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Short communication

Synthesis and antibacterial activity of bis-[2-hydroxy-3-(1,7,8,9,10-pentamethyl-3,5-dioxo-4-aza-tricyclo[5.2.1.$0^{2,6}$] dec-8-en-4-yloxy)-propyl]-dimethyl-ammonium chloride

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Abstract

A new quaternary ammonium compound, bis-[2-hydroxy-3-(1,7,8,9,10-pentamethyl-3,5-dioxo-4-aza-tricyclo[5.2.1.$0^{2,6}$]dec-8-en-4-yloxy)-propyl]-dimethyl-ammonium chloride (**4**), was synthesized. The compound was investigated for antibacterial activity, including Gram-positive cocci and Gram-negative rods, and antifungal activity. Compound **4** showed significant inhibition against *Staphylococcus aureus*. Research was carried out over 4 standard strains and 40 hospital strains. Elementary analysis and/or MS, ^{1}H NMR and ^{13}C NMR spectra confirmed the identity of the products. The molecular structure of **3** was determined by an X-ray analysis.

Keywords: Cyclic imide; Quaternary ammonium compound; Antimicrobial activity; X-ray crystal structure analysis

1. Introduction

Amphiphilic molecules have an ability to intercalate into phospholipid membranes and may consequently affect the biological processes [1]. For this reason such compounds are called membrane-active. It should be noted that a multitude of various amphiphilic substances can be beneficial or harmful to living cells. Some of amphiphilic compounds can be used as drugs [2], whereas some other have deleterious affect on living system.

Quaternary ammonium salts exist as amphiphilic cations in aqueous solution. The compounds are called bifunctional surfactants and they are applied as common pesticides, fertilizers or antioxidants [3,4]. These salts are used widely in paint, water treatment, textile, and food industries, because they have a relatively low toxicity and a broader antimicrobial spectrum [5,6].

Long chain quaternary ammonium compounds exert antibacterial activity against both Gram-positive and Gram-negative bacteria as well as against some pathogenic species of fungi and protozoa [7–9]. The bis-quaternary ammonium salts show the highest antimalarial activities [10].

2. Chemistry

Compound **1** was synthesized in the Diels–Alder reaction. Starting compound was 1,2,3,4,5-pentamethylcyclopentadiene (available from Aldrich), which was heated with maleic anhydride. 4-Hydroxy-1,7,8,9,10-pentamethyl-4-aza-tricyclo [5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione (**2**) was subjected to the reaction with hydroxylamine hydrochloride in water solution.

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Compound **3** was obtained by alkylation of hydroxyimide (**2**) with 2-chloromethyl-oxirane (in the presence of anhydrous potassium carbonate) to give the epoxy ether, and then it was condensed with dimethylamine. Resulting compound was transformed into corresponding quaternary ammonium salt (**4**) by HCl saturated methanol. The general synthetic pathway is given in Fig. 1.

Obtained compounds were purified by flash chromatography or crystallization. Elementary analysis and/or MS, ^{1}H NMR and ^{13}C NMR spectra confirmed the identity of the products. The molecular structure of **3** was confirmed by an X-ray crystallography.

3. Results and discussion

3.1. Chemistry

Based on one-dimensional ^{1}H and ^{13}C NMR spectra, as well as on DEPT, COSY and HSQC correlations, a full assignment of resonance signals in **2**, **3** and **4** has been completed, and the structure of above compounds was determined (NMR data are collected in Table 1).

The molecule **3**, as revealed by an X-ray analysis (Fig. 2 and Table 2), contains the tricyclodecane skeleton having the *anti–endo* configuration. The –C9═C10– bridge and the cyclic imide ring are cisoidally oriented while the substitution at the C1–C5 bond is *cis*. Geometry of the epoxide group is close to mean values found for other alkyl epoxide fragments.

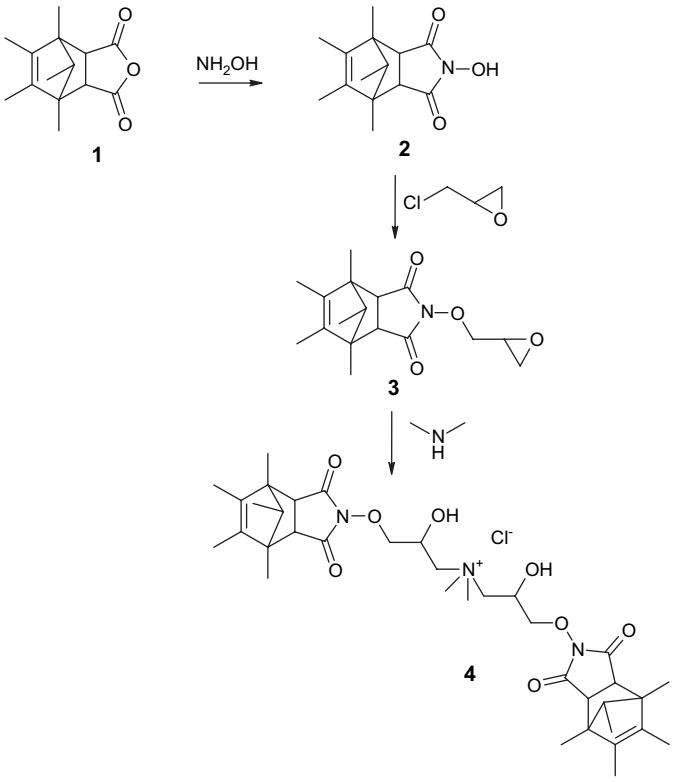

Fig. 1. Synthetic procedure for compounds **1**–**4**.

The *syn–endo* adduct [11] of 1,2,3,4,5-pentamethylcyclopentadiene cycloaddition was present as admixture (0–10%) and in the ^{1}H NMR spectra of **4** it was observed as a low-intensity CH_3CH doublet at 0.74 ppm.

During the synthesis of **4** two additional chiral centers *CHOH are formed, therefore *RR*, *SR* and *RS* (*SR*) diastereomers may occur. In fact, the NH_3N^+ group appears as three signals both in ^{1}H and in ^{13}C NMR spectra, their intensities equal 1:1:2.

In the chain $OCH_2{}^{*}CH(OH)CH_2$ the molecular asymmetry is manifested by duplication of signals assigned to the chiral and prochiral centers due to local configuration *R* and *S* in analyzed diastereomers. Therefore, the carbon resonances in above chain form pairs of signals, and in the proton spectrum the group $CH_AH_BN^+$ manifests by two overlapping ABC systems, arising from $CHCH_2N^+$ couplings in two chiral forms. The couplings are schematically shown in Fig. 3.

The composition of **4** has been also confirmed by mass spectral (ESI) high resolution measurements. The *m/z* value of molecular signal was obtained with an accuracy of 1.9 ppm.

3.2. Antimicrobial activity

Newly obtained compounds were tested in vitro against a number of bacteria including Gram-positive cocci and Gram-negative rods. The parental compounds **1**–**3** showed no antimicrobial activity. Test results of activity for compound **4** are summarized in Tables 3–5.

Preliminary test by disc-diffusion method showed antimicrobial activity against standard *Staphylococcus* strains, therefore next step was evaluation of compound's MIC values for standard and hospital strains of *Staphylococcus aureus*. Research was carried out over 4 standard strains and 40 hospital strains used for routine antimicrobial media susceptibility testing. Hospital strains were isolated from different biological materials of patients hospitalized in one of the Warsaw Medical School Hospitals. Among them, 20 strains showed methicillin susceptibility (MSSA) and 20 strains showed methicillin resistance (MRSA). Differences between activities of above strains (MSSA and MRSA) can be observed when we apply commonly used antibiotics (Tables 4 and 5).

MIC value of each standard *Staphylococcus* strain was 50 μg mL^{-1}. Eleven of twenty MSSA hospital strains had MIC value of 25 μg mL^{-1}, eight strains had 50 μg mL^{-1} and only one strain had 100 μg mL^{-1}. MRSA strains were more resistant to investigated compound: the MIC value for nine of them was 50 μg mL^{-1}, for six – 100 μg mL^{-1}, and for four – 200 μg mL^{-1}. Only one among investigated strains was more susceptible to the compound reaching MIC value of 25 μg mL^{-1}.

4. Experimental protocol

4.1. Chemistry

Chemicals and solvents were purchased from Sigma–Aldrich. Melting points (uncorr.) were measured in open

Table 1
The assignment of ^{1}H and ^{13}C NMR resonances in **2**–**4**

Atoms	Compound **2** (in $CDCl_3$)		Compound **3** (in $CDCl_3$)		Compound **4** (in D_2O)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
CH_3CH	0.598, d, $^{3}J = 6.4$ Hz	7.462	0.615, d, $^{3}J = 6.4$ Hz	7.165	0.603, d, $^{3}J = 6.4$ Hz	9.263
CH_3*C*H	1.524[a], q, $^{3}J = 6.4$ Hz	64.809	1.538, q	64.526	1.674, q, $J = 6.4$ Hz	67.217
CH_3C_{IV}	1.314, s	14.597	1.339[c], s 1.342[c], s	14.364	1.308, s	16.355
CH_3*C*$_{IV}$	–	57.574	–	57.329[g] 57.347[g]	–	60.426
CH_3C=	1.479, s	11.038	1.524, s	11.015[h] 11.032[h]	1.507, s	13.223
CH_3*C*=	–	133.527	–	133.358	–	136.183
*C*HC=O	2.826, s	50.093	2.854[d], d, $^{3}J = 7.5$ Hz 2.865[d], d, $^{3}J = 7.5$ Hz	49.686	3.100, s	52.662
CH*C*=O	–	173.089[b] 173.588[b]	–	171.517[i] 171.607[i]	–	177.882[l] 177.923[l] 177.958[l] 178.000[l]
NO*H*	4.900, br s	–	–	–	–	–
NO*C*H_2	–	–	H_A: 3.849, dd, $^{2}J = 11.2$ Hz, $^{3}J = 6.7$ Hz H_B: 4.075, dd, $^{2}J = 11.2$ Hz, $^{3}J = 3.4$ Hz	–	3.952, m	81.175[m] 81.408[m]
*C*HO	–	–	3.279, m	48.534	4.524, m	66.092[n] 66.277[n]
CH_2O	–	–	H_A[e]: 2.578, dd, $^{2}J = 4.9$ Hz, $^{3}J = 2.6$ Hz H_B[f]: 2.807, dd, $^{2}J = 4.9$ Hz, $^{3}J = 4.2$ Hz	43.744	–	–
N^+*C*H_2	–	–	–	–	H_A: 3.652, dd, $^{2}J = 14.2$ Hz, $^{3}J = 9.2$ Hz H_B: 3.760, dd, $^{2}J = 14.2$ Hz, $^{3}J \approx 1$ Hz $H_{A'}$: 3.669, dd, $^{2}J = 14.2$ Hz, $^{3}J = 9.4$ Hz $H_{B'}$: 3.790, dd, $^{2}J = 14.2$ Hz, $^{3}J \approx 1$ Hz[j]	69.014[o] 69.484[o]
N^+*C*H_3	–	–	–	–	3.293, s[k] 3.302, s[k] 3.326, s[k]	55.866[p] 56.010[p] 55.676[p]

The chemical shift values are given in parts per million (δ), from TMS as a standard.
Abbreviations for signals: s – singlet; br s – broad singlet; d – doublet; q – quartet; m – multiplet.
[a] From COSY, signal masked in proton spectrum.
[b] Differentiation into equal-intense signals.
[c] Differentiation into equal-intense signals.
[d] AB proton system.
[e] *Trans* in respect to the proton CH.
[f] *Cis*, as above.
[g] Differentiation into equal-intense signals.
[h] Differentiation into equal-intense signals.
[i] Differentiation into equal-intense signals.
[j] Ratio of signals $H_A/H_{A'}$ or $H_B/H_{B'}$ 1:1.
[k] Ratio of consecutive signals 1:1:2.
[l] Differentiation into equal-intense signals.
[m] Differentiation into equal-intense signals.
[n] Differentiation into equal-intense signals.
[o] Differentiation into equal-intense signals.
[p] Ratio of consecutive signals 1:1:2.

capillary tubes by the use of Kofler's melting point apparatus. Flash chromatography was performed on Merck silica gel 60 (200–400 mesh). Analytical TLC was carried out on silica gel F_{254} (Merck) plates (0.25 mm thickness).

The NMR spectra were performed on a Bruker DRX 500 Avance instrument at 30 °C operating at 500 MHz for proton. The solvents used were $CDCl_3$ or D_2O for particular compounds, as given in Table 1, and the concentrations were ca. 10 mg mL^{-1}. Double quantum filtered $^{1}H-^{1}H$-correlated spectroscopy (DQFCOSY), heteronuclear $^{1}H-^{13}C$-correlated single quantum coherence (HSQC), and distortionless enhancement by polarization transfer (DEPT) were performed according to standard pulse sequences. The chemical shifts are given in parts per million (δ), from tetramethylsilane (TMS) used as a standard, referred to the same spectrum, and shown in Table 1.

Mass spectral ESI (Electrospray Ionization) measurements were carried out on a Mariner PE Biosystems instrument with TOF detector. Methanol was used as solvent. The spectra were performed in the positive ion mode with a declustering potential 140–300 V.

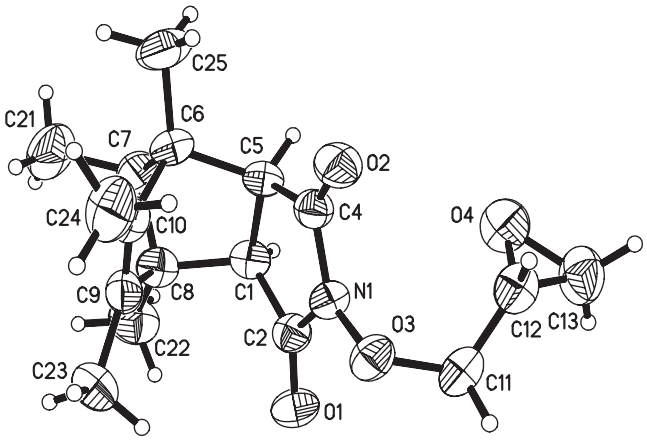

Fig. 2. Molecular structure of **3**.

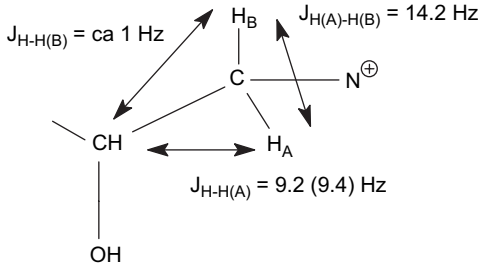

Fig. 3. Couplings within the $CHCH_2N^+$ system in **4**. The coupling constants are equal for both configurations within experimental error except of $J_{H-H(A)}$. Chemical shifts are given in Table 1.

*4.1.1. 1,7,8,9,10-Pentamethyl-4-oxa-tricyclo [5.2.1.0^{2,6}]dec-8-ene-3,5-dione **1***

1,2,3,4,5-Pentamethylcyclopentadiene (0.037 mol, 5.0 g) and maleic anhydride (0.037 mol, 3.61 g) were heated for 2 h with 20 mL of benzene, which was then removed on rotary evaporator. The residue was crystallized from heptane to give 8 g of compound **1**, which was identical with that obtained by another procedure [12,13]. Yield 81%. M.p. 132 °C. Anal. Calcd for $C_{14}H_{18}O_3$ (234.29): C, 71.77; H, 7.74. Found: C, 71.76; H, 7.63.

*4.1.2. 4-Hydroxy-1,7,8,9,10-pentamethyl-4-aza-tricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5-dione **2***

Compound **2**, was described previously [14], was obtained by heating compound **1** (0.021 mol, 5 g) with aqueous solution of hydroxylamine hydrochloride and by dissolving sodium carbonate (0.2 g) in aqueous solution of hydroxylamine hydrochloride (0.2 g in 15 mL of water). Mixture was heated at 60–70 °C for 1 h. Obtained solution was stored overnight in the refrigerator. The collected crystals were washed twice with 10 mL portions of ice-cold 0.5 M HCl. Yield 90%. M.p. 184–185 °C. Anal. Calcd for $C_{14}H_{19}NO_3$ (249.30): C, 67.47; H, 7.63; N, 5.63. Found: C, 67.46; H, 7.63; N, 5.55.

*4.1.3. 1,7,8,9,10-Pentamethyl-4-oxiranylmethoxy-4-aza-tricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5-dione **3***

A mixture of compound **2** (0.002 mol, 0.5 g) and 8 mL of 2-chloromethyl-oxirane was heated in the presence of 0.5 g K_2CO_3 for 50 h. The hot mixture was filtered. The solvent was distilled off, and then the oily residue was purified by column chromatography (chloroform). The compound was crystallized from ethanol. Yield 75%. M.p. 98 °C. Anal. Calcd for $C_{17}H_{23}NO_4$ (305.36): C, 66.86; H, 7.59; N, 4.59. Found: C, 66.81; H, 7.6; N, 4.42.

Crystal data for **3**: $C_{17}H_{23}NO_4$, $M_r = 305.36$, monoclinic, space group $P2_1/c$, $Z = 4$, $F(000) = 656$, $d_{calc} = 1.206$ g cm^{-3}, μ(Cu Kα) = 0.698 mm^{-1}. Unit cell parameters: $a = 11.287(2)$ Å, $b = 9.250(2)$ Å, $c = 16.671(3)$ Å, $\beta = 104.97\ (3)°$, $V = 1681.5(6)$ Å^{-3}.

The crystal with dimensions $0.13 \times 0.32 \times 0.6$ mm was used for data collection on a KM4 diffractometer. A total of 3713 reflections were collected ($\theta_{max} = 80.25°$) of which 3629 were independent ($R_{int} = 0.0357$). The structure was solved by direct methods and refined by full-matrix least squares method on F^2 using the SHELX-97 programs [15]. The structure was refined to final $R_1 = 0.0503$ for 1518 data [$I > 2\sigma(I)$] with 204 parameters, $wR_2 = 0.1983$ for all data,

Table 2
Selected geometric parameters for **3**

Bond lengths (Å)		Valence angles (°)		Torsion angles (°)	
N1–C2	1.380(3)	N1–O3–C11	110.9(2)	C2–C1–C8–C9	47.7(3)
N1–C4	1.381(3)	O4–C13–C12	59.7(2)	C4–C5–C6–C10	−46.9(3)
O1–C2	1.210(3)	C13–O4–C12	61.0(2)	C1–C8–C9–C10	70.9(3)
O2–C4	1.205(3)	O4–C12–C13	59.3(2)	C5–C6–C10–C9	−69.9(3)
N1–O3	1.383(2)	O4–C12–C11	117.2(3)	C2–N1–O3–C11	87.4(3)
O3–C11	1.439(3)			N1–O3–C11–C12	69.1(3)
C11–C12	1.466(4)			C13–O4–C12–C11	−110.6(3)
C12–C13	1.437(4)			C11–C12–C13–O4	105.6(3)
O4–C13	1.413(4)				
O4–C12	1.419(3)				
C1–C2	1.495(3)				
C1–C5	1.542(3)				
C4–C5	1.490(3)				
C9–C10	1.327(4)				

Table 3
Antibacterial and antifungal in vitro activity expressed as diameter of growth inhibitory area for compound **4**

Strain	Diameter of growth inhibitory area (mm)			
	Compound **4**		Ciprofloxacin[a]	Miconazole[b]
	100 μg/disc	400 μg/disc		
S. aureus ATCC 25923	16	20	26	–
S. aureus NCTC 4163	17	24	26	–
S. aureus ATCC 29213	14	24	22	–
S. aureus ATCC 6538P	17	23	28	–
B. subtilis ATCC 6633	25	30	40	–
E. hirae ATCC 10541	14	18	–	–
E. coli ATCC 25922	–	Trace	35	–
E. coli ATCC 10538	–	Trace	34	–
P. aeruginosa NCTC 6749	–	–	26	–
P. aeruginosa ATCC 15442	–	–	26	–
B. bronchiseptica ATCC 4617	13	21	31	–
C. albicans ATCC 10231	–	14	–	20

– Denotes lack of the growth inhibition area.
[a] Ciprofloxacin 5 μg/9 mm disc.
[b] Miconazole 10 μg/9 mm disc.

GOF = 1.007 and residual electron density was max/min = 0.16/−0.19 e Å^{-3}.

Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Center, CCDC No. 643698. Copies of the data may be obtained on application to The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (e-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).

4.1.4. Bis-[2-hydroxy-3-(1,7,8,9,10-pentamethyl-3,5-dioxo-4-aza-tricyclo[5.2.1.0^{2,6}]dec-8-en-4-yloxy)-propyl]-dimethyl-ammonium chloride **4**

A mixture of compound **3** (0.0016 mol, 0.5 g), 0.5 mL of dimethylamine, 10 mL of methanol and 0.5 mL of water was heated for 10 h. The solvent was distilled off, and then the oily residue was purified by column chromatography (chloroform/methanol; 9:1). Dried residue was dissolved in methanol, and then 10 drops of HCl saturated methanol were added. The mixture was kept for 12 h at 6 °C and after that time the solvent was distilled off. Yield 54%. M.p. 101 °C. Anal. Calcd for $C_{36}H_{54}N_3O_8Cl\cdot 2H_2O$ (727.85): C, 59.4; H, 7.98; N, 5.77. Found: C, 59.79; H, 7.7; N, 5.55.

MS ESI: *m/z* value was 656.39177 and the calculated was 656.39054 considering the mass of electron, giving 1.9 ppm error (acceptable 5.0 ppm).

4.2. Microbiology

4.2.1. In vitro evaluation of antimicrobial activity

Following microorganisms were used: (1) Gram-positive bacteria: *S. aureus* ATCC 25923, *S. aureus* NTC 4163, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633, *Enterococcus hirae* ATCC 10541; (2) Gram-negative bacteria: *Escherichia coli* ATCC 25922, *E. coli* ATCC 10538, *Pseudomonas aeruginosa* ATCC 15442, *P. aeruginosa* NCTC 6749, *Bordetella bronchiseptica* ATCC 4617; fungi: *Candida albicans* ATCC 10231. The microorganisms used were obtained from the collection of the Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

4.2.2. Media, growth conditions and antimicrobial activity assays

Antibacterial activity was examined by the disc-diffusion method and the MIC method under standard conditions using Mueller–Hinton II agar medium (Becton Dickinson) and antifungal activities were assessed using YNB-agar medium (Difco), according to the guidelines established by the CLSI [16,17].

For disc-diffusion assay, all tested solutions were aqueous. Sterile filter paper discs (10 mm diameter, Whatman No. 3 chromatography paper) were dripped with test compound solution to load 400 μg and 100 μg of a given compound per disc. The results were read after 24–48 h of incubation at 30 °C for fungi. Results of antibacterial activity were read after 18 h of incubation at 35 °C.

For MICs' determination all investigated solutions were prepared in water. After preparation they were added to liquid

Table 4
Activity of compound **4** against model and hospital strains *S. aureus* susceptive of methicillin (MSSA)

Bacteria strain	Compound **4** MIC (μg mL^{-1})	Ciprofloxacin[a] MIC (μg mL^{-1})	Bacteria strain	Compound **4** MIC (μg mL^{-1})	Ciprofloxacin[a] MIC (μg mL^{-1})
ATCC 25923	50	0.5	38/05	50	0.25
NCTC 4163	50	0.5	40/05	50	0.5
ATCC 29213	50	0.5	42/05	25	2
ATCC 6538P	50	0.5	49/05	25	0.25
2/04	25	>4	60/05	25	0.5
3/04	25	0.25	65/05	25	0.5
4/04	25	0.25	69/05	25	2
6/04	25	0.25	72/05	50	0.25
7/04	25	2	98/06	100	0.25
14/04	25	1	101/06	50	0.25
28/04	50	1	102/06	50	0.5
36/05	50	0.125	112/06	50	0.125

[a] Ciprofloxacin is used as reference drug.

Table 5
Activity of compound **4** against hospital strains *S. aureus* resistance of methicillin (MRSA)

Bacteria strain	Compound **4** MIC ($\mu g\ mL^{-1}$)	Ciprofloxacin[a] MIC ($\mu g\ mL^{-1}$)	Bacteria strain	Compound **4** MIC ($\mu g\ mL^{-1}$)	Ciprofloxacin[a] MIC ($\mu g\ mL^{-1}$)
1/04	50	64	56/05	50	32
5/04	200	16	79/05	200	128
19/04	200	64	80/05	50	16
29/04	200	64	83/05	50	32
46/05	50	64	84/05	50	16
47/05	50	128	85/06	100	16
48/05	100	128	90/06	100	16
53/05	50	128	91/06	100	>128
54/05	50	128	92/06	100	8
55/05	100	128	93/06	25	128

[a] Ciprofloxacin is used as reference drug.

solution of agar medium to form two series of dilutions, in the range 6.25–400 $\mu g\ mL^{-1}$. Next, solidified agar plates were inoculated using 2 μL aliquots. The final inocula of all studied organisms were 10^4 CFU mL^{-1}. Results of antibacterial activity were read after 18 h of incubation at 35 °C.

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