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Research Paper

Synthesis and Biological Evaluation of Asymmetrical dicoumarol Derivatives as Effective Anticancer agent

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Abstract
*A range of Asymmetrical dicoumarol was synthesized by reactingan appropriate 4-hydroxycoumarin (**1a-c**)with different aromatic alcohol in the presence of iridium metal catalyst under microwave conditions. The reaction which is generally known as 'Borrowing Hydrogen Methodology' gave products which were obtained in moderate to good yields (50-70%:(**2a-h**). The enzyme assay result revealed that these compounds (**2a-h**)were effective as potential NQO1 inhibitors. For drugs however, to gain access to their site of action, it must cross one or more barriers especially the plasma and the intracellular membranes of the cell. In view of this, these potent NQO1 inhibitors were re-modified as prodrugs (**4a-h**) by insertion of a cytotoxic agent(**3a**) derived from natural product (-)-quinic acid.The 'MTT assay'wascarried outusing A549 cancer cell line which has a high level of NQO1 activity.The result revealed that they were effective asanticancer agents.*

Keywords: *Synthesis, asymmetrical dicoumarol, enzyme assay, MTT assay, cytostatic activity, NQO1,*

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I. Introduction

Cancer is a disease of the cells in the body with many possible causes such as genetic or epigenetic factors and exposure to different types of chemicals. Most cancers occur when certain vital genes, which control how cells divide and proliferate, are damaged. For example: DNA mutation(Benstein, B. E., et al. 2000), oncogenes (Oppermann, H., et al. 1979), tumor suppressor protein (p53) (Takaoka, A., et al., 2003), reactive oxygen species (ROS) (Finkel, T., 2011), etc. Reactive oxygen species can be mutagenic, as well as causing damage to the cell membrane and consequently living organisms have a complex system of antioxidants. Antioxidants (Figure 1) are molecules that inhibit the oxidation of other molecules such as glutathione(**1**), vitamin C (**2**), vitamin E (**3**) and oxidoreductase enzymes such as the NQO1.

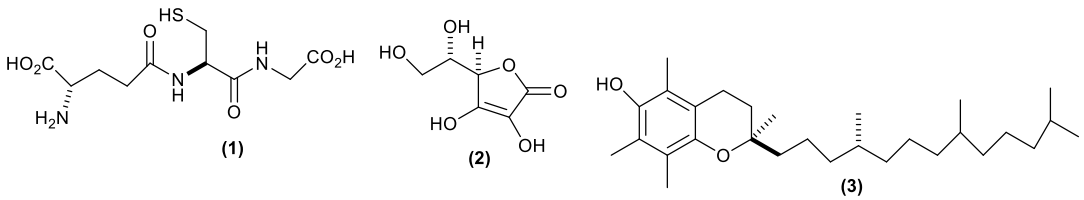
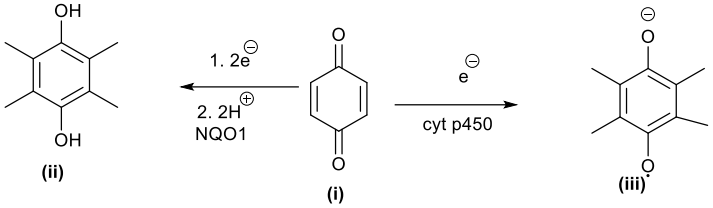


Figure 1: Structures of antioxidant: glutathione (**1**); vitamin C(**2**); vitamin E (**3**)

The antioxidants terminate chain reactions by removing the free radical intermediates, resulting in themselves being oxidized. Thus, insufficient levels of antioxidants, or inhibition of their activity, can cause accumulation of free radical intermediates leading to oxidative stress, which can result in DNA damage or cell apoptosis.

NQO1 is a detoxification enzyme, which catalyses an obligate 2-electron reduction of quinones(i)to stable hydroquinones(ii) using NADH or NAD(P)H at equal efficacy as electron donor (Sanchez-Cruz, P. and Alegria, A. E., 2009), by-passing toxic semi-quinone intermediate(iii) which can be formed by one electron

reduction by cytochrome p450 reductase (Vermeulen, N. P. E. and Boca Raton, F. L., 1996) as depicted in Scheme 1.



Scheme 1: One electron reduction of quinones(i)by cytochrome p^{450} reductase enzyme results in the formation of semi-quinone radical intermediate(iii), while two electron reduction forms hydroquinone (ii).

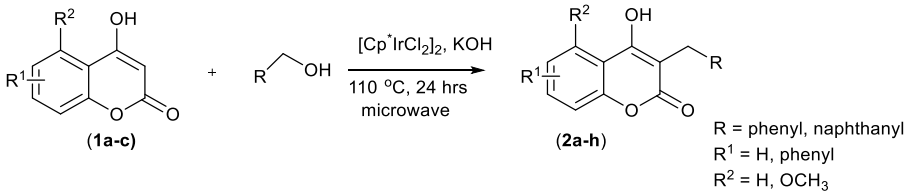
Recent research studies on NQO1 thus, revealed that the enzyme plays an antioxidant role through the reduction of natural quinones which helps in the protection against oxidative damage (Siegel, D., et al. 2004). A number of reports confirm that NQO1 plays additional roles apart from metabolic functions, for example, NQO1 controls the stability of p^{53} by inhibiting its degradation (Beyer, R. E., et al. 1996).The over expression of NQO1 will consequently enhance p^{53} activity, whereas the NQO1 lockdown will reduce the level of p^{53}. Inhibition of NQO1 activities therefore, will have some consequences as there will be a resulting over accumulation of reactive oxygen species resulting in oxidative stress and cell apoptosis. But the inhibition will be effective, however, in terms of *anti*cancer therapy if there is up-regulation of NQO1 activities in tumor tissues compared with the corresponding normal tissues as depicted in Table 1. Findings such as these therefore, have led to the chemotherapeutic targeting of NQO1 in some cancer treatments. The aim of this research work therefore, is to find drugs that cannot only inhibit the activities of NQO1 but also will kill the cancer cells.

Tissue	Normal	Tumor
Breast	50 ± 11	165 ± 43
Colon	17 ± 4	66 ± 13
Liver	17 ± 4	64 ± 32
Lung	10 ± 2	150 ± 45

Table 1: Level of NQO1 activity in normal and tumor tissues. The values represent the activity inhibited by 1 µM dicoumarol with dichloroindophenol (DCPIP) as the substrate. Units are mmol/min/mg protein (Schlager, J. J. and Powis, G., 1990).

II. Result and Discussion

The metal-catalyzed auto-transfer hydrogen, otherwise known as 'borrowing hydrogen methodology' is an effective tool for the functional group interconversion. This method has, to a significant extent, replaced the traditional coupling and reductive aminations of C-C and C-N bonds respectively (Fristrup, P., et al. 2012). Thus, the indirect functionalization of alcohols using catalytic amounts of metal complex such as $[^{Cp^*}IrCl_2]_2$ and base, which produces only water as a by-product, is eco-friendly compared to standard C-C and C-N forming reactions.In using borrowing hydrogen methodology, a range of asymmetrical dicoumarol (**2a-h**) was synthesized as shown in Scheme 2.



Scheme 2: General procedure for the synthesis of asymmetrical dicoumarol (**2a-h**) derived from 4-hydroxycoumarin and its derivatives (**1a-c**) using iridium metal complex.

Following the synthesis, enzyme assays were carried out in the absence of bovine serum albumin (BSA) to determine their potency as NQO1 inhibitors. An ideal enzyme inhibitor, in the context of a potential therapeutic agent, would have high specificity and potency but would also have few side effects and toxicity. Thus, the results revealed that some of the compounds(**2b, 2c, 2e, 2f, 2g and 2h**)were effective as NQO1 inhibitors as depicted in Figure 2.

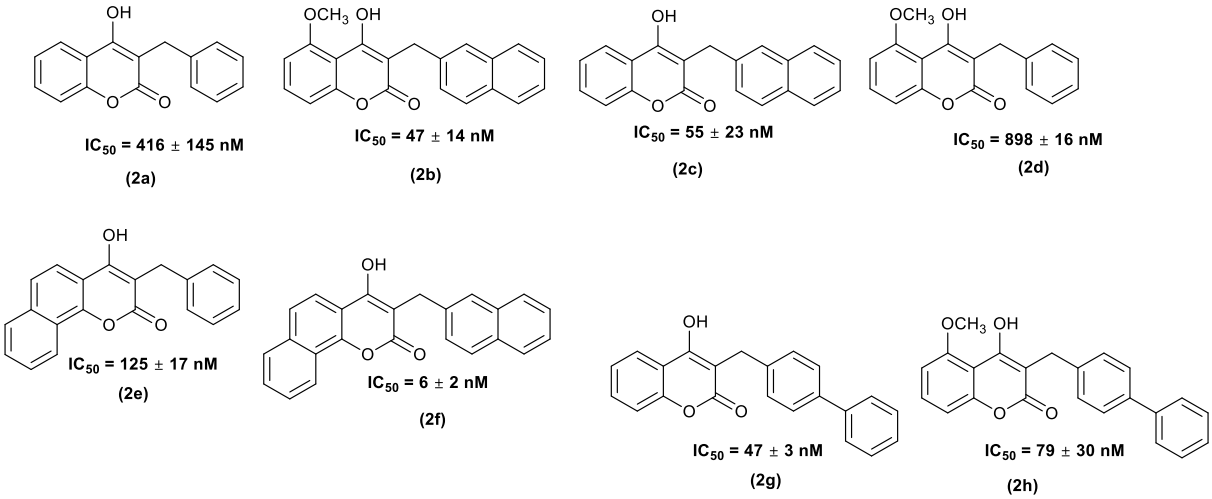


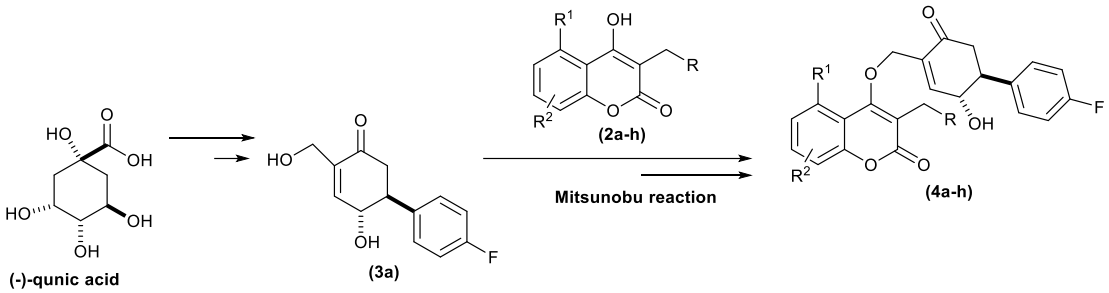
Figure 2: IC_{50} values of the synthesized asymmetrical dicoumarol (**2a-h**).

The compounds bearing a substituted naphthyl rings displayed higher inhibitory activity than those with a substituted phenyl rings such as (**2a** and **2d**).These differences could be attributed to the fact that naphthyl rings undergoing hydrophobic interactions with the NQO1 enzyme. Thephenyl ring is less hydrophobic than the naphthyl ringand thus less effective as NQO1 inhibitors. To make compounds (**2a and 2d**) more hydrophobic, compounds (**2b, 2c, 2e, 2f, 2g** and **2h**) were synthesized and assayed. Interestingly, the IC_{50}value of (**2d**) improve from 898 ± 16 nM to 47 ± 14 nM, hence confirming the importance of hydrophobic interactions of the inhibitors at the NQO1 active site.Introduction of phenyl group (**2g** and **2h**)also, gave a lower IC_{50} values which again suggested that the NQO1 active site binds successfully to hydrophobic compounds.

On the other hand, there may be the possibility of drug barriers to the target site with these inhibitors, therefore these inhibitors(**2a-h**) were re-modified as prodrugs(**4a-h**) in order to overcome some of these barriers such as chemical instability and poor penetration which may lead to low toxicity to cancer cells. Since the πGSTP1-1 enzyme, which uses GSH as a cofactor, is over expressed in most solidtumors, coupling of these inhibitors with an *anti*-cancer agent (**3a**)*via* the use of Mitsunobu reaction could produce compounds which will serve as prodrugs.These prodrugs(**4a-h**) could be activated by GSH in the presence of GST which would result in the release of the inhibitors (**2a-h**)of NQO1 as well as the cytotoxic agent(**3a**), thus leading to cell apoptosis. This reaction therefore, resulted in the synthesis of several novel prodrugs (**4a-h**) as shown in Scheme 3, which displayed good potency towards A549 cancer cell line.

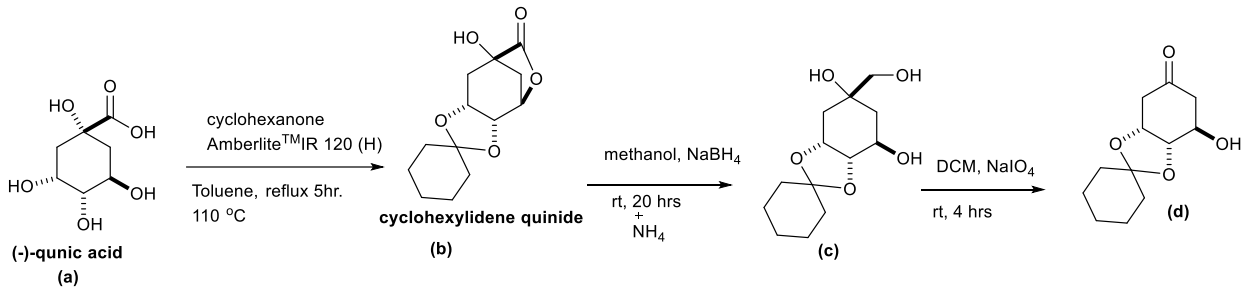
A non-small cell adenocarcinoma cell line A549 was chosen for this research as a result of the following:

- It has up-regulation of NQO1 activity (Huang, X., et al. 2012);
- It has elevated levels of glutathione and gluthatione transferase (GST/GSH).



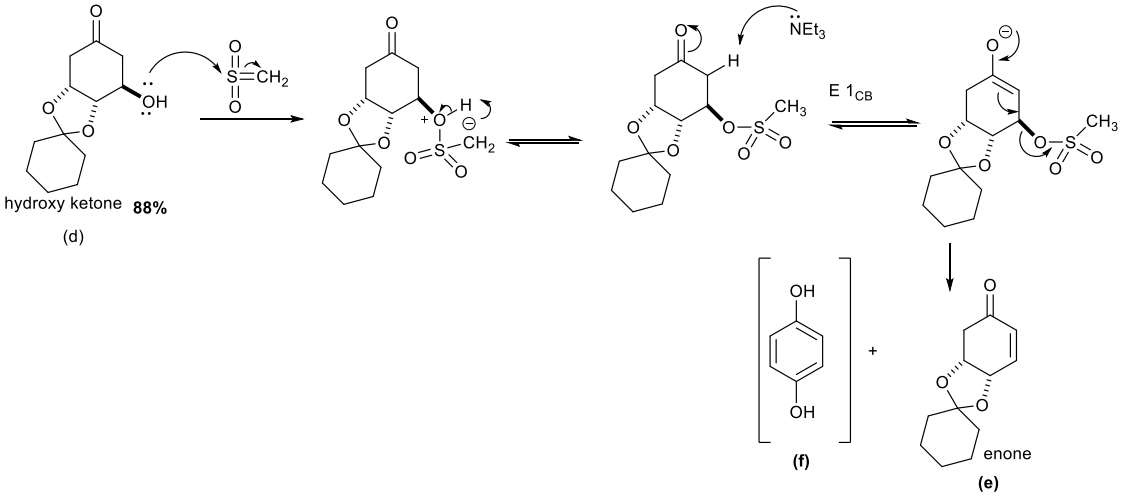
Scheme 3: Synthesis of *anti*-cancer agent (**3a**) and their corresponding Prodrugs (**4a-h**).

The cis-3,4-diol group of (-)-quinic acid (**a**)was selectively protected with cyclohexanone in an acid catalyst (Amberlite™ IR 120 (H) resin) as shown in Scheme 4.



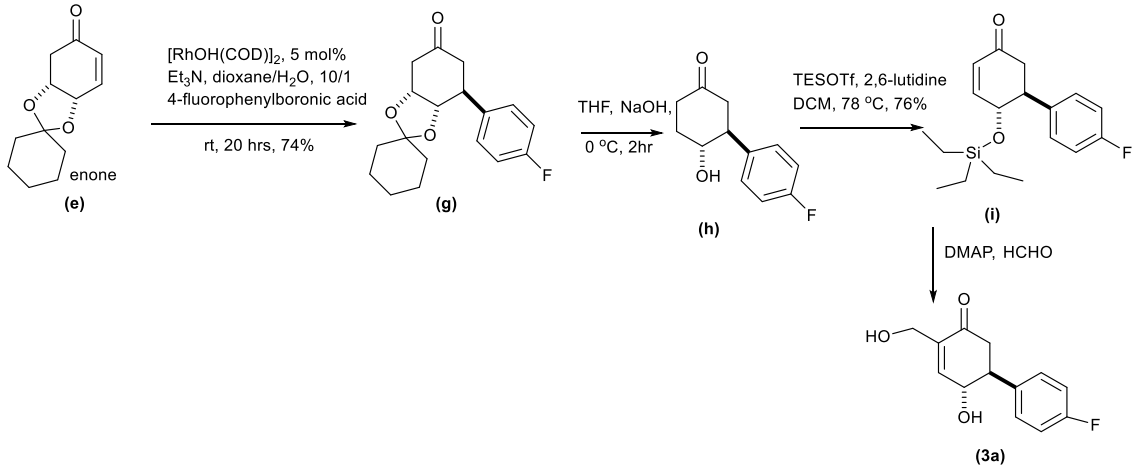
Scheme 4: Synthesis of cyclohexylidene quinide (**b**) using (-)-quinic acid (**a**) and conversion to hydroxyketone (**4d**).

The removal of water using a Dean and Strak trap assisted in driving the reaction to completion by application of Le Chatelier's principle. Reductive ring opening was also achieved using $NaBH_4$ in methanol solvent. The reduction was successful due to the presence of the electron withdrawing hydroxyl group adjacent to the carbonyl which increases its electrophilic nature and reactivity towards nucleophilic attack by the hydride ion to give vicinal diol moiety (**c**). The oxidative cleavage of the vicinal diol moiety (**c**)was accomplished using free-flowing powder form of silica gel-supported sodium periodate ($NaIO_4$) to give hydroxyketone (**d**).
The elimination of hydroxyl group involves its conversion to a better leaving group by reacting with methanesulfonyl chloride in the presence of triethylamine to give enone (**e**)and hydroquinone (**f**)as a by-product, Scheme 5.

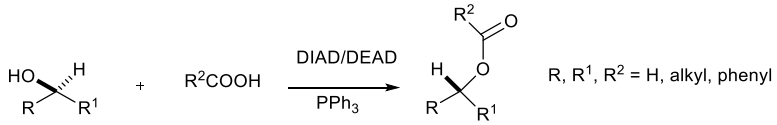


Scheme 5: Mechanism of synthesis of enone (**e**) and hydroquinone (**f**).

The reaction was then followed by 1,4-conjugate addition using organoboronic acid and rhodium complex in the presence of triethylamine (ET_3N) to give conjugate adduct (**g**) as depicted in Scheme 6.

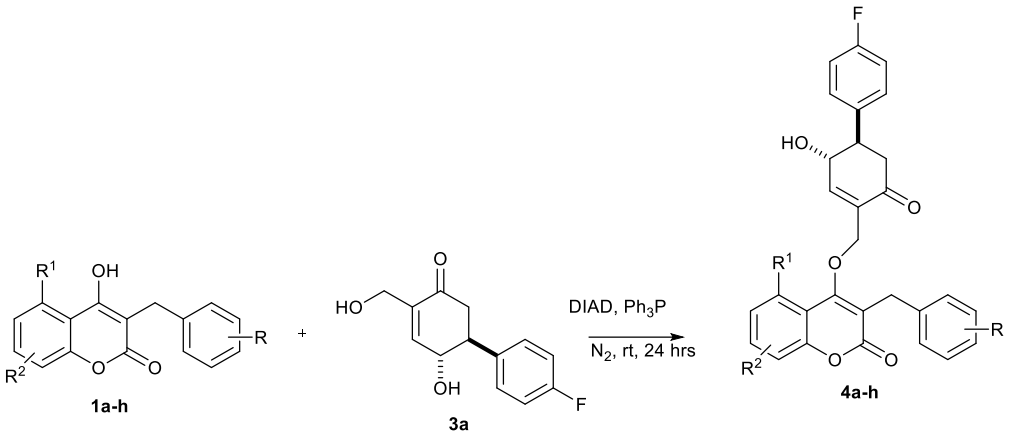


The utilization of Mitunobu reaction (Mitsunobu, O. Y., and Bull, M., 1967), helps in the conversion of primary and secondary alcohols to esters, ethers, thioethers and other compounds as shown in Scheme 7.



Scheme 7: General reaction scheme for the Mitsunobu reaction

For this reaction to occur therefore, one of the reacting components (nucleophile) must be adequately acidic in order to facilitate protonation of the DIAD/DEAD, thus preventing side reactions from occurring.
Using this method, a range of novel cytotoxic compounds (**4a-h**)thus were synthesized by the application of Morita-Baylis-Hilman reactions using DMAP and formaldehyde *via* a cross aldol reaction as demonstrated in Scheme 8.



Scheme 8: Synthesis of novel prodrugs(**4a-h**).

The targeted compounds and their cytotoxic values are thus summarized in Figure 3 below:

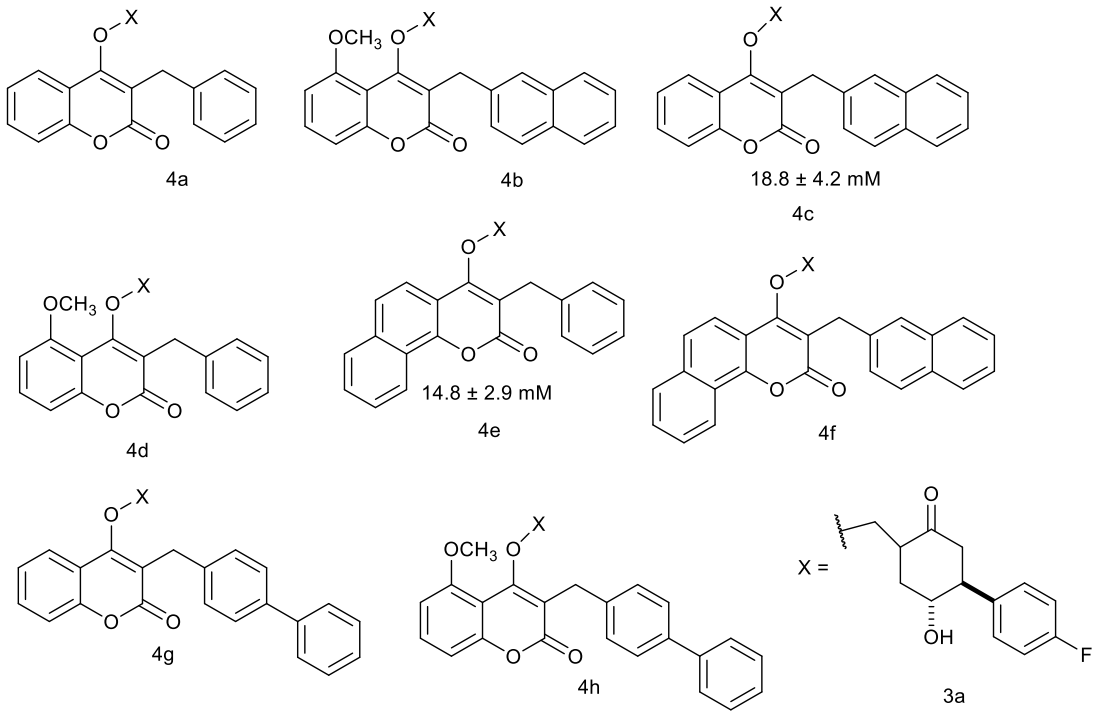


Figure 3: Structures of potential *anti*-cancer prodrugs (**4a-h**).

- Evaluation of MTT assay

MTT cell viability assay represents the concentration at which half of the cells seeded remain viable at the end of the analysis. MTT viability assay was therefore carried out on selected compounds (**4c** and **4e**) and the compounds displayed toxicity towards the A549 cancer line.

III. Conclusion

In the search for more potent inhibitors of NQO1, a range of novel potent inhibitors were synthesized using borrowing hydrogen methodology. Compounds containing naphthyl rings have proved to be more potent than those containing phenyl rings. Also, since the use of cytotoxic drugs is an unavoidable therapeutic method for the treatment of cancer, MTT assay was conducted on some of these inhibitors. Among the inhibitors assayed, compounds (**4c** and **4e**) are remarkable as they are not only effective as NQO1 inhibitors, but also displayed toxicity towards A549 cancer cell line.

Acknowledgement

I will forever remain grateful to TETfund Nigeria for sponsoring the research abroad. My appreciation also goes to the staff of Chemistry department, University of Manchester, England, for providing all the sophisticated equipments needed for this research.

Experimental

All the reagents used were obtained from commercial sources (Sigma-Aldrich Co., Alfa Aesar and Fisher Scientic). Melting point was measured using a Sanyo Gallenkamp MPD. 350 variable heater instrument and are uncorrected. IR spectra were recorded in solid state using a Bruker Alpha PFT-IR instrument. ^{13}C and ^{1}H NMR were recorded using Bruker Avance 400 spectrometers. Chemical shifts are given in ppm to the nearest 0.01 ppm and referenced to the solvent residual peak. Proton assignments were assisted by DEPT. HCOSY and ^{1}HMQC.

Synthesis of compound (**4b**)

Compound (**4b**) was isolated as a white solid (16.8 mg, 67%), R_f0.3: Mp 88-90 ^{o}C; $[\alpha]_D^{30}$ -25.5 (c 0.47 in CH_2Cl_2; V_{max}/cm^{-1} (film), 3435, 2931, 1675, 1600; δ_H (400 MHz; $CDCl_3$) 2.06 (1H), 2.50 (1H), 2.62 (1H), 3.13 (1H), 3.91 (3H), 4.12 (2H), 4.54 (1H), 4.59 (H), 4.67 (1H), 6.78 (1H), 7.01 (1H), 7.07 (2H), 7.16 (2H), 7.39-7.43 (2H), 7.45-7.49 (2H), 7.72 (1H), 7.74-7.79 (3H); δ_C (100 MHz; $CDCl_3$) 30.4, 43.2, 49.8, 56.4, 69.7, 71.9, 106.5, 107.4, 110.0, 115.9, 116.1, 117.1, 125.4, 126.0, 126.7, 127.3, 127.6, 128.0, 129.1, 129.2, 131.9, 132.2, 133.5, 134.5, 135.0, 136.9, 137.0, 148.8, 154.7, 156.1, 160.9, 163.4, 163.7, 196.2; δ_F (376 MHz; $CDCl_3$) -114.2; m/z (+ES) 551.4 ([M +H]$^+$, 100%); (Found 573.1689; $C_{34}H_{27}O_6NaF$ ([M+Na]$^+$, requires 573.1698).

Synthesis of compound (**4c**).

Compound (**4c**) was isolated as a white foam (23 mg, 70%), R_f0.3: Mp 128-130 ^{o}C; $[\alpha]_D^{30}$ -25.5 (c 0.47 in CH_2Cl_2; V_{max}/cm^{-1} (film), 3433, 1682 1605; δ_H (400 MHz; $CDCl_3$) 2.55 (1H), 2.66 (1H), 3.15 (1H), 3.71 (1H), 4.52 (1H), 4.66 (1H), 4.75 (1H), 7.05-7.10 (3H), 7.16-7.20 (2H), 7.33 (1H), 7.39 (1H), 7.42-7.47 (3H), 7.48 (1H), 7.56 (1H), 7.73 (1H), 7.76-7.80 (4H); δ_C (100 MHz; $CDCl_3$) 30.8, 43.1, 70.2, 71.8, 117.0, 124.3, 125.5, 126.1, 126.7, 127.1, 127.6, 127.7, 128.2, 129.1, 129.2, 131.8, 132.2, 133.5, 134.0, 136.5, 150.0152.9, 160.2, 163.1, 196.0; δ_F 248.6; m/z (+ES) 521.3 (M +H]$^+$, 100%; (Found 521.1759; $C_{33}H_{26}O_5F$ (M +H]$^+$), requires 521.1753).

Synthesis of compound (**4e**)

Compound (**4e**) was isolated as a white solid (16.4 mg, 57%), R_f 0.4. $[\alpha]_D^{29}$ -26.4 (c 0.48, in CH_2Cl_2; V_{max}/cm^{-1} (film), 3432, 1676, 1604; δ_H (400 MHz; $CDCl_3$) 2.76 (2H), 3.29 (1H), 4.06 (2H), 4.79 (1H), 7.08 (1H), 7.10-7.13 (1H), 7.19-7.22 (2H), 7.29-7.32 (4H), 7.38 (2H), 7.63-7.68 (2H), 7.74 (2H), 7.87-7.91 (1H), 8.5-8.58 (1H); δ_C (100 MHz; $CDCl_3$) 30.6, 43.3, 49.8, 70.3, 72.0, 116.2, 116.3, 116.8, 119.0, 122.5, 123.1, 123.4, 124.3, 124.3, 125.8, 126.5, 127.2, 127.8, 128.6, 128.7, 129.3, 134.2, 134.8, 139.1, 149.9, 152.3, 161.1, 196.0, 247.7; δ_F (MHz; $CDCl_3$) -113.9; m/z (+ES) 521.18 ([M +H]$^+$, 100%; (Found 521.1771; $C_{30}H_{27}O_6F$ (M +H]$^+$), requires 521.1776).

Synthesis of (**4f**)

Compound (**4f**) was isolated as an off white solid (16.8 mg, 67%), R_f 0.3: $[\alpha]_D^{30}$ -30.2 (c 0.45, in CH_2Cl_2; V_{max}/cm^{-1} (film), 3416, 1677, 1603; δ_H (400 MHz; $CDCl_3$) 2.55 (1H), 2.66 (1H), 3.16 (1H), 4.23 (2H), 4.53 (1H), 4.72 (1H), 4.81 (1H), 7.00 (1H), 7.05-7.09 (2H), 7.14-7.19 (2H), 7.40-7.46 (2H), 7.49 (1H), 7.65-7.69 (2H), 7.73-7.80 (6H), 7.88-7.92 (1H), 8.54-857 (1H); δ_C (100 MHz; $CDCl_3$) 30.8, 43.1, 49.6, 70.3, 71.8, 116.0, 116.2, 118.9, 122.4, 123.1, 124.6, 125.5, 126.1, 126.6, 127.1, 127.4, 127.6, 127.7, 127.8, 128.3, 128.9, 129.2, 132.3, 133.6, 134.0, 134.9, 136.5, 150.3, 196.3; δ_F (376 MHz; $CDCl_3$) -114.0; m/z (+ES) 593.3 ([M + Na]$^+$, 100%).

Synthesis of (4g)

Compound (**4g**) was isolated as a white foam (23 mg, 71%), R_f 0.2. $[\alpha]_D^{29}$ -26.4 (c 0.48, in CH_2Cl_2; V_{max}/cm^{-1} (film), 3401, 2961, 1678, 1607; δ_H (400 MHz; $CDCl_3$) 2.05 (1H), 2.72 (2H), 3.27 (1H), 4.05 (2H), 4.68 (1H), 4.71 (1H), 4.80 (1H), 7.08 (2H), 7.19 (1H), 7.24-7.28 (2H), 7.34-7.43 (5H), 7.51-7.57 (5H), 7.79 (1H); δ_C (100 MHz; $CDCl_3$) 30.2, 43.2, 49.7, 60.8, 70.3, 116.1, 116.3, 116.9, 117.1, 117.2, 123.4, 124.5, 127.0, 127.2,

127.3, 128.8, 128.9, 129.0129.2, 129.3, 131.9, 134.1, 134.7, 137.9, 139.4, 140.8, 150.3, 152.8, 161.3, 163.2, 164.1, 196.3; δ_F (MHz; $CDCl_3$) -114.0; m/z (+ES) 548.6 ($[M+2H]^+$, 100%; (Found 569.1740; $C_{35}H_{27}O_5NaF$ ($[M+Na]^+$), requires 569.1725).

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