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Probing the Mechanism of Action of Amphotericin B.

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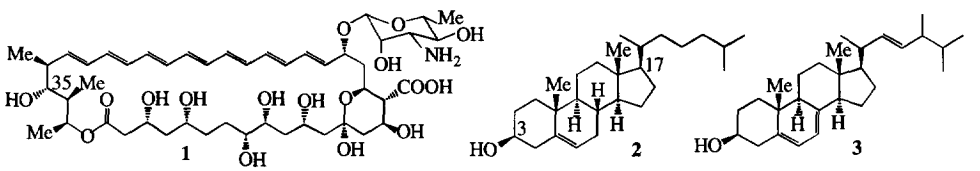
Key Words: Amphotericin B; ion channel.

Abstract: Transmembrane spanning dimers of cholesterol were unable to form ion channels with amphotericin B: We suggest that the two 'half pores' as proposed by De Kruijff[1] may be aligned unsymmetrically.

The polyene antibiotic amphotericin B **1** is a potent antifungal agent which has for over thirty years been used to treat serious systemic fungal infections.[2] However, its severe human toxicity, has limited its application to serious life threatening situations, or topical use. As a group, the polyene antibiotics are toxic to eukaryotic, but not prokaryotic, cells causing a loss of small molecules and ions from the cell, suggestive of damage to sterol containing cytoplasmic membranes. Mammalian membranes contain cholesterol **2**, resulting in amphotericin B's human toxicity, and fungal cell membranes contain ergosterol **3** as their principal steroid, whilst bacterial membranes do not normally contain steroids, rendering them insensitive to amphotericin B.



Amphotericin B also forms pores in steroid containing model membranes such as vesicles or black lipid membranes, and it has been shown, both *in vitro* and *in vivo*, that the structural requirements of the steroid for pore formation with amphotericin B are a 3β-OH, a planar ring system and a hydrophobic side chain at C-17.[2] Rychnovsky has recently shown that a model membrane containing *ent*-cholesterol did not form pores with the addition of amphotericin B, thus demonstrating that amphotericin B does indeed intimately bind to cholesterol in a specific manner in the ion channel structure.[3] De Kruijff in 1974 proposed that amphotericin B (A) and cholesterol (C) form a face to face complex (A-C) perpendicular to the plane of the bilayer membrane, with the polar carboxylic acid/amino-sugar end of amphotericin B on the hydrophilic surface of the membrane.[2] However, this results in the lipophobic hydroxyl groups being buried in the bilayer. This energetically unfavourable situation is alleviated by a second A-C complex aligning itself along the first, so the two sets of hydroxyl groups partially shield each other from the lipophilic core of the bilayer. De Kruijff's proposal is that further aggregation results in an $(\text{-A-C-})_8$ cylindrical complex (half pore), whose length approximates to one half that of the membrane diameter, that

is highly stabilised because now all the hydroxyl groups are directed inwards a central channel, or pore, and the hydrophilic polyene section is on the outer surface of the cylinder, in contact the hydrophilic portion of the bilayer. This cylinder, or 'half pore', would be quite mobile within one-half of the bilayer. To form a transmembrane pore, two such 'half pores' would need to align. Figure **1** show the half pores symmetrically aligned as in the models proposed by De Kruijff,[1] with the cholesterol molecules in line with each other, and the amphotericin B molecules in line with each other. However, visual inspection of computer generated models of the dimeric complex suggested to us that the two 'half pores' may instead be rotated 22.5° relative to each other, with an amphotericin B from one 'half pore' directly above a cholesterol from the other 'half pore'. This would allow closer, more intimate contact between the two 'half pores', as illustrated in Figure **2**, and allow the C-35 hydroxyl group of one amphotericin B molecule to come close enough to the C-35 hydroxyl group of an amphotericin B molecule in the other 'half pore' to form a hydrogen bond. This hydroxyl group is not involved in formation of the ion channel, and is a common structural feature in several other related polyenes.

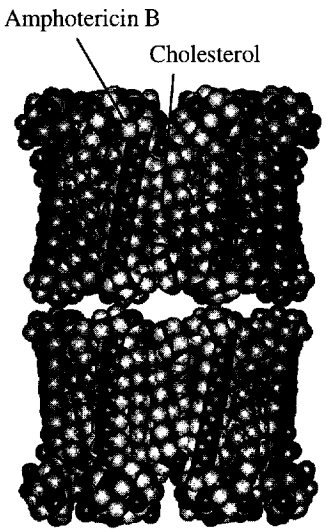


Figure 1

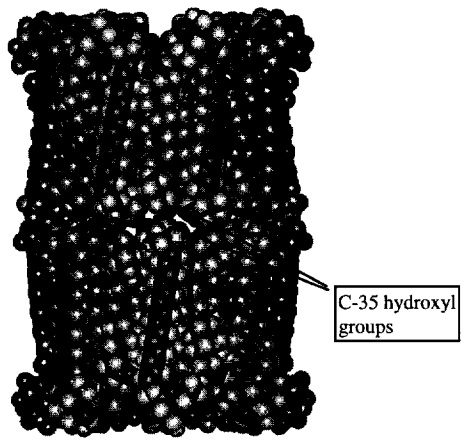
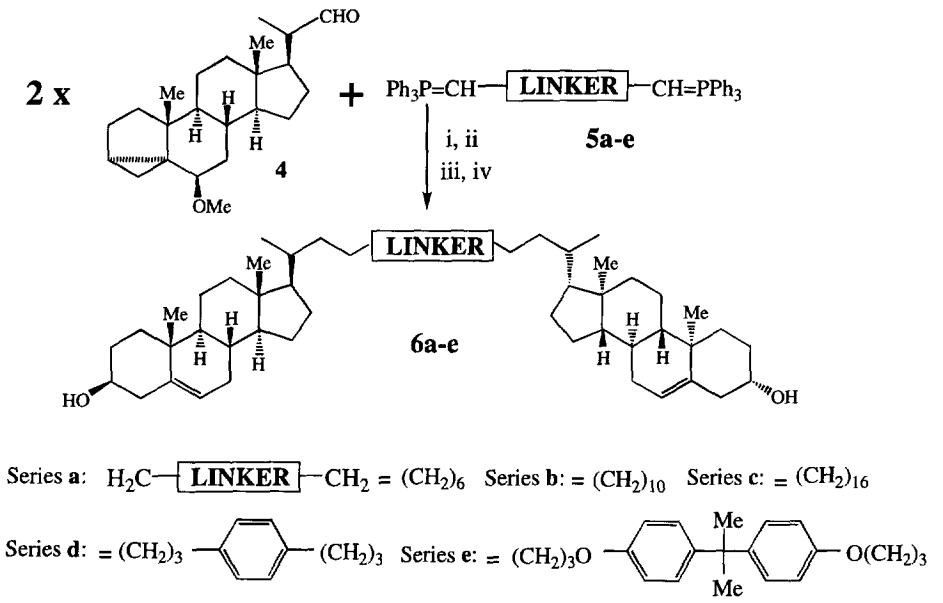


Figure 2

We decided to probe De Kruijff's original model by synthesising (Scheme **1**) a series of tail-to-tail linked dimers of cholesterol (**6a-e**) that should be able to participate in the formation of a symmetrically aligned pore (Figure **1**), but not a pore in which the two 'halves' are rotated relative to each other (Figure **2**). Five linker groups were chosen, three methylene chains (C_6, C_{10}, and C_{16}) that would span possible distance constraints between the two steroids, and two rigid linkers that would prevent the two steroid halves of a single dimer from folding back on itself and occupying the same side of the bilayer.

Stigmasterol was protected as its *i*-methyl ether (MsCl/Et_3N, then KOAc/MeOH; 90% yield),[4] and ozonolysed (O_3, then methanolic thiourea; 70%) to yield the aldehyde **4**.[5] Wittig coupling of two aldehydes **4** with an α,ω-bisylid **5a-e**, followed by hydrogenation of the resulting *trans* double bonds gave the *i*-

methyl ether protected dimer (50% overall yield). Deprotection gave the corresponding diacetate, which was hydrolysed to give the corresponding 'cholesterol dimer' **6a-e** (80% yield, 30% overall yield from stigmasterol), as extremely insoluble solids judged pure by proton and ^{13}C NMR. †



Scheme 1

i) Add aldehyde **4** to ylid **5a-e** in Et_2O (20°C); ii) H_2/Pd on C; iii) $Zn(OAc)_2$/HOAc; iv) KOH/MeOH.

Vesicle preparations containing intravesicular sodium ions and extravesicular lithium ions were prepared[6] as follows: Egg lecithin (29 mg) and steroid or steroid dimer (1mg; 6 or 3 mol% respectively) were dissolved in chloroform. Removal of the solvent *in vacuo* gave a thin film. Warming in aqueous buffer (200 mM NaCl, Tris-HCl/pH 7.8) containing detergent (*n*-octyl-β-D-glucopyranoside, 100 mg), with agitation, gave a clear solution which was dialysed three times at room temperature (3 x 8 h ; 200 mM NaCl) resulting in a cloudy suspension of vesicles, followed by further dialysis (3 x 8 h; 200 mM LiCl). Electron microscopy‡ showed that most of the material was as large unilamellar liposomes (*ca* 200 nm diameter), with some small unilamellar liposomes (ca 50 nm diameter) and multilamellar liposomes. Dysprosium polyphosphate (final conc. 3mM) was added to the resulting suspension (0.5 mL) which was then examined by ^{23}Na NMR.[7] Intravesicular sodium ions are unaffected by the shift reagent, whilst any that leak through the vesicular membrane are shifted upfield by several ppm.

A series of experiments were performed on vesicle preparations containing (i) no steroid; (ii) cholesterol **2**; (iii) ergosterol **3**; (iv) stigmasterol **4**; (v) *epi*-cholesterol (5-cholesten-3α-ol); (vi) **6a**; (vii) **6b**; (viii) **6c**; (ix) **6d**; (x) **6e.** ^{23}Na NMR of all ten samples showed a similar quantity of intravesicular sodium, no significant loss of intravesicular sodium ions having occurred during dialysis, with in each case very low concentrations of extravesicular sodium. To each vesicle sample, a solution of amphotericin B§ in DMSO (5μL; 0.2 mM) was added, and the efflux of sodium ions monitored by NMR (Figure 3)

immediately after the addition (Figure 3, Expt A) and after ten minutes (Expt B). Only when the vesicle preparation contained cholesterol **2**, ergosterol **3**, or stigmasterol **4** [experiments (ii), (iii) and (iv)], was there any significant efflux of sodium ions. Control experiments in which just DMSO was added to the vesicle suspension gave results similar to experiments (i), and (v)-(x).

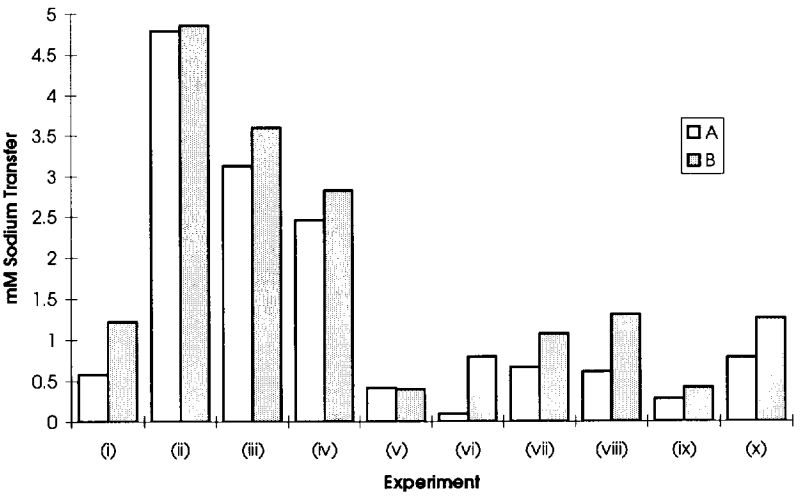


Figure 3: Efflux of sodium ions upon addition of amphotericin B.

The inability of the steroid dimers (**6a-e**) to participate in transmembrane pore formation with amphotericin B **1** supports the proposal that the two 'half pores' may be rotated relative to each other. Recent molecular mechanics calculations by Khutorsky[8] support the formation of the '$(A\text{-}C)_8$ half pore'. Using molecular modelling, we are examining the proposed unsymmetrical dimeric pore, as illustrated in Figure **2**.

† No crystals suitable for X-ray analysis, or combustion data on **6a-e** have yet been obtained, but using the above scheme with isovaleryl bromide, stigmasterol was cleanly converted to cholesterol acetate with no epimerisation at C-20.

‡ Electron microscopy was performed by Mrs E. Roberts of Leicester University's Electron Microscopy Unit.

§ Amphotericin B was kindly provided by Mr Steven Box of SmithKline Beecham Pharmaceuticals.

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REFERENCES:

1. DeKruijff, B.; and Demel, R. A. *Biochimica et Biophysica acta*, **1974**, *339*, 57.
2. Gale, E. F.; *Macrolide Antibiotics*: *Chemistry, Biology and Practice*; Omura, S., Ed.; Acad. Press: Orlando, 1984.
3. Mickus, D. E.; Levitt, D. G.; Rychnovsky, S. D. *J. Am. Chem. Soc.*, **1992**, *114*, 359.
4. Aburatani, M.; Takeuchi T.; Mori, K. *Synthesis*, **1987**, 181.
5. a) Nicotra, F.; Ronchetti, F.; Russo, G.; Toma, L.; Gariboldi P.; Ranzi, B. M. *J. Chem. Soc., Perkin Trans.*, **1985**, 521. b) Gupta, D.; Soman R.; Dev, S. *Tetrahedron*, **1982**, *38(20)*, 3013.
6. Mimms, L. T.; Zampighi, G.; Nozaki, Y.; Tanford, C.; Reynolds, J. A. *Biochemistry*, **1981**, *20*, 833.
7. Riddell F. G. and Hayer, M. K. *Biochimica et Biophysica Acta* **1985**, *817*, 313.
8. Khutovsky, V. E. *Biochimica et Biophysica Acta*, **1992**, *1108*, 123.

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