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Surface properties of new virginiamycin M_1 derivatives

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ABSTRACT

Three kinds of derivatives of the M_1 factor of virginiamycin have been synthesised: esters with long chain fatty acids, oximes with modified polar amino acids and bis-derivatives with both the ester and oxime function. The study of the surface tension time dependence of M_1 and its derivatives has shown that it is necessary to enhance simultaneously the hydrophobicity and the hydrophilicity of M_1 to render M_1 surface-active. A structure/function relationship study of the surface-active bis-derivatives has shown that enhancing the hydrophobicity of the molecule led to slower adsorption kinetics, higher stability of the monolayers formed and a better capacity to penetrate a membrane model. The repulsive electrostatic forces due to the presence of charges on the amino acids linked to M_1 lead to higher surface tensions, a greater molecular area at the interface and lower penetration into a membrane model.

This study has demonstrated that modifying systematically the hydrophobicity and hydrophilicity of a non surface-active molecule allows the production of surface-active derivatives.

1. Introduction

Virginiamycin is a streptogramin antibiotic produced by fermentation using *Streptomyces virginiae*. It is composed of two classes of structurally unrelated molecules, the M (M_1, M_2 and M_3) and S factors (S_1, S_2, S_3, S_4 and S_5) which are respectively, polyunsaturated macrolactones and cyclic hexadepsipeptides [1]. The M_1 factor is the major constituent and represents approximately 65 wt.% of the antibiotic. Virginiamycin inhibits the biosynthesis of proteins. The two types of factors used separately are bacteriostatic against Gram positive bacteria, while their mixture is bactericidal due to a synergistic effect. Although the ribosomes of Gram negative bacteria appear as sensitive to the antibiotic as those of Gram positive strains, Gram negative bacteria are resistant to virginiamycin. This could be due to an impermeability of the outer membranes of these bacteria [2].

The therapeutic applications of virginiamycin have been limited but it has been successfully used commercially as a growth promoting agent in animal husbandry for many years. However with the growing problem of antibiotic resistance, interest in this antibiotic has been renewed and new derivatives of virginiamycin have been developed, such as dalfopristine. Synercid, a 70/30 combination of dalfopristin/quinupristin has been used against *Staphylococcus aureus* and *Staphylococcus epidermidis* resistant to methycillin and *Enterococcus faecium* resistant to vancomycin, which are Gram positive bacteria particularly important in nosocomial infections [3–6].

The aim of this work was to produce and characterize surface-active virginiamycin M_1 derivatives. The planar formula of M_1 represented in Fig. 1(a) shows the presence of polar functions (hydroxyl, ketone, lactone, lactame etc.) and of more hydrophobic groups (methyls, isopropyl etc.) but no clear separation between a hydrophilic and a hydrophobic zone can be seen. To render M_1 surface-active it is necessary to enhance its amphiphilicity. As a first step, it was decided to evaluate the impact on M1's surface properties of increasing separately either its hydrophobicity or hydrophilicity or of increasing both characters simultaneously. Three types of derivatives have thus been synthesised: hydrophobic esters with fatty acids of increasing chain length (octanoic, dodecanoic, tetradecanoic acids), hydrophilic oximes with polar amino acids (serine, lysine, aspartic acid) and bis-derivatives with both an ester and an oxime function. The impact of these modifications on M_1's capacity to decrease the surface tension of water has been studied. In the second part of this work a more complete study of the surface-active derivatives was undertaken. The influence of the derivatives structure on their properties was investigated by studying the surface tension time dependence of their solutions and their compression isotherms. Finally, the ability of these compounds to penetrate into a membrane model has been evaluated.

Different studies have shown that surface activity may be linked to many biological properties (antibacterial, fungicidal, antiviral etc.) of biosurfactants. Examples would be the surfactin lipopeptide of *Bacillus subtilis* [7] and rhamnolipids produced by *Pseudomonas aeruginosa* [8]. New surface-active derivatives of M_1 may increase

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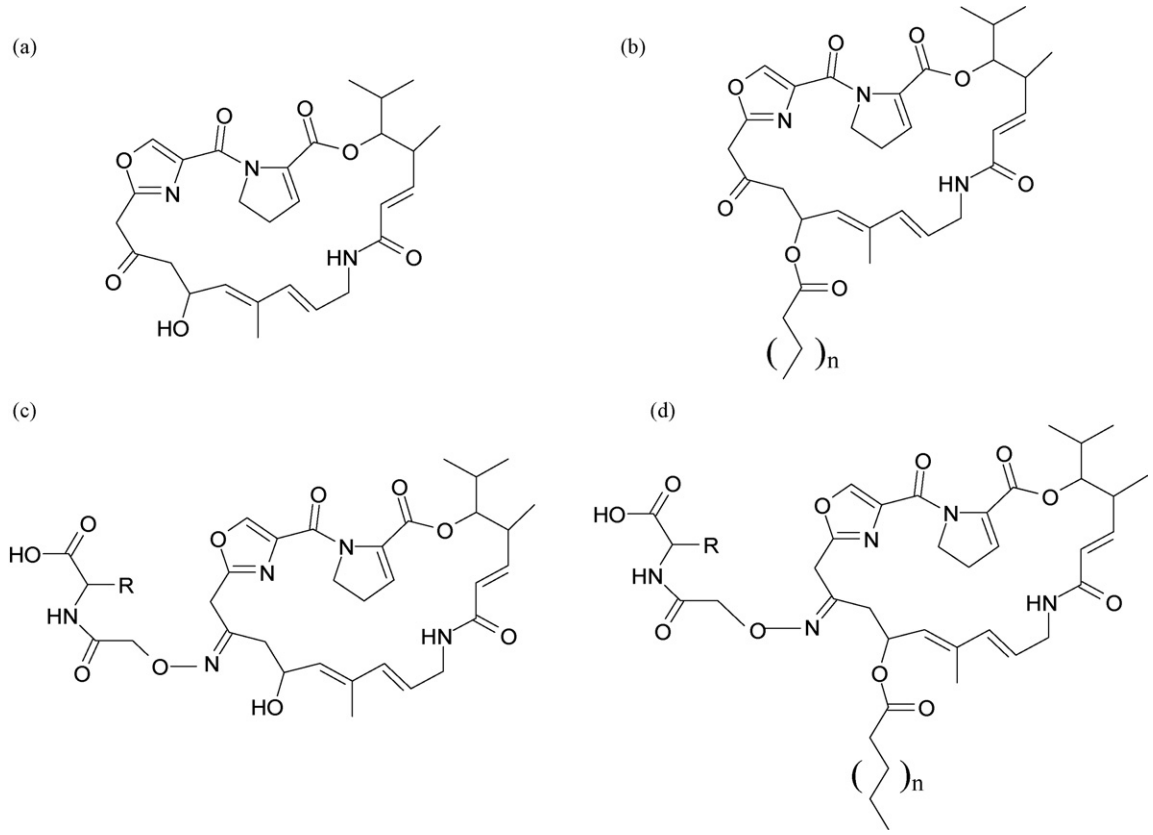

Fig. 1. Planar formula of M_1 (a) and general planar formula of M_1's derivatives: (b) the esters M_1C8 ($n=5$), M_1C12 ($n=9$) and M_1C14 ($n=11$); (c) the oximes M_1AOASer (R = CH_2OH), M_1AOALys (R = $(CH_2)_4NH_2$) and M_1AOAAsp (R = CH_2COOH); (d) the bis-derivatives M_1C8AOALys (R = $(CH_2)_4NH_2$ and $n=4$), M_1C8AOAAsp (R = CH_2COOH and $n=4$), M_1C12AOALys (R = $(CH_2)_4NH_2$ and $n=8$), M_1C12AOAAsp (R = CH_2COOH and $n=8$), M_1C14AOALys (R = $(CH_2)_4NH_2$ and $n=10$) and M_1C14AOAAsp (R = CH_2COOH and $n=10$)

the bioavailability of the antibiotic and may help it to penetrate the outer membrane of resistant Gram negative bacteria and reach their sensitive ribosomes. The association of biological and surfactant activities are particularly valued in the cosmetic and pharmaceutical domains.

2. Experimental

2.1. Chemicals

Virginiamycin M fraction was donated by Phibro Animal Health S.A. (Rixensart, Belgium). It had been produced on an industrial scale by fermentation using *S. virginiae*. After acidification of the broth, the antibiotic was extracted with methyl isobutyl ketone then precipitated by n-hexane. Finally, the M fraction was separated by successive crystallisation steps in methanol. Highly purified M_1 (HPLC-UV(214 nm) purity > 99%) was obtained via the method developed by Nott et al. [9]. The technique involved elimination of the impurities remaining from the M fraction by filtration on a silica gel cake followed by reversed-phase flash chromatography.

The following chemicals were purchased from: *Alexis* (*Switzerland*)*:* TBTU ($C_{11}H_{16}N_5OBF_4$) 98%; *Acros Organics* (*USA*): N,N-diisopropylethylamine ($C_8H_{19}N$) 98%; *Aldrich* (*Germany*): aminooxy acetic acid (AOA) (($H_2NOCH_2CO_2H)_2$.HCl) 98%; dimethylsulfoxide (C_2H_6OS) anhydrous 99.9%; dodecanoic anhydride ($C_{24}H_{46}O_3$) 98%; octanoic anhydride ($C_{16}H_{30}O_3$) 99%; piperidine ($C_5H_{11}N$) 99%; pyridine (C_5H_5N) anhydrous 99.8%; tetradecanoic anhydride ($C_{28}H_{54}O_3$) 95%; *Merck* (*Germany*): Acetic acid ($C_2H_4O_2$) for analysis 96%; 4-dimethylamino-pyridine ($C_7H_{10}N_2$) for synthesis; *Neosystem* (*France*): N-hydrobenzotriazole ($C_6H_5N_3O.H_2O$); *Scharlau* (*Spain*): Acetonitrile (CH_3CN) HPLC grade; chloroform ($CHCl_3$) multisolvent, stabilised with ca. 0.5% ethanol; dichloromethane (CH_2Cl_2) multisolvent stabilised with ca. 50 ppm amylene; isopropanol (C_3H_8O) multisolvent; methanol (CH_3OH) multisolvent; N-methylpyrrolidone (C_5H_9NO) for peptide synthesis; sodium acetate ($C_2H_3O_2Na.3H_2O$); *Sds*(*France*): trifluoroacetic acid (CF_3CO_2H) for synthesis; *Sigma* (*Germany*): DL-α-dipalmitoylphosphatidylcholine ($C_{40}H_{80}NO_8P$) 99%; *VWR* (*Belgium*): ethanol (C_2H_5OH) absolute for analysis.

The water used for measurement of the surface properties was milliQ and prepared with a Millipore Synthesis A10 apparatus.

The glassware was cleaned with a sulfochromic mixture and rinsed with distilled and milliQ water.

2.2. Synthesis and purification of virginiamycin M_1 derivatives

2.2.1. M_1 esters with fatty acids (octanoic, dodecanoic and tetradecanoic acid)

- Commercial chloroform contains ethanol as the stabilising agent. To avoid side reactions, the ethanol was eliminated by liquid–liquid extraction. One volume of the commercial chloroform was extracted five times with 1/2 volume of milliQ water. Anhydrous magnesium sulphate was then added to the $CHCl_3$ free of ethanol obtained. The mixture was stirred at room temperature overnight. The magnesium sulphate was eliminated by filtration.
- Synthesis: M_1 + 2 eq. fatty acid anhydrides and pyridine +0.5 eq. 4-(dimethylamino)-pyridine, in chloroform free of stabiliser were stirred magnetically at room temperature, for 1 h. The synthesis was stopped by adding one volume of cold (4 °C) methanol.
- Pre-purification: this was done via reversed-phase flash chromatography (octadecylsilicagel and elution with a mixture of methanol water in a ratio depending upon the length of the fatty

acid linked) followed by purification by reversed-phase HPLC (C18 column and isocratic elution using a mixture of acetonitrile and water with 0.1% trifluoroacetic acid in a ratio depending of the length of the chain).
- In the rest of the text, the abbreviations M_1C8, M_1C12 and M_1C14 will be used to refer to, respectively, the octanoate, dodecanoate and tetradecanoate of M_1. The general planar formula of these esters is shown in Fig. 1(b).

2.2.2. M_1 oximes with polar amino acids (serine (Ser), lysine (Lys), aspartic acid (Asp))

- The oximes of M_1 with serine, lysine and aspartic acid were synthesised and purified as previously described [10]. Briefly, the introduction of the aminooxy moiety on the amino acids by Fmoc solid phase peptide synthesis using AOA was followed by reaction between the modified amino acid and M_1 and purification via reversed-phase HPLC.
- In the rest of the text, the abbreviations M_1AOASer, M_1AOALys and M_1AOAAsp will be used to refer, respectively to the oxime of M_1 with serine, lysine and aspartic acid. The general planar formula of these oximes is shown in Fig. 1(c).
- Each of the oximes synthesised exists under two isomeric forms and they will be referred to as isomer 1(I1) and isomer 2 (I2) throughout the text. Both isomers of the oximes were purified and studied separately.

2.2.3. M_1 bis-derivatives

- M_1's esters were obtained as described above (Section 2.2.1)
- The oximes of the esters were synthesised using the same method as for M_1's oximes (Section 2.2.2) and purified by reversed-phase HPLC (C18 column and elution with a gradient of acetonitrile and water with 0.1% trifluoroacetic acid in a ratio depending of the length of the chain).
- In the rest of the text, the abbreviations M_1C8AOALys, M_1C8AOAAsp, M_1C12AOALys, M_1C12AOAAsp, M_1C14AOALys and M_1C14AOAAsp will be used to refer, respectively to the bis-derivatives of M_1 with lysine or aspartic acid with a fatty chain of 8, 12 or 14 carbon atoms. The general planar formula of these bis-derivatives is shown in Fig. 1(d).
- Each of the bis-derivatives synthesised exists under two isomeric forms and they will be referred to as isomer 1 (I1) and isomer 2 (I2) throughout the text. For the bis-derivatives with a chain length of 8 carbon atoms mixtures of both isomers were used. For the longer chain length derivatives (C12 and C14), both isomers were separated.

HPLC-UV (214 nm) purity levels of a minimum of 98% were achieved for both M_1 and its derivatives. The identity of all the derivatives was confirmed after synthesis and purification by a combination of spectral techniques (IR, mass and NMR).

2.3. Surface tension measurements

The surface tension time dependence of aqueous solutions of M_1 and its derivatives was measured with the automated drop volume tensiometer TVT 1 (Lauda, Germany). The design and procedures for use of this apparatus have been fully described by Miller et al. [11]. In the dynamic mode used, the volume of the drops formed by the apparatus is continuously increasing and each successive drop is formed more and more slowly. This allows measurement of the surface tension as a function of the quantity of surfactant having had time to reach the surface of the drop. The volume of the syringe was 2.5 mL and the radius of its capillary was 1.055 mm. All measurements were performed at $25 \pm 0.5\,^{\circ}C$. Each measurement was repeated twice.

M_1 and its derivatives were solubilised either in milliQ water or in an acetate buffer 25 mM at pH 5. The concentrations of the solutions were determined by HPLC-UV (214 nm) using a calibration curve.

2.4. Compression isotherms

The surface pressure measurements were done on a KSV Langmuir film balance (KSV instrument, Helsinki, Finland). The apparatus consists of a rectangular Teflon trough ($364 \times 75 \times 5$ mm) with a central well ($37 \times 37 \times 70$ mm), two mobile barriers, a Wilhelmy plate (19.62×10 mm), a temperature probe and a thermostatisation system linked to a water bath Julabo F12-MV (Julabo, Labortechnik GmbH, Seelbach, Germany). Between each analysis, the trough, the barriers, the temperature probe and the Wilhelmy plate were cleaned with pure isopropanol and rinsed thoroughly with milliQ water.

Before each compression isotherm, the trough was filled with acetate buffer 25 mM at pH 5, the temperature was stabilised at $20\,^{\circ}C$ and after compression the surface was cleaned by suction. The cleanliness of the system was checked with a blank.

The samples were dissolved in a mixture of $CHCl_3/CH_3OH$ (v/v; 2/1) at a concentration between 0.5 and 1 mM except for M_1 for which the concentration was 10 mM.

A precise volume (varying between 10 and 100 μL) of the sample was deposited drop by drop at the surface of the aqueous phase with a Hamilton syringe (Bonaduz AG, Switzerland).

After a waiting time of 15 min allowing for solvent evaporation and dispersion of the molecules at the surface, the film was compressed with the mobile barriers at a speed of 10 mm/min corresponding to a reduction of the surface of 15 cm^2/min. During the compression the surface pressure was recorded. At least three repetitions were done for each isotherm.

2.5. Study of the penetration of M_1 and its bis-derivatives into a membrane model

A monolayer of dipalmitoylphoshatidylcholine (DPPC) was chosen as a membrane model and the experiments were carried out with the Langmuir film balance used for the surface pressure measurements. The experimental set up was as described above except that the trough used was smaller ($205 \times 75 \times 5$ mm) with no well. The sub-phase was prepared in the same manner as for the compression isotherms.

The samples were dissolved in dimethylsulfoxide (DMSO) at a concentration chosen such that after injection of 40 μL in the 75 mL of sub-phase the concentration of the molecule in the latter reached 10 μM.

A solution of DPPC at 1 mM was prepared in a $CHCl_3/CH_3OH$ mixture (v/v; 2/1). It was deposited drop by drop at the surface of the sub-phase. The volume of DPPC deposited was chosen such that the area of the monolayer at the initial surface pressure (π_i) reached approximately 55 cm^2. After 15 min, the film was compressed until attaining the desired π_i. The film was then stabilised at a defined initial value until the change in the area was less than 0.10 cm^2/min and this during a minimum time of 15 min.

The sample was then injected into the sub-phase with a Hamilton syringe and the surface pressure was monitored until an equilibrium state was reached.

It is important to note that blanks where only 40 μl of DMSO were injected in the sub-phase showed no surface pressure variation during at least 24 h.

Experiments studying the adsorption of the molecules at the buffer/air interface were identical to those of the penetration study except that no DPPC was deposited at the surface of the buffer.

3. Results and discussion

3.1. Impact of the modification of M_1's structure on the surface tension of its aqueous solutions

The influence on the surface properties of the M_1 factor, of increasing either M_1's hydrophobicity (esters), hydrophilicity (oximes) or both of these properties simultaneously (bis-derivatives) was evaluated. The surface tension (γ) of M_1 solutions and its derivatives was determined using the drop volume tensiometer (TVT) in the dynamic mode.

The graph (a), Fig. 2, shows the evolution of $\gamma_{dyn\,\infty}$ of solutions of M_1 at different concentrations. M_1 decreases the surface tension slightly. The higher the concentration the greater the decrease. The curve shows no break and therefore no critical micellar concentration could be determined for the range of concentrations studied which was limited by the poor solubility of M_1. Furthermore the curves γ_{dyn} obtained as function of time (results not shown) did not indicate any tendency of M_1 to migrate towards the interface. The value of γ_{dyn} obtained after approximately 10 min was very close to that reached after less than 10 s. Thus the results suggest that M_1 has no or only slight surface-active characteristics and therefore behaves like any other organic molecule in solution in water. This is consistent with the fact that no clear separation between a hydrophilic and a hydrophobic region can be predicted by the planar formula of the antibiotic. However a certain degree of amphiphilicity could have been expected. NMR studies (ROESY and NOESY) of M_1 have shown that the antibiotic adopts different conformations in various solvents. In $CDCl_3$, which can be considered as a model system for an hydrophobic environment, the hydrophilic groups are oriented towards the interior of the molecule. On the contrary, in CD_3OD, the hydrophilic groups are pointed outwards and the more hydrophobic groups are oriented inwards [12,13]. These data suggests that M_1 could adopt an optimal 3D structure at a hydrophilic/hydrophobic interface. However, in view of the results obtained in the present study, this conformational flexibility does not seem sufficient to render M_1 clearly amphiphilic and thus surface-active. This flexibility may well contribute to enhance the surface activity of its derivatives.

The esters of M_1 with fatty acids (M_1C8, M_1C12 and M_1C14), were scarcely soluble in water. At saturation of the solutions, the γ_{dyn} reached was around 70 mN/m. Their effect on water's surface tension can thus be considered as negligible. They are either not sufficiently amphiphilic or their concentrations are not high enough to have a significant influence on the γ.

The graph (b), (c) and (d) of Fig. 2 show the evolution of the $\gamma_{dyn\,\infty}$ of the oximes of M_1 with polar amino acids (M_1AOASer, M_1AOALys and M_1AOAAsp), as a function of their concentrations. As for M_1, the surface tension decreases as the concentration of the derivatives increases but no CMC could be determined in the range of concentrations tested. The results obtained with M_1AOASer are similar to those obtained with M_1. The effects of M_1AOALys and of M_1AOAAsp on the surface tension are slightly less than that of M_1. For example, the $\gamma_{dyn\,\infty}$ of a 500 μM solution of M_1AOALys (I1) is approximately of 65 mN/m compared to 60 mN/m for M_1. The isomery of the oxime link does not affect the property studied here (data not shown).

These initial results show that the modification of only one characteristic of M_1, its hydrophobicity or its hydrophilicity, is not sufficient to render M_1 surface-active. To confer a clear amphiphile character to M_1, it is necessary to modulate both characteristics. Increasing its hydrophilicity is important to increase its water solubility and enhancing its hydrophobicity is also necessary in order that the molecules do not tend to stay in the bulk of the solution but migrate towards the interface.

The surface tension evolution of M_1C8AOALys (mixture of I1 and I2) solutions as a function of time is shown for different concentrations in Fig. 3. Their surface tension decreases with time

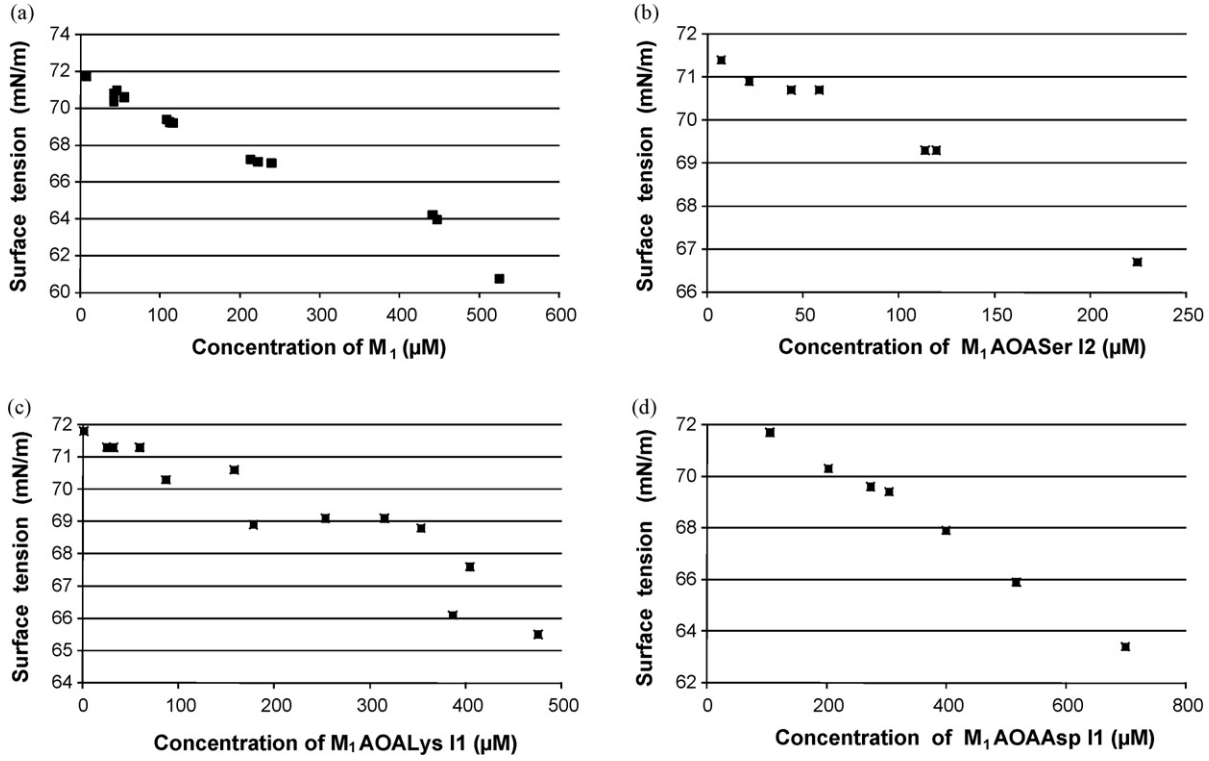

Fig. 2. Evolution of the dynamic surface tension ($\gamma_{dyn\,\infty}$) of solutions in milliQ water of M_1 (a) and its oximes with serine (b), lysine (c) and aspartic acid (d) in function of their concentrations. The dynamic surface tensions (γ_{dyn}) obtained were extrapolated to an infinite time ($\gamma_{dyn\,\infty}$) by linear regression of the curves of the γ_{dyn} as a function of the inverse of the square root of time ($t^{-1/2}$).

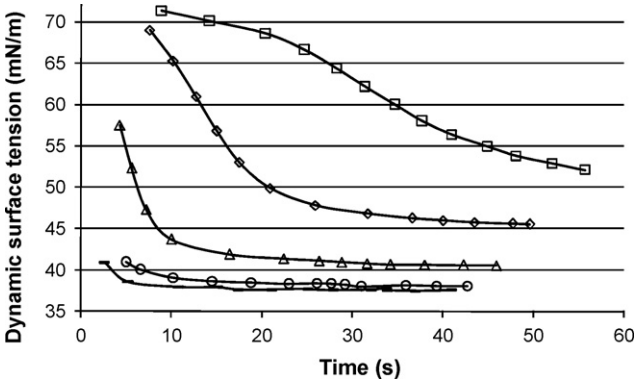

Fig. 3. Graph of $\gamma = f(t)$ recorded by TVT in dynamic mode for M_1C8AOALys (mixture of I1 and I2) in milliQ water at different concentrations: (□) 21 μM; (◊) 35 μM; (Δ) 42 μM; (○) 66 μM; (–) 132 μM.

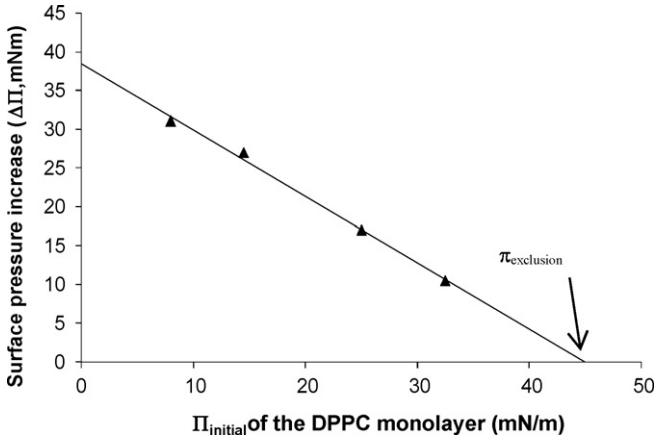

Fig. 4. Graph of the increase of surface pressure reached at equilibrium ($\Delta\pi = \pi_{eq} - \pi_i$) after injection of M_1C12AOAAsp (I1) as function of the initial surface pressure of the DPPC monolayer (π_i).

which indicates a tendency of the molecules to migrate towards the air/water interface. Similar results were obtained for all the bis-derivatives of M_1 synthesised. These derivatives thus behave clearly as surfactant molecules. The higher the concentration, the faster the decrease. For the lowest concentration of M_1C8AOALys, the first three regions characteristic of surfactant adsorption at an air/water interface are observed: the induction, the rapid fall and meso-equilibrium regions [14]. For the two intermediary concentrations, the induction zone is not visible and for the two highest concentrations, only the meso-equilibrium region can be seen. The graph also illustrates that the higher the concentration, the lower the surface tension reached at the end of the measurement. This is the case for all surfactants at concentrations below their CMC.

As the bis-derivatives show a clear surfactant character, a more detailed study of their properties has been carried out and is presented below.

3.2. Study of the relationship between the structure of the bis-derivatives and their surface properties

The influence of both the chain length (8, 12 or 14 carbon atoms) and the nature of the amino acid (Lys or Asp) of the bis-derivatives on three categories of properties has been studied: the surface tension time dependence of their solutions, the compression isotherms of their monolayers at an air/water interface and finally their capacity to penetrate into a membrane model. The two first categories of properties studied are important to better comprehend the fundamental surface properties of the derivatives and to link them to their structure. The third type of properties studied is a simple and rapid method to predict if the derivatives may have some interesting biological activities. Indeed, the active site for M_1 is at the ribosomes of the bacteria. However, to be active, the antibiotic must go through the outer layers of the cell. As a first approach, it seemed interesting to investigate if the modification of the hydrophobicity and hydrophilicity of M_1 achieved in this work has an influence on its capacity to interact with biological membranes. A monolayer of dipalmitoyl phosphatidylcholine, a frequently applied model in the literature [15–18], was used. Such a model allows easy control of its density by adjustment of its surface pressure and of the experimental conditions (pH and composition of the sub-phase, temperature) [19,20]. The molecule was injected in the sub-phase under the monolayer of DPPC which was compressed at a given surface pressure beforehand. The evolution of π was then followed in function of the time elapsed after the injection. It is generally considered that an increase of π indicates a penetration of the molecule in the monolayer. The surface pressure increase reached at the equilibrium as a function of the initial surface pressure of the DPPC monolayer allows the determination of the exclusion pressure ($\pi_{exclusion}$) of the compound understudy. $\pi_{exclusion}$ corresponding to the intersection of the regression line with the abscissa represents the surface pressure of the lipid monolayer at which the molecule injected in the sub-phase can no longer penetrate in the monolayer [19,21]. The Fig. 4 shows as an example the graph established for the determination of the exclusion pressure of the bis-derivative M_1C12AOAAsp (I1).

3.2.1. Influence of the chain length of the fatty acid (8, 12 or 14)

The influence of the bis-derivatives chain length on the surface tension time dependence of their solutions was studied using the drop volume tensiometer. Fig. 5 illustrates the results obtained for the bis-derivatives with lysine and a chain length of 8 and 12 carbon atoms at a low and high concentration. Similar results were obtained for those with aspartic acid. For a given amino acid, at similar concentrations, the longer the chain length of the bis-derivative the slower its adsorption kinetic at the interface. As it was explained by Razafindralambo et al. [22] with surfactins, this could be due to the fact that during the adsorption of the surfactant at the interface, the alkyl chain of the adsorbing molecules must pass through the layer of the molecules already adsorbed. The longer the chain, the more energy is necessary for that passage.

The surface tension reached is lower for the longer chain derivatives. This could be due to an increase in the attractive hydrophobic interactions between the alkyl chains of the molecules whilst the

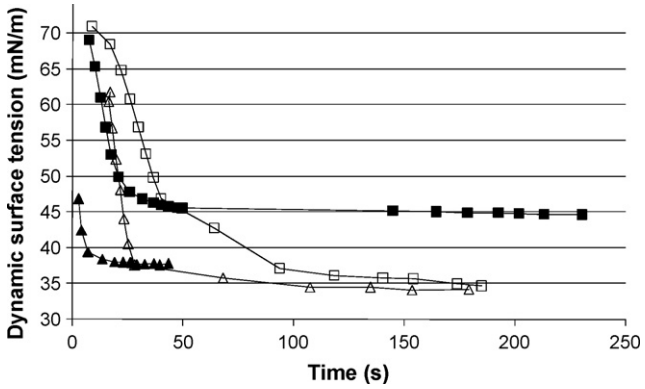

Fig. 5. Effect of the chain length of bis-derivatives on the surface tension time dependence of their solutions: example of the bis-derivatives with lysine: M_1C8AOALys (mixture of I1 and I2): (■) 35 μM and (▲) 107 μM in milliQ water, M_1C12AOALys (mixture of I1 and I2): (□) 49 μM and (Δ) 120 μM in milliQ water.

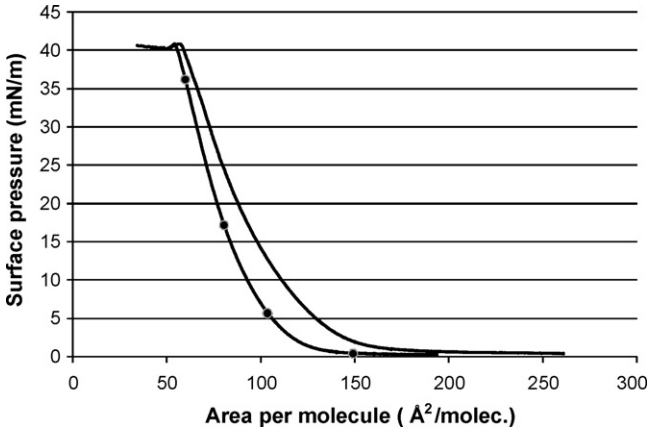

Fig. 6. Compression isotherms of the bis-derivatives M_1C12AOALys (I1) (-●-) and M_1C14AOAAsp (I1) (-). The compression isotherms of the other bis-derivatives have a similar form and for the sake of clarity they are not shown.

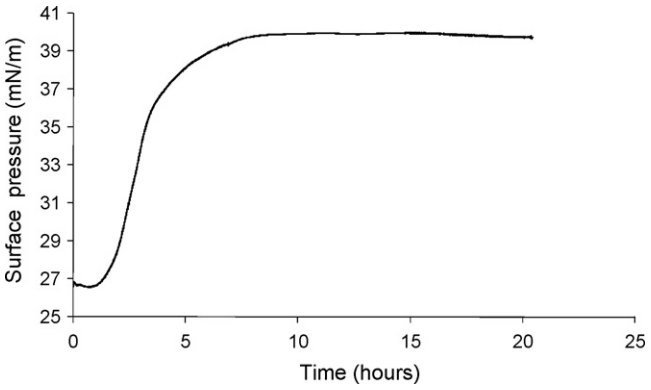

Fig. 7. Evolution of the surface pressure of a DPPC monolayer at the interface air/acetate buffer 25 mM at pH5 initially compressed at 26.5 mN/m as a function of the time elapsed after the injection in the buffer sub-phase of 40 μL of a DMSO solution of M_1C12AOALys (I1) (10 μM final concentration in the sub-phase).

electrostatic repulsion forces remained unchanged. This allows a more compact organisation at the interface and thus a higher surface concentration which is directly linked to surface tension. Similar tendencies have been frequently reported in the literature [22–24].

The monolayer properties of M_1 and its bis-derivatives at an air–aqueous medium interface were studied using the Langmuir trough technique. For M_1, no stable monolayer was formed and no compression isotherms could be observed under the experimental conditions used. For the bis-derivatives, compression isotherms have been recorded. Fig. 6 shows the compression isotherms obtained for M_1C12AOALys (I1) and M_1C14AOAAsp (I1). The isotherms of all the bis-derivatives have a similar shape and for the sake of clarity the other isotherms are not shown. Two parameters were used to characterize the compression isotherms: the limiting area (A_0) and the collapse pressure ($\pi_{collapse}$). A_0 is the area at which the surface pressure (π) begins to increase and differs from zero. It indicates that the molecules are close enough to begin to interact. $\pi_{collapse}$ is the highest pressure the film can reach. If the area is still further decreased the monolayer collapses and the molecules go into the solution or rearrange into multilayers. The characteristic parameters obtained for all the bis-derivatives are compiled in Table 1.

For a given amino acid, the results show no influence of the chain length of the fatty acid on the A_0 or the $\pi_{collapse}$. The results obtained also show no significant effect of the isomery of the oxime link.

Fig. 7 shows the evolution of the surface pressure of a monolayer of DPPC initially compressed to a value of 26.5 mN/m in function of the time elapsed since the injection of M_1C12AOALys (I1) in the sub-phase (final concentration of derivative of 10 μM). The pressure remains stable during the first 2 h and then increases steadily during more or less 5 h to approximately 39 mN/m. This value then remains stable for 10 h at least. This shows that the derivative is able to penetrate into the DPPC monolayer and that once in the lipid environment remains stable and no rearrangement occurs that may change the surface pressure. The kinetic of penetration of the bis-derivatives into DPPC monolayers is very slow compared to other bioactive amphiphile such as, for example, surfactin [18], colistin [25] and fengycin [26]. This could be due to a reorganisation of the molecules during their penetration or to the fact that a high energy barrier must be overcome.

Table 1
Characteristic parameters (average of three repetitions) of the compression isotherms of M_1's bis-derivatives at the interface air/acetate buffer 25 mM at pH 5.

bis-Derivative	A_0 (Å²/molecule)	$\pi_{collapse}$ (mN/m)
M_1C12AOALys (I1)	100.9 ± 4.2	41.1 ± 0.4
M_1C12AOALys (I2)	106.2 ± 0.7	40.6 ± 0.2
M_1C14AOALys (I1)	108.6 ± 3.6	41.5 ± 0.4
M_1C14AOALys (I2)	102.8 ± 0.9	42.1 ± 0.2
M_1C12AOAAsp (I1)	122.6 ± 4.1	39.7 ± 0.4
M_1C12AOAAsp (I2)	120.3 ± 0.9	38.4 ± 0.2
M_1C14AOAAsp (I1)	118.0 ± 3.6	40.5 ± 0.3
M_1C14AOAAsp (I2)	124.9 ± 1.1	40.4 ± 0.1

The experiments have shown that all the bis-derivatives synthesised are able to penetrate a DPPC monolayer as can M_1 to a limited extent. The exclusion pressure of M_1 and of the bis-derivatives were determined. Table 2 details the results obtained for the bis-derivatives with aspartic acid. Their adsorption equilibrium pressures at a clean interface (without DPPC) are also presented. They are lower than their exclusion pressures. This suggests that an interaction with DPPC occurs. This interaction maintains the molecules in the monolayer even at surface pressure higher than those attained after simple adsorption at the interface. If there was no interaction, the derivatives would fill the empty spaces in the DPPC monolayer only until reaching their adsorption equilibrium pressure [16,27]. Furthermore, increasing the chain length enhances the capacity of penetration into the DPPC monolayer. This is also confirmed by the fact that the exclusion pressure of M_1 ($\pi_{exclusion}$ = 20 mN/m) is smaller than those of all the derivatives studied. The same tendency was shown with the bis-derivatives with lysine. The results obtained in this study confirm that the increase of the hydrophobic forces is in favour of the penetration into lipidic monolayers [18,27].

3.2.2. *Influence of the amino acid (Lys or Asp)*

Fig. 8 shows the surface tension time dependence of solutions of bis-derivatives with the same chain length (eight carbon atoms) and either lysine or aspartic acid as the polar part. As for the molecules with 12 carbon atoms, no significant influence of the nature of the amino acid on the kinetics of the adsorption at the air/aqueous interface clearly appears.

However, the results obtained have shown that the bis-derivatives with lysine reach lower surface tensions than those with aspartic acid. This could be due to the existence of a higher adsorption barrier in the case of the Asp derivatives due to repulsion forces between the adsorbed molecules. Indeed at pH 5, Asp residue is predominantly charged twice negatively while Lys is zwiterrionic. Apparently the ions of the buffer cannot totally compensate the charges.

Table 2
Effect of the chain length of M_1's bis-derivatives on their exclusion pressure in a DPPC monolayer and on their adsorption pressure at the interface air/acetate buffer 25 mM at pH 5 (concentration of the bis-derivatives: 10 μM): an example with the bis-derivatives with aspartic acid. (N.D.: not determined).

bis-Derivative	M_1C8AOAAsp (I1 and I2)	M_1C12AOAAsp (I1)	M_1C12AOAAsp (I2)	M_1C14AOAAsp (I2)
$\pi_{exclusion}$ (mN/m)	29	45	44	50
$\pi_{adsorption}$ (mN/m)	17	34	N.D.	40

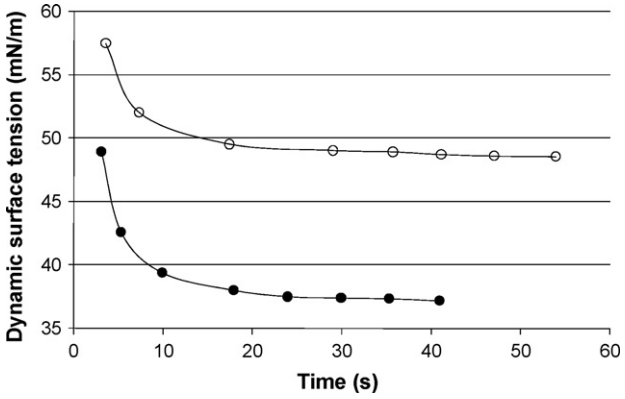

Fig. 8. Effect of the nature of the amino acid of M_1's bis-derivatives on the surface tension time dependence of their solutions: an example of the bis-derivatives with a chain length of eight carbon atoms: M_1C8AOALys (mixture of I1 and I2): (●) 69 μM in acetate buffer 25 mM at pH 5, M_1C8AOAAsp (mixture of I1 and I2): (○) 62 μM in acetate buffer 25 mM at pH 5.

Table 3
Effect of the nature of the amino acid of M_1's bis-derivatives on their exclusion pressure in a DPPC monolayer and on their adsorption pressure at the interface air/acetate buffer 25 mM at pH 5 (concentration of the bis-derivatives: 10 μM): an example with the bis-derivatives having a chain length of eight carbon atoms.

bis-Derivative	M_1C8AOALys (I1 and I2)	M_1C8AOAAsp (I1 and I2)
$\pi_{exclusion}$ (mN/m)	36	29
$\pi_{adsorption}$ (mN/m)	27	17

The results shown in Table 1 for the compression isotherms of the bis-derivatives indicate that, for a given chain length, the derivatives with aspartic acid are more expanded at the beginning of the compression than those with lysine. This could be due to higher electrostatic repulsion forces occurring between the twice negatively charged Asp derivatives than between the zwiterrionic Lys based molecules. Table 3 shows that the adsorption and exclusion pressure of the bis-derivative with lysine are higher than those with aspartic acid. Indeed, the molecules with aspartic acid are more extended (repulsion), their density at the surface is lower and thus the adsorption pressure reached is lower than in the case of the zwiterrionic lysine derivatives. In presence of DPPC which is zwiterrionic, the negative charges of Asp are not completely hidden and the exclusion pressures for the Asp derivatives are lower than those with lysine.

4. Conclusions and perspectives

In conclusion, this study has shown that virginiamycin M_1 is not surface-active. It is necessary to enhance both its hydrophobicity and hydrophilicity to render it surface-active as was shown by the properties of the bis-derivatives. A structure/function relationship study of the bis-derivatives was undertaken. The comparison of the properties of the bis-derivatives having a given amino acid and increasing chain length has shown that enhancing the hydrophobicity of the molecules leads to a slower adsorption kinetic, lower surface tensions, higher stability of the monolayers formed and better capacity to penetrate a membrane model. The comparison of the properties of the molecules having the same chain length but a different amino acid has shown that increase of the repulsive electrostatic forces lead to higher surface tensions, a greater molecular area at the interface and lower penetration into a membrane model.

The study of the penetration of M_1 and the bis-derivatives in a DPPC monolayer has shown that all these molecules, except M_1 and M_1C8AOAAsp, have an exclusion pressure greater than 30–35 mN/m which is generally considered as the value of the lateral pressure that reigns in biological membranes [28]. This suggests that these molecules, under the conditions used in this study, can potentially insert into biological membranes in vivo. In the future it would be very interesting to verify this hypothesis with more complex membrane models such as bilayers or liposomes but also in vitro by studying the minimum inhibitory concentrations of M_1 and the bis-derivatives towards bacteria. It would be interesting to know if the new surface properties of the bis-derivatives allow them to penetrate the outer membrane of the Gram negative bacteria.

It will be also important to test the activity of these new derivatives towards resistant bacteria as they may be as effective as dalfopristin.

Some studies have shown that M_1 and some of its derivatives (esters and carbamates) were antagonists of regulation peptides such as gastrin and cholecystokinin that are found in the gastrointestinal tissues and in the central nervous system. Such compounds could be useful to treat numerous illnesses such as ulcers, Zollinger-Ellison syndrome and different cancers [29,30]. It would be very interesting to test the new derivatives studied here for antagonist activity towards these regulatory peptides.

This work has shown that by systematically studying the impact of modifying the hydrophobicity or/and the hydrophilicity of a compound on its surface properties it is possible to produce surface-active derivatives from a non surfactant molecule. This strategy could be used with many other active agents and may lead to the obtention of new molecules combining the initial properties of the agent to new ones that may enlarge its field of applications. A possibility for example could be in the pharmaceutical or cosmetic field where the combination of formulation properties to biological activities is particularly looked for.

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