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Synthesis, characterization and antibacterial activity of bis-amidrazones and bis-triazolones derivatives

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Abstract: We present a new method for the synthesis of bis-triazolones **3**, which result from the processing of novel bis-amidrazones **2** with ethyl chloroformate. The bis-amidrazones **2** are prepared by the reaction of the N^1-tosylhydrazonates **1** with two equivalents of aliphatic diamine. The structures of the new products were determined through IR, ^{1}H and ^{13}C NMR studies as well as elemental analysis. The molecular structure of the compound **3c** was also determined via an X-ray analysis. The antimicrobial activity of the synthesized compounds was evaluated against a panel of 09 bacterial strains using broth microdilution methods. Results showed that compounds exhibited moderate to strong antibacterial activity against the tested species.

Key-words: N^1-tosylhydrazonates, Bis-Amidrazones, Bis-Triazolones, Antibacterial activity.

Introduction

Much attention has been devoted to the synthesis of bisheterocyclic compounds, which exhibit strong biological activities, including anticancer, antibacterial, anti-tumor, and anti-mycobacterial activities[1-10]. Among such compounds are the bistriazoles derivatives, which also possess significant biological activities[11-15]. In this paper, we propose the synthesis and *in vitro* antibacterial activity evaluation of a series of novel bis-amidrazones **2** and bis-triazolones **3**. The Bis-triazolones **3** were prepared from a novel series of bis-amidrazones **2**, the latter being synthesized from N^1-tosylhydrazonates **1**.

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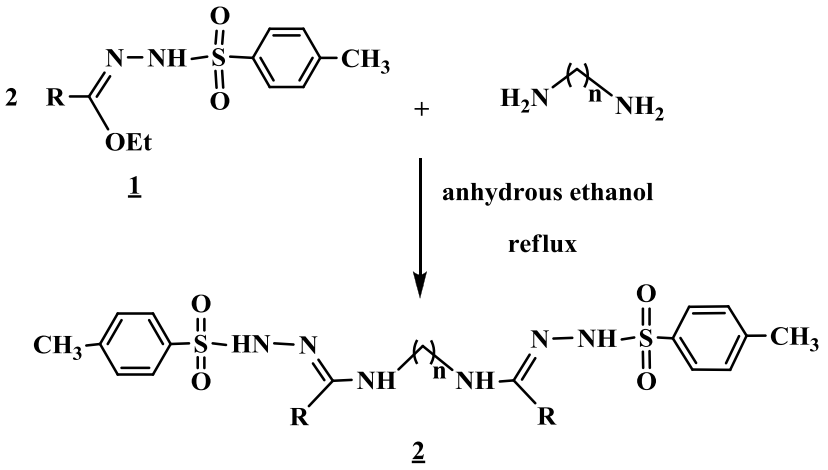
Results and Discussion

The required N^1-tosylhydrazonates **1** were prepared as described in the literature[16]. The N^1-tosylhydrazonates **1** have electrophilic centers and they are very reactive toward reactants having NH_2- moiety such as amines, hydrazide, etc[16-17]. In fact, refluxing in ethanol of two moles of N^1-tosylhydrazonates **1** with one mole of diamine derivatives yields bis-(1-tosylamidrazone) alkanes **2** (Scheme 1). Such a reaction consists of a double nucleophilic attack by the two nitrogen atoms of diamine on the electrophilic center of two moles of N^1-tosylhydrazonate derivatives.

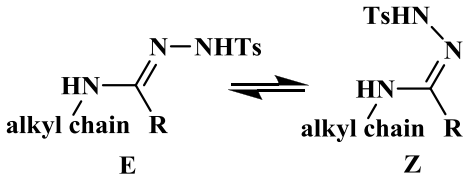


Compound	R	n	Compound	R	n
2a	CH_3	6	**2e**	CH_3	10
2b	Et	6	**2f**	Et	10
2c	CH_3	8	**2g**	CH_3	12
2d	Et	8			

Scheme 1. Synthetic pathway of bis-(1-tosylamidrazone) alkanes **2**.

The structural assignments of the new compounds were based on their elemental analysis and spectral (IR, ^{1}H NMR and ^{13}C NMR) data. The characterisation data of all the new compounds is presented in experimental section.

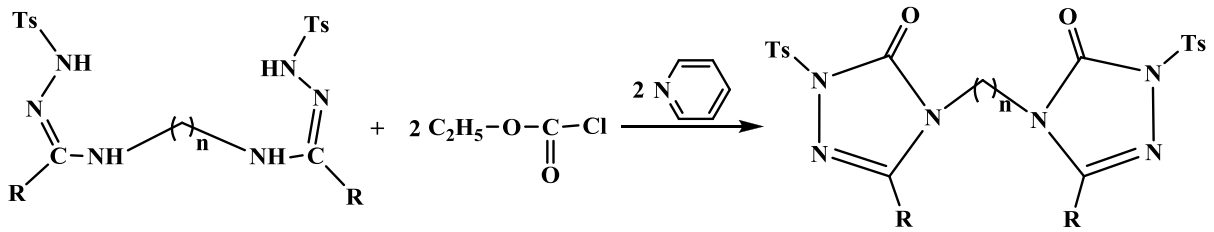
During the spectroscopic characterisation of bis-amidrazones **2**, we noted the doubling of each of the ^{1}H-NMR and ^{13}C-NMR signals, which implies the existence of two isomers in solution (DMSO-d_6), as shown in Scheme 2.



Scheme 2. Isomeric forms

IR spectra of compounds **2** shows absorption bands in the range of 1645-1655 cm^{-1}, which are attributed to the C=N group, whereas amino stretching vibration bands appear in the range of 3100 - 3255 cm^{-1}. The NMR spectrum of **2** shows essentially the absence of the signal of ethoxy group of the parent N^1-tosylhydrazonates **1**, and it shows the presence of the characteristic resonance signals of protons introduced by the diamine derivatives. The ^1H NMR spectrum reveals a singlet in the range of 6.33-6.81 ppm and broad peaks in the range of 8.52-8.75 ppm which are due to the NH protons. The ^{13}C NMR signals for the two –C==N- groups were recorded at about 159 and 164 ppm.

Reaction of bis-amidrazones **2,** in cold conditions, with two molar equivalents of ethyl choloroformate in anhydrous ethanol in the presence of two molar equivalents of pyridine, yields the desired bis-triazolones **3** (Scheme3).



3

Compound	R	n	Compound	R	n
3a	CH_3	6	**3e**	CH_3	10
3b	Et	6	**3f**	Et	10
3c	CH_3	8	**3g**	CH_3	12
3d	Et	8			

Scheme 3. Synthetic pathway of bis–triazolones **3**

The reaction involves a double nucleophilic attack of the nitrogen of the two amidrazones moiety on the carbonyl center of the chloroformate.

The assignment of selected characteristic IR bands provides significant indications for the formation of bis-triazolone **3**. No absorption was observed in the 3100-3250 cm^{-1} range, providing evidence for absence of the amino group. The appearance of an intense band at around 1735 cm^{-1} shows a (C=O) stretch of triazolone, and the absorption band in the range of 1590-1610 cm^{-1} can be attributed to the vibration of the -C=N group.

The structure of the compounds **3** is confirmed by ^1H and ^{13}C NMR. The NMR spectra shows clearly the symmetry that is present in these compounds. In the ^1H NMR spectra of bis-triazolone **3**, we note in particular the absence, in the ranges of 6.33-6.81 ppm and 8.52-8.75 ppm, of the characteristic signals of the NH groups, which existed in the parent bis-amidrazones **2**. The ^{13}C-NMR signal for the C=O group was observed at around 151 ppm, whereas the signal for the C=N group was observed at values in the range of 147-150 ppm.

The formation of bis-triazolones **3** was further confirmed by X-ray diffraction. The crystal data and parameters relevant to the structure determination and refinement for the bistriazolone **3c** are listed in Table 1.

Table 1. Crystal data and structure refinement for the compound **3c**

Formula : $C_{28}H_{36}N_6O_6S_2$
Formula weight : 608.68
Crystal system : triclinic
Space group : P -1
a = 8.4550(4)
b = 8.4970(4)
c = 11.7070(6)Å
α = 99.991(5)
β = 99.006(4)
γ = 109.721(4)°
V = 758.44(6)Å^3
D_x = 1.333
T = 293(2)K
Crystal size : 0.56 x 0.1x 0.1
Goodness of fit : 1.113
No. of unique data measured = 14248
No. of reflections used = 3398
No. of parameters : 191
2 θ_{max} = 56 with *Mo/ Kα*
R = 0.71073
$(\Delta\varsigma)_{max}$ = 0.317
$(\Delta\sigma)_{min}$ = -0.240
Measurement : Kappa CCD diffractometer

Selected bond lengths and bond angles are listed in Table 2.

The crystals **3c** were obtained by slow evaporation of an ethanol solution; a white block shaped crystal of this compound with approximate dimensions of 0.56 x 0.1 x 0.1 was mounted on a Bruker Smart diffractometer equipped with an Apex CCD area detector. Intensity data were collected using a graphite monochromated Mok α radiation (λ = 0.71073 Å) at 293 K.

The structure was solved by direct Methods (SHELXS 79)[18] and refined by full matrix least squares on F^2 using the program SHELXL 97[19]. The compound is triclinic with the following unit cell dimensions: a = 8.4550(4)Å, b = 8.4970(4)Å, c = 11.7070(6)Å, α = 99.991(5)°, β = 99.006(4)°, γ = 109.721(4)°, space group P1. All hydrogen positions were calculated. Hydrogens attached to carbons were located in their calculated positions (C-H = 0.9700 Å). The final refinements converged at R_1 = 0.0791, wR_2 = 0.1511 with w = 1 / [$\sigma 2(Fo^2)$ + (0.0000P)^2 + 1.2151P],], where P = Max [(Fo^2, o) + 2 Fc^2]/3.

The X-ray crystallographic analysis of the 1,8-bis-(5-methyl-2-tosyl-1,2,4-triazol-3-one-4-yl)octane **3c** revealed that the molecule is centrosymetric. In the crystal structure, the rings are coplanar, in the title compound, the first ring built up by the atoms (C3, C4, C5, C6, C7, C8)

is planar (rms deviation of fitted atoms equal to 0.002). The second ring built up by the atoms (N1, N3, C2, N2, C1,) is less planar (rms deviation of fitted atoms equal to 0.0128), but the plane of the triazolone ring forms greater angles to the molecular plane (N2-C9-C10 angle is 112°).

Table 2. Selected bond lengths (Å) and bond angles (°) for the compound **3c**

Bond	*Distance*	*Bond*	*Distance*	*Bond*	*Distance*
S1- O2	1.418(3)	N2- C1	1.378(4)	C6- C7	1.384(6)
S1- O1	1.417(3)	N2- C9	1.470(5)	C6- C13	1.509(6)
S1- N1	1.684(3)	N3- C2	1.300(5)	C7- C8	1.369(6)
S1- C3	1.743(4)	C2- C14	1.487(5)	C9- C10	1.518(5)
O3- C1	1.208(4)	C3- C4	1.378(6)	C10- C11	1.511(5)
N1- C1	1.397(5)	C3- C8	1.385(5)	C11- C12	1.521(5)
N1- N3	1.405(4)	C4- C5	1.380(6)	C12- C12	1.527(8)
N2- C2	1.374(5)	C5- C6	1.383(6)		
Bond angles	*Deg*	*Bond angles*	*Deg*	*Bond angles*	*Deg*
O2- S1- O1	120.94(2)	C2- N2- C9	128.4(3)	C4- C3- S1	119.8(3)
O2- S1- N1	105.49(2)	C1- N2- C9	122.4(3)	C8- C3- S1	120.2(3)
O1- S1- N1	105.57(2)	C2- N3- N1	103.7(3)	C3- C4- C5	119.3(4)
O2- S1- C3	110.4(2)	O3- C1- N2	129.0(3)	C4- C5- C6	121.5(4)
O1- S1- C3	109.68(2)	O3- C1- N1	128.4(3)	C5- C6- C7	117.9(4)
N1- S1- C3	103.01(2)	N2- C1- N1	102.6(3)	C5- C6- C13	121.0(4)
C1- N1- N3	112.0(3)	N3- C2- N2	112.7(3)	C7- C6- C13	121.0(4)
C1- N1- S1	124.0(2)	N3- C2- C14	123.4(4)	C8- C7- C6	121.4(4)
N3- N1- S1	120.7(2)	N2- C2- C14	123.9(4)	C7- C8- C3	119.7(4)
C2- N2- C1	108.8(3)	C4- C3- C8	120.0(4)		

The molecular structure of the crystal with atom labeling and a view of the packing diagram along the axis are shown in Figures 2 and 3, respectively.

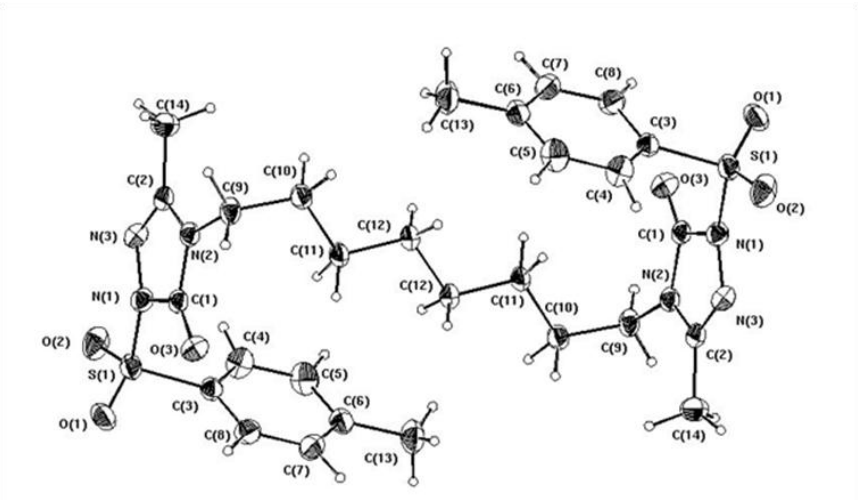


Figure 2. Molecular structure of the compound **3c** with atom numbering

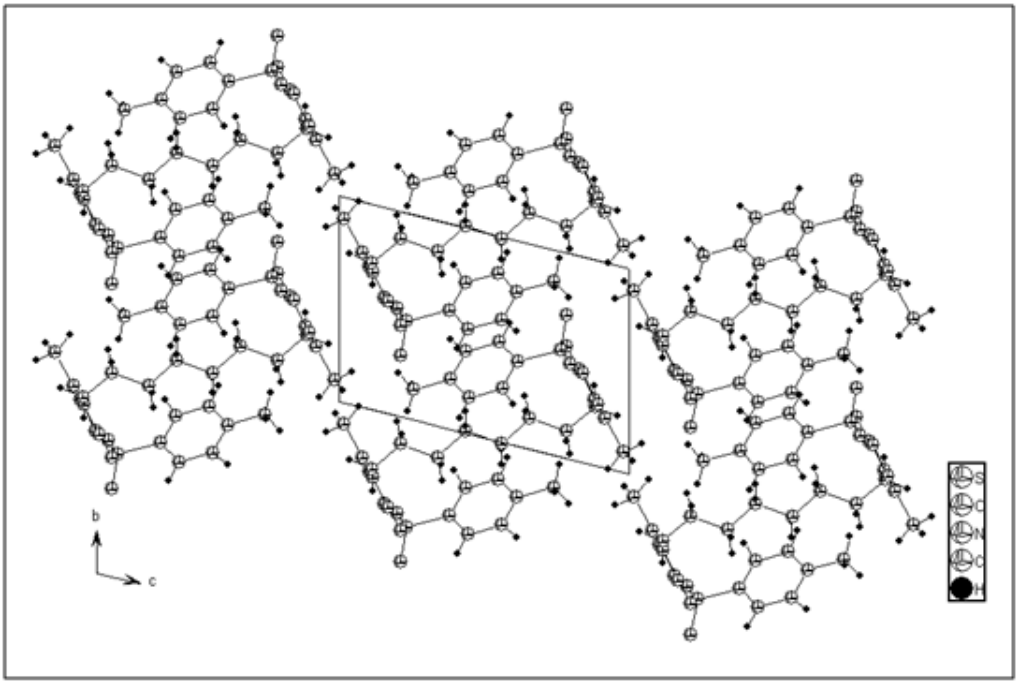


Figure 3. Projection along the axis of the atomic arrangements of compound **3c**

Antibacterial activity

All new compounds were evaluated for their *in vitro* antibacterial activities against human pathogens.

In the present study, we have tested the antibacterial activities against a panel of human pathogenic microorganisms representing different species of different ecosystems and known as opportunists for man and animals and causes food contamination. The *in vitro* antibacterial activities against Gram positive and negative bacteria were determined using broth microdilution method.

As shown in Table 3, all the new compounds possessed moderate antibacterial activities against both Gram-positive and Gram-negative bacteria. The activity of the new compounds depends on their concentration and the tested bacteria.

The MICs values were found for **2a-g** and **3a-g**, ranging from 0.46 to 4.72 mM. A comparative study of the MICs values indicates that the compound **2a** and **2f** are more active than the other bis-(1-tosylamidrazone)alkanes. The compounds **2a** and **2f** exhibited antibacterial activities with MICs values of 0.5 and 1.16 mM respectively, while the antibacterial activities of the others compounds were observed in 2.11 and 4.72 mM. Compound **3g** showed a high bactericidal activity against Gram positive bacteria (MICs: 0.46 and 0.92 mM) compared to the others bis–triazolones (MICs: 2.02 and 4.04 mM).

When examining the effectiveness of the compounds against the Gram positive and negative bacteria, we observed that the Gram positive are more susceptible to the synthesized compounds than Gram negative ones. This effect could be attributed in part to the great complexity of the double membrane-containing cell envelope in Gram negative bacteria compared to the single membrane structure of positive ones[20]. In the current study, the growth of *Enterococcus faecalis* was remarkably inhibited by the synthesized bis–triazolones and

bis-(1-tosylamidrazone)alkanes derivatives. These results showed that the synthesized compounds could be used to minimize problems of drug resistance and protect foods against microbial enterotoxins that cause gastroenteritis[21].

From our observations, we thought that increasing $(CH_2)_n$ unit into the ligand might provide interesting biological properties, like those of **3g** in bis–triazolones compounds. The **R** substitution by CH_3 or Et seemed to be a secondary parameters for antibacterial activity of bis–triazolones and bis-(1-tosylamidrazone)alkanes compounds.

Table 3. Minimum Inhibitory Concentration of the synthesized compounds in mM

Strains[a]	Gram positive bacteria					Gram negative bacteria			
	M. luteus	*L. monocytogenes*	*E. faecalis*	*B. cereus*	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*	*E. coli*	*S. enteritidis*
2a	1.16	2.33	1.16	1.16	2.33	1.16	2.33	2.33	1.16
2b	2.21	2.21	2.21	2.21	4.42	2.21	4.42	2.21	4.42
2c	1.10	2.21	1.10	2.21	4.42	2.21	4.42	2.21	2.21
2d	2.36	2.36	1.18	2.36	4.72	2.36	4.72	2.36	2.36
2e	2.11	2.11	1.05	2.11	2.11	1.05	2.11	2.11	1.05
2f	0.5	2.01	1.00	1.00	4.02	1.00	2.01	1.00	1.00
2g	2.01	2.01	1.00	2.01	0.50	2.01	4.02	2.01	2.01
3a	1.06	2.12	1.06	2.12	4.25	2.12	2.12	2.12	2.12
3b	2.02	1.01	1.01	2.02	4.04	2.02	2.02	2.02	2.02
3c	2.02	2.02	1.01	2.02	4.04	2.02	4.04	2.02	2.02
3d	1.94	1.94	1.94	1.94	3.88	3.88	3.88	1.94	3.88
3e	1.94	1.94	0.97	1.94	3.88	1.94	1.94	3.88	0.97
3f	3.71	1.85	1.85	1.85	3.71	1.85	1.85	1.85	1.85
3g	0.460	0.92	0.46	0.92	0.92	1.85	1.85	1.85	0.92
Positive control									
Gen[d]	2.61	5.22	5.22	2.61	1.30	5.22	5.22	2.61	5.22

Microorganisms[a]: Gram-positive bacteria: *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 1880, *Listeria monocytogenes (*food isolate 2132*)* and Gram-negative bacteria: *Salmonella enteritidis (* food isolate), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 10031.

Gen[d] : MICs of Gentamicin sulfate expressed in µM

Conclusion

This study reports the successful synthesis and characterization of new bis(1-tosylamidrazone-4-yl) alkane derivatives **2a-g** and bis(5-alkyl-2-tosyl-1,2,4-triazol-3-one-4-yl) alkane derivatives **3a-g**. The synthesized compounds were tested for their antibacterial activity. Results showed that the tested compounds exhibited moderate to strong antibacterial activity.

Acknowledgments

We gratefully acknowledge the Ministry of Higher Education, Scientific Research and Technology in Tunisia for financial support.

Experimental Section

Melting points were determined on Electrothermal 9100 apparatus and are uncorrected. IR Spectra were determined from KBr pellets on a JASCO FT-IR-420 spectrometer whose precision is of 2 cm^{-1} covering field 400 – 4000 cm^{-1}. The NMR spectra were recorded in $CDCl_3$ or in DMSO-d_6 on a Bruker Avance spectrometer (300 MHz for ^1H, 75 MHz for ^{13}C). ^1H and ^{13}C chemical shifts are given on the δ scale (ppm) and are referenced to internal TMS. The multiplicities of the signals are indicated by the following abbreviations: s: singlet, d: doublet, t: triplet, q: quadruplet, m: multiplet, br: broad. Elemental analyses were performed at the Service de Microanalyse, Nancy.

The reactions and the purity of substances were monitored by thin layer chromatography (TLC) (elution systems chloroform-ethanol, 9:1) using aluminium sheets with silica gel 60 F_{254} Merck. All reactions, unless otherwise stated, were carried out under nitrogen atmosphere in dry solvents under anhydrous conditions. N^1-tosylhydrazonates **1** were synthesized according to the literature procedures[16]. All other reagents were purchased and used without purification.

Single crystal X-ray diffraction studies were realized on a KPPACCD diffractometer. Solution and refinement: direct methods SHELXS-79[18] for structure solution and SHELXL-97[19] software package for refinement and data output.

General procedure for the synthesis of bis-(1-tosylamidrazone-4-yl)alkanes (2)

A mixture of N^1-tosylhydrazonate **1** (0,002 mol), diamino-alkane (0,001mol) and 30 mL of anhydrous ethanol was refluxed. After reaction completion (4-8 h), as indicated by TLC, the solvent was removed by evaporation and the precipitated product **2** obtained by addition of diethyl ether, was re-crystallised from methanol.

1,6-bis(1-tosylacetamidrazone-4-yl)hexane (2a): Yield: 75%; mp: 208-210 °C; IR (KBr, cm^{-1}): 3101-3235 (NH), 1655 (C=N); ^1H NMR (DMSO-d_6): δ (ppm): 1.02-1.32 (m, 8H), 1.87 (s,6H), 2.26-2.35 (m, 6H), 2.84-3.00(m, 4H), 6.38(s, 2NH), 7.20-7.66(m, 8H), 8.75(br, 2NH); ^{13}C NMR (DMSO-d_6): δ (ppm): (15.6 and 17.2, $2CH_3$), (157.2 and 163.8, 2C=N), (21.4 and 21.5, $2CH_3$(Ts)), (41.2 and 42.5, $2CH_2$-NH), (28.6 and 28.7, $2CH_2$), (26.2 and 26.8, $2CH_2$), C_{arom} 127.3-142.8. *Anal*. Calcd for $C_{24}H_{36}N_6S_2O_4$: C, 53.71; H, 6.76; N, 15.66. Found: C, 53.42; H, 6.87; N, 15.36.

1,6-bis(1-tosylpropioamidrazone-4-yl)hexane (2b): Yield: 78%; mp: 176-178 °C; IR (KBr, cm $^{-1}$): 3100-3221 (NH), 1645 (C=N); ^{1}H NMR (DMSO-d_6): δ (ppm): 0.94(t, 3H, J=7.2 Hz), 1.00-1.32 (m, 7H), 2.18(q, 2H, J= 7.2 Hz), 2.25-2.35(m, 12H), 2.38-3.00(m, 4H), 6.33(s, 2NH), 7.09-7.66(m, 8H), 8.65(br, 2NH); ^{13}C NMR (DMSO-d_6): δ (ppm): (11.8 and 12.0, 2$\underline{C}H_3$-CH_2), (26.1 and 26.3, 2$\underline{C}H_2$-CH_3), (160.9 and 168.8, 2C=N), (21.4 and 21.5, 2CH_3(Ts)), (41.0 and 42.2, 2CH_2-NH), (28.6 and 28.7, 2CH_2), (26.8 and 27.0, 2CH_2), C_{arom} 127.5-142.8. *Anal*. Calcd for $C_{26}H_{40}N_6S_2O_4$: C, 55.29; H, 7.14; N, 14.88. Found: C, 55.04; H, 7.11; N, 14.76.

1,8-bis(1-tosylacetamidrazone-4-yl)octane (2c): Yield: 82%; mp: 180-182°C; IR (KBr, cm $^{-1}$): 3170-3255 (NH), 1651 (C=N); ^{1}H NMR (DMSO-d_6): δ (ppm): 1.17-1.36 (m, 10H), 1.88(s,6H), 2.28-2.36 (m, 8H), 2.87-3.03(m, 4H), 6.39(s, 2NH), 7.10-7.67(m, 8H), 8.70(br, 2NH); ^{13}C NMR (DMSO-d_6): δ (ppm): (15.5 and 16.5, 2CH_3), (157.0 and 163.7, 2C=N), (21.2 and 21.3, 2CH_3(Ts)), (41.0 and 42.3, 2CH_2-NH), (29.1 and 29.2, 2CH_2), (28.5 and 29.0, 2CH_2), (26.3 and 27.0, 2CH_2), C_{arom} 124.7-142.6. *Anal*. Calcd for $C_{26}H_{40}N_6S_2O_4$: C, 55.29; H, 7.14; N, 14.88. Found: C, 55.17; H, 7.09; N, 14.80.

1,8-bis(1-tosylpropioamidrazone-4-yl)octane (2d): Yield: 77%; mp: 152-154 °C; IR (KBr, cm $^{-1}$): 3173-3245 (NH), 1647 (C=N); ^{1}H NMR (DMSO-d_6): δ (ppm): 0.93(t, 3H, J=7.3 Hz), 1.05(t, 3H, J=7.3 Hz), 1.17-1.36(m, 8H), 2.20(q, 2H, J= 7.3 Hz), 2.28-2.36(m, 12H), 2.84-3.02(m, 4H), 6.34(s, 2NH), 7.11-7.68(m, 8H), 8.55(br, 2NH); ^{13}C NMR (DMSO-d_6): δ (ppm): (11.7 and 11.9, 2$\underline{C}H_3$-CH_2), (26.2 and 26.3, 2$\underline{C}H_2$-CH_3), (160.8 and 168.6, 2C=N), (21.2 and 21.3, 2CH_3(Ts)), (40.9 and 42.1, 2CH_2-NH), (29.3 and 29.1, 2CH_2), (28.6 and 29.0, 2CH_2), (26.9 and 27.0, 2CH_2), C_{arom} 124.7-142.6. *Anal*. Calcd. for $C_{28}H_{44}N_6S_2O_4$: C, 56.73; H, 7.48; N, 14.18. Found: C, 56.37; H, 7.48; N, 13.86.

1,10-bis(1-tosylacetamidrazone-4-yl)decane (2e): Yield: 84%; mp: 198-200 °C; IR (KBr, cm $^{-1}$): 3171-3264 (NH), 1646 (C=N); ^{1}H NMR (DMSO-d_6): δ (ppm): 1.18-1.36 (m, 10H), 1.99 (s,6H), 2.1-2.36 (m, 8H), 2.87-3.03 (m, 4H), 3.64-3.71 (m, 4H), 6.35 (s, 2NH), 7.20-7.80 (m, 8H), 8.70 (br, 2NH); ^{13}C NMR (DMSO-d_6): δ (ppm): (15.5 and 16.5, 2CH_3), (154.1 and 163.7, 2C=N), (21.5 and 21.6, 2CH_3(Ts)), (41.9 and 43.0, 2CH_2-NH), (29.0 and 29.2, 2CH_2), (28.5 and 29.0, 2CH_2), (26.3 and 27.0, 2CH_2), (26.1 and 25.9, 2CH_2), C_{arom} 127.5-142.2. *Anal*. Calcd. for $C_{28}H_{44}N_6S_2O_4$: C, 56.73; H, 7.48; N, 14.18. Found: C, 56.49; H, 7.41; N, 13.94.

1,10-bis(1-tosylpropioamidrazone-4-yl)decane (2f): Yield: 87%; mp: 210-212 °C; IR (KBr, cm $^{-1}$): 3171-3268 (NH), 1644 (C=N); ^{1}H NMR (DMSO-d_6): δ (ppm): 0.99 (t, 3H, J=7.5 Hz), 1.04-1.36 (m, 13H), 2.24-2.47 (m, 16H), 2.99 (q, 4H, J=6.9 Hz), 6.81 (s, 2NH), 7.09-7.72 (m, 8H), 8.52 (br, 2NH); ^{13}C NMR (DMSO-d_6): δ (ppm): (11.6 and 11.7, 2$\underline{C}H_3$-CH_2), (28.1 and 28.2, 2$\underline{C}H_2$-CH_3), (153.5 and 164.6, 2C=N), (21.7 and 21.8, 2CH_3(Ts)), (39.7 and 42.9, 2CH_2-NH), (30.1 and 30.3, 2CH_2), (29.3 and 29.6, 2CH_2), (26.7 and 26.8, 2CH_2), (22.6 and 22.7, 2CH_2), C_{arom} 124.7-142.6. *Anal*. Calcd. for $C_{30}H_{48}N_6S_2O_4$: C, 58.03; H, 7.79; N, 13.54. Found: C, 57.96; H, 7.72; N, 13.46.

1,12-bis(1-tosylacetamidrazone-4-yl)dodecane (2g): Yield: 90%; mp: 178-180 °C; IR (KBr, cm $^{-1}$): 3110-3210 (NH), 1646(C=N); ^{1}H NMR (DMSO-d_6): δ (ppm): 1.22-1.34 (m, 12H), 1.86 (s,6H), 2.18-2.35 (m, 14H), 2.87-3.00 (m, 4H), 6.35 (s, 2NH), 7.09-7.66 (m,

8H), 8.65 (br, 2NH); ^{13}C NMR (DMSO-d_6): δ (ppm): (16.6 and 16.7, $2CH_3$), (157.2 and 163.8, 2C=N), (21.4 and 21.5, $2CH_3$(Ts)), (41.1 and 42.5, $2CH_2$-NH), (29.6 and 30.4, $2CH_2$), (29.4 and 29.6, $2CH_2$), (28.7 and 29.4, $2CH_2$), (27.1 and 27.2, $2CH_2$), (26.4 and 26.5, $2CH_2$), C_{arom} 127.3-142.7. *Anal*. Calcd. for $C_{30}H_{48}N_6S_2O_4$: C, 58.03; H, 7.79; N, 13.54. Found: C, 57.89; H, 7.73; N, 13.49.

General procedure of the synthesis of bis-(5-alkyl-2-tosyl-1,2,4-triazol-3-one-4-yl) alkane (3)

To a solution of 0,001 mole of bis-amidrazone **2** and 0,002 mole of pyridine with 20 mL of anhydrous ethanol, stirred magnetically in ice-cold water, was added dropwise a solution of 0,002 mole of ethyl chloroformate within 10 mL of anhydrous ethanol. Once the mixing is completed, the mixture was stirred during 24 hours (TLC monitoring, SiO_2, chloroform/ethanol (9/1)). After evaporation of the solvent, 30 ml of chloroform was added and the mixture was washed three times with 20 mL of distillated water. The chloroform layer separated, dried and the solvent was evaporated to give the resulting compound **3** precipitated soon by the addition of diethyl ether. The resulting compounds were re-crystallized from ethanol.

1,6-bis(5-methyl-2-tosyl-1,2,4-triazol-3-one-4-yl)hexane (3a): Yield: 70%; mp: 190-192 °C; IR (KBr, cm $^{-1}$): 1722 (C=O), 1601 (C=N); ^{1}H NMR ($CDCl_3$): δ (ppm): 1.24 (m, 4H), 1.54 (m, 4H), 2.18 (s, 6H), 2.40 (s, 6H), 3.45 (t, 4H, J=6.9 Hz), 7.31 (d, 4H, J= 7.8 Hz), 7.93 (d, 4H, J= 7.8 Hz); ^{13}C NMR ($CDCl_3$): δ (ppm): (151.3, 2C=O), (147.1, 2C=N), (21.8, $2CH_3$(Ts)), (12.2, $2CH_3$), (41.6, $2CH_2$-N), (28.4, $2CH_2$), (25.8, $2CH_2$), C_{arom} 128.2-145.9. *Anal*. Calcd. for $C_{26}H_{32}N_6O_6S_2$: C, 53.05; H, 5.48; N, 14.28. Found: C, 53.05; H, 5.62; N, 13.99.

1,6-bis (5-ethyl-2-tosyl-1,2,4-triazol-3-one-4-yl)hexane (3b): Yield: 65%; mp: 194-196 °C; IR (KBr, cm $^{-1}$): 1735 (C=O), 1594 (C=N); ^{1}H NMR ($CDCl_3$): δ (ppm): 1.20 (m, 10H), 1.49 (m, 4H), 2.35 (s, 6H), 2.42 (q, 4H, J=7.2 Hz), 3.39 (t, 4H, J=7.2 Hz), 7.27 (d, 4H, J= 8.1 Hz), 7.89 (d, 4H, J= 8.1 Hz); ^{13}C NMR ($CDCl_3$): δ (ppm): (152.0, 2C=O), (151.3, 2C=N), (22.2, $2CH_3$(Ts)), (19.9, $2\underline{C}H_2$-CH_3), (10.2, $2\underline{C}H_3$-CH_2), (41.8, $2CH_3$-N), (28.8, $2CH_2$), (26.2, $2CH_2$), C_{arom} 128.5-146.2. *Anal*. Calcd. for $C_{28}H_{36}N_6O_6S_2$: C, 54.53; H, 5.88; N, 13.63. Found: C, 54.49; H, 5.81, N, 13.42.

1,8-bis (5-methyl-2-tosyl-1,2,4-triazol-3-one-4-yl)octane (3c): Yield: 70%; mp: 162-164 °C; IR (KBr, cm $^{-1}$): 1732 (C=O), 1593 (C=N); ^{1}H NMR ($CDCl_3$): δ (ppm): 1.00 (m, 4H), 1.38 (m, 4H), 2.11 (s, 6H), 2.31 (s, 6H), 2.43 (m, 4H), 3.39 (t, 4H, J=6.8 Hz), 7.39 (d, 4H, J= 7.9 Hz), 7.74 (d, 4H, J= 7.9 Hz); ^{13}C NMR ($CDCl_3$): δ (ppm): (151.5, 2C=O), (149.1, 2C=N), (21.5, $2CH_3$(Ts)), (12.0, $2CH_3$), (41.5, $2CH_2$-N), (28.6, $2CH_2$), (28.0, $2CH_2$), (26.0, $2CH_2$), C_{arom} 127.8-146.2. *Anal*. Calcd for $C_{28}H_{36}N_6O_6S_2$: C, 54.53; H, 5.88; N, 13.63. Found: C, 54.50; H, 5.82; N, 13.59.

1,8-bis (5-ethyl-2-tosyl-1,2,4-triazol-3-one-4-yl)octane (**3d**): Yield: 72%; mp: 174-176 °C; IR (KBr, cm $^{-1}$): 1727 (C=O), 1588 (C=N); ^{1}H NMR ($CDCl_3$): δ (ppm): 1.04-1.15 (m, 14H), 1.49 (m, 4H), 2.38(s, 6H), 2.53(q, 4H, J=7.5 Hz), 3.45(t, 4H, J=7.3 Hz), 7.46(d, 4H, J= 8.2 Hz), 7.82(d, 4H, J= 8.2 Hz); ^{13}C NMR ($CDCl_3$): δ (ppm): (151.6, 2C=O), (151.0, 2C=N), (21.5,

CH_3(Ts)), (19.0, 2$\underline{C}H_2$-CH_3), (9.3, 2$\underline{C}H_3$-CH_2), (41.3, 2CH_2-N), (28.6, 2CH_2), (28.0, 2CH_2), (26.0, 2CH_2), C_{arom} 128.1-146.7. *Anal*. Calcd for $C_{30}H_{40}N_6O_6S_2$: C, 55.88; H, 6.25; N, 13.03. Found: C, 55.61; H, 6.23; N, 12.92.

1,10-bis (5-methyl-2-tosyl-1,2,4-triazol-3-one-4-yl)decane (3e): Yield: 80%; mp: 180-182 °C; IR (KBr, cm $^{-1}$): 1736 (C=O), 1603 (C=N); ^{1}H NMR ($CDCl_3$): δ (ppm): 1.20-1.28 (m, 12H), 1.54 (m, 4H), 2.18 (s, 6H), 2.40 (s, 6H), 3.45 (t, 4H, J=7.2 Hz), 7.31 (d, 4H, J= 8.4 Hz), 7.93 (d, 4H, J= 8.4 Hz); ^{13}C NMR ($CDCl_3$): δ (ppm): (151.3, 2C=O), (147.2, 2C=N), (21.8, 2CH_3(Ts)), (12.2, 2CH_3), (41.9, 2CH_2-N), (29.2, 2CH_2), (28.9, 2CH_2), (28.6, 2CH_2), (26.4, 2CH_2), C_{arom} 128.2-145.9. *Anal*. Calcd for $C_{30}H_{40}N_6O_6S_2$: C, 55.88; H, 6.25; N, 13.03. Found: C, 55.57; H, 6.19; N, 12.82.

1,10-bis (5-ethyl-2-tosyl-1,2,4-triazol-3-one-4-yl)decane (3f): Yield: 70%; mp: 188-190 °C; IR (KBr, cm $^{-1}$): 1735 (C=O), 1600 (C=N); ^{1}H NMR ($CDCl_3$): δ (ppm): 1.08-1.23 (m, 18H), 1.51 (m, 4H), 2.31-2.48 (m, 10H), 3.41 (t, 4H, J=6.5 Hz), 7.26 (d, 4H, J= 7.3 Hz), 7.92 (d, 4H, J= 7.3 Hz); ^{13}C NMR ($CDCl_3$): δ (ppm): (151.6, 2C=O), (150.9, 2C=N), (21.8, 2CH_3(Ts)), (18.2, 2$\underline{C}H_2$-CH_3), (10.9, 2$\underline{C}H_3$-CH_2), (41.9, 2CH_2-N), (29.2, 2CH_2), (28.9, 2CH_2), (28.6, 2CH_2), (26.4, 2CH_2), C_{arom} 128.2-145.6. *Anal*. Calcd for $C_{32}H_{44}N_6O_6S_2$: C, 57.12; H, 6.59; N, 12.49. Found: C, 56.99; H, 6.41; N 12.32.

1,12-bis (5-methyl-2-tosyl-1,2,4-triazol-3-one-4-yl)dodecane (3g): Yield: 80%; mp: 166-168 °C; IR (KBr, cm $^{-1}$): 1732 (C=O), 1605 (C=N); ^{1}H NMR ($CDCl_3$): δ (ppm): 1.14 (m, 16H), 1.50 (m, 4H), 2.14 (s, 6H), 2.36 (s, 6H), 3.41 (m, 4H), 7.26 (d, 4H, J= 6.9 Hz), 7.90(d, 4H, J= 6.9 Hz); ^{13}C NMR ($CDCl_3$): δ (ppm): (150.8, 2C=O), (146.6, 2C=N), (21.3, 2CH_3(Ts)), (11.7, 2CH_3), (41.5, 2CH_2-N), (28.8, 2CH_2), (28.5, 2CH_2), (28.5, 2CH_2), (28.3, 2CH_2), (26.0, 2CH_2), C_{arom} 127.7-145.3. *Anal*. Calcd for $C_{32}H_{44}N_6O_6S_2$: C, 57.12; H, 6.59; N, 12.49. Found: C, 57.11; H, 6.61; N; 12.58.

Antibacterial activity

Microorganisms and growth conditions

Authentic pure cultures of bacteria were obtained from international culture collections (ATCC) and the local culture collection of the Centre of Biotechnology of Sfax, Tunisia. They included Gram-positive bacteria: *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 1880, *Listeria monocytogenes (*food isolate 2132*)* and Gram-negative bacteria: *Salmonella enteritidis* (food isolate), *Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 9027.
The bacterial strains were cultivated in Muller-Hinton agar (MH) (Oxoid Ltd, UK) at 37 °C except for Bacillus which was incubated at 30 °C. Working cultures were prepared by inoculating a loopful of each test bacteria in 3 ml of Muller-Hinton broth (MH) (Oxoid Ltd, UK) and were incubated at 37°C for 12 h. For the test, final inoculum concentrations of 10^6 CFU/ml bacteria were used. DMSO was used as negative control.
Minimum inhibitory concentration measurement

Minimum inhibitory concentrations (MIC) of the synthesized compounds were determined according to Gulluce et al[22] with minor modifications against a panel of 08 microorganisms

representing different species of different ecosystems. The test was performed in sterile 96-well microplates with a final volume in each microplate well of 100 µL. For susceptibility testing, 100 µL of Mueller-Hinton broth was distributed from the second to the twelfth test wells. A stock solution of the synthesized compounds was prepared by dissolving 100 µL of the tested compounds in dimethyl sulfoxide and then adjusted to a final concentration of 50 mg/mL by Mueller-Hinton broth. The first well of the microplate was prepared by dispensing 160 µL of the growth medium and 40 µL of the synthesized compounds to reach a final concentration of 10 mg/mL and then 100 µL of scalar dilutions were transferred from the second to the ninth well. Thereafter and from each well, 10 µL of the suspension were removed and replaced by the bacterial suspensions to final inoculum concentrations of 10^6 CFU/ml for bacteria. The final concentrations of the synthesized compounds adopted to evaluate the antimicrobial activity were 0.039 to 10 mg/mL. The 10th well was considered as positive growth control containing Mueller-Hinton media for bacterial strains, since no of the synthesized compounds was added. The plates were then covered with the sterile plate covers and incubated at 37 °C for 24 h for bacterial strains. The MIC was defined as the lowest concentration of the total essential oil at which the microorganism does not demonstrate visible growth after incubation. As an indicator of microorganism growth, 25 µL of *p*-iodonitrotetrazolium violet (INT) (0.5 mg/mL) dissolved in sterile water were added to the wells and incubated at 37 °C for 30 min. Where microbial growth was inhibited, the solution in the well remained clear after incubation with INT. All experiments were performed in triplicate.

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