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The synthesis of two novel bicyclic haloketones and measurement of their biological activity

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Abstract: The electrophilic addition of bromine to bicyclo[4.2.0]oct-3-en-7-one in $CHCl_3$ at 0 °C led to 60% yield of *trans*- and 30% yield of *cis*-dibromide. The structure of the synthesized molecules was determined using ^1H and ^{13}C NMR spectra. The biological activity of *cis*- and *trans*-dibromide was investigated in terms of antibiotic and toxic effects at cellular level using microbiologic and cytogenetic test, respectively. The antimicrobial activity of *trans*-dibromide**9** and *cis*-dibromide**10** was tested against *Bacillus spizizenii ATCC 6633, Salmonella typhimurium ATCC 14028,Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 9027*. Peripheral blood lymphocytes culture assay was used for determining the cytotoxicity of *trans*-dibromide **9** and *cis*-dibromide **10** substrates. *Cis*- and *trans*-dibromide showed antibiotic activity and the toxic effects of *cis*- and *trans*-dibromide were measured at cellular level by mitotic index as the cell kinetic parameter in a peripheral lymphocyte culture assay.

Keywords: Dibromoketone; biological activity; antibiotic effect; toxic effect; mitotic index.

1. Introduction

Research related to the synthesis of bioactive natural products (Scheme 1) has attracted interest since the discovery of antibiotic activity by Fleming in 1929.[1] A large number of studies followed that discovery on the synthesis and activity of antibiotics.[2-7] In 2008, Zareef et al. synthesized some derivatives of acylhydrazine including benzene diazasulfonamides and newly synthesized molecules were screened in vitro for their antibacterial activity and some for their antifungal activity.[8] Balemans et al. identified new diarylquinoline drug candidates (**3**) with inhibitory activity against gram-positive pathogens and rapid bactericidal activity on *S.aureus* in 2012.[9] Organohalogen molecules frequently display the highest level of biological activity. Recently, we described the synthesis and biological activity of epoxide **4** containing a bicyclic skeletal structure in terms of antibiotic and toxic effects at cellular level.[10] Another recent study concerned the synthesis of potent macrolide antibiotics **5** with in situ click chemistry in which 70S E. coli

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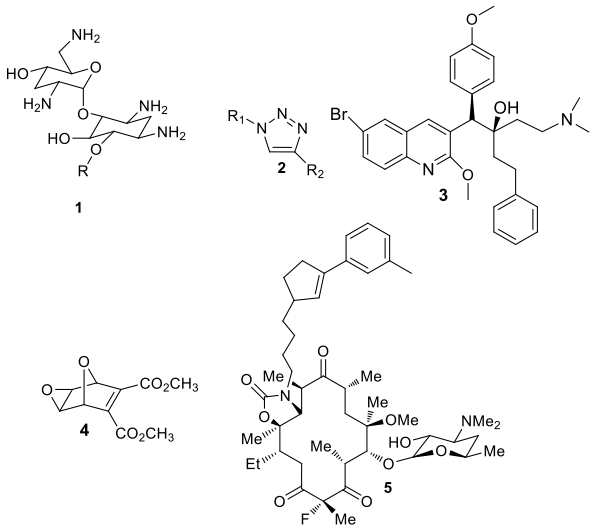
ribosomes and 50S ribosomal subunits were employed as platforms by Glassford et al.[11] MI is commonly used as the cell kinetic parameter in many studies.[12-18] It is considered as a parameter used in the measurement of biologic activities of the substances synthesized in many chemical synthesis studies.[10,19]

In this study, we aimed to synthesize the brominated molecules and investigate the activity of the synthesized molecules.



Scheme 1. Some of the biologically active molecules

2. Experimental

2.1. Materials and apparatus

RPMI 1640, Fetal Calf serum and phytohemagglutinin were purchased from Gibco. Mitomycin C was used as positive control for lymphocytes assay and purchased from Sigma. Other chemicals or solvents used in this study were of cell culture, HPLC, or analytical grade. Melting points were determined with a Mettler Toledo MP90 melting point system and were not corrected. Infrared spectra was recorded on a Perkin Elmer Win First® Satellite. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Ultrashield Plus Biospin GmbH 400 MHz spectrometer. Column chromatography was performed on silica gel (Kiesel 60, 230-400 mesh, Merck). TLC was carried out on Merck 0.2 mm silica gel 60 F_{254} analytical aluminum plates. All substances reported in this paper were in racemic form.

2.2. Chemistry

*2.2.1. Ketene Addition Reaction of 1,4-cyclohexadiene (**6**):* To a magnetically stirred solution of 1,4-cyclohexadien (**6**) (62.5 mmol, 5 g) in anhydrous diethyl ether (250 mL) at room temperature in a 1-L three-necked flask equipped with a condenser, addition funnel, and nitrogen atmosphere was added Zn-Cu (125 mmol, 8.15 g). The suspension was stirred under N_2 and with a solution of trichloroacetyl chloride (62.5 mmol, 11.35 g) and phosphoryl trichloride (62.5 mmol, 9.58 g) in diethyl ether (50 mL). The mixture was stirred for 24 h at room temperature. After the mixture was filtered on Celite, the ethereal solution was washed with saturated $NaHCO_3$ and dried with $MgSO_4$.[20] Chromatography of the residue on 50 g of silica gel eluting with diethyl ether/hexane (3:7) afforded dichlorobicyclic ketone **7** (87%, 54.4 mmol, 10.4 g) as a colorless liquid.

*8,8-dichlorobicyclo[4.2.0]oct-3-en-7-one (**7**)***:** Yield 87%. Colorless liquid, IR (cm^{-1}) 2983, 1804, 1735, 1445, 1372, 1237, 1044, ^{1}H (400 MHz, $CDCl_3$): δ 5.8 (m, 2H), 3.98 (ddd, *J*= 10.6, 6.8, 2.4 Hz, 1H), 3.2 ppm (ddd, *J*= 8 Hz, 2 Hz, 1H), 2.6-2 (m, 4H), ^{13}C (100 MHz, $CDCl_3$): 198.3, 127.3, 126.4, 53.7, 45.2, 23.1, 21.3.

*2.2.2. Reduction of 8,8-dichlorobicyclo[4.2.0]oct-3-en-7-one(**7**):* To a vigorously stirring suspension of Zn (168.4 mmol, 11 g) in 150 mL of glacial acetic acid at room temperature, was added dropwise a solution of dichlorobicyclic ketone **7** (42.11 mmol, 8 g) in 50 mL of acetic acid. After addition was complete, the temperature was raised to and maintained at 100 °C for 20 h. The reaction mixture was cooled and treated with diethyl ether, and the zinc residue was filtered. The ethereal layer was washed with water and a saturated solution of sodium bicarbonate to remove acetic acid and then it was dried with $MgSO_4$. The solvent was removed in an evaporator. Chromatography of the residue on 50 g of silica gel eluting with diethyl ether/hexane (1:1) afforded bicyclic ketone **8** (95%, 40 mol, 4.88 g) as a pale yellow viscous oil.[20]

*Bicyclo[4.2.0]oct-3-en-7-one (**8**):* Yield 95%. Pale yellow viscous oil; IR (cm^{-1}) 2950, 1788, 1654, 1420, 1248, 1166. ^1H NMR ($CDCl_3$, 400 MHz): δ 5.8 (m, 2H, H_3 and H_4), 3.45 (m, 1H, H_6), 2.55 (m, H_1), 2.4-2.2 (m, 4H, H_2, H_5),^{13}C NMR ($CDCl_3$, 100 MHz): δ 213.2, 127.4, 126.5, 56.6, 52.1, 26.5, 22.1, 21.8.

*2.2.3. Bromination of bicyclo[4.2.0]oct-3-en-7-one (**8**):* To a magnetically stirred solution of bicyclic ketone **8** (32.8 mmol, 4 g) in 20 mL of dry chloroform cooled to 25 °C was added dropwise a solution of bromine (39.4 mmol, 6.3 g) in 5 mL of chloroform for 10 min. After stirring for 5 minutes at 25 °C, the solution was slowly warmed to room temperature.[21] After removal of the solvent under reduced pressure, the oily residue was chromatographed over silica gel (20 g) with carbon tetrachloride as the eluent to giveof *trans*-dibromide **9** (60%, 18.4 mmol, 5.18 g) and *cis*-dibromide **10** (30%, 12.8 mmol, 3.6 g) as a pale yellow viscous oil.

*trans-3,4-Dibromobicyclo[4.2.0]octan-7-one (**9**):* Yield 60%. Pale yellow viscous oil; IR (cm^{-1}) 2922, 1779, 1424, 1359, 1316, 1248, 1167, 1089, 1050, 1007, 970, ^1H NMR ($CDCl_3$, 400 MHz): δ 4.54 (1H, m, H_3), 4.48 (1H, m, H_4), 3.27 (2H, m, H_6 and H_{8a}), 2.73 (1H, m, H_1), 2.62 (1H, m, H_{8b}), 1.35 (3H, m, H_{5a}, H_{5b} and H_{2b}), 2.35 (1H, ddd, *J*= 15.5, 9.84, 2.79 Hz, H_{2a}), ^{13}C NMR($CDCl_3$, 100 MHz): δ 207.4 (C=O), 52.99 (CH), 51.9 (CH_2), 50.1 (CH), 48.3 (CH), 31.5 (CH_2), 26.2 (CH_2), 19.7 (CH); GCMS *m/z* 239.9, (M^+, -Br), 159.0, (M^+, -Br), 79.1. Anal. Calcd. For $C_8H_{10}Br_2O$: C, 34.08, H, 3.57%. Found: C, 34.11, H, 3.59%.

*cis-3,4-Dibromobicyclo[4.2.0]octan-7-one (**10**):* Yield 30%.Pale yellow viscous oil; IR (cm^{-1}) 2931, 1774, 1435, 1400, 1338, 1209, 1170, 1097, 1067, 1023, 957. ^1H NMR ($CDCl_3$, 400 MHz): δ 4.22 (2H, m, H_3 and H_4), 3.41 (1H, m, H_6), 3.16 (1H, ddd, *J*= 16.7, 8.8, 3.1 Hz, H_{8a}), 2.84 (2H, m, H_{2a} and H_{8b}), 2.69 (1H, dt, *J*= 15.1, 3.7 Hz, H_{5a}), 2.61 (1H, m, H_1), 2.09 (1H, dt, *J*= 15.1, 8.8 Hz, H_{5b}), 1.84 (1H, m, H_{2b}), ^{13}C NMR ($CDCl_3$, 100 MHz): *δ* 206.4 (C=O), 55.7 (CH), 52.4 (CH_2), 52.3 (2 CH), 37.5 (CH_2), 29.9 (CH_2), 22.5 (CH). GCMS *m/z* 239.9, (M^+, -Br), 159.0, (M^+, -Br), Calcd. For $C_8H_{10}Br_2O$: C, 34.08, H, 3.57%. Found: C, 34.13, H, 3.55%.

2.3. Antimicrobial assay

The antimicrobial activity of *trans*-dibromide **9** and *cis*-dibromide **10** was tested against *Bacillus spizizenii ATCC 6633, Salmonella typhimurium ATCC 14028, Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 9027*.

2.3.1. Preparation of microbial cultures and for antimicrobial activity test of cis- and trans-3,4-dibromobicyclo[4.2.0]octan-7-one

Disc diffusion method was performed according to the standard method by Bauer et al. to assess the presence of antibacterial activities of the test compounds [22]. The antimicrobial activity of *trans*-dibromide **9** and *cis*-dibromide **10** was tested against *S. typhimurium ATCC 14028, P. aeruginosa ATCC 9027, B. spizizenii ATCC6633, E. coli ATCC 25922, and S. aureus ATCC 25923*. The bacteria strains were incubated in LBbroth (Difco). Temperature conditions set to 37 °C and incubation time was set to 24 hours. In accordance with the agar disc diffusion method, the bacteria were inoculated on Mueller-Hinton Agar (Merck). Test samples were dissolved using ethanol (96%). 10 µL was added to empty discs (6 mm)

(Bioanalyse) from each test solution and incubated for 10 minute. After that, the discs were were placed on the surface of the agar and then incubated for 24 hours. Ethanol absorbed discs were used for solvent control. The inhibition zones on the plates were measured in mm using tobramycin (10 µg) (Bioanalyse) as standard drug.

2.3.2. Time-kill Assay

The time-kill test was carried out using the macrodilution technique in accordance with the NCCLS standard and was performed in broth culture containing test substances which final concentration of 0,25x MIC, 1x MIC, 2xMIC, Tobramycin (10 µg/mL) and growth control.[23] 30 ml broths were inoculated to give a final inoculum of 105 cfu/mL which adjusted to 0,5 McFarland turbidity standard and diluted 1/100 with 0,85% sodium chlorid solution. Broths were incubated at 36°C with shaking and samples from broth were taken for varied time intervals (0, 2, 4, 6, 8, 10, 12 and 24 h) and plated nutrient agar (Merck). Viable counts were read manually after 24 h incubation. All antimicrobial activity tests were conducted in triplicate.

2.4. Eukaryotic system assay/ Lymphocyte cultures in peripheral blood lymphocytes and mitotic index

Peripheral blood lymphocytes culture assay was used for determining the cytotoxicity of *trans*-dibromide **9** and *cis*-dibromide **10** substrates. In vitro analysis was performed in human blood lymphocytes from three donors at four concentrations of each substrate (150 µg/mL, 200 µg/mL, 250 µg/mL and 300 µg/mL). The cytotoxicity of the substrates were evaluated by using mitotic index (MI), the parameter of cytotoxicity in human peripheral blood lymphocyte cultures.

The study was performed using blood samples from three healthy non-smoking male donors, aged 26, 24 and 25 years. In the three donors, the results of clinical routine laboratory analyses were within the normal ranges, and the absence of exposure to known genotoxicants was assumed. Lymphocyte cultures were prepared according to the technique described by Moorhead et al. with slight modifications.[16] Heparinized whole blood (1 mL) was added to 6 mL of RPMI 1640 medium (Gibco, USA), supplemented with 20 % fetal calf serum (Gibco, USA), 0.1 % mL phytohemagglutinin (Gibco,USA), and antibiotics (10,000 µg/mL penicillin and 10,000 IU/mL streptomycin). Lymphocytes were cultured for 72 h, and metaphases were blocked during the last 1.5 h with colcemid at a final concentration of 0.2 µg/mL. Mitomycin C (2 µg/mL) was used as positive control. The cells were harvested by replacing the culture medium with KCl (0.075 M) in which cells were incubated for 20 min at 37 °C. The cells were fixed in Carnoy's fixative (methanol:acetic acid, 3:1 v:v) five times, and the slides were kept at room temperature overnight. The air-dried slides were stained with 5 % Giemsa stain.

2.4.1. Microscopic evaluation

The MI was calculated as the proportion of metaphases among the total cell population by counting 1,000 cells for each dose treatment.

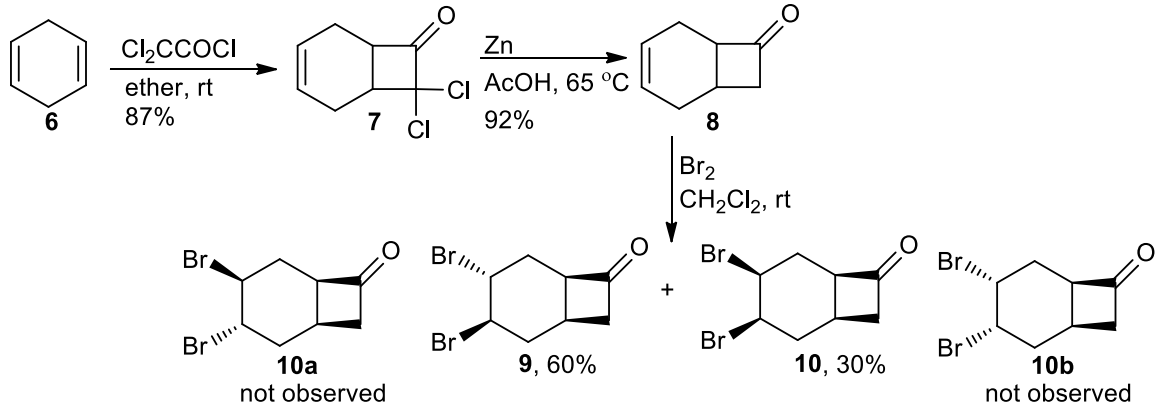
2.4.2. Statistical evaluation

The data were compared using one-way variance analysis. Statistical analysis was performed using SPSS for Windows 21.0 Post hoc analysis was performed using the least significant difference (LSD) test.

3. Results and discussion

Ketene cyclo addition reactions are reactions of diene of ketenes with unsaturated compounds to provide four-membered rings (Scheme 2).[24,25] The addition of dichloroketene to 1,4-cyclohexadiene (**6**) was reported by Liotta et al., (1987), Davis et al., (1996) and Kishali et al., (2011).[20,26,27] Bicyclic ketone **8** was obtained from elimination with Zn-Cu in acetic acid.[20,25] The ^{1}H and ^{13}C NMR spectra are in good agreement with addition product **7** and bicyclic ketone **8**. Later, *trans*- and *cis*-dibromide (**9**, **10**) were obtained at 0 °C and 25 °C as a result of the bromination of bicyclic ketone **8** which were separated by

column chromatography.[21] *Trans*-Isomer **9** (Rf= 0.95) has a larger Rf value than *cis*-isomer **10** (Rf= 0.83) [hexane/ ethyl acetate mixture (9:1)]. *trans*-Dibromide **9** was formed as the major product with 60% yield. The absorption bands at 1779cm^{-1} (**9**) and 1774 cm^{-1} (**10**) in the FT-IR spectra confirmed the cyclobutanone ring in the structure. In *trans*-dibromide **9**, H_4 and H_6 are in *trans* position relative to each other. The chemical shifts of H_{5a} and H_{5b} are close together (2.60 and 2.44 ppm). In *cis*-dibromide **10**, bromine and oxygen shifted the chemical shift of H_{5a} in the downward direction in NMR. Therefore, the chemical shifts of H_{5a} and H_{5b} are very far from each other (2.68 and 2.09 ppm). In molecule **9**, bromine atom bound to C_4 and cyclobutanone rings are in the same space since there is w-interaction; but bromine atom bound to C_3 and cyclobutanone rings are in the different space since there is no w-interaction. In molecule **10**, C_3 and C_4 protons did not give w-interaction with C_1 and C_6 protons. This shows that the bromines are *cis*-position according to each other and in the *trans*-position with the cyclobutanone ring. It showed that *cis*-dibromide **10** occurred over radicalic mechanism in this reaction.



Scheme 2. Ketene addition to 1,4-cyclohexadiene (**6**) and the bromination reaction of bicyclic ketone **8**

3.1. *The antimicrobial activity of trans- and cis-3,4-dibromobicyclo[4.2.0]octan-7-one (9 and 10)*

Inhibition zones were measured in mm compared with tobramycin (10 µg) (Bioanalyse) as standard drug (Table 1 and 2). The Minimal inhibitory concentration (MIC) of the test solution of *trans*-dibromide **9** and *cis*-dibromide **10** were detected using Macrodilution test method. The tubes contained LB medium, ranging from 50 to 300 µg/mL. The macrobroth dilutions were incubated for 24 h at 37 °C and spectrophotometrically measured (OD 600 nm) to verify the presence or absence of growth (Fig.1 and 2). Only the *Staphylococcus aureus* strain was susceptible to the tested compound and MIC values of *trans*-dibromide **9** and *cis*-dibromide **10** for 150 and 200 µg/mL were obtained, respectively.

Table 1. Inhibitory effects of *trans*-dibromide **9** on the tested bacterial strains.

Bacteria	Test (mm) **9**						Controls	
	50 µg	100 µg	150 µg	200 µg	250 µg	300 µg	Tobramycin (10 µg)	Ethanol (96%)
S. aureus	-	7.7	9.9	10.5	11.5	12.7	17.9	-
B.spizizenii	-	-	-	-	-	-	23.1	-
E.coli	-	-	-	-	-	-	11.05	-
P. aeruginosa	-	-	-	-	-	-	25.85	-
S. typhimurium	-	-	-	-	-	-	17.18	-

Radii of the inhibition zones on the plates were measured in mm. Tobramycin was used as standard drug for antibacterial activity

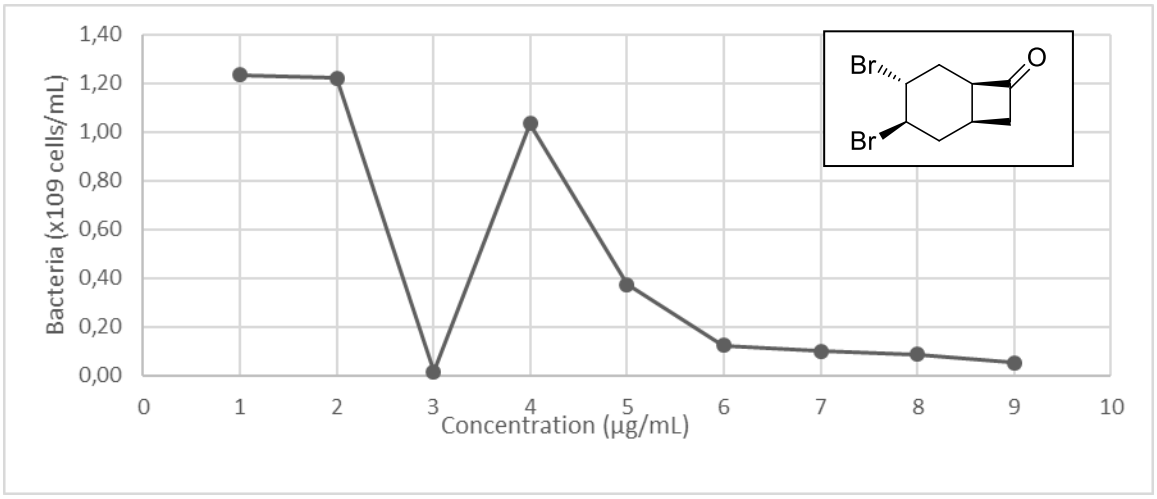


Figure 1. Effects of different concentrations of *trans*-dibromide **9** on the bacterial strain (*S. aureus*) tested.

Table 2. Inhibitory effects of *cis*-dibromide **10** on the tested bacterial strains.

Bacteria	Test (mm) **10**						Controls	
	50 µg	100 µg	150 µg	200 µg	250 µg	300 µg	Tobramycin (10 µg)	Ethanol (96%)
S. aureus	9.4	11.3	13.5	14.9	16.1	18.1	18.7	-
Bacillus spizizenii	-	-	-	-	-	-	23.1	-
E.coli	-	-	-	-	-	-	11.05	-
P. aeruginosa	-	-	-	-	-	-	25.85	-
S. typhimurium	-	-	-	-	-	-	17.18	-

Radii of the inhibition zones on the plates were measured in mm. Tobramycin was used as standard drug for antibacterial activity

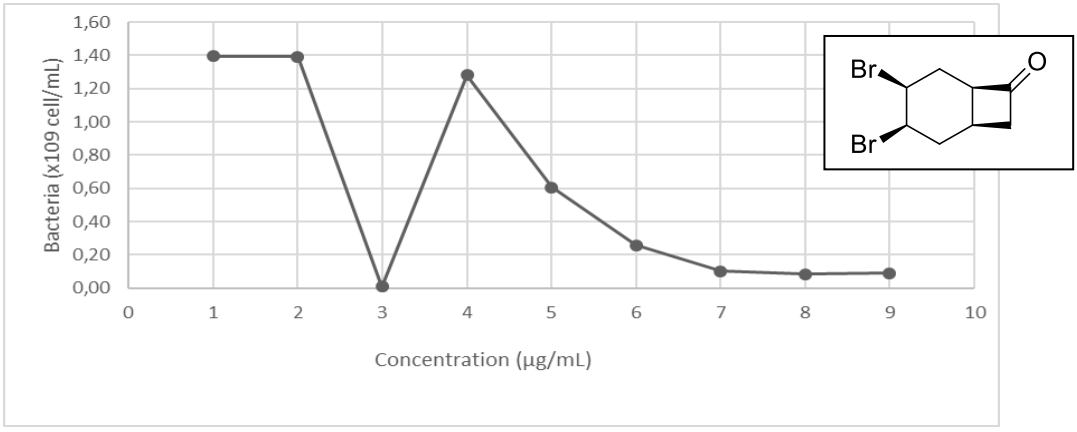


Figure 2. Effects of different concentrations of *cis*-dibromide **10** on the bacterial strain (*S. aureus*) q tested.

3.2. Time-Kill Assay

The time–kill curves of *trans*-dibromide 9 and *cis*-dibromide 10 against the *S. aureus* are shown in Figure 3 and 4. A bactericidal effect was defined as a ≥3 log10 cfu/mL decrease compared with the initial inoculum after 24 h of incubation.[28] *Trans*–dibromide **9** and *cis*-dibromide **10** showed no bactericidal effect at a concentration of 0.25xMIC. While concentration of 1xMIC *trans*-dibromide **9** reduced the amount of bacteria by less than 3 log10 CFU/ml-1, 2x MIC concentration reduced the amount of bacteria by more than 3 log10 CFU/ml-1 within 6 hours when compared to the growth control. Also, the concentration of

2xMIC of *cis*-dibromide **10** substance caused a decrease in the amount of bacteria over 3 log10 CFU/ml-1 within 6 hours. Nevertheless, 1xMIC concentrations of *cis*-dibromide **10** and *trans*-dibromide **9** showing bacteriostatic activity.

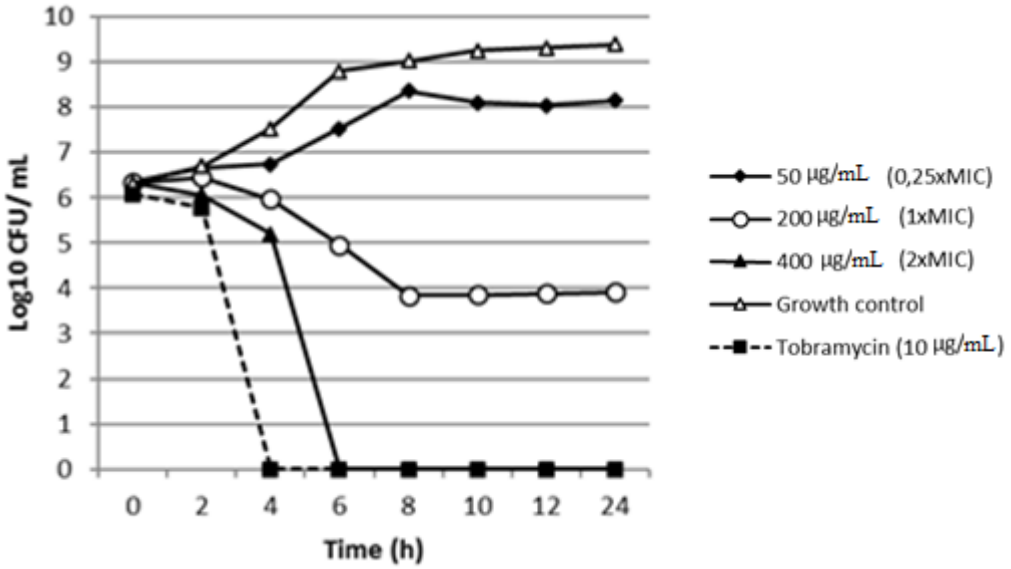


Figure 3. Time-Kill curve of *trans*-dibromide **9** (µg/mL)*

*Time-Kill curve of *trans*-dibromid **9** (ug/ml)against *S. aureus*. 0,25× MIC (black diamonds); 1xMIC (white circles); 2×MIC (black triangles); growth control (white triangles); and Tobramycin control (black square).

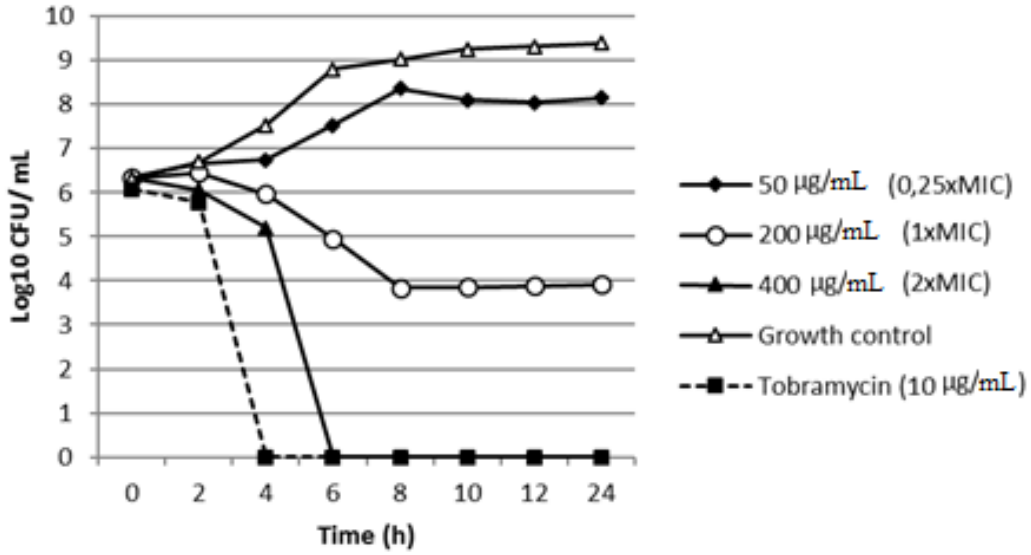


Figure 4. Time-kill curve of *cis*-dibromide **10** (µg/mL)*

*Time-Kill curve of *cis*-dibromid **10** (ug/ml)against *S. aureus*. 0,25× MIC (black diamonds); 1xMIC (white circles); 2×MIC (black triangles); growth control (white triangles); and Tobramycin control (black square)

3.3. Cytotoxicity Results /Cell kinetic parameter-mitotic index (MI)

Table 3 shows the values of MI in lymphocyte cultures treated with four different concentrations of *trans*-dibromide **9** and *cis*-dibromide **10** substrates (150 µg/mL, 200 µg/mL, 250 µg/mL and 300 µg/mL).

All the concentrations of *trans*-dibromide **9** decreased the MI. There is a statistically significant difference between concentrations of *trans*-dibromide**9** (150 µg/mL, 200 µg/mL, 250 µg/mL and 300 µg/mL) and the negative control ($p<0.05$, $p<0.001$). Two concentration (150 µg/mL, 200 µg/mL) of *cis*-dibromide **10** substrate increased the MI except for the concentration of 250 µg/mL of *cis*-dibromide **10**. The concentration of 250 µg/mL of *cis*-dibromide **10** did not cause any change in mitotic index values. This increase is statistically significant ($p<0.001$). Moreover, 300 µg/mL concentrations of *cis*-dibromide**10** substrate significantly decreased the MI ($p<0.001$).

Table 3. The values of MI in lymphocytes cultures treated with four different concentration of *trans*-dibromide **9** and *cis*-dibromide **10** in vitro.

	Groups	Donor 1	Donor 2	Donor 3	Mean ± SE
1	9-150 µg/mL	47	62	59	56.8±4.58*
2	9-200 µg/mL	20	42	38	33.3±6.76***
3	9-250 µg/mL	12	20	16	16.0±2.30***
4	9-300 µg/mL	5	10	11	8.66±1.85***
5	10-150 µg/mL	96	108	102	102.0±3.46***
6	10-200 µg/mL	92	100	84	92.0±4.61***
7	10-250 µg/mL	67	65	79	70.3±4.37
8	10-300 µg/mL	25	28	30	27.6±1.45***
9	NC	66	67	71	68.0±1.52
10	PC (MMC 2µg/mL)	34	41	33	36.0±2.51***

*$p<0.05$, ***$p<0.001$ compared with negative control

4. Conclusion

We synthesized dichlorobicyclic ketone **7** from 1,4-cyclohexadiene (**6**) in good yield by using a ketene addition reaction. After the reduction reaction of the chlorine, the addition of bromine to bicyclic ketone **8** was performed.

The in vitro antibacterial activity of the synthesized compounds were determined by broth macrodilution method using Gram-positive and Gram-negative organisms. Tobramycin used as a control antibiotic. It was observed that *trans*-dibromide **9** and *cis*-dibromide **10** showed antibiotic properties against *S. aureus ATCC 25923*. However, no antibacterial effects on the other test strains were observed. MIC values of *trans*-dibromide **9** and *cis*-dibromide **10** for 150 and 200 µg/mL were obtained, respectively.

Nowadays, people are exposed to various chemicals for many reasons such as environmental, occupational and therapeutic or lifestyle changes. Several chemicals have been shown to have toxic effects in many *in vivo* and *in vitro* studies including prokaryotic and eukaryotic systems.[12-14,17,18,29] Several studies have indicated that one of the most major response in cells exposed to chemicals, ionizing radiation, and/or other genotoxic agents is the inhibition or the delay of cell-cycle progression. MI is used as an indicator of cell cytotoxicity.[12,13,15]

According to the data reported in the present study, four different concentrations of *trans*-dibromide **9** and *cis*-dibromide **10** affected the MI, considered as a parameter of cell kinetic or cytotoxic activity. The fact that all concentrations of trans-dibromide9 cause a decrease in mitotic index values indicates that this substance can be considered as a mitotic inhibiting agent at the cellular level and in vitro. These data reveal that all the concentrations of *trans*-dibromide **9** had cytotoxic effects at cellular level and likewise, the 300 µg/ml concentrations of cis-dibromide **10** has mitotic inhibitory properties because it cause a decrease in mitotic index values. It can be said that two concentrations (150 µg/mL and 200 µg/mL) of *cis*-dibromide **10** have a mitotic stimulating effect because this concentrations cause an increase in mitotic index values.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/-bioorganic-medicinal-chemistry-reports

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