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Influence of net charge on the aggregation and solubility behaviour of amphotericin B and its derivatives in aqueous media

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Abstract. The poor solubility of polyene antibiotics in aqueous media limits their application in the therapy of systemic fungal infections. In the present paper we have demonstrated that the ionic state (net electrical charge) of the antibiotic molecule is an important factor in determining the aggregation and solubility properties of amphotericin B and its derivatives. A multi-step model of polyene self-association in aqueous media has been proposed as an explanation for the fact that some major differences are observed when aggregation is monitored by different techniques.

Key words: Amphotericin B – aggregation – solubility – aqueous media – mathematical model

Introduction

Amphotericin B (AMB), a potent antifungal agent, is a heptaene antibiotic produced by *Streptomyces nodosus*. The AMB molecule has a large lactone ring, one side of which is a non-polar conjugated heptaene, while the other side is polar, owing to the presence of hydroxyl groups (Table 1). Thus, the molecule is amphiphilic. It is also amphoteric, owing to the presence of a carboxyl and an amino group, both of which are charged at neutral pH. These molecular characteristics make AMB poorly soluble in water and even less so in salt solutions. This poor water solubility is one of the important factors limiting its application in the therapy of systemic fungal infections.

In polar organic solvents (methanol, ethanol, DMF, DMSO) AMB exists as a mono-molecular dispersion: its optical absorption spectra are not concentration-dependent and between 420 and 320 nm it exhibits strong vibronic structure with four bands at 409, 385, 365, and 347 nm (in ethanol) characteristic of the heptaene chromophore. In aqueous media the optical absorption spectrum is concentration-dependent (Bolard et al. 1980). At very low concentration ($c < 10^{-7} M$) the spectrum is similar to that in polar organic solvents, with only small red shifts. As the concentration increases, the spectrum is progressively modified and above $c = 10^{-4} M$ a totally new spectrum is observed. The change in the spectrum as a function of concentration is consistent with the formation of small oligomers, most probably dimers (Ernst et al. 1981; Mazerski et al. 1982; Straus and Kral 1982).

However, differential ultrafiltration (Straus and Kral 1982) and light scattering experiments (Rinnert et al. 1977) indicate that large species of molecular weight up to 10^6 daltons exist at high AMB concentration in aqueous media ($c > 10^{-5} M$). The comparison of results obtained by circular dichroism and light scattering techniques in hydro-alcoholic media suggests (Rinnert et al. 1977) that the formation of large, insoluble aggregates detected by light scattering is not reflected in the spectroscopic properties.

The optical absorption and CD spectra, as well as solubility and aggregation properties, are influenced by chemical modification of ionizable groups of the antibiotic molecule and by pH changes (Dupont et al. 1977; Ernst et al. 1981; Straus and Kral 1982).

In the present paper we have examined the role of the ionic state of AMB and some of its semi-synthetic derivatives (Table 1) on their aggregation behaviour in aqueous media. A multi-step model of aggregation has been proposed to describe polyene antibiotic self-association. In this model we take into consideration the fact that polyene antibiotic solutions in aqueous media contain many different species with molecular weights in the range one thousand (monomer) to a few million (coloid type micelles). Molecules of polyene antibiotics in the monomeric state are amphipathic and in aqueous media have a strong tendency to undergo lipophilic interaction with other monomeric molecules. Thus, dimerization of monomers to hydrophilic dimers is a first step in aggrega-

Abbreviations: DMF, dimethylformamide; DMSO, dimethylsulphoxide; CD, circular dichroism; MMC, maximal monomer concentration; MSC, maximal solute concentration

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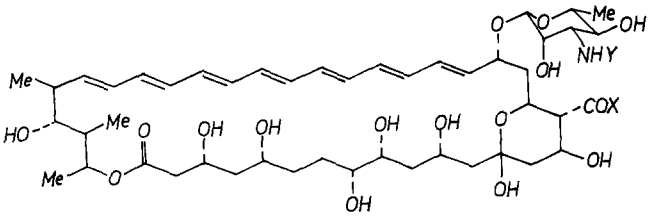
Table 1. Structure of amphotericin B and its derivatives



No.	Compound	Abbrev.	X	Y
1.	amphotericin B	AMB	OH	H
2.	amphotericin B methyl ester	AME	OCH_3	H
3.	N-acetylamphotericin B	Ac-AMB	OH	$COCH_3$
4.	N-acetylamphotericin B methyl ester	Ac-AME	OCH_3	$COCH_3$
5.	amphotericin B 3-(N′,N′-dimethylamino) propyl amide	AMA	$NH(CH_2)_3$-$N(CH_3)_2$	H

Table 2. The proposed model of polyene antibiotic self-association in aqueous media

$M+M \xrightarrow{K_d} D$		
$D+D \xrightarrow{K_a} A_1$		
$D+A_1 \xrightarrow{K_a} A_2$		soluble species
$D+A_2 \xrightarrow{K_a} A_3$		
$\vdots$		
$D+A_{n-1} \xrightarrow{K_a} A_n$		
$D+A_n \xrightarrow{K_a} A_{n+1}$		insoluble species
$\vdots$		
$D+A_{k-1} \xrightarrow{K_a} A_k$		
$\vdots$		

where: M = monomer, D = dimer, A_i = aggregate of i^{th} degree, K_d = dimerization constant, K_a = aggregation constant

tion. In subsequent steps, aggregates grow by addition of dimers. Growth by incorporation of monomers is less probable because in such a case the lipophilic part of the molecule would have to pass across the hydrophilic surface of the aggregate. Thus, the model may be described by the set of equilibrium equations presented in Table 2.

The presence of isosbestic points in the set of optical absorption and CD spectra of polyenes in aqueous media at different concentrations (Bolard et al. 1980; Ernst et al. 1981; Mazerski et al. 1982; Straus and Kral 1982) allows us to suppose that there are only two distinct spectra for all the species present. The variation in the spectrum with changes in antibiotic concentration suggests that the spectroscopic properties change drastically when dimers are formed. Further steps in association do not change the solution spectroscopic properties.

If the proposed model is valid the total antibiotic concentration, C, and the soluble antibiotic concentration, S, can be described as a function of monomer concentration $[M]$:

$$C = [M] + \frac{2 \cdot K_d \cdot [M]^2}{(1 - K_a \cdot K_d \cdot [M]^2)^2} \quad (1)$$

$$S = [M] + 2 \cdot K_d \cdot [M]^2 \cdot \frac{1 - (n+1) \cdot (K_a \cdot K_d \cdot [M]^2)^n + n \cdot (K_a \cdot K_d \cdot [M]^2)^{n+1}}{(1 - K_a \cdot K_d \cdot [M]^2)^2} \quad (2)$$

where n is the maximal degree of aggregation for which oligomers are medium soluble. For such aggregation one of the characteristics features is the existence of a Maximal Monomer Concentration, MMC, which is equal to:

$$\mathrm{MMC} = \lim_{C \to \infty} [M] = 1/(K_a \cdot K_d)^{1/2} \quad (3)$$

If only the first few oligomers formed are soluble in the medium a further characteristic feature of the proposed model is apparent: the presence of a Maximal Solute Concentration, MSC, which is equal to:

$$\mathrm{MSC} = \lim_{C \to \infty} \left\{ [M] + 2 \cdot [D] + \sum_{i=2}^{n} (2 \cdot i \cdot [A_i]) \right\} = \mathrm{MMC} + n \cdot (n+1)/K_a \quad (4)$$

In the present work we used this model for studying the changes in the spectroscopic properties of antibiotics tested, as well as the formation of high molecular weight insoluble aggregates.

Materials and methods

Antibiotics

Amphotericin B and Fungizon were obtained from Squibb & Sons, Inc. The AMB derivatives were synthesized in our laboratory by the methods previously described (Schaffner and Borowski 1961; Mechliński and Schaffner 1972; Jarzebski et al. 1982). Stock solutions of tested compounds ($c = 10^{-2} M$) were prepared daily, for Fungizon and AMA in water, for other antibiotics tested in DMF. Final solutions were obtained by adding the desired volume of the stock solution to 100 mM KCl solution adjusted to the desired pH using 50 mM buffers.

Buffers

Three types of buffers were used: (*1*) for pH between 2 and 5:50 mM acetic acid adjusted with HCl or KOH, (*2*) for pH between 5 and 8:50 mM Tris adjusted with HCl, (*3*) for pH between 8 and 10:50 mM triethanolamine adjusted with HCl.

Determination of monomer concentration

The significant differences between optical absorption spectra of monomeric and aggregated forms of AMB and

its derivatives made it possible to use this technique to determine the molecular ratio of monomers in solution. A wavelength of 408 nm was selected for the analysis because at this wavelength there is a large difference between the spectra of the monomeric and aggregated forms of the antibiotic. The molar ratio of monomer, x, was calculated according to the formula:

$$x = (\varepsilon - \varepsilon_a)/(\varepsilon_m - \varepsilon_a)$$

where: ε = observed molar absorption coefficient for tested solution, ε_m and ε_a = the molar absorption coefficients extrapolated to 0 (for monomer ε_m) or infinite (for aggregate ε_a) concentration.

Determination of solubility

In a methanol : water (1 : 1 v/v) mixture the absorption spectra of AMB and its derivatives are typical for monomeric form (Dupont et al. 1977) and are not concentration- and pH-dependent. The tested solutions were divided into three parts. One part was directly used to determine spectra in aqueous media and to calculate the monomer ratio. The second part was centrifuged at $2000 \cdot g$ for 30 min (for this time of centrifugation the spectroscopic properties of the supernatants do not change). 1 ml of the supernatant and 1 ml of the third part of the sample were separately mixed with 1 ml of methanol and the absorbance at 408 nm was determined. The % of soluble species in the sample was calculated according to the formula:

$$y = A/A_0 \cdot 100\%$$

where: A and A_0 = absorbance of centrifuged and non-centrifuged solutions, respectively.

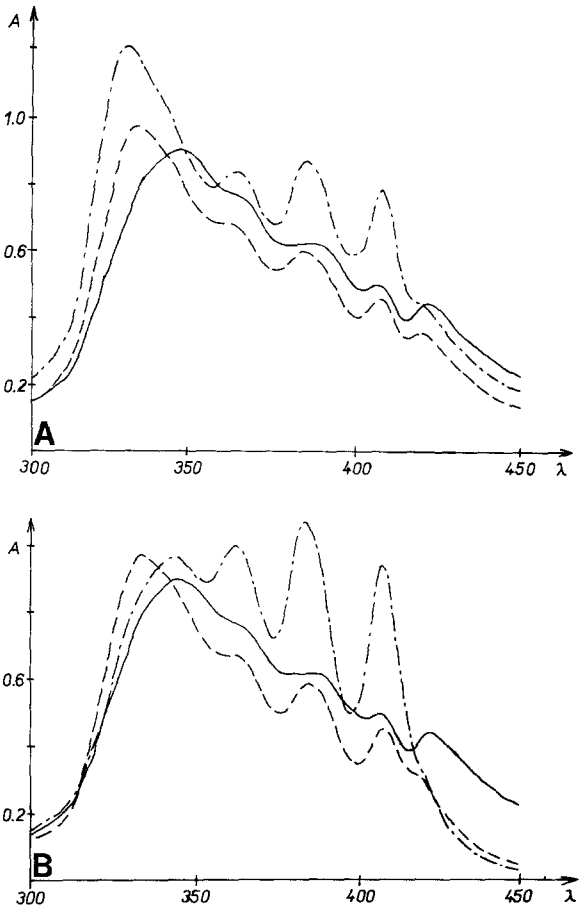


Fig. 1 A, B. The optical absorption spectra of aggregated forms of AMB and its derivatives in aqueous media. **A** the influence of pH on AMB aggregate spectra: —— pH 7, – – – – pH 2, – · – · – pH 10. **B** the influence of chemical modification of carboxyl and amino groups on aggregate spectra: —— AMB, – – – – AME, – · – · – Ac-AMB; pH 7.4

Determination of model constants

The experimental data for monomer concentration vs. total antibiotic concentration were used to determine the dimerization constant, K_d, and the association constant, K_a. These values and the experimental data on solubility from the same experiments were used to determine n, the maximal degree of aggregation for which oligomers are medium soluble. These model constants were calculated from least-squares fitting of experimental data to model Eqs. (1) and (2) by the simplex algorithm. The characteristic features of the model: MMC and MSC were calculated according to (3) and (4), respectively.

For each compound and pH tested the experiments were repeated at least three times.

Results

Influence of pH on the optical absorption spectra of antibiotic aggregates

The optical absorption spectrum of solutions of AMB and its tested derivatives in monomeric form, 1 : 1 v/v buffer : methanol mixture, are the same for all tested compounds and are not pH- and concentration-dependent. However, in aggregated form, in buffer at $C > 10^{-5} M$, these compounds exhibit characteristic differences in the positions and relative intensities of the bands. Figure 1 A shows spectra of the aggregated form of AMB at different pH. At neutral pH aggregates exhibit absorption bands at 420, 390, and 346 nm. In strong acidic buffer at pH = 2, these bands are shifted to 419, 384, and 334 nm, respectively. In basic buffer at pH = 10, these bands are observed at 418, 386, and 327 nm. Figure 1 B shows spectra of aggregated forms of AMB, AME, and Ac-AMB at neutral pH. Under these conditions the spectra of AMB and Ac-AMB are very similar, whilst the spectrum of AME is similar to that of AMB in acidic buffer. These results appear to suggest an influence of the ionic state of the antibiotic molecule in the aggregates on its optical absorption spectra. Figure 2 shows the position of the shortest wavelength band for AMB, AME, Ac-AMB, and Ac-AME for different pH in the medium. For AMB and Ac-AMB, i.e. compounds with a carboxyl group able to dissociate, major changes in the position of the studied band are observed between pH 3 and pH 4: the band is

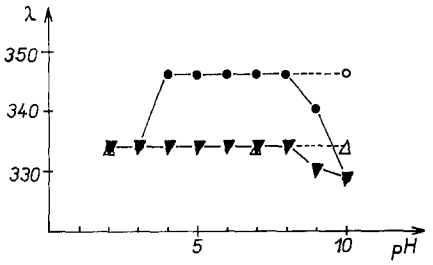


Fig. 2. The influence of pH on the position of the shortest wavelength band of aggregate spectra of AMB and its derivatives: ●–● AMB, ○–○ Ac-AMB, ▲–▲ AME, △–△ Ac-AME

shifted from 334 nm in strong acidic medium to 346 nm in weak acidic medium. For AME and Ac-AME, compounds without a free carboxyl group, no shift is observed in this pH range.

For AMB and AME, compounds with a protonable amino group, the studied band is shifted from 346 nm (AMB) or 334 nm (AME) in neutral or weak alkaline medium to 328 nm at pH ≥ 10. This is probably the result of the deprotonation of a charged nitrogen atom on an aminosugar. The plots of band position vs. pH confirm the supposition that the ionic state of aggregates determines their absorption spectra. The pK_a values of carboxylic and amino groups can be determined from these plots at about 3.5 and 9.5, respectively.

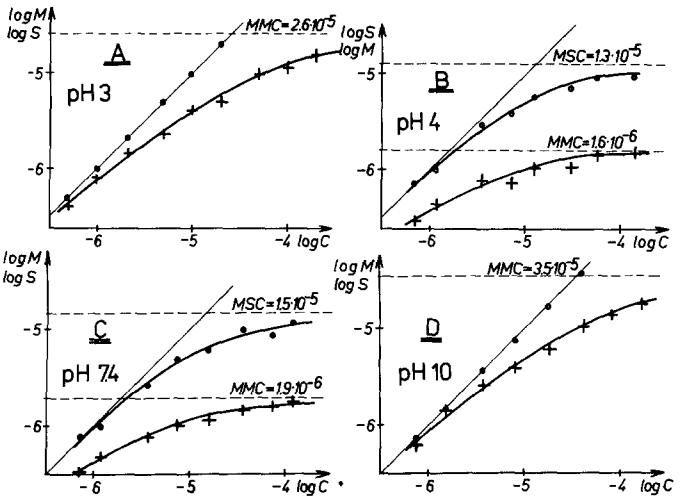


Fig. 3A–D. Examples of plots of monomer and soluble oligomer concentration vs. total AMB concentration at different pH

Influence of ionic state of AMB on its aggregation and solubility

Figure 3 shows examples of the variation of monomer concentration and the concentration of soluble species of AMB as a function of the total concentration at four different pH. In Table 3 the mean values of the model constants obtained and characteristic features are summarized.

At pH 7.4 antibiotic molecules are zwitter-ions. The MMC and MSC are equal to $1.5 \cdot 10^{-6} M$ and $9.1 \cdot 10^{-4} M$, respectively. The dimerization constant, K_d, determined from our model of self-association is equal to $1.6 \cdot 10^{6} M^{-1}$ and the association constant, K_a, is equal to $2.7 \cdot 10^{5} M^{-1}$. The n value equal to about 1 suggests that the highest aggregates soluble under these conditions are dimers. In a weak acidic medium, pH 4, at which antibiotic molecules still exist as zwitter-ions, the MMC and MSC as well as K_d, K_a, and n are practically the same.

In alkaline and strongly acidic media, pH 10 and pH 2 respectively, the antibiotic molecules have net charge. In this case the MMC significantly increases to $3.5 \cdot 10^{-5} M$. pH 10, and $2.6 \cdot 10^{-5} M$, pH 2. This means that the dimerization and association constants decrease to: $K_d = 1.1 \cdot 10^{5} M^{-1}$, $K_a = 7.6 \cdot 10^{3} M^{-1}$ at pH 10 and to $K_d = 2.1 \cdot 10^{5} M^{-1}$, $K_a = 6.9 \cdot 10^{3} M^{-1}$ at pH 2 owing to strong repulsive forces between molecules bearing net charges with the same sign. Under these conditions the MSC increases so strongly that up to a total concentration equal to $10^{-4} M$ all of the antibiotic is soluble. This suggests that net charged aggregates higher then dimers are also medium soluble.

Comparison of the aggregation state and solubility of Fungizon with AMA, a new potential antifungal drug

The method of examining the aggregation state of polyene antibiotics proposed in this paper has been used to analyse the behaviour of AMB molecules in aqueous medium when they are introduced as Fungizon: a commercial mixture of AMB, sodium deoxycholate and sodium phosphate. Fungizon is directly soluble in water even at a concentration of $10^{-2} M$. The absorption spectrum indicates that nearly all molecules are aggregated but after centrifugation no pellet is observed.

This solution has been used as a stock solution for the preparation of aqueous solutions in 100 mM KCl, pH 7.4, in the concentration range from $2 \cdot 10^{-7} M$ to $2 \cdot 10^{-4} M$. The change in absorbance at 408 nm suggests that the behaviour is very similar to that of AMB at the same pH (Table 3). MMC is equal to $1.7 \cdot 10^{-6} M$, $K_d = 2.2 \cdot 10^{6} M^{-1}$, and $K_a = 1.6 \cdot 10^{5} M$. However, the changes in the amount of antibiotic remaining in solution after centrifugtion are strange. Up to $C = 2 \cdot 10^{-5} M$ the samples show an increasing amount of insoluble aggregates. The MSC seems to be equal to $1.7 \cdot 10^{-6} M$. This value is similar to that obtained for pure AMB at the same pH. However, at higher concentrations the amount of insoluble aggregates decreases and for $C > 2 \cdot 10^{-4} M$ no insoluble aggregates are observed. Such untypical behaviour can be explained if one assumes that at AMB concentrations higher then $10^{-4} M$ solubilization of AMB aggregates by deoxycholate are observed.

The 3-(N′, N′-dimethylamino)propyl amide of amphotericin B, AMA (Jarzebski et al. 1982), prepared as an aspartate salt (Grzybowska et al. 1986) exhibits improved selective toxicity (Cheron et al. 1988) and pharmacological properties (Zaremba et al. 1988). Moreover, it is

Table 3. The mean values (±SD) of constants in the self-association model

Compound	pH	$K_d \cdot 10^{-6}$ $[M^{-1}]$	$K_a \cdot 10^{-5}$ $[M^{-1}]$	MMC $\cdot 10^6$ $[M]$	n	MSC $\cdot 10^5$ $[M]$
AMB	2	0.21 ±0.04	0.069±0.006	26 ±3	–	–
AMB	4	2.5 ±0.4	1.4 ±0.5	1.7±0.6	1.0 ±0.1	1.6 ±0.7
AMB	7.4	1.6 ±0.3	2.7 ±0.7	1.5±0.5	1.02±0.05	0.91±0.09
AMB	10	0.11 ±0.06	0.076±0.005	35 ±7	–	–
Fungizon	7.4	2.2 ±0.6	1.6 ±0.2	1.7±0.7	1.3 ±0.2	2.0[a] ±0.8
AMA	7.4	0.022±0.005	0.10 ±0.03	67 ±8		

[a] Theoretical value; in real solutions of Fungizon at concentrations higher than $10^{-4} M$ aggregations are solubilized by deoxycholate

directly soluble in water and saline. It has been very interesting to compare its aggregation ability with that of the parent antibiotic, AMB, as well as with Fungizon (Table 3). The change in absorbance at 408 nm indicates that for AMA at pH 7.4 the $K_d = 2.2 \cdot 10^4 M^{-1}$, the $K_a = 1.0 \cdot 10^4 M^{-1}$, and the MMC $= 6.7 \cdot 10^{-5} M$. This means that the dimerization ability of this compound is two orders of magnitude lower than the ability of AMB in the zwitter-ionic state or Fungizon and about ten times lower then ability of AMB bearing net charge, at pH 2 or pH 10. No insoluble aggregates of AMA are observed up to $C = 2 \cdot 10^{-4} M$ and for that reason the MSC is not measurable.

Discussion

The changes observed in optical absorption spectra in aqueous solution of aggregated forms of AMB and its derivatives at different pH show that the geometry of aggregates, with respect to the distance and angle between polyene chromophores, depends on the net charge of the molecules.

The similarity of the optical spectra of AMB (zwitter-ion) and Ac-AME (neutral) aggregates suggests that in polyene aggregates the electrostatic interactions between charges of neighbouring molecules are negligible. Thus, the forces responsible for the geometry of zwitter-ion and neutral aggregates are the same and are not electrostatic. We suppose that for such compounds the driving forces responsible for aggregation are hydrophobic interactions and formation of hydrogen bonds. The absence of aggregation in 4 M urea confirms the significant role of hydrogen bonds in self-association (Mazerski et al. 1982).

The molecules with net charge change the organization of the aggregate as a result of the repulsion forces between charges. Thus, the geometry of a charged aggregate is determined by an equilibrium between hydrophobicity and hydrogen bond formation (attractive forces) on the one hand and electrostatic repulsion on the other. A small difference observed between the positions of the shortest-wavelength band of aggregate spectra in strongly acidic and strongly alkaline conditions suggests also that the position of the charge in these rigid molecules with net charge plays some role in the equilibrium geometry of charged aggregates.

The observed relationship between the position of the shortest-wavelength band and the ionic state of the above discussed molecules makes it possible to detect this state in other conditions or for other amphotericin B derivatives. For example, Fungizon exhibits, in aqueous media of neutral pH, a short wavelength band at 329 nm, i.e. at a position typical for negatively charged AMB aggregates. The same position of this band is observed for the borate complex of AMB at pH 7 (Straus and Kral 1982). This may mean that in both cases the formation of the complex is associated with deprotonation of a nitrogen atom.

The changes of MMC and MSC values obtained for AMB at different pH as well as for Fungizon and AMA at neutral pH indicate that a net charge on the molecule increases solubility in two ways: first, it decreases the dimerization and association constants ten-fold or more, and secondly it increases the threshold of the degree of aggregation for which oligomers are water soluble. For these reasons we postulate that the presence of a net charge in the antibiotic molecule is the main factor which induces the solubility of polyenes. The majority of water soluble preparations of polyene antibiotics possess a net charge in the molecule; for example, salts of: esters, trimethylammonium derivatives of methyl esters (Falkowski et al. 1979), amides with diamines (Falkowski et al. 1980), and esters of N-aminoacyl derivatives (Wright et al. 1982). Even "neutral" complexes such as Fungizon or borate complexes are at least in part soluble owing to the presence of net charge, as discussed above. The formally zwitter-ionic derivatives such as some N-aminoacyl (Wright et al. 1982) as well as N-fructosyl (Falkowski et al. 1975) derivatives are more soluble in aqueous media only after the formation of salts. For AMB the formation of such salts is not possible between pH 4 and pH 9, probably due to the presence of strong intramolecular electrostatic interaction between charges of opposite signs. This interaction may be decreased by steric effects (N-fructosyl AMB) or by increasing the distance between charges (N-aminoacyl derivatives). This problem is not clear yet and will be a subject of our further studies.

The model of polyene self-association proposed in this paper explains well the observed saturation plots of monomer or soluble species concentration when total antibiotic concentration increases. The models explains also the discrepancies in the aggregation state obtained by different techniques.

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