IOSR Journal of Applied Chemistry (IOSR-JAC)
e-ISSN: 2278-5736.Volume 15, Issue 7 Ser. I (July 2022), PP 53-59
www.iosrjournals.org

Reduction of Unsymmetrical Dicoumarol Using Hanzsch's Ester and Their Measurment as Nqo1 Inhibitors

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Abstract
*Syntheses of unsymmetrical dicoumarol were carried out by reacting one equivalent of 4-hydroxycoumarin (**5a-c**) and one equivalent of an appropriate aromatic anhydride in ethanol solvent at a temperature of 80 ^{o}C for 45 minutes. The products (**1a-f**) were isolated as yellow compounds with poor yields (28 - 30%) due to competitive dimer formation (**1g**). Their after, six equivalents of Hanzsch's ester (**6a**) were added to a solution of the unsymmetrical dicoumarol (**1a-f**) in methanol solvent. In order to achieve complete conversion, the reaction were left to stir overnight and this gave compound (**2a- f**) in a poor to moderate yields (20- 74%).The identities of the compounds were confirmed using NMR, IR and mass spectrometry. Compounds (**2a- f**) were assayed in the absence of BSA and their IC_{50} values show moderate inhibitory potency towards NQO1 enzyme.*
Key words: *synthesis, unsymmetrical dicoumarol, Hanzsch's ester, enzyme assay, NQO1.*

Date of Submission: 28-06-2022 Date of Acceptance: 09-07-2022

I. Introduction

NADH:oxidoreductase qunone 1 (NQO1) is a ubiquitous flavoprotein, containing flavin adenine dinucleotide (FAD). NQO1 performs its function in a catalytic manner and can therefore be called flavoenzyme. The enzymatic activity of NQO1 was initially reported by Ernester and Navazio who discovered that it catalyzed the reduction of 2,6-dichlorophenolindophenol in the rat liver cytosol (Ernester, L. and Navazio, F., 1958). NQO1 was originally called DT-diaphorase (Ernester, L., Estabrook, R. W., et al. 1987) to express that the enzyme uses NADH or NAD(P)H as a source of reducing equivalents (Begleiter, A. and Fourie, J., 2004) and it is currently designated as NAD(P)H:oxidoreductase quinine 1, and in humans, it is encoded as NQO1 or QR1.

NQO1 is mostly found in the cytosol of human cells (Edlund, C., et al., 1982), although little fractions can be found in the mitochondrin, ribosomes and the Golgi apparatus (Ernester, L., 1998). NQO1 has attracted considerable awareness because of its ability not only to detoxify a variety of natural and unnatural compounds but also to activate certain *anti*-tumor agents, such as mitomycin C (MMC) (**3a**) (Ross, D., 1997), Qinolinquinone (**3b**) (Moore, H. W. and Czerniak, R., 1981), CB 1954 (**3c**) (Ross, D., et al. 2000), aziridinylbenzoquinone (**3d**), (Iyanagi, T. and Yamazaki, I., 1970), etc (Figure 1). NQO1 enzyme also protect healthy cells from oxidative stress (Kameyama, et al., 2017) and electrophilic attack (Li, ., et al., 2015 which could lead to genetic instability and cell apoptosis.

These findings therefore, attracted significant interest among many researchers to study the enzyme and its analogues, and their role in protection against free radical formation and cancer development. It is also predicted that NQO1 may possibly protect cancer cells by removing free radicals and thus making cancer cells more resistant to *anti*-cancer drugs (Zeekpudsa, 2014).

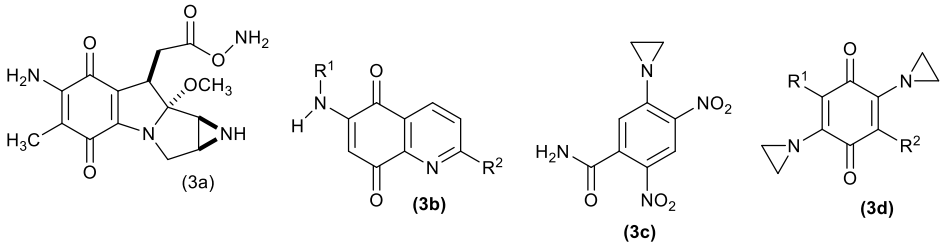


Figure 1: Structures of compounds considered as NQO1 directed *anti*-tumor agents.

The most potent inhibitor of NQO1 is dicoumarol (**4a**) (IC_{50} = 2.6 nM) (Ernester, 1967). Dicoumarol suffers a major predicament of poor selectivity to cancer cells and off target effect as it binds to other proteins in circulating blood such as serum albumin. As a result, an analogue of dicoumarol (**4b, 4c**) with high selectivity for cancer cells and without problematic off target effect in contrast to dicoumarol (**4a**) have been reported (Nolan, et al. 2009) (Figure 2). Due to up regulation of NQO1 in most solid tumors, it therefore believed that targeting this enzyme could help in the treatment of cancer. There is also the need for finding novel effective inhibitors of NQO1 without problematic off-target effects like symmetrical dicoumarol (4**a**).

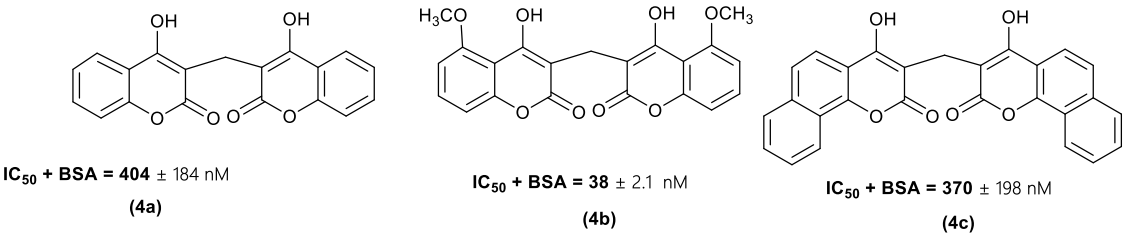
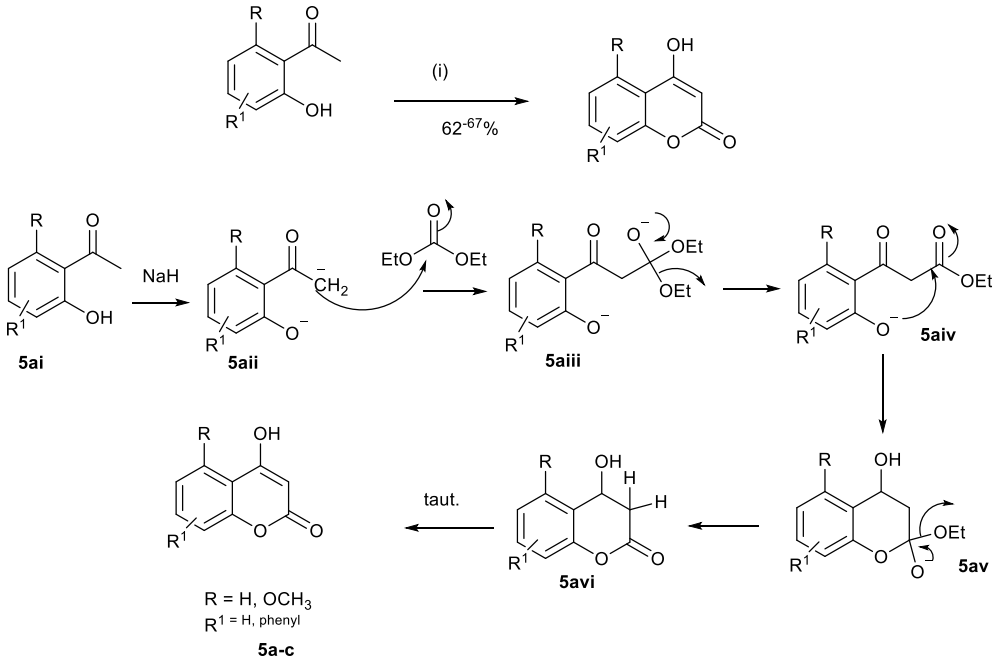


Figure 2: IC_{50} values of symmetrical dicoumarol (**4a**) and its derivatives (**4b** and **4c**).

II. Synthesis

2.1 Synthesis of 4-hydroxycoumarin derivatives

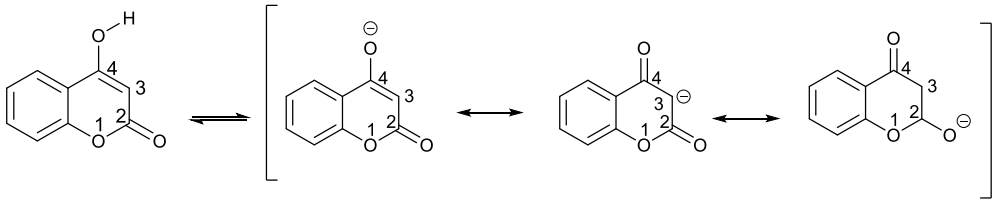
The 4-hydroxycoumarin derivatives (**5a-c**) which served as precursors for the synthesis of unsymmetrical compounds were synthesized by reacting appropriate acetophenone (**5ai**) with diethyl carbonate in the presence of NaH. The identities of all the cyclised products were confirmed by ^{1}H NMR which is in line with the data reported by Carbery et al., (1997). The presence of vinylogous carboxylic acid makes these compounds strong acids (Obi, J.C and Ezenwa, E. T., 2018). In this reaction sequence, the hydride ion from NaH abstracts the α-hydrogen of the acetophenone (**5ai**) to give the enolate anion (**5aii**). The second step involves the nucleophilic attack on the electrophilic carbon of the carbonyl of diethyl carbonate to form an intermediate β-keto ester (**5aiii**). This intermediate then undergoes cyclisation by addition and elimination of ethoxide to give compound (**5aiv**) which undergoes rapid keto-enol tautomerisation to give the targeted compounds (**5a-c**). The structural identities of the compounds were confirmed by ^{1}HNMR spectroscopy which revealed the presence of only one product as shown in Scheme 1.



Scheme 1: Reaction mechanism of base mediated cyclisation reaction to give 4-hydroxycoumarin and its derivatives.

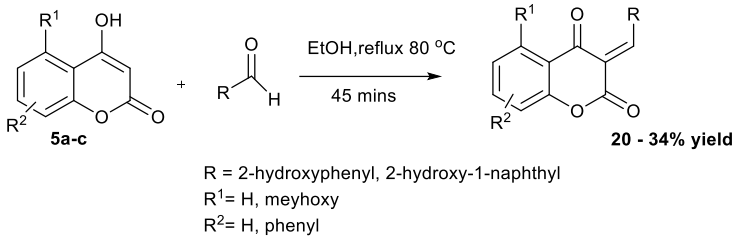
2.2 Synthesis of unsymmetrical dicoumarol.

The carbon 3 position of 4-hydroxycoumarin and its derivatives (**5a-c**) is reactive as a nucleophile because it is the central carbon atom of an enolic 1,3-dicarbonyl compound. It is this feature that facilitates the synthesis of the unsymmetrical dicoumarol as illustrated by their resonance structures in Scheme 2.



Scheme 2: Resonance structures of ionized 4-hydroxycoumarin.

The synthesis of unsymmetrical dicoumarol (**1a-f**) were carried out by reacting one equivalent of an appropriate 4-hydroxycoumarin (5**a-c**) and one equivalent of aromatic aldehyde using microwave conditions as depicted in scheme 3.



Scheme 3: General method for the preparation of unsymmetrical dicoumarol (**1a-f**).

Generally, the poor yields were obtained due to competitive dimer formation (**1g**) Figure 3.

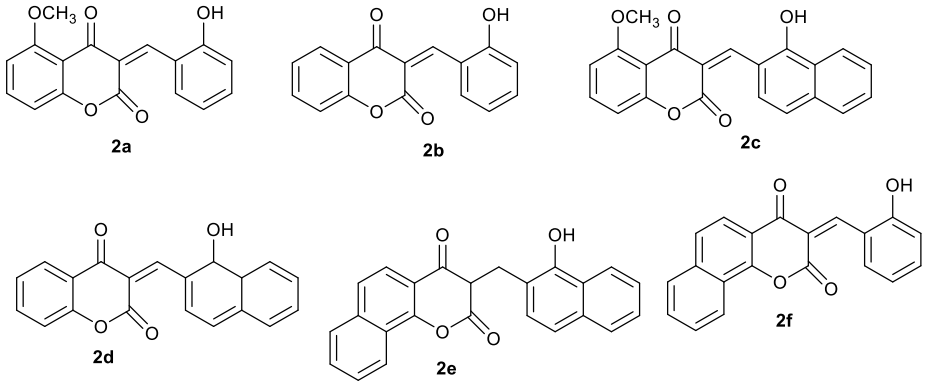
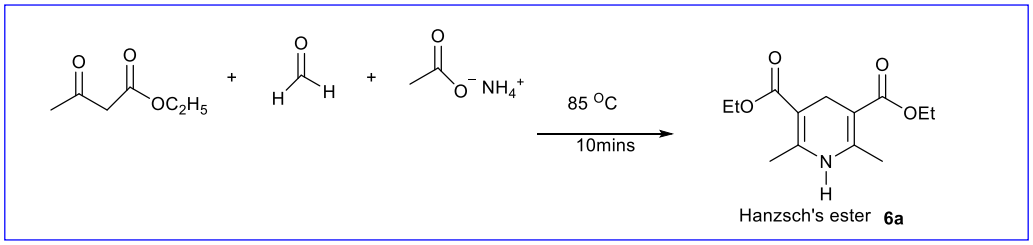


Figure 3: Structures of Unsymmetrical dicoumarol (**1a-f**).

2.3 Synthesis of Hanzsch's Ester

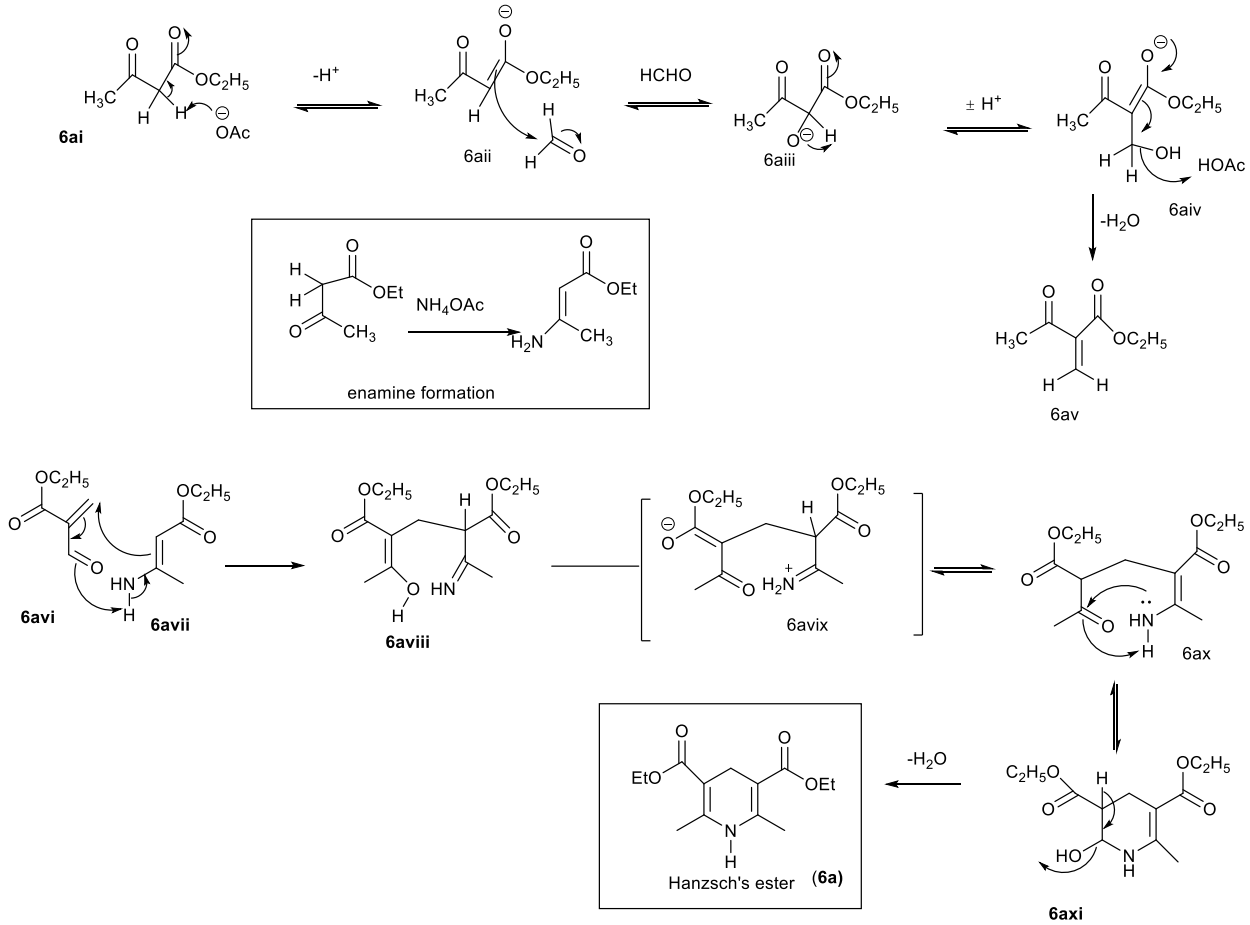
Hanzsch's ester (**6a**), which have been ultilized for efficient transfer hydrogenation of C=C, C=N and C=O bonds (Zhao, G.L. and Cordova, A., 2006), (Ouellet, S. G., Tuttle, J.B and MacMillan D.W.C. 2005) was first discovered in 1882 by Arthur Hanzsch (Lavilla, R., 2002). The compound was synthesized by the reaction of ethyl acetoacetate (**6ai**), formaldehyde (**6aii**) and ammonium acetate (**6aiii**) in a solvent-free single step reaction at 85 °C for 10 minutes as depicted in Scheme 4.



In this sequence, the most acidic proton (α-proton) of the ethyl acetoacetate (**6ai**) is abstracted by acetate to form the intermediate enolate anion (**6aii**). The enolate anion attacks the electrophilic carbonyl carbon of formaldehyde by an aldol condensation reaction to form an intermediate (**6aiii**). This intermediate

undergoes proton transfer to form (**6aiv**) which then subsequently undergoes elimination of water to give compound (**6av**).

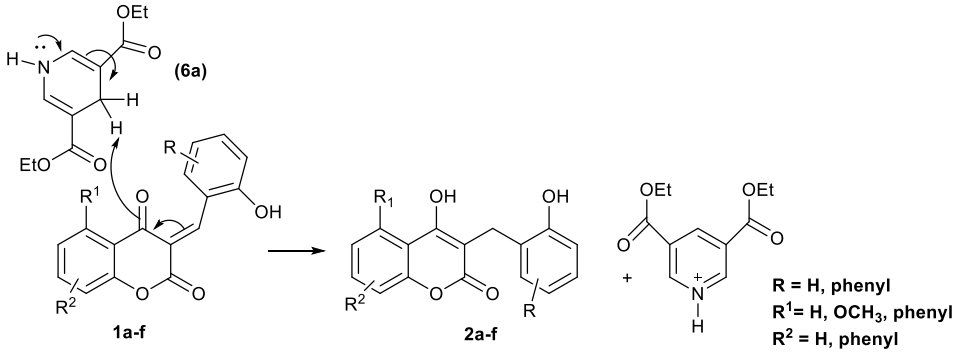
In the second step, the second molecule of ethyl acetoacetate undergoes enamine formation to give ethyl (*E*)-3-aminobut-2-enoate (**6avi**) which then reacts with (**6avi**) *via* Michael addition to enone to give (**6aviii**). Compound (**6aviii**) undergoes multiple proton transfer to give an intermediate (**6avix**) followed by internal cyclisation and elimination of water to give the final product, Hanzsch's ester (**6a**).



Scheme 4: Mechanism of Hanzsch's ester synthesis (**6a)**.

2.3 **Reduction of unsymmetrical dicoumarol (1a-f) using Hanzsch's ester (6a).**

NQO1 enzyme detoxifies its substrates through hydrogen transfer using NADH/NADPH as electron donor. Similarly, a biometric approach involving the use of Hanzsch's ester (**6a**), an analogue of NADH was utilized for the reduction of the unsymmetrical dicoumarol (**1a-f**) to give compound (**2a-f**). The successful reduction of compound (**1a-f**) with Hanzsch's ester (**6a**) was an indication that compound (**2a-f**) as illustrated in Scheme 5 may also likely undergo reduction by NQO1.



Scheme 5: Mmechanism of reduction of compound (**1a-f**) using Hanzsch's ester (**6a**) to give the targeted compound (**2a-f**).

III. Enzyme assay

According to Federal Drug Agency (FDA), the half maximal inhibitory concentration (IC_{50}) value represents the concentration of drug that is required for 50% inhibition in *vitro* (Bergmeyer, H. U., 1974). It is used to determine the effectiveness of a drug in inhibiting biological activity such as that of NQO1. The IC_{50} values were therefore measured using nonlinear curve fitting as implemented in the program Excel (GraphPad Prism 5). Each measurement was made in triplicate and the experiments were repeated three times. The concentration-response plots obtained displayed a sigmoid response curve which demonstrated moderate to good inhibitory potency for the NQO1 inhibitors. Thus, low IC_{50} values indicate that the compounds have good inhibitory potency while high values indicated poor inhibition. The IC_{50} values of the targeted compounds (**2a-f**) are summarized in Figure 4:

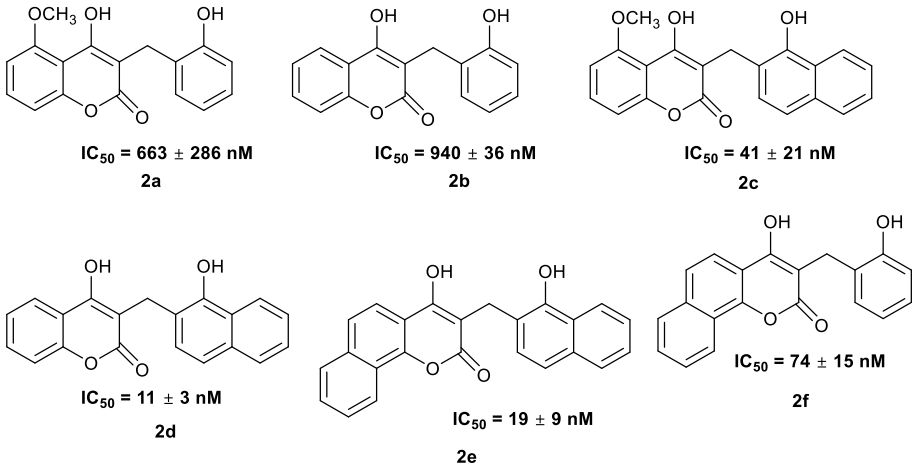


Figure 4: IC_{50} values of compounds (**2a-f**).

The IC_{50} value of compounds bearing a substituted naphthyl ring displayed higher inhibitory potency than those with a substituted phenyl ring. This could be as a result of naphthyl ring undergoing hydrophobic interactions with the NQO1 enzyme. The phenyl ring is less hydrophobic than the naphthyl ring and this may be the reason that the analogues are less effective as NQO1 inhibitors.

Therefore, to make compound (**2b)** more hydrophobic, compounds (**2e** and **2f**) were synthesized. Interestingly, the IC_{50} values improve from 940± 36 nM to 74 ± 15 nM hence confirming the importance of hydrophobic interactions of inhibitors at the NQO1active site.

IV. Experimental Part

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.

a. General method (B) for the synthesis of compounds (2**a-f**)

A solution of Hanzsch's ester (6 equivalents) was stirred in THF at room temperature under nitrogen gas for 10 minutes. A solution of an appropriate unsymmetrical dicoumarol (**1a-f**) (1 equivalent) in THF (5 mL) was added dropwise at 0 ^{o}C and the reaction mixture was then allowed to stir for 24 hours at room temperature. The reaction was quenched by pouring into cold HCl (0.1 M: 10 mL) and the reaction mixture was concentrated in *vacuo* to give dark brown oil. Ethyl acetate (20 mL) was added and the resulting suspension filtered. The residue was washed with ethyl acetate (3x10 mL) dried over MgSO4 and concentrated in *vacuo* to give a crude product. This crude product was further purified by flash silica chromatography (petroleum: ethyl acetate).

Synthesis of 4-hydroxy-3-(2-hydroxyl)-5-methoxy-2H-chromen-2-one (**2a**). Using method (B) gave the title compound as a white solid (20 mg, 50%): Mp 206-208 ^{o}C; V_{max}/cm^{-1} 3286, 1660, 1635; δ_H (400 MHz; $CDCl_3$) 3.79 (2H, s), 4.03 (3H, s), 6.73-6.77, m), 6.87 (1H, dd), 6.96 (1H, d), 7.03-7.07 (1H, m), 7.34-7.40 (2H, M), 8.52 (1H, s), 9.95 (1H, s); δ_C (100 MHz; $CDCl_3$) 24.2, 57.2, 104.8, 105.1, 106.0, 111.2, 117.6, 120.1, 125.6, 128.2, 131.5, 132.2, 153.1, 155.2, 155.8, 162.5, 166.4; *m/z* (+ES) 297.1 ($[M+H]^-$, 100%); Found 299.0905; ($C_{17}H_{15}O_5(M+H]^+$), requires 299.0919).

Synthesis of 4-hydroxy-3-(2-hydroxybenzyl)-2H-chromen-2-one (**2b**). Using (B) gave the title compound (**2a**) as a white solid, 70 mg, 18%). Mp 236-238 ^oC; V_{max}/cm^{-1} 2940, 1653, 1600; δ_H (400 MHz; DMSO-d_6) 3.78 (2H,s), 6.66 (1H, td), 6.8 (1H, dd), 6.83-6.85 (1H, m), 7.00 (1H,td), 7.35-7.39 (2H, m), 7.62 (1H, td), 7.94, (1H, dd); δ_C (100 MHz; DMSO-d_6) 23.8, 102.8, 114.7, 116.1, 116.4, 118.9, 123.3, 123.8, 125.4, 126.8, 128.0, 131.7, 152.0, 154.9, 161.1, 162.9; *m/z* (+ES) 269.1 ([M+H]$^+$, 100%); Found 291.0628; ($C_{16}H_{12}O_4Na]^+$), requires 291.0633.

Synthesis of 4-hydroxy-3-((2-hydroxynaphthalen-1-yl)methyl-5-methoxy-2H-chromen-2-one (**2c**). Using method (B) gave the title compound (**2c**) as a white solid (10 mg, 32%): Mp 218-220 ^oC; V_{max}/cm^{-1} 3233, 1660, 1635; δ_H (400 MHz; $CDCl_3$) 4.13 (3H, s), 4.21 (2H, s), 6.81 (1H, d), 7.01 (1H, d), 7.31-7.35 (2H, m), 7.43 (1H, t), 7.50-7.54 (1H, m), 7.67 (1H, d), 7.74 (1H, d), 8.51 (1H, d), 9.53 (1H, s)10.37 (1H, s); δ_C (100 MHz; $CDCl_3$) 19.0, 56.2, 103.4, 104.0, 104.9, 110.2, 116.9, 119.8, 121.7, 125.0, 127.4, 127.7, 128.5, 131.2, 132.7, 151.9, 152.9, 154.7, 161.2, 165.6; *m/z* (+ES) 371.3 ([M+Na]$^+$, 100%), 349.3 ([M+H]$^+$, 63%. Found 291.0628; ($C_{16}H_{12}O_4Na]^+$), requires 291.0633.

Synthesis of 4-hydroxy-3-((2-hydroxynaphthalen-1-yl)methyl-2H-chromen-2-one (**2d**). using method (B), gave title compound as a white solid (70 mg, 18%). Mp 236-238 ^oC; V_{max}/cm^{-1} 2940, 1653, 1600; δ_H (400 MHz; DMSO-d_6) 3.78 (2H, s), 6.66 (1H, td), 6.80 (1H, dd), 6.83-6.85 (1H, m), 7.00 (1H, td), 7.35-7.39 (2H, m), 7.62 (1H, td), 7.94 (1H, dd); δ_C (100 MHz; $CDCl_3$) 23.8, 102.8, 116.1, 116.4, 118.9, 123.3, 123.8, 125.4, 126.8, 128.0, 131.7, 152.0, 154.9, 161.1, 162.9; *m/z* (+ES) 269.1 ([M+H]$^+$, 100%); Found 291.0628; ($C_{16}H_{12}O_4$ Na([Na]$^+$), requires 291.0633.

Synthesis of 4-hydroxy-3((2-hydroxynaphthalen-1-yl)methyl)-2H-benzo[h]chromen-2-one, (**2e**). Using method (B) gave the title compound (2e) as a white solid (80 mg, 53%): Mp 284-286 ^oC; V_{max}/cm^{-1} 2999, 1657, 1596; δ_H (400 MHz; DMSO-d_6) 4.30 (2H, s), 7.24 (1H, d), 7.29 (1H, ddd), 7.47 (1H, ddd), 7.66-7.7); δ_C (100 MHz; DMSO-d_6) 20.2, 102.2, 103.6, 118.2, 119.7, 122.0, 122.5, 123.3, 124.1, 124.4, 126.8, 127.8, 128.5, 128.7, 129.0, 129.2, 133.9, 134.8, 149.2, 151.8, 163.5; ***m/z*** (+ES) 341.1 ([M+Na]$^+$, 100%); Found 369.1120; ($C_{24}H_{17}O_4$ ([M+H]$^+$), requires 369.11073).

Synthesis of 4-hydroxy-3-(2-hydroxybenzyl)-2H-benzo[h]chromen-2-one (**2f**). Using method B, gave the title compound (**2f**) as a brown crystal (90 mg, 74%): Mp 266-268 ^oC; V_{max}/cm^{-1} 3269, 15911526; δ_H (400 MHz; DMSO-d_6) 3.61 (2H, s), 6.58-6.61 (2H, m), 6.92 (1H, td), 7.24 (1H, dd), 7); δ_C (100 MHz; DMSO-d_6) 26.2, 98.8, 114.2, 116.5, 117.1, 117.9, 122.0, 126.1, 126.5, 127.0, 127.8, 129.9, 130.7, 133.9, 136.1, 156.6, 164.2; ***m/z*** (+ES) 341.1 ([M+Na]$^+$, 100%); Found 341.0793; ($C_{20}H_{14}O_4$ Na ([M+H]$^+$), requires 341.0790).

V. Conclusion

Due to up regulation of NQO1 in most solid tumors, it therefore believed that targeting this enzyme could help in the treatment of cancer. There is also the need for finding novel effective inhibitors of NQO1 without problematic off-target effects like symmetrical dicoumarol.

Acknowledgement

My special thanks goes to 'TETfund' Nigeria for funding this research work. I am deeply grateful to all the staff of Chemistry department, University of Manchester, England, for their support and also providing all the necessary materials and equipment for this research work.

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Juliana Chineze Obi. "Reduction of Unsymmetrical Dicoumarol Using Hanzsch's Ester and Their Measurment as Nqo1 Inhibitors." *IOSR Journal of Applied Chemistry (IOSR-JAC)*, 15(07), (2022): pp 53-59.