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SYNTHESIS AND EVALUATION OF ANTIBACTERIAL ACTIVITY OF DANAZOL-SUCCINATE-LISOZYME CONJUGATE ON *STAPHYLOCOCCUS AUREUS*.

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Abstract

In this work, danazol-succinate-lisozyme conjugate was synthesized (**5**); the route involved the preparation of danazol succinate (**3)** by esterification of danazol (**1**) with succinic anhydride (**2**) followed by the reaction of **3** with lisozyme (**4**) in order to obtain **5**. The structure of **3** was confirmed by spectroscopy and spectrometry data and **5** was characterized by spectroscopy ultraviolet and

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electrophoresis analysis. The antibacterial activity of compound **3** and **5** was evaluated *in* vitro on *Staphylococcus aureus*, using dilution method and the minimum inhibitory concentration. The results showed that 11 residues of steroid-derivative **(3)** were coupled on a lisozyme molecule (**4**), obtaining 5.23 x 10-5 mmol of danazol-succinate per milligram of **5**. Electrophoresis analysis showed a band of ≅ 16 Kda for **5**. Other experimental data indicate that bacterial growth of *Staphylococcus aureus* in presence of **3** and **5** it was inhibited following a behavior of the type dose-dependent. In conclusion, experimental data suggest that antibacterial activity of danazol-succinate-lisozyme depend on interaction of protein with danazol-succinate, in order to interact with the cell surface, and perturb bacterial growth of *Staphylococcus aureus*.

Key words: danazol: lisozyme; conjugate; antibacterial activity

Resumen

En este trabajo fue sintetizado el succinato de danazol-lisozima (**5**) conjugado; la ruta involucra la preparación del succinato de danazol (**3**) por esterificación del danazol (**1**) con anhídrido succínico (**2**), seguido por la reacción de **3** con lisozima (**4**) para obtener **5**. La estructura de **3** fue confirmada por datos espectrométricos y espectroscópicos y **5** fue caracterizado por espectroscopía de ultravioleta y electroféresis. La actividad antibacteriana del compuesto **3** y **5** fue evaluada *in vitro* sobre *Staphylococcus aureus* usando el método de dilución y la concentración mínima inhibitoria. Los resultados mostraron que 11 residuos del derivado esteroidal **(3)** fueron acoplados sobre una molécula de lisozima (**4**), obteniéndose 5.23 x 10^{-5} mmol de succinato-danazol por miligramo de **5.** El análisis electroforético mostró una banda de ≅ 16 Kda para **5**. Otros datos experimentales, indicaron que el crecimiento de la bacteria *Staphylococcus aureus* en presencia de **3** y **5** fue inhibida siguiendo un comportamiento del tipo dosis-dependiente. De acuerdo a los datos experimentales se demuestra que la actividad antibacterial del succinato de danazol-lisozima depende de la interacción de la proteína con el derivado de danazol, para interactuar en la superficie celular y perturbar el crecimiento bacterial de *Staphylococcus aureus*.

Palabras clave: danazol: lisozima; conjugado; actividad antibacteriana

Introduction

Infectious diseases are one of the main causes of morbidity-mortality in the world [1-3]. Several causal agents such as *Staphylococcus aureus*, have been shown to accelerate the progression of this pathologies [4-7]. Although there are many therapeutic agents for treating them [8-10], several data show that prolonged antibiotic therapy induces bacterial resistance [11-12]. Therefore, antibiotic resistance can be considered a serious threat for the human health, fact that requires an international approach to its management. In this direction, new drugs have been developed for control of bacterial resistance [13-15]. Particularly, there has been a resurgence of the interest in steroids associated with antibiotics as potential therapeutic agents for the infectious diseases [16]. In this sense, several steroid-antibiotics have been developed to mimic the antibacterial behavior of endogenous peptide antibiotics [17]. This task includes selective interaction of the steroid-antibiotic with disruption of bacterial membranes [18]. This interaction is related to chemical structural characteristics of the steroid-antibiotic agents such as cationic forms and facially amphiphilic conformations, which seems to be the key required for antibacterial activity. It has also been suggested that membrane selectivity is primarily derived from ionic recognition of negatively charged bacterial membranes [19]. Several studies suggest that functional groups of steroid-derivatives are involved in the bacterial activity [20]. For example, a series of 3-amino and polyaminosterol analogues of squalamine and trodusquemine [21] showed antibacterial activity on *Gram positive* and *Gram negative* bacteria. Other studies indicate that several 15-azasteroid analogues have antimicrobial activity on *Gram negative* bacteria [22]. Recently in our laboratory several pregnenolone-derivatives were synthesized and their antibacterial activity was evaluated on *Gram positive* and *Gram negative* bacteria [23-24]. Therefore, in this work our fundamental objective was to synthesize a new steroid-drug that can be used for treatment of infectious diseases. Our initial experimental design included the synthesis of danazol-succinate-

lisozyme conjugate; here it is important to mention that this *steroid-conjugate* has a spacer arm with amide group between the fragments of danazol and lisozyme involved in their chemical structure. This danazol-succinate-lisozyme conjugate was used to evaluate their antibacterial activity on *Staphylococcus aureus* using the microbial minimal inhibitory (MIC) method [25].

Experimental

General methods

Danazol-succinate was prepared according to a previously reported method by Figueroa and coworkers [26]. Lisozyme (single-chain mol wt 14.7 kDa) and the other compounds evaluated in this study were purchased from Sigma-Aldrich Co., Ltd. The melting points for the different compounds were determined on an Electrothermal (900 model). Ultraviolet spectroscopy (UV) was carried out in dry methanol on a Perkin-Elmer model 552 spectrophotometer and infrared spectra (IR) was recorded using KBr pellets on a Perkin Elmer Lambda 40 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75.4 MHz in $CDCl_3$ using TMS as internal standard. EIMS spectra were obtained with a Finnigan Trace GCPolaris Q. spectrometer. Elementary analysis data were acquired from a Perkin Elmer Ser. II CHNS/0 2400 elemental analyzer.

Synthesis of danazol succinate (5-(1-ethynyl-10a,12a-dimethyl-2,3,3a,3b,4,5,10,10a,10b,11,12, 12a-dodecahydro-1H-7-oxa-8-aza-dicyclopenta[a,h]-phenanthren-1-yl)-4-oxo-pentanoic acid.

A solution of danazol (17-Pregna-2,4-dien-20-yno[2,3-d]-isoxazol-17-ol) 200 mg (0.59 mmol), succinic anhydride 118 mg (1.18 mmol), 3 mL of pyridine in 10 mL of toluene was gently refluxed for 24 h and then cooled to room temperature. The reaction mixture was evaporated to a smaller volume, diluted with water, and extracted with chloroform. The organic phase was evaporated to dryness under reduced pressure. A solid was obtained which was purified by crystallization from hexane:methanol:water (1:2:1), yielding 190 mg, 60 % of product; mp 156 ^{o}C; UV (MeOH) λ_{max} (log) 210 (1.80) 284 (2.82) nm; IR V_{max} 3302, 2941, 2876, 1736 cm^{-1}; $^{1}HNMR$ (300 MHz, $CDCl_3$) δ_H: 0.90 (3H, s, 18-$\mathbf{H}_3$), 1.02 (3H, s,19-$\mathbf{H}_3$), 1.25-1.78 (1H, m), 1.88 (1H, s, C≡C**H**), 2.02 (1 H, m), 213-231 (1 H, m), 2.52 (2H, s, C$\mathbf{H}_2$-CO_2), 2.58 (2H, m, C$\mathbf{H}_2$-CO_2H), 3.06 (1H, m, **H**C-C≡C), 3.28 (1H, m), 4.86 (1 H, m, OC**H**, cyclopentane), 5.67 (1H, m, OC**H**, isoxazole-ring), 6.17 (1H, s, J = 3.63, C=C**H**), 8.01, (1H, s, N=C**H**), 10.02 (1H, s, CO_2**H**). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 12.61 (**C**-18),18.66 (**C**-19), 21.12 (**C**-11), 30.52 (CO_2-**C**H_2-**C**H_2-CO_2H), 32.13 (**C**-15), 32.48 (**C**-6), 33.21 (**C**-7), 36.02 (**C**-12), 36.81 (**C**-8), 37.48 (C-16), 38.75 (C-10) 40.91 (**C**-1), 46.59 (C-13), 49.70 (C-2), 51.20 (**C**-9), 53.71 (**C**-14), 67.55 (C≡**C**H), 83.28 (O**C**H, isoxazole-ring) 87.51 (**C**-17), 87.68 (**C**≡CH), 118.62 (H**C**=C), 148.54 (HC=**C**), 154.41 (N=**C**H), 164.80 (CH_2-**C**O_2), 171.02 (**C**O_2H). EIMS [M+] m/z 439.54 (12), 421.49 (100), 412.22 (8), 339.12 (15). Anal. found to $C_{26}H_{33}NO_5$: C, 71.18; H, 7.58; N, 3.20; O, 18.04. Calcd. C,71.05; H, 7.57; N, 3.19; O, 18.20.

Synthesis of danazol-succinate-lisozime conjugate.

A solution of danazol succinate (200 mg, 0.59 mmol) was added to a solution of *lisozyme* (200 mg, 1.40 x 10^{-2} mmol) and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (110 mg, 0.57 mmol) in acetonitrile-water (15 mL, 2:1). The mixture was stirred at room temperature for 72 h, the solvent was removed under vacuum and the crude product was purified by a method reported elsewhere with some modifications [27]. In this method the mixture was deposited on glass with a semi-permeable membrane with micropourus diameters between 10 and 400 Å. It is important to mention that the glass was deposited within a chamber with distilled water by 24 h and change of water was made three times.

Characterization of danazol-succinate-lisozime conjugate

Spectroscopy ultraviolet analyses

The absorbance of the steroid moiety of the danazol-succinate-lisozyme conjugate was determined by subtracting the absorption curve of danazol-succinate from that of steroid-lisozyme. It is important to mention that ultraviolet spectrum of lisozyme was determinate using the report done by Kumagai and coworkers [28] whom indicate a maximun in the absorbance of lisozyme at 280 nm. In order to determine the number of steroid molecules coupled a lisozyme molecule, the spectral differences methods reported by Dorobantu [29] and Erlanger [30] were used.

Polyacrilamide gel electrophoresis analyses

Analysis of danazol succinate-lisozyme conjugate by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was made using standard electrophoresis procedures [31]. Polyacrylamide gels were prepared using commercially available Bio-Rad 40% acrylamide solution as follows; acrylamide (3.75 mL) to give a 15 % final solution was mixed with pH 4.08 reservoir buffer (6.25 mL). It is important to mention that the reservoir buffer consist of glycine (28.1 g), glacial acetic acid (3.06 mL), and Milli-Q water as required to adjust the volume to 1 L at pH 4.08 as described by Hrkal [32].

After degassing, N,N,N´,N´,-tetramethylethylendiamine (15 μL) and ammonium persulfate (100 μL of a freshly prepared 50 mg/mL solution) were added and mixed before the gel was poured. Normally 20 μL de each samples were applied to obtained at least one protein band within the range of the lisozyme and steroid-conjugate. In addition, the gel was run with one line containing *phosphorylase b* marker. Electrophoresis was first run at 60 V for 20 min, and then at 100 V for 2 h. Gels were fixed in 12% trichloroacetic acid for at least 30 min and stained in 0.2% Coomasie G-250 (Serva, Heidelberg, Germany), 40% methanol, 10% acetic acid for at least 4 h in a glass petri dish. Distaining was in 12% methanol, 7% acetic acid with five changes of destainer during 24 h. After of this process, the gels were scanned with a laser scanner (Ultrascan XI, Pharmacie). The laser beam was adjusted for each lane to cover the whole band, which in most cases resulted in a laser track with a width of 5.6 mm and a length of about 15 mm. Boundaries for start and stop of integration were set for each lane.

Biological evaluation

Strains. The microorganisms employed in this study were obtained from the strain bank at the Department of Pharmaco-Chemistry of the Faculty of Chemical-Biological Sciences of the Universidad Autonoma de Campeche. The strains were certified by the Center for Disease Control of Atlanta and were as follows. *S. aureus* (ATCC 25923). The strains were kept under refrigeration at 4 ^{o}C in special gel (BBL).

Antimicrobial agents. Danazol-succinate compound was dissolved in methanol and diluted with distilled water. Danazol-succinate-lisozyme conjugate was dissolved in distilled water. Cefotaxime, gentamicin, methicillin and ciprofloxacin were used as control drugs.

Antimicrobial activity. The evaluation of antimicrobial effect of the different compounds on the bacterial species was made by the method described by Chiong *et al*. [25] The bacterial species were incubated on *Staphylococcus* 110 (*S. aureus*) agar for 24 hours at 37 ^{o}C. After this process, it was determined whether it had taken place the growth or not. In addition, a series of tubes were prepared, the first of which contained 2 mL of culture medium (tripticase soye) at double concentration and the remainder (11 tubes), contained the same quantity of medium at single concentrations. From the first tube (double concentration) an aliquot of 2 mL of the studied compound (1 mg/mL) was added and stirred, from this tube an aliquot of 2 mL was taken and added to the following tube (simple concentration) and the process was successively repeated until the last

2 mL of dissolution had been used up. After this process, each tube was inoculated with 0.1 mL of the bacterial suspension, whose concentration corresponded to McFarland scale (9×10^8 cells/mL) and all the tubes were incubated at 37 ^oC for 24 hours. Subsequently, a loop was taken from each of them and inoculated into the appropriate cultures for different bacterial organisms, and were incubated for 24 hours at 37 ^oC. After this process, the minimum inhibitory concentration (MIC) was evaluated to consider the antimicrobial effect of all compounds. In order to discard the effect of methanol (solvent) on the bacterial species studied, a series of the same number of tubes was prepared in parallel, to which 2 mL of methanol at 60% was added to the first and corresponding successive dilutions were added in the same way as before. In addition, a control series was also performed using distilled water at pH 7.0.

Results and Discussion

We report in this work a straightforward route for the synthesis of danazol-succinate-lisozyme conjugate (**5**). The first step (see Figure 1) involves the esterification of the hydroxyl group of danazol (**1**) to form danazol-succinate compound (**3**) by the method reported by Figueroa [26], using toluene as solvent to avoid hydrolysis in the new arm formed in cyclopentane-ring of danazol-succinate, which has like characteristic a free carboxyl group. The ^1H NMR spectrum of danazol succinate showed signals at 0.90 and 1.02 ppm corresponding to methyl groups in the steroid rings. In addition, signals at 1.88 ppm assigned to the proton of alkyne group (C≡C**H**), 2.58 ppm for methylene bound to carboxyl group, and two signal at 5.67 (OC**H**) and 8.01 ppm (N=C**H**) in the isoxazole-ring were found. Finally, a signal at 10.02 ppm corresponding to the acidic hydrogen of C(=O)-O**H** was detected.

On the other hand, ^{13}C NMR spectra of **3** displays chemical shifts at 12.61 and 18.66 ppm for the carbons of methyl groups present in the molecule. The chemical shift of the methylene bound to carboxyl group was found out at 30.52 ppm. In addition, were detected several signals in the range 32.13-53.71 ppm corresponding to carbons involved in the steroid rings and at 67.55 ppm (C≡**C**H) and 87.68 ppm (**C**≡CH) for alkyne group. Additionally, two characteristic signals of isoxazole-ring carbons at 83.28 ppm (O**C**H) and 154.41 ppm (N=**C**H) were also detected. Finally, a signal at 171.02 ppm for the carbon of **C**O_2H group was found. Additionally, the identity of the danazol succinate was further confirmed from mass spectrum which showed a molecular ion at m/z 439.54.

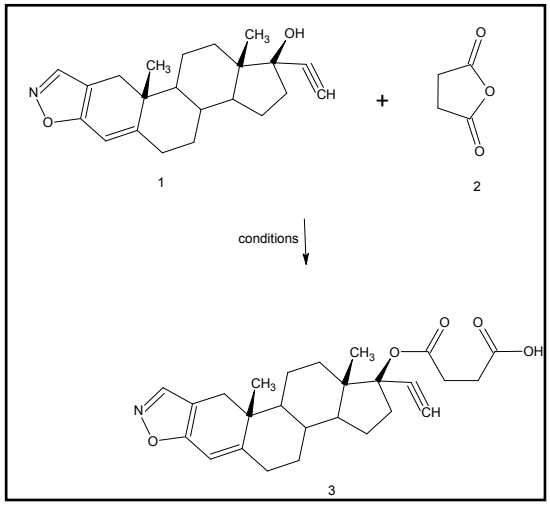

Figure 1. Synthesis of danazol-succinate compound (**3)**. Conditions; pyridine/toluene; refluxed for 48 h.

The second step of the synthesis of danazol-succinate-lisozyme conjugate (see Figure 2) was achieved using the method reported by Erlanger et al [30]. In this method it was synthesized the steroid-protein conjugate (**5**) by the reaction of danazol-succinate compound (**3)** with lisozyme (**4**) in presence of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide for amide bond formation in the danazol-succinate-lisozyme conjugate.

It is important to mention that many procedures for the formation of amide groups are known in the literature [33-35]. The most widely practiced method employs carboxylic acid chlorides as the electrophilic reactive which react with the amino group in the presence of an acid scavenger [36]. Despite its wide scope, the former protocol suffers of several drawbacks, being the most remarkable ones the limited stability of many acid chlorides and the need of hazardous reagents for their preparation (thionyl chloride) [37]. In this work it was used a derivative of carbodiimide [38] for amide bond formation in the danazol-succinate-lisozyme conjugate.

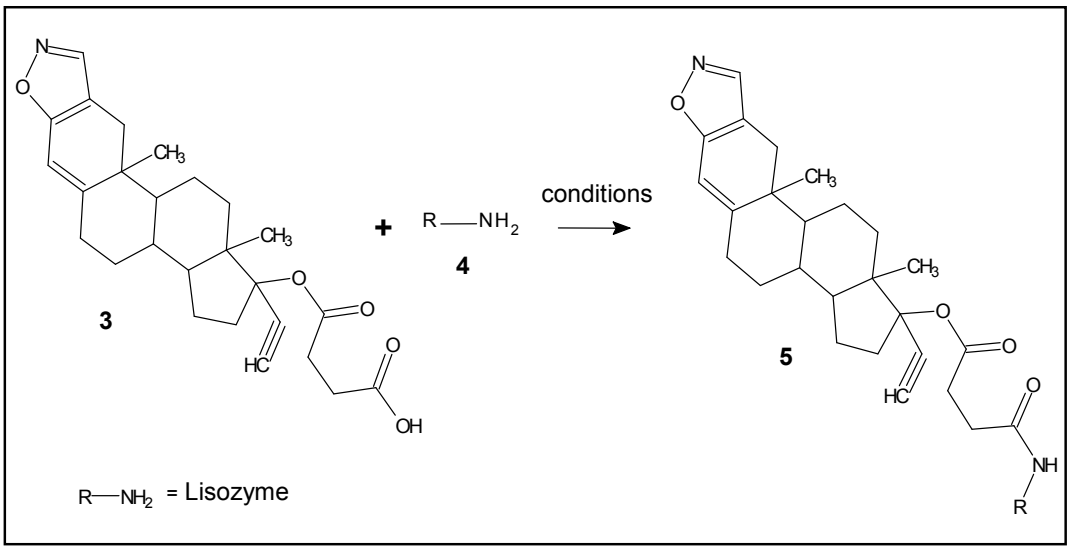

Figure 2. Synthesis of danazol-succinate-lisozyme conjugate (**5)**. Conditions; 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride; acetonitrile/water.

The obtained yield of steroid-lisozyme conjugate was 156 mg (39 %). In order to determine the number of steroid molecules coupled on lisozyme molecule, the methods reported by Dorobantu et al., [29] and Erlanger et al., [30] were used. It is important to mention that optical density values corresponding to the absorption maximum provide a basis for the estimation of the degree of steroid substitution in the danazol-succinate-lisozyme conjugate. Figure 3 shows the ultraviolet spectra of the danazol-succinate-lisozyme polymer in comparison with danazol-succinate, danazol and lisozyme. The results showed that danazol-succinate displays an absorption maximum at 287 nm and its molar extinction coefficient (ε) is 2.14 at this wavelength. Thus, the absorbance of the steroid-derivative moiety of the danazol-succinate-lisozyme conjugate was determined by subtracting the absorption curve of lisozyme (λ_{max} (ε) = 280 nm (2.41)) of the curve danazol-succinate-lisozyme polymer and was found to be maximal at 275 nm with a molar extinction coefficient of 1.02. Here it is important to mention that it has often been assumed that the aromatic side chains (tryptophan and tyrosine) of lisozyme contributes to absorbance properties in the far-ultraviolet region of lisozyme [39] and other proteins [40-41]. In the same way, it is important to

mention that amide group involved in the proteins, could also contributes to this phenomenon [42]. In this sense, we assumed that the presence of an amide-bound between the danazol-succinate and lisozyme would modify the interaction with the chromophoric group of the steroid-derivative and thus influencing its electronic configuration, resulting in a change of intensity and possibly a spectral shift of the ultraviolet absorption. This premise could be similar to works done by Westphal [43] who suggest that spectrophotometric analysis can indicate interactions between several steroid-derivatives with certain proteins. This assumption is in line with the spectrophotometric analysis done in this work (see Table 1) using the method reported by Donabatu et al [29]. This analysis showed that 11 residues of steroid-derivative-are coupled to a lisozyme molecule obtaining 5.23×10^{-5} mmol of danazol-succinate per milligram of danazol-succinate-lisozyme conjugate.

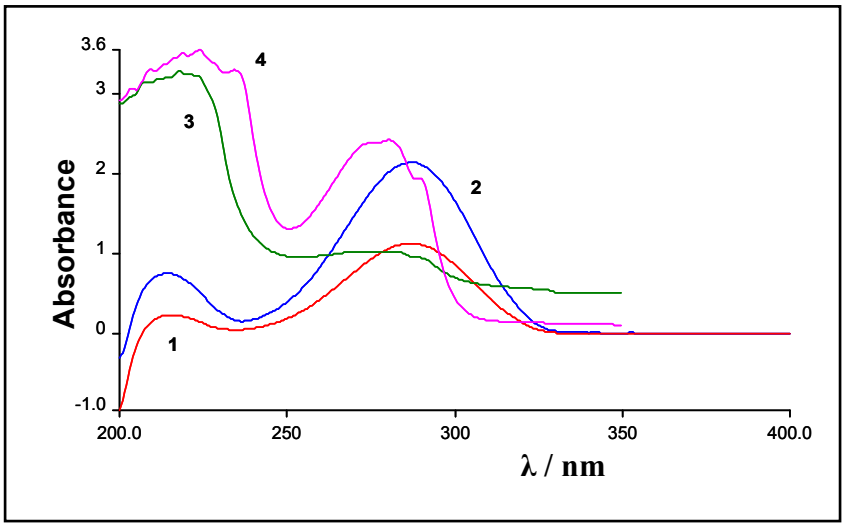

Figure 3. Ultraviolet spectra of danazol (**1**); danazol-succinate compound (**2**); danazolsuccinate-lisozyme conjugate (**3**); lisozyme (**4**).

SDS-PAGE analysis of the danazol-succinate-lisozyme conjugate showed evidence of resolution between the conjugate and lisozyme. The results (Figure 4) showed a band of ≅ 16 kDa for steroid-protein and ≅ 14 kDa for lisozyme. These experimental data suggest that modifications in the chemical structure of lisozyme by the reaction of Lys aminoacid situated on the surface of lisozyme with danazol-succinate bring consequently changes in the position of the lisozyme band. This phenomenon revealed that diminution relative degree of positive charges exerted by Lys aminoacid in lisozyme induces changes in the migration of the band associated with danazol-succinate-lisozyme. These data are supported with studies performed by Masuda and coworkers [44] that suggest that chemical modification of Lys aminoacid residues of lisozyme by interaction of estrone with the protein induce change in total charges.

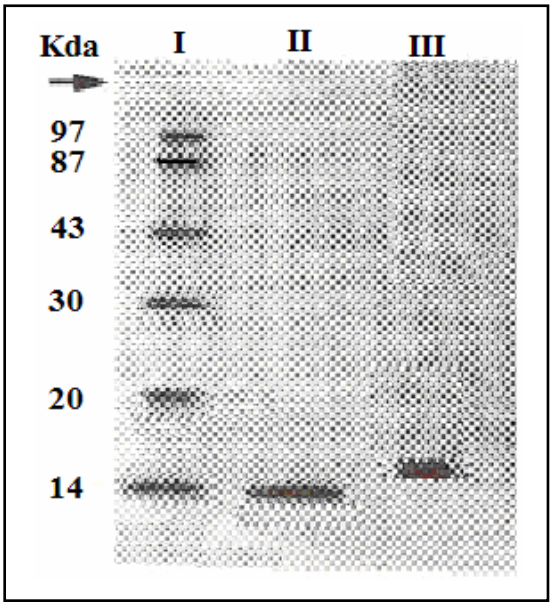

Figure 4. Polyacrilamide gel electrophoresis analyses (poliacrilamide-gel, pH 4.08) of lisozyme (II), danazol-hemisuccinate-lisozyme conjugate (III) and phosphorylase b marker (I).

Table 1. Experimental data obtained of extinction values and coupled ratio of danazolsuccinate-lisozyme conjugate.

Drugs	Concentration (μg)	Absorbance ε (λ)	Danazol-succinate-lisozyme (N)
Danazol	500	$\lambda_{215} = 0.23$	
		$\lambda_{287} = 1.12$	
Lisozyme	500	$\lambda_{280} = 2.41$	
		$\lambda_{223} = 3.54$	
Danazol-succinate	500	$\lambda_{214} = 0.74$	
		$\lambda_{287} = 2.14$	
Danazol-succinate-lisozyme	500	$\lambda_{217} = 3.28$	0.34
		$\lambda_{275} = 1.02$	21.55
Coupling ratio Danazol-succinate-lisozyme			$10.84 \cong 11$

On the other hand, the antibacterial activity of the *steroid-protein* conjugate on *Staphylococcus aureus* was evaluated by mean of dilution method. The minimum inhibitory concentration (MIC) of the conjugate was determined and its value compared to those of gentamicin, ampicillin, cefotaxime and ciprofloxacin. The results obtained (Figure 5) indicate that bacterial growth of *Staphylococcus aureus* was inhibited with cefotaxime (MIC = 0.25 mg/mL, 5.23 $\times 10^{-4}$ mmol), gentamicin (MIC = 0.0125 mg/mL, 2.68 $\times 10^{-5}$ mmol) and ciprofloxacin (MIC=3.77 $\times 10^{-4}$ mg/mL). Ampicillin does not inhibit the bacterial growth of *Staphylococcus* (data not shown). The bacterial growth of *Staphylococcus aureus* in presence of danazol-succinate-lisozyme conjugate (MIC = 0.5 mg/mL; equivalent at 3.39 x 10^{-5} mmol) was blocked. These data indicate that danazol-succinate-lisozyme conjugate had antibacterial potency similar to gentamicin (inhibitor of synthesis of protein). Nevertheless, in comparison with cefotaxime (β-lactam antibiotic) and ciprofloxacin (inhibitor of ADN-gyrase) the antibacterial activity on this pathogen microorganism was high, which can be due mainly to the different molecular mechanism involved and the characteristic chemical structure of the compound. In this sense, it is important to mention that this compound contains in the cyclopentane-ring of the steroid nucleus a spacer arm with both characteristics, ester and amide groups (-O-C=O-$(CH_2)_2$-CO- NH-) coupled to lisozyme, involving in addition an imine group in the isoxazole ring. Several reports have shown that compounds with nitrogen atom in their chemical structure, exert antibacterial activity against both Gram-positive through perturbation of lipid bi-layer membranes that constitute the bacterial cytoplasmic membrane and the outer-membrane of bacteria [45]. To evaluate this premise, the 17-Pregna-2,4-dien-20-yno[2,3-d]-isoxazol-17-ol (danazol) was used, since the nature of functional groups contained in the chemical structure a imine in the isoxazole ring. The results showed that in presence of *danazol* the bacterial growth of *Staphylococcus aureus* was not blocked (data not showed). The experimental data suggest that the imine group of free danazol by itself, does not have antibacterial activity on the pathogen microorganism studied and suggested that the steroid-derivative moiety could be the only responsible. In order to analyze this possibility, we evaluated the danazol-succinate fragment in this sense, alternative experiments in Staphylococcus aureus using danazol-succinate were made to compare its effects with those induced by the danazol-succinate-lisozyme conjugate. The obtained results showed that the steroid-derivative can block the bacterial growth of *Staphylococcus aureus* in a dose-dependent manner (MIC of 1 mg/mL (1.14 $\times 10^{-3}$ mmol). This experimental data suggest that antimicrobial effect induced by danazol-succinate can depend on the nature of the free carboxyl group contained in its chemical structure, which is a membrane-perturbing agent whose antibacterial activity is induced, possibly by the interaction with the positively charged amino groups contained in the D-alanyl moiety incorporated in the teichoic acids, essential polymers that plays a vital role in the growth and development of the gram-positive bacteria [46]. On the other hand, thinking that free protein (lisozyme) could induce by itself antibacterial activity, alternative experimental were done. The results indicate that in presence of lisozyme, the bacterial growth of *Staphylococcus aureus* was not blocked (data not shown).

Conclusions

In this study, we report an easy methodology to synthesize danazol-succinate-lisozyme (**5)** using carboddimide-derivative. The antibacterial activity induced by (**5)** depends on the interaction of protein with a hydrophobic region of danazol-succinate compound, in order to interact with the cell surface, and perturb bacterial growth of *Staphylococcus aureus*.

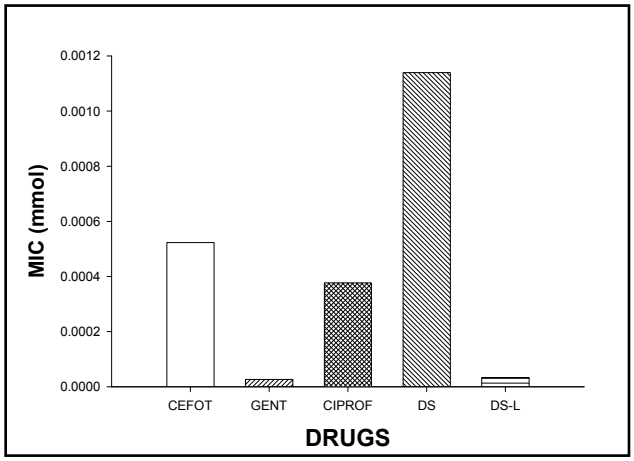

Figure 5. Effect induced by danazol-succinate-lisozyme and controls (cefotaxime, CEFOT, gentamicin, GENT, and ciprofloxacin, CIPROF) on Staphylococcus aureus. It is showed that exist differences of antibacterial activity of CEFOT (MIC = 5.23×10^{-4} mmol) and GENT (MIC =2.68 × 10-5 mmol) on *Staphylococcus aureus* in comparison with the danazol-succinate (DS) compound (MIC = 1.14×10^{-3} mmol) and danazolsuccinate-lisozyme conjugate (DS-L; MIC = 3.39×10^{-5} mmol). MIC = minimum inhibitory concentration.

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