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Synthesis and Evaluation of Antimicrobial Activity of New 3-Hydroxy-6-methyl-4-oxo-4*H*-pyran-2-carboxamide Derivatives

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The synthesis of a range of 3-hydroxy-4-oxo-4*H*-pyran-2-carboxamide with antimicrobial activity is described. Amide derivatives of pyranone were synthesised using TBTU [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] as a coupling agent and NMM (N-methylmorpholine) as a base. Antimicrobial activities were determined as MIC values using the microdilution broth method against *Staphylococcus aureus*, *Entrococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* for bacteria and *Candida albicans*, *C. krusei* and *C. parapsilosis* for fungi. 3-Hydroxy-6-methyl-4-oxo-4*H*-pyran-2-[*N*-(4′-methylcoumarin-7-yl)carboxamide] (**8c**) exhibited higher antibacterial activity against *S. aureus*, *E. faecalis* and *E. coli* than the other compounds. In addition, **8c** was more active against *C. krusei* than the other synthesised compounds.

Key Words: Chlorokojic acid, amide derivatives of pyranone, antibacterial activity, antifungal activity.

Introduction

Kojic acid (5-hydroxy-2-hydroxymethyl-pyran-4-one) is an antibiotic produced by many species of *Aspergillus* and *Penicillium* in an aerobic process from a wide range of carbon sources[1]. Some natural antibiotics contain a siderophore structure. Most siderophores contain either hydroxamate or catechol groups, which are used to sequester iron[2,3]. It is well known that kojic acid and its derivatives are reported to possess pharmacological effects such as herbicidal[4,5], antimicrobial[5−9], pesticidal and insecticidal[10], as well as being a antiacne[11] and skin whitening agents[12]. The bidentate chelating ligand kojic acid, which is analogous to the catechol-like

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function, forms stable complexes with several metal ions[13−14]. Hydroxypyranones and pyridinones that possess metal chelating ability have an inhibitory effect on the growth of *Eschericia coli*, *Listeria inocua* and *Staphylococcus aureus*[7,15,16]. Wolf et al. have previously demonstrated that 2-chloromethyl-5-hydroxy-pyran-4-one (chlorokojic acid, **1**) inhibited *Aeromonas aerogenes*, *Micrococcus pyogenes* var. *aureus*, *Salmonella typhosa*, *Penicilium digitalum*, *Russula nigricans* and *Saccharomyces cerevisiae* in the range of 0.02-0.07% [6].

The increasing numbers of pathogen bacteria and fungi that are resistant to the commonly used thera peutic agents is a major worldwide health problem. For these reasons the search for new antimicrobial agents with novel modes of action represents a major target in chemotherapy. In this paper, we describe the synthesis, structural properties and antimicrobial activities of 2 new amide series of 3-benzyloxy/hydroxy-6-methyl-4-oxo-4*H*-pyran (**7**,**8**) derivatives. Their antimicrobial activities were examined by using the microdilution broth method against various bacteria and fungi (Table).

Table. Antibacterial and antifungal activities of the synthesised compounds (MIC in μg/mL).

Compound Number	Bacteria (MIC-μg/mL)				Fungi (MIC-μg/mL)		
	S. aureus ATCC 29213	*E. faecalis* ATCC 29212	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 90028	*C. krusei* ATCC 6258	*C. parapsilosis* ATCC 22019
Kojic acid	256	256	128	128	128	128	128
1	256	256	256	64	128	32	32
2	256	256	256	128	128	128	128
3	256	256	128	128	128	128	128
4	256	256	256	256	128	128	128
5	64	256	256	128	128	64	64
6	256	256	256	128	128	128	128
7a	256	64	128	128	32	64	64
7b	256	128	128	128	32	64	32
7c	128	64	128	128	128	128	128
8a	64	64	64	128	128	128	128
8b	256	64	256	128	128	64	128
8c	8	32	32	128	8	4	128
Ceftazidime	4	-*	0.5	2			
Fluconazole					0.5	64	4

Experimental

Chemistry

All chemicals used in this study were supplied by Aldrich (England), or Fluka (England). Melting points were determined on a Thomas Hoover capillary melting point apparatus and were uncorrected. The IR spectra were recorded with a Perkin Elmer FT-IR spectrometer 1720 X as a KBr disc (γ, cm^{-1}). ^{1}H-NMR and ^{13}C-NMR spectra (DMSO-d$_6$) were recorded on Bruker AMX 400 MHz/ 52 MM NMR and R24B Perkin-Elmer 60 MHz NMR spectrometers. Chemical shifts (δ) are reported in ppm downfield from the internal

standard tetramethylsilane (TMS). Fast atom bombardment (FAB) mass spectra analyses were carried out by the Mass Spectrometry Facility, Department of Pharmaceutical and Biological Chemistry, The School of Pharmacy, London Mass spectra were recorded via VG Analytical ZAB-SE with Matrix m-nitrobenzylalcohol + Sodium and Xenon Gas @ 8 KV. Elemental analyses were performed by the Microanalytical Laboratories, Department of Chemistry, University of Manchester, U.K.

2-Chloromethyl-5-hydroxy-pyran-4-one (chlorokojic acid) (1), **5-hydroxy-2-methyl-pyran-4-one (allomaltol) (2)** and **3-hydroxy-2-hydroxymethyl-6-methyl-pyran-4-one (3)** were synthesised by following the methodology as described by Ellis et al[17].

3-Benzyloxy-2-hydroxymethyl-6-methyl-4-oxo-4*H*-pyran (4) (80%): mp 115-116 °C (lit. value[18] mp 115-116 °C). IR (KBr disc) 1651 cm^{-1} (C=O st). ^{1}H-NMR (DMSO-d_6, 60 MHz) δ 2.20 (3H; s; 6-CH_3), 4.20 (2H; d; J = 6 Hz; 2-CH_2OH), 5.00 (2H; s; -CH_2Ph), 5.25 (1H; t; J = 6 Hz; 2-CH$_2$OH), 6.05 (1H; s; H^5), 7.20 (5H; s; phenyl). MS (FAB): m/z 247 (M$^+$+H, 100%).

3-Benzyloxy-6-methyl-4-oxo-4*H*-pyran-2-carbaldehyde (5) (87%): mp 78-80 °C (lit. value[18] mp 78-81 °C). IR (KBr disc) 1694 (C=O st, CHO), 1646 cm^{-1} (C=O st). ^{1}H-NMR (DMSO-d_6, 60 MHz) δ 2.10 (3H; s; 6-CH_3), 5.10 (2H; s; -CH_2Ph), 6.20 (1H; s; H^5), 7.10 (5H; s; phenyl), 9.55 (1H; s; -CHO). MS (FAB): m/z 245 (M$^+$+H, 100%).

3-Benzyloxy-6-methyl-4-oxo-4*H*-pyran-2-carboxylic acid (6) (85%): mp 173-175 °C (lit. value[18] mp 173-175 °C). IR (KBr disc) 1632 cm^{-1} (C=O st, pyranone). ^{1}H-NMR (DMSO-d_6, 60 MHz) δ 2.15 (3H; s; 6-CH_3), 5.00 (2H; s; -CH_2Ph), 6.20 (1H; s; H^5), 7.20 (5H; s; phenyl). MS (FAB): m/z 261 (M$^+$+H, 100%).

General procedure for the preparation of amide derivatives (7a-c).

6 (1 g, 3.80 mmol) was dissolved in DMF (5 mL). NMM (0.76 g, 7.60 mmol), and TBTU (1.28 g, 3.99 mmol) were added in solution under nitrogen. The resulting solution was stirred at room temperature for 20 min. An aromatic amine (4.56 mmol) was added slowly into the reaction mixture and stirred at room temperature under nitrogen for 24 h. Solid was separated by filtration and after recrystallisation from CH_3OH to furnish the product as a white powder.

3-Benzyloxy-6-methyl-4-oxo-4 *H*-pyran-2-[*N*-(naphthyl)carboxamide] (7a).

Yield: 86%, mp 162-3 °C. IR (KBr disc) 1697 (C=O st, -CONH-), 1650 cm^{-1} (C=O st, pyranone). ^{1}H-NMR δ (DMSO-d_6, 60 MHz) 2.10 (3H; s; 6-CH_3), 5.00 (2H; s; 3-OCH_2-), 6.10 (1H; s; H^5), 6.80-7.70 (12H; m; naphthalene and phenyl). MS (FAB): 386 (M$^+$+H, 100%). MW: 385.41 Anal. calcd. for $C_{24}H_{19}NO_4$: C, 74.79; H, 4.96; N, 3.63. Found: C, 74.95; H, 4.60; N, 3.79.

3-Benzyloxy-6-methyl-4-oxo-4*H*-pyran-2-[*N*-(3′-quinolinyl)carboxamide] (7b).

Yield: 80%, mp 175-7 °C. IR (KBr disc) 1680 (C=O st, -CONH-), 1645 cm^{-1} (C=O st, pyranone). ^{1}H-NMR δ (DMSO-d_6, 60 MHz) 2.30 (3H; s; 6-CH_3), 5.40 (2H; s; 3-OCH_2-), 6.15 (1H; s;H^5), 7.25-8.30 (11H; m; quinoline and phenyl), 9.80 (1H; s; -NH-). MS (FAB): 387 (M$^+$+H, 100%). MW: 386.40 Anal. calcd. for $C_{23}H_{18}N_2O_4$ Cal: C, 71.49; H, 4.69; N, 7.24. Found: C, 71.96; H, 5.02; N, 7.24.

3-Benzyloxy-6-methyl-4-oxo-4*H*-pyran-2-[*N*-(4′-methyl-coumarin-7-yl)-carboxamide] (7c).

Yield: 71%, mp 234-5 °C. IR (KBr disc) 1736 (C=O st, coumarin), 1693 cm^{-1} (C=O st, -CONH-), 1645 (C=O st, pyranone). ^{1}H-NMR δ (DMSO-d_6, 60 MHz) 2.30 (6H; s; 6-C$H_3$and coumarin 4-CH_3), 5.01 (2H; s; 3-OCH_2-), 6.03 (1H; s; $H^{3'}$), 6.10 (1H; s; H^5), 7.05-7.70 (8H; m; coumarin and phenyl). MS (FAB): m/z

418 (M^++H, 100%). MW: 417.12 Anal. calcd. for $C_{24}H_{19}NO_6$ Cal: N, 69.04; H, 4.59; N, 3.35. Found: C, 68.71; H, 4.68; N, 3.30.

General procedure for debenzylation (8a-c)

Solutions of **7a-c** in DMF (15 mL) were subjected to hydrogenolysis (12 psi) in the presence of 5% Pd/C (5-10% w/w) for 1 h. The mixtures were warmed and filtered. The resulting solution was rotary evaporated to yield a white powder. Recrystallisation from CH_3OH gave pure products (**8a-c**) as white crystalline solids.

3-Hydroxy-6-methyl-4-oxo-4*H*-pyran-2-[*N*-(naphthyl)carboxamide] (8a).

Yield: 62%, mp 234-5 °C. IR (KBr disc) 1666 (C=O st, -CONH-), 1631 cm^{-1} (C=O st, pyranone). ^{1}H-NMR δ (DMSO-d_6, 400 MHz) 2.35 (3H; s; 6-CH_3), 4.10-4.40 (1H; broad; -OH), 6.44 (1H; s; H^5), 7.55-7.60 (3H; m; naphthalene $H^{5'}$, $H^{6'}$, $H^{7'}$), 7.62-8.01 (4H; m; naphthalene $H^{2'}$, $H^{3'}$, $H^{4'}$, $H^{8'}$), 10.78 (1H; s; -NH-). ^{13}C-NMR δ (DMSO-d_6) 19.9 (6-CH$_3$), 112.8C_5, 122.1$C_{2'}$, 122.3$C_{4'}$, 126.0$C_{8'}$, 126.7$C_{7'}$, 126.9$C_{3',5'}$, 127.8$C_{8a'}$, 128.8$C_{6'}$, 132.4$C_{4a'}$, 134.1$C_{1'}$, 137.7C_3, 147.4C_6, 161.1C_2,165.8C_4 and 174.3 (-CONH-). ^{13}C-DEPT δ (DMSO-d_6, 400 MHz) 19.9 (-CH$_3$) and 112.8, 122.1, 122.3, 126.1, 126.7, 126.9, 128.8 (-CH). MS (FAB): m/z 296 (M^++H, 100%). MW: 295.08. Anal. calcd. for $C_{17}H_{13}NO_4$ Cal: C, 69.13; H, 4.43; N, 4.75. Found: C, 68.79; H, 4.63; N, 4.83.

3-Hydroxy-6-methyl-4-oxo-4*H*-pyran-2-[*N*-(3′-quinolinyl)carboxamide] (8b).

Yield: 61%, mp 285 °C dec. IR (KBr disc) 1645 (C=O st, -CONH-), 1604 cm^{-1} (C=O st, pyranone). ^{1}H-NMR δ (DMSO-d_6, 400 MHz) 2.39 (3H; s; 6-CH_3), 3.30-3.70 (1H; broad; -OH), 6.40 (1H; s; H^5), 7.83 (1H; dd; J = 7.4 Hz; $H^{5'}$), 7.99 (1H; dd; J = 7.7 Hz; $H^{6'}$), 8.14 (2H; dd; J = 8.4 Hz; $H^{7'}$, $H^{4'}$), 8.74 (1H; d; J = 2.2 Hz; $H^{8'}$), 9.16 (1H; d; J = 2.4 Hz; H$^{2'}$), 10.87 (1H; s; -NH-). MS (FAB): m/z 297 (M^++H, 100%). MW: 296.08 Anal. calcd. for $C_{16}H_{12}N_2O_4$ Cal: C, 64.84; H, 4.08; N, 9.45. Found: C, 64.51; H, 4.12; N, 9.24.

3-Hydroxy-6-methyl-4-oxo-4*H*-pyran-2-[*N*-(4′-methyl-coumarin-7-yl)carboxamide] (8c).

Yield: 54%, mp 290 °C dec. IR (KBr disc) 3269 (OH st, broad), 1739 (C=O st, coumarin), 1641 (C=O st, -CONH-), 1606 cm^{-1} (C=O st, pyranone). ^{1}H-NMR δ (DMSO-d_6, 400 MHz) 2.34 (6H; s; 6-CH_3 and coumarin 4-CH_3), 6.28 (2H; s; H^5, $H^{3'}$), 7.54-7.88 (3H; m; coumarin). MS (FAB): m/z 328 (M^++H), 154 (100%). MW: 327.07 Anal. calcd. for $C_{17}H_{13}NO_6$ Cal: C, 62.37; H, 4.00; N, 4.28. Found: C, 62.68; H, 3.75; N, 4.36.

Microbiology

Minimal inhibitory concentrations (MICs) were determined by the microdilution broth method following the procedures recommended by the National Committee for Clinical Laboratory Standards[19,20]. Fluconazole and ceftazidime were used as the reference compounds for fungi and bacteria, respectively. Two Gram-positive (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212) and two Gram-negative (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) bacteria were used as quality control strains[19]. For testing antifungal activities, the following reference strains of *C. albicans* ATCC 90028, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were tested[20]. The MIC values of the compounds are presented in the Table. The reference compounds were dissolved in sterile distilled water. The stock solutions of compounds were prepared in DMSO. The dilutions in the test medium were prepared at the required concentration of 512-0.5

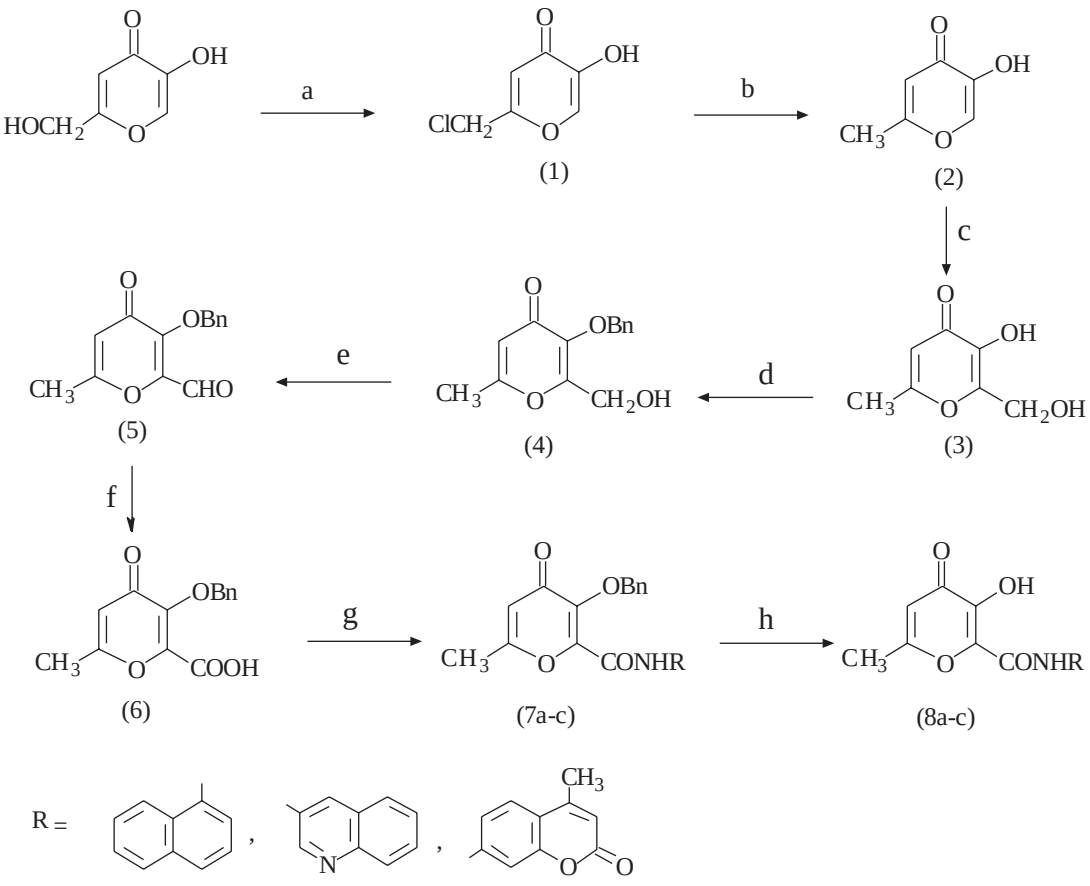
μg/mL, and for reference compounds at 64-0.0625 μg/mL. It was established that dilutions of DMSO lacked antimicrobial activity against any of the test micro-organisms. The microtiter plates were incubated at 35 °C and read visually after 24 h for bacteria and after 48 h for fungi.

Results and discussion

Chemistry

The general methodology adopted for the synthesis of 3-hydroxy-6-methyl-4-oxo-4*H*-pyran-2-carboxamide derivatives is summarised in the Scheme. Allomaltol (**2**) was synthesized from kojic acid by a using similar method to that described by Ellis et al[17]. Chlorination of the 2-hydroxymethyl moiety of commercially available kojic acid using neat thionyl chloride afforded chlorokojic acid (**1**) in good yield (75%), with the ring hydroxyl being unaffected. **2** was produced by reduction of chlorokojic acid with zinc dust in concentrated hydrochloric acid[17]. The 2-position of allomaltol can be functionalised using the aldol condensation whereby an enolate, in this case the pyrone anion, attacks a carbonyl compound, formaldehyde, under alkaline aqueous conditions to furnish the 2-hydroxymethylated product **3** in high yield (82%)[17,18]. The pH of the reaction solution was found to be critical, since highly alkaline conditions resulted in extensive aldehyde polymerisation. The optimal pH for this reaction was found to be 10.5[21,22]. The 3-hydroxy group was protected in order to allow modification of the adjacent primary alcohol. The 2-hydroxymethylated product **3** was refluxed with benzyl bromide in the presence of sodium hydroxide to give 2-hydroxymethyl-3-benzyloxy-6-methyl-4-oxo-4*H*-pyran, **4**[23]. The benzyl protecting group was selected because of facile removal under mild conditions by catalytic hydrogenolysis[22]. The direct conversion of the primary alcohol of the protected pyranone (**4**) to a carboxylic acid was initially attempted the Jones reagent. However, the pyranone moiety was found to be too unstable under these conditions, producing extensive degradation. Alternatively, the aldehyde was prepared before further oxidation to the carboxylic acid was attempted[18]. After this 2-step oxidation, the alcohol group at the 2 positions on the ring was converted into the corresponding acid moiety. The selective oxidation of the alcohol **4** to the aldehyde **5** (3-benzyloxy-6-methyl-4-oxo-4*H*-pyran-2-carbaldehyde) proceeded by with sulphur trioxide pyridine complex in a combination of triethylamine and dimethylsulphoxide in chloroform. For the second oxidation step, an sulfamic acid and sodium chlorite in acetone-water mixture at room temperature afforded 3-benzyloxy-6-methyl-4-oxo-4*H*-pyran-2-carboxylic acid, **6** in excellent yield (85%)[18,24]. The carboxylic acid **6** was reacted with TBTU [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] as the coupling reagent and NMM (N-methylmorpholine) as a base and an aromatic amine containing a naphthalene, quinoline or coumarin ring in a DMF solution under nitrogen[25,26]. Debenzylation of the compounds by catalytic hydrogenation at room temperature[27] afforded the desired products, the 3-hydroxy-6-methyl-4-oxo-4*H*-pyran-2-carboxamide derivatives, **8a-c**.

All compounds have C=O and -CONH- stretching bands at 1650-1645 and 1697-1641 cm^{-1}, respectively. The ^{1}H-NMR spectra of all compounds, demonstrated the presence of the characteristic singlet peaks of the pyranone (H^5) ring proton (6.15-6.55 ppm)[18,26]. The methylene protons of **7a-c** appeared as a singlet at 5.00-5.40 ppm. 6-Methyl-pyran-4-one derivatives showed methyl group protons as a singlet at 2.10-2.39 ppm. ^{13}C-NMR spectra of **8a** have characteristic peaks for methyl, carbonyl of pyranone ring and amide carbonyl at 19.85, 165.84 and 174.33 ppm, respectively. The FAB mass spectra of these compounds generally possess the M^++H peak as the base peak.



Bn = Benzyl

Reagents:

(a) $SOCl_2$; (b) Zn/HCl, H_2O; (c) HCHO/ NaOH-H_2O; (d) BnBr, NaOH, CH_3OH/H_2O; (e) SO_3.pyridine, Et_3N, DMSO, $CHCl_3$

(f) $NaClO_2$, NH_2SO_3H, $H_2O/(CH_3)_2CO$; (g) RNH_2, TBTU, NMM, CH_2Cl_2; (h) H_2, Pd-C, DMF

Scheme. Synthesis of the compounds.

Antimicrobial Activity

The synthetic route of an amide series of 3-benzyloxy-6-methyl-4-oxo-4*H*-pyran (**7a-c**) and 3-hydroxy-6-methyl-4-oxo-4*H*-pyran (**8a-c**) is presented in the Scheme. The antibacterial and antifungal activities of the molecules are reported in the Table. In this study, we compared the antimicrobial activity of kojic acid and all intermediate compounds **1-6** by using the microdilution broth method. Chlorokojic acid (**1**) (MIC: 32 μg/mL) was found to be potent against *C. krusei* when compared with the other synthetic intermediates. Indeed, **1** (MIC: 64 μg/mL) was found to be the most active compound among the entire series against *P. aeruginosa*. The other intermediate compounds had no appreciable inhibitory activity against bacteria and fungi. When the antibacterial activities of the amide derivatives (**8**) were investigated, **8c** (MIC: 8-32 μg/mL) was found to be the most active compound against *S. aureus*, *E. faecalis* and *E. coli*. All amide derivatives except **7b** (MIC: 128 μg/mL) possess similar antibacterial activity (MIC: 32-64 μg/mL) against *E. faecalis*. In addition, **5** and **8a** have moderate antibacterial activity (MIC: 64 μg/mL) against *S. aureus*.

The results of the antifungal tests demonstrated that **8c** (MIC: 4-8 μg/mL) was the most active compound against *C. albicans* and *C. krusei* in this amide series. **7a**, **7b** and **8b** (MIC: 64 μg/mL) have similar activity against *C. krusei*. The amide derivative **8c** is the most effective the compound in the entire series, although it is not effective against *P. aeruginosa*. The presence of the coumarin ring clearly has an important influence on the toxicity of this class of substances. It is possible that the lactone of the ring forms a covalent link with a receptor on the cell membrane. Experiments are in progress to further investigate this concept.

In conclusion, compound **8c** proved to be a promising antifungal agent. This preliminary study would suggest that a more detached structure activity study with this class of compounds could be useful.

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