Inorganica Chimica Acta, 108 (1985) 117–122

The Synthesis and Characterisation of Polyene Complexes with Divalent Metal Ions: Mg(II), Ca(II), Ni(II), Cu(II) and Zn(II)

ANTHONY E. BEEZER, PAUL O'BRIEN* and WAI L. SHAM

Chemistry Department, Royal Holloway and Bedford New College, University of London, Egham, Surrey, TW20 0EX, U.K.

Received June 20, 1985

Abstract

Metal ion (Mg(II), Ca(II), Zn(II), Cu(II), Ni(II)) complexes of nystatin and amphotericin B (polyene antibiotics) have been prepared as solids. The stoichiometry of the complexes has been established. IR, ESR investigation indicates the metal-ligating sites in the polyene molecules. The existence of such complexes is discussed in the light of polyene mode-of-action theories.

Introduction

The polyene antibiotics, of which nystatin and amphotericin B are prominent members, are used in the treatment of fungal infections [1]. Their properties both chemical, physical and biological have been adequately reviewed [2, 3, 4]. The consequences of polyene interaction with yeast cells include loss of membrane integrity (*i.e.* loss of selective permeability functions) and expression of cytoplasmic constituents to the suspending medium. The principal component of the yeast cell membrane which interacts with polyene antibiotics is generally held to be sterols, principally ergesterol. The conclusion drawn from kinetic and physical chemical studies of yeast cell/polyene interaction was that both uptake of nystatin and release of cytoplasmic constituents were controlled largely by the presence in the yeast of a cell wall [5]. The activation energies derived for these differing processes fell within the range anticipated for simple diffusion processes through viscous fluids. Thus if the yeast cell wall may be described as a viscous fluid then it would appear that the mechanism of cellular ingress or egress via the membrane is rapid, and, that it is the non-specific diffusion process through the cell wall which is rate determining. These conclusions have been substantiated by demonstration of linear ΔH–ΔS compensation plots and, more particularly, by linear ΔG–ΔH plots [5].

It is noteworthy that divalent metal ions have been shown to 'protect' yeast cells from the effects of nystatin [2, 3, 6] and also that no report exists of any residual enzymic activity in cells treated with nystatin. Thus cells not only lose viability but they also appear to lose all enzymic activity too.

Ca(II) ion has been shown to be important in determining membrane ridigity [7] and in controlling membrane permeability. Furthermore enzymic activity within yeast cells depends to a large degree upon the presence of divalent metal ions particularly Mg(II), Zn(II), Cu(II). Little attention has, however, been given to the existence of complexes of nystatin with these metal ions; and hence to their role in the mechanism of the nystatin/yeast cell interaction [2]. There have been reports of metal ion/nystatin complexes e.g. Cu(II) has been shown [8] to form complexes with nystatin as has Fe(II) and Fe(III). These complexes however were prepared only in solution and in only the Cu(II) complex was any attempt at characterisation made.

In an attempt to clarify the role of metal ions in cell/nystatin interactions we have therefore prepared solid complexes of nystatin with Cu(II), Ni(II), Zn(II), Mg(II) and Ca(II). These same metals have also been studied on reaction with amphotericin B a related polyene antibiotic widely used in clinical practice [1].

Due to the poor aqueous solubility of these polyene antibiotics the complexes have been prepared from dimethyl formamide (DMF)/water solutions.

The nystatin and amphotericin B molecules (Fig. 1) contain many functional groups capable of co-ordinating to metal ions. The amino, hydroxy and carboxylate groups are all fairly strong ligators. It might be expected that hard Lewis acids, such as Mg(II) would interact most strongly with the carboxylate group and other oxygen donors. The 'softer' metals, as typified by Cu(II) might be expected to interact strongly with the amino/hydroxy functions; there is here an obvious analogy with the well developed chemistry of ethanolamines [9, 10]. In this paper the preparation and characterisation of a number of polyene complexes with divalent metal

*Present address: Chemistry Department, Queen Mary College, Mile End Road, London, E1 4NS, U.K.

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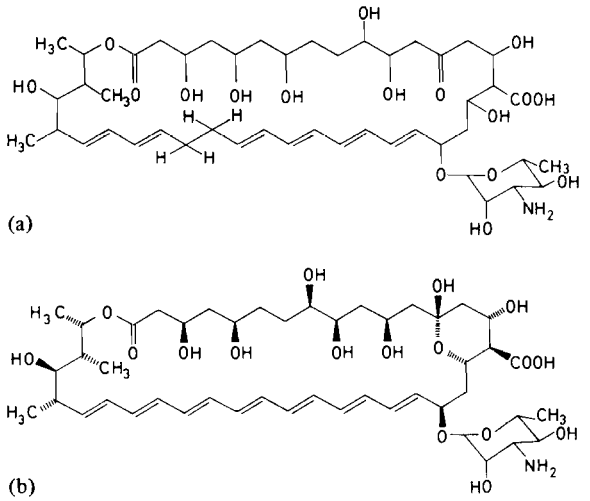

Fig. 1. Nystatin (a) and amphotericin B (b) molecules.

ions is described. The results are in accord with the suggestions made above.

A further objective in the manufacture of derivatives of polyene antibiotics is to improve clinical utility through improvements in solubility and biological potency. A further paper in this series reports the biological activity of these complexes upon interaction with *Saccharomyces cervisiae* and shows that potency may be improved by between ~2 to 10 fold compared to nystatin itself.

Experimental

Materials

Nystatin and amphotericin B were gifts from E. R. Squibb and Sons, Liverpool, U.K.; both were stored in a deep freeze (−24 °C) when not in use. Copper nitrate trihydrate and zinc nitrate hexahydrate were SLR grade. Nickel nitrate hexahydrate, calcium chloride dihydrate and magnesium nitrate hexahydrate were BDH AnalaR grade. Sodium hydroxide and hydrochloric acid (1M Factor), were also BDH AnalaR grade. Deionised water was used throughout, N,N′ dimethylformamide was Fisons A.R. grade.

Preparation of the Complexes

About 1 gm of the polyene antibiotic (nystatin or amphotericin B) was dissolved in 10 ml of DMF. An aqueous solution containing the stoichiometric amount of the metal ion in 10 ml of water was added dropwise to the polyene antibiotic solution whilst cooling on ice. The solution was shaken and ~0.1 mol of sodium hydroxide was added dropwise to neutralise the solution. A 'pickled' electrode* was used to check the pH. Excess water (30 ml) was then added to precipitate the solid complex. The complexes were separated by centrifugation, washed with several alternate aliquots of methanol and water, and dried under reduced pressure.

Atomic Absorption

A Perkin-Elmer 103 instrument was used. Standard solutions were prepared using BDH AnalaR grade metal salts.

The metal complexes were ground to a fine powder before weighing. The required quantity of complex was dissolved in the minimum volume of concentrated HNO_3; the resulting solution was diluted to contain a concentration of metal ion within the range of the studied curve (usually 0.5–5 ppm).

EDTA Titrations

Analar disodium dihydrogen ethylenediamine tetraacetate was used to prepare ~0.1 mol dm^{-3} solutions in water. The EDTA solutions were standardized using AnalaR zinc sulphate heptahydrate as primary standard. Titrations were then carried out on the complexes in DMF/water mixtures at values of pH between nine and ten, this preculded an analysis of the copper complex as a copper hydroxide precipitated.

The indicators used were E. Black T powder for zinc and magnesium and murexide for nickel and calcium analysis.

Infra-Red Spectroscopy

The IR spectra of the metal–polyene antibiotic complexes were studied as nujol or hexachlorobutadiene mulls using caesium iodide plates. A Perkin-Elmer model 21 spectrometer was used, spectra were scanned from 3500 cm^{-1} to 200 cm^{-1}.

ESR Spectroscopy

Electron spin resonance spectra were recorded with a Varian E4 instrument operated at room temperature (close to 298 K) and at 77 K. Diphenyl picrylhydrazyl (D.P.P.H.) was used as a standard. *g*-values were calculated by the method of Kneubahl [14]. A mixture of 2% copper–nystatin in the diamagnetic zinc–nystatin complex was prepared by adding 2% copper ion in the aqueous zinc solution which then followed the preparation described. The same procedure was used to prepare a mixture of 2% copper–amphotericin B in the zinc–amphotericin B complex.

Results and Discussion

The dissolution of nystatin or amphotericin B in DMF produced a clear yellow solution. In a typical

*Hydrogen-ion activities in DMF/water mixtures were measured with a specially prepared ('pickled') electrode, the details of which are described in the following paper.

experiment an aqueous solution of copper(II) (10 ml, 0.1 mol dm^{-3}) was added dropwise to the nystatin solution ($\sim 10^{-1}$ mol dm^{-3}). Heat was evolved on the mixing and, as nystatin is heat labile the reaction mixture was cooled on ice. The green copper(II) complex precipitated and was collected by centrifugation. The other metal complexes were orange.

The optimum conditions (molar ratios, pH) for the preparation of nystatin complexes with a number of metal-ions are summarized in Table I. The metal-ion content of the solid complexes prepared depended on both the pH of reaction and the ratio of metal-ion to polyene. Analytical results, in terms of percentage of metal-ion, are summarized in Table II. In general the complexes are of 1:1 or 1:2 (metal: ligand) stoichiometry. There are some anomalies (Table II), these are probably caused by coprecipitation and/or degradation of the polyene during the preparation. The complexes were of different colours from the parent polyenes and had a similar, but somewhat weaker, odour. X-ray powder diffraction photographs were obtained for the Mg(II) and Ca(II) species, these are hence microcrystalline. The complexes showed no photosensitivity and could be kept in a dessicator for long period.

The solubility in water of the complexes was slightly greater than that of the parent polyenes. The solubilities of nystatin and its metal complexes are summarized in Table III.

The metal complexes are very soluble in DMF and considerably more soluble than the polyenes in H_2O/MeOH mixtures.

TABLE I. The Optimum Molar Ratio and pH Condition for the Preparation of Metal–polyene Antibiotic Complexes.

Metal–polyene antibiotic	[metal][a]:[polyene antibiotic][b]	pH[c]
Copper–nystatin	1:1	5–6
Copper–(nystatin)$_2$	1:2	5–6
Zinc–nystatin	1:1	6–7
Zinc–(nystatin)$_2$	1:1	6–7
Zinc–(nystatin)$_2$	1:2	7
Nickel–nystatin	2:1	6–7
Nickel–(nystatin)$_2$	1:1	7
Magnesium–nystatin	10:1	6–7
Magnesium–(nystatin)$_2$	5:1	8
Calcium–nystatin	20:1	6–7
Calcium–(nystatin)$_2$	10:1	8
Copper–amphotericin B	1:1	5–6
Copper–(amphotericin B)$_2$	1:2	5–6
Zinc–amphotericin B	1:1	6–7
Zinc–(amphotericin B)$_2$	1:2	7
Nickel–amphotericin B	1:1	6–7
Nickel–(amphotericin B)$_2$	1:2	7
Magnesium–amphotericin B	30:1	7
Magnesium–(amphotericin B)$_2$	10:1	8
Calcium–amphotericin B	20:1	6–7
Calcium–(amphotericin B)$_2$	10:1	8

[a]In aqueous solution. [b]*ca.* 1×10^{-1} M in DMF solution. [c]pH measured is relative to 50% DMF/H_2O.

Infra-Red Spectroscopy

The IR spectra of nystatin and its complexes permit an assignment of most of the main absorptions. The assignments are tabulated in Table IV (C–H stretching frequencies are omitted). The $-COO^-$ and $-NH_2$ are the most likely to be involved in complexation.

The modification of carboxylate stretches on complexation to metal-ions has been discussed by Nakamoto [11]. The N–H stretching vibration gives rise to absorption in the range 3300–3200 cm^{-1}, the N–H bending absorption in the range 1600–1550 cm^{-1}, the N–H_2 twisting absorption band in the range 1030–1050 cm^{-1} and the N–H_2 rocking absorption in the range 850–800 cm^{-1}. The N–H_2 twisting and rocking modes are sensitive to the nature of the metal and are shifted to lower frequencies because the N–H bond order will be reduced on co-ordination [12].

Nystatin is a large molecule containing different functional groups and the N–H bands are obscured by absorptions due to the other groups in the molecule. For example, the N–H stretching at 3200 cm^{-1} is nearly coincident with O–H stretching frequencies. At 1560 cm^{-1}, the N–H bending absorption is interfered with by the OCO antisymmetric stretching and at 1072 cm^{-1}, the N–H_2 twisting mode is interfered with by COC stretching for the ether group. The sharp peak at 850 cm^{-1}, on the other hand which is not interfered with by the other group, is the N–H_2 out of plane rocking absorption and it is therefore this band which may be studied on complexation.

In Table IV the major assignments for the infrared spectra of metal–nystatin complexes are reported. For the magnesium complex both the symmetric and antisymmetric OCO stretching absorptions were absent. The strongly suggests that the hard magnesium(II) centre interacts with the carboxylate group. The COO antisymmetric may be shifted to higher energy but this band is obscured by the absorption of the polyene system. The spectrum also shows a broad peak in the range 1250–1035 cm^{-1} which is completely different in shape from any band in the nystatin spectrum. It is probable that the COO symmetric absorption would shift to around this region in a complex involving a monodentate carboxylate [11]; this is illustrated below:

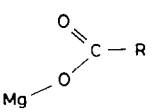

TABLE II. Metal–Ion Analysis of the Polyene Complexes.

Metal complexes ratio	Copper–nystatin		Zinc–nystatin		Nickel–nystatin		Magnesium–nystatin		Calcium–nystatin	
	1:1	1:2	1:1	1:2	1:1	1:2	1:1	1:2	1:1	1:2
Theoretical % metal content	6.42	3.32	6.59	3.41	5.96	3.07	2.56	1.30	4.15	2.12
% metal content from EDTA titration	–	–	6.61	3.35	5.83	2.81	2.65	1.35	3.80	2.09
			6.43	3.38	5.83	2.82	2.55	1.42	3.90	2.06
					5.75	2.82		1.22	3.93	1.91
% metal content from A.A. analysis	6.25	3.42	5.55	3.93	–	3.10	2.74	1.18	5.54	2.09
	5.88	3.65	5.36	3.68		3.61		1.18	6.82	
	6.25		5.20							

TABLE III. Solubility Data for Metal Ion/Nystatin Complexes.

Compound	Aqueous solubility (mg ml^{-1})
Nystatin	0.050
$CuNC_2$	0.47
$NiNC_2$	0.51
$ZnNC_2$	0.64
$CaNC_2$	0.72
$MgNC_2$	0.75

A small peak at 830 cm^{-1}, assigned as the NH_2 rocking absorption, is unaffected on complexation thus the hydroxy amino group of the Mg(II)–nystatin complex remains in a similar form to that in free nystatin. Further an absorption at 420 cm^{-1} tentatively assigned as an M–O stretching vibration may confirm the structure.

It was found that the OCO antisymmetric absorption was present in the Cu(II), Ni(II) and Zn(II) complexes. This band is present at 1560 cm^{-1} in the nystatin spectrum and appears as a shoulder in the spectrum of the metal-complexes. This suggests that the carboxylate group is not involved in the complexation of these metals. The NH_2 rocking absorptions have shifted from 850 cm^{-1} to lower energy in these complexes. It seems likely that these metal ions react with the hydroxy amine group of nystatin. New low energy bands in the infra-red spectra of these complexes are tentatively assigned as M–N and M–O stretching frequencies Table IV.

The infra-red spectra of the amphotericin B complexes which we have prepared are remarkably similar to those described above for nystatin. The same conclusions may hence be reacted for the mode of co-ordination of both polyenes with the metals, Mg(II), Ca(II), Ni(II), Cu(II), and Zn(II), in the solid-state.

TABLE IV. Major Frequencies (cm^{-1}) in Nystatin and Metal–Nystatin Complexes.

Nystatin	Cu–(Nystatin)$_2$	Cu–Nystatin	Ni–Nystatin
3400–3200(broad)	3500–3200(broad)	3600–3100(broad)	3500–3100(broad)
1740–1710(m)	1710(shoulder)	1710(shoulder)	1710(shoulder)
1620(small)	1650(sharp)	1650(broad)	1650(broad)
1560(m)	1560(shoulder)	1570(shoulder)	1560(shoulder)
1408(s)		1418(s)	
		1260(m)	
1175(s)	1160(shoulder)	1170(shoulder)	1160(shoulder)
		1105(m)	
1072(m)	1070(broad)	1065(shoulder)	1070(m)
1001(sharp)	1005(small)	1010(small)	1000(s)
930(m)	840	900(s)	
850(sharp)	770(s)	665(m)	620(broad)
	620(small)	375(m)	400(m)
	350(small)		

[a]Tentatively assigned.

Copper–amphotericin B		Zinc–amphotericin B		Nickel–amphotericin B		Magnesium–amphotericin B		Calcium–amphotericin B	
1:1	1:2	1:1	1:2	1:1	1:2	1:1	1:2	1:1	1:2
6.43	3.32	6.61	3.42	5.97	3.08	2.56	1.30	4.16	2.12
		6.55	3.48	5.75	3.03	2.51	1.28	3.89	2.01
–	–	6.44	3.48	5.85	3.05	2.50	1.22	3.92	2.05
					3.07				1.89
5.79	3.29	4.69	–	4.65	2.46	2.19	1.39	5.51	2.31
6.41	3.29						1.21	6.29	2.31
								7.34	2.47

TABLE V. ESR Parameters for Copper(II) Complexes.

	$g_{\parallel}$	$A_{\parallel}$[a]	g	Notes
Cu(amphotericin-B)$_2$	2.281	193	2.08	–
	2.278	193	2.08	~2% dope in Zn complex
Cu(nystatin)$_2$	2.288[b]	–	2.06	–
	2.246	178	2.06	~2% dope in Zn complex

[a]$A_{\parallel}$ $10^4 \times cm^{-1}$. [b]g_{av} estimated from a broad isotropic band.

Electron Spin Resonance Spectroscopy

The broad line ESR spectra of the 2:1 complexes of copper(II) with both nystatin and amphotericin B were recorded. No fine structure was observed at either room temperature or 77 K. The g-values (Table V) are consistent [13] with approximately axial CuN_2O_2 co-ordination at the copper atom, and hence support the conclusions of the infra-red investigation.

The preparation of metal complexes of polyene antibiotics has been suggested and tentatively reported previously [8]. However only solution phase preparations were described without any reference to the chemical or physical conditions which favour their synthesis.

The Tables indicate that solid complexes of nystatin and amphotericin B with Ca(II), Mg(II), Cu(II), Ni(II), Zn(II) can be formed. The complexes

Zn–Nystatin	Mg–Nystatin	Assignments
3500–3100(broad)	3500–3100(broad)	OH stretching and NH stretching
1710(shoulder)		C=O stretching for ester and ketone
1650(broad)	1650(broad)	C=C stretching for polyene system
1570(shoulder)		OCO antisym. stretching for CO_2^-, N–H bending (out of plane)
		OCO sym. stretching for CO_2^-
1160(shoulder)	1170(broad)	C–O stretching for ester
1100(broad)	1072(broad)	COC stretching for ether $N-H_2$ twisting mode
990(m)	1010(shoulder)	CO stretching for R_1R_2CHOH
		CH bending (out of plane) for C=CH
610(m)	830(s)	$N-H_2$ bending (out of plane) (rocking)
	870(s)	
		M–N stretching[a]
	420(s)	M–O stretching[a]

so formed are stable with respect to light, air exposure and in solution. Co-ordination by carboxylate is suggested for magnesium, whereas for the softer Cu(II), Zn(II) and Ni(II) centres the amino hydroxy function coordinates to the metal.

The aqueous solubilities of the metal complexes of nystatin together with the solubility of nystatin itself are displayed in Table III. The solubilities of the complexes are shown to be comparable to nystatin itself. The objectives of the manufacture of derivatives of polyene antibiotics are to:

(i) improve the solubility of the antibiotic and

(ii) to improve the biological activity or

(iii) to achieve both these objectives together.

It is apparent that solubility has not been significantly improved and hence improved clinical efficacy must be sought through improvement in biological activity. The results of such an examination on the complexes reported will be published elsewhere.

However, independently of any improvements in solubility and/or biological potency the fact that stable complexes of polyenes with metals exist requires consideration of their role in mode of action theories. The problem remains however of the solvent system which may mimic the biological milieu. The complexes reported here have been prepared from DMF/aqueous solution yet the metal ions *in vivo* occur in lipoid phases.

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