

Assessment of *in vitro* and *in vivo* Antimicrobial Activity of an Isonitrosomalnonitrile Silver(I) Salt

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Abstract

The design and evaluation of antimicrobial activities of an isonitrosomalnonitrile silver(I) salt was reported. This highly stable water-soluble silver salt shows Minimum Inhibitory Concentrations (MIC) values ranging from 0.15 to 5 µg/mL towards both sensitive and resistant Gram-positive and negative bacteria. Furthermore, this silver salt has been investigated for its ability to treat a *S. aureus* infected *Galleria mellonella* larvae animal model with promising results. Thus, our results demonstrated that 80% of the treated larvae survived after 24h with respect to 10% of the untreated ones, respectively.

Keywords: Antimicrobial drug; Isonitrosomalnonitrile silver complex; *Galleria mellonella*; *Staphylococcus aureus*

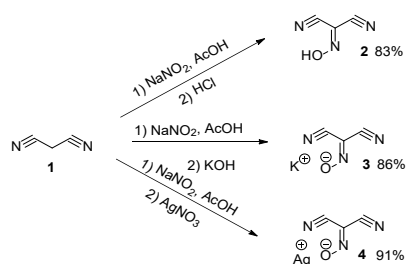
Introduction

Extensive misuse of antibiotics world wide lead to the emergence of antimicrobial resistance as a major threat to healthcare [1]. Thus, numerous scientists reported the end of the antibiotic age [2-4] and the emergence of multidrug-resistant bacteria (superbugs) such as vancomycin-resistant Enterococcus (VRE), *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (MRPA) [5-6]. Furthermore, the development of novel resistance should therefore be a prime activity of research in industry [7]. In this context, silver-based drugs [8-13] constitute an interesting area of research since one of their possible mechanism of action *in vivo* could be denaturing the deoxyribonucleic acid (DNA) molecule by disrupting the hydrogen bonding between the two anti-parallel strands [14]. Indeed, the antimicrobial activity of silver by limiting the growth of bacteria and biofilms [15] is well established [16] and its use applied into many medical products (e.g. catheters, plasters).

Thus, it has been suggested that such a class of derivatives such as carbene-silver acetates could be of interest to become drug candidates [17-18]. Furthermore, the development of more lipophilic benzimidazole-derived carbene-silver complexes presenting potent activities against numerous bacteria were recently reported [19-20]. This paper describes the synthesis and biological antimicrobial evaluation of a new isonitrosomalnonitrile silver salt against both Gram-positive and negative bacteria and demonstrates its *in vivo* efficiency against a *S. aureus* infected *G. mellonella* larvae animal model.

Results and Discussion

The preparation of the isonitrosomalnonitrile silver(I) salt 4 was easily achieved in a two steps synthesis as outlined in scheme 1. A solution of malononitrile 1 in acetic acid reacted with an aqueous solution of sodium nitrite to lead to the formation of the expected isonitrosomalnonitrile 2. This latter can subsequently react with an aqueous solution of silver nitrate to afford the expected isonitrosomalnonitrile silver(I) salt 4 as a stable yellow solid in 91% yield (¹³C NMR (63 MHz, CD₃OD) δ (ppm): 118.44, 112.50, 107.39). In parallel, we prepared the corresponding isonitrosomalnonitrile potassium salt 3 parent derivatives to compare the influence of the nature of the metal used on the potent antimicrobial activities encountered. In this case, the isonitrosomalnonitrile potassium salt was prepared by mixing as previously a solution of sodium nitrite solution in acetic acid in the presence of malononitrile 1 for 1h then quenched by adding 2N hydrochloric acid solution. After extraction, the oily residue can be purified by flash chromatography to afford isonitrosomalnonitrile 2 in 83% yield or treated by a potassium hydroxide solution to lead to the expected isonitrosomalnonitrile potassium salt 3 as yellow powder in 86% yield. This powder can be crystallized by slow cooling of an oversaturated water solution heated at 50°C. These afforded large crystals suitable for X-ray structure analysis.



A suitable yellow single crystal of 3 was carefully selected under a polarizing microscope and deposited on a single loop for single crystal X-ray diffraction analysis (Figure 1).

Atoms are drawn with their respective thermal ellipsoids. A photograph of crystals of 3 is shown in insert. The data were collected on a Bruker X8 APEX2 diffractometer using an Incoatec Ag-K α Microfocus source ($\lambda = 0.56086$ Å) at room temperature. The diffraction intensities were collected until $2\theta = 36.36^\circ$. This value is related to the absence of reflections at high angles which is linked to the crystallinity of the crystals. The intensities were extracted from the frames using SAINT V7 53a (SAINT: Area-Detector Integration Software. Madison: Siemens Industrial Automation, Inc., 1996). A monoclinic unit cell $a = 8.7459$ (4), $b = 8.8229$ (3), $c = 7.1182$ (3) and $\beta = 106.23$ (1) was deduced from all data set. Intensities were semi-empirically corrected from absorption effects using SADABS (SADABS: Area-Detector Absorption Correction. Madison: Siemens Industrial Automation, Inc., 1995). The observed systematic extinction conditions led to use the space group $P2_1/c$. The crystal structure was determined by using

the software SUPERFLIP [21] and further refined by full-matrix least-squares procedures using JANA2006 software [22]. At the end of the refinement, the final R value was 0.0253, obtained for 759 reflections ($I > 3\sigma(I)$). All experimental conditions as well as structure refinement details are given in table 1.

In the obtained structure, there is one independent crystallographic site for potassium and oxygen and three for both carbon and nitrogen. The atomic coordinates, isotropic and anisotropic displacement parameters are listed in table 2.

The lengthening of bond lengths is of 1.136 (4), 1.139 (3) and 1.322 (3) Å for respectively N1-C1, N2-C2 and N3-C3 which correspond perfectly with double and triple C-N bonds. Concerning single C-C distances, there are at 1.426 (4) and 1.425 (3) Å for C1-C3 and C2-C3 in good agreement with expected bond lengths. N3-O1 bond distance is at 1.286(2) Å corresponding to usual N-O distances [23] since isonitrosomalnonitrile molecule is almost planar. C1-C3-N3 angle of $122.0(2)^\circ$ and C1-C3-C2 of $119.5(2)^\circ$. N1-C1-C3 is almost linear ($177.1(3)^\circ$) as expected in case of C-C=C bond. O1-N3-C3 is at $115.8(2)^\circ$ which is a little bit further than common 120° . The potassium is coordinated by eight atoms, five azote and three oxygen with distances ranging from 2.811(2) to 3.022(3) Å. The potassium is capping the molecule from (O1) and nitrogen (N1, N2 and N3). The main distances are gathered in table 3.

The X-ray diffraction analysis of the synthesized powder of 3 confirmed the correlation with the determined crystal structure of isonitrosomalnonitrile potassium salt 3 and the purity of the sample. A Pattern matching performed using the same space group and unit cell parameters shows the perfect matching between both (Figure 2).

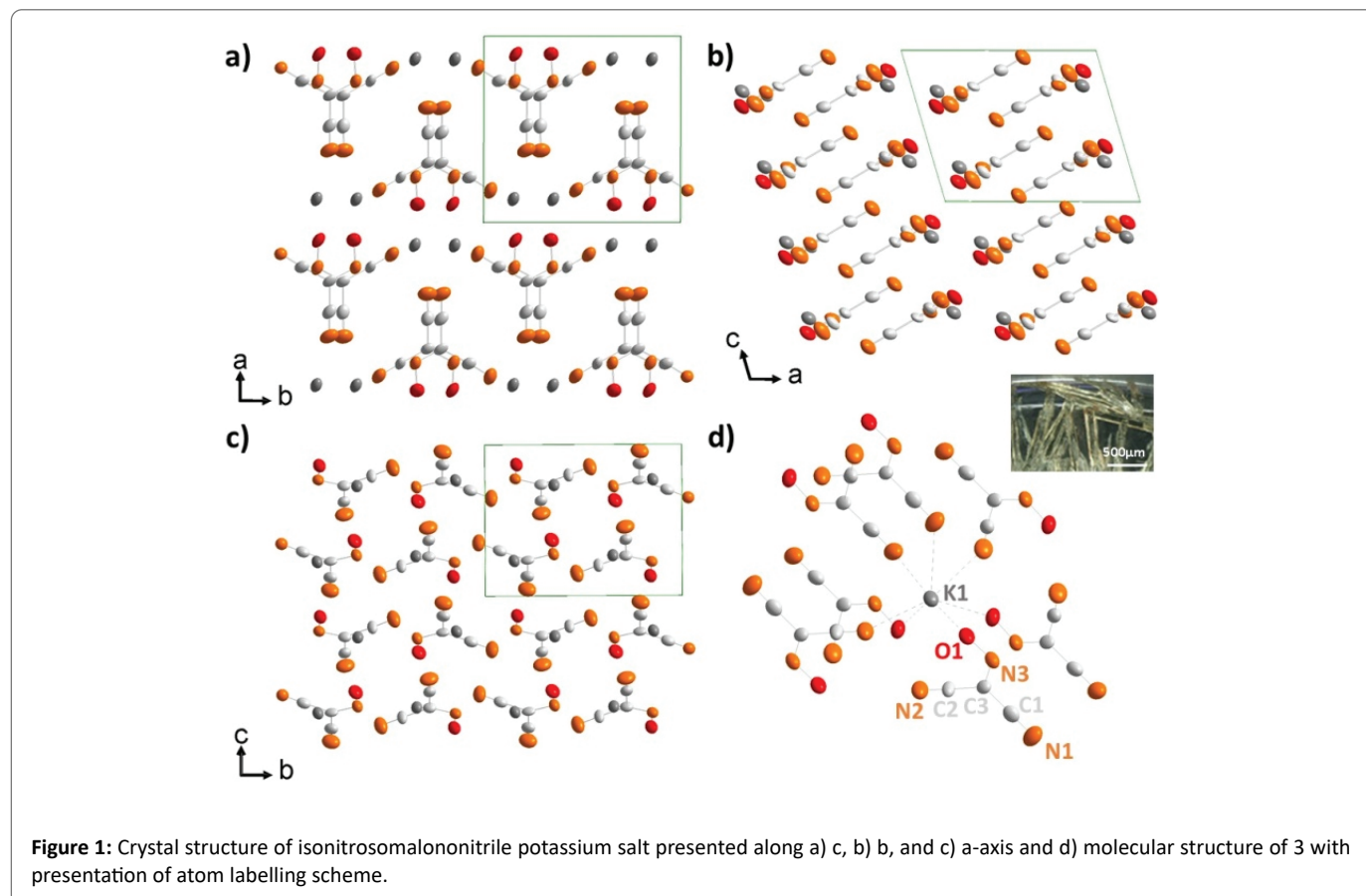
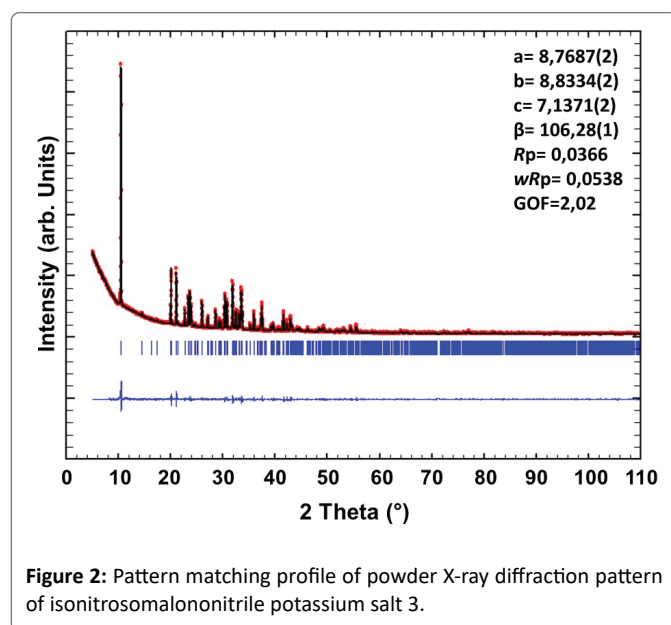


Figure 1: Crystal structure of isonitrosomalnonitrile potassium salt presented along a) c, b) b, and c) a-axis and d) molecular structure of 3 with presentation of atom labelling scheme.

Table 1: Crystal data and structure refinement details for 3.

CCD Number	2004760
Formula	K1C3N3O
Formula weight (g/mol)	133.2
Temperature (K)	300
Wavelength (Å)	0.56086, (Silver microsource)
Crystal system	Monoclinic
Space group	P2 ₁ /c (n°14)
a, b, c, (Å), β(°)	8.7459(4), 8.8229(3), 7.1182(3), 106.23(1)
Volume (Å ³)	527.39(26)
Z	4
Calculated density, (g/cm ³)	1.6775
Crystal size (mm ³)	0.50*0.19*0.21
θ range for data collection (°)	1.91-18.18
Limiting indices	-8 ≤ h ≤ 8, -8 ≤ k ≤ 8, -7 ≤ l ≤ 7
Reflections collected/unique	16460/759 [Rint= 0.1298]
Data/restraints/parameters	759/0/74
GOOF on I	1.00
Final R indices R[>3σ(I)]	R _{all} = 0.0253, wR _{all} = 0.0319
Final R indices for all data	R _{all} = 0.0405, wR _{all} = 0.0351



Now, despite all our attempts, we were unable to obtain crystals corresponding to the powder of isonitrosomalnonitrile silver salt 4 suitable for X-ray analysis. The X-ray powder diffraction pattern is also different from the one of isonitrosomalnonitrile potassium salt 3 which implies that the isonitrosomalnonitrile silver salt 4 is differently arranged, in strong relation with the rather big different size of the respective ionic radii.

The crystals obtained were nevertheless studied by X-ray diffraction. Their analysis shows a monoclinic unit cell with parameters $a=17.315(4)$ Å, $b=3.598(1)$ Å, $c=17.316(4)$ Å and $\beta=110.34(1)^\circ$, S.G. P2₁. The poor quality of the crystals only allowed to propose a hypothetical model of that unknown phase presented on figure 3 which confirmed the differences with the expected the isonitrosomalnonitrile silver salt 4.

All the synthesized water-soluble compounds were then screened for their potent antimicrobial activity against sensitive or resistant Gram-positive and negative bacteria strains as listed in table 4.

First, we must notice that isonitrosomalnonitrile 2 and isonitrosomalnonitrile potassium salt 3 do not possess any activity even at high concentrations against the tested bacterial strains. Nevertheless, isonitrosomalnonitrile silver salt 4 was found to be active against all the microorganisms tested with Minimum Inhibitory Concentrations (MICs) varying from 0.625 to 2.5 µg/mL whatever the considered bacterial strains. Thus, 4 is active in the same range against Gram-positive and negative bacteria and in no case, we have noticed a lack of activity. Thus, the great difference in terms of antimicrobial efficiency between the different tested compounds 1-4 seems to be exclusively related to the presence of a silver atom in the case of derivative 4. Indeed, the absence or replacement of this atom by a potassium atom leads to a loss of activity of the considered compound. Otherwise, since the MICs encountered are very close whatever the considered Gram-negative or Gram-positive bacteria as well as sensitive or resistant it is reasonable to envision that 4 possess a common mechanism of action against these pathogens. Furthermore, similar MICs were observed when targeting MDR bacteria or sensitive ones such as *P. aeruginosa* and PA01 as well as *S. aureus* ATCC25923 and CF Marseille. All these considerations could indicate that 4 possess a mechanism of action which circumvents the mechanisms of resistance involved by the bacteria towards conventional antibiotics.

The cytotoxic activity of isonitrosomalnonitrile silver(I) salt 4 was also assessed by investigating the cell viability. Thus, two different physiological mechanisms involving the ability of mitochondrial dehydrogenases to transform WST-1 to formazan as well as the lysosomal membranes capacity to concentrate Neutral Red. All the results indicated that isonitrosomalnonitrile silver(I) salt 4 has a moderate cytotoxicity with an IC₅₀ around 35 µM with respect to requested concentration necessary against all the bacteria tested. On the other hand, no differences are observed in the IC₅₀ values regarding the nature of the cell line, or the vital dye considered suggesting a global impact on different intracellular targets (Table 5). Moreover, IC₅₀'s in the same range than those obtained for well-known AgNO₃ were encountered opening the way for animal testing's.

Due to their ability to possess an immune system similar to mammals [24] cheap Greater Wax Moth, *Galleria mellonella* [25-26] were widely chosen to evaluate the *in vivo* efficacy and stability of new antimicrobial compounds and determine the virulence of microbial pathogens [27] in 24-48 h [28-29]. A preliminary study was performed by using *Galleria mellonella* larvae as an animal model to determine their tolerance to isonitrosomalnonitrile silver salt 4 as well as its *in vivo* activity against a *S. aureus* infection. Thus, no toxicity up to a concentration of 25 µg/mL (12 µM) was encountered whereas larvae inoculated with a dose of 50µg/mL showed a 90% reduction in viability after 24h. Finally, larvae inoculated with isonitrosomalnonitrile silver salt 4 (3.125, 6.25, 12.5, 25 µg/mL) do not present any signs of acute toxicity such cuticular darkening (Figure 4A).

To ensure to the preliminary *in vivo* activity of isonitrosomalnonitrile silver salt 4, larvae were infected with *S. aureus* ATCC25923 (20 µL of a

Table 2: Atomic positions, isotropic and anisotropic displacement parameters (\AA^3) for 3.

Atom	X	Y	Z	Ueq
K1	0.62374(6)	0.85209(6)	0.23261(8)	0.0353(2)
O1	0.5958(2)	0.1605(2)	0.1294(3)	0.0432(7)
C1	0.7397(3)	0.4258(3)	0.1995(3)	0.0341(9)
C2	0.9826(3)	0.2861(3)	0.3701(4)	0.0361(10)
C3	0.8185(3)	0.2857(3)	0.2627(3)	0.0298(9)
N1	0.6708(3)	0.5342(3)	0.1472(4)	0.0547(10)
N2	0.1137(3)	0.2870(3)	0.4561(3)	0.0542(11)
N3	0.7443(2)	0.1540(2)	0.2242(3)	0.0365(8)

Atom	U11	U22	U33	U12	U13	U23
K1	0.0335(4)	0.0271(4)	0.0411(4)	0.0003(2)	0.0035(2)	0.0012(3)
O1	0.0362(11)	0.0360(12)	0.0490(11)	-0.0052(8)	-0.0020(9)	-0.0003(8)
C1	0.0274(13)	0.0300(17)	0.0425(15)	-0.0084(13)	0.0059(12)	-0.0024(12)
C2	0.0344(16)	0.0417(18)	0.0316(14)	0.0048(12)	0.0084(12)	-0.0005(12)
C3	0.0276(13)	0.0286(16)	0.0301(13)	0.0009(11)	0.0031(11)	0.0020(10)
N1	0.0432(14)	0.0336(17)	0.0774(17)	0.0000(12)	0.0004(12)	0.0030(13)
N2	0.0355(15)	0.067(2)	0.0531(16)	0.0047(12)	0.0015(12)	-0.0046(13)
N3	0.0364(12)	0.0308(14)	0.0395(12)	0.0030(10)	0.0057(10)	0.0013(10)

Table 3: Main bond distances and length in crystal of 3.

Atoms	Bond distance (\AA)	Atoms	Bond angle ($^\circ$)
N1-C1	1.136(4)		120.9(2)
N1-K1	2.924(3)	C1-N1-K1	122.6(2)
O1-K1	2.752(2)	C2-N2-K1	177.1(3)
N2-C2	1.139(3)	N1-C1-C3	179.7(3)
N2-K1	2.974(2)	N2-C2-C3	119.5(2)
C1-C3	1.426(4)	C1-C3-C2	122.0(2)
C2-C3	1.425(3)	C1-C3-N3	118.4(2)
N3-K1	2.872(2)		
N3-C3	1.322(3)		

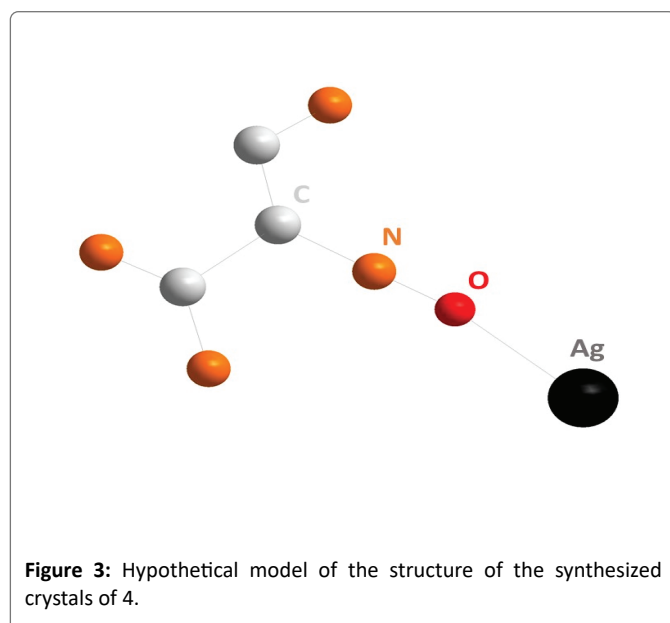


Figure 3: Hypothetical model of the structure of the synthesized crystals of 4.

solution containing 10^7 bacterial cells) and subsequently administered with or without isonitrosomalnonitrile silver salt 4 ($6.25 \mu\text{g/mL}$) 4h post infection. Our results demonstrated that 80% of the treated larvae survived after 24h with respect to 10% of the untreated ones, respectively (Figure 4B). These preliminary but promising data open the way to perform more detailed studies typically for atypical use towards *S. aureus* Skin infections.

Conclusion

The design and evaluation of antimicrobial activities of a water-soluble isonitrosomalnonitrile silver(I) salt with MIC values ranging from 0.15 to $5 \mu\text{g/mL}$ against both sensitive and resistant Gram-positive and negative bacteria was investigated. We have also demonstrated its ability to be used for the treatment of a *S. aureus* infected *Galleria mellonella* larvae animal model with promising results opening the way for a potent therapeutic use. Studies are under progress to evaluate and improve the potentiality of such silver salt derivatives as well as determine more precisely the potent mechanism of action of this new class of antimicrobial agents.

Experimental Section

All solvents were purchased from VWR and used without further purification. Reagents were used as commercially available. ^{13}C NMR spectra were recorded in CDCl_3 on a Bruker AC 300 spectrometer working at 75 MHz. Tetramethylsilane was used as internal standard. All chemical shifts are given in ppm.

Synthesis of isonitrosomalnonitrile 2

7g of sodium nitrite (101 mmol) were slowly added at 0°C to 4 g of malononitrile (65 mmol) dissolved in water (20 mL) and acetic acid (10 mL). Stirring was maintained at room temperature in the dark overnight then quenched by adding 50 mL of a 2N HCl solution. The solution was then extracted with diethyl ether and dried over Na_2SO_4 .

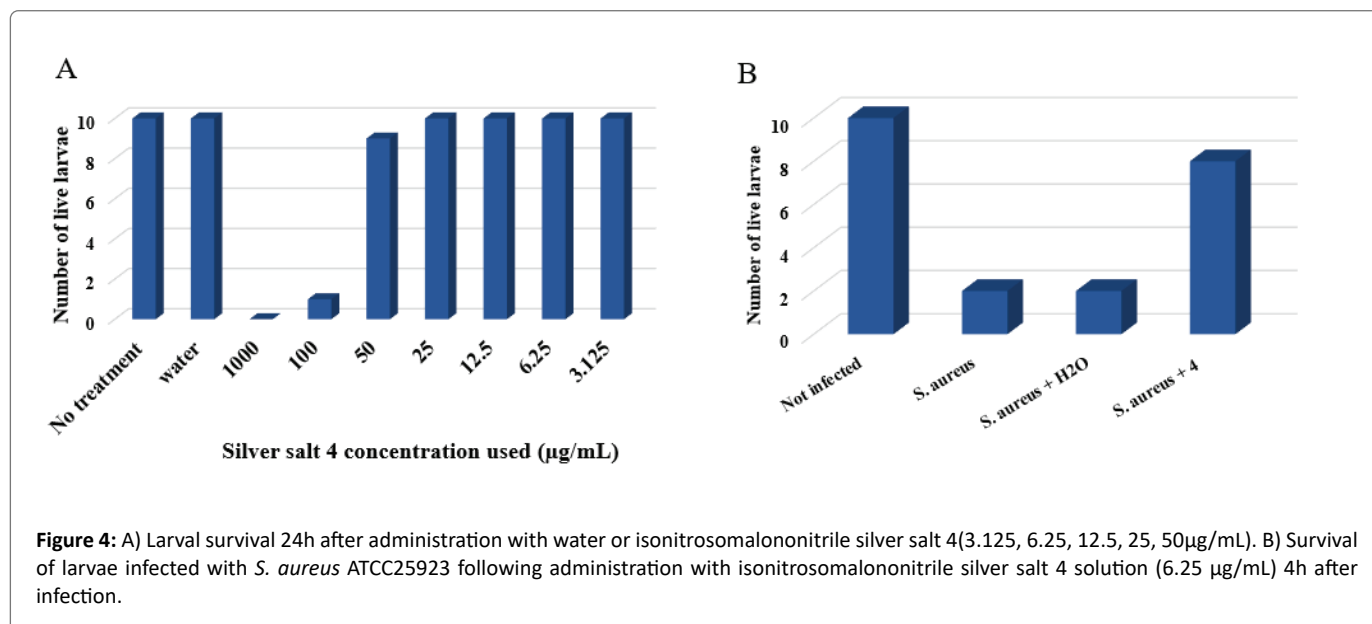


Figure 4: A) Larval survival 24h after administration with water or isonitrosomalnonitrile silver salt 4(3.125, 6.25, 12.5, 25, 50µg/mL). B) Survival of larvae infected with *S. aureus* ATCC25923 following administration with isonitrosomalnonitrile silver salt 4 solution (6.25 µg/mL) 4h after infection.

Table 4: Antibacterial activities of derivatives 2-4.

		MIC (µg/mL)			
		Colistin sulfate	2	3	4
Gram-Positive Bacteria	<i>S. aureus</i> CIP65.8	>32	>256	>256	1.25
	<i>S. aureus</i> ATCC25923	>32	>256	>256	1.25
	<i>S. intermedius</i> 1051997	>32	>256	>256	2.5
	<i>B. cereus</i> ATCC14579	>32	>256	>256	5
	<i>E. faecalis</i> ATCC29212	>32	>256	>256	5
	<i>S. australis</i> sp	>32	>256	>256	0.15
	<i>E. coli</i> ATCC 25922	16	>256	>256	2.5
Gram-Negative Bacteria	<i>E. coli</i> 0157H7	16	>256	>256	1.25
	<i>E. aerogenes</i> EA289(MDR)	16	>256	>256	5
	<i>S. maltophilia</i> ATCC51331	4	>256	>256	1.25
	<i>K. pneumoniae</i> NCTC13443	4	>256	>256	2.5
	<i>K. pneumoniae</i> ST258	8	>256	>256	2.5
	<i>K. pneumoniae</i> NASEY	8	>256	>256	2.5
	<i>P. aeruginosa</i> PA01	2	>256	>256	2.5
	<i>P. aeruginosa</i> PA14	4	>256	>256	1.25
	<i>P. aeruginosa</i> ATCC27853	4	>256	>256	1.25
	<i>P. aeruginosa</i> PA3337	4	>256	>256	1.25
<i>A. baumannii</i> ATCC17978	0.5	>256	>256	2.5	
<i>A. baumannii</i> AYE	4	>256	>256	2.5	

After filtration, the solvents are removed under vacuum to give an oily residue which was purified by flash chromatography on silica gel using petroleum ether/ethylacetate as eluent. The product 2 is obtained as a pale-yellow oil in 83% yield.

¹H NMR (300 MHz, CD₃OD) δ (ppm): 8.89 (s, 1H). ¹³C NMR (63 MHz, CD₃OD) δ (ppm): 113.4, 112.1, 107.1. Anal. For C₃H₃N₃O (95.0) Calcd: C 37.91, H 1.05, N 44.1. Found: C 37.90, H 1.15, N 43.98. MS (ESI) C₃H₃N₃O m/z: 96.012 (100%, (M + H+)).

Synthesis of isonitrosomalnonitrile potassium salt 3

7g of sodium nitrite (101 mmol) were slowly added at 0°C to 4 g of malononitrile (65 mmol) dissolved in water (20 mL) and acetic acid (10 mL). Stirring was maintained at room temperature in the dark overnight then quenched by adding at 0°C 50 mL of a KOH (4g, 71 mmol) methanolic solution. The reaction mixture is stirred at 0°C for 1h then excess of diethyl ether was added to afford the potassium salt precipitation. The precipitate was collected by filtration, and then washed with diethyl ether. The yellow solid was then dried at 80°C under vacuum for 4 hours affording the expected compound 3 in 86% yield.

¹³C NMR (63 MHz, CD₃OD) δ (ppm): 119.05, 112.93, 106.92. Anal. For C₃KN₃O (133.2) Calcd: C 27.03, N 31.53. Found: C 27.36, N 31.73.

Synthesis of isonitrosomalnonitrile silver salt 4

A solution of malononitrile (10 g, 0.15 mol) was dissolved in 60 mL of acetic acid and 60 mL of water and cooled at 0°C. 2. 11.5 g of sodium nitrite (0.16 mol) dissolved in 100 mL of water was then added drop wise. Stirring was maintained at room temperature in the dark overnight and then 100 mL of a water solution of silver nitrate (23 g, 0.15 mol) was added. The precipitate was collected by filtration, and then washed with water, methanol, and diethyl ether. The yellow solid was dried at 80°C under vacuum for 4 hours affording the expected compound 4 in 91% yield.

¹³C NMR (63 MHz, CD₃OD) δ (ppm): 118.44, 112.50, 107.39. UV (MeOH): 291 (4.06), 407 (1.90). Anal. For C₃AgN₃O (201.9) Calcd: C17.85, N 20.81. Found: C 17.46, N 20.63.

Table 5: Cytotoxic activities of isonitrosomalnonitrile silver(I) salt 4 and AgNO₃

Cpd	IC ₅₀ (μM) as assessed by WST1 vital dye				IC ₅₀ (μM) as assessed by neutral red vital dye			
	CHO	HEPG2	MDCK	3T3	CHO	HEPG2	MDCK	3T3
4	30.1 ± 2.2	31.3 ± 2.6	34.3 ± 1.6	37.4 ± 3.8	35.3 ± 2.4	39.8 ± 3.4	39.4 ± 6.5	32.6 ± 0.8
AgNO₃	35.1 ± 1.2	31.4 ± 1.6	36.2 ± 1.4	32.4 ± 3.8	35.4 ± 1.4	33.8 ± 6.4	38.8 ± 6.6	34.5 ± 1.1

IC₅₀ Inhibitory Concentration 50% (μM)

Inoculation of *Galleria mellonella* larvae

Ten larvae of *G. mellonella* (purchased at Animalis center) were stored in the dark at 15°C. Larvae of the same age and weighing around 0.2 g were inoculated with 20 μL of water containing 510⁷ *S. aureus* cells through the last pro-leg using a Myjector U100 insulin syringe (Terumo Europe, Leuven, Belgium).

In vivo toxicity assay

Larvae were injected with 20 μL of isonitrosomalnonitrile silver salt 4 solution (31.25, 62.5, 125, 250, 500 or 1000 μM) or water. Larvae were incubated at 30°C for 24 h prior to quantifying survival.

Effect of isonitrosomalnonitrile silver salt 4 on survival of larvae infected with *S. aureus*

Larvae were injected with 20 μL of isonitrosomalnonitrile silver salt 4 solution (62.5 μM) 4 h post-inoculation with *S. aureus*. The control consisted of larvae inoculated with *S. aureus* treated or not with water (20 μL). Larvae were incubated at 30°C and survival was assessed at 24 h.

Determination of minimal inhibitory concentrations

Antimicrobial activity of the compounds was studied by determination of minimal inhibitory concentrations (MIC) according to the NCCLS guidelines M7-A2 using the microbroth dilution methods. The bacteria strains were grown on trypticase soy agar (Becton Dickinson) at 37°C for 24h. Inocula were prepared in TCE (tryptone 0.1%, NaCl 8%, wt/vol) by adjusting the turbidity at 623 nm to obtain 1-3 10⁵ CFU/mL.

Antimicrobial activities of the compounds were determined by using a broth microdilution method performed in sterile 96-well microplates. All compounds were solubilized in water at a concentration of 10 mM and were transferred to each microplate well in order to obtain a two-fold serial dilution in 100 μL of broth and 100 μL of inoculum containing 2-6 10⁵ CFU of each bacterium were added to each well. Some wells were reserved for positive controls and inoculum viability. After 24 h incubation, MIC was defined for each agent from duplicate observations as the lowest concentration of compound allowing no visible growth.

Cytotoxicity assays

The cytotoxic activities of 4 were assessed on 5 cell lines provided from ATCC-LGC Standards Sarl (Molsheim, France): Chinese hamster Ovary cells (CHO-K1), Hepatocellular carcinoma cells (HepG2), Canine kidney cells (MDCK), primary mouse embryonic fibroblasts (3T3), and immortalized human keratinocytes (Hacat). Cells were maintained in Mc Coy's 5A (CHO) or DMEM (HEPG2, MDCK, 3T3, Hacat) media supplemented with 10% bovine calf serum, 2 mM glutamine, and 100 U.mL⁻¹/10 μg.mL⁻¹ penicillin/streptomycin

mixtures. For the cytotoxicity experiments, they were seeded in 96-well plates and incubated at 37°C in humidified atmosphere containing 5% CO₂ overnight, and then concentrations of compounds were incorporated in triplicate cultures. After a 24-hours incubation period at 37°C, cells were submitted to three successive washes in phosphate buffer saline (PBS) and cell viability was evaluated by two different vital dyes:

- A first set of cell cultures was incubated in PBS containing 10% WST-1 for 30 min at 37°C, 5% CO₂. Cell viability was evaluated by the assessment of WST-1 absorbance at 450 nm in a microplate spectrophotometer.

- A second set of cell cultures was placed into Neutral Red medium (50 μg/ml Neutral Red in complete medium) and incubated for 3 hours at 37°C, 5% CO₂. Then the Neutral Red medium was removed and the distaining solution (50% ethanol, 1% acetic acid, 49% distilled water; 50 μL per well) was added into the wells. The plates were shaken for 15-20 min at room temperature in the dark. Cell viability was evaluated by the assessment of absorbance at 540 nm in a microplate spectrophotometer.

Results were expressed as percentages of cell viability about the control (culture medium-only), which corresponded to 100% cell viability. Dose-response curves were calculated by non-linear regression analysis with Table Curve V2 software. The Inhibitory Concentration 50% (IC₅₀) was defined as the concentration of 4 that induced a 50% decrease of viable cells.

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