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CD and NMR studies on the aggregation of amphotericin-B in solution

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We report in this paper the aggregation properties of amphotericin-B (amp-B) in solution using CD and ^{1}H-NMR techniques. Our results indicate that the preferred structure of amp-B in dimethylsulfoxide is a monomer at low concentrations (10^{-4} M and below) and a stable dimer at higher concentrations (range $5 \cdot 10^{-3}$ M to 10^{-2} M). In a DMSO/ethanol mixture (1:1 (v/v)), the antibiotic is monomeric, irrespective of the concentration within the range studied. We propose a head-to-tail model based on NMR data. An understanding of the head-to-tail dimer, is, we believe important, particularly in view of the recent report wherein it is proposed that the drug inserts into bilayers as head-to-tail oligomers.

Introduction

Amphotericin-B is a membrane-active polyene antibiotic used extensively to treat life-threatening systemic mycotic infections [1,2] and in antitumor treatments [3]. Recent reports have implicated this molecule in delaying scrapie (a neurodegenerative disease) symptoms in test animals [4].

The molecule is a rectangular ring containing a transconjugated heptene chromophore juxtaposed by an acyl chain having many hydroxyls. The head end of the molecule has a ketal ring, a carboxyl group on the ketal ring at carbon 16 and an amino sugar attached to carbon 19. The tail-end of the molecule has a hydroxyl group at carbon 35. The poor solubility and amphiphilic properties of the drug molecule are attributed to its chemical nature. The chemical structure of amp-B is depicted in Fig. 1.

The antifungal effect of the antibiotic is believed to result mainly from its ability to interact with cell membranes containing sterols, especially fungal membranes with ergosterol [5]. The consequence of such an interaction is increased cell-membrane permeability that leads to cell death [6]. The generally-accepted model [7,8] that has attempted to explain the antifungal action of the antibiotic is that polyenes penetrate into the lipid matrix of the membrane where they interact with sterols, forming complexes. These polyene-sterol complexes organize themselves into pores or channels spanning the membrane. An alternative model considers a membrane defect mechanism [9] that changes the ionic permeability. That the drug has to aggregate within the membrane in order to influence the cell membrane permeability is certain from reports published so far.

An understanding of the structure and conformation of the antibiotic and its variability under different conditions, such as solvent polarity, presence of metal ions and presence of different types of sterols are important before proposing a molecular model for its action. Numerous reports directed towards understanding the structure and interaction of this molecule

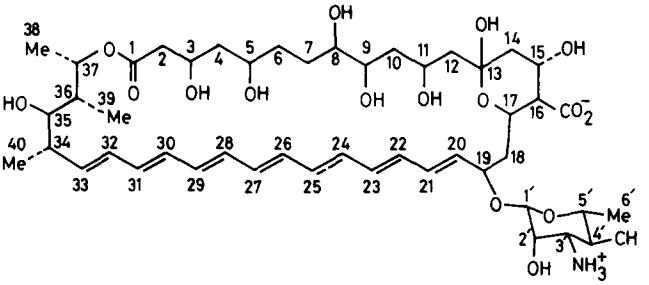

Fig. 1. Chemical structure of amphotericin-B.

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Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; amp-B, amphotericin B; DMSO, dimethylsulfoxide; DQF-COSY, double quantum filtered correlation spectroscopy; NOESY, 2D nuclear Overhauser enhancement spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; ROE, rotating frame Overhauser effect.

[10–14] are available in the literature. Aggregation of the antibiotic due to the presence of surfactants and cyclodextrin has been studied [15,16]. The single crystal X-ray structure reported is of *N*-iodoacetyl derivative of amp-B [17]. As part of a program in determining amp-B structure in solution under varied conditions and where possible using single crystal X-ray structural analysis, we report in this paper the aggregation properties of amp-B in dimethylsulfoxide (DMSO) using circular dichroism (CD) and proton nuclear magnetic resonance (^{1}H-NMR) techniques. Our results show that the preferred structure of amp-B in DMSO is a monomer at low concentrations of the drug and a stable dimer at higher concentrations (greater than $5 \cdot 10^{-3}$ M). Using NOE and ROE connectivities we propose a head-to-tail dimer structure for the antibiotic. Understanding of oligomeric structures of the antibiotic in solution is essential, as it reflects the first-level aggregated structure of the antibiotic before insertion into the lipid matrix. Our results support recent studies where head-to-tail dimer insertion and assembly in bilayers has been hypothesized [18].

Materials and Methods

Amp-B was obtained from Sigma (St. Louis, MO, USA) and was used without further purification. All solvents used for CD measurements were of spectroscopy grade. Ethanol was doubly-distilled just before each experiment. Deuterated solvents used for NMR work were from Sigma.

CD experiments were done on a Jasco J500A spectropolarimeter calibrated with camphorsulfonic acid-d_{10}. ^{1}H-NMR experiments were done on an AMX 400 MHz Bruker NMR spectrometer. Double quantum filtered correlation spectroscopy (DQFCOSY) experiment was used for assignment of various resonances. 2D ^{1}H-nuclear Overhauser effect and exchange spectroscopy (NOESY) experiments were done at different mixing times (400, 200 and 100 ms). Rotating frame Overhauser effect spectroscopy (ROESY) experiment was performed at 100 ms spin lock time. The natural line width in DMSO-d_6 of 7–8 Hz was reduced to 1.5 Hz by processing the spectra with sufficient resolution enhancement [10].

Stock solutions of amp-B were prepared in DMSO and used within two days. For studies in different solvents, the required aliquot of the stock was dispersed keeping DMSO amount to a minimum.

Results

Circular dichroism studies

Fig. 2 shows the CD spectra of amp-B in different solvents. As is evident in the figure, the spectra is highly-dependent on solvent polarity. Circular dichroism studies of amp-B in water and nonpolar solvents like chloroform and acetonitrile (10^{-5}–10^{-4} M) gave a strong couplet centered around 342 nm with an intense positive band at 328 nm and large ellipticity. The dichroic couplet centered at about 342 nm has been attributed to an aggregated structure of amp-B. The positive CD band that appears at around 328 nm is an indication of the levels of aggregation of the antibiotic. Light-scattering measurements in solutions of amp-B (concn. between 10^{-5}–10^{-4} M) indicated an aggregated system of about 2000 molecules of amp-B in the aqueous phase [19]. CD spectra of amp-B in chloroform and acetonitrile at approx. 10^{-4} M concentration is similar in characteristics to that of amp-B in water, except for a decrease in amplitude. Reduction in amplitude is because of the formation of aggregates with lesser number of antibiotics in these solvents [20]. The bisignate shape of the CD spectra is an indication of a multimolecular structure in which heptene chromophores are in excitonic interactions [21].

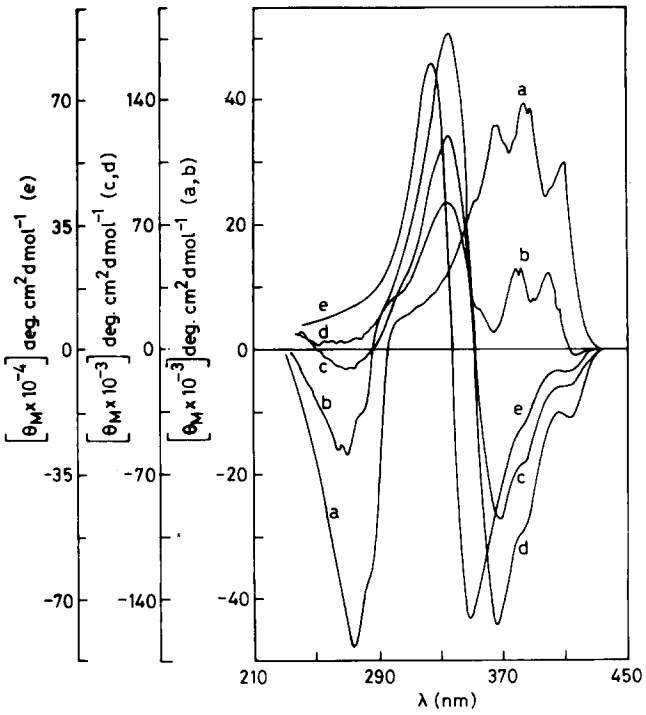

Fig. 2. CD spectra of amp-B in different solvents. (a) DMSO; (b) ethanol; (c) chloroform; (d) acetonitrile; (e) water. Concentration of amp-B = 10^{-4} M.

The CD spectra of amp-B in DMSO and 75% ethanol are different from that observed in water or nonpolar solvents. Spectrum of amp-B in DMSO at 10^{-4} M concentration (Fig. 2a) showed positive bands at 412 nm, 385 nm, 361 nm with a shoulder around 352 nm. These bands are characteristic of conjugated systems. The vibrational fine structure observed in this wavelength region show that the molecule exists as a monomer at concentrations of approx. 10^{-4} M and below. The negative CD band around 272 nm corre-

sponds to the acid carbonyl on the ketal ring at position 16 and to the keto-carbonyl at position 1. The CD spectra in DMSO were found to be concentration-dependent, as shown in Fig. 3. As the concentration of amp-B is increased from 10^{-4} M to 10^{-3} M (Fig. 3a–c), there is a continuous increase in ellipticity of the bands at 412, 385 and 361 nm (corresponding to the conjugated double-bond stretch) and an increase in the ellipticity of the 272 nm band. However, on further increase in the amp-B concentration $> 5 \cdot 10^{-3}$ M, the bands corresponding to the conjugated double-bond significantly decreased in magnitude, giving a near zero ellipticity with a simultaneous large increase in negative ellipticity of the 272 nm band. A positive CD band at around 328 nm appears at these concentrations. The dichroic couplet as observed in chloroform and acetonitrile centered around 342 nm does not appear. The appearance of the band at 328 nm coupled with the

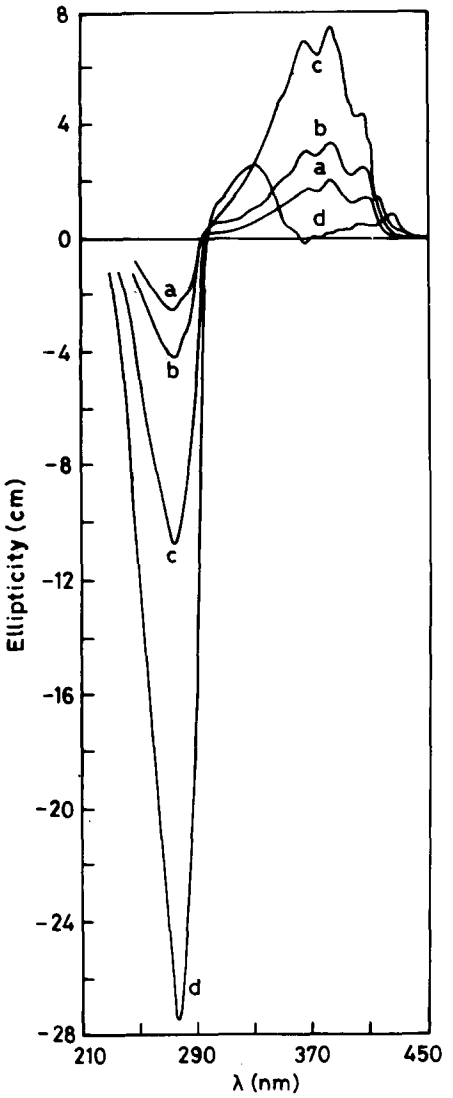

Fig. 3. CD spectra of amp-B in DMSO at different concentrations. (a) 10^{-4}M; (b) $5 \cdot 10^{-4}$M; (c) 10^{-3}M; (d) $5 \cdot 10^{-3}$ M. Cell length 0.02 cm; Sensitivity 2 m°/cm (Observed ellipticity values are given).

enormous increase in ellipticity of the 272 nm band suggests formation of dimeric structures. CD measurements done at $5 \cdot 10^{-3}$ M concentration at different temperatures showed a decrease in the band at 328 nm as the temperature is increased. This is an indication of the decrease in the oligomeric species with increase in temperature.

CD spectra of amp-B in ethanol vary depending on the water content in ethanol [19]. The spectra in Fig. 2b correspond to approx. 75% ethanol. The band at approx. 328 nm is clearly seen, the intensity of which decreases with decrease in water content. Comparing approx. 75% ethanol spectra at concentration 10^{-4} M and the DMSO spectra at concentration $5 \cdot 10^{-3}$ M of the drug, it is apparent that the origin of the induced band at approx. 328 nm is due to the presence of oligomeric associations of the molecule. That we observe increased ellipticity of the 272 nm band in the latter case points towards the presence of dimeric structures of the antibiotic in DMSO at high concentrations. This band begins to decrease in intensity as higher oligomers are formed (like in 75% ethanol) and completely disappears when aggregation is extensive. Fig. 4a shows the spectrum of amp-B (10^{-4} M) in approx. 75% ethanol titrated with DMSO. Fig. 4b shows spectra of amp-B in DMSO ($5 \cdot 10^{-3}$ M) titrated with ethanol. The CD spectra of amp-B in a mixture of DMSO/ethanol show no band at approx. 328 nm irrespective of the concentration of the drug, indicating the presence of a monomeric species in this solvent mixture.

NMR results

Fig. 5 is the 400 MHz ^{1}H-NMR spectrum of amp-B in DMSO-d_6 at 10^{-2} M concentration. The assignments made using a DQFCOSY (Fig. 6) were the same as reported earlier [10] and recently by Sowinski et al. [11] The observed coupling constants between the various protons are given in Table Ia. Near zero coupling constants are observed between protons 1′ and 2′, 15 and 16 and 19 with 18a and 18b. NOESY was done at various mixing times (400, 200 and 100 ms) to completely rule out spin diffusion [22]. ROESY was done at 100 ms spin lock time in order to confirm all observed NOEs. Fig. 7 is a NOESY spectrum of amp-B (10^{-2} M) in DMSO-d_6 at 100 ms mixing time. Analysis of the spectrum showed unusual NOE cross-peaks which are listed in Table Ib. Proton 21 showed NOE cross peaks with protons 33 (weak), 34, 37 and 40. 6′-Methyl group protons showed a NOE cross peak with proton 35. Fig. 8 is a NOESY spectrum of amp-B at the same concentration in a 1:1 DMSO/ethanol mixture (v/v) (100 ms mixing time). The spectrum showed solvent induced shifts and no unusual NOEs as observed under the previous solvent conditions. The resonances corresponding to 18b and 2b protons have

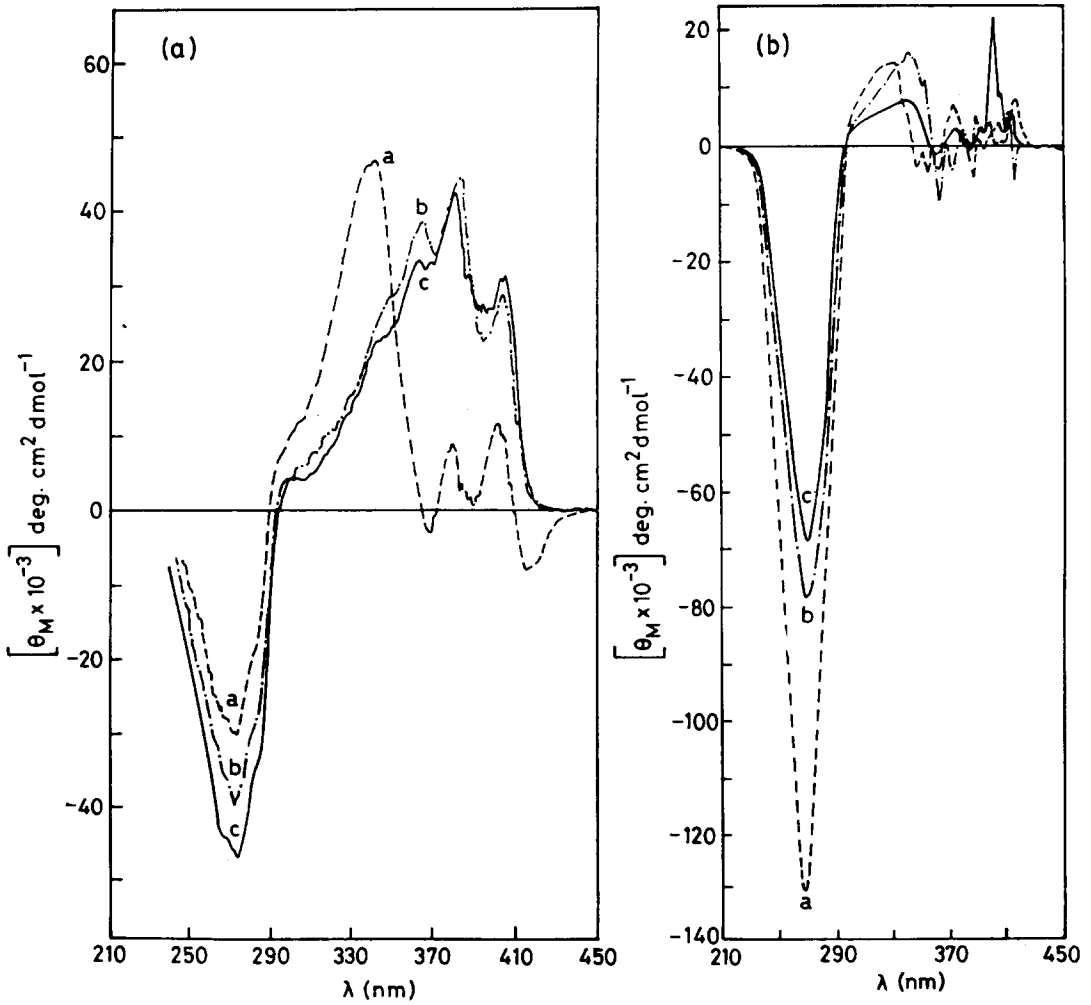

Fig. 4. (a) CD spectra of amp-B (a) in 75% ethanol; (b) in ethanol/DMSO 4:1 ratio; (c) ethanol/DMSO 3:2 ratio. Concentration of amp-B, 10^{-4} M. (b) CD spectra of amp-B (a) in DMSO; (b) DMSO/ethanol 1:0.5 ratio; (c) DMSO/ethanol 1:1 ratio. Concentration of amp-B, $5 \cdot 10^{-3}$ M.

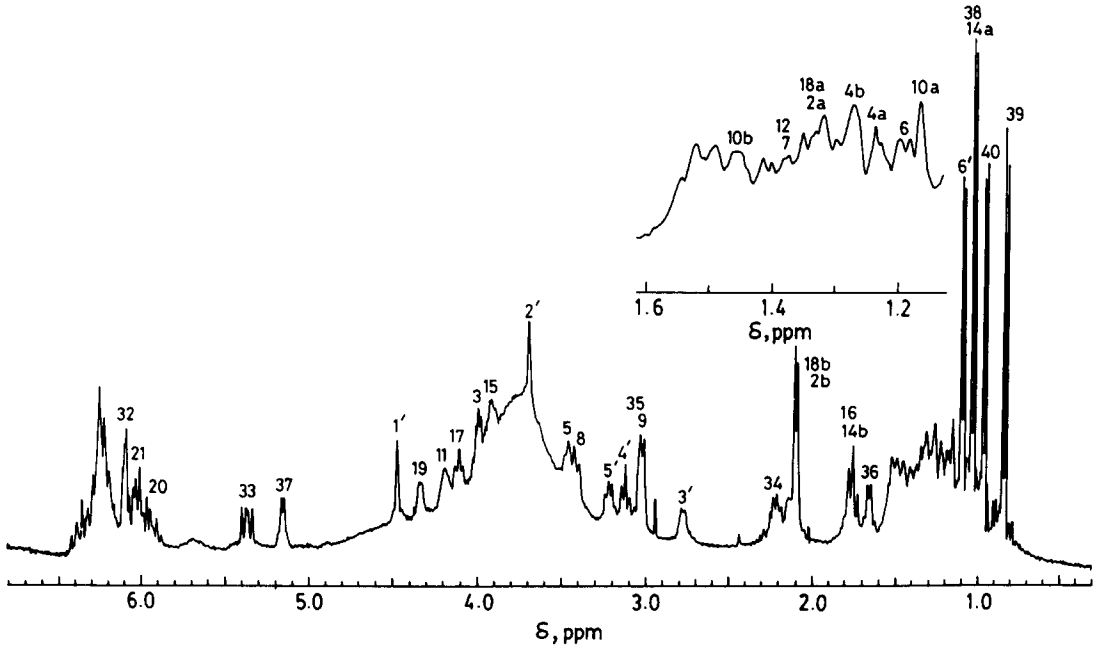

Fig. 5. 400 MHz ^{1}H-NMR spectrum of amp-B in DMSO-d_6. Concentration of amp-B 10^{-2} M.

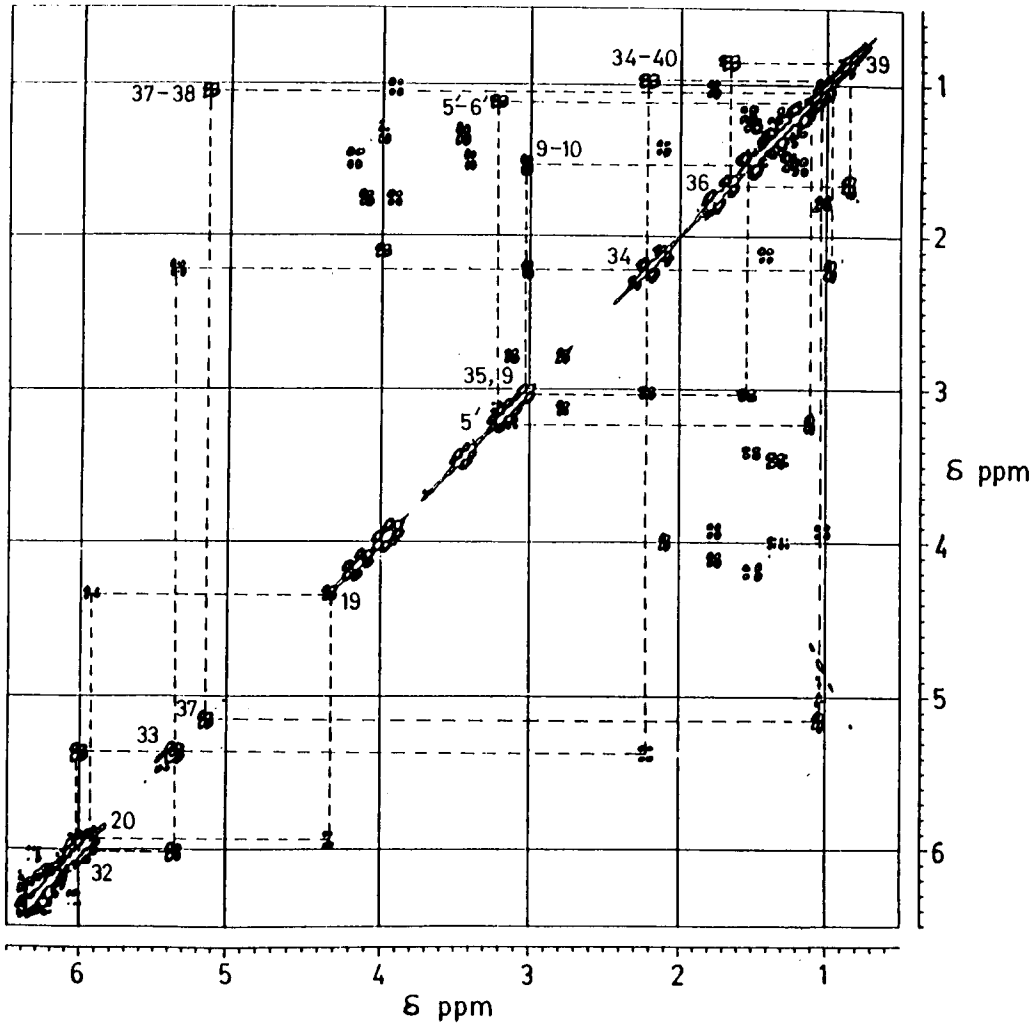

Fig. 6. 400 MHz DQFCOSY spectrum of amp-B in DMSO-d_6. Concentration of amp-B 10^{-2} M.

separated out. The shift in proton 18b resonance has brought it closer to proton 34. Protons 20, 21 overlap. Proton 20, therefore, shows a NOE cross peak with protons 18b (seen partially overlapping proton 34 cross-peak) and 18a.

TABLE I

(a) Observed proton coupling constants (± 0.5 Hz) for amphotericin B in DMSO-d_6

See Fig. 1 for the numbering scheme.

$J_{2,3}$ = 5.9 Hz; $J_{3,4}$ = 6.11 Hz; $J_{14,15}$ = 4.7 Hz; $J_{16,17}$ = 10.4 Hz; $J_{17,18}$ = 10.1 Hz; $J_{19,20}$ = 8.2 Hz; $J_{20,21}$ = 9.7 Hz; $J_{32,33}$ = 9.6 Hz; $J_{33,34}$ = 9.7 Hz; $J_{34,40}$ = 6.0 Hz; $J_{36,39}$ = 6.92 Hz; $J_{37,38}$ = 6.1 Hz; $J_{2',3'}$ = 2.75 Hz; $J_{3',4'}$ = 9.0 Hz; $J_{4',5'}$ = 8.72 Hz; $J_{5',6'}$ = 5.7 Hz.

(b) Observed unusual NOE / ROE:

Proton	NOE/ROE
21	33, 34, 37, 40
6' methyl	35

Discussion

The CD data clearly showed that amp-B in DMSO is a monomer at low concentrations and at concentrations of $5 \cdot 10^{-3}$ M and above it forms oligomeric aggregates (possibly dimers). The data also showed that in a solvent mixture of 1:1 (v/v) DMSO/ethanol the molecule exists as a monomer, irrespective of the concentration within the range studied (10^{-4}–10^{-2} M).

The possible conformation of the molecule has been worked out from the ^{1}H-NMR chemical shift and coupling-constant data (Table 1a). Reasons for near zero coupling observed between 1' and 2' protons of the sugar moiety (β-pyranose), in which the 1' proton on C-1 is axial and the 2' proton on C-2 is in an equatorial position, is because 2' proton is in an anti-periplanar relationship with the ring oxygen [23,24]. The low coupling between proton 19 with proton 18a and 18b is due to the glycosidic bond at the C-19 position. Low vicinal coupling $J_{15,16}$ is also because these protons are on the ketal ring and the ring oxygen

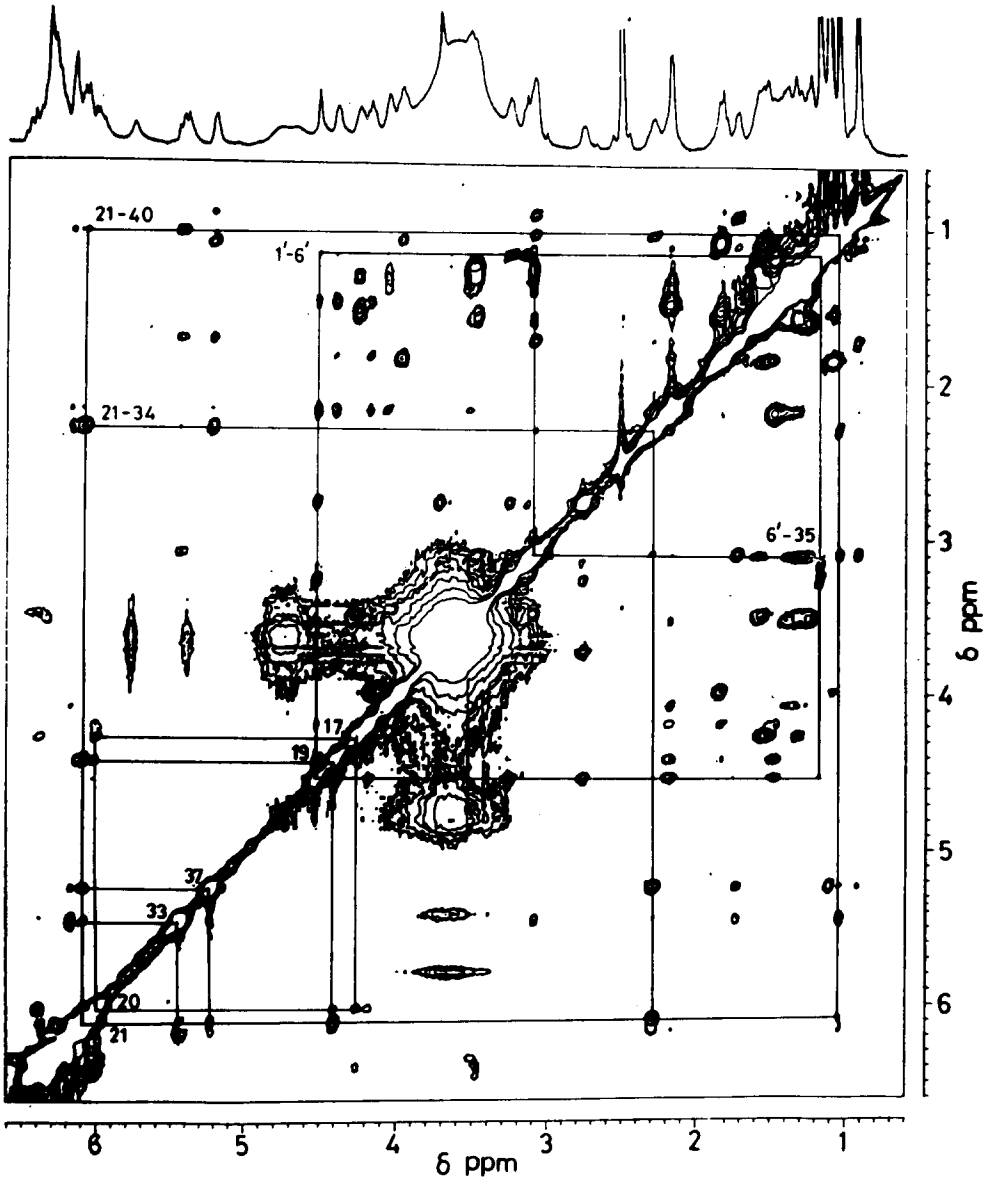

Fig. 7. 400 MHz NOESY of amp-B in DMSO-d_6 (100 ms mixing time). Concentration of amp-B 10^{-2} M.

relationship reduces the coupling constant. The observation of only one set of resonances corresponding to the protons in the molecule shows that the molecule exists as a single conformer in the NMR time-scale. The conformational model of the molecule based on NMR data has been constructed and the structures generated were energy minimized. The conformation of the molecule is consistent with a linear heptene unit with the chain torsional angles of approx. 180°. Torsional angles between ^{1}H-20–C-20–C-19–^{1}H19 = approx. 160°; ^{1}H-33–C-33–C-34–^{1}H-34 = approx. 160° and not approx. 180° as estimated from the coupling constants. The deviation is reasonable because these dihedrals are at the junction of the transconjugated double-bond system where the molecule begins cyclization.

NOESY spectra of amp-B in DMSO-d_6 at 100 ms mixing time (Fig. 7) and that of amp-B in DMSO-d_6/ethanol-d_6 mixture (1:1 ratio) at 100 ms mixing time (Fig. 8) show all NOE enhancements to be negative. This renders direct and indirect enhancements indistinguishable [25]. Even in the DMSO/ethanol mixture, viscosity is still high, DMSO viscosity being four times that of ethanol. Calculated $\omega\tau_c$ values are > 1.12 both in the case of DMSO and DMSO/ethanol mixture. Spectra of amp-B in DMSO-d_6 recorded at different mixing times of 400, 200 and 100 ms showed very little change. For all discussion herein we refer to the experiment done at 100 ms (Fig. 7). Analysis of the spectrum (Fig. 7) shows proton 21 having NOE cross peaks with protons 33 (weak), 34, 37 and 40 and 6′ methyl group protons (on sugar) with proton 35. These

two sets of NOEs are unexpected as they are between protons from one end of the molecule to the other end, which are spatially far apart (approx. 18 Å). It is likely that these NOEs observed are of intermolecular nature rather than intramolecular. CD studies carried out at 10^{-2} M concentration clearly showed the existence of oligomeric species (possibly dimeric) at this concentration. Considering unusual NOEs observed combined with CD results, makes it reasonable to assume the presence of dimeric species in DMSO at high concentrations of the drug. Due to dimerization, the correlation time (τ_c) for the isotropic tumbling of the molecule is reduced and this may be reflected in the 2D NOE spectrum as spin diffusion [22]. We have, however, ruled out the possibility of spin diffusion by doing NOESY experiments at different mixing times as well as by confirming the same by ROESY experiment. The observed unusual NOEs/ROEs are due to intermolecular dipolar coupling explained best by a dimeric structure where the molecules are in a head-to-tail orientation. The head end is hydrophilic, due to a β-pyranose (3-aminomannose) sugar and a carboxylic group. A head-to-tail arrangement favors a strong hydrophobic interaction between conjugated double-bond segments of the two molecules and probably gets stabilized by solvent-mediated interactions at the hydrophilic end. The NOE between 6′ protons in the sugar moiety of one molecule, with proton 35 in the tail end of another molecule arises probably due to hydrogen bonding between OH at C-35 of one molecule and any one of the sugar oxygens on the other. This might further stabilize the dimer. Analysis of 2D NOE of amp-B in DMSO-d_6/ethanol-d_6 (1:1) mixture (Fig. 8) at 100 ms mixing time showed no unusual NOE cross peaks. The CD experiments mentioned earlier showed that the antibiotic exists as a monomer in DMSO-ethanol mixture within the studied range of concentration. The absence of unexpected NOEs in the DMSO/ethanol mixture is due to disaggregation of dimers under these solvent conditions.

Comparison of the solution with the crystal structure

From crystal structure analysis reported [17], the following information is derived: The molecules of

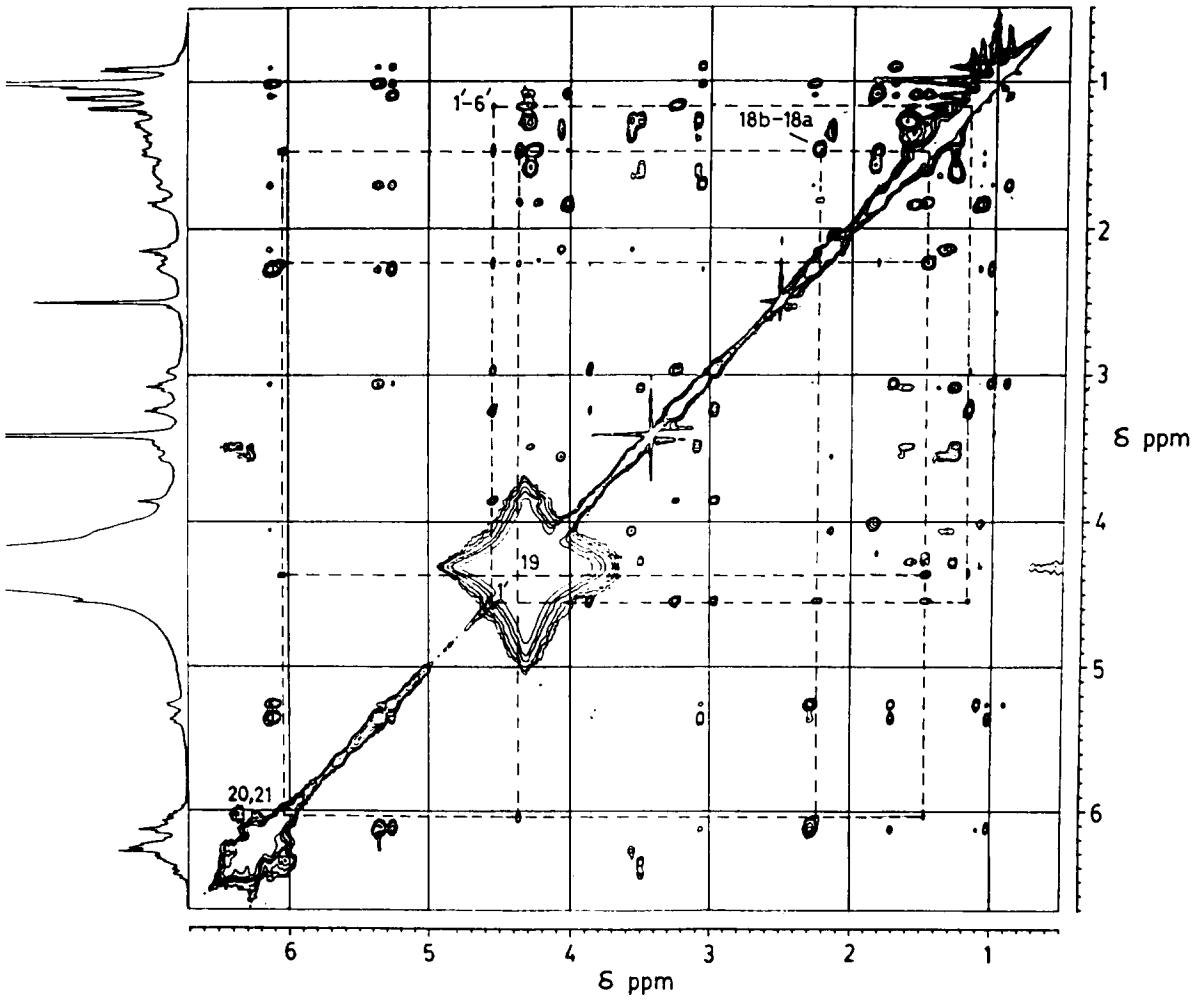

Fig. 8. 400 MHz NOESY of amp-B in DMSO-d_6/ethanol-d_6 (1:1 ratio). 100 ms mixing time. Concentration of amp-B 10^{-2} M.

N-iodoacetyl-amp-B are arranged in stackwise manner displaced pairwise by 0.5 Å from the two-fold screw axis. Each pair is a head-to-tail dimer. The lactone rings of the molecules are nearly parallel (4.3 Å) to each other with the long sequence of conjugated double bonds packed closely. The amino-sugar moiety is joined intramolecularly to the lactone ring by water-mediated hydrogen bonds. Several intermolecular hydrogen bonds are present. One with striking importance is in the region where the lactone rings overlap each other to form a weak hydrogen bond between O-35 on the tail end of one molecule, with O-42, -46 ring oxygen on the sugar moiety, of the other.

The head-to-tail dimer that explains the unusual NOEs/ROEs observed compares well with the way the molecules pack in the crystalline state. It is likely that the preferred aggregated state, when no other interaction takes over, is the head-to-tail dimeric state. In solution, the distances between the molecules are likely to be lesser within 4-Å units, and hence, detectable by NOE experiments.

Based on all the above data we propose a head-to-tail dimerization of amp-B stabilized by hydrophobic interactions between the conjugated double-bond system and also the weak hydrogen bond formed between O-35 and the sugar-ring oxygen. This structure also takes into account a strong intramolecular interaction, a solvent-mediated hydrogen bond between sugar O-43 and ketal ring oxygen O-13 on the macrocycle. Detailed work on the geometries and the effects that molecular dynamics may have on our NOE results are being studied by comparing our experimental results with predictions based on molecular dynamics trajectory of all sterically allowed head-to-tail dimers considered. A description of these results will be given elsewhere. One of the NMR-derived structures of the proposed head-to-tail dimer model is given in Fig. 9.

In conclusion, our results on the CD and NMR studies on the polyene antibiotic amp-B, provided a very interesting solvent-dependent structure for the molecule. In DMSO, the molecule exhibits a monomer conformation at low concentration of the drug ($< 10^{-4}$ M) and a dimer structure at higher concentration ($> 5 \cdot 10^{-3}$ M). However, in a mixed solvent, DMSO/ethanol (1:1 (v/v)), the molecule exists as a monomer irrespective of the concentration of the drug. The data are consistent with a dimer structure of the molecule as 'head-to-tail' type which compares well with that reported in the crystalline-packing state. The model suggests a strong hydrophobic interaction of the conjugated heptene stretch playing a dominant role in stabilizing the structure. Having the hydrophilic head group exposed favors possible inter pair and solvent interactions. Our observation of possible head-to-tail dimeric structure for amp-B in solution agrees well with that reported by Bolard et al. [18] where head-to-tail dimers

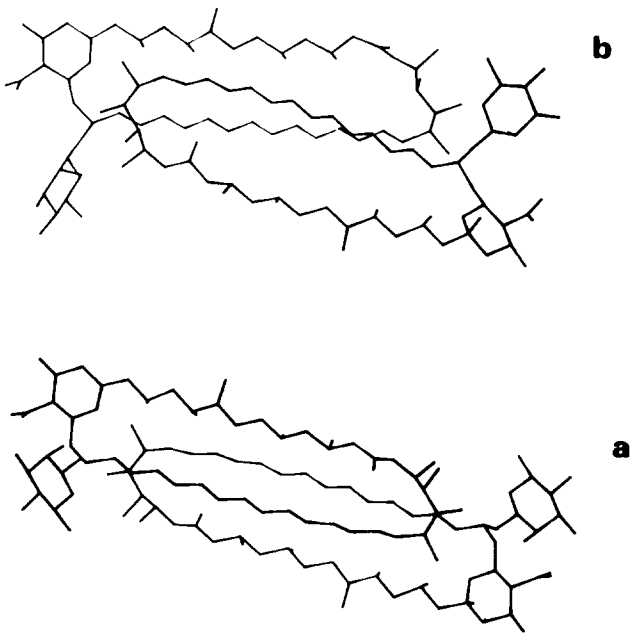

Fig. 9. Proposed model for head-to-tail dimer structure derived using NMR data. (a) Before minimization; (b) after minimization.

are considered to insert into bilayers, leading to higher aggregation due to other interactions. These studies also help reflect upon the smallest stable oligomeric unit in water, which might be a head-to-tail dimer. Accessing NMR information about the antibiotic molecules in water at low concentrations where such aggregates have been hypothesized to exist is next to impossible. Our studies add to the structural information relevant to the understanding of the aggregation properties of this very important polyene antibiotic molecule.

Acknowledgements

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