1,8-Substituted Anthraquinones, Anthrones and Bianthrones as Potential Non-Azole Leads against Fungal Infections

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Abstract

The synthesis of a variety of 1,8-substituted anthraquinones, anthrones and bianthrones and their potential as antifungal agents is evaluated. Preliminary screening against *Schizosaccharomyces pombe (S. pombe)*, a fission yeast, and *Saccharomyces cerevisiae (S. cerevisiae)*, a budding yeast, is reported. Both these yeast species demonstrate close homologue to a number of pathogenic fungi.

Keywords

Antifungal; Anthraquinones; Anthrones; Bianthrones; Non-Azole; Yeasts

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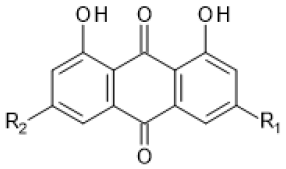
Fungal infections continue to be a serious problem for modern-day healthcare with incidences rising notably over the last decade, as resistance to classical antifungals has become more apparent.[1-3] This is combined with the increased susceptibility of immunocompromised patients to infection from emerging opportunistic fungal strains.[4-7] To counteract this, healthcare professionals and drug companies seek more aggressive forms of chemotherapeutic treatments with the azoles shown to be good candidates. There is no doubt that the azoles drugs have revolutionised clinical mycology, by targeting the biosynthesis of ergosterol. However, these drugs are not without problems including:

1. Toxicity – Although the azoles are competitive inhibitors for fungal CYP51, these drugs also affect mammalian xenobiotic-metabolising CYP enzymes,[8]
2. Resistance – The prolonged and overuse of the azoles has led to the emergence of resistant yeast strains.[9,10]

With the increase in microbial resistance, there is now a clear and direct need for novel drug candidates to combat this worrying situation with medicinal chemists now focusing their attention towards the areas of semi-synthetic and natural product chemistry.

The quinones, in particular the anthraquinones as shown in Figure 1, are one type of natural product which is well documented for having wide ranging biological properties, these include anti-bacterial,[11] anti-ulcer,[12] anti-cancer,[13] anti-inflammatory[14] and anti-viral[15] activity. Indeed, various natural and synthetic substituted anthraquinones and their phenanthroperylene cousin, hypericin, have also been recorded to have significant antifungal activity.[16-20]



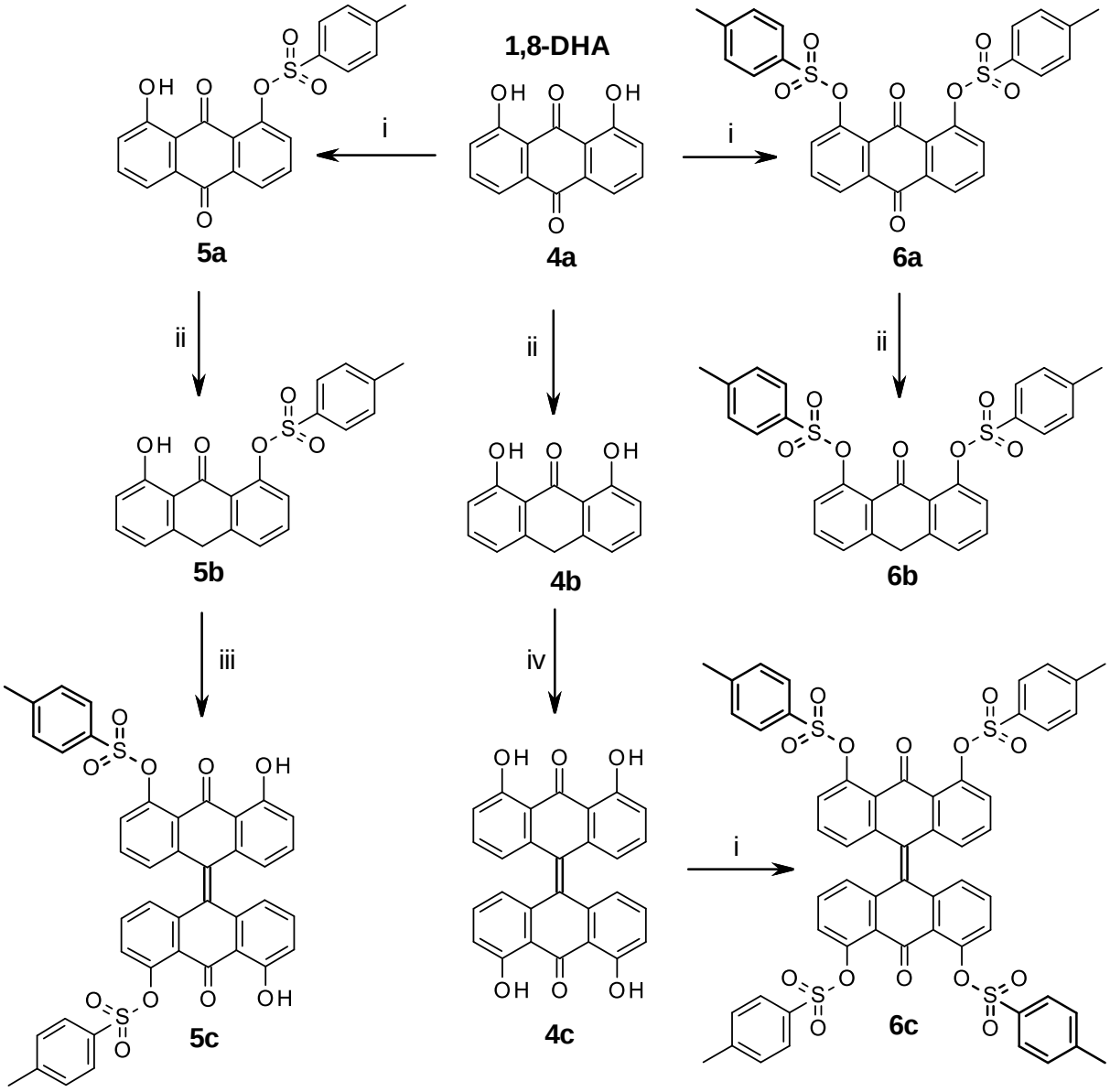
R_1	R_2	Name
COOH	H	Rhein
OCH_3	CH_3	Physcion
CH_2OH	H	Aloe-emodin
CH_3	H	Chrysophanol

Figure 1. Anthraquinones from *Rheum emodi* (Polygonaceae).

Surprisingly, there is a lack of data on the antifungal activity for the hypericin type intermediates, the anthones and bianthrones. Inspired by this, we have set out to create a small library of structurally similar anthraquinones, anthrones and bianthrones and study their antifungal activity in two different yeast species; *Schizosaccharomyces pombe (S. pombe)*, a fission yeast; *Saccharomyces cerevisiae (S. cerevisiae)*, a budding yeast, both used extensively in eukaryotic microbiological research. These yeast species demonstrate close homologue to a number of pathogenic fungi. For example, *S. cerevisiae* is a yeast closely related to *Candida albicans*, a widespread commensal and important pathogen of humans, and *S.pombe* is closely related to *Pneumocystis jiroveci* which is a fungal infection of the lungs.[21] *S. cerevisiae* itself is also emerging as an opportunistic pathogen, especially in immunosuppressed and immunocompromised patients and has been associated with fungemia, endocarditis,

peritonitis, meningitis, ventriculitis, and with polymicrobial fatal pneumonia in AIDS patients.[22, 23] These yeast can serve as models to learn more about pathogenic fungi, in particular with regard to regulatory features and drug therapy, because yeast as a fungal species shares many characteristics with its pathogenic relatives.[24] Therefore, the aforementioned yeast can be used as representative species for screening of chemical libraries for fungicidal activity, without exposure to fungi that are harmful to human health.[25]

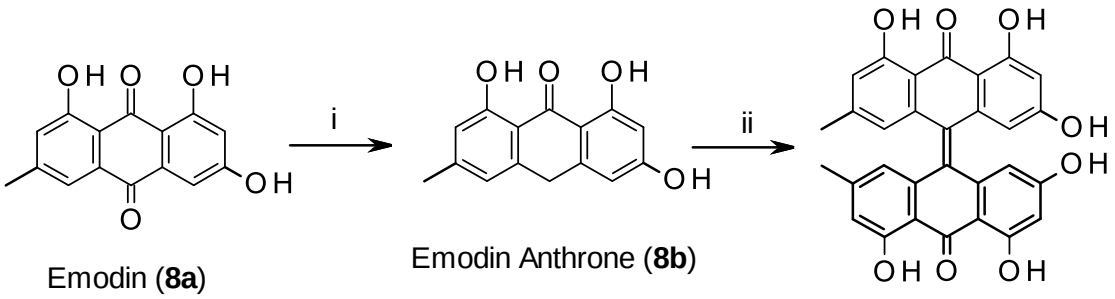
Detailed synthetic pathways to target the anthraquinones, anthrones and bianthrones are shown below in Schemes 1.



i. p-TsCl, Et_3N, MeCN; ii. $SnCl_2$, HCl, AcOH; iii. KtOBu, DMF, MW-130°C; iv. KtOBu, DMF, Reflux.

Scheme 1. Synthetic route to the anthroquinones (5a-6a), anthrones (4b-6b) and bianthrones (4c–6c).

All compounds made within this study followed standard published methodology. The mono and tosylation of 1,8-hydroxyanthroquinone **5a** and **6a** was accomplished using reported methods[30, 31] with minor modifications from 1,8-dihydroxyanthroquinone (**4a**). The anthrones (**4b–6b** and **8b**) were prepared by chemoselective reduction of the carbonyl group in the 10th position of the 1,8-substituted anthraquinones. This method of chemoselective reduction has been previously demonstrated by the NOE interaction of the newly introduced protons at the 10th position, with the protons in positions 4 and 5.[32] Finally, the bianthrones (**4c-6c** and **8c**) was formed either under under wet or microwave conditions using the newly reduced anthrone.[33] Scheme 2 shows the synthesis of emodin bianthrone (**8c**) from the starting emodin (**8a**) using the same method as above.

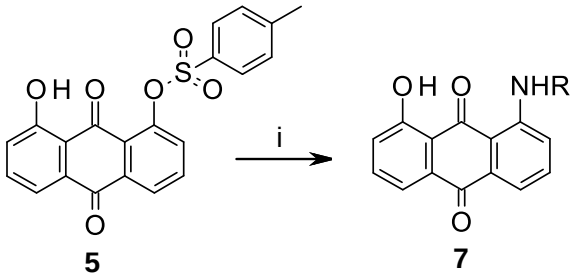


Emodin (**8a**) Emodin Anthrone (**8b**) Emodin Bianthrone (**8c**)

i. $SnCl_2$, HCl, AcOH; ii. KtOBu, DMF, MW-130°C.

Scheme 2. Synthesis of the emodin anthrone and emodin bianthrone.

One of the main driving factors for the introduction of the tosyloxy functionality was to determine if amino substituents could be added with relative ease. This was studied using the mono-protected **5a**, which was independently reacted with three 1° diamines (ethylenediamine, trimethylenediamine or putrescine) to yield monosubstituted compounds **7a-c** as shown in Scheme 3.



5 **7**

7a R = $(CH_2)_2NH_2$
7b R = $(CH_2)_3NH_2$
7c R = $(CH_2)_4NH_2$

i. R-NH_2, DCM, Reflux, 2h

Scheme 3. The synthetic route to the mono substituted aminoanthroquinones (7a-c).

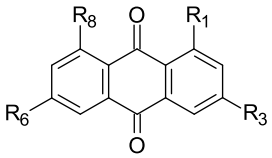
The diamines were used in a 4 molar excess; the ability to remove the tosylate groups being controlled by the selection of an appropriate solvent, which in this case was dichloromethane under reported conditions.[30] The only variation noted using this procedure being the 4 molar excess of the 1° diamine in comparison to a 250 molar excess of the amine which had been reported. The amine functionalities were not added to the anthrones (**4b-6b**) or bianthones (**5c-6c**) due to large scale reactivity and purification issues encountered.

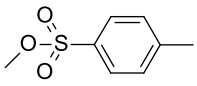
The synthesised compounds were tested *in vitro* to determine growth inhibitory activity using a standard method.[4, 36] Minimum inhibitory concentration (MIC) values were

determined in sets by comparison with the following compounds, which are intermediates towards the natural product hypericin; emodin (**8a**), emodin anthrone (**8b**) and emodin bianthrone (**8c**). Emodin itself is a well-known antifungal and has been used as a positive control for this study. Its mechanism of action is thought to be due to induction of DNA damage.[34]

Table 1 shown below gives the full set of antifungal data collected in this study. The substituted anthraquinones, showed varied results between each of the yeast species. In *S. pombe,* they were consistently inhibitive at concentrations greater than 1000 µM (**4a**, **5a** and **6a**). In *S. cerevisiae,* the results were varied, with emodin (**8a**) and compounds **5a** and **6a** having similar results to that of the *S. pombe*. However, the MIC for **4a** in *S. cerevisiae* was twice that for *S.pombe*, 2910µM compared to 1460µM, with the unsubstituted 9,10-anthroquinone also showing similar results to **4a**.
The amine substituted anthraquinones **7a-c** showed a range of activities against both yeast strains tested, but all MICs were lower than 900µM. When compared against the control emodin (**8a**), **7a-c** showed lower MICs, with both **7a** and **7b** being approx. 3.5 times more active against the *S. pombe* (440µM and 420µM) and approx. 1.5 times more active against the *S. cerevisiae* (886µM and 844µM). It's interesting to note **7c** showed the greatest activity of the three with the MIC being approx. 6.5 times higher against both yeast species (200µM), when compared to emodin (**8a**). It is postulated that this is due to increasing the length of amine chain, which will increase the lipophilicity of the molecule, allowing for better membrane penetration.[35] Finally, all compounds within this family were fungastatic apart from **7b** and **7c** which were shown to be fungicidal against *S. pombe* alone.
The growth inhibition concentrations for the anthrones are shown alongside **8b**. It is interesting to note that **8b** is 13 times more active against *S. pombe* in comparison to the control **8a** (97.6µM versus 1300µM). The anthones **4b**, **5b** and **6b** (55.3µM, 65.7µM and 46.8µM) are approx. 1.5 times more active against *S. pombe* when compared to the **8b** (97.6µM) and approx. 20 times more active when compared to **8a** (1300µM). It is also noteworthy that **8b** (1950µM) is less active against *S. cerevisiae* by a factor of 1.5 when compared against **8a** (1300µM), however, **4b** (553µM), **5b** (329µM) and **6b** (234µM) again show superior activity when compared to both **8a** and **8b** respectively. However, the MICs are still significantly higher than that seen in *S. pombe*. The unsubstituted anthrone followed the same trend as the other compounds but were less active against *S. pombe* in comparison to the other anthrones, whilst showing the same type of activity against *S. cerevisiae* as **4b**. All compounds within this family were shown to be fungistatic, apart from **8b** that was fungicidal, against *S. cerevisiae.*
Finally, the antifungal activity of the bianthrones show a variation in results. **8c**, the bianthrone substited emodin, was showed to be slightly more active than **8a**, the emodin anthraquinone, when compared against both yeast species, 980µM and 1300 µM respectively. Compound **4c** showed to be 13 times more active against *S. pombe* (140µM) and 2 times more active against *S. cerevisiae* (555µM) when compared to the control emodin (**8a**) and emodin bianthrone (**8c**). Compound **5c** again showed almost identical activity against both *S. pombe* and *S. cerevisiae*, 460µM and 461µM respectively. The unsubstituted bianthrone gave the lowest activity against both activity *S. pombe* and *S. cerevisiae,* 1355µM and 2168 µM respectively. Finally **6c**, showed no growth inhibition at the concentrations tested. All compounds tested within this study, except **6c**, were found to be fungistatic in both yeast species. It should be noted that the *cLogP* data given in Table 1 shows no correlation to the activity.

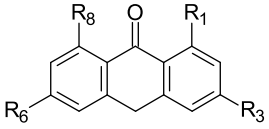
Anthraquinones

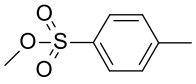


R = OTs = 

Compound	R^1	R^3	R^6	R^8	cLogP	MIC (μM)			
						S. pombe	*S/C*	*S. cerevisiae*	*S/C*
9,10-Anthraquinone	H	H	H	H	3.67	1250	S	2001	S
Emodin (8a)	OH	OH	CH_3	OH	3.01	1300	S	1300	S
4a	OH	H	H	OH	3.13	1460	S	2910	S
5a	OH	H	H	OTs	4.77	1770	S	1770	S
6a	OTs	H	H	OTs	6.40	1280	S	1280	S
7a	OH	H	H	$NH(CH_2)_2NH_2$	0.9	440	S	886	S
7b	OH	H	H	$NH(CH_2)_3NH_2$	1.44	420	C	844	S
7c	OH	H	H	$NH(CH_2)_4NH_2$	1.98	200	C	201	S

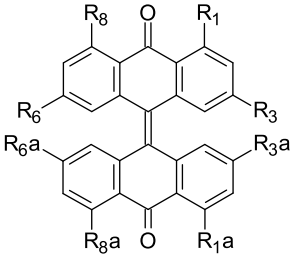
Anthrones

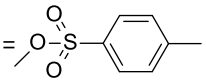


R = OTs = 

Compound	R^1	R^3	R^6	R^8	cLogP	MIC (μM)			
						S. pombe	*S/C*	*S. cerevisiae*	*S/C*
Anthrone	H	H	H	H	3.49	267	S	534	S
Emodin Anthrone (8b)	OH	OH	Me	OH	3.25	97.6	S	1950	C
4b	OH	H	H	OH	3.38	55.3	S	553	S
5b	OH	H	H	OTs	4.80	65.7	S	329	S
6b	OTs	H	H	OTs	6.22	46.8	S	234	S

Bianthrones



R = OTs = 

Compound	R^1&R^1a	R^3&R^3a	R^6&R^6a	R^8& R^8a	cLogP	MIC (μM)			
						S. pombe	*S/C*	*S. cerevisiae*	*S/C*
Bianthrone	H	H	H	H	7.69	1355	S	2168	S
Emodin Bianthrone (8c)	OH	OH	Me	OH	6.37	980	S	980	S
4c	OH	H	H	OH	6.62	140	S	555	S
5c	OH	H	H	OTs	9.06	460	S	461	S
6c	OTs	H	H	OTs	9.82	α	-	α	-

Minimal Inhibitory Growth Concentration (MIC) of synthesised compounds tested in *Schizosaccharomyces pombe* (*S.pombe*), *Saccharomyces cerevisiae* (*S. cerevisiae*). Cells were inoculated at a concentration of 3 x 10^4/ml. Culture media tested were in yeast extract broth (YE) for *S.pombe* and complex growth media (YPD) *for S. cerevisiae*. Growth of yeast was determined visually after 24 hours incubation at 30 ˚C. The MIC of the compounds were determined to be the well before yeast growth was first seen. The experiment was repeated three times to ensure reproducibility of the results. Compounds were determined to be fungicidal (C), if no growth was observed, and fungistatic (S), if normal growth was seen after inoculation into fresh media. .α - Growth seen in all wells up to 600 μM concentration.

Table 1. Structures and growth inhibitory data for the 1,8-substituted anthroquinones.

In conclusion, the anthraquinones (**5a** and **6a**), anthrones (**4b-6b** and **8b)** and bianthrones (**4c-6c** and **8c)** were all prepared using literature methods in varying yields from pre-purchased compounds (**4a** and **8a**). The synthesis of the monoamino substituted anthraquinones (**7a-c)** was also successfully accomplished using literature methods with minor modifications. The target anthraquinones, anthrones and bianthrones are important from a structural viewpoint; the toluenesulphonyl functionality allows easy addition of amino functionalities, this has the advantage of greater intrinsic water solubility and allows for further attachments to take place such as amino acids, mono-clonal antibodies etc. This would be advantageous as it would be a route to specific cell targeting. Preliminary screen of all the compounds in this study showed varying levels of antifungal activity, with the control emodin, often showing poorer activity when compared to the other compounds in this study. As highlighted earlier in this study, the mechanism of action of emodin (**8a**) is thought to be due to induction of DNA damage.[34] However, the compounds shown in this study are fungistatic, thus suggesting an alternative mode of action is highly plausible
It should also be noted that there is no correlation between *cLogP* data given in Table 1 and compound activity, which might suggest the growth inhibition is not the result of an intracellular mechanism of action. It is clear from these results that the anthrones (**4b-6b**), the bianthrones (**4c-5c**) and the amino substituted anthraquinones (**7a-c**) show promising antifungal activity and should form the structural basis towards possible new antifungal leads.

Notes

The authors declare no competing financial interest.

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36. The growth inhibitory activity of the compounds were determined by screening *S. pombe* and *S.cerevisiae* using the following method: Yeast species were inoculated into relevant media; *S. pombe* (NJ2 *h*$^{-}$ *ura4-D18 leu1-32 ade6-M210 his7-366*)[26] into yeast extract broth (YE),[27] and *S.cerevisiae* (strain BY4741a, a derivative of S288C, (*MAT***a***his3*Δ1 *leu2*Δ0 *met15*Δ0*ura3*Δ0)[28] into complex media (YPD).[29] The culture was then incubated for 12 hours at 30^{o}C with shaking at 200 rpm. Stock solutions of the compounds were prepared in 20% (v/v) DMSO and culture media. DMSO and culture media were used as controls for the experiment. 3 x 10^4 exponential growing yeast cells were transferred into the wells of a 96-well plate. A 1:2 serial dilution of the compounds was then performed. The wells were inspected visually for growth of yeast after 24 hours of incubation at 30 $^{\circ}$C. Growth was indicated by full or partial white appearance of yeast on the bottom of the wells. The MIC values of the compounds were determined to be the well before yeast growth was first seen. The experiment was repeated three times to ensure reproducibility of the results. To determine whether compounds had fungistatic or fungicidal activity, cells were taken from the well on the microtitre plate at the concentration where lack of yeast growth was first observed. These cells were inoculated into fresh culture media, to strongly dilute the presence of the compound, and grown for 24 hours at 30^{o}C with shaking at 200rpm. Compounds were determined to be fungicidal, if no growth was observed, and fungistatic, if normal growth was seen.