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Synthesis and antimicrobial properties of steroid-based imidazolium salts

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ABSTRACT

Imidazolium salts reveal interesting biological properties, especially regarding antitumor and antimicrobial activities. Two series of imidazolium salts based on steroids were obtained in an efficient and convenient synthesis. They were biologically tested to evaluate their antibacterial and antifungal properties. The activities of new salts, especially in relation to Gram-positive bacterial strains are comparable to the activities of known antibiotics. The most promising activity was that against *C. albicans*, which exceeded the antifungal activity of commonly used drugs. Some of the new salts exhibited improved antifungal activities against phytopathogenic fungi: *B. cinerea* and *C. beticola*. Our research showed that new compounds could be potentially useful as antifungal antibiotics or inhibiting agents against pathogenic fungi.

1. Introduction

Imidazolium salts are very important imidazole derivatives that consist of discrete cation and anion pairs [1]. They are widely utilized in organic synthesis, especially as ionic liquids [2] or precursors of *N*-heterocyclic carbenes [3]. They have a tremendous potential in biological applications, because of their antitumor [4–6] and antimicrobial activities [7–9] or antioxidative properties [10,11]. They are also widely utilized in bioengineering as drug/gene delivery systems [12] or biosensors [13]. Imidazolium salts were also reported to exhibit fungicidal activity [14]. These biological activities are related to their ionic structure, the presence of azole core [15] and various substituents attached to nitrogen atoms. The intrinsic biological activity of an azole moiety is often expressed when it is introduced to some bioactive compounds [16]. Moreover, it should be noted that combining two bioactive molecules as a way to improve biological properties of starting compounds is an emerging practice in medicinal chemistry. In this context, it was expected that a hybrid compound formed by attaching an imidazole moiety to a biologically active steroid may enhance the biological properties of both fragments [17,18]. The basicity and hydrophilicity of an azole moiety might alter the biological function of a steroid. Lithocholic acid (LCA, **1**) was chosen because of its wide range of biological activities such as α-2,3-sialyltransferase inhibition [19], vitamin D receptor modulation [20], antibacterial and antifungal effect [21], and antitumor activity [22,23]. These interesting properties are connected with its large, rigid, and curved steroidal skeleton, enantiomeric purity and unique amphiphilicity. The pharmacological interest in lithocholic acid is directly related to the fact that liver cells can specifically recognize such a natural ligand, which makes LCA ideal building block for the synthesis of novel molecules that can be recognized at the molecular level [24]. Derivatives of **1** with oxazole fragment itself display some antifungal activity against *Candida albicans* [25].

Syntheses of some steroids with an imidazole ring attached to different positions of the skeleton were reported by substitution of halogeno- or epoxy-steroids with lithiated imidazole either under standard conditions [26,27] or using microwave irradiation [28]. These compounds exhibit cytotoxic activity against cancer cells [29,30], inhibit 17α-lyase [31], show potent skeletal muscle relaxant and neuromuscular blocking properties [32]. They can also be utilized as receptors for fluoride ion recognition [33]. However, to the best of our knowledge, there is no report about antimicrobial activity, including activity against plant pathogens, of imidazolium salts, especially steroid derivatives substituted in the side chain (22- or 24-imidazolo). We designed and synthesized two series of imidazolium salts starting from lithocholic acid and a steroid compound similar to one of LCA metabolites [34] with a 4-en-3-one group in ring A and a shorter side chain: 3-oxo-23,24-dinorchol-4-en-22-al (**2**) (Fig. 1).

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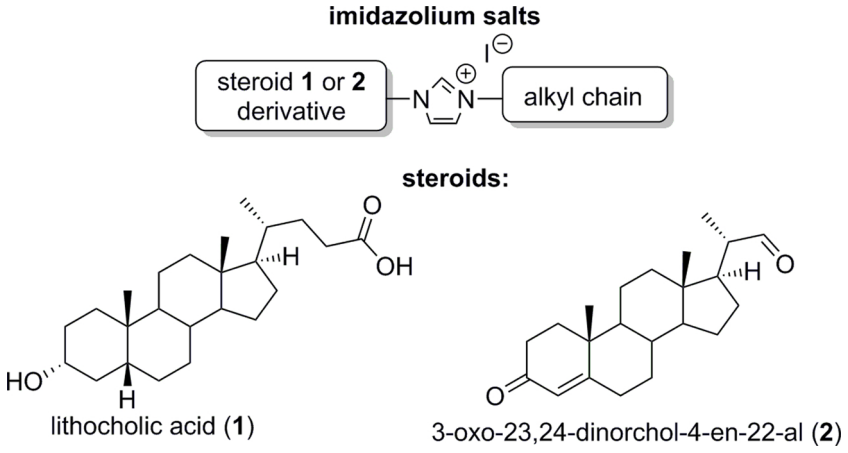


Fig. 1. The target imidazolium salts and starting steroids.

2. Materials and methods

2.1. General remarks

Melting points were determined on an MP70 (Mettler Toledo) apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II spectrometer (400 and 100 MHz, respectively). Spectra are referenced relative to the chemical shift of TMS. Mass spectra were obtained with Micromass LCT TOF and Accurate-Mass Q-TOF LC/MS 6530 spectrometers. IR spectra were recorded on a Nicolet series II Magna-IR 550 FT-IR spectrometer. Column chromatography was performed on silica gel 230–400 mesh. CH_2Cl_2 was dried by distillation over CaH_2, THF over Na/benzophenone. Steroidal compounds were synthesized according to literature procedures: 5β-cholane-3α,24-diol [35], 24-*p*-toluenesulfonyloxy-5β-cholan-3α-ol and 24-iodo-5β-cholan-3α-ol (**3**) [36,37], 22-hydroxy-23,24-dinorchol-4-en-3-one [38]. Other chemicals are commercially available and used as received.

2.2. General procedures A-D

2.2.1. Synthesis of N-imidazolyl substituted steroid – general procedure A

Imidazole (5 eq) and NaH (5 eq) were stirred in dry THF (3 mL) for 30 min at room temperature under argon. To this mixture, the steroid iodide (1 eq) in dry THF (5 mL) was added and the reaction was stirred for 16 h at room temperature. Then water was added (20 mL) and the mixture was extracted with CH_2Cl_2 (3 x 15 mL). The combined extracts were washed with brine (2 x 15 mL) and water (15 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The resulting product was sufficiently pure to use in the next step or was purified by flash chromatography.

2.2.2. Synthesis of imidazolium salts – general procedure B

To the solution of *N*-imidazolyl steroid in dry CH_2Cl_2 (1 mL), alkyl iodide (excess) was added under argon. The reaction was carried out at room temperature for 48 h, protected from light. Then the solvent and excess of alkyl iodide were evaporated. The remaining residue was dissolved in CH_2Cl_2 (2 mL), the product was precipitated with Et_2O (10 mL) and filtered. The crystallization (CH_2Cl_2/Et_2O) was repeated two more times.

2.2.3. Synthesis of imidazolium salts – general procedure C

N-Imidazolyl steroid was dissolved in alkyl iodide (excess) under argon. The reaction was carried out at room temperature for 48 h while protected from light. Then the excess of alkyl iodide was evaporated. The remaining residue was dissolved in CH_2Cl_2 (2 mL), the product was precipitated with Et_2O (10 mL) and filtered. The crystallization from CH_2Cl_2/Et_2O was repeated two more times.

2.2.4. Synthesis of imidazolium salts – general procedure D

To the solution of *N*-imidazolyl steroid in dry CH_2Cl_2 (0.5–1 mL), alkyl bromide (excess) and NaI (2 eq) were added under argon. The reaction was carried out at room temperature for 48 h and protected from light. Then inorganic solid was filtered off and the filtrate was evaporated. The remaining residue was dissolved in CH_2Cl_2 (2 mL), the product was precipitated with Et_2O (10 mL) and filtered. The crystallization (CH_2Cl_2/Et_2O) was repeated two more times.

2.3. Synthesis of imidazolium salts

2.3.1. 24-(N-imidazolyl)-5β-cholan-3α-ol (*4*)

General procedure A was followed using imidazole (144 mg, 2.1 mmol), NaH (84 mg, 60% suspension in mineral oil, 2.1 mmol) and 24-iodo-5β-cholan-3α-ol (**3**, 200 mg, 0.42 mmol) to produce a white solid in 100% yield (175 mg). Mp = 163–165 °C (from MeOH); IR (ATR) ν = 3104, 2927, 2852, 1506, 1445, 1363, 1067 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 7.48 (s, 1H, $H_{imidazole}$), 7.05 (s, 1H, $H_{imidazole}$), 6.91 (s, 1H, $H_{imidazole}$), 3.83 (m, 2H, CH_2N), 3.62 (m, 1H, H-3), 0.91 (s, 3H, CH_3), 0.89 (d, J = 6.6 Hz, 3H, CH_3), 0.63 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.0 ($CH_{imidazole}$), 129.2 ($CH_{imidazole}$), 118.7 ($CH_{imidazole}$), 71.6 (CH, C-3), 56.4 (CH), 55.9 (CH), 47.5 (CH_2), 42.7 (C), 42.0 (CH), 40.3 (CH), 40.1 (CH_2), 36.4 (CH_2), 35.8 (CH), 35.3 (CH), 34.5 (C), 32.7 (CH_2), 30.5 (CH_2), 29.6 (CH_2), 28.2 (CH_2), 27.6 (CH_2), 27.1 (CH_2), 26.4 (CH_2), 24.1 (CH_2), 23.3 (CH_3), 20.8 (CH_2), 18.5 (CH_3), 12.0 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M+H]^+$ $C_{27}H_{45}N_2O^+$ 413.3526, found 413.3513.

2.3.2. N-(5β-cholan-3α-ol-24-yl)-N'-methylimidazolyl iodide (*5a*)

General procedure B was followed using **4** (50 mg, 0.12 mmol), methyl iodide (0.5 mL, 8 mmol) to produce a white salt in 83% yield (55 mg). Mp = 179–181 °C (from CH_2Cl_2/Et_2O); IR (ATR) ν = 3342, 2927, 2861, 1555, 1444, 1370, 1156, 1031 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 10.12 (s, 1H, H-$2_{imidazole}$), 7.44 (s, 1H, $H_{imidazole}$), 7.34 (s, 1H, $H_{imidazole}$), 4.29 (brs, 2H, CH_2N), 4.13 (s, 3H, NCH_3), 3.62 (m, 1H, H-3), 0.93 (d, J = 6.6 Hz, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.63 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.6 ($CH_{imidazole}$), 123.7 ($CH_{imidazole}$), 122.0 ($CH_{imidazole}$), 71.6 (CH, C-3), 56.3 (CH), 55.7 (CH), 50.6 (CH_2), 42.6 (C), 41.9 (CH), 40.3 (CH), 40.0 (CH_2), 37.1 (CH_3), 36.3 (CH_2), 35.7 (CH), 35.3 (CH), 34.4 (C), 32.2 (CH_2), 30.4 (CH_2), 30.3 (CH_2), 28.3 (CH_2), 27.1 (CH_2), 27.0 (CH_2), 26.3 (CH_2), 24.1 (CH_2), 23.3 (CH_3), 20.7 (CH_2), 18.6 (CH_3), 12.0 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M-I]^+$ $C_{28}H_{47}N_2O^+$ 427.3683, found 427.3695.

2.3.3. N-(5β-cholan-3α-ol-24-yl)-N'-ethylimidazolyl iodide (*5b*)

General procedure B was followed using **4** (50 mg, 0.12 mmol), ethyl iodide (0.5 mL, 6.3 mmol) to produce a white salt in 64% yield

(44 mg). Mp = 176–178 °C (from CH_2Cl_2/Et_2O); IR (ATR) ν = 3380, 2920, 2858, 1561, 1444, 1378, 1166, 1035 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 10.14 (s, 1H, H-$2_{imidazole}$), 7.56 (s, 1H, $H_{imidazole}$), 7.44 (s, 1H, $H_{imidazole}$), 4.45 (d, J = 6.6 Hz, 2H, CH_2N), 4.30 (brs, 2H, CH_2N), 3.62 (brs, 1H, H-3), 1.63 (brs, 3H, CH_3), 0.90 (brs, 6H, $2CH_3$), 0.62 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.1 ($CH_{imidazole}$), 122.0 ($2CH_{imidazole}$), 71.6 (CH, C-3), 56.4 (CH), 55.7 (CH), 50.5 (CH_2), 45.4 (CH_2), 42.6 (C), 42.0 (CH), 40.3 (CH), 40.0 (CH_2), 36.3 (CH_2), 35.7 (CH), 35.3 (CH), 34.5 (C), 32.2 (CH_2), 30.5 (CH_2), 30.4 (CH_2), 28.3 (CH_2), 27.1 (CH_2), 27.0 (CH_2), 26.3 (CH_2), 24.1 (CH_2), 23.3 (CH_3), 20.7 (CH_2), 18.6 (CH_3), 15.7 (CH_3), 12.0 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M\text{-}I]^+$ $C_{29}H_{49}N_2O^+$ 441.3839, found 441.3851.

2.3.4. N-(5β-cholan-3α-ol-24-yl)-N'-pentylimidazolyl iodide (5c)

General procedure D was followed using **4** (50 mg, 0.12 mmol), pentyl bromide (0.5 mL, 4 mmol) and NaI (36 mg, 0.24 mmol) to produce a pale yellow salt in 48% yield (35 mg). Mp = 182–184 °C (from CH_2Cl_2/Et_2O) (decomposition); IR (ATR) ν = 3324, 2925, 2859, 1561, 1448, 1374, 1161, 1037 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 10.37 (s, 1H, H-$2_{imidazole}$), 7.43 (s, 1H, $H_{imidazole}$), 7.40 (s, 1H, $H_{imidazole}$), 4.35 (m, 4H, CH_2N), 3.61 (brs, 1H, H-3), 0.89 (brs, 6H, $2CH_3$), 0.61 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.2 ($CH_{imidazole}$), 121.9 ($CH_{imidazole}$), 121.7 ($CH_{imidazole}$), 71.6 (CH, C-3), 56.4 (CH), 55.8 (CH), 50.4 (CH_2), 50.0 (CH_2), 42.6 (C), 42.0 (CH), 40.3 (CH), 40.1 (CH_2), 36.3 (CH_2), 35.7 (CH), 35.29 (CH), 35.27 (CH_2), 34.5 (C), 32.2 (CH_2), 30.4 (CH_2), 30.0 (CH_2), 28.3 (CH_2), 28.2 (CH_2), 27.1 (CH_2), 27.0 (CH_2), 26.3 (CH_2), 24.1 (CH_2), 23.3 (CH_3), 22.0 (CH_2), 20.7 (CH_2), 18.5 (CH_3), 13.8 (CH_3), 12.0 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M\text{-}I]^+$ $C_{32}H_{55}N_2O^+$ 483.4309, found 483.4321.

2.3.5. N-(5β-cholan-3α-ol-24-yl)-N'-hexylimidazolyl iodide (5d)

General procedure D was followed using **4** (50 mg, 0.12 mmol), hexyl bromide (0.5 mL, 3.6 mmol) and NaI (36 mg, 0.24 mmol) to produce a pale yellow salt in 43% yield (32 mg). Mp = 129–130 °C (from CH_2Cl_2/Et_2O) (decomposition); IR (ATR) ν = 3320, 2924, 2860, 1562, 1441, 1368, 1152 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 10.44 (s, 1H, H-$2_{imidazole}$), 7.40 (s, 1H, $H_{imidazole}$), 7.38 (s, 1H, $H_{imidazole}$), 4.37 (m, 4H, CH_2N), 3.62 (m, 1H, H-3), 0.92 (d, J = 7.0 Hz, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.62 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.3 ($CH_{imidazole}$), 121.8 ($CH_{imidazole}$), 121.7 ($CH_{imidazole}$), 71.6 (CH, C-3), 56.4 (CH), 55.8 (CH), 50.5 (CH_2), 50.1 (CH_2), 42.7 (C), 42.0 (CH), 40.3 (CH), 40.1 (CH_2), 36.4 (CH_2), 35.8 (CH), 35.3 (CH), 34.5 (C), 32.2 (CH_2), 31.0 (CH_2), 30.5 (CH_2), 30.2 ($2CH_2$), 28.3 (CH_2), 27.1 (CH_2), 27.0 (CH_2), 26.4 (CH_2), 25.8 (CH_2), 24.1 (CH_2), 23.3 (CH_3), 22.3 (CH_2), 20.7 (CH_2), 18.6 (CH_3), 13.9 (CH_3), 12.0 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M\text{-}I]^+$ $C_{33}H_{57}N_2O^+$ 497.8315, found 497.8328.

2.3.6. 22-Iodo-23,24-dinorchol-4-en-3-one (6)

To the solution of triphenylphosphine (1.09 g, 4.17 mmol, 1.5 eq) in dry CH_2Cl_2 cooled to 0 °C, iodine (1.06 g, 4.17 mmol, 1.5 eq) and imidazole (0.28 g, 4.17 mmol, 1.5 eq) were added. After stirring for 30 min at room temperature while protected from light, a solution of 22-hydroxy-23,24-dinorchol-4-en-3-one (0.92 g, 2.78 mmol, 1 eq) in dry CH_2Cl_2 was added. The reaction was carried out at room temperature for 30 min. Then an aqueous solution of $Na_2S_2O_3$ was added (20 mL) and the mixture was extracted with CH_2Cl_2 (3 × 15 mL). The combined extracts were washed with aqueous solution of $NaHCO_3$ (2 x 15 mL) and water (15 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The obtained solid was purified by flash chromatography (hexane–AcOEt, v/v 9:1) to give a white solid in 81% yield (996 mg). Mp = 142–144 °C (from MeOH); IR (ATR) ν = 2924, 2850, 1666, 1611, 1447 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 5.72 (s, 1H, CH = C), 3.32 (dd, J = 9.5, 1.4 Hz, 1H, CH_2I), 3.16 (dd, J = 9.4, 4.5 Hz, 1H, CH_2I), 1.18 (s, 3H, CH_3), 1.02 (d, 3H, J = 5.3 Hz, CH_3), 0.75 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.5 (C, C = O), 171.3 (C, C=CH), 123.8 (CH, C=CH), 55.5 (CH), 55.3 (CH), 53.6 (CH), 42.4 (C), 39.2 (CH_2), 38.5 (C), 36.8 (CH), 35.7 (CH_2), 35.6 (CH), 33.9 (CH_2), 32.8 (CH_2), 31.9 (CH_2), 27.5 (CH_2), 24.0 (CH_2), 21.0 (CH_2), 20.9 (CH_2), 20.7 (CH_3), 17.3 (CH_3), 12.7 (CH_3) ppm, ESI-HRMS *m/z*: calcd for $[M+H]^+$ $C_{22}H_{34}IO^+$ 441.1659, found 441.1649.

2.3.7. 22-(N-Imidazolyl)-23,24-dinorchol-4-en-3-one (7)

General procedure A was followed using imidazole (155 mg, 2.3 mmol), NaH (92 mg, 60% suspension in mineral oil, 2.3 mmol) and 22-iodo-23,24-dinorchol-4-en-3-one (**6**, 200 mg, 0.45 mmol) to produce, after column chromatography (FC, dichloromethane – methanol, v/v 12:1), a white solid in 53% yield (91 mg). Mp = 176–177 °C (from MeOH); IR (ATR) ν = 2935, 2864, 1668, 1614, 1506, 1445, 1378, 1228 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 7.41 (s, 1H, $H_{imidazole}$), 7.03 (s, 1H, $H_{imidazole}$), 6.86 (s, 1H, $H_{imidazole}$), 5.71 (s, 1H, CH = C), 4.00 (dd, J = 13.8, 3.6 Hz, 1H, CH_2N), 3.52 (dd, J = 13.8, 9.3 Hz, 1H, CH_2N), 1.17 (s, 3H, CH_3), 0.84 (d, J = 6.6 Hz, 3H, CH_3), 0.75 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.4 (C, C = O), 171.1 (C, C=CH), 137.7 ($CH_{imidazole}$), 129.2 ($CH_{imidazole}$), 123.8 (CH, C=CH), 119.3 ($CH_{imidazole}$), 55.5 (CH), 53.6 (CH_2), 53.5 (CH), 52.7 (CH), 42.6 (C), 39.3 (CH_2), 38.5 (CH), 38.4 (C), 35.6 (CH_2), 35.5 (CH), 33.9 (CH_2), 32.8 (CH_2), 31.9 (CH_2), 28.2 (CH_2), 24.2 (CH_2), 20.9 (CH_2), 17.3 (CH_3), 16.9 (CH_3), 11.9 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M+H]^+$ $C_{25}H_{37}N_2O^+$ 381.2900, found 381.2915.

2.3.8. N-(3-Oxo-23,24-dinorchol-4-en-22-yl)-N'-methylimidazolyl iodide (8a)

General procedure C was followed using **7** (50 mg, 0.13 mmol), methyl iodide (1 mL, 16 mmol) to produce a white salt in quantitative yield (68 mg). Mp = 223–225 °C (from CH_2Cl_2/Et_2O); IR (ATR) ν = 3418, 2933, 1666, 1610, 1575, 1446, 1383, 1170 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 10.07 (s, 1H, H-$2_{imidazole}$), 7.57 (s, 1H, $H_{imidazole}$), 7.37 (s, 1H, $H_{imidazole}$), 5.69 (s, 1H, CH = C), 4.34 (dd, J = 13.6, 3.6 Hz, 1H, CH_2N), 4.13 (s, 3H, NCH_3), 4.00 (dd, J = 13.6, 9.7 Hz, 1H, CH_2N), 1.16 (s, 3H, CH_3), 0.96 (d, J = 6.5 Hz, 3H, CH_3), 0.76 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.4 (C, C = O), 171.2 (C, C=CH), 137.2 ($CH_{imidazole}$), 123.7 (CH, C=CH), 123.6 ($CH_{imidazole}$), 122.5 ($CH_{imidazole}$), 55.5 (CH_2), 55.4 (CH), 53.4 (CH), 53.2 (CH), 42.8 (C), 39.2 (CH_2), 38.4 (C), 37.5 (CH), 37.0 (CH_3), 35.6 (CH_2), 35.4 (CH), 33.9 (CH_2), 32.7 (CH_2), 31.8 (CH_2), 28.1 (CH_2), 24.1 (CH_2), 20.8 (CH_2), 17.3 (CH_3), 16.7 (CH_3), 12.1 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M\text{-}I]^+$ 395.3057, found 395.3069.

2.3.9. N-(3-Oxo-23,24-dinorchol-4-en-22-yl)-N'-ethylimidazolyl iodide (8b)

General procedure C was followed using **7** (50 mg, 0.13 mmol), ethyl iodide (1 mL, 12.5 mmol) to produce a pale yellow salt in 90% yield (63 mg). Mp = 151–152 °C (from CH_2Cl_2/Et_2O); IR (ATR) ν = 3440, 2935, 1661, 1561, 1445, 1352, 1164 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 10.31 (s, 1H, H-$2_{imidazole}$), 7.49 (s, 1H, $H_{imidazole}$), 7.31 (s, 1H, $H_{imidazole}$), 5.72 (s, 1H, CH = C), 4.47 (q, J = 7.3 Hz, 2H, CH_2N), 4.39 (dd, J = 13.6, 3.4 Hz, 1H, CH_2N), 4.01 (dd, J = 13.5, 9.9 Hz, 1H, CH_2N), 1.63 (t, J = 7.3 Hz, 3 H), 1.18 (s, 3H, CH_3), 0.94 (d, J = 6.4 Hz, 3H, CH_3), 0.79 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.2 (C, C = O), 171.1 (C, C=CH), 136.2 ($CH_{imidazole}$), 123.5 (CH, C=CH), 122.6 ($CH_{imidazole}$), 122.0 ($CH_{imidazole}$), 55.2 (CH_2), 55.2 (CH), 53.3 (CH), 53.1 (CH), 45.2 (CH_2), 42.6 (C), 39.0 (C), 38.3 (CH_2), 37.3 (CH), 35.4 (CH_2), 35.2 (CH), 33.7 (CH_2), 32.5 (CH_2), 31.6 (CH_2), 27.8 (CH_2), 24.0 (CH_2), 20.7 (CH_2), 17.1 (CH_3), 16.4 (CH_3), 15.0 (CH_3), 12.0 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M\text{-}I]^+$ $C_{27}H_{41}N_2O^+$ 409.3213, found 409.3225.

2.3.10. N-(3-Oxo-23,24-dinorchol-4-en-22-yl)-N'-pentylimidazolyl iodide (8c)

General procedure D was followed using **7** (50 mg, 0.13 mmol), pentyl bromide (1 mL, 8 mmol) and NaI (39 mg, 0.26 mmol) to produce a yellow salt in 78% yield (59 mg). Mp = 143–145 °C (from CH_2Cl_2/

Et_2O) (decomposition); IR (ATR) ν = 3433, 2933, 2853, 1663, 1613, 1561, 1448, 1378, 1163 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 10.44 (s, 1H, H-$2_{imidazole}$), 7.50 (s, 1H, $H_{imidazole}$), 7.37 (s, 1H, $H_{imidazole}$), 5.68 (s, 1H, CH = C), 4.35 (m, 3H, CH_2N), 4.04 (m, 1H, CH_2N), 1.15 (s, 3H, CH_3), 0.88 (m, 7 H), 0.75 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.4 (C, C = O), 171.1 (C, C=CH), 137.5 ($CH_{imidazole}$), 123.7 (CH, C=CH), 122.4 ($CH_{imidazole}$), 122.0 ($CH_{imidazole}$), 55.4 (CH), 55.3 (CH_2), 53.4 (CH), 53.3 (CH), 50.0 (CH_2), 42.8 (C), 39.2 (CH_2), 38.4 (C), 37.5 (CH), 35.5 (CH_2), 35.4 (CH), 33.8 (CH_2), 32.7 (CH_2), 31.8 (CH_2), 29.9 (CH_2), 28.1 (CH_2), 27.9 (CH_2), 24.1 (CH_2), 21.9 (CH_2), 20.8 (CH_2), 17.2 (CH_3), 16.5 (CH_3), 13.8 (CH_3), 12.1 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M\text{-}I]^+$ $C_{30}H_{47}N_2O^+$ 451.3683, found 451.3682.

2.3.11. N-(3-Oxo-23,24-dinorchol-4-en-22-yl)-N'-hexylimidazolyl iodide (8d)

General procedure D was followed using **7** (50 mg, 0.13 mmol), hexyl bromide (1 mL, 7 mmol) and NaI (39 mg, 0.26 mmol) to produce a yellow salt in 42% yield (32 mg). Mp = 148–149 °C (from CH_2Cl_2/Et_2O) (decomposition); IR (ATR) ν = 3730, 3393, 2933, 2854, 1665, 1559, 1444, 1161 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 10.58 (s, 1H, H-$2_{imidazole}$), 7.37 (s, 1H, $H_{imidazole}$), 7.27 (s, 1H, $H_{imidazole}$), 5.72 (s, 1H, CH = C), 4.39 (m, 3H, CH_2N), 4.05 (m, 1H, CH_2N), 1.17 (s, 3H, CH_3), 0.88 (d, J = 6.6 Hz, 3H, CH_3), 0.78 (s, 3H, CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.5 (C, C = O), 171.1 (C, C=CH), 137.8 ($CH_{imidazole}$), 123.8 (CH, C=CH), 122.2 ($CH_{imidazole}$), 121.7 ($CH_{imidazole}$), 55.5 (CH_2), 55.4 (CH), 53.5 (CH), 53.3 (CH), 50.2 (CH_2), 42.8 (C), 39.3 (CH_2), 38.5 (C), 37.6 (CH), 35.6 (CH_2), 35.5 (CH), 33.9 (CH_2), 32.7 (CH_2), 31.8 (CH_2), 31.0 (CH_2), 30.2 (CH_2), 28.0 (CH_2), 25.8 (CH_2), 24.2 (CH_2), 22.3 (CH_2), 20.9 (CH_2), 17.3 (CH_3), 16.5 (CH_3), 13.9 (CH_3), 12.1 (CH_3) ppm; ESI-HRMS *m/z*: calcd for $[M\text{-}I]^+$ $C_{31}H_{49}N_2O^+$ 465.3839, found 465.3837.

2.3.12. Bis-N,N'-(3-oxo-23,24-dinorchol-4-en-22-yl)imidazolyl iodide (8e)

Imidazole (15 mg, 0.22 mmol, 1 eq) and NaH (9 mg, 60% suspension in mineral oil, 0.22 mmol, 1 eq) were stirred in dry THF (2 mL) for 30 min at room temperature under argon. To this mixture, steroid **6** (200 mg, 0.45 mmol, 2 eq) in dry THF (3 mL) was added and the reaction was refluxed for 16 h. Then the inorganic solid was filtered off and the filtrate was evaporated. The residue was dissolved in DCM (2 mL), then the product was precipitated with Et_2O (10 mL) and filtered. The crystallization from DCM/Et_2O was repeated two more times to produce a pale yellow salt in 34% yield (64 mg). Mp = 287–289 °C (from CH_2Cl_2/Et_2O) (decomposition); IR (ATR) ν = 2937, 2851, 1681, 1447, 1379, 1233 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ 10.45 (s, 1H, H-$2_{imidazole}$), 7.26 (s, 2H, $H_{imidazole}$), 5.73 (s, 2H, CH = C), 4.43 (dd, J = 13.6, 3.6 Hz, 2H, CH_2N), 4.04 (dd, J = 13.6, 9.8 Hz, 2H, CH_2N), 1.18 (s, 6H, $2CH_3$), 0.94 (d, J = 6.4 Hz, 6H, $2CH_3$), 0.80 (s, 6H, $2CH_3$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.4 (C, C = O), 171.1 (C, C=CH), 137.4 ($CH_{imidazole}$), 123.7 (CH, C=CH), 122.5 ($CH_{imidazole}$), 55.5 (CH_2), 55.4 (CH), 53.5 (CH), 53.4 (CH), 42.8 (C), 39.3 (CH_2), 38.4 (C), 37.5 (CH), 35.6 (CH_2), 35.4 (CH), 33.9 (CH_2), 32.7 (CH_2), 31.8 (CH_2), 28.0 (CH_2), 24.2 (CH_2), 20.9 (CH_2), 17.3 (CH_3), 16.5 (CH_3), 12.2 (CH_3) ppm, ESI-MS *m/z*: 693 ($M\text{-}I^+$). ESI-HRMS *m/z*: calcd for $[M\text{-}I]^+$ $C_{47}H_{69}N_2O_2^+$ 693.5354, found 693.5345.

2.4. Anti-microbial studies

Antibacterial and antifungal activities of imidazolium salts were evaluated by broth microdilution assay in 96 well plates. The two-fold serial microdilution assay, described by the Clinical and Laboratory Standards Institute, was performed for the measurements of the minimal inhibitory concentrations (MICs) expressed in μg/mL. The imidazolium salt was first dissolved in DMSO and incorporated into Mueller-Hinton broth (MHB) to obtain a concentration of 1024 μg/mL with the final solution composition being 95% MHB and 5% DMSO by volume. The salts were then serially two-fold diluted to obtain concentrations ranging from 512 to 0.125 μg/mL in wells containing MHB. Then each diluted sample (50 μL) was mixed with 50 μL of inoculums of the tested microorganisms to achieve an initial inoculum of approximately 10^6 CFU/mL and incubated at 35 °C for 24 h. The experiments were performed in duplicates. The MIC value was determined as the lowest concentration of the salt that inhibits visible growth after incubation. Four reference strains were tested: *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Escherichia coli* DSM 10233 and *Candida albicans* ATCC 10231.

Activities of imidazolium salts on growth inhibition level of plant pathogenic fungi were tested on four widespread species – *Alternaria alternata*, *Botrytis cinerea*, *Cercospora beticola* and *Fusarium culmorum*. The strains were provided by Mycology Department of Plant Protection Institute – National Research Institute (Poznań, Poland). All the isolates were incubated on a PDA medium (Potato Dextrose Agar; Becton, Dickinson and Co.) for 1 week at 21 °C. Mycelial plugs (0.5 cm^2) taken from the edge of the colonies and placed on a PDA medium containing imidazolium salts at a concentration of: 0.1, 1, 5, 10, 25, 50 and 100 μg/mL and on PDA without amendment. The salts were dissolved in methanol at 5 mg/mL before being added to the medium. The samples were incubated for 5–8 days at 21 °C. The assays were repeated three times and the mean percentage of growth inhibition rate and EC50 value (half maximal effective concentration) of the tested isolates was calculated.

3. Results and discussion

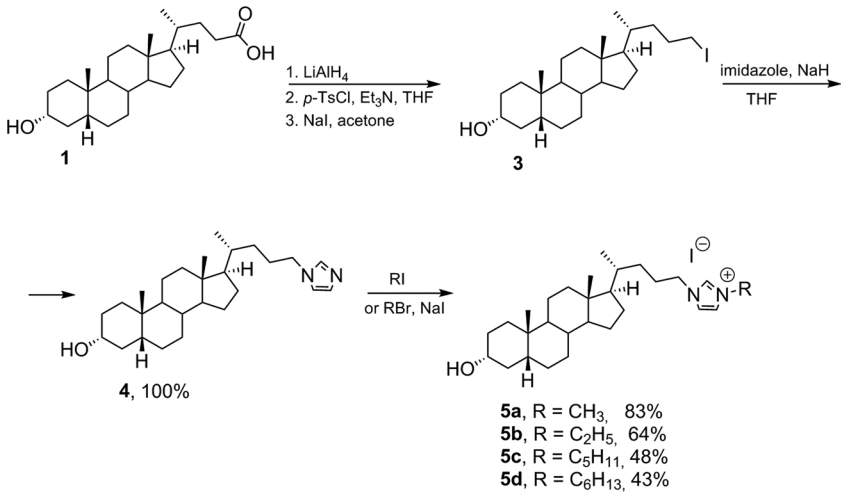
3.1. Synthesis of imidazolium salts

Two series of imidazolium iodides based on lithocholic acid (**1**, Scheme 1) and 3-oxo-23,24-dinorchol-4-en-22-al (**2**, Scheme 2) were prepared. LCA was subjected to reduction with $LiAlH_4$ [35] followed by *p*-tosylation of the 24-hydroxy group. Selective tosylation was achieved using Et_3N as a base at 0–4 °C in THF [36,37]. The crude product needed chromatographic purification due to contamination by 3,24-ditosylate (20%) then was isolated in 60% yield. The 24-iodide (**3**, Scheme 1) was synthesized by substitution of pure 24-tosylate with NaI [36]. The reaction of **3** with an excess of anion generated *in situ* from imidazole with NaH afforded *N*-steroid substituted imidazole (**4**) in quantitative yield. The product was subjected to reaction with alkyl iodide or bromide of different chain lengths. In the case of alkyl bromides, an activator (NaI) was added. As a result of these reactions, four imidazolium salts **5a-d** were obtained. The most efficient reaction was for short-chain alkyl halides (*e.g.* for preparation of **5a**) and yields slightly decreased with increasing chain lengths of the substituent. 3-Iodo-23,24-dinorchol-4-en-22-al (**6**) was obtained upon $NaBH_4$ reduction of steroid **2** followed by the Appel iodination (Scheme 2). Selective reduction of the 22-carbonyl group **2** was achieved using one equivalent of $NaBH_4$ (r.t. 30 min) [38]. Subsequent transformations of iodide **6** were analogous with those for LCA derivatives. Although the synthesis of *N*-steroid substituted imidazole **7** was less efficient, the formation of salts **8a-d** proceeded in higher yields than those of LCA derivatives. Additionally, symmetrically substituted steroid salt **8e** was obtained directly from iodide **6**.

It should be emphasized that the synthesis of new salts proved very efficient and quite convenient, especially the last step, where chromatographic purification was not needed. Salts with sufficient purity were obtained after triple crystallization. They were air-stable solids and may be handled under normal laboratory conditions.

3.2. The activity of imidazolium salts against human pathogens

Several reports have recently shown that imidazolium salts possess promising biological activities [1]. Encouraged by these results, we



Scheme 1. Synthesis of imidazolium salts based on lithocholic acid.

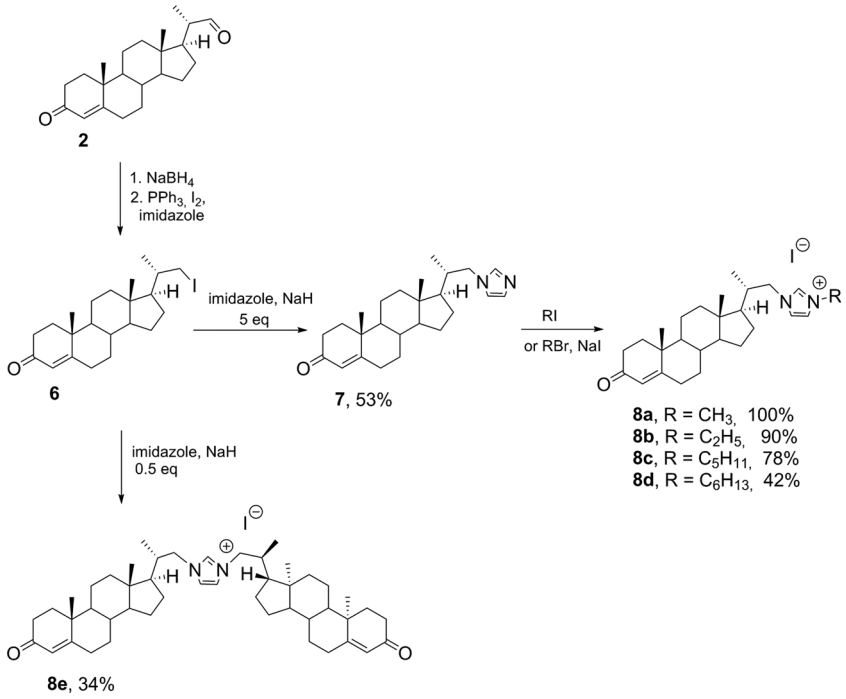
evaluated the antimicrobial activities of steroidal imidazolium salts against bacterial species causing nosocomial and healthcare-associated infections (*S. aureus, E. coli*). A severe causative agent for food poisoning (*S. aureus*, *B. cereus*) and *C. albicans*, an opportunistic fungal pathogen that is most frequently isolated from immunocompromised patients, were also used to examine effects of imidazolium salts.

The results of MIC experiments showed that all the tested imidazolium salts have a great effective activity against bacteria with MIC values from 0.5 to 64 μg/mL and 2- to 32-fold more potent activity against fungi with MIC values from 0.25 to 2 μg/mL (Table 1). Comparing both series of imidazolium salts, those based on lithocholic acid (salts **5a–d**) demonstrated better activity than those based on 3-oxo-23,24-dinorchol-4-en-22-al (salts **8a–d**) against *S. aureus*, *B. cereus* and *C. albicans*, respectively. However, compounds **8a–d** were found to be 2- to 4-fold more effective against *E. coli* than compounds **5a–d**. The symmetrically substituted disteroidal imidazolium salt (**8e**) exhibited excellent activity against *S. aureus*, *B. cereus* and *C. albicans*, but moderate to weak activity against *E. coli*.

The antimicrobial activity of new salts presented as a relationship between the number of carbon atoms in the alkyl substituent and the log MIC values are shown in Fig. 2 (LCA derivatives) and Fig. 3 (steroid **2** derivatives). In general, the antibacterial efficacy of all tested imidazolium salts increased with increasing chain length of the substituent. However, the chain length of the substituent had no significant effect on the antifungal activity of both series of imidazolium salts. It should be noted that in both cases antifungal activity is even better than antibacterial one. The obtained MIC values for *C. albicans* of all tested salts were significantly lower than that for commercial Fluconazole. In addition, in the case of LCA derivatives, the antifungal effect was better than that observed for Amphotericin B, which is the most effective but highly toxic antifungal drug. Therefore, new salts seem to be very promising compounds.

3.3. Evaluation of activity against phytopathogenic fungi

Encouraged by the bioassays above, especially promising results



Scheme 2. Synthesis of imidazolium salts based on 3-oxo-23,24-dinorchol-4-en-22-al.

Table 1
MIC values of imidazolium salts.

Imidazolium salt	MIC							
	Staphylococcus aureus		*Bacillus cereus*		*Escherichia coli*		*Candida albicans*	
	[μg/mL]	[μM]	[μg/mL]	[μM]	[μg/mL]	[μM]	[μg/mL]	[μM]
5a	4	7.2	32	58	16	29	0.25	0.5
5b	2	3.5	16	28	16	28	0.5	0.9
5c	1	1.6	4	6.5	16	26	0.5	0.8
5d	0.5	0.8	2	3	16	26	0.5	0.8
8a	32	61	64	122	16	31	2	3.8
8b	16	30	32	60	8	15	1	1.9
8c	4	7	4	7	4	7	2	3.5
8d	2	3.4	2	3.4	4	6.7	1	1.7
8e	1	1.2	1	1.2	64	78	2	2.4
Ampicillin[†]	0.25-1	0.7-2.9	0.25-0.5	0.7-1.4	0.8-2	5.7-22.9	–	–
Fluconazole[‡]	–	–	–	–	–	–	2	6.5
Amphotericin B[‡]	–	–	–	–	–	–	1	1.1

[†] Clinical and Laboratory Standards Institute (CLSI), approved guideline M45A2E and supplement M100-S23.
[‡] http://www.eucast.org/clinical_breakpoints.

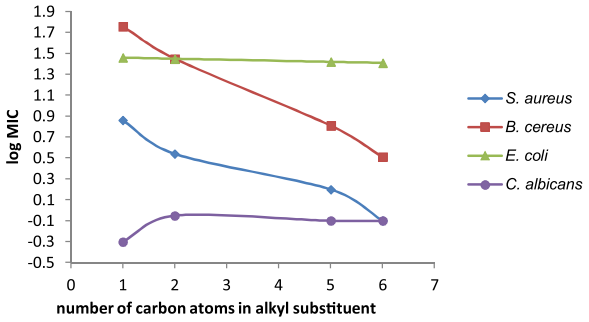


Fig. 2. Antimicrobial activity of salts **5a–d**.

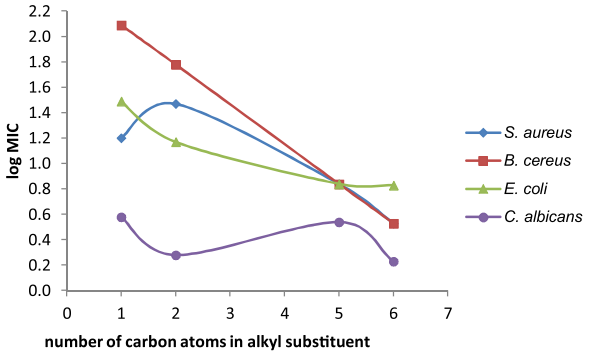


Fig. 3. Antimicrobial activity of salts **8a–d**.

Table 2
Percent inhibition of mycelial growth induced by imidazolium salts (concentration 0.1 mg/mL)[†].

Imidazolium salt	*Botrytis cinerea*	*Cercospora beticola*	*Alternaria alternata*	*Fusarium culmorum*
5a	100 ± 0	66.2 ± 3.4	54.1 ± 5.1	27.5 ± 6.8
5b	94.1 ± 5.8	69.0 ± 2.0	46.1 ± 7.0	2.2 ± 0.8
5c	100 ± 0	65.3 ± 4.6	61.9 ± 2.3	29.4 ± 1.2
5d	94.1 ± 0	58.0 ± 2.3	28.6 ± 1.1	6.0 ± 3.5
8a	100 ± 0	83.3 ± 0	50.5 ± 7.2	13.7 ± 3.4
8b	100 ± 0	75.6 ± 4.2	66.0 ± 3.9	52.4 ± 2.7
8c	100 ± 0	67.4 ± 0	44.2 ± 3.7	49.0 ± 9.0
8d	100 ± 0	68.2 ± 2.1	49.6 ± 2.8	44.8 ± 1.0

[†] Values are the mean ± standard deviation of three replicates.

against *C. albicans*, antifungal activity was additionally assessed by *in vivo* experiments on a set of phytopathogenic fungi causing widespread diseases on crops (*Botrytis cinerea, Fusarium culmorum, Alternaria alternata, Cercospora beticola*). The activity data for compounds **5a–d** and **8a–d** at a concentration of 0.1 mg/mL against different fungi are reported in Table 2. The best activity of all new compounds was observed against *B. cinerea*, which causes gray mold. It is a very common disease that affects about 200 species of different botanical families and has shown resistance to some currently used fungicides [39].

Due to the fact that the salts activity at a concentration of 0.1 mg/mL (0.16-0.19 mM) was very high, we also evaluated the activity at lower concentrations: 0.1, 1, 5, 10, 25 and 50 μg/mL (representative test for salt **5b** is presented in Fig. 4). Although new compounds showed no significant activity against *F. culmorum* and *A. alternata* at a concentration of less than 0.1 mg/mL, their activity against *B. cinerea* was surprisingly high. Additionally, EC50 vales were determined and the results of the selected salts are summarized in Table 3. The most active compounds were **5a** and **5b**, which showed 45.1% and 86.7% of growth inhibition for *B. cinerea*, respectively, at a concentration as low as 5 μg/mL. Compounds **5c** and **8b** showed high activity (> 70%) at a concentration of 10 μg/mL. The trend in results obtained for *B. cinerea* is

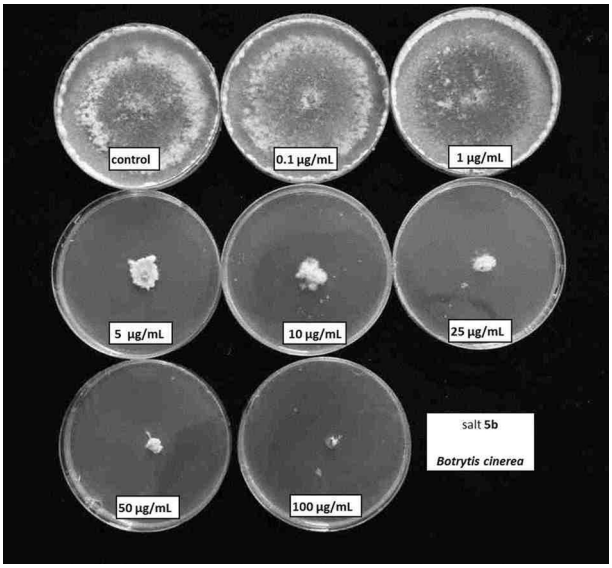


Fig. 4. Evaluation of the activity of **5b** against *B. cinerea* at different concentrations.

Table 3
EC50 values and percent inhibition of mycelial growth *B. cinerea* and *C. beticola* induced by selected salts[†].

Imidazolium salt	concentration		*Botrytis cinerea*		*Cercospora beticola*	
	[μg/mL]	[μM]	% of inhibition[a]	EC50	% of inhibition[a]	EC50
5a	5	9	45.1 ± 2.5	5.5 μg/mL (10 μM)	51.6 ± 5.2	4.6 μg/mL (8 μM)
	10	18	87.4 ± 2.4		53.8 ± 1.9	
	25	45	92.5 ± 0.7		60.6 ± 6.2	
	50	90	100 ± 0		65.0 ± 1.9	
	100	180	100 ± 0		66.2 ± 3.4	
5b	5	9	86.7 ± 2.4	3.0 μg/mL (5 μM)	47.6 ± 4.1	6.0 μg/mL (11 μM)
	10	18	93.7 ± 0.7		53.5 ± 3.6	
	25	44	94.5 ± 0.7		55.9 ± 4.1	
	50	88	89.8 ± 8.8		64.3 ± 3.6	
	100	176	94.1 ± 5.9		69.0 ± 2.0	
5c	5	8	17.6 ± 2.0	7.2 μg/mL (12 μM)	42.7 ± 2.3	12.0 μg/mL (20 μM)
	10	16	77.6 ± 4.7		48.0 ± 4.0	
	25	41	100 ± 0		57.3 ± 2.3	
	50	82	100 ± 0		57.4 ± 4.6	
	100	164	100 ± 0		65.3 ± 4.6	
8b	5	9	27.4 ± 4.3	6.8 μg/mL (13 μM)	49.9 ± 5.6	5.0 μg/mL (9 μM)
	10	18	73.3 ± 1.9		59.7 ± 6.3	
	25	47	100 ± 0		65.8 ± 5.6	
	50	93	100 ± 0		67.0 ± 3.6	
	100	186	100 ± 0		75.6 ± 4.2	
8d	5	8	0.6 ± 0.6	18.5 μg/mL (31 μM)	36.5 ± 4.2	8.0 μg/mL (13 μM)
	10	17	5.8 ± 1.2		53.6 ± 2.1	
	25	42	72.5 ± 6.5		60.9 ± 4.2	
	50	84	94.5 ± 0.7		63.4 ± 3.7	
	100	169	100 ± 0		68.2 ± 2.1	

[†] Values are the mean ± standard deviation of three replicates.

similar to that of *C. albicans*, where LCA-based salts were better than steroid **2** based ones. In the case of compounds **8a–d**, salts with an even number of carbon atoms in the alkyl substituent (**8b** and **8d**) showed better activity than those with an odd amount (**8a** and **8c**). In Table 3, the results of salts against *C. beticola* were at a similar level and did not exceed 70%, with one exception; **8b** at a concentration of 100 μg/mL showed 75.6% of growth inhibition.

In summary, new salts showed similar or even greater activity than the agricultural fungicides used against *B. cinerea*. For example, commercial drazoxolon inhibited mycelium growth in 91% at 42 μM [40], whereas salt **5b** showed similar inhibition at a concentration more than 4 times lower (9 μM). Another known fungicide, procymidone, completely inhibited the growth at 88 μM [41] just like salts **5c** and **8b**. The EC50 values of imidazole-based fungicide, imazalil, against *B. cinerea* (isolates collected from table and wine grapes) ranged from 0.4 to 34 μM [42] and were similar to those of salts **5a-c**, **8b** and **8d**. It should be noted that the ethyl substituent is optimal for achieving the highest antifungal activity (**5b** and **8b**). Although tetraconazole, showing total inhibition at 27 μM [41], proved to be more effective against *C. beticola* than new salts, it should be noted that **5a–c**, **8b** and **8d** displayed EC50 in the range of 8 to 20 μM. To our knowledge, this is the first report on the possibility of using steroid-based imidazolium iodides as fungicides. It should be noted that new iodides are very stable, easy to handle and soluble in organic solvents (*e.g.* in alcohols). In addition, imidazolium salts generally show low toxicity to humans. New salts may serve as potential fungicides or at least as structural templates in the search for new and highly efficient fungicides.

4. Conclusion

Two series of steroid-based imidazolium salts were synthesized in a short, straightforward route and their antimicrobial activities were investigated. New compounds were tested against human and plant pathogens. The most promising finding was the activity against *C. albicans*, which exceeded the antifungal activity of commonly used antibiotics. Additionally, new salts with *N*-ethyl substituent at the imidazole moiety showed greater activity compared to known fungicides against phytopathogenic fungi. In contrast, salts with longer alkyl chains were highly active against Gram-positive bacteria. In summary, new compounds showed a broad spectrum of activity and had promising antimicrobial activities, especially antifungal effect against either human or plant pathogens, at a level comparable to commercial drugs and fungicides.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2019.02.006.

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