

Synthesis and Antibacterial Activity of Pregnenolone-Vitamin B1 Conjugate

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Abstract. In this work was synthesized a pregnenolone derivative; the route involved preparation of pregnenolone hemisuccinate (**2**) by esterification of pregnenolone (**1**) with succinic anhydride followed by formation of the hemisuccinate of pregnenolone-vitamin B1 conjugate (**3**). The antibacterial activity of compound **3**, as well as **2** and vitamin B1, was evaluated *in vitro* on *S. aureus*, *K. pneumoniae* and *E. coli* using dilution method and the minimum inhibitory concentration (MIC). The structure of **3** was confirmed by spectroscopy and spectrometry data. The ¹H NMR spectrum showed, up field shifts at 2.52 and 2.58 ppm for methyls present in the heterocycles rings; at 2.90 and 3.77 methylenes of the hydroxietilen moiety bonded to thiazol ring, in addition the hydrogens of the methylene between the pyrimidine and thiazol ring appears at 4.39 ppm. At down field there are two chemical shifts (7.54, 8.33) corresponding to protons in the heterocycles. The results of the biological activity indicate that the bacterial growth of the microorganisms studied was inhibited by **3**, and **2** in a manner dose-dependent, but not by vitamin B1. These data suggest that quaternary amine group involved in the HPVB1 conjugate requires the hydrophobic region of the steroid in order to interact with some components of bacterial cell, disturbing the bacterial growth and to cause cell death.

Key words: Pregnenolone, Hemisuccinate-pregnenolone-vitamine B1, Quaternary amine.

Resumen. En este trabajo fue sintetizado un derivado de pregnenolona, la ruta involucra la preparación de hemisuccinato de pregnenolona (**2**) por esterificación de pregnenolona (**1**) con anhídrido succínico, seguido por formación del conjugado hemisuccinato de pregnenolona-vitamina B1 (**3**). La actividad antibacteriana del compuesto **3**, así como la de **2** y de la vitamina B1 fue evaluada *in vitro* sobre *S. aureus*, *K. pneumoniae* and *E. coli* usando el método de dilución y la concentración mínima inhibitoria (CMI). La estructura **3** fue confirmada por datos espectroscópicos y espectrométricos. El espectro de ¹H NMR mostró cambios a 2.52 y 2.58 ppm para metilos presentes en los anillos heterocíclicos; a 2.90 y 3.77 para metilenos del hidroxietilén enlazado al anillo de tiazol; además, los hidrógenos del metileno entre la piridina y el anillo de tiazol aparecen a 4.39 ppm. A campos bajos hay dos cambios químicos (7.54, 8.33) correspondientes a protones en los heterocíclicos. Los resultados de la actividad biológica indican que el crecimiento bacteriana de los microorganismos estudiados fue inhibido por **3** y **2** en una manera dosis-dependiente pero no por la vitamina B1. Esos datos sugieren que el grupo amino cuaternario envuelto en el conjugado hemisuccinato de pregnenolona-vitamina B1 posiblemente requiere la región hidrófoba del esteroide para interactuar con algunos componentes de la célula bacteriana perturbando el crecimiento bacteriana y causar muerte celular.

Palabras clave: Pregnenolona, Hemisuccinato-pregnenolona-vitamina B1, Quaternary amine.

Introduction

Infectious diseases are one of the main causes of morbidity-mortality in the world [1-3]. Several causal agents, such as *S. aureus*, *K. pneumoniae* and *E. coli* have been shown to accelerate the progression of these pathologies [4-7]. Although there are many therapeutic agents for treating them [8-10], data exist showing that prolonged antibiotic therapy induce bacterial-resistance [11-12], because some bacteria have developed ways to circumvent the effects of antibiotics [13-14] for example, several studies indicate that β -lactamic antibiotics (ie. methicilin/oxacillin) induce resistance in *S. aureus* [15-16]. Other reports showed that antibiotic-resistant strains have emerged among Gram-negative bacilli such as *K. pneumoniae* [17] and *E. coli* [18]. Therefore, antibiotic resistance can be considered as a serious threat for health, and an international approach to its management is required, in this sense, new drugs have been developed for control of bacterial resistance [19-21] for example, the synthesis of cholic acid-derivate compounds that have antibacterial activity by increasing the permeability of the outer membrane of Gram negative bacteria have shown promising results [22]. In addition, other studies

showed a correlation of the antibacterial activities between cationic peptides and cationic steroids on Gram-negative and Gram-positive bacteria [23]. Cationic steroid-antibiotics were developed to mimic the antibacterial behavior of endogenous peptide antibiotics, this task include selective association of the steroid-antibiotic with disruption of bacterial membranes [24-25]. The association involves structural characteristics of the steroids-antibiotic such as cationic forms and facially amphiphilic conformations, which appear to be the key requirement for antibacterial activity, and membrane selectivity is primarily derived from ionic recognition of negatively charged bacterial membranes [26].

In this work our initial design included the synthesis of pregnenolone-Vitamin B1 compound that contains in the A-ring of the steroid nucleus a spacer arm with both ester and amide functional groups (-O-C=O-(CH₂)₂-CO-NH-) coupled to pyrimidin ring, and involve a quaternary amine in the thiazole ring with positive charge. This compound was made with the purpose to evaluate their antibacterial activity on *S. aureus*, *K. pneumoniae* and *E. coli* using the microbial minimal inhibitory method [27], because several data exist indicating that quaternary amine compounds exert antibacterial activity against both

Gram-positive and Gram-negative bacteria by the perturbation of the bacterial membrane. In addition to evaluate this premise, we used as pharmacological tool the 3-((4-Amino-2-methyl-5-pyrimidinyl)methyl)-5-(2-hydroxyethyl)-4-methylthiazolium chloride compound (Vitamin B1), since the nature of functional groups contained in the chemical structure of this compound involve a quaternary amine in the thiazole ring.

Results and Discussion

In this work we report a straightforward route for the synthesis of hemisuccinate of pregnenolone-vitamin B1 conjugate (see Scheme 1). The first step involves the esterification of the *hydroxyl* group (C-3 A-ring) of pregnenolone to form the pregnenolone hemisuccinate (5-Pregnen-20-one, 3-(3-carboxy-1-oxopropoxy) by the method reported by Figueroa [28], using *toluene* to avoid hydrolysis in the new arm formed in A-ring of the *pregnenolone-derivative*, that has both characteristic ester and carboxyl groups. The results indicate that the ^1H NMR spectrum of pregnenolone hemisuccinate showed a signal at 10.03 corresponding to the acidic hydrogen of $\text{C}(=\text{O})-\text{OH}$. The presence of the pregnenolone hemisuccinate was further confirmed from mass spectrum which showed a molecular ion at m/z 416.

The second step was achieved by reacting 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride (Vitamin B1) with pregnenolone hemisuccinate resulting in amide bond formation. It is important to mention that many procedures for the formation of amide groups are known in the literature [29-31]. The most widely practiced method employs carboxylic acid chlorides as the electrophiles which react with the amino group in the presence of an acid scavenger [32]. Despite its wide scope, the former protocol suffers from several drawbacks; most notable are the limited stability of many acid chlorides and the need for hazardous reagents for their preparation (thionyl chloride) [33]. In this work was used, a derivative of carbodiimide [34] for amide bond formation in the pregnenolone-Vitamin B1 conjugate. The ^1H NMR spectra of the pregnenolone-Vitamin B1 conjugate shows in addition of the characteristic chemical shifts of the pregnenolone hemisuccinate, upfield shifts at 2.52 and 2.58 ppm for methyls present in the heterocycles rings; at 2.90 and 3.77 methylenes of the hydroxietilen moiety bonded to thiazol ring; the hydrogens of the methylene between the pyrimidine and thiazol ring appears at 4.15 ppm. At down field there are two chemical shifts (7.54, 8.33) corresponding to protons in the heterocycles. On the other hand, ^{13}C NMR spectra displays chemical shifts at 11.46 and 25.30 ppm for the carbons of the methyls groups presents in the heterocycles. The

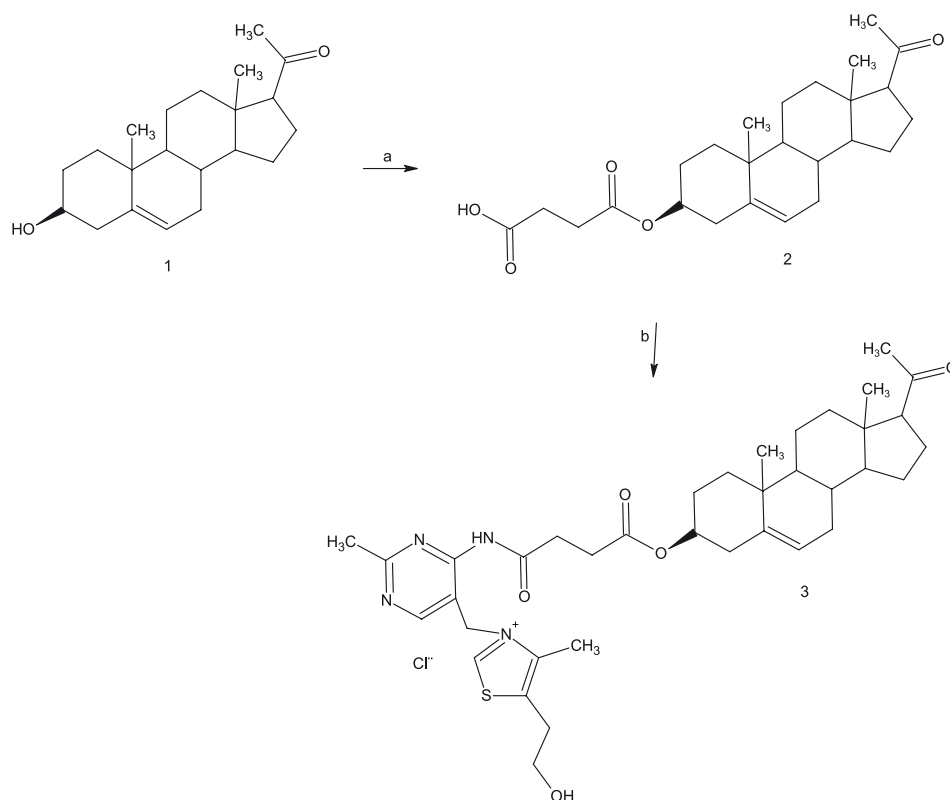


Fig. 1. Synthesis of hemisuccinate-pregnenolone-Vitamin B1 conjugate (3). The route involved preparation of pregnenolone hemisuccinate compound (2) by the esterification of (1) pregnenolone, followed by formation of pregnenolone-vitamin B1 conjugate (3). Conditions: a) succinic anhydride, pyridine/toluene; b) 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride, acetonitrile/water.

chemical shift of the methylene joined the pyrimidine and thiazol rings is found out at 59.9 ppm. Finally, at down field there are several signals (117.42, 122.69, 131.47, 148.70, 154.14, 160.44 and 166.89) corresponding to the carbons of the heterocycles and at 174.81 the chemical shift of the carbon of amide. Additionally, the mass spectra displays a molecular ion of m/z 664.08 corresponding to $M^+ \cdot Cl$ which confirm the structure of the pregnenolone-Vitamin B1 conjugate obtained.

On the other hand, the antibacterial activity of pregnenolone-Vitamin B1 conjugate on *S. aureus*, *K. pneumoniae* and *E. coli* was evaluated by means of dilution method and the minimum inhibitory concentration (MIC), using gentamicin, ampicillin and cefotaxime as control in this study. The results obtained (Figure 2) indicate that bacterial growth of *S. aureus* was inhibited with cefotaxime (MIC = 0.25 mg/mL, 5.23×10^{-4} mmol) and gentamicin (MIC = 0.0125 mg/mL, 2.68×10^{-5} mmol). In presence of ampicillin, the bacterial growth of *S. aureus* was not blocked (data not shown). In addition, the bacterial growth of *S. aureus* in presence of hemisuccinate of pregnenolone-vitamin B1 conjugate (MIC = 0.25 mg/mL, 3.66×10^{-4} mmol) was blocked. All this data indicate that pregnenolone-vitamin B1 compound had antibacterial potency similar to cefotaxime (β -lactam antibiotic), nevertheless in comparison with gentamicin (inhibitor of synthesis of protein) the antibacterial activity on this pathogen microorganism was lower; this can be due mainly to the different molecular mechanism involved and the characteristic chemical structure of the compounds studied in this work. In this sense, it is interesting to consider the different molecular mechanisms involved in the hemisuccinate of pregnenolone-vitamin B1 conjugate induced effects, here is important to mention that this compound contains in the A-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 pyrimidin ring of vitamin B1, in addition involve a quaternary amine in the thiazole ring with positive charge.

Here is important to mention that several reports have shown that quaternary amine compounds exert antibacterial activity against both Gram-positive and Gram-negative bacteria through perturbation of lipid bi-layer membranes that constitute the bacterial cytoplasmic membrane and the outer-membrane of bacteria [35]. To evaluate this premise, we used the 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2 hydroxyethyl)-4-methyl-thiazolium chloride (vitamin B1), since the nature of functional groups contained in the chemical structure a quaternary amine in the thiazole ring. The results showed that in presence of vitamin B1 the bacterial growth of *E. coli*, *S. aureus* and *K. pneumoniae* was not blocked (data not showed). The experimental data suggest that quaternary amine of free vitamin B1 by itself, does not have antibacterial activity on the pathogen microorganism studied and suggested that the steroid moiety could be the only responsible, in order to analyze this possibility, we evaluated the hemisuccinate of pregnenolone fragment. In this sense, alternative experimental in *S. aureus*, *K. pneumoniae* and *E. coli* using hemisuccinate of pregnenolone were made to compare its effects with those induced by the hemisuccinate

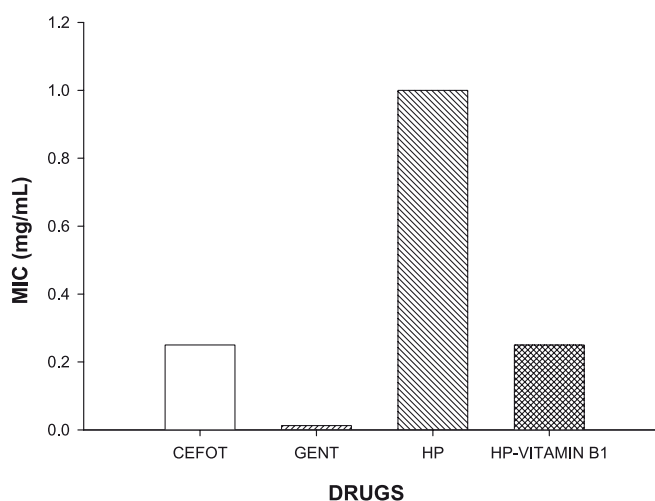


Fig. 2. Antibacterial effects induced by pregnenolone-derivates and control (cefotaxime, CEFOT and gentamicin, GENT) on *S. aureus*. Data showed that *S. aureus* was susceptible to cefotaxime (MIC of 0.25 mg/mL, 5.23×10^{-4} mmol) and gentamicin (MIC of 0.0125 mg/mL, 2.68×10^{-5} mmol). The bacterial growth of this microorganism in presence of the hemisuccinate-pregnenolone compound (MIC of 1 mg/mL, 2.40×10^{-3} mmol) and the hemisuccinate of pregnenolone-vitamin B1 conjugate (MIC of 0.25 mg/mL, 3.66×10^{-4} mmol) was inhibited. MIC = minimum inhibitory concentration.

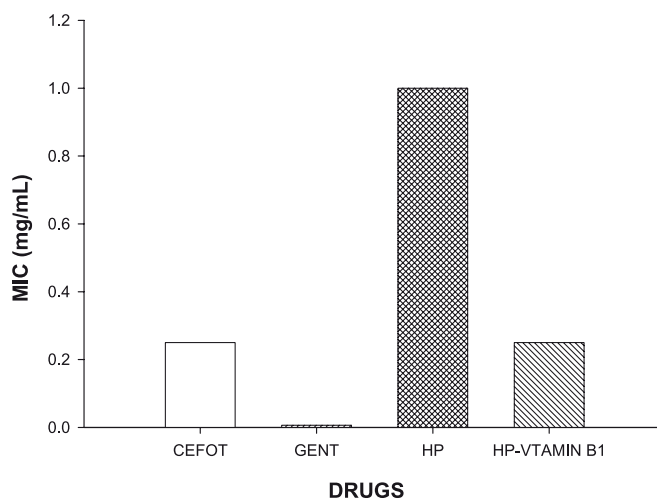


Fig. 3. Effect induced by pregnenolone-derivates and control (cefotaxime, CEFOT and gentamicin, GENT) on *E. coli*. It is showed that exist differences of antibacterial activity of CEFOT (MIC = 0.25 mg/mL, 5.23×10^{-4} mmol) and GENT (MIC = 6.25×10^{-3} mg/mL, 1.34×10^{-5} mmol) on *E. coli* in comparison with the hemisuccinate-pregnenolone compound (MIC = 2.40×10^{-3} mmol) and pregnenolone-vitamin B1 conjugate (MIC of 0.25 mg/mL, 3.66×10^{-4} mmol). MIC = minimum inhibitory concentration.

of pregnenolone-Vitamin B1 conjugate. The obtained results showed that the steroid can blocked the bacterial growth of *S. aureus*, *E. coli*, and *K. pneumoniae* in a dose-dependent manner (MIC of 1 mg/mL (2.40×10^{-3} mmol)). This experimental data suggest that antimicrobial effect induced by hemisuccinate-pregnenolone 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 incorporated in the teichoic acids, essential polymers that plays a vital role in the growth and development of the gram-positive bacteria [36]. Other possibility involve the intramolecular interaction of hemisuccinate-pregnenolone via divalent cations (Mg^{2+} and Ca^{2+}), involved in the membrane cell providing a substantial increase the permeability of the outer membrane of Gram-negative bacteria include bactericidal/ permeability increasing protein.

Nevertheless, it is important to mention that when hemisuccinate-pregnenolone is bound with Vitamin B1 to form the hemisuccinate of pregnenolone-vitamin B1 conjugate, the antibacterial activity seems to be greater, possibly because the quaternary amine compounds require only a strong positive charge together with a hydrophobic region in order to interact with the cell surface and integrate into the cytoplasmic membrane. Such integration into the membrane is sufficient to perturb bacterial growth to cause the membrane to lose fluidity and for the cell to die. This phenomenon can be associated by interaction of hemisuccinate of pregnenolone-vitamin B1 conjugate with teichoic acid that is an element of Gram-positive bacteria and with the lipopolysaccharide of Gram-negative bacteria. In addition, this phenomenon can induce, as consequence, an increase in the permeability of the outer membrane and induce growth bacterial inhibition on these pathogen microorganisms. This premise is supported by some mechanisms, based in experimental data, which proposed that steroid-antibiotics can adopt cationic conformations to induce bacterial death [37].

Conclusions

Experimental data suggest that quaternary amine group involved in the hemisuccinate of pregnenolone-vitamin B1 conjugate require only positive charge together with a hydrophobic region, in order to interact with the cell surface, and perturb bacterial growth.

Experimental

Chemistry Evaluation

General methods

The 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride (Vitamin B1) were purchased from Sigma-Aldrich Co., Ltd. 5-Pregnen-20-one,3-(3-carboxy-1-oxopropoxy (hemisuccinate of pregnenolone) was prepared according to a previously reported method by Figueroa *et al* [28]. 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. 1H 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/O 2400 elemental analyzer.

Synthesis of hemisuccinate of pregnenolone (5-Pregnen-20-one-3-(3-carboxy-1-oxopropoxy). A solution of pregnenolone (3 β -hydroxypregn-5-en-20-one) 200 mg (0.95 mmol), succinic anhydride 142 mg (1.42 mmol), 3 mL of pyridine in 10 mL of toluene was gently refluxed for 8 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, the residue was purified by crystallization from hexane:methanol:water (1:2:1) to give 235 mg (80%), mp 165 °C; UV (MeOH) λ_{max} (log) 215 (2.74) nm; IR V_{max} 3505, 1700 cm^{-1} ; 1H NMR (300 MHz, $CDCl_3$) δ_H : 0.62 (3H, s, H3-18), 0.92-1.14 (1H, m), 1.01(3H, s, H3-19), 1.12-1.35 (4H, m), 1.45-1.57 (3H, m), 1.58-1.73 (4H, m), 1.93-2.19 (2H, m), 2.06, (3H, s, H3-21), 2.35 (5H, m), 3.42 (1H, m, H-3), 5.33 (1H, d, J = 4.5 Hz, H-6), 10.03 (1H, br, CO_2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C : 13.40 (C-18), 19.52 (C-19), 21.92 (C-11), 22.90 (C-15), 23.61 (C-21), 27.80 (C-2), 29.45 (RO2C-C-CO $_2$ H), 31.40 (C-16), 31.80 (C-8), 32.6 (C-7), 36.74 (C-10), 37.01 (C-1), 38.29 (C-4), 38.80 (C-12), 43.90 (C-13), 49.94 (C-9), 56.78 (C-14), 63.60 (C-17), 73.95 (C-3), 122.69 (C-6), 139.60 (C-5), 173.85 (CO_2R), 177.30 (CO_2H); EIMS(30 eV) m/z (rel. int.), 416 (12, M+), 298 (64), 283 (30), 255 (27), 213 (58), 209 (27), 161 (28), 147 (46), 105 (66), 91 (100). Anal. $C_{25}H_{36}O_5$: C, 72.08; H, 8.71; O, 19.20. Calcd. for C, 72.01; H, 9.02, O, 19.22.

Synthesis of 3-{4-[3-(17-acetyl-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopentaphenanthren-3-yloxy-carbonyl)-propionylamino]-2-methyl-pyrimidin-5-ylmethyl}-5-(2-hydroxy-ethyl)-4-methyl-thiazol-3-ium;chloride. The pregnenolone hemisuccinate (100 mg, 0.24 mmol) was added to a solution of 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride (122 mg, 0.37 mmol) and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (55 mg, 0.28 mmol) in acetonitrile-water (15 mL, 2:1). The mixture was stirred at room temperature for 48 h, the solvent was removed under vacuum and the crude product was purified by crystallization from methanol:hexane:water (3:2:1) to give 73 mg (39%), mp 138 °C; UV (MeOH) λ_{max} (log e) 215 (2.74) nm; IR V_{max} 3330, 3200, 3100, 2851, 1738 cm^{-1} ; NMR 1H (300 MHz, $CDCl_3$) δ_H : 0.64 (s, 3H, H19), 1.02 (s, 3H, H18), 1.08-1.18 (m, 1 H, H14), 1.21-1.50 (m), 1.55-1.74 (m), 1.83-2.00 (m), 2.05-2.08 (m), 2.13 (s, 3H H21), 2.16-2.22 (m), 2.28 (s, 3H, CH3 en 4), 2.26-2.41 (m), 2.44-2.50 (m), 2.52-2.58 (s, 3 H, CH3 2), 2.61-2.71 (m), 2.90 (t, 2H, thiazol ring-CH $_2$ -CH $_2$ -OH), 3.77 (t, 2H, thiazol ring-CH $_2$ -CH $_2$ -OH), 4.39 (s,

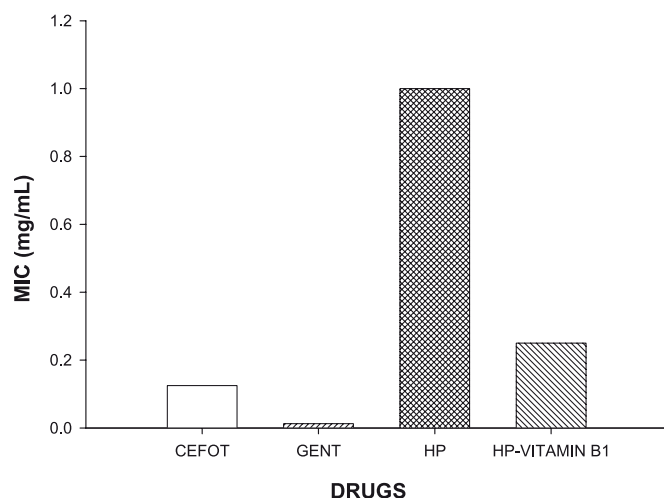


Fig. 4. Differences in antibacterial effects between the pregnenolone-derivatives and controls (cefotaxime, CEFOT and gentamicin, GENT) on *K. pneumoniae*. Experimental data showed that *K. pneumoniae* was susceptible to cefotaxime (MIC = 0.125 mg/mL, 2.61×10^{-4} mmol) and gentamicin (MIC = 0.012 mg/mL, 2.68×10^{-5} mmol). In presence of hemisuccinate-pregnenolone compound the MIC was of 1 mg/mL (2.40×10^{-3} mmol) and for pregnenolone-vitamin B1 conjugate its of MIC of 0.25 mg/mL, 3.66×10^{-4} mmol.

2H, pyrimidin ring -CH₂-thiazol ring), 5.13, m, 6.87 broad, m, 7.84 (s, 1H, H6 pyrimidin ring), 8.33(s, 1H H2 thiazol ring); NMR ¹³C (75.4 MHz, CDCl₃) δ_C: 11.56 (CH₃-C4 thiazole ring), 13.17 (CH₃-C19), 19.52 (CH₃-C18), 21.92 (C11), 22.90 (C15), 23.61 (C21), 25.30 (CH₃-C2 pyrimidine ring), 27.80 (C2), 27.81 (CH₂-C5 thiazole ring), 29.50 (OC-CH₂-), 31.40 (C16), 31.44 (-CH₂-CONH-), 31.80 (C7, C8), 37.74 (C1), 38.20 (C4), 38.80 (C12), 43.90 (C13), 49.94 (C9), 56.78 (C14), 59.92 (-thiazol ring-C-pyrimidine ring), 62.28 (C-OH), 63.60 (C17), 73.95 (C3), 117.42 (C5-pyrimidine ring), 122.69 (C6), 122.89 (C5-thiazol ring), 131.47 (C4-thiazol ring), 139.60 (C5), 148.20 (C6-pyrimidine ring), 154.14 (C2-thiazole ring), 160.44 (C4-pyrimidine ring), 166.39 (C2-pyrimidine ring), 171.39 (CO₂-), 174.81 (CONH-), 209.27 (C20), EIMS(30 ev) *m/z* (rel. int.), 664.08 (5.3%, M⁺-Cl), 316.17 (28%), 298.17 (49%), 264.00 (32%), 231 (100%), 213.22 (90%), 187.24 (80%), 145.28 (86%), 91.24 (95%). Anal. C₃₇H₅₁N₄O₅S: C, 66.94; H, 7.74; N, 8.44; O, 12.05; S, 4.83. Calcd. for C, 66.82; H, 7.66; N, 8.40; O, 12.00; S, 4.80

Microbiological Evaluation

Strains. The microorganisms in this study belonged to the strain bank at the Departamento de Fármaco-Química de la Facultad de Ciencias Químico-Biológicas de la Universidad Autónoma de Campeche. The strains are certified by Center for Disease Control in Atlanta and were as follows. *S. aureus* (ATCC 25923), *K. pneumoniae* (ATCC 700603) and *E. coli* (ATCC 25922). The strains are kept under refrigeration at 4 °C in special gel (BBL).

Antimicrobial agents. The pregnenolone-derivates were dissolved in methanol and diluted with distilled water. Cefotaxime, gentamicin and methicillin were used as positive controls. Freshly prepared solutions of the test compounds and control drugs were used in each assay.

Antimicrobial activity. The evaluation of antimicrobial effect of the different compounds on the bacterial species was made using the method described by Chiong *et al* [27]. The bacterial species were incubated on Mc-Conkey (*E. coli* and *K. pneumoniae*) and Staphylococcus 110 (*S. aureus*) agars for 24 hours at 37 °C, after such time, it could be determined whether growth had taken place or not.

In the other hand, a series of tubes were prepared, where the first one contained 2 mL culture medium (tripticase soya protein) at double concentration and the remainder (11 tubes), contained the same quantity of medium at normal concentrations. From the first tube (double concentration) an aliquot of 2 mL of compound studied was added and stirred, and 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 °C 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 °C. After such time, the minimum inhibitory concentration (MIC) was evaluated to consider the antimicrobial effect of the pregnenolone-derivates.

In order to discard the effect of methanol 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 (pH = 7).

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