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Synthesis and Antibacterial Activity of Pregnenolone-Carbamazepine Conjugate on *Proteus mirabilis*

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In this work, the steroid-carbamazepine conjugate was synthesized. The route involve preparation of carbamazepine-aminocaproic acid compound (**3**) followed by coupling of hemisuccinate-pregnenolone (**4**) to the **3** compound and formation of pregnenolone-carbamazepine conjugate (**5**). In addition, the evaluation of antimicrobial effect of the different compounds on *Proteus mirabilis* was made by the method of microbial minimal inhibitory (MIC). The structure from **5** was confirmed by spectroscopy and spectrometry data. The ^{1}H NMR spectrum showed, up field shifts at 0.60 and 0.98 ppm for methyls substituents in the steroid nucleus. In addition, other signals display chemical shifts at 1.40-1.60 and 1.80-2.07 ppm for methylens present in the steroid nucleus. In addition, at down field there is a signal at 6.8 ppm for the proton involved in central seven-membered azepine and two chemical shifts at 7.7 and 7.9 ppm corresponding to protons in the benzene rings. Finally, a signal at 9.02 ppm for functional amide groups involved in the spacer arm between the steroid nucleus and carbamazepine. Other results showed that bacterial growth of *Proteus mirabilis* was inhibited with cefotaxime (MIC = 5.23×10^{-4} mmol), gentamicin (MIC = 2.68×10^{-5} mmol), ciprofloxacin (3.01×10^{-3}) and pregnenolone-carbamazepine conjugate (MIC = 3.18×10^{-4} mmol). All this data indicate that pregnenolone-carbamazepine conjugate had different antibacterial potency in comparison with cefotaxime (β-lactam antibiotic), gentamycin (inhibitor of synthesis of protein) and ciprofloxacin (inhibitor of DNA gyrase). In order to develop new strategies to synthesize the pregnenolone-carbamazepine conjugate, that could be used as antibiotic-drugs on *Proteus mirabilis* bacteria.

Key Words: Pregnenolone-carbamazepine, Conjugate, *Proteus mirabilis*.

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INTRODUCTION

Pregnenolone is a potent neuromodulator that is formed by an oxidative side-chain cleavage from cholesterol and its biological activity depends on structural features of the steroid A and D-rings[1]. In this context, several investigators have prepared a number of compounds in order to verify the hypothesis that specific conformation of the functional groups in pegnenolone is required for high biological activity, as example, Jiang and co-workers[2] synthesized several analogs of 3α-hydroxy-5α-pregnan-20-one and evaluated their activity in electrophysiological experiments using rat R1,2Á2L GABAA receptors expressed in *Xenopus laevis* oocytes. Other studies have shown the synthesis of 6-oxa-analogs of pregnenolone and their interaction with GABAA receptor[3]. In this regard, it has been reported the synthesis of 19-[O-(carboxymethyl)oxime] of pregnanolone designed for the development of immunoassays of the corresponding parent neuroactive steroids[4]. Other studies also showed the synthesis of 20-amino and 20,21-aziridinyl pregnene steroids; developed as potent inhibitors of 17-hydroxylase/C17,20-lyase (P450 17)[5]. Recently, we synthesized several pregnenolone derivatives and evaluated their antibacterial effect[6,7]. In this work, we report a method that involves the modification of the carboxyl group of hemisuccinate of pregnenolone, in order to develop new strategies to synthesize the pregnenolone-carbamazepine conjugate with spacer groups, between pregnenolone and carbamazepine. This compound was synthesize with the purpose to evaluate their antibacterial activity on *Proteus mirabilis* using the microbial minimal inhibitory method[8].

EXPERIMENTAL

5-Pregnen-20-one,3-(3-carboxy-1-oxopropoxy (hemisuccinate of pregnenolone) was prepared according to a previously reported method by Figueroa *et al.*[6]. Carbamazepine (5*H*-dibenzo[b,f]azepine-5-carboxamide) and 6-aminohexanoic acid were purchased from Sigma-Aldrich Co., Ltd. The melting points for the different compounds were determined on an Electrothermal (900 model). Ultraviolet spectroscopy was carried out in dry methanol on a Perkin-Elmer model 552 spectrophotometer and infrared spectra 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 Dibenzo [b,f]azepine-5-carboxyl acid (6-amino-hexanoyl)amide

Method-A: The 5*H*-dibenzo[b,f]azepine-5-carboxamide (200 mg, 0.85 mmol) was added to a solution of 6-aminohexanoic acid (221 mg, 1.70 mmol) and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (243 mg, 1.70 mmol) in 1,4-dioxane:water (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 crystallization from methanol:hexane:water (3:2:1) to give 143 mg (46 %), m.p. 136 ºC; UV (MeOH) λ_{max} (log ε) 284 (1.35) and 2.16 (2.24) nm; IR, ν_{max} 3482, 3338, 1678, 1590 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H; 1.25 (2 H, m), 1.70 (2 H, m), 1.77 (2 H, m), 2.30 (2H, t, *J* = 6-9 Hz), 2.7 (2 H. t, *J* = 7.0 Hz), 5.50 (3 H, m), 6.71 (2 H, s), 7.27 (2 H, d, *J* = 8.0 Hz), 7.30 (2 H, m), 7.47 (2 H, m),7.67 (2 H, m). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C; 24.15, 25.71, 32.24, 38.16. 41.37, 119.11, 121.73. 122.01, 123.85, 124.50, 125.21, 129.05, 132.63, 136.04, 140.76, 155.01, 164.31. EIMS(70 ev) m/z (rel. int.), 349 (M^+, 18), 220 (25), 192 (100). Anal. $C_{21}H_{23}N_3O_2$: C, 72.08; H, 6.52;N, 12.00. Calcd. for C, 72.18; H, 6.63; N, 12.03.

Method-B: A solution of 5*H*-dibenzo[b,f]azepine-5-carboxamide 200 mg (0.846 mmol), 6-aminohexanoic acid 221 mg (1.70 mmol) and boric acid 105 mg (1.70 mmol) in 1,4-dioxane:water (2:1). After stirring at room temperature for 72 h, the solution was concentrated and the product was extracted with chloroform and water (2:1) to give carbamazepine-pregnenolone derivative (**2**) (131 mg, 43 % yield). Similar ^{1}H and ^{13}C NMR data were obtained compared with method A product.

Synthesis of N-{6-[(Dibenzo[b,f]azepine-5-carbonyl)-amino]-6-oxo-hexyl}-succinamic acid 17-acetyl-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopentaphenanthren-3-yl ester: The pregnenolone hemisuccinate (100 mg, 0.24 mmol) was added to a solution of dibenzo[b,f]azepine-5-carboxyl acid (6-amino-hexanoyl)amide (60 mg, 0.25 mmol) and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (96 mg, 0.50 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 55 mg (32 %), m.p. 116-118 ºC; UV (MeOH) λ_{max} (log ε) 284 (1.48) and 2.12 (2.4) nm; IR, ν_{max} 2933, 1730, 1682, 1590 cm^{-1}; ^{1}H NMR 300 MHz δ_H; 0.60 (1 H, s), 0.96 (1 H, s), 1.35 (1 H, d), 1.60-1.66 (1 H, dd), 1.69 (2 H, m), 1.71 (1 H, s), 1.76 (2 H, m), 1.80-2.08 (1 H, m) 2.10 (3 H, s), 2.20-2.30 (1 H, s), 2.33 (2 H, s), 2.38 (1 H, s) 2.50-254 (2 H, m), 2.92 (2 H, m, *J* = 6 Hz), 4.60 (1 H, s), 5.70 (1 H, s), 6.80 (1 H, s), 7.42-760 (1H, s), 9.02 (1 H, s). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C; 13.78, 19.58, 21.90 22.92 23.61. 24.16, 26.46, 27.20 27.78, 29.42, 30.74, 31.40, 31.78, 37.02, 37.82, 38.10, 38.20, 38.80, 39.30, 43.90, 50.02, 56.80, 63.60, 73.90, 119.10, 121.73, 122.62, 123.90, 124.60, 125.20, 129.00, 132.60, 136.02, 139.60, 140.80, 157.00, 164.30, 172.20, 173.30, 209.30. EIMS (70 eV) m/z (rel. int.), 747 M+, 5), 432 (25), 399 (16), 192 (100). Anal. $C_{46}H_{57}N_3O_6$: C, 73.08; H, 7.61; N, 5.58. Calcd. for C, 73.87; H, 7.68; N, 5.62.

Microbiological evaluation

Strains: The microorganism in this study belonged to the strain bank at the departamento de Fármaco-Química de la Facultad de Ciencias Químico-Biológicas de la Universidad Autonóma de Campeche. The strains are certified by Center for Disease Control in Atlanta and were as follows. *Proteus mirabilis* (ATCC 43071). The strain was kept under refrigeration at 4 ºC in special gel (BBL).

Antimicrobial agents: The pregnenolone-derivates were dissolved in methanol and diluted with distilled water. Cefotaxime, gentamycin, methicyllin and ciprofloxacin 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.*[8]. *Proteus mirabilis* was incubated on McKonkey agar for 24 h at 37 ºC, after such time, it could be determined whether growth had taken place or not.

On 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 ºC for 24 h. Subsequently, a loop was taken from each of them and inoculated into the appropriate cultures for different bacterial organisms and were incubated for 24 h at 37 ºC. After such time, the minimum inhibitory concentration (MIC) was evaluated to consider the antimicrobial effect of the pregnenolone-carbamazepine conjugate and controls.

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).

Determination of log P: To estimates the logarithmic octanol-water partition coefficient (log P) of organic compounds was used the molinspiration software[9].

RESULTS AND DISCUSSION

In this work, a straightforward route for the synthesis of pregnenolone-carbamazepine conjugate (**5**) is reported. The first step involves the coupling of 6-aminohexanoic acid (**2**) to 5*H*-dibenzo[b,f]azepine-5-carboxamide (**1**) resulting in amide bond formation contained in the chemical structure of **3** (Fig. 1). Many procedures for the formation of amide groups are known in the literature[10-12]. 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[13]. Despite its wide scope, the former protocol suffers from several drawbacks such as limited stability of many acid chlorides and the need for hazardous reagents for their preparation (thionyl chloride)[14]. In this work two different methods for amide formation were employed, in the first one the technique reported by Pingwah[15] for boric acid

catalyzed amidation of carboxylic acids and amines (method A) was used. In the second one, a derivate of carbodiimide was used (method B) as catalyzer[16] for amide bond formation in the fragment connected to central seven-membered azepine. It's important to mention here that the use of carbodiimide derivative results in higher yields compared to the amide bond formed with method A. ^{1}H NMR spectra of the carbamazepine-aminocaproic acid compound (**3**) showed to upfield shifts at 2.30 for -CH_2-C=O and at 2.70 ppm for proton of methylene bonded to amino group (CH_2-NH_2). The resonance of the proton for secondary amide was concluded to coincide with the amino group at 5.50 ppm. The ^{1}H NMR spectra of the secondary amides are usually more complex than the primary amides due to the presence of a substituent bonded to the amide nitrogen atom. These substituents produce a much wider range of chemical shifts for the amide proton which may, in addition, display coupling to aliphatic groups bonded to it. In addition, the chemical shifts of aliphatic groups bonded to the carbonyl group are similar to those observed for the primary amides, while those groups bonded to the nitrogen resonate at slightly lower field than the corresponding amines[17].

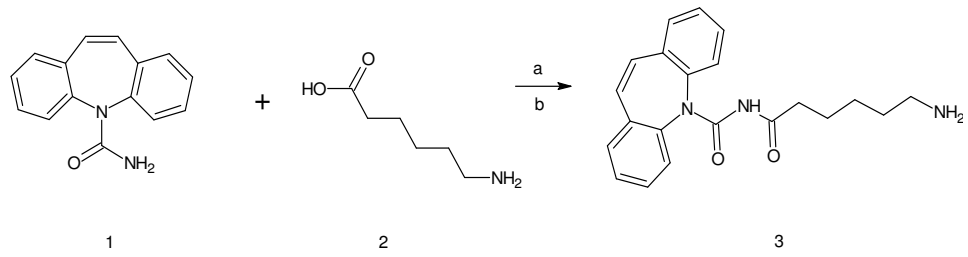

Fig. 1. Reaction of carbamazepine (**1**) with aminocaproic acid (**2**) to form carbamazepine-amino caproic acid compound (**3**)

Other signals showed at down field a signal at 6.71 ppm to protons involved in the central seven-membered azepine, in addition several signals between 7.27 and 7.67 ppm corresponding to protons in the benzene rings was found.

On the other hand, the ^{13}C NMR spectral assignments at 38.10 ppm for -CH_2-C=O and at 41.30 ppm to CH_2-NH_2 were found. The spectra at down field showed several signals (119.11, 121.73, 122.63, 125.20) corresponding to carbons of the benzene rings. Finally, the methylenes joined the seven-membered azepine were displayed at 123.80 and 140.76 ppm and another signals for the carbons at 155.01 ppm for -N-C=O- and at 164.31 ppm for the -NH-C=O fragment connected to azepine ring were found. The presence of the pregnenolone-derivate (**3**) was further confirmed from the mass spectrum which showed a molecular ion at m/z 349 corresponding to M^+ which confirm the structure of the pregnenolone-amino caproic acid compound.

The second step (Fig. 2) was achieved by reacting carbamazepine-amino caproic acid compound (**3**) with 3β-hydroxypregn-5-en-20-one hydrogen succinate (**4**) using carbodiimide-derivative to form N-{6-[(dibenzo[b,f]azepine-5-carbonyl)amino]-6-oxohexyl}succinamic acid 17-acetyl-10,13-dimethyl-2,3,4,7,8,9,10, 11,12,13,14, 15,16, 17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl ester (**5**). The ^{1}H NMR spectra showed upfield shifts at 0.60 and 0.98 ppm for methyls substituents in the steroid rings, in addition, other signals display chemical shifts at 1.40-1.60 and 1.8-2.07 ppm for methylens present in the steroid nucleus. Other result showed a signal at 2.11 ppm for the protons involved in the -CH_3-C=O fragment and another signal at 4.9 ppm for -CH-O- was found.

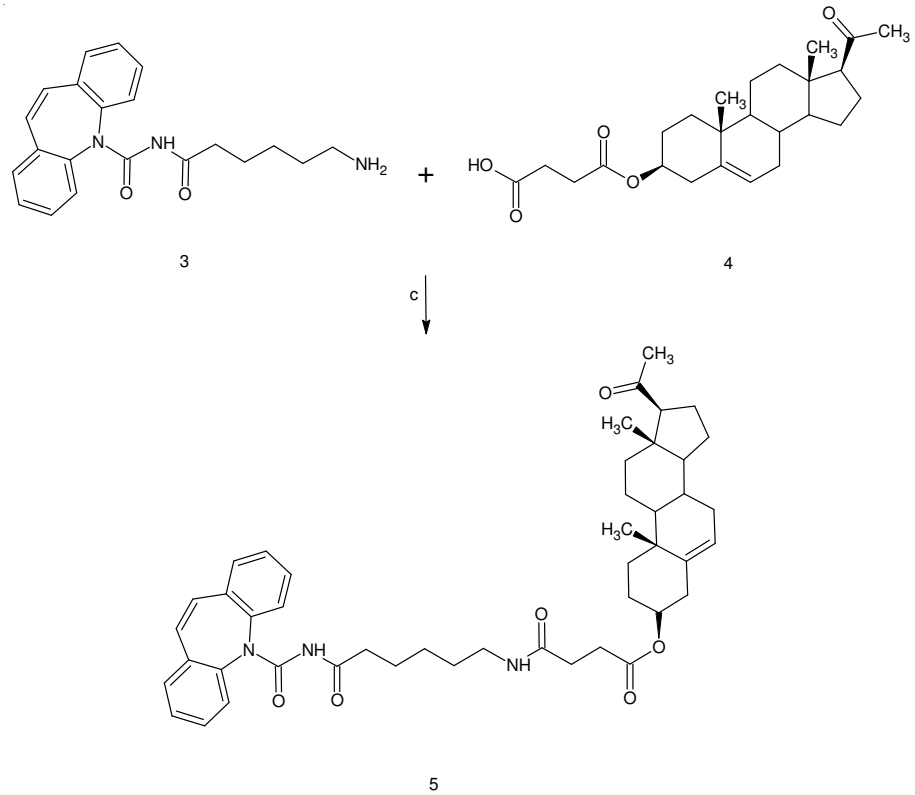

Fig. 2. Reaction of carbamazepine-amino caproic acid compound (**3**) with hemisuccinate-pregnenolone (**4**) to form pregnenolone-carbamazepine conjugate (**5**)

At down field there is a signal at 6.8 ppm for the proton involved in central seven-membered azepine and two chemical shifts at 7.7 and 7.9 ppm corresponding to protons in the benzene rings. Finally, a signal at 9.02 ppm for functional amide groups involved in the spacer arm between the steroid nucleus and carbamazepine was found whereas the signal of amino group is absent. The chemical shift of protons involved in amide group is known to vary strongly with the measurement of conditions

of change in molecular structure. This premise is supported with the works made by Jin and coworkers[18] who showed that the amide groups involved in the chemical structure of perfluoro-heterocyclic derivatives were displayed at 9.06 and 9.34 ppm. On the other hand, ^{13}C NMR spectra display two signals at 13.78 and 19.58 ppm for methyl substituents of steroid and signals at 21.9, 22.9 ppm for methylens of steroid nucleus were found. In addition, the spectra showed shifts at 23.61 ppm for $COCH_3$ (steroid) and 30.7 ppm for methylene involved in the $NHCOCH_2CH_2$ fragment. Several signals at 73.90 ppm for CHOCO and 119.1-122.6 ppm corresponding to protons in the benzene rings are displayed. Finally the spectra display shift at 157.0 ppm for NCONH, 164.3 for $NHCOCH_2$, 172.2 for CH_2CO_2, 173.3 for NH-CO-CH_2 and 209.3 ppm for -$COCH_3$ (steroid). Finally, the structure of **5** was further confirmed from the mass spectrum which showed a molecular ion at m/z 747 corresponding to M^+ which confirm the structure of the pregnenolone-carbamazepine conjugate.

On the other hand, the antibacterial activity of pregnenolone-carbamazepine conjugate (**5**) on *Proteus mirabilis* was evaluated by means of dilution method and the minimum inhibitory concentration (MIC), using gentamycin, ampicillin, cefotaxime and ciproflaxin as control in this study. The results obtained (Fig. 3) indicate that bacterial growth of *Proteus mirabilis* was inhibited with cefotaxime (MIC = 5.23×10^{-4} mmol), gentamycin (MIC = 2.68×10^{-5} mmol) and ciprofloxacin (3.01×10^{-3}). In presence of ampicillin, the bacterial growth of *Proteus mirabilis* was not blocked (data not shown). All this data indicate that pregnenolone-carbamazepine conjugate had different antibacterial potency in comparison with cefotaxime (β-lactam antibiotic)[19], gentamycin (inhibitor of synthesis of protein)[20] and ciprofloxacin (inhibitor of DNA gyrase)[21]. 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, possibly the antibacterial activity of pregnenolone-carbamazepine could be mainly by selective association of the steroid-antibiotic with some factor involved in the bacterial membrane. This hypothesis is suggested by Ding *et al.*[22,23]. These authors suggest that association relatives to the chemical structural characteristics of the steroid-antibiotic agents such as, facially amphiphilic conformations, which seems to be the key required for antibacterial activity. Nevertheless, thinking that possibly the liposolubility of all compounds studied could be a factor involved in their antibacterial activity, in this work the liposolubility was calculated (Fig. 4) using the descriptor log P[9]. Is important to mention that log P estimates the logarithmic octanol-water partition coefficient; therefore the log P represents the lipophilic effects of a molecule which includes the sum of the lipophilic contributions of the parent molecule and its substituent[24-26]. The results obtained in this work indicate that lipophilic effect of pregnenolone-carbamazepine conjugate was greater in comparison with the controls. This data indicate that possibility of a greater lipophilic effect could induce a substantial increase in the permeability of the outer membrane of the outer membrane of *Proteus mirabilis* bacteria include bactericidal/permeability increasing protein.

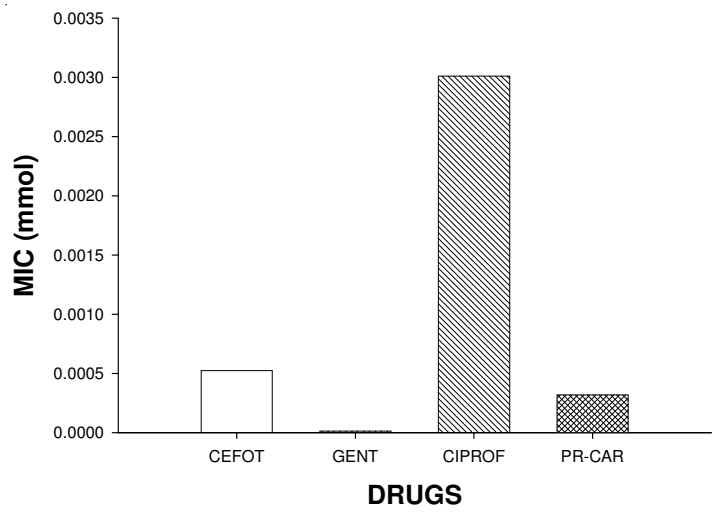

Fig. 3. Antibacterial activity of cefotaxima (CEFOT), gentamycin (GENT), ciproflaxin (CIPROF) and pregnenolone-carbamazepine conjugate (PR-CAR). The results showed that bacterial growth of *Proteus mirabilis* was inhibited with cefotaxime (MIC = 5.23×10^{-4} mmol), gentamycin (MIC = 2.68×10^{-5} mmol), ciprofloxacin (3.01×10^{-3}) and pregnenolone-carbamazepine conjugate (MIC = 3.18×10^{-4} mmol). MIC = minimum inhibitory concentration.

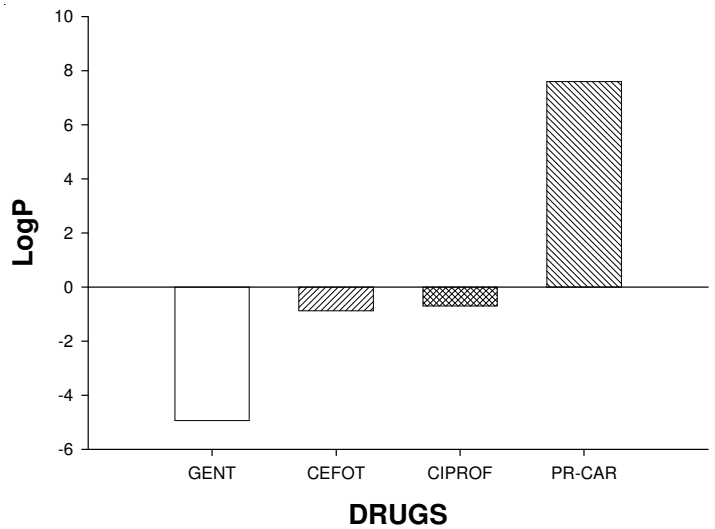

Fig. 4. Physico-chemical parameter (log P) of the compounds studied. The results calculated were for gentamicin (GENT) of -4.9350, cefotaxime (CEFOT) of -0.8710, ciprofloxacin (CIPROF) of -0.7010 and for pregnenolone-carbamazepine conjugate (PR-CAR) of 7.5990. This data indicate that pregnenolone-carbamazepine conjugate had greater lipophilic properties in comparison with the controls

Conclusion

An easy procedure has been reported for the preparation of pregnenolone-carbamazepine complex (**5**) using carbodiimide-derivative and boric acid as catalyzers. In order to develop new strategies to synthesize the pregnenolone-carbamazepine conjugate that could be used as antibiotic-drugs on *Proteus mirabilis*.

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