

Effects of Novel Silver (I) N-Heterocyclic Carbene Complexes on Mycotoxin Producing Fungi and Biofilm Forming Microorganisms

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ABSTRACT

Ag (I) complexes were prepared for a series of NHC substituents. Firstly, NHC precursors (1-9) were synthesized. In the second step, Ag (I) -NHC complexes (10-15) were synthesized. Structure of silver (I) N-heterocyclic carbene complexes was analysed using ¹H- and ¹³C-NMR spectroscopy. The activity of synthesized and characterized silver complexes on mycotoxin producing fungi and biofilm-forming Gram negative or positive bacteria and *Candida albicans* was investigated. As a result, it was determined that the most effective compound was 14, 15 and 13 respectively. Especially, compound 14 has been shown to have a high effect (12-15mm/16-64µgmL⁻¹) against *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 35032, *Listeria monocytogenes* ATCC 19112, *Candida albicans* ATCC 10231, *Aspergillus parasiticus* NRLL 502. The results of antimicrobial activity and microfungus micelle development were also found to be compatible.

Keywords: Silver (I) N-Heterocyclic Carbene Complexes, Mycotoxin, Fungi, Biofilm, Microorganisms

1. INTRODUCTION

Foods are a suitable source for the growth of microorganisms. But it is also important that people can eat healthy and long-term food. Bacterial and fungal contaminants that may occur in foods cause food degradation and also threaten human health. In particular, fungi that cause toxin formation in foods produce mycotoxins as secondary metabolites. The mycotoxins are a serious problem in dried foods such as figs, peanuts, peppers, apricots, hazelnuts (Goyal et al., 2016). Furthermore, biofilm-forming microorganisms both threaten the lives of people and cause economic losses in the health and food sector (Rabin et al., 2015).

Recently, there has been a need to seek alternative agents that can inhibit the growth of fungi that produce mycotoxins and traditional antibiotics used against infectious diseases that are bacterial resistant and difficult to treat (Chen et al., 2013; Ait et al., 2015). For this purpose, when metal-N-heterocyclic carbene (NHC) chemistry has been examined since 2006, it is seen that there are many synthesized Ag (I) -NHC complexes (Ivan and Chandra, 2007).

In this study, the effects of synthesized silver(I) N-heterocyclic carbene (Ag(I)-NHC) complexes were investigated on mycotoxin producing fungi and biofilm-forming microorganisms.

2. MATERIALS AND METHODS

2.1. Synthesis of Imidazolium Salts (1-3,5-7)

Compounds (1-3) and benzyl bromides were prepared by following a previously described method (Fırıncı et al., 2018); (Figure 1). 2,4,6-trimethylbenzyl chloride/2,3,5,6-tetramethylbenzyl bromide/2,3,4,5,6-pentamethylbenzyl chloride (1.25 mmol) was added over (N-octadecyl-imidazole) (4, 1.25 mmol) in toluene (15 mL) at 80°C. The resulting mixture was heated to 80°C and stirred at this temperature for 24 h. After, pentane (15 mL) was added to the mixture and a white solid crystal was obtained. The solid obtained was washed with pentane (3x10 mL) and the crude product was recrystallized from CH₂Cl₂/pentane (Rohini et al., 2013); (Figure 2).

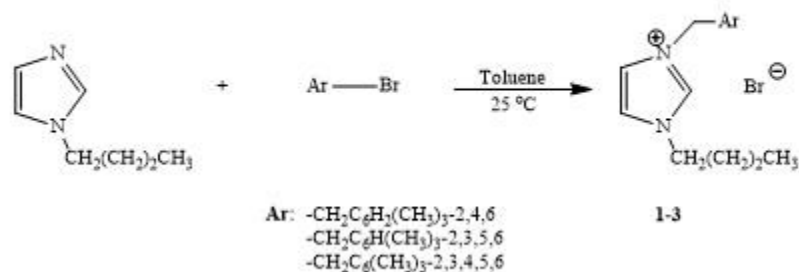


Fig. 1 Synthesis of NHC precursors 1-3

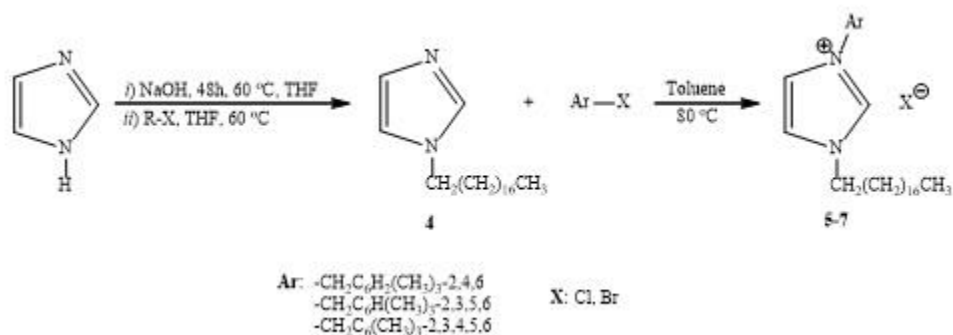


Fig. 2 Synthesis of NHC precursors 4-7

N-octadecyl-N'-(2,4,6-trimethylbenzyl)imidazolium chloride, (5)

Yield: (0.5189 g, 85%). m.p.: 80-81°C. ¹H-NMR (δ, 400 MHz, CDCl₃): 0.82-0.85 (t, 3H, J = 7.0 Hz, CH₃(CH₂)₁₆CH₂N); 1.18-1.27 (m, 30H, CH₃(CH₂)₁₆CH₂N); 1.85-1.88 (m, 2H, CH₃(CH₂)₁₆CH₂N); 2.24 (s, 6H, NCH₂C₆H₂(CH₃)₃-o-CH₃); 2.25 (s, 3H, NCH₂C₆H₂(CH₃)₃-p-CH₃);

4.29-4.33 (t, 2H, $J = 7.5$ Hz, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 5.58 (s, 2H, $\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3$); 6.77 (s, 1H, NCHCHN); 6.89 (s, 2H, $\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3$); 7.22 (s, 1H, NCHCHN); 10.62 (s, 1H, NCHN). ^{13}C -NMR (δ , 100 MHz, CDCl_3): 14.1 ($\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 19.7 ($\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3\text{CH}_3$); 21.0 ($\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3\text{CH}_3$); 22.6, 26.2, 28.9, 29.9, 29.3, 29.4, 29.5, 29.6, 29.6, 30.2, 31.8 ($\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 47.8 ($\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3$); 50.2 ($\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 120.4 (NCHCHN); 121.4 (NCHCHN); 125.4, 129.8, 137.6, 138.1 ($\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3$); 139.8 (NCHN).

N-octadecyl-N'-(2,3,5,6-tetramethylbenzyl)imidazolium bromide, (6)

Yield: (0.5751 g, 84%). m.p.: 101-102 °C. ^1H -NMR (δ , 400 MHz, CDCl_3): 0.82-0.85 (t, 3H, $J = 7.0$ Hz, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 1.21-1.28 (m, 30H, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 1.82-1.94 (m, 2H, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 2.16 (s, 6H, $\text{NCH}_2\text{C}_6\text{H}(\text{CH}_3)_{4-o-\text{CH}_3}$); 2.21 (s, 6H, $\text{NCH}_2\text{C}_6\text{H}(\text{CH}_3)_{4-m-\text{CH}_3}$); 4.30-4.34 (t, 2H, $J = 7.0$ Hz, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 5.65 (s, 2H, $\text{NCH}_2\text{C}_6\text{H}(\text{CH}_3)_4$); 6.82 (s, 1H, NCHCHN); 7.05 (s, 1H, $\text{NCH}_2\text{C}_6\text{H}(\text{CH}_3)_4$); 7.34 (s, 1H, NCHCHN); 10.55 (s, 1H, NCHN). ^{13}C -NMR (δ , 100 MHz, CDCl_3): 14.1 ($\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 15.8 ($\text{CH}_3\text{CH}_2(\text{CH}_2)_{15}\text{CH}_2\text{N}$); 16.9 ($\text{NCH}_2\text{C}_6\text{H}(\text{CH}_3)_4\text{CH}_3$); 20.4 ($\text{NCH}_2\text{C}_6\text{H}(\text{CH}_3)_4\text{CH}_3$); 22.6, 26.2, 28.9, 29.0, 29.0, 29.3, 29.3, 29.4, 29.5, 29.6, 29.6, 29.7, 30.3, 30.4, 31.9 ($\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 48.6 ($\text{NCH}_2\text{C}_6\text{H}(\text{CH}_3)_4$); 50.3 ($\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 120.7 (NCHCHN); 121.4 (NCHCHN); 128.0, 133.5, 134.1, 135.0, 135.8 ($\text{NCH}_2\text{C}_6\text{H}(\text{CH}_3)_4$); 137.1 (NCHN).

N-octadecyl-N'-(2,3,4,5,6-pentamethylbenzyl)imidazolium bromide, (7)

Yield: (0.6272 g, 975%). m.p.: 105-106 °C. ^1H -NMR (δ , 400 MHz, CDCl_3): 0.82-0.85 (t, 3H, $J = 6.5$ Hz, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 1.20-1.28 (m, 30H, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 1.85-1.92 (m, 2H, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 2.19 (s, 12H, $\text{NCH}_2\text{C}_6(\text{CH}_3)_{5-o,m-\text{CH}_3}$); 2.23 (s, 6H, $\text{NCH}_2\text{C}_6(\text{CH}_3)_{5-p-\text{CH}_3}$); 4.30-4.34 (t, 2H, $J = 7.0$ Hz, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 5.64 (s, 2H, $\text{NCH}_2\text{C}_6(\text{CH}_3)_5$); 6.83 (s, 1H, NCHCHN); 7.32 (s, 1H, NCHCHN); 10.51 (s, 1H, NCHN). ^{13}C -NMR (δ , 100 MHz, CDCl_3): 14.1 ($\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 16.7 ($\text{NCH}_2\text{C}_6(\text{CH}_3)_5\text{CH}_3$); 16.8 ($\text{NCH}_2\text{C}_6(\text{CH}_3)_5\text{CH}_3$); 17.2 ($\text{NCH}_2\text{C}_6(\text{CH}_3)_5\text{CH}_3$); 22.6, 26.2, 28.9, 29.3, 29.3, 29.4, 29.6, 29.6, 29.7, 30.3, 31.9 ($\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 49.1 ($\text{NCH}_2\text{C}_6(\text{CH}_3)_5$); 50.2 ($\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 120.6 (NCHCHN); 121.1 (NCHCHN); 125.4, 133.6, 133.7, 137.2 ($\text{NCH}_2\text{C}_6(\text{CH}_3)_5$); 137.5 (NCHN).

2.2. Synthesis of Ag(I)-NHC Complexes (8-13)

A solution of imidazolium salt (**1-3,5-7**) (1.0 mmol), Ag_2O (0.5 mmol) and activated 4 Å molecular sieves in dry dichloromethane (30 mL) was stirred at room temperature for 18h in the dark condition. The mixture was filtered and the solvent removed under vacuum. The filtrate was recrystallized with dichloromethane / n-pentane solution at room temperature. The synthesis of Ag(I)-NHC complexes **8, 9** and **10** were reported by our group (Fırıncı et al., 2018); (Fig.3).

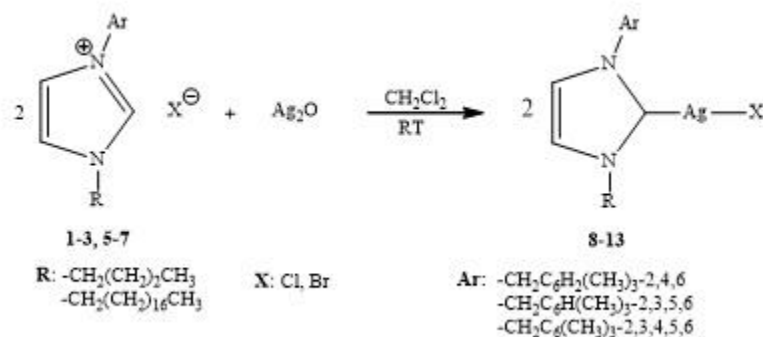


Fig. 3 Synthesis of Ag(I)-NHC complexes 8-13

(Bis[N-octadecyl-N'-(2,4,6-trimethyl)imidazole-2-ylidene]Ag(I))[AgCl₂], (11)

Yield: (0.3133 g, 50%). m.p.: 65-66 °C. ^1H -NMR (δ , 400 MHz, CDCl_3): 0.85-0.88 (t, 6H, $J = 6.5$ Hz, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 1.24-1.28 (m, 60H, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 1.77-1.80 (m, 4H, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 2.23 (s, 12H, $\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_{3-o-\text{CH}_3}$); 2.29 (s, 6H, $\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_{3-p-\text{CH}_3}$); 4.05-4.09 (t, 4H, $J = 7.0$ Hz, $\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{N}$); 5.26 (s, 4H, $\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3$); 6.50 (s, 2H, NCHCHN); 6.86 (s, 2H, NCHCHN); 6.91 (s, 4H, $\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3$). ^{13}C -NMR (δ , 100 MHz, CDCl_3): 14.1 ($\text{NCH}_2(\text{CH}_2)_{16}\text{CH}_3$); 19.9 ($\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3$); 21.0 ($\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3$); 22.7, 26.4, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 31.5, 31.9 ($\text{NCH}_2(\text{CH}_2)_{16}\text{CH}_3$); 49.6 ($\text{NCH}_2\text{C}_6\text{H}_2(\text{CH}_3)_3$); 52.3 ($\text{NCH}_2(\text{CH}_2)_{16}\text{CH}_3$); 119.7 (NCHCHN); 120.4 (NCHCHN); 127.6 (Ar-C); 129.7 (Ar-C); 137.7 (Ar-C); 139.0 (Ar-C); 181.0 (Ag-C_{carbene}).

[Bis{N-octadecyl-N'-(2,3,5,6-tetramethyl)imidazole-2-ylidene}Ag(I)][AgBr₂], (12)

Yield: (0.5170 g, 79%). m.p.: 70-71 °C. ¹H-NMR (δ, 400 MHz, CDCl₃): 0.85-0.88 (t, 6H, *J* = 7.0 Hz, CH₃(CH₂)₁₆CH₂N); 1.24-1.28 (m, 60H, CH₃(CH₂)₁₆CH₂N); 1.77-1.80 (m, 4H, CH₃(CH₂)₁₆CH₂N); 2.15 (s, 12H, NCH₂C₆H(CH₃)₄-*o*-CH₃); 2.24 (s, 12H, NCH₂C₆H(CH₃)₄-*m*-CH₃); 4.06-4.09 (t, 4H, *J* = 7.0 Hz, CH₃(CH₂)₁₆CH₂N); 5.33 (s, 4H, NCH₂C₆H(CH₃)₄); 6.53 (d, 2H, *J* = 2.0 Hz, NCHCHN); 6.85 (d, 2H, *J* = 2.0 Hz, NCHCHN); 7.03 (s, 2H, NCH₂C₆H(CH₃)₄). ¹³C-NMR (δ, 100 MHz, CDCl₃): 14.1 (NCH₂(CH₂)₁₆CH₃); 15.8 (NCH₂C₆H(CH₃)₄); 20.5 (NCH₂C₆H(CH₃)₄); 22.6, 26.4, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 31.4, 31.9 (NCH₂(CH₂)₁₆CH₃); 50.1 (NCH₂C₆H(CH₃)₄); 52.3 (NCH₂(CH₂)₁₆CH₃); 120.0 (NCHCHN); 120.2 (NCHCHN); 130.3 (Ar-C); 132.7 (Ar-C); 133.7 (Ar-C); 134.7 (Ar-C); 180.9 (Ag-C_{carbene}).

[Bis{N-octadecyl-N'-(2,3,4,5,6-pentamethyl)imidazole-2-ylidene}Ag(I)][AgBr₂], (13)

Yield: (0.5155 g, 77%). m.p.: 85-86 °C. ¹H-NMR (δ, 400 MHz, CDCl₃): 0.84-0.87 (t, 6H, *J* = 7.0 Hz, CH₃(CH₂)₁₆CH₂N); 1.23-1.27 (m, 60H, CH₃(CH₂)₁₆CH₂N); 1.76-1.80 (m, 4H, CH₃(CH₂)₁₆CH₂N); 2.19 (s, 12H, NCH₂C₆(CH₃)₅-*o*-CH₃); 2.22 (s, 6H, NCH₂C₆(CH₃)₅-*p*-CH₃); 2.26 (s, 12H, NCH₂C₆(CH₃)₅-*m*-CH₃); 4.05-4.09 (t, 4H, *J* = 7.5 Hz, CH₃(CH₂)₁₆CH₂N); 5.33 (s, 4H, NCH₂C₆H(CH₃)₄); 6.55 (s, 2H, *J* = 2.0 Hz, NCHCHN); 6.85 (s, 2H, *J* = 2.0 Hz, NCHCHN). ¹³C-NMR (δ, 100 MHz, CDCl₃): 14.1 (NCH₂(CH₂)₁₆CH₃); 16.8 (NCH₂C₆(CH₃)₅); 16.9 (NCH₂C₆(CH₃)₅); 17.2 (NCH₂(CH₂)₁₆CH₃); 22.6 (NCH₂C₆(CH₃)₅); 26.4, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 31.5, 31.9 (NCH₂(CH₂)₁₆CH₃); 50.6 (NCH₂C₆(CH₃)₅); 52.3 (NCH₂(CH₂)₁₆CH₃); 120.0 (NCHCHN); 120.1 (NCHCHN); 127.6 (Ar-C); 133.3 (Ar-C); 133.5 (Ar-C); 136.4 (Ar-C); 177.5 (Ag-C_{carbene}).

2.3. Antimicrobial Screening

The antimicrobial activities of all the synthesized compounds were determined by the disc diffusion method (Collins et al., 2004; CLSI, 2015) and the minimum inhibitory concentrations (MIC) were obtained by broth dilution method (Jorgensen and Ferraro, 2009; CLSI, 2009).

2.3.1. Cultivation Condition of Microorganisms

Three mycotoxin producing fungi (*Aspergillus flavus* NRRL 500, *Aspergillus parasiticus* NRRL 502 and *Aspergillus ochraceus* NRRL 398) and four bacteria (*Pseudomonas aeruginosa* ATCC 35032, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 35218) and one yeast culture (*Candida albicans* ATCC 10231) as biofilm-forming microorganisms were used in this study.

Aspergillus flavus NRRL 500, *Aspergillus parasiticus* NRRL 502 and *Aspergillus ochraceus* NRRL 398 used in the study were obtained from the Agricultural Research Service (ARS, USA). The biofilm-forming microorganisms were provided from the American Type Culture Collection (ATCC, Rockville, MD, USA).

The fungi strains were cultured in Sabouraud Dextrose Agar (SDA) at 27°C for 3-5 days. The bacteria strains were cultured in Tryptic Soy Agar (TSA) at 37°C for 24 h. The yeast strains were cultured in Malt Extract Agar (MEA) at 30°C for 24 h (Abbaszadeh et al., 2014; Çoban et al., 2017; Oyeka et al., 2018).

2.3.2. Preparation of Silver Ion Solutions

The stock solutions (1000 µg mL⁻¹) of all the synthesized compounds were prepared in dichloromethane. Besides, commercial AgNO₃ (Sigma-Aldrich) solutions prepared at the same concentration was used as negative control.

2.3.3. Disc Diffusion Method

Synthesized silver carbene complexes were tested against biofilm-forming bacteria or yeast and mycotoxin producing fungi by agar well diffusion method (Collins et al., 2004; Clinical and Laboratory Standards Institute, 2015).

Bacteria and yeasts are activated in Tryptic Soy Broth medium at 30-37°C for 24 h. The concentrations of the activated cultures were adjusted to 0.5 Mc Farland standard tubes to give a concentration of 1×10⁸ bacterial cells and 1×10⁶ yeast cells/mL (Babahan et al., 2014; Çoban et al., 2017; Oyeka et al., 2018).

The fungi isolates were grown on Sabouraud Dextrose Agar (SDA; Merck) at 27°C for 5-7 days. After, 0.1% Tween 20 solution was added on the growing fungus culture and shaken. So, fungus spores were enabled to pass into the solution. The conidial suspensions were counted and adjusted as 1×10⁶ conidia/mL using Thoma Slide (Abbaszadeh et al., 2014; Ismaiel and Tharwat, 2014).

To test the antibacterial and antifungal of silver (I)-NHC complexes, Mueller Hinton Agar plates were used and 0.1 mL of suspensions were homogeneously spread over the surface of the agar medium. Then a hole of 6 mm in diameter and depth was made on top with a sterile stick and filled with 50 µL of the silver carbene complexes.

Plates inoculated with *Pseudomonas aeruginosa* ATCC 35032, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermis* ATCC 12228, *Escherichia coli* ATCC 35218 were incubated at 37°C for 24 h and *Candida albicans* ATCC 10231 were incubated at 30°C for 24 h and *Aspergillus flavus* NRRL 500, *Aspergillus parasiticus* NRRL 502 ve *Aspergillus ochraceus* NRRL 398 were incubated at 27°C for 5 days. The diameter of the inhibition zone was then measured. For bacteria, discs of Chloramphenicol (C30, Oxoid), Gentamycin (GN10 Oxoid), Tetracycline (TE30), Erythromycin (E15), Ampicillin (AMP10) and for yeast, disc of Nystatine (NS100) and for microfungi, discs of were used as Amphotericin B (AMB100), Clotrimazole (KTC10), Fluconazole (FCA25), Ketoconazole (CTL10), Nystatin (NS100) were as reference antibiotics (Ismail and Tharwat, 2014).

2.3.4. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

Minimum inhibitory concentration (MIC) was determined by the reported method (Jorgensen, and Ferraro, 2009; CLSI, 2009). Yeasts and bacteria were grown in Malt Extract Broth medium and Tryptic Soy Broth medium at 27-37°C for 24 h. The microfungi were inoculated in Sabouraud Dextrose Broth (SDA; Merck) at 27°C for 5-7 days.

The inoculums were adjusted according to 0.5 McFarland standard tubes. 96-well sterile Elisa Plate on was used for this test. 100 μL of Mueller Hinton Broth medium was added into each well. The stock solutions ($2000 \mu\text{g}\cdot\text{mL}^{-1}$) were obtained by dissolving the substances in dichloromethane. Two fold dilutions ($256\text{-}0.5 \mu\text{g}\cdot\text{mL}^{-1}$) were applied for MIC analysis. The lowest concentration in which the microorganism grows was evaluated as the MIC value. As positive controls, Streptomycin for bacteria and Fluconazole for yeast and microfungi were used in the dilution method.

The MBC and MFC is the lowest concentration of compounds desired to kill the microorganisms (Abbaszadeh et al., 2014; Saquib et al., 2019). In order to determine the MBC and MFC value, 0.01 mL of the mixture is taken from the wells where no growth is observed and spread onto Tryptic Soy Agar, Malt Extract Agar and Sabouraud Dextrose Agar plates. The plates were incubated at 27-30-37°C.

2.3.5. Effect of Ag(I)-NHC Compounds on Mycelium Growing

Sabouraud Dextrose Broth containing 50 mL medium in conical flasks of 250 mL was prepared and sterilized at 121°C for 15 min. Different concentrations of Ag(I)-NHC compounds were prepared as 0.0, 8.0, 12, 24, 36, 48, 60 $\mu\text{g}\cdot\text{mL}^{-1}$. Spore suspension (0.2 mL) was added to the medium in each flask. The inoculated flasks were incubated at 27°C for 10 days (Ismail and Tharwat, 2014).

2.3.6. Determination of Mycelial Dry Weights

At the end of the incubation period, the fungal culture flasks were filtered by Whatman no.389 filter papers and dried at 80°C for 24 h (Ismail and Tharwat, 2014).

2.3.7. Statistical Analyses

Results were given using analysis of variance (ANOVA, SPSS software version 22).

3. RESULTS AND DISCUSSION

3.1. Preparation of The Imidazolium Salts (1-3,5-7)

The asymmetrically substituted imidazolium salts (1-3,5-7) were prepared from the treatment of N-butylimidazole/imidazole and benzyl bromide derivative (Figure 1, 2). Salts are stable to air and moisture both in the solid-state and in solution. The salts are soluble in $\text{C}_2\text{H}_5\text{OH}$, CH_2Cl_2 , and CHCl_3 , but insoluble in diethyl ether and hexane.

The chemical structure of the salts obtained was determined using $^1\text{H-NMR}$. Results were consistent with the predicted structures. The resonances for NCHN protons give as a sharp singlet between at δ 10.50 and 10.85 ppm.

3.2. Preparation of the Ag(I)-NHC Complexes (8-13)

The general procedure for the preparation of Ag(I)-NHC complexes is shown in Figure 3. Complexes (8-13) were prepared from the treatment of the imidazolium salts with Ag_2O in dry CH_2Cl_2 at room temperature for 18 h in dark condition. The formation of the complexes was confirmed by the disappearance of the characteristic imidazolium ^1H NMR proton signals of the NHC precursors. The signals for the carbene carbons in Ag(I)-NHC complexes (11-13) appears 181.0, 180.9 and 177.5 ppm, respectively.

3.3. Antimicrobial Screening

The antibacterial and antifungal effects of Ag(I)-NHC complexes (compounds **13**, **14**, **15**) against biofilm-forming bacteria or yeast and mycotoxin producing fungi were tested using agar well diffusion method. Inhibition zones (mm) of the Ag(I)-NHC compounds and AgNO₃ as negative control were listed in Table 1. The MIC test was applied to the compounds showing antimicrobial activity. Results were indicated in Table 2. In addition, inhibition zones (mm) of the reference antibiotics as positive control were given in Table 3.

Table 1: Antimicrobial activities of compounds **13**, **14**, **15** and negative control (1000 µg.mL⁻¹) (Inhibition zone mm)

Test Microorganisms	Inhibition zone (mm)			
	Compounds			Negative control
	13	14	15	AgNO ₃
<i>Escherichia coli</i> ATCC 35218	8	8	10	9
<i>Staphylococcus aureus</i> ATCC 25923	10	11	11	9
<i>Staphylococcus epidermidis</i> ATCC 12228	-	9	8	9
<i>Pseudomonas aeruginosa</i> ATCC 35032	15	12	-	-
<i>Listeria monocytogenes</i> ATCC 19112	-	11	12	7
<i>Candida albicans</i> ATCC 10231	-	15	12	15
<i>Aspergillus parasiticus</i> NRLL 502	10	12	14	15
<i>Aspergillus ochraceus</i> NRLL 398	-	-	-	15
<i>Aspergillus flavus</i> NRRL 500	-	-	-	11

(-): No zone

(-): Zone did not occur.

NT: Not tested.

Table 2: Antimicrobial activities of compounds (MIC, MBC/MFC µg.mL⁻¹)

Test Microorganisms	Compound 13		Compound 14		Compound 15		AgNO ₃	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
<i>Escherichia coli</i> ATCC 35218	256	≥256	64	128	256	≥256	256	≥256
<i>Staphylococcus aureus</i> ATCC 25923	256	≥256	64	128	256	≥256	256	≥256
<i>Staphylococcus epidermidis</i> ATCC 12228	NT	NT	256	≥256	128	256	256	≥256
<i>Pseudomonas aeruginosa</i> ATCC 35032	32	64	64	128	256	≥256	NT	NT

<i>Listeria monocytogenes</i> ATCC 19112	128	256	64	128	64	128	256	≥256
<i>Candida albicans</i> ATCC 10231	256	≥256	16	32	256	≥256	64	128
<i>Aspergillus parasiticus</i> NRLL 502	256	≥256	64	32	32	64	16	32
<i>Aspergillus ochraceus</i> NRLL 398	NT	NT	NT	NT	NT	NT	64	128
<i>Aspergillus flavus</i> NRRL 500	NT	NT	NT	NT	NT	NT	64	128

MIC: Minimum inhibitory concentration

MBC: Minimum bactericidal concentration

MFC: Minimum fungicidal concentration

NT: Not tested

Table 3: References antibiotics (Inhibition zone mm)

Test Microorganisms	Inhibition zone (mm)										
	Reference antibiotics (Positive control)										
	C30	CN10	TE30	E15	AMP 10	P10	AMB 100	KTC 10	FCA 25	CTL 10	NS 100
<i>Escherichia coli</i> ATCC 35218	24	21	15	11	-	16	NT	NT	NT	NT	NT
<i>Staphylococcus aureus</i> ATCC 25923	23	20	22	23	20	12	NT	NT	NT	NT	NT
<i>Staphylococcus epidermidis</i> ATCC 12228	22	17	19	11	17	11	NT	NT	NT	NT	NT
<i>Pseudomonas aeruginosa</i> ATCC 35032	22	20	20	21	-	14	NT	NT	NT	NT	NT
<i>Listeria monocytogenes</i> ATCC 19112	20	15	20	23	-	10	NT	NT	NT	NT	NT
<i>Candida albicans</i> ATCC 10231	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	22
<i>Aspergillus parasiticus</i> NRLL 502	NT	NT	NT	NT	NT	NT	-	30	-	21	13
<i>Aspergillus ochraceus</i> NRLL 398	NT	NT	NT	NT	NT	NT	-	27	-	30	13
<i>Aspergillus flavus</i> NRRL 500	NT	NT	NT	NT	NT	NT	-	30	-	23	13

C30: Chloramphenicol (30 mg Oxoid), CN10: Gentamycin (10 mg Oxoid), TE30: Tetracycline (30 mg Oxoid), Penicilin (10 mg Oxoid), E15: Erythromycin (15 mg Oxoid), AMP10: Ampicillin (10 mg Oxoid), NS: Nystatin (100 mg Oxoid), AMB100: Amphotericin B, KTC10: Clotrimazole, FCA25: Fluconazole, CTL10: Ketoconazole.

(-): Zone did not occur.

NT: Not tested.

According to Table 1, the compound **13** showed high effect (15 mm) against *Pseudomonas aeruginosa* ATCC 35032 while the same compound demonstrated moderate effect (10 mm) against *Staphylococcus aureus* ATCC 25923 and *Aspergillus parasiticus* NRLL 502.

Besides, the compound had no effect against *Staphylococcus epidermidis* ATCC 12228, *Listeria monocytogenes* ATCC 19112, *Candida albicans* ATCC 10231, *Aspergillus ochraceus* NRRL 398, *Aspergillus flavus* NRRL 500, while the compound revealed slightly effect (8 mm) against *Escherichia coli* ATCC 35218. The compound **14** indicated high effect (15 mm) against *Candida albicans* ATCC 10231, while the same compound demonstrated moderate effect (11-12 mm) on *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 35032, *Listeria monocytogenes* ATCC 19112, *Aspergillus flavus* NRRL 500. Besides it had low effect (8-9 mm) against *Escherichia coli* ATCC 35218 and *Staphylococcus epidermidis* ATCC 12228, while the compound **14** had no effect on *Aspergillus ochraceus* NRRL 398 and *Aspergillus flavus* NRRL 500. The compound **15** remarked high effect (14 mm) against *Aspergillus parasiticus* NRRL 502, while the compound expressed moderate effect (10-11-12 mm) on *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19112, and *Candida albicans* ATCC 10231. Nevertheless, the compound had no effect on *Pseudomonas aeruginosa* ATCC 35032, *Aspergillus ochraceus* NRRL 398 and *Aspergillus flavus* NRRL 500, while it inferred slightly effect (8 mm) against *Staphylococcus epidermidis* ATCC 12228. On the other hand, AgNO₃ as negative control displayed remarkable effect (11-15 mm) against biofilm-forming *Candida albicans* ATCC 10231 and mycotoxin producing *Aspergillus flavus* NRRL 500, *Aspergillus ochraceus* NRRL 398 and *Aspergillus flavus* NRRL 500. The negative control (AgNO₃) screened very low effect (7-9 mm) against the other biofilm-forming bacteria, while it had no effect on *Pseudomonas aeruginosa* ATCC 35032.

According MIC, MBC/MFC values, some of the compounds assayed signified noteworthy activity on the tested microorganisms (Table 2). For example, *Pseudomonas aeruginosa* ATCC 35032 (compound **13**= 32 µg mL⁻¹, compound **14**= 64 µg mL⁻¹), *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 35032, *Listeria monocytogenes* ATCC 19112, *Aspergillus parasiticus* NRRL 502 (compound **14**=64 µg mL⁻¹), *Candida albicans* ATCC 10231 (compound **14**=16 µg mL⁻¹), *Listeria monocytogenes* ATCC 19112, *Aspergillus parasiticus* NRRL 502 (compound **15**=64 µg mL⁻¹ and 32 µg mL⁻¹, respectively). *Candida albicans* ATCC 10231, *Aspergillus ochraceus* NRRL 398, *Aspergillus flavus* NRRL 500 and *Aspergillus parasiticus* NRRL 502 (compound AgNO₃=64 µg mL⁻¹ and 16 µg mL⁻¹, respectively).

3.4. Effect of Ag(I)-NHC Compounds on Mycelium Growing

The effect of silver carbene complexes (compounds **13**, **14**, **15**) in different concentrations on mycelium growing was given in Table 4. Compound **14** had appreciable effect on mycelium growing of *A. parasiticus* NRLL 502. As the compound concentration increased, mycelium growing decreased. When compound **14** was examined, while dry cell weight is 0.94 g100mL⁻¹ at the lowest concentration (8 µg.mL⁻¹), dry cell weight is low at the highest concentration applied in this study. Compounds (**13**, **14**, **15**) reduced *A. parasiticus* NRLL 502 mycelium growing by 12-36%. However, these compounds did not significantly affect the mycelium growing of other fungi (Table 4).

Table 4: Effects of silver carbene complexes ion in different concentrations on mycelium growing

Ag-NHC	Mycelium weights of fungi													
	Ag(I)-NHC solutions in different concentrations (µg.mL ⁻¹)													
	Wet Cell Weights (g100 mL ⁻¹)							Dry Cell Weights (g100 mL ⁻¹)						
	Control	8	12	24	36	48	60	Control	8	12	24	36	48	60
	<i>Aspergillus parasiticus</i> NRLL 502													
13	5,31 (±0,15)	3,23 (±0,14)	6,45 (±0,03)	5,73 (±0,07)	5,63 (±0,04)	4,69 (±0,03)	5,55 (±0,04)	0,94 (±0,01)	0,92 (±0,01)	0,90 (±0,02)	0,90 (±0,02)	0,88 (±0,01)	0,84 (±0,02)	0,82 (±0,02)
14	4,88 (±0,03)	5,01 (±0,04)	3,90 (±0,02)	5,35 (±0,05)	3,96 (±0,15)	5,43 (±0,04)	4,32 (±0,13)	0,96 (±0,03)	0,94 (±0,02)	0,80 (±0,02)	0,76 (±0,01)	0,70 (±0,01)	0,64 (±0,01)	0,60 (±0,01)
15	5,37 (±0,06)	5,59 (±0,06)	5,40 (±0,05)	4,63 (±0,04)	5,63 (±0,05)	4,76 (±0,04)	4,76 (±0,05)	0,94 (±0,04)	0,92 (±0,02)	0,88 (±0,01)	0,80 (±0,01)	0,75 (±0,03)	0,70 (±0,00)	0,62 (±0,01)
	<i>Aspergillus ochraceus</i> NRLL 398													
13	5,59 (±0,05)	5,71 (±0,06)	4,83 (±0,04)	4,72 (±0,04)	5,21 (±0,05)	5,64 (0,06)	5,01 (±0,05)	0,96 (±0,01)	0,96 (±0,02)	0,94 (±0,01)	0,96 (±0,02)	0,96 (±0,02)	0,95 (±0,01)	0,95 (±0,01)

14	5,61 (±0,06)	5,09 (±0,05)	5,47 (±0,06)	6,09 (±0,07)	5,52 (±0,05)	6,12 (±0,08)	6,69 (±0,09)	0,98 (±0,02)	0,96 (±0,03)	0,98 (±0,03)	0,95 (±0,04)	0,96 (±0,03)	0,95 (±0,03)	0,96 (±0,04)
15	6,14 (±0,06)	6,17 (±0,07)	5,77 (±0,05)	5,61 (±0,05)	6,09 (±0,06)	6,12 (±0,06)	6,23 (±0,06)	0,98 (±0,02)	0,98 (±0,03)	0,94 (±0,03)	0,96 (±0,03)	0,95 (±0,02)	0,95 (±0,03)	0,95 (±0,04)
Aspergillus flavus NRLL 500														
13	5,70 (±0,00)	5,38 (±0,01)	5,50 (±0,02)	5,75 (±0,06)	5,37 (±0,05)	5,60 (±0,03)	5,45 (±0,00)	0,100 (±0,00)	0,98 (±0,01)	0,98 (±0,00)	0,95 (±0,01)	0,96 (±0,10)	0,96 (±0,01)	0,96 (±0,00)
14	5,80 (±0,00)	6,30 (±0,01)	5,60 (±0,00)	5,74 (±0,14)	5,75 (±0,21)	5,43 (±0,16)	5,62 (±0,17)	0,100 (±0,00)	0,101 (±0,01)	0,98 (±0,02)	0,98 (±0,00)	0,98 (±0,11)	0,98 (±0,13)	0,98 (±0,05)
15	5,48 (±0,15)	6,05 (±0,00)	5,50 (±0,15)	5,08 (±0,21)	5,15 (±0,01)	5,42 (±0,02)	6,15 (±0,06)	0,98 (±0,00)	0,100 (±0,02)	0,98 (±0,05)	0,98 (±0,00)	0,98 (±0,01)	0,98 (±0,02)	0,98 (±0,01)

Calculated mean is for triplicate measurements from two independent experiments \pm SD.

Silver (I)-N-heterocyclic carbene compounds were characterized and researched activity against some bacteria (Habib et al., 2020). The substances had remarkable effect against *Bacillus subtilis*, *Bacillus cereus* and *Macroccoccus brunensis*. Boubakri et al. (2019) demonstrated that Ag(I)-N-heterocyclic carbene complexes had significant effect against *Staphylococcus aureus* ATCC 6538 and *Listeria monocytogenes* ATCC 19117. Gök et al. (2019) showed that naphthalen -1-ylmethyl substituted silver N-heterocyclic carbene complexes had high effect against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans* and *Candida tropicalis*. Shahini et al. (2018) expressed that benzoxazole and dioxolane substituted benzimidazolebased N-heterocyclic carbene-silver(I) complexes had considerable effect against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Ismaiel ve Tharwat (2014) examined effect of silver ions against mycotoxin producing *Aspergillus flavus* OC1 and obtained appreciable data.

4. CONCLUSION

In this research, we examined antimicrobial activity of silver (I) N-heterocyclic carbene complexes on biofilm forming bacteria or yeast and mycotoxin producing fungi. For this purpose, synthesis of NHC precursors was carried out. N-butyl imidazole and imidazole were purchased commercially and used without purification. The chemical structure of the compounds was revealed using ¹H- and ¹³C-NMR spectroscopy. We found that the most effective substances were compounds 14, 15 and 13, respectively. Because these compounds damage the metabolism of Gram negative or positive bacteria and fungi, they inhibit biofilm formation and mycotoxin synthesis. The results obtained will contribute to the pharmaceutical industry as novel drug discovery.

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Author's contributions

Esin Poyrazoğlu Çoban: Design of study, Testing microbial studies, Analysis and interpretation of results

Halil Bıyık: Supply of mycotoxin-producing fungi, Testing microbial studies, Analysis of results

Engin Ertuğrul: Synthesis of silver (I) N-heterocyclic carbene complexes

Rukiye Fırınacı: Analysis of silver (I) N-heterocyclic carbene complexes, Analysis of data

Muhammet Emin Günay: Analysis of data

Conflict of interest

There is no conflict of interest in the content of this manuscript.

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Data and materials availability:

All data associated with this study are present in the paper.

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