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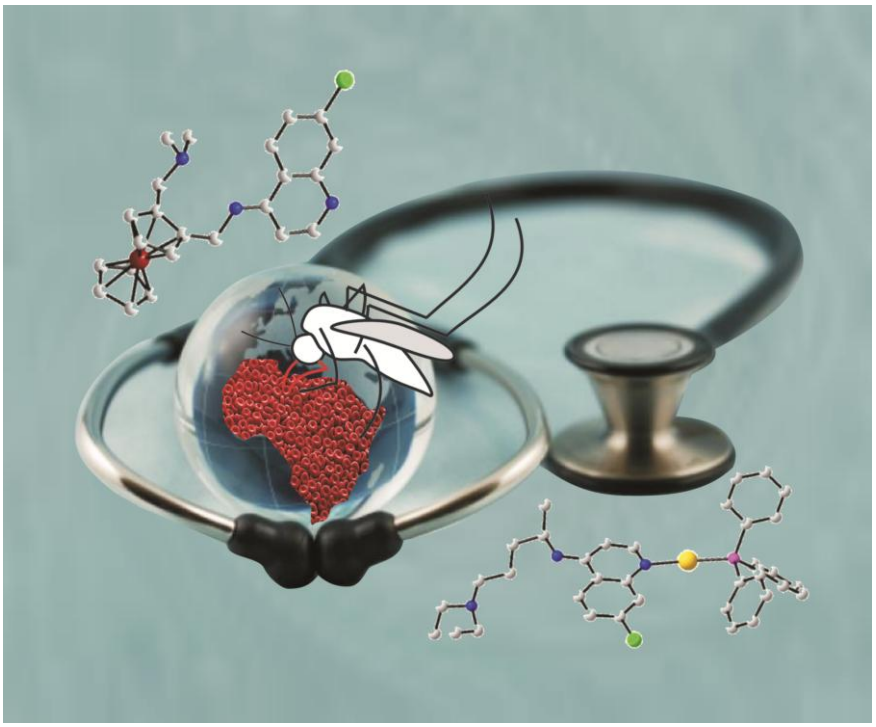

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PAPER

In vitro inhibitory properties of ferrocene-substituted chalcones and aurones on bacterial and human cell cultures

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Two series of ten chalcones and ten aurones, where ferrocene replaces the C ring and with diverse substituents on the A ring were synthesized. The compounds were tested against two antibiotic-sensitive bacterial strains, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, and two antibiotic-resistant strains, *S. aureus* SA-1199B and *S. epidermidis* IPF896. The unsubstituted compound and those with methoxy substitution showed an inhibitory effect on all bacterial strains at minimum inhibitory concentrations ranging between 2 and 32 mg L^{-1}. For four of these compounds, the effect was bactericidal, as opposed to bacteriostatic. The corresponding organic aurones did not show growth inhibition, underscoring the role of the ferrocene group. The methoxy-substituted aurones and the unsubstituted aurone also showed low micromolar (IC_{50}) activity against MRC-5 non-tumoral lung cells and MDA-MB-231 breast cancer cells, suggesting non-specific toxicity.

Introduction

Bacterial infection and disease is a ubiquitous health hazard and despite having clinically efficacious antibiotics the issue of bacterial resistance poses a strong necessity for the design and development of novel antibacterial agents. In 2004, methicillin-resistant *Staphylococcus aureus* (MRSA) was one of the most common causes of nosocomial infections accounting for more than 60% of *S. aureus* infections in intensive care units in the USA,[1] and from 40% to 70% in India.[2] Of particular concern, these nosocomial MRSA infections commonly have reduced susceptibility to classes of antibiotics other than the β-lactams. Furthermore, the rising incidence of community-associated MRSA is alarming since infections have occurred in otherwise healthy individuals with no known health risks.[3] There is thus a critical need for new therapeutics to combat these evolving pathogens. In the search for biologically active compounds, one potential strategy is to look for unnatural molecules. These are less likely to induce bacterial resistance than biosynthetized molecules like some penicillins or streptogramins, providing their medical use remains under control, as shown by the worldwide spread of fluoroquinolone resistance.

Aurones, 2-benzylidenebenzofuran-3(*2H*)-ones, are naturally occurring yellow-colored flavonoids, structurally isomeric to the more well-studied flavones. Besides their role in pigmentation, aurones have also been found to possess variety of biological properties, some of which have been recently reviewed in the literature.[4] Aurones play a primarily protective role in plants, possessing insect antifeedent,[5] antiparasitic[6] and antifungal activity.[7] In humans, there is interest in developing aurones for anticancer[8,9] and antileishmanial[10,11] applications.

Aurones have demonstrated *in vitro* antibacterial activity and some structural aspects have been identified by structure–activity relationship (SAR) studies. Recently, QSAR analysis of a class of (*Z*)-2-(5-nitrofuran-2-yl-methylene)-3(*2H*)-benzofuranones (Chart 1, **1a**) identified the benzofuranic core as a key structural feature contributing to antibacterial properties,[12] and a recent publication concluded that *Z* stereochemistry around the double bond is preferred.[13] The modification of ring A has also been demonstrated to influence the antibacterial activity of aurones. For example, compounds in series **1a** possessing electron-donating groups showed better antibacterial activity than those with electron-withdrawing groups.[12] In another study, minimum

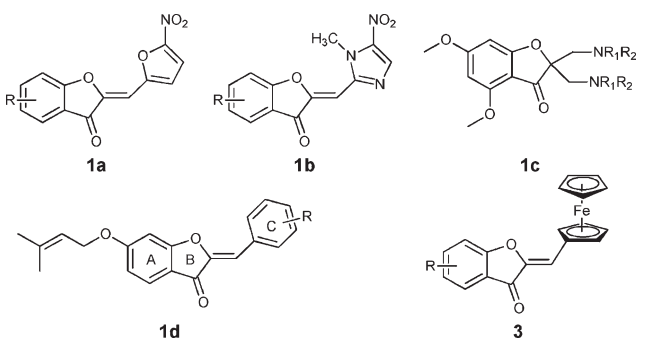

Chart 1 Aurones from the literature showing antibacterial activity (**1a–1d**); ferrocenyl aurones studied in this report (**3**).

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inhibitory concentration (MIC) values ranged between 0.8 to >100 mg L^{-1} against Gram positive bacteria in a series of substituted (*Z*)-2-arylidene-3(*2H*)-benzofuranones[14] possessing 1-methyl-5-nitroimidazole (Chart 1, **1b**). On the other hand, the effect of replacement of the phenyl group C by another function cannot be simply generalized. For instance, for 2,2-bisaminomethylated aurone analogues (Chart 1, **1c**) the activity varied only by a factor of 2 within the series.[7] However, in the case of series **1b**, the changing of ring C from a 5-nitroimidizole to a 4-nitroimidazole completely suppressed the antibacterial properties of the former.[14] Increased lipophilicity also may improve antibacterial activity; prenyloxy-substituted aurones (Chart 1, **1d**)[15] showed enhanced activity probably due to better interaction with cellular membranes.

Our group is interested in how the modification of natural products or drugs with organometallic units can influence their biological properties. Ferrocene, in particular, has gained considerable interest as a substituent in medicinal chemistry due to its stability in aqueous and aerobic media, its lipophilicity, the ease of substitution and its redox activity. The literature on ferrocene in medicinal chemistry is vast and diverse; highlights include work on cancer[16] and malaria.[17] Some of the first applications of ferrocene in medicinal chemistry were in the context of antibacterial studies, when Edwards *et al.* demonstrated an improvement in the therapeutic spectrum of penicillin and cephalosporin by ferrocene substitution.[18] Similarly, ferrocene derivatized penem antibiotics showed increased activity *vs.* the standard faropenem.[19] Ferrocene itself, as well as (dimethylaminomethyl)ferrocene have shown moderate inhibitory activities against *E. coli* ATCC 25922.[20] In other work, ferrocene-substituted tamoxifen derivatives,[21] pyrazoles[22] antibiotics[23] and acylhydrazones[24] have been studied for their antibacterial effects.

Taking into consideration the antibacterial properties of aurones and the often favorable effects of ferrocene substitution, we reasoned that aurones, where the phenyl group C is replaced by ferrocene, may show promising antibacterial properties, particularly against antibiotic-resistant strains. As part of our ongoing project studying ferrocene-modified biomolecules, we have recently obtained access to such ferrocenyl aurones in high yields.[25,26] We here report the synthesis of a series of ten ferrocene aurones and their chalcone precursors and their antibacterial effects on two antibiotic-sensitive bacterial cell strains, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, and two resistant strains, *S. aureus* SA-1199B and *S.epidermidis* IPF 896 as compared to selected organic aurones. To judge their utility for further development in animals, we also tested selected compounds' cytotoxicity against MRC-5 non-tumoral lung cells and MDA-MB-231 breast cancer cells.

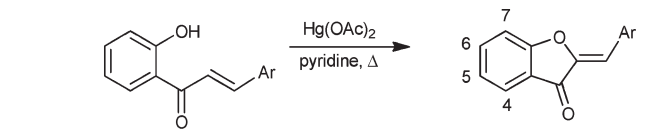
Results and discussion

Synthesis

Ferrocene chalcones **2a–j** were obtained by a standard Claisen-Schmidt condensation of ten different hydroxyacetophenones and ferrocenecarboxaldehyde with NaOH in refluxing EtOH, as previously reported.[27] All the chalcones were found to have an *E* configuration based on their ^{1}H NMR coupling constants ($J \approx$ 15 Hz). Treatment of **2a–j** with mercury(II) acetate in pyridine at 80 °C for 3–5 h cleanly yielded **3a–j** in good yield (60–80%) after purification on silica gel. The configuration of the aurones was found to be *Z* as confirmed by 2D NMR studies along with the X-ray crystallographic characterization of **3b**.[26] The organic analogs of **3a** and **3g–j** were similarly obtained in 70–80% yield after purification on silica gel.[13] In all cases, a single *Z* geometric isomer was obtained as confirmed by proton and carbon NMR spectra and IR spectra.[28]

Antibacterial activities

Ferrocenyl chalcones **2a–j**, ferrocenyl aurones **3a–j** and the organic aurones **5a**, **5g–j** were tested against four bacterial strains (Table 1). Two strains are antibiotic-sensitive: *Escherichia coli* ATCC 25922, a Gram negative strain, and *Staphylococcus aureus* ATCC 25923, a Gram positive strain. Two laboratory



Ar = Fc **2a-j** **3a-j**
Ph **4a, 4g-j** **5a, 5g-j**

Scheme 1 Synthesis of aurones from precursor chalcones; **a**: unsubstituted; **b**: 5-Cl; **c**: 5-Br; **d**: 5,7-diF; **e**: 5,7-diCl; **f**: 5,7-diBr; **g**: 6-OMe; **h**: 5-OMe; **i**: 4-OMe; **j**: 4,6-diOMe.

Table 1 MIC values for **2a–j**, **3a–j** and **5a**, **5g–j** against *E. coli*, *S. aureus and S. epidermidis* strains

	Minimum Inhibitory Concentration (MIC, mg L^{-1})[a]			
	E. coli ATCC 25922	*S. aureus* ATCC 25923	*S. aureus* SA-1199B	*S. epidermidis* IPF 896
2a	>32	>32	>32	>32
2b	>32	>32	>32	>32
2c	>32	>32	>32	>32
2d	>32	>32	>32	>32
2e	>32	>32	>32	>32
2f	>32	>32	>32	>32
2g	>100[b]	>100[b]	>100[b]	n.d.[c]
2h	>32	>32	>32	>32
2i	>100[b]	>100[b]	>100[b]	n.d.[c]
2j	>100[b]	>100[b]	>100[b]	n.d.[c]
3a	32	8–16	8–16	8–16
3b	>32	>32	>32	>32
3c	>32	>32	>32	>32
3d	>32	>32	>32	>32
3e	>32	>32	>32	>32
3f	>32	>32	>32	>32
3g	8	2–4	2–4	4
3h	32	8–32	8–32	16–32
3i	8	2–8	4–8	4–8
3j	8	2–8	4–8	4–8
5a	>32	>32	>32	n.d.[c]
5g	>32	>32	>32	n.d.[c]
5h	>32	>32	>32	n.d.[c]
5i	>32	>32	>32	n.d.[c]
5j	>32	>32	>32	n.d.[c]

[a] When only one value is given, results for the two experiments were identical. [b] Tests carried out using 10 mg mL^{-1} stock solution. [c] Not determined.

Table 2 Time kill assay: Reduction in pathogen concentration following a 24 h antibiotic exposure at the MIC[a]

	$\log_{10}$ CFU/mL (t = 0) − $\log_{10}$ CFU/mL (t = 24 h)				
	3a	**3g**	**3h**	**3i**	**3j**
E. coli ATCC 25922	>3.5	>3.5	>3.7	>3.5	>3.5
S. aureus ATCC 25923	>3.8; 1.2 ± 0.1	1.5 ± 0.3;>3.8	>3.8	>3.8	2.9 ± 0.3
S. aureus SA-1199B	>3.9	1.2 ± 0; 3.4 ± 0.3	3.0 ± 0.0	>3.9	3.2 ± 0.2
S. epidermidis IPF896	>3.6	0.2 ± 0.3; >3.5	>3.5	3.5 ± 0.2	>3.6

[a] Molecules were tested at their MIC (from Table 1) in a time-kill endpoint assay. Bactericidal activity is defined as a 1000-fold or greater reduction in bacterial concentration, expressed as colony forming unit (CFU)/mL. When [$\log_{10}$ CFU/mL (t = 0) − $\log_{10}$ CFU/mL (t = 24 h)] ≥ 3, the compound is considered bactericidal. Experimental error is mentioned only for counting above the detection limit. When only one value is given, results for the two experiments were identical, except for **3h** where only one experiment was performed.

strains with known resistance mechanisms were also used. The fluoroquinolone resistant strain SA-1199B of *S. aureus* harbors the *norA* gene that encodes a membrane-associated protein that mediates the active efflux of fluoroquinolones.[29] *S. epidermidis* IPF 896 is resistant to erythromycin, by overexpressing Msr(A), an ATP-binding cassette (ABC) protein involved in macrolide efflux, which is one of the two most prevalent mechanisms of resistance to this widely used antibiotic in *Streptoccoci*. NorA, the most studied pump among the major facilitator superfamily efflux system, is involved in the so-called multidrug resistant (MDR) bacterial phenotype and exports an extensive range of structurally unrelated compounds while Msr(A) is specific to 14 and 15 membered macrolides and steptogramins B. Both efflux pumps are widely found in resistant *Staphyloccoci*.[30]

None of the ferrocenyl chalcones showed activity at the maximum concentration of 32 mg L^{-1} or even at 100 mg L^{-1} for **2g** and **2i–j**. This result suggests that the benzofuranic core is one of the key structural features contributing to antibacterial properties since, unlike the chalcones, some of the tested aurones efficiently inhibited bacterial growth. For the ferrocenyl aurones, the halogenated-substituted derivatives were inactive. However, the unsubstituted compound **3a** as well as all of the methoxy-substituted compounds **3g–j** showed significant activity, ranging from 2–32 mg L^{-1} against both *Staphyloccoci* and *E. coli*, Gram positive and Gram negative bacteria, respectively, demonstrating activity on a wide bacterial spectrum. The compounds were generally slightly more active on Gram positive bacteria and showed a similar activity against antibiotic-sensitive and antibiotic-resistant strains. A 10 mg L^{-1} MIC is generally recognized as a maximal value in the pharmaceutical industry for a valuable hit to be further developed as a potential drug. The methoxy substituted ferrocenyl aurones thus show an interesting potential in this regard.

It should be mentioned that these results are consistent with previous observations that electron-donating groups on ring A tend to enhance antibacterial effects.[12,14] Here, however, a position effect is noted; the unsubstituted ferrocenyl compound **3a** and that with methoxy substitution in position 5 (**3h**) exhibited lower activities (MIC = 8–32 mg L^{-1}) than those methoxy-substituted aurones in position 4 and/or 6 (**3g**, **3i**, **3j**, MIC = 2–8 mg L^{-1}). We recorded the redox potential for the ferrocenyl group on each of the molecules by cyclic voltammetry in CH_2Cl_2. The most active compounds, **3g**, **3i** and **3j** had the lowest $E_{1/2}$ (0.118, 0.125 and 0.103 ± 0.003 V *vs.* Fc^+/Fc respectively) compared to **3a** and **3h** (0.144 and 0.147 ± 0.003 V respectively), suggesting that a role of the redox properties of ferrocene in the inhibitory effects of the ferrocenyl aurones cannot be excluded.

In order to further assess the role of the ferrocenyl group in the antimicrobial activity, the organic aurones representative of the most active ferrocenyl derivatives were tested (Scheme 1). Their MIC was found to be higher than 32 mg L^{-1} against three bacterial strains (Table 1), demonstrating that the organometallic group is required for the antibacterial activity.

The antibacterial activities of the most active aurones **3a**, **3g–j**, were characterized using 24 h-time-kill assays (Table 2). Time-kill studies allow the quantification of bacterial death over a specified amount of time. This type of methodology has an advantage over traditional MIC testing in that it allows for typing the antibiotic activity as bactericidal or bacteriostatic. Bactericidal activity (99.9% kill) is defined as a ≥3-$\log_{10}$ colony forming unit (CFU)/mL reduction in colony count from the initial inoculum.[31] That is, after 24 h, if there is more than a 1000-fold reduction in bacterial CFU, the compound is considered bactericidal.

Bacterial counting after 24 h incubation showed that **3a** and **3h–j** were bactericidal against all strains at their MIC, having values of $\log_{10}$ CFU/mL greater than 3. In a first test, compound **3g** demonstrated bacteriostatic activity against Gram positive bacteria, while the compound was found to be bactericidal against *Staphylococci* in a second test. A similar situation for **3a** was observed for antibiotic-sensitive *S. aureus*. Such variability in the obtained results was clearly due to the fact that the time-kill assays were performed at the MIC, that is, at the lowest concentration where the compounds show inhibition of bacterial growth. The bactericidal activity evidenced for the aurone compounds is noteworthy from a clinical perspective. When treating severe infections, a rapid clearance of a pathogen may be necessary to achieve a better prognosis, and therefore a bactericidal agent is preferred.

Cytotoxic effects on MRC-5 and MDA-MB-231 cells

In order to evaluate the selectivity of the most active aurones against bacterial cell strains, compounds **3a**, **3g–3j** were evaluated against MRC-5 human fetal lung cells and estrogen receptor negative MDA-MB-231 breast carcinoma (Table 3). Cell growth inhibition was determined by the MTT test at 10^{-5} M and 10^{-6} M in triplicate, followed by the evaluation of the IC_{50} values at concentrations ranging from 100 to 0.005 μM on a 96-well plate. Stock solutions were freshly prepared 30 min before cell testing.

Table 3 Cell growth inhibition for non-tumoral and cancer cells for **3a** and **3g–j**

	MRC-5 IC_{50} (μM)	MDA-MB-231 IC_{50} (μM)
3a	3.5 ± 0.0	2.8 ± 0.5
3g	3.5 ± 0.1	4.1 ± 0.2
3h	1.0 ± 0.3	1.8 ± 0.0
3i	1.0 ± 0.3	1.1 ± 0.2
3j	3.7 ± 0.1	3.8 ± 0.1

All compounds showed almost complete inhibition of cell growth at 10^{-5} M against both cell lines, while no significant cell growth inhibition was observed at 10^{-6} M (data not shown). IC_{50} values differed only slightly between compounds, with IC_{50} values ranging from 1 to 4 μM. The difference between cell lines was even smaller (±1 μM), suggesting non-specific toxicity. It should be mentioned however that the SAR observed in the anti-bacterial tests was not observed here. Nonetheless, due to the relatively high value of the ratio MIC (μM)/IC_{50} (μM) of these compounds their future development as therapeutic agents appears to be therefore rather in jeopardy.

Experimental

General remarks

All reactions were carried out under argon. THF was distilled over sodium/benzophenone and all other chemical reagents and solvents were used as received without further purification. Silica gel chromatography was performed with Merck 60 (40–63 μm) silica. ^1H and ^{13}C NMR spectra were recorded with a 300 or 400 MHz Bruker Avance spectrometer, and δ are given in ppm and referenced to the residual solvent peaks (^1H, δ 7.26 and $^{13}C\{^1H\}$, δ 77.1 for $CDCl_3$ and ^1H, δ 2.05 and $^{13}C\{^1H\}$, δ 29.7 for acetone-d_6). Mass spectra were measured on a Thermo-scientific ITQ1100 spectrometer using the direct exposure probe method by the mass spectrometry service at the École Nationale Supérieure de Chimie de Paris. High resolution mass spectra were determined by ESI/ESCI-TOF using a Waters LCT Premier XE or a Thermo Fischer LTQ-Orbitrap XL. Melting points were determined using an Electrothermal 9100 apparatus. IR data were collected on a JASCO FT/IR-4100 using a KBr pellet.

Synthesis of ferrocenyl chalcones

Ferrocenyl chalcones **2a–2j** were synthesized by combining equimolar quantities of the appropriate 2-hydroxyacetophenone with ferrocenecarboxaldehyde in EtOH with 3 equivalents of NaOH. The synthetic procedure and their structural characterization has been reported.[27]

Synthesis of ferrocenyl aurones

Ferrocenyl aurones **3a–3j** were synthesized by refluxing the appropriate chalcone in pyridine with 2 eq. $Hg(OAc)_2$ according to the reported procedure.[26] The aurone synthesis was performed on a 200 mg scale (in chalcone). The characterization of ferrocenyl aurones **3a**, **3c** and **3h** has been previously reported.[26] Elemental analyses for **3a–3f** reprinted from ref. 25.

(*Z*)-5-chloro-2-(ferrocenylidene)benzofuran-3-one, 3b

(65%); mp 159 °C; Found: C 61.65; H 3.67%. $C_{19}H_{13}O_2FeCl{\cdot}0.25\ H_2O$ requires: C 61.66, H 3.69%; UV/VIS (CH_2Cl_2): λ_{max} = 394 nm (ε = 16620 $L.mol^{-1}.cm^{-1}$), 565 nm (5490 $L.mol^{-1}.cm^{-1}$); IR (KBr): 1700 (C═O), 1639 (C═C) cm^{-1};^1H NMR (300 MHz; $CDCl_3$) 4.24 (s, 5H, C_5H_5), 4.63 (s, 2H, C_5H_4), 4.91 (s, 2H, C_5H_4), 6.92 (s, 1H, H_{vinyl}), 7.24 (m, 1H, H_{arom}), 7.57 (dd, ^4J = 2.1 Hz, ^3J = 8.7 Hz, 1H, H_{arom}), 7.77 (d, J = 2.1 Hz, 1H, H_{arom}); ^{13}C NMR (75 MHz; $CDCl_3$) 70.0 (C_5H_5), 71.6 (C_5H_4), 72.2 (C_5H_4), 74.7 (C_5H_4 *ipso*), 114.2 (C_{arom}), 117.9 (C_{vinyl}), 123.8 (C_{arom}), 124.0 (C_{arom}), 128.8 (C_{arom}), 135.8 (C_{arom}), 146.1 (C_{vinyl}), 163.5 (C_{arom}), 181.4 (C═O); MS (APCI) *m*/*z* 364.91 $[M+H]^+$.

(*Z*)-5,7-difluoro-2-(ferrocenylidene)benzofuran-3-one, 3d

(66%); mp 171 °C; Found: C 61.04; H 3.22%. $C_{19}H_{12}O_2FeF_2{\cdot}0.5\ H_2O$ requires: C 60.83; H 3.49%; UV/VIS (CH_2Cl_2): λ_{max} = 395 nm (ε = 16940 $L.mol^{-1}.cm^{-1}$), 575 nm (5630 $L.mol^{-1}.cm^{-1}$); IR (KBr): 1701 (C═O), 1654 (C═C) cm^{-1}; ^1H NMR (400 MHz; $CDCl_3$) 4.22 (s, 5H, C_5H_5), 4.65 (s, 2H, C_5H_4), 4.91 (s, 2H, C_5H_4), 7.02 (s, 1H, H_{vinyl}), 7.22 –7.15 (m, 1H, H_{arom}), 7.31–7.29 (m, 1H, H_{arom}); ^{13}C NMR (100 MHz; $CDCl_3$) 70.2 (C_5H_5), 71.9 (C_5H_4), 72.6 (C_5H_4), 74.3 (C_5H_4 *ipso*), 105.5 (dd, ^2J = 23.8 Hz, ^4J = 4.1 Hz, C_{arom}), 110.9 (dd, ^2J = 19.7 Hz, ^2J = 24.3 Hz, C_{arom}), 119.8 (C_{vinyl}), 125.6 (d, ^3J = 7.7 Hz, C_{arom}), 145.9 (C_{vinyl}), 148.1 (dd, ^1J = 254.7 Hz, ^3J = 11.2 Hz, C_{arom}), 148.8 (d, ^2J = 11.3 Hz, C_{arom}), 157.9 (dd, ^1J = 246.5 Hz, ^3J = 7.3 Hz, C_{arom}), 180.7 (C═O); MS (APCI) *m*/*z* 367.07 $[M+H]^+$.

(*Z*)-5,7-dichloro-2-(ferrocenylidene)benzofuran-3-one, 3e

(64%); mp 181 °C; Found: C 56.05; H 3.31%. $C_{19}H_{12}O_2FeCl_2{\cdot}0.5\ H_2O$ requires: C 55.93; H 3.21%; UV/VIS (CH_2Cl_2): λ_{max} = 397 nm (ε = 15700 $L.mol^{-1}.cm^{-1}$), 581 nm (12380 $L.mol^{-1}.cm^{-1}$); IR (KBr): 1697 (C═O), 1635 (C═C) cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) 4.22 (s, 5H, C_5H_5), 4.66 (s, 2H, C_5H_4), 4.93 (s, 2H, C_5H_4), 7.06 (s, 1H, H_{vinyl}), 7.64 (d, J = 2.1 Hz, 1H, H_{arom}), 7.69 (d, J = 2.1 Hz, 1H, H_{arom}); ^{13}C NMR (75 MHz; $CDCl_3$) 69.2 (C_5H_5), 71.0 (C_5H_4), 71.7 (C_5H_4), 73.3 (C_5H_4 *ipso*), 118.4 (C_{arom}), 118.9 (C_{vinyl}), 121.4 (C_{arom}), 124.1 (C_{arom}), 127.9 (C_{arom}), 134.0 (C_{arom}), 144.8 (C_{vinyl}), 158.2 (C_{arom}), 179.3 (C═O); MS (APCI) *m*/*z* 398.96 $[M+H]^+$.

(*Z*)-5,7-dibromo-2-(ferrocenylidene)benzofuran-3-one, 3f

(60%); mp 122 °C; Found: C 46.47; H 2.60%. $C_{19}H_{12}O_2FeBr_2{\cdot}0.25\ H_2O$ requires: C 46.34; H 2.56%; UV/VIS (CH_2Cl_2): λ_{max} = 394 nm (ε = 14400 $L.mol^{-1}.cm^{-1}$), 570 nm (4400 $L.mol^{-1}.cm^{-1}$); IR (KBr): 1697 (C═O), 1639 (C═C) cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) 4.22 (s, 5H, C_5H_5), 4.67 (s, 2H, C_5H_4), 4.93 (s, 2H, C_5H_4), 7.03 (s, 1H, H_{vinyl}), 7.86 (d, J = 1.5 Hz, 1H, H_{arom}), 7.92 (d, J = 1.5 Hz, 1H, H_{arom}); ^{13}C NMR (75 MHz; $CDCl_3$) 69.2 (C_5H_5) 71.0 (C_5H_4), 71.7 (C_5H_4), 73.3 (C_5H_4 *ipso*), 115.0 (C_{arom}), 119.0 (C_{vinyl}), 124.5 (C_{arom}), 125.0 (C_{arom}), 129.8 (C_{arom}), 139.2 (C_{arom}), 144.4 (C_{vinyl}), 159.9 (C_{arom}), 179.3 (C═O); MS (EI) *m*/*z* 485.99 $[M]^+$.

(*Z*)-6-methoxy-2-(ferrocenylidene)benzofuran-3-one, 3g

(75%); mp 142 °C; HRMS (ESI) Found: 360.04434. $C_{20}H_{16}FeO_3^+$ requires: 360.04489; UV/VIS (CH_2Cl_2): λ_{max} = 365 nm (ε = 16560 L.mol^{-1}.cm^{-1}), 534 nm (3810 L.mol^{-1}.cm^{-1}); IR (KBr): 1685 (C═O), 1639 (C═C) cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$) 3.93 (s, 3H, OMe), 4.19 (s, 5H, C_5H_5), 4.53 (s, 2H, C_5H_4), 4.85 (s, 2H, C_5H_4), 6.74 (s, 1H, H_{vinyl}), 6.72–6.79 (m, 2H, H_{arom}), 7.71 (d, *J* = 8.1 Hz, 1H, H_{arom}); ^{13}C NMR (100 MHz; $CDCl_3$) 55.7 (OMe), 69.5 (C_5H_5), 70.9 (C_5H_4), 71.2 (C_5H_4), 74.8 (C_5H_4 *ipso*), 96.3 (C_{arom}), 111.8 (C_{arom}), 114.4 (C_{vinyl}), 115.4 (C_{arom}), 125.2 (C_{arom}), 146.5 (C_{vinyl}), 166.6 (C_{arom}), 167.5 (C_{arom}), 181.1 (C═O); MS (APCI) *m*/*z* 361.15 [M+H]$^+$.

(*Z*)-4-methoxy-2-(ferrocenylidene)benzofuran-3-one, 3i

(66%); mp 174 °C; HRMS (ESI) Found: 383.03411. $C_{20}H_{16}FeO_3Na^+$ requires: 383.03411; UV/VIS (CH_2Cl_2): λ_{max} = 390 nm (ε = 17350 L.mol^{-1}.cm^{-1}), 536 nm (4550 L.mol^{-1}.cm^{-1}); IR (KBr): 1700 (C═O), 1643 (C═C) cm^{-1}; ^{1}H NMR (300 MHz; Acetone d_6) 4.00 (s, 3H, OMe), 4.24 (s, 5H, C_5H_5), 4.59 (t, *J* = 1.8 Hz, 2H, C_5H_4), 4.95 (t, *J* = 1.8 Hz, 2H, C_5H_4), 6.68 (s, 1H, H_{vinyl}) 6.78 (d, *J* = 8.4 Hz, 1H, H_{arom}) 6.98 (d, *J* = 8.4 Hz, 1H, H_{arom}) 7.66 (t, *J* = 8.4 Hz, 1H, H_{arom}); ^{13}C NMR (100 MHz; $CDCl_3$) 55.8 (OMe), 69.5 (C_5H_5), 70.9 (C_5H_4), 71.2 (C_5H_4), 74.8 (C_5H_4 *ipso*), 104.2 (C_{arom}), 104.4 (C_{arom}), 110.8 (C_{arom}), 114.4 (C_{vinyl}), 137.3 (C_{arom}), 145.4 (C_{vinyl}), 157.9 (C_{arom}), 165.9 (C_{arom}), 180.3 (C═O); MS (APCI) *m*/*z* 361.15 [M+H]$^+$.

(*Z*)-4,6-dimethoxy-2-(ferrocenylidene)benzofuran-3-one, 3j

(68%); mp 166 °C; HRMS (ESI) Found: 413.04467. $C_{20}H_{16}FeO_3Na^+$ requires: 413.04467; UV/VIS (CH_2Cl_2): λ_{max} = 368 nm (ε = 17300 L.mol^{-1}.cm^{-1}), 522 nm (3600 L.mol^{-1}.cm^{-1}); IR (KBr): 1693 (C═O), 1643 (C═C) cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$) 3.90 (s, 3H, OMe), 3.93 (s, 3H, OMe), 4.15 (s, 5H, C_5H_5), 4.47 (s, 2H, C_5H_4), 4.78 (s, 2H, C_5H_4), 6.11 (s, 1H, H_{arom}), 6.35 (s, 1H, H_{arom}), 6.72 (s, 1H, H_{vinyl}); ^{13}C NMR (75 MHz; $CDCl_3$) 56.0 (OMe), 56.1 (OMe), 69.7 (C_5H_5), 70.9 (C_5H_4), 71.1 (C_5H_4), 75.3 (C_5H_4 *ipso*), 89.0 (C_{arom}), 93.7 (C_{arom}), 105.9 (C_{arom}), 113.2 (C_{vinyl}), 117.1 (C_{arom}), 146.8 (C_{vinyl}), 159.2 (C_{arom}), 168.3 (C_{arom}), 179.3 (C═O); MS (APCI) *m*/*z* 391.19 [M+H]$^+$.

Synthesis of organic chalcones

Organic chalcones **4a** and **4g–j** were synthesized by the same method as for the ferrocenyl chalcones **2a–j**.

Synthesis of organic aurones

Organic aurones **5a** and **5g–j** were synthesized by refluxing the appropriate chalcone (100 mg) in pyridine with 2 eq. $Hg(OAc)_2$ according to a literature procedure.[13] For the aurones **5a** and **5j**, the structural characterization was found in good agreement with the reported values.[32,33] The aurones were found to be the *Z*-isomer based on the chemical shift of the vinylic proton (<7 ppm).

(*Z*)-6-methoxy-benzofuran-3-one, 5g

(73%); mp 140 °C; IR (KBr) 1697, 1650, 1604, 1496 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$) 3.92 (s, 3H, OMe), 6.75 (m, 2H, H_{arom}), 6.82 (s, 1H, H_{vinyl}), 7.35–7.47 (m, 3H, H_{arom}), 7.70 (dd, *J* = 8.2 Hz, 0.9 Hz, 1H, H_{arom}), 7.88 (dd, *J* = 8.2 Hz, 0.9 Hz, 2H, H_{arom}). ^{13}C NMR (75 MHz; $CDCl_3$) 56.0, 96.6, 111.9, 112.2, 114.8 (C_q), 125.8, 128.8, 129.6, 131.3, 132.4 (C_q), 147.8 (C_q), 167.4 (C_q), 168.6 (C_q), 183.0 (C═O); MS (APCI) *m*/*z* 253.08 [M+H]$^+$.

(*Z*)-5-methoxy-benzofuran-3-one, 5h

(76%); mp 122 °C; IR (KBr) 1704, 1646, 1596, 1488 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$) 3.83 (s, 1H, OMe), 6.87 (s, 1H, H_{vinyl}), 7.20–7.24 (m, 3H, H_{arom}), 7.36–7.48 (m, 3H, H_{arom}), 7.90 (dd, *J* = 8.2 Hz, 1.5 Hz, 2H, H_{arom}); ^{13}C NMR (75 MHz; $CDCl_3$) 55.9, 105.2, 113.1, 113.8, 121.7 (C_q), 126.2, 128.9, 129.9, 131.5, 132.3 (C_q), 147.7 (C_q), 156.1 (C_q), 161.3 (C_q), 185.0 (C═O); MS (APCI) *m*/*z* 253.08 [M+H]$^+$.

(*Z*)-4-methoxy-benzofuran-3-one, 5i

(71%); mp 144 °C. IR (KBr) 1704, 1654, 1596, 1488 cm^{-1};^{1}H NMR (300 MHz; $CDCl_3$) 4.00 (s, 3H, OMe), 6.61 (d, *J* = 8.2 Hz, 1H, H_{arom}), 6.83 (s, 1H, H_{vinyl}), 6.86 (dd, *J* = 8.2 Hz, 0.6 Hz, 1H, H_{arom}), 7.34–7.46 (m, 3H, H_{arom}), 7.56 (t, *J* = 8.2 Hz, 1H, H_{arom}), 7.89 (dd, *J* = 8.2 Hz, 1.5 Hz, 2H, H_{arom}); ^{13}C NMR (75 MHz; $CDCl_3$) 56.2, 104.7, 105.1, 110.8 (C_q), 111.9, 128.8, 129.6, 131.3, 132.4 (C_q), 138.4, 146.9 (C_q), 158.5 (C_q), 167.0 (C_q), 182.4 (C═O); MS (APCI) *m*/*z* 253.08 [M+H]$^+$.

Bacterial strains and media

Two sensitive strains (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923) and two resistant strains (*Staphylococcus aureus* SA-1199B and *Staphylococcus epidermidis* IPF 896) were used. The strain SA-1199B is resistant to fluoroquinolones due notably to the overexpression of the NorA efflux pump, a membrane-associated protein that mediates the active efflux of fluoroquinolones.[28,30] *S. epidermidis* IPF 896 is resistant to 14- and 15-membered macrolides, including erythromycin, and steptogramins B by overexpressing Msr(A), an ATP-binding cassette (ABC) protein.[31] All strains were grown at 37 °C in Mueller Hinton Broth (MH, Bio Rad) or spread on MH agar plates for counting. In case of *S. epidermidis* IPF 896, 10 μg mL^{-1} of chloramphenicol was added in the overnight culture used to prepare the antibacterial susceptibility test inoculum.

Test compound preparation

Ferrocenyl chalcones (**2a–j**) and aurones (**3a–j**) are colored (violet for those with the most electron-donating substituents to blue for those with the most electron-withdrawing substituents), thus interfering with the optical density measured at 620 nm to assess the bacterial growth. In addition, their solubility assessment in DMSO was difficult at high concentration (10 mg mL^{-1}). As a compromise, compounds were prepared at

1 mg mL^{-1} in DMSO (corresponding to a concentration range of 0.03 to 32 mg L^{-1} in the MIC test) except for **2g** and **2i–j** which were tested from stock solutions at 10 mg mL^{-1}. The organic molecules (**5a**, **5g–j**) were tested in the same conditions as their corresponding ferrocenyl derivatives.

Determination of antibacterial susceptibility

Flavonoid derivatives were dispensed in a 96-wells microplate by two fold serial dilutions in Muller-Hinton medium using a Biomek 2000 (Beckman) handling robot. 100 µL of an overnight bacterial culture diluted to achieve a bacterial concentration of 5 × 10^5–1 × 10^6 CFU/mL (as checked by colony counting) in each well was then added. The final volume in each well was 200 µL. Growth was assayed with a microplate reader by monitoring absorption at 620 nm at 0, 2, 4, 6 and 24 h incubation at 37 °C. In addition, the plates were read visually after 24 h incubation. The MIC was defined as the minimal concentration of the flavonoid compound that completely inhibited cell growth during 24 h incubation at 37 °C. 3.2% DMSO was used as a negative control. MIC determination of reference antibiotics - ampicillin for *E. coli* ATCC 25922 (MIC 4 mg L^{-1}), ciprofloxacin for *S. aureus* ATCC 25923 and 1199B (MIC 0.25 mg L^{-1} and 8 mg L^{-1}, respectively), and erythromycin for *S. epidermidis IPF89* (MIC 64-128 mg L^{-1}) was used as a positive control, in the same run as the flavonoids MIC measurement.

The accepted variance on MIC value can be estimated to a 2-fold difference. All experiments were performed in duplicate.

Determining time-kill endpoint at 24 h

CFU monitoring was carried out by enumerating colonies in 10 µl of a serial log dilutions of the tested bacterial culture spotted in duplicate on MH agar plates. Plates were examined for growth after 24 h of incubation at 37 °C. This methodology has a lower limit of detection of 2 log10 CFU/mL. Bacterial counting was performed in duplicate, except for **3h**.

Cytotoxicity assay

The human cell line MRC5-SV2 (fetal lung), purchased from ECACC, was grown in complete D-MEM medium, supplemented with 10% fetal calf serum, in the presence of penicillin, streptomycin and fungizone in a 75 cm^3 flask under 5% CO_2. The human cell line MDA-MB-231 (breast adenocarcinoma), purchased from ATCC, was grown in RPMI medium supplemented with 10% fetal calf serum, in the presence of penicillin, streptomycin and fungizone in a 75 cm^3 flask under 5% CO_2. For cytotoxicity determinations, cells were plated in 96-well tissue culture microplates in 200 µl complete medium and treated 24 h later with 2 µL stock solution of compounds dissolved in DMSO using a Biomek 3000 (Beckman-Coulter). Controls received the same volume of DMSO (1% final volume). After 72 h exposure, MTS reagent (Promega) was added and incubated for 3 h at 37 °C: the absorbance was monitored at 490 nm and results expressed as the inhibition of cell proliferation calculated as the ratio {[1-(OD490 treated/OD490 control)]×100} in triplicate experiments. For IC_{50} experiments performed in duplicate, compounds were added in the range 5 nM–100 µM in a fixed volume of DMSO.

Electrochemistry

Cyclic voltammograms (CVs) were obtained using a three electrode cell with a 0.5 mm Pt working electrode, stainless steel rod counter electrode, and Ag/AgCl ethanol reference electrode, with an µ-Autolab 3 potentiostat driven by GPES software (General Purpose Electrochemical System, Version 4.8, EcoChemie B.V., Utrecht, the Netherlands). Solutions consisted of 10 mL CH_2Cl_2, approximately 1 mM analyte, and 0.1 M Bu_4NPF_6 supporting electrolyte.

Conclusions

Two series of ten chalcones and ten aurones possessing a ferrocene group in lieu of the phenyl ring were synthesized and evaluated for their antibacterial activity against *E. coli*, antibiotic-sensitive and antibiotic-resistant strains of *S. aureus* and a resistant strain of *S. epidermidis*. The unsubstituted aurone **3a** and the methoxy-substituted aurones **3g–j** showed minimum inhibitory concentrations between 2 and 32 mg L^{-1}, with higher activity against Gram positive bacteria and similar activities against antibiotic-sensitive and antibiotic-resistant strains, while their organic analogs showed no effect at concentrations of 32 mg L^{-1}. The most active compounds, **3g**, **3i** and **3j** have methoxy substitution in the 4 and/or 6 position and all showed a bactericidal effect from time-kill assays. All of the active compounds were also active *in vitro* against human fetal lung cells and human breast carcinoma, with IC_{50} values in the low micromolar range.

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