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Determination of antimicrobial activity of novel synthesized N-Heterocyclic Carbene Silver complex

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KEYWORDS

Silver (I)-NHC complexes,
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Minimum Inhibitory concentration,
Antibiotics.

A B S T R A C T

The anti bacterial activity of newly synthesized silver (I)-NHC complexes has been determined on both Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria. The minimum inhibitory concentration of the compounds was measured on *Escherichia coli.* The comparative anti bacterial activity of the synthesized compounds are measured with commercially available antibiotics like Ornof, Sepmax, Norflox, Tetramycin and Enteromycin. In every case the inhibition zone diameter was measured and calculated from the means of five determinations and expressed in terms of susceptibility.

Introduction

"Antimicrobial agent" is a general term used to refer to any compound which include antibiotics, food antimicrobial agents, sanitizers, disinfectants, and other substances that acts against microorganisms.

There are two groups of antimicrobial agents: antibiotics and chemotherapeutic agents**.** Antibiotics are natural substances produced by certain groups of microorganisms and chemotherapeutic agents are chemically synthesized.

Unregulated and unscientific use of antimicrobial agents has resulted in resistance to antimicrobial agents which resulted in morbidity and mortality from treatment failures and increased health care costs (Yetkin *et. al*.2013). Although defining the precise public health risk and estimating the increase in costs is not simple, there is no doubt that present

antibiotic resistance is a serious global problem (Napoli *et. al*, 2013).

Among several synthetic antimicrobial compounds Silver salts have enjoyed a long history as antimicrobial agents and have proved to exhibit low toxicity for humans (Patrick *et.al*, 2013). These complexes have been used in a variety of applications like water purification, wound management, eye-drops, and anti-infective coatings in medical devices and in burn treatment (Bishra *et.al*, 2006). It is reported that the Ag (I) imidazolate complex has antibacterial and antifungal properties (Jered C. Garrison, 2005). In addition, an anticancer activity of silver and silver complexes has been demonstrated recently (Medvetz *et al*, 2012). The present study was undertaken to determine the antimicrobial activity of newly synthesized silver (I)-NHC complexes has been studied on both Gram positive and Gram negative bacteria.

Materials and Methods

Synthesis of Ligand

A 284 mg (1mili mole) α α 'Di bromo P-Xylene was taken in 10ml dry THF solvent and stirred for 45minutes at room temperature in 25ml Round bottom flask.164mg (2mili mole) 1-Methyl imidazole was taken and poured in to THF solution and reflux at 80^{o} C for 5 hours yield a colorless white solid compound 3, 3'-P-phynelene Bis (1-methyl imidazolium) Bromide (1.1).yield:434mg (97%).

Synthesis of NHC Silver Bromide Complex

A 45mg (0.1milimole) ligand (1.1) was taken in 10ml dry Dichloromethane (DCM) solvent and, 25mg (0.1mili mole) silver oxide (Ag2O) was taken and added in to DCM solution and stirred for10 hours at room temperature in 25ml Round bottom flask. That gives a colorless solution and filtered and the filtrate was collected and the DCM solvent was removed by rotary evaporator gives colorless white powder (1.2).Yield:58 mg (93%)

Synthesis of NHC Silver -acetate complex

A 45mg (0.1milimole) ligand (1.1) was taken in 15ml dry Methanol solvent and, 67mg (0.4mili mole) silver acetate (AgCO2CH3) was taken and added in to Methanol solution and stirred for 3 hours at room temperature in 50ml Round bottom flask. That gives a colorless solution and filtered and filtrate was collected and Methanol solvent was removed by rotary evaporator gives colorless white powder (1.2).Yield:34mg (78%).

Bacterial Strains and Growth Conditions

The gram positive bacteria *Staphylococcus aureus* and gram negative bacteria *Escherichia coli* and *Klebsiella pneumoniae* used in this study were obtained from the department of Microbiology, Vidyasagar University,West Bengal, India. Bacterial cultures were maintained in Mueller-Hinton Broth (MHB) and a total inoculums load of ca. 105 cells per well-maintained.

Antimicrobial Assays

The antibacterial properties of LT 2/8 AgBr (water insoluble, dissolve in DMSO) and LT 2/8 AgoAc (water soluble) has been evaluated against both gram positive (*Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae).*

The clear zone Test

At first the stock concentration (5mg/ml) of antimicrobial agent is prepared. Then two concentration gradient of antimicrobial agent is made by serial dilution method i.e 10^{-1} and $10^{-2.}$

Agar plates are kept in an incubator (right side up) and heated to 37°C for 10-20 minutes with the covers adjusted so that the plates are slightly opened. Covers of all plates are labeled with the name of the test organism to be inoculated. All the plates are inoculated with respective test organism. Culture plates are allowed to dry for about 5 minutes. Then three wells are made in each nutrient agar plate by using sterile cup borer and 40 µl of each concentration of antimicrobial agent is added into individual well of each plate. All the plates are kept in incubator for 24 hr at 37^0C.

The MIC Test

The MIC is the lowest concentration of an antimicrobial agent that inhibits the growth of test organism. The broth tube dilution method is used to determine the minimum inhibitory concentration of the antimicrobial agents against the selected microorganisms.

10mg LT2/08 AgBr antimicrobial agent was dissolved in 10 ml DMSO. So the concentration was 1mg /ml. 2 ml of sterile nutrient broth was transferred into 9 test tubes with the help of 10 ml sterile pipette (except 1st test tube). 2 ml of antimicrobial agent (1mg/ml) was added to first test tube and mixed properly, so net antimicrobial agent concentration was formed 500 µl / ml. 2 ml of antimicrobial agent solution was taken from stock (1mg/ml) and added into test tube 2 and mixed homogenately. Net antimicrobial agent concentration of test tube 2 was 500 µl /ml. Similarly by serial dilution method 2 ml of mixture from test tube 2 was transferred to test tube 3. Then concentration was 250 µl /ml. 2 ml from the last test tube was discharged, so net volume remains 2 ml. In positive control no antimicrobial agent was added. It contained only 2 ml of sterile media and 2 ml of test culture. In negative control the test tube was contained only 2 ml of antimicrobial agent stock and 2 ml of test culture. Finally 2 ml of young *E.coli* culture was added in every test tube aseptically. All test tubes were incubated for 24 hours in incubator at 37^0C and the turbidity was measured by spectrophotometer.

Result and Discussion

Synthesis of the Complexes

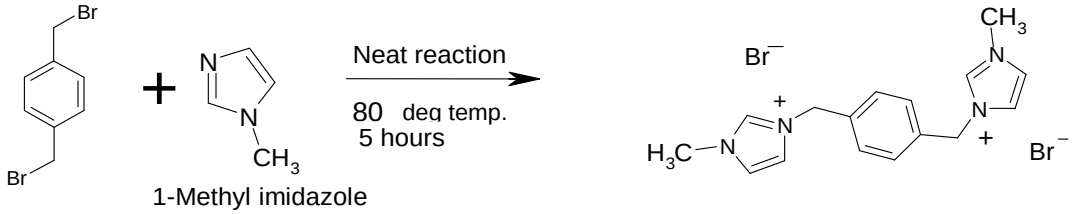
Silver has been used as an antimicrobial agent for centuries and has been proven effective against a wide range of pathogens (Hsuan-Jui Huang,2014). The interest in Ag-NHC complexes is largely due to their ease of synthesis and their ability to serve as useful to other NHC-metal complexes by NHC transfer reactions (Samantaray, 2012). The preparation of the compound is possible without the use of solvent, that's why it is environment friendly and follows the green chemistry. The reaction yield is very high, >95%.The compound is very stable and the preparation time is very short.

Antimicrobial activity

Clear Zone Test

The antibacterial properties of LT 2/8 AgBr (water insoluble, dissolve in DMSO) and

Step Diagram of Synthesis of Silver (I) NHC Compounds



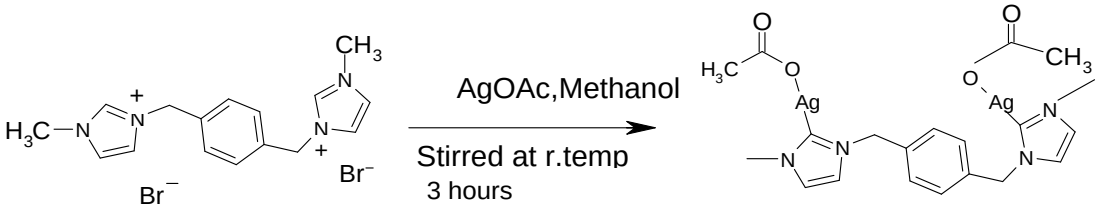
1-Methyl imidazole

alfa alfa' di bromo-p-xylene

(1.1)

33'-P-phynelene Bis (1-methyl imidazolium) Bromide

(1.2)



(1.3)

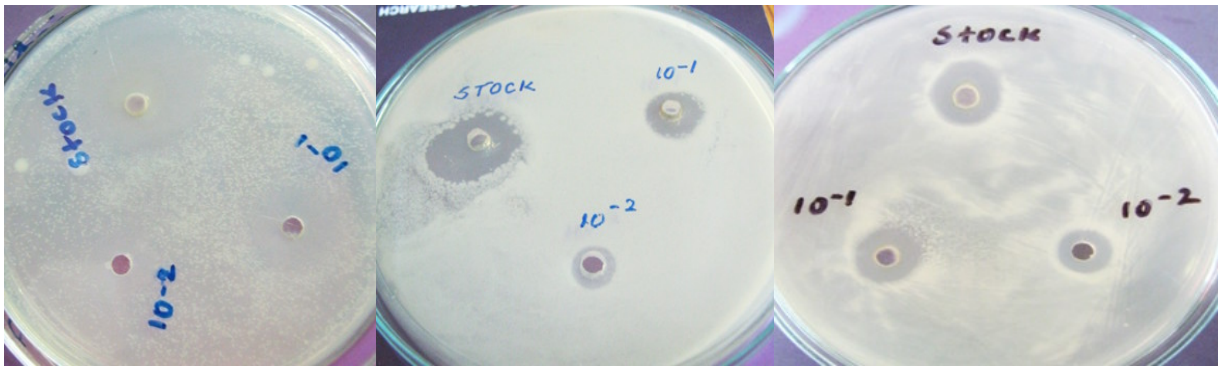


Fig: I a Fig: I b Fig: I c

Fig:Ia : Clear zone test of the chemical against *Klebsiella pneumoniae*

Fig: Ib: Clear zone test of the chemical against *Escherichia coli*

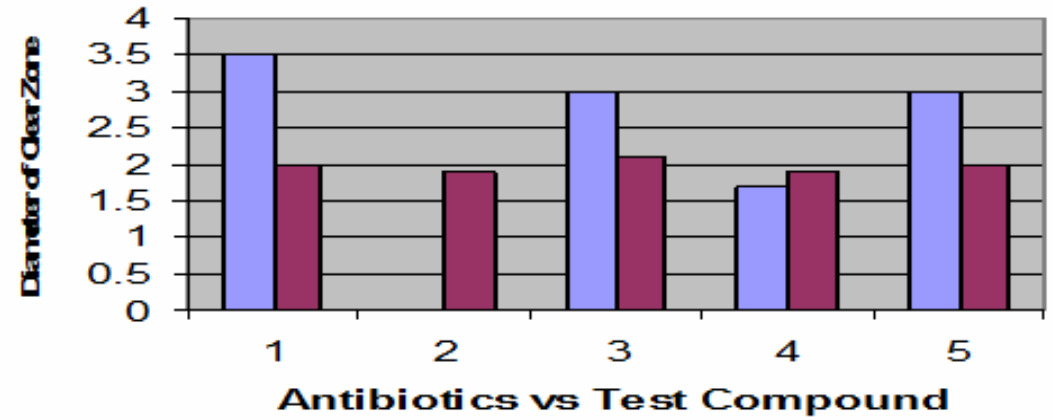
Fig:Ic: Clear zone test of the chemical against *Staphylococcus aureus* .

Name of the compound	Test organism	Concentration	Diameter of clear zone	Bore diameter	Chemicals in each bore
LT 2/8 AgBr	i. *Escherichia coli*	i. Stock(5mg/ml)	3.3cm	0.5cm	40µl
		ii. 0.5mg/ml	2.0cm		
		iii. 0.05mg/ml	1.5cm		
	i. *Staphylococcus aureus*	i. Stock(5mg/ml)	2cm		
		ii. 0.5mg/ml	1.4cm		
		iii. 0.05mg/ml	0.8cm		
	i. *Klebsiella pneumoniae*	i. Stock(5mg/ml)	1.5cm		
		ii. 0.5mg/ml	1.3cm		
		iii. 0.05mg/ml	1.1cm		
LT 2/8 AgoAc	i. *Escherichia coli*	i. Stock(5mg/ml)	3.0cm	0.5cm	40µl
		ii. 0.5mg/ml	1.6cm		
		iii. 0.05mg/ml	1.3cm		
	i. *Staphylococcus aureus*	i. Stock(5mg/ml)	1.7cm		
		ii. 0.5mg/ml	1.3cm		
		iii. 0.05mg/ml	0.8cm		
	i. *Klebsiella pneumoniae*	i. Stock(5mg/ml)	1.4cm		
		ii. 0.5mg/ml	1.3cm		
		iii. 0.05mg/ml	1.1cm		

Table.I Result of Clear zone test on selected micro organism

Test organism	Concentration of chemical	Bore diameter	Chemicals in each bore	Antibiotics vs test compound		Diameter of clear zone (in cm)	
				antibiotics	test chemical	antibiotics	LT 2/8 AgBr
Escherichia coli	**0.5mg/ml**	**0.5cm**	**40µl**	**1.Ornof**	**LT 2/8 AgBr**	**3.5**	**2.0**
				2.Sepmax	**do**	**0**	**1.9**
				3. Norflox	**do**	**3.0**	**2.1**
				4.Teramycin	**do**	**1.7**	**1.9**
				5.Enteromycin	**do**	**3.0**	**2.0**

Table.II Comparative antimicrobial activity of available antibiotics and synthesized compounds



Graph: I: Along X axis 1 indicates Ornof & Test compound, 2 indicates Sepmax & Test compound, 3 indicates Norflox & Test Compound, 4 indicates Teramycin & Test Compound, 5 indicates Enteromycin & Test Compound. Red Colour indicates Test compound & Violet colour indicates different Antibiotics.

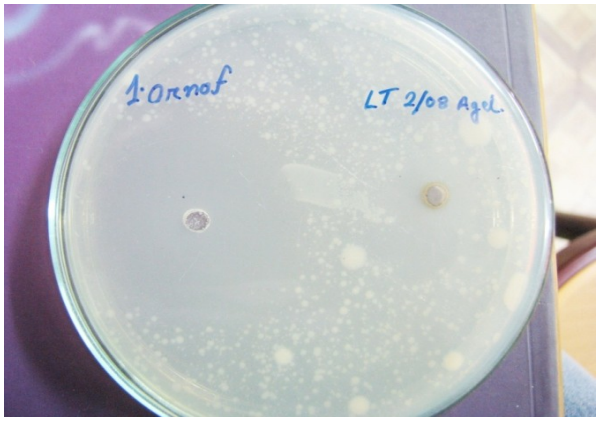


Fig:II a

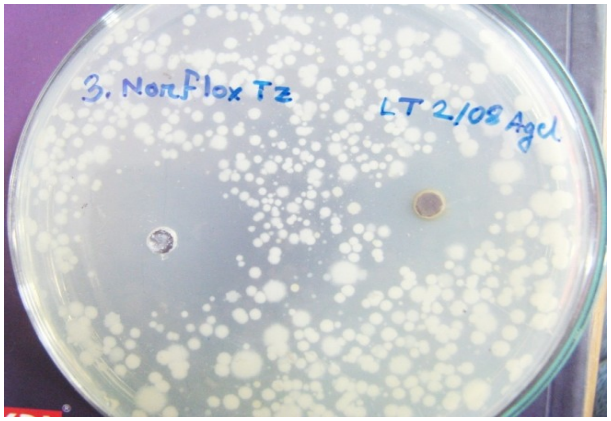


Fig:II b

Fig:II c

Fig: II d

Fig:II a: Comparison of Clear zone between Ornof and LT 2/08 AgBr, Fig:II b Comparison of Clear zoneBetween Norflox TZ and LT 2/08 AgBr, Fig:II c Comparison of Clear zone between Teramycin and LT 2/08 AgBr, Fig: II d Comparison of Clear zone between Enteromycin and LT 2/08 AgBr.

Test tube No.	1	2	3	4	5	6	7	8	9	10
Chemical concentration(µg/ml)	**500 (+) control)**	**500**	**250**	**125**	**62.5**	**31.2**	**15.6**	**7.8**	**3.9**	**0(-) control)**
Optical density	**0.40**	**0.41**	**0.41**	**0.42**	**0.43**	**0.85**	**0.89**	**0.91**	**0.94**	**0.94**
Visual inference	-	-	-	-	-	+	+	+	+	+

LT 2/8 AgoAc (water soluble) has been evaluated against both gram positive (*Staphylococcus aurous*) and gram negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae)* (Fig: Ia,Ib and Ic). The Inhibition zone diameter was measured and calculated from the means of five determinations and expressed in terms of susceptibility (Table-I).

Comparative study

To know the efficiency of Lt 2/8 AgBr, it has been compared with different well known antibiotics available in the market. Clear zone test is performed and the distance of the clear zone is measured (Fig: II a, b, c, d and Table II).

The MIC Test

To know the effective minimum inhibitory concentration of both compounds standard MIC test (test organism- *Escherichia coli*, optical density measured after 24hrs of incubation) has been done. The result of MIC test for LT 2/8 AgBr is shown in the table III.

The synthesized Silver (I) N heterocyclic carbene Bromin complex is a novel compound with various activities. The most important prospect of the compound is that it can be used as alternative to antibiotics, antitumor agent and anticancer agent (Berner et al, 1988). Other applications may be water disinfection in hotels and hospitals, post harvest cleaning of oysters and crabs, inhibition of bacterial growth on farms, application to eyes of newborn babies to prevent infection etc.

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