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ORIGINAL PAPER

Newly synthesized indolizine derivatives – antimicrobial and antimutagenic properties

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A series of indolizine derivatives have been synthesized and subjected to antibacterial screening studies. Antibacterial activity of 21 derivatives was investigated against *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Salmonella typhimurium* and *Escherichia coli*; also, the sensitivity of model yeast *Candida parapsilosis* and some model filamentous fungi *Aspergillus fumigatus*, *Alternaria alternata*, *Botrytis cinerea* and *Microsporum gypseum* was tested. Newly synthesized indolizine derivatives have shown selective toxicity to Gram-positive bacteria *S. aureus* and were also considered to be able to inhibit the acidoresistant rod *M. smegmatis*. Derivative *XXI* has shown the highest inhibition effect with the bacteriostatic effect on the cells at the concentration of 25 μg mL^{-1}. The best antifungal activity has been detected in the presence of derivative *XIII*. Derivative *XIII* did also affect the morphology of hyphal tips of *B. cinerea*, which led to enhanced ramification of hyphae. Finally, the antimutagenic activity of derivatives was investigated. Significant antimutagenic activity was registered in case of derivative *VIII*. The number of induced revertants by mutagen [2-(5-nitrofuryl)acrylic acid] was decreased almost to the level of spontaneous revertants in the lowest applied concentration (50 μg per plate).

Keywords: indolizine derivative, antimicrobial activity

Introduction

Treatment of microbial diseases remains an important and challenging worldwide problem. In spite of a large number of antibiotics and chemotherapeutics available for medical use, the emergence of microbial resistance reveals a substantial medical need for new classes of antimicrobial agents. There is a real perceived need for the discovery of new compounds endowed with antimicrobial activity possibly acting through different mechanisms than those of the well-known classes of antibacterial agents. Indolizines, nitrogen containing heterocyclic systems, are widely distributed in nature. Synthesis of biologically active indolizine derivatives continues to attract the attention of organic chemists as potent pharmaceutical drugs due to their wide spectra of activities e.g. antioxidative, antiinflamatory, antibacterial, antifungal, antitumor, antiherpes and antinociceptive (Vemula et al., 2011; Nasir et al., 1998; Teklu et al., 2005; Grundersen et al., 2003; Kubo et al., 1996; Pearson & Guo, 2001; Foster et al., 1995; Vaught et al., 1990; Couture et al., 2000). Toyota et al. (2003) have recently reported that some indole alkaloids show antiviral properties due to the presence of the indolizine ring system in their structure. Polycyclic indolizine derivatives have been found to provide high-efficiency long-wavelength fluorescence quantum yield (Vlahovici et al., 2002). The synthesis of polycyclic indolizine derivatives has recently attracted research interest in the search for

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new opto-electronic materials (Mitsumori et al., 2004). Several polyhydroxylated indolizines have been proved to be promising inhibitors of glycosides (Hempel et al., 1993; Brandi et al., 1995). Thus, indolizine derivatives represent an important class of bioactive compounds with a wide range of applications such as potential central nervous system depressants, calcium entry blockers, cardiovascular agents, spectral sensitizers, biological markers and novel dyes (Gubin et al., 1992; Gupta et al., 2003; Sonnenschein et al., 2000; Hema et al., 2003). Other well-known pharmacological applications associated with this ring system are well documented in literature (Jørgensen et al., 2000; Wavefunction, 2006). Owing to the increasing importance of indolizine heterocycles in the field of biology and pharmacology, a novel series of 21 indolizine derivatives was synthesized and subjected to antibacterial screening studies against model bacteria, yeasts and filamentous fungi.

Experimental

Antimicrobial activity and antimutagenic assay in vitro

Antibacterial activity on acid fast bacteria *Mycobacterium smegmatis* (Collection of Microorganisms of Department of Microbiology, University of Natural Science, Comenius University, Bratislava, Slovakia), Firmicutes *Staphylococcus aureus* CCM 3953 and γ-proteobacteria *Escherichia coli* CCM 3988, *Salmonella typhimurium* CCM 4763 (Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic) and antifungal activity against model yeast *Candida parapsilosis* were evaluated by the microdilution method (M10-A5, M27-A3) (Clinical Laboratory Standard Institute, 2014).

Antifungal activity on filamentous fungi *Botrytis cinerea* CCM F-16 (Czech Collection of Microorganisms), *Alternaria alternata* (Collection of Microorganisms of Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology STU, Bratislava, Slovakia), *Microsporum gypseum* (Laboratory of Medical Mycology, Postgraduate Medical Institute, Bratislava, Slovakia) was studied by the macrodilution method according the protocol of Dudová et al. (2002).

Subcultures of microorganisms were prepared separately in Petri dishes containing appropriate agar medium and incubated at 37 ℃ for 24 h (bacteria); 48 h (model yeast) and at 25 ℃ for 96 h (filamentous fungi).

Assessment of antibacterial and antifungal activities was expressed as the concentration of the derivative inhibiting the growth of bacteria by 50 % (MIC_{50}) and MIC_{100} values that represent the minimal concentration at which full inhibition of the microbial growth (by 100 %) is observed. The MIC_{50} and MIC_{100} values were derived from the toxicity curves. Chromatographically pure compounds were dissolved in dimethylsulfoxide (DMSO) Sigma–Aldrich (Germany) and their final concentrations never exceeded 1.0 vol. % either in control or in treated samples. Morphological changes of *B. cinerea* were observed microscopically according to the protocol of Hudecová et al. (1994).

Assessment of mutagenicity and antimutagenicity was performed by the classic plate incorporation method (Maron & Ames, 1983) without metabolic activation using *S. typhimurium* TA 98 and TA100 (antimutagenicity was assayed only on *S. typhimurium* TA 98). (*E*)-3-(5-Nitrofuran-2-yl)prop-2-enoic acid (NFAA; Sigma–Aldrich) was used as a positive mutagen. Positive response was defined as a reproducible two fold increase of revertans with dose response relationship and statistical evaluation using the *t*-test.

Synthesis of indolizines

Melting points were determined on a Stuart SMP-30 melting-point apparatus (Fisher, Slovakia). All other solvent (Sigma–Aldrich) were used as supplied (analytical or HPLC grade) without prior purification. Reactions performed under argon (Messer Tatragas, Slovakia) were performed using an inflated balloon. All air- and moisture-sensitive reactions were carried out under positive argon atmosphere and applying magnetic stirring. Evaporation of solvents was performed on a Heidolph (Sigma–Aldrich) rotavapor (water aspirator) followed by static evaporation using an oil pump. Ascending flash column liquid chromatography (FLC) was performed on silica gel Kieselgel 60 (40–63 μm, 230–400 mesh; Lambda Life, Slovakia) as the stationary phase and analytical thin-layer chromatography (TLC) was performed on TLC silica gel 60 F254 glass plates (2.5–7.5 cm; Merck, Germany) or 0.25 mm silica gel 60 F254 (ALUGRAM-SIL G/UV254; Macherey–Nagel, Germany) and by dipping the plates in an aqueous solution of $KMnO_4$, K_2CO_3 and NaOH (3 : 20 : 5 mass %; all Sigma–Aldrich), followed by charring with a heat gun. Optical rotations were measured using a P-2000 Polarimeter (PTC-203; Jasco, Germany) with a water-jacketed 10 cm cell at the wavelength of the sodium D line ($\lambda = 589$ nm). Specific rotations are given in units of 10^{-1}° cm^2 g^{-1} and concentrations in mg mL^{-1}. IR spectra were recorded on a Nicolet 5700 FT-IR spectrometer (MKS Instruments, USA) as KBr discs or as thin films on KBr plates (film). ^{1}H NMR and ^{13}C NMR spectra were recorded on a VXR 300 and an Inova 600 Varian (USA) spectrometers in CD_3OD or $CDCl_3$. Solvents and chemical shifts are given in δ relative to TMS as the internal standard. COSY, NOESY and DIFFNOE techniques were used in the assignment of ^{1}H–^{1}H relationships and the determination of relative configurations. HSQC and HMBC techniques

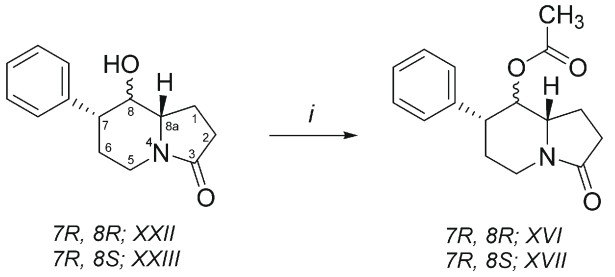

Fig. 1. Synthesis of (7*R*,8*R*)- and (7*R*,8*S*)-phenyloctahydroindolizines (*XVI* and *XVII*). Reagents and conditions; *i*) Ac_2O, Et_3N, CH_2Cl_2, 4-dimethylaminopyridine (0.1 eq.), 20 °C, 20 h, then $NaHCO_3$.

were used throughout the study for the assignment of the ^1H–^{13}C relationships.

Preparation of compounds *I–IV*, *VIII–XV*, *XVIII–XXI* from L-glutamic acid was introduced in our previous papers (Marchalin et al., 1999, 1993; Šafář et al., 2009a, 2009b).

All reagents and solvents used were purchased from Sigma–Aldrich, Lambda Life or Centralchem.

General procedure for the preparation of acetoxy derivatives V, VI, XVI and XVII

A solution of the corresponding alcohols *III*, *IV* and *XXII*, *XXIII* (2 mmol) and triethylamine (5 mL) in dichloromethane (25 mL) was cooled to + 5 °C. Acetic anhydride (4 mL) was added dropwise and the reaction was stirred for 10 h at room temperature. Then, the reaction mixture was diluted with dichloromethane (10 mL) and washed twice with 2 M hydrochloric acid (5 mL) and with saturated $NaHCO_3$ (2 × 25 mL). The organic layer was further washed with water (3 × 10 mL), and brine (10 mL), dried with $MgSO_4$ and concentrated under reduced pressure to give the crude product (Fig. 1–3).

(7R,8R,8aS)-3-Oxo-7-phenyloctahydro-indolizin-8-yl acetate (XVI)

This compound was obtained from alcohol *XXII* by recrystallization from toluene providing the acetate derivative *XVI* (0.46 g, 85 %) as colorless crystals: mp 146–148 °C; $[\alpha]_D^{22}$ = –29.8° (*c* 1.05, MeOH); IR, $\tilde{\nu}/cm^{-1}$ (KBr): 3500, 3440, 2927, 1734, 1644, 1464, 1456, 1424, 1375, 1273, 1234, 1057, 1046, 953, 872, 757, 698, 611, 578, 540, 492, 459. ^1H NMR (600 MHz, $CDCl_3$), δ: 1.77 (s, 3H, CH_3CO), 1.81 (dt, 1H, H_{6ax}; J = 4.8 Hz and 13.3 Hz), 1.89 (dddd, 1H, H_1; J = 5.8 Hz, 7.9 Hz, 8.4 Hz and 10.6 Hz), 1.92–1.97 (m, 1H, H_{6ax}), 2.18 (dddd, 1H, H_1; J = 5.1 Hz, 7.9 Hz, 10.1 Hz and 13.1 Hz), 2.40 (dt, 1H, H_2; J = 8.7 Hz and 17.5 Hz), 2.48 (ddd, 1H, $H_{2'}$; J = 4.7 Hz, 10.2 Hz and 17.0 Hz), 2.77 (dd, 1H, H_{5ax}; J = 2.0 Hz and 12.9 Hz), 2.81 (dt, 1H, H_{7ax}; J = 3.6 Hz and 10.4 Hz), 3.52 (dd, 1H, H_{8a}; J = 7.3 Hz and 7.9 Hz), 4.22 (dd, 1H, H_{5eq}; J = 3.6 Hz and 10.4 Hz), 4.87 (t, 1H, H_{8ax}; J = 10.0 Hz), 7.18 (d, 2H, 2 × H_{Ar}; J = 7.2 Hz), 7.23 (t, 1H, H_{Ar}; J = 7.3 Hz), 7.29 (t, 2H, 2 × H_{Ar}; J = 7.3 Hz); ^{13}C NMR (150 MHz, $CDCl_3$), δ: 20.5 (q, CH_3), 21.9 (t, C_1), 30.0 (t, C_2), 31.5 (t, C_6), 39.6 (t, C_5), 48.3 (d, C_7), 60.5 (d, C_{8a}), 76.4 (d, C_8), 127.2, 127.5 and 128.5 (d, C_{Ar}), 140.2 (s, C_{Ar}), 170.0 (s, OCO), 173.8 (s, C_3). HRMS calcd for $C_{16}H_{19}NO_3$ (273.33) $[M + 1]^+$: 274.1365, found 274.1358.

(7R,8S,8aS)-3-Oxo-7-phenyloctahydro-indolizin-8-yl acetate (XVII)

Recrystallization of alcohol *XXIII* from toluene gave the acetate derivative *XVII* (0.44 g, 80 %) as colorless crystals: mp 141–142 °C; $[\alpha]_D^{22}$ = –72.6° (*c* 1.08, MeOH); IR, $\tilde{\nu}/cm^{-1}$ (KBr): 3440, 2935, 1732, 1671, 1525, 1498, 1455, 1428, 1417, 1388, 1279, 1250, 1231, 1215, 1190, 1151, 1076, 1010, 935, 874, 798, 760, 703, 663, 573, 559, 474, 424. ^1H NMR (300 MHz, $CDCl_3$), δ: 1.57–1.67 (m, 2H, 2 × H_6), 1.84 (s, 3H, CH_3CO), 1.97–2.12 (m, 1H, H_1), 2.15 (dq, 1H, H_1; J = 5.0 Hz and 13.0 Hz), 2.29 (tt, 1H, 2 × H_2; J = 1.4 Hz and 9.0 Hz), 2.81 (dt, 1H, H_{5ax}; J = 3.4 Hz and 12.9 Hz), 2.97 (td, 1H, H_{7ax}; J = 2.9 Hz and 13.1 Hz), 3.81 (ddd, 1H, H_{8a}; J = 2.2 Hz, 4.8 Hz and 8.6 Hz), 4.30 (ddd, 1H, H_{5eq}; J = 1.6 Hz, 4.9 Hz and 13.3 Hz), 5.19 (t, 1H, H_{8eq}; J = 2.0 Hz), 7.10 (td, 2H, 2 × H_{Ar}; J = 1.6 Hz and 6.9 Hz), 7.13–7.27 (m, 3H, 3 × H_{Ar}); ^{13}C NMR (75 MHz, $CDCl_3$), δ: 19.7 (t, C_1), 20.7 (q, CH_3), 23.4 (t, C_6), 30.2 (t, C_2), 39.5 (t, C_5), 45.1 (d, C_7), 59.9 (d, C_{8a}), 72.3 (d, C_8), 127.2, 127.4 and 128.5 (d, C_{Ar}), 140.6 (s, C_{Ar}), 170.0 (s, OCO), 173.9 (s, C_3). HRMS calcd for $C_{16}H_{19}NO_3$ (273.33) $[M + 1]^+$: 274.1365, found 274.1360.

(4R,4aS)-7-Oxo-4,4a,5,6,7,9-hexahydrothieno[2,3-f]indolizin-4-yl acetate (V)

Recrystallization of alcohol *III* (Fig. 2) from toluene/hexane (φ_r = 15 : 85) resulted in the acetate derivative *V* (0.40 g, 79 %) as colorless crystals: mp 102–103 °C; $[\alpha]_D^{22}$ = –27.9° (*c* 1.18, MeOH); IR, $\tilde{\nu}/cm^{-1}$ (KBr): 3111, 2951, 1725, 1677, 1435, 1420, 1374, 1321, 1263, 1230, 1141, 1033, 997, 967, 946, 929, 837, 802, 712, 666, 608, 527, 498, 432. ^1H NMR (300 MHz, $CDCl_3$), δ: 1.91–2.04 (m, 1H, H_5), 2.13 (s, 3H, CH_3CO), 2.18–2.37 (m, 1H, $H_{5'}$), 2.38–2.55 (m, 2H, 2 × H_6), 3.72 (dt, 1H, H_{4a}; J = 3.9 Hz and 8.2 Hz), 4.13 (d, 1H, H_{9ax}; J = 16.9 Hz), 5.00 (d, 1H, H_{9eq}; J = 16.9 Hz), 5.71 (d, 1H, H_4; J = 8.7 Hz), 6.71 (d, 1H, H_3; J = 5.2 Hz), 7.14 (d, 1H, H_2; J = 5.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$), δ: 21.0 (q, CH_3), 22.1 (t, C_5), 29.5 (t, C_6), 39.5 (t, C_9), 57.7 (d, C_{4a}), 70.6 (d, C_4), 124.7, 124.9 (d, C_2 and C_3), 133.3, 134.2 (s, C_{9a} and C_{3a}), 170.8 (s, OCO), 174.0 (s, C_7). HRMS calcd. for $C_{12}H_{13}NO_3S$ (251.30) $[M + 1]^+$: 252.0616, found 252.0611.

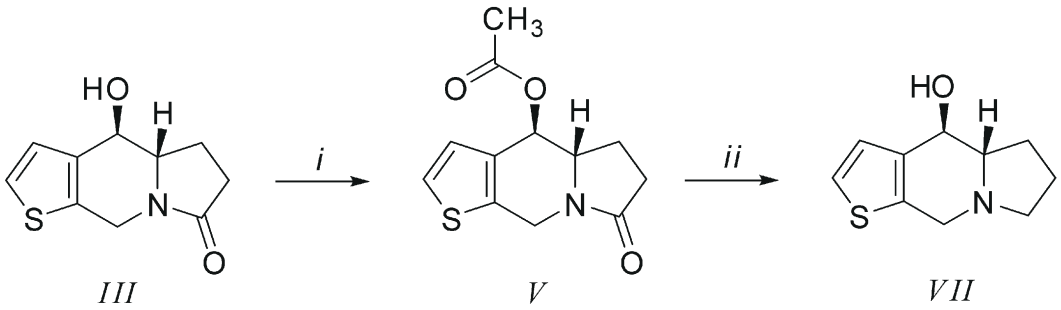

Fig. 2. Synthesis of *VII*; *i*) Ac_2O, Et_3N, CH_2Cl_2, DMAP cat., 20 °C, 12 h, then $NaHCO_3$; *ii*) $LiAlH_4$, Glauber's salt, Celite®.

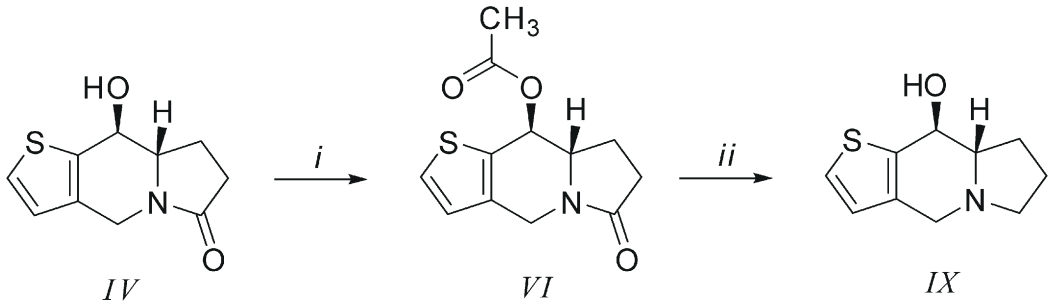

Fig. 3. Synthesis of *IX*; *i*) Ac_2O, Et_3N, CH_2Cl_2, DMAP cat., 20 °C, 12 h, then $NaHCO_3$; *ii*) $LiAlH_4$, Glauber's salt, Celite®.

(4R,4aS)-4,4a,5,6,7,9-Hexahydrothieno[2,3-f] indolizin-4-ol (VII)

Lithium aluminum hydride (0.38 g, 10 mmol) was added to a solution of acetoxy derivative *V* (0.50 g, 2 mmol) in dry tetrahydrofuran (THF; 20 mL) at room temperature and the mixture was heated under reflux for 1 h (Fig. 2). The resulting mixture was cooled and water was added cautiously until the lithium complex was destroyed. The mixture was then diluted with water (20 mL) and dichloromethane (50 mL). The dichloromethane layer was separated and the aqueous layer was extracted with dichloromethane (2 × 20 mL). The combined extracts were washed with water, brine, dried over $MgSO_4$ and concentrated in vacuum to give a residue. Recrystallization of the solid from hexane gave pure indolizinol *VII* (0.32 g, 82 %); mp 71–73 °C; $[\alpha]_D^{22}$ = +43.3° (*c* 1.04, MeOH); IR, $\tilde{\nu}/cm^{-1}$ (KBr): 3103, 2956, 2870, 2808, 1668, 1433, 1367, 1288, 1254, 1209, 1133, 1111, 1069, 1009, 975, 941, 906, 835, 789, 692, 625, 573, 455. ^{1}H NMR (300 MHz, $CDCl_3$), δ: 1.52–1.69 (m, 1H, H_5), 1.70–1.96 (m, 2H, 2 × H_6), 2.04–2.17 (m, 2H, H_5 and H_{4a}), 2.27 (q, 1H, H_7; J = 8.9 Hz), 2.47 (s, 1H, OH), 3.12 (t, 1H, H_7; J = 7.8 Hz), 3.28 (d, 1H, H_{9ax}; J = 14.3 Hz), 4.00 (d, 1H, H_{9eq}; J = 14.3 Hz), 4.29 (d, 1H, H_4; J = 7.0 Hz), 6.95 (d, 1H, H_3; J = 4.8 Hz), 7.06 (d, 1H, H_2; J = 4.8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$), δ: 22.1 (t, C_6), 28.3 (t, C_5), 51.6 (t, C_9), 54.2 (t, C_7), 68.2 (d, C_{4a}), 72.5 (d, C_4), 123.6, 125.1 (d, C_2 and C_3), 135.1, 139.3 (s, C_{3a} and C_{9a}). HRMS calcd for $C_{10}H_{13}NOS$ (195.28) $[M + 1]^+$: 196.0718, found 195.0711.

(8aS,9S)-6-Oxo-4,6,7,8,8a,9-hexahydrothieno[3,2-f] indolizin-9-yl acetate (VI)

Recrystallization of alcohol *IV* from toluene/hexane (φ_r = 15 : 85) provided the acetate derivative *VI* (0.41 g, 82 %) as colorless crystals: mp 105–106 °C; $[\alpha]_D^{22}$ = –25.8° (*c* 1.12, MeOH); IR, $\tilde{\nu}/cm^{-1}$ (KBr): 2951, 2906, 1727, 1676, 1416, 1369, 1261, 1229, 1194, 1168, 1140, 1025, 929, 833, 800, 710, 661, 617, 584, 500, 439. ^{1}H NMR (300 MHz, $CDCl_3$), δ: 1.91–2.07 (m, 1H, H_8), 2.13 (s, 3H, CH_3CO), 2.24–2.39 (m, 1H, $H_{8'}$), 2.40–2.50 (m, 2H, 2 × H_7), 3.76 (dt, 1H, H_8a; J = 4.4 Hz and 8.2 Hz), 4.00 (d, 1H, H_{4ax}; J = 16.8 Hz), 4.87 (dd, 1H, H_{4eq}; J = 1.4 Hz and 16.8 Hz), 5.77 (td, 1H, H_9; J = 1.8 Hz and 8.6 Hz), 6.76 (d, 1H, H_3; J = 5.2 Hz), 7.23 (d, 1H, H_2; J = 5.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$), δ: 20.9 (q, CH_3), 22.4 (t, C_8), 29.6 (t, C_7), 40.2 (t, C_4), 58.2 (d, C_{8a}), 70.7 (d, C_9), 124.4, 126.6 (d, C_2 and C_3), 133.6, 133.8 (s, C_{9a} and C_{3a}), 170.7 (s, OCO), 174.2 (s, C_6). HRMS calcd. for $C_{12}H_{13}NO_3S$ (251.30) $[M + 1]^+$: 252.0616, found 252.0611.

(8aS,9S)-4,6,7,8,8a,9-Hexahydrothieno[3,2-f] indolizin-9-ol (IX)

Lithium aluminum hydride (456 mg, 12 mmol) was added to a solution of acetoxy derivative *VI* (600 mg,

Table 1. Antibacterial activity of tested compounds characterized by IC_{50} and MIC (μg mL^{-1})

Compound	*S. aureus*		*M. smegmatis*		*S. typhimurium*		*E. coli*	
	MIC_{50}	MIC_{100}	MIC_{50}	MIC_{100}	MIC_{50}	MIC_{100}	MIC_{50}	MIC_{100}
	μg mL^{-1}							
1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	350	> 400	400	> 400	n.a.	n.a.	n.a.	n.a.
3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
6	100	400[a]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
10	300	> 400	400	> 400	n.a.	n.a.	n.a.	n.a.
11	100	400[a]	250	> 400	n.a.	n.a.	n.a.	n.a.
12	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
13	400	> 400	100	400[a]	n.a.	n.a.	n.a.	n.a.
15	25	100[a]	150	400[a]	n.a.	n.a.	n.a.	n.a.
16	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
17	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
18	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
19	100	400[a]	75	100[f]	n.a.	n.a.	n.a.	n.a.
20	25	400[a]	75	100[a]	n.a.	n.a.	25	400[a]
21	12	25[a]	12	25[a]	n.a.	n.a.	35	100[a]
standard	0.001[b]	0.004[f,a]	0.12[c]	1[c,f]	0.005[d]	0.003[d,f]	0.0006[e]	0.001[f,e]

a) Bacteriostatic effect; *b*) ampicillin; *c*) streptomycin; *d*) ciprofloxacin; *e*) gentamicin; *f*) minimal bactericidal concentration; n.a. – not active.

2,4 mmol) in dry THF (30 mL) at room temperature and the mixture was heated under reflux for 1 h (Fig. 3). After additional 40 min, it was carefully quenched with $NaSO_4 \cdot 10H_2O$/Celite® (10 g; 2 : 1 mass ratio). Gas evolution was observed. Dry diethyl ether (20 mL) was then added and, after 30 min, the suspension was dried over $MgSO_4$ (3 g), filtered and concentrated in vacuo to give a residue (451 mg, 90 %). Recrystallization of the solid from hexane gave pure indolizinol *IX* (369 g, 79 %); mp 144.8–145.9 °C; $[\alpha]_D^{22} = +93.3°$ (*c* 1.02, MeOH); IR, $\tilde{\nu}/cm^{-1}$ (KBr): 3289, 3076, 2972, 2954, 2903, 2801, 1459, 1401, 1309, 1182, 1171, 1150, 1097, 1057, 1050, 1014, 983, 962, 923, 909, 869, 850, 838, 772, 707, 686, 631, 603, 580, 514, 495, 442. ^{1}H NMR (300 MHz, $CDCl_3$), δ: 1.90–1.71 (m, 2H, H_7 and H_8), 2.08–1.98 (m, 1H, H_8), 2.55 (dt, 1H, H_6; J = 9.8 Hz, 6.9 Hz), 3.18–3.08 (m, 2H, H_6 and H_9), 3.23 (s, 1H, OH), 3.73 (d, 1H, H_4; J = 17.1 Hz), 3.93 (d, 1H, H_4; J = 17.1 Hz), 5.17 (d, 1H, H_{9a}; J = 7.1 Hz), 6.87 (d, 1H, H_3; J = 7.6 Hz, 1H), 7.25 (d, 1H, H_2; J = 7.3 Hz,). ^{13}C NMR (75 MHz, $CDCl_3$), δ: 27.2 (t, C_6), 24.0 (t, C_5), 49.7 (t, C_9), 57.0 (t, C_7), 66.6 (d, C_{4a}), 70.0 (C_4), 124.6, 124.6 (d, C_2 and C_3), 129.6, 133.9 (s, C_{3a} and C_{9a}). HRMS calcd for $C_{10}H_{13}NOS$ (195.28) $[M + 1]^+$: 196.0718, found 195.0711.

Results and discussion

Antimicrobial activity in vitro

Table 1 summarizes antimicrobial activities of all tested compounds (characterized by IC_{50} and MIC values in μg mL^{-1}). Among the tested compounds, *VI*, *XI*, *XV*, *IXX*, *XX*, and *XXI* were proved to fully inhibit the growth of *S. aureus* with bacteriostatic activity on the cells (Table 1). Compound *XXI* (Fig. 4) demonstrated the best antistaphylococcal activity.

The growth of *S. aureus* was fully inhibited at the concentration of 25 μg mL^{-1} with bacteriostatic effect on the cells. The MIC_{50} value was determined at the concentration of 12 μg mL^{-1}. Good antistaphylococcal activity was also noticed in the presence of compound *XV*. At the concentration of 100 μg mL^{-1}, the growth of *S. aureus* was fully inhibited. According to the results of previous studies, indolizine derivatives were able to inhibit the growth of mycobacteria (Gundersen et al., 2007), therefore, the acidoresistant rod *M. smegmatis* was chosen as the model microbe for antimycobacterial activity assay. The best growth inhibition was observed in the presence of derivatives *XXI* and *XX*; MIC_{100} = 25 μg mL^{-1} (derivative *XXI*) and MIC_{100} = 100 μg mL^{-1} (derivative *XX*). Also deriva-

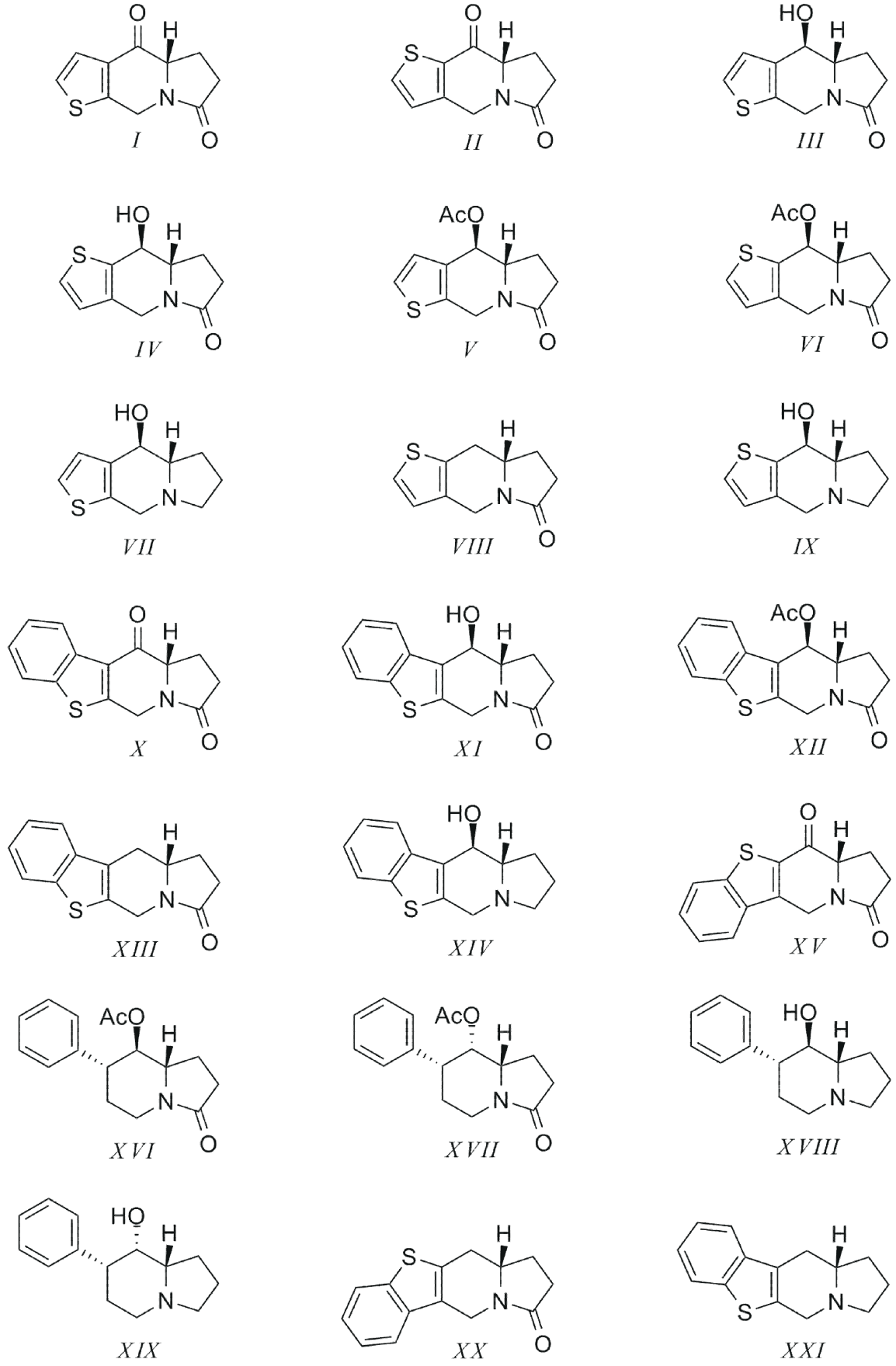

Fig. 4. Newly synthesized indolizine derivatives tested for antimicrobial activity.

Table 2. Antifungal activity of active newly synthesized indolizine derivatives characterized by IC_{50} and MIC (μg mL^{-1})

Compound	*A. flavus*		*M. gypseum*		*A. alternata*		*B. cinerea*		*C. parapsilosis*	
	MIC_{50}	MIC_{100}	MIC_{50}	MIC_{100}	MIC_{50}	MIC_{100}	MIC_{50}	MIC_{100}	MIC_{50}	MIC_{100}
	μg mL^{-1}									
11	n.a.	n.a.	100	400s	400	> 400	400	> 400	n.a.	n.a.
12	100	400[a]	n.a.	n.a.	200	> 400	n.a.	n.a.	n.a.	n.a.
13	100	400[a]	73	200[a]	75	200[a]	100	400[b]	145	200[b]
20	75	400[a]	40	100[b]	40	400[a]	140	400[a]	n.a.	n.a.
21	183	> 400	50	400[b]	200	400[b]	75	400[a]	n.a.	n.a.
standard[c]	0.1	1.0[b]	0.05	0.1[b]	1.0	2.5[b]	0.8	25[a]	0.05	0.1[b]

a) Bacteriostatic effect; *b*) fungicide effect on spores; *c*) terbinafine; n.a.– not active.

tives *XIII* and *XV* were able to fully inhibit the growth of this bacterium (Table 1). All these derivatives have related structures. Based on our results it can be assumed that the antimicrobial activity depends on the presence of a benzothienoindolizine skeleton and that the position of the sulfur heteroatom and the presence of substituents on the basic skeleton are critical for the antimicrobial potency. In this newly synthesized set of derivatives, two structural analogues were presented, derivatives *IXX* and *XVIII*. While derivative *IXX* was proved to be able to inhibit the growth of *M. smegmatis* by 100 %, its structural analogue (isomer), derivative *XVIII*, did not affect the growth of *M. smegmatis* at all. No inhibitory effect was found even at its concentration of 400 μg mL^{-1}. Further study of these two derivatives uncovered rapid induction of resistance to derivative *IXX* that was combined with the cross-resistance to a wide spectra of clinical relevant antibiotics and chemotherapeutics (Olejníková et al., 2013).

Some researchers, e.g. Hazra et al. (2011) or Darwish (2008), had reported on the synthesis and antibacterial activity of functionalized indolizine derivatives. For bacterial strains, the MIC values were in the range of 32–500 μg mL^{-1}. These authors concluded that the phenyl substituent on the basic indolizine skeleton of the active compound might be responsible for its good antimicrobial properties. Also, Gundersen et al. (2003) had synthesized 1-substituted indolizine derivatives and reported on their antimicrobial activity against *Mycobacterium tuberculosis* H37Rv. Interestingly, the authors claimed one of the tested derivatives as the first antimycobacterial indolizine with the MIC value of 6.25 μg mL^{-1}. These authors highlighted the presence of hydroxyl and phenyl group(s), which might be responsible for the antitubercular activity. However, no elucidation of the mechanism of action of this active compound has been provided. Another series of indolizine-1-carbonitrile derivatives was also investigated for their antimicrobial and antimycobacterial activities and were suggested as phosphatase inhibitors. Tyrosine phosphatases are responsible for the virulent character of many pathogenic bacteria (Weide et al., 2006). Specifically, two putative tyrosine phosphatases, MPTPA and MPTPB, are virulence factors of *M. tuberculosis* (Koul et al., 2000). Weide et al. (2006) concluded that the benzoyl substituent on the indolizine scaffold is critical for the phosphatase inhibitory activity. Moreover, the 5-(phenoxymethyl)indolizine skeleton present in this active derivative might be a promising lead structure.

Hence, it is evident that the substituted indolizine derivatives are potent candidates for extensive (range of) antibacterial activities. The synthesized derivatives are able to inhibit the growth of *M. tuberculosis* strain (H37RV) and they show good antimicrobial activity on Gram-negative bacteria such as *E. coli*, *Pseudomonas aeruginosa*, *Enterebacter fecalis* as well as on Gram-positive *S. aureus* (Vemulla et al., 2011).

Our tested compounds did not affect the growth of model Gram-negative bacteria (*E. coli, S. typhimurium*) significantly excluding derivatives *XX* and *XXI*, which fully inhibited the growth of *E. coli* (Table 1); antimicrobial potency of derivative *XXI* was higher compared to that of the other derivatives studied. (Table 1)

The previous works of Hazra et al. (2011) and Darwish (2008) on antimicrobial activity had also taken into account the antifungal activity on both yeasts and filamentous fungi. All the tested derivatives showed just moderate effect on the growth of *Aspergillus niger*, *Candida albicans*, and *Candida tropicalis*. The MIC values of most active compound ranged from 500 μg mL^{-1} to 1000 μg mL^{-1}.

Concerning the antifungal activity of the studied set of derivatives, only derivative *XIII* has shown antifungal effect on model yeast *C. parapsilosis* (MIC_{100} = 200 μg mL^{-1}). The growth of model filamentous fungi was inhibited by derivatives *XI*, *XII*, *XIII*, *XX*, *XXI* (MIC_{50} = 75–400 μg mL^{-1}; MIC_{100} = 200–400 μg mL^{-1}). The activity of these four derivatives on model filamentous fungi is comparable (Table 2).

The most sensitive fungus was the dermatophyte *M. gypseum* (MIC_{100} = 100 μg mL^{-1}). Derivative *XIII* had inhibited the growth by 100 % with fungicidal effect on conidia (spore). Partial growth inhibi-

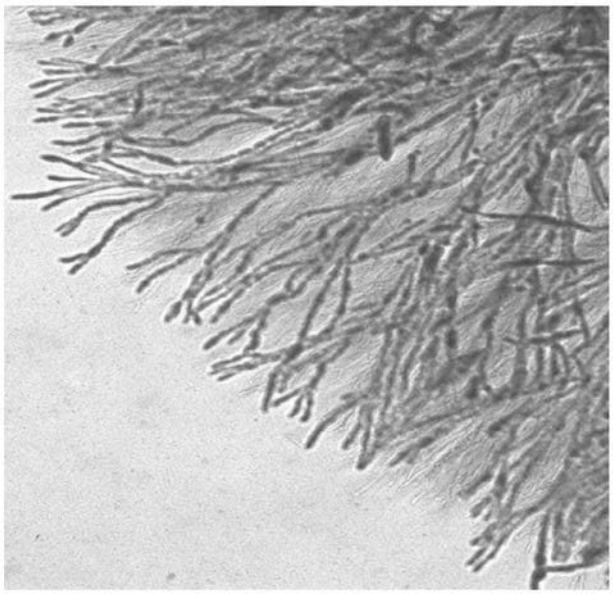

Fig. 5. Changes in the morphology of *B. cinerea* hyphal tips induced by derivative *XIII*, (magnification × 200).

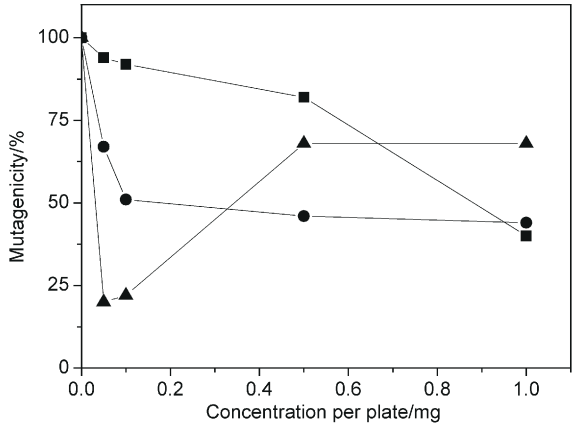

Fig. 6. Antimutagenic activity of: ● – *II*, ■ – *VII*, ▲ – *VIII*.

tion in addition to significant morphological changes of *B. cinerea* was also observed. In the presence of derivative *XIII,* the effect of increased ramification of the hyphal tips of *B. cinerea* were manifested (Fig. 5).

Comparing the structure of the tested derivatives, it seems that the addition of the OH group (*XI*) and CH_3COO (*XII*) on the basic indolizine skeleton decreases the antifungal activity. Finally, the derivatives were tested for their potential mutagenic activity. Based on our data it can be concluded that the newly synthesized derivatives have not increased the number of revertants of *S. typhimurium* TA98 or TA100. This means that they do not induce point and frameshift mutations at any of the tested concentrations and are considered to be non-mutagenic. Finally, the derivatives were also investigated for their antimutagenic activity using *S. typhimurium* TA 98, in particular, whether these structures are able to disturb the effect of the positive mutagen NFAA. Significant antimutagenic activity was registered in case of derivative *VIII*, in which the number of induced revertants decreased almost to the level of spontaneous revertants in the lowest applied concentration (50 μg per plate; Fig. 6). With the increasing concentration of this derivate, its antimutagenicity decreased. Derivative *II* reduced the effect of mutagen NFAA only moderately, especially at the concentration of 100 μg per plate (Fig. 6). These two derivatives, *VIII* and *II*, showed similar tendency to diminish the antimutagenic effect with their increasing concentration, which can be caused by some interaction of NFAA and the derivative at its higher concentrations. Partial decrease of induced revertants by NFAA was observed also in the presence of derivative *VII*, which showed significant antimutagenic trend with the increasing concentration. The highest decrease (55 %) of mutagenicity was observed at the concentration of 1 mg per plate (Fig. 6). The trend of antimutagenic activity can be related to the derivative structure. Both derivatives, *II* and *VIII*, with a similar trend in the antimutagenic activity, have a sulfur atom in position 5 and oxygen in position 11. Moreover, derivative *VIII* has no substituent in position 7 and it showed the highest decrease (80 %) of revertants, almost to the level of spontaneous revertants. Dose dependent antimutagenic effect (derivative *VII*) was caused by a structure with a sulfur atom in position 3, the position 7 was substituted with OH group and no substituent was presented in the position 11.

All other tested compounds account for a very nominal or almost no antimutagenic effect.

Conclusions

Antimicrobial activity of 21 newly synthesized indolizine derivatives is described. Derivative *XXI* (Fig. 4) showed the best antibacterial activity on both *S. aureus* and *M. smegmatis* (Table 1). The tested derivatives did not inhibit the growth of Gram-negative bacteria and only moderate growth inhibition was observed in case of *C. parapsilosis* except for derivative *XIII* (Table 2). Derivative *XIII* provided the best antifungal activity on all model filamentous fungi (Table 2). Considering the structure it seems that the antibacterial activity is caused by the presence the benzothienoindolizine skeleton, while the position of a sulfur heteroatom and a substituent on the basic skeleton are critical for the biopotency. Based on our results it seems that the addition of an OH group (*XI*) and CH_3COO (*XII*) to the active structure (*XIII*) results in the reduction of antifungal activity. Finally, antimutagenic activity of these compounds was also investigated. Some of the indolizine derivatives (*II*, *VII*, *VIII*) were able to suppress the activity of mutagen (NFAA). Antimutagenic effect of

indolizine derivatives was proved to be dependent on their concentration (Fig. 5). Perhaps there is a need for interaction of these compounds to achieve the antimutagenic effect demonstrated only at defined concentrations. Further studies are necessary in order to detect the antibacterial mode of action and also to explain the decrease of antimutagenic activity of the tested derivatives.

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