SANDWICH COMPLEXES AS A FUNCTIONAL FORM OF THE ENNIATIN IONOPHORES

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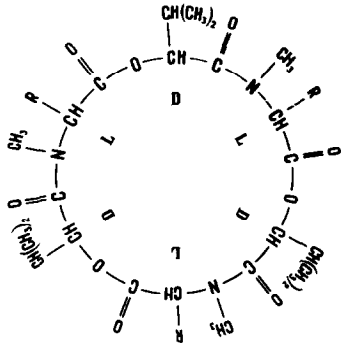
1. Introduction

The ability of the enniatin cyclodepsipeptides (CDP) (fig. 1) to form complexes with alkali metal ions (M^+) and induce ionic permeability in artificial and biological membranes has been described in a number of papers [1, 2]. The complexes were found to be equimolar in both solutions and in the crystalline state; by analogy with valinomycin and the nactins the role of the M^+ carriers across the membrane was ascribed to them [3, 4]. In the present paper evidence is produced showing that an important part in the functioning of this group of ionophores is played by complexes with 2:1 and 3:2 macrocycle:cation ratios.



	R
Enniatin A	$-CH(CH_3)C_2H_5$
Enniatin B	$-CH(CH_3)_2$
Enniatin C	$-CH_2CH(CH_3)_2$
Beauvericin	$-CH_2C_6H_5$

Fig. 1. Antibiotics of the enniatin group.

2. Materials and methods

The study was carried out on synthetic samples of enniatin B [5], beauvericin [6] and '*bis*-enniatin B' (see fig. 8). '*bis*-Enniatin B' was synthesized in a 26% yield by the acid chloride method starting from enniatin B analogues with an *N*-methyl-L-glutamic acid or *N*-methyl-L-lysine residue instead of one of the *N*-methyl L-valine residues [7]; $[\alpha]_D^{20} -23.3$ (c 0.1% in EtOH).

The NMR-^{1}H spectra were obtained on JNM-4H-100 and Varian XL-100 instruments with an operating frequency of 100 MHz. Circular dichroism (CD) curves were recorded on a Cary-60 spectropolarimeter with a CD-6001 attachment. The bilayer lipid membranes (BLM) from total lipids of the white matter of bovine brain were prepared according to Mueller et al. [8]. A methanol solution of the cyclodepsipeptide was added to the aqueous solution. The methanol content in the aqueous solution did not exceed 0.4 vol % and so had practically no effect on the electrical properties of the BLM which are determined at 26°C ± 1 C° with the aid of Ag/AgCl electrodes in an apparatus described in [9]. The membrane conductivity (g_0) was evaluated from the slope of the current–voltage curve at zero current, stationary state values being reported here. In the following the initial CDP and salt concentrations are designated c_0 and b_0 (M), respectively.

3. Results and discussion

Analysis of the NMR spectra of enniation B titrated by alkali salts showed that an increase in salt

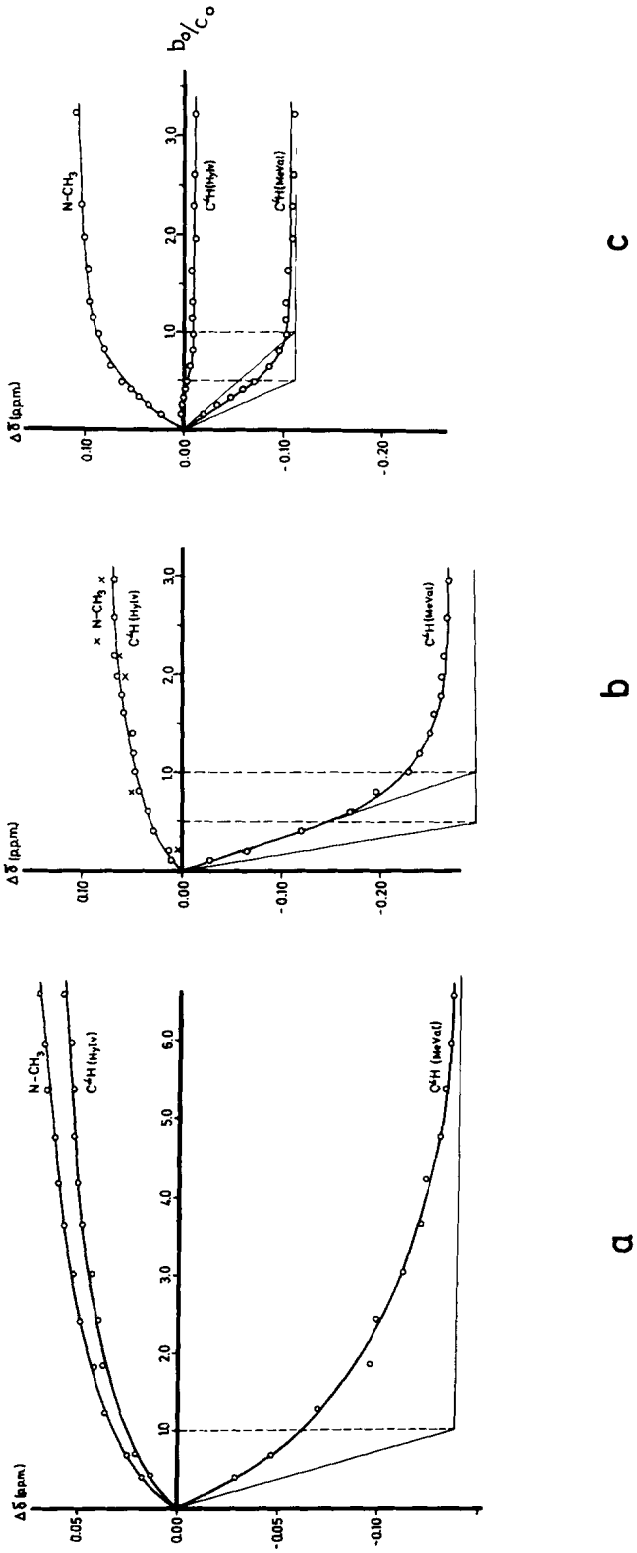


Fig. 2. Enniatin B titration curves in CD_3OD: a) with LiCl, $c_0 = 4.44 \times 10^{-2}$; b) with NaNCS, $c_0 = 4.17 \times 10^{-2}$; c) with CaNCS, $c_0 = 3.95 \times 10^{-2}$; $\Delta\delta = \delta_{complex} - \delta_{free\ CDP}$ so that $\Delta\delta > 0$ indicates a downfield shift.

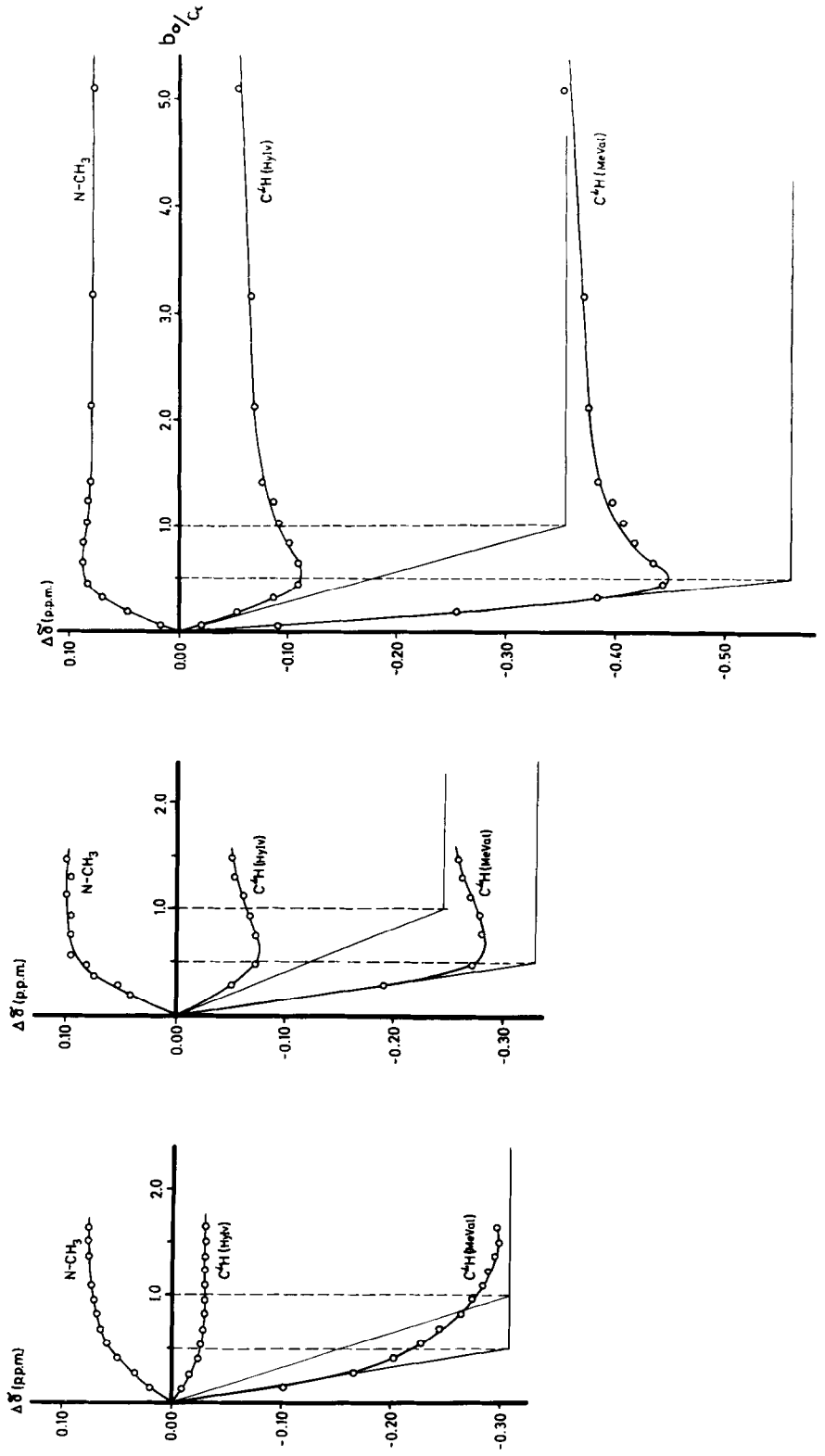


Fig. 3. Enniatin B titration curves with KNCS: a) $(CD_3)_2SO$, $c_0 = 7.36 \times 10^{-2}$; b) CD_3OD, $c_0 = 3.54 \times 10^{-2}$; c) CD_3CN:$CDCl_3$ (1:1), $c_0 = 4.32 \times 10^{-2}$.

concentration and formation of complexes are accompanied by changes in the chemical shifts ($\Delta\delta$) and the $^3J_{C^\alpha H - C^\beta H}$ spin–spin coupling constants to a limiting value corresponding to complete complex formation. As one can see from figs. 2 and 3, the shapes of the $\Delta\delta = f(b_0/c_0)$ curves depend upon the nature of the complexed cation. With Li^+ smooth curves are observed the slopes which are less than for the curves computed for a 1:1 complex with an infinitely high stability constant (fig. 2a). In contrast, when enniatin B interacts with K^+ the $\Delta\delta$ vs. b_0/c_0 curves are steeper than the predicted curve in all the solvents studied (fig. 3). A similar phenomenon was observed when enniatin C was titrated with KNCS in CD_3OD. This suggests that for the relatively high concentrations of the macrocyclic compound required for the NMR experiments, the equilibrium involves a significant amount of complexes with a higher than 1:1 macrocycle:M^+ ratio. Since in the titration with KNCS the experimental $\Delta\delta$ vs. b_0/c_0 plots closely approximate those computed for 2:1 complexes but none of them is steeper, we assume that 2:1 complexes are most probably formed. This is also supported by the non-monotonic change of the proton chemical shifts for enniatin B in CD_3OD and in a 1:1 $CDCl_3CD_3CN$ mixture showing extrema in the region of $b_0/c_0 = 0.5$ (figs. 3b and c). The curves reach a plateau at $b_0/c_0 \approx 3$ (fig. 3b), which indicated that, with such excess of salt, the 2:1 complex practically completely changes to the 1:1 complex. Determination of the stability constant K_2 for the 2:1 complex on the basis of these data leads to values by 1–2 orders of magnitude less than K_1 (that is, approx. $10^{-2}M^{-1}$ in ethanol since the K_1 lie within the range from 10^3 to 10^4M^{-1} [1, 2]).

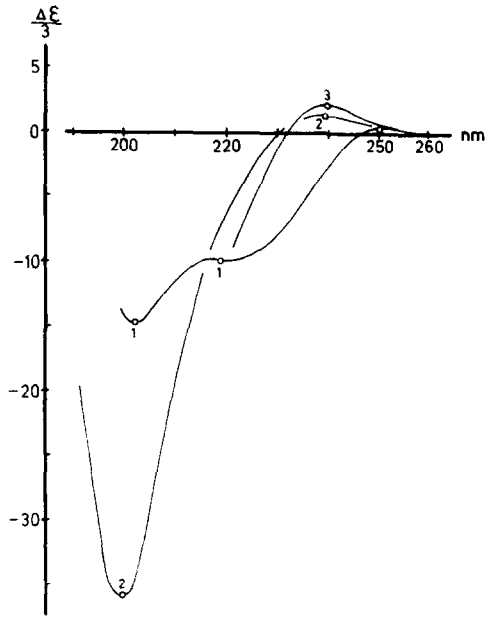


Fig. 4. CD curves in CH_3CN of: 1) enniatin B; 2) 1:1 (enniatin B): K^+ complex, $c_0 = 3.17 \times 10^{-4}$, $b_0/c_0 = 4.5$, and 3) 2:1 (enniatin B):K^+ complex, $c_0 = 3.44 \times 10^{-2}$, $b_0/c_0 = 0.5$

$$CDP + M^+ \rightleftarrows CDP{\cdot}M^+; \qquad K_1 = \frac{[CDP{\cdot}M^+]}{[CDP]\cdot[M^+]}$$

$$CDP + CDP{\cdot}M^+ \rightleftarrows (CDP)_2{\cdot}M^+; \quad K_2 = \frac{[(CDP)_2{\cdot}M^+]}{[CDP]\cdot[CDP{\cdot}M^+]}$$

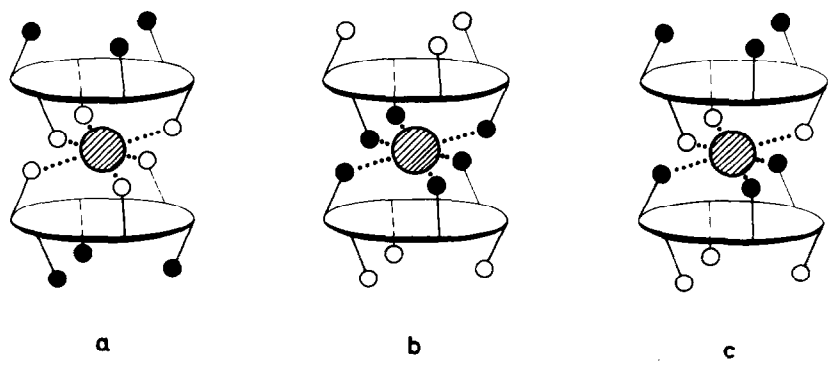


Fig. 5. Possible sandwich complexes for the enniatin antibiotics. • – Amide oxygen; ○ – ester carbonyl oxygen.

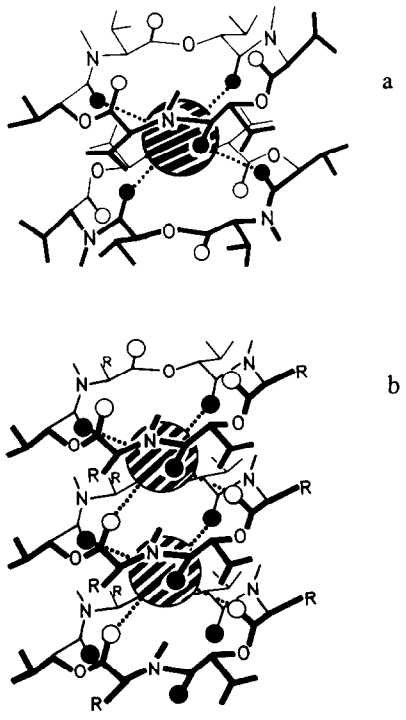


Fig. 6. Possible conformations of a) 2:1 and b) 3:2 complexes.

Comparison of the enniatin B titration curves in CD_3OD with salts of various metals (figs. 2 and 3b) suggests that the stability of the 2:1 complexes decreases in the order $K^+ > Cs^+ > Na^+$, no 2:1 complexes being observed for Li^+. It should be noted that the initial slopes of the experimental curves for titration of enniatin B with CsNCS, have values between those for the 2:1 and 1:1 complexes, i.e. bear evidence of the presence of 3:2 complexes. The formation of such complexes is in accord with the results of bilayer experiments (see below).

As may be inferred from the similarity of the corresponding CD curves (fig. 4), the CDP conformation in the 2:1 complexes is similar to that of the well-studied equimolecular complexes [10].

The results obtained suggest that the 2:1 complexes are of a sandwich structure with 'amide' (fig. 5a), 'ester' (fig. 5b) or 'mixed' (fig. 5c) types of coordination. The last two are the least probable because of spatial hindrance by the *N*-methyl groups. The 'amide' type of sandwich is shown in fig. 6a. In the 3:2 complexes the ligands are, apparently, both amide and ester carbonyl groups (fig. 6b).

Undoubtedly, in the sandwich complexes the cation is more effectively screened from interaction with the

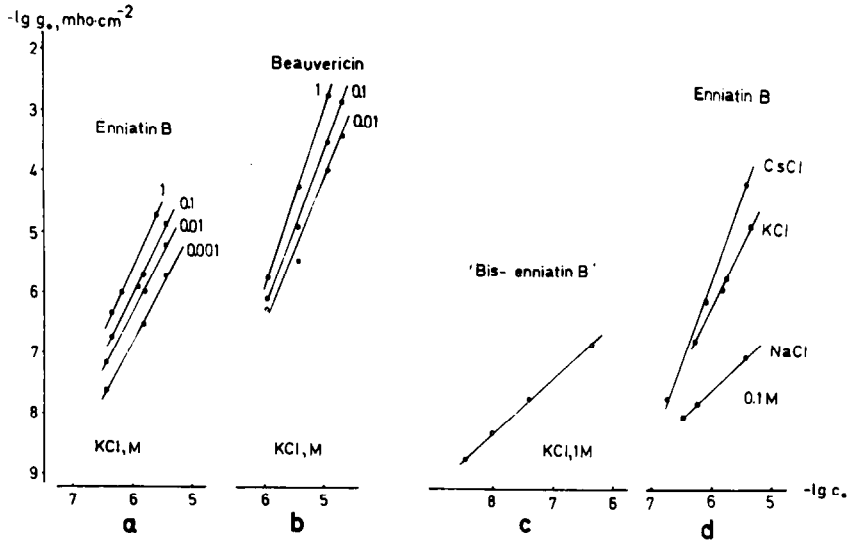


Fig. 7. Bilayer membrane conductivities (g_0) in the presence of enniatins.

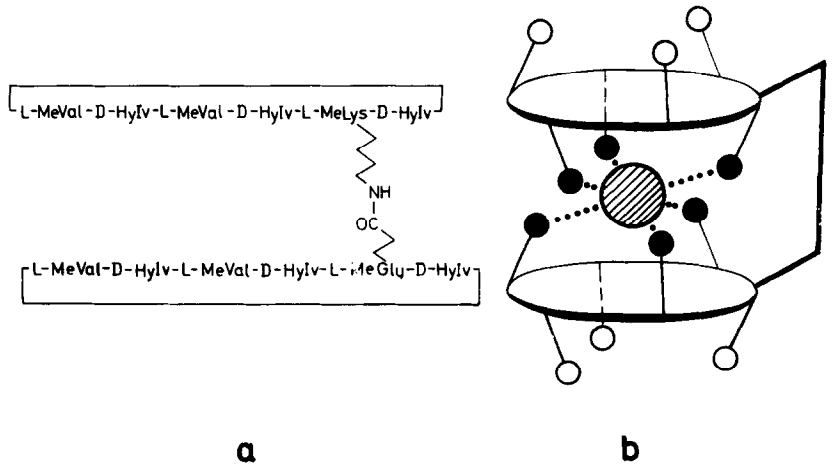


Fig. 8. a) '*bis*-Enniatin B' and b) the assumed structure of its equimolecular complex.

solvent and anion than in the equimolecular complexes, thus suggesting the possible participation of the former in ion transport across membranes despite their low stability constants. To test this possibility, we studied the effect of enniatin B and beauvericin on the ionic conductivity of BLM. The results obtained are shown in fig. 7.

First of all attention is drawn to the fact that the dependence of potassium conductivity on enniatin B concentration is of the second power over a wide range of salt concentration (fig. 7a). This suggests that the event of potassium ion transfer across a membrane in fact involves two molecules of enniatin B. However, it was not certain whether the cation is transported in the sandwich form or whether the second power of the g_0 vs. c_0 dependence is associated with other features of the transport mechanism. To shed more light on this problem we synthesized '*bis*-enniatin B' – an analog in which two depsipeptide rings are connected by a sufficiently long and flexible chain, that is, which has the pre-requisites for sandwhich formation within a single molecule of the ionophore (fig. 8). If such an analog would show a linear g_0 vs. c_0 plot, the observed second power dependence for enniatin B could be in all probability attributed to the stoichiometry. Such a linear dependence has indeed been observed (fig. 7c) lending support to the main proposal of this paper.

It should also be noted that the power of the c_0-dependence of the enniatin B induced conductivity is lower for sodium salts and higher for cesium salts than for the potassium salts (fig. 7d). It seems that the main contribution to the Na^+-conductivity is provided by a mechanism involving equimolecular complexes, whereas for Cs^+ three antibiotic molecules are involved in the transport. Based on the titration results, it may be assumed that the Cs^+ complexes participating in the transport are of a 3:2 stoichiometry. The high power of the c_0-dependence for the beauvericin induced conductivity (fig. 7b) also suggests formation of 'club-sandwiches' by this antibiotic. In the limiting case, when the complexes are stacked to sufficient length, it is possible to think in terms of a channel mechanism of cation transfer by the enniatin cyclodepsipeptides (similar to that suggested by Urry for gramicidin A [11]).

Thus, the mode of action of enniatins is markedly different from that of the other naturally occurring ionophores (viz. valinomycin [3], the nactins [4] which mediate the transport in the form of 1:1 complexes. At the same time the enniatins resemble the synthetic cyclopolyethers which form non-equimolecular complexes in solution [12] and in transporting the ions across phospholipid bilayers [13].

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