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[F0005]

Synthesis and characterization of a new *gemini* surfactant derived from 3α,7α,12α-trihydroxy-5β-cholan-24-amine (steroid residue) and ethylenediamintetraacetic acid (spacer)

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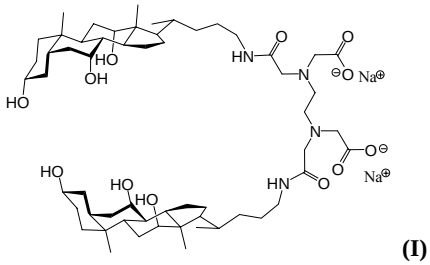
Abstract

A new *gemini* steroid surfactant derived from 3α,7α,12α-trihydroxy-5β-cholan-24-amine (steroid residue) and ethylenediamintetraacetic acid (spacer) was synthesized and characterized in aqueous solution by surface tension measurements and fluorescence intensity of pyrene. These techniques evidence the existence of a threshold concentration, *cac*, below which a three layers film is formed at the air-water interface. At high concentrations, the intensity ratio of the vibronic peaks of pyrene, I_1/I_3, (= 0.81) is very close to published values for sodium cholate micelles, indicating that the probe is located in a region with a very low polarity and far from water.

Introduction

During the past few years, an increasing number of papers have been published on the surface and micellar properties of *gemini* surfactants.[1,1,2] This is mainly due to their better efficiency in decreasing both the surface tension of water and the critical micelle concentration (*cmc*) in comparison to their corresponding monomeric analogs. Most of them contain two hydrophobic long alkyl chains and two hydrophilic groups which are linked through a flexible or rigid spacer.[3]

Although bile salts are very well known surfactants[4,5] and good solubilizers of hydrophobic compounds (including drugs[6] and cholesterol[7]), little attention has been paid to their potential use as amphiphile residues to design new *gemini* surfactants. Only a few examples of *gemini* surfactants formed by two bile acid residues have been published.[8-11] Here we have designed, synthesized and characterized a dicarboxylic *gemini* steroid surfactant derived from 3α,7α,12α-trihydroxy-5β-cholan-24-amine (*i. e.*, a 24-amino derivative of cholic acid), as surfactant residue, and ethylenediamintetraacetic acid, as spacer (Figure 1).



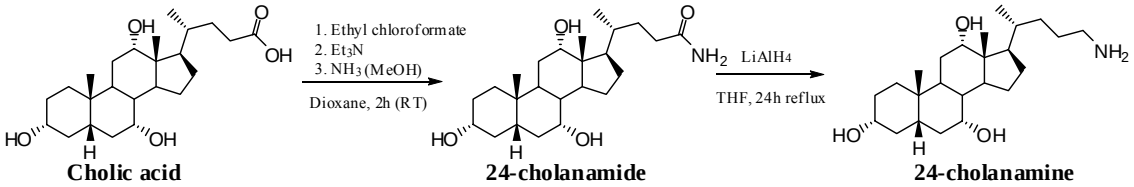
(I)

Figure 1.- Structure of the *g-2C$_{24}$-EDTA gemini*-compound (I)-, derived from 3α,7α,12α-trihydroxy-5β-cholan-24-amine and ethylenediaminetetraacetic acid.

Experimental section

Synthesis.

The synthesis of the cholic *gemini* was carried out by following schemes 1 and 2.



Scheme 1: Synthesis path of 24-cholanamine.[12]

The 24-cholanamide and 24-cholanamine were characterized by NMR (Figure 2and 3 respectively).

24-Cholanamide characterization: ^{13}C NMR (300 MHz, MeOD): C1 (CH_2) 36.53, C2 (CH_2) 31.22, C3 (CH) 72.92, C4 (CH_2) 40.50, C5 (CH) 43.23, C6 (CH_2) 35.92, C7 (CH_2) 69.09, C8 (CH) 41.05, C9 (CH) 27.92, C10 (C) 35.94, C11 (CH_2) 29.63, C12 (CH) 74.08, C13 (C) 47.53, C14 (CH) 43.04, C15 (CH_2) 24.27, C16 (CH_2) 28.71, C17 (CH) 48.05, C18 (CH_3) 13.03, C19 (CH_3) 23.21, C20 (CH) 36.98, C21 (CH_3) 17.73, C22 (CH_2) 33.41, C23 (CH_2) 33.26, C24 (C) 180.32 ppm. **^{1}H NMR (300 MHz, MeOD):** 0.71 (s, 3H, H18); 0.91 (s, 3H, H19); 0.8 to 2.4 (m, $H_{aliphatic}$); 3.34 (bs, 1H, H3); 3.79 (bs, 1H, H7); 3.95 (bs, 1H, H12) ppm.

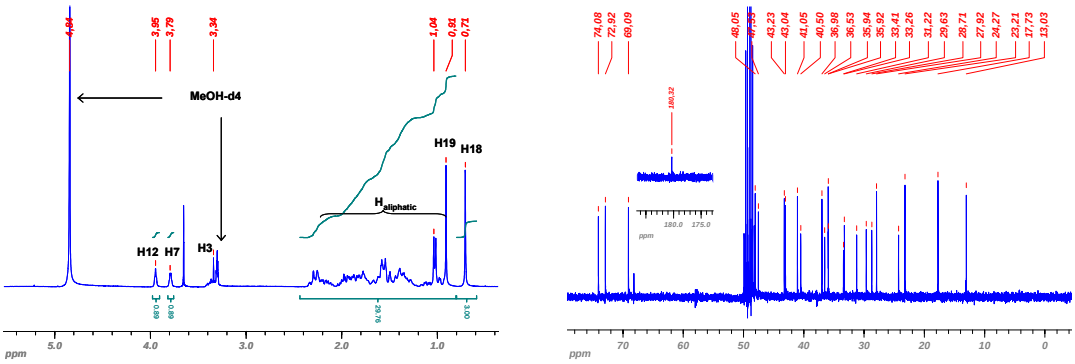


Figure 2.- ^{1}H and ^{13}C-NMR spectra of 24-cholanamide in MeOD.

24-Cholanamine characterization: **^{13}C NMR (300 MHz, MeOD):** C1 (CH_2) 40.47, C2 (CH_2) 31.19, C3 (CH) 72.88, C4 (CH_2) 40.47, C5 (CH) 43.19, C6 (CH_2) 35.91, C7 (CH_2) 69.09, C8 (CH) 41.00, C9 (CH) 27.90, C10 (C) 35.93, C11 (CH_2) 29.63, C12 (CH) 74.10, C13 (C) 47.45, C14 (CH) 43.04, C15 (CH_2) 24.28, C16 (CH_2) 28.70, C17 (CH) 48.13, C18 (CH_3) 13.00, C19 (CH_3) 23.21, C20 (CH) 37.09, C21 (CH_3) 17.98, C22 (CH_2) 34.13, C23 (CH_2) 27.83, C24 (CH_2) 42.21 ppm. **^{1}H NMR (300 MHz, MeOD):** 0.62 (s, 3H, H18); 0.82 (s, 3H, H19); 0.8 to 2.4 (m, $H_{aliphatic}$); 3.53 (bs, 1H, H3); 3.70 (bs, 1H, H7); 3.86 (bs, 1H, H12); 2.65 (m, 2H, H24) ppm.

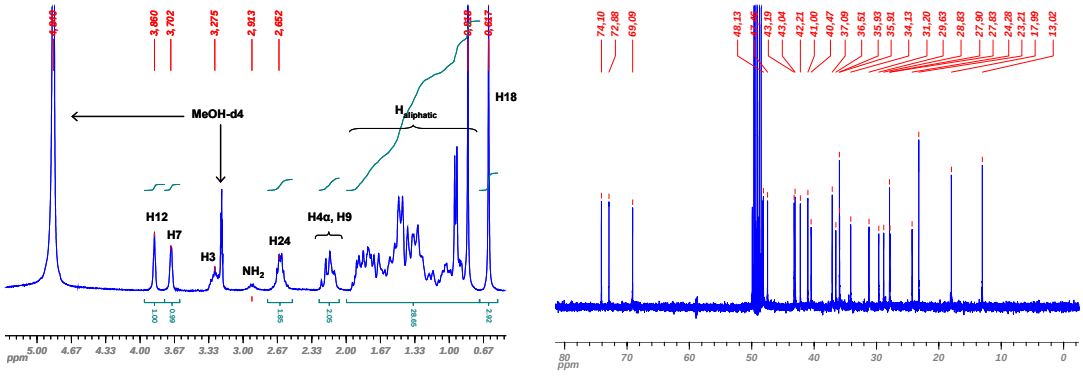
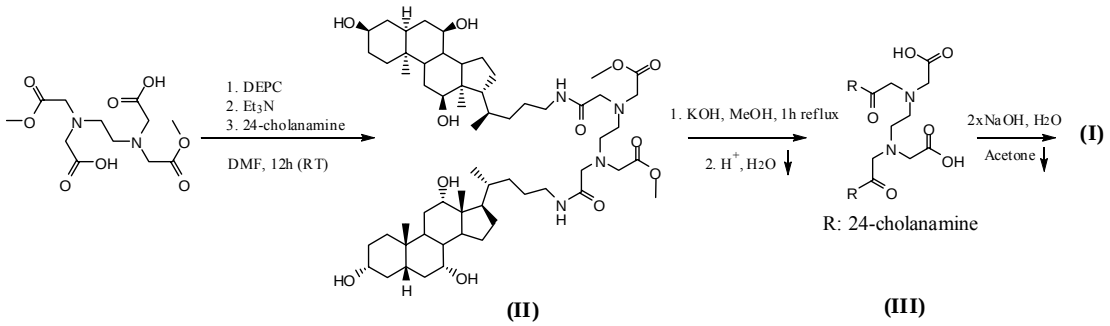


Figure 3.- ^{1}H and ^{13}C-NMR spectra of 24-cholanamine in MeOD.



Scheme 2: Synthesis path of *g-2C$_{24}$-EDTA*.

Dimethyl ester of EDTA[11] (0.60 g, 1.87 mmol) was dissolved in a mixture of 5 mL of dried DMF and 10 mL of dried THF. Diethyl cyanophosphate, DEPC, (0.65 mL, 4.28 mmol) was added to this solution. After 30 min, the solution was cooled to 0ºC and a solution of 3α,7α,12α-trihydroxy-5β-cholan-24-amine (1.55 g, 3.94 mmol) and triethylamine (0.6 mL, 4.30 mmol) in 20 mL of dried THF was added dropwise with stirring. After 90 min the ice bath was removed and the reaction was maintained for 6 h at r.t. The solvent was evaporated in vacuum. Then 200 mL of chloroform were added and washed twice with water (50 mL) to remove all DMF. The organic phase was dried (Na_2SO_4) and partially evaporated under reduced pressure. Finally the product was

purified by column chromatography (silica gel 70-230 mesh; eluent 7:3 ethyl acetate:methanol, R_f=0.41). Identity of the compound was confirmed by NMR and MALDI-TOF. Overall yield 56%.

To remove the methyl groups of the ester in the spacer, the compound was refluxed with KOH 1M in methanol for one hour at 80 ºC. The solvent was evaporated and the solid redissolved in water (200 mL) and acidified with HCl (pH≈ 1). When the solution is cooled, the compound precipitates in its diacid form. The precipitate was filtered and dried in a vaccum oven. The disodium salt was obtained by adding the stoichiometric amount of NaOH. Both the diacid and the disodium salts were repeatedly crystallized to guarantee the purity of the *gemini* compound.

***g-2C$_{24}$-EDTA* characterization: ^{13}C NMR (300 MHz, MeOD):** C1 (CH_2) 36.03, C2 (CH_2) 31.12, C3 (CH) 71.16, C4 (CH_2) 40.25, C5 (CH) 42.26, C6 (CH_2) 35.59, C7 (CH_2) 67.01, C8 (CH) 40.22 C9 (CH) 26.94, C10 (C) 35.10, C11 (CH_2) 29.27, C12 (CH) 71.79, C13 (C) 46.46, C14 (CH) 42.03, C15 (CH_2) 23.50, C16 (CH_2) 28.02, C17 (CH) 47.00, C18 (CH_3) 13.04, C19 (CH_3) 23.29, C20 (CH) 37.79, C21 (CH_3) 18.05, C22 (CH_2) 33.57, C23 (CH_2) 26.63, C24 (CH_2) 39.58, -N-$\underline{C}H_2$-CH_2-N- 53.14, -$\underline{C}H_2$-COOH 56.23, -$\underline{C}H_2$-CNH- 58.40, -COOH 170.80, -CNH 173.20 ppm. **^{1}H NMR (300 MHz, MeOD):** 0.59 (s, 3H, H18); 0.85 (s, 3H, H19); 0.8 to 2.4 (m, $H_{aliphatic}$); 2.70 (s, 4H, -N-C$\underline{H}_2$-CH_2-N-); 3.04 (m, 4H, H24); 3.19 (s, 6H, -C$\underline{H}_2$-COOH + H3); 3.35(s, 4H, -C$\underline{H}_2$-CNH-); 3.62 (bs, 1H, H7); 3.79 (bs, 1H, H12); 7.95 (m, 2H, Hamide) ppm.

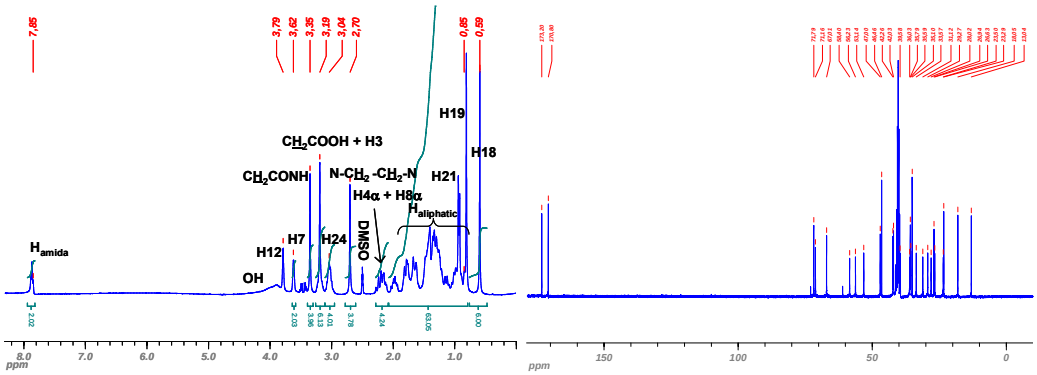


Figure 4.- ^{1}H and ^{13}C-NMR spectra of *g-2C$_{24}$-EDTA* (acid form) in DMSO.

Instrumental techniques. Surface tension measurements were carried out in a Kruss K10ST tensiometer by the Wilhelmy method. Fluorescence measurements were carried out in a Hitachi model F-3010 spectrofluorimeter at an excitation wavelength of

336 nm, and excitation and emission slit widths of 5 nm. Samples were thermostated at 25 ºC.

Results and Discussion

In Figure 5 surface tension data, γ, are plotted against log *C* for 24-cholanamine (*$C_{24}NH2$*) and *g-2C_{24}-EDTA*. The absence of a minimum in the surface tension versus concentration curves of both compounds (see Fig. 5) must be noticed. This indicates the absence of any strong surface-active trace impurity in the medium.[13,14] The surfactants were purified by repeated crystallization until no impurities could be detected by thin layer chromatography, by NMR-spectroscopy or FAB-MS.

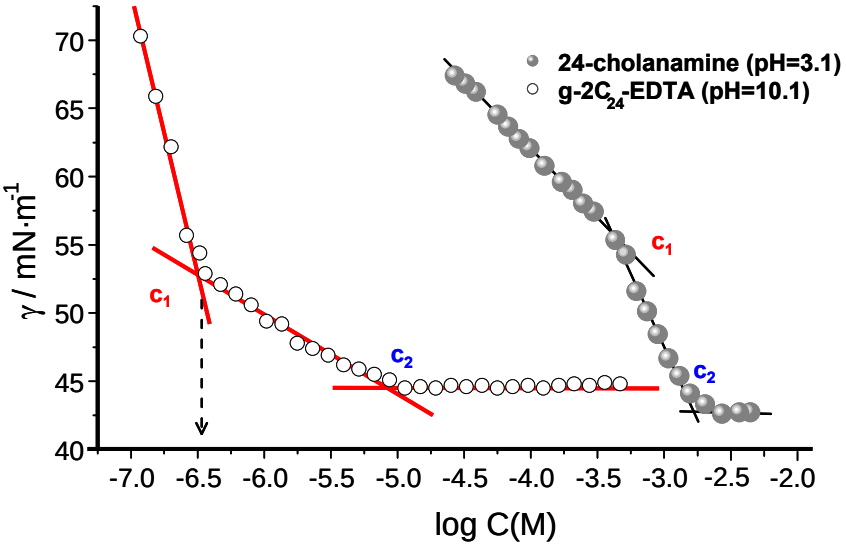


Figure 5.- Plots of surface tension *vs* log[bile salt] concentration for [●] 24cholanamine in HCl solution at pH=3.1 and [¤] *g-2C_{24}-EDTA* in 0.15M bicarbonate/carbonate buffer, pH=10.1. T = 25.0±0.5ºC

Prosser and Franses[15] have reviewed the application of the Gibbs adsorption isotherm to surface tension of ionic surfactants at the air–water interface. For a strong ionic surfactant of ν_+ free positive ions and ν_- free negative ions of charges z_+ and z_-, respectively, the surfactant surface density, $\bar{\Gamma}$, is given by

$$\bar{\Gamma} = -\frac{1}{RTm(c,c_s)}\left(\frac{d\gamma}{d\ln C}\right)_{c_s} \qquad [1]$$

where $m(c,c_s)$ is a function of ν_+, ν_-, the surfactant concentration, C, the concentration of added inert salt, C_s, and stoichiometry coefficient of the counterion of the surfactant in the supporting electrolyte, ν_+^s. T is absolute temperature and R=8.314 Jmol^{-1}K^{-1}. $m(c,c_s)$ is given by

$$m(c,c_s) = \nu_- + \frac{\nu_+^2}{\nu_+ + \nu_+^s \frac{C_s}{C}} \qquad [2]$$

So, $m(c,c_s)$ can be calculated at any particular experimental conditions. It is not a function of the coion valence of the supporting electrolyte ν_-^s. In the absence of inorganic electrolyte, $m = (\nu_- + \nu_+)$ and the surface excess density is inversely proportional to the total number of free ions in solution. Moreover, when the electrolyte concentration is high, the term involving ν_+ becomes negligible and the surface excess density is inversely proportional to only the number of surfactant ions ν_-. For highly surface active surfactants in dilute solutions, the surface excess density may be approximated by the adsorbed surface density, $\Gamma \approx \Gamma = 1/(A_s N_A)$, ($N_A$ is Avogadro's number).

For the *C_{24}NH2*, below (c_1=0.4 mM), A_S is ~102 Å^2/molecule, and from the straight line between c_1 and c_2, A_S is ~89 Å^2/molecule. Both values are very close to the theoretical surface value per molecule calculated from a spacefilling model (*Figure 6*). This suggests that the bile ions are lying flat at the water interface with a tighter packing of the molecules above c_1. In this case c_2 (1.8 mM) would correspond to the concentration above which aggregates are formed. This value is one order of magnitude lower than the one published by Fini *et al.*[12]

The analysis of the surface tension *vs* concentration for the *g-2C_{24}-EDTA* evidences some noticeable differences. In agreement with the literature on *gemini* surfactants,[16] c_1 (0.4 μM, in water) is three orders of magnitude lower than *cmc* values of the structurally closely related single tail surfactants as *C_{24}NH2* (see above) and cholate (10.4±4.5 mM, calculated from compiled values by Coello *et al*).[4] Below c_1, γ varies linearly with log *C* as for many classical and *gemini* surfactants, but the straight line above this threshold concentration has a lower slope. This is just the opposite of what was observed for *C_{24}NH2,* suggesting a different change of the packing or orientation of the *gemini* on the air/water interface in comparison to *C_{24}NH2*. In other words, between c_1 and c_2 each *g-2C_{24}-EDTA* molecule occupies more space that below c_1 (A_S being 28 Å^2/molecule and 159 Å^2/molecule, respectively). For these calculations a value of $m(c,c_s) = 1$ was used since $C_s >> C$. None of these experimental values is close to the theoretical values for different orientations of the surfactant (Figure 6) . The area occupied for the fully extended *g-2C_{24}-EDTA* molecule with the two steroid

residues lying flat on the surface is 230 Å^2. For an upright orientation of the *gemini* (ionic carboxylic groups oriented towards the water and steroid moities oriented towards the aerial phase) the area occupied by a molecule depends on the angle formed by the two branches of the *gemini*. For a maximum packing of the steroids (minimum angle), the projected area on the surface is 94 Å^2/molecule.

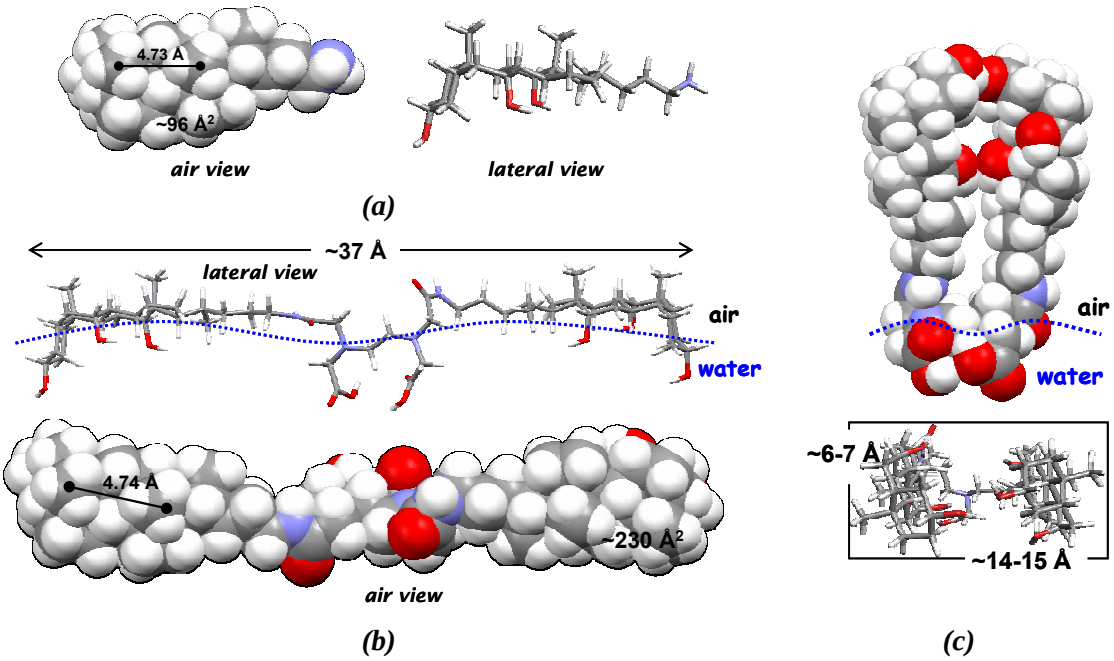


Figure 6.- Representation of the surface configuration of: **(a)** *$C_{24}NH2$* molecule lying flat. **(b)** *g-$2C_{24}$-EDTA* lying flat (maximum angle between cholate backbones). **(c)** *g-$2C_{24}$-EDTA* in upright orientation (ionic carboxylic groups oriented towards the water and steroid moities oriented towards the aerial phase). The area occupied by a molecule depends on the angle formed by the two branches of the *gemini*.

The first value is identical to the one published for the similar gemini *g-$2DC_{24}$-EDTA* in which the starting bile residue is deoxycholic acid,[11] and was interpreted as corresponding to a film structure at the air-water interface with three layers. The length of the steroid side chain plus the EDTA bridge (~11.7 Å), which is almost twice the length of the steroid nucleus, would allow the formation of the multilayer without preventing the interaction of the ionic groups of upper layers with water. Rosen *et al*[17] Tsubone *et al*[18] have also proposed the formation of multilayer structures to explain the aberrant behavior of some *gemini* surfactants. Fifty years ago Ekwall and Ekholm[19] suggested that lithocholic acid forms a single bulk phase made up of a trilayer of bile acid molecules.

Since above c_1 the slope diminishes, each molecule has more space at the interface since A_S increases. This behaviour has been associated with the existence and growth of premicellar aggregates,[20] and in fact premicellization seems to be a rather general effect in *gemini* surfactant solutions.[3,21,22] Therefore the increase of A_S suggests that the three layers film is broken and molecules from the film incorporate into aggregates which start to form in the bulk solution because of the increment of the surfactant concentration above c_1.

Figure 7 shows the pyrene I_1/I_3 ratio plots for *g-2C$_{24}$-EDTA* at 25ºC. It can be noticed that I_1/I_3 decreases gradually with increasing concentration of the *gemini* over a wide range of concentration, from log C=-5.7 (C=1.9 μM; blue line in the Figure) to log C=-3 (C=1 mM; red line in the Figure). These values are close to c_1 and c_2 determined from surface tension measurements. The gradual decrease in I_1/I_3 has been observed for other surfactants showing premicellar association.[20] It contrasts with sharp drops at a particular concentration observed for typical surfactants as SDS. Above of ~1 mM I_1/I_3 reaches a plateau equal to 0.81. This value is close to published values for pyrene included in sodium cholate micelles[23] and reflect a very apolar micro-environment for the fluorescent probe. Fitting the experimental data to a Boltzmann type equation[24] gives values of 1.3 μM and 1.2 mM for the two threshold concentrations.

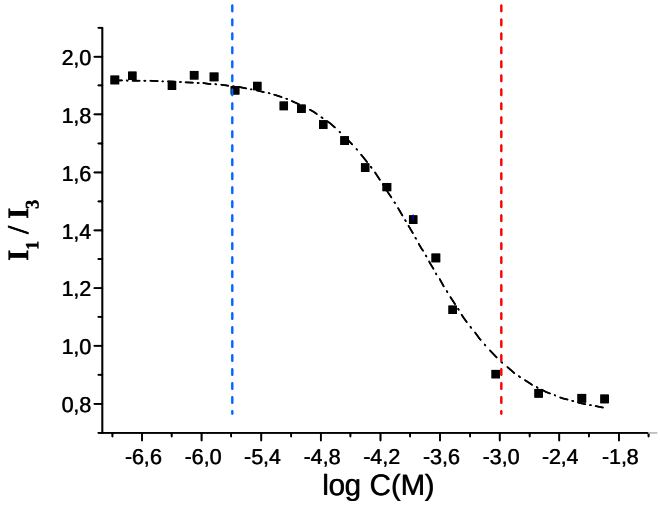


Figure 7.- Fluorescence intensity ratio I_1/I_3 of pyrene vs log [*g-2C$_{24}$-EDTA*]/M at 25±0.5 ºC in water at pH=9.3. [Pyrene]=1.2 μM.

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