Effects of Plant Lactones on the Production of Biofilm of *Pseudomonas aeruginosa*

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Sixteen plant sesquiterpene lactones, thirteen from four species of the Family Asteraceae, and three from a species of Hepaticae, as well as seven annonaceous acetogenins isolated from the seeds of the tropical tree *Annona cherimolia* (Family Annonaceae), were evaluated for their ability to inhibit or stimulate the production of biofilm by a strain of *Pseudomonas aeruginosa*. The tested compounds carry a γ-lactone moiety in their structures. This structural feature is similar to the lactone moiety present in *N*-acyl homoserine lactones, compounds that play the important role of "quorum sensors" in the mechanisms of biofilm formation observed in many gram-negative bacteria. A special assay was employed to evaluate the influence of the tested plant compounds to inhibit or stimulate the production of biofilm in a *P. aeruginosa* wild strain. Most of the tested compounds affected the biofilm formation mechanism. Six sesquiterpene lactones isolated from *Acanthospermum hispidum* and one from *Enydra anagallis* as well as an acetogenin from *Annona cherimolia* strongly inhibited (69—77%) the biofilm formation when incorporated to a bacterial culture at a concentration of 2.5 μg/ml. However, one of the acetogenins, squamocin, stimulated the biofilm formation even at a concentration of 0.25 μg/ml. The study of substances affecting the biofilm formation can lead to the design of new strategies to control *P. aeruginosa* infections.

Key words sesquiterpene lactone; annonaceous acetogenin; *Pseudomonas aeruginosa;* biofilm

Diverse Gram-negative bacteria produce *N*-acylated homoserine lactones. These compounds serve as intercellular signals that facilitate a phenomenon termed "quorum sensing." In quorum sensing, bacteria monitor their own population density and activate specific sets of genes when a sufficiently high cell density has been reached.[1] The expression of many virulence factors produced by the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa*, is regulated by this cell density-dependent mechanism that utilizes a transcriptional activator protein which acts in concert with a small signalling molecule, known as the autoinducer, to stimulate the expression of target genes. Most autoinducers are *N*-acylated homoserine lactones with structures that differ only in the length and substitution of their acyl side chain (Fig. 1). This cellular signalling mechanism plays a critical role in *P. aeruginosa* virulence and in its survival in hostile environments.[2]

Apparently, quorum sensing plays a key role in the regulation of virulence gene expression in *P. aeruginosa*, and has been shown to be involved in the differentiation of *P. aeruginosa* biofilms. Morphologically, *P. aeruginosa* has flagellum and pili, and produces an exopolysaccharide. These characters are important in the pathogenicity because they allow the bacteria to build biofilms. Biofilms are extremely difficult to eradicate, since they are shielded from host defences such as phagocytes or antibodies, as well as antibiotics and are responsible for chronic infections.[3]

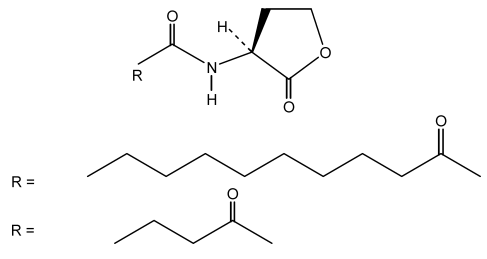

Fig. 1. Autoinducers Produced by *Pseudomonas aeruginosa* Strains[15]

Because it has been demonstrated that the γ-lactone moiety plays an important role in the quorum sensing mechanism,[4] we decided to test the influence of natural compounds that carry γ-lactones with a variety of substitution patterns, on the biofilm production of *P. aeruginosa*.

Two groups of compounds distributed in certain groups of plants possess a γ-lactone moiety in their structures. They are the annonaceous acetogenins found in the family Annonaceae and the sesquiterpene lactones, present in many genera of the family Compositae.

We report herein the effects on the biofilm production of the acetogenins **1**—**7** isolated in our laboratory from the seeds of *Annona cherimolia*,[5] as well as the sesquiterpene lactones **8**—**16** coming from *Acanthospermum hispidum*,[6] **17** from *Enydra anagallis*, **18** obtained from a lactone present in our collection of *E. anagallis*, by simple derivatization,[7] **19** came from *Vernonathura pinguis*,[8] **20** from *Cyrtocymura cincta*,[9] and **21**—**23** were obtained from the liverwort *Frullania brasiliensis*.[10]

Results and Discussion

All the tested compounds share the structural feature of a γ-lactone moiety. In the acetogenins this group is located at the end of a fatty acid type hydrocarbon chain, while in the sesquiterpenes tested, the lactone is part of a bicyclic or tricyclic carbon skeleton. Among the tested acetogenins, only two produce important effects. Compound **3**, squamocin, is a potent stimulator of the biofilm formation at both tested concentrations (2.5, 0.25 μg/ml). Biofilm production has been calculated through the measurement of the absorbance at

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560 nm for treated and control experiments. Treatments of 1 h and 6 h of bacteria with squamocin displayed significant differences in relation to control. After 6 h of contact with bacteria, squamocin produced increments of more than 200% and 100% in the production of biofilm in relation to control at 2.5 and 0.25 μg/ml. In fact, at 2.5 μg/ml the absorbance rate was $Abs_T/Abs_C = 3.0720 \pm 0.8827$, while at 0.25 μg/ml, $Abs_T/Abs_C = 2.2348 \pm 0.6459$ as shown in Table 1. On the other hand, the acetogenin **6**, motrilin, with a structure very similar to squamocin, inhibited 70% and 50% the production of biofilm when incorporated to the culture at concentrations of 2.5 and 0.25 μg/ml, respectively ($Abs_T/Abs_C = 0.3068 \pm 0.0522$ for 2.5 μg/ml and 0.4902 ± 0.1466 for 0.25 μg/ml, Table 1). Previous studies on the bioactivities of acetogenins (inhibition of Complex I in the mitochondrial respiratory chain) suggested that small differences in hydrocarbon chain length and in the position of OH groups had strong effects in the binding mechanisms to cell receptors.[11] On the other hand, these results agree with previous reports that indicated that small structural changes in the autoinducer molecules, responsible for the biofilm formation, result in significant modifications of the biofilm production mechanism.[4,12] After 1h of contact with the bacterial culture, 2.5 μg/ml of squamocin produced an increment of 86% in the biofilm formation ($Abs_T/Abs_C = 1.8646 \pm 0.5362$) while 0.25 μg/ml gave an increment of 40% ($Abs_T/Abs_C = 1.3985 \pm 0.4458$), as shown in Table 1. On the other hand, motrilin displayed no detectable effects on the biofilm formation after 1 h of contact.

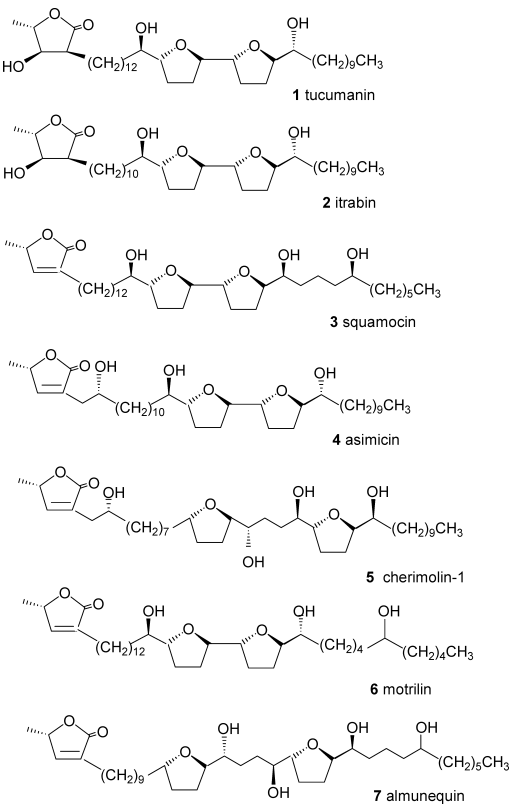


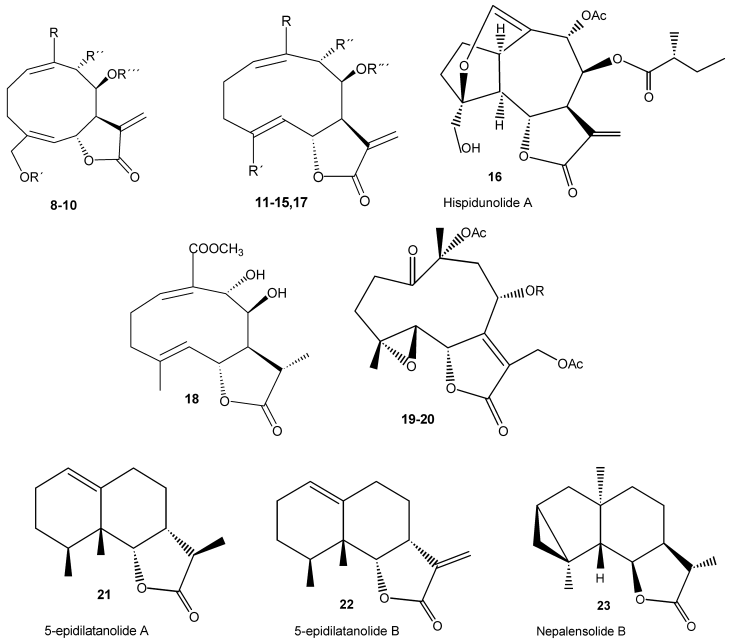
Fig. 2. Annonaceous Acetogenins

Only 6 of the 16 sesquiterpene lactones tested, produced any effect in the "rapid biofilm formation assay (1 h)." Compounds **8**, **9**, **14**, **15**, and **17** inhibited the biofilm formation at both concentrations while **13** only at 2.5 μg/ml, and the inhibition was not dose-dependant. The production of biofilm was reduced to around 50% in relation to the control experi-

Table 1. Compounds Tested at Two Concentrations (2.5, 0.25 μg/ml) on the Biofilm Formation of *P. aeruginosa*, Measured after 1 h and 6 h of Bacteria-Substance Contact

Compound	Biofilm formation (1 h)		Biofilm formation (6 h)	
	Abs_T/Abs_C [2.5 μg/ml][a)]	Abs_T/Abs_C [0.25 μg/ml][a)]	Abs_T/Abs_C [2.5 μg/ml][a)]	Abs_T/Abs_C [0.25 μg/ml][a)]
1	NSD	NSD	NSD	NSD
2	NSD	NSD	NSD	NSD
3	1.8646±0.5362	1.3985±0.4458	3.0720±0.8827	2.2348±0.6459
4	NSD	NSD	NSD	NSD
5	NSD	NSD	NSD	NSD
6	NSD	NSD	0.3068±0.0522	0.4902±0.1466
7	NSD	NSD	NSD	NSD
8	0.5787±0.1858	0.5656±0.2025	0.2941±0.0575	0.2679±0.0490
9	0.4845±0.1121	0.5529±0.1643	0.3113±0.0451	0.4257±0.1529
10	NSD	NSD	0.2770±0.0445	0.2929±0.0697
11	NSD	NSD	NSD	NSD
12	NSD	NSD	NSD	0.6593±0.1589
13	0.4613±0.1759	NSD	0.2941±0.0582	0.3186±0.0389
14	0.4494±0.1428	0.3483±0.2192	0.2346±0.0321	0.2316±0.0468
15	0.3654±0.1296	0.4476±0.1958	0.2409±0.0402	0.2885±0.0815
16	NSD	NSD	NSD	NSD
17	0.4092±0.1341	0.4573±0.1154	0.3407±0.0548	0.2990±0.0371
18	NSD	NSD	NSD	NSD
19	NSD	NSD	NSD	NSD
20	NSD	NSD	NSD	NSD
21	NSD	NSD	1.4470±0.1202	NSD
22	NSD	NSD	0.6212±0.1115	NSD
23	NSD	NSD	NSD	NSD

Abs_T=absorbance of the treated experiment, Abs_C=absorbance of the control. *a*) Mean values±S.E.M. (triplicate). NSD=Non significant differences between treatment and control.



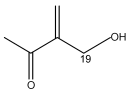
	R	R´	R´´	R´´´	Name
8	CHO	Ac	H	*i*-Bu	15-Acetyloxy-8β- isobutanoyloxy-14-oxo-(4Z)-acanthospermolide
9	CHO	Ac	H	Ang	15-Acetyloxy-8β-angeloyloxy-14-oxo-(4Z)-acanthospermolide
10	CHO	H	OAc	MeBu	9α-Acetyloxy-8β-(2-methylbutanoyloxy)-14-oxo-(4Z)-acanthospermolide
11	CHO	CH_2OH	OAc	MeBu	Acanthospermal B
12	CHO	CH_2OH	OAc	Ang	Lecocarpinolide B
13	CH_2OH	Me	OAc	Ang	9α-Acetyloxy-8β-angeloyloxy-14-hydroxyacanthospermolide
14	CH_2OH	CH_2OH	OAc	MeBu	9α-Acetyloxy-14,15-dihydroxy-8β-(2-methylbutanoyloxy)-acanthospermolide
15	CH_2OH	CH_2OH	OAc	Ang	9α-Acetyloxy-14,15-dihydroxy-8β-angeloyloxy-acanthospermolide
17	$COOCH_3$	Me	OAc		19-hydroxy-15-desoxy-orientalide
19		-	-	-	Glaucolide A
20	Pro	-	-	-	1-oxo-4,5-epoxy-8-propanoyloxy-10,13-diacetoxygermacr-7(11)-en-6,12-olide

Fig. 3. Sesquiterpene Lactones

ment, as described in Table 1. All the sesquiterpene lactones that resulted effective after 1 h of contact were also active after 6 h, with an increment of the inhibition rates. Compounds **10**, **12** and **22** inhibited the biofilm production only after 6 h of contact. Just the sesquiterpene lactone **21** stimulated the formation of biofilm ($Abs_T/Abs_C = 1.4470 \pm 0.1202$). Noteworthy, **21** lacks the exocyclic double bond to the lactone ring and possesses no other oxygenation but the lactone. Apparently biofilm inhibition is associated with highly oxygenated γ-lactones. In fact, lactones **21** and **23**, with no oxygenation except for the lactone moiety, have no inhibitory effect. On the other hand, the active compounds **8**, **9**, **10**, **12**—**15**, **17** and **22** possess exomethylene-γ-lactone moieties. Compounds **18**—**20**, and **23** lacking the exocyclic double bond to the lactone ring, do not display any activity ($p > 0.05$). It is important to point out that the dihydroxy melampolides **14** and **15** were the most active compounds at 2.5 μg/ml. Our results indicate that most of the lactones analyzed, produce an effect opposite to that of the autoinducers. On the other hand it is important to point out that no bactericidal effects were detected at the tested doses.

Conclusions

This is the first report on the activity of plant secondary metabolites on *P. aeruginosa* biofilm formation. The present results indicate that sesquiterpene lactones may be good candidates for the control of *Pseudomonas* strains through biofilm production inhibition.

Experimental

Compounds The twenty three lactones tested on their effects on the biofilm formation are shown in Figs. 2 and 3.

Bacterial Strains and Media The *P. aeruginosa* wild strain PA100, isolated from an infectious process (Collection of Cátedra de Inmunología, Facultad de Bioquímica, Química y Farmacia, UNT, Argentina), was grown in Luria-Bertani (LB) medium (Cabeo, Rockville, MD, U.S.A.) and employed for the bioassays.

Biofilm Formation Assay (6 h) The assay is based on the ability of bacteria to form biofilms[13,14] on polystyrene microplates. Biofilm formation was assessed by its adhesion to the wells of 96-well microtitre dishes.

LB medium (170 μl) and 10 μl of an ethanol solution (50, 5 μg/ml) of each compound were placed in the wells and cells of *P. aeruginosa* were

then inoculated in a 1 : 8 dilution from an overnight LB culture (20 μl). After inoculation, plates were incubated at 37 °C for 6 h and the biofilm formation was later quantified. Experiments were conduced in triplicate for each concentration.

Rapid Biofilm Formation Assay (1 h) To assess the rapid formation of biofilms, 170 μl of LB medium and 10 μl of an ethanol solution (50, 5 μg/ml) of each compound were placed in the wells. Cells of *P. aeruginosa* were then inoculated (20 μl) from an overnight LB culture (not diluted) resulting in a viable count of approximately 10^8 cfu/ml. After inoculation, plates were incubated at 37 °C for 1 h and the biofilm formation was later quantified. Experiments were conduced in triplicate for each concentration.

Quantitation of Biofilm Formation After incubation (6 h or 1 h), 25 μl of a 1% solution of crystal violet (CV) was added to each well (this dye stains the cells but not the polystyrene). The plates remained at room temperature for approximately 15 min and were rinsed thoroughly and repeatedly with water to leave only the biofilm adhered to the wells walls. The biofilm was removed by the addition of 2×200 μl of 95% ethanol to each CV-stained microtitre dish well. This ethanol solution was transferred to a 1.5 ml Eppendorf tube, the volume brought to 1 ml with H_2O, and the absorbance determined at 560 nm in a spectrophotometer (Shimadzu UV-160A spectrophotometer).

Statistical Analysis Results are reported as Abs_T/Abs_C (Abs_T=absorbance of the treated experiment, Abs_C=absorbance of the control). The differences in the mean values (Abs±S.E.M.) were evaluated by analysis of variance (ANOVA). The Tukey test was used for all pair wise multiple comparisons of groups. In all statistical analysis, values of $p>0.05$ were considered not significant (Statistix 7.1, 2002).

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