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# New 1,4-anthracenedione derivatives with fused heterocyclic rings: synthesis and biological evaluation†

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Several terpenylquinones derived from 1,4-anthracenedione (1,4-anthracenequinone, AQ) have been prepared by addition or substitution nucleophilic reactions and further transformed into extended polycyclic systems, which mainly kept the 1,4-quinone moiety fused to different nitrogen-heterocyclic rings (pyrrole, imidazole, pyrazine or quinoxaline) into the structure. The compounds synthesized were evaluated for their antineoplastic, antifungal and antiviral activities. GI<sub>50</sub> antineoplastic values remained under  $\mu\text{M}$  levels for AQs, while the heterocyclic derivatives showed antifungal MIC values in the low  $\mu\text{g mL}^{-1}$  range against yeasts and filamentous fungi. Only few compounds displayed a discrete non-selective antihyperthermic activity in the  $\mu\text{g mL}^{-1}$  range.

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## 1. Introduction

The quinone/hydroquinone moiety is a common structural feature in a large number of natural and synthetic compounds with a wide range of biological activities such as anticancer,<sup>1–4</sup> antiparasitic,<sup>5</sup> antimicrobial,<sup>6</sup> antifungal,<sup>7</sup> antiviral,<sup>8</sup> anti-oxidant,<sup>1b,9</sup> etc., and it is possible to find agents, derived from benzoquinone (BQ), naphthoquinone (NQ) or anthracenequinone (AQ), used clinically as anticancer agents<sup>10</sup> such as daunorubicin or mitomycin C (Fig. 1A). The mechanisms of the antineoplastic cytotoxicity of quinone derivatives have been widely studied, and it is known that they usually participate in important biological redox processes and can also act as intercalating or alkylating agents.<sup>11</sup> The potential of quinones to generate reactive oxygen species (ROS) has been described in the literature,<sup>12</sup> as well as the different targets that might be involved in its molecular mechanisms of action depending on the tumour cell type.<sup>13</sup> For instance, the DNA cross-linking and

monoalkylation by mitomycin C require the reduction of the quinone ring, while furo-naphthoquinones are able to modify the mitochondrial permeability through ROS generation.<sup>12c</sup> In this sense, quinones can be considered as multi-target drugs.<sup>14</sup> Several of those bioactive quinones bore a heterocyclic fragment fused to the quinone moiety, including five and six-membered heterocycles with nitrogen,<sup>15</sup> oxygen<sup>16</sup> and/or sulphur<sup>17</sup> atoms.

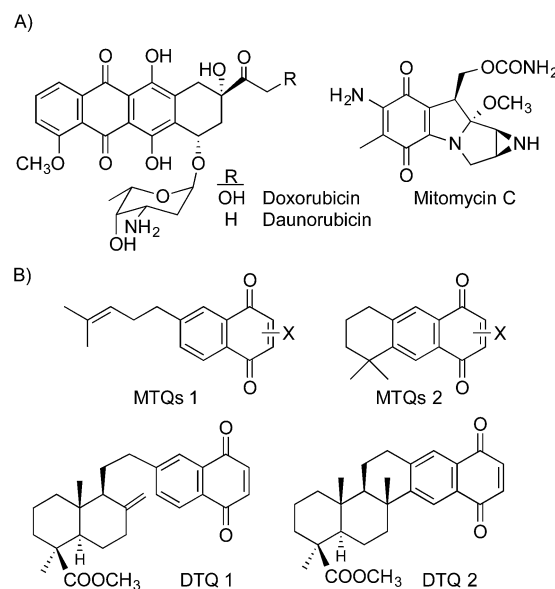


Fig. 1 (A) Structure of clinically used anticancer quinones. (B) Structure of several monoterpeno-derived (MTQ) and diterpene-derived (DTQ) antineoplastic quinones.

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† Electronic supplementary information (ESI) available: <sup>13</sup>C NMR data and complete assignments for the <sup>1</sup>H and <sup>13</sup>C NMR signals are included together with HMQC and HMBC spectra for several separated regioisomers. Complementary antifungal, antiviral and Vero cytotoxicity data for all the compounds tested. See DOI: 10.1039/c4ra11726c

It has also been reported that the presence of an *N*-heterocyclic moiety in a polycyclic planar structure contributes to enhance their activity,<sup>18</sup> being important too the number and position of the nitrogen atoms in the heterocyclic system.<sup>19</sup>

Among the bioactive quinones, 1,4-NQs and 9,10-AQs have been extensively studied. In contrast, AQs with the quinone carbonyls located at other positions are much less frequent and only the antitumor activity<sup>20,21</sup> and the promising anti-trypanosomal profile<sup>22</sup> of a limited number of 1,4-AQs has been described in the last few years. These facts prompted us to design and prepare new 1,4-AQ derivatives, including a number of heterocycle-fused quinones for evaluating them, not only for their expected antineoplastic cytotoxicity, but also for their antifungal activity, based on previous results reported by us for NQs and AQs<sup>23</sup> and prospectively, against viruses, based on the assumption of their wide involvement in bio-redox processes, on their structural novelty and on the absence of previous evaluations in such field. Complementarily, it can be noted that antifungal and antiviral activities reported for quinones were often related to their reduction potential,<sup>6c,8b,11</sup> and that recent mechanistic studies revealed that the antifungal activity of several NQs against various *Candida* spp. strains was related to the generation of ROS,<sup>24</sup> in the same way as other antifungals as amphotericin B, miconazole or histatin 5 also do.<sup>25</sup>

In our search focused to the induction of antineoplastic and antimicrobial activities on natural inactive terpenoids, we prepared and evaluated a large number of 1,4-NQs, with the model structures shown in Fig. 1B, starting from natural terpenoids such as myrcene and myrcenol.<sup>26,27</sup> They were synthesized through Diels–Alder cycloaddition between the natural terpenoids and *p*-benzoquinone. The preliminary biological results indicated that monoterpenylquinones (MTQs) and diterpenylquinones (DTQs) (Fig. 1B) were cytotoxic and good candidates to further structural optimization, becoming our lead compounds in order to improve their bioactivity profiles. Several structural modifications, dealing with size and oxidation degree of the quinone ring, were performed on those derivatives, including changes of substituents in the side chain or in the quinone ring.<sup>26,27</sup>

From those studies, we deduced that the presence of a terpenyl residue attached to the benzenoid ring of the NQ moiety improved the cytotoxicity of 1,4-NQ itself, and we attained to obtain some derivatives with GI<sub>50</sub> values in the μM range against several tumour cell lines. Among them, we also prepared a number of 1,4-AQs by cyclization of the side chain in the MTQs **1** to get MTQs **2** (Fig. 1B), which also showed a very interesting cytotoxicity.<sup>21</sup>

In a prospective manner, several representative MTQs were evaluated against human pathogenic yeasts and filamentous fungi, with promising antifungal results found for several chlorinated derivatives.<sup>23</sup>

The incidence of opportunistic infections caused by microorganisms including fungi and viruses have increased significantly in recent decades, mainly in immunocompromised patients as those under anticancer treatment, who are susceptible to opportunistic infections. Among fungal pathogens, the species from *Candida*, *Aspergillus* and *Cryptococcus* genera are

the most frequent in human infections.<sup>28</sup> With the increase of these infections, has also increased the occurrence of secondary resistance by prolonged treatment with antifungals or by using them in prophylactic treatments.<sup>29</sup> Also, infections caused by other emerging fungi as *Fusarium* spp. and *Scedosporium* spp. are increasing. Within these genera there are multiresistant strains or with variable susceptibility to antifungal drugs.<sup>30</sup>

Infections by Human Herpes virus type 1 (HHV-1) and Human Herpes virus type 2 (HHV-2) are also important due to morbidity that they can cause<sup>31</sup> in immunocompromised patients. In this sense, the multitarget approach could be advantageous over drug combination, as it has been recently discussed,<sup>22a,32</sup> and would reinforce the convenience of evaluating the new compounds for their antifungal and antiviral potential, in addition to the antineoplastic evaluation. Discovering entities that can be effective against two or more pathologies improves the chance of efficacy, and could prevent the development of the more and more frequent appearance of multi-drug resistant strains.<sup>33,34</sup>

Keeping in mind that quinone systems are involved in a wide range of biological activities and that the presence of nitrogen atoms can contribute to improve the bioactivity of quinone systems, together with our previous results with terpenylquinones, we planned to synthesize several new 1,4-AQs bearing fused nitrogen-heterocyclic rings very common in drug molecules, such as pyrrole, imidazole, pyrazine or quinoxaline, in order to analyze the influence of the enlargement of the heteropolycyclic planar system on their bioactivity. The synthesis of such new heterocycle-fused 1,4-AQ derivatives and the results of their evaluation in the three proposed pathogenic areas are reported and discussed here.

## 2. Results and discussion

### 2.1. Chemistry

The heterocyclic-fused quinones objective of this study included naphthoindole, anthraimidazole, anthraoxazole, naphthophenazine, naphthoquinoxaline and anthraimidazopyridine derivatives. We have used as starting compounds the AQs **1**, **2** and **3** previously obtained by us through Diels–Alder cycloaddition between myrcene and *p*-benzoquinone derivatives as reported<sup>21,23</sup> (Fig. 2).

The actual precursors of the heterocyclic systems were obtained through nucleophilic processes,<sup>35</sup> either addition or substitution reactions, using *N*-nucleophiles as it is represented in Schemes 1–9. As the starting quinones are non-symmetrically substituted, the corresponding reaction products consisted of approximately 1 : 1 mixtures of the two expected regioisomers.

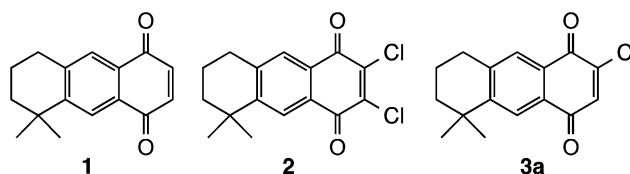
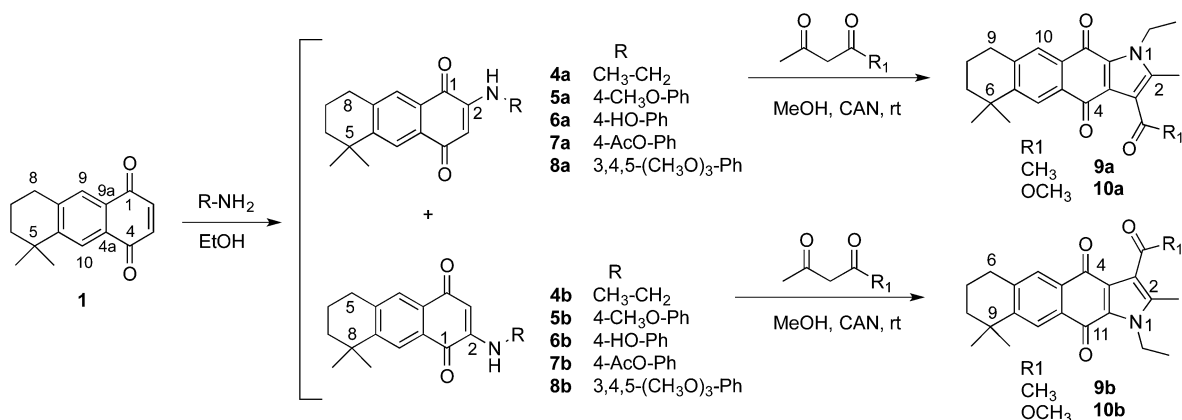
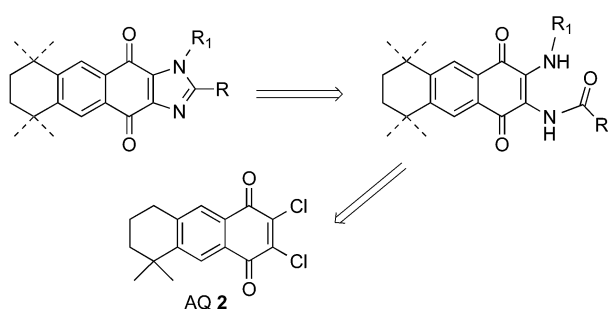


Fig. 2 Anthracene-1,4-quinones used as starting materials.



Scheme 1 Preparation of aminoanthracenediones 4–8 and naphtho[2,3-*f*]indole-4,11-diones 9 and 10.

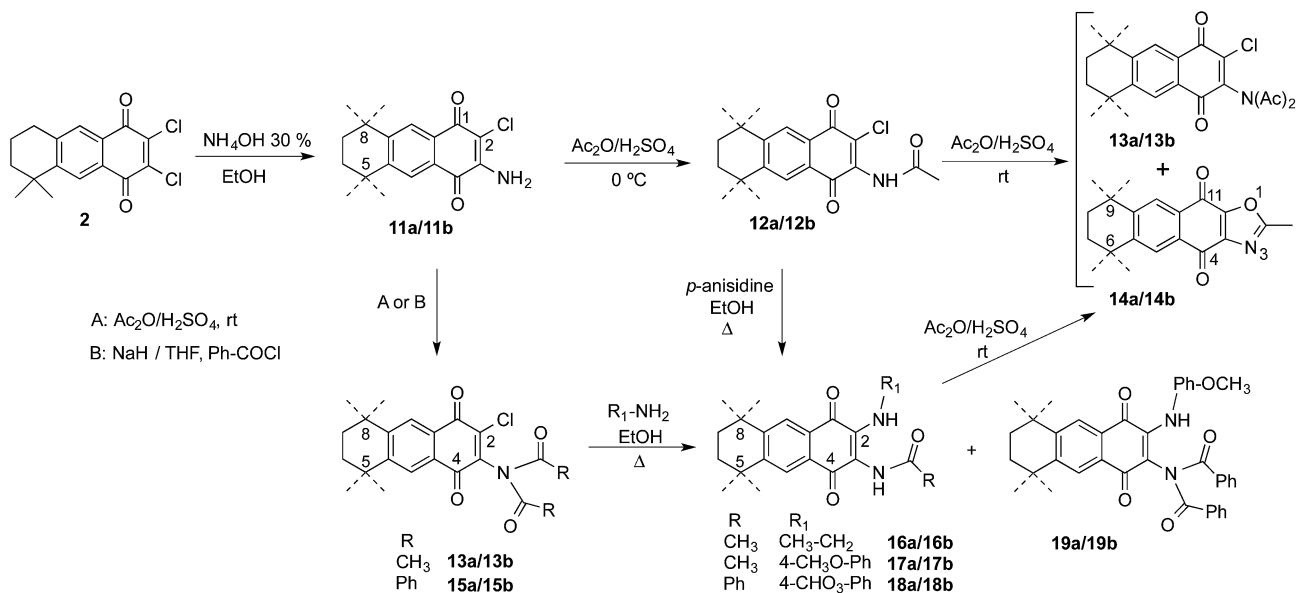


Scheme 2 Retrosynthetic scheme for imidazole-fused AQs.

However, in several cases they were separated by column chromatography in quantities enough to facilitate their independent spectroscopic characterization, and to perform further chemical transformations and their biological evaluation. NMR

spectra of representative compounds and complete <sup>1</sup>H and <sup>13</sup>C NMR data assignments are included in the ESI.†

**2.1.1. Pyrrole fused systems.** Michael addition of aliphatic or aromatic amines to AQ 1 gave the monosubstituted AQs 4–8 as 1 : 1 mixtures of regioisomers, which were separated by CC to allow independent characterization and biological evaluation<sup>23</sup> (Scheme 1). The regioisomers **4a** and **4b**, which were unequivocally identified in our previous work,<sup>23</sup> were used to obtain the naphtho[2,3-*f*]indole-4,11-diones **9** and **10** by condensation with β-dicarbonyl compounds *via* a cerium(IV) mediated oxidative free radical reaction.<sup>36</sup> The condensation reaction was carried out with each regioisomer independently. Thus, **4a** was treated with 2,4-pentanedione or ethyl acetoacetate and CAN in methanol at room temperature to yield **9a** and **10a** respectively. When AQ **4b** was used as the starting material, derivatives **9b** and **10b** were obtained in moderate yields. The formation of the pyrrole ring fused to the quinone system was deduced from the analysis of <sup>1</sup>H NMR spectra, in which the signal at 5.6 ppm



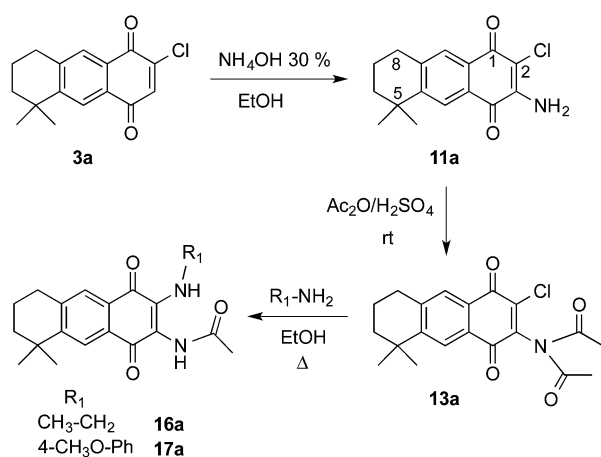
Scheme 3 Preparation of intermediates and heterocycle-fused 1,4-anthraquinones.

corresponding to the hydrogen H-3 in compounds **4**, disappeared from the spectrum and new singlets at 2.4 and 2.7 (and 3.9) ppm were observed respectively for compounds **9** (and **10**) (see ESI†). The correlations observed in two-dimensional NMR spectra obtained for **9a** and **9b** confirmed the structures assigned to both naphthoindoleiones.

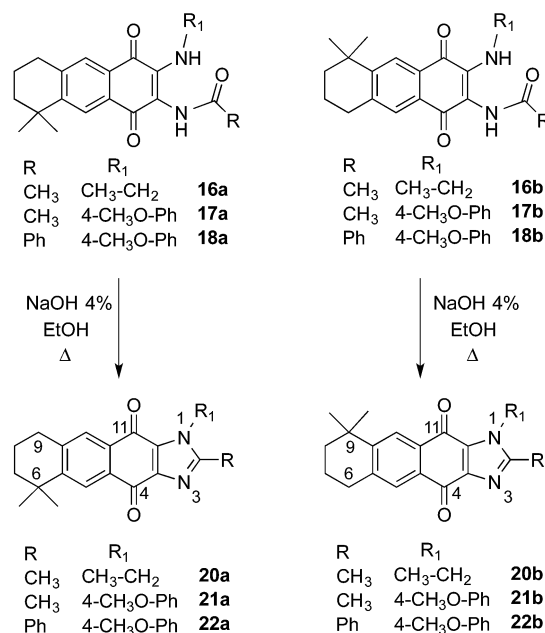
**2.1.2. Imidazole-fused systems.** The imidazole ring can be formed through the condensation of an acylamino and an alkylamino groups attached to the quinone fragment. Such groups would be previously introduced by substitution of the two chlorine atoms on the dichloro-AQ **2** as stated in the retrosynthetic Scheme 2.

Dichloro-AQ **2** was obtained from **1**,<sup>23</sup> and both halogen atoms were subsequently substituted using *N*-nucleophiles. It is known that aliphatic amines and anilines smoothly substitute one chlorine atom, but such substitution induces electronic enrichment of the quinone system and makes difficult the replacement of the second chlorine atom.<sup>35,37</sup> The second reaction step could be favoured if a substituent with electron-withdrawing effect is attached to the quinone ring. Due to that, we chose to acylate the monoaminoquinone, previously to the introduction of the alkylamino substituent. For such proposes, two different acyl groups, acetyl and benzoyl, were chosen.

Thus, treatment of **2** with sodium azide in acetic acid, followed by reduction with NaBH<sub>4</sub> yielded the mixture of regioisomers **11** in low yield (data not shown). However, when ammonia was used as nucleophile, AQs **11** were obtained in 95% yield (Scheme 3). Reaction of **11** with acetic anhydride, in the presence of sulphuric acid at 0 °C, afforded the monoacylated AQs **12** in moderate yield. In order to improve the result, the acylation reaction was performed at room temperature and the imide derivatives **13** were obtained in good yield. When the monoacylated AQs **12** were treated in the same conditions, the same imide analogues **13** were obtained together with the oxazoles **14**. The formation of the later can be easily explained through the intramolecular displacement of the halogen by the oxygen atom of the tautomerized amide function.

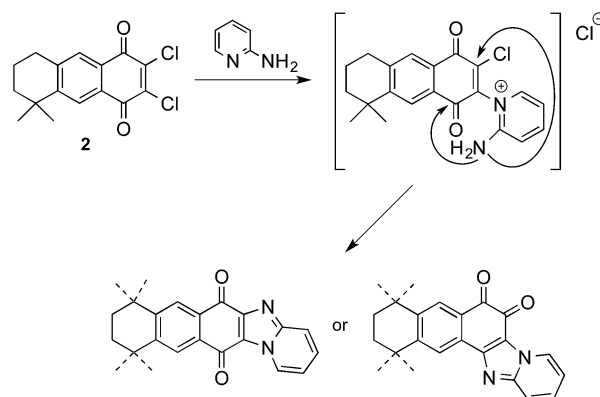


Scheme 4 Synthesis of AQ regioisomers **16a** and **17a**.

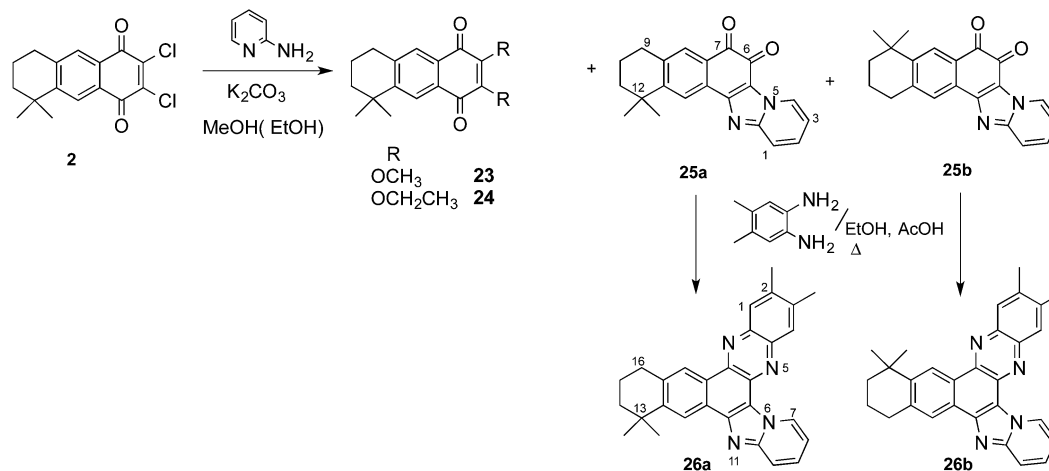


Scheme 5 Preparation of anthra[2,3-*d*]imidazole-4,11-diones.

When chloroquinones **11** were treated with benzoyl chloride in basic media, only the dibenzoylated AQs **15** were detected (Scheme 3). The next step was the substitution of the second chlorine atom using alkylamines and anilines. The corresponding monoacylated derivatives **16–18** were obtained when AQs **13** and **15** were submitted to the substitution reaction, in which one acyl group was transferred to the amine, used in excess in the reaction, as can be deduced from the acetyl-*p*-anisidine isolated during the chromatographic purification of the reaction products. In the case of **15**, the imides **19** were also formed and the reaction of **12** with *p*-anisidine also gave the monoacylated analogues **17**. Aiming to obtain the diacylated analogues from **16** and **17**, both were treated with acetic anhydride in the presence of sulphuric acid but instead the expected products, the oxazoles **14** were formed again as the main products (up to 60% yield).



Scheme 6 Possible reaction products of anthracenequinone **2** with 2-aminopyridine.



Scheme 7 Preparation of 1,2-AQ derivatives **25a/b** and extended condensation products.

The reactions performed until this point gave mixtures of the two regioisomers in a 1 : 1 ratio. In some cases they were separated by CC, but the exact position of the substituents in the AQ ring was undetermined, so that the same reactions were applied to the major regioisomer **3a**, obtained previously at our laboratory.<sup>21b</sup> When **3a** was treated with ammonia followed by acetic anhydride, the imide **13a** was obtained in quantitative yield. The <sup>1</sup>H NMR spectrum of **13a** showed only two singlet signals over 6 ppm ( $\delta$  7.83 and 8.04 ppm) indicating that an addition reaction took place at the position C-3, instead of the substitution reaction of the chlorine atom. Further substitution with primary amines afforded **16a** and **17a**, which allowed us to identify regioisomer “a” as the one with the acyl group on the same side as the geminal methyl groups (Scheme 4).

Once in our hands the disubstituted AQs **16–18**, the regioisomers **16a**, **16b**, **17a**, **17b** separately and the mixture **18a/18b** were treated with NaOH to give the imidazole derivatives **20–22** in good yields (Scheme 5).

**2.1.3. Other heterocycle-fused systems.** In a previous work<sup>21b</sup> we found that 1,2-naphthoquinones (1,2-NQs) fused to a furan ring were more cytotoxic than the corresponding 1,4-NQs. Similar results were obtained with 1,2-naphthohydroquinone diacetates<sup>38</sup> and after that, we explored the formation of 1,2-quinone analogues fused to nitrogenated rings. To get such type of compounds, we chose 2-aminopyridine as nucleophile to react with the dichloro-AQ **2**. It was expected that the aromatic nitrogen promoted first the nucleophilic substitution of one chlorine atom, and then the reaction could evolve in two different ways, depending on if the amino group would produce the substitution of the second chlorine atom or the condensation with the close quinone carbonyl. In the later case, the remaining chlorine atom would be replaced by a hydroxyl group, which should favour the formation of the *o*-quinone.<sup>39</sup> Both possible products are represented in Scheme 6.

Thus, when AQ **2** was treated with 2-aminopyridine in methanol or ethanol, in the presence of K<sub>2</sub>CO<sub>3</sub>, complex

reaction products were obtained from which the major products were the dimethoxy and diethoxy derivatives **23** and **24** respectively, resulting from the substitution of both chlorine atoms by the alcohol used as solvent, along with a small amount of the corresponding *o*-quinones **25** (Scheme 7). The formation of the alkoxy derivatives **23** and **24** should be due to the presence of base, so that the reaction was repeated without base to obtain in moderate yield only the *o*-quinone derivatives **25**. Both regioisomers were separated by CC and their spectroscopic characterization was done on the basis of two-dimensional COSY and HMBC NMR experiments (see ESI†). Representative correlations observed between aromatic hydrogens and some carbonyl and quaternary carbons are shown in Fig. 3. These correlations served to unequivocally assign the structures of **25a** and **25b**. The presence of the 1,2-quinone system was also demonstrated through its reaction with 4,5-dimethyl-*o*-phenylenediamine to give compounds **26a** and **26b**. In order to achieve the formation of 1,2-quinones with six-membered fused-heterocyclic rings, we used aliphatic and aromatic 1,2-diamines in the addition and substitution reactions.

When ethylenediamine was added to the AQ **1** in short times (10 min) only the addition products **27** were detected (Scheme 8); however with longer reaction times (30 min) or under microwave irradiation, mixtures of the addition products **27**

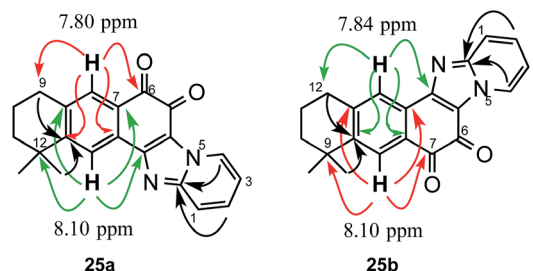
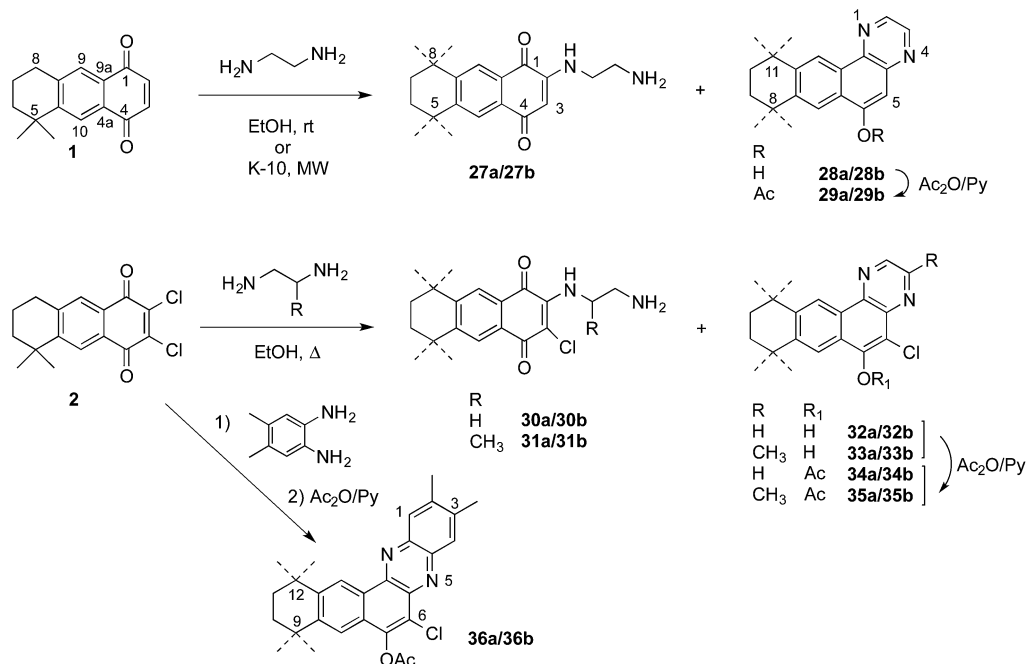


Fig. 3 Selected long-range H/C connectivities for the heterofused *ortho*-quinones **25a** and **25b**.



Scheme 8 Preparation of angular naphthoquinoxaline and naphthophenazine derivatives.

and the further condensation/aromatization products **28** were obtained, giving rise to a polycyclic naphtho[2,3-*a*]quinoxaline system in low yield. The same quinoxaline arrangement was formed when dichloroquinone **2** was treated with ethylenediamines. In these cases, the compounds first formed were the substitution products of one chlorine atom, **30** or **31**, followed by the condensation of the second amine group with one of the carbonyl groups of the quinone system, leading to compounds **32** and **33**. The presence of the phenolic hydroxyl group was confirmed by acetylation to obtain the acetates **34** and **35**. A similar result was attained when 4,5-dimethyl-*o*-phenylenediamine was used as the nucleophile, and only the naphthophenazines **36** were obtained after acetylation of the reaction product.

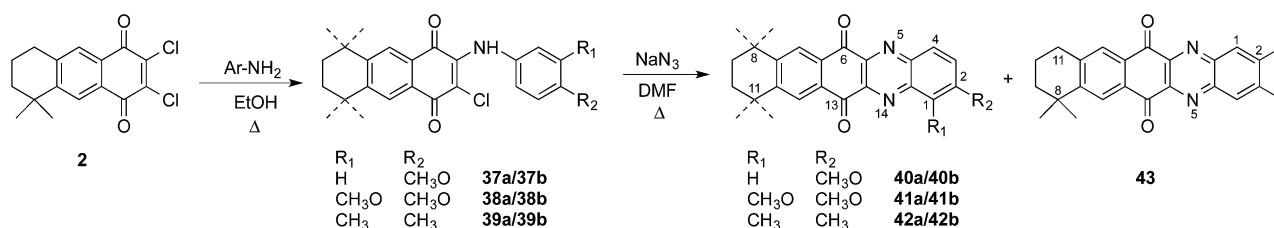
Finally, linear naphthophenazines retaining the *p*-quinone system were prepared in two steps by reaction of the dichloroquinone **2** with arylamines to obtain the mononitrogenated quinones **37–39**, in very good yields,<sup>21</sup> from which further reaction with sodium azide afforded the expected naphthophenazinediones **40–43** in moderate to low yields (Scheme 9).

## 2.2. Antineoplastic cytotoxicity

Most of the compounds prepared were evaluated *in vitro* to establish their cytotoxicity against the following tumour cell lines: A-549 lung carcinoma, HT-29 colon adenocarcinoma and SK-BR3 breast carcinoma using a colorimetric cell growth inhibition assay.<sup>40</sup> The results are shown in Table 1, expressed as GI<sub>50</sub> values (drug concentration in  $\mu\text{M}$  causing 50% growth inhibition). The common anticancer quinone drug doxorubicin was included in the assays as reference. Most of the compounds tested were cytotoxic and from the results obtained, some general observations can be made for each class of heterocycles in comparison with the parent compounds **1** and **2**.

If we considered the 1,4-AQ derivatives without any fused-heterocycle at the quinone ring (Table 1), the presence of an alkyl/aryl/acylamino groups at position C-2 with or without a chlorine atom at C-3, in general improved the cytotoxicity in comparison with the unsubstituted AQ **1** as we have already described for similar 1,4-NQs.<sup>27b</sup>

The better cytotoxicity results, on one or more tumour cell lines tested, were found for those derivatives bearing an arylamino group at C-2, AQs **5**, **6**, **7** and **8**, which showed GI<sub>50</sub> values



Scheme 9 Preparation of linear naphthophenazinequinones.

Table 1 Cytotoxicity data (GI<sub>50</sub> μM)<sup>a</sup> for AQ and AQ-heterofused derivatives

	Compound	A-549	HT-29	SK-BR3
AQs without fused-heterocycle	<b>1</b>	>12.5	9.16	9.95
	<b>2</b>	—	9.64	6.27
	<b>3a</b>	>10.9	>10.9	>10.9
	<b>4a</b>	6.28	1.64	9.88
	<b>4b</b>	6.81	2.07	>10.6
	<b>5a</b>	<b>0.17</b>	<b>0.15</b>	5.70
	<b>5b</b>	8.31	<b>0.14</b>	>8.30
	<b>6a/6b</b>	4.52	<b>0.19</b>	>8.64
	<b>7a/7b</b>	>7.7	1.09	<b>0.38</b>
	<b>8a/8b</b>	5.72	<b>0.18</b>	>7.12
	<b>12a</b>	2.98	3.92	2.47
	<b>12b</b>	4.82	5.42	4.22
	<b>13a</b>	4.55	7.22	3.74
	<b>15a/15b</b>	6.09	9.39	3.81
	<b>16a</b>	>29.4	28.2	>29.4
	<b>16b</b>	>29.4	29.1	13.2
	<b>17a</b>	15.3	11.5	10.5
	<b>19a/19b</b>	>17.1	>17.1	>17.1
	<b>30a/30b</b>	2.79	3.71	>30.1
	<b>37a/37b</b>	4.04	3.54	3.03
<b>38a/38b</b>	10.1	19.7	9.39	
<b>39a/39b</b>	12.7	21.6	11.4	
AQs with linear fused-heterocycle	<b>9a</b>	14.0	11.8	15.7
	<b>9b</b>	>27.2	12.1	17.1
	<b>10a</b>	>26.4	11.6	>26.4
	<b>10b</b>	>26.4	>26.4	17.1
	<b>14a</b>	1.79	5.76	1.93
	<b>14b</b>	1.93	6.09	1.93
	<b>20a</b>	8.68	11.5	52.7
	<b>20b</b>	12.1	13.0	14.9
	<b>21a</b>	9.24	10.2	9.74
	<b>21b</b>	23.0	>25	12.2
	<b>40a/40b</b>	5.10	4.83	4.83
	<b>42a/42b</b>	>27	>27	>27
	<b>43</b>	>27	>27	15.7
	AQs with angular fused-heterocycles	<b>25a</b>	1.06	12.4
<b>25b</b>		8.17	>30.3	>30.3
<b>26a</b>		>23.2	16.3	>23.2
<b>26b</b>		>23.2	>23.2	>23.2
<b>32a/32b</b>		13.1	13.1	13.1
<b>34a/34b</b>		11.3	9.02	13.5
<b>35a/35b</b>		>27.2	>27.2	>27.2
<b>36a/36b</b>		>23.1	>23.1	>23.1
Doxorubicin		0.07	0.10	0.09

<sup>a</sup> GI<sub>50</sub> values are expressed as the mean from triplicate determinations on A-549 lung carcinoma, HT-29 colon adenocarcinoma and SK-BR3 breast carcinoma. Values under 1 μM are bold-faced to facilitate comparisons.

under de μM level (bold-faced in Table 1), very close to those of doxorubicin. When the quinone ring was disubstituted at C-2 and C-3 with an alkyl/aryl amino group and a chlorine atom, the cytotoxicity slightly decreased with respect to those mono-substituted (37 vs. 5), although they were more cytotoxic than the unsubstituted **1**, keeping the GI<sub>50</sub> values in the μM range. Additionally, the simultaneous presence of an alkyl/aryl amino and an acylamino groups at neighbouring positions led to a decrease in cytotoxicity, as in AQs **16**, **17** and **19**.

Some of these derivatives were several times more potent on the HT-29 cell line than on the other cell lines tested, thus indicating a certain degree of selectivity, which is always an

objective in the design of better drugs. When possible, the regioisomers were tested separately, but no significant differences were observed between them, except on the A-549 cell line for AQ **5**, whose **5a** isomer was nearly fifty times more potent than the **5b**.

Those analogues with a heterocycle fused to the quinone ring at positions C-2/C-3 (linearly fused-heterocycle, Table 1) showed from moderate to poor cytotoxicity values, with a certain dependence on the ring size and on the number and type of heteroatoms present. Those with pyrrole- or imidazole-fused rings were cytotoxic in the same level than the parent compound **1**, giving slightly better results with the

imidazole-fused than with the pyrrole-fused rings. However, the presence of two different heteroatoms, as in compound **14**, which bear an oxazole ring, led to a potency increase in comparison with the parent AQ **1**.

Considering the six-membered fused rings, the nature of the substituent present in the additional aromatic rings seemed important for the bioactivity, and the presence of a methoxy group was much better than the presence of methyl groups (**42**, **43** vs. **40**).

Heterocycles fused at C-1/C-2 positions of the quinone ring (angularly fused-heterocycle, Table 1) did not improve at all the cytotoxicity, and only compound **25a**, which contains an *o*-quinone system, showed better cytotoxicity, though only against the A-549 cell line, as compared with the imidazole linearly-fused 1,4-quinones **20** and **21**, being worth to notice the difference in potency observed for both regioisomers **25a** and **25b**. The other analogues in this group did not contain the 1,2-quinone system and this could explain their poor cytotoxicity. Again, the presence of methyl groups on the added heterocyclic system led to lesser potent compounds.

### 2.3. Antifungal activity

Recently we have described the antifungal activity of several 1,4-NQs and 1,4-AQs related to those reported here.<sup>23</sup> Continuing this line of research, and in order to analyse the influence of the fused-heterocycles on the antifungal activity of 1,4-AQs, several representative compounds obtained in this work were also evaluated against *Candida* spp., *Aspergillus* spp., dermatophytes, and *Fusarium oxysporum*. The minimum inhibitory concentration 90 (MIC<sub>90</sub>), was determined following the standard microdilutions method AFST-EUCAST and CLSI M38-A,<sup>41,42</sup> with some modifications, for yeast and filamentous fungi, respectively. Minimum inhibitory concentrations 90 were expressed as the Geometric mean (GM-MIC<sub>90</sub>). Only those derivatives that showed MIC values lower than 32.0 μg mL<sup>-1</sup> (~100 μM) against one or more strains were considered active.

The selected results are shown in Table 2. Three standard drugs, itraconazole (ITZ), amphotericin B (AMB) and terbinafine (TRB), were used as reference drugs to cover the fungal spectrum. Complete evaluation data for all the compounds tested are given as ESI.† Also cytotoxicity was evaluated in Vero cells (African green monkey kidney-*Cercopithecus aethiops* ATCC: CCL 81 line) and the cytotoxic concentrations 50% (IC<sub>50</sub>) were calculated and included in Tables 2 and 3.

Seven of seventeen evaluated compounds were actives against *Trichophyton* spp. while five compounds showed activity against *Candida* spp. and three against *A. fumigatus*. The other *Aspergillus* spp. and *F. oxysporum* were less susceptible. Only compound **2** resulted active against *A. flavus* and **3a** and **12a** against *F. oxysporum*. These two later compounds showed fairly wide antifungal spectra. The most potent compounds against *Candida* spp. were **1**, **2**, **3a**, **12a** and **14**. The AQ **12a** was the best against both tested strains of *C. albicans*. This yeast is the most important in infections caused by fungi. AQ **12a** was also active against other pathogenic yeasts like *C. parapsilosis*, *C. krusei*, *C. lusitaniae* and *C. tropicalis* with GM-MIC<sub>90</sub> values from 2.2 to 8.0 μg mL<sup>-1</sup>.

Compound **2** showed important activity against *C. parapsilosis*, *C. krusei* and *C. lusitaniae*. These results are important considering the intrinsic resistance to fluconazole and echinocandin of *C. krusei* and *C. parapsilosis*, respectively and also because *C. lusitaniae* has the ability to rapidly develop resistance to AMB.<sup>34</sup> This antifungal is the most important available agent for treating candidiasis and actually the only antifungal that has a broad spectrum of activity.

AQs **2**, **3a** and **12a** and oxazole **14**, showed moderate activity against *Aspergillus fumigatus*. The compound **2** was the most active against this filamentous fungus and also was active against *A. flavus*. None of the tested compounds were active against *A. terreus* and *A. niger*. *A. terreus* is intrinsically resistant to AMB and less susceptible to other antifungals<sup>43</sup> for this

**Table 2** *In vitro* susceptibilities of *Candida* spp. and filamentous fungi (GM-MIC<sub>90</sub>, μg mL<sup>-1</sup>) and Vero cell cytotoxicity (IC<sub>50</sub>, μg mL<sup>-1</sup>) for selected AQs and heterofused derivatives<sup>a</sup>

Comp	<i>Cp</i>	<i>Ck</i>	<i>Cl</i>	<i>Ct1</i>	<i>Ct2</i>	<i>Ca1</i>	<i>Ca2</i>	HSI ( <i>C. spp.</i> )	<i>Afu</i>	<i>Afl</i>	<i>At</i>	<i>An</i>	<i>Fo</i>	HSI ( <i>A. spp.</i> )	<i>Tm</i>	<i>Tr</i>	HSI ( <i>T. spp.</i> )	Vero
<b>1</b>	<b>4.8</b>	<b>4.0</b>	<b>5.0</b>	12.7	<b>5.0</b>	18.0	22.6	2.2 ( <i>Ck</i> )	>32	>32	>32	>32	>32	na	<b>1.3</b>	<b>4.0</b>	6.6 ( <i>Tm</i> )	8.6
<b>2</b>	<b>2.0</b>	<b>1.0</b>	<b>2.0</b>	>32	<b>5.0</b>	>32	<b>8.0</b>	<b>42.2</b> ( <i>Ck</i> )	<b>8.0</b>	<b>4.8</b>	>32	>32	>32	<b>8.8</b> ( <i>Afl</i> )	<b>6.1</b>	<b>8.0</b>	6.9 ( <i>Tm</i> )	42.2
<b>3a</b>	<b>4.0</b>	<b>2.0</b>	<b>5.0</b>	12.7	<b>8.0</b>	20.2	16.0	<b>54.5</b> ( <i>Ck</i> )	16	>32	>32	>32	16	6.8 ( <i>Afu</i> )	<b>3.2</b>	<b>5.0</b>	<b>34.1</b> ( <i>Tm</i> )	109
<b>4b</b>	>32	>32	>32	>32	>32	>32	>32	na	>32	>32	>32	>32	>32	na	<b>5.0</b>	<b>3.2</b>	<b>11.1</b> ( <i>Tr</i> )	35.6
<b>12a</b>	<b>8.0</b>	<b>2.8</b>	<b>2.2</b>	<b>6.4</b>	<b>4.0</b>	<b>4.0</b>	<b>8.0</b>	<b>10.1</b> ( <i>Cl</i> )	22.6	>32	>32	>32	32	0.9 ( <i>Afu</i> )	<b>3.2</b>	<b>5.0</b>	7.0 ( <i>Tm</i> )	22.3
<b>14</b>	<b>6.7</b>	19.0	20.2	>32	32	>32	>32	0.7 ( <i>Cp</i> )	16	>32	>32	>32	>32	0.3 ( <i>Afu</i> )	32	<b>8.0</b>	0.6 ( <i>Tr</i> )	4.6
<b>38</b>	>32	>32	>32	>32	>32	>32	>32	na	>32	>32	>32	>32	>32	na	25.4	25.4	2.0	50.5
ITZ	0.25	0.5						>100 ( <i>Ck</i> )										>50
AMB									2.0	4.0				7.1 ( <i>Afl</i> )				28.6
TRB															0.03	0.08	386 ( <i>Tr</i> )	30.9

<sup>a</sup> Minimum inhibitory concentrations 90 (MIC<sub>90</sub>) values are expressed as the geometric mean (GM). Cytotoxic Concentrations 50% (IC<sub>50</sub>, Vero cells) found in triplicate experiments for each compound. *Cp*: *Candida parapsilosis*; *Ck*: *C. krusei*; *Cl*: *C. lusitaniae*; *Ct1* and *Ct2*: *C. tropicalis*; *Ca1* and *Ca2*: *C. albicans*; *Afu*: *Aspergillus fumigatus*; *Afl*: *A. flavus*; *At*: *A. terreus*; *An*: *A. niger*; *Fo*: *Fusarium oxysporum*; *Tm*: *Trichophyton mentagrophytes*; *Tr*: *T. rubrum*. Vero cells (African green monkey kidney-*Cercopithecus aethiops*). na: not applicable. ITZ: itraconazole; AMB: amphotericin B; TRB: terbinafine. HSI: Highest Selectivity Index (IC<sub>50</sub> Vero/lowest MIC<sub>90</sub> found for each compound) rounded values. MIC values under 10 μg mL<sup>-1</sup> and HSI over 8 are bold-faced to facilitate comparisons. Complete table with MIC values ± SD are given in ESI and for fungal strain characterizations, see experimental part.



Table 3 Antiviral activity of selected AQ derivatives

Compound	Vero ATCC CCL-81 <sup>a</sup>						Vero cells IC <sub>50</sub> (μg mL <sup>-1</sup> )
	1 TCID <sub>50</sub> <sup>b</sup> HHV-1			10 TCID <sub>50</sub> <sup>c</sup> HHV-2			
	(R <sub>f</sub> ) <sup>d</sup>	Antiviral activity <sup>e</sup> (μg mL <sup>-1</sup> )	SI <sup>f</sup>	(R <sub>f</sub> ) <sup>d</sup>	Antiviral activity <sup>e</sup> (μg mL <sup>-1</sup> )	SI <sup>f</sup>	
<b>1</b>	10 <sup>0.5</sup>	6.25	1.37	nd	>50	nd	8.6
<b>2</b>	10 <sup>0.5</sup>	50	0.85	10 <sup>2</sup>	25	1.7	42.2
<b>3a</b>	10 <sup>2</sup>	6.25	17.5	nd	>50	nd	109.3
<b>10a</b>	nd	>50	nd	10 <sup>1</sup>	50	1.39	69.5
<b>12a</b>	nd	>50	nd	10 <sup>1</sup>	12.5	1.78	22.3
<b>14</b>	nd	>50	nd	10 <sup>1</sup>	25	0.18	4.6
<b>37a/b</b>	10 <sup>1</sup>	50	1.42	nd	>50	nd	71.0
<b>38a/b</b>	10 <sup>1</sup>	50	1.01	nd	>50	nd	50.5
<b>39a/b</b>	10 <sup>1</sup>	50	≤0.5	nd	>50	nd	≤25
<b>43</b>	nd	>50	nd	10 <sup>1</sup>	50	0.33	16.4
Dextran sulphate	10 <sup>2</sup>	0.5	nd	10 <sup>2</sup>	0.5	nd	
Acyclovir	10 <sup>4</sup>	1.5	nd	10 <sup>4</sup>	1.5	nd	

<sup>a</sup> Vero *Cercopithecus aethiops* African green monkey kidney cell line ATCC CCL 81. <sup>b</sup> 1TCID<sub>50</sub>: 1 cell culture infectious dose fifty percent. <sup>c</sup> 10TCID<sub>50</sub>: 10 cell Culture infectious dose fifty percent. <sup>d</sup> R<sub>f</sub>: value obtained by dividing the viral titer in the absence of the compound on the titer obtained in presence of the compound. <sup>e</sup> Maximum non-toxic concentration of compound which had the highest factor of reduction of viral titer. <sup>f</sup> SI: selectivity index (IC<sub>50</sub> Vero/TCID<sub>50</sub>); nd: not determined.

reason it would be important to find new active molecules against this fungus.

The AQ **3a**, in addition to be fairly active against *Candida* spp. and *A. fumigatus*, showed moderate activity against *F. oxysporum*. This result is relevant because, compared with other filamentous fungi, *Fusarium* species are intrinsically resistant to ITZ and echinocandins and the response to AMB and voriconazole is variable depending on the particular species.<sup>44</sup>

All compounds listed in Table 2 were active against both dermatophytes strains. AQs **3a**, **4** and **12a**, showed similar good activity against both *Trichophyton* species. AQ **1** was the most active against *T. rubrum* and *T. mentagrophytes*. This compound may be of interest as a lead compound for further studies in order to develop new molecules with activity against this group of fungi.

According to this data and the previous results with similar NQs,<sup>23</sup> we can conclude that, in general, the precursor 1,4-AQs showed better antifungal activity than their heterofused derivatives. Only the oxazole-fused compound **14** was active against several strains, although its selectivity was poor. The MIC value for compound **14** against *C. parapsilosis* (MIC<sub>90</sub> = 6.7 μg mL<sup>-1</sup>) was approximately three times lower than for the other *Candida* species. These data are important because *C. parapsilosis* exhibits a consistent trend of decreasing fluconazole susceptibility over the treatment time.<sup>45</sup> AQ **14** also showed activity against *A. fumigatus*, but not against other *Aspergillus* spp. or *F. oxysporum*. However, it was also active against both *Trichophyton* species.

The viability of the Vero cell line in the presence of the seven 1,4-AQ derivatives selected was evaluated and the corresponding cytotoxicity calculated (Tables 2 and 3). All the tested compounds produced a dose-dependent inhibition on the Vero cell line, with linear regression coefficients higher than 0.7

(ESI†). According to the results obtained, the compounds reported here should be considered as low cytotoxic for normal monkey cells and the highest selectivity index (HSI = IC<sub>50</sub> for Vero cells/lowest GM-MIC<sub>90</sub> value of a compound against any pathogenic fungus) was calculated in order to quantify their respective selectivity and to facilitate comparisons.

According to literature, SI values higher than 8 are considered indicative of a potential therapeutic agent that merit further attention.<sup>46</sup> To this respect, AQs **2**, **3a** and **12a** were the most selective against *Candida* spp. (HSI = 10.1–54.5), AQ **2** was also selective against *A. flavus* and compounds **3a** and **4b** were selective against dermatophytes. At this point, it is important to note that HSI values should be higher than those calculated and included in Table 2 if we have in mind that MIC values refer to nearly total (90–100%) fungal growth inhibition and IC<sub>50</sub> values refer to the inhibition of only 50% of Vero cells.

#### 2.4. Antiviral activity

It has been reported that the most effective drugs against herpetic infections, acyclovir and its related prodrugs and analogues, have developed a number of drug-resistant strains after prolonged or inadequate use.<sup>33,47</sup> Consequently, the search for new therapeutic agents, with non-related structures and different mechanisms of action, seems necessary to maintain efficacious treatments, and can justify the antiviral evaluation of some representative AQS synthesised in this work.

The antiviral activity was evaluated against the 50% Tissue Culture Infective Dose, 1TCID<sub>50</sub> and 10TCID<sub>50</sub>, of Human Herpes virus 1 (HHV-1 CDC Atlanta acyclovir-sensitive strain) and Human Herpes virus 2 (HHV-2 VR-734-G acyclovir-sensitive strain) respectively, by determining the reduction factor (R<sub>f</sub>), which corresponds to the value obtained by dividing the viral titer in the absence of compound over the viral titer obtained in

the presence of compound.<sup>48</sup> Two positive controls, dextran sulfate and acyclovir, were included in this assay. Dextran sulfate was included because this sulfated polysaccharide can inhibit viral entry by a competitive effect with heparan sulfate receptors present on the cell surface. In addition, acyclovir was included because is effective in the inhibition of viral DNA polymerase. Compounds that showed reduction of viral titer respect to control ( $R_f$ ) at concentrations lower or equal than  $50 \mu\text{g mL}^{-1}$ , were considered active and are collected in Table 3. Complete evaluation data for all the compounds tested are given as ESI.†

In this study, compounds that showed mild activity ( $R_f \leq 1 \times 10^1$ ) against HHV-1 at a concentration of  $50 \mu\text{g mL}^{-1}$  were **2**, **37**, **38** and **39**, while AQ **1** attained the same  $R_f$  at a concentration of  $6.25 \mu\text{g mL}^{-1}$ . Notably, AQ **3a** showed better anti HHV-1 activity ( $R_f = 1 \times 10^2$ ) at a concentration of  $6.25 \mu\text{g mL}^{-1}$ . The AQ **10a** and the naphthophenazine **43** showed mild activity against HHV-2 at a concentration of  $50 \mu\text{g mL}^{-1}$ . Moreover, compounds **12a** and **14** were active at a concentration of 12.5 and  $25 \mu\text{g mL}^{-1}$ , respectively. Only AQ **2** showed moderate anti HHV-2 activity ( $R_f = 1 \times 10^2$ ) at a concentration of  $25 \mu\text{g mL}^{-1}$ .

In our study, the parameter established to define antiviral selectivity was the selectivity index (SI) value. SI is defined as the relative effectiveness of a product to inhibiting viral replication compared to the capacity for inducing cell death. It is desirable to have a high therapeutic index ( $SI \geq 10$ ) giving maximum antiviral activity with minimal cell toxicity.<sup>48</sup> The antiviral SI for each active compound was calculated and listed in Table 3. Our results showed that only compound **3a** showed a high antiviral SI (17.5) against HHV-1 strain. This value could be considered indicative of a potential lead compound for further antiviral studies.<sup>49</sup>

### 3. Conclusions

As a global consideration, the best biological results were principally found for 1,4-AQs and their substituted derivatives rather than for their heterocycle-fused analogues. Among the 1,4-AQs, those bearing an arylamino group at C-2 were the most cytotoxic against the tumour cell lines tested, while the presence of a chlorine atom at the quinone moiety seemed to improve the antifungal and antiviral activities. Though the heterofused AQs were generally less potent than their corresponding AQ precursors and intermediates, the oxazole-fused compound **14** showed positive results on the three biological tests.

The relevance of the antifungal activity varies with the pathogenicity of the fungus. In general, filamentous fungi are more resistant than yeasts to antifungal agents. Then, the discovery of AQ **2**, active against *A. flavus* and *A. fumigatus* is considered enough relevant. Furthermore, the finding of several molecules (AQs **1**, **2**, **3a**, and **12a** and the oxazole-fused AQ **14**), active against yeasts as *C. parapsilosis* and *C. krusei*, with lower susceptibility to one or several standard antifungal drugs, could support further studies to establish their mechanism(s) of action.

Finally, among the compounds tested as antiviral, the chlorinated 1,4-AQ **3a**, as an active compound against HHV-1 with

the highest selectivity index, could be considered as a lead compound for further research.

At the current level of our research, we cannot formulate consistent proposals for possible mechanisms or targets to the new type of compounds, with novel structural arrangements directly attached to the quinone ring. Further experimental research will be planned with such objectives.

## 4. Experimental section

### 4.1. Chemistry

NMR spectra were recorded on a Bruker AC 200 at 200 MHz for  $^1\text{H}$  and 50.3 MHz for  $^{13}\text{C}$  in deuteriochloroform with TMS as internal standard. Chemical shift ( $\delta$ ) values are expressed in ppm followed by multiplicity and coupling constants ( $J$ ) in Hz. IR spectra were obtained on a Nicolet Impact 410 spectrophotometer in NaCl film, and wavenumbers are given in  $\text{cm}^{-1}$ . UV spectra were determined on a Hitachi 100-60 spectrophotometer in  $\text{CHCl}_3$  solution, and  $\lambda_{\text{max}}$  are given in nm. HRMS were run in a VG-TS-250 spectrometer working at 70 eV. Column chromatography (CC) was performed on silica gel (Merck no. 9385) and TLC was carried out on silica gel 60 F<sub>245</sub> (Merck, 0.25 mm thick). Solvents and reagents were purified by standard procedures as necessary.

$^1\text{H}$  NMR data are given for the new compounds described here and complete  $^1\text{H}$  and  $^{13}\text{C}$  NMR assignments are included in the ESI.†

Anthracene-1,4-diones **1–8**, **12**, **24**, and **37–39** were obtained by means of previously described procedures.<sup>21,23</sup> Other compounds were prepared as follows.

**4.1.1. 3-Acetyl-1-ethyl-2,6,6-trimethyl-1H-6,7,8,9-tetrahydronaphtho[2,3-f]indole-4,11-dione 9a.** To a solution of **4a** (22 mg, 0.08 mmol) and 2,4-pentanedione (32  $\mu\text{L}$ , 0.29 mmol) in MeOH (1 mL) and dichloromethane (0.2 mL) stirred at rt was added CAN (154 mg, 0.28 mmol) in four portions at 10 min intervals. The reaction mixture was stirred for another 30 min and then diluted with EtOAc, washed with sat aq sodium bicarbonate and brine, dried over  $\text{Na}_2\text{SO}_4$  and vacuum-evaporated to yield a product, which was purified by column chromatography (eluent: Hex/EtOAc 8 : 2) affording **9a** (12 mg, 43%): mp 128–132 °C (from  $\text{CH}_2\text{Cl}_2$ ); UV  $\lambda_{\text{max}}$ (log  $\epsilon$ ): 270 (4.6), 297 (4.2), 388 (3.9); IR  $\nu_{\text{max}}$  (film): 1657, 1596, 1495, 1463, 1265, 1026  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 1.34 (6H, s), 1.39 (3H, t,  $J = 7.1$ ), 1.70–1.82 (4H, m), 2.42 (3H, s), 2.72 (3H, s), 2.88 (2H, t,  $J = 6.3$ ), 4.52 (2H, m), 7.78 (1H, s), 8.09 (1H, s); HRMS (ES, M + Na) calcd: 386.1726, found: 386.1736.

**4.1.2. 3-Acetyl-1-ethyl-2,9,9-trimethyl-1H-6,7,8,9-tetrahydronaphtho[2,3-f]indole-4,11-dione 9b.** Following the above procedure, treatment of **4b** and 2,4-pentanedione with CAN, gave **9b** (47%): mp 137–140 °C (from  $\text{CH}_2\text{Cl}_2$ ); IR  $\nu_{\text{max}}$  (film): 1660, 1596, 1495, 1465, 1265, 1017  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 1.35 (6H, s), 1.41 (3H, t,  $J = 7.1$ ), 1.71–1.83 (4H, m), 2.43 (3H, s), 2.70 (3H, s), 2.88 (2H, t,  $J = 6.3$ ), 4.52 (2H, m), 7.79 (1H, s), 8.09 (1H, s); HRMS (ES, M + Na) calcd: 386.1726, found: 386.1734.

**4.1.3. Methyl 1-ethyl-2,6,6-trimethyl-4,11-dioxo-1H-6,7,8,9-tetrahydronaphtho[2,3-f]indole-3-carboxylate 10a.** Following the above procedure, treatment of **4a** and methyl acetylacetate

with CAN, gave **10a** (40%): mp 160–163 °C (from CH<sub>2</sub>Cl<sub>2</sub>); IR  $\nu_{\max}$  (film): 1711, 1660, 1597, 1464, 1263, 1018 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.32 (6H, s), 1.40 (3H, t, *J* = 7.1), 1.69–1.81 (4H, m), 2.48 (3H, s), 2.86 (2H, t, *J* = 6.2), 3.95 (3H, s), 4.52 (2H, m), 7.78 (1H, s), 8.07 (1H, s); HRMS (ES, M + Na) calcd: 402.1675, found: 402.1691.

**4.1.4. Methyl 1-ethyl-2,9,9-trimethyl-4,11-dioxo-1H-6,7,8,9-tetrahydronaphtho[2,3-*f*]indole-3-carboxylate 10b.** Following the above procedure, treatment of **4b** and methyl acetylacetate with CAN, gave **10b** (22%): mp 150–154 °C (from CH<sub>2</sub>Cl<sub>2</sub>); IR  $\nu_{\max}$  (film): 1710, 1668, 1597, 1465, 1270, 1108 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.33 (6H, s), 1.39 (3H, t, *J* = 7.1), 1.72–1.82 (4H, m), 2.48 (3H, s), 2.87 (2H, t, *J* = 6.0), 3.97 (3H, s), 4.52 (2H, m), 7.77 (1H, s), 8.11 (1H, s).

**4.1.5. 3-Amino-2-chloro-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones 11a(11b).** To a solution of AQ 2 (1.82 mmol) in EtOH (30 mL), 30% NH<sub>4</sub>OH (17 mL) was added dropwise and kept stirring at room temperature for 1 h. Then, the solvent was vacuum-evaporated and the residue was redissolved in ethyl acetate. The organic layer was washed with 2 N HCl and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated till dryness, affording a 1 : 1 mixture of **11a/11b**, that was used in the next steps without purification.

Treatment of **3a** (0.71 mmol) with 30% NH<sub>4</sub>OH (7 mL), following the above procedure, afforded **11a**.

**4.1.6. 3-Acetamido-2-chloro-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones 12a(12b).** AQ **11a/11b** (0.36 mmol) were acetylated at 0 °C with Ac<sub>2</sub>O–H<sub>2</sub>SO<sub>4</sub> (6 mL taken from a previously solution prepared with 50 mL of Ac<sub>2</sub>O and 0.15 mL of H<sub>2</sub>SO<sub>4</sub>) for 30 min. The reaction was quenched with ice and extracted with EtOAc. The organic layer was washed with satd aq NaHCO<sub>3</sub>, and brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and vacuum-evaporated yielding a crude product, which was purified by column chromatography (eluent: Hex/EtOAc 8 : 2) to afford **12a/12b**.<sup>23</sup>

**4.1.7. 2-Chloro-3-diacetyl-amino-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones 13a(13b).** AQ **11a/11b** (1.73 mmol) were acetylated at rt with Ac<sub>2</sub>O–H<sub>2</sub>SO<sub>4</sub> (31 mL taken from a solution previously prepared with 50 mL of Ac<sub>2</sub>O and 0.15 mL of H<sub>2</sub>SO<sub>4</sub>) for 1 h. The reaction was worked up as described above to afford **13a/13b** (80%): IR  $\nu_{\max}$  (film): 1670, 1599, 1493, 1288, 1253, 1048 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.34 (6H, s), 1.71–1.86 (4H, m), 2.28/2.36 (6H, s), 2.90/2.91 (2H, t, *J* = 6.2), 7.83/8.17 (1H, s), 8.04/7.82 (1H, s); HRMS (ES, M + Na) calcd: 396.0973, found: 396.0986.

Treatment of **11a** with Ac<sub>2</sub>O–H<sub>2</sub>SO<sub>4</sub>, in the same way described above yielded **13a** (96%).

**4.1.8. 2-Chloro-3-dibenzoylamino-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones 15a(15b).** AQ **11a/11b** (0.83 mmol) and NaH (1.8 mmol) were stirred at room temperature until compound **12** disappeared by TLC control. Then, benzoyl chloride (1.23 mmol) was added and stirred for another 5 min. The reaction mixture was quenched with water and extracted with EtOAc. The organic layer was washed with satd aq NaHCO<sub>3</sub>, and brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and vacuum-evaporated yielding a crude product, which was purified by column chromatography (eluent: Hex/EtOAc 8 : 2) to afford AQs

**15a/15b** (30%): <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.30/1.33 (6H, s), 1.71–1.81 (4H, m), 2.83–2.93 (2H, m), 7.27–7.47 (6H, m), 7.73–7.77 (4H, m), 7.84/8.12 (1H, s), 8.07/7.78 (1H, s); HRMS (ES, M + Na) calcd: 520.1286, found: 520.1277.

**4.1.9. General procedure for the nucleophilic substitution to obtain compounds 16–19.** AQ **12**, **13** or **15** and the corresponding amine in EtOH were stirred at reflux for a specify time. Then, the solvent was vacuum-evaporated and the residue was redissolved in ethyl acetate. The organic layer was washed with 2 N HCl and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated till dryness, affording a crude product, which was purified through column chromatography over silica gel.

**4.1.10. 3-Acetamido-2-ethylamino-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones 16a(16b).** Following the above procedure, treatment of **13a/13b** (0.33 mmol) with ethylamine (0.8 mmol) for 45 min, gave out, after preparative TLC (eluent EtOAc): (a) 15 mg (13%) of **16a**: mp 160–165 °C (from MeOH); IR  $\nu_{\max}$  (film): 3300, 1674, 1594, 1558, 1505, 1341, 1257, 1013, 809 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.24 (2H, t, *J* = 7.2), 1.31 (6H, s), 1.69–1.80 (4H, m), 2.23 (3H, s), 2.83 (2H, t, *J* = 6.1), 3.45 (2H, m), 7.68 (1H, s), 7.99 (1H, s); HRMS (ES, M + Na) calcd: 363.1679, found: 363.1689. (b) 9 mg (8%) of **16a/16b**. (c) 16 mg (14%) of **16b**: mp 154–159 °C (from MeOH); IR  $\nu_{\max}$  (film): 3283, 1673, 1600, 1595, 1515, 1341, 1300, 1005, 919 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.24 (2H, t, *J* = 7.0), 1.31 (6H, s), 1.66–1.80 (4H, m), 2.22 (3H, s), 2.85 (2H, t, *J* = 6.1), 3.45 (2H, m), 7.69 (1H, s), 7.97 (1H, s); HRMS (ES, M + Na) calcd: 363.1679, found: 363.1666.

Following the same procedure, treatment of **13a** (0.7 mmol) with ethylamine (0.7 mmol) afforded **16a** (48%) after CC.

**4.1.11. 3-Acetamido-2-(*p*-methoxyanilino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones 17a(17b).** Following the above procedure, treatment of **13a/13b** (0.2 mmol) with *p*-anisidine (0.5 mmol) for 20 h at rt, gave out, after two consecutive preparative TLC (eluents Hex/EtOAc 3 : 7 and benzene/methanol 9 : 1): (a) 3 mg (4%) of **17a**: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.33 (6H, s), 1.62 (3H, s), 1.67–1.85 (4H, m), 2.87 (2H, t, *J* = 6.4), 3.80 (3H, s), 6.81 (2H, d, *J* = 9.1), 6.89 (2H, d, *J* = 9.1), 7.75 (1H, s), 8.02 (1H, s). (b) 30 mg of **17a/17b** impurified with *N*-acetylanisidine. (c) 7 mg (9%) of **17b**: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.34 (6H, s), 1.61 (3H, s), 1.67–1.85 (4H, m), 2.88 (2H, t, *J* = 6.4), 3.80 (3H, s), 6.80 (2H, d, *J* = 9.0), 6.90 (2H, d, *J* = 9.0), 7.76 (1H, s), 8.04 (1H, s).

Treatment of **12a/12b** (0.08 mmol) with *p*-anisidine (0.11 mmol) following the general procedure for 1.5 h, afforded the mixture **17a/17b** in quantitative yield.

Treatment of **13a** (0.3 mmol) with *p*-anisidine (0.7 mmol) following the same procedure, afforded **17a** impurified with *N*-acetylanisidine.

**4.1.12. 3-Benzamido-2-(*p*-methoxyanilino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones 18a(18b) and 3-dibenzoylamino-2-(*p*-methoxyanilino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones 19a(19b).** Following the above procedure, treatment of **15a/15b** (0.26 mmol) with *p*-anisidine (0.56 mmol) for 1 h, gave out, after column chromatography (eluent: toluene/EtOAc 8 : 2): (a) 29 mg of **19a/19b** with *N*-acetylanisidine, which were submitted to PTLC (eluent: benzene/EtOAc 8 : 2) to yield 14 mg (11%) of **19a/19b**: IR  $\nu_{\max}$  (film): 3346, 1664, 1599, 1508, 1474, 1334, 1249, 1029 cm<sup>-1</sup>; <sup>1</sup>H NMR

(CDCl<sub>3</sub>)  $\delta$ : 1.30/1.33 (6H, s), 1.67–1.82 (4H, m), 2.86 (2H, m), 3.66 (3H, s), 6.56 (2H, d,  $J$  = 8.8), 6.89 (2H, d,  $J$  = 8.8), 7.21–7.76 (10H, m), 7.76 (1H, s), 8.03/8.04 (1H, s); HRMS (ES, M + Na) calcd: 607.2203, found: 607.2200. (b) 19 mg of **18a/18b** (19%): IR  $\nu_{\max}$  (film): 3300, 1667, 1596, 1515, 1472, 1343, 1284, 1031 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.30/1.33 (6H, s), 1.68–1.83 (4H, m), 2.88 (2H, t,  $J$  = 6.2), 3.59 (3H, s), 6.64 (2H, d,  $J$  = 8.7), 6.91 (2H, d,  $J$  = 8.7), 7.30–7.80 (5H, m), 7.75/7.77 (1H, s), 8.05/8.07 (1H, s); HRMS (ES, M + Na) calcd: 503.1941, found: 503.1924.

**4.1.13. 2,6,6(2,9,9)-Trimethyl-6,7,8,9-tetrahydroanthra[2,3-*d*]oxazole-4,11-dione 14a(14b).** Treatment of **16a/16b** (0.06 mmol) with Ac<sub>2</sub>O–H<sub>2</sub>SO<sub>4</sub> as described above for **12a/12b**, yielded a reaction product that was chromatographed on PTLC (eluent Hex/EtOAc 1 : 1) to give: (a) 4 mg (24%) of **14a**: mp 154–156 °C (from CH<sub>2</sub>Cl<sub>2</sub>); IR  $\nu_{\max}$  (film): 1674, 1588, 1453, 1381, 1240, 1089, 1031, 803 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.35 (6H, s), 1.71–1.83 (4H, m), 2.63 (3H, s), 2.82 (2H, d,  $J$  = 6.2), 7.57 (1H, s), 7.81 (1H, s); HRMS (ES, M + Na) calcd: 318.1100, found: 318.1041. (b) 6 mg (36%) of **14b**: mp 135–138 °C (from CH<sub>2</sub>Cl<sub>2</sub>); IR  $\nu_{\max}$  (film): 1681, 1592, 1462, 1420, 1264, 1091, 1032, 736 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.31 (6H, s), 1.67–1.86 (4H, m), 2.61 (3H, s), 2.82 (2H, m), 7.32 (1H, s), 8.10 (1H, s); HRMS (ES, M + Na) calcd: 318.1100, found: 318.1096.

Treatment of **17a/17b** (0.09 mmol) with Ac<sub>2</sub>O–H<sub>2</sub>SO<sub>4</sub> as described above, yielded **14a/14b** (40%).

Treatment of **12a/12b** (0.09 mmol) with Ac<sub>2</sub>O–H<sub>2</sub>SO<sub>4</sub> as described above, yielded a complex reaction product containing **13a/13b** and **14a/14b**.

**4.1.14. General procedure for the preparation of compounds 20–22.** The corresponding AQ was dissolved in EtOH (1–3 mL) and 4% NaOH (3–6 mL) was added dropwise and kept under reflux for 30 min. Then, the organic solvent was evaporated off, the residue was redissolved in EtOAc, washed with 2 N HCl and brine, and dried over Na<sub>2</sub>SO<sub>4</sub> to give the corresponding imidazole.

**4.1.15. 1-Ethyl-2,6,6-trimethyl-6,7,8,9-tetrahydro-1H-anthra[2,3-*d*]imidazole-4,11-dione 20a.** Following the above general procedure, treatment of **16a** (0.71 mmol) with 4% NaOH (3 mL) yielded **20a** (77%): mp 104–108 °C (from CH<sub>2</sub>Cl<sub>2</sub>); UV  $\lambda_{\max}$ (log  $\epsilon$ ): 259 (4.5), 267 (4.6), 299 (4.1), 347 (3.4); IR  $\nu_{\max}$  (film): 1665, 1594, 1517, 1473, 1436, 1291, 1243, 1172, 1091, 1012, 922 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.34 (6H, s), 1.43 (3H, t,  $J$  = 7.2), 1.70–1.82 (4H, m), 2.57 (3H, s), 2.88 (2H, t,  $J$  = 6.3), 4.43 (2H, m), 7.77 (1H, s), 8.17 (1H, s); HRMS (ES, M + Na) calcd: 345.1578, found: 345.1593.

**4.1.16. 1-Ethyl-2,9,9-trimethyl-6,7,8,9-tetrahydro-1H-anthra[2,3-*d*]imidazole-4,11-dione 20b.** Following the above general procedure, treatment of **16b** (0.04 mmol) with 4% NaOH (3 mL) yielded **20b** (63%): mp 158–161 °C (CH<sub>2</sub>Cl<sub>2</sub>); IR  $\nu_{\max}$  (film): 1662, 1594, 1517, 1473, 1436, 1289, 1244, 1172, 1088, 921 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.34 (6H, s), 1.45 (3H, t,  $J$  = 7.1), 1.70–1.82 (4H, m), 2.56 (3H, s), 2.87 (2H, t,  $J$  = 6.3), 4.43 (2H, m), 7.89 (1H, s), 8.08 (1H, s); HRMS (ES, M + Na) calcd: 345.1578, found: 345.1562.

**4.1.17. 1-(*p*-Methoxyphenyl)-2,6,6-trimethyl-6,7,8,9-tetrahydro-1H-anthra[2,3-*d*]imidazole-4,11-dione 21a.** Following the above general procedure, treatment of **17a** (0.03 mmol) with 4%

NaOH (3 mL) yielded **21a** (86%): mp 98–102 °C (from CH<sub>2</sub>Cl<sub>2</sub>); IR  $\nu_{\max}$  (film): 1670, 1597, 1515, 1454, 1251, 1171, 1027, 802 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.34 (6H, s), 1.65–1.79 (4H, m), 2.38 (3H, s), 2.82 (2H, t,  $J$  = 6.2), 3.89 (3H, s), 7.06 (2H, d,  $J$  = 8.8), 7.24 (2H, d,  $J$  = 8.8), 7.65 (1H, s), 8.18 (1H, s); HRMS (ES, M + 1) calcd: 401.1859, found: 401.1879.

**4.1.18. 1-(*p*-Methoxyphenyl)-2,9,9-trimethyl-6,7,8,9-tetrahydro-1H-anthra[2,3-*d*]imidazole-4,11-dione 21b.** Following the above general procedure, treatment of **17b** (0.03 mmol) with 4% NaOH (3 mL) yielded **21b** (97%): mp 130–133 °C (from CH<sub>2</sub>Cl<sub>2</sub>); IR  $\nu_{\max}$  (film): 1671, 1596, 1515, 1456, 1253, 1171, 1025, 800 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.27 (6H, s), 1.61–1.84 (4H, m), 2.39 (3H, s), 2.87 (2H, t,  $J$  = 6.3), 3.90 (3H, s), 7.07 (2H, d,  $J$  = 6.8), 7.25 (2H, d,  $J$  = 6.8), 7.91 (1H, s), 7.99 (1H, s); HRMS (ES, M + 1) calcd: 401.1859, found: 401.1869.

**4.1.19. 1-(*p*-Methoxyphenyl)-2-phenyl-6,6(9,9)-dimethyl-6,7,8,9-tetrahydro-1H-anthra[2,3-*d*]imidazole-4,11-dione 22a(22b).** Following the above general procedure, treatment of **18a/18b** (0.04 mmol) with 4% NaOH (3 mL) yielded **22a/22b** (93%): IR  $\nu_{\max}$  (film): 1670, 1596, 1514, 1429, 1246, 1174, 1030, 840 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.28/1.36 (6H, s), 1.67–1.84 (4H, m), 2.86 (2H, m), 3.88 (3H, s), 6.96–7.59 (9H, m), 7.69/8.03 (1H, s), 8.23/7.95 (1H, s); HRMS (ES, M + Na) calcd: 485.1835, found: 485.1832.

**4.1.20. 12,12(9,9)-Dimethyl-9,10,11,12-tetrahydroanthra[1',2':4,5]imidazo[1,2-*a*]pyridine-6,7-dione 25a(25b).** A mixture of AQ 2 (0.65 mmol) and 2-aminopyridine (1.3 mmol) in EtOH (8 mL) was stirred under reflux for 72 h. The solvent was vacuum evaporated and the residue was redissolved in EtOAc, washed with 2 N HCl and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated till dryness yielding a reaction product that was purified by column chromatography (eluent Hex/EtOAc 1 : 1) to afford: (a) 22 mg (10%) of **25a**: mp 270–275 °C (from CH<sub>2</sub>Cl<sub>2</sub>); UV  $\lambda_{\max}$ (log  $\epsilon$ ): 283 (4.4), 351 (3.7), 402 (3.7); IR  $\nu_{\max}$  (film): 1644, 1455, 1428, 1391, 1249, 1039, 913 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.39 (6H, s), 1.70–1.85 (4H, m), 2.82 (2H, t,  $J$  = 6.4), 7.16 (1H, dd,  $J$  = 6.2 and 7.6), 7.60 (1H, dd,  $J$  = 7.6 and 8.8), 7.79 (1H, s), 7.80 (1H, d,  $J$  = 8.9), 8.10 (1H, s), 9.29 (1H, d,  $J$  = 6.2); HRMS (ES, M + Na) calcd: 353.1260, found: 353.1260. (b) 26 mg (12%) of **25a/25b**. (c) 7 mg (3%) of **25b**: mp 279–282 °C (from CH<sub>2</sub>Cl<sub>2</sub>); IR  $\nu_{\max}$  (film): 1641, 1457, 1427, 1388, 1250, 1111, 1041, 769 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.34 (6H, s), 1.70–1.85 (4H, m), 2.89 (2H, t,  $J$  = 6.4), 7.17 (1H, dd,  $J$  = 6.3 and 7.4), 7.61 (1H, dd,  $J$  = 7.4 and 8.7), 7.78 (1H, d,  $J$  = 8.7), 7.84 (1H, s), 8.10 (1H, s), 9.29 (1H, d,  $J$  = 6.3); HRMS (ES, M + Na) calcd: 353.1260, found: 353.1275.

A mixture of AQ 2 (0.43 mmol), 2-aminopyridine (0.95 mmol) and K<sub>2</sub>CO<sub>3</sub> (64 mg) in MeOH (2 mL) was stirred under reflux for 19 h. Following the procedure described above and after column chromatography (eluent Hex/EtOAc 8 : 2 and 1 : 1) of the complex reaction product, 18 mg (14%) of **23** were obtained: IR  $\nu_{\max}$  (film): 1665, 1602, 1455, 1338, 1296, 1041, 917, 734 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.32 (6H, s), 1.71–1.80 (4H, m), 2.86 (2H, t,  $J$  = 6.2), 4.08 (6H, s), 7.71 (1H, s), 8.00 (1H, s).

A mixture of AQ 2 (0.65 mmol), 2-aminopyridine (1.3 mmol) and K<sub>2</sub>CO<sub>3</sub> (200 mg) in EtOH (5 mL) was stirred under reflux for 14 h. Following the procedure described above and after column chromatography (eluent Hex/EtOAc 8 : 2 and 1 : 1) of

the reaction product, the following compounds were obtained: 44 mg (20%) of **24** (ref. 23) and 22 mg (10%) of **25a/25b**.

**4.1.21. 2,3,13,13-Tetramethyl-13,14,15,16-tetrahydronaphtho[2,3-*a*]pyrido[1',2':1,2]imidazo[4,5-*c*]phenazine 26a.** A mixture of **25a** (0.07 mmol) and glacial acetic acid (400  $\mu$ L) in EtOH (1.5 mL) was stirred at rt for 30 min. Then, 4,5-dimethyl-1,2-phenylenediamine (0.07 mmol) was added and heated under reflux for 6 h. The reaction mixture was diluted with water and extracted with EtOAc. The organic phase was washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , filtered and evaporated off to yield a reaction product, which was purified by column chromatography (eluent Hex/EtOAc 1 : 1) affording **26a** (50%): mp 240–245  $^\circ\text{C}$  (from  $\text{CH}_2\text{Cl}_2$ ); IR  $\nu_{\text{max}}$  (film): 1450, 1334, 1264, 1114, 1028, 735  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.48 (6H, s), 1.84–1.97 (4H, m), 2.46 (3H, s), 2.49 (3H, s), 3.14 (2H, t,  $J = 6.2$ ), 7.10 (1H, dd,  $J = 6.7$  and 7.0), 7.51 (1H, dd,  $J = 7.0$  and 8.2), 7.80 (1H, s), 7.90 (1H, s), 7.94 (1H, d,  $J = 8.2$ ), 8.70 (1H, s), 8.94 (1H, s), 10.12 (1H, d,  $J = 6.7$ ); HRMS (ES, M + H) calcd: 431.2230, found: 431.2260.

**4.1.22. 2,3,16,16-Tetramethyl-13,14,15,16-tetrahydronaphtho[2,3-*a*]pyrido[1',2':1,2]imidazo[4,5-*c*]phenazine 26b.** A mixture of **25b** (0.05 mmol) and glacial acetic acid (300  $\mu$ L) in EtOH (1.5 mL) was stirred at rt for 30 min. Then, 4,5-dimethyl-1,2-phenylenediamine (0.06 mmol) was added and heated under reflux for 2 h. The reaction mixture was diluted with water and extracted with EtOAc. The organic phase was washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , filtered and evaporated off to yield a reaction product, which was purified by column chromatography (eluent Hex/EtOAc 1 : 1) affording **26b** (32%): mp 205–210  $^\circ\text{C}$  (from  $\text{CH}_2\text{Cl}_2$ ); IR  $\nu_{\text{max}}$  (film): 1454, 1336, 1261, 1116, 1043, 735  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.57 (6H, s), 1.75–1.95 (4H, m), 2.49 (3H, s), 2.52 (3H, s), 3.11 (2H, t,  $J = 6.2$ ), 7.08 (1H, dd,  $J = 6.7$  and 7.0), 7.48 (1H, m), 7.85 (1H, d,  $J = 8.4$ ), 7.85 (1H, s), 7.98 (1H, s), 8.39 (1H, s), 9.31 (1H, s), 10.13 (1H, d,  $J = 6.7$ ); HRMS (ES, M + H) calcd: 431.2230, found: 431.2225.

#### 4.1.23. General procedure for the preparation of compounds 27–36

**Method A.** The AQs **1** or **2** and the corresponding 1,2-diamine were dissolved in EtOH and kept stirring for the specified time. Then, the organic solvent was evaporated off and the residue was chromatographed over silica-gel to give the corresponding derivative.

**Method B.** The AQs **1** or **2** and the corresponding 1,2-diamine were dissolved in dichloromethane and mortmorillonite K10, as solid support (3 times the reactants weight), was added and well mixed. Then, the organic solvent was evaporated under vacuum and the dried material was subjected to microwave irradiation in a domestic microwave oven for a specified time. The solid mixture was extracted with EtOAc and MeOH and after evaporation of the organic solvent, the reaction product was obtained and purified by column chromatography.

**4.1.24. 2-(2-Aminoethylamino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-dione 27a(27b) and 8,8(11,11)-dimethyl-8,9,10,11-tetrahydronaphtho[2,3-*f*]quinoxalin-6-yl acetate 29a(29b).** Following the above method A, a mixture of **1** (1.19 mmol) and 1,2-ethylenediamine (1.20 mmol) dissolved in EtOH, was stirred at rt for 10 min. Column chromatography of the reaction product (eluent Hex/EtOAc 1 : 1) afforded a 1 : 1 mixture of **27a/**

**27b** (30%): IR  $\nu_{\text{max}}$  (film): 3296, 3246, 1609, 1572, 1543, 1458, 1335, 1297, 1104, 732  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.30 (6H, s), 1.72–1.80 (4H, m), 2.81 (2H, t,  $J = 6.1$ ), 3.28 (2H, t,  $J = 6.2$ ), 4.02 (2H, t,  $J = 6.2$ ), 5.63 (1H, s), 7.73 (1H, s), 7.99 (1H, s); HRMS (ES, M + Na –  $\text{H}_2\text{O}$ ) calcd: 303.1473, found: 303.1483.

Following the method A, a mixture of **1** (1.21 mmol) and 1,2-ethylenediamine (1.73 mmol) dissolved in EtOH, was stirred at rt for 30 min. Column chromatography of the reaction product (eluent Hex/EtOAc 7 : 3) afforded: (a) a 1 : 1 mixture of **28a/28b** (8%). (b) A 1 : 1 mixture of **27a/27b** (6%). Acetylation of **28a/28b** with acetic anhydride and pyridine yielded the acetates **29a/29b** (61%): IR  $\nu_{\text{max}}$  (film): 1766, 1605, 1441, 1371, 1299, 1194, 1093, 733  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.42/1.44 (6H, s), 1.78–1.95 (4H, m), 2.54/2.53 (3H, s), 3.05/3.12 (2H, t,  $J = 6.3$ ), 7.64/7.63 (1H, s), 7.90/7.62 (1H, s), 8.84 (1H, s), 8.86 (1H, s), 8.89/9.22 (1H, s); HRMS (ES, M + H) calcd: 321.1603, found: 321.1616.

Following the method B, a mixture of **1** (0.96 mmol) and 1,2-ethylenediamine (1.76 mmol) adsorbed on mortmorillonite K10 was irradiated at 700 W for 3 min at 1 min intervals. Column chromatography of the reaction product (eluent Hex/EtOAc 8 : 2 and 7 : 3) afforded: (a) a 1 : 1 mixture of **28a/28b** (13%). (b) A 1 : 1 mixture of **27a/27b** (4%).

**4.1.25. 2-(2-Aminoethylamino)-3-chloro-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-dione 30a(30b), 5-chloro-8,8(11,11)-dimethyl-8,9,10,11-tetrahydronaphtho[2,3-*f*]quinoxalin-6-ol 32a(32b) and 5-chloro-8,8(11,11)-dimethyl-8,9,10,11-tetrahydro-naphtho[2,3-*f*]quinoxalin-6-yl acetate 34a(34b).** Following the above method A, a mixture of **2** (0.45 mmol) and 1,2-ethylenediamine (0.48 mmol) dissolved in EtOH, was stirred at reflux for 16 h. Column chromatography of the reaction product (eluent Hex/EtOAc 7 : 3) afforded a 1 : 1 mixture of **30a/30b** (30%): IR  $\nu_{\text{max}}$  (film): 3290, 1605, 1567, 1554, 1433, 1337, 1287, 1110, 734  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.33/1.34 (6H, s), 1.60–1.82 (4H, m), 2.85 (2H, m), 3.45 (2H, m), 4.14 (2H, m), 7.80/8.10 (1H, s), 8.12/7.81 (1H, s); HRMS (ES, M –  $\text{H}_2\text{O}$  + H) calcd: 315.1258, found: 315.1272.

Following the method A, a mixture of **2** (0.65 mmol) and 1,2-ethylenediamine (0.68 mmol) dissolved in EtOH, was stirred at reflux for 96 h. Column chromatography of the reaction product (eluent Hex/EtOAc 8 : 2) afforded a 1 : 1 mixture of **32a/32b** (10%): IR  $\nu_{\text{max}}$  (film): 3306, 1582, 1434, 1375, 1331, 1151, 1118, 1082, 844, 735  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.44/1.46 (6H, s), 1.75–1.93 (4H, m), 3.06 (2H, m), 8.28/7.99 (1H, s), 8.77/9.11 (1H, s), 8.84 (1H, d,  $J = 2.2$ ), 8.74 (1H, d,  $J = 2.2$ ); HRMS (ES, M + H) calcd: 313.1108, found: 313.1148. Acetylation of **32a/32b** with acetic anhydride and pyridine yielded the acetates **34a/34b**: IR  $\nu_{\text{max}}$  (film): 1779, 1594, 1447, 1369, 1174, 1098, 1010, 871, 736  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.46/1.40 (6H, s), 1.76–1.92 (4H, m), 2.59/2.57 (3H, s), 3.03/3.08 (2H, t,  $J = 6.3$ ), 7.83/7.55 (1H, s), 8.83/9.17 (1H, s), 8.91/8.88 (1H, d,  $J = 2.1$ ), 8.95 (1H, d,  $J = 2.1$ ); HRMS (ES, M + H) calcd: 355.1207, found: 355.1216.

Following the method B, a mixture of **2** (0.3 mmol) and 1,2-ethylenediamine (0.47 mmol) adsorbed on mortmorillonite K10 was irradiated at 500 W for 1 min and at 750 W for 2 min (1 min intervals). Column chromatography of the reaction product (eluent Hex/EtOAc 8 : 2) afforded: (a) **2** (11%). (b) A 1 : 1 mixture of **32a/32b** (24%). (c) A 1 : 1 mixture of **30a/30b** (20%).

**4.1.26. 2-((2-Amino-1-methyl)ethylamino)-3-chloro-5,5(8,8)-dimethyl-5,6,7,8-tetrahydro-anthracene-1,4-dione 31a(31b), 5-chloro-3,8,8(3,11,11)-trimethyl-8,9,10,11-tetrahydro-naphtho[2,3-*f*]quinoxalin-6-ol 33a(33b) and 5-chloro-3,8,8(3,11,11)-trimethyl-8,9,10,11-tetrahydronaphtho[2,3-*f*]quinoxalin-6-yl acetate 35a(35b).** Following the method A, a mixture of **2** (0.45 mmol) and 1,2-propanediamine (0.48 mmol) dissolved in EtOH, was stirred at reflux for 7 days. Column chromatography of the reaction product (eluent Hex/EtOAc 8 : 2) afforded: (a) a 1 : 1 mixture of **33a/33b** (11%):  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.45/1.49 (6H, s), 1.76–1.94 (4H, m), 2.80/2.82 (3H, s), 3.08 (2H, m), 8.28/7.99 (1H, s), 8.82/9.14 (1H, s), 8.73 (1H, s). (b) A 1 : 1 mixture of **31a/31b** (20%): IR  $\nu_{\text{max}}$  (film): 3311, 1641, 1605, 1570, 1436, 1272, 1175, 1093, 734  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.32/1.34 (6H, s), 1.65–1.93 (4H, m), 1.66/1.69 (3H, d,  $J = 3.3$ ) 2.85 (2H, t,  $J = 6.0$ ), 3.13 (2H, m), 3.51 (1H, m) 7.80/8.10 (1H, s), 8.14/7.83 (1H, s); HRMS (ES, M – H<sub>2</sub>O + H) calcd: 329.1421, found: 329.1427. Acetylation of **33a/33b** with acetic anhydride and pyridine yielded the acetates **35a/35b**: IR  $\nu_{\text{max}}$  (film): 1779, 1595, 1457, 1362, 1190, 1096, 966, 869, 735  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.41/1.48 (6H, s), 1.77–1.94 (4H, m), 2.59/2.58 (3H, s), 2.85/2.87 (3H, s), 3.04/3.10 (2H, t,  $J = 6.3$ ), 7.81/7.54 (1H, s), 8.88/9.20 (1H, s), 8.83/8.82 (1H, s); HRMS (ES, M + H) calcd: 369.1364, found: 369.1373.

**4.1.27. 6-Chloro-2,3,9,9(2,3,12,12)-tetramethyl-9,10,11,12-tetrahydronaphtho[2,3-*a*]phenazin-7-yl acetate 36a(36b).** Following the method B, a mixture of **2** (0.3 mmol) and 4,5-dimethyl-1,2-phenylenediamine (0.47 mmol) adsorbed on mortmorillonite K10 was irradiated at 650 W for 0.5 min and an additional 1 min. The reaction product was acetylated and column chromatography of the acetylation product (eluent Hex/EtOAc 8 : 2) afforded a 1 : 1 mixture of **36a/36b** (22%): IR  $\nu_{\text{max}}$  (film): 1778, 1492, 1357, 1179, 1097, 1015, 921, 869  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.42/1.52 (6H, s), 1.78–1.94 (4H, m), 2.56/2.58 (3H, s), 2.59 (6H, s), 3.04/3.13 (2H, t,  $J = 6.2$ ), 7.52/7.81 (1H, s), 8.07 (1H, s), 8.13 (1H, s), 9.34/9.01 (1H, s); HRMS (ES, M + H) calcd: 433.1683, found: 433.1687.

**4.1.28. General procedure for the preparation of compounds 40–43.** To a solution of the corresponding arylamino-AQ **37–39** (ref. 23) in DMF, sodium azide, suspended in a little amount of water, was added and heated under reflux overnight. The reaction mixture was diluted with EtOAc and the organic phase was washed with brine, dried over  $\text{Na}_2\text{SO}_4$  and the solvent evaporated off, affording a crude product that was purified by column chromatography to yield the corresponding phenazines.

**4.1.29. 2-Methoxy-11,11(8,8)-dimethyl-8,9,10,11-tetrahydronaphtho[2,3-*b*]phenazine-6,13-dione 40a(40b).** From **37a/37b** (0.53 mmol) in DMF (5 mL) and  $\text{NaN}_3$  (1.07 mmol) and following the general procedure, a 1 : 1 mixture of **40a/40b** (21%) was obtained: IR  $\nu_{\text{max}}$  (film): 1685, 1616, 1487, 1417, 1334, 1248, 1127, 1097, 1020, 841, 731  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.41 (6H, s), 1.76–1.87 (4H, m), 2.97 (2H, t,  $J = 6.5$ ), 4.03 (3H, s), 7.62/7.60 (1H, d,  $J = 9.4/9.1$ ), 7.69/7.71 (1H, s), 8.33/8.31 (1H, d,  $J = 9.1/9.4$ ), 8.13/8.41 (1H, s) 8.41/8.13 (1H, s); HRMS (ES, M + Na) calcd: 395.1366, found: 395.1363.

**4.1.30. 1,2-Dimethoxy-11,11(8,8)-dimethyl-8,9,10,11-tetrahydronaphtho[2,3-*b*]phenazine-6,13-dione 41a(41b).** From **38a/38b** (0.35 mmol) in DMF (4 mL) and  $\text{NaN}_3$  (0.77 mmol) and following the general procedure, a 1 : 1 mixture of **41a/41b** (13%) was obtained: IR  $\nu_{\text{max}}$  (film): 1686, 1599, 1494, 1465, 1384, 1261, 1071, 1018, 732  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.41/1.42 (6H, s), 1.41–1.80 (4H, m), 2.99 (2H, t,  $J = 6.2$ ), 4.14 (3H, s), 4.32/4.33 (3H, s), 7.83 (1H, d,  $J = 9.3$ ), 8.13/8.42 (1H, s), 8.25/8.26 (1H, d,  $J = 9.3$ ), 8.42/8.15 (1H, s); HRMS (ES, M + H) calcd: 403.1652, found: 403.1670.

**4.1.31. 1,2,11,11(1,2,8,8)-Tetramethyl-8,9,10,11-tetrahydronaphtho[2,3-*b*]phenazine-6,13-dione 42a(42b) and 2,3,8,8-tetramethyl-8,9,10,11-tetrahydro-naphtho[2,3-*b*]phenazine-6,13-dione 43.** From **39a/39b** (0.60 mmol) in DMF (6 mL) and  $\text{NaN}_3$  (1.29 mmol), following the general procedure, and after column chromatography, the following compounds were separated: (a) a 1 : 1 mixture of **42a/42b** (13%): IR  $\nu_{\text{max}}$  (film): 1686, 1597, 1458, 1334, 1252, 1018, 753  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.42 (6H, s), 1.64–1.77 (4H, m), 2.60 (3H, s), 2.91 (3H, s), 2.99 (2H, m), 7.79 (1H, d,  $J = 8.6$ ), 8.20/8.21 (1H, d,  $J = 8.6$ ), 8.12/8.41 (1H, s), 8.42/8.14 (1H, s); HRMS (ES, M + H) calcd: 371.1754, found: 371.1759. (b) Compound **43** (8%): mp 104–107 °C (from  $\text{CH}_2\text{Cl}_2$ ); UV  $\lambda_{\text{max}}$ (log  $\epsilon$ ): 312 (4.6), 351 (4.0); IR  $\nu_{\text{max}}$  (film): 1688, 1596, 1481, 1363, 1334, 1311, 1248, 1022, 873, 734  $\text{cm}^{-1}$ ;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$ : 1.41 (6H, s), 1.73–1.90 (4H, m), 2.56 (6H, s), 2.98 (2H, t,  $J = 6.3$ ), 8.13 (1H, s), 8.18 (1H, s), 8.20 (1H, s), 8.41 (1H, s); HRMS (ES, M + H) calcd: 371.1754, found: 371.1766.

## 4.2. Cytotoxicity studies on tumour cell lines

A colourimetric assay using sulforhodamine B (SRB) was adapted for a quantitative measurement of cell growth and viability, following a previously described method.<sup>40</sup> Cells were seeded in 96-well microtitre plates, at  $5 \times 10^3$  cells per well in aliquots of 195  $\mu\text{L}$  of RPMI medium, and they were allowed to attach to the plate surface by growing in a drug-free medium for 18 h. Afterwards, samples were added in aliquots of 5  $\mu\text{L}$  (dissolved in DMSO/ $\text{H}_2\text{O}$ , 3 : 7). After 72 h exposure, the antitumor effect was measured by the SRB methodology: cells were fixed by adding 50  $\mu\text{L}$  of cold 50% (wt/vol) trichloroacetic acid (TCA) and incubating for 60 min at 4 °C. Plates were washed with deionised water and dried; 100  $\mu\text{L}$  of SBR solution (0.4% wt/vol in 1% acetic acid) was added to each microtitre well and incubated for 10 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried and bound stain was solubilized with Tris buffer. Optical densities (OD) were read on an automated spectrophotometer plate reader at single wavelength of 490 nm. Data analyses were generated automatically by the LIMS implementation. Using control OD values ( $C$ ), test OD values ( $T$ ) and time zero OD values ( $T_0$ ), the drug concentration that caused a 50% growth inhibition ( $\text{GI}_{50}$  value) was calculated from the equation:  $100 \times [(T - T_0)/(C - T_0)] = 50$ . Each value represents the mean from triplicate determinations.

### 4.3. Cytotoxicity studies on normal Vero cells

Vero cells (African green monkey kidney-*Cercopithecus aethiops*, ATCC: CCL 81 line) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% inactivated Fetal Bovine Serum (FBS), 100 units per mL of penicillin, 100  $\mu\text{g mL}^{-1}$  of streptomycin, 100  $\mu\text{g mL}^{-1}$  of L-glutamine, 0.14%  $\text{NaHCO}_3$ , and 1% of each non-essential amino acids and minimum essential medium vitamin solution (choline chloride, D-calcium pantothenate, folic Acid, nicotinamide, pyridoxal hydrochloride, riboflavin, thiamine hydrochloride and i-Inositol). Cells were incubated at 37 °C in humidified 5%  $\text{CO}_2$  atmosphere.

The cytotoxicity on Vero cells, was examined *in vitro* using an MTT (dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma, New Jersey, USA) assay as described by Betancur-Galvis *et al.*<sup>50</sup> Briefly, Vero (African green monkey kidney, ATCC: CCL 81) cell line was seeded into 96-well plates at  $2.0 \times 10^3$  cells per well and incubated for 24 h at 37 °C. After incubation, each diluted compound was added to the appropriate well and the plates were incubated for 48 h at 37 °C in a humidified 5%  $\text{CO}_2$  atmosphere. Subsequently, the culture medium was removed and the MTT solution (24  $\mu\text{L}$ , 2 mg  $\text{mL}^{-1}$ ) was added to each well. The minimal dilution of compound that induced 50% growth inhibition of the cells was expressed as Inhibitory Concentration 50% ( $\text{IC}_{50}$ ). The  $\text{IC}_{50}$  values for each compound were obtained by linear regression analysis of the dose-response curves generated from the absorbance data with the statistical package R (Development Core Team, Vienna, Austria, 2008).  $\text{IC}_{50}$  values were expressed as the mean  $\pm$  standard deviation ( $M \pm \text{SD}$ ) of two independent experiments done in quadruplicate.

### 4.4. Antifungal assay

The antifungal activity were evaluated following the standard method of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing for yeasts (AFST-EUCAST)<sup>41</sup> and the Clinical and Laboratory Standards Institute (CLSI) M38-A protocol<sup>42</sup> for filamentous fungi with some modifications. The yeast *Candida parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258), *C. tropicalis* (Ct1, CECT 11901), *C. tropicalis* (Ct2, ATCC 200956), *C. albicans* (Ca1, ATCC 10231), *C. albicans* (Ca2, ATCC 90028), *C. lusitanae* (ATCC 200951), and filamentous fungi *Aspergillus fumigatus* (ATCC 204305), *A. flavus* (ATCC 204304), *A. terreus* (CDC 317), *A. niger* (ATCC 16404), *Fusarium oxysporum* (ATCC 48112), and the dermatophytes *Trichophyton rubrum* (ATCC 28188) and *T. mentagrophytes* (ATCC 24198) were used to evaluate antifungal activity.

Briefly, 100  $\mu\text{L}$  of five serial dilutions of the compounds were dispensed into 96-well microtitration plates (Becton Dickinson, New Jersey, USA) in duplicate at final concentrations between 2–32  $\mu\text{g mL}^{-1}$ . The same volume of inoculum of each strain was added to the dilutions of the compounds to achieve a final inoculum size of  $0.5\text{--}2.5 \times 10^5$  and  $0.4\text{--}5 \times 10^4$  CFU  $\text{mL}^{-1}$  for yeast and filamentous fungi, respectively. *Aspergillus fumigatus* and *A. flavus* were tested with amphotericin B (AmB, Sigma

Chemical Co, MO, USA), *C. krusei* ATCC and *C. parapsilosis* ATCC with itraconazole (ITZ, Sigma Chemical Co, MO, USA) and *T. mentagrophytes* and *T. rubrum* with terbinafine (TRB, Recalcine Laboratories, Santiago de Chile, Chile) as positive control. The range of antifungal was 0.031 to 16.0  $\mu\text{g mL}^{-1}$ . For the AFST-EUCAST method, the Minimal Inhibitory Concentrations (MICs) were determined after 24 h of incubation at 35 °C by spectrophotometric reading at 405 nm and it was defined as the lowest concentration that resulted in a 90% reduction of growth. For filamentous fungi MICs were determined after 48 h of incubation at 35 °C for *Aspergillus* spp. and at 28 °C to *F. oxysporum*. The MIC values for *T. rubrum* and *T. mentagrophytes* were defined after 6 days of incubation at 28 °C. MICs were the lowest drug concentrations that produced growth inhibition in a 90% of inhibition. Each compound was tested in duplicate on three different assays and MIC results were expressed as their geometric mean (GM).

### 4.5. Antiviral assay

Both Human Herpes virus 1 (HHV-1 CDC Atlanta acyclovir-sensitive strain) and Human Herpes virus 2 (HHV-2 VR-734-G acyclovir-sensitive strain) were from the Center for Disease Control (CDC, Atlanta, GA) and they were amplified in Vero cells. Virus stocks were titrated in Vero cells by plaque assay and expressed as plaque forming units (PFU  $\text{mL}^{-1}$ ).

The antiviral activity evaluation of 1,4-AQ derivatives on Vero cells (*Cercopithecus aethiops* African green monkey kidney cell line ATCC CCL 81) against  $1\text{TCID}_{50}$  of HHV-1 and  $10\text{TCID}_{50}$  of HHV-2, were carried out using the end-point titration technique (EPTT).<sup>47</sup> Vero cells were harvested in 96-well plates at a density of  $2.0 \times 10^4$  cells per well at 37 °C in a humidified 5%  $\text{CO}_2$  atmosphere until constituted  $\geq 80\%$  of the cell monolayer. Then  $\text{TCID}_{50}$  viral suspensions of HHV-1 or HHV-2 with the compounds at concentrations of 50 to 6.25  $\mu\text{g mL}^{-1}$  were performed in Minimum Essential Medium eagle (MEM) 2% FBS supplemented containing carboxymethylcellulose 1% and 0.5% for HHV-1 and HHV-2 respectively and incubated for 15 minutes at room temperature before being added to cell monolayer. Virus/compound mixture was added later to the confluent monolayer of Vero cells. After 48 h of a humidified 5%  $\text{CO}_2$  atmosphere at 37 °C, the cytopathic effect was examined, and then the microplates cells were stained with a solution of 3.5% formaldehyde with 0.2% of crystal violet. The activity was evaluated by determining the reduction factor ( $R_f$ ), which corresponds to the value obtained by dividing the viral titer in the absence of compound over the viral titer obtained in the presence of compound.<sup>47</sup> Two independent experiments by quadruplicate were carried out for each viral serotype and for each compound. Controls were included untreated cells, cells treated with compounds and cells infected with HHV-1 or HHV-2. Two positive controls were included in this assay: dextran sulfate and acyclovir. According to the parameters established by Vlietinck *et al.*,<sup>51</sup> the relevant or moderate antiviral activity of a purified natural product, is one whose  $R_f$  of viral titer is respectively of  $\geq 1 \times 10^3$  or  $1 \times 10^2$ . In this study, the mild or moderate antiviral activity is one whose  $R_f$  of viral titer of  $\geq 1 \times$

$10^1$  or  $1 \times 10^2$ , respectively. The antiviral SI was determinate and defined as the ratio between the inhibitory concentration 50 (IC<sub>50</sub>) in Vero cells and the concentration that inhibits 50% of the cytopathic effect caused by the virus on cells (EC<sub>50</sub>). A compound with a SI  $\geq 10$  was considered selective.

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