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Synthesis, characterisation and antimicrobial activity of copper(II) and manganese(II) complexes of coumarin-6,7-dioxyacetic acid ($cdoaH_2$) and 4-methylcoumarin-6,7-dioxyacetic acid (4-$MecdoaH_2$): X-ray crystal structures of $[Cu(cdoa)(phen)_2]\cdot 8.8H_2O$ and $[Cu(4\text{-}Mecdoa)(phen)_2]\cdot 13H_2O$ (phen = 1,10-phenanthroline)

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Abstract

Two novel coumarin-based ligands, coumarin-6,7-dioxyacetic acid (**1**) ($cdoaH_2$) and 4-methylcoumarin-6,7-dioxyacetic acid (**2**) (4-$MecdoaH_2$), were reacted with copper(II) and manganese(II) salts to give $[Cu(cdoa)(H_2O)_2]\cdot 1.5H_2O$ (**3**), $[Cu(4\text{-}Mecdoa)(H_2O)_2]$ (**4**), $[Mn(cdoa)(H_2O)_2]$ (**5**) and $[Mn(4\text{-}Mecdoa)(H_2O)_2]\cdot 0.5H_2O$ (**6**). The metal complexes, **3**–**6**, were characterised by elemental analysis, IR and UV–Vis spectroscopy, and magnetic susceptibility measurements and were assigned a polymeric structure. **1** and **2** react with Cu(II) in the presence of excess 1,10-phenanthroline (phen) giving $[Cu(cdoa)(phen)_2]\cdot 8.8H_2O$ (**7**) and $[Cu(4\text{-}Mecdoa)(phen)_2]\cdot 13H_2O$ (**8**), respectively. The X-ray crystal structures of **7** and **8** confirmed trigonal bipyramidal geometries, with the metals bonded to the four nitrogen atoms of the two chelating phen molecules and to a single carboxylate oxygen of the dicarboxylate ligand. The complexes were screened for their antimicrobial activity against a number of microbial species, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* and *Candida albicans*. The metal-free ligands **1** and **2** were active against all of the microbes. Complexes **3**–**6** demonstrated no significant activity whilst the phen adducts **7** and **8** were active against MRSA ($MIC_{80} = 12.1$ μM), *E. coli* ($MIC_{80} = 14.9$ μM) and *Patonea agglumerans* ($MIC_{80} = 12.6$ μM). Complex **7** also demonstrated anti-*Candida* activity ($MIC_{80} = 22$ μM) comparable to that of the commercially available antifungal agent ketoconazole ($MIC_{80} = 25$ μM).

Keywords: Coumarin-6,7-dioxyacetic acid; 1,10-Phenanthroline; Copper(II); Manganese(II); X-ray structure; Antimicrobial

1. Introduction

The phenomenum of drug-resistance in microbial strains emerged within a few years of penicillin being put on the market when scientists began noticing the emergence of a penicillin-resistant strain of *Staphylococcus aureus*, a common bacterium which causes a variety of suppurative (pus-forming) infections and toxinoses in humans. From

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that first case of resistant *Staphylococcus*, the problem of antimicrobial resistance has snowballed into a serious public health concern with economic, social and political implications. Of particular concern has been the emergence of methicillin-resistant *S. aureus* (MRSA) and recent reports indicate that community-associated MRSA now has reached epidemic proportions in many areas and has become a worldwide problem [1–7].

Drug-resistance to antifungal agents is also of increasing concern. *Candida albicans* is the major fungal pathogen in humans and is carried by over 50% of the population. Although in healthy individuals the pathogen may cause relatively minor health problems, such as oral thrush, the interactions of a fungus with its host can be disturbed by hormonal or immunological imbalances in the host to the point of provoking serious superficial infections and life-threatening systemic infections [8–10]. The increasing prevalence of fungal infections, especially hospital-acquired infections and infections in immunocompromised patients, has heightened the need for new anti-fungal treatments [11–14]. Drug-resistant fungal isolates have been reported for all known cases of antifungal drugs [15–17]. Thus, there is an urgent need to develop new and more effective antifungal therapies.

In an earlier study by our group it was found that the metal-based drugs $[Cu(phen)_2(mal)]\cdot 2H_2O$, $[Mn(phen)_2(mal)]\cdot 2H_2O$ and $[Ag_2(phen)_3(mal)]\cdot 2H_2O$ (phen = 1,10-phenanthroline, $malH_2$ = malonic acid) demonstrated a different mode of action compared to the commonly used commercial polyene and azole antifungal drugs [18]. It was established that both metal-free phen and the metal–phen complexes affect mitochondrial function, by retarding the synthesis of cytochromes *b* and *c* and uncoupling cellular respiration. Treatment of fungal cells with the Cu(II) and Ag(I) complexes resulted in a reduced amount of ergosterol in the cell membrane and a subsequent increase in its permeability. Cells exposed to metal-free phen and the Cu(II) and Mn(II) complexes (but not the Ag(I) complex) demonstrated an elevation in oxygen uptake.

Lately, a number of metal complexes of coumarins have been synthesised and their biological activity determined. Triorganotin(IV) derivatives of 7-hydroxycoumarin have shown good antimicrobial activity against *S. aureus* and *Bacillus subtilis*, *C. albicans* and *Microsporum gypseum*, and this activity was slightly enhanced upon adduct formation with phen [19]. Kostova et al. have shown the cytotoxic potential of coumarins complexed to cerium, lanthanum, zirconium and neodymium ions [20–24]. Whilst our initial work concentrated on the anticancer activity of a series of substituted coumarins [25–31], more recently we have investigated the antimicrobial activity of a number of coumarin-based Ag(I) and Cu(II) complexes [32,33], with the activity of a number of complexes against *C. albicans* being comparable to that of commercially used drugs such as ketoconazole. We have also reported an Ag(I) coumarin complex which showed significant activity against the clinically important bacterial strain MRSA ($MIC_{80} = 0.63\ \mu M$) [33].

In the present work, we have prepared coumarin-derived dioxyacetic acid ligands and isolated their Cu(II) and Mn(II) complexes as well as the phen adducts of the Cu(II) complexes. The metal-free ligands, their metal complexes and the simple salts, $Mn(ClO_4)_2\cdot 6H_2O$ and $Cu(ClO_4)_2\cdot 6H_2O$ were tested against clinical isolates of both Gram-positive and Gram-negative bacteria and also against a clinical isolate of *C. albicans*. The modes of action of selected compounds, showing anti-*Candida* activity, were also investigated.

2. Experimental

2.1. Materials/instrumentation

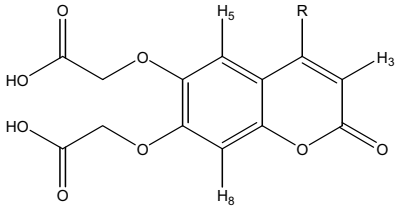
Reagent grade chemicals and solvents were purchased from Sigma–Aldrich Co. (Dorset, UK) and were used without further purification. Infrared spectra of solids (in a KBr matrix) were recorded in the region 4000–400 cm^{-1} on a Nicolet Impact 410 Fourier-Transform Infrared Spectrophotometer. Melting points were recorded on a Stuart Scientific SMP-1 apparatus (up to 300 °C). A JEOL JNM-LA300 FT NMR spectrometer was used to record ^1H (300 MHz, −5 to 15 ppm from TMS) and ^{13}C (79 MHz, −33 to 233 ppm from TMS) NMR spectra of the ligands as solutions in d_6-DMSO. Atomic absorption spectroscopy measurements were recorded on a Perkin–Elmer 460 AAS instrument (emission wavelength 324.8 nm). Microanalytical data were provided by the Microanalytical Laboratory, National University of Ireland, Belfield, Dublin 4. Solid state magnetic susceptibility measurements were carried out at room temperature using a Johnson Matthey Magnetic Susceptibility Balance with $[HgCo(SCN)_4]$ being used as a reference standard. UV–Vis spectra were recorded using a Cary IE Varian UV–Vis spectrophotometer. Sterol concentrations were determined using a gas chromatographic system (Hewlett–Packard 5890, Series 11) with a flame ionisation detector and a Chromopack capillary column (Chromopack International BV, Middleburg, The Netherlands) operated isothermally at 300 °C. Injector and detector temperatures were 320 °C and the carrier gas was N_2.

2.2. Ligand synthesis

Assignments of ^1H NMR spectra of ligands are based on the numbering scheme shown in Fig. 1.

2.2.1. Coumarin-6,7-dioxyacetic acid (2-(2-oxo-2H-chromen-6-yloxy-7-carboxymethyloxy)acetic acid) ($cdoaH_2$) (**1**)

A suspension of 6,7-dihydroxycoumarin (2.00 g, 11.2 mmol) and potassium carbonate (3.41 g, 24.7 mmol) in acetone (100 mls) was refluxed for 0.5 h. Upon cooling, methyl bromoacetate (4.29 g, 2.7 ml, 28.1 mmol) was added over a 5 min period and the resulting solution was refluxed for a further 4 days. After cooling, the mixture



1. R = H,
2. R = Me

Fig. 1. Numbering scheme used for ^{1}H NMR assignment of ligands used in this study.

was filtered and the filtrate was then evaporated to leave an off-white, crude solid. This solid was recystallised from methanol to give the white solid dimethyl coumarin-6,7-dioxyacetate. The purity of the product was confirmed by TLC and ^{1}H NMR analysis (Yield 2.2 g, 56%).

A mixture of dimethyl coumarin-6,7-dioxyacetate (2.50 g, 7.75 mmol) in distilled water (50 ml) and concentrated hydrochloric acid (37%, 5 ml) was refluxed for 5 h. On cooling, the white solid cdoaH_2 (**1**) precipitated. The solid was filtered off, washed with cold ethanol and water and then dried for 4 days in vacuo at 50 °C. Yield: 2.2 g (96%); m.p. 218–220 °C; TLC 0.14 [(70:30) ethyl acetate:hexane]; *Anal.* Calc. for $C_{13}H_{10}O_8$: C, 53.07; H, 3.42. Found: C, 52.92; H, 3.34%. ^{1}H NMR (d_6-DMSO, ppm; s, single; d, doublet): 4.72 (s, 2H, –OCH_2COOH), 4.84 (s, 2H, –OCH_2COOH), 6.27 (d, $J = 9.3$ Hz, 1H, vinyl-H3), 7.93 (d, $J = 9.5$ Hz, 1H, vinyl-H4), 6.98 (s, 1H, Ar-H5), 7.21 (s, 1H, Ar-H8); ^{13}C NMR (d_6-DMSO, ppm): 65.1, 65.4 (CH_2–O), 101.4 (Ar C–H8), 111.4 (Q, Ar C_{fused}), 111.6 (Ar C–H5), 113.2 (C–H_{vinyl}), 144.2 (C–H_{vinyl}), 144.1 (Q, Ar C_{fused}–O), 149.4 (Q, Ar C_{fused}–O), 150.9 (Q, Ar C_{fused}–O), 160.2 (Q, C=$O_{lactone}$), 169.5, 169.9 (Q; C=O_{acid}). IR (cm^{-1}): ν_{OH} 3271, $\nu_{C=O}$(acid) 1755, $\nu_{C=O}$(lactone) 1730. Soluble in: DMSO, alcohols, hot water.

2.2.2. 4-Methylcoumarin-6,7-dioxyacetic acid (2-2-oxo-2H-chromen-4-methyl-6-yloxy-7-carboxymethyloxy)acetic acid, (4-MecdoaH$_2$) (**2**)

This white compound was synthesised by the same method as that employed to prepare (**1**) except 4-methyl-6,7-dihydroxycoumarin was used instead of 6,7-dihydroxycoumarin. Yield: 1.65 g (91%); m.p. 240–242 °C; TLC 0.11 [(70:30) ethyl acetate:hexane]; *Anal.* Calc. for $C_{14}H_{12}O_8$; C, 54.55; H, 3.92. Found: C, 54.29; H, 3.82%. ^{1}H NMR (d_6-DMSO, ppm, s, singlet: d, doublet): 2.38 (d, $J = 1.1$ Hz, 3H, CH_3), 4.82 (s, 2H, –OCH_2COOH), 4.88 (s, 2H, –OCH_2-COOH), 6.23 (d, $J = 1.1$ Hz, 1H, vinyl-H3), 7.01 (s, 1H, Ar-H5), 7.18 (s, 1H, Ar-H8); 13.11 (COOH); ^{13}C NMR (d_6-DMSO, ppm): 18.3 (CH_3), 65.1, 65.8 (CH_2–O), 101.4 (Ar C–H8), 109.1 (Q, Ar C_{fused}), 112.4 (C–H_{vinyl}), 111.7 (Ar C–H5), 144.1 (Q, Ar C_{fused}–O), 148.8 (C–H_{vinyl}), 151.0, 153.2 (Q, Ar C–O), 160.2 (Q, C=O_{lac}) 169.5, 170.0 (Q, C=O_{acid}). IR (cm^{-1}): ν_{OH} 3046, $\nu_{C=O}$(acid) 1760, $\nu_{C=O}$(lactone) 1741. Soluble in: DMSO, alcohols, hot water.

2.3. Syntheses of Cu(II) and Mn(II) complexes

2.3.1. [Cu(cdoa)(H$_2$O)$_2$] · 1.5H$_2$O (**3**)

cdoaH_2 (**1**) (0.200 g, 0.68 mmol) was dissolved in an ethanol:water mixture (4:1, 50 ml) with heating and stirring. Dicopper(II) tetraacetate dihydrate (0.135 g, 0.340 mmol) was added with stirring and the resulting solution was then refluxed with stirring for 2 h. A pale blue precipitate formed during reflux. The mixture was filtered whilst hot and the blue solid was washed with water and hot ethanol and then air-dried. Yield: 0.24 g (86%); *Anal.* Calc. for $C_{13}H_{14}CuO_{11}$: C, 37.28; H, 3.61; Cu, 15.17. Found: C, 37.42; H, 3.44; Cu, 14.95%. IR (cm^{-1}): ν_{OH} 3052, $\nu_{C=O}$(lactone) 1685, ν_{asym}(OCO) 1607, ν_{sym}(OCO) 1395, ν_{asym}(COC) 1277, ν_{sym}(COC) 1048, $\nu_{M\text{-}O}$ 819. μ_{eff}: 1.98 B.M.; Soluble in: DMSO. UV–Vis (DMSO): $\lambda_{291\ nm}$ $\varepsilon = 9770$ M^{-1} cm^{-1}, $\lambda_{340\ nm}$ $\varepsilon = 12{,}800$ M^{-1} cm^{-1}, $\lambda_{741\ nm}$ $\varepsilon = 24.6$ M^{-1} cm^{-1}: UV–Vis (Nujol): $\lambda = 747$ nm.

2.3.2. [Cu(4-Mecdoa)(H$_2$O)$_2$] (**4**)

This blue complex was synthesised by the same method as that employed for (**3**), using 4-MecdoaH$_2$. Yield: 0.21 g (79%); *Anal.* Calc. for $C_{14}H_{14}CuO_{10}$: C, 41.44; H, 3.48; Cu, 15.60. Found: C, 41.26; H, 3.31; Cu, 15.45%. IR (cm^{-1}): ν_{OH} 3309, $\nu_{C=O}$(lactone) 1752, ν_{asym}(OCO) 1605, ν_{sym}(OCO) 1397, ν_{asym}(COC) 1279, ν_{sym}(COC) 1049, $\nu_{M\text{-}O}$ 844, 819. μ_{eff}: 1.93 B.M.; Soluble in: DMSO. UV–Vis (DMSO): $\lambda_{285\ nm}$ $\varepsilon = 13{,}100$ M^{-1} cm^{-1}, $\lambda_{336\ nm}$ $\varepsilon = 17{,}200$ M^{-1} cm^{-1}, $\lambda_{796\ nm}$ $\varepsilon = 26.7$ M^{-1} cm^{-1}: UV–Vis (Nujol): $\lambda = 787$ nm.

2.3.3. [Mn(cdoa)(H$_2$O)$_2$] (**5**)

This white compound was synthesised by the same method as that employed for (**3**) using $MnCl_2 \cdot 4H_2O$. Yield: 0.110 g (25%); *Anal.* Calc. for $C_{13}H_{12}MnO_{10}$; C, 40.74; H, 3.15. Found: C, 40.26; H, 3.01%. IR (cm^{-1}): ν_{OH} 3421, $\nu_{C=O}$(lactone) 1746, ν_{asym}(OCO) 1616, ν_{sym}(OCO) 1395, ν_{asym}(COC) 1280, ν_{sym}(COC) 1037, $\nu_{M\text{-}O}$ 832. μ_{eff}: 5.88 B.M.; Soluble in: DMSO. UV–Vis (DMSO): $\lambda_{273\ nm}$ $\varepsilon = 4575$ M^{-1} cm^{-1}, $\lambda_{303\ nm}$ $\varepsilon = 4943$ M^{-1} cm^{-1}.

2.3.4. [Mn(4-Mecdoa)(H$_2$O)$_2$] · 0.5H$_2$O (**6**)

This white compound was synthesised by the same method as that employed for (**5**), using 4-MecdoaH$_2$. Yield: 0.150 g (64%); *Anal.* Calc. for $C_{14}H_{15}MnO_{10.5}$; C, 43.32; H, 3.38. Found: C, 43.27; H, 3.28%. IR (cm^{-1}): 3401, $\nu_{C=O}$(lactone) 1727, ν_{asym}(OCO) 1601, ν_{sym}(OCO) 1392, ν_{asym}(COC) 1275, ν_{sym}(COC) 1048 cm^{-1}; $\nu_{M\text{-}O}$ 820. μ_{eff}: 5.93 B.M.; Soluble in: DMSO. UV–Vis (DMSO): $\lambda_{279\ nm}$ $\varepsilon = 3677$ M^{-1} cm^{-1}, $\lambda_{380\ nm}$ $\varepsilon = 2687$ M^{-1} cm^{-1}.

2.3.5. [Cu(cdoa)(phen)$_2$]·6H$_2$O (7)

A solution of cdoaH$_2$ (0.200 g, 0.680 mmol), dicopper(II) tetraacetate dihydrate (0.135 g, 0.340 mmol) and phen (0.247 g, 1.37 mmol) in an ethanol:water mixture (4:1, 50 mls) was refluxed for 3 h and upon cooling a green precipitate formed. The solid was isolated by filtration, washed with water and ethanol and dried in a vacuum oven at 50 °C for 2 days. Yield: 0.311 g (52%); *Anal.* Calc. for $C_{37}H_{36}CuN_4O_{14}$, C, 53.92; H, 4.40; N, 6.80. Found: C, 53.55; H, 4.35; N, 6.36%. IR (cm^{-1}): ν_{OH} 3036, $\nu_{C=O}$(lactone) 1707, ν_{asym}(OCO) 1617, 1565, ν_{sym}(OCO) 1395, 1426, ν_{asym}(COC) 1277, ν_{sym}(COC) 1037. μ_{eff}: 1.86 B.M. Soluble in: DMSO, alcohols, hot water. UV–Vis (DMSO): $\lambda_{270\ nm}$ $\varepsilon = 68{,}300\ M^{-1}\ cm^{-1}$, $\lambda_{335\ nm}$ $\varepsilon = 13{,}300\ M^{-1}\ cm^{-1}$, $\lambda_{696\ nm}$ $\varepsilon = 77\ M^{-1}\ cm^{-1}$: UV–Vis (Nujol): $\lambda =$ 803 nm. Crystals suitable for X-ray diffraction studies were obtained by redissolving the solid in a 1:1 methanol:water mixture and allowing the solution to stand for several days.

2.3.6. [Cu(4-MeCdoa)(phen)$_2$]·7H$_2$O (**8**)

This green compound was synthesised by the same method as that employed for (**7**) using 4-MecdoaH$_2$. Yield: 0.286 g (48%); *Anal.* Calc. for $C_{38}H_{40}CuN_4O_{15}$; C, 53.3; H, 4.71; N, 6.54. Found: C, 53.97; H, 4.54; N, 6.43%. IR (cm^{-1}): ν_{OH} 3411, $\nu_{C=O}$(lactone) 1712, ν_{asym}(OCO) 1616, 1565, ν_{sym}(OCO) 1391, 1426, ν_{asym}(COC) 1277, ν_{sym}(COC) 1037. μ_{eff}: 1.91 B.M.; Soluble in: DMSO, alcohols, hot water. UV–Vis (DMSO): $\lambda_{290\ nm}$ $\varepsilon = 54{,}208\ M^{-1}\ cm^{-1}$, $\lambda_{342\ nm}$ $\varepsilon = 14{,}776\ M^{-1}\ cm^{-1}$, $\lambda_{742\ nm}$ $\varepsilon = 67.2\ M^{-1}\ cm^{-1}$: UV–Vis (Nujol): $\lambda = 820$ nm. Crystals suitable for X-ray diffraction studies were obtained by redissolving the solid in a 1:1 methanol:water mixture and allowing the solution to stand for several days.

2.4. X-ray crystallography

Single crystals of **7** and **8** were analysed at 150 K using a Nonius Kappa CCD diffractometer equipped with graphite monochromated Mo Kα radiation. Details of the data collections, solutions and refinements are given in Table 1. Both structures were solved using SHELXS-97 [34] and refined using full-matrix least-squares in SHELXL-97 [35]. Convergence was uneventful, with the following points of note. The asymmetric unit of **7** consists of one copper complex molecule and 8.8 water molecules. Hydrogens were located for all full water molecules (O10, O11, O12, O13, O14, O16) and for the partial water based on O15 (75% occupancy). These solvent hydrogens were refined at a distance of 0.89 Å from the relevant parent atoms. The remaining water hydrogen atoms could not be readily located, even by considering a difference Fourier electron density map computed on low Bragg angle data, and hence they were omitted from the refinement. In addition to one

Table 1
Crystal data and structure refinement for **7** and **8**

Complex	**7**	**8**
Empirical formula	$C_{37}H_{41.60}CuN_4O_{16.80}$	$C_{38}H_{52}CuN_4O_{21}$
Formula weight	874.68	964.38
Temperature (K)	150(2)	150(2)
Wavelength (Å)	0.71073	0.71073
Crystal system	Triclinic	Triclinic
Space group	$P\bar{1}$	$P\bar{1}$
a (Å)	10.9390(3)	11.0260(2)
b (Å)	11.5730(3)	13.4450(3)
c (Å)	15.9080(4)	16.0120(4)
Volume (Å^3)	1916.51(9)	2208.62(8)
Z	2	2
Density (calculated, Mg/m^3)	1.516	1.450
Absorption coefficient (mm^{-1})	0.653	0.580
$F(000)$	910	1010
Crystal size (mm)	$0.35 \times 0.25 \times 0.10$	$0.25 \times 0.20 \times 0.10$
Theta range (°)	3.57–27.43	4.17–27.45
Index ranges	$-14 \leqslant h \leqslant 13$; $-14 \leqslant k \leqslant 14$; $-19 \leqslant l \leqslant 20$	$-14 \leqslant h \leqslant 14$; $-16 \leqslant k \leqslant 17$; $-20 \leqslant l \leqslant 20$
Reflections collected	33,659	41,143
Independent reflections [R_{int}]	8615 [0.0695]	10038 [0.0526]
Reflections observed	6312	7296
Data Completeness	0.987	0.993
Absorption correction	Semi-empirical from equivalents	None
Maximum and minimum transmission	0.9544	0.870
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data/restraints/parameters	8615/15/607	10038/39/683
Goodness-of-fit on F^2	1.081	1.075
Final R indices [$I > 2\sigma(I)$]	$R^1 = 0.0617$ $wR_2 = 0.1388$	$R_1 = 0.0495$ $wR_2 = 0.1249$
R indices (all data)	$R^1 = 0.0942$ $wR_2 = 0.1535$	$R_1 = 0.0783$ $wR_2 = 0.1385$
Largest difference in peak and hole (eÅ^{-3})	1.136 and −0.816	0.594 and −0.503

molecule of the copper complex, the asymmetric unit in **8** was seen to also contain 13 full occupancy waters. The solvent hydrogens were universally located and refined in a similar manner to those in **7**.

Crystallographic data for **7** and **8** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publications CCDC 622270 and 622271, respectively. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax(+44) 1223 336033, e-mail: deposit@ccdc.cam.ac.uk].

2.5. Antimicrobial studies

2.5.1. Assessment of antibacterial activity

All bacterial isolates were obtained clinically: *S. aureus* (urinary track infection), methicillin resistant *S. aureus* (wound infection), *Staphylococcus simulans* (facial skin), *Micrococcus luteus* (facial skin), *Escherichia coli* (gastrointestinal tract), *Bacillus oleronius* (facial skin), *Patonea agglumerans* (facial skin).

Bacterial strains were grown overnight in nutrient broth medium at 30 °C and 200 rpm in an orbital incubator. The absorbance of these cultures was measured at 660 nm and cultures were diluted to an optical density of 0.1. The cell suspension (100 μl) was added to the wells of a 96 well plate containing test compound dissolved in nutrient broth medium in serial dilutions from 100 to 0.25 μg/ml. Test compounds for all the antimicrobial studies were initially prepared in DMSO and then diluted with nutrient broth, as above, so that the final concentration of DMSO in the cell suspension was never more than 1%. Plates were incubated at 30 °C for 24 h and the optical density was measured spectrophotometrically (Dynex Technology) at 450 nm.

2.5.2. Antifungal susceptibility testing

C. albicans ATCC 10231 (obtained from the American Type Culture Collection, MD, USA) was maintained on YEPD agar [2% (w/v) glucose (Sigma–Aldrich Chemical Co Ltd., Dublin, Ireland), 2% (w/v) bactopeptone (Difco Laboratories, Detroit, USA), 1% (w/v) yeast extract (Oxoid Ltd., Basingstoke, England), 2% (w/v) agar] plates and subcultured every 6–8 weeks and stored at 4 °C. Fresh cultures were grown at 30 °C in YEPD broth (as above but without agar). All cultures were grown to the stationary phase (approximately 1×10^8 cells/ml) overnight in 50 ml Antibiotic Medium 3 (Oxoid Ltd.) broth at 30 °C and 200 rpm.

Antifungal susceptibility testing was performed using broth microdilution assays according to the National Committee for Clinical Laboratory standards (Document M27-A2) protocol with slight modifications. The M27-A2 method was altered by substituting antibiotic medium 3 for RPMI 1640 medium. Using this method MIC_{80} values were determined spectrophotometrically at 405 nm by comparing the turbidity of growth in each well. MIC_{80} is defined as the lowest concentration of drug that inhibits fungal growth by 80% relative to the control.

2.5.3. Measurement of oxygen uptake

The respiration rate of yeast cells was determined using a Clark-type oxygen electrode (Techne, Rank Brothers Ltd., Cambridge, UK). This electrode consists of a platinum cathode and silver anode. Both the cathode and anode were immersed in a potassium chloride buffer and are separated from the test solution by a Teflon membrane. A disc of lens tissue with a hole in the middle was placed over the anode and cathode, in order to leave the cathode uncovered. The lens tissue had previously been immersed in 0.5 M potassium chloride. Following this, a Teflon membrane was placed over the electrode and the test chamber was placed on top of the electrode and screwed to place. This chamber was maintained at 30 °C by circulating water around the walls of the chamber.

Prior to the determination of respiration rates, the electrode was calibrated using sodium dithionate, thus allowing determination of 100% and 0% oxygen levels. Yeast cells in the late exponential phase (10 h), were grown in the presence of test agent, using a concentration equivalent to half the MIC_{80} at 30 °C, and in an orbital incubator (Certomat R).

Yeast cells at a density of 1×10^8 cells were harvested and washed in PBS and then resuspended in 0.025 M phosphate buffer, pH 7.2. Phosphate buffer (0.025 M) was prepared following the addition of 72 ml of 0.05 M disodium hydrogen phosphate, and 28 ml of 0.05 M sodium dihydrogen phosphate, to a final volume of 100 ml in distilled water. Oxygen consumption was determined following the addition of 100 μl of cell sample into the oxygen electrode sample chamber, which previously contained 1.9 ml of phosphate buffer. The rate of oxygen consumption was measured for 30 s, with data recorded using a strip chart recorder. Oxygen consumption was calculated using the following formulae, and was expressed as μM of oxygen consumed/10^8 cells. Oxygen consumption = number of units travelled/span (100% level − 0% level) × 468.75 μM.

2.5.4. Cytochrome analysis

Test agent was added to yeast cells and incubated for 10 h. Cells with a density of 2×10^{10} cells/ml were harvested by centrifugation at 3000*g* for 5 min, and washed twice with PBS. The cells were divided into two equal volumes, one half was oxidised by suspending in 0.2% (w/v) sodium hypochlorite solution followed by harvesting using centrifugation, and subsequently resuspended in 50% (v/v) glycerol. The second half of the sample was resuspended in 50% (v/v) glycerol and reduced by adding a few crystals of sodium-dithionate. Differential spectra of reduced and oxidised cytochrome were immediately recorded using a double beam UV–Vis spectrophotometer at 500–650 nm (Cary IE Varian).

2.5.5. Sterol extraction

Yeast cells were grown in the presence of half the MIC_{80} concentration of test agent in antibiotic medium 3 at 30 °C in an orbital incubator (Certomat R), until cells reached the late exponential phase (ca. 10 h). Cells at a density of

2×10^9 cells/ml were harvested and washed with PBS. Cells were resuspended in 1.5 ml of a solution containing 20% (w/v) potassium hydroxide and 60% (v/v) ethanol, and placed in a shaking water bath at 90 °C for 1.5–2 h. Heptane was added to this solution and it was then vortexed for 10 s. The upper layer containing sterols was removed according to the method of Arthington-Skaggs et al. [36].

2.5.6. Analysis of yeast sterols

Sterol analysis was determined by using double beam UV–Vis spectrophotometer (Cary IE Varian) over the wavelength range of 240–320 nm. An ergosterol standard curve was constructed (0.25–100 μg/ml). In addition, sterol concentration was also determined using a gas chromatograph.

3. Results and discussion

3.1. Chemical synthesis and characterisation

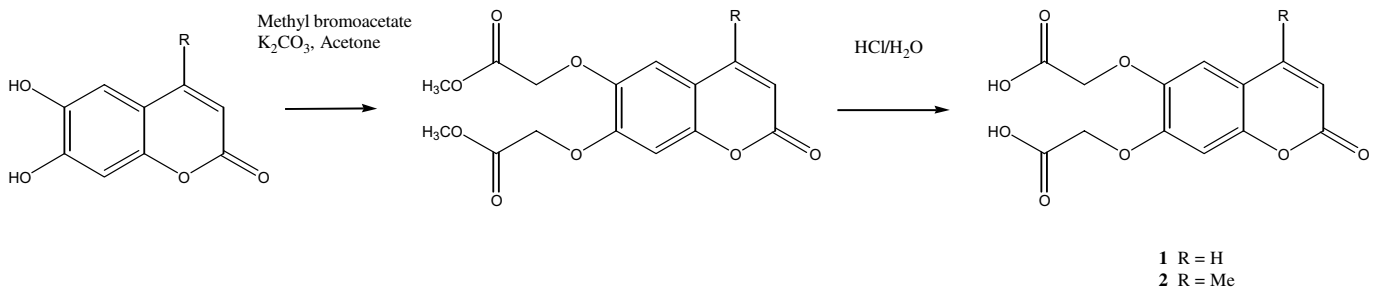
The dicarboxylate coumarin ligands **1** and **2** were prepared by etherification of the precursor dihydroxycoumarins followed by acid hydrolysis (Scheme 1). The Cu(II) and Mn(II) dicarboxylate complexes **3**–**6** were prepared by simple ligand anion exchange reactions by refluxing the appropriate dicarboxylate ligand with dicopper(II) tetraacetate dihydrate or manganese(II) chloride tetrahydrate in water (Scheme 2). These dicarboxylate complexes were insoluble in water and all common solvents (except DMSO) suggesting they may be polymeric in nature, and repeated attempts to recrystallise them failed. Elemental analyses indicated a metal to ligand ratio of 1:1 and with either two or three water molecules in the formulation.

The Cu(II) dicarboxylate/phen complexes **7** and **8** were obtained by refluxing the respective coumarin-6,7-dioxyacetic acid with phen and dicopper(II) tetraacetate dihydrate in ethanol:water (4:1). These phen complexes were soluble in hot water, alcohols and DMSO. The microanalytical data reported shows loss of water molecules from the crystal lattice of both complexes over time. Attempts to prepare the Mn(II) analogues were unsuccessful.

The IR ν_{asym}(OCO) and ν_{sym}(OCO) stretching frequencies and the $\Delta\nu$(OCO) values for the metal complexes (**3**–**8**) are listed in Table 2. The spectra of complexes **3**–**6** were very similar and all had a $\Delta\nu(\mathrm{OCO}) > 200\ \mathrm{cm}^{-1}$, a value normally considered to indicate unidentate carboxylate coordination [37]. These data are also consistent with those previously reported for Mn(II) and Cu(II) complexes of benzene-1,2-dioxyacetic acid ($\mathrm{bdoaH_2}$) [38]. Ethereal (COC) asymmetric and symmetric vibrational frequencies for complexes **3**–**8** (Table 2) are also similar to those published for benzene-1,2-dioxyacetate complexes [39–41]. The X-ray structures of bdoa^{2-} complexes indicated an octahedral geometry about the metal centre, with the bdoa^{2-} ligand coordinated in a quadridentate fashion via a carboxylate oxygen from each of the acid functions and from the two ethereal oxygen atoms [38]. However, while a similar structure for complexes **3**–**6** could be possible, the general insolubility of complexes **3**–**6** suggests that there may also be intermolecular interactions between individual monomeric units (possibly via a carboxylate oxygen and/or the lactone carbonyl oxygen) leading to polymeric structures. Indeed, it is also possible that the quadridentate binding environment about the metal centre could be generated from carboxylate groups of alternating dioxyacetic acid ligands. The IR spectra of the complexes **3**–**6** also contain bands between 840 and 820 cm^{-1}, which are not present in the free ligands, which are characteristic of coordinated water [42]. The UV–Vis spectra of complexes **3** and **4**, recorded in DMSO solution as well as in the solid state as a Nujol mull show approximately similar shapes and positions of the absorption bands, indicating no appreciable change in the geometry of these complexes in solution. The room temperature magnetic moment (μ_{eff}) values of complexes **3**–**6** (calculated by assuming a monomeric formulation) are in agreement with those expected of simple mononuclear complexes in which there are no metal–metal interactions.

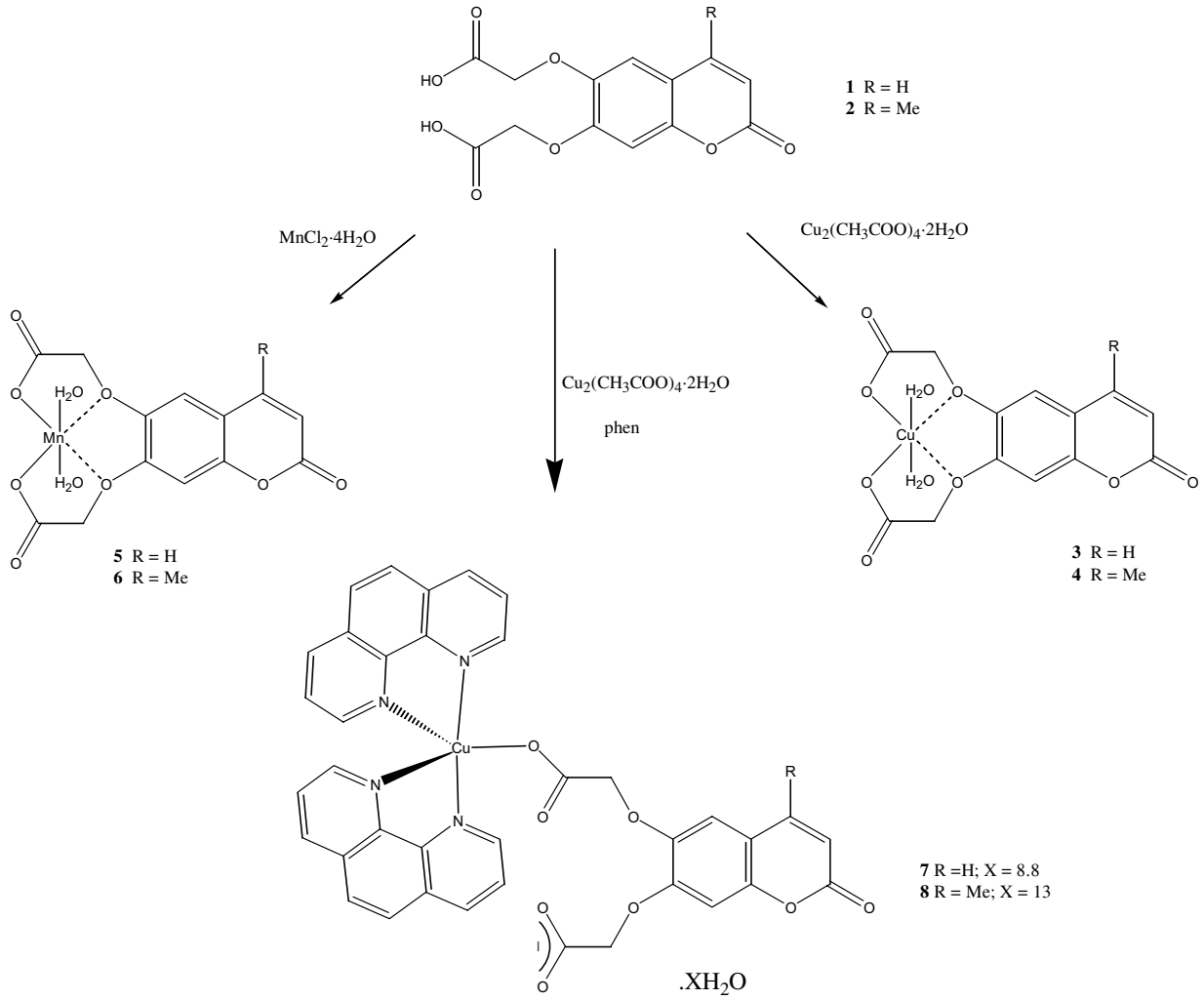
The IR spectra of the phen-containing complexes **7** and **8** were similar to each other and showed distinct bands for the phen ligands at 1426, 850 and 723 cm^{-1}. For these two complexes there were two separate sets of ν_{asym}(OCO) and ν_{sym}(OCO) vibrations. One set of values, with $\Delta\nu(\mathrm{OCO}) > 200\ \mathrm{cm}^{-1}$, was indicative of unidentate coordination to the Cu(II) centre. Two additional bands at ca. 1426 and 1565 cm^{-1}, were assigned to ν_{asym}(OCO) and ν_{sym}(OCO) vibrations of the uncoordinated carboxylate group [43].

The UV–Vis spectra of **7** and **8**, recorded in DMSO solution and as a Nujol mull do show appreciable



1 R = H
2 R = Me

Scheme 1. Synthesis of the coumarin-6,7-dioxyacetic acid ligands.



Scheme 2. Synthesis of the coumarin-6,7-dioxyacetic acid complexes shown with proposed structures for complexes. Complexes **3**–**6** are likely to be polymeric in nature.

Table 2
Selected IR data and magnetic moments for complexes **3**–**8**

Compound	ν_{asym}(OCO) cm^{-1}	ν_{sym}(OCO) cm^{-1}	$\Delta\nu$OCO cm^{-1}	ν_{asym}(COC) cm^{-1}	ν_{sym}(COC) cm^{-1}	μ_{eff} (B.M.)
[Cu(cdoa)(H$_2$O)] · 1.5H$_2$O (**3**)	1607	1395	212	1277	1048	1.98
[Cu(4-Mecdoa)(H$_2$O)$_2$] (**4**)	1605	1397	208	1279	1049	1.93
[Mn(cdoa)(H$_2$O)$_2$] (**5**)	1616	1395	221	1280	1037	5.88
[Mn(4-Mecdoa)(H$_2$O)$_2$)] · 0.5H$_2$O (**6**)	1600	1392	208	1275	1048	5.94
[Cu(cdoa)(phen)$_2$] · 8.8H$_2$O (**7**)	1617	1390	227	1277	1037	1.86
	1565	1426	139			
[Cu(4-Mecdoa)(phen)$_2$] · 13H$_2$O (**8**)	1616	1391	225	1277	1037	1.91
	1565	1426	139			

differences, indicating a change in stereochemistry about the copper centre on dissolution [44].

3.2. Crystal structure analysis

The X-ray crystal structures of the Cu(II) complexes **7** and **8** are shown in Figs. 2 and 3, respectively, with pertinent bond lengths and angles listed in Tables 3 and 4, respectively. The packing diagram for **7** is shown in Fig. 4. There is extensive hydrogen bonding in both structures, involving available acceptors in the copper complexes and the lattice waters.

Complex **7** has an approximate trigonal bipyramidal geometry ($\tau = 0.663$) [45] with the metal coordinated by two chelating phens and one oxygen (O1) of a carboxylate function on the meridional trigonal plane (O1, N2, N4). The acid group coordinated to the metal centre is the one attached to the pendant ethereal arm 6-position of the

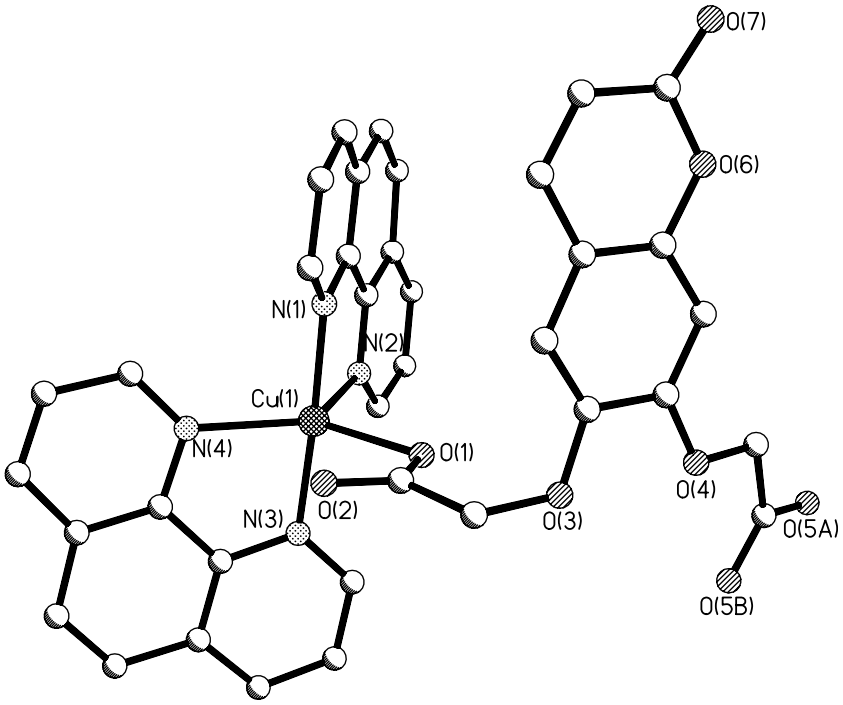


Fig. 2. Molecular structure of **7**. Solvent and hydrogen atoms are omitted for clarity.

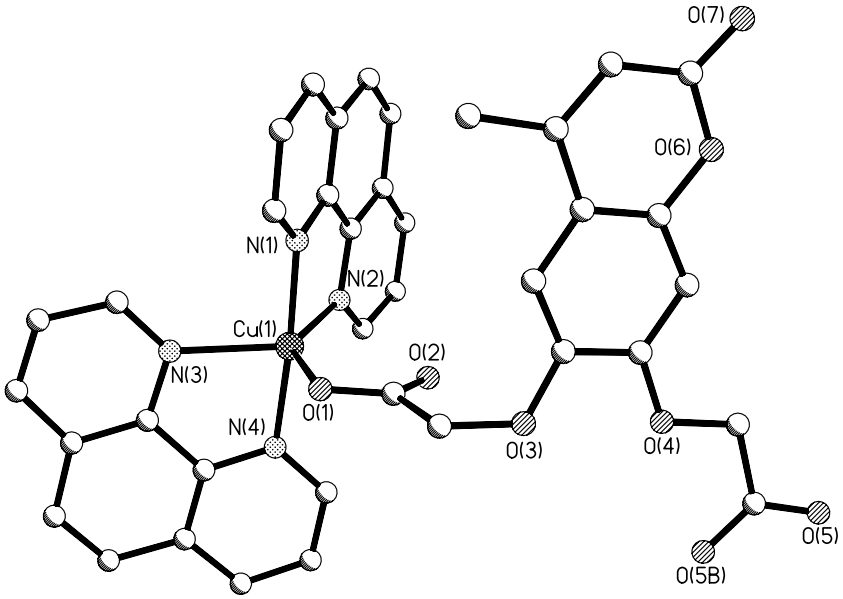


Fig. 3. Molecular structure of **8**. Solvent and hydrogen atoms are omitted for clarity.

coumarin ligand. Cu–N bond lengths range from 1.984(3) to 2.123(3) Å and are similar to those reported for other Cu–N bonds with *N*,*N*′-donor ligands [46]. The copper to oxygen bond length is 2.076(2) Å which is also similar to other unidentate copper(II) carboxylate bond lengths [47]. The bond angles of the meridian atoms illustrate deviation from ideal trigonal bipyramidal geometry; O1–Cu–N2, 102.54(10)°, N4–Cu–N2, 120.03(10)° and O1–Cu–N4, 137.39(10)°. The axial groups are close to perpendicular relative to the meridian plane, with N3–Cu–O1 = 90.56(10)°; N1–Cu–N4 = 95.72(11)°; N3–Cu–N1 = 177.19(11)°. There are also 8.8 water molecules per copper centre. The second acid function of the diacid is uncoordinated and deprotonated. The lactone oxygen of the coumarin, the ethereal oxygens of the dioxyacetic acid groups, the deprotonated uncoordinated carboxylate group attached to position 7 on the coumarin and the lattice waters are involved in an extensive network of hydrogen bonding (Fig. 4). Aryl stacking between the coordinated phen groups affords added stability and rigidity in the solid state.

Table 3
Selected bond lengths (Å) and angles (°) for $[Cu(Cdoa)(phen)_2]\cdot 8.8H_2O$ (**7**)

Cu(1)–N(3)	1.984(3)	Cu(1)–N(1)	1.988(3)
Cu(1)–O(1)	2.076(2)	Cu(1)–N(4)	2.086(3)
Cu(1)–N(2)	2.123(3)		
N(3)–Cu(1)–N(1)	177.19(11)	N(3)–Cu(1)–O(1)	90.56(10)
N(1)–Cu(1)–O(1)	92.12(10)	N(3)–Cu(1)–N(4)	81.72(11)
N(1)–Cu(1)–N(4)	95.72(11)	O(1)–Cu(1)–N(4)	137.39(10)
N(3)–Cu(1)–N(2)	99.19(12)	N(1)–Cu(1)–N(2)	81.06(12)
O(1)–Cu(1)–N(2)	102.54(10)	N(4)–Cu(1)–N(2)	120.03(10)

Table 4
Selected bond lengths (Å) and angles (°) for $[Cu(4\text{-Mecdoa})(phen)_2]\cdot 13H_2O$ (**8**)

Cu(1)–N(1)	1.995(2)	Cu(1)–N(4)	2.004(2)
Cu(1)–O(1)	2.065(2)	Cu(1)–N(2)	2.075(2)
Cu(1)–N(3)	2.144(2)		
N(1)–Cu(1)–N(4)	176.06(10)	N(1)–Cu(1)–O(1)	90.21(9)
N(4)–Cu(1)–O(1)	91.66(9)	N(1)–Cu(1)–N(2)	81.45(9)
N(4)–Cu(1)–N(2)	99.39(9)	O(1)–Cu(1)–N(2)	138.09(9)
N(1)–Cu(1)–N(3)	95.67(9)	N(4)–Cu(1)–N(3)	80.53(9)
O(1)–Cu(1)–N(3)	102.97(8)	N(2)–Cu(1)–N(3)	118.64(9)

The fused benzene rings of the coumarins may also be engaged in aryl stacking. The structure of **7** is similar to that reported for bis(2,2′-bipyridine)*p*-(phenylenedioxy)diacetatocopper(II) tetrahydrate [48].

The X-ray crystal structure of **8** (Fig. 3) is very similar to **7**, with the Cu(II) center in a distorted trigonal bipyramidal environment ($\tau = 0.633$) with two chelating phen ligands. The 4-methylcoumarin-6,7-dioxyacetic in **8** acid has also got one unidentate coordinated pendant carboxylate arm (position-6) but unlike complex **7** it is coordinated through the opposite oxygen of the carboxylate group.

3.3. Antibacterial screening

Manganese(II) and copper(II) dicarboxylates and their phen adducts have previously been shown to have antifungal activity against *C. albicans* [49–52]. The metal-free ligands (**1**, **2**), the metal complexes (**3**–**8**) and the simple salts $Cu(ClO_4)_2\cdot 6H_2O$ and $Mn(ClO_4)_2\cdot 6H_2O$ were screened for their antimicrobial activity (Table 5). The growth inhibition results are expressed as MIC_{80} values (minimum inhibitory concentration (μM) required to inhibit 80% of the growth of the microbe).

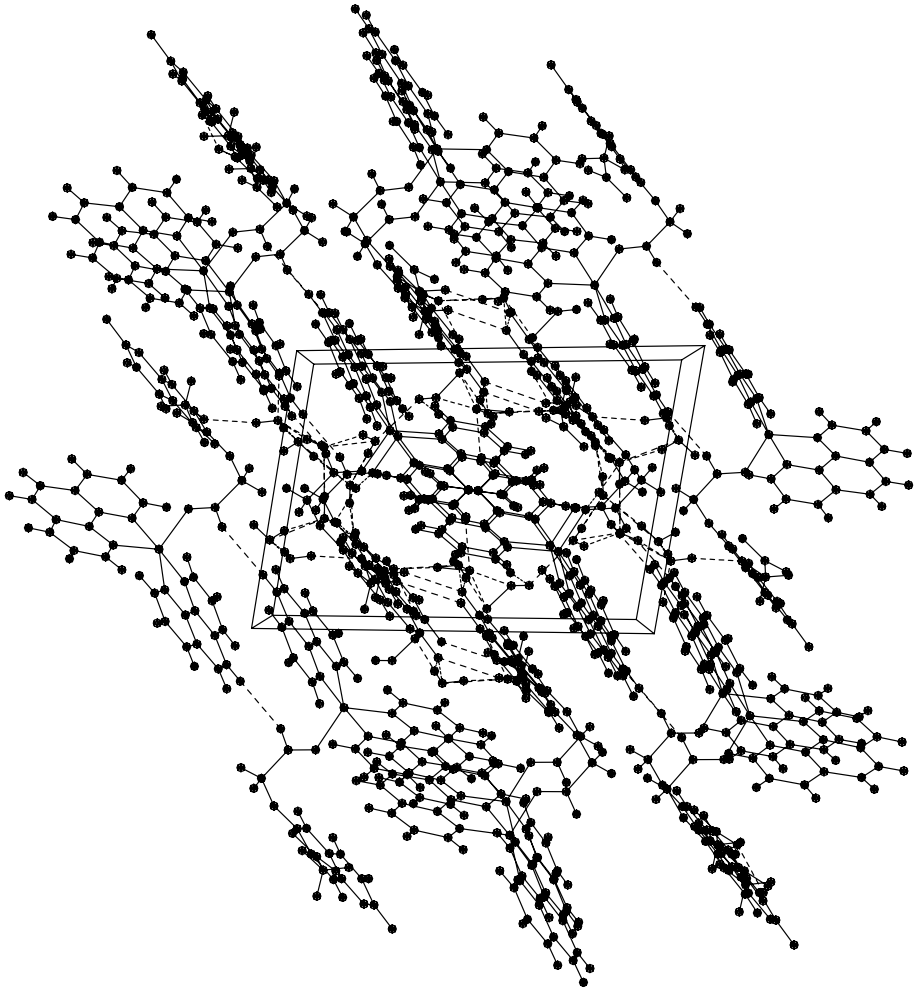


Fig. 4. Packing diagram for $[Cu(cdoa)(phen)_2]\cdot 8.8H_2O$ (**7**).

Table 5
Antimicrobial activity of test compounds [MIC_{80} (μM)]

Compound	SA	MRSA	SS	Ml	*E. Coli*	BO	PA	Can
1	282.5	194.9	267.2	84.2	153.3	240.2	167.3	87.74
2	264.4	208.1	293.6	92.6	149.5	159.6	153.7	98.5
3	>500	>500	>500	>500	>500	>500	>500	>500
4	>500	>500	>500	>500	>500	>500	>500	>500
5	>500	>500	>500	>500	>500	>500	>500	>500
6	>500	>500	>500	>500	>500	>500	>500	>500
7	19.9	12.1	17.8	22.8	14.9	31.9	12.6	22.2
8	24.3	37.5	28.3	43.7	44.7	27.5	74.1	42.7
Phen	259	150	223	315	>500	>500	448	16.7
$Cu(ClO_4)_2 \cdot 6H_2O$	>500	>500	>500	>500	>500	>500	>500	>500
$Mn(ClO_4)_2 \cdot 6H_2O$	>500	>500	>500	>500	>500	>500	>500	>500

(SA), *Staphylococcus aureus*: (MRSA), methicillin-resistant *Staphylococcus aureus*: (*S. Sim*) *Staphylococcus simulans*: (Ml) *Micrococcus luteus*: (*E. Coli*) *Escherichia coli*: (BO) *Bacillus olenius*: (PA) *Patonea agglumerans*: (Can) *Candida albicans*.

The water-soluble metal-free dicarboxylate ligands **1** and **2** showed a similar and wide range of antibacterial activity (MIC_{80} values ranging from 93 to 294 μM). However, the water-insoluble Cu(II) and Mn(II) dicarboxylate complexes **3**–**6** and the water–soluble Cu(II) and Mn(II) simple salts were inactive against all of the organisms. These data indicate that decreasing the ligand solubility (via complex formation) lowers the bioavailablility of the dicarboxylates.

Complexes **7** and **8** had significantly greater antimicrobial activity than both the metal-free dicarboxylic acids and phen, in particular against the clinically important MRSA and *E. coli* bacteria (Table 5). Complexation with phen or bipyridine has been used to enhance the antimicrobial activity of several metal ions, including copper [53–56]. Moreover, the phen ligand has been shown previously to act as a potential anti-tumour agent [57] and that anti-tumour activity can be increased on forming water-soluble copper complexes [58]. In addition, copper complexes containing phen are of considerable interest in nucleic acids chemistry, following the discovery of the 'chemical nuclease' activity of bis(phen)copper(I) complexes [59] and more recently that of a copper(II) complex of a phen-derived ligand [60]. A similar pattern is noted here in that the antibacterial activity of metal-free phen is considerably enhanced by the formation of Cu(II) complexes. Surprisingly, **7** was universally more active than the methylated analogue **8**, implying that the methyl group in the 4-position was having a deactivating effect. Overall, the activity of **7**, particularly against the clinically important MRSA and *P. agglumerans* ($MIC_{80} = 12.1$ and 12.6 μM, respectively), was considerably greater than the activity of phen, the coumarin ligand or the copper salt suggesting that it may well have therapeutic potential.

3.4. Antifungal activity

Against *C. albicans* metal-free phen and the phen-containing Cu(II) complexes **7** and **8** had significantly lower MIC_{80} values than the metal-free ligands **1** and **2** (Table 5). The values for **7** and **8** were comparable to those of the commercial antifungal agent ketoconazole (25 μM/ml) [33] but interestingly were not as active as the metal-free phen. Thus, unlike the antibacterial studies, whereby formation of a Cu(II) phen complex enhances the antibacterial activity of the phen ligand, the anti-*Candida* activity is reduced upon complex formation. Given our previous mechanistic studies on Cu(II)–phen complexes [18], some preliminary mode of antifungal action studies on **7** and **8** were undertaken.

3.4.1. Respiration rates

Previous studies have shown that fungal respiration is affected when cells are exposed to metal-based drugs [18,61,62]. Stationary phase *Candida* cells were exposed to **7** and **8** for 10 h and then oxygen-uptake measurements were made (Fig. 5). The complexes show a modest

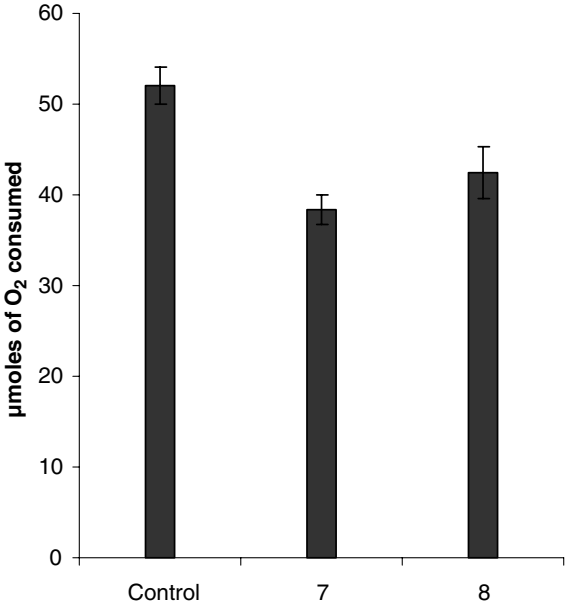


Fig. 5. Oxygen consumption of *C. albicans*. Fungal cells were treated with the complexes **7** and **8** at half of their MIC_{80} concentrations for 10 h. Bars indicate ±SEM. Control cells are those grown with no added complex.

reduction in O_2 consumption, in comparison to the control. An earlier study by our group has shown that metal-free phen induces a modest increase in oxygen uptake [18].

3.4.2. Cytochrome analysis

Cytochromes are an important component of the mitochondrial electron transfer chain. Previous work has indicated that impairment of cytochrome synthesis and/or function leads to a reduction in respiration rates in *Candida* cells following exposure to metal-based drugs [18,63]. The spectroscopic cytochrome profile of control cells indicates the presence of cytochromes *aa*3 (602 nm), *b* (564 nm) and *c* (550–554 nm) (Fig. 6). However, their spectroscopic profiles were altered when cells were exposed to **7** and **8**. In particular, the cytochrome *aa*3 peak was greatly reduced in yeast cells treated with **8**. Disruption of the mitochondrial cytochrome content of a cell has the potential to reduce its respiratory efficacy. Again a previous study had shown that metal-free phen retards the synthesis of cytochromes *b* and *c* [18].

3.4.3. Ergosterol content

Fungal cells require oxygen in order to synthesise the membrane sterol ergosterol. Thus, either a reduction in respiratory efficiency or an inability to respire leads to reduced levels of ergosterol [63]. Reduction in the ergosterol content in *C. albicans* has been identified previously as a mechanism for increased growth in the presence of the potent anti-fungal drug amphotericin B [63,64]. The requirement for a functional mitochondrion in ergosterol biosynthesis is well established and arises from the provision of NADPH for squalene dimerisation [63]. The relative ergosterol contents of drug-treated and control cells were determined (Fig. 7). *C. albicans* cells exposed to complexes **7** and **8** showed diminished levels of ergosterol, with complex **7** inflicting the most deleterious effect. As sterol synthesis is dependent upon a fully functional cellular respiratory system the decrease in ergosterol levels observed with these drugs indicate a disabled mitochondria.

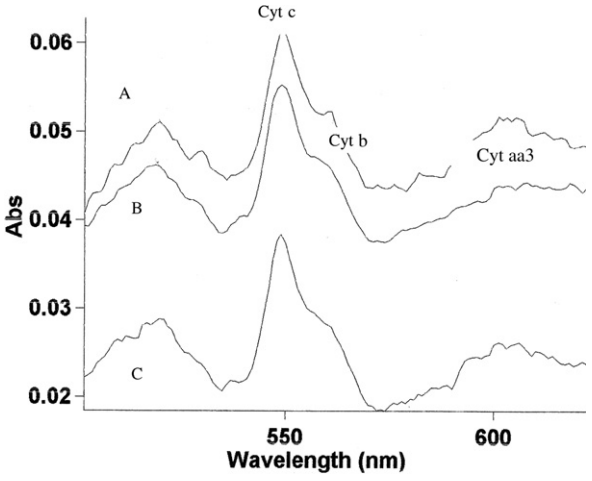


Fig. 6. Cytochrome profile of *C. albicans*. Fungal cells were treated with the metal complexes **7** (B) and **8** (C) at half of their MIC_{80} concentrations for 10 h. Control cells are those grown with no added complex (A).

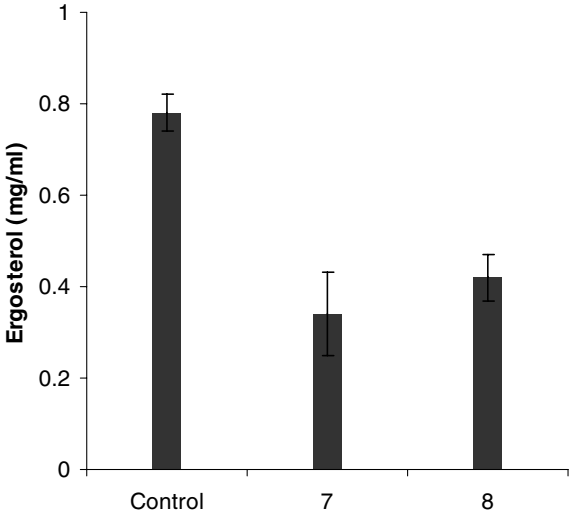


Fig. 7. Ergosterol content of *C. albicans*. Fungal cells were treated with **7** and **8** at half of their MIC_{80} concentrations for 10 h. Bars indicate ±SEM. Control cells are those grown with no added complex.

4. Conclusions

Two new coumarin-6,7-dioxyacetic acid ligands were prepared and each complexed to a Mn(II) and Cu(II) centre. Mixed-ligand dicarboxylate/phen complexs of Cu(II) were also isolated. The most active complexes, $[Cu(cdoa)(phen)_2]\cdot 8.8H_2O$ (**7**) and $[Cu(4\text{-}Mecdoa)(phen)_2]\cdot 13H_2O$ (**8**) displayed significant broad spectrum antimicrobial activity with particularly good activity against MRSA, *E. coli* and *P. agglumerans*.

It has previously been shown that N-donor derivatives of the dicarboxylate complexes of a range of metals are more effective antifungal agents than the simple dicarboxylate complexes and possess significantly different modes of action to the state-of-the-art prescription drugs [53,60,18] and the results presented here show a similar trend. When administered to *C. albicans* **7** and **8** inhibited respiration, reduced the levels of ergosterol in the membrane and altered cytochrome content. These results suggest that the antifungal effect of these complexes is mediated through the disruption of mitochondrial function, which is different to the mode of action of the conventional azole and polyene drugs.

In light of their antimicrobial activities and their distinct antifungal mode of action, **7** and **8** may find application as novel drugs for the treatment of microbial infections, and they may also be employed in conjunction with existing drugs for the treatment of infections demonstrating resistance to conventional agents.

Acknowledgements

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