Microbiological Research 165 (2010) 617—626

Available online at www.sciencedirect.com

ScienceDirect

Microbiological Research

www.elsevier.de/micres

Antibacterial properties of zeylasterone, a triterpenoid isolated from *Maytenus blepharodes*, against *Staphylococcus aureus*

L. de León, M.R. López, L. Moujir*

Departamento de Microbiología y Biología Celular, Facultad de Farmacia, Universidad de La Laguna, 38206 Tenerife, Canary Islands, Spain

Received 28 September 2009; received in revised form 17 December 2009; accepted 21 December 2009

KEYWORDS
Staphylococcus aureus;
Antibacterial;
Natural products;
Maytenus blepharodes;
Macromolecular synthesis

Summary

The anti-staphylococcal properties of zeylasterone and demethylzeylasterone, two 6-oxophenolic triterpenoids isolated from *Maytenus blepharodes*, were investigated. Zeylasterone was more active than demethylzeylasterone on *Staphylococcus aureus* cells, showing bactericidal activity at 30 μg/ml (6 × MIC) in less than three hours and bacteriostatic at lower concentrations. At the same cell density, a more drastic reduction in CFU count was obtained when the triterpenoid was incorporated into cultures growing actively. Zeylasterone at 3 × MIC added on *S. aureus* cultures showed an early inhibitory effect on incorporation of radiolabeled thymidine, uridina and N-acetyl-glucosamine, and later on leucine. It also caused cell membrane disruption in *S. aureus*, as shown by the inhibition of radiolabeled precursor uptake, rapid potassium leakage, inhibition of NADH oxidation, and formation of mesosome-like structures around the septa. The structural features of the molecule, the blockage of solute transport through the membrane and changes in its permeability, suggest that zeylasterone acts mainly on cytoplasmic membrane.

Introduction

In the past few decades, most of the new drugs brought onto the market have been derivatives of older compounds. Some of them have increased activity or improved pharmacological properties against many hazardous pathogens, but can only temporarily overcome the problem of resistance (Kunin and Ellis, 2000). An example of this is the increasing resistance of Gram-positive bacteria such us staphylococci to known bactericides (Diekema et al., 2001; Kim et al., 2004; Appelbaum

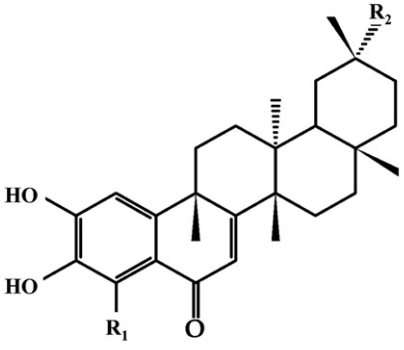
*Corresponding author. Tel.: +34 922 318513; fax: +34 922 318477.
E-mail address: lmoujir@ull.es (L. Moujir).

0944-5013/$ - see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2009.12.004

and Jacobs, 2005). Moreover, strategies to control infection also include the use of biocides in the form of antiseptics and disinfectants in the hospital environment (Weber et al., 2007) that may contribute to the development of bacterial resistance. In response to this important challenge, the search for new antimicrobials has been intensified (Chopra et al., 1997).

Historically, plants have provided a rich source of anti-infective agents, contributing to human health and well-being. Their primary benefits are that they are generally safer, offering powerful therapeutic benefits and more affordable treatment (Iwu et al., 1999). In this context, the *Celastraceae* family has a long history in traditional medicine since they produce an extraordinary variety of bioactive metabolites of medical interest such as triterpenoid quinonemethides or phenolic triterpenes (González et al., 2000; Alvarenga and Ferro, 2006). Phenolic-type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties (McDonnell and Russell, 1999; Katsura et al., 2001). It has been proposed that they have membrane-active properties which contribute to their overall potency (Denyer, 1995).

As a part of our ongoing search for biologically active metabolites we have reported a series of 6-oxophenolic triterpenoids (zeylasteral, demethylzeylasteral, zeylasterone and demethylzeylasterone) (Figure 1) from *Maytenus blepharodes* Lundell, whose branches are considered to have antitumoral properties (Gupta, 1995). These phenolic triterpenoids were isolated previously from *Kokoona zeylanica* (Gunaherath and Gunatilaka, 1983; Gamlath et al., 1986) whose outer stem bark is used to obtain ''kokum soap'', a paste prepared by mixing powdered bark with water to form flat pieces used as toilet soap with antiseptic properties (Gunatilaka, 1996). The antimicrobial activity and action mechanism of these triterpenoids against spore-forming bacteria have been described (De León et al., 2005; De León and Moujir, 2008) but, to our knowledge, studies on the anti-staphylococcal action of these compounds have not been documented.



	R_1	R_2
Zeylasterone	COOH	COOMe
Demethylzeylasterone	COOH	COOH
Zeylasteral	COH	COOMe
Demethylzeylasteral	COH	COOH

Figure 1. Structure of 6-oxophenolic triterpenoids cited in this study.

In order to further our understanding on the biological effect of 6-oxophenolic triterpenoids, the aims of this study were to establish their structure–activity relationship, evaluate the anti-staphylococcal properties of zeylasterone, elucidate its mechanism of action on *S. aureus*, and obtain information about its potential applications.

Materials and methods

Material and bacterial strains

Zeylasterone and demethylzeylasterone, were isolated from the root of *Maytenus blepharodes* as previously reported (Rodríguez et al., 2005). In all experiments, the phenolic triterpenoids were added pre-dissolved in dimethylsulphoxide (DMSO). The reference antibacterial agents, ciprofloxacin, penicillin, rifampin, tetracycline, vancomycin, bacitracin and clofoctol (Sigma-Aldrich) were used according to the Clinical and Laboratory Standards Institute (CLSI, 2006).

Staphylococcus aureus ATCC 6538 (used for the following experiments), *S. aureus* LMM1 (clinical isolate, University of La Laguna) and *Escherichia coli* ATCC 9637 strains were used in this study. All bacterial cultures were developed in nutrient broth (NB) from an overnight liquid culture prepared in YP medium (yeast extract 10 g; peptone 10 g per liter). All media were purchased from Oxoid.

Antibacterial activity

The minimal inhibitory and bactericidal concentrations (MIC and MBC) were determined in triplicate for zeylasterone, demethylzeylasterone and known antibiotics by the broth microdilution method in 96-well microtiter plates as previously described (De León and Moujir, 2008). Wells with the same proportions of DMSO were used as controls, and never exceeded 1% (vol/vol). The starting bacterial density was approximately

$1\text{-}5 \times 10^5$ colony-forming units (CFU/ml), and growth was monitored by measuring the increase in optical density at 550 nm (OD_{550}) with a microplate reader (Multiskan Plus II) and viable count in agar plates.

Killing curves

Liquid cultures of *S. aureus* in lag-phase (10^6 CFU/ml) and log-phase of growth ($OD_{550}\sim0.2$) were treated with zeylasterone at 15 μg/ml ($3 \times$ MIC) and incubated at 37 °C in a rotatory shaker. Cultures with known antibiotics and without drugs were used as positive and negative controls, respectively. Cultures in log-phase of growth were also exposed to zeylasterone at 5, 15 and 30 μg/ml for 3 h of incubation at 37 °C.

Furthermore, the effect of zeylasterone added at 15 μg/ml was evaluated on different inoculum concentrations of *S. aureus* (approximately from 10^3 to 10^7 CFU/ml).

All cultures were incubated at 37 °C in a rotatory shaker and growth was monitored by measuring the optical density at 550 nm and by determination of the viable cell count on nutrient agar plates. The assays were repeated three times.

Transmission electron microscopy

Suspensions of *S. aureus* in the log-phase of growth (10^7 CFU/ml) were treated with zeylasterone at 15 μg/ml ($3 \times$ MIC) for 1 h at 37 °C. Control experiments (without product) were performed in parallel. Organisms were harvested by centrifugation at 6500*g* for 8 min at 4 °C, and the pellet was resuspended in 1 ml of phosphate buffer 0.1 M (pH 7.4). After centrifugation (10,000*g* for 10 min, 4 °C) the pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 day.

Subsequently, bacteria were washed in fixative buffer, post-fixed in 1% OsO_4 in fixative buffer and washed with distilled water. Bacteria were dehydrated with a graded series of ethanol, embedded in Epon 812 resin and polymerized at 70 °C. Semi-thin (1 μm) sections were cut with a Reichert-Ultracut ultramicrotome and stained with toluidine blue; ultrathin sections were contrasted with uranyl acetate and lead. Observations were made under a Zeiss EM 912 transmission electron microscope. Pictures were taken with a Proscan Slow-scan CCD-Camera for TEM and Soft Imaging System software.

Measurement of radioactive precursor incorporation

The total synthesis of DNA, RNA, protein and peptidoglycan were measured by quantifying the incorporation of radiolabeled precursors (thymidine, uridine, leucine and N-acetylglucosamine, from Amersham Biosciences Europe GmbH) into acid-insoluble material. Cultures of *S. aureus* ($\sim10^6$ CFU/ml) in NB medium were grown at 37 °C in a rotatory shaker for approximately 3 h until the optical density at 550 nm (OD_{550}) reached 0.2. Aliquots of the culture (10 ml) were then transferred to prewarmed flasks and incubated at 37 °C with zeylasterone at 15 μg/ml and the precursors of DNA (1 μCi/ml [6-^{3}H]+2 μg/ml unlabeled thymidine), RNA (1 μCi/ml [5-^{3}H]+2 μg/ml unlabeled uridine), protein (5 μCi/ml [4,5-^{3}H]+2 μg/ml unlabeled leucine), and cell-wall peptidoglycan (0.1 μCi/ml N-acetyl-D-[1-^{14}C]glucosamine), respectively. Parallel experiments with the same proportions of DMSO or a specific inhibitor with a known action mechanism were included as negative and positive control, respectively.

At different times (up to 30 min), 0.5 ml samples were precipitated with 2 ml ice-cold 10% trichloroacetic acid (TCA). After 30 min in cold TCA, samples were filtered on GF/C glass microfiber filters (Whatman) and washed three times with 5 ml cold 10% TCA and once with 5 ml of 95% ethanol. The dried filters were placed in vials, covered with 4 ml of scintillation cocktail and counted in LKB Wallac 1214 Rackbeta. The assays were repeated three times.

Measurement of initial uptake of solutes in *S. aureus*

Initial uptake activity was measured on exponentially growing cultures ($OD_{550}\sim0.2$) as total cell-associated counts after addition of zeylasterone, and labeled compounds (thymidine, uridine, leucine, N-acetyl-D-glucosamine and glucose). Exponentially growing cells in NB were transferred to flasks containing radiolabeled and unlabeled precursors at the concentrations indicated above. For determination of glucose uptake 2 μCi/ml D-[1-^{14}C]-glucose was added to *S. aureus* cultures grown in Davis–Mingoli minimal medium (Davis and Mingoli, 1950) with glucose (1%), asparagine (0.1 g/liter) and casamino acids (2 g/liter). At different times (up to 30 min), 0.5 ml of samples were taken and filtered (Millipore Corp., Bedford, Mass., type HA; 0.45 μm). Filters were washed three times with 5 ml of phosphate buffer, dried

and radioactivity measured as previously. Parallel experiments with the same proportions of DMSO or clofoctol (15 μg/ml) were used as negative and positive controls, respectively.

Furthermore, the thymidine uptake was also determined when DNA synthesis was inhibited. *S. aureus* cultures prepared as previously were treated with ciprofloxacin (1.5 μg/ml) for 15 min and 1 μCi/ml of [6-^{3}H]+2 μg/ml unlabeled thymidine was added. After 5 min, zeylasterone at 15 μg/ml was incorporated and thymidine uptake measured at different times. A sample without phenol but with the same proportion of DMSO was used as control. The assays were repeated three times.

Fluorescence labeling

The effect of zeylasterone at 15 μg/ml on cytoplasmic membrane of *S. aureus* in log-phase was determined using the LIVE/DEAD BacLight kit (catalog no. L-7012; Molecular Probes, Eugene, Oreg.), after 30 min and 1 h of treatment, as described in the manufacturer's instructions. Assays were carried out three times in parallel with cell cultures treated with clofoctol at 10 μg/ml, and the same proportion of DMSO as positive and negative control, respectively. The cells were observed after a 15 min dark-staining period at ×1000 magnification with an epifluorescence microscope (Leica DM4B) provided with a fluorescein–rhodamine dual filter.

Leakage of cellular constituents absorbing at 260 and 280 nm

The experimental procedure of Chou and Pogell (1981) was adopted. Cells in log-phase were harvested, washed with 0.05 M potassium phosphate buffer (pH 7.3) containing 0.05 M sucrose, and resuspended in the same buffer to reach a cell density of 10^7 CFU/ml (OD_{550}~0.3). The suspension was divided into two equal parts, then zeylasterone (15 μg/ml) and a similar proportion of DMSO were added, respectively, before incubating at 37 °C under shaking. Cellular constituents liberation was determined by measuring optical density of supernatant (after removing cells by centrifugation at 8000*g* for 10 min, 4 °C) at 260 and 280 nm.

Release of intracellular potassium

S. aureus cells in log-phase of growth (OD_{550}~0.5) were harvested, washed twice with saline buffer, and resuspended in the same buffer. The suspension was divided into three equal parts: zeylasterone (15 μg/ml), clofoctol (15 μg/ml) as positive control, and DMSO in the same proportion as negative controls were added. Samples were removed over a 30-min period, membrane-filtered (0.2 μm pore size, Whatman) and K^+ release was measured in duplicate by means of an atomic absorption spectrophotometer (Mod. S/Series, Thermo). The assays were repeated three times.

Effect of zeylasterone on NADH oxidation

Cultures of *S. aureus* and *E. coli* were grown for 24 h in YP medium at 37 °C under aeration. Subcellular preparations were obtained by sonic disruption as previously described by Moujir et al. (1991). Aliquots (0.2 ml) were incubated at room temperature in 2.8 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing NADH (0.1 mM). The oxidation of NADH was followed by measuring the decrease in absorbance at 340 nm over a period of 20 min, with and without zeylasterone (15 μg/ml). Clofoctol at 120 μg/ml was used as control.

Results

Susceptibility of *S. aureus* to zeylasterone

The MIC and MBC values (Table 1) of the 6-oxophenolic compounds showed a higher activity of zeylasterone against the two *S. aureus* strains evaluated, while demethylzeylasterone was inactive (MIC > 40 μg/ml).

Table 1. Comparison of minimal inhibitory and bactericidal concentrations (MIC/MBC) in μg/ml of 6-oxophenolic triterpenoids and standard antibiotics against *Staphylococcus aureus*.

Antibacterial substances	Bacterial strains	
	S. aureus ATCC 6538	*S. aureus* LMM1
Zeylasterone	5/30	10-5/15
Demethylzeylasterone	>40/>40	>40/>40
Zeylasteral	40/>40	20/30
Demethylzeylasteral	25/>40	>30/>30
Ciprofloxacin	0.5/1	-
Penicillin	0.5/0.8	-
Rifampin	0.005/0.006	-
Tetracycline	0.6/>10	-
Bacitracin	12.5/50	-
Vancomycin	1.56/50	-
Clofoctol	5/>40	-

(-): not determined.

The killing kinetic assays were done with *S. aureus* ATCC 6538 in presence of zeylasterone added at different growth phases (Figure 2).

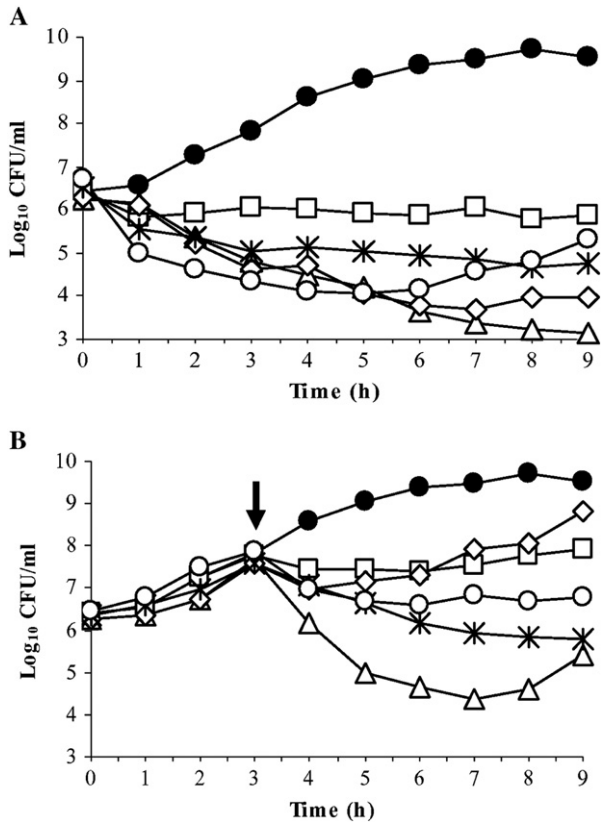


Figure 2. Time-kill curves of *S. aureus* measured by CFU counts after treatment with different antimicrobial substances added in lag-phase (A) and log-phase (B, time of addition indicated by arrow) of growth. Antimicrobial compounds: zeylasterone (○) at 15 μg/ml (3 × MIC), ciprofloxacin (Δ) at 1.5 μg/ml (3 × MIC), penicillin (◇) at 5 μg/ml (10 × MIC), rifampin (×) at 0.015 μg/ml (3 × MIC), tetracycline (□) at 1.8 μg/ml (3 × MIC) and negative control without drug and with the maximum proportion of DMSO (●).

Inclusion of zeylasterone at 15 μg/ml (3 × MIC) in lag-phase produced a reduction of 2.2-$\log_{10}$ in the initial inoculum of *S. aureus* in the first two hours of treatment (Figure 2A). This effect was higher than that obtained with the known antibiotics like ciprofloxacin, penicillin, rifampin or tetracycline. However, when the phenol was incorporated during the log-phase of growth, a lesser reduction in CFU count (Figure 2B) and no decrease in optical density (data not shown) were obtained. In these growth conditions, zeylasterone added at 5 μg/ml (1 × MIC) also showed a bacteriostatic effect, but bactericidal when added at 30 μg/ml (> 3-$\log_{10}$ in CFU reduction) (data not shown).

The anti-staphylococcal activity following exposure to zeylasterone was not dependent on the starting inoculum. At lower concentrations ($\leq 10^6$ CFU/ml), bacterial populations remained constant during the first 6 hours of incubation; whereas at 10^7 CFU/ml, growth was observed in the first three hours of treatment. Note that when the phenolic triterpenoid was added at 10^7 CFU/ml in log-phase of growth (Figure 2B), the result was different, since a reduction in CFU count (1.4 $\log_{10}$) was obtained at the same time.

Ultrastructural changes in *S. aureus* after treatment with zeylasterone

Thin sections of *S. aureus* cells treated with zeylasterone for 1 h at 15 μg/ml (3 × MIC) were prepared, to observe ultrastructural changes (Figure 3). These electron micrographs showed the formation of intracellular lamellar membranes (mesosomes) (Figure 3A), that were not observed in untreated cells (Figure 3B).

Often these mesosomes occurred around the septum. Cytoplasmic membrane and cell wall

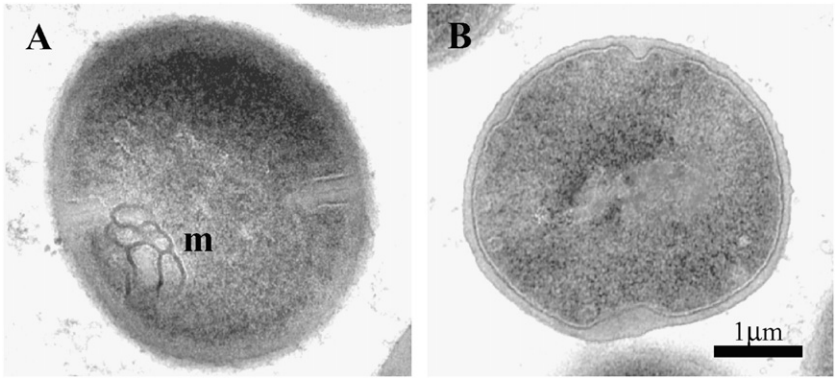


Figure 3. Transmission electron microscopy of *S. aureus* after exposure for 1 h to zeylasterone at 15 μg/ml (A). In treated cells the image show the presence of mesosome-like structures (m) and conspicuous damage to cytoplasmic membrane and cell wall which appeared diffuse compared to the untreated cells (B).

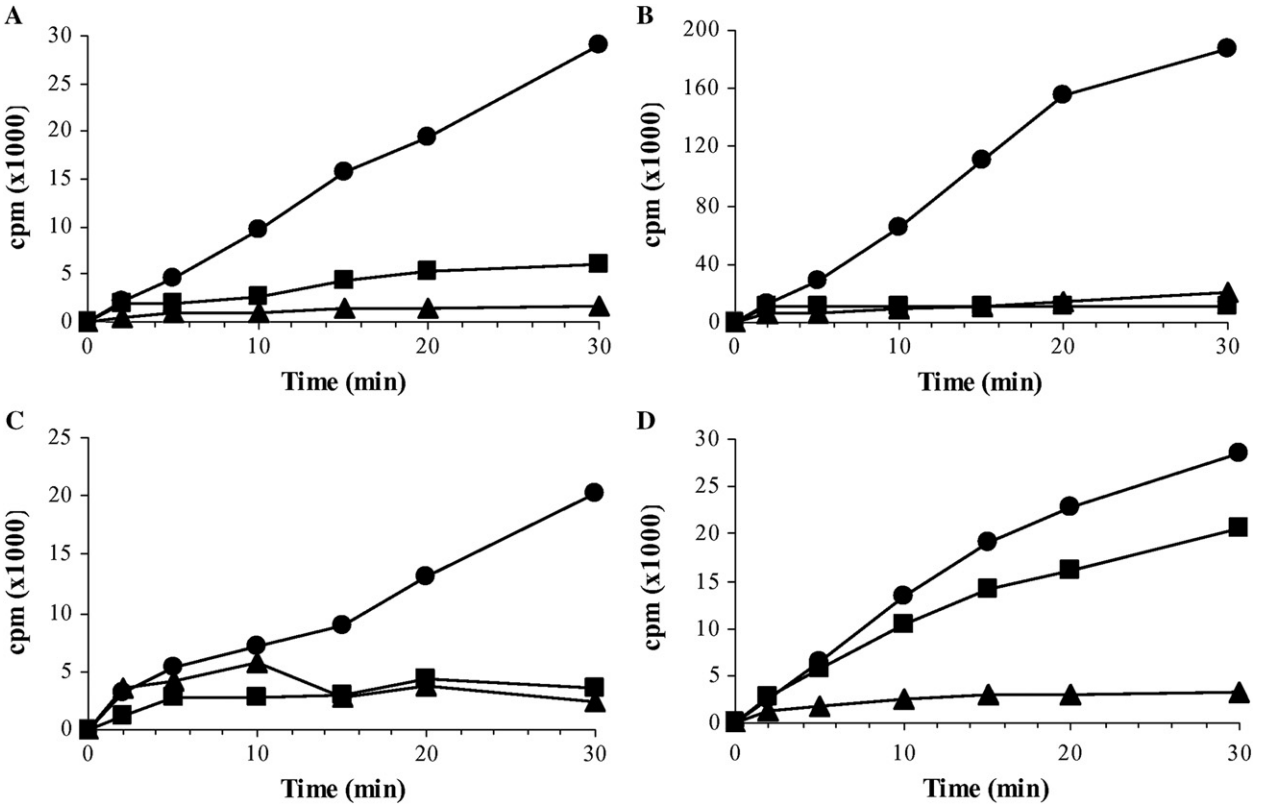


Figure 4. Inhibition by zeylasterone of precursor incorporation into macromolecules in *S. aureus*. Synthesis inhibition was measured for DNA (A), RNA (B), protein (C) and cell wall (D): zeylasterone at 15 μg/ml (3 × MIC) (▲) or untreated control (●) with the same proportion of DMSO. Known inhibitors were included in each experiment as positive controls (■): ciprofloxacin at 1.5 μg/ml (3 × MIC); rifampin at 0.015 μg/ml (3 × MIC); tetracycline at 1.8 μg/ml (3 × MIC); and penicillin at 5 μg/ml (10 × MIC).

became diffuse but no apparent lysis or gross leakage of cytoplasmic contents was observed.

Effects on macromolecular synthesis

The effect of zeylasterone on the incorporation of radiolabeled precursors into DNA, RNA, protein and cell-wall synthesis on *S. aureus* cells was evaluated (Figure 4).

Addition of triterpenoid at 15 μg/ml (3 × MIC) stopped all incorporation processes but the blockage did not occur simultaneously. The incorporation of [6-^{3}H] thymidine ceased within 2 min with 74% inhibition (Figure 4A) while blockage of incorporation of [5-^{3}H] uridine (76%) and N-acetyl-D-[1-^{14}C] glucosamine (71%) occurred 5 min after addition of the triterpenoid (Figures 4B and D). Up to 20 min were necessary to obtain a similar effect on [4,5-^{3}H] leucine incorporation in protein synthesis (Figure 4C). Zeylasterone had a stronger effect than penicillin, ciprofloxacin and rifampin, used as specific inhibitors of peptidoglycan, DNA and RNA synthesis, respectively. Clofoctol, a cytoplasmic membrane disruptor (Yablonsky, 1983), blocked all biosynthetic processes after 5–10 min (data not shown).

Measurements of initial uptake of macromolecular precursors

The uptake of solutes by *S. aureus* was evaluated as the total cell-associated counts after cells were separated from the labeled precursors in the incubation medium. Addition of zeylasterone at 15 μg/ml to *S. aureus* cultures inhibited the uptake of [6-^{3}H] thymidine after 5 min (72%). The uptake of [4,5-^{3}H] leucine and N-acetyl-D-[1-^{14}C]glucosamine (~60%) was inhibited after 10 min with only slight inhibition initially. Up to 30 min was required to obtain the same rate of uptake inhibition on D-[1-^{14}C]-glucose. Uptake of [5-^{3}H] uridine was only marginally affected by the triterpenoid and the cell-associated radioactivity increased progressively during the incubation period (Figure 5). Clofoctol, used as control, inhibited the initial uptake (>70%) of all precursors, between 2 and 20 min after addition.

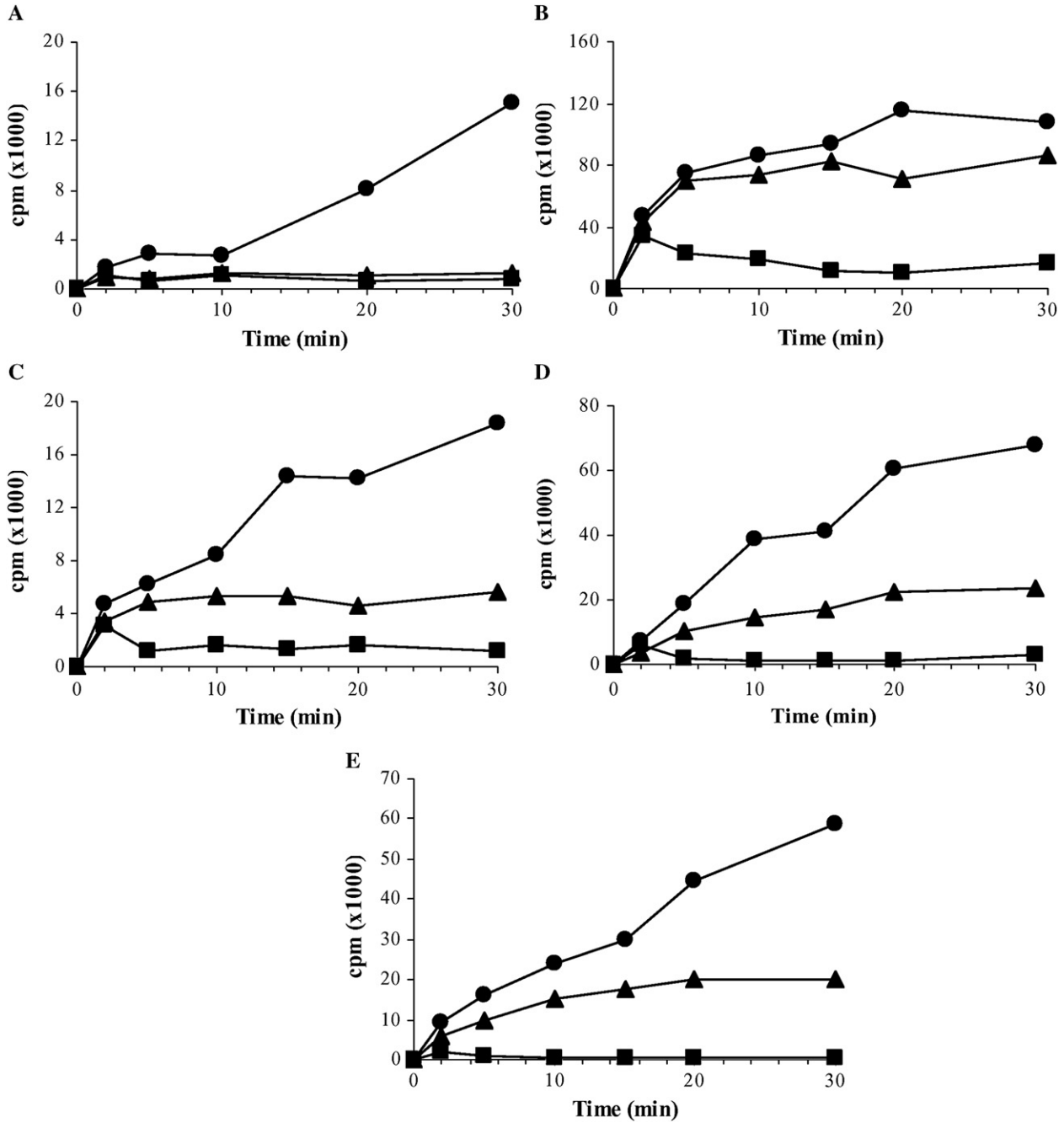


Figure 5. Inhibition of timidine (A), uridine (B), leucine (C), N-acetyl-D-glucosamine (D) and glucose (E) uptake in *S. aureus* cells after treatment with zeylasterone (▲) added at 15 μg/ml (3 × MIC). Negative controls (●) with the same proportion of DMSO and positive controls (■) with clofoctol at 30 μg/ml (3 × MIC) were also included in each experiment.

The effect of zeylasterone on thymidine uptake was also evaluated in cultures of *S. aureus* previously treated with ciprofloxacin (Figure 6). The thymidine uptake-rate increased with time when DNA synthesis was inhibited specifically in the control cells. In those conditions, addition of zeylasterone at 15 μg/ml blocked [6-^{3}H] thymidine uptake, revealed when the precursor was no longer accumulated inside the cells. In fact, a slight leakage of accumulated [6-^{3}H] thymidine in *S. aureus* cells followed the triterpenoid addition.

Membrane damage following exposure to zeylasterone

Microscopic observations after BacLight assays and measurement of optical density revealed that

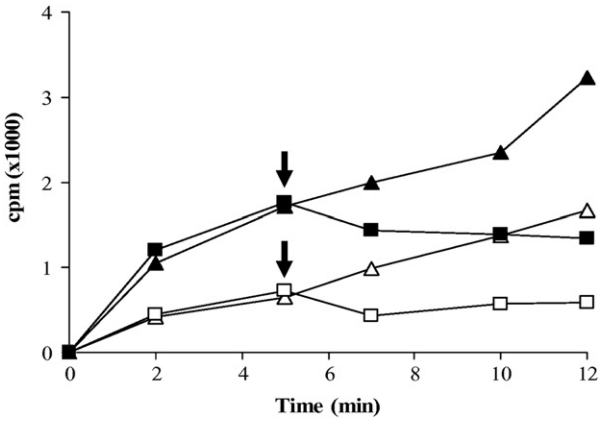


Figure 6. Inhibition of thymidine uptake by zeylasterone in absence of DNA synthesis. Ciprofloxacin at 1.5 μg/ml was added to *S. aureus* cultures and after 15 min, 1 μCi/ml of [6-^{3}H]thymidine was added (0 min). Zeylasterone at 15 μg/ml and the same proportion of DMSO, used as negative control, were added at the time indicated by the arrow. Total counts in presence of zeylasterone (■) or DMSO (▲), and trichloroacetic acid-insoluble counts in presence of zeylasterone (□) or DMSO (Δ) were determined during the incubation.

the cells treated with zeylasterone at 15 μg/ml for 1 h maintained membrane integrity, showing green fluorescence and no release of UV-absorbing material. However, a rapid leakage of intracellular potassium was detected after this treatment. Cultures treated with 15 μg/ml of clofoctol, used as positive control, revealed damage to the cytoplasmic membrane showing red fluorescence in BacLight assays and UV-absorbing material and potassium release (data not shown).

Membrane preparations from both *S. aureus* and *E. coli* oxidized NADH. In both cases, when zeylasterone (15 μg/ml) was added, the slope of the substrate oxidation rate leveled off, as observed when clofoctol was added at 15 μg/ml.

Discussion

This study describes the anti-staphylococcal activity and mechanism of action of zeylasterone, a 6-oxophenolic triterpenoid, showing its highest activity against *S. aureus* when compared with demethylzeylasterone or other phenolic triterpenoids like zeylasteral and demethylzeylasteral (De León et al., 2005). Zeylasterone showed similar activity to clofoctol, an active membrane antibacterial, but higher than bacitracin, a bactoprenolphosphate inhibitor located in cytoplasmic membrane. However, it had less activity than other antibiotics with an intracellular action mechanism.

Structural analyses of these 6-oxophenolic triterpenoids suggest that a hydrogen-bond-donor (HBD) group at C-4 and increased lipophilicity are essential requirements for their activity, as observed in other terpenoids (Urzúa et al., 2008). Lipophilicity of drugs is associated with solubility, permeability through cell membrane, and ability to interact with the hydrophobic region (Lipinski et al., 1997). Thus, the increased lipophilicity caused by esterification of the carboxylic acid at C-29 leads to enhanced activity (zeylasterone vs. demethylzeylasterone). However, the absence of a HBD group strategically positioned at C-4 resulted in reduced activity against *S. aureus*, which is in line with the results for other related molecules like zeylasteral and demethylzeylasteral (De León et al., 2005).

The killing curves showed that zeylasterone was bactericidal on *S. aureus* cells at high concentrations but cell damage appears to be sublethal at lower ones, indicating a bacteriostatic action. This effect was observed independently of growth phase and inoculum size, although actively growing cells were more sensitive, which could mean macromolecular synthesis is affected.

Studies on macromolecular synthesis verified that all precursor incorporation ceased in *S. aureus* after addition of zeylasterone, but pathways like DNA synthesis were inhibited preferentially. In contrast, we previously demonstrated that zeylasterone and other related phenolic triterpenoids had an early effect on peptidoglycan synthesis on spore-forming bacteria and a later effect on the incorporation of thymidine (De León et al., 2005; De León and Moujir, 2008).

Interference with solute transport would also lead to the inhibition of macromolecular synthesis, so initial cellular uptake of solutes is generally used as a rough measure of transport in bacteria (Chou and Pogell, 1981). Phenolic compounds have been shown to inhibit glucose uptake into the cells (Evans and Martin, 2000) as well as DNA, RNA and protein synthesis (Ness and Eklund, 1983). Indeed, zeylasterone stopped thymidine uptake, whereas the inhibition of leucine, N-acetyl-glucosamine or glucose uptake was more gradual. However, uptake of uridine was only marginally affected by zeylasterone.

In addition, the inhibition of precursor transport into the cells could also be caused by its blocking their incorporation into macromolecular synthesis, if these processes are in fact coupled (Chou and Pogell, 1981). Our observations revealed that when DNA synthesis was blocked by a specific inhibitor

the processes were not closely linked and thymidine uptake was inhibited immediately by zeylasterone as a consequence of transport inhibition, and not as a direct effect on DNA synthesis. A slight leakage of radiolabeled precursor was also noted, probably due to simultaneous treatment with ciprofloxacin plus zeylasterone. Thus, a disturbance in the transport mechanism through the membrane seems to be evident, although other processes such as RNA synthesis cannot be ruled out since its inhibition was not dependent on the uptake process.

Disruption of the cytoplasmic membrane is often indicated by leakage of intracellular constituents, including potassium as a first sign of membrane damage (Lambert and Hammond, 1973). In fact, zeylasterone produced a marked and rapid efflux of intracellular potassium in *S. aureus*, as well as an inhibition of NADH oxidation in membrane preparations from *S. aureus* and *E. coli*. This is particularly interesting because it suggests that cell membranes of both Gram-positive and Gram-negative bacteria are sensitive to its action. Therefore, the insensitivity of the intact Gram-negative bacteria may be due to a permeability barrier, probably the outer membrane of these bacteria. Besides this, the presence of mesosome-like structures in treated cells, which had been regarded as indicative of cytoplasmic membrane alteration (Friedrich et al., 2000), reinforces the hypothesis that zeylasterone causes membrane disruption in *S. aureus*.

It has previously been reported that phenolic and diterpenoid compounds primarily target the cytoplasmic membrane due to their hydrophobic nature and therefore preferentially partition into the lipid bilayer (Sikkema et al., 1994; Weber and de Bont, 1996; Kyrikou et al., 2005). Although further studies are necessary to fully understand the precise mechanism involved, the results obtained here and the structural features of zeylasterone indicate that its mechanism of action against *S. aureus* indeed mainly lies in its ability to damage the cytoplasmic membrane. The present work provides valuable information that may serve as a base-line in the future development of 6-oxophenolic triterpenoids as antiseptic and disinfectant agents.

Acknowledgements

This study was supported by the Spanish Grants SolSubC2008010000049. We thank Felix Rodriguez of the Instituto Universitario de Bio-Orgánica (IUBO) "Antonio González" for the isolation of phenolic compounds, Antonio Padrón of the Departamento de Edafología (Universidad de La Laguna) and the Servicio de Microscopía de la Universidad de Las Palmas de Gran Canaria.

References

Alvarenga NL, Ferro EA. Bioactive triterpenes and related compounds from Celastraceae. In: Rahman A, editor. Studies in Natural Products Chemistry, Bioactive Natural Products, 33. Amsterdam: Elsevier Science Publisher; 2006. p. 239–308.

Appelbaum PC, Jacobs MR. Recently approved and investigational antibiotics for treatment of severe infections caused by Gram-positive bacteria. Curr Opin Microbiol 2005;8:510–7.

Chopra I, Hodgson J, Metcalf B, Poste G. The search for antibacterial agents effective against bacteria resistant to multiple antibiotics. Antimicrob Agents Chemother 1997;41:497–503.

Chou WG, Pogell BM. Mode of action of pamamycin in *Staphylococcus aureus*. Antimicrob Agents Chemother 1981;20:443–54.

Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS), National Committee for Clinical Laboratory Standards, Performance standards for Antimicrobial Susceptibility Testing; 16th Informational supplement. CLSI document M7-A7, 940 West Valley Road, Wayne, Pennsylvania 19087; 2006.

Davis BD, Mingoli ES. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J Bacteriol 1950;**60**:17–28.

De León L, Beltrán B, Moujir LM. Antimicrobial activity of 6-oxophenolic triterpenoids. Mode of action against *Bacillus subtilis*. Planta Med 2005;71:313–9.

De León L, Moujir LM. Activity and mechanism of the action of zeylasterone against *Bacillus subtilis*. J Appl Microbiol 2008;104:1266–74.

Denyer SP. Mechanism of action of antibacterial biocides. Int Biodeter Biodegr 1995;36:227–45.

Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, et al. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clin Infect Dis 2001;32:S114–32.

Evans JD, Martin SA. Effects of thymol on ruminal microorganisms. Curr Microbiol 2000;41:336–40.

Friedrich CL, Moyles D, Beveridge TJ, Hancock RE. Antibacterial action of structurally diverse cationic peptides on Gram-positive bacteria. Antimicrob Agents Chemother 2000;44:2086–92.

Gamlath GR, Gunaherath GM, Gunatilaka AA. Isolation and structures of some novel phenolic triterpenoids of Sri Lankan Celastraceae. In: Rahman A, LeQuesne PW, editors. New Trends In Natural Products Chemistry. Amsterdam: Elsevier Science Publisher; 1986. p. 109–21.

González AG, Bazzocchi IL, Moujir LM, Jiménez IA. Ethnobotanical uses of Celastraceae. Bioactive Metabolites. In: Rahman A, editor. Studies in Natural Products Chemistry, Bioactive Natural Products (Part D). Amsterdam: Elsevier Science Publisher;; 2000. p. 649–738.

Gunaherath GM, Gunatilaka AA. Studies on terpenoids and steroids. Part 3: structure and synthesis of a new phenolic D: a-friedo-24-noroleanane triterpenoid, zeylasterone, from *Kokoona zeylanica*. J Chem Soc Perkin 1983;T1:2849–54.

Gunatilaka AA. Triterpenoid quinonemethides and related compounds (celastroids). In: Hertz W, Kirby GW, Moore RE, Steglich W, Tamm C, editors. Progress in the Chemistry of Organic Natural Products. New York: Springer; 1996. p. 1–123.

Gupta MP. Panamanian flora: Source of bioactive molecules. In: Hostettmann K, Marston A, Maillard M, Hamburger M, editors. Phytochemistry of Plants Used in Traditional Medicine. New York: Oxford University Press; 1995. p. 359–98.

Iwu MW, Duncan AR, Okunji CO. New antimicrobials of plant origin. In: Janick J, editor. Perspectives on New Crops and New Uses. Alexandria, VA: ASHS Press; 1999. p. 457–62.

Katsura H, Tsukiyama RI, Suzuki A, Kobayashi M. In vitro antimicrobial activities of bakuchiol against oral microorganisms. Antimicrob Agents Chemother 2001;45:3009–13.

Kim HB, Jang HC, Nam HJ, Lee YS, Kim BS, Park WB, et al. In vitro activities of 28 antimicrobial agents against *Staphylococcus aureus* isolates from tertiary-care hospitals in Korea: a nationwide survey. Antimicrob Agents Chemother 2004;48:1124–7.

Kunin CM, Ellis WY. Antimicrobial activities of mefloquine and a series of related compounds. Antimicrob Agents Chemother 2000;44:848–52.

Kyrikou I, Georgopoulos A, Hatziantoniou S, Mavromoustakos T, Demetzos CA. Comparative study of the effects of cholesterol and sclareol, a labdane type diterpene, on phospholipid bilayers. Chem Phys Lipids 2005;133:125–34.

Lambert PA, Hammond SM. Potassium fluxes, first indications of membrane damage in micro-organisms. Biochem Biophys Res Commun 1973;54:796–9.

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Delivery Rev 1997;23(1–3):3–25.

McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action and resistance. Clin Microbiol Rev 1999;12:147–79.

Moujir L, Gutiérrez-Navarro AM, González AG, Ravelo AG, Luis JG. Mode of action of netzahualcoyone. Antimicrob Agents Chemother 1991;35:211–3.

Ness IF, Eklund T. The effect of parabens on DNA, RNA and protein synthesis in *Escherichia coli* and *Bacillus subtilis*. J Appl Bacteriol 1983;54:237–42.

Rodríguez FM, López MR, Jiménez IA, Moujir L, Ravelo AG, Bazzocchi IL. New phenolic triterpenes from *Maytenus blepharodes*. Semisynthesis of 6-deoxoplepharodol from pristimerine. Tetrahedron 2005;61:2513–9.

Sikkema J, Bont JAM, Poolman B. Interaction of cyclic hydrocarbons with biological membranes. J Biol Chem 1994;269:8022–8.

Urzúa A, Rezende MC, Mascayano C, Vásquez L. A structure-activity study of antibacterial diterpenoids. Molecules 2008;13:882–91.

Weber DJ, Rutala WA, Sickbert-Bennett EE. Outbreaks associated with contaminated antiseptics and disinfectants. Antimicrob Agents Chemother 2007;51: 4217–24.

Weber FJ, de Bont JAM. Adaptation mechanism of microorganisms to the toxic effect of organic solvents on membranes. Biochim Biophys Acta 1996;1286: 225–45.

Yablonsky F. Alteration of membrane permeability in *Bacillus subtilis* by clofoctol. J Gen Microbiol 1983;129:1089–95.