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Simple avarone mimetics as selective agents against multidrug resistant cancer cells

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ABSTRACT

In this work, synthesis of alkylamino and aralkylamino derivatives of sesquiterpene quinone avarone and its model compound *tert*-butylquinone was described. For all obtained derivatives biological activity was studied. Cytotoxic activity of the synthesized derivatives towards multidrug resistant MDR human non-small cell lung carcinoma NCI-H460/R cells, their sensitive counterpart NCI-H460 and human normal keratinocytes (HaCaT) as well as detection of cell death superoxide anion generation were investigated. Antimicrobial activity towards Gram positive and Gram negative bacteria and fungal cultures was determined. The results showed that strong cytotoxic activity toward cancer cells was improved with simple avarone mimetics. Some derivatives were selective towards MDR cancer cells. The most active derivatives induced apoptosis in both cancer cell lines, but not in normal cells. Superoxide production was induced by 2,6-disubstituted compounds in MDR cancer cells and not by less active 2,5-disubstituted compounds and was accompanied by the collapse of the mitochondrial transmembrane potential. Two *tert*-butylquinone derivatives were particularly selective towards MDR cancer cells. Some *tert*-butylquinone derivatives exhibited a strong antimicrobial activity.

Introduction.

A major factor preventing successful cancer treatment is development of resistance to chemotherapeutics. The mechanisms of drug resistance include: overexpression of transmembrane drug efflux pumps, alterations in drug targets, activation of prosurvival pathways and ineffective induction of cell death [1]. Besides intrinsic resistance developed during carcinogenesis, initially sensitive cancer cells can develop acquired resistance after administration of single chemotherapeutic. The occurrence of multidrug resistance (MDR) phenotype is a serious obstacle to efficient cancer treatment. Therefore, targeting MDR cells is one of principal goals in the development of new anticancer drugs.

Marine sponges are well known as prolific sources of bioactive metabolites with diversity of structures. Sesquiterpene quinones, in particular, are known as compounds with a wide range of biological activities, including antibacterial activity [2], promotion of TNF-α production [3], antidiabetic activity due to inhibition of tyrosine phosphatase 1B (PTP1B) [4,5], inhibition of farnesyl diphosphate synthase [6], antiproliferative activity under normoxic and hypoxic conditions [7,8], and protein kinase inhibitory activity [9]. Activity in various cancer cell lines has been also in focus for the investigation of this class of compounds. Numerous isolated and synthesized sesquiterpenoid quinones displayed anticancer activity against, among others, HeLa and HepG2 cell lines [10,11], multiple myeloma [12], and NCI-H460 cell line [13].

Avarol/avarone, a redox pair with versatile biological activity was isolated from marine sponge *Dysidea avara*. Both compounds displayed strong cytotoxic activity toward K-562 and Fem-X cell lines [14], T-lymphocyte DNA inhibition activity [15], and anti-HIV activity [16].

Our previous results have shown that some alkylamino derivatives of avarone, in particular 4'-(methylamino) derivative were selective toward cancer cell lines in comparison with normal cell

lines [14]. In addition, a significant difference in the activity between two regioisomers was found, likely precluding non-specific mode of action.

When dealing with natural products derivatives, the source availability has to be considered. Avarol, the precursor compound, is the major constituent of the sponge *Dysidea avara* (3 g/kg sponge) [17]. In order to secure sustainable production of avarol, various attempts were made on cultivation of sponge and/or cell culture [18,19]. Still, the problem of supplying sufficient amount of avarol remains.

One solution to this problem is to consider avarol/avarone as model for design of active substances. In this study, a relatively crude model was used, based on a simple quinone, *tert*-butylquinone (TBQ). *tert*-Butyl group was selected for several reasons. First, it is a hydrophobic group, but with fewer carbon atoms, so that modifications in the substituents can be performed, affording derivatives with different hydrophobicites. Second, this group is voluminous, which is important since there is a considerable steric bulk in avarone sesquiterpene moiety. Third, the group is achiral, avoiding problems with molecular asymmetry. Fourth, since the mechanisms of action of avarone derivatives might include radical intermediates, the bulky *tert*-butyl group might help in stabilization of semiquinone radicals. Finally, the starting compound, *tert*-butylquinone, is readily available.

Both TBQ and its hydroquinone (TBHQ) induce cytotoxicity, with TBQ having a greater effect. Both compounds induce decreased mitochondrial membrane potential, disruption of mitochondrial structure with formation of cytosolic vacuoles, release of cytochrome c from mitochondria, caspase activation, PARP cleavage and the decrease of intracellular GSH and ATP [20,21].

Biological activity of quinones is dual. It is a combination of Michael addition to enone system and generation of oxygen reactive species (ROS) in redox cycle between quinone and its hydroquinone counterpart via semiquinone anion radical. Studies have shown that TBQ at a low dose induces DNA damage by forming 8-hydroxydeoxyguanosine due to generation of reactive oxygen species (ROS) [22]. TBHQ is $O_2^{\cdot-}$ scavenger with IC_{50} of 18.1 μM [23,24].

In this work, a series of of alkylamino and aralkylamino derivatives of avarone and TBQ were synthesized, and their cytotoxic activity was tested against three cell lines, non-small cell lung cancer, both sensitive and MDR, NCI-H460 and NCI-H460/R, respectively, and normal human keratinocytes HaCaT. The type of cell death was discriminated by AV/PI staining, while DHE fluorescent probe was employed to assess superoxide anion generation ability. Mitochondrial transmembrane potential ($\Delta\Psi$m) was also assessed. Cyclic voltammetry was used to measure half-wave potentials of synthesized compounds in two-stage redox system quinone–semiquinone radical–hydroquinone. In addition, antibacterial and antifungal activities were determined.

Results and Discussion.

Hydroquinones (*tert*-butylhydroquinone and avarol) were oxidized by silver(I) oxide to corresponding quinones **1** and **2**. Quinones were treated with amines to yield two regioisomers of aminoquinones **3a-i** and **4a-i**. The reaction steps are addition of an amine to form the substituted hydroquinone intermediate, which is immediately oxidized by the excess of the starting quinone. With *tert*-butylquinone 2,6-disubstituted quinone product by far dominated 2,5-disubstituted product in the mixture, while with avarone the excess of 2,6- product was much less pronounced, so that with phenethyl amine the major product was 2,5- product. The exception in the synthetic route is preparation of ethylamino derivatives **3b** and **4b**, which were obtained in the reaction of

the quinone with diethylamine, in which the initial addition product undergoes Hoffman-like elimination [25] which results in low yields of products.

All **3** derivatives display long-range W coupling between quinone protons with ^4J around 2 Hz, resulting with signals of these hydrogens to be dublets. On the other hand, **4** derivatives' quinone protons are five bonds away and their long-range coupling is too small to be detected, resulting in their signals to be singlets. Additionally, experimentally measured ^{13}C shifts of quinone core carbons showed good correlation with expected values calculated from basic benzoquinone known shifts and adding alkyl and alkylamino/aralkylamino substituents contributions, showing different patterns for **3** and **4** series of compounds. This way it was possible to make unambiguous distinction between **3** and **4** regioisomers (Scheme 1).

For all the synthesized compounds, half-wave potentials were recorded at glassy carbon disk (3mm diameter) in dimethyl sulfoxide against silver wire immersed in electrolyte solution containing 0.01 M silver ions as the reference electrode, and ferrocene as reference compound. Results are given in Table 1 and Fig. 1.

Cyclic voltammograms of avarone amino derivatives show typical behavior of quinone/hydroquinone redox pair. Two waves, corresponding to reversible or quasireversible one electron processes were observed. The reversibility of the first wave attributed to quinone/semiquinone anion radical redox system, was not perturbed by derivatization. Similarly, second wave assigned to semiquinone anion radical/hydroquinone dianion redox system behaves as quasireversible.

As expected, amino derivatization of avarone moiety led to negative shift of first reduction peak potentials with respect to avarone. *tert*-Butylquinone derivatives were reduced at slightly more

negative potential, due to a greater electron donating ability of *tert*-butyl group. Aralkyl derivatives were reduced at a slightly less negative potential probably due to a slight electron withdrawing effect of phenyl group. Changes in length of the side chain did not produce significant effect on the redox properties, as expected. Finally, 2,5-disubstituted derivatives were always, except for the small methylamino substituent, reduced at a more negative potential. Consequently, similar behavior of avarone and its *tert*-butyl analogue justifies application of the latter as model system of avarone.

Anticancer activity. The anticancer effects of several TBQ derivatives were compared with their avarone counterparts (**3c-4c** vs. **3g-4g**, **3d-4d** vs. **3h-4h**, and **3f-4f** vs. **3i-4i**). Cytotoxic activity was investigated in MDR human non-small cell lung carcinoma NCI-H460/R cells, their sensitive counterpart NCI-H460 and human normal keratinocytes (HaCaT). The differences in response between cancer and normal cells were assessed by MTT after 72 h treatment (Table 2). Most of 2,5-disubstituted quinone derivatives, except **4a** and **4b** showed selectivity towards cancer cell lines (Table 2, Fig. S1). Derivative **4h** was the least active in all tested cell lines. Although less selective, 2,6-disubstituted derivatives exhibited higher cytotoxicity than the corresponding 2,5-disubstituted derivatives (Table 2, Fig. S1). Importantly, the presence of MDR phenotype in NCI-H460/R cells did not diminish the efficacy of tested compounds, with the exception of **4c**. Even more, some derivatives exerted prominent activity against MDR cells (**3e** and **4e, 3f**, **3g** and **4g**, **3i** and **4i**). The IC_{50} values for two compounds, 2-*tert*-butyl-6-(phenethylamino)-1,4-benzoquinone (**3f**) and 3'-(butylamino)avarone (**3g**) were less than 10 μM in cells with the MDR phenotype.

When comparing activities of *tert*-butylquinone derivatives with avarone counterparts, it can be noticed that the former ones are generally more selective to cancer cell lines. Among TBQ

derivatives aralkyl ones showed the best activity and selectivity to both sensitive and MDR cancer cells. Within the avarone series, large alkylamino derivatives were less active, probably due to excessively high log *P*. According to cytotoxicity evaluation, the most promising derivatives are: 2-*tert*-butyl-6-(phenethylamino)-1,4-benzoquinone (**3f**), 3'-(butylamino)avarone (**3g**) and 3'-(phenethylamino)avarone (**3i**) due to their high activity and good selectivity to the MDR cancer cell line (Table 2). For comparison, parent compounds TBQ (**1**) and avarone (**2**) were less potent than their derivatives, while cisplatin (CDDP), an FDA-approved drug for non-small cells lung carcinoma treatment [26], showed strong cytotoxicity towards MDR cancer cells, but even stronger cytotoxicity towards normal human keratinocytes (Table 2).

Then, the type of cell death induced by 25 μM of *tert*-butyl compounds **3d-4d**, **3f-4f** and by their sesquiterpene counterparts **3h-4h, 3i-4i** was compared to parent compounds **1**, **2** and control compound CDDP (Table 3, Fig. S2). The results analyzed after 72 h showed the highest increase in necrotic cells (AV–PI+). *tert*-Butyl compounds, particularly **3f**, significantly increased a portion of apoptotic cells (AV+PI– and AV+PI+). Therefore, it is reasonable to assume that increase in fraction of dead cells after 72 h is a consequence of apoptotic cell death in case of *tert*-butyl compounds. On the contrary, necrosis seems to be the type of death induced by avarone derivatives **3h** and **3i** (Figs. S2 and S4). Also, with *tert*-butyl compounds, 2,6-derivatives (**3d, 3f** and **3i**) induced a significantly higher fraction of apoptotic cells than 2,5-derivatives (**4d, 4f** and **4i**). Selectivity towards cancer cells was additionally confirmed with **3d** and **3f,** while **3f** was considerably more active against MDR cancer cells (Table 3, Figs. S2 and S4). CDDP and **2** showed pro-apoptotic activity against all three cell lines showing no selectivity to cancer cells (Table 3, Fig. S3).

In addition to genomic DNA damage, many anticancer drugs act on multiple cellular levels affecting different organelles and signaling pathways. One of the proposed mechanisms behind their toxicity is the generation of ROS, which mostly affect the mitochondrial function [27]. Quinones are generally thought to exhibit their toxicity through oxygen activation by redox cycling and alkylation of essential macromolecules [28]. However, their biological effect can be cell type-dependent as it was reported that *p*-benzoquinone cytotoxicity correlated with ROS formation in primary rat hepatocytes, but not in PC12 cells. Furthermore, *tert*-butylhydroquinone was shown to exhibit anticancer effect through the ability to induce phase II xenobiotic metabolizing enzymes via an Nrf2-dependent pathway [29].

Therefore, in this work the potential of quinone derivatives to generate superoxide anions in resistant NCI-H460/R cells was examined by dihydroethidium (DHE) staining (Fig. 2).

Mean DHE fluorescence intensity showed significant potential of 2,6-disubstituted derivatives (**3d**, **3h**, **3f** and **3i**) to generate superoxide anions in MDR cancer, while treatment with 2,5-disubstituted derivatives (**4d**, **4h**, **4f** and **4i**) was not prooxidative compared to untreated control (Fig. 2A). This effect was more pronounced with avarone derivatives (**3h** and **3i**). The higher activity of 2,6-disubstituted compounds cannot be directly ascribed to differences of redox properties, since there is no significant difference in their electrochemical parameters (E^{0}_{1} is more negative for 2,5-disubstituted compounds for only 0.01–0.02 V). Therefore, the elevation of ROS levels could be the consequence of the antioxidant enzymes activity modulation. As described previously [30], MDR cancer cells (NCI-H460/R) have lower antioxidant capacity than their corresponding sensitive counterpart (NCI-H460). Particularly, the expression of MnSOD enzyme (responsible for superoxide anion metabolism) is significantly decreased in MDR cancer cells. Consequently, **3d**, **3f** and **3h** showed different pattern of action in sensitive NCI-H460 by

lowering the superoxide anion content (Fig. 2A) which is in accordance with previous findings

that TBHQ acts as $O_2^{\cdot-}$ scavenger [23]. A putative target responsible for different mode of action

in MDR cancer cells and assumed as a mechanism of selectivity towards MDR cancer cells

remains to be identified, which will be a subject of further studies. In normal cells, TBQ

derivatives **3d** and **3f** did not change superoxide anion content, while corresponding avarone

derivatives **3h** and **3i** increased its production although showing weaker pro-oxidative effect than

that observed in MDR cancer cells (Fig. 2A). Control compound **2** was pro-oxidative in MDR

cancer cells, while CDDP induced increase in $O_2^{\cdot-}$ production in all tested cell lines (Fig. 2A).

The representative flow-cytometric profile of **3d** and **3f**-induced DHE fluorescence is shown in

Fig. 2B.

Besides regulation of fundamental cellular processes such as respiration and oxidative

phosphorylation, mitochondria also provide signals that trigger apoptosis [31]. Cytochrome *c*

release from mitochondria is a central event in apoptosis initiation, which induces the assembly

of the apoptosome required for activating downstream caspases [32,33]. Mitochondrial

membrane potential (ΔΨm) is vital for ATP generation and major loss of ΔΨm is generally

followed by substantial cell death. ΔΨm and ROS levels give valuable insight into the

physiological condition of the cell and are good indicators of mitochondrial dysfunction and

oxidative stress. Several *p*-benzoquinones were reported to disrupt mitochondrial membrane

potential in rat hepatocytes and ROS formation in PC12 cells [28]. *tert*-Butylhydroquinone and 2-

tert-butyl-1,4-benzoquinone were shown to display a strong anticancer potential [29]. These

compounds decreased the mitochondrial membrane potential, disturbed the mitochondrial

structure, caused the mitochondrial release of cytochrome *c*, activated caspases and induced poly

ADP ribose polymerase (PARP) cleavage in human monocytic leukemia U937 cells [20]. Our study has shown that superoxide production induced by **3d** was accompanied by the collapse of the mitochondrial transmembrane potential ($\Delta\Psi$m). The results were assessed by JC-1 assay in cancer (NCI-H460 and NCI-H460/R) and normal (HaCaT) cells (Fig. 3). As quantified by flow-cytometry, 25 μM **3d** and **3f** induced prominent depolarization of mitochondrial membrane in cancer cells, while normal cells mitochondria remained unaffected. It is striking that the loss of mitochondrial potential after **3d** and **3f** application was detected in sensitive as well in MDR cancer cells despite different pattern of action (anti- vs. pro-oxidative) observed in NCI-H460 and NCI-H460/R cells. Thus, we assume that mitochondria of both sensitive and MDR cancer cells are susceptible to redox imbalance no matter whether it was caused by decrease or increase in superoxide anion content. CDDP and **1** did not change mitochondrial membrane potential. However, it must be emphasized that dead cells after CDDP treatment were removed during experimental procedure and not analyzed. Therefore, the majority of survived cells were resistant to CDDP showing no difference in comparison with control untreated cells. The change in JC-1 fluorescence from red to green was visualized on a fluorescent microscope (Fig. 3B).

Antimicrobial activity. Compounds **3a-i** and **4a-i** were assayed for their antimicrobial activity against Gram-negative and Gram-positive bacterial and fungal strains. Their activity was compared to commercial antibiotic, amikacin, and antimicotic, nystatin. Results are shown in Tables 4 and 5.

Antibacterial activity evaluation showed that the most sensitive bacterial strains were *E. coli*, *S. aureus* and *M. luteus* (ATCC 10240). Antibacterial activity similar to that of amikacin was found for compounds **3c**, **3e** and **3f** against *E. coli* and *S. aureus*, while against *M. luteus* (ATCC 10240)

derivatives **3c** and **3e** exhibited better antibacterial activity than amikacin. Slightly weaker activity but still similar to that of amikacin was achieved by **4c** against *E. coli,* by **3b** and **4a** against *S. aureus* and by **3b** and **4e** against *M. luteus* (ATCC 10240). Other derivatives showed a weak activity, while compound **3d** and derivatives with sesquiterpene moiety did not show any antibacterial activity. Based on the results, the structural requirements for good antibacterial activity are *tert*-butylquinone scaffold, 2,6-disubstitution and aralkylamino or medium-sized alkylamino substituent. It should also be pointed out that MIC values for **3c**, **3e** and **3f** are lower than IC$_{50}$ values against normal keratinocytes, which makes them promising candidates for antibacterial drugs.

The reported compounds were also tested for activity against three fungal strains: *C. albicans*, *S. cerevisae* and *A. brasiliensis*. Of all the compounds **3c** and **3f** showed the most potent and the broadest activity versus all three strains, significantly higher than nystatin. Interesting behaviour was noted for regioisomeric pairs **3c**-**4c** and **3f**-**4f**. Pair **3c**-**4c** displayed the same activity toward *C. albicans* and *S. cerevisae* but **3c** was much more active against *A. brasiliensis*. On the other hand, **3f**-**4f** pair showed the same activity toward *A. brasiliensis*, with **3f** being much more active against the other two strains. 3-Substituted compounds generally showed a stronger activity than their 4-substituted counterparts. Avarone derivatives again showed a low activity, with their MIC values falling beyond the concentration range used in the assay. Besides that, the structure requirements were not as clear-cut as for the antibacterial activity.

Conclusion.

Some general conclusions can be made regarding the optimization of scaffold structure, position and nature of substituents. Most importantly, *tert*-butyl derivatives showed at least the same or

better cytotoxic activity and selectivity as avarone counterparts. This is if great importance in view of the availability of the precursor compound.

2,6-Disubstituted quinones were shown to be more active than the corresponding 2,5-disubstituted quinones in both series of compounds, due to differences in redox status. Selectivity to MDR cell line occurred with medium sized or longer alkylamino as well as with aralkylamino substituents. The most promising derivatives are 2-*tert*-butyl-6-(octylamino)-1,4-benzoquinone and 2-*tert*-butyl-6-(phenethylamino)-1,4-benzoquinone, because of their selectivity towards both sensitive and MDR cancer cells in comparison with normal cells confirmed on several different levels: apoptosis, changes in redox balance and loss of mitochondrial membrane potential.

Since several compounds showed a good selectivity to MDR cells, the results of this study might contribute to selecting candidates for overcoming the problem of multidrug resistance in cancer cells.

EXPERIMENTAL SECTION

General procedure. Reagents and solvents were obtained from commercial sources (Fluka, Sigma-Aldrich, Merck, Acros Organics). Solvents were distilled before use, while the other chemicals were used as received. All of the reactions were carried out under a normal atmosphere with magnetic stirring unless stated otherwise. The compounds were visualized under UV light (254 nm). Column chromatography was performed on silica gel with pore size 60 Å, 70-230 mesh, 63-200 μm particle size, with the indicated solvents. Preparative thin-layer chromatography was performed on silica gel GF$_{254}$ (with UV active indicator). The yields refer to purified products. ^{1}H NMR spectra were recorded at on Varian Gemini 2000 200 MHz instrument and Bruker Avance III 500 MHz instrument. Measurements were made at temperature 298 K and are reported in ppm using tetramethylsilane as internal standard (CDCl$_3$ at 7.26 ppm). The coupling constants (*J*) are given in Hz, and the splitting patterns are designated as s, singlet;

bs, broad singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. ^{13}C NMR spectra were recorded on Varian Gemini 2000 200 MHz instrument and Bruker Avance III 500 MHz instrument. Measurements were made at temperature 298 K and are reported in ppm using tetramethylsilane as internal standard (CDCl$_3$ at 77.0 ppm). All mass spectra were recorded using electrospray ionisation. Optical rotations were measured on a Rudolph Research Analytical Autopol IV polarimeter. UV−Vis spectra were recorded using Cintra 40 UV−Visible spectrometer.

tert-Butyl-1,4-benzoquinone (**1**). tert-Butylhydroquinone (2 g; 12 mmol) was dissolved in diethyl ether (100 mL). Silver(I) oxide (3.74 g; 16.14 mmol) was added in portions to the reaction mixture, and the mixture was stirred for 2 hours at room temperature. After this period stirring was stopped, sodium sulphate was added and the mixture was left overnight. Silver, excess of silver(I) oxide and sodium sulphate were removed by filtration over Kieselgur. Diethyl ether was removed by evaporation and tert-butyl-1,4-benzoquinone used without further purification. The reaction yield was quantitative.

Avarone (**2**). Avarol (1.1 g; 3.5 mmol) was dissolved in diethyl ether (100 mL). Silver(I) oxide (1.1 g; 4.7 mmol) was added in portions to the reaction mixture, and the mixture was stirred for 2 h at room temperature. The reaction mixture work-up was the same as above. The reaction yield was quantitative.

General synthetic procedure. Quinones (300mg, unless stated otherwise; 1.83 mmol **1**; 0.96 mmol **2**) were dissolved in ethanol (50 mL). Hydrochloride salts (in large excess; 22.22 mmol for **1**; 21.12 mmol for **2**, unless stated otherwise) were prepared from alkyl and aralkyl amines as aqueous solutions if amines were soluble in water, or solutions in mixture of water and ethanol if amines were not soluble in water. pH of the solution was adjusted to pH 7−8 by adding solid

sodium bicarbonate, and added into solution of a quinone. Water and ethanol were added to the reaction mixture to the final ratio water–ethanol (1:1, 300 mL). Reaction mixture was stirred at room temperature for 16 h. Ethanol was removed by vacuum evaporation, and the reaction mixture was extracted by methylene chloride, two times with half the volume of the water. Organic phase was separated, dried with anhydrous calcium chloride, and the solvent removed by evaporation under vacuum. Crude products were separated by column chromatography and purified by preparative thin-layer chromatography, with the indicated solvents.

2-*tert*-butyl-6-(methylamino)-1,4-benzoquinone (**3a**). The compound was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent and purified by preparative thin layer chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.24). The product was obtained as reddish brown crystal, m.p. 178^0C. Yield 105 mg; 59.5 %.

^{1}H NMR (500 MHz, CDCl$_3$) δ 1.26 (s, 9H, C2-C(C**H$_3$**)$_3$), 2.85 (d, J = 5.5 Hz, 3H, C6-NHC**H$_3$**), 5.41 (d, J = 2 Hz, 1H, C5-**H**), 5.79 (bs, 1H, C6-N**H**CH$_3$) 6.47 (d, J = 2.5 Hz 1H, C3-**H**).

^{13}C NMR (125 MHz, CDCl$_3$) δ 29.0 (3C, C2-C(**C**H$_3$)$_3$) 29.2 (1C, C6-NH**C**H$_3$) 34.7 (1C, C2-**C**(CH$_3$)$_3$), 96.7 (1C, **C5**), 135.0 (1C, **C3**), 148.6 (1C, **C6**), 151.2 (1C, **C2**), 183.1 (1C, **C1**) 186.0 (1C, **C4**). IR (ATR) 3310.7 3070.8 3003.1 2962.9 2921.9 2874.1 2803.2 1674.7 1625.9 1575.2 1499.9 1449.2 1411.6 1373.8 1339.6 1261.1 1206.6 1176.5 1138,6 1068.4 907.1 796.1 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 276 (7.68x10^4), 482 (2.50x10^4). MS C$_{11}$H$_{15}$NO$_2$, exact mass = 193.1103, [M+H]$^+$, calculated m/z = 194.11756; found 194.11742. E_{c1} = −1.200 V; E_{a1} = −1.104 V; E_{c2} = −1.910 V; E_{a2} = −1.785 V; $E°_1$/Fc = −1.213 V.

2-*tert*-Butyl-5-(methylamino)-1,4-benzoquinone (**4a**). The compound was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent and purified by preparative thin layer chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.46).

The product was obtained as reddish brown crystal, m.p. 94^{0}C. Yield 8.9 mg; 5.0 %. ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.30 (s, 9H, C2-C(C**H**$_3$)$_3$), 2.82 (d, J = 5 Hz, 3H, C5-NHC**H**$_3$) 5.38 (s, 1H, C6-**H**), 5.44 (bs, 1H, C5-N**H**CH$_3$), 6.43 (s, 1H, C3-**H**). ^{13}C NMR (125 MHz, CDCl$_3$) δ 28.9 (1C, C5-NH**C**H$_3$), 29.7 (3C, C2-C(**C**H$_3$)$_3$), 35.7 (1C, C2-**C**(CH$_3$)$_3$), 100.2 (1C, **C6**), 127.4 (1C, **C3**), 146.3 (1C, **C5**), 159.7 (1C, **C2**), 184.8 (1C, **C4**), 185.8 (1C, **C1**). IR (ATR) 3302.6 3069.1 3007.3 2959.5 2914.6 2816.9 1669.9 1621.3 1578.5 1504.2 1458.1 1420.1 1342.8 1240.7 1195.1 1148.6 1060.0 1023.1 885.0 832.2 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 274 (7.62x10^4), 478 (1.62x10^4). MS: C$_{11}$H$_{15}$NO$_2$, exact mass = 193.1103; [M+H]$^+$, calculated m/z = 194.11756; found 194.11785. E_{c1} = −1.200 V, E_{a1} = −1.124 V, E_{c2} = −1.906 V, E_{a2} = −1.766 V, $E°_1$/Fc = −1.223 V.

2-*tert*-Butyl-6-(ethylamino)-1,4-benzoquinone (**3b**). The compound was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.32) and purified by preparative thin layer chromatography using hexane–acetone (8:2) as eluent (Rf = 0.43). The product was obtained as reddish brown oil. Yield 10.2 mg; 5.3 %. ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.26 (s, 9H, C2-C(C**H**$_3$)$_3$), 1.29 (t, J = 7 Hz, 3H, C2'-**H**$_3$), 3.14 (quintet, J = 7 Hz, 2H, C1'-**H**$_2$), 5.42 (d, J = 2 Hz, 1H, C5-**H**), 5.64 (bs, 1H, C6-N**H**Et), 6.46 (d, J = 2 Hz, 1H, C3-**H**). ^{13}C NMR (125 MHz, CDCl$_3$) δ 13.4 (**C2'**), 29.0 (3C, C2-C(**C**H$_3$)$_3$), 34.7 (1C, C2-**C**(CH$_3$)$_3$), 37.3 (**C1'**), 97.0 (**C5**), 135.0 (**C3**), 147.4 (**C6**), 151.2 (**C2**), 183.2 (**C1**), 186.1 (**C4**). IR (ATR) 3386.0 3320.5 2962.3 2871.9 1729.3 1670.7 1631.5 1584.7 1506.5 1466.8 1362.3 1338.0 1257.5 1169.9 1095.0 1062.9 907.4 804.4 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 278 (6.69x10^4) 484 (2.38x10^4). MS C$_{12}$H$_{17}$NO$_2$, exact mass = 207.1259 [M+H]$^+$, calculated m/z = 208.13321, found 208.13318, [2M+H]$^+$, calculated m/z = 415.25913, found 415.25877. E_{c1} = −1.160 V, E_{a1} = −1.080 V, E_{c2} = −1.896 V, E_{a2} = −1.746 V, $E°_1$/Fc = −1.191 V.

2-*tert*-Butyl-5-(ethylamino)-1,4-benzoquinone (**4b**). The compound was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.54) and purified by preparative thin layer chromatography using hexane–acetone (8:2) as eluent (Rf = 0.58). The product was obtained as reddish brown crystal, m.p. 136^0C. Yield 4.5 mg; 2.4 %.

^{1}H NMR (500 MHz, CDCl$_3$) δ 1.27 (t, J = 7 Hz, 3H, C2'-**H$_3$**), 1.30 (s, 9H, C2-C(C**H$_3$**)$_3$), 3.11 (quintet, J = 7 Hz, 2H, C1'-**H$_2$**), 5.34 (bs, 1H, C5-N**H**Et), 5.38 (s, 1H, C6-**H**), 6.43 (s, 1H, C3-**H**). ^{13}C NMR (125 MHz, CDCl$_3$) δ 13.5 (**C2'**), 29.7 (3C, C2-C(**C**H$_3$)$_3$), 35.7 (C2-**C**(CH$_3$)$_3$), 37.0 (**C1'**), 100.4 (**C6**), 127.4 (**C3**), 145.2 (**C5**), 159.7 (**C2**), 184.8 (**C4**), 185.9 (**C1**). IR (ATR) 3391.6 3057.3 2962.3 1669.7 1644.4 1625.9 1590.6 1513.3 1475.6 1364.1 1265.7 1221.2 1183.8 1108.3 1018.1 894.2 865.0 831.9 739.2 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 266 (4.16x10^4) 480 (0.39x10^4). MS C$_{12}$H$_{17}$NO$_2$, exact mass = 207.1259 [M+H]$^+$, calculated m/z = 208.13321, found 208.13342; E_{c1} = −1.170 V, E_{a1} = −1.102 V, E_{c2} = −1.860 V, E_{a2} = −1.672 V $E°_1$/Fc = − 1.211 V.

2-*tert*-Butyl-6-(butylamino)-1,4-benzoquinone (**3c**). The compound was synthesized by reaction of 1,4-benzoquinone **1** (312 mg, 1.9 mmol) with *n*-butylamine(1.68 g, 2.28 mL, 23 mmol). It was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent and purified by preparative thin layer chromatography using toluene-ethylacetate (9:1) as eluent (Rf = 0.44). The product was obtained as reddish brown oil. Yield 124.9 mg; 55.9 %. ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.96 (t, J = 3 Hz, 3H, C4'-**H$_3$**), 1.26 (s, 9H, C2-C(C**H$_3$**)$_3$), 1.42 (sextet, J = 3 Hz, 2H, C3'-**H$_2$**), 1.64 (quintet, J = 3 Hz, 2H, C2'-**H$_2$**), 3.08 (q, J = 2.8 Hz, 2H, C1'-**H$_2$**), 5.42 (d, J = 2.5 Hz, 1H, C5-**H**), 5.69 (bs, 1H, C6-N**H**^{n}Bu), 6.46 (d, J = 2.5 Hz, 1H, C3-**H**). ^{13}C NMR (125 MHz, CDCl$_3$) δ 13.7 (1C, **C4'**), 20.2 (1C, **C3'**), 29.0 (3C, C2-C(**C**H$_3$)$_3$), 30.2 (1C, **C2'**), 34.7 (1C, C2-**C**(CH$_3$)$_3$), 42.3 (1C, **C1'**), 97.0 (1C, **C5**), 135.1 (1C, **C3**),

147.6 (1C, **C6**), 151.2 (1C, **C2**), 183.2 (1C, **C1**), 186.0 (1C, **C4**). IR (ATR) 3387.2 3302.5 2993.1 2959.9 2932.2 2871.0 2363.4 1670.3 1632.4 1586.0 1506.9 1465.7 1363.2 1338.8 1255.9 1206.4 1164.9 1075.9 964.9 905.5 807.1 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 278 , (5.81x10^4), 488 (2.12x10^4). MS C$_{14}$H$_{21}$NO$_2$, exact mass = 235.1572 [M+H]$^+$, calculated m/z = 236.16451 found 236.16460. E_{c1} = −1.160 V, E_{a1} = −1.081 V, E_{c2} = −1.892 V, E_{a2} = −1.759 V, $E°_1$/Fc = −1.192 V.

2-*tert*-Butyl-5-(butylamino)-1,4-benzoquinone (**4c**). The compound was synthesized by reaction of 1,4-benzoquinone **1** (312 mg, 1.9 mmol) with *n*-butylamine (1.68 g, 2.28 mL, 23 mmol). It was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent and purified by preparative thin layer chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.70). The product was obtained as reddish brown crystal, m.p. 59^{0}C. Yield 28.7 mg; 12.8 %. ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.95 (t, J = 3 Hz, 3H, C4'-**H$_3$**), 1.30 (s, 9H, C2-C(C**H$_3$**)$_3$), 1.40 (sextet, J = 3 Hz, 2H, C3'-**H$_2$**), 1.61 (quintet, J = 3 Hz, 2H, C2'-**H$_2$**), 3.06 (q, J = 2.6 Hz, 2H, C1'-**H$_2$**), 5.38 (s, 2H, C4-N**H**^{n}Bu, C6-**H**), 6.43 (s, 1H, C3-**H**). ^{13}C NMR (125 MHz, CDCl$_3$) δ 13.7 (1C, **C4'**), 20.2 (1C, **C3'**), 29.7 (3C, C2-C(**C**H$_3$)$_3$), 30.3 (1C, **C3'**), 35.7 (1C, C2-**C**(CH$_3$)$_3$), 42.0 (1C, **C1'**), 100.3 (1C, **C6**), 127.4 (1C, **C3**), 145.4 (1C, **C5**), 159.7 (1C, **C2**), 184.9 (1C, **C4**), 185.8 (1C, **C1**). IR (ATR) 3734.1 3294.1 3081.5 3003.2 2958.7 2929.5 2869.7 2195.6 2156.3 2025.5 1970.3 1671.7 1624.5 1578.0 1509.8 1459.3 1349.3 1297.6 1237.7 1191.0 1066.0 1015.8 893.5 841.2 672.0 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 274 (5.93x10^4), 484 (1.93x10^4). MS C$_{14}$H$_{21}$NO$_2$, exact mass = 235.1572 [M+H]$^+$, calculated m/z = 236.16451, found 236.16462, [2M+H]$^+$, calculated m/z = 474.32173, found 474.32154.

E_{c1} = −1.182 V, E_{a1} = −1.096 V, E_{c2} = −1.921 V, E_{a2} = −1.773 V, $E°_1$/Fc = −1.210 V.

2-*tert*-Butyl-6-(octylamino)-1,4-benzoquinone (**3d**). The compound was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent and purified by preparative thin layer chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.56). The product was obtained as reddish brown oil. Yield 138.5 mg; 52.0 %. ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.89 (t, *J* = 2.8 Hz, 3H, C8'-**H$_3$**), 1.26 (m, 19H), 1.64 (quintet, *J* = 3 Hz, 2H, C2'-**H$_2$**), 3.07 (q, *J* = 2.8 Hz, 2H, C1'-**H$_2$**), 5.41 (d, *J* = 2.5 Hz, 1H, C5-**H**), 5.70 (bs, 1H, C5-N**H**^{n}Oct), 6.46 (d, *J* = 2.5 Hz, 1H, C3-**H**). ^{13}C NMR (125 MHz, CDCl$_3$) δ 14.0 (1C, **C8'**), 22.6 (1C, **C7'**), 27.0 (1C, **C3'**), 28.2 (1C, **C2'/C6'**), 29.0 (3C, C2-C(**C**H$_3$)$_3$), 29.1 (1C, **C4'/C5'**), 29.2 (1C, **C4'/C5'**), 31.7 (1C, **C2'/C6'**), 34.7 (1C, C2-**C**(CH$_3$)$_3$), 42.6 (1C, **C1'**), 97.0 (1C, **C5**), 135.1 (1C, **C3**), 147.5 (1C, **C6**), 151.2 (1C, **C2**), 183.2 (1C, **C1**), 186.0 (1C, **C4**). IR (ATR) 3390.5 3295.0 2957.4 2927.3 2856.5 1669.7 1631.8 1585.7 1506.4 1465.0 1362.9 1338.9 1255.8 1160.4 1074.0 906.4 807.2 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 276 (6.79x10^4) 488 (2.52x10^4).

MS C$_{18}$H$_{29}$NO$_2$, exact mass = 291.2198 [M+H]$^{+}$, calculated *m/z* = 292.22711, found 292.22811, [2M+H]$^{+}$, calculated *m/z* = 583.44693, found 583.44804. E_{c1} = −1.183 V, E_{a1} = −1.095 V, E_{c2} = −1.917 V, E_{a2} = −1.806 V, $E°_{1}$/Fc = −1.200 V.

2-*tert*-Butyl-5-(octylamino)-1,4-benzoquinone (**4d**). The compound was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent and purified by preparative thin layer chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.76). The product was obtained as reddish brown oil. Yield 25.0 mg; 9.4 %. ^{1}H NMR (500 MHz, CDCl$_3$) δ 0.88 (t, *J* = 3 Hz, 3H, C8'-**H$_3$**), 1.30 (m, 19H), 1.62 (quintet, *J* = 2.8 Hz, 2H, C2'-**H$_2$**), 3.05 (q, *J* = 2.8 Hz, 2H, C1'-**H$_2$**), 5.38 (s, 1H, C6-**H**), 5.40 (bs, 1H, C5-N**H**^{n}Oct); 6.43 (s, 1H, C3-**H**). ^{13}C NMR (125 MHz, CDCl$_3$) δ 14.0 (1C, **C8'**), 22.6 (1C, **C7'**), 27.0 (1C, **C3'**), 28.2 (1C, **C2'/C6'**), 29.1 (1C, **C4'/C5'**), 29.2 (1C, **C4'/C5'**), 29.7 (3C, C2-C(**C**H$_3$)$_3$), 31.7 (1C, **C2'/C6'**),

35.7 (1C, C2-$\underline{\textbf{C}}$(CH$_3$)$_3$), 42.3 (1C, $\underline{\textbf{C1'}}$), 100.2 (1C, $\underline{\textbf{C6}}$), 127.4 (1C, $\underline{\textbf{C3}}$), 145.3 (1C, $\underline{\textbf{C5}}$), 159.7 (1C, $\underline{\textbf{C2}}$), 184.9 (1C, $\underline{\textbf{C4}}$), 185.8 (1C, $\underline{\textbf{C1}}$). IR (ATR) 3392.8 2925.6 2856.0 1735.9 1669.2 1625.9 1587.4 1514.1 1462.0 1364.7 1338.1 1226.9 1187.8 1019.9 952.4 798.3 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 274 (9.08x10^4), 484 (2.18x10^4). MS C$_{18}$H$_{29}$NO$_2$, exact mass = 291.2198 [M+H]$^+$, calculated m/z = 292.22711, found 292.22813. E_{c1} = −1.199 V, E_{a1} = −1.116 V, E_{c2} = −1.933 V, E_{a2} = −1.785 V, $E°_1$/Fc = −1.219 V.

2-(Benzylamino)-6-(*tert*-butyl)-1,4-benzoquinone (**3e**). The compound was synthesized by reaction of 1,4-benzoquinone **1** (300 mg, 1.83 mmol) with benzylamine (4.3 g, 4.4 mL, 40.2 mmol). It was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.39) and purified by preparative thin layer chromatography using hexane–acetone (8:2) as eluent (Rf = 0.41). The product was obtained as reddish brown crystal, m.p. 132^{0}C. Yield 102.1 mg; 41.5 %. ^{1}H NMR (200 MHz, CDCl$_3$) δ 1.27 (s, 9H, C6-C(C$\underline{\textbf{H}}_3$)$_3$), 4.26 (d, J = 4 Hz, 2H, C1'-$\underline{\textbf{H}}_2$), 5.48 (d, J = 2 Hz, 1H, C3-$\underline{\textbf{H}}$), 6.01 (bs, 1H, C2-N$\underline{\textbf{H}}$CH$_2$Ph), 6.46 (d, J = 2 Hz, 1H, C5-$\underline{\textbf{H}}$), 7.32 (m, 5H, C3'-$\underline{\textbf{H}}$, C4'-$\underline{\textbf{H}}$, C5'-$\underline{\textbf{H}}$, C6'-$\underline{\textbf{H}}$, C7'-$\underline{\textbf{H}}$). ^{13}C NMR (50 MHz, CDCl$_3$) δ 29.0 (3C, C6-C($\underline{\textbf{C}}$H$_3$)$_3$), 34.8 (1C, C6-$\underline{\textbf{C}}$(CH$_3$)$_3$), 46.9 (1C, $\underline{\textbf{C1'}}$), 98.0 (1C, $\underline{\textbf{C3}}$), 127.7 (2C, $\underline{\textbf{C3'}}$, $\underline{\textbf{C7'}}$), 128.1 (1C, $\underline{\textbf{C5'}}$), 129.0 (2C, $\underline{\textbf{C4'}}$, $\underline{\textbf{C6'}}$), 134.8 (1C, $\underline{\textbf{C5}}$), 136.0 (1C, $\underline{\textbf{C2'}}$), 147.3 (1C, $\underline{\textbf{C2}}$), 151.4 (1C, $\underline{\textbf{C6}}$), 183.2 (1C, $\underline{\textbf{C1}}$), 186.2 (1C, $\underline{\textbf{C4}}$). IR (ATR) 3566.5 3511.2 3362.5 3199.2 3063.5 3031.6 2998.8 2965.5 2873.6 1671.8 1634.9 1587.5 1504.2 1456.7 1364.2 1340.1 1257.8 1163.4 1061.1 909.6 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 276 (5.35x10^4), 480 (1.83x10^4). MS C$_{17}$H$_{19}$NO$_2$, exact mass = 269.1416 [M+H]$^+$, calculated m/z = 270.14886, found 270.14903, [2M+H]$^+$, calculated m/z = 539.29043, found 539.28999. E_{c1} = −1.128 V, E_{a1} = −1.053 V, E_{c1} = −1.800 V, E_{a2} = −1.619 V, $E°_1$/Fc = −1.159 V.

2-(Benzylamino)-5-(*tert*-butyl)1,4-benzoquinone (**4e**). The compound was synthesized by reaction of 1,4-benzoquinone **1** (300 mg, 1.83 mmol) with benzylamine (4.3 g, 4.4 mL, 40.2 mmol). It was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.51) and purified by preparative thin layer chromatography using hexane–acetone (8:2) as eluent (Rf = 0.49). The product was obtained as reddish brown oil. Yield 17.1 mg; 7.0 %. ^{1}H NMR (200 MHz, CDCl$_3$) δ 1.29 (s, 9H, C5-C(C**H$_3$**)$_3$), 4.26 (d, J = 6 Hz, 2H, C1'-**H$_2$**), 5.45 (s, 1H, C3-**H**), 5.74 (bs, 1H, C2-N**H**CH$_2$Ph), 6.45 (s, 1H, C6-**H**), 7.32 (m, 5H,C3'-**H**, C4'-**H**, C5'-**H**, C6'-**H**, C7'-**H**). ^{13}C NMR (50 MHz, CDCl$_3$) δ 29.6 (3C, C5-C(**C**H$_3$)$_3$), 35.7 (1C, C5-**C**(CH$_3$)$_3$), 46.6 (1C, **C1'**), 101.2 (1C, **C3**), 127.5 (1C, **C5'**), 127.6 (2C, **C3'**, **C7'**), 128.0 (1C, **C6**), 128.9 (2C, **C4'**, **C6'**), 136.1 (1C, **C2'**), 145.1 (1C, **C2**), 159.5 (1C, **C5**), 184.8 (1C, **C1**), 186.0 (1C, **C4**). IR (ATR) 3384.0 3061.9 3029.6 3003.0 2960.7 2920.8 2868.3 1668.5 1626.6 1587.3 1513.4 1455.3 1389.0 1360.7 1337.5 1229.2 1182.8 1062.3 1021.6 895.4 836.0 803.8 741.1 700.4 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 276 (8.60x10^4), 476 (1.62x10^4). MS C$_{17}$H$_{19}$NO$_2$, exact mass = 269.1416 [M+H]$^+$, calculated m/z = 270.14886, found 270.14910. E_{c1} = −1.141 V, E_{a1} = −1.071 V, E_{c2} = −1.803 V, E_{a2} = −1.593 V, $E°_1$/Fc = −1.174 V.

2-(*tert*-Butyl)-6-(phenethylamino)-1,4-benzoquinone (**3f**). The compound was synthesized by reaction of 1,4-benzoquinone **1** (300 mg, 1.83 mmol) with phenethylamine (4.87 g, 5.05 mL, 40.2 mmol). It was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.38) and purified by preparative thin layer chromatography using hexane–acetone (7:3) as eluent (Rf = 0.44). The product was obtained as reddish brown oil. Yield 91.9 mg; 35.5 %. ^{1}H NMR (200 MHz, CDCl$_3$) δ 1.26 (s, 9H,C2-C(C**H$_3$**)$_3$), 2.94 (t, J = 7 Hz, 2H, C2'-**H**), 3.35 (q, J = 7 Hz, 2H, C1'-**H$_2$**), 5.47 (d, J = 3 Hz, 1H, C5-**H**), 5.76 (bs, 1H, C6-N**H**CH$_2$CH$_2$Ph), 6.45 (d, J = 3 Hz, 1H, C3-**H**), 7.22-7.34 (m, 5H, {C4'-C8'}-**H**). ^{13}C NMR (50

MHz, CDCl$_3$) δ 28.9 (3C, C2-C($\underline{\textbf{C}}$H$_3$)$_3$), 34.3 (1C, C2-$\underline{\textbf{C}}$(CH$_3$)$_3$), 34.7 (1C, $\underline{\textbf{C2'}}$), 43.7 (1C, $\underline{\textbf{C1'}}$), 97.3 (1C, $\underline{\textbf{C5}}$), 126.9 (1C, $\underline{\textbf{C6'}}$), 128.6 (2C, $\underline{\textbf{C4'}}$, $\underline{\textbf{C8'}}$), 128.9 (2C, $\underline{\textbf{C5'}}$, $\underline{\textbf{C7'}}$), 134.9 (1C, $\underline{\textbf{C3}}$), 137.8 (1C, $\underline{\textbf{C3'}}$), 147.3 (1C, $\underline{\textbf{C6}}$), 151.3 (1C, $\underline{\textbf{C2}}$), 183.1 (1C, $\underline{\textbf{C1}}$), 186.1 (1C, $\underline{\textbf{C4}}$). IR (ATR) 3378.8 3304.2 3062.4 3027.5 2961.2 2870.6 1669.4 1632.6 1585.9 1505.4 1461.8 1362.7 1339.1 1255.5 1196.7 1157.0 905.4 700.9 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 276 (6.59x10^4), 486 (2.37x10^4). MS C$_{18}$H$_{21}$NO$_2$, exact mass = 283.1572 [M+H]$^+$, calculated m/z = 284.16451, found 284.16454, [2M+H]$^+$, calculated m/z = 567.32173, found 567.32097.

E_{c1} = −1.135 V, E_{a1} = −1.069 V, E_{c2} = −1.889 V, E_{a2} = −1.749 V, $E°_1$/Fc = −1.174 V.

2-(*tert*-Butyl)-5-(phenethylamino)-1,4-benzoquinone (**4f**). The compound was synthesized by reaction of 1,4-benzoquinone **1** (300 mg, 1.83 mmol) with phenethylamine (4.87 g, 5.05 mL, 40.2 mmol). It was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.62) and purified by preparative thin layer chromatography using hexane–acetone (7:3) as eluent (Rf = 0.51). The product was obtained as reddish brown crystal, m.p. 100^{0}C. Yield 21.0 mg; 8.1 %. ^{1}H NMR (200 MHz, CDCl$_3$) δ 1.30 (s, 9H, C2-C(C$\underline{\textbf{H}}$$_3$)$_3$), 2.91 (t, J = 6.8 Hz, 2H, C2'-$\underline{\textbf{H}}$$_2$), 3.34 (q, J = 6.6 Hz, 2H, C1'-$\underline{\textbf{H}}$$_2$), 5.44 (s, 1H, C6-$\underline{\textbf{H}}$), 5.46 (bs, 1H, C5-N$\underline{\textbf{H}}CH_2CH_2$Ph), 6.42 (s, 1H, C3-$\underline{\textbf{H}}$), 7.17-7.37 (m, 5H, {C4'-C8'}-$\underline{\textbf{H}}$).

^{13}C NMR (50 MHz, CDCl$_3$) δ 29.6 (3C, C2-C($\underline{\textbf{C}}$H$_3$)$_3$), 34.3 (1C, C2-$\underline{\textbf{C}}$(CH$_3$)$_3$), 35.7 (1C, $\underline{\textbf{C2'}}$), 43.4 (1C, $\underline{\textbf{C1'}}$), 100.6 (1C, $\underline{\textbf{C6}}$), 126.9 (1C, $\underline{\textbf{C6'}}$), 127.5 (1C, $\underline{\textbf{C3}}$), 128.6 (2C, $\underline{\textbf{C4'}}$, $\underline{\textbf{C8'}}$), 128.8 (2C, $\underline{\textbf{C5'}}$, $\underline{\textbf{C7'}}$), 137.9 (1C, $\underline{\textbf{C3'}}$), 145.1 (1C, $\underline{\textbf{C5}}$), 159.6 (1C, $\underline{\textbf{C2}}$), 184.7 (1C, $\underline{\textbf{C4}}$), 185.9 (1C, $\underline{\textbf{C1}}$). IR (ATR) 3278.2 3065.1 3029.6 3005.1 2959.5 2871.0 1670.7 1622.8 1583.3 1508.1 1455.9 1360.2 1284.0 1236.5 1197.8 1068.2 1021.3 895.2 841.4 749.3 699.6 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 274 (9.02x10^4), 482 (2.14x10^4). MS C$_{18}$H$_{21}$NO$_2$, exact mass = 283.1572

[M+H]$^+$, calculated m/z = 284.16451, found 284.16424. E_{c1} = −1.147 V, E_{a1} = −1.084 V, E_{c2} = −1.873 V, E_{a2} = −1.728 V, $E°_1$/Fc = −1.188 V.

2-(Butylamino)-6-(((1*R*,2*S*,4a*S*,8a*S*)-1,2,4a,5-tetramethyl-1,2,3,4,4a,7,8,8a-octahydronaphthalen-1-yl)methyl)cyclohexa-2,5-diene-1,4-dione (**3g**). The compound was synthesized by reaction of avarone **2** (220 mg, 0.705 mmol) with *n*-butylamine (1.13 g, 1.54 mL, 15.51 mmol). It was separated from its regioisomer by column chromatography using toluene−ethyl acetate (9:1) as eluent and purified by preparative thin layer chromatography using toluene−ethyl acetate (9:1) as eluent (Rf = 0.40). The product was obtained as reddish brown oil. Yield 41.6 mg; 30.8 %. ^{1}H NMR (200 MHz, CDCl$_3$) δ 0.80-1.10 (m, 9H, C4''-**H$_3$**, C13-**H$_3$**, C14-**H$_3$**), 1.20-1.70 (m, 16H, C1-**H$_2$**, C7-**H$_2$**, C8-**H**, C10-**H**, C11-**H$_3$**, C12-**H$_3$**, C2''-**H$_2$**, C3''-**H$_2$**), 1.80-2.20 (m, 4H, C2-**H$_2$**, C6-**H$_2$**), 2.37 (d, J = 13.4 Hz, 1H, C15-**Ha**), 2.62 (d, J = 13.4 Hz, 1H, C15-**Hb**), 3.07 (dd, 2H, C1''-**H$_2$**), 5.14 (s, 1H, C3-**H**), 5.42 (d, J = 2.2 Hz, 1H, C4'-**H**), 5.63 (bs, 1H, C3'N-**H**), 6.37 (d, J = 2.2 Hz, 1H, C6'-**H**). ^{13}C NMR (50 MHz, CDCl$_3$) δ 13.7 (1C, **C4''**), 16.6 (1C, **C13**), 17.7 (1C, **C14**), 18.0 (1C, **C11**), 19.3 (1C, **C12**), 20.0 (1C, **C1**), 20.1 (1C, **C3''**), 26.5 (1C, **C2**), 27.3 (1C, **C7**), 30.1 (1C, **C2''**), 34.9 (1C, **C15**), 35.9 (1C, **C6**), 36.5 (1C, **C8**), 38.4 (1C, **C9**), 42.0 (1C, **C5**), 42.3 (1C, **C1''**), 46.7 (1C, **C10**), 97.6 (1C, **C4'**), 120.6 (1C, **C3**), 139.9 (1C, **C6'**), 142.0 (1C, **C1'**), 144.0 (1C, **C4**), 146.8 (1C, **C3'**), 184.0 (1C, **C2'**), 185.5 (1C, **C5'**). IR (ATR) 3389.7 3301.5 2958.9 2931.8 2870.2 1671.4 1635.4 1588.0 1508.1 1464.5 1380.1 1346.8 1287.9 1250.3 1190.5 1124.5 1090.0 1040.6 914.5 802.1 737.7 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 292 (4.81x10^4), 496 (2.14x10^4). MS C$_{25}$H$_{37}$NO$_2$, exact mass = 383.2824 [M+H]$^+$, calculated m/z = 384.28971, found 384.28892, [2M+H]$^+$, calculated m/z = 767.57214, found 767.57256. E_{c1} = −1.138 V, E_{a1} = −1.067 V, E_{c2} = −1.876 V, E_{a2} = −1.747 V, $E°_1$/Fc = −1.172 V. [α]^{20} = −40° (c = 0.083 in MeOH).

2-(Butylamino)-5-(((1*R*,2*S*,4a*S*,8a*S*)-1,2,4a,5-tetramethyl-1,2,3,4,4a,7,8,8a-octahydronaphthalen-1-yl)methyl)cyclohexa-2,5-diene-1,4-dione (**4g**). The compound was synthesized by reaction of avarone **2** (220 mg, 0.705 mmol) with *n*-butylamine (1.13 g, 1.54 mL, 15.51 mmol). It was separated from its regioisomer by column chromatography using toluene−ethyl acetate (9:1) as eluent and purified by preparative thin layer chromatography using toluene−ethyl acetate (9:1) as eluent (Rf = 0.60). The product was obtained as reddish brown oil. Yield 17.0 mg; 12.6 %. ^{1}H NMR (200 MHz, CDCl$_3$) δ 0.80-1.10 (m, 9H, C4''-**H$_3$**, C13-**H$_3$**, C14-**H$_3$**), 1.20-1.70 (m, 16H, C1-**H$_2$**, C7-**H$_2$**, C8-**H**, C10-**H**, C11-**H$_3$**, C12-**H$_3$**, C2''-**H$_2$**, C3''-**H$_2$**), 1.80-2.20 (m, 4H, C2-**H$_2$**, C6-**H$_2$**), 2.46 (d, 1H, C15-**Ha**), 2.65 (d, 1H, C15-**Hb**), 3.07 (dd, 2H, C1''-**H$_2$**), 5.15 (s, 1H, C3-**H**), 5.43 (s, 1H, C3'-**H**), 5.51 (bs, 1H, C4'N-**H**), 6.36 (s, 1H, C6'-**H**).

^{13}C NMR (50 MHz, CDCl$_3$) δ 13.6 (1C, **C4''**), 16.8 (1C, **C13**) 17.8 (1C, **C14**) 18.0 (1C, **C11**), 19.4(1C, **C12**), 20.0 (1C, **C1**), 20.1 (1C, **C3''**), 26.5 (1C, **C2**), 27.5 (1C, **C7**), 30.2 (1C, **C2''**), 35.7 (1C, **C15**), 36.1 (1C, **C6**), 37.0 (1C, **C8**), 38.5 (1C, **C9**), 42.0 (1C, **C5**), 43.1 (1C, **C1''**), 47.2 (1C, **C10**), 98.2 (1C, **C3'**), 120.8 (1C, **C3**), 131.7 (1C, **C6'**), 144.0 (1C, **C1'**), 146.1 (1C, **C4**), 151.3 (1C, **C4'**), 183.7 (1C, **C5'**), 185.2 (1C, **C2'**). IR (ATR) 3354.6 2959.1 2932.1 2868.3 1665.5 1628.5 1593.4 1514.3 1463.3 1380.6 1318.2 1241.7 1222.4 1197.9 1130.0 1099.9 898.7 842.9 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 292 (6.14x10^4), 490 (1.66x10^4). MS C$_{25}$H$_{37}$NO$_2$, exact mass = 383.2824 [M+H]$^+$, calculated *m/z* = 384.28971, found 384.28848, [2M+H]$^+$, calculated *m/z* = 767.57214, found 767.57067. E_{c1} = −1.148 V, E_{a1} = −1.078 V, E_{c2} = −1.888 V, E_{a2} = −1.749 V, $E°_1$/Fc = −1.182 V. $[\alpha]^{20}$ = 80º (*c* = 0.083 in MeOH).

2-(Octylamino)-6-(((1*R*,2*S*,4a*S*,8a*S*)-1,2,4a,5-tetramethyl-1,2,3,4,4a,7,8,8a-octahydronaphthalen-1-yl)methyl)cyclohexa-2,5-diene-1,4-dione (**3h**). The compound was separated from its regioisomer by column chromatography using toluene−ethyl acetate (9:1) as

eluent and purified by preparative thin layer chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.57). The product was obtained as reddish brown oil. Yield 98.6 mg; 46.6 %.

^{1}H NMR (200 MHz, CDCl$_3$) δ 0.80-1.10 (m, 9H, C8''-**H$_3$**, C13-**H$_3$**, C14-**H$_3$**), 1.20-1.70 (m, 24H, C1-**H$_2$**, C7-**H$_2$**, C8-**H**, C10-**H**, C11-**H$_3$**, C12-**H$_3$**, C2''-**H$_2$**, C3''-**H$_2$**, C4''-**H$_2$**, C5''-**H$_2$**, C6''-**H$_2$**, C7''-**H$_2$**), 1.80-2.20 (m, 4H, C2-**H$_2$**, C6-**H$_2$**), 2.37 (d, J = 14 Hz, 1H, C15-**Ha**), 2.62 (d, J = 14 Hz, 1H, C15-**Hb**), 3.06 (dd, 2H, C1''-**H$_2$**), 5.14 (s, 1H, C3-**H**), 5.41 (d, J = 2.2 Hz, 1H, C4'-**H**), 5.62 (s, 1H, C3'N-**H**), 6.37 (d, J = 2.2 Hz, 1H, C6'-**H**). ^{13}C NMR (50 MHz, CDCl$_3$) δ 14.0 (1C, **C8''**), 16.6 (1C, **C13**), 17.7 (1C, **C14**), 18.0 (1C, **C11**), 19.3 (1C, **C1**), 20.0 (1C, **C12**), 22.6 (1C, **C7''**), 26.5 (1C, **C2**), 27.0 (1C, **C3''**), 27.3 (1C, **C7**), 28.1 (1C, **C4''**), 29.1 (1C, **C5''**), 29.1 (1C, **C2''**), 31.7 (1C, **C6''**), 34.9 (1C, **C15**), 35.9 (1C, **C6**), 36.5 (1C, **C8**), 38.4 (1C, **C9**), 42.0 (1C, **C5**), 42.6 (1C, **C1''**), 46.7 (1C, **C10**), 97.6 (1C, **C4'**), 120.6 (1C, **C3**), 139.9 (1C, **C6'**), 142.0 (1C, **C1'**), 144.0 (1C, **C4**), 146.8 (1C, **C3'**), 184.0 (1C, **C2'**), 185.5 (1C, **C5'**). IR (ATR) 3390.2 3314.7 2927.6 2856.3 1671.0 1635.4 1588.3 1507.5 1465.6 1345.7 1249.8 1193.0 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 292 (4.87x10^4), 496 (2.28x10^4). MS C$_{29}$H$_{45}$NO$_2$, exact mass = 439.3450 [M+H]$^+$, calculated m/z = 440.35231, found 440.35203, [2M+H]$^+$, calculated m/z = 879.69734, found 879.69826. E_{c1} = −1.132 V, E_{a1} = −1.059 V, E_{c2} = −1.852 V, E_{a2} = −1.711 V, $E°_1$/Fc = −1.165 V. [α]^{20} = 10° (c = 0.083 in MeOH).

2-(Octylamino)-5-(((1R,2S,4aS,8aS)-1,2,4a,5-tetramethyl-1,2,3,4,4a,7,8,8a-octahydronaphthalen-1-yl)methyl)cyclohexa-2,5-diene-1,4-dione (**4h**). The compound was separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.75) and purified by preparative thin layer chromatography using chloroform as eluent (Rf = 0.60). The product was obtained as reddish brown oil. Yield 46.6 mg; 22.0 %.

^{1}H NMR (200 MHz, CDCl$_3$) δ 0.80-1.10 (m, 9H, C8''-**H$_3$**, C13-**H$_3$**, C14-**H$_3$**), 1.20-1.70 (m, 24H, C1-**H$_2$**, C7-**H$_2$**, C8-**H**, C10-**H**, C11-**H$_3$**, C12-**H$_3$**, C2''-**H$_2$**, C3''-**H$_2$**, C4''-**H$_2$**, C5''-**H$_2$**, C6''-**H$_2$**, C7''-**H$_2$**), 1.80-2.20 (m, 4H, C2-**H$_2$**, C6-**H$_2$**), 2.46 (d, J = 13 Hz, 1H, C15-**Ha**), 2.65 (d, J = 13.6 Hz, 1H, C15-**Hb**), 3.06 (dd, 2H, C1''-**H$_2$**), 5.15 (s, 1H, C3-**H**), 5.42 (s, 1H, C3'-**H**), 5.50 (bs, 1H, C4'N-**H**), 6.35 (s, 1H, C6'-**H**). ^{13}C NMR (50 MHz, CDCl$_3$) δ 14.0 (1C, **C8''**), 16.8 (1C, **C13**), 17.8 (1C, **C14**), 18.0 (1C, **C11**), 19.4 (1C, **C1**), 20.0 (1C, **C12**), 22.6 (1C, **C7''**), 26.5 (1C, **C2**), 27.0 (1C, **C3''**), 27.5 (1C, **C7**), 28.2 (1C, **C4''**), 29.1 (1C, **C5''**), 29.2 (1C, **C2''**), 31.7 (1C, **C6''**), 35.7 (1C, **C15**), 36.1 (1C, **C6**), 37.0 (1C, **C8**), 38.5 (1C, **C9**), 42.3 (1C, **C5**), 43.1 (1C, **C1''**), 47.2 (1C, **C10**), 98.2 (1C, **C3'**), 120.8 (1C, **C3**), 131.7 (1C, **C6'**), 144.0 (1C, **C1'**), 146.1 (1C, **C4**), 151.3 (1C, **C4'**), 183.7 (1C, **C5'**), 185.2 (1C, **C2'**). IR (ATR) 3391.3 3359.3 2957.7 2928.7 2857.1 1666.6 1628.9 1594.0 1512.7 1467.9 1382.4 1312.8 1221.6 cm^{-1}. UV–Vis (MeOH, λ_{max}/nm, (ε/ dm^{2}mol^{-1})): 290 (7.35x10^4), 490 (2.01x10^4). MS C$_{29}$H$_{45}$NO$_2$, exact mass = 439.3450 [M+H]$^+$, calculated m/z = 440.35231, found 440.35181, [2M+H]$^+$, calculated m/z = 879.69734, found 879.69842. E_{c1} = −1.144 V, E_{a1} = −1.076 V, E_{c2} = −1.849 V, E_{a2} = −1.684 V, $E°_1$/Fc = −1.179 V. $[\alpha]^{20}$ = 50° (c = 0.083 in MeOH).

2-(Phenethylamino)-6-(((1R,2S,4aS,8aS)-1,2,4a,5-tetramethyl-1,2,3,4,4a,7,8,8a-octahydronaphthalen-1-yl)methyl)cyclohexa-2,5-diene-1,4-dione (**3i**). The compound was separated from regioisomer by column chromatography using toluene–ethyl acetate (9:1) as eluent (Rf = 0.58) and purified by preparative thin layer chromatography using hexane−acetone (8:2) as eluent (Rf = 0.38). The product was obtained as reddish brown oil. Yield 43.7 mg; 21.1 %. ^{1}H NMR (200 MHz, CDCl$_3$) δ 0.80-1.10 (m, 6H, C13-**H$_3$**, C14-**H$_3$**), 1.20-1.80 (m, 12H, C1-**H$_2$**, C7-**H$_2$**, C8-**H**, C10-**H**, C11-**H$_3$**, C12-**H$_3$**), 1.80-2.20 (m, 4H, C2-**H$_2$**, C6-**H$_2$**), 2.34 (d, J = 14 Hz, 1H, C15-**Ha**), 2.63 (d, J = 14 Hz, 1H, C15-**Hb**), 2.92 (t, J = 7 Hz, 2H, C2''-**H$_2$**), 3.35 (dd,

2H, C1''-**H₂**), 5.12 (s, 1H, C3-**H**), 5.46 (d, J = 2.4 Hz, 1H, C4'-**H**), 5.68 (bs, 1H, C3'N-**H**), 6.37

(d, J = 2.4 Hz, 1H, C6'-**H**), 7.10-7.40 (m, 5H, C4''-**H**, C5''-**H**, C6''-**H**, C7''-**H**, C8''-**H**).

¹³C NMR (50 MHz, CDCl₃) δ 16.6 (1C, **C13**), 17.7 (1C, **C14**), 18.0 (1C, **C11**), 19.3 (1C, **C1**),

20.0 (1C, **C12**), 26.5 (1C, **C2**), 27.3 (1C, **C7**), 34.2 (1C, **C15**), 35.9 (1C, **C2''**), 36.0 (1C, **C6**),

36.5 (1C, **C8**), 38.4 (1C, **C9**), 42.0 (1C, **C5**), 43.7 (1C, **C1''**), 46.7 (1C, **C10**), 97.9 (1C, **C4'**),

120.6 (1C, **C3**), 126.9 (1C, **C6''**), 128.6 (2C, **C4''**, **C8''**), 128.8 (2C, **C5''**, **C7''**), 137.8 (1C,

C3''), 139.7 (1C, **C6'**), 142.2 (1C, **C1'**), 144.0 (1C, **C4**), 146.6 (1C, **C3'**), 183.8 (1C, **C2'**), 185.6

(1C, **C5'**). IR (ATR) 3381.8 2931.7 1733.0 1671.1 1635.8 1587.9 1507.0 1456.9 1380.3 1347.2

1253.4 1197.6 1096.4 1028.3 913.0 889.1 803.6 744.7 700.5 cm⁻¹. UV–Vis (MeOH, λ_{max}/nm, (ε/

dm²mol⁻¹)): 288 (4.44x10⁴), 494 (2.08x10⁴). MS $C_{29}H_{37}NO_2$, exact mass = 431.2824 [M+H]⁺,

calculated m/z = 432.28971, found 432.28613, [2M+H]⁺, calculated m/z = 863.57214, found

863.56985. E_{c1} = −1.123 V, E_{a1} = −1.052 V, E_{c2} = −1.876 V, E_{a2} = −1.747 V, $E°_1$/Fc = −1.156 V.

$[\alpha]^{20}$ = 50° (c = 0.083 in MeOH).

2-(Phenethylamino)-5-(((1*R*,2*S*,4a*S*,8a*S*)-1,2,4a,5-tetramethyl-1,2,3,4,4a,7,8,8a-

octahydronaphthalen-1-yl)methyl)cyclohexa-2,5-diene-1,4-dione (**4i**). The compound was

separated from its regioisomer by column chromatography using toluene–ethyl acetate (9:1) as

eluent (Rf = 0.76) and purified by preparative thin layer chromatography using hexane–acetone

(8:2) as eluent (Rf = 0.49). The product was obtained as reddish brown crystal, m.p. 122-123⁰C.

Yield 52.4 mg; 25.2 %. ¹H NMR (200 MHz, CDCl₃) δ 0.80-1.10 (m, 6H, C13-**H₃**, C14-**H₃**),

1.20-1.80 (m, 12H, C1-**H₂**, C7-**H₂**, C8-**H**, C10-**H**, C11-**H₃**, C12-**H₃**), 1.80-2.20 (m, 4H, C2-**H₂**,

C6-**H₂**), 2.46 (d, J = 13 Hz, 1H, C15-**Ha**), 2.65 (d, J = 13 Hz, 1H, C15-**Hb**), 2.93 (t, J = 7.4 Hz,

2H, C2''-**H₂**), 3.35 (dd, 2H, C1''-**H₂**), 5.15 (s, 1H, C3-**H**), 5.47 (s, 1H, C3'-**H**), 5.57 (bs, 1H,

C4'N-**H**), 6.35 (s, 1H, C6'-**H**), 7.10-7.40 (m, 5H, C4''-**H**, C5''-**H**, C6''-**H**, C7''-**H**, C8''-**H**).

^{13}C NMR (50 MHz, CDCl$_3$) δ 16.8 (1C, **C13**), 17.7 (1C, **C14**), 18.0 (1C, **C11**), 19.3 (1C, **C1**), 20.0 (1C, **C12**), 26.4 (1C, **C2**), 27.5 (1C, **C7**), 34.4 (1C, **C15**), 35.7 (1C, **C2''**), 36.0 (1C, **C6**), 37.0 (1C, **C8**), 38.5 (1C, **C9**), 43.1 (1C, **C5**), 43.4 (1C, **C1''**), 47.1 (1C, **C10**), 98.5 (1C, **C3'**), 120.7 (1C, **C3**), 126.9 (1C, **C6''**), 128.6 (2C, **C4''**; **C8''**), 128.8 (2C, **C5''**; **C7''**), 131.8 (1C, **C6'**), 137.8 (1C, **C3''**), 144.0 (1C, **C1'**), 145.8 (1C, **C4**), 151.2 (1C, **C4'**), 183.4 (1C, **C5'**), 185.3 (1C, **C2'**). IR (ATR) 3360.9 2932.4 2361.1 1666.3 1628.8 1592.8 1513.6 1454.4 1380.1 1317.6 1248.3 1221.8 1098.8 1032.4 898.8 800.1 747.2 700.8 cm^{-1}. UV–Vis (MeOH, λ$_{max}$/nm, (ε/dm^{2}mol^{-1})): 290 (7.01x10^4), 488 (1.93x10^4). MS C$_{29}$H$_{37}$NO$_2$, exact mass = 431.2824 [M+H]$^+$, calculated m/z = 432.28971, found 432.29161, [2M+H]$^+$, calculated m/z = 863.57214, found 863.57121. E_{c1} = −1.131 V, E_{a1} = −1.061 V, E_{c2} = −1.869 V, E_{a2} = −1.729 V, $E°_{1}$/Fc = −1.164 V. [α]^{20} = 40° (c = 0.083 in MeOH).

Cyclic voltammetry. Cyclic voltammetry experiments were performed at room temperature under nitrogen atmosphere in a three-electrode cell at 25°C and using a CHI1760B workstation (CH Instruments, Austin TX, USA). The working electrode was glassy carbon disk (3mm diameter). The counter electrode was a platinum wire, and a silver wire immersed in electrolyte solution containing 0.01 M silver ions was used as the reference electrode. The quinone derivatives were used as 2 mM solution in dimethyl sulfoxide, with 0.1 M tetrabutylammonium perchlorate as an electrolyte. Ferrocene was used as reference compound.

Biology.

Anticancer activity. Chemicals. RPMI 1640 medium, DMEM medium, penicillin–streptomycin solution, antibiotic–antimycotic solution, L-glutamine and trypsin/EDTA were purchased from PAA, Vienna, Austria. Fetal bovine serum (FBS), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were obtained from Sigma–Aldrich Chemie GmbH, Germany. Propidium iodide (PI) and annexin V–FITC (AV) were

purchased from Abcam, Cambridge, UK. Dihydroethidium (DHE) was obtained from Molecular Probes®, Invitrogen, CA, USA. JC-1 was purchased from BD Pharmingen™, BD Biosciences, San Jose, CA, USA.

Drugs. All compounds were diluted in ethanol and 20 mM aliquots were stored at 4°C. Drugs were thawed and diluted in fresh water to 1 mM before use. CDDP was obtained from Pfizer (Perth) Pty Ltd, Bentley, Australia and stored as 1 mM stock in H_2O.

Cell culture. NCI-H460 cell line was purchased from the American Type Culture Collection, Rockville, MD. NCI-H460/R cells were selected originally from NCI-H460 cells and cultured in a medium containing DOX [34]. HaCaT cell line (normal human keratinocytes obtained from CLS - Cell Lines Service, Eppelheim, Germany) was a generous gift from Prof. Jörg Andrä, Division of Biophysics, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany. NCI-H460 and NCI-H460/R cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, and 10,000 U/ml penicillin, 10 mg/ml streptomycin, 25 μg/ml amphotericin B solution. HaCaT cells were cultured in DMEM supplemented with 10% FBS, 4 g/L glucose, L-glutamine (2 mM) and 5000 U/ml penicilin, 5 mg/mL streptomycin solution. NCI-H460 and NCI-H460/R cells were subcultured at 72 h intervals using 0.25% trypsin/EDTA and seeded into a fresh medium at 8,000 and 16,000 cells/cm^2, respectively. HaCaT cells were subcultured at 144 h intervals using 0.25% trypsin/EDTA and seeded into a fresh medium at 64,000 cells/cm^2.

Cytotoxicity by MTT assay. Cell viability was assessed by MTT assay based on the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide into formazan dye by active mitochondria of living cells. Briefly, cells were placed in 96-well microplates at following densities: 1000 cells/well for NCI-H460, 2000 cells/well for NCI-H460/R and 4000 cells/well for

HaCaT in 100 μL of adequate medium. After 24 h, cells were treated with compounds and incubated for additional 72 h. MTT was added to final concentration of 0.4 mg/mL in each well of a 96-well microplate and plates were incubated at 37 °C for 4 h. Media were discarded and 100 μL DMSO was added to dissolve formazan product, the amount of which was proportional to the number of live cells. The absorbance of the dissolved dye was measured at 540 nm using an automatic microplate reader (LKB 5060-006 Micro Plate Reader, Vienna, Austria).

Growth inhibition (I) was determined according to the following equitation:

I (%) = (1 − ($A_{\text{treated sample}}$/$A_{\text{untreated control}}$)) x 100, where A is absorbance.

IC_{30} and IC_{50} values were defined as concentration of each drug that inhibited cell growth by 30% and 50%, respectively. These values were calculated by linear regression analysis using Excel software.

Cell death detection. The percentages of apoptotic, necrotic and viable cells were determined by annexin V–FITC (AV) and propidium iodide (PI) labeling. NCI-H460 and NCI-H460/R cells were plated and incubated overnight in 6-well plates at density of 50,000 cells/well, while HaCaT cells were seeded at density of 100,000 cells/well. Cells were subjected to single treatments with 25 μM TBQ (**1**) and its derivatives as well as avarone (**2**) and its derivatives. CDDP was used as a control compound in concentration of 5 μM. After 72 h, the attached and floating cells were collected by centrifugation. The cells pellet was re-suspended in 100 μL of binding buffer supplemented with 5 μl AV and 5 μl PI according to manufacturer's instructions. After the incubation period (5 min at room temperature), additional 400 μL of binding buffer was added and AV/PI staining was analyzed within 1 h by flow-cytometry. The fluorescence intensity (green FL1-H and red FL2-H) was measured on FACSCalibur flow-cytometer (Becton Dickinson, Oxford, United Kingdom). In each sample, 10,000 cells were recorded (gated to

exclude cell debris), and the percentages of viable (AV–PI–), early apoptotic (AV+PI–), apoptotic and necrotic (AV+PI+), and already dead (AV–PI+) cells were analyzed by CellQuest Pro data analysis software.

Superoxide anion detection. Flow-cytometric analysis of DHE fluorescence intensity was used to detect superoxide anion level in cells. NCI-H460 and NCI-H460/R cells were plated and incubated overnight in 6-well plates at density of 50,000 cells/well, while HaCaT cells were seeded at density of 100,000 cells/well. Cells were treated with 25 μM of TBQ (**1**) and its derivatives as well as avarone (**2**) and its derivatives. CDDP was used as a control compound in concentration of 5 μM. After 72 h, adherent cells were harvested by trypsinization and incubated in adequate medium with 1 μM DHE for 30 min at 37 °C in the dark. Cells were subsequently washed twice in PBS and DHE fluorescence was analyzed by flow-cytometry (excitation 488 nm, and emission 585 nm, FL2-H channel). Mean fluorescence intensity (MFI) was calculated after correction for auto-fluorescence.

JC-1 assay. JC-1 is a cationic dye whose accumulation in mitochondria is dependent on the mitochondrial membrane potential. Under normal conditions, JC-1 accumulates in healthy mitochondria as red aggregates detectable in FL2-H channel in flow-cytometer while in mitochondria with depolarized $\Delta\Psi$m the dye remains in the cytoplasm in monomeric form detectable in FL1-H channel as green fluorescence. Thus, in mitochondria undergoing a transition from polarized to depolarized $\Delta\Psi$m, JC-1 leaks out of the mitochondria into the cytoplasm resulting in an increase in green fluorescence and a decrease in red fluorescence.

According to the manufacturer's instructions, cells were incubated with a JC-1 reagent for 15 min at 37 °C in CO_2 incubator. After two washings in 1 x Assay Buffer, the cells were re-suspended in PBS prior flow-cytometric analysis.

JC-1 stained live cells were also examined under the Zeiss Axiovert fluorescent microscope (Carl Zeiss Foundation, Oberkochen, Germany) using an AxioVision4.6 software.

Statistical analysis. Statistical analysis was performed by GraphPad Prism 6 Software. In MTT assay analysis, the differences between groups were tested by Student's t-test and were considered statistically significant if $p < 0.05$. Flow cytometry results were examined by Two-way ANOVA and analyzed by Tukey's multiple comparisons test.

Antimicrobial Activity Determination. Antimicrobial activity was evaluated using a broth microdilution method according to NCCLS [35]. Test compounds were dissolved in DMSO to a stock concentration of 10 mg/mL and dilluted in the Mueller Hinton broth for bacteria or Sabouraund dextrose broth for fungi. Amikacin served as positive control for bacteria, while nystatin served as positive control for fungi. Gram-negative bacterial strains used were: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus hauseri* (ATCC 13315) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076). Gram-positive bacterial strains used were: *Bacillus subtilis* (ATCC 6633), *Clostridium sporogenes* (ATCC 19404), *Streptosporangium longisporum* (ATCC 25212), *Micrococcus luteus* (ATCC 10240), *Micrococcus luteus* (ATCC 4698), *Kocuria rhizophila* (ATCC 9341), and *Staphylococcus aureus* (ATCC 6538). Fungal species used were: *Candida albicans* (ATCC 10231), *Saccharomyces cerevisiae* (ATCC 9763) and *Aspergillus brasiliensis* (ATCC 16404).
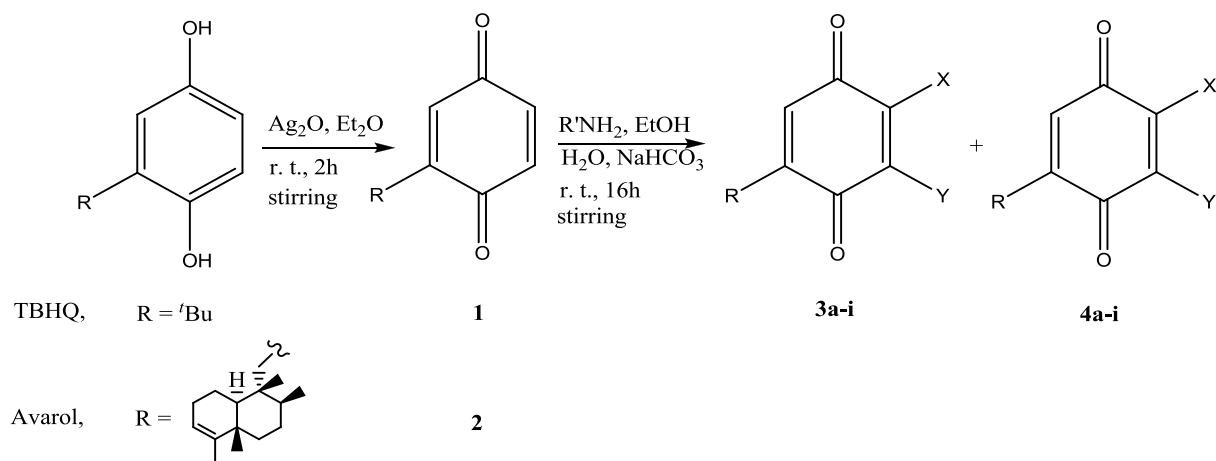
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Scheme 1.

Table 1. Voltammetric half-peak potentials and standard redox potential of the synthesized compounds

	Ec1	Ea1	Ec2	Ea2	E^0_1/Fc
3a	-1.200	-1.104	-1.910	-1.785	-1.213
4a	-1.200	-1.124	-1.906	-1.766	-1.223
3b	-1.160	-1.080	-1.896	-1.746	-1.191
4b	-1.170	-1.102	-1.860	-1.672	-1.211
3c	-1.160	-1.081	-1.892	-1.759	-1.192
4c	-1.182	-1.096	-1.921	-1.773	-1.210
3d	-1.183	-1.095	-1.917	-1.806	-1.200
4d	-1.199	-1.116	-1.933	-1.785	-1.219
3e	-1.128	-1.053	-1.800	-1.619	-1.159
4e	-1.141	-1.071	-1.803	-1.593	-1.174
3f	-1.135	-1.069	-1.889	-1.749	-1.174
4f	-1.147	-1.084	-1.873	-1.728	-1.188
3g	-1.138	-1.067	-1.876	-1.747	-1.172
4g	-1.148	-1.078	-1.888	-1.749	-1.182
3h	-1.132	-1.059	-1.852	-1.711	-1.165
4h	-1.144	-1.076	-1.849	-1.684	-1.179
3i	-1.123	-1.052	-1.876	-1.747	-1.156
4i	-1.131	-1.061	-1.869	-1.729	-1.164

Table 2. Cytotoxic activity of avarone, TBQ and their derivatives in non-small cell lung carcinoma cell lines (NCI-H460 – sensitive and NCI-H460/R – MDR variant) as well as in human normal keratinocytes HaCaT

Compounds	NCI-H460		NCI-H460/R		HaCaT	
	IC_{30}	IC_{50}	IC_{30}	IC_{50}	IC_{30}	IC_{50}
CDDP[c]	2.2 ± 0.3	5.2 ± 0.4	0.7 ± 0.1	1.7 ± 0.1	0.3 ± 0.1	0.7 ± 0.1
1[c]	>100	>100	31 ± 2	72 ± 8	41 ± 2	95 ± 4
2[a,c]	36 ± 2	84 ± 9	10 ± 1	24 ± 4	16 ± 1	37 ± 8
3a	14 ± 1	25 ± 3	15 ± 1	23 ± 4	8 ± 3	10 ± 4
4a	34 ± 1	63 ± 2	31 ± 1	58 ± 2	17 ± 1	28 ± 1
3b[a]	4 ± 2	13 ± 2	5 ± 3	14 ± 5	22 ± 5	30 ± 5
4b	45 ± 2	64 ± 2	45 ± 1	65 ± 1	50 ± 3	75 ± 4
3c	4 ± 3	14 ± 3	4 ± 1	13 ± 1	7 ± 2	11 ± 2
4c[a,b]	5 ± 2	36 ± 1	19 ± 1	72 ± 3	50 ± 2	>100
3d[a]	5 ± 1	20 ± 3	8 ± 1	21 ± 3	18 ± 2	30 ± 2
4d[a]	18 ± 3	70 ± 1	21 ± 1	65 ± 3	>100	>100
3e[a,c]	5 ± 1	14 ± 2	3 ± 2	10 ± 2	12 ± 2	21 ± 1
4e[a,c]	13 ± 1	19 ± 4	5 ± 1	15 ± 2	12 ± 1	28 ± 2
3f[a,c]	7 ± 2	14 ± 1	3 ± 1	6 ± 1	19 ± 1	23 ± 2
4f[a]	17 ± 2	35 ± 3	11 ± 1	33 ± 2	>100	>100
3g[c]	9 ± 2	16 ± 2	3 ± 1	6 ± 2	11 ± 1	16 ± 4
4g[a,c]	91 ± 1	>100	24 ± 2	>100	86 ± 2	>100
3h	21 ± 1	38 ± 3	13 ± 1	35 ± 1	13 ± 2	17 ± 7
4h[a]	>100	>100	>100	>100	>100	>100
3i[a,c]	14 ± 1	21 ± 4	6 ± 1	11 ± 1	18 ± 3	23 ± 4
4i[a,c]	75 ± 1	>100	10 ± 1	91 ± 1	76 ± 3	>100

[a] Selectivity towards cancer cells (IC_{30} or IC_{50} of cancer cells either sensitive or MDR ≤ 1.5 fold than IC_{30} or IC_{50} of normal cells. The activity od **4h** is shown in Fig. S1)
[b] Resistance (lower efficacy in NCI-H460/R compared to NCI-H460) (IC_{30} or IC_{50} of sensitive cancer cells ≤ 1.5 fold than IC_{30} or IC_{50} of corresponding MDR cells)
[c] Selectivity towards MDR cells (higher efficacy in NCI-H460/R compared to NCI-H460) (IC_{30} or IC_{50} of sensitive cancer cells ≥ 1.5 fold than IC_{30} or IC_{50} of corresponding MDR cells)

Table 3. Cell death induction by *tert*-butyl compounds **3d, 4d, 3f, 4f** and their sesquiterpene moiety counterparts **3h, 4h, 3i, 4i** compared with parent compounds **1** and **2** and control compound CDDP

Cell Line	Live Cells	Apoptotic Cells		Dead Cells
	AV–PI–	AV+PI–	AV+PI+	AV–PI+
NCI-H460				
control	98.3	0.3	0.2	1.2
CDDP	66.0	1.6	5.2	27.2
1	95.2	0.5	0.5	3.8
2	74.0	3.4	16.3	6.3
3d	54.9	0.2	2.9	42.0
4d	80.9	0.1	1.7	17.3
3f	66.1	2.4	9.5	22
4f	80.8	0.8	5.4	13
3h	95.7	0.1	0.3	3.9
4h	95.1	0.0	0.3	4.6
3i	71.7	0.1	0.2	28
4i	92	0.3	0.5	7.2
NCI-H460/R				
control	96.4	1.1	1	1.5
CDDP	55.3	3.8	13.3	27.6
1	95.2	0.5	1.2	3.1
2	65.8	2.6	22.7	8.9
3d	61.4	0.1	2.1	36.4
4d	83.9	0.2	1.9	14.0
3f	45.9	2	11.8	40.3
4f	81	1.3	3.6	14.1
3h	92.6	0.0	0.1	7.3
4h	96.1	0.1	0.7	3.1
3i	53	1.2	3.5	42.3
4i	94.9	0.4	0.6	4.1
HaCaT				
control	96.8	0.2	0.2	2.8
CDDP	53.5	1.4	9.0	36.1
1	86.8	1.9	3.8	7.5
2	78.3	2.5	10.1	9.1
3d	77.0	0.6	2.6	19.8
4d	93.8	0.0	0.5	5.7
3f	74.8	4.1	6.9	14.2
4f	89.2	0.8	1.7	8.3
3h	85.3	0.3	0.6	13.8
4h	93.4	0.1	0.7	5.8
3i	58.1	0.2	0.4	41.3
4i	89.5	0.3	1	9.2

Table 4. Antibacterial *in vitro* activity against Gram-positive and Gram-negative bacteria

	MIC (mM)									
Compounds	*S. aureus*	*K. rhizophila*	*B. subtilis*	*M. luteus* (ATCC 10240)	*M. luteus* ATCC 4698)	*C. sporogenes*	*E. coli*	*P. hauseri*	*S. enterica*	*P. aeruginosa*
3a	0.051	0.813	1.621	0.202	1.621	1.621	0.202	1.621	1.621	3.238
4a	0.025	0.409	0.813	0.103	0.831	0.813	0.104	0.813	0.813	1.621
3b	0.024	0.381	0.758	0.024	0.758	0.758	0.024	0.758	0.381	0.758
4b	0.096	0.758	1.512	0.048	1.512	1.512	0.188	1.512	0.758	3.019
3c	0.009	/	1.237	0.009	0.154	1.237	0.009	/	0.62	0.62
4c	0.038	2.47	1.237	0.038	2.47	1.237	0.019	1.237	0.62	0.62
3d	/	/	/	/	/	/	/	/	/	/
4d	4.295	4.295	4.295	2.147	4.295	2.147	4.295	2.147	2.147	2.147
3e	0.009	/	2.323	0.009	4.646	2.323	0.009	/	4.646	2.323
4e	0.072	4.646	4.646	0.018	2.323	2.323	0.036	2.323	4.646	4.646
3f	0.009	17.667	4.416	0.034	2.208	4.416	0.009	17.667	4.416	4.416
4f	/	2.208	4.416	4.416	2.208	2.208	/	2.208	4.416	2.208
3g	/	/	/	/	/	/	/	/	/	/
4g	/	/	/	/	/	/	/	/	/	/
3h	/	/	/	/	/	/	/	/	/	/
4h	/	/	/	/	/	/	/	/	/	/
3i	/	/	/	/	/	/	/	/	/	/
4i	/	/	/	/	/	/	/	/	/	/
Amikacin	0.019	0.003	0.072	0.014	0.003	0.026	0.009	0.012	0.014	0.085

Table 5. Antifungal *in vitro* activity

Compound	MIC (mM)		
	C. albicans	*S. cerevisiae*	*A. brasiliensis*
3a	1.621	3.238	0.103
4a	1.621	1.621	0.051
3b	1.512	1.512	0.048
4b	1.512	3.019	0.381
3c	0.077	0.154	0.620
4c	0.077	0.154	2.464
3d	0.067	0.539	2.147
4d	4.295	4.295	2.147
3e	2.323	2.323	2.323
4e	/	/	/
3f	0.034	0.034	1.106
4f	2.208	2.208	1.106
3g	/	/	/
4g	/	/	/
3h	/	/	/
4h	/	/	/
3i	/	/	/
4i	/	/	/
Nystatin	2.700	1.350	1.350

Figure captions

Figure 1. Cyclic voltammogram of compounds **3d** and **3h**

Figure 2. The impact of quinone derivatives on superoxide anion production**.** Mean DHE fluorescence detected in NCI-H460, NCI-H460/R and HaCaT cells treated with 5 μM of CDDP and 25 μM of **1**, **2**, **3d**, **4d**, **3h**, **4h**, **3f**, **4f 3i** and **4i** evaluated by flow-cytometry (**A**). Statistically significant difference compared to corresponding untreated control: $p < 0.001$ (***). Flow-cytometric profile of cells untreated and treated with 25 μM of **3d** and **3f** (B)

Figure 3. Selective loss of mitochondrial transmembrane potential ($\Delta\Psi$m) in cancer cells. Mitochondria of NCI-H460, NCI-H460/R and HaCaT cells untreated and treated with 5 μM CDDP and 25 μM TBQ (**1**) and its derivatives **3d** and **3f** were labeled with JC-1 for 15 min at 37 °C for flow-cytometric analysis (**A**) and live imaging on a fluorescent microscope (**B**). Scale bar = 50 μm. Statistically significant difference compared to corresponding untreated control: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)
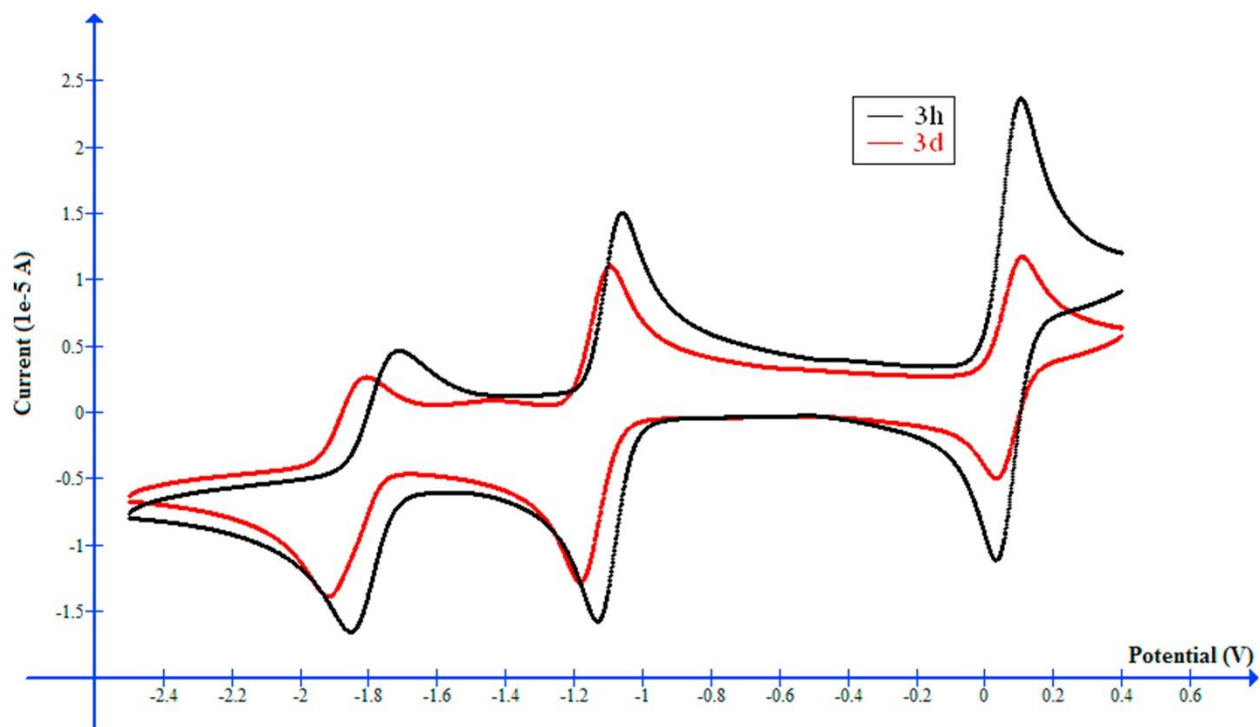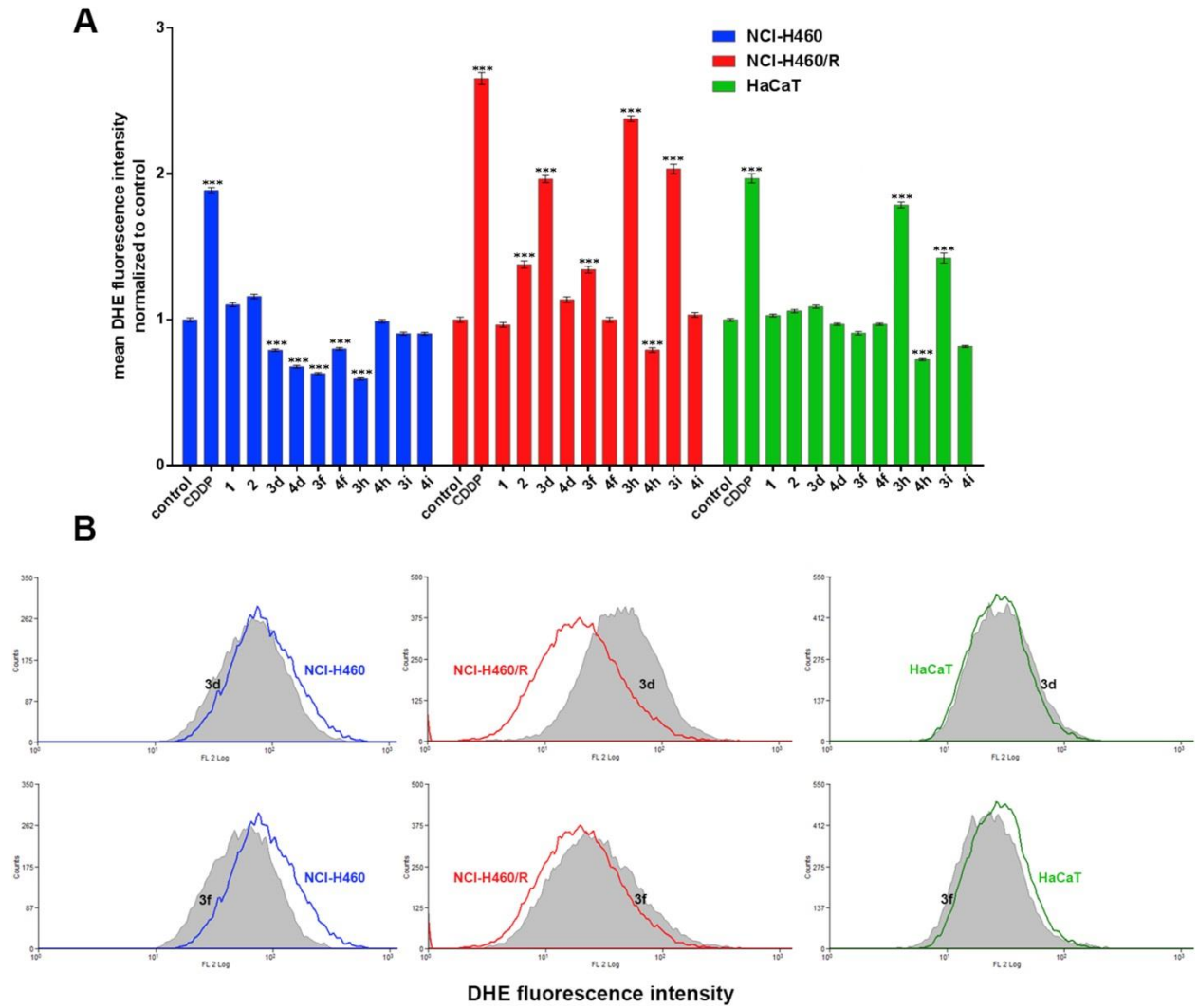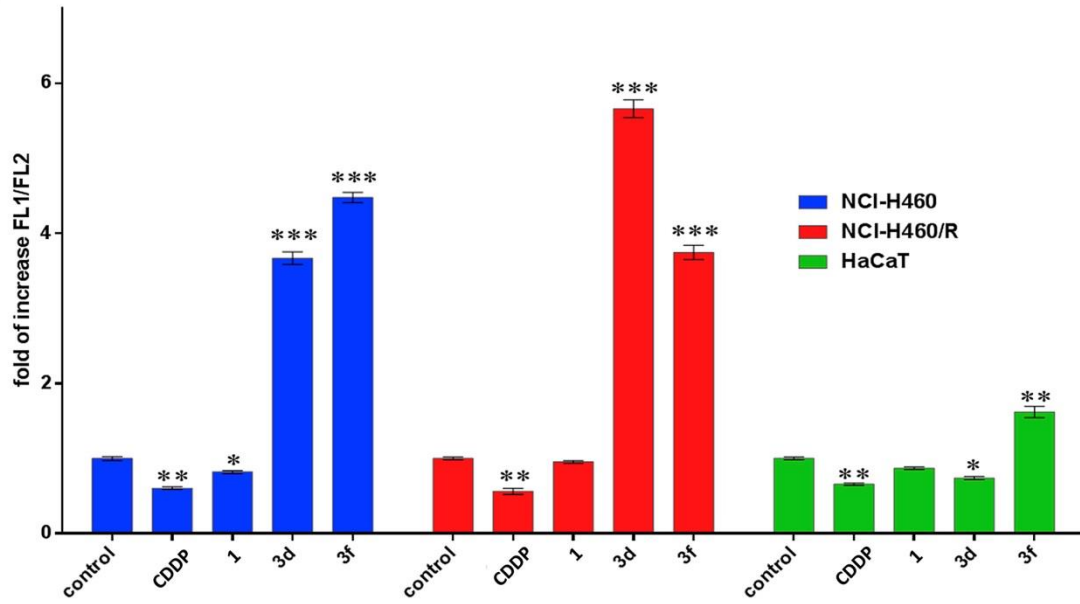
Figure 1.

Figure 2.

DHE fluorescence intensity

Figure 3.