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## Accepted Manuscript

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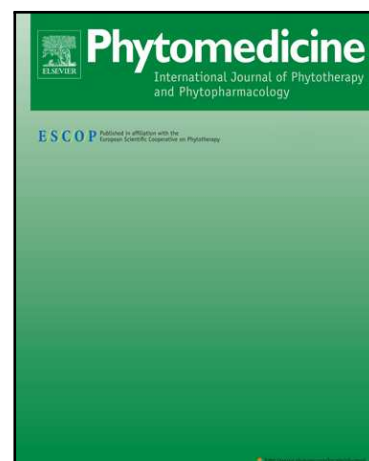
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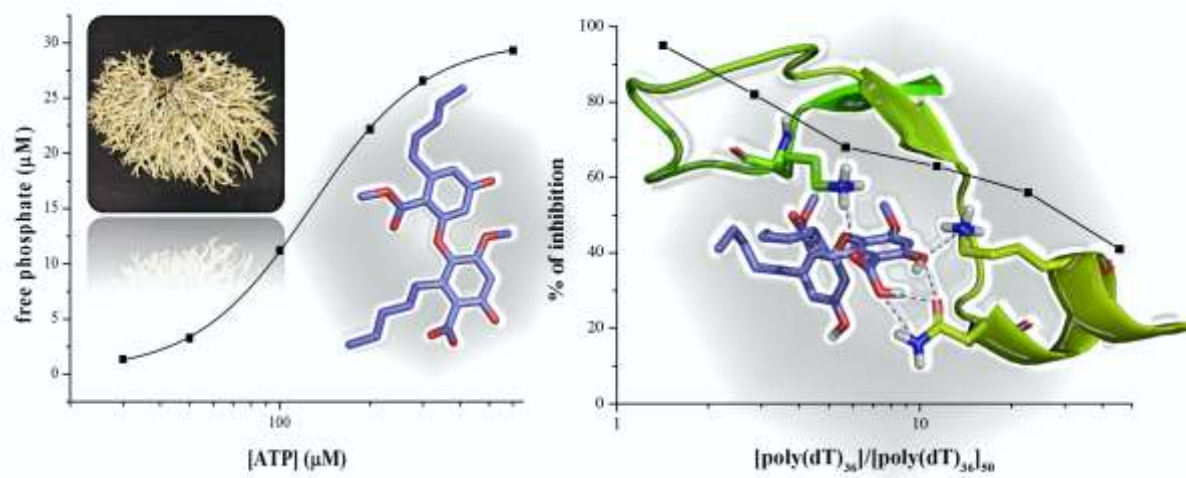
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## Graphical abstract



ACCEPTED MANUSCRIPT

## SOS response in bacteria: inhibitory activity of lichen secondary metabolites against *Escherichia coli* RecA protein

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### ABSTRACT

**Background:** RecA is a bacterial multifunctional protein essential to genetic recombination, error-prone replicative bypass of DNA damages and regulation of SOS response. The activation of bacterial SOS response is directly related to the development of intrinsic and/or acquired resistance to antimicrobials. Although, recent studies directed towards RecA inactivation *via* ATP binding inhibition described a variety of micromolar affinity ligands, inhibitors of the DNA binding site are still unknown.

**Purpose:** Twenty-seven secondary metabolites classified as anthraquinones, depsides, depsidones, dibenzofurans, diphenyl-butenolides, paraconic acids, pseudo-depsidones, triterpenes and xanthones, were investigated for their ability to inhibit RecA from *Escherichia coli*. They were isolated in various Chilean regions from 14 families and 19 genera of lichens.

*Methods:* The ATP hydrolytic activity of RecA was quantified detecting the generation of free phosphate in solution. The percentage of inhibition was calculated fixing at 100  $\mu\text{M}$  the concentration of the compounds. Deeper investigations were reserved to those compounds showing an inhibition higher than 80%. To clarify the mechanism of inhibition, the semi-log plot of the percentage of inhibition *versus* ATP and *versus* ssDNA, was evaluated.

*Results:* Only nine compounds showed a percentage of RecA inhibition higher than 80% (divaricatic, perlatolic, alpha-collatolic, lobaric, lichesterinic, protolichesterinic, epiphorellic acids, sphaerophorin and tumidulin). The half-inhibitory concentrations ( $\text{IC}_{50}$ ) calculated for these compounds were ranging from 14.2  $\mu\text{M}$  for protolichesterinic acid to 42.6  $\mu\text{M}$  for sphaerophorin. Investigations on the mechanism of inhibition showed that all compounds behaved as uncompetitive inhibitors for ATP binding site, with the exception of epiphorellic acid which clearly acted as non-competitive inhibitor of the ATP site. Further investigations demonstrated that epiphorellic acid competitively binds the ssDNA binding site. Kinetic data were confirmed by molecular modelling binding predictions which shows that epiphorellic acid is expected to bind the ssDNA site into the L2 loop of RecA protein.

*Conclusion:* In this paper is describe the first RecA ssDNA binding site ligand. Our study set epiphorellic acid as a promising hit for the development of more effective RecA inhibitors. In our drug discovery approach natural products in general and lichen specifically, represent a successful source of active ligands and structural diversity.

*Keywords:*

Bacterial resistance; SOS response; RecA; Lichen secondary metabolite; Natural source inhibitor;

*Abbreviations:*

MDR, Multi-Drug Resistant; ATP, Adenosine triphosphate; DMSO, dimethyl sulfoxide; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; TLC, thin layer chromatography; dNTP, deoxynucleoside triphosphate;  $\text{IC}_{50}$ , half-maximal inhibitory concentration; ssDNA, single stranded DNA.

## Introduction

Multi Drug Resistant (MDR) bacteria to all available antibiotics, represent a global emergency, limiting *de facto*, the effective treatment of bacterial infections. In the 2014 Global Report on Antimicrobial Resistance, the World Health Organization stated that we are entering a post-antibiotic era in which "common infections and minor injuries can kill.", as in the recent past (WHO, 2014). Since WHO considers bacterial resistance among the three main risks for human health, and the identification of alternative targets and the development of new drugs to circumvent resistance, appears as strongly necessary.

Bacteria possess an extraordinary ability to adapt and evolve in response to antibiotics, acquiring and implementing multiple resistant mechanisms. Among these, the SOS pathway plays a crucial role. Bacteria utilise the SOS response pathway to counteract a variety of adverse and stressful conditions, antimicrobials included. SOS response was proposed by Miroslav Radman in 1975 (Radman, 1975) and defined as "an inducible DNA repair system ("SOS repair") which is also responsible for induced mutagenesis".

Bacteria exposed to agents that interfere with DNA replication, or damage DNA, induces the expression of a number of genes related to SOS response. In bacteria, most of the genes involved in the SOS response are under control of two SOS regulators: RecA, which can be considered the "stress sensor protein", and LexA, the "effector protein" (Culyba et al., 2015). Briefly, as a consequence of damage to DNA, RecA induces, upon ATP binding, self-cleavage of LexA, a transcriptional repressor that binds a specific palindromic sequence of 16-19 base pairs called *lexA* binding box. LexA self-cleavage induces the expression of more than 40 genes involved in a variety of functions, among them, DNA repair and mutagenesis (Lenhart et al., 2012; Patel et al., 2010). Furthermore, the activation of the SOS response is directly related to bacterial ability to acquire resistance from outside by horizontal gene transfer. For instance, gene encoding integrases in integrons are under LexA control (Cirz and Romesberg, 2007). As a result, the SOS pathway under RecA/LexA regulation is directly involved in several mechanisms by which pathogens can tolerate antimicrobials and acquire drug resistance.

Recently the SOS response has been validated as an important target for combating the evolution of antibiotic resistance: its inactivation, by deletion of RecA or by engineering a noncleavable LexA into the bacteria, inevitably slows bacterial acquisition of drug resistance (Cirz and Romesberg, 2007; Leite et al., 2016). When SOS activity is reduced, the antibiotic-induced mutation is suppressed (Mo et al., 2016). Moreover, in infection models, the inactivation of LexA self-cleavage decreases considerably the bacterial ability in acquiring resistance (Culyba et al., 2015).

RecA is a highly conserved ~38 kDa protein characterised by the presence of three domains: a central core RecA ATPase domain surrounded by a smaller N-terminal regulatory domain (NTD) and a large C-terminal regulatory domain (CTD). ATPase domain contains the nucleotide binding site, the conserved Walker A and B motifs, and the putative DNA binding site(s), designed loops L<sub>1</sub> and L<sub>2</sub> (Leite et al., 2016). RecA monomers can form extended nucleoprotein filaments on single stranded DNA (ssDNA) across thousands of base pairs *via* oligomerization mediated by the core RecA fold. Filamentous RecA has a helical groove that envelope DNA, preparing it for homology searching and DNA strand exchange. ATP is required

for filament formation and simple DNA strand exchange reactions. The *core* RecA fold binds ATP at the monomer-monomer interface.

ATP is required for filament formation and simple DNA strand exchange reactions. RecA also catalyses ATP hydrolysis, important for filament depolymerization and other recombinative activities. Moreover, ATP binding, but not hydrolysis, is required for RecA co-protease activity on LexA self-cleavage stimulation *via* RecA allosteric modulation.

Blocking ATP binding on RecA might have strong effects on RecA recombinant activities and SOS response activation. The inhibition of LexA self-cleavage blocks the induction of the SOS response, reducing the acquisition and integration of new resistance genes and as a result the ability of bacteria to mutate. As a consequence, RecA inhibition would delay the development of resistant bacteria by acting at the level of horizontal and vertical transfer of resistance genes (Delmas and Matic, 2005).

Nowadays, several studies targeting *E. coli* RecA ATP binding site have been undertaken, and few potential inhibitors of RecA ATPase activity have been identified; interestingly many of them comes from a variety of natural sources (Alam et al., 2016; Bellio et al., 2014; Lee et al., 2005; Nautiyal et al., 2014; Sexton et al., 2010; Wigle et al., 2006; Wigle et al., 2009; Wigle and Singleton, 2007; Xiao et al., 2006a, b).

We recently reported the synergistic activity of lichen derivatives in combination with “old” antimicrobials. Lichen secondary metabolites can increase the efficacy of antimicrobials against Multi Drug Resistance (MDR) methicillin-resistant *Staphylococcus aureus* (Bellio et al., 2015; Celenza et al., 2013; Segatore et al., 2012). Secondary metabolites from lichens, comprising more than 20,000 species, represent an immense resource of starting scaffolds for the development of new drugs (Ranković and Kosanić, 2015). To date, the functions of the majorities of secondary metabolites are not still deeply understood in the lichens symbioses (Hager et al., 2008) and most of them are not absolutely essential for their survival and growth (Bentley, 1999). The environments where lichens implement their life cycle is very complex and varied and the synthesis of secondary metabolites is strictly connected with defence mechanisms against competitors, herbivores, pathogens but also to counteract all the variable environmental factors as temperature fluctuation, elevation and UV exposition. Cultures across the world have used lichens in traditional medicine for centuries, commonly for treating wounds, skin and respiratory infections, digestive issues, as well as obstetric and gynaecological disorders (Crawford, 2015). Recently, secondary metabolites from lichens have been well investigated for their biological activities as anticancer (Brisdelli et al., 2016; Stanojković, 2015), anti-inflammatory, antifungal, antimicrobial (Hemaiswarya et al., 2008; Kosanić and Ranković, 2015a) and antioxidant (Kosanić and Ranković, 2015b).

Unlike the studies previously mentioned, this paper addresses these molecules to a specific target. Twenty-seven secondary metabolites from lichens were tested as potential inhibitors of RecA activity and for the most active one, the mechanism of action and binding mode deeply investigated.

## Materials and methods

### Reagents

Adenosine triphosphate (ATP), dimethyl sulfoxide (DMSO), isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), Tris-HCl, imidazole, glycerol, turbonuclease,  $\beta$ -mercaptoethanol and kanamycin were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, Missouri, USA). The oligonucleotide named poly(dT)<sub>36</sub> was purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA), BIOMOL Green was obtained from Enzo Life Sciences Inc., subsidiary of Enzo Biochem. Inc. (Farmingdale, NY, USA) and Lysozyme from EuroClone S.p.A. (Pero, MI, Italy).

### Lichen secondary metabolites

All the compounds were isolated from lichens collected in various Chilean regions, including the Chilean Antarctic Territory (Table 1). Lichen compounds were isolated and structurally determined as previously described (Garbarino et al., 1987; Garbarino et al., 1991; Piovano et al., 1985; Piovano et al., 1991; Piovano et al., 1993; Piovano et al., 2001; Quilhot et al., 1983; Quilhot et al., 1989) and their structures are reported in Table S1 (supplementary content). The degree of purity for the compounds was >98% as determined by HPLC analysis which chromatograms are shown in Table S2 (supplementary content).

### Preparation of DNA construct

The *recA* gene was amplified by PCR from genomic DNA of *Escherichia coli* clinical isolate with specific oligonucleotides, NdeI\_for, 5'-GGGGGG**CATATG**GCTATCGACGAAAAC-3' containing NdeI restriction site (in bold underlined) and XhoI\_rev, 5'-GGGGG**CTCGAG**TAAAAATCTTCGTTAGTTT-3' containing XhoI restriction site (in underlined bold). The amplification was carried out in 100  $\mu$ l reaction: 2 ng of DNA template, 800 pmol of each oligonucleotide, 2  $\mu$ l of 10 mM dNTPs mix, 2 units of Q5 Hot Start High-Fidelity DNA Polymerase from New England Biolabs (Ipswich, MA, USA), 1X of Q5 Reaction Buffer and 1X of Q5 High GC Enhancer.

The His-tagged N-terminal affinity-fusion protein was over-expressed in pET-28b(+) vector from Merck Millipore, subsidiary of Merck KGaA (Darmstadt, Germany). PCR products and pET-28b(+) were subjected to double enzymatic digestion using NdeI and XhoI restriction enzymes followed by ligation using T4 DNA ligase (New England Biolabs). *E. coli* JM109(DE3) competent cells, having genotype *endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17* ( $r_k^-$ ,  $m_k^+$ ), *relA1*, *supE44*,  $\lambda^-$ ,  $\Delta(lac-proAB)$ , [F', *traD36*, *proAB*, *lacI<sup>q</sup>Z $\Delta$ M15*],  $\lambda$ DE3, were transformed with the pET28b-*recA* plasmid. The construct was extracted and purified from these cells using Exprep Plasmid SV mini kit from GeneAll Biotechnology Co., LTD (Dongnam-ro, Songpa-gu, Seoul, Korea) and the integrity of the cloned fragment was confirmed by DNA sequencing.



### *Expression and purification of RecA protein*

The transformed recombinant bacterial strain was grown in Terrific Broth (TB) medium with 50 mg/l kanamycin. The culture was incubated at 37°C in orbital shaker at 220 rpm. When the optical density at 600 nm reached 0.4-0.5, 1 mM of IPTG was added and the culture incubated overnight at 20°C. Cells were pelleted (4,000 rpm, 30 min, 4°C) and washed twice with washing buffer containing 20 mM Tris-HCl (pH 8.0). The obtained pellet was resuspended in lysis buffer (20 mM Tris-HCl, 45 mM imidazole, 10% glycerol, at pH 8.0) added with Turbonuclease and Lysozyme (EuroClone) and incubated at room temperature for 1 h. Crude protein extract was obtained by sonication, followed by centrifugation (20,000 rpm, 1 h, 4°C). The supernatant containing the soluble protein fraction was added with 500 mM of NaCl and then stored at 4°C.

Protein purification was performed by using two His Trap FF 1 ml columns in series (GE Healthcare, Little Chalfont, Buckinghamshire, UK) pre-equilibrated with binding buffer (20 mM Tris-HCl, 45 mM imidazole, 10% glycerol, 500 mM NaCl, at pH 8.0). Bound proteins were eluted in a single step with elution buffer containing a high imidazole concentration (500 mM) and the protein quantified by Bradford assay. To evaluate the purity of RecA protein, the highest concentration fractions were resolved on a SDS-Page and those containing highly purified RecA were pooled and dialyzed against 20 mM Tris-HCl, 5% glycerol, 5 mM  $\beta$ -mercaptoethanol, pH 8.0. After dialysis, the sample was further concentrated up to 1 mg/ml using Corning Spin-X UF Concentrators (Sigma-Aldrich) and frozen stored at -80 °C.

### *Colorimetric assay for determination of ATPase activity*

The ATPase activity of RecA protein was quantified by BIOMOL Green, an analogue of the malachite green reagent. This method allows the detection, in a single point step, of free phosphate in solution. Reactions were performed as previously described (Lee et al., 2007) using an assay buffer containing 20 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, pH 8.0.

### *Determination of RecA parameters*

In order to describe RecA activity, the parameters [ATP]<sub>50</sub> and [poly(dT)<sub>36</sub>]<sub>50</sub> were determined. They are defined as the half maximal effective concentrations for ATP and poly(dT)<sub>36</sub>, respectively. [ATP]<sub>50</sub> has been calculated as the result of a dose-response plot obtained using a saturating poly(dT)<sub>36</sub> concentration (2  $\mu$ M) and a concentration of ATP ranging from 30  $\mu$ M to 600  $\mu$ M. [poly(dT)<sub>36</sub>]<sub>50</sub> has been determined fixing the concentration of ATP at 300  $\mu$ M and a concentration of poly(dT)<sub>36</sub> ranging from 0.015  $\mu$ M to 15  $\mu$ M. RecA (0.5  $\mu$ M) was incubated in the assay buffer and the ATPase activity monitored in clear, flat-bottom 96-well microplate (Freitag and McEntee, 1988).

### *Inhibition assay, IC<sub>50</sub> determination and mechanism of inhibition*

The inhibition assays were conducted with a fixed concentration of each lichen compound (100  $\mu$ M). Reactions were performed in a 96-well microplate using 0.5  $\mu$ M of RecA, 300  $\mu$ M of ATP and 2  $\mu$ M of

poly(dT)<sub>36</sub>. The ATPase activity in each well was revealed by BIOMOL Green, following manufacturer instructions, and quantified spectrophotometrically at 655 nm by microplate reader iMark, BioRad (Milan, Italy). The percentage of inhibition was calculated as the percentage residual activity of RecA incubated 10 min with lichen compound with respect to the untreated reaction. The half inhibitory concentration (IC<sub>50</sub>) for each lichen compound, showing a percentage of inhibition higher than 80%, was further investigated following the above conditions, with concentrations of each compound ranging from 1 μM to 100 μM.

The possible mechanism of inhibition *vs* ATP was determined by fixing poly(dT)<sub>36</sub> at a saturating concentration (2 μM), lichen compounds at the IC<sub>50</sub> value previously determined and ATP ranging from 30 to 600 μM. RecA at 0.5 μM was incubated 10 min with lichen compound in 96-well microplate and the ATPase activity was spectrophotometrically monitored at 655 nm. The semi-log plot of the percentage of inhibition *versus* the ratio [ATP]/[ATP]<sub>50</sub> allowed to determine the possible mechanism of inhibition. For instance, for competitive inhibitors, the affinity decreases at higher [ATP]/[ATP]<sub>50</sub> values. Inversely, for the uncompetitive inhibitors, the affinity increases at higher [ATP]. A flat line is, indeed, the distinctive attribute of a non-competitive inhibitor.

The mechanism of inhibition for poly(dT)<sub>36</sub> was determined fixing the concentration of ATP at 300 μM, and poly(dT)<sub>36</sub> ranging from 0.0156 μM to 2.0 μM. As described above, the plot of the percentage of inhibition as function of [poly(dT)<sub>36</sub>]/[poly(dT)<sub>36</sub>]<sub>50</sub> was used to determine the mechanism of inhibition.

#### *Statistics and data analysis*

All the experiments were performed in triplicate. All the parameters, experimental errors and the non-linear fitting were estimated and calculated using the software OriginPro 8.5.1 and Microsoft Excel.

#### *Molecular modelling*

*In silico* bind prediction for epiphorellic acid *vs* RecA was performed by using Autodock4 (version 4.2.6). Docking was conducted with the x-ray crystallographic coordinates of *E. coli* RecA protein (PDB code 3CMW). Hydrogens were added and Amber charges calculated for the protein and for epiphorellic acid. Crystallographic waters were removed. Docking parameter imposed flexibility for the catalytic pocket residues while the remaining part of the protein was kept rigid.

## Results and discussion

In order to investigate the potential inhibitory activity of the lichen secondary metabolites listed in Table 1, RecA protein was cloned into pET-28b(+) overexpression vector and purified by His Trap columns, yielding about 30 mg of protein *per* litre of culture with a degree of purity higher than 95% as determined by SDS-PAGE.

In our experimental conditions, the half maximal effective concentration for ATP ( $[ATP]_{50}$ ) (Fig. 1A) and poly(dT)<sub>36</sub> ( $[poly(dT)_{36}]_{50}$ ) (Fig. 1B) measured for RecA has been determined to be  $127.7 \pm 1.5 \mu\text{M}$  and  $0.044 \pm 0.005 \mu\text{M}$ , respectively.

In this study, twenty-seven lichen secondary metabolites were screened as potential inhibitors of RecA protein. As shown in Table 1, the molecules can be classified on the base of their structure in nine classes: anthraquinones, depsides, depsidones, dibenzofurans, diphenyl-butenolides, paraconic acids, pseudo-depsidones, triterpenes and xanthones. All of them were previously isolated and characterised from lichens belonging to nineteen genera from fourteen families collected in various Chilean regions, included the Chilean Antarctic Territory (Garbarino et al., 1987; Garbarino et al., 1991; Piovano et al., 1985; Piovano et al., 1991; Piovano et al., 1993; Piovano et al., 2001; Quilhot et al., 1983; Quilhot et al., 1989).

All twenty-seven lichen secondary metabolites were first of all tested for their ability to inhibit the production of free phosphate coming from the ATP hydrolytic activity of RecA protein. The percentage of inhibition was evaluated by fixing the concentration of each compound at 100  $\mu\text{M}$  and measuring the production of free phosphate. As reported in Table 1, only two compounds over twenty-seven did not show any inhibitory activity; specifically diffractaic acid and arthothelin, belonging to depside and xanthone classes, respectively. Nine compounds exhibited an inhibitory activity higher than 80%: four depsides (divaricatic acid, perlatolic acid, sphaerophorin and tumidulin), two depsidones (alpha-collatolic acid and lobaric acid), two paraconic acids (lichesterinic acid and protolichesterinic acid) and one pseudo-depsidone (epiphorellic acid). Unfortunately the high chemical variability and the limited number of compounds makes the structure-activity relationship (SAR) analysis difficult, and it cannot be possible to relate the inhibitory activity to a specific class of compound.

A deeper investigation was reserved to only those previously mentioned compounds showing a percentage of inhibition higher than 80%; for each of them the half inhibitory concentration ( $IC_{50}$ ) and the mechanism of inhibition was determined.

The  $IC_{50}$  was calculated using concentrations of the compounds ranging from 1  $\mu\text{M}$  to 200  $\mu\text{M}$ . As shown in Table 2 the  $IC_{50}$  values were about 25-30  $\mu\text{M}$  with the exception of protolichesterinic acid, with an  $IC_{50}$  value of  $14.2 \pm 1.4 \mu\text{M}$ , and sphaerophorin, with an  $IC_{50}$  value of  $42.6 \pm 4.6 \mu\text{M}$ .

As previously mentioned, RecA preferentially binds ssDNA forming a nucleoprotein filament, which triggers the SOS response by inducing the autoproteolysis of the transcriptional repressor LexA (Bell and Kowalczykowski, 2016; Bianco and Kowalczykowski, 2001; Story et al., 1992). In presence of DNA, RecA shows a high ATP hydrolytic activity, which is critical for nucleofilament assembly and disassembly, but not for simple DNA strand exchange reactions (Kim et al., 2014; Kowalczykowski, 1991; Story et al., 1992).

Since ATP binding site is located at the monomer-monomer interface, the only active form able to hydrolyse ATP is, indeed, the nucleofilament. ATP hydrolysis occurs throughout the whole RecA-DNA complex (Brenner et al., 1987). In RecA can be identified a N-terminal domain (residues 6–33) a *core* domain (residues 34–269) and a C-terminal domain (residues 270–328). Two loops of the *core* domain, L1 (residues 157–164) and L2 (residues 195–209), constitute the ssDNA binding site (Bell, 2005).

Since the ATP hydrolysing inhibitory activity can be the result of the inhibition of the ATP hydrolysing activity itself, or the occupancy of the ssDNA binding site, the mechanism of inhibition of each compound reported in Table 2 was assessed by observing the semi-log plot of the percentage of RecA inhibition *versus*  $[ATP]/[ATP]_{50}$ .

All compounds behaved as uncompetitive inhibitors with the exception of epiphorellic acid, which results to act as non-competitive inhibitor for ATP (Fig. 2A, Table 2). It is plausible, for the only epiphorellic acid, that the inhibitory ATP hydrolysing activity can be ascribed to the ability of this compound to occupy the ssDNA binding site. In order to verify the hypothesis, the activity of RecA has been measured as function of poly(dT)<sub>36</sub> concentrations. The semi-log plot of the percentage of inhibition of RecA *versus*  $[\text{poly}(\text{dT})_{36}]/[\text{poly}(\text{dT})_{36}]_{50}$  clearly shows a competitive model of inhibition (Fig. 2B).

The kinetic data seems to be confirmed by the molecular modelling. In fact, as shown in Fig. 3A epiphorellic acid was predicted *in silico* to bind into the L2 loop, where ssDNA binds (Fig. 3B). The carboxylic group of epiphorellic acid interacts *via* three hydrogen bonds involving the  $\epsilon$ -amino group of Lys-198 (3.57 Å), the  $\gamma$ -amino group of Asn-213 (3.07 Å) and the  $\gamma$ -oxy group of the same amino acid (2.82 Å). Additionally, the hydroxyl-group located on the benzene ring can hydrogen bond to the  $\gamma$ -oxy group of Asn-213 (2.61 Å) and to the  $\epsilon$ -amino group of Lys-216 (2.94 Å).

It is reasonable to suppose that the presence of epiphorellic acid into the L2 loop interferes with the binding of ssDNA, hindering the initiation of the nucleofilament, thus preventing the formation of the ATP binding site. It is worth to remark that Lys-216 lies adjacently to Phe-217 that plays a critical role in regulating the flow of ATP-mediated information throughout the protein filament structure (Kelley De Zutter et al., 2001). It is possible that the presence of epiphorellic acid into the L2 loop can constrain the loop itself, interfering with the position of Phe-217.

Although, several molecules have been previously identified as potential inhibitors of RecA, (Alam et al., 2016; Nautiyal et al., 2014; Sexton et al., 2010; Wigle et al., 2006; Wigle et al., 2009), none of them were addressed to the study of lichen secondary metabolites as potential RecA binders. Moreover, the mechanism of inhibition *versus* the target protein, including the prediction of the binding interactions for the best inhibitor, was deeply investigated for the first time in the present study.

## Conclusion

Bacteria possess an extraordinary ability to adapt and evolve in response to antibiotics, acquiring and implementing multiple resistant mechanisms. While in the past bacterial drug resistance was mainly considered the consequence of spontaneous mutations accumulated during replication and resulting in inconsequential, harmful or accidental benefit to the bacteria themselves, recent evidences have proven that spontaneous mutations are not the only way by which bacteria acquire resistance to antibiotics. Nevertheless, new targets are needed to circumvent resistance, which has emerged against every antibiotic class (Culyba et al., 2015; Kohanski et al., 2010).

Bacteria play an active role in their own evolution and this new prospective of looking at bacterial resistance must change our way of facing the problem. The development of new antimicrobial strategies should overcome the *paradigm* of the most efficient weapon against the target. Under this point of view the inhibition of SOS response represents a novel and attractive strategy. In the iterative cycle of antibiotic drug discovery and antimicrobial resistance onset, we are dramatically facing the opportunity to control bacterial evolution. About that, lichens, as source of important secondary metabolites for pharmaceutical industries, may play a central role. As demonstrated in this study, the potentialities of lichen secondary metabolites are still underexploited. The identification of an inhibitor of RecA, one of the pivotal protein in bacterial SOS system, confirms once again the importance of lichen metabolites in drug discovery.

While we were looking for potential inhibitors of the ATP binding site, we surprisingly described for the first time an inhibitor of the DNA binding site. The pseudo-depsidone epiforellic acid represents a good scaffold for the development of new and more efficacious molecules targeting RecA protein. Epiforellic acid, does not represent the finish line but the preliminary and important step for the development of new drugs, paving the way to a novel approach to withstand antimicrobial resistance phenomenon in pathogenic bacteria. In our drug discovery approach natural products in general and lichen specifically, represent a successful source of active ligands and structural diversity. Moreover, the methodologies used in this study can be extended to other natural compounds, in order to well characterise novel potential RecA inhibitors, as well as their mechanisms of inhibition.

## Conflict of interest

There was no conflict of interest.

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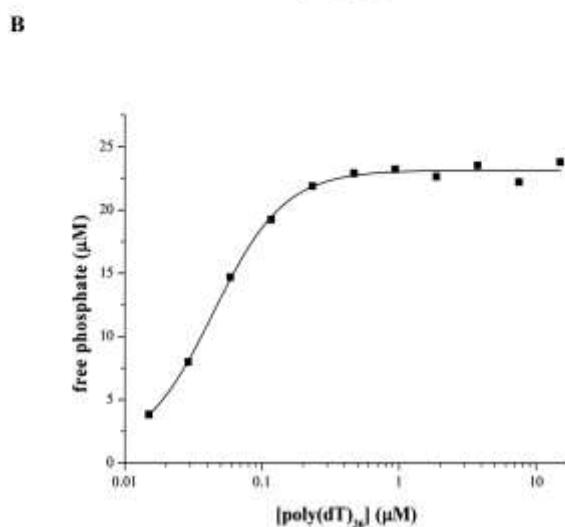
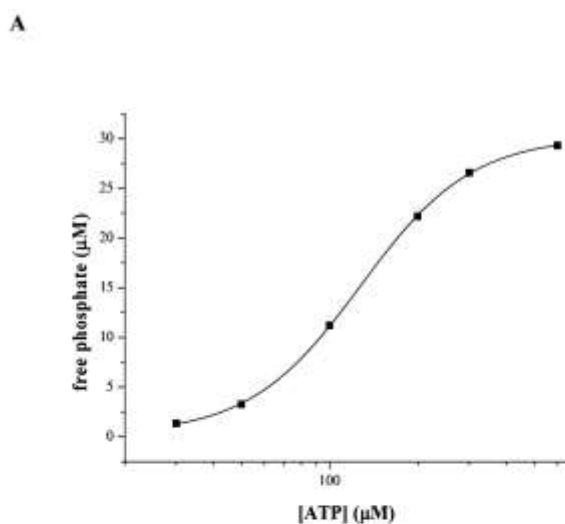
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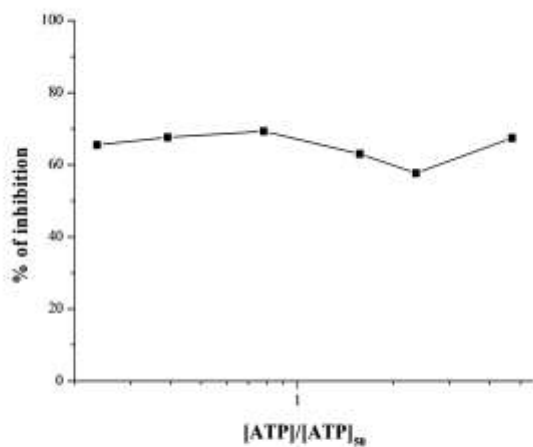
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**Fig. 1.** Semi-log plot of free phosphate produced by RecA ATPase activity as function of A) concentrations of ATP, B) concentrations of poly(dT)<sub>36</sub>. All the experiments were performed in triplicate. Experimental error never exceeds 5%.

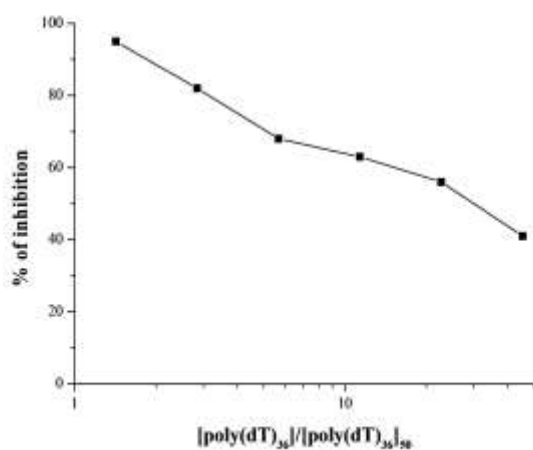


**Fig. 2.** Semi-log plot of the percentage of activity of RecA incubated with epiphorellic acid *versus* A) ATP and *versus* B) poly(dT)<sub>36</sub>. All the experiments were performed in triplicate. Experimental error never exceeds 5%.

A

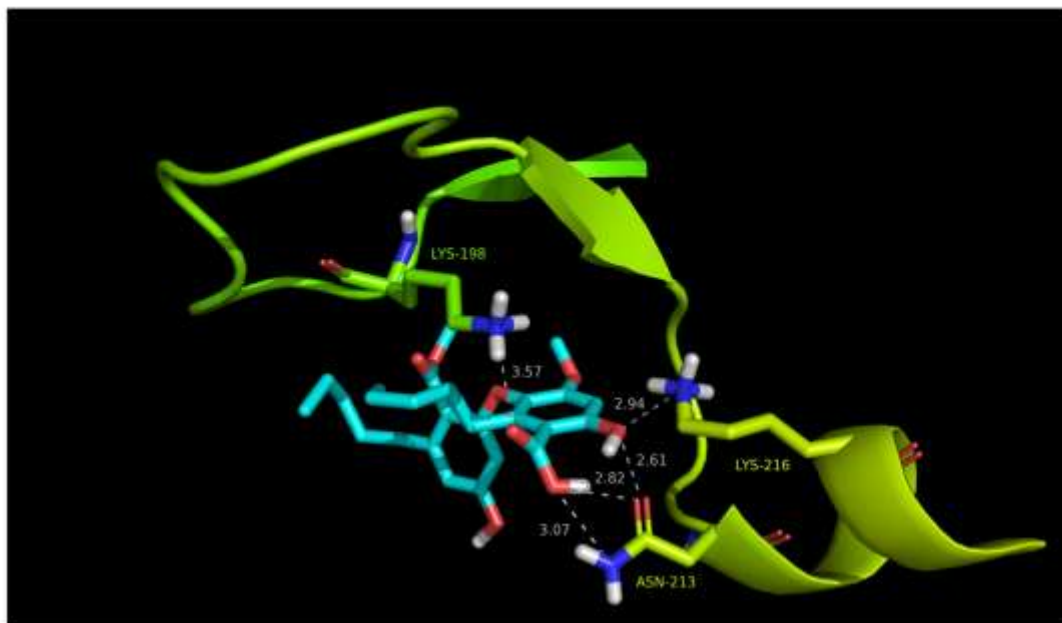
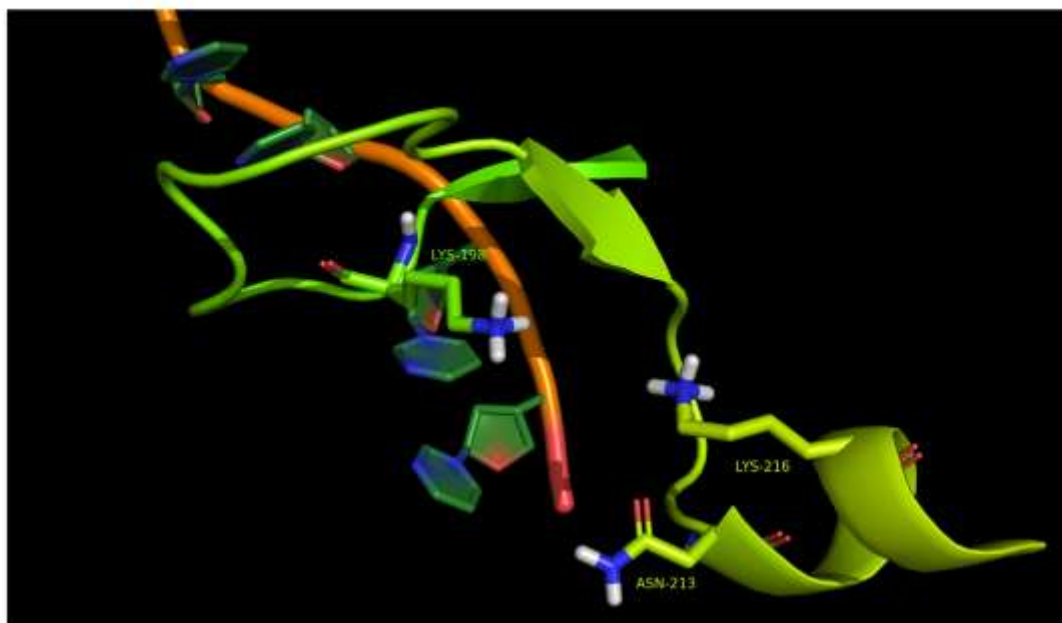


B



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**Fig. 3.** Molecular modelling of RecA protein A) in presence of epiphorellic acid, B) in presence of ssDNA.**A****B****Table 1.** Percentage of the inhibition of the lichen secondary metabolites used in this study with the identification of the species and the geographic regions of collection.

Lichen compound	% of inhibition <sup>a</sup>	Species (Family)	Chilean geographic region
<b>Anthraquinones</b>			
Parietin	14.2 ± 0.7	<i>Caloplaca regalis</i> (Vain) Zahlbr. (Teloschistaceae)	King George Island, Islas Shetland del Sur, Antarctica

Lichen compound	% of inhibition <sup>a</sup>	Species (Family)	Chilean geographic region
<b>Depsides</b>			
Diffractaic acid	N.I.	<i>Protousnea magellanica</i> (Mont.) Krog (Parmeliaceae)	Laguna Icalma, Region de La Araucanía
Divaricatic acid	93.1 ± 2.8	<i>Protousnea magellanica</i> (Mont.) Krog (Parmeliaceae)	Laguna Icalma, Region de La Araucanía
Gyrophoric acid	28.5 ± 0.31	<i>Placopsis contortuplicata</i> Lamb. (Trapeliaceae)	Robert Island, Shetland del Sur, Antarctica
Perlatolic acid	108.6 ± 6.2	<i>Stereocaulon</i> sp. (Stereocaulaceae)	Parque Nacional Puyehue, Región de Los Lagos
Sphaerophorin	104.6 ± 5.2	<i>Sphaerophorus globosus</i> (Huds.) Vain (Sphaerophoraceae)	Robert Island, Shetland del Sur, Antarctica
Tenuiorin	10.0 ± 0.2	<i>Pseudocyphellaria nitida</i> (Taylor) Malme, <i>P. meyenii</i> (Trevisan) D. Galloway, <i>P. exanthematica</i> Lamb, <i>P. divulsa</i> (Taylor) Imshaug (Lobariaceae)	Puyehue National Park, Region de Los Lagos
Tumidulin	85.4 ± 0.9	<i>Ramalina cactacearum</i> Follm. (Ramalinaceae)	Enco, Choshuenco, Region de Los Rios
<b>Depsidones</b>			
Alpha-collatolic acid	103.4 ± 2.1	<i>Lecanora atra</i> (Hudson) Acharius (Lecanoraceae)	Robert Island, Shetland del Sur, Antarctica
Lobaric acid	96.8 ± 1.0	<i>Stereocaulon alpinum</i> Laurer ex Funck (Stereocaulaceae)	Ardley Cove, King George Island, Shetland del Sur, Antarctica
Norstictic acid	18.2 ± 0.5	<i>Rhizoplaca aspidophora</i> (Vain) Redon (Lecanoraceae)	Coppermine Cove, Robert Island, Shetland del Sur, Antarctica.
Pannarin	13.1 ± 0.5	<i>Psoroma</i> sp. (Pannariaceae)	Los Lagos y de Los Ríos Regions.
Protocetraric acid	11.5 ± 0.4	<i>Hypogymnia lugubris</i> (Pers.)Krog (Parmeliaceae)	King George Island, Islas Shetland del Sur, Antarctica
Salazinic acid	8.4 ± 0.1	<i>Parmelia saxatilis</i> (L.) Ach. (Parmeliaceae)	Robert Island, Shetland del Sur, Antarctica.
Stictic acid	16.7 ± 0.8	<i>Rhizoplaca aspidophora</i> (Vain) Redon (Lecanoraceae)	Coppermine Cove, Robert Island, Shetland del Sur, Antarctica.
Variolaric acid	3.2 ± 0.3	<i>Ochrolechia deceptionis</i> (Hue) Darb. (Ochrolechiaceae)	King George Island, near Teniente Marsh Base, Shetland del Sur, Antarctica
Vicanicin	73.7 ± 2.2	<i>Psoroma pallidum</i> Nyl., <i>P. pulchrum</i> Malme (Pannariaceae)	Villarrica, Region de La Araucania
<b>Dibenzofurans</b>			
Usnic acid	11.4 ± 0.6	<i>Cladonia lepidophora</i> Ahti & Kashiw (Cladoniaceae)	Robert Island, Shetland del Sur, Antarctica.
<b>Diphenyl-butenolides</b>			
Rhizocarpic acid	11.6 ± 0.5	<i>Rhizocarpon geographicum</i> (L.) D.C. (Rhizocarpaceae)	Coppermine Cove, Robert Island, Shetland del Sur, Antarctica.
Calycin	7.4 ± 0.4	<i>Pseudocyphellaria encoensis</i> SANT. (Lobariaceae)	Enco, Panguipulli, Region de Los Rios

Lichen compound	% of inhibition <sup>a</sup>	Species (Family)	Chilean geographic region
Vulpinic acid	8.2 ± 0.2	<i>Pseudocyphellaria encoensis</i> SANT. (Lobariaceae)	Enco, Panguipulli, Region de Los Rios
<b>Paraconic acids</b>			
Lichesterinic acid	92.5 ± 1.8	<i>Cetraria chlorophylla</i> (Willd.) Vain (Parmeliaceae)	Gil de Vilches, Talca, Region del Maule
Protolichesterinic acid	103.8 ± 3.1	<i>Cornicularia aculeata</i> (Schreb.) Ach. (Parmeliaceae)	Ardley Cove, King George Island, Shetland del Sur, Antarctica
Roccellic acid	4.2 ± 0.2	<i>Lobodirina cerebriformis</i> (Mont) Follm. syn. <i>Roccellina cerebriformis</i> (Roccellaceae)	Coastal rocks at the mounth of Limari River, Ovalle, Region de Coquimbo
<b>Pseudo-depsidones</b>			
Epiphorellic acid	96.6 ± 4.9	<i>Cornicularia epiphorella</i> (Nyl.) Du Rietz (Parmeliaceae)	Conguillío National Park, Región de la Araucanía
<b>Triterpene</b>			
Zeorin	47.7 ± 1.2	<i>Rhizoplaca aspidophora</i> (Vain) Redon (Lecanoraceae)	Coppermine Cove, Robert Island, Shetland del Sur, Antarctica.
<b>Xanthones</b>			
Arthothelin	N.I.	<i>Buellia anisomera</i> Vain (Buellioideae)	Robert Island, Shetland del Sur, Antarctica

<sup>a</sup>N.I., Not Inhibited.

**Table 2.** IC<sub>50</sub> and mechanism of inhibition vs ATP hydrolytic activity

Compound	IC <sub>50</sub> (μM)	Mechanism of inhibition
Protolichesterinic acid	14.2 ± 1.4	uncompetitive
Divaricatic acid	23.5 ± 2.1	uncompetitive
Tumidulin	25.1 ± 2.6	uncompetitive
Lichesterinic acid	25.6 ± 2.1	uncompetitive
Epiphorelic acid	29.8 ± 2.2	noncompetitive
Alpha-collatolic acid	31.7 ± 4.2	uncompetitive
Lobaric acid	32.5 ± 1.8	uncompetitive
Perlatolic acid	34.6 ± 3.0	uncompetitive
Sphaerophorin	42.6 ± 4.6	uncompetitive