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Natural Sesquiterpene Lactones Enhance Oxacillin and Gentamicin Effectiveness Against Pathogenic Bacteria Without Antibacterial Effects on Beneficial Lactobacilli

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This is a report on the synergistic interactions (SIs) between melampolide-type sesquiterpene lactones 1–8 from *Acanthospermum hispidum* DC., and oxacillin or gentamicin, against four pathogenic strains of *Staphylococcus aureus* and *Enterococcus faecalis*; two of them were multi-resistant strains obtained from chronic infectious processes. Our results showed that all associations of 1–8 with antibiotics (ATBs) are more effective than pure ATBs to control pathogenic strains of *S. aureus* and *E. faecalis*. The most relevant SIs were observed when the major lactone of *A. hispidum*, acanthospermal B [5], was combined with gentamicin (protein synthesis inhibitor) against an *ex vivo* culture of methicillin-resistant *S. aureus* SAR 1, displaying a significant MIC reduction in 5 (312.5 to 78.1 μg/mL), and gentamicin (120 μg/mL to 3 μg/mL). Compound 4 improved the antibiotic potency of oxacillin (cell wall synthesis inhibitor) against ampicillin-resistant *E. faecalis* (60 μg/mL to 1.5 μg/mL). It is important to remark that three beneficial lactobacilli were resistant to 1–8 and their mixtures with gentamicin or oxacillin in effective concentrations against pathogenic bacteria. Synergism between ATBs and phytochemicals is a therapeutically helpful concept to improve ATB efficacy and prevent resistance. The present results show that selective SIs occur between melampolides and gentamicin or oxacillin, and open a new field of research.

Keywords: melampolide-type sesquiterpene lactones; synergistic and selective interactions; pathogenic strains; *Lactobacillus*; approach to antibacterial mechanism of lactones.

INTRODUCTION

The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and dissemination of resistant strains. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections not only in Argentina but also in the whole world. MRSA infections are very difficult to cure because MRSA strains are resistant to almost all clinically available antibiotics (ATBs). Consequently, there is a pressing need to identify new types of antibacterial agents or new effective ways for the treatment of infectious diseases caused by drug-resistant bacteria including MRSA (Taylor *et al.,* 2002; Adwan and Mhanna, 2008).

In search of more effective chemotherapeutic agents for treating microbial infections, combination therapy becomes an important strategy as synergistic interactions can potentially increase efficacy, reduce toxicity, cure faster, prevent the emergence of resistance, and provide broader spectrum of activity than monotherapy (Olajuyigbe and Afolayan, 2012). Synergistic effects occur when the constituents of a mixture affect different targets or interact with one another in order to enhance the bioavailability of one or several substances of the mixture. A special synergy effect is detected when ATBs are combined with an agent that antagonizes bacterial resistance mechanisms (Wagner and Ulrich-Merzenich, 2009).

Acanthospermum hispidum DC. is a shrub indigenous to northern Argentina. The infusion of the aerial parts is employed in folk medicine against infections, and as a diuretic, abortive, and insect repellent. Our previous research on the chemistry and antibacterial action of sesquiterpene lactones (SLs) from this species (Cartagena *et al.,* 2000, 2007, 2008; Arena *et al.,* 2011) indicated that the antibacterial activity was selective in a group of Gram positive human pathogenic strains, being harmless on three *Lactobacillus* strains. These previous results lead us to investigate *in vitro* SIs of eight SLs [**1–8**] from *A. hispidum* with two antibiotics on pathogenic and non pathogenic bacteria.

In addition, SLs [**1–8**] were submitted to a chemical reaction with 1,4-dithiothreitol (DTT) in order to assess the ability of **1–8** to react with SH groups of bacterial enzymes or proteins in a biomimetic reaction. A possible SL antibacterial mechanism could be derived from the results of this reaction.

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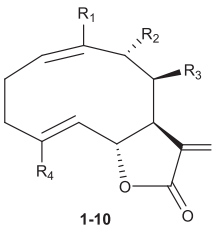

1-10

	R_1	R_2	R_3	R_4
1	CHO	H	MeBut	CH_2OH
2	CHO	H	*i*-Val	CH_2OH
3	CHO	OAc	*i*-But	CH_2OH
4	CHO	OAc	Ang	CH_2OH
5	CHO	OAc	MeBut	CH_2OH
6	CHO	OH	MeBut	CH_2OH
7	CH_2OH	OAc	*i*-But	CH_2OH
8	CH_2OH	OAc	Ang	CH_2OH
9	$COOCH_3$	OH	OH	CH_3
10	$COOCH_3$	OH	Meacr	CH_3

Figure 1. Sesquiterpene lactones structures.

MATERIALS AND METHODS

Extraction, purification, and identification of natural SLs. Aerial parts of *A. hispidum DC.* were collected at the flowering stage in Vipos, Tucumán (Argentina). A voucher specimen LIL # 604458 is on deposit at the herbarium of Fundación Miguel Lillo, Tucumán, Argentina. The melampolide-type SLs [**1–8**] (Fig. 1) were isolated from a chloroform extract and purified by RP-HPLC using C-8 and C-18 columns, and MeOH-H_2O, as a mobile phase. Compounds were unambiguously identified by their IR, NMR, and MS spectra by comparison with previously reported data (Cartagena *et al.,* 2000).

Bacterial strains. Representative isolates from clinically relevant bacterial strains, collected during clinical trials at Hospital Avellaneda, Tucumán, were used in this study. The Gram-positive microorganisms were wild strains of *S. aureus* (MRSA, SAR 1), *Enterococcus faecalis* (ampicillin-resistant, F 208), and collection strains of *S. aureus* ATCC (American Type Culture Collection) 6538 P and *E. faecalis* ATCC 39212. Non pathogenic strains *Lactobacillus paracasei* ssp. *paracasei* CE 75 and *L. plantarum* CE 105 were isolated from regional cheeses, while *L. plantarum* CE 358 came from high mountain soils of the Argentine northwest (Collection of Centro de Referencia de Lactobacilos, CERELA, Tucumán, Argentina).

Evaluation of antibacterial and synergistic effects of the mixtures of ATBs with compounds 1–8. SIs between gentamicin or oxacillin (Sigma Aldrich) and natural SLs [**1–8**] were evaluated in order to support the future use of these commercial antibiotics in a combination therapy with natural products that display a selective anti-pathogenic action. Antibacterial activity tests were conducted by the micro dilution method using Mueller–Hinton as a culture medium (NCCLS, 2000) in polystyrene microplates of 96 wells. The DMSO-PEG 400 system was used to dissolve melampolides [**1–8**] and to improve their solubility in water (Cartagena *et al.,* 2008). The DMSO–PEG 400 mixture, incorporated to the culture medium under identical experimental conditions, displayed no antibacterial effect and was used as control. Antibacterial action of SLs [**1–8**] was evaluated at doses ranging from 625 to 19.5 μg/mL, while the ATBs were tested in amounts that went from 120 to 0.09 μg/mL in order to determine the minimum inhibitory concentrations (MICs). After incorporation of each substance to microbroth dilution plates, they were inoculated with each microorganism to yield the appropriate bacterial density (10^5 CFU/mL) in a final volume of 100 μL. Then, they were incubated for 24 h at 37 °C.

Synergistic effects of the mixtures of each SL and each ATB were determined according to the following formula: FIC index = $FIC_A + FIC_B = [A]/MIC_A + [B]/MIC_B$. FIC_A, FIC_B: Fractional inhibitory concentration of drug A and B, respectively. MIC_A, MIC_B: Minimum inhibitory concentration of drug A and B, respectively. [A], [B]: Concentration of drug A and B, respectively. FIC index by checkerboard method is interpreted as follows: ≤ 0.5 synergy; >0.5 and ≤ 4 additivity and > 4 antagonism (Jayaraman *et al.,* 2010).

Effects of the mixtures of ATBs with compounds 1–8 on non pathogenic *Lactobacillus* cultures. The ATB (gentamicin or oxacillin) associations with SLs [**1–8**] that showed synergistic interactions against pathogenic strains (Table 2) were also evaluated on the three *Lactobacillus* strains using the micro dilution method at the highest MICs obtained against *S. aureus* and *E. faecalis*. The ATBs (120 to 6 μg/mL) and **1–8** were also individually screened, at the concentrations reported in Table 3. Bacterial growth was corroborated by plating 0.1 mL of the inoculation onto agar, and viable cell colonies were counted.

An approach to antibacterial mechanism displayed by melampolide-type SLs [1–8]. To evaluate one of the SL molecular mechanisms of antibacterial action, a *in vitro* reaction of SLs with 1,4-dithiothreitol (DTT) was carried out. DTT reacts with SLs as does

Table 1. Antibacterial activity of SL [1–8] and ATB

	MIC [μg/mL]									
Microorganisms	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	GEN	OXA
S. aureus ATCC 6538 P	1250	625	1000	312.5	1250	1250	156.2	156.2	6	1.5
S. aureus SAR 1	1250	625	1000	78.1	312.5	1000	156.2	156.2	120	25
E. faecalis ATCC 39212	1250	1250	1000	312.5	1250	1000	156.2	156.2	6	6
E. faecalis F 208	1250	1250	1000	625	1250	1000	312.5	312.5	120	60

S.: *Staphylococcus*. *E.*: *Enterococcus*. GEN: Gentamicin. OXA: Oxacillin. Compound numbers in bold.

Table 2. MIC [μg/mL] and FIC index (FICI) of associations of SL [1–8] with ATB

	S. aureus ATCC 6538 P			*S. aureus* SAR 1			*E. faecalis* ATCC 39212			*E. faecalis* F 208		
Mixtures	**1**	**GEN**	**FICI**	**1**	**GEN**	**FICI**	**1**	**GEN**	**FICI**	**1**	**GEN**	**FICI**
1 + GEN	420	3	0.8 (NI)	500	3.5	0.4 (S)	250	1.5	0.4 (S)	250	1.5	0.2 (S)
	2			**2**			**2**			**2**		
2 + GEN	78.1	0.38	0.2 (S)	312.5	1.5	0.5 (S)	625	3	1 (NI)	312.5	1.5	0.3 (S)
	3			**3**			**3**			**3**		
3 + GEN	125	0.78	0.2 (S)	500	3.5	0.5 (S)	500	3.5	1 (NI)	500	3.5	0.5 (S)
	4			**4**			**4**			**4**		
4 + GEN	78.1	1.5	0.5 (S)	78.1	1.5	1.1 (NI)	78.1	1.5	0.5 (S)	312.5	3	0.5 (S)
	5			**5**			**5**			**5**		
5 + GEN	250	1.5	0.5 (S)	78.1	3	0.3 (S)	500	1.5	0.7 (NI)	500	1.5	0.4 (S)
	6			**6**			**6**			**6**		
6 + GEN	250	1.5	0.4 (S)	500	3	0.5 (S)	500	3	1 (NI)	500	3	0.5 (S)
	7			**7**			**7**			**7**		
7 + GEN	39.1	1.5	0.5 (S)	78.1	3	0.6 (NI)	39.1	1.5	0.5 (S)	39.1	1.5	0.3 (S)
	8			**8**			**8**			**8**		
8 + GEN	19.5	0.75	0.2 (S)	78.1	1.5	0.5 (S)	78.1	3	1 (NI)	156.2	3	0.5 (S)
	1	**OXA**		**1**	**OXA**		**1**	**OXA**		**1**	**OXA**	
1 + OXA	125	0.78	0.6 (NI)	500	3	0.5 (S)	125	0.78	0.2 (S)	250	1.5	0.2 (S)
	2			**2**			**2**			**2**		
2 + OXA	78.1	0.38	0.2 (S)	312.5	1.5	0.6 (NI)	625	3	1 (NI)	625	3	0.6 (NI)
	3			**3**			**3**			**3**		
3 + OXA	250	1.5	1.3 (NI)	500	3	0.6 (NI)	250	1.5	0.5 (S)	250	1.5	0.3 (S)
	4			**4**			**4**			**4**		
4 + OXA	78.1	1.5	1.2 (NI)	78.1	1.5	2 (NI)	78.1	1.5	0.5 (S)	78.1	1.5	0.1 (S)
	5			**5**			**5**			**5**		
5 + OXA	250	1.5	1.2 (NI)	156	3	0.6 (NI)	500	3	0.9 (NI)	250	1.5	0.2 (S)
	6			**6**			**6**			**6**		
6 + OXA	250	1.5	1.2 (NI)	500	3	0.6 (NI)	500	3	1 (NI)	500	3	0.6 (NI)
	7			**7**			**7**			**7**		
7 + OXA	39.1	1.5	1.2 (NI)	78.1	3	0.6 (NI)	39.1	1.5	0.5 (S)	39.1	1.5	0.2 (S)
	8			**8**			**8**			**8**		
8 + OXA	19.5	0.38	0.4 (S)	156.2	3	1.1 (NI)	78.1	3	0.8 (NI)	156.2	3	0.5 (S)

S. aureus: *Staphylococcus aureus. E. faecalis*: *Enterococcus faecalis*. GEN: Gentamicin. OXA: Oxacillin. FICI: Fractional inhibitory concentration index. NI: Non interaction or additive effect. S: Synergistic effect. Compound numbers in bold type.

2-mercaptoethanol used as a biomimetic model of the topoisomerase II-DNA complex (Neder *et al.,* 1998). DTT (1 μM) and α-methylene-γ-lactones **1–8** (0.5 μM) were dissolved in 100μL EtOH and kept with constant shaking for 1 min at room temperature. The reaction mixtures containing the adducts of DTT-SL were detected by TLC with $Ce(SO_4)_2$ as chromatographic reagent. The reaction mixtures were then incorporated to a hole plate in the antibacterial activity assay. They were placed in the 3 mm holes that contained microorganisms in a dilution of 10^8 CFU/mL. A 0.5 μM solution of each SL [**1–8**] was used as a positive control, while DTT (1 μM) was the negative control under the same experimental conditions. It is important to remark that synthetic SL **9**, which lacks the α-methylene-γ-lactone group (Cartagena *et al.,* 2008), was also incorporated to the test. *S. aureus* MRSA (SAR 1), *E. faecalis* ampicillin-resistant (F 208), and collection strains of *S. aureus* ATCC 6538 P and *E. faecalis* ATCC 39212 were employed in the bioassay.

RESULTS AND DISCUSSION

Antibacterial and synergistic activities

Antibacterial effects of SLs were reported by Picman in Picman (1986). Particularly, the antibacterial properties of **1–8** were informed by Cartagena *et al.* in Cartagena *et al.* (2008).

The results of this research show that the associations of SLs [**1–8**] with ATBs are more effective than pure ATBs to control pathogenic strains of *S. aureus* and *E. faecalis* (Tables 1 and 2). The most relevant SIs were observed when the major SL of *A. hispidum,* acanthospermal B [**5**], was combined with gentamicin (protein synthesis inhibitor) against an *ex vivo* culture of methicillin-resistant *S. aureus* (SAR 1), displaying a significant MIC reduction in **5** (312.5 to 78.1 μg/mL), and gentamicin (120 μg/mL to 3 μg/mL); therefore, FIC index = 0.3. Compound **4** improved the antibiotic potency of oxacillin (cell wall synthesis inhibitor) against ampicillin-resistant *E. faecalis* (FIC index = 0.1) as shown in Table 2. All SLs (except for **1**) produced synergistic effects in mixtures with gentamicin on *S. aureus* ATCC 6538 P. In addition, all SLs with gentamicin displayed synergistic effects on ampicillin-resistant *E. faecalis* (Table 2).

The present is a new example of natural products that generate antibacterial synergistic effects in combination with clinical ATBs as previously reported for *A. hispidum* essential oil (Alva *et al.,* 2012), and other plant extracts (Yam *et al.,* 1998; Yang *et al.,* 2005; Esimone *et al.,* 2006; Sibanda and Okoh, 2008; Adwan and Mhanna, 2008; Hijleh *et al.,* 2009). Based on previous studies, plants are sources of potential resistance modifying agents (Gibbons *et al.,* 2003; Sibanda and Okoh, 2007; Adwan and Mhanna, 2008). The discovery of synergistic combinations between plant-derived natural products and ATBs would be explained by a double attack of both agents on different target sites of the bacteria (Esimone *et al.,* 2006; Adwan and Mhanna, 2008; Jayaraman *et al.,* 2013). Gershenzon and Dudareva (2007) reported that the lipophilic nature of many terpenoid compounds suggests that their principal targets are cell membranes and that their toxicity is caused by loss of chemiosmotic control. Terpenes might also synergize the effects of other toxins by acting as solvents to facilitate their passage through membranes. This phenomenon is now being exploited by pharmacologists seeking new ways to achieve drug delivery through the skin (Kanikkannan *et al.,* 2000).

In addition, further studies using effluxing strains (Gibbons *et al.,* 2003) are required to verify if the observed synergism is due to the inhibition of bacterial efflux pumps that play a main role in the multidrug resistance.

Effects of SLs [1–8] and their ATB mixtures on *Lactobacillus* cultures

The SLs [**1–8**] and their associations with ATBs, in effective concentrations for pathogenic bacteria (Tables 1 and 2), resulted innocuous for the three strains of lactobacilli (in all experiments and controls 1–5 10^5 CFU/mL was recovered). On the other hand, pure ATBs, at higher doses than in the mixtures, were toxic as expected (Table 3). It is important to note that probiotic *Lactobacillus* strains, which were isolated from natural environments of northern Argentina, were resistant to SLs obtained from plants collected in the same region, as published in a previous report (Cartagena

Table 3. Effects of SLs [1–8], ATBs, and their associations on *Lactobacillus* cultures*

SLs, ATBs, and associations	[μg/mL]	*Lactobacillus* growth
1	1250	+
2	1250	+
3	1000	+
4	625	+
5	1250	+
6	1250	+
7	312.5	+
8	312.5	+
GEN	6–120	–
OXA	6–60	–
1 + GEN	500 + 3.5	+
1 + OXA	500 + 3.5	+
2 + GEN	625 + 3	+
2 + OXA	625 + 3	+
3 + GEN	500 + 3.5	+
3 + OXA	500 + 3	+
4 + GEN	312.5 + 3	+
4 + OXA	78.1 + 3	+
5 + GEN	500 + 1.5	+
5 + OXA	500 + 3	+
6 + GEN	500 + 3	+
6 + OXA	500 + 3	+
7 + GEN	78.1 + 3	+
7 + OXA	78.1 + 3	+
8 + GEN	156.2 + 3	+
8 + OXA	156.2 + 3	+

**Lactobacillus paracasei* ssp. *paracasei* CE 75, *L. plantarum* CE 105, and *L. plantarum* CE 358. GEN: Gentamicin, OXA: Oxacillin. (+): Bacterial growth (1–5 10^5 CFU/mL were counted). (–): Bacterial growth inhibition (no colonies were detected). Compound numbers in bold type.

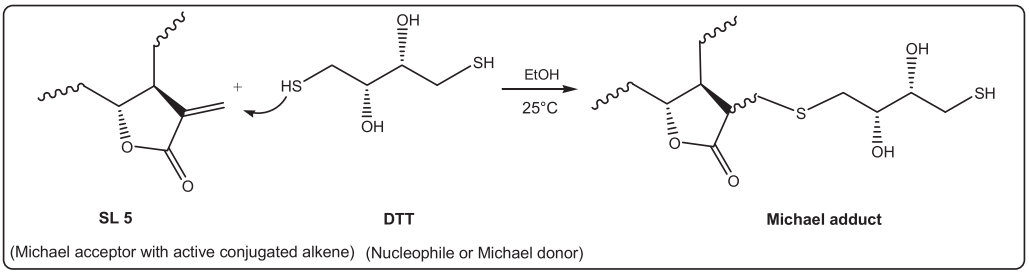

Figure 2. Biomimetic reaction model.

et al., 2008). Indeed, lactobacilli can be found in plants or material of plant origin, silage, ferments, as well as in oral cavities, gastrointestinal tracts (GIT), and human and animal vaginas. In particular, the *Lactobacillus* species found in the GIT have received tremendous attention due to their health-promoting properties. They are commonly used as probiotics, which are defined by the FAO/WHO as live microorganisms that confer a health benefit on the host when administered in adequate amounts. It has become more and more evident that shifts in gut commensal populations and an aberrant immune reaction toward these microbes are associated with several disease conditions such as allergies, inflammatory bowel disease, obesity, and colon cancer. Redress of these ecological and immunological imbalances, for instance by probiotics, has the potential to ameliorate and prevent disease (Walter, 2008).

An approach to molecular mechanism involved in the antibacterial effects of melampolide-type SLs

Our results indicate that the adducts resulting from the reaction of SLs and DTT (Fig. 2) produce no antibacterial effects, as DTT blocks the antibacterial effects of compounds **1–8**. A similar reaction might occur between SLs and biological nucleophilic targets like proteins and enzymes containing cysteine. SLs might inactivate proteins and enzymes involved in bacterial resistance mechanisms. It is generally accepted that the α-methylene-γ-lactone moiety (Michael acceptor) of SLs is the key group that correlates with biological activity (Picman *et al.,* 1979; Mares, 1987; Jodynis-Liebert *et al.,* 2000; Özçelik *et al.,* 2009). In fact, the semi synthetic SL **9**, previously obtained in our laboratory by reduction of the α-methylene-γ-lactone group of compound **10** (Cartagena *et al.,* 2008), did not react with DTT.

Selectivity and antibacterial synergistic effects of combinations of melampolide-type SLs with ATBs might be important for the design of a new generation of antibiotics innocuous for the lactic acid bacteria present in native microbiota.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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