Božinović, N.; Šegan, S.; Vojnovic, S.; Pavic, A.; Šolaja, B. A.; Nikodinovic-Runic, J.; Opsenica, I. M. Synthesis and Anti-Candida Activity of Novel Benzothiepino[3,2-c]Pyridine Derivatives. *Chemical Biology and Drug Design* **2016**, *88* (6), 795–806. https://doi.org/10.1111/cbdd.12809

Received Date : 13-Apr-2016
Revised Date : 13-Jun-2016
Accepted Date : 15-Jun-2016
Article type : Research Article

Synthesis and anti-*Candida* Activity of Novel Benzothiepino[3,2-c]pyridine Derivatives

Nina Božinović,[a] Sandra Šegan,[b] Sandra Vojnovic ,[c] Aleksandar Pavic,[c] Bogdan A. Šolaja,*[a] Jasmina Nikodinovic-Runic,*[c] Igor M. Opsenica,*[a]

[a] *Faculty of Chemistry, University of Belgrade, Studentski trg 16, P.O. Box 51, 11158, Belgrade, Serbia;*

[b]*Institute of Chemistry, Technology, and Metallurgy, University of Belgrade, Njegoševa 12,11000 Belgrade, Serbia*

[c] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11000 Belgrade, Serbia;

*Corresponding author:

Bogdan A. Šolaja, bsolaja@chem.bg.ac.rs

Igor M. Opsenica, igorop@chem.bg.ac.rs

Jasmina Nikodinovic-Runic, jasmina.nikodinovic@imgge.bg.ac.rs

Running title: Thiepines as efficient antifungals

Key words*:* Thiepine, Pd-catalyzed S-arylation, *Candida* sp., Antifungal, Zebrafish

Abstract

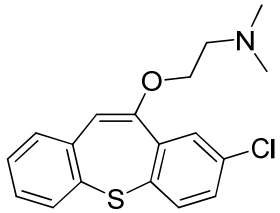
A novel series of thiepine derivatives were synthesized and evaluated as potential antimicrobials. All the synthesized compounds were evaluated for their antimicrobial activities in vitro against the fungi *Candida albicans* (ATCC 10231), *C. parapsilosis* (clinical isolate), Gram-negative bacterium *Pseudomonas aeruginosa* (ATCC 44752), and Gram-positive bacterium *Staphylococcus aureus* (ATCC 25923). Synthesized compounds showed higher antifungal activity than antibacterial activity indicating that they could be used as selective antimicrobials. Selected thiepines efficiently inhibited *Candida* hyphe formation, a trait necessary for their pathogenicity. Thiepine 8-phenyl[1]benzothiepino[3,2-c]pyridine (**16**) efficiently killed *Candida albicans* at 15.6 µg/mL and showed no embryotoxicity at 75 µg/mL. Derivative 8-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl][1]benzothiepino[3,2-*c*]pyridine (**23**) caused significant hemolysis and in vitro DNA interaction. The position of the phenyl ring was essential for the antifungal activity, while the electronic effects of the substituents did not significantly influence activity. Results obtained from in-vivo embryotoxicity on zebrafish (*Danio rerio*) encourage further structure optimizations.

Introduction

Notable biological activities have been described for the members of the dibenzo[*b*,*f*]thiepine series (1). Zotepine **1** (Figure 1) is a thiepine antipsychotic drug that has been used in clinical practice for the treatment of schizophrenia for several decades (2). The results published recently have shown that dibenzo[*b*,*f*]thiepines and pyridobenzothiepines exhibit anticancer (3) and antifungal activity (4). These results strongly support that thiepines are an important class of biologically active heterocyclic molecules.

Having transitioned from a rare incidence to an everyday problem, invasive fungal infections are a rapidly increasing global threat to human health. In the developed world, fungal infections predominantly occur in the context of increasingly aggressive immunosuppressive therapies. Invasive candidiasis is the fourth most common bloodstream infection (surpassing many bacterial pathogens) in the United States and the statistics are similar for Europe (5). *Candida* species now account up to 9% of all bloodstream infections with mortality rates remaining disturbingly high at 40%, despite the advent of new diagnostic and therapeutic strategies (6). More than 17 different *Candida* species are known to be etiological agents of human infection, however, more than 90% of invasive infections are caused by *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. krusei* (7). The expanding population of immune-compromised patients that use intravenous catheters, total

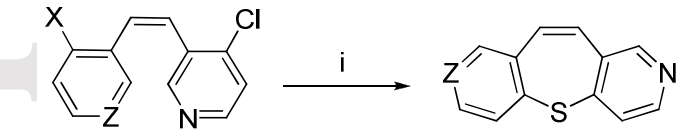
parenteral nutrition, invasive procedures and the growing use of broad-spectrum antibiotics, are factors that contribute to the increase of *Candida* infections and consequently to the increased need of new therapeutic options.



1

Figure 1. Structure of zotepine.

Different approaches have been used for the synthesis of the thiepine derivatives,(1,8,9,10,11) including the two that involve palladium-catalyzed reactions as a ring-closing step. Nielsen and the coworkers published the protocol with the Mizoroki-Heck cyclisation of the corresponding diaryl (thio)ethers as a final step (12). In our previous work we described the ring closure using the double palladium-catalyzed S-arylation of the *ortho*-halogen-substituted *Z*-stilbenes (13). The described methodology for the C-S bond formation and the previously synthesized thiepines **2**-**5** are shown in Scheme 1.



X = Cl, Br
Z = N, CH, CCl, $COCH_3$

2 Z = N
3 Z = CH
4 Z = CCl
5 Z = $COCH_3$

Scheme 1. Reagents and conditions: (i) $Pd(OAc)_2$, dppf, KSAc, NaO*t*-Bu, PhMe, 175 °C, µW.

In the preliminary disc-diffusion assay have shown that initial benzothiepino[3,2-*c*]pyridines had weak antibacterial activity and better antifungal activity (4). Encouraged by these results, we synthesized a series of novel benzothiepino[3,2-c]pyridine derivatives, and for all new thiepines, and previously synthesized derivatives we evaluated their antimicrobial potential in detail, as well as toxicity in vertebrate model organism (*Danio rerio*). All tested compounds have exhibited higher

antifungal than antibacterial activity. Some of the synthesized compounds efficiently inhibited the growth of *C. parapsilosis* that showed resistance to common antifungal drugs. *In-vivo* embryotoxicity on zebrafish *(Danio rerio*) showed improved toxicity profile of the three most active derivatives in comparison to that of voriconazole.

Experimental Section

Melting points were determined on a Boetius PMHK apparatus and were not corrected. IR spectra were recorded on a Thermo-Scientific Nicolet 6700 FT-IR Diamond Crystal. NMR: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Ultrashield Advance III spectrometer (at 500 and 125 MHz, respectively) using tetramethylsilane (TMS) as the internal standard. The NMR solvents are specified individually for each compound. Chemical shifts are expressed in parts per million (ppm) on the (δ) scale. Chemical shifts were calibrated relative to those of the solvents. ESI MS spectra of the synthesized compounds were recorded on an Agilent Technologies 6210 Time-of-Flight LC/MS instrument in positive ion mode using CH_3OH/H_2O = 1:1 with 0.2 % HCOOH as the carrying solvent solution. The samples were dissolved in pure methanol (HPLC grade). The selected values were as follows: capillary voltage = 4 kV; gas temperature = 350 °C; drying gas = 12.l min^{-1}; nebulizer pressure = 45 psig; fragmentator voltage = 70 V. All yields reported refer to isolated yields. Compounds were analyzed for purity using: Agilent 1200 HPLC system equipped with Quat Pump (G1311B), Injector (G1329B) 1260 ALS, TCC 1260 (G1316A) and Detector 1260 DAD VL+ (G1315C), and Waters 1525 HPLC dual pump system equipped with an Alltech Select degasser system, and a dual λ 2487 UV-VIS detector.

The synthesis of compounds **2**, **3**, **4**, **5**, and **16** were previously described (4,13).

10,11-dibromo-10,11-dihydro[1]benzothiepino[3,2-c]pyridine (6)

A solution of Br_2 in carbon tetrachloride (0.2 mL, 1.1 eq, 0.1 M) was added to the solution of alkene **3** (4.2 mg, 0.020 mmol) in carbon tetrachloride (0.1 mL). After stirring at room temperature for 16 hours, the reaction mixture was washed with saturated aqueous $Na_2S_2O_3$ and water, dried over $MgSO_4$ and concentrated in vacuum. Crystallization from ethanol yielded pale red crystals (7.3 mg, 100%), mp 138-140 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.45 (s, 1H), 8,34 (d, *J* = 5.0 Hz, 1H), 7.44 (dd, *J* = 7.8 Hz, *J* = 1.8 Hz, 1H), 7.38-7.26 (m, 3H), 7.23 (d, *J* = 5.5 Hz, 1H), 5.79 (d, *J* = 7.0 Hz, 1H), 5.71 (d, *J* = 7.0 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 153.0, 148.2, 145.4, 136.6, 132.8, 132.6, 131.4, 129.8, 129.3, 127.4, 122.0, 52.7, 50.8. IR (ATR, cm^{-1}): 3405w, 3169w, 3043m, 2958s, 2928s, 2852m, 2585m,

2361m, 1714w, 1626s, 1574m, 1513w, 1458m, 1263s, 1099m, 1021m, 805m, 765w, 735s, 699w, 619w. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 369.88848 (error: -2.81 ppm).

11-bromo[1]benzothiepino[3,2-c]pyridine (7) and 10-bromo[1]benzothiepino[3,2-c]pyridine (8)

In an oven-dried flask, vicinal dibromide **6** (61.0 mg, 0.164 mmol) was disolved in *tert*-butyl alcohol (2.2 mL) and potassium *tert*-butoxide (22.1 mg, 0.197 mmol, 1.2 equiv.) was added. After heating under reflux for 2 h, reaction mixture was poured onto ice and concentrated in vacuum. Water and diethyl ether were added, and the ether phase was separated and washed with saturated aqueous $Na_2S_2O_3$ solution. The organic extract was dried over Na_2SO_4, concentrated in vacuum and purified by preparative column chromatography (SiO_2, hexane/EtOAc = 9:1) to afford the compound **7** (37.7 mg, 78%) as the colorless oil and compound **8** (5.7 mg, 12%) as the colorless oil.

7: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.97 (s, 1H), 8.49 (d, *J* = 5.0 Hz, 1H), 7.82 (s, 1H), 7.51-7.46 (m, 1H), 7.38-7.35 (m, 1H), 7.35-7.30 (m, 2H), 7.28-7.24 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.2, 150.5, 146.1, 138.7, 138.3, 136.0, 133.4, 133.0, 129.8, 129.2, 128.9, 126.2, 122.8. IR (ATR, cm^{-1}): 3362m, 3048m, 2924s, 2852m, 1729w, 1604w, 1561s, 1535m, 1467s, 1432w, 1396m ,1271m, 1166w, 1055w, 910w, 830w, 768w, 749m. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 289.96339 (error: 0.11 ppm). The compound was > 95% pure based on HPLC purity analysis.

8: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.53-8.43 (m, 2H), 7.85-7.77 (m, 1H), 7.70 (s, 1H), 7.50-7.45 (m, 1H), 7.42-7.31 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 149.6, 149.4, 145.4, 140.2, 134.6, 134.2, 133.2, 131.4, 131.0, 129.0, 128.6, 126.3. IR (ATR, cm^{-1}): 3359m, 3056m, 3026s, 2924s, 2852m, 1732w, 1612w, 1565s, 1539m, 1492w, 1469s, 1444m, 1396m, 1367w, 1260w, 1239w, 1177w, 1056w, 948w, 924w, 828w, 772s, 750m, 739m, 702s. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 289.96228 (error: -3.71 ppm).

General procedure for the Suzuki reactions (with 11-bromo[1]benzothiepino[3,2-*c*]pyridine or 10-bromo[1]benzothiepino[3,2-c]pyridine):

The reaction tube containing a stirring bar was evacuated and backfilled with Ar. The tube was charged with $Pd(OAc)_2$ (3 mol %), triphenylphosphine (12 mol %) and 1,2-dimethoxyethane. After stirring at room temperature for 10 min, appropriate vinyl bromide and sodium carbonate (aq, 2 M) were added and stirring was continued for 10 min. A solution of boronic acid (1.25 equiv.) in ethanol was added, the tube was capped and the reaction mixture was heated to 90 °C and stirred at the same temperature overnight. The products were purified by preparative column chromatography: SiO_2, hexane/EtOAc = 8:2.

11-phenyl[1]benzothiepino[3,2-*c*]pyridine (9)

Following general procedure, a mixture of $Pd(OAc)_2$ (0.2 mg, 3 mol%), triphenylphosphine (0.9 mg, 12 mol %), Na_2CO_3 (aq) (2 M, 20 µL), 11-bromo[1]benzothiepino[3,2-*c*]pyridine (8.0 mg, 0.028 mmol), phenylboronic acid (4.2 mg, 0.034 mmol), ethanol (100 µL) and 1,2-dimethoxyethane (150 µL) was stirred at 90 °C for 15 hours. White solid (3.9 mg, 50%), mp 134-135 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.47 (d, *J* = 5.0 Hz, 1H), 8.21 (s, 1H), 7.53-7.50 (m, 1H), 7.49-7.47 (m, 1H), 7.46-7.44 (m, 1H), 7.42-7.28 (m, 8H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 151.6, 149.5, 146.6, 143.4, 143.0, 139.8, 137.5, 134.1, 133.7, 132.6, 129.6, 129.1, 128.8, 128.6, 128.5, 128.2, 126.7. IR (ATR, cm^{-1}): 3055s, 3028s, 2924m, 2852w, 1608w, 1563s, 1538m, 1493m, 1469s, 1445m, 1394m, 1369w, 1272m, 1068w, 1032w, 833m, 762s, 700m. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 288.08333 (error: -2.58 ppm). The compound was > 95% pure based on HPLC purity analysis.

11-(4-methoxyphenyl)[1]benzothiepino[3,2-*c*]pyridine (10)

Following general procedure, a mixture of $Pd(OAc)_2$ (0.3 mg, 3 mol%), triphenylphosphine (1.3 mg, 12 mol %) Na_2CO_3 (aq) (2 M, 30 µL), 11-bromo[1]benzothiepino[3,2-*c*]pyridine (12.0 mg, 0.041 mmol), 4-methoxyphenylboronic acid (7.8 mg, 0.052 mmol) and 1,2-dimethoxyethane (220 µL) was stirred at 90 °C for 15 hours. White solid (11.9 mg, 92%), mp 153-155 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.46 (d, *J* = 5.0 Hz, 1H), 8.23 (s, 1H), 7.52-7.46 (m, 2H), 7.42-7.26 (m, 6H), 6.97-6.89 (m, 2H), 3.85 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.7, 151.7, 149.4, 146.5, 142.9, 140.0, 137.6, 135.5, 134.1, 132.6, 132.5, 130.0, 129.4, 128.9, 128.5, 126.7, 114.0, 55.4. IR (ATR, cm^{-1}): 3278w, 3033m, 3003m, 2954m, 2926s, 2851m, 1734w, 1605m, 1565m, 1511s, 1468m, 1394w, 1370w, 1294m, 1252s, 1181m, 1034m, 832m, 752m. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 318.09501 (error: 0.95 ppm). The compound was > 95% pure based on HPLC purity analysis.

11-(4-fluorophenyl)[1]benzothiepino[3,2-*c*]pyridine (11)

Following general procedure, a mixture of $Pd(OAc)_2$ (0.5 mg, 3 mol%), triphenylphosphine (2.2 mg, 12 mol %), Na_2CO_3 (aq) (2 M, 60 µL), 11-bromo[1]benzothiepino[3,2-*c*]pyridine (20.0 mg, 0.069 mmol), 4-fluorophenylboronic acid (12.0 mg, 0.086 mmol), ethanol (300 µL) and 1,2-dimethoxyethane (450 µL) was stirred at 90 °C for 15 hours. White solid (12.6 mg, 60%), mp 131-133 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.48 (d, *J* = 5.0 Hz, 1H), 8.19 (s, 1H), 7.54-7.47 (m, 2H), 7.43-7.28 (m, 6H), 7.14-7.05 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 162.8 (d, *J* = 246.5 Hz), 151.5, 149.6, 146.6, 142.3, 139.6, 139.1, 137.3, 134.1, 133.7, 132.7, 130.5 (d, *J* = 8.0 Hz), 129.5, 129.2, 128.6, 126.8, 115.6 (d, *J* = 20.8 Hz). IR (ATR, cm^{-1}): 3301w, 3223w, 3048w, 2923s, 2852m, 1734m, 1600w, 1564w, 1539w,

1507m, 1468m, 1394w, 1268m, 1232m, 1160w, 834m, 740m. $[M + H]^+$ 306.07480 (error: 0.23 ppm). The compound was > 95% pure based on HPLC purity analysis.

4-[1]benzothiepino[3,2-*c*]pyridin-11-ylbenzonitrile (12)

Following general procedure, a mixture of $Pd(OAc)_2$ (0.9 mg, 3 mol%), triphenylphosphine (4.4 mg, 12 mol %), Na_2CO_3 (aq) (2 M, 120 µL), 11-bromo[1]benzothiepino[3,2-*c*]pyridine (40.0 mg, 0.138 mmol), 4-cyanophenylboronic acid (25.3 mg, 0.172 mmol), ethanol (600 µL) and 1,2-dimethoxyethane (1.0 mL) was stirred at 90 °C for 15 hours. Pale yellow oil (24.9 mg, 58%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.51 (d, *J* = 5.0 Hz, 1H), 8.13 (s, 1H), 7.74-7.68 (m, 2H), 7.56-7.50 (m, 4H), 7.50-7.47 (m, 1H), 7.42-7.32 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 151.0, 150.0, 147.3, 147.2, 141.6, 139.0, 136.6, 135.6, 134.2, 132.8, 132.5, 129.8, 129.8, 129.4, 128.7, 127.0, 118.5, 111.9. IR (ATR, cm^{-1}): 3056m, 2926m, 2679w, 2227s, 1606s, 1566m, 1540w, 1508m, 1470m, 1433w, 1396m, 1274m, 1171w, 1062w, 836s, 772w, 749m, 701w. $[M + H]^+$ 313.07878 (error: -1.96 ppm). The compound was > 95% pure based on HPLC purity analysis.

11-pyridin-4-yl[1]benzothiepino[3,2-*c*]pyridine (13)

Following general procedure, a mixture of $Pd(OAc)_2$ (0.5 mg, 3 mol%), triphenylphosphine (2.2 mg, 12 mol %), Na_2CO_3 (aq) (2 M, 50 µL), 11-bromo[1]benzothiepino[3,2-*c*]pyridine (20.0 mg, 0.069 mmol), 4-pyridinylboronic acid (10.6 mg, 0.086 mmol), ethanol (250 µL) and 1,2-dimethoxyethane (700 µL) was stirred at 90 °C for 15 hours. White film (10.6 mg, 53%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.70-8.65 (m, 2H), 8.52 (d, *J* = 5.0 Hz, 1H), 8.17 (s, 1H), 7.56-7.52 (m, 2H), 7.51 (d, *J* = 5.0 Hz, 1H), 7.42-7.31 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 151.0, 150.3, 150.1, 150.0, 147.3, 140.8, 139.0, 136.2, 135.6, 134.3, 132.9, 129.9, 129.8, 128.7, 127.0, 123.2. IR (KBr, cm^{-1}): 3426s, 3028w, 2922s, 2852m, 1730w, 1632w, 1593m, 1564w, 1541w, 1469m, 1400w, 1276w,1100m, 821w, 754w, 615w, 549m. (+)ESI-HRMS (m/z): $[M + H]^+$ 289.07948 (error: 0.29 ppm).

10-phenyl[1]benzothiepino[3,2-*c*]pyridine (14)

Following general procedure, a mixture of $Pd(OAc)_2$ (0.5 mg, 3 mol%), triphenylphosphine (2.2 mg, 12 mol %), Na_2CO_3 (aq) (2 M, 50 µL), 10-bromo[1]benzothiepino[3,2-*c*]pyridine (20.0 mg, 0.069 mmol), phenylboronic acid (10.5 mg, 0.086 mmol), ethanol (250 µL) and 1,2-dimethoxyethane (700 µL) was stirred at 90 °C for 15 hours. White solid (12.7 mg, 64%), mp 134 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.59 (s, 1H), 8.44 (d, *J* = 5.5 Hz, 1H), 7.61-7.55 (m, 1H), 7.47-7.36 (m, 6H), 7.35-7.28 (m, 2H), 7.23-7.17 (m, 1H), 7.05-7.00 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 150.0, 149.0, 148.6, 145.9, 143.6, 141.7, 135.7, 135.0, 133.3, 131.2, 129.8, 128.9, 128.4, 128.4, 128.3, 128.1, 126.0. IR (ATR, cm^{-1}):

3054w, 3021w, 2919w, 2851w, 1562w, 1539w, 1464m, 1390w, 1236w, 1174w, 916w, 864w, 825m, 748m, 691m, 636w, 551w, 518w. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 288.08472 (error: 1.98 ppm). The compound was > 95% pure based on HPLC purity analysis.

10-(4-methoxyphenyl)[1]benzothiepino[3,2-*c*]pyridine (15)

Following general procedure, a mixture of $Pd(OAc)_2$ (0.5 mg, 3 mol%), triphenylphosphine (2.2 mg, 12 mol %), Na_2CO_3 (aq) (2 M, 50 µL), 10-bromo[1]benzothiepino[3,2-*c*]pyridine (20.0 mg, 0.069 mmol), 4-methoxyphenylboronic acid (13.1 mg, 0.086 mmol), ethanol (250 µL) and 1,2-dimethoxyethane (700 µL) was stirred at 90 °C for 15 hours. White solid (20.3 mg, 93%), mp 145-146 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.58 (s, 1H), 8.43 (d, *J* = 5.5 Hz, 1H), 7.60-7.54 (m, 1H), 7.41-7.35 (m, 3H), 7.35-7.30 (m, 1H), 7.26 (s, 1H), 7.24-7.19 (m, 1H), 7.07-7.04 (m, 1H), 6.95-6.91 (m, 2H), 3.86 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.7, 150.0, 148.8, 148.1, 145.8, 141.9, 136.1, 135.9, 135.0, 133.3, 131.3, 130.0, 129.8, 128.3, 127.2, 126.0, 113.8, 55.4. IR (ATR, cm^{-1}): 3031m, 2954m, 2930m, 2835w, 1605m, 1566m, 1537w, 1511s, 1467m, 1394w, 1365w, 1295m, 1249s, 1180m, 1113w, 1034m, 833m, 776w, 750w. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 318.09449 (error: -0.70 ppm). The compound was > 95% pure based on HPLC purity analysis.

General procedure for Suzuki reactions (with 8-chloro[1]benzothiepino[3,2-*c*]pyridine):

The reaction tube containing a stirring bar was evacuated and backfilled with Ar. The tube was charged with $Pd(OAc)_2$ (5 mol %), SPhos (10 mol %), boronic acid (1.2 equiv.) and anhydrous K_3PO_4 (2.0 equiv.). The tube was capped with a rubber septum and filled with argon. Dry toluene was added through the septum and the resulting mixture was stirred at room temperature for 2 min. 8-Chloro[1]benzothiepino[3,2-*c*]pyridine was added and the tube was sealed. The reaction mixture was heated at 100 °C overnight. The reaction mixture was allowed to cool to room temperature. The products were purified by preparative column chromatography (SiO_2, hexane/EtOAc = 8/2).

8-(4-methoxyphenyl)[1]benzothiepino[3,2-*c*]pyridine (17)

Following general procedure, a mixture of $Pd(OAc)_2$ (0.3 mg, 1 mol%), SPhos (1.0 mg, 10 mol %), anhydrous K_3PO_4 (51.8 mg, 0.224 mmol), 8-chloro[1]benzothiepino[3,2-*c*]pyridine (30.0 mg, 0.122 mmol), 4-methoxyphenylboronic acid (22.3 mg, 0.146 mmol) and toluene (1.5 mL) was heated at 100 °C overnight. White solid (24.0 mg, 62%), mp 133-134 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.50-8.42 (m, 2H), 7.52-7.44 (m, 4H), 7.42-7.38 (m, 1H), 7.36-7.33 (m, 1H), 7.16 (d, *J* = 12.5 Hz, 1H), 7.02-6.93 (m, 3H), 3.83 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.5, 149.9, 149.8, 144.7, 141.5, 139.9, 136.1, 135.4, 133.8, 132.7, 130.8, 130.5, 128.0, 128.0, 127.9, 126.2, 114.3, 55.3. IR (ATR, cm^{-1}): 3023m,

2959w, 2842w, 1607m, 1567m, 1543w, 1510s, 1467s, 1390w, 1301m, 1265m, 1242s, 1171w, 1025w, 835m, 806m. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 318.09491 (error: 0.63 ppm). The compound was > 95% pure based on HPLC purity analysis.

4-[1]benzothiepino[3,2-*c*]pyridin-8-ylbenzonitrile (18)

Following general procedure, a mixture of $Pd(OAc)_2$ (0.3 mg, 1 mol%), SPhos (1.0 mg, 10 mol %), anhydrous K_3PO_4 (51.8 mg, 0.224 mmol), 8-chloro[1]benzothiepino[3,2-*c*]pyridine (30.0 mg, 0.122 mmol), 4-cyanophenylboronic acid (21.4 mg, 0.146 mmol) and toluene (2 mL) was heated at 100 °C overnight. Yellow oil (14.0 mg, 36%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52-8.45 (m, 2H), 7.76-7.70 (m, 2H), 7.68-7.61(m, 2H), 7.59-7.50 (m, 2H), 7.47-7.44 (m, 1H), 7.37 (d, *J* = 5.0 Hz, 1H), 7.18 (d, *J* = 12.0 Hz, 1H), 7.06 (d, *J* = 12.0 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 150.1, 150.0, 144.4, 144.1, 140.3, 139.8, 135.6, 135.2, 133.7, 133.4, 132.7, 131.3, 128.4, 127.6, 126.3, 118.6, 111.6. IR (ATR, cm^{-1}): 3311w, 2954m, 2921m, 2851m, 2226w, 1771w, 1735m, 1651m, 1561m, 1540m, 1519m, 1465m, 1373w, 1266w, 1178w, 824w, 737w. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 313.07895 (error: -1.42 ppm). The compound was > 95% pure based on HPLC purity analysis.

General procedure for C-S coupling:

11-(phenylthio)[1]benzothiepino[3,2-*c*]pyridine (19)

The reaction tube containing a stirring bar was evacuated and backfilled with Ar. The tube was charged with Pd_2dba_3 (5.5 mg, 5 mol %), dppf (6.7 mg, 10 mol %), sodium *tert*-butoxide (13.9 mg, 0.145 mmol, 1.2 equiv.) and toluene (2.5 mL). The tube was evacuated and backfilled with argon. After stirring at room temperature for 10 min, 11-bromo[1]benzothiepino[3,2-*c*]pyridine (**7**) (35.0 mg, 0.121 mmol) and thiophenol (13 µL, 0.133 mmol, 1.1 equiv.) were added. The tube was capped and the reaction mixture was heated to 90 °C and stirred at the same temperature overnight. The product was purified by preparative column chromatography: SiO_2, hexane/EtOAc = 9:1. Yellow solid (30.0 mg, 77%), mp 108-110 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.03 (s, 1H), 8.36 (d, *J* = 5.0 Hz, 1H), 7.67 (s, 1H), 7.52-7.47 (m, 1H), 7.43-7.37 (m, 2H), 7.35-7.32 (m, 1H), 7.32-7.28 (m, 2H), 7.25-7.19 (m, 3H), 7.16-7.11 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 151.0, 150.1, 146.7, 139.0, 138.7, 136.9, 135.1, 134.1, 134.0, 132.7, 130.1, 129.5, 129.2, 129.0, 128.7, 127.1, 126.4. IR (ATR, cm^{-1}): 3056w, 3023w, 1712w, 1562w, 1466w, 1388w, 1266w, 1184w, 1081w, 1026w, 948w, 883w, 832w, 742m, 697w, 619w. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 320.05694 (error: 2.24 ppm). The compound was > 95% pure based on HPLC purity analysis.

4-([1]benzothiepino[3,2-c]pyridin-11-ylthio)benzonitrile (20)

Following general procedure, a mixture of Pd_2dba_3 (11.0 mg, 5 mol %), dppf (11.9 mg, 10 mol %), sodium *tert*-butoxide (28.6 mg, 0.289 mmol, 1.2 eq), 11-bromo[1]benzothiepino[3,2-*c*]pyridine (70.0 mg, 0.241 mmol), 4-mercaptobenzonitrile (50.0 µL, 0.366 mmol, 1.5 equiv.) and toluene (5.0 mL) was stirred at 90 °C overnight. The product was purified by preparative column chromatography: SiO_2, hexane/EtOAc = 8:2. Yellow solid (55.6 mg, 67%), mp 158-160 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.99 (s, 1H), 8.41 (d, *J* = 5.0 Hz, 1H), 7.92 (s, 1H), 7.56-7.52 (m, 1H), 7.46-7.33 (m, 7H), 7.32-7.29 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 150.6, 150.5, 146.7, 143.4, 142.2, 138.5, 134.9, 134.2, 133.2, 132.9, 132.4, 130.3, 129.2, 128.9, 127.9, 126.5, 118.4, 109.5. IR (ATR, cm^{-1}): 3072w, 3041w, 2936w, 2320w, 1589m, 1558s, 1464m, 1428w, 1396m, 1269w, 1188w, 1082w, 951w, 825s, 753m. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 345.04998 (error: -4.30 ppm). The compound was > 95% pure based on HPLC purity analysis.

General procedure for the synthesis 4,5-dihydro-1*H*-imidazole derivatives

11-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl][1]benzothiepino[3,2-*c*]pyridine (21)

A mixture of sulfur (1.2 mg, 0.038 mmol) and **12** (12.0 mg, 0.038 mmol) was treated with ethylene diamine (0.5 mL), then irradiated with microwave at 110 °C for 80 min. The mixture was suspended in water, the phases were separated using centrifugation and the precipitate was rinsed with water (3×). The residue was then dried under vacuum, affording the product as a yellow film (12.0 mg, 88%). ^{1}H NMR (500 MHz, *d*-DMSO, CF_3COOD) δ 8.77 (d, *J* = 6.0 Hz, 1H), 8.38 (s, 1H), 8.16 (d, *J* = 6.0 Hz, 1H), 8.00 (d, *J* = 8.5 Hz, 2H), 7.91 (s, 1H), 7.72 (d, *J* = 8.5 Hz, 2H), 7.61-7.56 (m, 1H), 7.55-7.50 (m, 1H), 7.44-7.38 (m, 2H), 3.98 (s, 4H). ^{13}C NMR (125 MHz, *d*-DMSO) δ 163.3, 150.7, 150.0, 146.1, 144.1, 141.7, 139.3, 136.6, 134.2, 133.4, 132.5, 130.4, 130.2, 129.7, 129.0, 128.4, 127.5, 126.9, 54.9. IR (ATR, cm^{-1}): 3302w, 3197m, 3025m, 2929m, 2874m, 1670w, 1605m, 1562m, 1514m, 1468m, 1395w, 1347w, 1272m, 979w, 844m, 758w. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 356.12178 (error: 0.51 ppm). The compound was > 95% pure based on HPLC purity analysis.

11-{[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]thio}[1]benzothiepino[3,2-*c*]pyridine (22)

Following general procedure, a mixture of sulfur (2.0 mg, 0.064 mmol), **20** (22.0 mg, 0.064 mmol) and ethylene diamine (0.8 mL) was irradiated with microwave at 110 °C for 80 min. Yellow solid (21.1 mg, 85%), mp 156-158 °C. ^{1}H NMR (500 MHz, *d*-DMSO, CF_3COOD) δ 9.00 (s, 1H), 8.53 (d, *J* = 5.0 Hz, 1H), 8.19 (s, 1H), 7.78 (d, *J* = 8.5 Hz, 2H), 7.71 (d, *J* = 5.0 Hz, 1H), 7.61-7.58 (m, 1H), 7.58-7.45 (m, 5H), 3.94 (s, 4H). ^{13}C NMR (125 MHz, *d*-DMSO, CF_3COOD) δ 164.5, 149.2, 148.6, 148.3, 144.7, 143.3, 143.3, 138.3, 135.7, 133.1, 131.8, 131.2, 130.2, 129.6, 129.4, 127.8, 127.6, 120.0, 44.5, 44.4. IR (ATR,

cm^{-1}): 3439w, 3323w, 3247m, 3138m, 3062m, 2917m, 2869m, 1704w, 1605m, 1557m, 1466m, 1392w, 1351w, 1272m, 1180w, 1122w, 1092w, 831m, 756m. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 388.09197 (error: -4.38 ppm).

8-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl][1]benzothiepino[3,2-*c*]pyridine (23)

Following general procedure, a mixture of sulfur (0.8 mg, 0.025 mmol), **18** (8.5 mg, 0.025 mmol) and ethylene diamine (0.5 mL) was irradiated with microwave at 110 °C for 80 min. Yellow film (7.0 mg, 80%). ^{1}H NMR (500 MHz, *d*-DMSO, CF_3COOD) δ 8.91 (s, 1H), 8.80 (d, *J* = 6.0 Hz, 1H), 8.06-7.97 (m, 5H), 7.95-7.90 (m, 2H), 7.62 (d, *J* = 8.0 Hz, 1H), 7.49 (d, *J* = 12.5 Hz, 1H), 7.23 (d, *J* = 12.5 Hz, 1H), 4.02 (s, 4H). ^{13}C NMR (125 MHz, *d*-DMSO, CF_3COOD) δ 164.7, 152.5, 144.2, 143.8, 143.0, 139.8, 139.7, 138.2, 137.5, 134.0, 131.1, 129.6, 129.3, 129.2, 128.8, 127.6, 121.7, 121.6, 44.6, 44.4. IR (KBr, cm^{-1}): 3429m, 2924w, 1618m, 1565w, 1518w, 1466w, 1388w, 1275w, 1243w, 1196w, 1021w, 827w, 669w, 617w. (+)ESI-HRMS (*m/z*): $[M + H]^+$ 356.12141 (error: -0.52 ppm).

Antimicrobial activity assay and microscopy

Test organisms for the antibacterial assays were obtained from the National Collection of Type Cultures (NCTC) and the American Type Culture Collection (ATCC). They included *Candida albicans* (ATCC 10231) and *C. parapsilosis* C27 (voriconazole resistant clinical isolate; from the collection of National Reference Medical Mycology Laboratory (Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, Belgrade))), Gram negative strain *Pseudomonas aeruginosa* PAO1 (NCTC 10332) and Gram positive *Staphylococcus aureus* (NCTC 6571). Broth microdilution assays were carried out in RPMI medium (Sigma Aldrich, Germany) according to the standards recommended by the National Committee for Clinical Laboratory Standards (M07-A8) for bacteria and Standards of European Committee on Antimicrobial Susceptibility Testing (EDef7.1.) (14). Tested compounds were dissolved in DMSO. The highest concentration used was 500 μg/mL. The inoculums were 10^5 colony forming units (cfu) per mL for bacteria, and 10^4 cfu/mL for *Candida* sp..

To study the effect of selected thiepines on *C. albicans* cells in time, overnight fungal culture grown in RPMI medium (Sigma Aldrich, Germany) supplemented with 2 % glucose and 10 % FCS (fetal calf serum) was diluted to optical density OD600 of 0.5 and the thiepines or DMSO were added in MIC concentrations. Culture was further incubated at 37 °C, and the aliquots were analysed by microscopy after 1, 5 and 24 h incubation. Culture aliquots were stained with FUN 1 yeast cell stain ([2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium

iodide]) used under the manufacturer's instructions (Molecular Probes, Inc.), and observed using fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, USA) at 40× magnification.

Hemolysis assay (15)

Sheep red blood cells in PBS, pH 7.4 (1% v/v, Torlak, Belgrade, Serbia) were treated for 1 h with concentration of compounds that corresponded to determined MIC and IC_{50} values at 37°C. Hemoglobin absorbance was measured at 405 nm (Tekan Infinite 200 Pro multiplate reader; Tecan Group Ltd., Männedorf, Switzerland). The hemolysis percentage was calculated using the following equation: hemolysis (%) = 100[(Abs_{405nm} (treated) - Abs_{405nm} (non treated) / (Abs_{405nm} (0.1% Triton X-100 lysed) - Abs_{405nm} (non treated)].

In vitro DNA interaction

Genomic DNA (gDNA) from *Candida albicans* was purified with a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). The quality and the concentration of DNA were estimated by measuring UV absorbance with NanoVue Plus spectrophotometer (GE Healthcare, Freiburg, Germany). The ability of test compounds to bind gDNA from *C. albicans* was examined by agarose gel electrophoresis. For the gel electrophoresis experiments, gDNA (500 ng) was treated with the complexes (25 µg/mL) in phosphate buffer (pH 7.4), and the contents were incubated for 12 h at 37 °C, then subjected to gel electrophoresis on 0.8% (w/v) agarose gel containing 0.1 µg/mL of ethidium bromide in TAE buffer (40 mM Tris acetate/1 mM EDTA, pH 7.4) buffer at 60 V for 2 h.

Zebrafish embryotoxicity assay

The assessment of toxicity (lethality and teratogenicity) of selected thiepines on zebrafish embryos have been performed following general rules of the OECD Guidelines for the Testing of Chemicals (OECD, 2013). All experiments involving zebrafish were performed in compliance with the European directive 86/609/EEC and the ethical guidelines of Guide for Care and Use of Laboratory Animals of Institute for Molecular Genetics and Genetic Engineering, University of Belgrade.

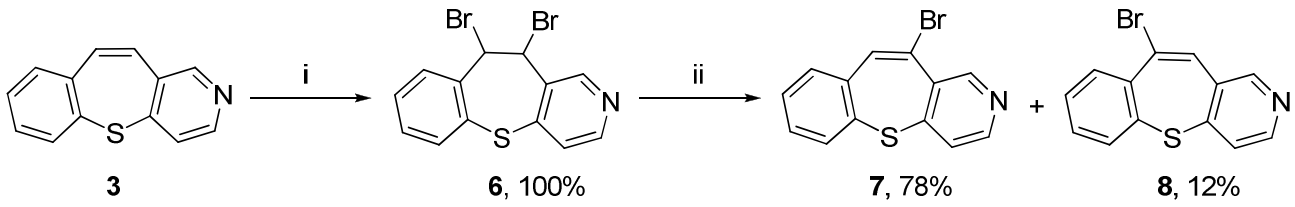
Adult zebrafish (*Danio rerio*, wild type) were maintained in the fish medium (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM $NaHCO_3$, 0.07 mM KCl) at 27 ± 1°C and 14 h light/10 h dark cycle, and regularly fed twice daily with commercially dry flake food supplemented with *Artemia nauplii* (TetraMin™ flakes; Tetra Melle, Germany). Eggs at 6 hours post fertilization (hpf) were treated with six different concentrations of selected thiepines (1, 5, 10, 25, 50 and 75 µg/mL) and 0.15% (v/v) DMSO as negative control. Embryos were then individually transferred into 24-well plates containing 1000 µL test solution, 10 embryo per well, and incubated at 28°C. Experiments were repeated two times, using 30 embryos per concentration.

Apical endopoints (Table S1) for toxicity evaluation were recorded at 24, 48, 72, and 96 hpf using an inverted microscope (CKX41; Olympus, Tokyo, Japan. At 96 hpf, the embryos were anesthetized by addition of 0.1% (w/v) tricaine solution (Sigma-Aldrich, St. Louis, MO), photographed and killed by freezing at −20°C for ≥ 24 h.

Results and Discussion

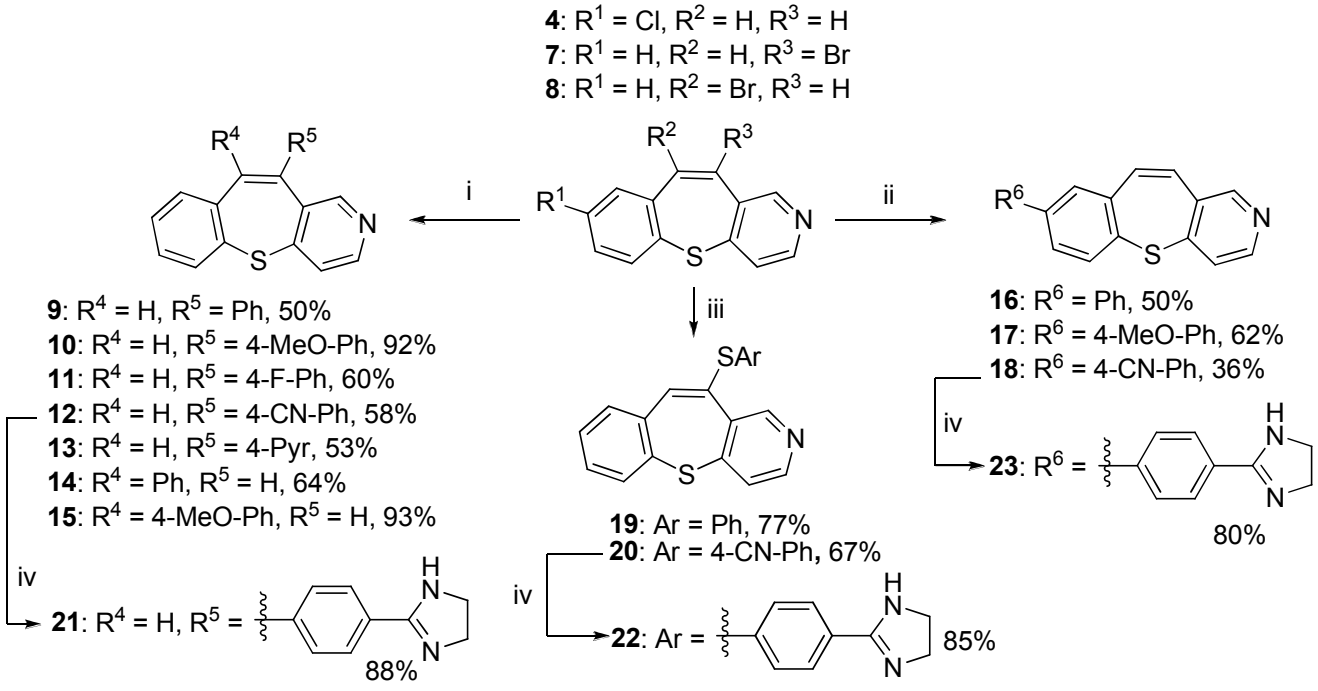
Chemistry

Thiepine derivatives **3** and **4** were used as the multifunctional building blocks for the synthesis of the differently substituted benzothiepino[3,2-*c*]pyridines. We performed the simple alkene bromination reaction on thiepine derivative **3**. The base-promoted HBr-elimination of the vicinal dibromide **6** gave vinyl bromides **7** and **8** with good regioselectivity (Scheme 2) (16). DBU/DMF (17), afforded the same product distribution of the elimination reaction.



Scheme 2. Reagents and conditions: (i) Br_2, CCl_4, r.t.; (ii) KO*t*-Bu, *t*-BuOH, 85 °C.

Since the isomeric products **7** and **8** could be easily separated, we proceeded with further transformations. Vinyl bromides **7** and **8** and the previously synthesized thiepine **4** were successfully coupled with a series of arylboronic acids under Suzuki cross-coupling reaction conditions (18), affording compounds **9**-**18** in moderate to excellent yields (Scheme 3). The compound **7** was coupled with thiophenols via the palladium catalyzed reaction, using the 1,1'-bis(diphenylphosphino)ferrocene (dppf) as a ligand, as shown in the Scheme 3. All the products containing the cyano group were subjected to the reaction with ethylenediamine in the presence of sulphur to give 2-substituted 2-imidazolines **21**-**23** (19).



Scheme 3. Reagents and conditions: (i) $ArB(OH)_2$, $Pd(OAc)_2$, PPh_3, DME, Na_2CO_3 aq, EtOH, 90 °C; (ii) $ArB(OH)_2$, $Pd(OAc)_2$, SPhos, K_3PO_4, PhMe, 100 °C; (iii) ArSH, Pd_2dba_3, dppf, NaO*t*-Bu, PhMe, 90 °C; (iv) $H_2NCH_2CH_2NH_2$, S, 110 °C, μW.

Antimicrobial activity

All synthesized thiepines were assessed for antimicrobial activity against two fungi (*Candida albicans* and clinical strain *C. parapsilosis*) and two bacterial strains (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) by determination of minimum inhibitory concentration (MIC) using the broth dilution method in comparison with clinically used antifungals voriconazole and nystatin and antibacterial kanamycin (14).From the MIC concentrations, it was obvious that the most synthesized

of thiepines did not show significant antibacterial activity (MICs≥500 μg/mL), suggesting selectivity towards fungi (Table 1). MIC values against fungal strains were between 15.62 and 250 μg/mL, with the most potent derivative being **16** (Table 1). Thiepines **4** and **5**, with chlorine and methoxy-substituent on the phenyl ring, showed better antifungal activity than corresponding unsubstituted analog **3**. Interestingly, in our previous study **4** did not show significant antifungal activity in the disc diffusion assay (4), suggesting possible interaction with cellulose discs. Antifungal activity of **4** and **5** was not significantly dependent on the electronic effect of substituent. In the case of compound **16** introduction of the phenyl ring on thiepine core was beneficial for activity in comparison to **3**. Nevertheless, in the case of compounds **17** and **18** additional substituent on phenyl ring reduced the antifungal activity.

Compounds **9** and **10** showed a four-fold greater potency (MIC = 31.25 μg/mL) than unsubstituted thiepine **3** in inhibiting the growth of the *C. albicans*. On the other hand, thiepines **11**, **12**, **19** had the same activity, while pyridine analog **13** was less potent than **3**. Among the isomeric thiepines **16** was 2 times more active than **9**, and 16 times more potent than **14** (Table 1). Similar difference in activity was observed for **10** and **15** analogs. Based on the obtained results it is obvious that position of the phenyl ring was essential for the antifungal activity.

It is known that 2-substituted 2-imidazolines are potent antimicrobials (20). In the case of thiepine **21** a lower antifungal activity was observed in comparison to **9**. Nevertheless, **23** showed to be potent with similar activity to **16** against *C. albicans*, and 8-fold greater potency than **16** in inhibiting the growth of *C. parapsilosis*. This is important due to the fact that *C. parapsilosis* spp. are often inherently resistant and are prone to acquired resistance to antifungal drugs (in this case C27 is voriconazole resistant clinical isolate). MIC concentrations determined in this study were quite comparable to MIC concentrations obtained for recently reported 1,5-benzodiazepine (21) and eugenol (22) derivatives against selection of fungal strains.

The effect of thiepines with antifungal MIC values ≤ 31.2 μg/mL on the *C. albicans* hyphal growth has also been evaluated on Spider medium as previously described (23) (Figure 2). The hyphal formation is essential for *Candida* spp. penetration into human cells during the initial phases of infection, as well as for biofilm formation. Therefore, compounds that could inhibit hyphal formation are sought after. The tested compounds were compared with VOR as a standard and showed variable results. Thiepines **4** and **23** successfully prevented hyphal formation while compounds **9** and **10** showed the opposite effect, significantly stimulating hyphal formation, which we considered as a negative trait that eliminated these two derivatives from further assessments (Figure 2).

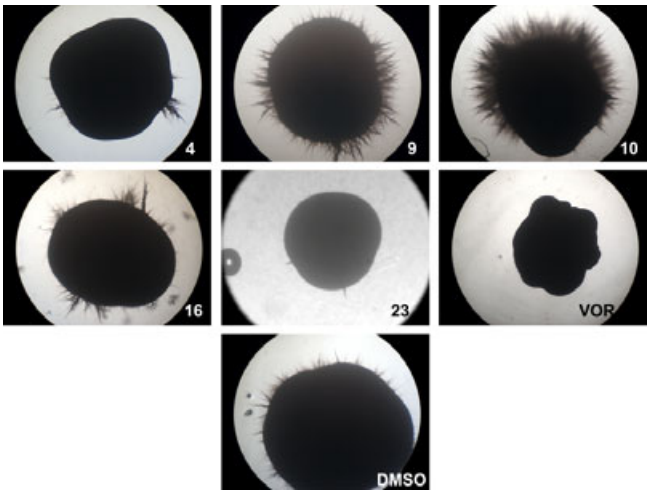


Figure 2. Effect of subinhibitory amounts (MIC70; 70 % of MIC determined against *C. albicans* for each compound was present in the Spider medium) of selected thiepines on *C. albicans* hyphal formation.

We next carried out the hemolysis assay in order to explore the potential interactions of selected thiepines with the cell membrane (Figure 3A) and observed the activity of **4**, **16** and **23** on *C. albicans* cells over 5 h using fluorescent microscopy (Figure 3B). The sheep red blood cells and thiepines were co-incubated in buffer at physiologic pH whereby erythrocyte membrane served as a model for the lipid bilayer. The percent of red blood cell disruption was quantified relative to positive control samples lysed with a Triton X detergent as described previously (24). Low hemolysis of up to 10% at physiologic pH (7.4) was observed using **4**, **16** and VOR at determined *C. albicans* MIC concentrations (Figure 3A). This implied that membrane was not the primary target of these molecules, which has been known for VOR. On the contrary, **23** efficiently caused between 30 and 40% hemolysis comparable to that of NYS that was previously shown for antifungal polyenes including nystatin due to the ability to cause large nonselective perforations in the cell membranes (25). This was mirrored in the microscopic study, which showed that NYS and **23** efficiently caused reduction in total cell numbers within first 5 h of treatment, while **4**, **16** and **VOR** caused reduction in metabolic activity in comparison to untreated sample, seen as lesser amount of cells containing orange-red or yellow-orange fluorescent intravacuolar structures (Figure 3B), and needed prolonged incubation time of up to 24 h, to cause complete fungal culture death (data not shown). Thus possible activity on the fungal membrane was concluded for thiepine **23** and ruled out for **4** and **16**.

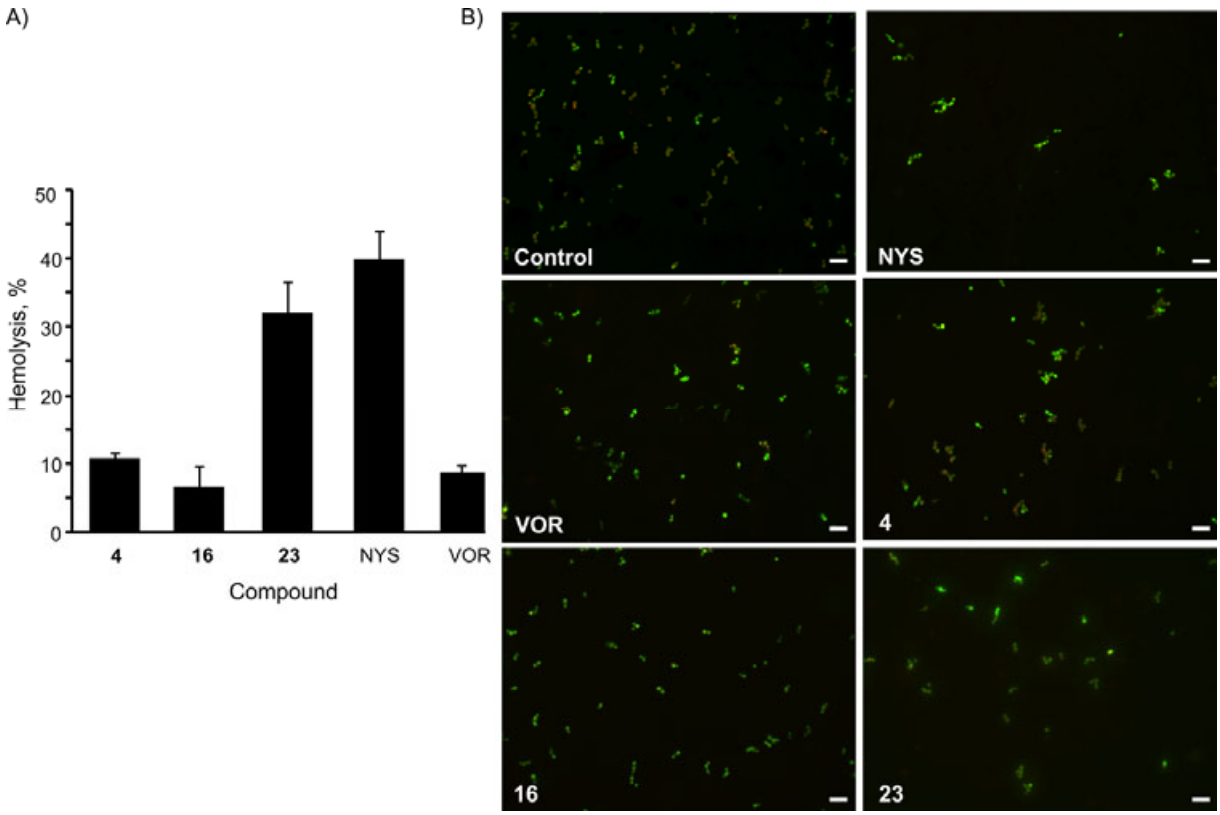


Figure 3. Activity of thiepines **4**, **16**, **23**, nystatin (NYS) and voriconazole (VOR) on A) washed sheep red blood cells in hemolysis assay using MIC concentrations of all tested compounds, and B) *C. albicans* culture monitored by fluorescent microscopy after 5 h treatment (white bar represents 10 µm; × 40 magnification).

Further, we studied *in vitro* interaction of selected thiepines with *C. albicans* genomic DNA using gel electrophoresis assay (22) after prolonged incubation (12 h) of compounds (25 µg/mL) with purified DNA (Figure 4). Under tested conditions, thiepines did not cause DNA degradation, while significant interacalation with DNA based on lower UV-luminescence upon exposure to ethidium bromide was exhibited by **4** and **23** by 30 and 60% of the DMSO treated control, respectively (Figure 4) and hence it was reasonable to attribute some of the activity of these compounds to interaction with this biomolecule. The DNA binding property of 2-imidazoline derivatives is well described in the literature (26), and therefore our result for compound **23** is well correlated with the literature data.

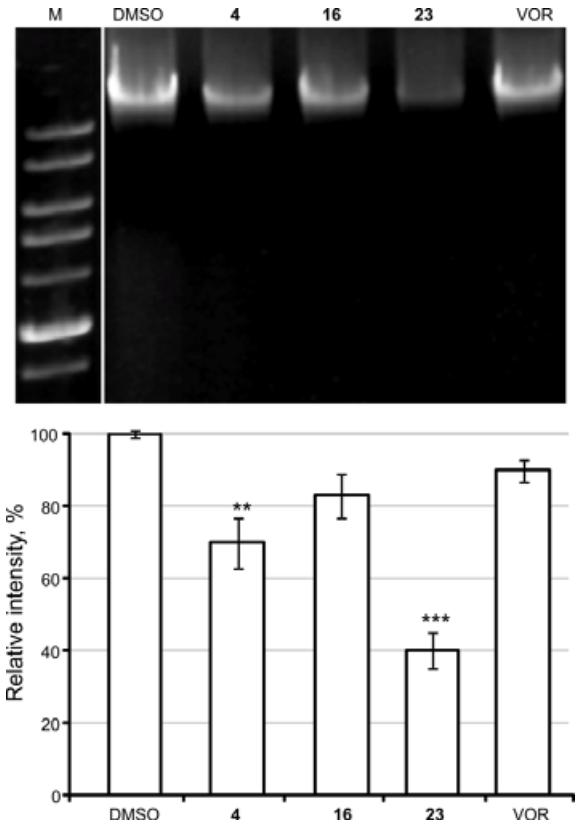
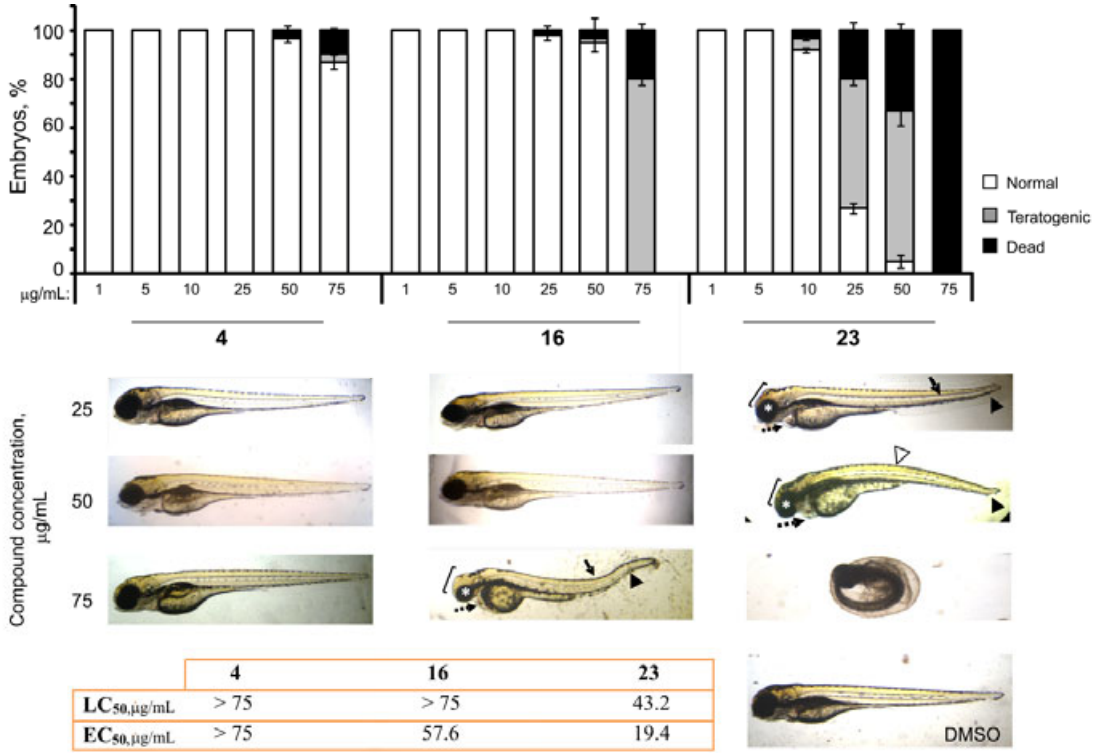


Figure 4. *In vitro* interaction of the thiepines **4**, **16**, **23** and voriconazole (VOR) with genomic DNA (gDNA) from *C. albicans* (results are considered significant when compared to the DMSO control: ***p<0.001 and **p<0.01; M is molecular weight standard).

In vivo embryotoxicity assessment

Zebrafish has emerged as a versatile platform for drug discovery, as findings from experiments including this organism translate more accurately into the context of human body in terms of toxicity and bioavailability simplifying the path to clinical trials and reducing the failure of potential therapeutics at later stages of testing (27). Therefore, the toxicity of the most promising thiepines (the lethality and teratogenicity; see Supporting Information Table S1) was evaluated. Obtained results revealed different toxicity-profiles of tested compounds, and according to LC_{50} and EC_{50} values they were ranked by toxicity: **23>16> 4** (Figure 5; Table S2). Embryos exposed to **4** were developed without signs of toxicity at concentrations up to 50 µg/mL, while at 75 µg/mL only 13% of embryos were affected (Figure 5). All alive embryos upon exposure to 75 µg/ml of **16** were reduced in growth and had serious skeletal deformities (lordosis, deformed tail, small head, malformed eyes) accompanied with pericardial edema, while at lower concentrations **16** exhibited very weak toxicity (Figure 5). Completely lethal outcome before hatching was caused by **23** at 75 µg/mL, while lower

concentrations of **23** caused pericardial edema and skeletal abnormalities of embryos (scoliosis, deformed eyes, head and tail tip). At 50 µg/mL, **23** prevented hatching of 58% embryos (Figure 5; Table S2). All three thiepines exhibited desirable higher LC_{50} value on zebrafish embryos in comparison to MIC against *C. albicans* (Figure 5; Table 1). From this test, only **23** showed undesirable high teratogenicity with EC_{50} value higher than MIC against *C. albicans* (Figure 5; Table 1). In our recent study, we have shown high teratogenic effect of clinically used VOR.[22] Thus, these findings imply that selected thiepines could be further developed for the antifungal treatment.



	4	16	23
LC_{50},µg/mL	> 75	> 75	43.2
EC_{50},µg/mL	> 75	57.6	19.4

Figure 5. *In-vivo* toxicity of thiepines **4**, **16** and **23** on zebrafish embryos. LC_{50} and EC_{50} values (µg/mL) were derived from the concentration-response curves for thiepines in zebrafish toxicity assay at 96 hpf. Teratogenic effect on embryos: dashed arrow – pericardial edema, arrow – lordosis, black arrowhead – malformed tail, empty arrowhead – kyphosis, asterisk – malformed eyes, bracket – malformed head.

Conclusion

A series of thiepines was designed, synthesized and their antimicrobial properties were evaluated. Analyzed thiepines showed higher antifungal activity than antibacterial activity. From biological activity screening it was evident that the position of the phenyl ring was essential for the antifungal

activity. On the other hand, the electronic effects of substituents did not significantly influence activity. The most potent thiepine, **16**, had an MIC of 15.6 µg/mL and showed no embryo toxicity at 75 µg/mL, providing evidence that this chemotype provides a good platform for further optimization.

Acknowledgments

Support from the Serbian Ministry of Education, Science and Technological Development (Grants no. 172008 and 173048) and the Serbian Academy of Sciences and Arts is greatly appreciated. We also acknowledge Prof Valentina Arsic-Arsenijevic, head of the National Reference Medical Mycology Laboratory, Belgrade, Serbia for support.

Conflict of Interest

The authors declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Lethal and teratogenic effects observed in zebrafish (*Danio rerio*) embryos at different hours post fertilization (hpf).

Table S2. Effects of different concentrations of selected thiepines (4, 16 and 23) and voriconazole on the development of zebrafish (*Danio re*rio) embryos assessed at 96 hours post fertilization (hpf).

Appendix S1. Spectrum data of final compounds (NMR, HPLC)

References

1. Protiva, M. (1996) The place of dibenzo[b,f]thiepin derivatives in medicinal chemistry: structures and activities. J. Heterocycl. Chem.;33:497–521, and references cited therein.

2. Ueda, I., Sato, Y., Maeno, S., Umio, S. (1978) Synthesis and pharmacological properties of 8-chloro-10-(2-dimethylaminoethoxy)dibenzo[b,f]thiepin and related compounds. Neurotropic and psychotropic agents. III. Chem. Pharm. Bull.;26:3058–3070.

3. Ansari, M.I., Hussain, M.K., Arun, A., Chakravarti, B., Konwar, R., Hajela, K. (2015) Synthesis of targeted dibenzo[b,f]thiepines and dibenzo[b,f]oxepines as potential lead molecules with promising anti-breast cancer activity. Eur. J. Med. Chem.;99:113–124.

4. Božinović, N., Novaković, I., Kostić Rajačić, S., Opsenica, I.M., Šolaja, B.A. (2015) Synthesis and antimicrobial activity of azepine and thiepine derivatives. J. Serb. Chem. Soc.;80:839–852.

5. Sardi, J.C., Scorzoni, L., Bernardi, T., Fusco-Almeida, A.M., Mendes Giannini, M.J. (2013) *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J. Med. Microbiol.;62:10–24.

6. Ostrosky-Zeichner, L., Casadevall, A., Galgiani, J.N., Odds, F.C., Rex, J.H. (2010) An insight into the antifungal pipeline: selected new molecules and beyond. Nat. Rev. Drug Discov.;9:719–727.

7. Pfaller, M.A., Messer, S.A., Moet, G.J., Jones, R.N., Castanheira, M. (2011) *Candida* bloodstream infections: comparison of species distribution and resistance to echinocandin and azole antifungal agents in Intensive Care Unit (ICU) and non-ICU settings in the SENTRY Antimicrobial Surveillance Program (2008–2009). Int. J. Antimicrob. Agents;38:65–69.

8. Matsuda, T., Sato, S. (2013) Synthesis of dibenzoheteropines of group 13−16 elements via ring-closing metathesis. J. Org. Chem.;78:3329–3335.

9. Shirani, H., Janosik, T. (2007) A new concise strategy for synthesis of dibenzo[*b,f*]thiepins and related fused symmetrical thiepin derivatives. J. Org. Chem.;72:8984–8986.

10. Shirani, H., Bergman, J., Janosik, T. (2009) New syntheses of unsymmetrical thiepins and their selenium analogues. Tetrahedron;65:8350–8353.

11. Saito, M., Yamamoto, T., Osaka, I., Miyazaki, E., Takimiya, K., Kuwabara, H., Ikeda, M. (2010) Facile synthesis of [1]benzothieno[3,2-b]benzothiophene from *o*-dihalostilbenes. Tetrahedron Lett.;51:5277–5280.

12. Jepsen, T.H., Larsen, M., Jørgensen, M., Nielsen M.B. (2012) Three-step synthesis of (thio)xanthene and dibenzothiepine/dibenzoxepine by an intramolecular Mizoroki–Heck reaction of diaryl (thio)ethers. Synlett;23:418–422.

13. Božinović, N., Opsenica, I., Šolaja, B.A. (2013) Double palladium-catalyzed synthesis of azepines. Synlett;24:49–52.

14. Casey, J. T., O'Cleirigh, C., Walsh, P. K., O'Shea, D. G. (2004) Development of a robust microtiter plate-based assay method for assessment of bioactivity. J. Microbiol. Methods;58: 327–334.

15. Evans, B. C., Nelson, C. E., Yu, S. S., Beavers, K. R., Kim, A. J., Li, H., Nelson, H.M., Giorgio, T. D., Duvall, C. L. (2013) *Ex vivo* red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. J. Vis. Exp.;73:50166.

16. Tochtermann, W., Oppenländer, K., Nguyen-Duong Hoang M. (1967) Untersuchungen an siebenringsystemen, III. Über das intermediäre auftreten von 4.5-dehydro-dibenzo-[*b,f*] thiepin-*S.S*-dioxid[2]) und 4.5-dehydro-dibenzo-[*b,f*]-oxepin. Justus Liebigs Ann. Chem.;701:117–125.

17. Kutsumura, N., Toguchi, S., Iijima, M., Tanaka, O., Iwakura, I., Saito, T. (2014) DBU-promoted regioselective HBr-elimination of vicinal dibromides: effects of the adjacent oxygen and/or other heterofunctional groups. Tetrahedron;70:8004–8009.

18. Opsenica, I.M., Verbić, T.Ž., Tot, M., Sciotti, R.J., Pybus, B.S., Djurković-Djaković, O., Slavić, K., Šolaja, B. A. (2015) Investigation into novel thiophene- and furan-based 4-amino-7-chloroquinolines afforded antimalarials that cure mice. Bioorg. Med. Chem.;23:2176–2186.

19. Wang, C, Widom, J., Petronijevic, F., Burnett, J.C., Nuss, J.E, Bavari, S., Gussio R., Wipf P. (2009) Synthesis and biological evaluation of inhibitors of botulinum neurotoxin metalloprotease. Heterocycles;79:487–520.

20. Maciejewska, D., Żabiński, J., Kaźmierczak, P., Wójciuk, K., Kruszewski, M., Kruszewska, H. (2014) In vitro screening of pentamidine analogs against bacterial and fungal strains. Bioorg. Med. Chem. Lett.;24:2918–2923.

21. An, Y.-S., Hao, Z.-F., Zhang, X.-J., Wang, L.-Z. (2016) Efficient synthesis and biological evaluation of a novel series of 1,5-benzodiazepine derivatives as potential antimicrobial agents. Chem. Biol. Drug Des. doi: 10.1111/cbdd.12739

22. Abrão, P.H. O., Pizi, R.B., de Souza, T.B., Silva, N.C., Fregnan, A.M., Silva, F.N., Coelho, L.F., Malaquias, L.C., Dias, A.L., Dias, D.F., Veloso, M.P., Carvalho, D.T. (2015) Synthesis and Biological Evaluation of New Eugenol Mannich Bases as Promising Antifungal Agents. Chem. Biol. Drug Des.;86:459–465.

23. Mohamed, B.S.R., Subramanian, M., Shunmugiah, K. P. (2014) Inhibition of *Candida albicans* virulence factors by novel levofloxacin derivatives. Appl. Microbiol. Biotechnol.;98:6775–6785.

24. Ajdačić, V., Senerovic, L., Vranić, M., Pekmezovic, M., Arsic-Arsnijevic, V., Veselinovic, A., Veselinovic, J., Šolaja, B.A.; Nikodinovic-Runic, J., Opsenica, I.M. (2016) Synthesis and evaluation of thiophene-based guanylhydrazones (iminoguanidines) efficient against panel of voriconazole-resistant fungal isolates. Bioorg. Med. Chem.;24:1277–1291.

25. Knopik-Skrocka, A., Bielawski, J. (2002) The mechanism of the hemolytic activity of polyene antibiotics. Cell. Mol. Biol. Lett.;7:31–38.

26. Racané, L, Tralić-Kulenović, V., Kraljević Pavelić, S., Ratkaj, I., Peixoto, P., Nhili, R., Depauw, S., Hildebrand, M.-P., David-Cordonnier, M.-H., Pavelić, K., Karminski-Zamola, G. (2010) Novel diamidino-substituted derivatives of phenyl benzothiazolyl and dibenzothiazolyl furans and thiophenes: synthesis, antiproliferative and DNA binding properties. J. Med. Chem.;53:2418–2432.

27. MacRae, C. A., Peterson, R. T. (2015) Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov.;14:721–731.

Figure Legends and Tables

Figure 1. Structure of zotepine.

Scheme 1. Reagents and conditions: (i) $Pd(OAc)_2$, dppf, KSAc, NaO*t*-Bu, PhMe, 175 °C, µW.

Scheme 2. Reagents and conditions: (i) Br_2, CCl_4, r.t.; (ii) KO*t*-Bu, *t*-BuOH, 85 °C.

Scheme 3. Reagents and conditions: (i) $ArB(OH)_2$, $Pd(OAc)_2$, PPh_3, DME, Na_2CO_3 aq, EtOH, 90 °C; (ii) $ArB(OH)_2$, $Pd(OAc)_2$, SPhos, K_3PO_4, PhMe, 100 °C; (iii) ArSH, Pd_2dba_3, dppf, NaO*t*-Bu, PhMe, 90 °C; (iv) $H_2NCH_2CH_2NH_2$, S, 110 °C, µW.

Figure 2. Effect of subinhibitory amounts (MIC70) of selected thiepines on *C. albicans* hyphal formation.

Figure 3. Activity of thiepines **4**, **16**, **23**, nystatin (NYS) and voriconazole (VOR) on A) washed sheep red blood cells in hemolysis assay using MIC concentrations of all tested compounds, and B) *C. albicans* culture monitored by fluorescent microscopy after 5 h treatment (white bar represents 10 µm; × 40 magnification).

Figure 4. *In vitro* interaction of the thiepines **4**, **16**, **23** and voriconazole (VOR) with genomic DNA (gDNA) from *C. albicans* (results are considered significant when compared to the DMSO control: ***$p<0.001$ and **$p<0.01$; M is molecular weight standard).

Figure 5. *In-vivo* toxicity of thiepines **4**, **16** and **23** on zebrafish embryos. LC_{50} and EC_{50} values (µg/mL) were derived from the concentration-response curves for thiepines in zebrafish toxicity assay at 96 hpf. Teratogenic effect on embryos: dashed arrow – pericardial edema, arrow – lordosis, black arrowhead – malformed tail, empty arrowhead – kyphosis, asterisk – malformed eyes, bracket – malformed head.

Table 1. *In-vitro* antimicrobial activity of thiepines in comparison to clinically used drugs given as minimal inhibitory concentrations (MICs, μg/mL)

Compound	Antifungal activity		Antibacterial activity	
	C. albicans ATCC10231	*C. parapsilosis* C27	*P. aeruginosa*	*S. aureus*
2	250	250	500	>500
3	125	>500	250	200
4	31.25	250	500	>500
5	62.5	125	250	250
7	125	>500	>500	200
9	31.2	250	>500	>500
10	31.2	62.5	>500	>500
11	125	500	>500	>500
12	125	62.5	500	>500
13	500	250	500	>500
14	250	>500	250	125
15	250	250	500	>500
16	15.6	250	>500	>500
17	125	>500	125	250
18	250	250	500	250
19	125	>500	>500	>500
20	125	500	500	500
21	250	250	250	100
22	125	62.5	250	500
23	31.2	31.2	125	500
VOR[a]	0.5	R	/	/
NYS	2	2	/	/
KAN	/	/	50	10

[a] Control drug: voriconazole (VOR); nystatin (NYS); kanamycin (KAN)