Phytomedicine xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

# Achyrofuran is an antibacterial agent capable of killing methicillin-resistant vancomycin-intermediate *Staphylococcus aureus* in the nanomolar range

Carina Casero[a,b], Ana Estévez-Braun[a,c], Ángel G. Ravelo[a,c], Mirta Demo[b], Sebastián Méndez-Álvarez[d,e], Félix Machín[c,d,*]

[a] *Instituto Universitario de Bio-Orgánica "Antonio González", Departamento de Química Orgánica, Universidad de La Laguna, Tenerife, Spain*
[b] *Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Córdoba, Argentina*
[c] *Instituto Canario de Investigación del Cáncer (ICIC), Canarias, Spain*
[d] *Unidad de Investigación, Hospital Universitario Nuestra Señora de Candelaria, Tenerife, Spain*
[e] *Departamento de Microbiología y Biología Celular, Universidad de La Laguna, Tenerife, Spain*

ARTICLE INFO

*Keywords:*
*Achyrocline satureioides*
*Staphylococcus aureus*
MRSA
Phytoalexins
Achyrofuran
Antimicrobial

ABSTRACT

Currently, there is a pressing need for novel antibacterial agents against drug-resistant bacteria, especially those which have been common in our communities and hospitals, such as methicillin-resistant *Staphylococcus aureus* (MRSA). The South American plant *Achyrocline satureioides* ("Marcela") has been widely used in traditional medicine for a number of diseases, including infections. Several crude extracts from this plant have shown good antimicrobial activities *in vitro*. In the search for the active principle(s) that confers these antimicrobial activities, we have processed the dichloromethane extract from the aerial parts of the plant. One of the isolated compounds showed extraordinary antibacterial activities against a set of clinically relevant Gram-positive strains that widely differ in their antibiogram profiles. This compound was identified as achyrofuran on the basis of its spectroscopic and physical data. We determined the MIC to be around 0.1 μM (0.07 μg/ml) for the reference methicillin-resistant and vancomycin-intermediate *S. aureus* strain NRS402. Moreover, nanomolar concentrations of achyrofuran killed $10^6$ bacteria within 12 h. Based on the presence of the 2,2′-biphenol core, we further studied whether achyrofuran killed bacteria through a mechanism of action similar to that reported for the naturally occurring antibiotic MC21-A. Indeed, we found that achyrofuran was not bacteriolytic by itself although it greatly compromised membrane impermeability as determined by increased SYTOX Green uptake.

© 2012 Elsevier GmbH. All rights reserved.

## Introduction

The selective pressure resulting from the extensive use of antibiotics over the last decades has led to the emergence of bacterial resistance and to the dissemination of resistance genes among pathogenic microorganisms. The progressive emergence and rapid dissemination of antibiotic resistance in staphylococci constitute a major health concern (Boucher and Corey 2008). Staphylococci are ubiquitous microorganisms present in the respiratory tract and on the skin of a high percentage of healthy adults. Within the genus *Staphylococcus*, *S. aureus* is the causal agent of most staphylococcal infections and is frequently associated with community-acquired and nosocomial diseases. Serious complications occur because of multiple-antibiotic-resistant *S. aureus*. A breakthrough during the weapon race between novel antibiotics and concomitant resistance was the onset of resistance against the penicillin-like β-lactamase-resistant antibiotic methicillin (Rolinson 1961). Thus, these methicillin-resistant *S. aureus* (MRSA) cannot be fought against with any β-lactam antibiotics. Thereafter, glycopeptide-based antibiotics (i.e., vancomycin) have been the main weapon against MRSA. Nevertheless, resistance against vancomycin has appeared as well; first as intermediate resistance (VISA) and later against high concentrations of the antibiotic (VRSA) (Hiramatsu et al. 1997; CDC 2002). Although novel antibiotics such as linezolid and daptomycin were then introduced to combat MRSA, VISA and VRSA, they suffer from multiple limitations, including adverse side-effect and organ-specific inactivation; yet more dramatically, *S. aureus* rapidly acquired resistance against them (Falagas et al. 2008; Baltz 2009). Therefore, there is an increased risk of finding untreatable infections by *S. aureus*, which makes urgent the study of the antibacterial potential of new molecules that could become new active antibiotics against this multiresistant infective agent.

The South American medicinal plant *Achyrocline satureioides* (Lam.) D. C. (Asteraceae) has been used in herbal medicine for different human diseases, including infections (Anesini and Perez 1993;

* Corresponding author at: Unidad de Investigación, Hospital Universitario Nuestra Señora de Candelaria, Tenerife, Spain. Tel.: +34 922 602951; fax: +34 922 600562.
*E-mail address:* fmacconw@gmail.com (F. Machín).

0944-7113/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.phymed.2012.11.003

Retta et al. 2012). Aqueous extracts from that plant have been broadly studied and a plethora of biomedical properties, mainly attributed to flavonoids and polyphenols, have been described (Retta et al. 2012). Interestingly, organic extracts from that plant have been poorly studied, even though some novel compounds have been isolated. One of them, achyrofuran, was first identified in a bioassay-guided fractionation searching for antihyperglycemic activities using a mouse model (Carney et al. 2002). Herein, we report that achyrofuran also possesses a high bactericidal activity against multiresistant Gram-positive bacteria, including MRSA and VISA.

## Materials and methods

### *Plant material*

*A. satureioides* (Lam) D. C. (Asteraceae) was collected from Alpa Corral, Córdoba, Argentina (S32°41′ W64°42′) in September 2008. Plant samples were identified in the Systematical Botany Area of the Department of Natural Sciences, National University of Rio Cuarto. A voucher specimen was deposited in the herbarium of Natural Sciences in the same university (RCV 1921).

### *General phytochemical procedures*

Macherey-Nagel polygram Sil G/UV254 and Analtech silica gel GF preparative layer with UV254 were used for TLC. Column chromatography separations and purifications were performed on silica gel (0.063–0.200 mm) from Macherey-Nagel or on Sephadex LH-20 (Pharmacia Fine Chemicals). Chromatotron Harrison Research 7924T was also used in the purification of some samples. Optical rotation was measured with a Perkin-Elmer 241 polarimeter. UV spectrum was recorded in absolute Methanol on a JASCO V-560 spectrophotometer. IR spectrum was obtained using a Bruker IFS28/55 spectrophotometer. $^1$H and $^{13}$C NMR spectra were recorded in deuterated chloroform at 400 and 100 MHz respectively, with TMS as the internal reference. High- and low-resolution mass spectra were obtained on a VG Micromass ZAB-2F spectrometer.

### *Extraction, isolation and characterization of achyrofuran*

The aerial parts (branches and leaves) of *A. satureioides* were dried in shade and grounded mechanically. Plant material (4.7 kg) was then extracted via maceration with dichloromethane for three successive 48 h periods. The extracts were filtered, combined and dried under reduced pressure to afford a dark extract (93 g). This residue was chromatographed on silica gel eluted with mixtures of *n*-hexane/ethyl acetate of increasing polarity (0–100%). Collected fractions (28 fractions of 200 ml each) were monitored by TLC using *n*-hexane/ethyl acetate (4:1, v/v or 1:1, v/v) as mobile phase. Achyrofuran (150 mg) was isolated from the fractions eluted with 20 and 30% *n*-hexane/ethyl acetate after repeated chromatographies on silica gel, Sephadex LH-20 and Chromatotron as a viscous yellow oil. It was thoroughly characterized by spectroscopic studies and it showed the following data: $[\alpha]_D^{20} = +47.9$ (c 0.29; $CHCl_3$); UV (MetOH) $\lambda_{max}$ (log $\varepsilon$): 341 (2.69); 243 (2.67) nm. IR ($cm^{-1}$): 3296, 2968, 2929, 2877, 1727, 1617, 1451, 1373, 1236, 1192, 1086, 1041, 905, 849. $^1$H NMR ($\delta$, $CDCl_3$): 15.62 (1H; bs; OH-7); 13.98 (1H; s; OH-3); 9.80 (2H; bs; OH-1, OH-9); 5.30 (1H; m; H-2′); 5.09 (1H; s; H-4a‴); 4.96 (1H; s; H-4b‴); 4.51 (1H; m; H-2‴); 4.00 (1H; sext; *J* = 6.5; H-2⁗); 3.79 (1H; sext; *J* = 6.5; H-2″); 3.44 (2H; d; *J* = 6.1; H-1′); 3.24 (1H; dd; *J* = 15.2; H-1a‴); 3.04 (1H; dd; *J* = 15.2; H-1b‴); 1.90–1.87 (2H; m; H-3b″, H-3b⁗); 1.88 (3H; s; 5‴); 1.83 (3H; s; H-5′); 1.69 (3H; s; H-4′); 1.54 (1H; m; H-3a″); 1.48 (1H; m; H-3a⁗); 1.26 (3H; d; *J* = 6.2; H-5″); 1.23 (3H; d; *J* = 6.8; H-5⁗); 0.95(3H; m; H-4⁗); 0.92 (3H; m; H-4″). $^{13}$C NMR ($\delta$, $CDCl_3$): 213.6 (s, C-1⁗); 207.2 (s, C-1″); 164.1 (s, C-3); 158. 4 (s, C-5a); 157.7 (s, C-7); 155.7 (s, C-9); 154.7 (s, C-1); 154.3 (s, C-4a); 145.9 (s, C-3‴); 132.3 (s, C-3′); 122.4 (d, C-2′); 111.8 (s, C-2); 111.8 (t, C-4‴); 107.6 (s, C-8); 105.4 (s, C-9b); 104.9 (s, C-9a); 102.5 (s, C-6); 101.5 (s, C-4); 78.2 (d, C-2‴); 46.4 (d, C-2⁗); 45.6 (d, C-2″); 30.5 (t, C-1‴); 27.2 (t, C-3⁗); 26.6 (t, C-3″); 25.9 (q, C-4′); 21.9 (t, C-1′); 18.3 (q, C-5‴); 18.0 (q, C-5′); 17.2 (q, C-5″); 16.5 (q, C-5⁗); 11.9 (q, C-4″; C-4⁗). EIMS *m/z* (%): 552 ($M^+$, 17); 534 (11); 479 (31); 425 (100); 389 (11).HREIMS: 552.2740 (calc. for $C_{32}H_{40}O_8$ $[M]^+$, 552.2723). These data fit with achyrofuran previously isolated from the same species (Carney et al. 2002).

### *Bacterial strains*

We picked a set of representative bacteria whose clinical importance in multiple antibiotic resistance is broadly acknowledged. These bacteria consisted of three collection strains of Gram-positive bacteria: methicillin-sensitive *Staphylococcus aureus* ATCC25923 (MSSA); methicillin-resistant *Staphylococcus aureus* NRS402, which is also intermediate resistant to vancomycin (VISA); and *Enterococcus faecalis* ATCC29212. We also added two Gram-negative bacteria: *Escherichia coli* ATCC35218 and *Pseudomonas aeruginosa* ATCC27853. Finally, a collection of clinical isolates belonging to the genus *Staphylococcus* and that differ in their antibiotic resistance profiles were used as well.

### *Minimum inhibitory concentrations (MIC)*

We followed the standard broth microdilution method described by the National Committee for Clinical Laboratory Standards (CLSI 2008). We determined MICs by measuring bacterial growth after 24 h under the presence of 1:2 serial dilutions of each compound ranging from $1.67 \times 10^{-5}$ to 70 μg/ml. Aside from the tested compounds, we also included the antibiotics ampicillin (Sigma Chemical Co.) and kanamycin (Roche Applied Biosciences) as controls using the following concentration windows: $5.06 \times 10^{-5}$ to 212 μg/ml for ampicillin and $7.94 \times 10^{-5}$ to 333 μg/ml for kanamycin. The inoculum size was $1 \times 10^5$ CFU/ml for all bacteria.

### *Time-kill experiment*

The time-kill experiment was conducted by the method of Aeschlimann and Rybak (1998). Overnight cultures of both MSSA and VISA strains were harvested when cell density reached $10^8$ CFU/ml. The experiments were conducted in 25 ml Erlenmeyer flasks containing 5 ml of fresh Brain Heart Infusion (BHI) medium (Difco Laboratories) inoculated with the overnight cultures to give an initial bacterial density of $10^6$ CFU/ml. For each strain four conditions were assayed. Achyrofuran was added at final concentrations that consisted of the MIC and two and four times the MIC for the MSSA strain. The fourth subculture was left with just the vehicle (1% (v/v) DMSO). The flasks were then incubated at 37 °C in an orbital incubator at 200 rpm for another day. The viable cell counts of the strains were estimated at various incubation times by the plating method. To minimize the effect of achyrofuran carryover, the samples were centrifuged at 1600 × *g* for 15 min. Then, the medium was replaced with fresh BHI medium, serially diluted 10-fold, and plated on BHI-agar medium. The plates were incubated at 37 °C for 24 h, and then the colonies were counted. These experiments were repeated three times with similar results.

Please cite this article in press as: Casero, C., et al., Achyrofuran is an antibacterial agent capable of killing methicillin-resistant vancomycin-intermediate *Staphylococcus aureus* in the nanomolar range. Phytomedicine (2012), http://dx.doi.org/10.1016/j.phymed.2012.11.003

*Bacteriolytic assay*

We followed the protocol previously described in (Isnansetyo and Kamei 2003) as a reference. Thus, MSSA and VISA overnight cultures were washed twice with sterile 0.9% (w/v) NaCl, and the absorbance adjusted to 0.15 at 620 nm (~$10^8$ CFU/ml). The bacterial cell suspension was then divided into aliquots of 5 ml each, placed into sterile test tubes, and exposed to achyrofuran at various concentrations or 0.2 μg/ml lysostaphin (Sigma Chemical Co.) as a positive control. These test tubes were incubated at 37 °C while shaking at 120 rpm. The absorbance at 620 nm was measured at different time points. The relative absorbance was calculated by dividing each absorbance by that for the time zero.

*Plasma membrane permeability assay*

We followed the SYTOX uptake methodology (Roth et al. 1997). MSSA and VISA strains were grown for 18 h at 37 °C and 120 rpm in BHI. The cultures were then harvested by 8800 × g centrifugation for 3 min. After two washing steps with 0.85% (w/v) NaCl plus 5% Trypticase Soy Broth media, bacterial density was adjusted to $3 \times 10^7$ CFU/ml in the same media and aliquoted to carry out the permeabilization experiment. Thus, bacteria cells were treated with increasing concentrations of achyrofuran or the vehicle alone as the negative control. Then, samples were taken after 6 h of drug addition and further incubated for 15 min with 5 μM SYTOX green (Invitrogen) before harvesting and washing the sample twice with 0.85% NaCl. The bacterial pellet was then observed under a Leica DMI6000 fluorescence microscope. Micrographs were taken with either the bright field (BF) or the green fluorescence channel (in order to see SYTOX green). A positive control for SYTOX-stained bacteria was also included. This control consisted in bacteria treated with 70% (v/v) isopropyl alcohol for 2 h before the 15 min SYTOX staining step.

## Results

In order to identify the metabolite(s) responsible for the antibacterial activities described for *Achyrocline satureioides*, the dichloromethane extracts from the aerial parts of *A. satureioides* were chromatographed using different methods until the isolation of a set of pure metabolites. We then tested the antibacterial activities of these metabolites against a selected panel of Gram-positive and -negative bacteria (see below) through a rapid screening based on the CLSI standard determination of MICs in microplates (CLSI 2008). Thus, we identified a few chromatographic samples of pure compounds with similar high antibacterial activities. These samples were more than 30 times stronger (in μg/ml) than the next most active compound within this set (data not shown). When we further characterized these active samples through spectroscopic analysis we found that all of them were the same metabolite, termed achyrofuran (Fig. 1A). Achyrofuran is a prenylated dibenzofuran previously isolated from this plant and reported as an antihyperglycemic drug in mice (Carney et al. 2002). Remarkably, achyrofuran shares distinct structural features with several compounds previously identified as potent antimicrobials (Fig. 1B, see also the discussion chapter).

In our hands, achyrofuran was active against the three Gram-positive bacteria we included in this assay: methicillin-sensitive *Staphylococcus aureus* ATCC25923 (MSSA); methicillin-resistant *Staphylococcus aureus* NRS402, which is also intermediate resistant to vancomycin (VISA); and *Enterococcus faecalis* ATCC29212 (Table 1). Achyrofuran had a MIC of 0.25 μM for MSSA, 0.12 μM for VISA and 4 μM for *E. faecalis*. Since we also used two commercial antibiotics in these assays, ampicillin and kanamycin,

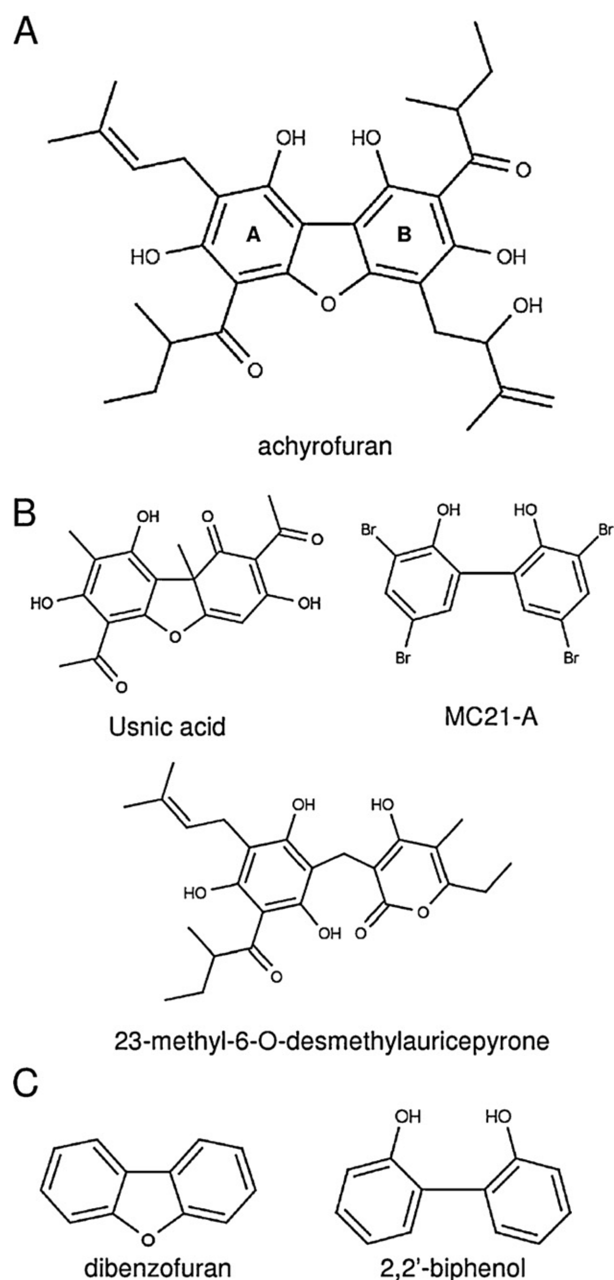


**Fig. 1.** (A) Chemical structure of achyrofuran. (B) Chemical structures of three antimicrobials that share structural similarities with achyrofuran. (C) Putative antibacterial moieties found in achyrofuran.

we were able to compare the relative antibacterial activity of achyrofuran. Thus, achyrofuran was as good as ampicillin against MSSA and *E. faecalis*, and was much better than kanamycin against these two bacteria (Table 1). On the other hand, and as expected, the VISA strain was highly resistant to both commercial antibiotics. Nevertheless, the VISA isolate was as sensitive to achyrofuran as the MSSA (in the submicromolar range). Achyrofuran had no effect on the growth of the two assayed Gram-negative bacteria: *Escherichia coli* ATCC35218 and *Pseudomonas aeruginosa* ATCC27853 (MIC > 127 μM [70 μg/ml]).

We also performed time-kill experiments to assess whether achyrofuran was a bacteriostatic or a bactericidal agent. Bacteria were grown and diluted to ~$1 \times 10^6$ CFU/ml and then treated with achyrofuran at the MIC concentration for the MSSA, and twice and four times of it (Fig. 2). Achyrofuran soon inhibited bacterial growth

Please cite this article in press as: Casero, C., et al., Achyrofuran is an antibacterial agent capable of killing methicillin-resistant vancomycin-intermediate *Staphylococcus aureus* in the nanomolar range. Phytomedicine (2012), http://dx.doi.org/10.1016/j.phymed.2012.11.003

**Table 1**
Minimum inhibitory concentrations (MICs) of achyrofuran, ampicillin and kanamycin against the three selected Gram-positive bacterial strains.

| Bacterial strain | MIC (μM and [μg/ml]) | | |
|---|---|---|---|
| | Achyrofuran | Ampicillin | Kanamycin |
| *S. aureus* ATCC25923 (MSSA) | 0.25 [0.14] | 0.50 [0.19] | 2.5 [1.46] |
| *S. aureus* NRS402 (VISA) | 0.12 [0.07] | 150 [56] | 150 [87] |
| *E. faecalis* ATCC29212 | 3.96 [2.19] | 10.0 [3.71] | 200 [116] |

and started killing bacteria after just 2 h. All bacteria had been killed by 12 h.

We next carried out bacteriolytic assays to determine if the killing activity correlated with the lysis of the cocci (Fig. 3). Since the bacterial concentration had to be at least $10^8$ CFU/ml in order to follow optical density readings, we accordingly scaled up the concentration of achyrofuran during this assay. Thus, reduction of the absorbance of cell suspensions was not observed in the presence of achyrofuran at up to a hundred times the MIC (10 μM) and all during the incubation period (16 h). In contrast, the absorbance of all cultures treated with lysostaphin (a specific lytic enzyme for Staphylococci) dropped drastically early in the incubation period (within the first hour).

After observing the nonlytic bactericidal activities of achyrofuran, we then tried to get further insights into its mode of action. By comparing the structure of achyrofuran to other chemically related antimicrobials, it became clear that achyrofuran shares some similarities to usnic acid (Fig. 1B), although there is an important modification at the core of the molecule (see below). Many biological activities have been attributed to usnic acid, including antiproliferative against multiresistant *S. aureus* (Lauterwein et al. 1995). Nevertheless, its actual mechanism of action remains unknown. A much simpler naturally occurring drug, 3,3′,5,5′-tetrabromo-2,2′-biphenyldiol (i.e., MC21-A, Fig. 1B), has been shown to exert its nonlytic bactericidal activity through a mechanism that involves membrane permeabilization (Isnansetyo and Kamei 2003). Taking into account that achyrofuran also carries a 2,2′-biphenol moiety at the core of the molecule, we decided to explore whether achyrofuran was also able to permeabilize the cell membrane. Indeed, we found that cell impermeability was highly compromised after a short treatment with achyrofuran, and allowed uptake of the SYTOX green nucleic acid fluorescent stain (Fig. 4).

Finally, we wondered whether resistance to most clinically relevant antibiotics conferred cross-resistance to achyrofuran. To answer this question we picked a broader set of clinical isolates

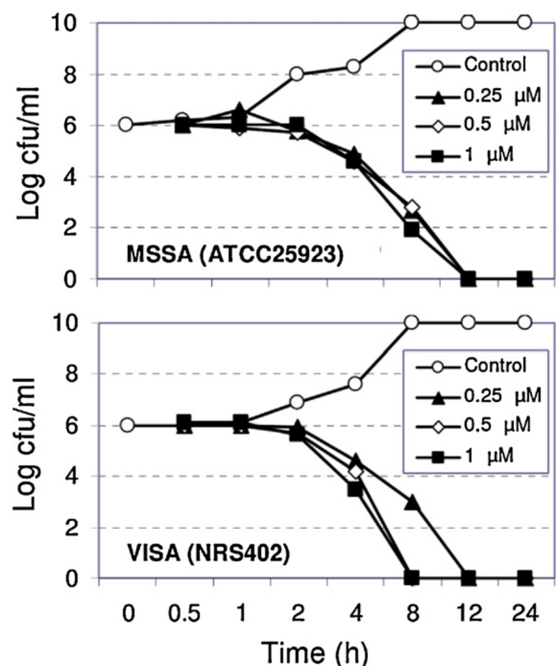


**Fig. 2.** Achyrofuran kills methicillin-resistant and vancomycin-intermediate *Staphylococcus aureus* in the nanomolar range. Cultures of MSSA (ATCC25923) and VISA (NRS402) strains were treated with achyrofuran for different intervals and surviving CFU were counted afterwards. Note how no surviving bacteria were rescued after just 12 h.

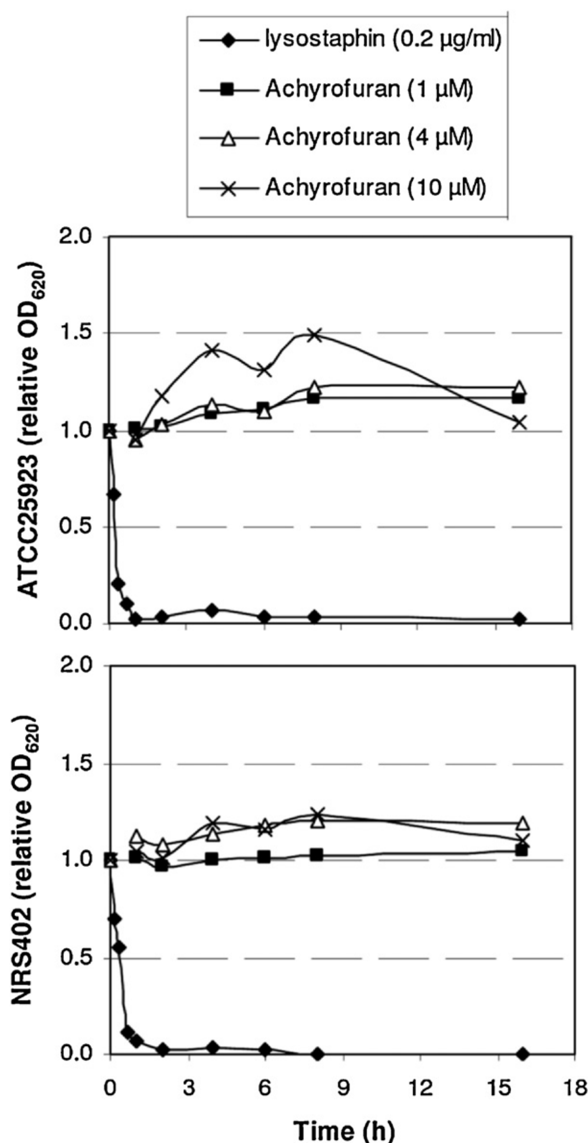


**Fig. 3.** Achyrofuran is not bacteriolytic. Cultures of MSSA and VISA *S. aureus* were split and treated as indicated in a sterile 0.9% (w/v) NaCl solution. Then, $OD_{620}$ was measured at the indicated times and compared to the initial value. Note how only lysostaphin, the positive control for lysis, quickly decreases the relative $OD_{620}$.

Please cite this article in press as: Casero, C., et al., Achyrofuran is an antibacterial agent capable of killing methicillin-resistant vancomycin-intermediate *Staphylococcus aureus* in the nanomolar range. Phytomedicine (2012), http://dx.doi.org/10.1016/j.phymed.2012.11.003

**Table 2**
Antibiotic profiles and susceptibility to 1 μM achyrofuran for clinical isolates of the genus *Staphylococcus*.

| SPECIES | STRAIN[b] | Commercial antibiotic[a] | | | | | | | | ACHYRO[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | OX | PE | CL | ER | GE | MU | VA | TE | |
| *S. aureus* | ATCC25923 | R | R | S | R | nd | S | S | S | 0.050 ± 0.007 |
| | HUNSC526(ST88) | R | R | S | S | S | S | S | S | 0.018 ± 0.013 |
| | HUNSC265(ST80) | R | R | S | S | S | S | S | S | 0.015 ± 0.014 |
| | HUNSC283(ST239) | R | R | R | R | R | S | S | S | 0.024 ± 0.023 |
| | HUNSC182(ST247) | R | R | R | R | R | R | S | S | 0.015 ± 0.010 |
| | HUNSC262(ST8) | R | R | R | R | S | S | S | S | 0.024 ± 0.010 |
| | HUNSC381(ST22) | R | R | R | R | S | S | S | S | 0.048 ± 0.016 |
| | HUNSC188(ST36) | R | R | R | R | S | S | S | S | 0.036 ± 0.014 |
| | HUNSC195(ST30) | R | R | R | R | S | S | S | S | 0.039 ± 0.013 |
| | HUNSC281(ST125) | R | R | R | R | S | S | S | S | 0.023 ± 0.010 |
| | HUNSC261(ST146) | R | R | R | R | S | S | S | S | 0.021 ± 0.022 |
| | HUNSC266(ST471) | R | R | S | S | S | S | S | S | 0.020 ± 0.022 |
| | USA100(ST5) | R | R | R | R | S | S | S | S | 0.037 ± 0.019 |
| | USA200(ST36) | R | R | R | R | S | S | S | S | 0.036 ± 0.019 |
| | USA300(ST8) | R | R | R | R | S | S | S | S | 0.038 ± 0.021 |
| | USA500(ST8) | R | R | R | R | S | S | S | S | 0.024 ± 0.011 |
| | USA700(ST72) | R | R | R | R | S | S | S | S | 0.024 ± 0.013 |
| | USA800(ST5) | R | R | R | R | S | S | S | S | 0.026 ± 0.007 |
| | HUNSC2721 | S | R | S | S | S | S | S | S | 0.025 ± 0.013 |
| | HUNSC9462 | S | R | S | S | S | S | S | S | 0.021 ± 0.003 |
| | HUNSC4152(ST152) | S | R | S | S | S | S | S | S | 0.029 ± 0.018 |
| | NRS402 | R | R | R | R | S | S | I = 8 | I = 16 | 0.022 ± 0.020 |
| *S. saprophyticus* | HUNSC539 | S | R | S | R | S | S | S | S | 0.033 ± 0.025 |
| | HUNSC541 | S | S | S | S | S | S | S | S | 0.039 ± 0.023 |
| *S. lugdunensis* | HUNSC8824 | S | R | S | S | S | S | S | S | 0.052 ± 0.030 |
| | HUNSC9286 | S | S | S | S | S | S | S | S | 0.041 ± 0.016 |

R, resistant; S, susceptible; I, intermediate (with value in μg/ml); nd, non-determined. OX, oxacillin; PE, penicillin; CL, clindamicin; ER, eritromycin; GE, gentamycin; MU, mupirocin; VA, vancomycin; TE, teicoplanin; ACHYRO, achyrofuran.

[a] For each bacterial isolate the susceptibility testing to the included antibiotics was performed with the automatic Vitek2 system (BioMérieux) according to the manufacturer's instructions. The antimicrobial susceptibility profiles were interpreted according to the Clinical and Laboratory Standards Institute.

[b] Strains with the HUNSC prefix were recovered by the Microbiology Service of the Hospital Universitario Nuestra Señora de Candelaria (Tenerife, Spain) and have been previously described (Pérez-Roth et al. 2004). The ST genetic profile is included between brackets.

[c] Relative growth (mean ± SEM, $n = 3$) was calculated as [Growth with 1 μM ACHYRO − control without cells]/[Growth with 1% (v/v) DMSO − control without cells]. Growth as measured at $OD_{620}$ after 24 h.

within the genus *Staphylococcus*, mostly *S. aureus*, that not only differ in their antibiotic resistance profile but also belong to important clonal families in the nosocomial infections (Table 2). We found that all strains were equally susceptible to achyrofuran.

## Discussion

In this work we show that achyrofuran is as good as some of the best antibiotics at inhibiting Gram-positive bacterial growth (Tables 1 and 2), and that can give a complete bactericidal response in the nanomolar range (Fig. 2). We also show that achyrofuran does not kill the bacterium by simply lysing it (Fig. 3), but it rather disrupts membrane impermeability (Fig. 4). The nonlytic permeabilization action of achyrofuran points out that the compound might either bind to the plasma membrane or enter the bacterium to exert its bactericidal action. This in turn would likely explain the observed Gram-positive specificity since Gram-negative bacteria are much more resistant to drug uptake (Kumar and Schweizer 2005).

Chemically, achyrofuran belongs to the dibenzofuran family of compounds (Fig. 1C). Some naturally occurring dibenzofurans exhibit moderate anti-staphylococcal activities (Shiu and Gibbons 2009), whereas others have been recently reported as phytoalexins (i.e., induced under pathogenic attack) (Chizzali et al. 2012). In this respect, anti-staphylococcal activity of achyrofuran appears to be much stronger (>1000-fold) than other dibenzofurans, although direct comparisons of MICs for all these dibenzofurans should be performed under the same experimental conditions. Besides, the 2,2′-biphenol moiety can be recognized within the dibenzofuran nucleus of achyrofuran (Fig. 1C). This moiety is also found in antimicrobials that permeabilize *S. aureus* membrane without lysing the bacterium (Isnansetyo and Kamei 2003).

On the other hand, a closely related compound, 23-methyl-6-O-desmethyllauricepyrone (Fig. 1B), was recently isolated from the ethanol extract of *A. satureioides* through antibacterial activity-guided fractionation (Joray et al. 2011). This compound resembles achyrofuran structure in many ways, especially in the prenylated branches of achyrofuran ring A (Fig. 1). However, the compound does not have the dibenzofuran nucleus and presents a 4-hydroxypyran-2-one group and a trihydroxy phenyl moiety instead of the two dihydroxy phenols. Importantly, authors reported this compound to have a MIC of about 6 μg/ml, still 100-fold higher than the one we report for achyrofuran in this work.

Another related compound is usnic acid (Fig. 1B), an antibacterial metabolite found in many lichens (e.g., *Usnea* and *Cladonia*) and used in a number of industrial applications as a preservative (e.g., toothpastes, hair shampoos, deodorants, etc.) (Ingólfsdóttir 2002). Usnic acid presents a central furan ring fused to an hydroxycyclohexa-2,4-dien-1-one ring and a dihydroxy phenyl group with similar substitution pattern to that of ring A in achyrofuran. Previous antimicrobial studies with usnic acid also found anti-Gram-positive specificity with MIC values equivalent to that of ampicillin (Lauterwein et al. 1995), as we report here for achyrofuran (Table 1). Critically, usnic acid appeared to be bacteriostatic (Lauterwein et al. 1995). Nevertheless, we show that achyrofuran is bactericidal in the submicromolar range (Fig. 2). This might be a key improvement of achyrofuran versus usnic acid to take into account for further development as antimicrobial.

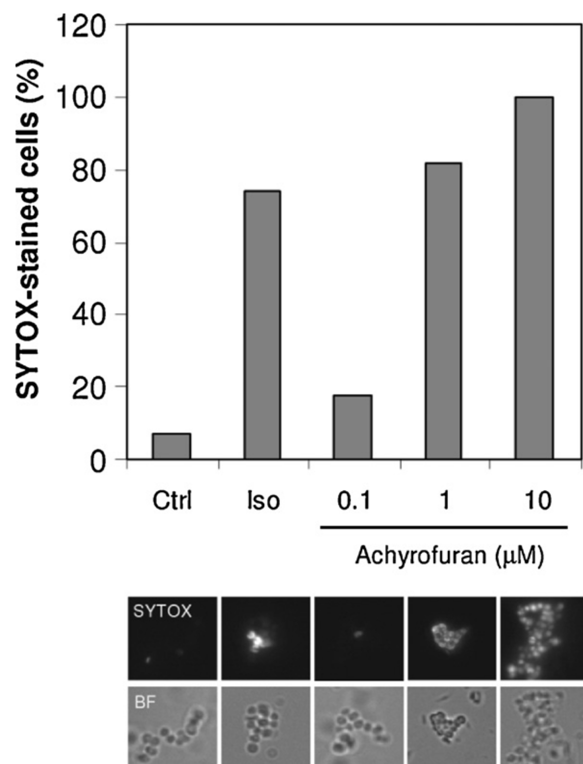


**Fig. 4.** Achyrofuran permeabilizes *S. aureus* membrane. MSSA and VISA strains were treated as indicated for 2 h before adding the SYTOX green stain (Ctrl, 1% (v/v) DMSO; Iso, 70% (v/v) isopropyl alcohol). Then, positively stained cocci were counted under the microscope. Representative micrographs are also shown underneath (SYTOX, green fluorescence channel; BF, bright field).

An analysis of the structures of all mentioned compounds (Fig. 1) seems to indicate the importance of free hydroxyl groups in the activity which could act as hydrogen bond donors. From the high antimicrobial activity exhibited by achyrofuran we can also assume the importance of the planar dibenzofuran core as well. The furan ring confers rigidity to the 2,2′-biphenol moiety, which might lead to an appropriate spatial rearrangement for the antibiotic activity.

In the future, it would be interesting to see whether other members of all these families share the high antibacterial activity seen with achyrofuran. Increasing the amount of compounds within these families will further help in establishing structure–antibacterial activity relationships to determine the corresponding pharmacophore.

Taking into account that: (i) achyrofuran has been used against diabetes in a mouse model, where it was orally administered at 20 mg/kg without acute toxicity (Carney et al. 2002) and (ii) the structural similarities to other commercial antimicrobials, especially usnic acid, we believe that our results encourage further research onto the cytotoxicity, pharmacokinetics and molecular mechanism of action in order to develop achyrofuran as a candidate for novel antibiotics against Gram-positive bacteria.

## Funding

This work was supported by Instituto de Salud Carlos III (PS09/00106 to F.M. and PI10/00125 to S.M.-A.) and MICINN (SAF 2009-13296-C02-01 to A.E.-B.), all co-founded by the European Regional Development Fund (ERDF). C.C. thanks CONICET for a predoctoral fellowship.

## Conflict of interest

There is a Spanish patent application (P201130432) by the same authors as inventors for the use of achyrofuran and related compounds as antimicrobials.

## Acknowledgements

We are thankful to NARSA (Network on Antimicrobial Resistance in *Staphylococcus aureus*, VA, USA) by kindly providing the vancomycin-intermediate strain NRS402 and the epidemic USA clones.

## References

Aeschlimann, J.R., Rybak, M.J., 1998. Pharmacodynamic analysis of the activity of quinupristin-dalfopristin against vancomycin-resistant *Enterococcus faecium* with differing MBCs via time-kill-curve and postantibiotic effect methods. Antimicrobial Agents and Chemotherapy 42, 2188–2192.

Anesini, C., Perez, C., 1993. Screening of plants used in Argentine folk medicine for antimicrobial activity. Journal of Ethnopharmacology 39, 119–128.

Baltz, R.H., 2009. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. Current Opinion in Chemical Biology 13, 144–151.

Boucher, H.W., Corey, G.R., 2008. Epidemiology of methicillin-resistant *Staphylococcus aureus*. Clinical Infectious Diseases 46, S344–S349.

Carney, J.R., Krenisky, J.M., Williamson, R.T., Luo, J., 2002. Achyrofuran, a new antihyperglycemic dibenzofuran from the South American medicinal plant *Achyrocline satureioides*. Journal of Natural Products 65, 203–205.

Chizzali, C., Khalil, M.N.A., Beuerle, T., Schuehly, W., Richter, K., Flachowsky, H., Peil, A., Hanke, M.-V., Liu, B., Beerhues, L., 2012. Formation of biphenyl and dibenzofuran phytoalexins in the transition zones of fire blight-infected stems of Malus domestica cv. “Holsteiner Cox” and Pyrus communis cv. “Conference”. Phytochemistry 77, 179–185.

CDC (Centers for Disease Control and Prevention), 2002. *Staphylococcus aureus* resistant to vancomycin – United States. Morbidity and Mortality Weekly Report 51, 565–567.

CLSI (Clinical and Laboratory Standards Institute), 2008. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard, 8th ed. Wayne, PA.

Falagas, M.E., Siempos, I.I., Vardakas, K.Z., 2008. Linezolid versus glycopeptide or beta-lactam for treatment of Gram-positive bacterial infections: meta-analysis of randomised controlled trials. Lancet Infectious Diseases 8, 53–66.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., Tenover, F.C., 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. Journal of Antimicrobial Chemotherapy 40, 135–136.

Isnansetyo, A., Kamei, Y., 2003. MC21-A, a bactericidal antibiotic produced by a new marine bacterium, *Pseudoalteromonas phenolica* sp. nov. O-BC30(T), against methicillin-resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 47, 480–488.

Ingólfsdóttir, K., 2002. Usnic acid. Phytochemistry 61, 729–736.

Joray, M.B., del Rollán, M.R., Ruiz, G.M., Palacios, S.M., Carpinella, M.C., 2011. Antibacterial activity of extracts from plants of central Argentina – isolation of an active principle from *Achyrocline satureioides*. Planta Medica 77, 95–100.

Kumar, A., Schweizer, H.P., 2005. Bacterial resistance to antibiotics: active efflux and reduced uptake. Advanced Drug Delivery Reviews 57, 1486–1513.

Lauterwein, M., Oethinger, M., Belsner, K., Peters, T., Marre, R., 1995. In vitro activities of the lichen secondary metabolites vulpinic acid, (+)-usnic acid, and (−)-usnic acid against aerobic and anaerobic microorganisms. Antimicrobial Agents and Chemotherapy 39, 2541–2543.

Pérez-Roth, E., Lorenzo-Díaz, F., Batista, N., Moreno, A., Méndez-Alvarez, S., 2004. Tracking methicillin-resistant *Staphylococcus aureus* clones during a 5-year period (1998–2002) in a Spanish hospital. Journal of Clinical Microbiology 42, 4649–4656.

Retta, D., Dellacassa, E., Villamil, J., Suárez, S.A., Bandoni, A.L., 2012. Marcela, a promising medicinal and aromatic plant from Latin America: a review. Industrial Crops and Products 38, 27–38.

Rolinson, G.N., 1961. “Celbenin” – resistant Staphylococci. British Medical Journal 1, 125–126.

Roth, B.L., Poot, M., Yue, S.T., Millard, P.J., 1997. Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. Applied and Environment Microbiology 63, 2421–2431.

Shiu, W.K.P., Gibbons, S., 2009. Dibenzofuran and pyranone metabolites from *Hypericum revolutum* ssp. revolutum and *Hypericum choisianum*. Phytochemistry 70, 403–406.

Please cite this article in press as: Casero, C., et al., Achyrofuran is an antibacterial agent capable of killing methicillin-resistant vancomycin-intermediate *Staphylococcus aureus* in the nanomolar range. Phytomedicine (2012), http://dx.doi.org/10.1016/j.phymed.2012.11.003