kastner 1981; Tóth et al. 2011; Tóth 2012) and are generally used in echinocandin fermentations to enhance the formation of the acyl side-chains of the antimycotics. However, they may have pleiotropic effects on echinocandin production: For example, in the case of *A. rugulosus*, sunflower oil can be a good source of linoleic acid but it is also a suitable carbon source supporting a relatively fast growth even at lower temperatures (Tóth et al. 2011). *A. rugulosus* showed a surprisingly high sensitivity to ECB at 37 °C, a temperature at which no ECB production takes place (Tóth et al. 2012). The addition of sunflower oil (or paraffin oil) to the culture medium decreased the antifungal effect of echinocandin B on the producer strain (Tóth 2012), and this beneficial feature of oils may also influence positively the production of echinocandin B at lower temperatures.

## Semisynthetic echinocandins

The natural echinocandins have certain unfavorable properties, which may limit their clinical applications. For example, depending on their structure, many of these compounds show a strong hemolytic activity, they have relatively narrow spectra and some of them even show low water solubility and/or stability. Among the natural echinocandins, pneumocandins are reported to possess minute hemolytic effects (Schmatz et al. 1992), and even the most hemolytic pneumocandins, pneumocandin $A_2$ and $C_0$, showed only modest hemolytic activity (Schmatz et al. 1992). The hemolytic property of other echinocandins—similarly to their antifungal activity—depends greatly on the nature of the acyl-side chain. In the case of echinocandin B, the replacement of the linoleoyl side-chain with either octyloxybenzoyl (cilofungin) or pentyloxyterphenyl (anidulafungin) side chains significantly reduced its hemolytic activity without affecting significantly their antifungal properties (Ghannoum and Maurissa 2005; Gordee et al. 1988). Similarly, substitution of the palmytoyl-group with either octyloxybenzoyl, decaoxybenzoyl or tetraoxyterphenyl acyl side-chains or, alternatively, with side chains containing both benzene and heterocyclic rings (e.g. the isoxazole ring in micafungin) markedly decreased the hemolytic activity of FR901379, a "catechol-sulfate" echinocandin, without decreasing its antifungal activity (Fujie et al. 2001, Fujie 2007).

"Catechol-sulfate" echinocandins are water-soluble owing to their sulfate moiety (Fujie 2007; Iwamoto et al. 1994; Kanasaki et al. 2006a, b, c). The water solubility of other

echinocandins can also be increased, e.g. by replacing the 3*S*-hydroxyl,4*S*-methyl-L-Pro residue with 4*R*-amino-L-Pro (Klein et al. 2000). In the case of pneumocandins, the replacement of the 3*R*-hydroxyl-L-Gln residue with 3*R*-hydroxyl-L-Orn and/or the substitution of the 5*R*-hydroxyl group of 4*R*,5*R*-dihydroxyl-L-Orn residue with 5R-2-aminoethoxy or 5*S*-2-aminoethylamino groups (like in caspofungin) also increased the solubility (Bouffard et al. 1994; Kurtz et al. 1994a; Vazquez et al. 1995). Alternatively, the modification of the 4*R*,5*R*-dihydroxy-L-Orn residue *via* diol-keto transposition to 4-(2-aminoethyl)amino-L-Orn (in aminocandin) also resulted in a water-soluble compound (Aszodi et al. 2002). The water stability of echinocandins greatly depends on the presence of the hemiaminal group. Substitution of the 5R-hydroxyl group of the 4*R*,5*R*-dihydroxyl-L-Orn residue increases the stability of the compound, as was demonstrated with mulundocandin and pneumocandins (Bouffard et al. 1994; Kurtz et al. 1994a; Lal et al. 2003;Vazquez et al. 1995).

The antifungal activity of natural echinocandins can also be enhanced by either the incorporation of an appropriate (and non-hemolytic) side chain or the modification of the hexapeptide ring. The above mentioned modifications aiming at either the improvement of water solubility or the reduction of the hemolytic activity also increased the antifungal activity of the compounds and the increased antifungal activity concomitantly widened the spectra of sensitive fungi. From a therapeutic point of view, it is worth noting that although the increased anti-*Candida* activity coincided with an elevated activity against both *Aspergillus fumigatus* and *P. carinii* these semisynthetic derivatives are still inactive against *C. neoformans*.

Among the numerous semisynthetic echinocandins developed in the last decades, caspofungin (trade name: Cancidas, Merck and Co.), micafungin (trade name: Mycamine, Astellas Pharmaceuticals) and anidulafungin (trade name: Eraxis, Pfizer Pharmaceuticals) are Food and Drug Administration (FDA)-approved echinocandins, and aminocandin is undergoing clinical evaluation (Mishra and Tiwari 2011) (Fig. 3). These semisynthetic echinocandins are produced from pneumocandin $B_0$ (caspofungin), echinocandin B (anidulafungin), FR901379 (micafungin) and deoxymulundocandin (aminocandin) via chemical modification of the hexapeptide scaffold (caspofungin), substitution of the fatty acid side chain (anidulafungin, micafungin) or both (aminocandin) (Fig. 3). The substitution of the fatty acyl side-chain is carried out by the enzymatic hydrolysis of the fatty acid-Orn amide bond by aculeacin A acylase and the subsequent chemical acylation of the deacylated hexapeptide ring. Aculeacin A acylase is an extracellular enzyme of *Actinoplanes utahensis* NRRL12052, which is composed of two non-identical subunits (Takeshima et al. 1989). It is encoded by a single gene, shares sequence similarity with β-lactam acylases (Inokoshi et al. 1992), and shows penicillin acylase activity as well (Torres-Bacete et al. 2007). Hydrolytic enzymes similar to aculeacin A acylase have been isolated from *Streptomyces* spp. and even from filamentous fungi (Ueda et al. 2010, 2011b). Both aculeacin A acylase and FR901379 acylase from *Streptomyces* sp. no. 6907 were efficiently expressed in *Streptomyces lividans* (Inokoshi et al. 1992; Ueda et al. 2011b), and aculeacin A acylase showed superb catalytic properties in an immobilized form (Hormigo et al. 2010). In the case of FR901379-acylase, large-scale production was also reported (Ueda et al. 2011a).

## Clinical significance

All three semisynthetic echinocandins used in human therapy, caspofungin, micafungin and anidulafungin, are approved for the treatment of oesophageal candidiasis and invasive candidiasis in adults and in children (caspofungin) over three months of age. Micafungin has been FDA-approved for antifungal prophylaxis in hematopoietic cell transplantation and caspofungin has been approved for empirical therapy of febrile neutropenia and for salvage and primary therapy of invasive aspergillosis. All of them have limited oral bioavailability and are therefore administered by intravenous infusion.

The FDA-approved echinocandins show good fungicidal or fungistatic activity against the human pathogenic fungi, *Candida* and *Aspergillus* species, in vitro at clinically attainable concentrations (Chen et al. 2011; Pappas et al. 2009; Perlin et al. 2007; Pfaller et al. 2009, 2011b). Echinocandins show concentration-dependent fungicidal activity either in RPMI-1640 or antibiotic medium 3 against many *Candida* species including *C albicans*, *C. glabrata*, *C tropicalis*, *C. dubliniensis,* C *krusei*, *C. lustitaniae*, *C. parapsilosis* and *C. guilliermondii* (Chen et al. 2011; Perlin et al. 2007; Pfaller et al. 2011b; Sóczó et al. 2007). The majority of these species (e.g. *C. albicans* and *C. tropicalis*) are innately susceptible to echinocandins with minimum inhibitory concentrations (MICs) in the range of 0.015-0.25 mg/L. Other species (e.g. *C. parapsilosis*, *C. lusitaniae* and *C. guilliermondii*) show higher MIC values to echinocandins in RPMI-1640 as test medium which is explained by their naturally occurring Fks1 polymorphisms (see below) (Chen et al. 2011; Garcia-Effron et al. 2008; Pfaller et al. 2009, 2011b; Perlin et al. 2007). Semisynthetic echinocandins show a strong fungistatic effect against *Aspergillus* species including *A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger* (Calvo et al. 2011, 2012; Chandrasekar and Sobel 2006; Chen et al. 2011, Mukherjee et al. 2011). Since echinocandins are unable to completely inhibit the growth of these species, clear determination of MIC is difficult. Therefore, an alternative

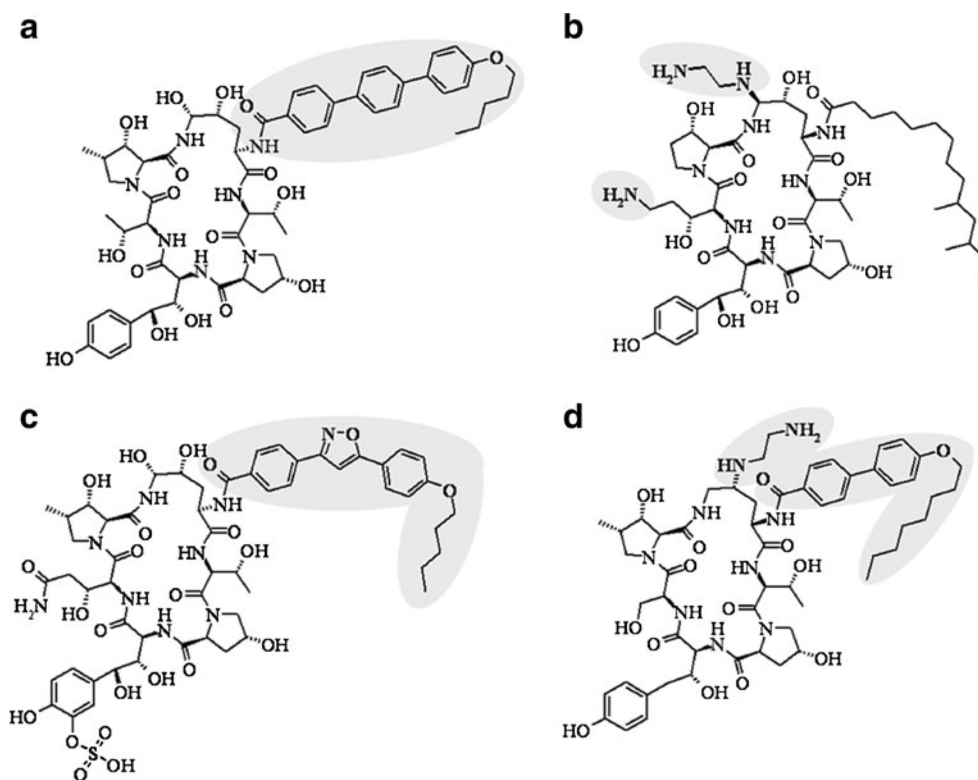



**Fig. 3** Semisynthetic echinocandins—chemical structure of anidulafungin (**a**), caspofungin (**b**), micafungin (**c**) and aminocandin (**d**). *Highlighted areas* indicate the site of modifications

method—determination of the minimum effective concentration (MEC)—was introduced to describe the activity of echinocandins against *Aspergillus* strains. MEC is defined as the lowest drug concentration which causes severe morphological changes (Arikan et al. 2001). In the case of the human pathogenic *Aspergillus* species the measured MEC values are in the range of 0.015-0.25 mg/L (Martos et al. 2010). The clinical importance of these data is debated, as successful treatment of these species with echinocandins has been reported multiple times (Pappas et al. 2009; Pfaller et al. 2011b).

Besides *Candida* and *Aspergillus* species, FDA-approved echinocandins show certain antifungal activity against *P. carinii* as well as against the filamentous form of *Penicillium marneffei*, *P. brasiliensis*, *H. capsulatum*, *Blastocystis dermatitidis* and *Coccidioides immitis* as well (Chandrasekar and Sobel 2006; Chen et al. 2011; Cushion and Collins 2011). However, some other clinically significant species are innately less susceptible to these drugs. These species include certain *Fusarium*, *Scedosporium*, *Paecilomyces*, *Scopulariopsis*, *Trichosporon*, *Cryptococcus*, *Madurella* and *Zygomycetes* strains (Bal 2010; Espinel-Ingroff 2003; Kanafani and Perfect 2008, Katiyar and Edlind 2009; Maligie and Selitrennikoff 2005; van de Sande et al. 2010.) Involvement of reduced β-1,3-glucan synthase sensitivity to echinocandins was demonstrated in the intrinsic resistance of *Fusarium* and *Scedosporium* strains (Johnson et al. 2011; Katiyar and Edlind 2009). In contrast, the β-1,3-glucan synthase complex of *C. neoformans* is sensitive to echinocandins; however echinocandins are ineffective against this species which may be explained by its melanin production (Maligie and Selitrennikoff 2005; van Duin et al. 2002). Inducible melanin production was also observed in *H. capsulatum* and *Madurella mycetomatis* and this phenomenon was accompanied by reduced susceptibility to echinocandins in these species (Maligie and Selitrennikoff 2005; van de Sande et al. 2010). Although there is no direct evidence, other mechanisms including enzymatic degradation, reduced uptake or activation of efflux systems may also play a role.

Echinocandins are highly protein-bound agents (from 97 % to 99.8 %), which leads to decreases in drug concentration available in the serum and tissues (Chen et al. 2011; Garcia-Effron et al. 2011; Odabasi et al. 2007; Paderu et al. 2007; Pfaller et al. 2011b). Echinocandin MIC values against *Candida* and *Aspergillus* species using the broth microdilution method increased by 32–1024 times in RPMI-1640 containing 50 % serum as a test medium when compared to RPMI-1640 alone (Földi et al. 2012; Garcia-Effron et al. 2011; Odabasi et al. 2007; Paderu et al. 2007). Decreased activity of echinocandins in the presence of serum against clinically important *Candida* species was also confirmed using time-kill methodology (Földi et al. 2012). However, it is supposed that some part of the protein-bound drug maintains its activity against *Candida* species (Garcia-Effron et al. 2011).

Echinocandins exert a postantifungal effect (inhibition of growth after short exposure to the echinocandins) in a

concentration-dependent manner in RPMI-1640 (Chen et al. 2011; Clancy et al. 2006; Pfaller et al. 2011b). One hour (or shorter) exposure to different echinocandins may produce more than 12 hours growth inhibition against the clinically most important *Candida* species (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. parapsilosis*) (Clancy et al. 2006; Shields et al. 2011a, b). Prolonged post-antifungal effects may contribute to the excellent efficacy of this antifungal group against *Candida* species; moreover, drugs with a marked post-antifungal effect may need less frequent administration (Chen et al. 2011; Shields et al. 2011a). Unfortunately the post-antifungal effect is less than 0.5 hours in the case of *Aspergillus* species (Chen et al. 2011).

Another important phenomenon influencing the outcome of echinocandin treatment is the paradoxical growth (PG) or Eagle effect observed with both *Candida* and *Aspergillus* species (Chamilos et al. 2007; Wiederhold 2007). PG is defined as reduced echinocandin activity well above the MIC, or a decreased killing activity of the high drug concentrations in time-kill studies (Chamilos et al. 2007; Sóczó et al. 2007). PG proved to be echinocandin-specific and species dependent. Occurrence of PG strongly depends on the test medium used, because PG observed in RPMI-1640 could be eliminated in antibiotic medium 3 as well as by bovine or human serum (Sóczó et al. 2007; Szilágyi et al. 2012). PG was rarely detected in *C. glabrata*, but was detected with all echinocandins in the case of *C. tropicalis* isolates (Chamilos et al. 2007). The precise mechanism of PG is still not well-defined, but the growing body of data suggests that echinocandin exposure as a stress signal activates cell wall remodeling *via* protein kinase C (PKC), HOG, $Ca^{2+}$-calcineurin and HSP90 pathways (Fortwendel et al. 2010; Singh et al. 2009; Singh-Babak et al. 2012; Stevens et al. 2006; Walker et al. 2008, 2012; Wiederhold 2007). Echinocandin stress increases the chitin content in the cell wall (compensatory chitin synthesis), which seems to be a common response among the most important *Candida* species (Walker et al. 2012) and *A. fumigatus* (Fortwendel et al. 2010) but also induce *fks2* in *C. glabrata* (Singh-Babak et al. 2012). Although different *Candida* species possess different basal chitin levels, treatment of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii* and some *C. krusei*, but not *C. glabrata,* clinical isolates with sub-MIC caspofungin led to activation of chitin synthesis (Walker et al. 2012). Moreover, *C. albicans*, *C. krusei*, *C. parapsilosis* and *C. guilliermondii* isolates stimulated with $CaCl_2$ and Calcofluor White were able to increase their chitin content and become less susceptible to caspofungin. The compensatory increased chitin response is reversible and fully inhibited with the chitin synthesis inhibitor nikkomycin Z (Szilágyi et al. 2012; Walker et al. 2012). The clinical importance of PG is still questionable (Bayegan et al. 2011; Clemons et al. 2006). However, echinocandin-induced increased chitin levels may explain the therapeutic failures noticed in some clinical situations, especially when elevated daily doses were administered (Betts et al. 2009; Perlin et al. 2007; Pfaller et al. 2011b). The high mortality rate due to *Candida* and *Aspergillus* species inspired clinicians to use higher-dose echinocandins for the treatment of invasive fungal infections, especially among neutropenic patients or patients suffering from invasive *C. parapsilosis* infections (Betts et al. 2009; Pappas et al. 2009). Interestingly, higher doses did not significantly improve the outcome among patients treated with 150 mg daily doses of caspofungin when compared to the traditional, 50 mg daily dosing regimen (Betts et al. 2009). Moreover, in cases of invasive infections caused by *C. glabrata* and *C. tropicalis* Betts et al. (2009) noticed numerically but not statistically higher clinical failures in the 150 mg daily dose group when compared to the 50 mg daily group, suggesting the possible role of the PG in clinical failures.

## Acquired echinocandin resistance

Acquired (secondary) resistance against echinocandins is still rare, although some increases have been detected among *C. glabrata* strains in the last few years (Pfaller et al. 2011a, 2012). This small, but clearly detectable, increase, as well as the growing number of reports on breakthrough infections during echinocandin therapy (Walker et al. 2010) have attracted attention to the molecular background of acquired echinocandin resistance.

One of the benefits of echinocandins is that they are poor substrates for most multi-drug efflux transporters (Niimi et al. 2006) and, hence, they are active against pathogenic fungal stains expressing high levels of these transporters (Bachmann et al. 2002a, b). Not surprisingly, activation of efflux systems has not been described so far as a reason for decreased susceptibility. In contrast, point mutations of the *fks1* gene encoding the catalytic subunit of the β-1,3-glucan synthase complex (the target of echinocandins) have been observed in *C. albicans* and several other *Candida* spp. including *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. tropicalis* and *C. dubliniensis*, and these mutants showed decreased susceptibilities against echinocandins. These point mutations are clustered into two hot spot regions, namely, HS1 at amino acid residues 641–649 and HS2 at residues 1345–1365 (Park et al. 2005; Perlin 2007). The most frequently observed mutations are the substitutions of Ser at position 645 with Phe, Pro or Tyr, and these alleles were proved to be dominant (Balashov et al. 2006; Park et al. 2005; Perlin 2007). Enzyme kinetics studies demonstrated that these mutations increased the inhibitory constant ($K_i$) of the enzymes (Garcia-Effron et al. 2009b; Park et al. 2005) and Garcia-Effron et al. (2009b) clearly indicated that there is a positive correlation between $K_i$ and echinocandin MIC

values. In yeasts—like in *C. glabrata*—where Fks1 and Fks2 are redundant, a hot spot mutation in Fks2 was also accompanied by an elevated echinocandin MIC (Garcia-Effron et al. 2009a; Katiyar et al. 2006). Hot spot mutation of Fks2 was detected in echinocandin resistant *S. cerevisiae* and *C. guilliermondii* strains as well (Garcia-Effron et al. 2009a; Park et al. 2005). As was mentioned earlier, mutations in the *fks1* gene are responsible for the innately decreased echinocandin susceptibilities of some *Candida* spp., e.g. *C. parapsilosis*, *C. lusitaniae*, *C. guilliermondii*, and those of certain *Fusarium* and *Scedosporium* spp. as well (Chen et al. 2011; Johnson et al. 2011; Katiyar and Edlind 2009).

Alterations in the cell wall composition and cell wall remodeling by mutations, e.g. increases in the cell wall chitin content, may be an alternative mechanism of acquired echinocandin resistance. Mutants with elevated chitin content showed a decreased susceptibility in vitro (Plaine et al. 2008), and the increased chitin content also conferred echinocandin resistance in vivo (Lee et al. 2012). Although all the echinocandin-resistant *Candida* isolates tested to date harbored hot spot *fks* mutations, Imtiaz et al. (2012) reported on an echinocandi-nresistant *C. albicans* strain, which had concomitantly both *fks1* mutation and an elevated chitin level. Moreover, Lee et al. (2012) suggested that in *C. albicans*, the increased chitin content increased the likelihood of acquiring an *fks1* hot spot mutation even in the absence of any echinocandin. These findings also suggest that therapies based on the combination of echinocandins with chitin synthase inhibitors or drugs inhibiting the function of cell wall remodeling signaling (Hsp90, calcineurin, PKC) could be promising to further increase the efficiency of these antimycotics and prevent the development of acquired resistance against them (Gansen et al. 2004; Lamoth et al. 2012).

In the case of *A. fumigatus*, the introduction of point mutations within the *fks1* gene caused elevated MEC values (Gardiner et al. 2005; Rocha et al. 2007). However, in spontaneous laboratory mutants and clinical isolates, the decreased susceptibility was not a consequence of mutations in the *fks1* gene (Arendrup et al. 2008; Gardiner et al. 2005). Overexpression of Fks1 (Arendrup et al. 2008), mutations in the *ecm33* gene encoding a cell wall protein, which is important in cell wall organization (Romano et al. 2006), and increases in chitin synthesis (Gardiner et al. 2005) may be responsible for the emerging resistance in these filamentous fungus pathogens. These data suggest that although *fks* hot spot mutations are known as the predominant source of resistance in *Candida* species, mechanisms outside *fks* mutations could be responsible for the observed elevations in the MEC values in the Aspergilli (Howard and Arendrup 2011). In the industrially important *A. rugulosus*, which is surprisingly sensitive to echinocandin B and caspofungin under non-echinocandin B producing conditions (Tóth et al. 2012), the echinocandin resistance induced under echinocandin B production was the consequence of a series of physiological changes (Tóth et al. 2012). For example, besides the induction of echinocandin transporter genes (Cacho et al. 2012), elevated chitin synthesis, increased β-1,3-glucan synthase activity as well as reduced growth rates were observed, which all contributed to the successful adaptation to increasing echinocandin B concentrations (Tóth et al. 2012).

Ben-Ami et al. (2011) demonstrated that the mutations in the *fks1* gene, which were responsible for echinocandin resistance, also decreased the fitness and virulence of *C. albicans* strains, which may limit their epidemiological and clinical impacts (Ben-Ami et al. 2011). Tóth et al. (2012) found that the echinocandin B producer *A. rugulosus* did not possess any constitutive echinocandin resistance and the adaptation to echinocandin B also enhanced its sensitivity to cell wall stress and was accompanied by a reduced growth rate. These observations may explain why the emergence of echinocandin resistance in sensitive yeasts and fungi is limited, especially when it is compared to the wide-spread azole resistance.

## Concluding remarks

The introduction of echinocandins—as the first antifungal drugs inhibiting fungal cell wall biosynthesis—into the daily clinical routine was a milestone in the development of antifungal therapies, which had previously been dominated by polyenes and azoles. The foreseeable FDA approval of new semisynthetic compounds such as aminocandin will broaden the spectrum of the clinically available echinocandin drugs in the near future. The whole genome sequencing of *G. lozoyensis* has been completed (Youssar et al. 2012) and further genome sequencing programs including echinocandin producer Aspergilli like *Aspergillus sydowi* and *Aspergillus aculeatus* are also progressing. New genome-annotation-based data may help us to shed light on the genetic and biochemical background of echinocandin biosynthesis, and may stimulate and facilitate the development of high echinocandin producer industrial strains, which will significantly decrease the costs of natural and semisynthetic echinocandins. The increasing knowledge of the mechanisms of intrinsic and acquired resistances as well as the expanding databases originating from SAR experiments can support the search for new-type semisynthetic derivatives suitable for oral administration or with better activity against *P. carinii*. To find optimal dosing strategies and/or to introduce suitable drug combination therapies to prevent the development of resistance and to improve the outcome of these antifungal treatments are also among the main objects of future research in this field.

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