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Laboratory note

Synthesis and investigation of antimicrobial activity of some bisbenzimidazole-derived chelating agents

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Abstract

The 1,2-bis(2-benzimidazyl)-1,2-ethanediol (**1**), 1,4-bis(2-benzimidazyl)-1,2,3,4-butanetetraol (**2**), 1,3-bis(2-benzimidazyl)-2-thia-propane (**3**), 1,3-bis(2-benzimidazyl)-2-thia-propane-dihydrochloride (**4**), 1,5-bis(2-benzimidazyl)-3-thiapentane (**5**), and 1,5-bis(2-benzimidazyl)-3-thiapentane dihydrochloride (**6**) chelating ligands are synthesised and characterised by using analytical data and modem spectroscopic methods such as FT-Raman, FT-IR, ^{1}H- and ^{13}C-NMR spectrometers. Their antimicrobial activities are reported by comparing the in vitro activities, with those of ofloxacin, ciprofloxacin, piperacillin, ampicillin and cefazolin antibacterial agents against fresh clinical isolates. Antifungal activities are reported on *Candida albicans*, *Candida utilis*, *Cryptococcus neoformans* fungi, and the results are referenced with amphotericin-B, fluconazole and flucytosine antifungal agents. It has been found that all the compounds have broad spectra activity and was either more active or equipotent to those compared antibiotic and antifungal agents.

Keywords: Ampicillin; Bisbenzimidazole; Chelating ligands; Equipotent; Ofloxacin

1. Introduction

Benzimidazole and bis-benzimidazole derivatives are key components in a great many bioactive compounds of both natural and synthetic origin. These ligands and their derivatives display a wide range of pharmacological activity, and their inhibitory properties as regards the replication of polio viruses, adenosine deaminase, and casein kinase have been fully demonstrated [1,2]. While others are important potent antiviral agents [3,4], many are active components of biocides such as fungicides, and insecticides [5,6]. For instance it is known that 1,2-bis(2-benzimidazyl)-1,2-ethanediol (**1**) shows antifungal and anti-polio viruses character, and that ligand such as 1,4-bis(2-benzimidazyl)-1,2,3,4-butanetetraol (**2**) have a negative effect on intracellular viruses [7,8]. Other examples include 5-aminoimidazole ribonucleotide, a key intermediate on the de novo purine biosynthetic pathway [9] and 5-(formylamino)imidazole ribonucleoside, a competitive inhibitor of adenosine deaminase.

In addition to their biological importance, these ligands are strongly coordinating agents and form stable complexes with various metals [10–12]. Metal ions play a vital role in vast number of different biological processes through co-enzymatic system. The interaction of these ions with biological active ligands, for instance in drugs, is a subject of great interest. Some of the biological active compounds do act via chelation, but for most of them little are known about how metal coordination influences their activity. It is believed that they react selectively towards certain biological systems [13,14].

We are interested in the microbiological activity of these ligands towards many bacterial and yeast isolates both representing several species.

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2. Chemistry

The compounds are prepared either by modified literature procedures Philips [15] or fused [16] method. Full general procedures for synthesis of these compounds are given in the experimental and the reactions sequence are shown in Figs. 1–4. The spectroscopic data of ^{1}H- and ^{13}C-NMR, FT-Raman and FT-IR do provide useful information for their formation and structural identification. The results of the spectroscopic data are summarized in the end of each experimental. Apart from few exceptions, the analysis of these spectra is straightforward. Exception arise that for all the ligands (**1**, **2**) in ^{1}H-NMR amino proton resonances are observed as broad signals at δ_H 12.24 and 12.07 ppm, respectively. Both of these disappear due deuterium exchange. For the ligands (**3**–**6**) the amino proton resonance was not detected due to the fast tautomeric equilibrium of the proton atom in the imidazole ring.

3. Pharmacological

Fresh clinical isolates are obtained from Faculty of Medicine, Uludag University (Turkey), and are supplemented by stock cultures of recent clinical isolates of some species to achieve target numbers. The total numbers of isolates are 115 bacteria and 30 yeasts representing 10 and 3 species, respectively.

Standardised powders of floxacin (an effective broad spectrum bactericidal, with inhibition of DNA gyrase activity), ciprofloxacin (antibaterial), piperacillin (β-lactamase a sensitive penicillin antibiotic), ampicillin (broad spectrum antibiotic), cefazolin (for intravenous and intramuscular administration), were purchased together with NAD from Sigma Chemicals. Amphotericin-B (antifungal and antiprotozoal), fluconazole (broad-spectrum bis-triazole antifungal) and flucytosine are obtained from Fluka. Mueller–Hinton media was purchased from Difco and the yeast extracts were obtained from Oxoid.

4. Results and discussion

Bisbenzimidazole derived compounds are evaluated by broth macro dilution procedure for their in vitro antibacterial activity against 115 pathogenic bacteria and 30 yeast-isolates representing 10 and 3 species, respectively. The results of these assays are summarised in Tables I and II. The data for ofloxacin, ciprofloxacin, piperacillin, ampicillin and cefazolin antibacterial agents and amphotericin, fluconazole and flucytosine antifungal agents are used for comparison. Apart from few exceptions, all the compounds are more active as compared to antibacterial and antifungal agents against all the tested micro-organisms. These compounds activity toward tested micro-organisms are equipotent to that of ofloxacin and requires over 64 μg ml^{-1} doses for the inhibition of *Enterococcus faecalis* and *Proteus species*.

It could be seen from the data that if the result of only few micro-organisms, namely *Escherichia coli*, *Salmonella thyphi*, *Bacillus subtilis* and *Stapylococcus aureus* are considered for the basis of this study, this would have resulted in the failure of achieving, respectable and valuable information. In this respect, we have used a reasonable number of micro-organisms, 10 (Gram-positive and Gram-negative) total, 115 bacteria and 30 yeast isolates, to obtain broad antimicrobial effect spectrum.

In classifying the antibacterial activity as Gram-positive or Gram-negative, it would generally be expected that a much greater number would be active against Gram-positive than Gram-negative bacteria [17]. However, in this study, the compounds are active against both Gram-positive and Gram-negative bacteria and highly active against yeasts. The activity against both types of bacteria and yeasts may be indicative of the presence of broad spectrum.

The results of our study indicate that the compounds have the potential to generate novel metabolites. The compounds demonstrating especially anticandidal activity could result in the discovery of novel anticandidal agents, showing demonstrating broad spectrum activities, this may help to discover new chemical classes of antibiotics that could serve as selective agents against infectious diseases.

5. Experimental protocols

5.1. Chemistry

All chemicals and solvents were reagent grade and used without further purification, unless otherwise indicated. Purity of the compounds was tested on thin layer chromatography (TLC) plates (silica gel 60 F_{254} Merck) in the solvent system ethanol:water (5:3). Spots

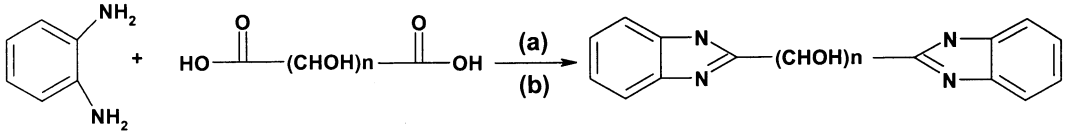

Fig. 1. Syntheses of ligands (**1**–**2**), (a) refluxed in 5 M and neutralised with dilute base, (b) fused at high temperature.

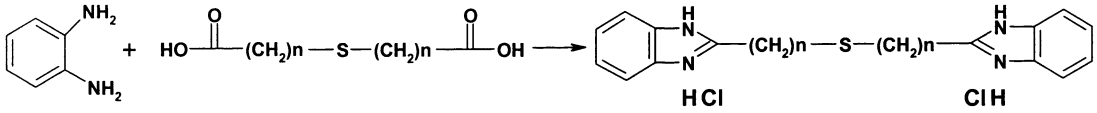

Fig. 2. Syntheses of ligands $n = 1$ (**4**) and $n = 2$ (**6**), refluxed in 5 M HCl.

were located under UV light or by exposure to iodine vapours. Melting points were determined with electrothermal 9100 melting point apparatus. Analytical data for ligands **5** and **6** were obtained with a Carlo Erba 1106 analyser. Infrared (IR) spectra were recorded (as KBr pellets or as mulls in Nujol) on a Mattson Satellite FT-JR spectrometer. FT-Raman spectra were obtained from powdered samples placed in a Pyrex tube using the Bruker RFS 100/S spectrometer (Nd: YAG-Laser, 1064 nm, 200 mW). Routine solution ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded at ambient temperature (20 °C), on a Bruker DPX 400 ultra-shield NMR spectrometer in DMSO-d_6. Chemical shifts (δ) were expressed in units of ppm relative to TMS. The residual DMSO-d_6 signal was also used as an internal reference. The NMR peaks are expressed as: singlets (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broad (br).

5.1.1. General procedure for the preparation of the compounds *1–6*

*5.1.1.1. The 1,3-bis(2-benzimidazyl)-2-thiapropane-dihydrochloride (**4**) and 1,3-bis(2-benzimida-zyl)-2-thiapropane (**3**) ligands are synthesized according to Philip's method [15].* Aqueous HC1 (5 M, 50 mL) was added to a mixture of 2,2′-thiodiethanoic acid (3.0 g, 20.0 mmol) and freshly sublimed *o*-phenylenediamine (4.32 g, 40.0 mmol). The solution mixture was refluxed for 24 h, followed by immediate filtration. As the filtrate cooled using ice-bath, large light-green crystals formed. These are collected by vacuum filtration, washed several times with cold water, dried in vacuo, and identified as the dihydrochloride (**4**), decompose at 246° C.

The free base ligand (**3**) was obtained by treatment of the **4** with excess aqueous 10% ammonia solution. The dark brown oily residue, which separated are dissolved in warm ethanol. The solution mixture was decolourised with charcoal at reflux temperature for about 10 min, filtered and crystallised by gradual addition of water. Fine colourless crystals are deposited when the solution was left standing overnight. These crystals are isolated by filtration and dried in vacuo (4.2 g, 70%), m.p. 221° C. ^{1}H-NMR (400 MHz): δ 3.85 (4H, s, CH_2), 6.96 (s, 4H), 7.32 (s, 4H), (2H, NH not detected); ^{13}C{^{1}H}-NMR (100 MHz): 6 29.04 (2C, CH_2), 115.17 (4C), 122.19 (4C), 138.93 (4C), 152.10 (2C); JR (KBr pellets): ν_{max}/cm^{-1} includes 1670 (C=N), 1620 (C=C), 742 (C=C); Raman (solid): ν_{max}/cm^{-1} includes 3055 (C–H), 2916 (C–H), 1590 (C=C), 770 (C=C).

*5.1.1.2. Ligand **4** is also synthesized using the direct reaction route, commonly applied for solid-state reactions according to the Fused method [16].* In a mullite mortar, a freshly sublimed *o*-phenylenediamine (2.00 g, 16.66 mmol) was ground together with 2,2′-thiodiethanoic acid (1.4 g, 9.0 mmol). To obtain dense pellets, about a quarter of the finely ground powder was introduced into a stainless steel die (Ø = 13 mm) which was then evacuated shortly to remove the air between the particles. The powder was then pressed into a disc between stainless steel anvils at a pressure of 10–12 tons. The pellets were transferred into a flame dried Schlenk tube, which was connected to a vacuum line and heated up to about 180 °C or until it melted, with occasional opening the vacuum line to remove condensed water. The completion of the reactions took 2–3 h. The bluish-purple solid product was formed, which was finely ground, and either sublimed at 160 °C or crystallized as above (yield 60%).

*5.1.1.3. 1,5-bis(2-Benzimidazyl)-3-thiapentane (**5**).* A mixture of 2,2′-thiodiethanoic acid (3.0 g, 20.0 mmol) and freshly sublimed *o*-phenylenediamine (3.64 g, 40.0

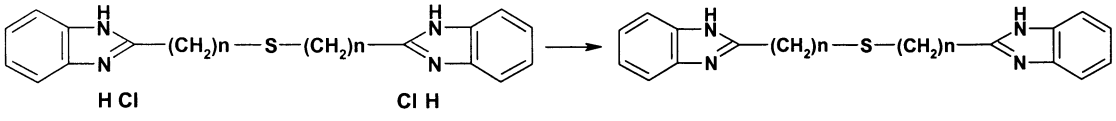

Fig. 3. Syntheses of ligands $n = 1$ (**3**) and $n = 2$ (**5**), neutralised with dilute base.

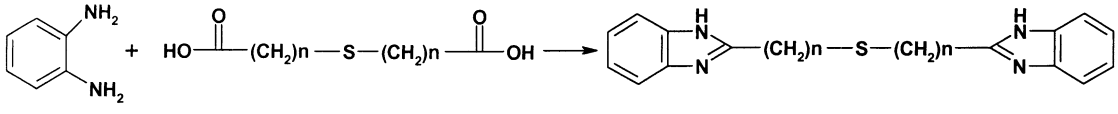

Fig. 4. Syntheses of ligands $n = 1$ (**3**) and $n = 2$ (**5**), fused at high temperature.

Table I
MICs of compounds **1**–**3** for 115 bacteria and 30 yeasts and their rates of susceptibility to five compared antibiotics

A	B	MIC (μg ml^{-1}) [a]									Isolates % for MICs < 64 μg ml^{-1}			% of isolates susceptible to [b]							
	No	Compound **1**			Compound **2**			Compound **3**			1	2	3	AMP	CIP	CFZ	OFL	PIP	AM	FLU	FL
		50%	90%	Range	50%	90%	Range	50%	90%	Range											
A	15	32	64	32–256	32	128	32–128	64	256	64–256	85	96	88	7	60	70	50	60	Nd	Nd	Nd
B	15	8	16	8–16	8	16	8–32	16	32	16–32	100	100	100	67	97	93	100	67	Nd	Nd	Nd
C	15	16	16	8–16	8	16	8–32	8	16	8–16	100	100	1000	100	6	100	88	Nd	Nd	Nd	Nd
D	10	4	8	4–8	8	8	4–8	8	8	4–8	100	100	12	100	96	100	88	Nd	Nd	Nd	Nd
E	10	16	32	16–32	16	64	16–64	32	64	32–64	100	100	100	100	80	0	80	100	Nd	Nd	Nd
F	10	32	128	32–128	64	128	64–128	16	64	16–64	96	96	100	16	100	4	92	96	Nd	Nd	Nd
G	10	64	128	64–128	64	128	64–256	64	128	64–128	96	96	96	100	100	100	97	100	Nd	Nd	Nd
H	10	> 256	> 256	256– > 256	> 256	> 256	256– > 256	> 256	> 256	256– > 256	0	0	0	100	96	85	92	100	Nd	Nd	Nd
I	10	> 256	> 256	256– > 256	> 256	> 256	256– > 256	> 256	> 256	256– > 256	0	0	0	100	96	85	94	100	Nd	Nd	Nd
J	10	> 256	> 256	256– > 256	> 256	> 256	256– > 256	> 256	> 256	256– > 256	0	0	0	100	87	0	83	100	Nd	Nd	Nd
K	10	4	8	4–8	4	8	4–8	4	8	4–8	100	Nd	Nd	Nd	Nd	Nd	100	100	100	100	100
L	10	4	8	4–8	8	8	4–8	4	8	4–8	100	100	100	Nd	Nd	Nd	Nd	Nd	100	100	100
M	10	8	8	4–8	4	8	4–8	2	4	0.125–4	100	100	100	Nd	Nd	Nd	Nd	Nd	98	100	92

A: microorganisms; B: number of isolates. (A) *Staphylococcus aureus*, (B) *Escherichia coli*, (C) *Enterobacter aerogenes*, (D) *Klebsiella pneumoniae*, (E) *Alcaligenes faecalis*, (F) *Citrobacter freundii*, (G) *Streptococcus pyogenes*, (H) *Proteus vulgaris*, (I) *Proteus mirabilis*, (J) *Enterococcusfaecalis*, (K) *Candida albicans*, (L) *Candida utilis*, (M) *Cryptococcus neoformans*.

[a] 50% and 90%, MICs at which 50 and 90% of the isolates are inhibited, respectively.

[b] Susceptibility breakpoints are in parentheses. OFL, ofloxacin (< 2.0 μg ml^{-1}); AMP, ampicillin (< 8.0 μg ml^{-1}); CIP, ciprofloxacin (< 1.0 μg ml^{-1}); CFZ, cefazolin (< 8.0 μg ml^{-1}); PIP, piperacillin (< 16 μg ml^{-1}); AM, amphotericin-B (< 16 μg ml^{-1}); FLU, fluconazoie (< 8.0 μg ml^{-1}); FL, flucytosine (< 16 μg ml^{-1}).

Table II
MICs of compounds **4**–**6** for 115 bacteria and 30 yeasts and their rates of susceptibility to compared antibiotics and fungi

A	B	MIC (μg ml^{-1}) [a]									Isolates % for MICs < 64 μg ml^{-1}			% of isolates susceptible to [b]							
	No	Compound **4**			Compound **5**			Compound **6**			1	2	3	AMP	CIP	CFZ	OFL	PIP	AM	FLU	FL
		50%	90%	Range	50%	90%	Range	50%	90%	Range											
A	15	32	64	32–64	64	128	64–128	64	256	64–256	85	96	88	7	60	70	50	60	Nd	Nd	Nd
B	15	8	16	8–16	8	16	8–16	8	16	8–32	100	100	100	67	97	93	100	67	Nd	Nd	Nd
C	15	8	16	8–16	8	16	8–16	8	16	8–16	100	100	100	0	100	6	100	88	Nd	Nd	Nd
D	10	4	8	4–8	8	8	4–8	8	8	4–8	100	100	100	12	100	96	100	88	Nd	Nd	Nd
E	10	32	54	32–64	32	32	32–64	32	64	32–64	100	100	100	100	80	0	80	100	Nd	Nd	Nd
F	10	32	64	32–64	32	128	32–128	64	128	64–128	96	96	100	16	100	4	92	96	Nd	Nd	Nd
G	10	64	128	32–128	64	128	64–256	64	128	32–128	96	96	96	100	100	100	97	100	Nd	Nd	Nd
H	10	> 256	> 256	256– > 256	> 256	> 256	256– > 256	> 256	> 256	256– > 256	0	0	0	100	96	85	92	100	Nd	Nd	Nd
I	10	> 256	> 256	256– > 256	> 256	> 256	256– > 256	> 256	> 256	256– > 256	0	0	0	100	96	85	94	100	Nd	Nd	Nd
J	10	> 256	> 256	256– > 256	> 256	> 256	256– > 256	> 256	> 256	256– > 256	0	0	0	100	87	0	83	100	Nd	Nd	Nd
K	10	2	4	≤ 0.125–4	4	8	4–8	2	4	≤ 0.125–4	100	100	100	Nd	Nd	Nd	100	100	100	100	100
L	10	2	4	2–4	8	8	4–8	2	4	≤ 0.125–44–8	100	100	100	Nd	Nd	Nd	Nd	Nd	100	100	100
M	10	4	8	4–8	8	8	4–8	4	8	4–8	100	100	100	Nd	Nd	Nd	Nd	Nd	98	100	92

A: microorganisms; B: number of isolates. (A) *Staphylococcus aureus*, (B) *Escherichia coli*, (C) *Enterobacter aerogenes*, (D) *Klebsiella pneumoniae*, (E) *Alcaligenes faecalis*, (F) *Citrobacter freundii*, (G) *Streptococcus pyogenes*, (H) *Proteus vulgaris*, (I) *Proteus mirabilis*, (J) *Enterococcusfaecalis*, (K) *Candida albicans*, (L) *Candida utilis*, (M) *Cryptococcus neoformans*.

[a] 50% and 90%, MICs at which 50 and 90% of the isolates are inhibited, respectively.

[b] Susceptibility breakpoints are in parentheses. OFL, ofloxacin (< 2.0 μg ml^{-1}); AMP, ampicillin (< 8.0 μg ml^{-1}); CIP, ciprofloxacin (< 1.0 μg ml^{-1}); CFZ, cefazolin (< 8.0 μg ml^{-1}); PIP, piperacillin (< 16 μg ml^{-1}); AM, amphotericin-B (< 16 μg ml^{-1}); FLU, fluconazoie (< 8.0 μg ml^{-1}); FL, flucytosine (< 16 μg ml^{-1}).

mmol) in 5 M aqueous HCl (50 mL) was refluxed overnight. After cooling to room temperature a light grey product was collected by filtration and dried in vacuo (4.6 g, 70%), m.p. 145 °C. Anal. Found: C, 63.6; H, 5.4; N, 13.8; S, 7.9. Calc. for $C_{18}H_{20}N_4C1_2S$: C, 54.7; H, 5.1; N, 14.2; S, 8.1%. The free base ligand was crystallised (3.2 g, 85%), m.p. 183–184 °C. Anal. Found: C, 66.7; H, 5.9 N, 17.0; S, 9.4. Calc. for $C_{18}H_{18}N_4S$: C, 67.1; H, 5.6; N, 17.4; S, 9.9%. ^{1}H-NMR (400 MHz): δ 3.03 (4H, t, CH_2), 3.12 (4H, t, CH_2), 7.12 (in, 4H), 7.48 (in, 4H), (2H, NIH not resolved); $^{13}C\{^1H\}$-NMR (400 MHz): δ 29.96 (2C, C–CH_2), δ 30.05 (2C, S–CH_2), 115.34 (4C), 122.12 (4C), 139.55 (4C), 154.29 (2C); JR (KBr pellets): ν_{max}/cm^{-1} includes 3055 (C–H), 2916 (CH_2), 1620 (C=N), 1540 (C=C), 1450, 1270 and 735 (C=C); Raman (solid): ν_{max}/cm^{-1} includes 3055 (C–H), 2916 (C–H), 1590 (C=C), 1540 (C=C), 1450, 1270 and 770 (C=C).

*5.1.1.4. 1,2-bis(2-Benzimidazyl)-1,2-ethanediol (**1**).* A mixture of DL-tartaric acid (1.50 g, 10 mmol) and freshly sublimed *o*-phenylenediamine (2.16 g, 20.0 mmol) in 5 M aqueous HC1 (30 mL) was refluxed overnight. After cooling to room temperature a light grey product was collected by filtration and dried in vacuo. After neutralisation with dilute alkali, the free base ligand was crystallised (1.95 g, 70%), m.p. 268 °C. ^{1}H-NMR (400 MHz): aliphatic; δ_H 5.33 (1H, d), 5.85 (1H, d), aromatic; 7.10–7.15 (2H, m), 7.49–7.52 (2H, m); 12.24 (1H, br, NH); deuteriated with D_2O, aliphatic; δ 5.32 (1H, s), aromatic; 7.10–7.15 (2H, m), 7.49–7.52 (2H, m); $^{13}C\{^1H\}$-NMR (100 MHz): δ_C 70.80 (CHOH), 114.83 (2C), 138.75 (2C), 155.45 (1C); IR (KBr pellets): ν_{max}/cm^{-1} includes 3431 (N–H), 3184 (CH), 1623 (C=N), 1540 (C=C), 1270 and 735 (C=C); Raman (solid): ν_{max}/cm^{-1} includes 3060 (C–H), 2930 (C–H), 1623 (C=N), 1594 (C=C), 1532 and 1271.

*5.1.1.5. 1,4-bis(2-Benzimidazyl)-1,2,3,4-butanetetraol (**2**).* A mixture of mucic acid (1.80 g, 10 mmnol) and freshly sublimed *o*-phenylenediamine (2.16 g, 20.0 mmol) in 5 M aqueous HC1 (30 mL) was refluxed overnight. After neutralisation with dilute alkali, the free base ligand was crystallised (2.50 g, 75%), m.p. 282 °C. ^{1}H-NMR (400 MHz): aliphatic; δ_H 4.11 (1H, s), 4.755.85 (1H, s), 5.16 (1H, d), 5.44 (1H, d) aromatic; 7.10–7.13 (2H, m), 7.49–7.51 (2H, m); 12.07(1H, br, NH); deuteriated with D_2O, aliphatic; δ_H 4.11 (1H, s), 5.16 (1H, s), aromatic; 7.11–7.13 (2H, m), 7.49–7.51 (2H, m); $^{13}C\{^1H\}$ (not soluble enough); IR (KBr pellets): ν_{max}/cm^{-1} includes 3438 (N–H), 3200br, 1615 (C=N), 1592 (CrC), 1276 and 745 (CrC); Raman (solid): ν_{max}/cm^{-1} includes 3065 (C–H), 2938 (C–H), 1620 (C=N), 1594 (C=C) and 1278.

5.2. Pharmacology

5.2.1. The experiments were carried out by preparing a broth micro dilution, following the procedure outlined by the National Committee for Clinical Laboratory Standards—NCCLS [18]

A cation adjusted Mueller–Hinton Broth was used for testing the susceptibility of microorganisms to antimicrobial agents. For streptocci and enterococci, the medium was supplemented with 5% lysed horse blood, 5 mg ml^{-1} of yeast extract, and 15 μg ml^{-1} of NAD. The final inoculation (inoculums) approximately was 10^5 cfu ml^{-1}. For 50 of the isolates, bactericidal endpoints are determined, following the method recommended by the NCCLS [19]. Susceptibilities of yeasts are performed by the broth macro dilution testing method using RPMI 1640 broth [20]. Tested concentrations of the compounds are serial twofold dilutions ranging from 256 to 0.125 μg ml^{-1}.

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