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Novel fungicidal benzylsulfanyl-phenylguanidines

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ABSTRACT

A series of substituted benzylsulfanyl-phenylamines was synthesized, of which four substituted benzylsulfanyl-phenylguanidines (**665**, **666**, **667** and **684**) showed potent fungicidal activity (minimal fungicidal concentration, MFC $\leqslant$ 10 μM for *Candida albicans* and *Candida glabrata*). A benzylsulfanyl-phenyl scaffold with an unsubstituted guanidine resulted in less active compounds (MFC = 50–100 μM), whereas substitution with an unsubstituted amine group resulted in compounds without fungicidal activity. Compounds **665**, **666**, **667** and **684** also showed activity against single *C. albicans* biofilms and biofilms consisting of *C. albicans* and *Staphylococcus epidermidis* (minimal concentration resulting in 50% eradication of the biofilm, BEC50 $\leqslant$ 121 μM for both biofilm setups). Compounds **665** and **666** combined potent fungicidal (MFC = 5 μM) and bactericidal activity (minimal bactericidal concentration, MBC for *S. epidermidis* $\leqslant$ 4 μM). In an in vivo *Caenorhabditis elegans* model, compounds **665** and **667** exhibited less toxicity than **666** and **684**. Moreover, addition of those compounds to *Candida*-infected *C. elegans* cultures resulted in increased survival of *Candida*-infected worms, demonstrating their in vivo efficacy in a mini-host model.

The increasing number of immunocompromised patients, combined with advances in medical technology, has led to an increase in fungal infections, with *Candida albicans* as the major fungal pathogen. These infections are, especially in immunocompromised patients, an important cause of morbidity and mortality, despite aggressive treatment with new or more established licensed antifungal agents.[1] Apart from their existence under free-living or planktonic form, fungi and bacteria are known to form biofilms upon contact with various surfaces. Fungal biofilms, especially those of *C. albicans*, can cause infections associated with medical devices like indwelling intravascular catheters. Such infections are particularly serious because biofilm-associated *Candida* cells are relatively resistant to a wide spectrum of antifungal drugs.[2] Due to this resistance, removal of the catheter is often required to cure the infection and this can be a serious risk for the patient.[3] Biofilms can also consist of mixed species, like *C. albicans* and *Staphylococcus epidermidis*.[4] Such mixed fungal-bacterial biofilms are more resistant to antimycotics, like fluconazole, compared to single species *C. albicans* biofilms.[5] Due to toxicity, drug–drug interactions and the increasing occurrence of resistance of current antimycotics, there is an urgent need to identify novel fungicidal compounds, preferentially with activity against fungal and mixed species biofilms.[6]

In the present study, we identified novel fungicidal compounds with activity against single *C. albicans* and mixed biofilms, consisting of *C. albicans* and *S. epidermidis*, two organisms commonly found in catheter-associated infections.[5] We focused on the class of piperazine-1-carboxamidine compounds, which were recently shown to exhibit fungicidal activity against *C. albicans*.[7] Their mode of action comprises the induction of endogenous reactive oxygen species, resulting in apoptosis in susceptible yeast.[7,8] These piperazine-1-carboxamidines share overall 3D structural similarity with abafungin, a compound belonging to a new class of microbiocidal arylguanidines. Abafungin is characterized by broad fungicidal activity against various species of pathogenic fungi and dermatophytes.[9] Based on this overall 3D structural similarity between piperazine-1-carboxamidines and arylguanidines, we hypothesized that benzylsulfanyl-phenylamines, uniting structural features of piperazine-1-carboxamidines and arylguanidines (Fig. 1), are characterized by increased fungicidal activity compared to piperazine-1-carboxamidines against *C. albicans* (MFC >100 μM).[7]

Abbreviations: MFC, minimal fungicidal concentration; MBC, minimal bactericidal concentration; BEC, biofilm eradicating concentration.

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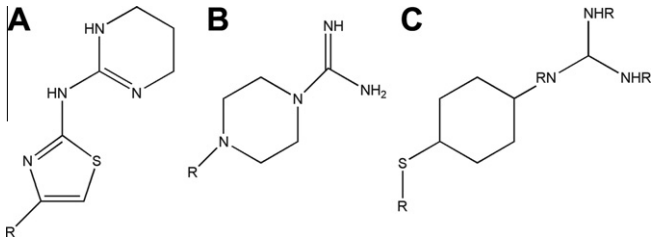


Figure 1. Overall 3D structural similarity of abafungin (A), piperazine carboxamidine (B) and benzylsulfanyl-phenylguanidine derivatives (C).

This hypothesis was further substantiated by a report on the antifungal activity of structurally related alkylsulfanyl-pyridinylguanidines.[10] In this study, we synthesized a series of benzylsulfanyl-phenylamines and assessed their fungicidal activity against *C. albicans* and *Candida glabrata*. Next, we assessed the bactericidal activity of the four most potent fungicidal benzylsulfanyl-phenylamines against *S. epidermidis*, as well as their potential to eradicate single and mixed species biofilms. Furthermore, we assessed toxicity and efficacy of these molecules in the mini-host *Caenorhabditis elegans* model.[11] The important advantage of such mini-host models is the possibility to test toxicity and efficacy of compounds in vivo on a small scale (in microtiter plates). Inclusion of the *C. elegans* model system in early stages of antifungal drug development allows the determination of in vivo nontoxic doses, the selection of the in vivo most promising molecules and the assessment of the effective concentration in vivo in a single assay, hence reducing animal testing considerably.

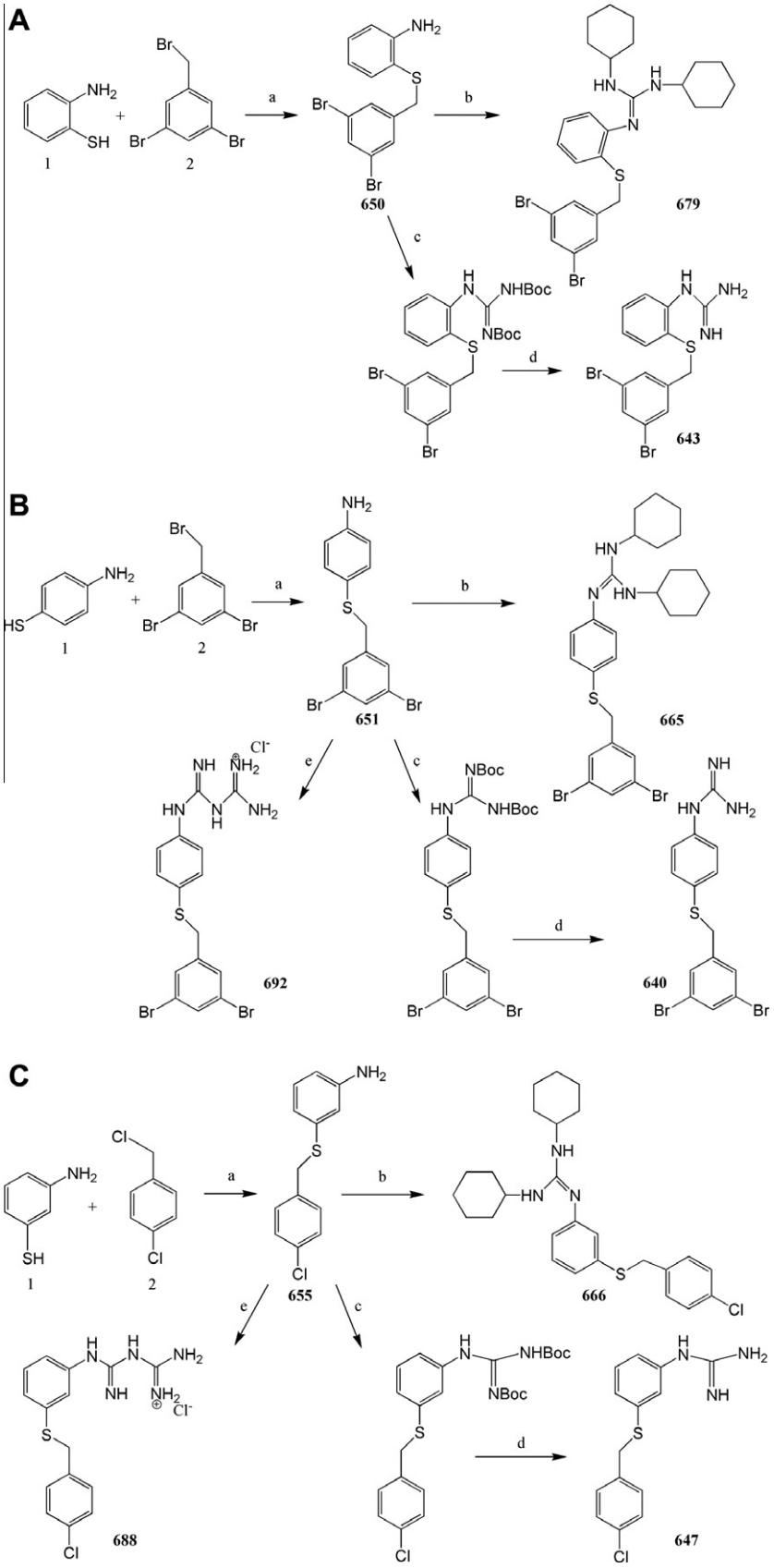
The synthetic route of the different compounds is outlined in Scheme 1. Compound **1** (1 equiv) was added to a solution of Et_3N (1 equiv) dissolved in DMF (dimethyl formamide, 3 mL mmol^{-1}), followed by compound **2** (1 equiv). The mixture was stirred at rt for 5 h. AcOEt (20 mL mmol^{-1}) and brine (20 mL mmol^{-1}) were added and the layers were separated. The organic layer was dried ($MgSO_4$), filtered and concentrated. The residue was purified by flash-chromatography on SiO_2 (Hex/AcOEt; 10:1) yielding pure *o*-, *m*- or *p*-amino derivatives **650** (Scheme 1, panel A), **651** (Scheme 1, panel B), **655** (Scheme 1, panel C), **657** (Scheme 1, panel D), **669** (Scheme 1, panel E) and **670** (Scheme 1, panel F). To synthesize compounds **640**, **641**, **642**, **643**, **647** and **649**, Di-Boc thiourea, DIPEA (diisopropyl ethyl amine) and EDCI (1:1.2:1.2) were added to respective solutions of the *o*-, *m*- and *p*-amino derivatives (compounds **651**, **670**, **669**, **650**, **655** and **657**) dissolved in DMF (3 mL mmol^{-1}). The mixture was stirred at rt for 48 h. Afterwards, additional Di-Boc thiourea, DIPEA and EDCI (1.2:1.2:1.2) were added to the mixture and stirred for 24 h. AcOEt (20 mL mmol^{-1}) was added and the solution was washed with H_2O (20 mL mmol^{-1}), $NaHCO_3$ (3×) (20 mL mmol^{-1}) and brine (20 mL mmol^{-1}). The organic layer was dried ($MgSO_4$), filtered and concentrated to give an intermediate compound, which was used in the next step without purification. This compound was dissolved in CH_2Cl_2 (1.5 mL mmol^{-1}) and cooled on an ice-bath. TFA (1.5 mL mmol^{-1}) was added and the reaction mixture was stirred for 6 h at 0 °C. The reaction mixture was concentrated in vacuo and the residue was purified by RP-HPLC to yield pure *o*-, *m*- or *p*-guanidyl derivatives **640**, **641**, **642**, **643**, **647** and **649**, respectively (Scheme 1).[12]

To synthesize *o*-, *m*- and *p*-1,2-dicyclohexylguanidyl derivatives **665**, **666**, **667**, **677**, **679** and **680** dicyclohexyl carbodiimide (DCC; 2 equiv) were added to respective solutions of the *o*-, *m*- and *p*-amino derivatives (compounds **651**, **655**, **657**, **670**, **650** and **669**) dissolved in dioxane (3 mL mmol^{-1}). The reaction mixture was heated at 130 °C and stirred for 12 h. Additional DCC (0.5 equiv) was added and the mixture was stirred at 130 °C for 4 h. At rt, AcOEt (20 mL mmol^{-1}) was added and washed sequentially with brine (20 mL mmol^{-1}), H_2O (20 mL mmol^{-1}), brine (20 mL mmol^{-1}), H_2O (20 mL mmol^{-1}) and NH_4Cl (20 mL mmol^{-1}). The organic layer was dried ($MgSO_4$), filtered and concentrated. The residue was purified by flash-chromatography on SiO_2 (Hex/AcOEt; 14:1) yielding pure *o*-, *m*- or *p*-1,2-dicyclohexylguanidyl derivatives **665**, **666**, **667**, **677**, **679** and **680**, respectively (Scheme 1).

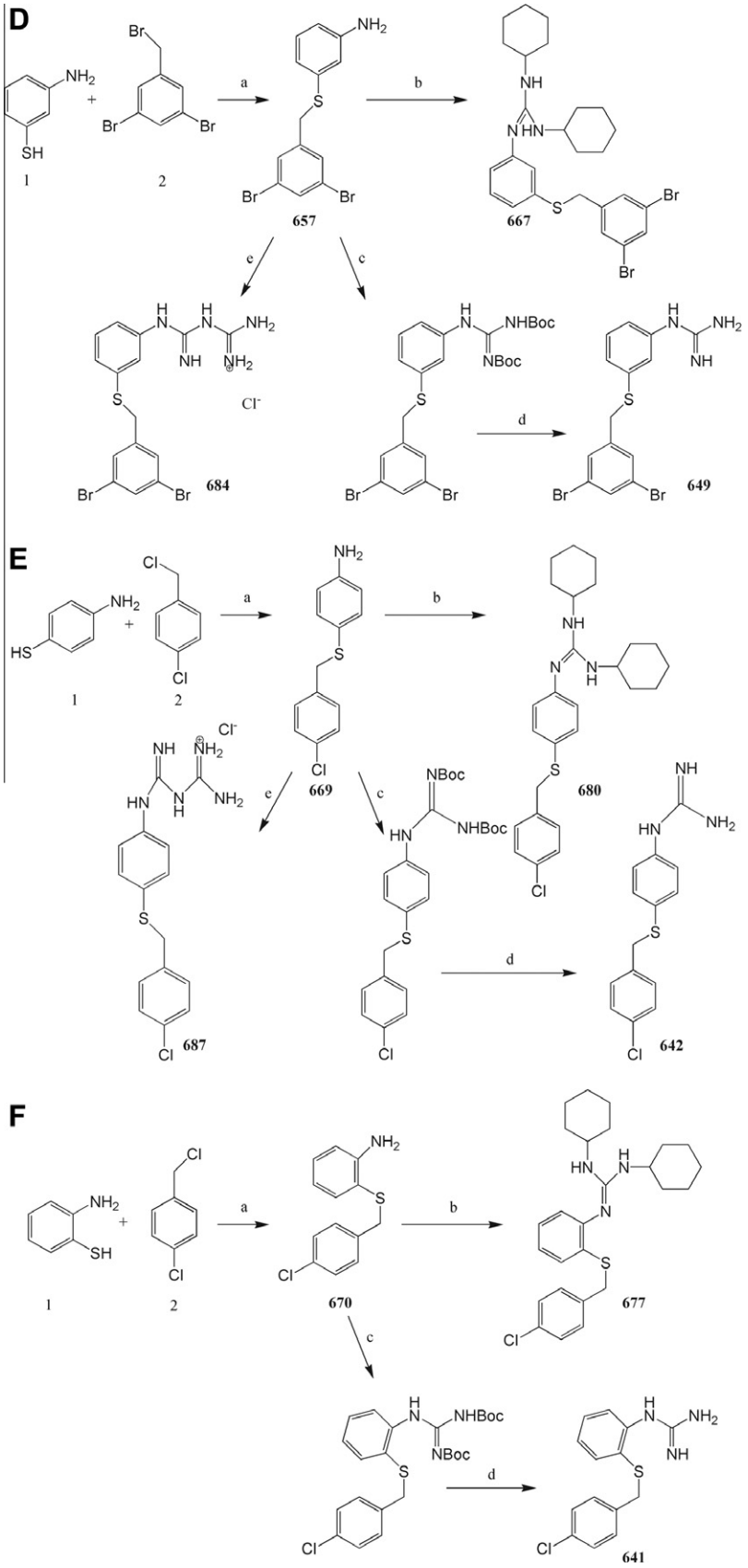
To synthesize *m*- and *p*-1-formimidamideguanidyl derivatives **684**, **687**, **688** and **692** cyanoguanidine and HCl (1:1) were added to respective solutions of the *m*- and *p*-amino intermediates (compounds **657**, **669**, **655** and **651**) dissolved in MeCN (4 mL mmol^{-1}). The reaction mixture was heated under microwaves at 150 °C for 15 min. After cooling to rt, the solid precipitate was filtered and washed with MeCN yielding pure *m*- and *p*-1-formimidamideguanidyl derivatives **684**, **687**, **688** and **692**, respectively (Scheme 1).[13]

The fungicidal activity of these newly synthesized compounds was tested against the human fungal pathogens *C. albicans* (strain SC5314)[14] and *C. glabrata* (strain BG2).[15] The minimal fungicidal concentration (MFC) of the benzylsulfanyl-phenylamines is shown in Table 1.[16–19] Fluconazole (Sigma, St. Louis, MO) was used as reference compound. We identified four potent fungicidal compounds, namely **665**, **666**, **667** and **684**, with MFC $\leqslant$ 10 μM for both pathogens. Compounds with moderate fungicidal activity (MFC = 10–50 μM) were compounds **640**, **642**, **643**, **649**, **679**, **687** and **688** (Table 1). The MFC of fluconazole for both species was >100 μM. Hence, compounds **665**, **666**, **667**, **684**, **640**, **642**, **643**, **649**, **679**, **687** and **688** are all characterized by an increased fungicidal activity compared to the most potent piperazine-1-carboxamidine derivatives.[7] In general, benzylsulfanyl-phenylguanidines displaying the highest fungicidal activity were characterized by either R^1 benzyl substituted with bromine on positions 3 and 5 in combination with R^2 thiophenyl substituted with 1,2-dicyclohexylguanidine in *meta* (**667**) or *para* position (**665**) or imidodicarbonimidic diamide in *meta* position (**684**), or R^1 benzyl substituted with chlorine on position 4 and R^2 thiophenyl substituted with 1,2-dicyclohexylguanidine in *meta* position (**666**). This structure–activity relationship (SAR) study revealed that to detect high fungicidal activity against *Candida* species (MFC $\leqslant$ 10 μM), the benzylsulfanyl-phenyl scaffold should bear a R^1 benzyl substituted with bromine on positions 3 and 5 and a substituted guanidine at the *meta* or *para* position of the R^2 thiophenyl moiety. A benzylsulfanyl-phenyl scaffold with an unsubstituted guanidine resulted in less active compounds (MFC = 50–100 μM), whereas substitution with an (unsubstituted) amine group resulted in compounds without fungicidal activity (Table 1). Whether the increased fungicidal activity of such substituted benzylsulfanyl-phenylguanidines as compared to unsubstituted benzylsulfanyl-phenylguanidines or benzylsulfanyl-phenylamines results from an increased interaction with their fungal target, or alternatively, results from increased hydrophobicity of the molecules and, consequently, increased intracellular uptake, needs to be determined.

The four substituted benzylsulfanyl-phenylguanidines with MFC $\leqslant$ 10 μM for both pathogens were selected for subsequent biological evaluation (Table 2). Their bactericidal activity against *S. epidermidis* was determined as well as their potential to eradicate single and mixed species biofilms. Compounds **665** and **666** were characterized by high bactericidal activity against *S. epidermidis* (MBC $\leqslant$ 4 μM), while compounds **667** and **684**showed moderate bactericidal activity (35 μM $\leqslant$ MBC $\leqslant$ 75 μM).[20] Activity of these compounds was further tested against single *C. albicans* biofilms and mixed biofilms using the crystal violet quantification method.[19,21] The biofilm-eradicating concentration (BEC50), that is, the concentration of a compound resulting in 50% eradication of the biofilm, was determined (Table 2).[22] The BEC50 for fluconazole against single and mixed biofilms was above 240 μM. Compounds **666**, **667**, **684** and **665** were all active against single as



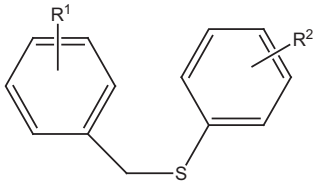
Scheme 1. Synthesis of benzylsulfanyl-phenylamines. (A) Synthesis of **650**, **643** and **679**. (B) Synthesis of **651**, **640**, **692** and **665**. (C) Synthesis of **655**, **647**, **666** and **688**. (D) Synthesis of **657**, **649**, **667** and **684**. (E) Synthesis of **669**, **687**, **680** and **642**. (F) Synthesis of **670**, **641** and **677**. Reagents and conditions: (a) Et_3N/DMF/rt; (b) dioxane/Δ, dicyclohexyl carbodiimide; (c) DMF/Di-Boc thiourea/DIPEA/EDCI; (d) CH_2Cl_2/TFA; (e) MeCN, cyanoguanidine, HCL (c), 150 °C, μw. See text for more details.



Scheme 1 (*continued*)

well as mixed biofilms (BEC50 $\leqslant$ 121 μM). Compounds **666**, **667** and **684** were most active (BEC50 $\leqslant$ 55 μM) in this respect. The in vivo toxicity and efficacy of the compounds was further assessed in a *C. elegans* model system as described by Breger and

Table 1
Antifungal activity of substituted benzylsulfanyl-phenylamines (μM)



Compound	R^1	R^2	MFC (*Ca*)[a]	MFC (*Cg*)[a]
641	4-Chlorine	*o*-Guanidyl	>100	>100
677	4-Chlorine	*o*-1,2-Dicyclohexylguanidyl	>100	50
670	4-Chlorine	*o*-Amino	>100	>100
647	4-Chlorine	*m*-Guanidyl	100	100
666	4-Chlorine	*m*-1,2-Dicyclohexylguanidyl	5	5
688	4-Chlorine	*m*-1-Formimidamideguanidyl	50	50
642	4-Chlorine	*p*-Guanidyl	50	50
680	4-Chlorine	*p*-1,2-Dicyclohexylguanidyl	>100	>100
687	4-Chlorine	*p*-1-Formimidamideguanidyl	50	50
669	4-Chlorine	*p*-Amino	>100	>100
643	3,5-Dibromine	*o*-Guanidyl	50	50
679	3,5-Dibromine	*o*-1,2-Dicyclohexylguanidyl	5	50
650	3,5-Dibromine	*o*-Amino	>100	>100
649	3,5-Dibromine	*m*-Guanidyl	50	50
667	3,5-Dibromine	*m*-1,2-Dicyclohexylguanidyl	5	5
684	3,5-Dibromine	*m*-1-Formimidamideguanidyl	5	10
640	3,5-Dibromine	*p*-Guanidyl	50	50
665	3,5-Dibromine	*p*-1,2-Dicyclohexylguanidyl	5	5
692	3,5-Dibromine	*p*-1-Formimidamideguanidyl	>100	>100
651	3,5-Dibromine	*p*-Amino	>100	>100
Fluconazole			>100	>100

[a] Minimal fungicidal concentration (μM) for *Candida albicans* (*Ca*) and *Candida glabrata* (*Cg*).

Table 2
Biological evaluation of substituted benzylsulfanyl-phenylguanidines

Compounds	MBC (*Se*)[a]	BEC50 (*Ca*)[b]	BEC50 (*Ca/Se*)[b]
665	3.0 ± 0.5	121.0 ± 17.2	84.0 ± 17.2
666	4.0 ± 1.1	18.0 ± 5.5	22.0 ± 6.8
667	75.0 ± 5.0	19.0 ± 12.1	55.0 ± 23.3
684	35.0 ± 8.8	31.0 ± 6.3	55.0 ± 15.1
Fluconazole	nd[c]	>240	>240

[a] Minimal bactericidal concentration (μM) of the compounds for *Staphylococcus epidermidis* (*Se*).
[b] Biofilm eradicating concentration (μM) of the compounds for *C. albicans* biofilms (*Ca*) or mixed species (*C. albicans/S. epidermidis*) biofilms (*Ca/Se*).
[c] Not determined.

co-workers.[11,23] Administration of DMSO (0.5%), the compound's solvent, resulted in 59 ± 1.5% nematode survival after 11 days. Compounds (50 μM) **665** and **667** exhibited minor toxicity (51 ± 0.0% and 33 ± 0.3% survival, respectively) whereas50 μM of compounds **666** and **684** showed higher toxicity in the *C. elegans* model (19 ± 5.0% and 5 ± 2.4% survival, respectively) upon the same incubation period. To test the efficacy of the compounds in the *C. elegans* infection model, survival of the *Candida* infected worms was monitored in the absence (DMSO control) or presence of 60 μM of compounds **665**, **666**, **667** and **684**.[24] When a chemical compound has no antifungal activity in the *C. elegans* in vivo infection model, only an average of 10–20% of the worms are still alive on day 5.[25] As shown in Figure 2, survival of the worms in the presence of DMSO (0.6%) was 20.0 ± 2.8% after 5 days of incubation. Addition of compounds **665** or **667** increased survival of the *Candida*-infected worms (49.0 ± 6.8% and 55.5 ± 4.0%, respectively) after 5 days of incubation and these survival percentages leveled off over time. Addition of compounds **666** or **684** had no significant

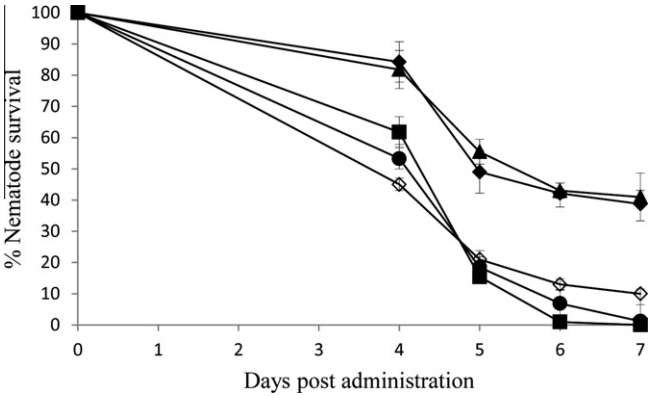


Figure 2. In vivo performance of benzylsulfanyl-phenylguanidines in a *C. elegans* model for *C. albicans* infection. Nematodes were infected with *C. albicans* for 4 h and then moved to pathogen-free liquid media in the presence of 60 μM of compounds **665** (black triangles), **666** (black circles), **667** (black diamonds), **684** (black squares) or DMSO (open diamonds) in PBS. Living worms were counted daily and percentage survival was calculated relative to the survival at day 0. Data are means of ±SEM of duplicate measurements and experiments were performed at least twice.

effect on survival of the *Candida*-infected worms as compared to DMSO treatment (18.5 ± 5.3% and 15.4 ± 0.3%, respectively, after 5 days of incubation). These data indicate that, using the miniaturized host model *C. elegans* infected with *C. albicans*, compounds **667** and **665** show in vivo activity against *C. albicans*.

In summary, based on all above in vitro and in vivo data, compounds **665** and **666** combine potent fungicidal and bactericidal activity, whereas compound **666** additionally exerts high activity against single *C. albicans* and mixed *C. albicans/S. epidermidis* species biofilms. Moreover, addition of **665** (Fig. 3A) and **667** to *Candida*-in-

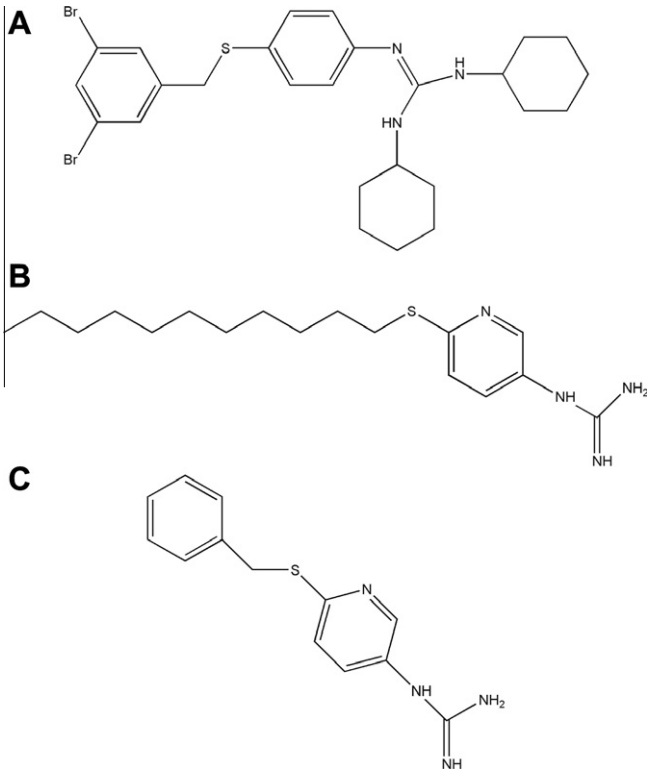


Figure 3. Chemical structure of benzylsulfanyl-phenylguanidine derivative **665** (A) and related alkylsulfanyl-pyridinylguanidines (B, C).

fected *C. elegans* cultures increased survival of the *Candida*-infected worms and proved not toxic in an in vivo *C. elegans* model system. Although use of this *C. elegans* model allows the evaluation of toxicity and antifungal activity of the compounds, it is unlikely that this will completely eliminate all toxic compounds,[11] so further toxicity test will be performed to determine the clinical potential of these compounds. In this respect, the most toxic compound, **684**, was used in a preliminary toxicity experiment in mice. The data indicated that a single intraperitoneal dose of 10 mg/kg, a dose level that is frequently used to assess in vivo efficacy of the antifungals in a murine candidiasis model,[26,27] was well tolerated. This indicates that this dose may be suitable to test the in vivo efficacy of the different compounds in *Candida*-infected mice in the future. Based on the results that will be obtained using this model, it can be decided if further optimization of these compounds is required. Until now, only one report describing the antifungal activity of structurally related compounds, namely alkylsulfanyl-pyridinylguanidines, is available.[10] Apparently, the antifungal activity of substituted alkylsulfanyl-pyridinylguanidines improved by increasing the length of the aliphatic chain: derivatives with C7 and longer aliphatic chains showed antifungal activity, with 6-(undecylsulfanyl)pyridin-3-ylguanidinium dinitrate being the most potent compound (Fig. 3B). However, substitution of the pyridinylguanidine scaffold with a benzylsulfanyl moiety, as in 6-(benzylsulfanyl)pyridin-3-ylguanidinium dinitrate (Fig. 3C), resulted in loss of the antifungal activity.[10] Hence, as substituted guanidines with an alkylsulfanyl moiety are characterized by increased antifungal activity as compared to guanidines with a benzylsulfanyl moiety,[10] future research will be directed at assessing the fungicidal activity of alkylsulfanyl-phenylguanidines.

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12. HPLC was carried out on a YMC-Pack ODS-AQ column (3 μm, 4.6 × 50 mm) with a column temperature set at 35 °C, a flow rate of 2.6 mL min^{-1} and an injection volume of 10 μL; or on a Zorbax SB-C18 column (1.8 μm, 4.6 × 30 mm) with a column temperature set at 65 °C, a flow rate of 4 mL min^{-1} and an injection volume of 1 μL. Solvent: acetonitrile/H_2O containing 0.1% of/HCOOH. The used detection method is UV detection at 254 nm.
13. Purity of all compounds was checked by LC–MS and was >95%. LC–MS data were obtained on a LC-MS agilent 1100 series instrument. Mass spectra were obtained in API-ES (Atmospheric Pressure Ionization, Electro Spray) mode and were acquired by scanning from 50 to 1500 mass units. The capillary needle voltage was 4 kV and the gas temperature was maintained at 140 °C. Nitrogen was used as the nebulizer gas.
^{1}H NMR spectra were recorded in $CDCl_3$with TMS as internal reference at room temperature on a 300 MHz Bruker spectrometer. ^{13}C NMR spectra were recorded in $CDCl_3$ with TMS as internal reference at room temperature on a 600 MHz Bruker spectrometer.
Compound **665**: ^{1}H NMR (300 MHz, DMSO): δ 9.29 (1H, s), 7.70 (1H, s), 7.57 (2H, s), 7.33–7.04 (4H, dd), 4.22 (2H, s), 3.47 (2H, s), 1.80 (4H, s), 1.69 (4H, s), 1.56 (2H, d), 1.32–1.03 (10H, m). ^{13}C NMR (300 MHz, DMSO): δ 143.40, 132.42, 131.19, 130.66, 129.79, 127.81, 124.29, 122.65, 99.99, 40.93, 32.65, 25.28, 24.98, 22.61.
Compound **666**: ^{1}H NMR (300 MHz, DMSO): δ 9.51 (1H, s), 7.38 (4H, dd), 7.27 (1H, m), 7.09 (1H, d), 7.01 (1H, s), 6.88 (1H, d), 4.24 (2H, s), 3.50 (2H, s), 1.80 (4H, s), 1.69 (4H, s), 1.55 (2H, d), 1.30–0.96 (10H, m). ^{13}C NMR (300 MHz, DMSO): δ 152.22, 137.3, 137.07, 132.15, 131.19, 131.02, 130.26, 128.83, 124.06, 122.69, 120.80, 51.48, 40.95, 36.14, 32.70, 25.37, 24.99.
Compound **667**: ^{1}H NMR (300 MHz, DMSO): δ 70 (1H, s), 7.71 (1H, s), 7.57 (2H, s), 7.42 (1H, s), 7.34 (4H, s), 7.24–6.99 (4H, m), 4.23 (2H, s). ^{13}C NMR (300 MHz, DMSO): δ 161.17, 154.92, 142.61, 139.21, 135.29, 132.25, 132.07, 130.82, 129.22, 122.25, 120.29, 118.50, 35.14.
Compound **684**: ^{1}H NMR (300 MHz, DMSO): δ 9.37 (1H, s), 7.71 (1H, s), 7.59 (2H, s), 7.32 (1H, m), 7.15 (1H, d), 7.02 (1H, s), 6.94 (1H, d), 4.26 (2H, s), 3.50 (2H, s), 1.80 (4H, s), 1.71 (4H, s), 1.57 (2H, d), 1.31–1.01 (10H, m). ^{13}C NMR (300 MHz, DMSO): δ 150.30, 141.31, 134.80, 130.48, 129.09, 128.42, 127.76, 126.89, 120.97, 119.11, 49.76, 49.67, 33.50, 30.1, 23.30, 23.07.
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16. An overnight culture was diluted in PBS (5×10^5 CFU/ml) and incubated for 2 h with different concentrations of the compounds. Cells were washed, plated on YPD (1% yeast extract, 2% peptone, 2% glucose) and after incubation, CFUs (Colony forming units) were determined. MFC, defined as the minimal concentration of the compound resulting in less than 0.1% survival of the yeast culture, was determined for both yeast species, relative to the DMSO control.
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20. To determine the bactericidal activity against *S. epidermidis*, an overnight culture of *S. epidermidis* in TSB (5% Tryptic Soy Broth; BD Diagnostics, MD, USA) was diluted in PBS (2×10^5 CFU/ml) and incubated for 2 h with different concentrations of the compounds. After the incubation period, cells were

washed, plated on TSB and CFUs were determined. Afterwards, MBC (Minimal bactericidal concentration), defined as the minimal concentration resulting in less than 0.1% survival of the bacterial culture, relative to the DMSO control treatment, was determined.
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22. To assess the activity of the compounds against mixed biofilms, composed of *C. albicans* SC5314 and *S. epidermidis* in a 50/50 ratio, overnight cultures of both organisms were suspended in 1/20 TSB at OD_{590nm} = 0.5 and 50 μL of both cultures were mixed in the wells of a 96-well plate. After an adhesion phase of 24 h, planktonic cells were removed and fresh 1/20 TSB-medium was added for the 48-h growth phase. Mature biofilms were incubated for 24 h in PBS containing the fungicidal compounds and biofilm mass was quantified using the crystal violet staining.[18,20] To assess the number of bacterial and fungal cells in these biofilms, biofilm cells were suspended and plated on media promoting growth of both *C. albicans* and *S. epidermidis* (YPD) or of *C. albicans* alone (YPD + 100 μg mL^{-1} ampicillin) after which the colony forming units (CFUs) were determined.
23. Larvae of a double mutant (*glp-4Δsek-1Δ*) of *C. elegans* were grown on NGM/OP50 agar plates (NGM agar plates on the surface inoculated with 100 μL of an overnight culture of OP50 *Escherichia coli* and incubated for 16 h at 37 °C) until all larvae had reached the L4 stage. Worms were collected and washed with M9 buffer (3 g L^{-1} KH_2PO_4, 6 g L^{-1} Na_2HPO_4, 5 g L^{-1} NaCl, 1 mM $MgSO_4$, 10 μg mL^{-1} cholesterol and 100 μg mL^{-1} kanamycin). For toxicity testing, 40 to 50 worms were suspended in 1 mL M9 buffer in each well of 24-well microtiter plates, in the presence or absence (DMSO control) of the fungicidal compounds **665**, **666**, **667** and **684** (50 μM). Survival of the worms was monitored daily. The percentage survival of the worms in the presence or absence of antifungal compounds was calculated each day relative to the survival at day 0.
24. The efficacy of the compounds in the *C. elegans* infection model was tested using L4 larvae which were fed for 4 h on *C. albicans* SC5314 agar plates (YPD agar plates on the surface inoculated with 100 μL of an overnight culture in YPD and incubated for 16 h at 37 °C).[11,28] Worms were collected and washed with M9 buffer. Survival of the worms in absence (DMSO control) or presence of compounds **665**, **666**, **667** and **684** (60 μM) was monitored daily. The percentage survival of the worms in presence or absence of antifungal compounds was calculated relative to the survival at day 0.
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