Increased antibacterial properties of indoline-derived phenolic Mannich bases

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Abstract

The search for antibacterial agents for the combat of nosocomial infections is a timely problem, as antibiotic-resistant bacteria continue to thrive. The effect of indoline substituents on the antibacterial properties of aminoalkylphenols was studied, leading to the development of a library of compounds with minimum inhibitory concentrations (MICs) as low as 1.18 µM. Two novel aminoalkylphenols were identified as particularly promising, after MIC and minimum bactericidal concentrations (MBC) determination against a panel of reference strain Gram-positive bacteria, and further confirmed against 40 clinical isolates (*Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis*, *E. faecium*, and *Listeria monocytogenes*). The same two aminoalkylphenols displayed low toxicity against two *in vivo* models (*Artemia salina* brine shrimp and *Saccharomyces cerevisiae*). The *in vitro* cytotoxicity evaluation (on human keratinocytes and human embryonic lung fibroblast cell lines) of the same compounds was also carried out. They demonstrated a particularly toxic effect on the fibroblast cell lines, with IC_{50} in the 1.7-5.1 µM range, thus narrowing their clinical use. The desired increase in the antibacterial properties of the alkylaminophenols, particularly indoline-derived phenolic Mannich bases, was reached by introducing an additional nitro group in the indolinyl substituent or by the replacement of a methyl by a bioisosteric trifluoromethyl substituent. Notably, the introduction of an additional nitro moiety did not confer added toxicity to the alkylaminophenols.

Keywords: antibacterials; alkylaminophenols; Gram-positive; nosocomial infections
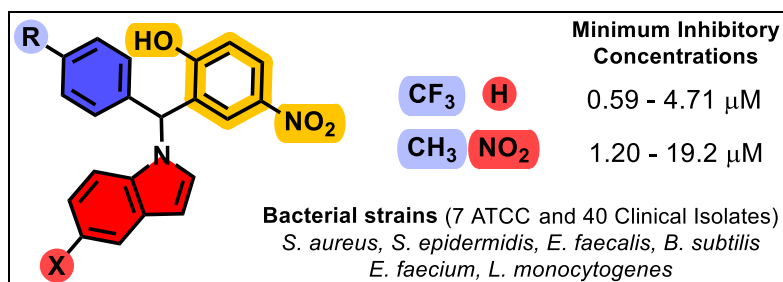
1. Introduction

The emergence of antimicrobial-resistant bacteria is a matter of concern for public health. The increased rate of bacterial resistance allied with the rather slow development of new antibacterial agents foreshadows a crisis. The actual global antibacterial clinical pipeline is composed of many agents that are modifications of existing classes of antibiotics.[1] Notwithstanding the efforts done by the scientific community to address the WHO´s Global Action Plan on Antimicrobial Resistance,[2] especially in tackling Gram-negative bacteria, more antibacterials and of narrow scope are necessary.[3] ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are the leading cause of nosocomial infections worldwide.[4,5] From these pathogens, *E. faecium* and *S. aureus* are Gram-positive, and second-generation glycopeptides such as dalbavancin and oritavancin or oxazolidinone linezolid have been described as antibacterial agents against infections by methicillin-resistant *S. aureus* (MRSA)[6,7] and vancomycin-resistant enterococci (VRE).[5,8] Many recent developments have been done towards the treatment of multidrug-resistant Gram-positive pathogens, however, the anticipated fast evolution of resistance makes searches for new antibacterials a timely challenge.[9-11]

S. aureus, *E. faecalis*, *E. faecium* and *S. epidermidis* cause important nosocomial or healthcare-associated infections, a major cause of mortality and morbidity worldwide.[12] *Enterococcus* spp. is frequently isolated from the surgical site and bloodstream infections, but rarely found in the respiratory tract.[13] On the other hand, *S. aureus* is the primary cause of lower respiratory tract and surgical site infections, and the second leading cause of nosocomial bacteremia, pneumonia and cardiovascular infections. Coagulase-negative staphylococci (CoNS) (especially *S. epidermidis*) are isolated almost twice as often as *S. aureus* in bloodstream infections. Hospital-acquired listerioses are often-fatal foodborne outbreak infections affecting mainly pregnant and immunocompromised patients, whose sources are difficult to identify.[14,15]

Besides the patient's underlying condition, several factors contribute to the success of these nosocomial pathogens. For instance, the widespread use of broad-spectrum antibiotics promotes the emergence and re-emergence of difficult-to-treat MDR strains, with VRE, MRSA, vancomycin-resistant *S. aureus* (VRSA) and methicillin-resistant *S. epidermidis* (MRSE) on the top of the list.[16,17] Pathogens' persistence on surfaces in the hospital environment, water system, or foreign body devices (such as catheters, implants, vascular grafts, intravenous devices, respiratory equipment, prostheses, etc.) is also a determinant factor for their success. Moreover, the capacity of these pathogens to form biofilms on inert surfaces that are highly resistant to antibiotic treatments and host immune response[18,19] [20] contributes even more for their persistence and dissemination in the health care setting.

The ability of phenolic Mannich bases to interact with living organisms[21] has been documented in several reports, biological properties such as antibacterial[22] and antitumoral,[23-28] to name a few, have been described. We have previously reported the antibacterial properties of a family of aminoalkylphenols in which the indoline amine counterpart and a *para*-nitrophenol group were deemed important in conferring antimicrobial properties (Figure 1). Tests against selected Gram-positive bacterial strains led to the identification of **1a** as a promising antibacterial agent.[29] In our follow-up work,[30] the potency of such derivatives was increased by the introduction of a chlorine substituent in the *para*-position of the phenyl ring (**2a**), thus lowering the minimum inhibitory concentration (MIC) to values as low as 1.23 µM for multidrug-resistant *S. aureus* and *E. faecalis*. While the influence of diverse substituents in both the phenol and phenyl rings has been evaluated in previous works, the effect of introducing different substituents on the aromatic ring of indoline was not assessed. In the present work, we compile our findings on this missing link to deepen our understanding of the structure-activity relationship of this underexplored class of antimicrobial agents.
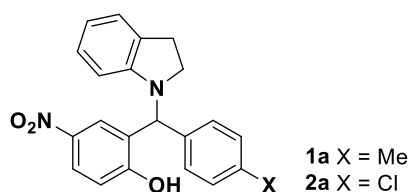

Figure 1. Indoline-derived phenolic Mannich bases previously identified[29,30] as antibacterials

2. Results and discussion

The compounds have been prepared as previously reported, using the multicomponent Petasis reaction[31,32] from 5-nitrosalicylaldehyde, substituted indolines **3**, and boronic acids **4**. Some indolines were obtained through the reduction of commercially available indoles with sodium cyanoborohydride or triethylsilane in trifluoroacetic acid. Specifically, sodium cyanoborohydride was used for the reduction of substituted indoles, being replaced by Et_3SiH and trifluoroacetic acid for deactivated indoles such as in the preparation of **3d** and **3e** (obtained in 96 and 70% yields, respectively). The condensation of 5-nitrosalicylaldehyde with indolines **3** in 1,2-dichloroethane, followed by reaction of the *in situ* formed iminium with the suitable boronic acid at 50 °C, provided the desired aminoalkylphenols **1**, **5**, and **6** in good to excellent yields. The procedure was successfully applied to the preparation of aminoalkylphenols derived from three different boronic acids: 4-tolyl (**4a**), 4-(trifluoromethyl)phenyl (**4b**) and 2-tolyl (**4c**) boronic acids providing aminoalkylphenols **1**, **5** and **6** respectively. All compounds were isolated by column chromatography, their purity assessed by ^{1}H NMR and their structures confirmed through NMR and HRMS characterization.

Scheme 1. Preparation of aminoalkylphenols studied
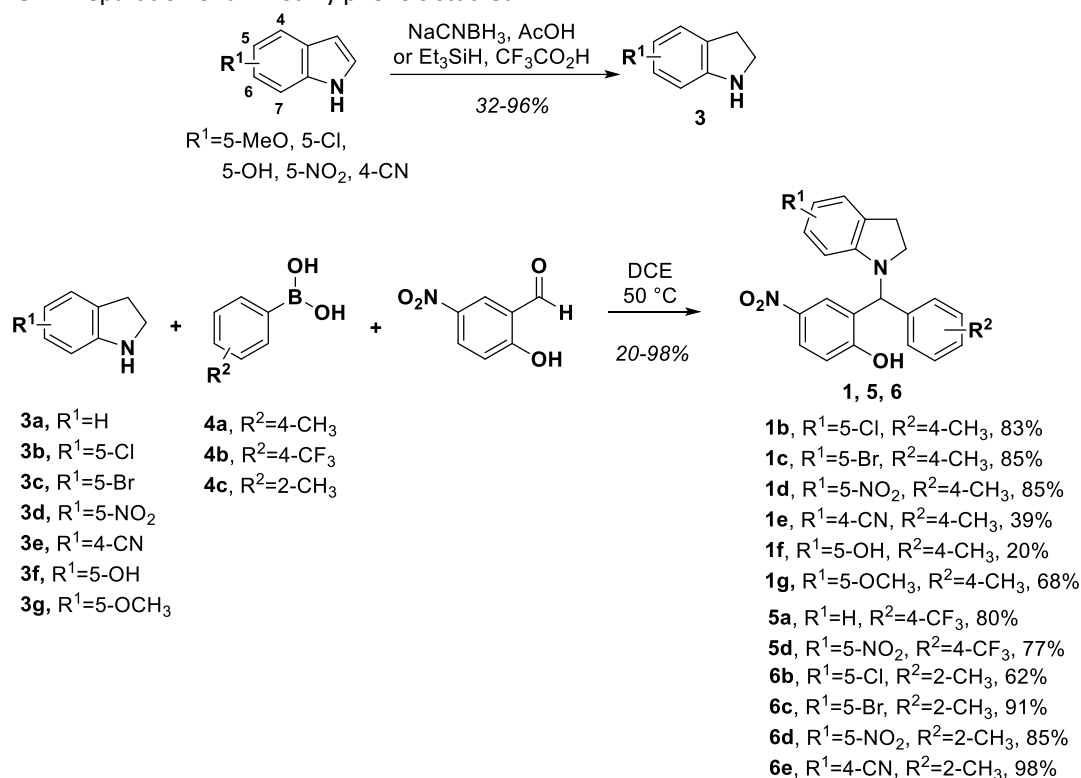
For the sake of comparison, the synthesized aminoalkylphenols were tested against a similar panel of Gram-positive microorganisms as for the previously reported compounds **1a** and **2a**.[30] Specifically, three *S. aureus* (the reference methicillin-sensitive ATCC 25923, the methicillin-resistant CIP106760, and the methicillin-sensitive ATCC 6538), two *E. faecalis* (the reference ATCC 29212 and the vancomycin-resistant ATCC 51299), a *Staphylococcus epidermidis* (the vancomycin-sensitive ATCC 12228), and a *Bacillus subtilis* (the ATCC 6633) strains were considered and the results of antibacterial activity presented in Table 1.

Table 1. Antimicrobial activity of aminoalkylphenols **1**, **2a**, **5,** and **6** against laboratory-adapted strains. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) are shown in µM.

Compd	*S. aureus* ATCC 25923 (MSSA)		*S. aureus* ATCC 6538		*S. aureus* CIP106760 (MRSA)		*E. faecalis* ATCC 29212		*E. faecalis* ATCC 51299 (VRE)		*S. epidermidis* ATCC 12228		*B. subtilis* ATCC 6633	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1a[a]	21.3	347	10.8	43.4	2.71	5.42	10.8	86.7	21.7	173	-[b]	-[b]	-[b]	-[b]
1b	2.30	36.8	-[b]	-[b]	1.15	4.59	1.15	9.19	2.30	9.19	75.4	37.7	37.7	151
1c	10.1	81.1	-[b]	-[b]	5.07	20.3	5.07	81.1	10.1	162	9.43	4.71	4.71	9.43
1d	1.20	38.6	-[b]	-[b]	1.20	4.82	1.20	9.64	2.41	154	18.9	37.7	18.9	37.7
1e	10.7	85.8	-[b]	-[b]	2.68	21.4	2.68	42.9	5.36	172	37.8	302	4.71	9.43
1f	67.7	542	-[b]	-[b]	67.7	108	135	108	135	108	37.8	302	2.4	>9.43
1g	20.0	160	20.0	80.0	10.0	40.0	10.0	160	20.0	160	<1.18	4.71	<1.18	4.71
2a[a]	2.47	9.86	1.23	2.47	1.23	<1.23	1.23	>9.86	1.23	19.7	-[b]	-[b]	-[b]	-[b]
5a	1.18	37.7	1.18	2.36	1.18	1.18	1.18	9.43	1.18	151	<1.18	<1.18	<1.18	<1.18
5d	2.22	35.6	-[b]	-[b]	1.11	1.11	1.11	8.89	4.45	142	<1.18	2.36	9.43	18.9
6b	2.47	39.6	1.24	4.95	1.24	2.47	1.24	9.89	2.47	158	<1.18	2.36	<1.18	4.71
6c	2.22	35.6	1.11	4.45	1.11	1.11	1.11	8.89	4.45	142	<1.18	<1.18	<1.18	<1.18
6d	1.20	38.6	2.41	9.64	1.20	4.82	1.20	9.64	2.41	154	<1.18	4.71	<1.18	2.36
6e	10.1	81.1	5.07	20.3	5.07	20.3	5.07	81.1	10.1	162	2.36	18.9	4.71	18.9
CP[c]	5.40	-[b]	1.35	-[b]	<1.50	-[b]	3.07	-[b]	<5.83	-[b]	1.95	-[b]	1.95	-[b]

[a] Previously reported data[30]; [b] not tested; [c] CP – positive control (Vancomycin).

Notwithstanding the moderate to good minimum inhibitory concentrations previously determined for **1a**, the new 4-tolyl derivatives **1b-g** shown similar to better antibacterial properties. The presence of the nitro group in 5-position of the indolinyl makes it particularly active and compound **1d** resulted in MICs as low as 1.20 µM. On the other hand, the presence of an oxygen atom in the same 5-position of indolinyl had a detrimental effect on the antibacterial properties. Therefore, compounds **1f** and **1g**, decorated with a hydroxy or a methoxy in the 5-positions of indolinyl were generally the least active in the series of 4-tolyl containing aminoalkylphenols **1**. A bioisoster replacement of the methyl group by a trifluoromethyl substituent increased the bacterial growth inhibition as observed for compounds **5a** and **5d**. Despite the higher antibacterial effect of 5-nitro in **1d** and 4-CF$_3$ in **5a** when compared with **1a**, the presence of both substituents in **5d** did not confer significantly better antibacterial properties than **5a**. Moving the methyl substituent from the 4- to the 2-position had little or no effect on the MICs of *S. aureus* or *E. faecalis* strains but inhibit considerably the growth of *S. epidermidis* and *B. subtilis*. Similar levels of growth inhibition were observed for 4-tolyl and 2-tolyl containing aminoalkylphenols **1** and **6.** Among the indolinyl substituents, the presence of chlorine and nitro in the heterocycle has a clear positive effect in increasing the inhibition properties, as observed for **1b**, **1d**, **6b,** and **6d**. Apart from the VRE strain tested, compounds **5a, 5d**, and **6b-d** were in general stronger bactericides than compounds **1**.

Considering that phenol compounds may exhibit antioxidant properties, scavenging the excessive amount of free radicals accumulated in the course of microbial infections and that can lead to cellular damage,[33,34] we wonder if a relation between the electronic character of the indolinyl substituents and such property could be established. Therefore, the antioxidant ability of compounds **1a-1f** was investigated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Figure 2). Indeed, reduced antioxidant activity was observed for highly deactivated indolinyl derivatives **1d** (5-nitro substituted) and **1e** (4-nitrile substituted) in 8% and 19%, respectively. On the other hand, unsubstituted **1a**, halides substituted **1b** and **1c,** and hydroxy substituted **1f** indolinyl derivatives showed high scavenging properties, with antioxidant activities higher than 80%. Despite the determined relationship between the electronic properties of the indolinyl substituents and the ability of the studied alkylaminophenols to scavenge free radicals, the antioxidant property does not correlate with the antibacterial effect observed. This corroborates our previous observations, suggesting a mechanism of action that does not involve the scavenging of free radicals.[30]
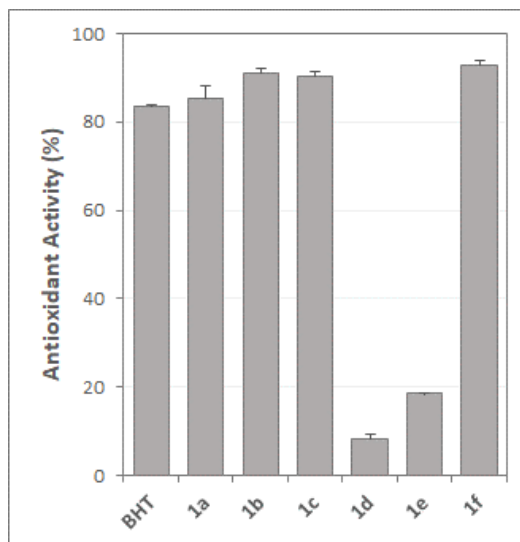
Figure 2. Antioxidant activity of **1a-f** by DPPH method. Concentrations of 10 µg/mL (22-26 µM) of each compound were subjected to free radical scavenging test. The change in optical density of DPPH was monitored at 517 nm. Butylated hydroxytoluene (BHT) was used as the reference standard. Results are reported as percentage mean values ± SD, based on three independent experiments.

In order to disclose potential applications of the newly synthesized compounds as antibacterial agents, the most active ones, namely **5a**, **5d**, and **6b-d** were considered for further studies regarding their biocompatibility and safety. The same properties were also determined for **1d** for the sake of comparison with compounds **5d** and **6d**, as the three molecules contain a nitro group at the 5-position of the indoline moiety, which might confer additional toxicity.[35]

Over the past decades, toxicity testing in pharmaceutical development has been centered on animal models.[36,37] However, besides expensive and laborious, these models cannot always be translated into the human *in vivo* responses and, more recently, have reverberated greatly social and ethical dilemmas.[38] Therefore, the use of alternative models at early phases of drug development is advised. In the present study, we select three distinct approaches to screen the general toxicity of our compounds, namely: *i)* an invertebrate animal model (*Artemia salina* brine shrimp); *ii)* a unicellular fungal model (*Saccharomyces cerevisiae*), and; *iii)* two *in vitro* human cell culture models (a keratinocyte cell line and embryonic lung fibroblast cell line). In contrast to the conventional animal models, these approaches are easy to follow, rapid, cost-effective, and have been increasingly used to screen the general toxicity in a broad spectrum of substances (such as synthetic chemicals, heavy metals, natural products, or engineered nanomaterials), constituting a convenient starting point to prioritize only the best candidates for further screening of vertebrates.[38-42]

Due to their simple anatomy, brief life-cycle, and small size, which allow large-scale screenings,[39] we started by evaluating the lethality of each compound on the *Artemia salina* brine shrimp (Figure 3). Compared with the previously studied compounds **1a** and **2a**,[30] we were pleased to observe that the newly developed molecules exhibited less toxicity in *A. salina* than **1a**, and were also generally less toxic than **2a**. Indeed, with exception of both **1d** and **5d**, the remaining compounds promoted a larvae lethality rate below the 15% observed for **2a**, with **6d** displaying a toxicity level similar to that seen for the artificial seawater solution (ca 3%). The most potent antibacterial agent prepared in this study (**5a**) showed very little toxicity against *A. salina* (ca. 8%), even at a concentration more than one order of magnitude higher than the MICs determined for the several strains. The brine shrimp mortality rates observed after treatment with the 5-nitro indolinyl derivatives **1d**, **5d**, and **6d** points out this last compound as being considerably less toxic. The different mortality rates observed for these three compounds indicate that the level of toxicity (even if residual) shall not be attributed to the presence of the second nitro group.
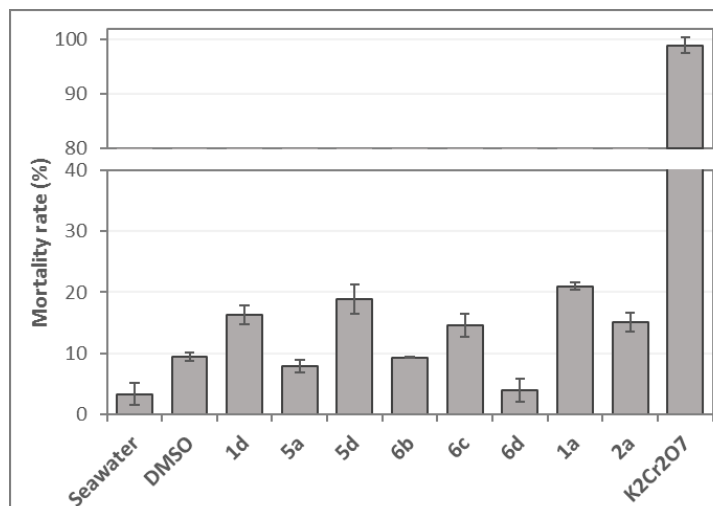
Figure 3. General toxicity of **1d**, **5a**, **5d,** and **6b-d** in the mortality rate of *A. salina* brine shrimp model. Concentrations of 10 µg/mL (22-28 µM) of each compound were tested in an artificial seawater solution (NaCl 3.5%) with 1% of dimethylsulfoxide (DMSO, v/v). Potassium dichromate ($K_2Cr_2O_7$) at 10 mg/mL was used as positive toxic control, while both artificial seawater and DMSO (1%) were used as negative controls. The previously studied compounds **1a** and **2a**[30] were included for comparative purposes. The number of dead larvae was recorded after 24 hours of exposure and used to calculate the lethal concentration (%). Results are reported as mean values ± SD, based on three independent experiments.

On the other hand, the yeast *S. cerevisiae* is one of the most popular and widely used eukaryotic models. Besides its rapid growth and ease of replica plating, it can be easily manipulated to evaluate multiple biological effects (inhibition of cellular growth, cytotoxicity and genotoxicity) induced by the drugs considered.[40,43] According to our results (Figure 4), all tested compounds showed no relevant general toxicity (IC_{50} > 100 µM) when compared to the positive control Nystatin (IC_{50} = 3.31 µM). Overall, the obtained IC_{50} values ranged from 121 µM for **6d** to 292 µM for **5d**, with the less active compounds (**5d**, **1a**, and **6c**) exhibiting, on average, IC_{50} values ~2-fold higher than the others (**1d**, **5a**, **6b**, **6d** and **2a**). Moreover, the MIC values of **1d** (1.18 – 18.9 µM) and **5a** (up to 1.18 µM) are 1-2 orders of magnitude lower (161.8 and 124.3 µM, respectively) than the IC_{50} values determined.
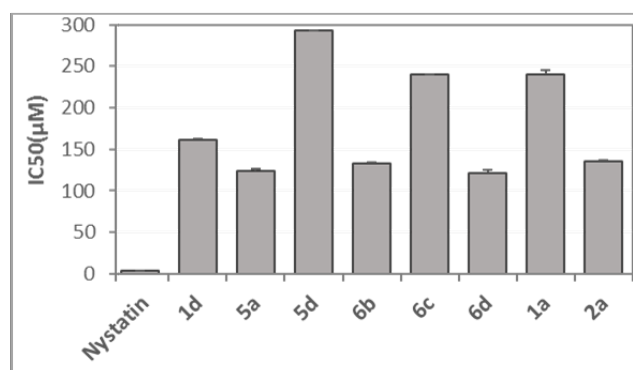
Figure 4. General toxicity of **1d**, **5a**, **5d,** and **6b-d** in *Saccharomyces cerevisiae* model. The graph depicts the concentration at which each substance induced inhibition of 50% (IC_{50}) in *S. cerevisiae* viability. Nystatin was used as the positive control. The previously studied compounds **1a** and **2a**[30] were included for comparative purposes. Results are expressed as mean IC_{50} values ± SD, based on three independent experiments.

Despite both organism-based methods being commonly used for initial screening of general toxicity, different toxicity trends were observed for the two species. For instance, among the most active antibacterial compounds tested, *A. salina* was found to be more sensitive to **5d**, which was revealed to be the least active compound in *S. cerevisiae*. On the other hand, *S. cerevisiae* was mostly sensitive to **6d**, the least toxic compound seen for *A. salina*. Considering the different characteristics of each organism, both methods hold limitations that may underline the observed toxicity disparities. For instance, it is known that, in the brine shrimp lethality bioassay, there may be a decrease in the solubility of some chemical substances in the saline medium producing false positives due to the

toxicity of the solvent itself.[39,44] Notwithstanding that no ultimate conclusions on the general toxicity of these compounds can be drawn from these protocols, the information collected is valuable in assessing the viability of this family of phenolic Mannich bases in the development of new drugs.

Finally, to unveil some of the potential applications these compounds might have, cytotoxicity was further characterized by using two widely used *in vitro* cell models of human origin: the HaCat immortalized epidermal keratinocyte line and the MRC-5 embryonic lung fibroblast line. While the former has proved to help evaluate the mechanisms of the cytotoxic and pharmacological action of various agents on the skin,[45] MRC-5 cells are a suitable human lung cell model.[46]

Figure 5 shows the viability of HaCat human keratinocytes after 24 h exposure at 50 μM of each compound under evaluation. Overall, the cytotoxic potential observed on the HaCat cells was highly heterogeneous among compounds, with cell viability ranging from 3% for **6b** (the same magnitude level as the 5% DMSO positive control) to 94% for **1d** (similar to that exhibited by non-treated cells). Among 2-tolyl analogs **6**, compound **6d** was the least cytotoxic to HaCat cells, exhibiting a cytotoxic potential 5.5- and 17.6-fold lower than **6c** and **6b**, respectively. The presence of a halide in the indolinyl moiety is the apparent cause for the increased cytotoxicity to **6b** and **6c** of the 2-tolyl series.

By comparing the 4-tolyl derivative **1d** and its isomer **5d** becomes evident that the cytotoxic disparities observed on HaCat viability may also rely on the influence of the substituent on the phenyl ring (Scheme 1), as the simple exchange of methyl from the 4- to the 2-positions has a prominent increasing effect on the toxicity. Moreover, likewise previously seen in the *A. salina* model (Figure 3), the additional presence of the 5-nitro substituent in **5d** seems to confer higher toxicity in HaCat cells, when compared to its trifluoromethyl analog **5a** (Figure 5). The most effective antibacterial, **1d**, and **5a** showed medium to almost none toxicity for HaCat cells, respectively, with **5a** displaying a cytotoxic potential 0.6-fold lower than that presented by **1d**. Notably, this represents a significant improvement when comparing with the previously studied compound **1a**,[29] as 20% cell viability was observed at a 28 μM concentration of that compound. The replacement of the methyl substituent by trifluoromethyl ameliorates the toxicity profile of the compound (*i.e.* **1a** vs **5a**) while also greatly increasing its antibacterial properties.
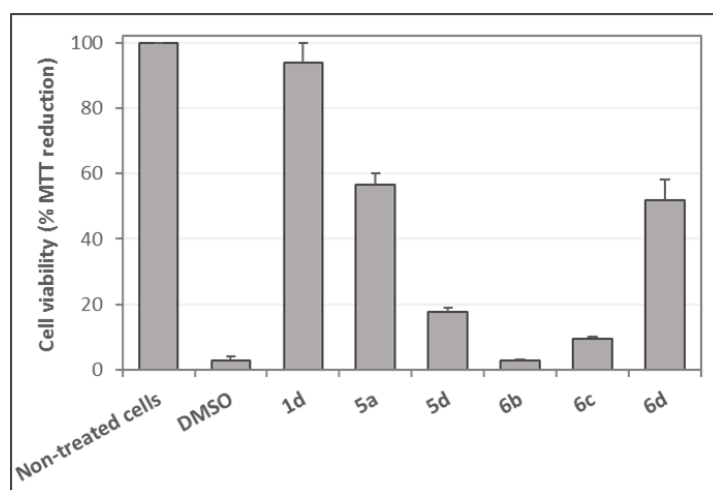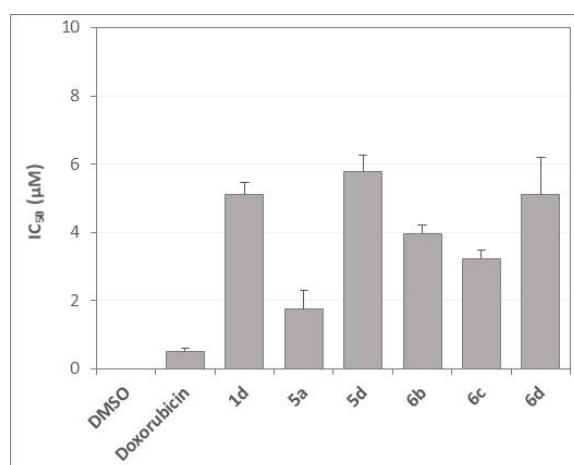

Figure 5. Effect of the synthesized aminoalkylphenols derivatives **1d**, **5a**, **5d,** and **6b-d** on the viability of HaCat human keratinocytes, evaluated by the MTT reduction assay. Cells were treated with each compound at a final concentration of 50 μM for 24h and then submitted to the MTT colorimetric assay. Dimethylsulfoxide (DMSO 5%) was used as the toxic positive control. Results (reported as mean ± SD) are expressed as percentages relative to non-treated control cells and are based on two independent experiments, each comprising four replicate cultures.

In contrast to that seen for HaCat cells, the cytotoxicity activity of the synthesized aminoalkylphenols derivatives under evaluation was more homogenous and efficacious on MRC-5 cells (Figure 6), with mean IC_{50} values ranging from 1.7 μM for **5a** to 5.8 μM for **5d**. Surprisingly, **1d**, **5d,** and **6d**, the three molecules containing a nitro group at the 5-position of the indoline moiety, exhibited the lowest toxic effect to MRC-5 cells. Among the 2-tolyl analogs **6**, compound **6d** was the least toxic, followed by **6b** and **6c**. Despite **5a** being found to be the most toxic to MRC-5 cells, it shown an IC_{50} value only 0.3-

fold lower than the other most effective antibacterial compound **1d**. Generally, the compounds considered for tests on MRC-5 were observed to have IC_{50} values higher than the concentration required to inhibit visible bacterial growth. While less cytotoxic than doxorubicin, the similarity between the determined MICs and IC_{50} values hamper the clinical use of these compounds in treating lower respiratory infections.

Figure 6. IC_{50} of synthesized aminoalkylphenols derivatives **1d**, **5a**, **5d**, and **6b-d** on MRC-5 human embryonic lung fibroblast cell line. The cell viability was measured by MTT. Doxorubicin was used as a positive toxic control. IC_{50} values (reported as mean ± SD) are based on 2 independent experiments, each comprising 4 replicate cultures.

Notwithstanding the lack of a possibility to draw clear conclusions on the toxicity of the compounds tested, as no trends could be established with the toxicity models considered, compounds **1d** and **5a**, having the strongest antibacterial properties were not particularly toxic against *A. salina*, *S. cerevisiae,* and HaCat. The high and consistent toxicity of the set of compounds tested on MRC-5 can indicate that the class of alkylaminophenols is generally toxic for these cell lines. Nevertheless, motivated by the very good bacterial growth inhibitory properties of **1d** and **5a** (previously performed with reference strains), we extended the antibacterial assays of such molecules against a larger collection of Gram-positive bacterial strains with dissimilar resistance phenotypes, including MRSA, VRE, and multidrug-resistant (MDR) strains. Overall, 40 strains from *S. aureus*, *S. epidermidis*, *E. faecalis*, *E. faecium*, and *Listeria monocytogenes* (eight clinical isolates of each species) were tested (Figure 7 and Supplemental Table 1)
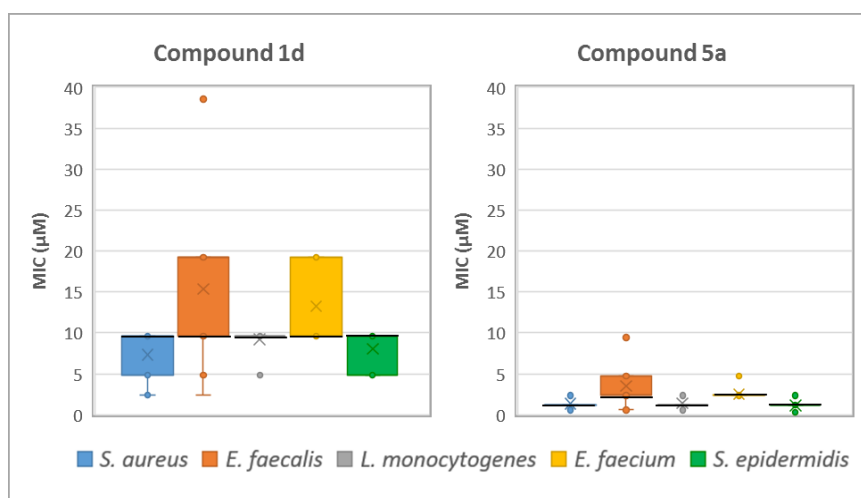
Figure 7. MIC values of compounds **1d** and **5a** for diverse clinical isolates of *S. aureus*, *E. faecalis*, *L. monocytogenes*, *E. faecium,* and *S. epidermidis*. Boxplot chart was generated using R statistical software v. 3.4.2 and consists of boxes (median and interquartile range) and whiskers that extend to the most extreme data points that were no more than 1.5 times the interquartile range from the box. Boxes represent the variability of MIC values found among all strains from each species. The horizontal black line marks the median, while the cross ("x") within each box represents the medium MIC value. The lowest and the highest coverage values observed

are represented by the extremes of the whisker below and above each box, respectively. Outliers are indicated by filled circles.

Similarly, to what was previously observed for the ATCC strains, compound **5a** revealed, in general, a higher and homogeneous antibacterial effect against all clinical isolates than compound **1d.** Indeed, lower median MIC values of 4-fold for *E. faecium* and 8-fold for the remaining species (Figure 7) were determined for **5a**, contrasting to the milder effects determined for **1d**. The median MIC values displayed by compound **5a** ranged from 1.18 µM for *S. aureus*, *S. epidermidis* and *L. monocytogenes* to 2.36 µM for both *Enterococcus* species, while compound **1d** exhibited median MICs of 9.64 µM for all species' clinical isolates except for *E. faecalis*, for which the median MIC was 19.27 µM. Moreover, whereas compound **5a** exhibited comparable MIC values between the ATCC adapted strains and clinical isolates of both *Staphylococcus* species and *E. faecalis*, compound **1d** revealed median MIC values 2-fold higher for the *S. epidermidis* ATCC adapted strains, but at least an 8-fold lower difference for *E. faecalis* clinical isolates. Such intra-species discrepancies observed for compound **1d** may be due to: *i)* the dissimilar genetic background of the clinical isolates, *ii)* to the low number of ATCC strains tested, and *iii)* to the extensive *in vitro* passaging of ATCC strains.

3. Conclusions

In summary, this work presents the preparation of new phenol Mannich bases, easily prepared from the multicomponent Petasis reaction, the assessment of their antibacterial properties, as well as their toxicity *in vivo* (on *A. salina* and *S. cerevisiae*) and *in vitro* (on keratinocyte and embryonic lung fibroblast cell lines).

This work corroborates the previous observations that this class of compounds is generally effective against multi-resistant Gram-positive bacteria. The introduction of different substituents in the indoline moiety contributed to a significant change in the antibacterial properties of previously reported analogs. The presence of an additional nitro substituent at the 5-indolinyl position conferred additional antibacterial activity and decreased the antioxidant activity. Despite the noticeable effect of the indoline's substituents on the antioxidant activity, a lack of correlation between the antibacterial properties of aminoalkylphenols and such property was also corroborated.

Apart from the increased toxic effect observed by **5d** on HaCat cell lines, the 5-nitroindolinyl derivatives did not show increased toxicity when compared with other analogs. Moreover, 5-nitroindolinyl derivative **1d** was determined to have increased antibacterial properties while not significantly increasing its toxicity to the eukaryotic systems considered. The installation of a trifluoromethyl substituent in the *para*-position of the aromatic ring also led to the improvement of the antibacterial properties. The 5-nitroindolinyl and trifluoromethylated derivatives (**1d** and **5a**, respectively) are efficient growth inhibitors of the five Gram-positive bacterial strains tested (*S. aureus*, *S. epidermidis*, *E. faecalis*, *E. faecium*, and *L. monocytogenes*). In particular, the antibacterial efficacy of compound **5a** translates into MIC's lower than 5 µM for tested reference strains and clinical isolates. While these compounds demonstrated trivial toxicity on the *in vivo* models tested, they exerted a toxic effect on normal human embryonic lung fibroblast cells indicating that the *in vivo* use of the compounds may be limited. Nevertheless, given the excellent antibacterial properties of these compounds and the urgent need to find new chemical entities toxic to pathogenic microorganisms, efforts are being carried out to find a suitable system for their delivery on living organisms, understand their mechanism of action, and evaluate their toxicity in other cell lines presents an opportunity for further research.

4. Materials and methods

4.1 Synthesis

4.1.1. General Remarks

All reagents were obtained from Sigma-Aldrich or TCI and were used without further purification. Reactions were performed under argon atmosphere and monitored by thin-layer chromatography carried out on precoated (Merck TLC silica gel 60 F254) aluminum plates by using UV light as a visualizing agent and cerium molybdate solution or ninhydrin as developing agents. Flash column

chromatography was performed on silica gel 60 (Merck, 0.040–0.063 mm). NMR spectra were recorded with Varian Mercury 300 MHz or JEOL ECZR 500 instruments using $CDCl_3$, DMSO-d_6, or methanol-d_4 as solvents and calibrated using tetramethylsilane as an internal standard. Chemical shifts (δ) are reported in ppm referenced to the non-deuterated residual peak (δ 7.26 for $CDCl_3$, δ 2.50 for DMSO-d_6 and δ 3.31 for MeOH-d_4) or TMS peak (δ 0.00) for ^{1}H NMR. The same approach was used for 13C NMR, and the chemical shifts are referenced to the non-deuterated residual peak (δ 77.16 for $CDCl_3$, δ 39.50 for DMSO-d_6, and δ 49.00 for MeOH-d_4). The following abbreviations were used to describe peak splitting patterns: s = singlet, d = doublet, t = triplet, m = multiplet. Coupling constants, *J*, were reported in hertz. High-resolution mass spectra were recorded on a Waters ESI-TOF MS spectrometer. All compounds tested for antibacterial activity were established to be >95% pure upon NMR analysis.

4.1.2 Preparation of indolines **3**
All indolines, except indoline (**3a**) and 5-bromoindoline (**3c**) which were obtained commercially, were synthesized by reducing commercially available indoles with sodium cyanoborohydride (Method A) or triethylsilane (Method B).
Method A: Acetic acid (10 mL) was added to a flask charged with sodium cyanoborohydride (3 mmol, 3 equiv.) and indole (1 mmol, 1 equiv.) at 0 °C, under argon. The partially frozen mixture was stirred fast for 5 minutes and moved to ambient temperature. The reaction was stirred until TLC indicated completion of the reaction. Acetic acid was evaporated under reduced pressure and the residue was dissolved in 15 mL of DCM. The resulting mixture was washed with 15 mL of saturated aqueous Na_2CO_3 and the aqueous layer from the washing was extracted with 3 x 5 mL of DCM. Organic extracts were combined, dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography to obtain the desired indoline.
Method B: Triethylsilane (2.6 mmol, 2.6 equiv.) was added dropwise to a stirred suspension of indole (1 mmol, 1 equiv.) in 1.5 mL of trifluoroacetic acid at ambient temperature, under argon. The reaction was heated to 50 °C and stirred until TLC indicated completion of the reaction. Trifluoroacetic acid was evaporated under reduced pressure and the residue was diluted with 5 mL of DCM. The resulting mixture was washed with 5 mL of saturated aqueous $NaHCO_3$ and the aqueous layer from the washing was extracted with 2 x 5 mL of DCM. Organic extracts were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography to obtain the desired indoline.
5-chloroindoline (**3b**): Method A provided the titled compound in 89% yield after 3 h reaction time, with similar spectral data to previously reported.[47] ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.99 (d, J=1.8 Hz, 1H), 6.88 (dd, J=2.3, 8.2 Hz, 1H), 6.46 (d, J=8.2 Hz, 1H), 3.50 (t, J=8.2 Hz, 2H), 2.94 (t, J=8.5 Hz, 2H).
5-nitroindoline (**3d**): Method B provided the titled compound in 96% yield after 30 min reaction time, with similar spectral data to previously reported.[48] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (dd, J=2.3, 8.8 Hz, 1H), 7.94 (s, 1H), 6.47 (d, J=8.8 Hz, 1H), 4.53 (br. s, 1H), 3.76 (t, J=8.8 Hz, 2H), 3.12 (t, J=8.8 Hz, 2H).
Indoline-4-carbonitrile (**3e**): Method B provided the titled compound in 70% yield after 30 min reaction time, with similar spectral data to previously reported.[49] ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.05 (t, J=7.7 Hz, 1H), 6.83 (d, J=7.4 Hz, 1H), 6.72 (d, J=8.0 Hz, 1H), 6.02 (br. s, 1H), 3.51 (td, J=8.6, 1.1 Hz, 2H), 3.08 (t, J=8.6 Hz, 2H).
Indolin-5-ol (**3f**)**:** Sodium cyanoborohydride (189 mg, 3 mmol, 3 equiv.) was added in small portions to a stirred solution of 1H-indol-5-ol (133 mg, 1 mmol, 1 equiv.) in 10 mL of acetic acid at ambient temperature, under argon. The reaction was stirred for 80 minutes and the acetic acid was evaporated under reduced pressure. The residue was diluted with 2.5 mL of hexane, 2.5 mL of DCM, and finally basified with 3 mL of triethylamine. Solvents and the remaining triethylamine were evaporated under reduced pressure and the residue was suspended in 10 mL of diethyl ether. The suspension was filtered through a celite pad and the pad was washed with 2x10 mL of Et_2O. Filtrates were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography to obtain the title compound with 32% yield, with similar spectral data to previously reported.[50] ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.59 (s, 1H), 6.50 (d, J=8.2 Hz, 1H), 6.43 (dd, J=2.3, 8.2 Hz, 1H), 4.53 (br. s, 3H), 3.46 (t, J=8.2 Hz, 2H), 2.92 (t, J=8.5 Hz, 2H).

5-methoxyindoline (**3g**): Method A provided the titled compound in 82% yield after 1 h reaction time, with similar spectral data to previously reported.[51] ^{1}H NMR (300 MHz, CDCl$_3$) δ 6.69 (s, 1H), 6.52-6.52 (m, 2H), 3.67 (s, 3H), 3.46 (t, J=8.2 Hz, 2H), 3.40 (br. s, 1H), 2.93 (t, J=8.5 Hz, 2H).

4.1.3 Preparation of Phenol Mannich bases **1, 5** and **6**

General procedure: Aldehyde (0.5 mmol, 1 equiv.) and arylboronic acid (0.5 mmol, 1 equiv.) were dissolved in 5 mL of DCE and heated to 50 °C. After stirring for 5 minutes, indoline (0.5 mmol, 1 equiv.) was added in one portion. Stirring was continued until TLC indicated completion of the reaction. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography to obtain the desired aminoalkylphenol.

2-((5-Chloroindolin-1-yl)(p-tolyl)methyl)-4-nitrophenol (**1b**): Obtained in 83% yield as an off-white solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 11.39 (br. s, 1H), 8.10 (dd, J=2.6, 9.1 Hz, 1H), 7.95 (d, J=2.9 Hz, 1H), 7.28 (d, J=8.2 Hz, 2H), 7.12-7.18 (m, 3H), 6.95 (d, J=8.8 Hz, 2H), 6.38 (d, J=8.2 Hz, 1H), 5.29 (s, 1H), 3.20-3.27 (m, 1H), 3.09 (q, J=9.6 Hz, 1H), 2.84-3.00 (m, 2H), 2.34 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 162.39, 148.82, 140.83, 138.89, 134.63, 134.33, 129.87, 128.56, 127.27, 126.48, 125.17, 124.73, 117.61, 113.00, 70.06, 53.57, 28.24, 21.11. HRMS (ESI/TOF): m/z calcd for C$_{22}$H$_{19}$ClN$_2$O$_3^+$ [M]$^+$, 394.1079; found 394.1061.

2-((5-Bromoindolin-1-yl)(p-tolyl)methyl)-4-nitrophenol (**1c**): Obtained in 85% yield as a pale yellow solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 11.30 (br. s, 1H), 8.10 (dd, J=2.9, 8.8 Hz, 1H), 7.95 (d, J=2.9 Hz, 1H), 7.26-7.30 (m, 3H), 7.16-7.18 (m, 2H), 7.10 (dd, J=2.1, 8.5 Hz, 1H), 6.96 (d, J=8.8 Hz, 1H), 6.34 (d, J=8.8 Hz, 1H), 5.30 (s, 1H), 3.20-3.27 (m, 1H), 3.05-3.14 (m, 1H), 2.85-3.01 (m, 2H), 2.34 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 162.35, 149.31, 140.86, 138.92, 134.72, 134.56, 130.20, 129.88, 128.58, 128.00, 126.44, 125.19, 124.73, 117.62, 114.62, 113.49, 69.88, 53.47, 28.18, 21.12. HRMS (ESI/TOF): m/z calcd for C$_{22}$H$_{19}$BrN$_2$O$_3^+$ [M]$^+$, 438.0574; found 438.0571.

4-Nitro-2-((5-nitroindolin-1-yl)(p-tolyl)methyl)phenol (**1d**): Obtained in 85% yield as an orange red solid. ^{1}H NMR (500 MHz, CDCl$_3$) δ 8.17 (s, 1H), 8.13 (dd, J = 8.9, 2.6 Hz, 1H), 7.98 (d, J = 2.9 Hz, 1H), 7.91-7.94 (m, 2H), 7.18 (q, J = 8.6 Hz, 4H), 7.03 (d, J = 9.2 Hz, 1H), 6.25 (d, J = 8.6 Hz, 1H), 6.02 (s, 1H), 3.44-3.54 (m, 2H), 3.10 (t, J = 8.6 Hz, 2H), 2.36 (s, 3H). ^{13}C NMR (126 MHz, CDCl$_3$) δ 160.38, 156.40, 141.22, 138.88, 138.53, 133.55, 130.90, 129.84, 128.07, 126.58, 126.42, 125.51, 125.15, 120.80, 116.65, 106.05, 60.25, 51.24, 26.96, 21.11. HRMS (ESI/TOF): m/z calcd for C$_{22}$H$_{19}$N$_3$O$_5^+$ [M]$^+$, 405.1319; found 405.1302.

1-((2-Hydroxy-5-nitrophenyl)(p-tolyl)methyl)indoline-4-carbonitrile (**1e**): Obtained in 39% yield as a pale yellow solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 10.37 (br. s, 1H), 8.12 (dd, J=2.9, 8.8 Hz, 1H), 7.98 (d, J=2.9 Hz, 1H), 7.27 (d, J=7.6 Hz, 2H), 7.19 (d, J=8.2 Hz, 2H), 7.05-7.14 (m, 2H), 6.98 (d, J=8.8 Hz, 1H), 6.62 (dd, J=1.8, 7.0 Hz, 1H), 5.41 (s, 1H), 3.31-3.39 (m, 1H), 3.09-3.27 (m, 3H), 2.35 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 161.73, 151.07, 141.10, 139.11, 136.66, 134.11, 130.01, 128.58, 128.52, 126.29, 125.33, 124.81, 124.32, 117.60, 117.13, 115.42, 109.14, 68.59, 52.81, 27.93, 21.13. HRMS (ESI/TOF): m/z calcd for C$_{23}$H$_{19}$N$_3$O$_3^+$ [M]$^+$, 385.1421; found 385.1425.

1-((2-Hydroxy-5-nitrophenyl)(p-tolyl)methyl)indolin-5-ol (**1f**): Obtained in 20% yield as an orange solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.09 (dd, J=2.9, 9.4 Hz, 1H), 7.93 (d, J=2.9 Hz, 1H), 7.29 (d, J=8.2 Hz, 2H), 7.15 (d, J=8.2 Hz, 2H), 6.94 (d, J=9.4 Hz, 1H), 6.69 (d, J=2.3 Hz, 1H), 6.45 (dd, J=2.9, 8.8 Hz, 1H), 6.29-6.36 (m, 1H), 5.17 (s, 1H), 3.16-3.23 (m, 1H), 2.96-3.05 (m, 1H), 2.79-2.94 (m, 2H), 2.33 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 163.04, 151.63, 143.67, 140.57, 138.73, 135.29, 134.48, 129.78, 128.53, 126.91, 125.06, 124.66, 117.59, 113.58, 113.35, 112.60, 71.63, 53.95, 28.64, 21.12. HRMS (ESI/TOF): m/z calcd for C$_{22}$H$_{19}$N$_2$O$_4^-$ [M-H]$^-$, 375.1350; found 375.1343.

2-((5-Methoxyindolin-1-yl)(p-tolyl)methyl)-4-nitrophenol (**1g**): Obtained in 68% yield as a pale yellow solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.01 (dd, J=2.3, 8.8 Hz, 1H), 7.87 (d, J=2.9 Hz, 1H), 7.22 (d, J=8.2 Hz, 2H), 7.08 (d, J=7.6 Hz, 2H), 6.87 (d, J=8.8 Hz, 1H), 6.69 (d, J=2.3 Hz, 1H), 6.46 (dd, J=2.9, 8.8 Hz, 1H), 6.32 (d, J=8.8 Hz, 1H), 5.12 (s, 1H), 3.65 (s, 3H), 3.10-3.16 (m, 1H), 2.84-2.96 (m, 1H), 2.75-2.81 (m, 2H), 2.26 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 163.03, 155.85, 143.71, 140.61, 138.70, 135.34, 134.26, 129.79, 128.53, 126.93, 125.04, 124.65, 117.59, 113.19, 111.86, 111.68, 71.61, 55.65, 53.99, 28.73, 21.12. HRMS (ESI/TOF): m/z calcd for C$_{22}$H$_{19}$N$_2$O$_3^+$ [M]$^+$, 390,1574; found 390.1574.

2-(Indolin-1-yl(4-(trifluoromethyl)phenyl)methyl)-4-nitrophenol (**5a**): Obtained in 80% yield as a pale yellow solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.13 (dd, J=2.6, 9.1 Hz, 1H), 7.95 (d, J=2.9 Hz, 1H), 7.56-7.66 (m, 4H), 7.20 (d, J=7.0 Hz, 1H), 6.92-7.05 (m, 3H), 6.49 (d, J=7.6 Hz, 1H), 5.42 (s, 1H), 3.19-3.26 (m, 1H),

2.93-3.08 (m, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 162.46, 149.89, 141.95, 140.95, 132.23, 131.27, 129.07, 127.63, 126.29, 126.24, 126.20, 126.14, 125.94, 125.49, 125.11, 124.55, 122.70, 117.91, 112.24, 70.07, 53.79, 28.47. HRMS (ESI/TOF): m/z calcd for $C_{22}H_{18}F_3N_2O_3^+$ [M+H]$^+$, 415.1264; found 415.1286.

4-Nitro-2-((5-nitroindolin-1-yl)(4-(trifluoromethyl)phenyl)methyl)phenol (**5d**)**:** Obtained in 77% yield as an orange red solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.11 (dd, J=2.6, 9.1 Hz, 1H), 7.89-7.91 (m, 3H), 7.60 (d, J=8.2 Hz, 2H), 7.37 (d, J=8.2 Hz, 2H), 6.94 (d, J=8.8 Hz, 1H), 6.21 (d, J=9.4 Hz, 1H), 6.01 (s, 1H), 3.36-3.43 (m, 2H), 3.07 (t, J=8.8 Hz, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 159.96, 156.12, 141.45, 141.13, 139.18, 130.61, 128.46, 126.40, 126.15, 125.89, 125.77, 125.17, 120.93, 116.65, 105.75, 59.44, 51.12, 27.04. HRMS (ESI/TOF): m/z calcd for $C_{22}H_{15}F_3N_3O_5^-$ [M-H]$^-$, 458.0969; found 458.0962.

2-((5-Chloroindolin-1-yl)(o-tolyl)methyl)-4-nitrophenol (**6b**)**:** Obtained in 62% yield as a pale yellow solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.11 (dd, J=2.6, 9.1 Hz, 1H), 7.87 (d, J=2.9 Hz, 1H), 7.35 (d, J=7.0 Hz, 1H), 7.13-7.23 (m, 4H), 6.93-6.98 (m, 2H), 6.27 (d, J=8.2 Hz, 1H), 5.69 (s, 1H), 3.23-3.28 (m, 1H), 2.92-3.10 (m, 3H), 2.38 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 162.34, 149.10, 141.08, 136.21, 136.00, 133.68, 131.25, 128.65, 128.29, 127.42, 127.17, 126.84, 126.61, 125.20, 124.49, 117.60, 112.06, 110.00, 64.22, 53.19, 28.26, 20.46. HRMS (ESI/TOF): m/z calcd for $C_{22}H_{19}ClN_2O_3^+$ [M]$^+$, 394.1079; found 394.1096.

2-((5-Bromoindolin-1-yl)(o-tolyl)methyl)-4-nitrophenol (**6c**)**:** Obtained in 91% yield as an orange red solid. ^{1}H NMR (300 MHz, CDCl$_3$) δ 10.65 (br. s, 1H), 8.12 (dd, J=2.9, 8.8 Hz, 1H), 7.88 (d, J=2.9 Hz, 1H), 7.35 (d, J=7.6 Hz, 1H), 7.15-7.28 (m, 4H), 7.09 (dd, J=2.1, 8.5 Hz, 1H), 6.97 (d, J=8.8 Hz, 1H), 6.23 (d, J=8.8 Hz, 1H), 5.70 (s, 1H), 3.23-3.31 (m, 1H), 2.93-3.11 (m, 3H), 2.38 (s, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 162.22, 149.60, 141.11, 136.15, 136.03, 134.01, 131.25, 130.32, 128.66, 128.26, 128.01, 127.16, 125.20, 124.50, 117.54, 112.41, 63.82, 53.04, 28.20, 20.43. HRMS (ESI/TOF): m/z calcd for $C_{22}H_{20}BrN_2O_3^+$ [M+H]$^+$, 439.0652; found 439.0645.

4-Nitro-2-((5-nitroindolin-1-yl)(o-tolyl)methyl)phenol (**6d**): Obtained in 85% yield as a dark red solid. ^{1}H NMR (500 MHz, DMSO-d$_6$) δ 11.58 (s, 1H), 8.15 (dd, J=8.9, 2.6 Hz, 1H), 7.91 (dd, J=8.6, 2.3 Hz, 1H), 7.87 (d, J=1.1 Hz, 1H), 7.71 (d, J=2.9 Hz, 1H), 7.27 (d, J=4.0 Hz, 2H), 7.21-7.24 (m, 1H), 7.15 (d, J=7.4 Hz, 1H), 7.08 (d, J=8.6 Hz, 1H), 6.19 (s, 1H), 6.17 (d, J=9.2 Hz, 1H), 3.37-3.48 (m, 3H), 3.06-3.16 (m, 2H), 2.15 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d$_6$) δ 161.92, 156.10, 139.67, 137.09, 136.27, 136.12, 130.93, 130.47, 128.02, 126.74, 126.50, 126.37, 125.86, 125.81, 124.35, 120.28, 115.85, 103.80, 54.66, 50.67, 26.25, 18.81. HRMS (ESI/TOF): m/z calcd for $C_{22}H_{18}N_3O_5^-$ [M-H]$^-$, 404.1252; found 404.1221.

1-((2-Hydroxy-5-nitrophenyl)(o-tolyl)methyl)indoline-4-carbonitrile (**6e**): Obtained with 98% yield as a pale yellow solid. ^{1}H NMR (300 MHz, Methanol-d$_4$) δ 8.10 (dd, J=2.6, 9.1 Hz, 1H), 7.96 (d, J=2.3 Hz, 1H), 7.14-7.22 (m, 4H), 6.95-7.01 (m, 2H), 6.79 (d, J=7.6 Hz, 1H), 6.22 (d, J=8.2 Hz, 1H), 6.06 (s, 1H), 3.40 (q, J=8.8 Hz, 1H), 3.25-3.33 (m, 1H), 3.12-3.18 (m, 2H), 2.23 (s, 3H). ^{13}C NMR (75 MHz, Methanol-d$_4$) δ 162.84, 153.10, 141.85, 138.44, 137.82, 135.75, 131.83, 129.58, 128.87, 128.80, 128.50, 127.25, 126.10, 125.95, 120.22, 118.68, 116.28, 111.02, 109.14, 57.13, 51.71, 28.58, 19.45. HRMS (ESI/TOF): m/z calcd for $C_{23}H_{19}N_3O_3^+$ [M]$^+$, 385.1421; found 385.1427.

4.2 Antibacterial Assays

4.2.1 Determination of MIC

For selective purposes, the antimicrobial activity of the diverse compounds was preliminarily tested against seven bacterial Gram-positive strains obtained from American Type Culture Collection (ATCC): *S. aureus* ATCC 25923 (MSSA), *S. aureus* ATCC 6538 (MSSA), *S. aureus* CIP106760 (MRSA), *E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299 (VRE), *S. epidermidis* ATCC 12228 and *B. subtilis* ATCC 6633 strains. For the most promising compounds (i.e., **1d** and **5a**), the antimicrobial activity was further evaluated against eight distinct clinical isolates of *S. aureus*, *S. epidermidis E. faecalis*, *E. faecium,* and *L. monocytogenes* from the wide collection of pathogenic Gram-positive strains of the Portuguese National Institute of Health (NIH) (Supplementary Table 1).

Before each experiment, frozen stocks of all isolates were subcultured three times to check strain viability and to avoid any negative growth effect from congelation. *S. aureus*, *S. epidermidis*, *E. faecalis*, *E. faecium* and *B. subtilis* were subcultured in Mueller-Hinton (MH) agar at 35 ºC whereas *L. monocytogenes* was subcultured in MH agar supplemented with 5% defibrinated horse blood and 20mg/L β-NAD (MH-F) at 35ºC in 5% CO_2.[52]

For each compound, the minimum inhibitory concentration (MIC) was determined by the broth dilution method,[53] according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.[52]

Twofold serial dilutions of the concentrated stock compound solution (1 mM) were prepared in the required growth medium for each bacterial species into a 96-well plate. For ATCC strains, vancomycin was added as the positive control. Cation-Adjusted Mueller Hinton Broth (CAMHB, BD BBL) was used for *S. aureus*, *S. epidermidis*, *E. faecalis*, *E. faecium*[52], and *B. subtilis* while CAMHB supplemented with 20mg/L β-NAD was used for *L. monocytogenes*. For all bacterial species, inocula of 1.5×10^8 CFU/ml (one for each strain/isolate) were prepared by direct colony saline–phosphate-buffered saline (PBS) suspension, equivalent to a 0.5 McFarland standard, using colonies from the respective overnight agar plates. A 1:20 dilution of each prepared bacterial inoculum in the appropriate broth medium was subsequently used. Controls without the compound and bacterial inocula were also prepared. Plates were incubated for 16 to 24h at 35°C (and with 5% CO_2 for *L. monocytogenes*). Purity check and colony or viable cell counts of the inoculum suspensions were also evaluated in order to ensure that the final inoculum density closely approximates the intended number. This was obtained by subculturing a diluted aliquot from the growth control-well (without compound) immediately after inoculation onto a suitable nonselective agar plate for simultaneous incubation. The MIC was determined as the lowest compound concentration at which no visible growth was observed. The bacterial growth was measured with an absorbance microplate reader (Thermo Scientific Multiskan FC, Loughborough, UK) set to 600 nm. Assays were carried out in triplicate for each ATCC strain and in sextuplicate for each isolate.

4.2.2 Determination of MBC

After MIC assessment, MBC was also evaluated for each ATCC strain. Briefly, the bacterial suspension on the wells was homogenized, serial diluted, triplicate spread on appropriate medium, and incubated. The MBC attributed to the compound concentration resulting in a 99.9% reduction in bacterial numbers. All assays were carried out in triplicate for each tested microorganism.

4.3 Toxicity evaluation

4.3.1 Brine Shrimp Lethality Bioassay (Artemia salina)

For the most active antibacterial agents (**1d**, **5a**, **5d**, and **6b-d**), the toxicity was evaluated using the *Artemia salina* model, as previously described.[54] Each compound was tested at a fixed concentration of 10 ppm in artificial seawater (3.5% NaCl solution) with 1% DMSO (v/v). Potassium dichromate ($K_2Cr_2O_7$) at 10mg/mL was used as positive toxic control, while individual artificial seawater and DMSO (1%) were included as negative controls. After 24h of treatment, the number of survived larvae was recorded and the mortality rate (%) was determined using the following equation:

$$Mortality\ rate\ (\%) = \frac{Total_{A.\ salina} - Alive_{A.\ salina}}{Total_{A.\ salina}}\ x\ 100\% \qquad (1)$$

where,
$Total_{A.\ salina}$ = the total number of larvae in the assay
$Alive_{A.\ salina}$ = the number of surviving *A. salina* larvae in the assay
The assay was carried out in triplicate for each tested compound.

4.3.2 Saccharomyces cerevisiae Bioassay

General toxicity studies using *Saccharomyces cerevisiae* (ATCC 2601) model were performed in a microdilution method adapted from a previous report.[54] Briefly, a volume of 100 μL was added to all the wells containing Sabouraud culture medium, and 100 μL of the samples at a concentration of 1 mg/mL were added to the first well. Serial dilutions were made in a proportion of 1:2 and 10 μL of yeast suspension was added to each well. The plates were incubated for 24 h at 37 °C and Optical Density at 525 nm was measured using a Microplate Reader (Thermo Scientific Multiskan FC,

Loughborough, UK). Nystatin (NYS) was used as positive control and the assays were carried out in triplicate. Inhibition of cell growth was determined according to the formula:

$$IC_{50} = 100 - \left[\frac{OD_{\text{ sample}} - OD_{\text{ medium control}}}{OD_{\text{ cell control}} - OD_{\text{ medium control}}}\right] x\ 100 \qquad (2)$$

4.3.3 MTT reduction assay
4.3.3.1 Cytotoxicity in HaCat cells
The cytotoxicity assessment in the human keratinocyte HaCat cell line was carried out using the colorimetric MTT assay, as previously described.[55] Briefly, 5 x 10^3 cells/well were seeded in 200 μL of culture medium (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin in 96-well plates, and incubated at 37°C under an atmosphere with 5% CO_2 in air.
After 24h, cells were exposed to each compound at a final concentration of 50 μM for a 24h-period. All compounds were initially solubilized in DMSO and then further diluted in PBS, so that the final concentration of DMSO in culture medium was 0.5% in all samples, except for the positive control (DMSO 5% (v/v)). After treatment, cells were washed with culture medium, followed by the addition of MTT (0.5 mg/mL). The cells were then incubated for a further 2.5h period and carefully washed with PBS, prior to the DMSO addition (200 μL/well) to solubilize the purple formazan crystals resultant from the MTT reduction by mitochondrial enzymes in metabolically active cells. The MTT reduction (proportional to the number of viable cells) was measured using an absorbance microplate reader (Thermo Scientific Multiskan FC, USA) set to 595 nm. Two independent experiments were performed, each comprising four replicate cultures. Cytotoxicity results (mean ± SD) were expressed as percentages relative to non-treated control cells.

4.3.3.2 Cytotoxicity in MRC-5 cells
The effects of increasing concentrations of each compound on MRC-5 cell (ATCC CCL-171) growth were tested in 96-well flat-bottomed microtiter plates. The cell line was purchased from LGC Promochem (Teddington, UK) and was cultured in Eagle's Minimal Essential Medium (EMEM) supplemented with non-essential amino acid mixture, a selection of vitamins, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1mM Na-pyruvate, nystatin and penicillin-streptomycin mixture (Sigma-Aldrich, USA) in concentrations of 100 U/L and 10 mg/L, respectively. The cell line was incubated at 37°C, in a 5% CO_2, 95% air atmosphere. Each compound was diluted in 100 μL of EMEM medium. Then, 1.5×10^4 cells in 100 μL of medium were added to each well, except for the medium control wells. The culture plates were further incubated at 37°C for 24h, prior to the addition of 20 μL of MTT (Sigma-Aldrich, Spain) (from a 5 mg/mL stock solution) to each well. After incubation at 37°C for 4 h, 100 μL of sodium dodecyl sulfate solution (Sigma-Aldrich, Spain) (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37°C overnight. Cell growth was determined by measuring the optical density (OD) at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader. Inhibition of cell growth was determined according to formula (2) (see section 4.3.2). Results are expressed in terms of IC_{50}, defined as the inhibitory dose that reduced the growth of the cells exposed to the tested compounds by 50%, and presented as an average of 2 independent experiments and 4 replicates.

4.4 Antioxidant Activity Evaluation (DPPH method)
The antioxidant activity of compounds **1d** and **5a** was tested as previously described.[30] Briefly, a concentration of 10 μg/mL (22-26 μM) of each compound was subjected to the free radical scavenging test (the DPPH method). The change in optical density (OD) of DPPH radicals was monitored at 517 nm against a corresponding blank. The antioxidant activity was calculated using the following equation:

$$Scavenging\ activity\ (\%) = \frac{OD_{\text{ control}} - OD_{\text{ sample}}}{OD_{\text{ control}}} x\ 100 \qquad (3)$$

The reference standard used for this procedure was butylated hydroxytoluene (BHT). Tests were carried out in triplicate.

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