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Posted Date: 16 October 2023

doi: 10.20944/preprints202310.0907.v1

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Abstract: Antibiotic resistance is now a first-order health problem, which makes the development of new families of antimicrobials an imperative. These compounds should ideally be inexpensive, readily available, highly active and non-toxic. Here we present the results on the antimicrobial activity of a series of natural and synthetic polyamines with different architectures (linear, tripodal and macrocyclic) and their derivatives containing the oxygen containing aromatic functional groups 1,3-benzodioxol, ortho/para phenol or 2,3-dihydrobenzofuran. The new compounds were prepared through a non-expensive process and their activity was tested against selected strains of yeast, gram-positive and gram-negative bacteria. In all cases, the conjugated derivatives showed antimicrobial activity higher than the unsubstituted polyamines. Several factors, such as the overall charge at physiological pH, the lipophilicity and the topology of the polyamine scaffold were relevant to their activity. The nature of the lipophilic moiety was also determinant on human cell toxicity. The lead compounds showed to be bactericidal and fungistatic, and they were synergic with the commercial antifungals fluconazole, cycloheximide and amphotericin B against the yeast strains tested.

Keywords: polyamine; antimicrobial; bacteria; yeast; synergy; bactericidal; fungistatic

1. Introduction

Antimicrobial resistance (AMR) has become a major global health problem. The estimates made in 2016 painted a grim feature in which AMR would become the leading cause of death, with a likelihood of 10 million deaths per year by 2050 [1]. However, other studies published in 2022 are more pessimistic on the situation, demonstrating that those predictions were probably an underestimation, and that this issue is spreading at a worrying pace: there were close to 5 million deaths associated with bacterial AMR already in 2019 [2]. The classical solution against AMR consists in developing new families of antimicrobials. However, there are several drawbacks for many of the compounds currently under clinical trials such as their high cost of production, related to the complexity of their synthesis, and, in the case of natural products, the scarcity of the sources [3].

 doi:10.20944/preprints202310.0907.v1

Biogenic polyamines, such as putrescine, spermidine, and spermine, are simple molecules with two or more amine groups positively charged at physiological pH. They are widely distributed in all organisms in which they develop multiple cellular functions. Their metabolic synthesis and concentration are highly regulated due to their role in cell proliferation and in apoptotic processes. Polyamines are also involved in several signalling pathways, can act as neuromodulators, and influence the properties of several neurotransmitters pathways involved in mental disorders [4]. The multiple activities developed by polyamines as consequence of their interaction with different biological objectives, support the proposal of polyamine skeleton being a universal template model in which appropriate structural modifications would provide selectivity on specific processes [5,6].

Exogenous polyamines are efficiently absorbed by transport systems, and as endogenous ones, they can also affect different biological targets. In recent decades, polyamine research is an important field for drug development and their potential application as anticancer and antiproliferative agents, agonist/antagonist's receptor ligands, or antiparasitic compounds, between others, has been studied [4]. The activity of novel polyamines against gram-positive and gram-negative bacteria has been analysed by different research groups, which have found promising compounds with antibacterial and/or antibiofilm activity, and sometimes with synergic effects on other known antimicrobial compounds. The positive charges of the amino groups have been considered a key factor in their antimicrobial activity [7,8,9,10,11]. Conjugated polyamines with lipophilic organic molecules have also been found effective in this sense; in these cases, the structure and characteristics of the organic moiety plays an important role in the antimicrobial activities [12,13,14,15,16].

1,3-Benzodioxole is a naturally occurring organic group found in many plant-based food products, such as vanilla (piperonal), black pepper (piperine) and cinnamon (safrole), among others. More importantly, its aldehyde (2H-1,3-benzodioxole-5-carbaldehyde) is mass produced, cheap and readily available since it is widely used by the industry of artificial flavours and perfumes. 1,3-Benzodioxole is also exploited in the pharmaceutical industry and it can be found as a component of many bioactive molecules. Among the pharmaceuticals bearing this unit there are examples in the literature such as anticancer drugs [17], HIV treatments [18], antiparkinsonian agents [19], anti-inflammatory or analgesic [20,21] and anticonvulsant remedies [22]. It is also present in a natural pesticide, with activity both as algicide and as herbicide [23]. Another group of bioactive compounds bearing this moiety is represented by psychoactive and stimulant drugs [24, 25]. There are also examples of derivatives that show antimicrobial activity [26,27,28,29,30].

In this context, with the purpose of developing new antimicrobial compounds, we set out to study the activities of various polyamines containing the 1,3-benzodioxole moiety. The polyamine core varied in number of amine groups and in its topology (linear, tripodal, and macrocyclic). Therefore, we determined the antimicrobial activity against two different types of bacteria (*E. coli* and *S. aureus*) and yeast. The possible synergies between them and commercial antimicrobials and their toxicity against a human cell line were also determined. These results allowed us to conclude that the topology of the polyamine scaffold was a determinant factor on the activity. We then extended the study to three other derivatives in which we conjugated the most promising polyamine macrocyclic core with three alternative oxygen-containing aromatic groups. It is also worth mentioning that all these compounds were prepared in good yields through a simple one-pot synthetic method, which is robust and easily scalable.

2. Results and discussion

2.1. Synthesis

The structures of the first polyamines used in this work are shown in Figure 1. **1b**-**3b** were commercially purchased. The synthesis of **4b** and **5b** has been optimized in our laboratory and are systematically prepared [31,32,33,34]. The conjugated derivatives **1a**-**5a** were obtained in the multigram scale in one-pot reactions and under mild conditions.

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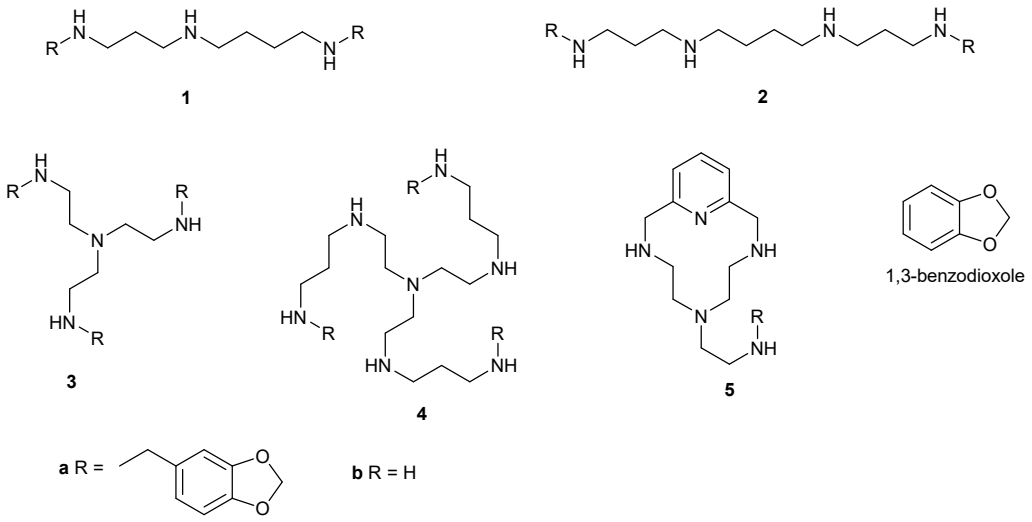
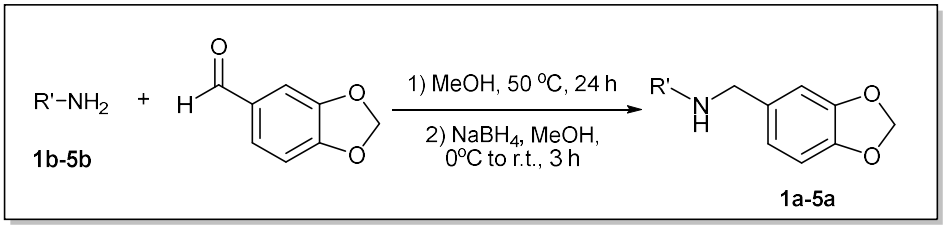


Figure 1. Structures of the compounds studied in this work.

For the conjugation of the aromatic moiety, selective condensation between 3,4-methylenedioxybenzaldehyde (commonly known as piperonal or heliotropin) and the primary amino groups of the corresponding polyamine (**1b**-**5b**) in the proper stoichiometry was carried out. Then, the intermediate imine was reduced *in situ* with $NaBH_4$ (Scheme 1). The purification to obtain spectroscopically pure compounds was easily achieved by simple precipitation and recrystallization of the hydrochloride salts.



Scheme 1. Synthetic route of the compounds **1a-5a**.

To our knowledge, the synthesis of compounds **1a-5a** is reported here for the first time, except for **3a**, which was described before by Sumoto's group [26], but following a different procedure to the one reported here. The ^1H spectra of the new compounds, as well as the X-ray diffraction structure of compound **3a**, can be found in the Electronic Supplementary Information.

2.2. Basicity of the receptors

The capacity of a given compound to interact with its biomolecular target depends heavily on factors such as the overall charge of the molecule and the number of hydrogen bond donor/acceptor atoms. Since the polyamines presented here are polyprotic systems, it is fundamental to understand what species are present in solution at the experimental pH values. For this reason, potentiometric titrations in water were carried out.

The stepwise protonation constants obtained are shown in Table 1. As expected, in the studied pH range ($2.5 < pH < 10.5$) the compounds display as many protonation constants as secondary amino groups there are in each molecule. The protonation reactions of the central tertiary nitrogen atoms (in the case of 3a, 4a and 5a) or the pyridine nitrogen (in 5a) were not detected since they tend to happen at a pH that falls outside the lower range of the technique. This was expected in account of the known lower basicity of tertiary amines in water and the electrostatic repulsion of the surrounding ammonium groups [35].

Table 1. Stepwise and cumulative protonation constants of the 1,3-benzodioxole derivatives.[a]

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Reaction[b]	1a	2a	3a	4a	5a
$L + H \rightleftarrows HL$	10.433(2)	10.81(3)	9.12(2)	10.95(1)	10.48(1)
$LH + H \rightleftarrows H_2L$	8.964(2)	9.481(8)	8.35(3)	9.63(1)	8.78(1)
$H_2L + H \rightleftarrows H_3L$	7.699(2)	8.245(7)	6.93(3)	9.12(1)	7.30(1)
$H_3L + H \rightleftarrows H_4L$		7.41(1)		8.07(1)	
$H_4L + H \rightleftarrows H_5L$				7.48(1)	
$H_5L + H \rightleftarrows H_6L$				6.35(2)	
log β [c]	**27.09**	**35.94**	**24.29**	**51.60**	**26.56**
n[d]	**2.7**	**3.4**	**2.1**	**4.5**	**2.4**

[a] Logarithms of the stepwise (K_{HjL}) and cumulative (β) protonation constants are reported. Values were determined at 298 K in 0.15 mol/dm^3 NaCl. Numbers in parentheses are the estimated errors in the last significant figure. [b] Charges have been omitted. [c] Calculated as $\log \beta = \sum_j \log K_{HjL}$. [d] Average number of positive charges at pH 7.4.

As it could be anticipated, the overall basicity correlates with the number of secondary amino groups, with **4a** having the higher value, in account of its six amino groups. Compounds **1a**, **3a** and **5a**, with three secondary amino groups in their structures, have similarly low log β values. Whereas **2a**, with four secondary amino groups, show an intermediate basicity. These protonation constants allowed us to build the corresponding distribution diagrams (Figure S3 in the Supplementary Information) and to calculate the percentage of species and the average number of positive charges (**n**) at pH 7.4 (last row in Table 1).

2.3. Determination of the antimicrobial activity

The antimicrobial activity of the polyamines was determined using two different types of microorganisms: bacteria and yeasts. Regarding the former, gram-negative *E. coli* and gram-positive *S. aureus* were considered. In the case of yeasts, we used the laboratory strain BY4741 carrying the YEplac195 plasmid or its derived one YEplac195-PDR5 (named PDR5 from now on). This plasmid allows the overexpression of the gene encoding Pdr5, an ATP-binding cassette (ABC) transporter, which can export toxic substances out of the cell (see [36] and references therein). For comparison purposes, commercial antibacterial (doxycycline, gentamicin, ciprofloxacin and ampicillin) and antifungal (fluconazole, cycloheximide and amphotericin B) agents were also included as control in our analyses. The organic compound 1,3-benzodioxole was also considered with both kinds of microorganisms. To obtain information about their antimicrobial activities, experiments to determine the minimal inhibitory concentration (MIC), the minimal microbicidal concentration (MMC) and possible synergies with the control substances were carried out.

2.3.1. Determination of the minimal inhibitory concentration (MIC)

The results obtained for the MIC are shown in Table 2. The most relevant observation, regarding the antibacterial MIC values, was the important differences between the unsubstituted polyamines **1b-4b** and the ones combined with 1,3-benzodioxole **1a-4a**. Only polyamine **5b** had, in some cases, a similar or better behaviour than the conjugated compounds. This fact, together with the higher MIC value measured for the lowest molecular mass compound 1,3-benzodioxole seems to indicate that the activity in the conjugated derivatives is mainly due to the polyamine moiety. It is also worth to mention that for the biogenic polyamines spermidine (**1b**) and spermine (**2b**), the results obtained agree with the data found in the literature about their antimicrobial activity. According to them and to the results presented herein, spermidine **1b**, and its conjugated derivative **1a**, showed similar or higher MIC values than spermine **2b** and its derivative **2a** against *E. coli* [37], *S. aureus* and the yeast strains. Considering the conjugated polyamines, the best antibacterial activity was found for **4a** and **5a**, followed by **3a** and **2a**. Polyamine **5b** was also very effective against *E. coli*.

Table 2. MIC values obtained for the control and test compounds in the *S. cerevisiae*, *E. coli* and *S. aureus* strains considered in this work.

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Compound	MIC (μg/mL), yeast[a]		MIC (μg/mL), bacteria[a]		ClogD[b]
	BY4741/YEplac195	BY4741/PDR5	*E. coli* JM101	*S. aureus* BHI	pH 7.4
1a	> 400	> 400	50	150	-2.53
1b	> 400	> 400	400	> 400	-7.33
2a	250	250	10	50	-3.92
2b	> 400	> 400	100	400	-8.74
3a	> 400	> 400	25	15	-1.15
3b	> 400	> 400	250	400	-8.02
4a	150	150	5	4	-4.85
4b	> 400	> 400	400	250	-12.72
5a	60	60	5	35	-3.04
5b	110	110	10	200	-5.36
1,3-benzodioxole	>500	>500	>500	>500	+1.6
Fluconazole	10	60	-	-	
Cycloheximide	0.5	0.5	-	-	
Amphotericin B	1	1			
Doxycycline	-	-	1	0.25	
Ampicillin	-	-	2	0.14	
Gentamicin	-	-	0.02	0.04	
Ciprofloxacin	-	-	0.04	0.1	

[a] Average values from at least three independent experiments are shown. Standard deviation was lower than 5% of the average value. [b] Calculator Plugins were used for logD calculation, Marvin 22.15.0, 2022, ChemAxon (https//plugins.calculators.cxn.io/logd).

In yeast, like in bacteria, conjugated compounds **4a** and **5a** and the polyamine **5b** showed the best results. For the commercial drug fluconazole, the susceptibility of the strain overexpressing Pdr5 protein was much higher due to its involvement in the efflux of this drug [38]. However, in the case of the new compounds considered here no differences between the two yeast strains were found, which demonstrates that Pdr5 is not involved in their efflux.

The fact that, in most cases, the antimicrobial activity was much higher in the conjugated polyamines containing 1,3-benzodioxole than in each one of both components seems indicative that their association improves some properties, probably related to the increased lipophilicity provided by the 1,3-benzodioxole fraction. Only the results of polyamine **5b** are comparable (and even better especially in yeasts) to those obtained with conjugated polyamines. This could also be explained by considering that it is the most lipophilic polyamine, as deduced from its ClogD value (see the last column in Table 2, in which ClogD values at physiological pH are indicated).

According to their topology, the studied polyamines can be classified as linear (**1** and **2**), tripodal (**3** and **4**) and macrocyclic (**5**). Besides, regarding the whole charge that they have at physiological conditions (pH = 7.4), we found that compounds **1a**, **3a** and **5a** have between +2.1 and +2.7 charges, **2a** has +3.4, and **4a** has +4.5. In the case of both tripodal compounds (**3a** and **4a**), or both linear ones (**1a** and **2a**), the activity was higher in the derivative with an upper charge. However, the main factor determining the activity seems to be the topology, because comparing the three compounds with similar charge (**1a**, **3a** and **5a**), higher activity is shown (except for *S. aureus*) by the macrocyclic compound **5a** followed by the tripodal one **3a** (see Figure 2). Moreover, it was similar or higher for macrocyclic derivative **5a,** with a whole charge of +2.4, than for to the tripodal **4a** and the linear **2a** with charges of +4.5 and +3.4 respectively.

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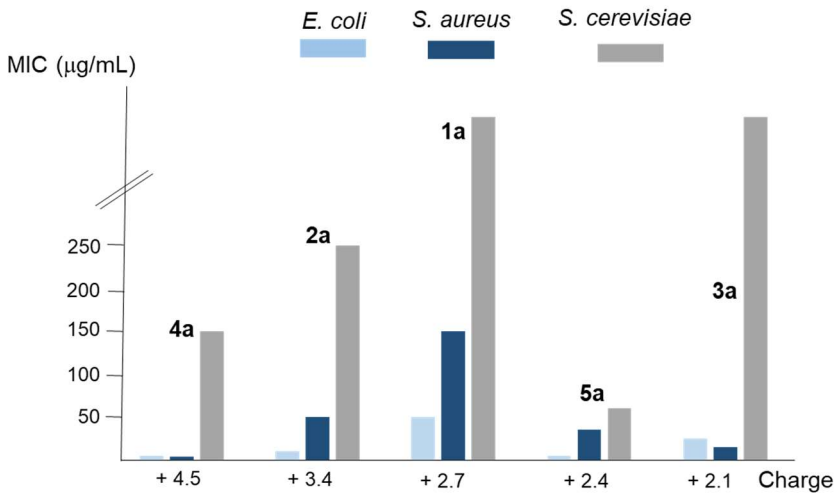

Figure 2. Analysis of the relationship between the average MIC values of the compounds considered in this work for the *E. coli*, *S. aureus* and *S. cerevisiae* strains employed and their whole charge.

Figure 3 represents the MIC value for the conjugated polyamines in each microorganism in relation to its ClogD. Again, the core polyamine structure provides a better explanation about the activity against bacteria and yeast than their lipophilia. For instance, **5a** has a ClogD value between **1a** and **2a** but showed greater antimicrobial activity than both. Besides, the behaviour of **4a** and **5a,** as well as the pure polyamine **5b** (except for *S. aureus*)**,** was similar despite the differences in lipophilia. In summary, the presence of a lipophilic component in the drug seems necessary to guarantee the accessibility to the membrane of the microorganisms, but once this is achieved, the most important factor that determines its activity is the topology of the polyamine.

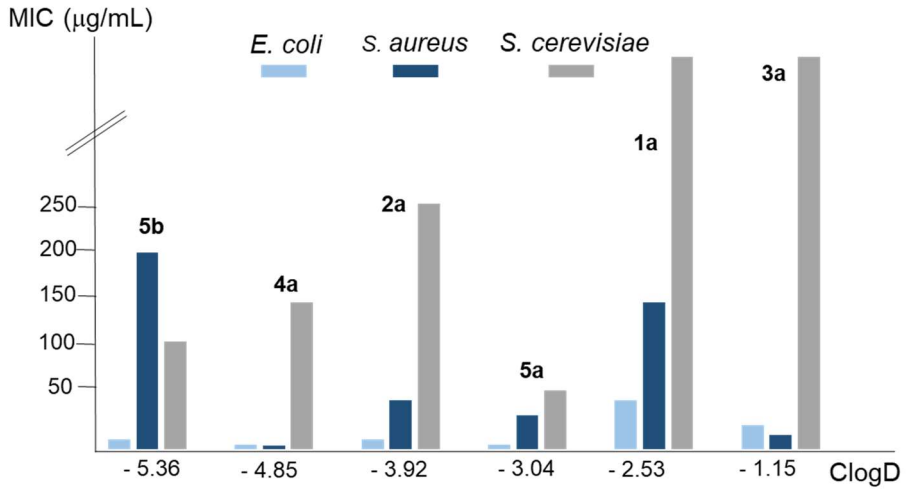

Figure 3. Analysis of the relationship between the average MIC values of the compounds considered in this work for the *E. coli*, *S. aureus* and *S. cerevisiae* strains employed, and their ClogD values.

2.3.1. Determination of the minimal microbicidal (bactericidal/fungicidal) concentration (MBC or MFC)

Determination of MBC/MFC was carried out for those compounds with an outstanding antimicrobial activity. The results revealed that those displaying antibacterial action showed to be bactericide for both *E. coli* and *S. aureus* strains (the MBC values are indicated in Table 3). In the case of *S cerevisiae*, compounds **4a**, **5a** and **5b** showed to be fungistatic: growth was observed after inoculation on SC-ura plates of an aliquot of a sample resulting from the overnight incubation of

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yeast cells with each one of these compounds at a concentration of four-times the MIC value. Representative images of these results are shown in Figure S4 in the Supplementary Material.

Table 3. Minimal bactericidal or fungicidal concentration (MBC, MFC) of the compounds described in this work.

Compound	*S. cerevisiae* strains	*E. coli JM101*	*S. aureus BHI*
1a	n.d.[a]	100[b]	n.d.[a]
2a	n.d.[a]	20[b]	100[b]
3a	n.d.[a]	50[b]	30[b]
4a	Fungistatic	10[b]	8[b]
5a	Fungistatic	10[b]	70[b]
5b	Fungistatic	20[b]	n.d.[a]

[a] Not determined due to the high MIC values previously described. [b] Values (in μg/mL) correspond to the MBC.

2.3.3. Determination of the synergies between compounds and control antifungal and antibacterial substances by the checkerboard titration approach

Synergy effect among different drugs is shown when the overall therapeutic action of their combination is greater than the sum of effects caused by each individual component [39]. Synergistic combinations allow to increase beneficial results, because the use of lower doses of each constituent can reduce side effects and toxicity related with high quantities of single drugs. Combination therapies are widely used for the treatment of the most awful diseases, such as cancer or AIDS [40 and references therein].

To determine the possibility of synergy between the lead compounds and the commercial antimicrobials essayed, each one was tested by the checkerboard titration method [41,42]. For the interpretation of the results, the criteria of the Loewe's additive theory were followed and, for this purpose, the fractional inhibitory concentration index (FICI) was calculated, as described in the Materials and Methods section. In the case of bacteria, the experiments were made between each active polyamine and every one of the commercial antibacterial drugs indicated in Table 1; we only found synergy between **5b** and ciprofloxacin in *E. coli*. Conversely, for yeast strains, positive results were found between compounds **4a** and **5a** and the known antifungal agent amphotericin B, and between **4a, 5a** and **5b** and cycloheximide. Besides, in the BY4741 transformed with the YEplac195 control plasmid, **4a**, **5a** and **5b** showed to be synergic with fluconazole. (Table 4). Figure 4 shows representative images of the results obtained after growth in plate of aliquots corresponding to the most meaningful tubes prepared for the checkerboard titration approach.

Table 4. Synergies between the most active compounds considered in this work and antifungal drugs already described.

BY4741/YEplac195	4a	5a	5b
Fluconazole	+	+	+
Cycloheximide	+	+	+
Amphotericin B	+	+	-
BY4741/PDR5			
Fluconazole	n.d.[a]	n.d.[a]	n.d.[a]
Cycloheximide	+	+	+
Amphotericin B	+	+	-

[a] Not determined due to the high MIC values previously described.

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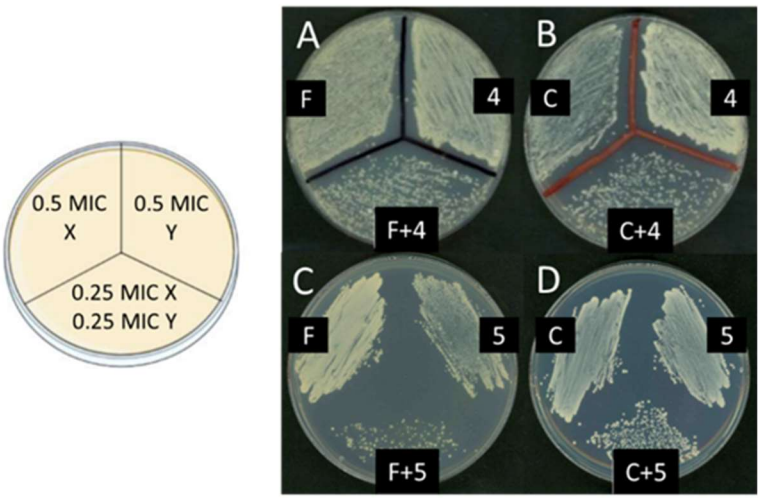

Figure 4. Sinergy between compounds **4a** or **5a,** and fluconazole or cycloheximide in the strain BY4741/YEplac195. On the left, a scheme of the plate used for these experiments is shown. In each sector 30 µL of a ten-fold dilution of each one of the tubes prepared as described in the Materials and Methods section were applied. On the right, a representative image of the resulting growth in plate is shown. (A) Fluconazole and compound **4a**, (B) Cycloheximide and compound **4a**, (C) Fluconazole and compound **5a**, (D) Cycloheximide and compound **5a**.

2.5. Compound cytotoxicity on human cells

Table 5 and Figure S5 in Supplementary Material show the results of the viability assay carried out to determine the cytotoxicity of the conjugated compounds **1a-5a** and the pure polyamine **5b** on the human Jurkat cell line. As can be seen in Table 5, the most polar one, **5b** (ClogD -5.36) showed the lowest cytotoxicity (IC_{50} 1252 µg/mL), while the most lipophilic one, **3a** (ClogD -1.15) was clearly the most cytotoxic (IC_{50}1.86 µg/mL). The remaining compounds showed intermediate cytotoxicity, with two different ranges of values, one for the lineal compounds **1a** and **2a** and the second one for compounds **4a** and **5a**. Figure 5 shows the IC_{50} values (black column) for each compound, together with the corresponding MIC values for the microorganisms considered (light blue, dark blue and grey columns). Considering all these data together, macrocyclic polyamine **5b** is the one that showed the highest therapeutic index in all cases. The conjugated polyamines **4a** and **5a** presented also acceptable values in *E. coli*, in contrast to the other compounds (**1a**, **2a** and **3a**) that turned out to be very toxic to human cells.

Table 5. Cytotoxicity on the human Jurkat cell line of the compounds described in this work, as determined by flow cytometry viability assay with Propidium Iodide. MIC values for each compound in the strains considered are also shown.

Compound/ClogD	IC_{50} (µg/mL)[a]	MIC *E. coli* (µg/mL)	MIC *S. aureus* (µg/mL)	MIC yeast (µg/mL)
1a/-2.53	11.41	50	150	>400
2a/-3.92	8.80	10	50	250
3a/-1.15	1.86	25	15	>400
4a/-4.85	76.86	5	4	150
5a/-3.04	37.84	5	35	60
5b/-5.36	1252	10	200	110

[a]Data shown corresponds to the average of three independent experiments, being the standard deviation lower than 5% in all cases.

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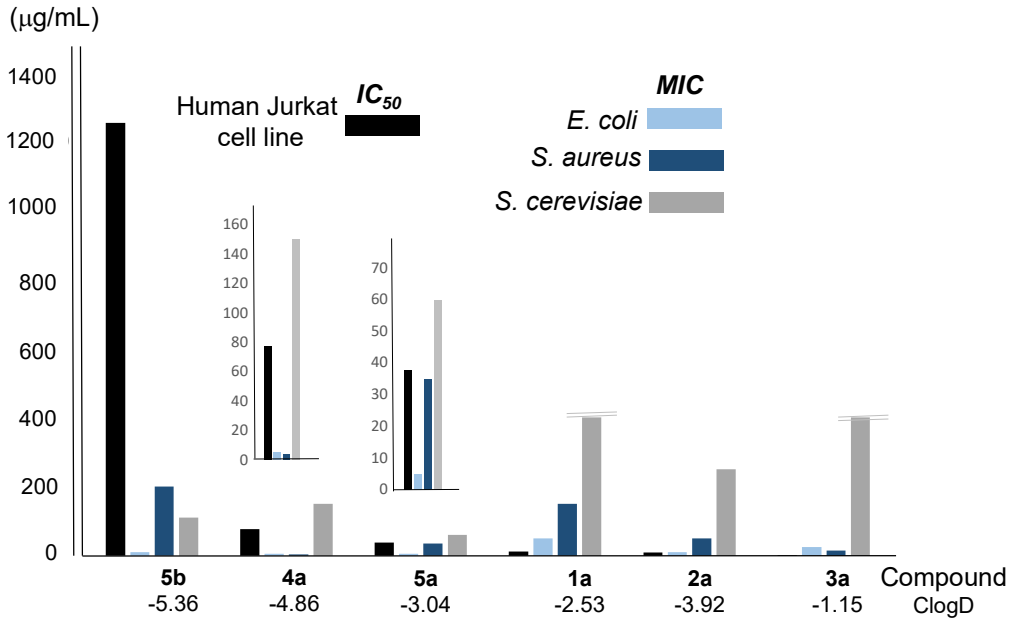

Figure 5. Analysis of the relationship between the average MIC and IC_{50} values of the compounds considered in this work, and their ClogD. Extended plots for **4a** and **5b** are also included to better appreciate the differences.

2.6. Antimicrobial potential of other derived compounds from polyamine 5b

Although the benzodioxole fragment is a component of some drugs, its inhibitory effect on the activity of CYP (Cytochrome P450)-dependent enzymes has been described in mammals and other species [43 and references therein]. For this reason, and in order to find active compounds in which toxicity could be lower than in the conjugated polyamines described herein till now, we extended the study to other 3 polyamines based in the macrocyclic scaffold conjugated with other groups different from benzodioxole, but with similar physicochemical characteristics and properties. Figure 6 shows the structure of these compounds (**6a**, **7a** and **8a**) in which polyamine **5b** was condensed, as is described in Scheme 1, with 2-hydroxybenzaldehyde, 4-hydroxybenzaldehyde and 2,3-dihydrobenzofuran-5-carbaldehyde, respectively. The synthesis of compounds **6a** and **7a** was recently reported by some of us [44], whereas compound **8a** is reported here for the first time.

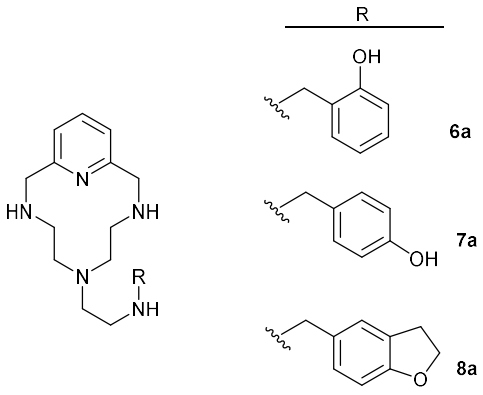

Figure 6. Structures of the conjugated polyamines **6a**, **7a** [44] and **8a**, based on macrocyclic scaffold **5b** considered in this work.

As with the other compounds, the acid-base behaviour of these new derivatives was studied by potentiometric titrations in water. As for **5a** and **5b,** the protonation of the central tertiary nitrogen

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atoms and the pyridine ones was not detected. The percentage of species, and the average number of positive charges, **n**, at pH 7.4 were calculated (Table 6).

Table 6. Stepwise and cumulative protonation constants of polyamines **6a**, **7a** [44] and **8a**[a].

Reaction[(b)]	6a	7a	8a
$L + H \rightleftarrows HL$	9.93(1)	10.153(9)	9.943(3)
$LH + H \rightleftarrows H_2L$	9.11(1)	9.473(9)	8.736(3)
$H_2L + H \rightleftarrows H_3L$	7.92(1)	8.516(8)	7.296(4)
$H_3L + H \rightleftarrows H_4L$	6.67(2)	7.341(9)	
log β [(c)]	**33.72**	**35.48**	**26.17**
n[(d)]	**1.91**	**2.4**	**2.4**

[a] Logarithms of the stepwise (K_{HjL}) and cumulative (β) protonation constants are reported. Values were determined at 298 K in 0.15 mol/dm NaCl. Numbers in parentheses are the estimated errors in the last significant figure. [b] Charges have been omitted. [c] Calculated as $\log \beta = \sum_j \log K_{HjL}$. [d] Average number of positive charges at pH 7.4.

The susceptibility of the microorganisms to these compounds was measured by determining the minimum inhibitory concentration (MIC) and the minimum microbicidal concentration (MMC) (Tables 7 and 8). To assess whether the antimicrobial activity of the tested compounds was relevant, it was compared with the value determined previously for **5a** and **5b**. It can be observed that **6a** exhibits the highest antimicrobial activity for yeast and *E. coli*. In the case of *S. aureus*, it was similar to that for **5a**. On the other hand, **7a** and **8a** only offered results like those of **5a** and **5b** in the case of *E. coli*, and better than **5b** in *S. aureus*.

Table 7. MIC values obtained for polyamines derived from **5b** in the *S. cerevisiae*, *E. coli* and *S. aureus* strains considered in this work.

Compound	MIC (μg/mL), yeast[a]		MIC (μg/mL), bacteria[a]		CLogD[b]
	BY4741/YEplac195	BY4741/PDR5	*E. coli JM101*	*S. aureus BHI*	pH 7.4
5a	60	60	5	35	-3.04
5b	110	110	10	200	-5.36
6a	45	45	2.5	40	-3.36
7a	295	295	13	135	-3.12
8a	400	400	15	100	-3.01

[a] Average values from at least three independent experiments are shown. Standard deviation was lower than 5% of the average value. [b] Calculator Plugins were used for logD calculation, Marvin 22.15.0, 2022, ChemAxon (https//plugins.calculators.cxn.io/logd).

Table 8. Minimal bactericidal or fungicidal concentration (MBC, MFC) of **6a**, **7a** and **8a.**

Compound	*S. cerevisiae* strains	*E. coli JM101*	*S. aureus BHI*
6a	Fungistatic	10[b]	160[b]
7a	n.d.[a]	Bacteriostatic	n.d.[a]
8a	n.d.[a]	60[b]	400[b]

[a] Not determined due to the high MIC values previously described. [b] Values (in μg/mL) correspond to the MBC.

Next, we investigated possible interactions with commercial antimicrobials by the checkerboard assay as described before. The results of these experiments, displayed in **Table 9,** showed no synergy in the case of bacteria with **6a**, but positive results were found with **7a** and gentamicin, and with **8a** and ampicillin, doxycycline and gentamicin in *E. coli*. In the case of yeast strains, **6a** offered positive results with cycloheximide and amphotericin B. Experiments were not carried out in those cases in which the MIC values were very high.

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Table 9. Synergies between polyamines based in the macrocyclic scaffold and commercial antimicrobials.

	6a	7a	8a
BY4741/YEplac195			
Fluconazole	-	n.d.[a]	n.d.[a]
Cycloheximide	+	n.d.[a]	n.d.[a]
Amphotericin	+	n.d.[a]	n.d.[a]
BY4741/PDR5			
Fluconazole	n.d.[a]	n.d.[a]	n.d.[a]
Cycloheximide	+	n.d.[a]	n.d.[a]
Amphotericin	+	n.d.[a]	n.d.[a]
E. coli			
Ampicillin	-	-	+
Ciprofloxacin	-	-	-
Doxycycline	-	-	+
Gentamicin	-	+	+
S. aureus			
Ampicillin	-	n.d.[a]	n.d.[a]
Ciprofloxacin	-	n.d.[a]	n.d.[a]
Doxycycline	-	n.d.[a]	n.d.[a]
Gentamicin	-	n.d.[a]	n.d.[a]

[a] Not determined due to the high MIC values previously described.

These results were confirmed by plating samples corresponding to the incubation of the microorganisms with concentrations corresponding to half of the MIC of every one of the antimicrobials and with a mixture of one-quarter of the MIC of both (data not shown).

To understand the level of safety in human cells, the cytotoxicity of polyamines **6a**, **7a**, and **8a** was determined using the Jurkat cell line of human lymphocytic leukaemia (ATCC TIB-152). The data obtained are shown in Table 10, in Figure 7, and in Figure S5 in the Supplementary Material, which compilates also the results for compounds **5a** and **5b** to clarify.

Table 10. Cytotoxicity on the human Jurkat cell line of the compounds described in this section, as determined by flow cytometry viability assay with propidium iodide. MIC values for each compound in the strains considered are also shown.

Compound/ClogD	IC_{50} (μg/mL)[a]	MIC *E. coli* (μg/mL)	MIC *S. aureus* (μg/mL)	MIC yeast (μg/mL)
5a/-3.04	37.84	5	35	60
5b/-5.36	1252	10	200	110
6a/-3.36	97.95	2.5	40	45
7a/-3.12	950.61	13	135	295
8a/-3.01	127.95	15	100	400

[a] Data shown corresponds to the average of three independent experiments, being the standard deviation lower than 5% in all cases.

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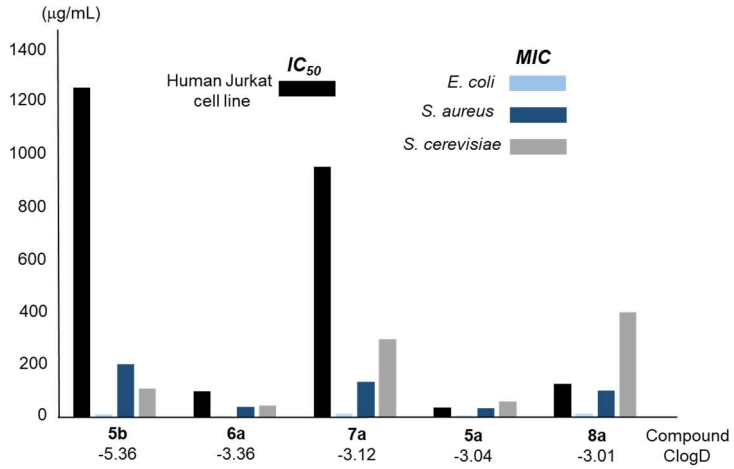

Figure 7. Cytotoxicity in the human Jurkat cell line was evaluated for the compounds described in this section using a propidium iodide viability assay by flow cytometry. The MIC values for each compound in the three tested strains are also shown. Data shows the average of three independent experiments.

As observed, **6a** and **8a**, and specially **7a**, were found to be less toxic than **5a**. This demonstrates that the substitution of the benzodioxole group by others aromatic groups with similar physicochemical properties (e.g., size and lipophilicity) produces a significant and positive impact. This fact could be attributed to the inhibitory effect of 1,3-benzodioxole on CYP-dependent enzymes, as mentioned above and demonstrated with other drugs, although further experiments would be necessary to confirm this [28].

In all cases, and particularly against the bacterium *E. coli*, the increase in the therapeutic index makes all these compounds potentially useful. It is important to note that although **5b** and **7a** may appear to be less suitable based on their higher MIC values, when considering the whole data, they appear to be very promising candidates.

2.7. Intracellular ATP concentrations and microbiolytic activity.

To gain further understanding of the effect of the compounds of relevant antimicrobial activity described in this work containing the same polyamine scaffold (**5a** and **6a**), we carried out two additional experiments: determination of the intracellular ATP values and analysis of their capacity to produce cell lysis.

2.7.1. Analysis of the intracellular ATP concentration in cells treated with the most active compounds considered in this work.

Lehtinen et al. [45] have described the use of bioluminescence-based techniques for measuring bacterial viability. Since ATP can be produced only in catabolically active cells, in the absence of external ATP, bioluminescence generated in an ATP-dependent reaction provides information about cell metabolic condition.

Following the procedure described in the Materials and Methods section, luciferase activity was used to determine the intracellular ATP concentration in bacterial and yeast cells treated with polyamines **5a** and **6a**. Studies were carried out in parallel with other compounds with antimicrobial activity (ampicillin and doxycycline in bacterial strains; fluconazole, amphotericin B and SDS in the case of yeasts).

According to the results shown in Figure 8, in yeast cells treated with a concentration 50-fold the MIC value of amphotericin B a dramatic decrease in ATP intracellular levels occurred after overnight treatment. Under the same conditions, treatment with **5a** and **6a**, resulted in ATP levels higher and similar to the ones measured in the presence of SDS at a concentration of 0.1 % (w/v). In these cases,

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ATP amounts were reduced to around 50 to 70% relative to untreated cells. The effect of fluconazole in the depletion of nucleotide intracellular levels was very reduced, although it is worth mentioning that a concentration 30-fold the MIC value was used due to its lower solubility in aqueous solutions.

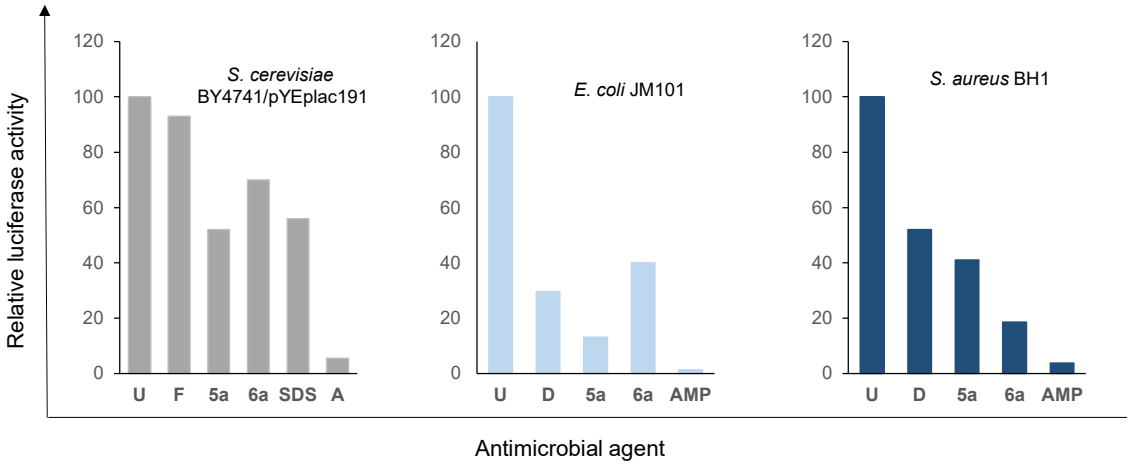

Figure 8. Determination of the ATP intracellular levels of cells treated with the compounds **5a** and **6a** during 20 h by bioluminiscence. Experiments were carried out as described in the Materials and Methods section. 50 OD_{600} units of yeasts cultures and 1.5 OD_{600} units of the bacterial ones were used. Experiments were carried out at least in triplicate and data shown correspond to the normalization of each average value to that found in untreated cells; standard deviation was lower than 5% in all cases. U refers to untreated cells, F to fluconazole, A to amphotericin B, D to doxycycline and AMP to ampicillin.

Regarding *E. coli* cells, the effect of all the compounds at 5-fold MIC concentrations was a reduction in the ATP intracellular levels, to 40% or less. The decrease was especially relevant in the case of the treatments with ampicillin and **5a**, followed by doxycycline and **6a**. For *S. aureus* similar results were found, although in this case **6a** displayed a higher effect than **5a** and doxycycline. These results confirm the important influence of **5a** and **6a** on cell metabolic activity in bacterial cells.

2.7.2. Determination of microbiolytic activity of the microbicidal compounds considered in this work using Time-Kill kinetics.

Although the precise mechanism of action of antibacterial polyamines has not been fully elucidated, the similarity found between the structural requirements for them and the known antimicrobial peptides (AMPs) seem to be indicative that both kind of compounds share the same pharmacophore model (presence of several positive charges and hydrophobic fragments) [46]. This suggests that both, at least in bacteria, could have similar action mechanism, being the negatively charged bacterial membrane the primary target, and the electrostatic interaction between it and the positively charged polyamine backbone the major driving force that finally leads to disruption of membrane integrity [15,16].

The experiments carried out with the studied polyamines revealed for most of them a bactericidal behaviour (Tables 3 and 8). In order to determine if these compounds were capable to induce cell lysis in bacteria, Time-Kill assays were performed following the procedure described in the Materials and Methods section. For these experiments, compounds **5a** and **6a** were used again. Cell growth was followed by the OD_{600} measurement in the absence and presence of the polyamines of interest and also using control agents with known lytic effects (ampicillin) [47] and non-lytic properties (doxycycline) [48 and references therein] (Figure 9).

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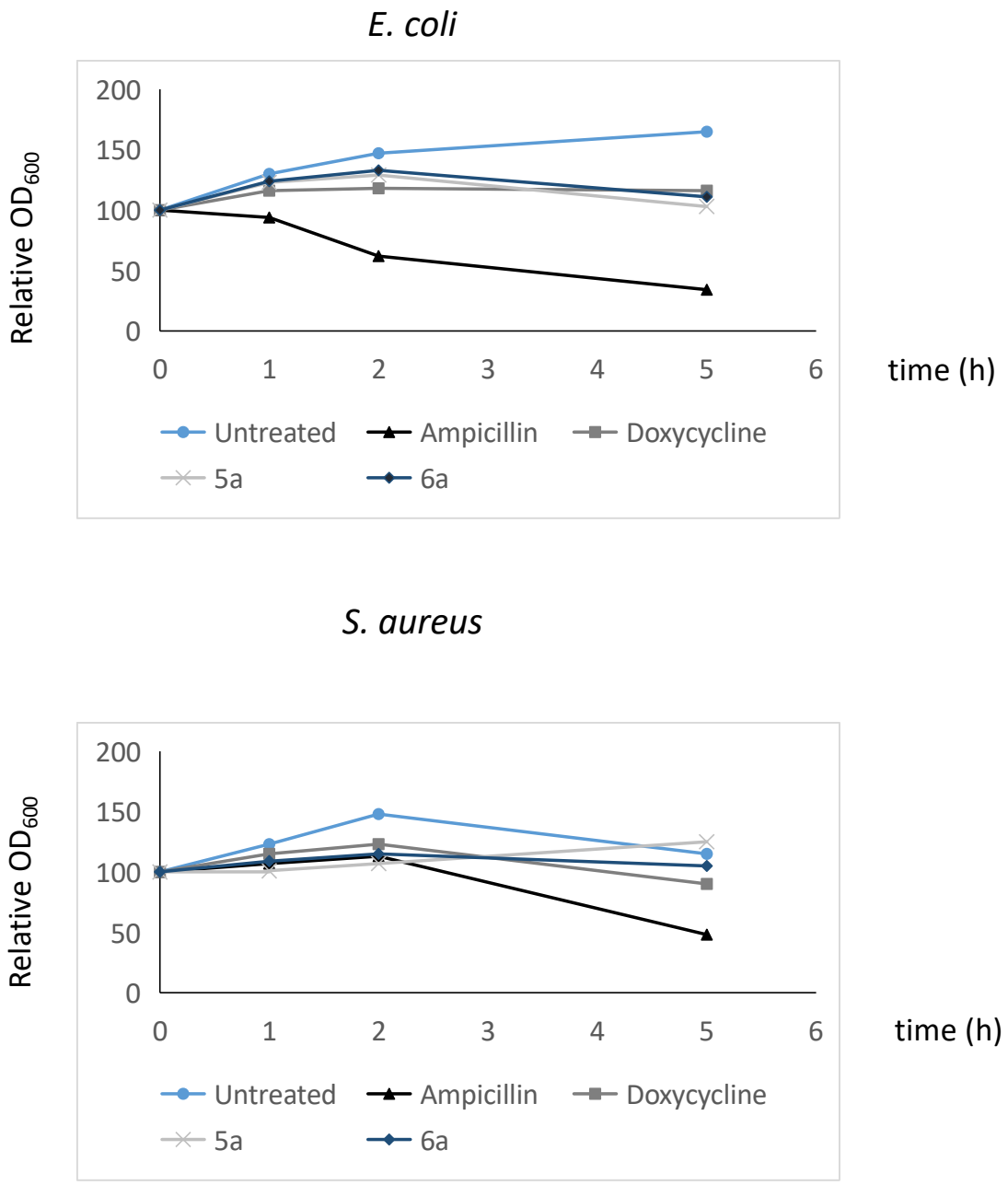

Figure 9. Average data obtained from three independent experiments to determine the lytic activity of the polyamines studied in this work. Time-kill analyses were carried out as described in the Materials and Methods section. Values shown are relative to those found at time 0 in the untreated samples, for which 100 was assigned. The standard deviation was lower than 5% in all cases.

In the case of *E. coli*, when cells were treated with ampicillin, and in accordance with its lytic activity, growth was not observed along time; actually, OD_{600} significantly decreased as incubation time increased. The obtained results for **5a** and **6a** demonstrate that they do not exhibit a lytic effect on this bacterium and behave in a similar way as doxycycline. Regarding *S. aureus*, the ampicillin treatment results also in cellular lysis, which is not the case for doxycycline and the polyamines considered in this study. However, with the methodology used in these experiments, some effect in membrane integrity cannot be completely ruled out.

Experiments were also carried out with yeast cells and, as expected according to its fungistatic character, results demonstrated that these compounds do not produce lysis, despite what occurred with amphotericin B (data not shown).

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3. Materials and Methods

3.1. Synthesis

All reagents and chemicals were obtained from commercial sources and were used as received. Solvents employed for the chemical synthesis were of analytical grade and were utilized without further purification. Polyamines **1b**-**3b** were commercially purchased, whereas **4b**, **5b, 6a** and **7a** were prepared following procedures previously described [24,32-34,44]. All compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy and elemental analysis.

For the synthesis of conjugated polyamines **1a-8a**, the corresponding polyamine (between 0.5-1 g) was first dissolved in 30-60 mL of methanol and placed in a 250 mL round flask with magnetic stirring. The number of equivalents of each aldehyde (2H-1,3-benzodioxole-5-carbaldehyde, 2-hydroxybenzaldehyde, 4-hydroxybenzaldehyde or 2,3-dihydrobenzofuran-5-carbaldehyde) corresponding to the number of primary amines was dissolved in another 30 mL of methanol and added dropwise to the reaction flask. The temperature was then slowly increased to 50°C and left under magnetic stirring for 24 h. After this time, it was lowered to 0°C, and an excess of $NaBH_4$ was slowly added (8 equivalents x No. of imines). The solution was left to stir until it reached room temperature, and then the solvent was eliminated. The crude of the reaction was dissolved in 100 mL of water and extracted four times with 25 mL of dichloromethane**.** The product was recrystalized by adding an excess of HCl 4 M in dioxane, centrifuged and dried under vacuum (60-90% yields).

N^1-(benzo[d][1,3]dioxol-5-ylmethyl)-N^4-(3-((benzo[d][1,3]dioxol-5-ylmethyl)amino)propyl)butane-1,4-diamine (**1a**). ^{1}H RMN (D_2O, 300 MHz): δ_H 6.88 (m, 3H); 5.91 (s, 6H); 4.09 (s, 6H); 3.05 (m, J = 5, 6H); 2,03 (m, 6H); 1.68 (m, 6H). ^{13}C RMN (D_2O, 75 MHz): δ_C 148.8, 148.1, 124.2, 109.9, 108.9, 101.6, 51.0, 46.9, 45.5, 44.1, 43.1, 22.1. Anal. Calc. for $C_{23}H_{34}N_3O_4Cl_3$: C, 52.8; H, 6.6; N, 8.0; O, 12.2; Cl, 20.4. Exp.: C, 51.9; H, 6.3; N, 7.2; O, 12.6; Cl, 22.0.

N^1,$N^{1'}$-(butane-1,4-diyl)bis(N^3-(benzo[d][1,3]dioxol-5-ylmethyl)propane-1,3-diamine) (**2a**). ^{1}H RMN (D_2O, 300 MHz): δ_H 6.88 (m, 3H); 5.92 (s, 6H); 4.11 (s, 6H); 3.06 (m, 6H); 2,03 (m, 6H); 1.70 (m, 6H). ^{13}C RMN (D_2O, 75 MHz): δ_C 148.3, 147.7, 124.2, 123.9, 118.1, 109.9, 108.9, 101.6, 51.1, 47.0, 44.4, 43.6, 22.7. Anal. Calc. para $C_{26}H_{42}N_4O_4Cl_4 \cdot H_2O$: C, 49.9; H, 6.9; N, 9.0; O, 11.5; Cl, 22.7. Exp.: C, 50.0; H, 6.7; N, 8.3; O, 11.1; Cl, 23.9.

N^1-(benzo[d][1,3]dioxol-5-ylmethyl)-N^2,N^2-bis(2-((benzo[d][1,3]dioxol-5-ylmethyl)amino)ethyl)ethane-1,2-diamine (**3a**) ^{1}H RMN (D_2O, 300 MHz): δ_H 6.86 (m,10H); 5.92 (s, 6H); 4.06 (s, 6H); 3.04 (m, J=6, 6H); 2.86 (t, J = 6, 5H). ^{13}C RMN (D_2O, 75 MHz): δ_C 148.2, 147.7, 124.3, 123.9, 109.5, 109.1, 101.6, 51.0, 49.1, 44.7, 44.5, 43.8, 22.4. Anal. Calc. for $C_{30}H_{39}N_4O_6Cl_3(H_2O)$: C, 53.3; H, 6.11; N, 8.29. Exp.: C, 53.0; H, 8.7; N, 8.1.

N^1-(benzo[d][1,3]dioxol-5-ylmethyl)-N^3-(2-((2-((3-((benzo[d][1,3]dioxol-5-ylmethyl)amino)propyl)amino)ethyl)(2-((3-(((2,3-dihydrobenzofuran-6-yl)methyl)amino)propyl)amino)ethyl)amino)ethyl)propane-1,3-diamine (**4a**). ^{1}H RMN (D_2O, 300 MHz): δ_H 6.86 (m,10H); 5.87 (s, 6H); 4.07 (s, 6H); 3.1 (m, J=6, 16H); 2.79 (t, J = 6, 5H); 2.11 (m, 6H). ^{13}C RMN (D_2O, 75 MHz): δ_C 148.2, 147.7, 124.3, 123.9, 109.5, 109.1, 101.6, 51.0, 49.1, 44.7, 44.5, 43.8, 22.4. Anal. Calc. for $C_{39}H_{63}N_7O_6Cl_6(H_2O)$: C, 49.90; H, 6.77; N, 10.45; O, 10.23. Exp.: C, 48.74; H, 6.63; N, 9.67; O, 11.73.

2-(3,6,9-triaza-1(2,6)-pyridinacyclodecaphane-6-yl)-N-(benzo[d][1,3]dioxol-5-ylmethyl)ethan-1-amine (**5a**). ^{1}H NMR (D_2O, 300 MHz): δ_H 7.97 (t, J=7.80, 1H); 7.46 (d, J=7.80, 2H); 6.99 (m, 3H); 6.03 (s, 2H); 4.64 (s, 4H); 4.21 (s, 2H); 3.28 (m, 6H); 3.06 (t, J=5.3, 2H); 2.92 (t, J=5.3, 4H). ^{13}C NMR (D_2O, 75 MHz): δ_C 148.96, 148.20, 147.68, 139.62, 124.42, 124.00, 122.02, 110.11, 106.91, 101.51, 51.12, 50.93, 50.43, 49.51, 45.84, 42.21. Anal. Calc. for $C_{21}H_{32}N_5O_2Cl_3(H_2O)$: C, 49.37; H, 6.71; N, 13.71; O, 9.39. Exp.: C, 47.87; H, 6.33; N, 11.96; O, 9.60.

2-(3,6,9-triaza-1(2,6)-pyridinacyclodecaphane-6-yl)-N-((2,3-dihydrobenzofuran-5-yl)methyl)ethan-1-amine (**8a**). ^{1}H NMR (D_2O, 300 MHz): δ_H 7.89 (t, J=7.80, 1H); 7.37 (d, J=7.80, 2H); 7.30 (s, 1H); 7.18 (dd, J= 8.3, 1H); 6.79 (d, J=8.3, 1H); 4.56 (m, 5H); 4.14 (s, 2H); 3.17 (m, 6H); 2.97 (m, 2H); 2.84 (t, J=5.3, 4H). ^{13}C NMR (D_2O, 75 MHz): δ_C 160.06, 148.84, 139.73, 130.19, 128.96, 127.04, 122.77, 122.14, 109.51, 72.10,

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66.53, 51.28, 50.85, 50.44, 49.42, 45.85, 42.07. Anal. Calc. for $C_{22}H_{34}N_5OCl_3(H_2O)_2$: C, 50.14; H, 7.27; N, 13.29; O, 9.11. Exp.: C, 49.69; H, 6.61; N, 12.58; O, 8.19.

3.2. Potentiometric titrations

The potentiometric titrations were carried out at 298.1 ± 0.1 K using NaCl 0.15 M as supporting electrolyte. The experimental procedure (burette, potentiometer, cell, stirrer, microcomputer, etc.) has been fully described elsewhere [50]. The acquisition of the EMF data was performed with the computer program PASAT [51]. The reference electrode was an Ag/AgCl electrode in saturated KCl solution. The glass electrode was calibrated as a hydrogen-ion concentration probe by titration of previously standardized amounts of HCl with CO_2-free NaOH solutions and the equivalent point determined by the Gran´s method [52,53], which gives the standard potential, E^{Θ}', and the ionic product of water (pK_w = 13.73(1)).

The computer program HYPERQUAD was used to calculate the protonation constants [54]. The pH range investigated was 2.5-11.0 and the concentration of the ligands ranged from $5x10^{-4}$ to $5x10^{-3}$ mol/dm. The different titration curves for each system (at least two) were treated either as a single set or as separated curves without significant variations in the values of the stability constants. Finally, the sets of data were merged and treated simultaneously to give the final stability constants.

3.3. Antimicrobial activity

3.3.1. Bacterial and yeast strains and growth conditions

The microorganisms used for the susceptibility tests were the bacteria *Escherichia coli* JM101 and *Staphylococcus aureus* BHI, and the yeast *Saccharomyces cerevisiae* strains BY4741 (*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (EUROSCARF)) carrying YEplac195 (from EUROSCARF) or YEPlac195-PDR5 (provided by Ayse Banu Demir, Izmir University of Economics, Turkey) plasmids.

Bacterial strains were usually maintained and grown in LB medium (0.5% (w/v) yeast extract, 1% (w/v) bactotryptone, 1% (w/v) NaCl) at 37°C. For MIC (Minimal Inhibitory Concentration) and MMC (Minimal Microbicidal Concentration) assays, and for the determination of sinergies, overnight growth was carried out in 2% (w/v) bactopeptone (BP), and then cultures were diluted, and incubated for several hours, in 2% (w/v) BP for ensuring logarithmic growth. Yeast strains were grown in SC-ura medium (0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulphate, 0.2% (w/v) Drop-out without uracil, 0.5% (w/v) $(NH_4)_2SO_4$, 2% (w/v) glucose) at 30°C. Liquid cultures were incubated with shaking (200 rpm). Solid media contained also 2% (w/v) agar.

3.3.2. Preparation of stock solutions of test and control compounds

Control drugs used in our experiments were the commercial antibacterial doxycycline, gentamicin, ciprofloxacin and ampicillin, and the antifungal fluconazole, amphotericin B and cycloheximide. All of them were purchased from Sigma-Aldrich. Stock solutions of these drugs and the compounds to be tested were prepared in phosphate buffer 20 mM pH 7.4.

3.3.3. Determination of the Minimum Inhibitory Concentration (MIC)

For the analysis of the antimicrobial activity the strategies and indications of Cushnie et al. were considered [55]. The standardized method of microdilution in culture medium was followed to determine the MIC [56]. In bacterial strains, serial dilutions of the compounds to be tested and the control antibiotics were prepared in phosphate buffer (20 mM, pH 7.4, 0.5 mL), and were mixed with a suspension of bacterial cells in logarithmic growth phase (in 1% BP, 0.5 mL). The final OD_{600} of the whole solutions were 0.004 and 0.008 for *E. coli* and *S. aureus* cultures respectively. After 20 h of incubation at 37°C with shaking, the MIC was determined as the lowest concentration of each compound in which no visible bacterial growth was observed.

Determination of MIC for yeast strains was carried out following a similar procedure with the polyamines and the antifungal reference agents: serial dilutions of each one in phosphate buffer (20

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mM, pH 7.4, 0.5 mL) were prepared and mixed with a suspension of yeast cells (OD_{600} 0.04 in SC-ura 2X, 0.5 mL). After 24 h of incubation, at 30°C with shaking, the MIC was determined as the lowest concentration of each compound that resulted in the same optical density of the solution than prior to the incubation. Experiments were carried out at least three times in all cases.

3.3.4. Determination of the Minimum Microbicidal Concentration (MMC)

Once the MIC was known, samples were prepared as described in the previous section with polyamine concentrations corresponding to the MIC and half, double and four times its value. After the overnight incubation with the microorganisms, 30 µL of a ten-fold dilution of each one was spread in LB (in the case of bacterial strains) or SC-ura (for yeast strains) plates. They were incubated for 20 h at 37°C (bacteria) or 48 h at 30°C (yeasts), and the level of growth was determined. Since microbiostatic activity has been defined as a ratio of MMC to MIC > 4 [57], we classified as microbiostatic those compounds for which growth was observed in plates proceeding from samples containing 4 times the MIC concentration, and microbicides those in which no growth was detected at this concentration. In the case of microbicidal substances, the Minimal Fungicidal/Bactericidal Concentration (MFC/MBC) was determined as the lowest concentration of the tested agent that either totally prevents growth or results in a >99.9% decrease of the inoculum. Experiments were carried out at least three times.

3.3.5. Determination of the synergies between the polyamines and control antifungal and antibacterial substances by the checkerboard titration approach.

The combined antimicrobial activity of the test compounds with the antibacterial or antifungal control agents was performed under the same conditions used for the MIC determination, and following the procedures described by Carton Herrán [58], Reis de Sá *et al*. [59] and Zharkova *et al.* [56]. For each experiment 16 samples (4 rows x 4 columns) in a total volume of 0.5 mL were prepared in tubes using an array of combinations between the MIC value and 0 µg/mL of the compound to be tested in each row, and between the MIC value and 0 µg/mL of the reference drug in each column. In this way, a variety of mixtures with different concentrations of them alone or in combination was obtained. 0.5 mL of a microorganism culture was added to each one of the samples and after incubation for 24 h with shaking at the appropriate temperature (37° or 30°C), the tubs were visually inspected and the OD_{600} was measured. From this information the Fractional Inhibitory Concentration Index (FICI) was calculated according to the formula: $FIC\ index = ([A]/[CMI\ A]) + ([B]/[CMI\ B])$, where [A] and [B] are respective concentrations of tested polyamine and reference drug in their combination effectively inhibiting growth of bacteria or yeast, and [MIC A] and [MIC B] are the individual MICs of the tested polyamine and reference drug when they are used alone. An interaction is considered synergistic when the FICI value is ≤0.5 [41,42].

To confirm the data obtained in the checkerboard assay, 30 µL of a ten-fold dilution of the tubes containing concentrations of half of the MIC of each one of the compounds alone (polyamine to be tested and reference drug) and one fourth of the MIC of each one in combination were also spread in LB or SC-ura plates (according to the microorganism considered in each case) and incubated at 37° or 30°C. Visualization of these plates was made after 24 or 48 hours respectively. All these experiments were carried out in triplicate.

3.3.6. Analysis of the intracellular ATP concentration in bacterial and yeast cultures treated with the tested compounds.

The procedure described by Lehtinen et al. was followed [45], with the modifications indicated herein. In the case of bacteria, 1.5 OD_{600} units were incubated overnight in 1 mL of final volume with 5-fold MIC concentrations. Then cells were collected, washed 3 times in 20 mM phosphate buffer pH 7.4, and lysed in 200 µL of LS1 (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) containing 5 mg/mL of lysozyme and the same volume of glass beads (425-600 µm), by stirring for 15 seconds during 10 times. Then the solution was centrifuged at 14800 x g for 15 min and the

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supernatant was used for a luciferase assay. For this purpose, in each well of a microtiter plate, 100 µL of luciferase assay buffer (0.015 M $MgSO_4$, 0.015 M KH_2PO_4 pH 8, 0.004 M EGTA pH 8, 2 mM DTT) was added together to a 50 µL of 0.3 mg/mL luciferine solution in 0.01% (w/v) Triton X-100 and 50 µL of the cell extract. Finally, 15 µL of a 67 µg/mL aqueous solution of luciferase was added. Light emission was immediately detected by the Luminoskan Ascent instrument (Thermo Scientific, Waltham, USA) for 1 s per well. Glass-beads, Lysozyme, luciferine and luciferase were purchased from Sigma-Aldrich.

In the case of yeasts, 50 OD_{600} units were incubated overnight in 1 mL of final volume with 50-fold MIC concentrations. Then cells were collected and washed as above and resuspended in 500 µL of LS2 (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.0, 0.5% (v/v) NP40). Cells were lysed in the presence of 1 volume of glass beads as described above. Next steps were carried out as in the case of bacterial samples.

Experiments were carried out at least in triplicate in all cases and control drugs and SDS (0.1% (w/v) of final concentration) were also included.

3.3.7. Analysis of the lytic activity of microbicidal compounds.

To determine if the compounds showing microbicidal activity produce cell lysis, the procedure described by Fleeman et al. was followed [60], with the variations indicated below. The *E. coli* and *S. aureus* strains were grown as in the experiments described earlier. Cells corresponding to 1.5 units of OD_{600} were collected and resuspended in 1% BP after washing in the same medium. They were incubated in a final volume of 1 mL with concentrations 5 times higher than the MIC of the analyzed compounds, and OD_{600} was monitored over time. Other antimicrobial compounds were also used in this study as a controls. The experiments were performed in triplicate.

3.4. Determination of compound cytotoxicity on human cells.

The cytotoxicity of the compounds was assessed by flow cytometry with a viability assay using propidium iodide (PI), a DNA-binding fluorescent probe not permeant to live cells, commonly used to detect dead cells in a population [61]. The human lymphocytic leukemia Jurkat cell line (ATCC TIB-152) was obtained from the American Type Cell Culture (Sigma Aldrich) and grown in suspension in RPMI 1640 medium (ThermoFisher Scientific) supplemented with L-Glutamine, antibiotics and 10% heat-inactivated fetal calf serum (FCS, ThermoFisher Scientific). For the viability assay, cells were seeded in plastic 96-well plates at 250,000 cells/µL in RPMI 1640 medium supplemented with 5% FCS. Cells were treated for 24 hours with a range of concentrations of the test compounds or with the same volume of phosphate buffer (PBS) in control wells. After incubation, cell suspensions were washed, resuspended in 400 µL fresh medium and incubated for 5 minutes at room temperature in the dark with 3.75 µM PI (Sigma Aldrich). Cell viability was determined by flow cytometry using a Gallios flow cytometer (Beckman Coulter). PI fluorescence was excited by an argon-ion laser at 488 nm and collected through a 620/630 nm band-pass emission. Cellular events were discriminated from debris using forward scatter (FS) and side scatter (SS) signals. Cell-aggregates were excluded from analysis by FS-Integral and FS-peak. Gates applied for population discrimination were set manually based on control samples. For each sample, 10,000 events were collected. For further analysis, the cytometric data were exported to Kaluza 2.1 software (Beckman Coulter). Dead cells were identified and quantified as PI-positive events. The cytotoxic potency of test compounds was quantified by their IC_{50} values, i.e., the concentration of compound at which cell viability is inhibited by 50%, as calculated by curve fitting and determination by the correlation coefficient (R2) with GraphPad Prism 9.0 software.

4. Conclusions

In this work, we describe a collection of compounds based in different polyamine scaffolds that are easily synthetized and display antibacterial/antifungal activity. In overall, addition of a lipophilic moiety to the polyamine backbone makes the resulting compounds more effective. Structural

characteristics such as total charge and the polyamine topology, seem to be determinant factors in the antimicrobial activity. Moreover, these substances are interesting antifungal agents in combination with other known drugs. Macrocyclic polyamine 5b, its derivatives 5a and 6a, and tripodal 4a resulted to be the most effective as antimicrobials. However, the nature of the lipophilic component was determinant in the toxicity of these derivatives on human cells, and in these sense, polyamines 5b and 7a resulted also promising as leads due to its low harmfulness. It would be interesting to advance in the research of similar compounds, designed by rational modification of their structure that could be more selective and effective. Further analyses would also be required to understand their mechanism of action on the cells.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: ^{1}H NMR spectra of the new compounds; Figure S2: Scheme and X-ray crystallographic structure of **3a**·3HCl; Table S1: Crystal data, data collection parameters, and results of the analysis; Figure S3: Molar fraction species distribution diagrams; Figure S4: Determination of the minimal microbicidal concentration; Figure S5: Cytotoxicity determination by FACS.

Author Contributions: Conceptualization, M.I., E.G.E. and C.A. Preparation of the compounds to be analysed, M.I., A.M.S. Development of most experimental work, N.T., A.M. and G.T. X-Ray Analyses, S.B. Experimental design, supervision, and review, M.I., C.A. and M.D.O. Flow cytometry experiments, B.J. and J.O. Manuscript writing: M.I., C.A., M.D.O. Funding acquisition: E.G.E. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Conselleria de Innovación, Universidades, Ciencia y Sociedad Digital, Generalitat Valenciana (PROMETEO Grant CIPROM/2021/030), Spanish MICINN and MEC and FEDER funds (grants PID2019-110751RB-I00, Universitat de València (grants UV-INV-AE19-1199043 and UV-INV-AE-1560151), Red de Excelencia de Química Supramolecular (RED2018-102331-T) and Unidad de Excelencia María de Maeztu (CEX2019-000919). Mario Inclán thanks Generalitat Valenciana and the European Social Fund for the financial support (APOSTD2019).

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Acknowledgments: We are grateful to Dr. Ayse Banu Demir for sending us the YEPlac195-PDR5 plasmid.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds presented here are available upon request from the corresponding authors.

References

1. O. 'Neill, J. Tackling drug-resistant infections globally: Final report and recommendations. *Review on Antimicrobial Resistance*; Wellcome Trust and the UK Department of Health: London, **2016**, 80 pp.
2. Murray, C.J.; Ikuta, K.S.; Sharara, F.; Swetschinski, L.; Robles Aguilar, G.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; Johnson, S.C.; Browne, A.J.; Chipeta, M.G.; Fell, F.; Hackett, S.; Haines-Woodhouse, G.; Kashef Hamadani, B.H.; Kumaran, E.A.P.; McManigal, B.; Naghavi, M. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet*. **2022**, *399*, 629–655. DOI: 10.1016/S0140-6736(21)02724-0
3. 2020 Antibacterial agents in clinical and preclinical development: an overview and analysis; Geneva: World Health Organization: Geneva, **2021**. Licence: CC BY-NC-SA 3.0 IGO.
4. Melchiorre, C.; Bolognesi, M.L.; Minarini, A.; Rosini, M.; Tumiatti, V. Polyamines in drug discovery: From the universal template approach to the multitarget-directed ligand design strategy. *J. Med. Chem.* **2010**, *53*, 5906–5914. DOI: 10.1021/jm100293f
5. Melchiorre, C.; Angeli, P.; Brasili, L.; Giardina, D.; Pigini, M.; Quaglia, W. Polyamines: a possible "passe-partout" for receptor characterization. *Actual. Chim. Ther.* **1988,** 149–168.
6. Bolognesi, M.L.; Minarini, A.; Budriesi, R.; Cacciaguerra, S.; Chiarini, A.; Spampinato, S.; Tumiatti, V.; Melchiorre, C. Universal template approach to drug design: polyamines as selective muscarinic receptor antagonists. *J. Med. Chem.* **1998**, *41*,4150–4160. DOI: 10.1021/jm981038d
7. Bottcher, T.; Kolodkin-Gal, I.; Kolter, R.; Losick, R.; Clardy, J. Synthesis and activity of biomimetic biofilm disruptors. *J. Am. Chem. Soc.* **2013**, *135*, 2927–2930. DOI: 10.1021/ja3120955
8. Goytia, M.; Dhulipala, V.L.; Shafer, W.M. Spermine impairs biofilm formation by *Neisseria gonorrhoeae*. *FEMS Microbiol. Lett.* **2013**, *343*, 64–69. DOI: 10.1111/1574-6968.12130

9. Balakrishna, R.; Wood, S.J.; Nguyen, T.B.; Miller, K.A.; Suresh Kumar, E.V.; Datta, A.; David, S.A. Structural correlates of antibacterial and membrane-permeabilizing activities in acylpolyamines. *Antimicrob. Agents Chemother.* **2006**, *50*, 852–861. DOI: 10.1128/AAC.50.3.852-861.2006
10. Alkhzem, A.H.; Shuxian, S.; Wonfor, T.; Woodman, T.J.; Laabei, M.; Blagbrough, I.S. Practical synthesis of antimicrobial long linear polyamine succinamides. *ACS Bio. & Med. Chem. Au.* **2022**, *2*, 607-616. DOI: 10.1021/acsbiomedchemau.2c00033
11. Négrel, S.; Brunel, J.M. Synthesis and biological activities of naturally functionalized polyamines: An overview. *Curr. Med. Chem.* **2021**, *28*, 3406-3448. DOI: 10.2174/0929867327666201102114544
12. Djouhri-Bouktab, L.; Vidal, N.; Rolain, J.M.; Brunel, J.M. Synthesis of new 3,20-bispolyaminosteroid squalamine analogues and evaluation of their antimicrobial activities. *J. Med. Chem.* **2011**, *54*, 7417–7421. DOI: 10.1021/jm200506x
13. Djouhri-Bouktab, L.; Alhanout, K.; Andrieu, V.; Raoult, D.; Rolain, J.M.; Brunel, J.M. Squalamine ointment for *Staphylococcus aureus* skin decolonization in a mouse model. *J. Antimicrob. Chemother.* **2011**, *66*, 1306–1310. DOI: 10.1093/jac/dkr114
14. Salmi, C.; Loncle, C.; Vidal, N.; Letourneux, Y.; Brunel, J.M. New stereoselective titanium reductive amination synthesis of 3-amino and polyaminosterol derivatives possessing antimicrobial activities. *Eur. J. Med. Chem.* **2008**, *43*, 540–547. DOI: 10.1016/j.ejmech.2007.04.006
15. Khusnutdinova, E.F.; Sinou, V.; Babkov, D.A.; Kazakova, O.; Brunel, J.M. Development of new antimicrobial oleanonic acid polyamine conjugates. Antibiotics **2022**, *11*, 94. DOI: 10.3390/antibiotics11.010094
16. Wang, B.; Pachaiyappan, B.; Gruber, J.D.; Schmidt, M.G.; Zhang, Y.-M.; Woster, P.M. Antibacterial diamines targeting bacterial membranes. J. Med. Chem. 2016, 59, 3140–3151. DOI: 10.1021/acs.jmedchem.5b01912
17. Chiappori, A.A.; Haura, E.; Rodriguez, F.A.; Boulware, D.; Kapoor, R.; Neuger, A.M.; et al. Phase I/II study of atrasentan, an endothelin A receptor antagonist, in combination with paclitaxel and carboplatin as first-line therapy in advanced non-small cell lung cancer. *Clin. Cancer Res.* **2008**, *14*, 1464–1469. DOI: 10.1158/1078-0432.CCR-07-1508
18. Curreli, F.; Belov, D.S.; Ahmed, S.; Ramesh, R.R.; Kurkin, A.V.; Altieri, A.; Debnath, A.K. Synthesis, antiviral activity, and structure-activity relationship of 1,3-benzodioxolyl pyrrole-based entry inhibitors targeting the Phe43 Cavity in HIV-1 gp120. *ChemMedChem*. **2018**, *13*, 2332–2348. DOI: 10.1002/cmdc.201800534
19. Millan, M.J.; Cussac, D.; Milligan, G.; Carr, C.; Audinot, V.; Gobert, A.; Lejeune, F.; Rivet, J.M.; Brocco, M.; Duqueyroix, D.; Nicolas, J.P.; Boutin, J.A.; Newman-Tancredi, A. Antiparkinsonian agent piribedil displays antagonist properties at native, rat, and cloned, human alpha(2)-adrenoceptors: cellular and functional characterization. *J. Pharmacol. Exp. Ther.* **2011**, *297*, 876–887.
20. Abd El Razik, H.A.; Badr, M.H.; Atta, A.H.; Mouneir, S.M.; Abu-Serie, M.M. Benzodioxole-pyrazole hybrids as anti-inflammatory and analgesic agents with COX-1,2/5-LOX inhibition and antioxidant potential. *Arch. Pharm. (Weinheim).* **2017**, *350*, 1700026. DOI: 10.1002/ardp.201700026
21. Armenian, P.; Vo, K.T.; Barr-Walker, J.; Lynch, K.L. Fentanyl, fentanyl analogs and novel synthetic opioids: A comprehensive review. *Neuropharmacology* **2017**, *134*, 121–132. DOI: 10.1016/j.neuropharm.2017.10.016
22. Nickels, K.C.; Wirrell, E.C. Stiripentol in the management of epilepsy. *CNS Drugs* **2017**, *31*, 405–416. DOI: 10.1007/s40263-017-0432-1
23. Gleason, F.K.; Case, D.E. Activity of the natural algicide, cyanobacterin, on angiosperms. *Plant Physiol.* **1986**, *80*, 834–837. DOI: 10.1104/pp.80.4.834
24. Freudenmann, R.W.; Öxler, F.; Bernschneider-Reif, S. The origin of MDMA (ecstasy) revisited: the true story reconstructed from the original documents. *Addiction* **2006**, *101*, 1241–1245. DOI: doi:10.1111/j.1360-0443.2006.01511.x
25. Kalant, H. The pharmacology and toxicology of "ecstasy" (MDMA) and related drugs. *CMAJ.* **2001**, *165*, 917–928.
26. Mibu, N.; Yokomizo, K.; Murakami, K.; Ono, Y.; Ishimaru, M.; Otsubo, M.; Inao, H.; Ono, Y.; Zhou, J.R.; Sumoto, K. Antiviral activity and molecular geometry of some new symmetrical tris(aminoalkyl)amine derivatives. *Chem. Pharm. Bull.* **2016**, *64*, 1769–1780. DOI: 10.1248/cpb.c16-00682
27. Leite, A.C.L.; Peixoto Da Silva, K.; De Souza, I.A.; Magali De Araújo, J.; Brondani, D.J. Synthesis, antitumour and antimicrobial activities of new peptidyl derivatives containing the 1,3-benzodioxole system. *Eur. J. Med. Chem.* **2004**, *39*, 1059–1065. DOI: 10.1016/j.ejmech.2004.09.007
28. Song, D.; Cao, X.; Wang, J.; Ke, S. Discovery of γ-lactam derivatives containing 1,3-benzodioxole unit as potential anti-phytopathogenic fungus agents. *Bioorg. Med. Chem. Lett.* **2020**, *30*, 126826. DOI: 10.1016/j.bmcl.2019.126826
29. Al-Wabli, R.I.; Al-Ghamdi, A.R.; Ghabbour, H.A.; Al-Agamy, M.H.; Attia, M.I. Synthesis, structure elucidation, and antifungal potential of certain new benzodioxole–imidazole molecular hybrids bearing ester functionalities. *Drug. Des. Devel. Thery.* **2019**, *13*, 775–789. DOI: 10.2147/DDDT.S199135

30. Yang, R.; Li, Z.; Xie, J.; Liu, J.; Qin, T.; Liu, J.; Du, H.; Ye, H. 4-Aminoquinolines bearing a 1,3-benzodioxole moiety: Synthesis and biological evaluation as potential antifungal agents. *Chem. Biodivers.* **2021**, *18*, 2–9. DOI: 10.1002/cbdv.202100106.
31. Lomadze, N.; Schneider, H.-J.; Albelda, M.T.; García-España, E.; Verdejo, B. Dramatic selectivity differences in the association of DNA and RNA models with new ethylene- and propylene diamine derivatives and their copper complexes. *Org. Biomol. Chem*, 2006, *9*, 1755–1759. DOI: 10.1039/B517386H
32. Albelda, M.T.; García-España, E.; Jiménez, H.R.; Llinares, J.-M.; Soriano, C.; Sornosa Ten, A.; Verdejo, B. Cu^{2+} and AMP complexation of enlarged tripodal polyamines. *Dalton Trans*. 2006, *37*, 4474–4481. DOI: 10.1039/b605639c
33. Sornosa Ten, A.; Humbert, N.; Verdejo, B.; Llinares, J.M.; Elhabiri, M.; Jezierska, J.; Soriano, C.; Kozlowski, H.; Albrecht-Gary, A.-M.; García-España, E. Cu^{2+} Coordination properties of a 2-pyridine heptaamine tripod: Characterization and binding mechanism. *Inorg. Chem.* 2009, *48*, 8985–8997. DOI: 10.1021/ic9010955
34. Verdejo, B.; Ferrer, A.; Blasco, S.; Castillo, C.E.; González, J.; Latorre, J.; Máñez, M.A.; Basallote, M.G.; Soriano, C.; García-España, E. Hydrogen and copper ion-induced molecular reorganizations in scorpionand-like ligands. A potentiometric, mechanistic, and solid-state study. *Inorg. Chem.* **2007**, *46*, 5707−5719. DOI: 10.1021/ic700643n
35. Bencini, A.; Bianchi, A.; García- España, E.; Micheloni, M.; Ramírez, J.A. Proton coordination by polyamine compounds in aqueous solution. *Coord. Chem. Rev.* **1999**, *188*, 97−156. DOI: 10.1016/S0010-8545(98)00243-4
36. Swagatika, S.; Tomar, R.S. ABC transporter Pdr5 is required for cantharidin resistance in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun*. **2021**, *553*, 141-147. DOI: 10.1016/j.bbrc.2021.03.074
37. Kwon, D.H.; Lu, C.D. Polyamine effects on antibiotic susceptibility in bacteria. *Antimicrob. Agents Chemother*. **2007**, *51*, 2070–2077. DOI: 10.1128/AAC.01472-06
38. Kolaczkowski, M.; van der Rest, M.; Cybularz-Kolaczkowska, A.; Soumillion, J.P.; Konings, W.N.; Goffeau, A. Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p. *J. Biol. Chem.* **1996**, *271*, 31543-31548. DOI: 10.1074/jbc.271.49.31543
39. Sucher NJ. Searching for synergy in silico, in vitro and in vivo. *Synergy* **2014**, *1*, 30-43. DOI: 10.1016/j.synres.2014.07.004
40. García-Fuente, A., Vázquez, F., Viéitez, J.M. et al. CISNE: An accurate description of dose-effect and synergism in combination therapies. *Sci. Rep*. **2018**, *8*, 4964. DOI: 10.1038/s41598-018-23321-6
41. Loewe, S. The problem of synergism and antagonism of combined drugs. *Arzneimittelforschung* **1953**, *3*, 285–290.
42. Meletiadis, J.; Pournaras, S.; Roilides, E.; Walsh, T.J. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, monte carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug combinations against *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2010**, *54*, 602-609. DOI: 10.1128/AAC.00999-09
43. Hodgson, E. Metabolic Interactions of Pesticides. In *Pesticide Biotransformation and Disposition*; Hodgson, E., Ed.; Academic Press, Cambridge, U.S.A., 2012; Chapter 7, pp. 149-178. DOI: 10.1016/B978-0-12-385481-0.00007-1
44. Verdejo, B.; Inclán, M.; Blasco, S.; Ballesteros-Garrido, R.; Savastano, M.; Bianchi, A.; García-España, E. Selective recognition of neurotransmitters in aqueous solution by hydroxyphenyl aza-scorpiand ligands. *Org. Biomol. Chem*. **2023**, 5424–5432, doi:10.1039/d3ob00562c
45. Lehtinen, J.; Järvinen, S.; Virta, M.; Lilius, E.M. Real-time monitoring of antimicrobial activity with the multiparameter microplate assay. *J. Microbiol. Methods*. **2006**, *66*, 381-389. DOI: 10.1016/j.mimet.2006.01.002
46. Giménez, D.; Andreu, C.; del Olmo, M.; Varea, T.; Diaz, D.; Asensio, G. The introduction of fluorine atoms or trifluoromethyl groups in short cationic peptides enhances their antimicrobial activity. *Bioorg. Med. Chem*. **2006**, *14*, 6971-6978. DOI: 10.1016/j.bmc.2006.06.027
47. Moradpour, Z.; Yousefi, N.; Sadeghi, D.; Ghasemian, A. Synergistic bactericidal activity of a naturally isolated phage and ampicillin against urinary tract infecting *Escherichia coli* O157. *Iran. J. Basic Med. Sci.* **2020**, *23*, 257–263. DOI: 10.22038/IJBMS.2019.37561.8989
48. Chopra, I.; Roberts, M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 232-260. DOI: 10.1128/MMBR.65.2.232-260.2001
49. Albelda, M.T.; García-España, E.; Jiménez, H.R.; Llinares, J.M.; Soriano, C.; Sornosa-Ten, A.; Verdejo, B. Cu^{2+} and AMP complexation of enlarged tripodal polyamines. *Dalton Trans.* **2006**, *37*, 4474–4481. DOI: 10.1039/b605639c
50. García-España, E.; Ballester, M.-J.; Lloret, F.; Moratal, J.M.; Faus, J.; Bianchi, A. Low-spin six-co-ordinate cobalt(II) complexes. A solution study of tris(violurato)cobaltate(II) ions. *Dalton Trans.* **1988**, *1*, 101-104. DOI: 10.1039/DT9880000101
51. Fontanelli, M.; Micheloni, M. Proceedings of the I Spanish-Italian Congress on Thermodynamics of Metal Complexes, Peñíscola, Castellón, **1990**. *Program for the automatic control of the microburette and the acquisition of the electromotive force readings*.

52. Gran, G. Determination of the equivalence point in potentiometric titrations. Part II. *Analyst*, **1952** *77*, 661-671.
53. Rossotti, F.J.; Rossotti, H. Potentiometric titrations using Gran plots: A textbook omission. *J. Chem. Educ.* **1965**, *42*, 375-378.
54. Gans, P.; Sabatini, A.; Vacca, A. Investigation of equilibria in solution. Determination of equilibrium constants with the HYPERQUAD suite of programs. *Talanta*, **1996**, *43*, 1739-1753. DOI: 10.1016/0039-9140(96)01958-3
55. Cushnie, T.P.T.; Cushnie, B.; Echeverría, J.; Fowsantear, W.; Thammawat, S.; Dodgson, J.L.A.; Law, S.; Clow, S.M. Bioprospecting for antibacterial drugs: a multidisciplinary perspective on natural product source material, bioassay selection and avoidable pitfalls. Pharm. Res. 2020, 37, 125. DOI: 10.1007/s11095-020-02849-1
56. Zharkova, M.S.; Orlov, D.S.; Golubeva, O.Y.; Chakchir, O.B.; Eliseev, I.E.; Grinchuk, T.M.; Shamova, O.V. Application of Antimicrobial Peptides of the Innate Immune System in Combination With Conventional Antibiotics—A Novel Way to Combat Antibiotic Resistance? *Front. Cell. Infect. Microbiol.* **2019**, *9*, 128. DOI: 10.3389/fcimb.2019.00128.
57. Pankey, G.A.; Sabath, L.D. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-Positive bacterial infections. *Clin. Infect. Dis.* **2004**, *38*, 864-870. DOI: 10.1086/381972
58. Carton Herrán, J.D. Actividad antifúngica de la combinación de fluconazol con otros fármacos: Un enfoque terapéutico alternativo de las candidiasis. PhD Thesis, Facultad de Medicina y Enfermería, Universidad del País Vasco, Leioa (Vizcaya), 2018.
59. Reis De Sá, L.F.; Toledo, F.T.; De Sousa, B.A.; Gonçalves, A.C.; Tessis, A.C.; Wendler, E.P.; Comasseto, J.V.; Dos Santos, A.A.; Ferreira-Pereira, A. Synthetic organotelluride compounds induce the reversal of Pdr5p mediated fluconazole resistance in *Saccharomyces cerevisiae*. *BMC Microbiol.* **2014,** *14*, 1–9. DOI: 10.1186/s12866-014-0201-y.
60. Fleeman, R.; LaVoi, T.M.; Santos, R.G.; Morales, A.; Nefzi, A.; Welmaker, G.S.; Medina-Franco, J.L.; Giulianotti, M.A.; Houghten, R.A.; Shaw, L.N. Combinatorial libraries as a tool for the discovery of novel, broad-spectrum antibacterial agents targeting the ESKAPE pathogens. *J. Med. Chem.* 2015, *58*, 3340–3355. DOI: 10.1021/jm501628s 26.
61. Clothier, R.; Gómez-Lechón, M.; Kinsner-Ovaskainen, A.; Kopp-Schneider, A.; O'Connor, J.E.; Prieto, P.; Stanzel, S. Comparative analysis of eight cytotoxicity assays evaluated within the ACuteTox Project. *Toxicology in vitro* **2013**, *27*, 1347–1356. DOI: 10.1016/j.tiv.2012.08.015