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ORIGINAL ARTICLE

Activity and mechanism of the action of zeylasterone against *Bacillus subtilis*

L. de León and L. Moujir

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

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Correspondence
Laila Moujir, Departamento de Microbiología y Biología Celular, Facultad de Farmacia Universidad de La Laguna, 38206, Tenerife, Canary Islands, Spain. E-mail: lmoujir@ull.es

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Abstract

Aims: To investigate the antimicrobial properties of 6-oxophenolic triterpenoids isolated from *Maytenus blepharodes* against different micro-organisms and the mode of action on *Bacillus subtilis*.

Methods and Results: The activity of zeylasterone and demethylzeylasterone was evaluated by microdilution method. Zeylasterone showed a higher activity, being active against Gram-positive bacteria (minimum inhibitory concentration 3–20 μg ml^{-1}) and *Candida albicans* (10 μg ml^{-1}). Killing curves revealed a bacteriostatic effect on *B. subtilis* that was dependent on the growth phase and inoculum size. Zeylasterone caused cell membrane alterations in *B. subtilis*, as shown by potassium leakage and formation of mesosome-like structures. However, membrane disruption was not revealed by either LIVE/DEAD Baclight assay or measurement of intracellular constituent efflux. Zeylasterone showed an early effect on *N*-acetyl-glucosamine and uridine incorporation and later on that of thymidine and leucine.

Conclusions: Diverse micro-organisms exhibit sensitivities towards compounds studied. The permeability changes in the cytoplasmic membrane and nonsimultaneous ceasing of macromolecular synthesis suggest that zeylasterone could act on multiple targets on *B. subtilis*.

Significance and Impact of the Study: The activity showed against *B. subtilis* as a model of spore-forming bacteria would provide valuable information for further studies in the development of 6-oxophenolic triterpenoids as antiseptic and disinfectant properties.

Introduction

Although biocides have been used for a century, the number of products containing biocides has recently increased dramatically with public awareness of hygiene issues (Maillard 2002). In the same way, reduced susceptibility to biocides is also apparently increased and different species of bacteria could gradually become less susceptible to most biocides over long periods of time (Russell 2002). Thus, the emergence of resistances force to the development of new compounds with antimicrobial properties. Investigation of the antimicrobial activity of new biocides, especially form natural products (e.g. plant extracts), has recently regained momentum. In this context, the Celastraceae family has a long history in traditional medicine (Muñoz *et al.* 1996; González *et al.* 2000). As part of an intensive investigation into biologically active metabolites from these species, we have previously reported phenolics (Alvarenga *et al.* 1996), triterpenoid quinonemethides named as celastroloids (Moujir *et al.* 1991; González *et al.* 1996b), and dimeric triterpenes (González *et al.* 1996a) from *Maytenus* species, all showing antimicrobial and/or cytotoxic activities.

In our search for antimicrobial agents from natural sources, we previously isolated a series of 6-oxophenolic triterpenoids (zeylasteral, demethylzeylasteral, zeylasterone and demethylzeylasterone) from *Maytenus blepharodes* Lundell, which grows in Panama and whose branches are considered to have antitumoural properties (Gupta 1995). However, these compounds were isolated previously from

Kokoona zeylanica (Gunaherath and Gunatilaka 1983; Gamlath *et al.* 1986) from the Asia-Pacific region, whose outer stem bark is used to relieve headaches and for the treatment of diabetic disorders (Batugal *et al.* 2004). A paste prepared by mixing powdered yellow bark with water is dried and formed into flat pieces to produce 'kokum soap' and used as toilet soap with antiseptic properties (Gunatilaka 1996). Demethylzeylasteral has been reported to exhibit inhibitory action on hepatitis C virus (Jo *et al.* 2006), and antifertility (Bai *et al.* 2003), antitumoural (Ushiro *et al.* 1997), immunosuppressive and anti-inflammatory (Tamaki *et al.* 1997; Lin *et al.* 2003) effects. Furthermore, in a previous work, we demonstrated the antimicrobial action of zeylasteral and demethylzeylasteral (de León *et al.* 2005). However, zeylasterone and demethylzeylasterone have so far been scarcely investigated, and only an inhibitory effect on the enzyme topoisomerase IIα was reported, besides antitumoural activity against the breast cancer cell line MCF-7 (IC_{50} 12·5 μmol l^{-1}) for demethylzeylasterone (Furbacher and Gunatilaka 2001).

The phenolic-type antimicrobial agents have long been used for the antiseptic, disinfectant or preservative properties depending on the compound. Our purpose in this work is to describe the antimicrobial activity of these 6-oxophenolic triterpenoids (Fig. 1) against different micro-organisms, and to investigate the mode of action of zeylasterone on *Bacillus subtilis* cells as model of spore-forming bacteria.

Materials and Methods

Micro-organisms

Strains used for determining antimicrobial activity included *B. subtilis* ATCC 6051, *Bacillus alvei* ATCC 6344, *Bacillus cereus* ATCC 21772, *Bacillus megaterium* ATCC 25848, *Bacillus pumilus* ATCC 7061, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* ATCC 6538, *S. aureus* LMM1 (clinical isolate, University of La Laguna), *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus saprophyticus* ATCC 15305, *Staphylococcus warneri* ATCC 27836, *Enterococcus faecalis* ATCC 29212, *Mycobacterium smegmatis* ATCC19420, *Escherichia coli* ATCC 9637, *Proteus mirabilis* CECT170 (from Type Culture Spanish Collection), *Pseudomonas aeruginosa* AK958 (from the University of British Columbia, Department of Microbiology collection), *P. aeruginosa* ATCC 25668, *Salmonella* sp. CECT456, *Salmonella typhimurium* UBC1 (from the University of British Columbia, Department of Microbiology collection), *Klebsiella oxytoca* LMM2 (clinical isolate, University of La Laguna) and *Candida albicans* UBC2 (from the University of British Columbia, Department of Microbiology collection).

The bacteria cultures were developed in nutrient broth (NB) or brain heart infusion broth (for *E. faecalis* and *M. smegmatis* containing 0·06% Tween 80) and the yeast was cultured in Sabouraud liquid medium at 37°C. All media were purchased from Oxoid.

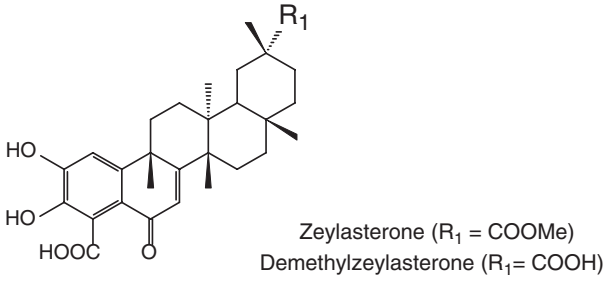

Figure 1 Structure of 6-oxophenolic triterpenoids.

Plant material and isolation of triterpenoids

Maytenus blepharodes was collected at Volcán Varu, Chirique, Panama, in August 1991, and a voucher specimen (FLORPAN n° 1587) is on file in the Department of Medicinal Chemistry and Pharmacognosy, University of Panama. The 6-oxophenolic triterpenoids, zeylasterone and demethylzeylasterone, were isolated at the Instituto Universitario de Bio-Orgánica (IUBO) 'Antonio González' (Tenerife, Canary Islands), from the root of the plant (1·54 kg) and extracted with *n*-hexane–Et_2O (1 : 1), yielding 68 g of extract. The extract was chromatographed on sephadex LH-20 using *n*-hexane–$CHCl_3$–MeOH (2 : 1 : 1) as eluent, followed by repeated chromatography on silica gel with mixtures of different solvents, yielding zeylasterone (440 mg) and demethylzeylasterone (53 mg). The compounds were identified by spectroscopic (IR, UV, NMR) and spectrophotometric (MS, HRMS (high-resolution mass spectrometry)) methods and by comparison with reported data.

In all experiments, the phenolic triterpenoids were added predissolved in dimethylsulfoxide (DMSO).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC were determined for each triterpenoid in triplicate by the broth microdilution method (range 0·08–40 μg ml^{-1}) in 96-well microtitre plates (Chraïbi *et al.* 1990). Wells with the same proportions of DMSO were used as controls, and never exceeded 1% (v/v). The starting micro-organism concentration was approximately 1×10^5 to 5×10^5 CFU ml^{-1}, and growth was monitored by measuring the increase in optical

density (OD) at 550 nm (OD_{550}) with a microplate reader (Multiskan Plus II; Tritertek, Huntaville, AL).

The MIC was defined as the lowest concentration of compound at which growth was inhibited after 24 h – except for *M. smegmatis* (48 h) – of incubation in a rotatory shaker at 37°C. All wells with no visible growth were subcultured by transferring in duplicate (100 μl) to nutrient, BHI or Sabouraud agar plates. After overnight incubation, colony counts were performed and the MBC was defined as the lowest triterpenoid concentration that produced ≥99·9% killing of the initial inoculum.

Growth curves

Overnight liquid cultures of *B. subtilis* were diluted in Monod's flasks containing 10 ml of NB medium, to give a working concentration of 10^6 CFU ml^{-1}. At time 0 (lag-phase) or after 3 h of incubation (log-phase, OD_{550} ~0·4), zeylasterone and demethylzeylasterone were added at 10 and 26 μg ml^{-1}, respectively. These suspensions were incubated at 37°C in a rotatory shaker and growth was monitored by measuring the OD at 550 nm and by counting the viable cells on nutrient agar plates. Cultures prepared under the same conditions but without drugs were used as negative controls. The assays were repeated three times.

Inoculum effect

Overnight liquid cultures of *B. subtilis* were diluted in NB medium to give different inoculum concentrations (approx. 5×10^4, 5×10^5, 5×10^6 and 5×10^7 CFU ml^{-1}), and zeylasterone and demethylzeylasterone were added at concentrations corresponding to 10 and 26 μg ml^{-1}, respectively. These new cultures were incubated at 37°C in a rotatory shaker and aliquots (100 μl) were removed at 0 (inoculum control), 3 and 6 h of incubation. Culture growth was monitored as described earlier. The assays were repeated three times.

Transmission electron microscopy

Suspensions of *B. subtilis* in the log-phase of growth (10^7 CFU ml^{-1}) were treated with zeylasterone at 6 μg ml^{-1} (2 × 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 mol l^{-1} (pH 7·4). After centrifugation (10 000 ***g*** for 10 min, 4°C), the pellets were fixed with 4% glutaraldehyde in 0·1 mol l^{-1} phosphate buffer for 2 h at 4°C. Microbial pellets were embedded in 2% low fusion-point agarose (Serva Electrophoresis GmbH, Germany) and maintained at 4°C for 40 min. The cells were then postfixed in 1% buffered osmium tetroxide for 10 h, stained with 1% uranyl acetate, dehydrated in a graded alcohol series, washed twice in propylene oxide and embedded in durcupan resin (Aname Instrumentación Científica, Madrid, Spain). Ultrathin sections were examined with a Jeol JEM 1010 transmission electron microscope at an accelerated voltage of 100 kV.

Measurement of radioactive precursor incorporation

The total synthesis of DNA, RNA, protein and peptidoglycan were measured by quantifying the incorporation of radiolabelled precursors (thymidine, uridine, leucine and *N*-acetylglucosamine, from Amersham Biosciences Europe GmbH) into acid-insoluble material. An overnight liquid culture of *B. subtilis* was diluted 100 times (~10^6 CFU ml^{-1}) in Davis–Mingoli minimal medium (Davis and Mingoli 1950) with glucose (1%), asparagine (0·1 g l^{-1}) and casamino acids (2 g l^{-1}) (pH 7). The culture was grown at 37°C in a rotatory shaker for approximately 3 h until the OD at 550 nm (OD_{550}) reached 0·4. Aliquots of the culture (10 ml) were then transferred to prewarmed flasks and incubated at 37°C with zeylasterone at 10 μg ml^{-1} and the precursors of DNA [1 μCi ml^{-1} (6-^{3}H)] + 2 μg ml^{-1} unlabelled thymidine], RNA [1 μCi ml^{-1} (5-^{3}H) + 2 μg ml^{-1} unlabelled uridine], protein [5 μCi ml^{-1} (4,5-^{3}H) + 2 μg ml^{-1} unlabelled leucine), and cell-wall peptydoglycan [0·1 μCi ml^{-1} *N*-acetyl-D-(1-^{14}C) glucosamine], respectively. Parallel experiments with the same proportions of DMSO were used as negative control. In each case, a specific inhibitor with a known mechanism of action was included as a positive control.

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 microfibre 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 scintillation cocktail and counted in LKB Wallac 1214 Rackbeta (Perkin Elmer, Courtaboeuf, France). The assays were repeated three times.

Fluorescence labelling

The effect of zeylasterone at 10 and 15 μg ml^{-1} on cytoplasm membrane of *B. subtilis* in exponential phase after 1 h of treatment was determined using the LIVE/DEAD BacLight kit (catalog no. L-7012; Molecular Probes, Eugene, OR, USA) as described in the manufacturer's instructions. BacLight is composed of two nucleic acid-binding stains: SYTO 9 and propidium iodide. SYTO 9 penetrates all bacterial membranes and stains the cells

green, while propidium iodide penetrates only bacteria with damaged membranes, and the combination of the two stains produces red fluorescing cells. Assays were carried out three times in parallel with cell cultures treated with clofoctol at 6 μg ml^{-1} or 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; Leica Microsystems GmbH, Wetzlar, Germany) provided with a fluorescein–rhodamine dual filter.

Leakage of cellular constituents absorbing at 260 nm

The experimental procedure of Chou and Pogell (1981) was adopted. Cells in log-phase were centrifuged at 15 000 ***g*** for 10 min at 4°C, washed with 0·05 mol l^{-1} potassium phosphate buffer (pH 7·3) containing 0·05 mol l^{-1} sucrose, and resuspended in the same buffer to reach a cell density of 10^7 CFU ml^{-1} (OD_{550} ~0·3). The suspension was divided into two equal parts, and zeylasterone (10 μg ml^{-1}) and a similar proportion of DMSO were added, respectively, before incubating at 37°C under shaking. The liberation of the cellular constituents was determined by measuring the OD of the suspensions and supernatant (after removing cells by centrifugation at 8000 ***g*** for 10 min, 4°C) at intervals of 20 min at 550 nm and 260 nm, respectively.

Release of intracellular potassium

Bacillus subtilis cultures in log-phase of growth (OD_{550} ~0·8) were centrifuged at 15 000 ***g*** for 10 min at 4°C, and washed twice with saline buffer. Subsequently, the pellet was resuspended in the same buffer to obtain a bacterial concentration of 1–2 × 10^8 CFU ml^{-1}. The culture was divided into three equal parts, and zeylasterone at 10 μg ml^{-1}, DMSO in the same proportion (used as negative control), and clofoctol at 6 μg ml^{-1} (as positive control) were added, respectively. The samples were removed over a 30-min period, membrane-filtered and K^+ release measured in duplicate by means of an atomic absorption spectrophotometer (Mod. S/Series, Thermo Fisher Scientific, Cambridge, UK).

Results

Antimicrobial activity

The MIC values listed in Table 1 clearly show that the effect of zeylasterone is limited to Gram-positive bacteria and the yeast *C. albicans*, whereas demethylzeylasterone was only weakly active against *B. subtilis* and *B. cereus* (MIC 13 and 30 μg ml^{-1}, respectively). Both compounds were inactive against Gram-negative bacteria (MIC over 40 μg ml^{-1}).

Table 1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of phenolic compounds against different micro-organisms

	MIC/MBC (μg ml^{-1})*	
Bacteria	Zeylasterone	Demethylzeylasterone
Bacillus alvei	4/20	>40/>40
Bacillus cereus	5/>25	30/>40
Bacillus megaterium	4/20	>40/>40
Bacillus pumilus	6/>25	>40/>40
Bacillus subtilis	3/16	13/20
Micrococcus luteus	20/>40	>40/>40
Staphylococcus aureus	6/>25	>40/>40
Staphylococcus aureus LMM1	10/15	>40/>40
Candida albicans	10/>40	>40/>40

*Values represent average obtained from a minimum of three experiments.
The phenolic compounds were inactive against *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus warneri*, *Enterococcus faecalis*, *Mycobacterium smegmatis*, and the Gram-negative bacteria assayed (MIC > 40 μg ml^{-1}).

Effects of the triterpenoids on the growth of *Bacillus subtilis*

The killing kinetic assays were done with *B. subtilis* in the presence of triterpenoids added at different growth phases (Fig. 2). Inclusion of zeylasterone at 10 μg ml^{-1} (~3 × MIC) and demethylzeylasterone at 26 μg ml^{-1} (2 × MIC) in lag-phase produced a 2 $\log_{10}$ reduction in the initial inoculum of *B. subtilis* in the first 2 h of treatment (Fig. 2a). However, when the drugs were incorporated in the log-phase of growth, the same reduction in CFU was obtained for zeylasterone but less (<1 $\log_{10}$) with demethylzeylasterone. Only when the zeylasterone was added in lag-phase, no regrowth was observed after 24 h (data not shown). In addition, a slight decrease from the initial OD (OD_{550}) was observed in the presence of both triterpenoids after 2-h exposure (Fig. 2b), which indicated their lytic nature.

Inoculum effect

Figure 3 shows the activity of the phenolic compounds against *B. subtilis* at different inoculum sizes. The bactericidal activity following exposure to zeylasterone was dependent on the starting inoculum. At low inoculum size (10^4–10^5 CFU ml^{-1}), a greater decrease in CFU ml^{-1} (≥3 $\log_{10}$) was obtained from the starting inoculum, whereas bacteriostatic activity (<3 $\log_{10}$) at higher inocu-

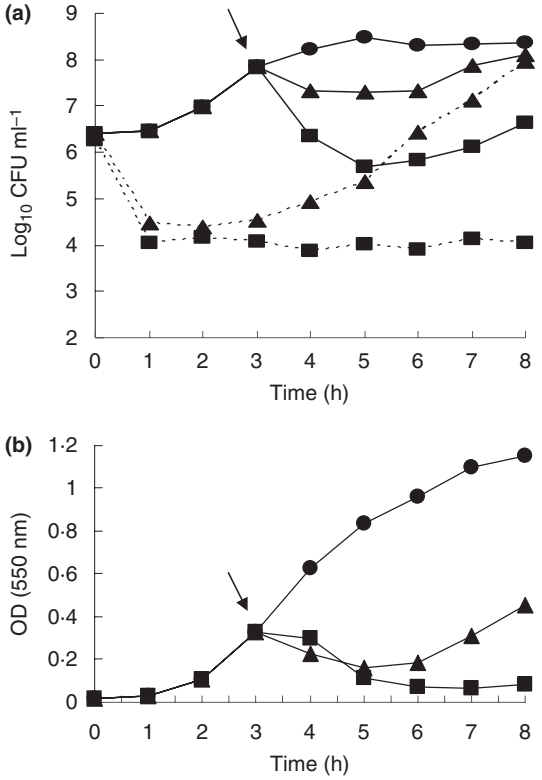

Figure 2 Time-kill curves of *Bacillus subtilis* measured by CFU counts (a) and optical density (b) in the presence of zeylasterone at 10 μg ml^{-1} (■), demethylzeylasterone at 25 μg ml^{-1} (▲) and negative control (●) with the same proportion of dimethylsulfoxide. The compounds were added in lag-phase (dotted line) and log-phase (solid line) of growth (time indicated by arrow).

lum sizes (10^6–10^7 CFU ml^{-1}) was observed. Demethylzeylasterone showed a bacteriostatic effect although higher killing rates were observed at the low inocula (10^4–10^5 CFU ml^{-1}). Note that at 10^7 CFU ml^{-1}, the result differed from that observed when the triterpenoids were added in the log-phase of growth (Fig. 2), as less effect on the cell viability and no fall in OD were seen during the experimentation time.

After 24 h of treatment with zeylasterone, there were morphological changes, where colonies looking like fried eggs were observed on the agar plates.

Ultrastructural changes in *Bacillus subtilis* after treatment with zeylasterone

Transmission electron microscopy was performed on thin sections of bacteria treated for 1 h at 6 μg ml^{-1} (2 × MIC). Treated cultures showed abnormally long cells (some up to 5 μm), variability in wall thickness, compact ribosomes underlying the plasma membrane and mesosomes arising from the septa and cell wall (Fig. 4a,b).

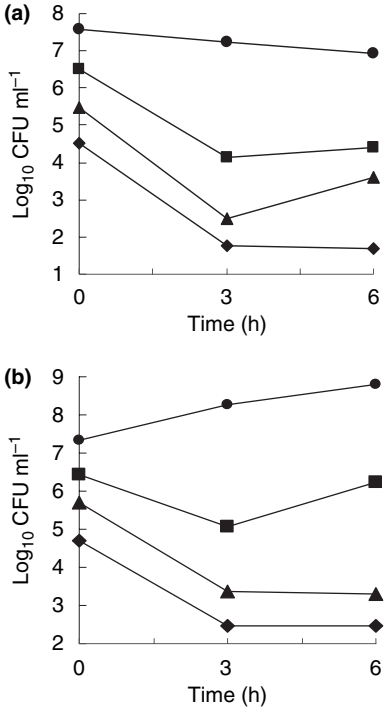

Figure 3 Effect of zeylasterone at 10 μg ml^{-1} (a) and demethylzeylasterone at 25 μg ml^{-1} (b) on *Bacillus subtilis* inocula of: 10^7 CFU ml^{-1} (●); 10^6 CFU ml^{-1} (■); 10^5 CFU ml^{-1} (▲); and 10^4 CFU ml^{-1} (◆).

Control untreated bacteria did not have detectable mesosome structures showing spore-forming cells with normal walls and cytoplasm containing evenly dispersed ribosomes (Fig. 4c,d).

Effects on macromolecular synthesis

The effect of zeylasterone on the incorporation of radiolabelled precursors into DNA, RNA, protein and cell wall synthesis on *B. subtilis* cells was evaluated (Fig. 5).

After addition of triterpenoid at 10 μg ml^{-1}, all processes were blocked but not simultaneously. Within 2 min, incorporation of *N*-acetyl-D-[1-^{14}C] glucosamine into peptidoglycan ceased (Fig. 5d), whereas cell-wall synthesis by penicillin (at 10 × MIC) was not inhibited so rapidly. Zeylasterone also produced a blockage of RNA synthesis resulting in a complete inhibition of [5-^{3}H] uridine incorporation (Fig. 5b), but this effect occurred at around 5 min after addition. Rifampin, a specific inhibitor of RNA synthesis, demonstrated comparable kinetics. The incorporation of [6-^{3}H] thymidine and [4,5-^{3}H] leucine continued throughout the experimentation time, reaching 54% inhibition of DNA and 68% for protein synthesis, after 30 min of incubation with zeylasterone. Nalidixic acid and tetracycline, specific inhibitors of DNA

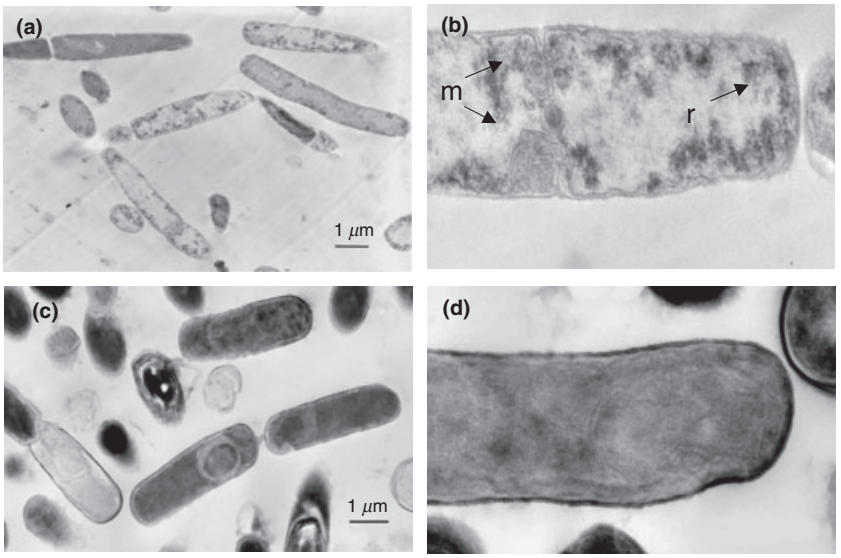

Figure 4 Transmission electron microscopy of *Bacillus subtilis* after exposure for 1 h to zeylasterone at 6 μg ml^{-1} (a and b). Images show the presence of mesosome-like structures (m) arising from the septa and compact ribosomes (r) underlying the plasma membrane. Detail of untreated cells (c and d).

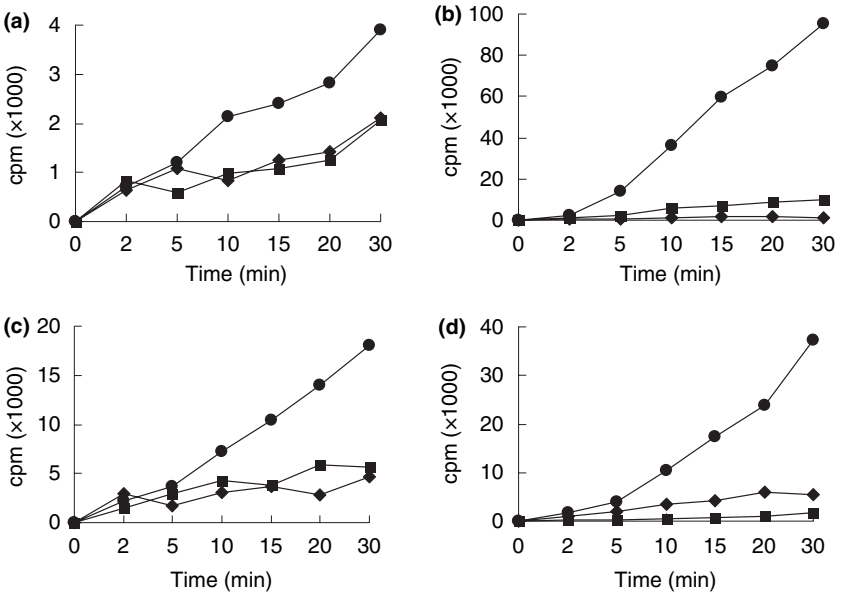

Figure 5 Inhibition by zeylasterone of precursor incorporation into macromolecules in *Bacillus subtilis*. Synthesis inhibition was measured for DNA (a), RNA (b), protein (c) and cell wall (d): untreated control (●) with the same proportion of dimethylsulfoxide; zeylasterone at 10 μg ml^{-1} (■). Known inhibitors were included in each experiment as positive controls (◆): nalidixic acid at 22·5 μg ml^{-1} [2 × minimum inhibitory concentration (MIC)]; rifampin at 0·10 μg ml^{-1} (2 × MIC); tetracycline at 5 μg ml^{-1} (2 × MIC); and penicillin at 30 μg ml^{-1} (10 × MIC).

and protein synthesis, respectively, caused comparable inhibition of incorporation. Clofoctol, a cytoplasmic membrane disruptor (Yablonsky 1983), blocked all biosynthetic processes (70% of inhibition), but at times over 10 min (data not shown).

Membrane damage following exposure to zeylasterone

The effect of zeylasterone on macromolecular synthesis may be related to a membrane-damaging activity. This membrane damage was examined in cultures of *B. subtilis* treated with this compound using the BacLight assay, loss of 260-nm-absorbing materials and measuring the leakage of intracellular potassium. Microscopic observations revealed that the cells treated with zeylasterone at 10 μg ml^{-1} for 1 h maintained membrane integrity, showing green fluorescence as untreated cells. In contrast, red fluorescence was exhibited by cultures treated with 15 μg ml^{-1}, as occurred with clofoctol, revealing damage to the cytoplasmic membrane. Furthermore, when the supernatant fluids from cells treated with zeylasterone at 10 μg ml^{-1} were read, the release of 260-nm-absorbing material was similar to that from untreated cells used as control (data not shown). However, zeylasterone induced marked leakage of intracellular potassium from the bacteria after 10 min, compared with cultures untreated or treated with clofoctol (Fig. 6).

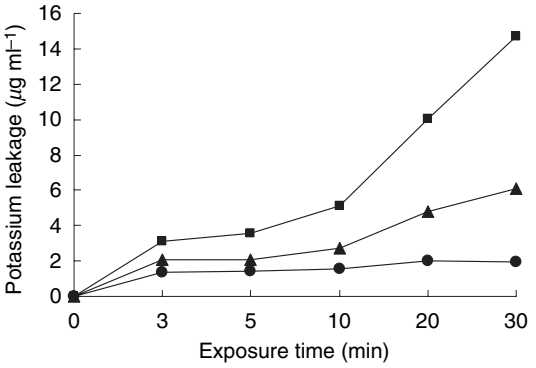

Figure 6 Potassium (K^+) leakage from *Bacillus subtilis* cells exposed to zeylasterone at 10 μg ml^{-1} (■), clofoctol at 6 μg ml^{-1} as positive control (▲) and negative control with the same proportion of dimethylsulfoxide (●).

Discussion

The phenolics are a group of chemicals used as preservatives and disinfectants in the medical and agricultural fields. They have anti-Gram-positive and -negative activity, but are poorly active against nonenveloped viruses and spores (Fraise 2002). Zeylasterone was active against Gram-positive bacteria and the yeast *C. albicans* as were zeylasteral and demethylzeylasteral (de León *et al.* 2005).

Zeylasterone showed higher activity, while demethylzeylasterone was only active against *B. subtilis* and *B. cereus*. The difference between the MIC and MBC of these compounds was less than three twofold dilutions for *B. subtilis*, suggesting cidal activity against this species. Chemically, these triterpenoids differ in the functional groups in ring E. For example, zeylasterone has a methyl ester group at C-29 (R1 = COOMe), whereas demethylzeylasterone has a carboxylic group (R1 = COOH). The difference in the MIC suggests that the increased electronegativity of demethylzeylasterone owing to the carboxylic group in C-29, reduces its antimicrobial potency, as occurred with demethylzeylateral (de León *et al.* 2005).

The killing curves show that both phenolic compounds had bacteriostatic activity (<3 $\log_{10}$ in CFU ml^{-1} reduction), and a slight lytic effect when added in log-phase of growth. The reduction in CFU varies according to the bacterial concentration. At the lowest inoculum (10^4–10^5 CFU ml^{-1}), there was a greater decrease in CFU ml^{-1} from the starting concentration, and zeylasterone showed a bactericidal effect under these conditions. Furthermore, the result obtained at 10^7 CFU ml^{-1} differed from that when phenolic compounds were added in the log-phase, as a less drastic reduction in CFU and no lytic effect were observed. This suggests that actively growing *B. subtilis* is more sensitive to zeylasterone.

Electron microscopy of cultures treated with zeylasterone at 6 μg ml^{-1} did not reveal lysis. Nevertheless, outer-surface alterations were distinguishable, such as cell-wall thinning and membrane thickening that could explain the lyses obtained after exposure of cultures to higher concentrations. Furthermore, the electron micrograph revealed cell membrane bending and mesosome structures arising from the septa, similar to those obtained with cationic peptides (Friedrich *et al.* 2000) and rifampin (Beveridge 1989). Mesosomes have been considered as structural artefacts induced by the chemical fixatives used on the cells prior to plastic embedding and thin sectioning (Beveridge 1989). However, in our case, these structures must be regarded as indicative of some change or damage to the cytoplasmic membrane, as untreated cells did not contain them.

For phenols and phenolic compounds, functional injury to membranes has been proposed as a mechanism of action (Hamilton 1971; Davidson and Branen 1981). Disruption of the cytoplasmic membrane is often indicated by the leakage of intracellular constituents, including potassium release, as first index of membrane damage, followed by inorganic phosphates, the aminoacid pool, materials absorbing at 260 nm, nucleic acids and proteins (Lambert and Hammond 1973). In addition, the fluorescent dyes in the LIVE/DEAD BacLight bacterial viability kit are used for evaluating the physiological condition of bacteria, such as membrane integrity and cell viability (Boulos *et al.* 1999; Sani *et al.* 2001; Alonso *et al.* 2002). Marked efflux of intracellular potassium after 10 min of treatment with zeylasterone (3 × MIC) confirm a membrane disruption. However, measurements of intracellular constituent efflux did not confirm this effect on the cytoplasmic membrane, as no leakage of materials absorbing at 260 nm was detected at least up to 60 min. It is important to emphasize that treatments with zeylasterone at 3 × MIC resulted in green fluorescence as did untreated cells using the LIVE/DEAD BacLight assay, but not at 5 × MIC, indicating membrane disruption at higher concentrations. This suggests a concentration-dependent effect on the cytoplasmic membrane, which may explain the bacteriostatic activity of this phenol at 3 × MIC.

Macromolecular synthesis studies further verified that the incorporation of all radiolabelled precursors did not cease simultaneously, as expected, and if this was merely membrane disruption resulting in leakage of essential molecules (Friedrich *et al.* 2001). In fact, the macromolecular synthesis of peptidoglycan was stopped immediately by the addition of zeylasterone, while 5 min was necessary to block RNA synthesis. Lesser effects were observed in thymidine and leucine incorporation.

The term 'membrane active agent' is used for compounds that, like phenols, are active at the cytoplasmic membrane level of the bacterial cells. In contrast with this, several studies have shown that phenolics might have other targets (Maillard 2002) e.g. DNA, RNA, protein and cell-wall synthesis (Ness and Eklund 1983) and enzyme inhibitors. The results obtained in the present study support the hypothesis that zeylasterone could act on multiple targets in *B. subtilis* even though further works are needed to fully understand the precise mechanism of the action of 6-oxophenolic triterpenoids. The antimicrobial activity showed by these compounds on different microorganisms and the mode of action showed on *B. subtilis* cells, provide useful information for a potential use of these compounds as antiseptic, disinfectant and preservative.

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