Synthesis of Unsymmetrical Dicoumarol and Its Measurement as Inhibitor of NQO1

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Abstract. Unsymmetrical analogues of dicoumraol (**1a-f**) were synthesized using a simple two-steps protocol. The 'half-way stage' analogues of dicoumarol (**4a-f**) were synthesized and later reduced using $NaBH_4$/$LiBH_4$ to give the unsymmetrical analogues of dicoumarol (**1a-f**). The structural identities of all the synthesized compounds were confirmed via melting point, mass spectrometry, IR and ^1H NMR spectroscopy. Enzyme assay was carried out in order to determine their inhibitory potency towards NAD(P)H:oxidoreductase quinone 1 (NQO1) enzyme. Remarkably, all the compounds containing 2-hydroxyl-1-naphthyl ring (**1a-c**) with (IC_{50} value ranges from 19 – 41 nM) are more effective as NQO1 inhibitors.

Key words: synthesis, unsymmetrical dicoumarol, inhibitors of NQO1, enzyme assay.

Introduction

NQO1 is a detoxifying flavoprotein, meaning it contains flavin adenine dinucleotide (FAD). Studies have revealed that about 80% of it is mainly found in cytosol of human cells (Edlund et al., 1982: 861-865), while the remaining fractions can be found in mitochondria, ribosomes and golgi apparatus (Ernester, 1998: 149-158). NQO1 activities were first reported in 1958 by Ernester and Navazio in rat liver cytosol and was originally called DT-diaphoorase (Ernester et al., 1962: 171-188; Ernester et al., 1987: 1-207) but currently the enzyme is designated as NAD(P)H:oxidoreductase quinine 1 and in human it is encoded as NQO1 or QR1.

NQO1 as a flavoenzyme that catalyses an obligate 2-electron reduction of quinone (**2a)** to stable hydroquinone (**2c**) by-passing toxic semi-quinone radical (**2b**) formed by cytochrome p450 using NADH or NAD(P)H at equal efficacy as electron donor (Sanchez-Cruz and Alegria, 2009: 818-823). The X-ray structures of NQO1, containing either the electron donor (NADH) or the substrate (quinone) bound to the active site verify that reaction occurs *via* a 'ping pong' mechanism (Deller et al., 2008: 141-160). That is, the reduced NAD(P)H or NADH binds and reduces the FAD cofactor and the oxidized NAD^+ or $NADP^+$ is released prior to the binding of the substrate (quinone). Evidence of this mechanism of action of NQO1 has been obtained from electron spin resonance experiments which did not detect the presence of semi-quinone radicals during the metabolism of benzoquinone (**2d**) or naphthoquinone (**2e**) substrates (Segura-Aguilar and Lind, 1989: 309-324) (Fig. 1).

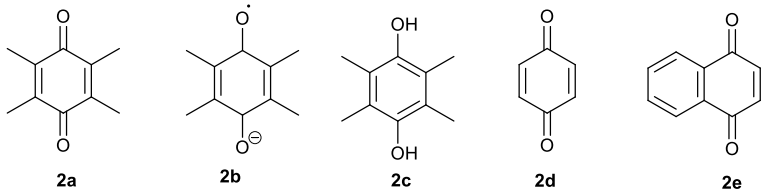

Fig. 1. Structures of NQO1 co-substrates

Recent research work on NQO1 revealed that the enzyme also plays an antioxidant role through reduction of natural quinones which helps in the protection against oxidative damage (Landi et al., 1997: 329-335; Siegel et al., 2004: 1238-1247). In view of this, it is

assumed that NQO1 may protect cancer cells by removing free radicals and hence making cancer cells more resistant to anticancer drugs (Zeekpudsa, 2014: 11-23). In addition, NQO1 enzyme has been linked with many types of human cancers, signifying that the enzyme plays vital roles in tumor occurrences and progression (Li et al., 2015: 207-215). Thus NQO1 is a potential target in order to enhance the susceptibility of tumor cells to chemotherapeutic drugs.

In 1967 Ernester reported that the most potent inhibitor of NQO1 is Dicoumarol (**3a**) (IC_{50} = 2.6 nM) (Ernester, 1967: 309). Dicoumarol, although suffers a major problem of poor selectivity to cancer cells and off target effect as it binds to other proteins in circulating blood such as serum albumin. Recently, an analogue of dicoumarol (**3b**) with high selectivity for cancer cells and without problematic off target effect in contrast to dicoumarol have been reported (Nolan et al., 2009: 7144-7145) in Fig. 2.

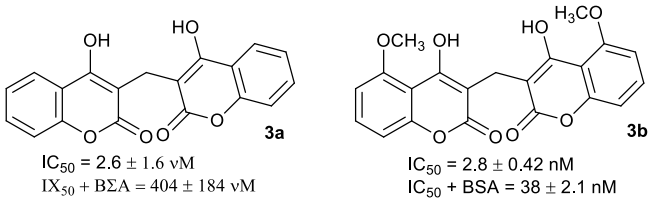

Fig. 2. Potent inhibitors of NQO1

The active site of NQO1 is hydrophobic and elastic, hence enabling compounds of different molecular sizes to bind. Chemists discovered this advantage to produce different inhibitors of the enzyme comparable to dicoumarol itself.

Synthesis

In our previous research, 'half-way stage' analogues of dicoumarol (**4a-f**) were synthesized by reacting 4-hydroxycoumarin and its derivatives (1 eq) with the appropriate aldehyde (1 eq) in ethanol solvent using microwave irradiation (Obi and Ezenwa, 2018: 1-7) as illustrated in Fig. 3.

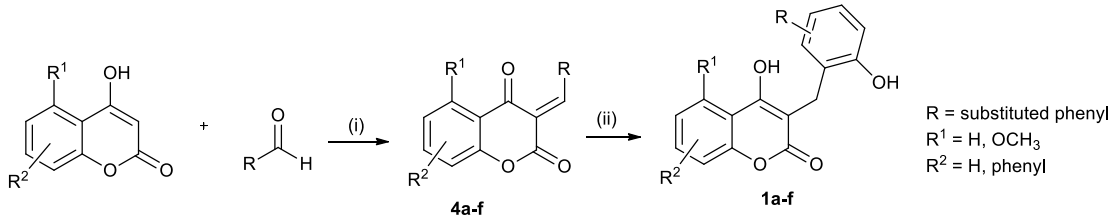

Fig. 3. General procedure for the synthesis of 'half-way stage' analogues (4a-f) of dicoumarol

Note: reaction conditions are as follows: (i) Ethanol, 80 ºC, 30 mins; (ii) NaBH4, methanol, reflux 80 oC, 24 hrs

Generally, 'half-way stage' analogues of dicoumarol (**4a-f**) Fig. 4, were obtained in poor yields (20-26%) which was believed to be due to competitive dimer formation (**4g**). A plausible mechanism for this conversion is depicted in Scheme in Fig. 5.

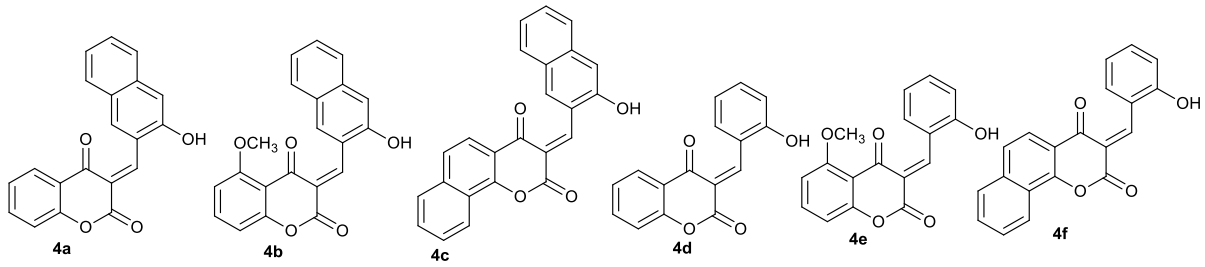

Fig. 4. Structures of 'half-way stage' analogues of dicoumarol (**1a-f**)

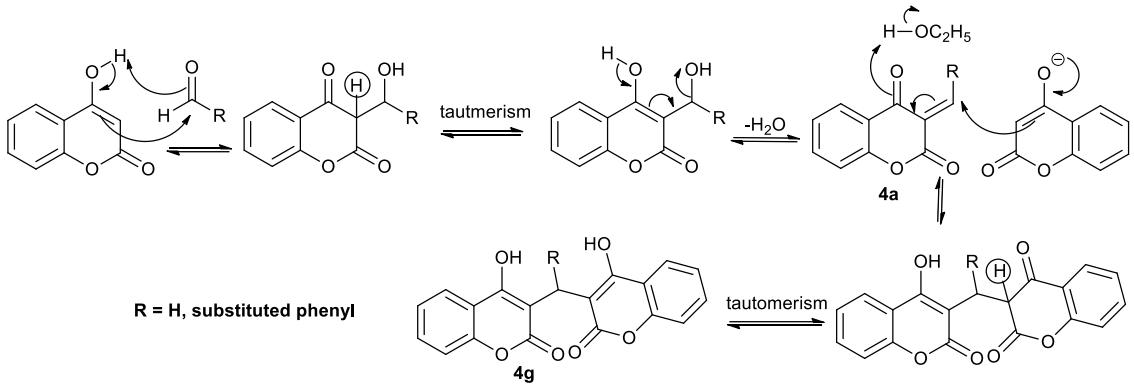

Fig. 5. General mechanism for condensation and dimerization reaction

In the second step of the reaction, sodium borohydride ($NaBH_4$) or $LirBH_4$ reagents were used for the reduction of the 'half-way stage' analogues of dicoumarol (**4a-f**). BH_4^- reagents were preferred due to the high reactivity of $LiAlH_4$ which reacts violently with protic solvent such as ethanol, methanol and water. Although, esters can be reduced with $NaBH_4$ this occurs at a much lower rate due to their natural electrophilicity. Six equivalents of sodium borohydride were added to a solution of the 'half-way stage' analogues of dicoumarol (**4a-f**) in methanol as solvent. In order to achieve complete conversion, the reactions were left to stir overnight to give compounds in moderate yields (40-55%). A plausible mechanism for the reaction is illustrated in Fig. 6.

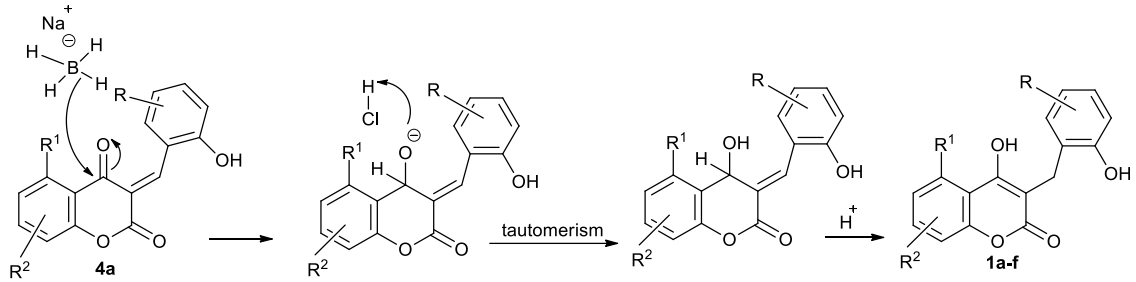

Fig. 6. Mechanism of reduction of 'half-way stage' analogues of dicoumarol (4a-f) with $NaBH_4$ or $LiBH_4$ to give unsymmetrical analogues of dicoumarol (1a-f)

Enzyme assay

An IC_{50} value, in terms of an enzyme assay, represents the concentration of a drug that is required for 50% inhibition in *vitro*. The unsymmetrical analogues (**1a-f**) were assayed and the result revealed a significant difference in the IC_{50} values, Fig. 7. The unsymmetrical analogues of dicoumarol bearing 2-hydroxyl naphthyl ring (**1a-c**) exhibited higher inhibitory potency compared to 2-hydroxyl phenyl ring (**1d-f**). The phenyl ring is less hydrophobic than the naphthyl ring, and the NQO1 active is hydrophobic in nature, therefore, the naphthyl ring could be undergoing hydrophobic interactions with the

enzyme. This is a proof that NQO1 active site binds more successfully to hydrophobic compounds.

Serial dilution of the stock solutions of the synthetic unsymmetrical analogues of dicoumarol (**1a-f**) (10 mM concentration) were prepared in six cuvettes using DMSO to give concentration ranging from 0.1 µM – 1000 µM. NQO1 enzyme was diluted in 50 mM phosphate buffer to give an enzyme activity within the 0.085 – 0.14 nM range. The IC50 values were calculated using nonlinear fitting as implemented in the Excel (Graph Pad Prism 5). Each measurement was made in triplicate and the experiments were repeated three times.

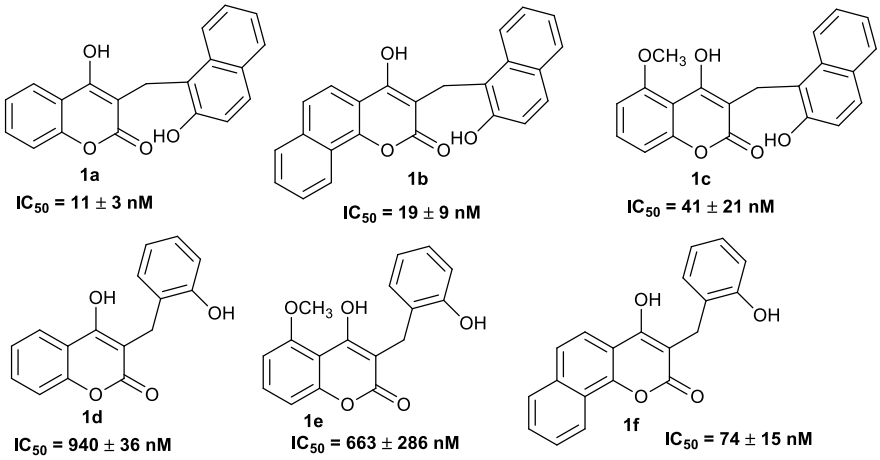

Fig. 7. IC_{50} values of unsymmetrical analogues of dicoumarol (1a-f)

Results

A Biotage Initiator TM microwave reactor (maximum power output of 300 W; operating frequency (2450 MHz) was used. Melting point was measured using a Sanyo Gallenkamp MPD 350 variable heater instrument and are uncorrected. IR spectra were recorded in the solid state using a Bruker Alpha P FT-IR instrument. ^{1}H NMR spectra 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. The abbreviations used are s-singlest, d-doublet, t-triplet, dd-doublet of doublets, td-triplet of doublets, m-multiplet.

General method for the synthesis of 'half-way stage' analogues of dicoumarol (4a-f)

Method A

The appropriate 4-hydroxycoumarin and its derivatives (1 equivalent) was reacted with the appropriate aromatic aldehyde (1 equivalent). Ethanol was added to give a solution of 0.5 M concentration with respect to coumarin. The reaction mixture was subjected to microwave irradiation at 80 ^{o}C for 30 mins. The resulting mixture was allowed to cool and the precipitate formed collected by filtration, washed with methanol and dried (Obi and Ezenwa, 2018: 1-7).

General method for the synthesis of unsymmetrical analogues of dicoumarol (1a-f)

Method B

A solution of $LiBH_4$ (2 eq) or $NaBH_4$ (6 eq) was stirred in THF or methanol respectively at room temperature under nitrogen gas for 10 minutes. A solution of an appropriate 'half-way stage' analogues of dicoumarol **(4a-f)** (1 eq) in THF or methanol (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 onto a cold HCl (0.1M: 10 mL) and the resulting 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), dried over $MgSO_4$ and concentrated in *vacuo* to give a crude product. This crude material was further purified by flash silica column chromatography eluting with petroleum: ethyl acetate.

Synthesis of 4-hydroxy-3-((2-hydroxynaphthalen-1-yl)methyl)-2H-chromen-2-one (1a)

Using method B: Reaction of compound (**4a**) (350 mg, 1.11 mmol) and sodium borohydride (251.4, 6.65 mmol) in methanol (5 mL) gave the title compound (**1a**) as a white solid (150 mg, 43%):Mp 250-252 ^oC; v_{max}/cm^{-1} 2980 (br, w, OH), 1650 (s, C=O), 1600 (s, C=C); δ_H (400 MHz; DMSO-d_6) 4.23 (2H, s), 7.20 (1H, d), 7.26-7.35 (3H, m), 7.43 (1H, ddd), 7.57 (1H, ddd), 7.70 (1H, d), 7.77 (1H, dd), 7.87 (1H, dd), 8.35 (1H, d); *m/z* (-ES) 317.2 ([M-H]$^-$, 100%); (+ES) (Found 319.0970; $C_{20}H_{15}O_4$ ([M+H]$^+$), requires 319.0975).

Synthesis of 4-hydroxy-3-((2-hydroxynaphthalen-1-yl)methyl-5-methoxy-2H-chromen-2-one (1b)

Using method B: Reaction of sodium borohydride (230 mg, 6.07 mmol) and compound (**4b**) (350 mg, 1.01 mmol) in methanol (12 mL) gave the title compound (**1b**) as white solid (140 mg, 40%). Mp 218-220 ^oC; v_{max}/cm^{-1} 3233 (br, w, OH), 1660 (s, C=O), 1635 (s, C=C); 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); *m/z* (+ES) 371.3 ([M+Na]$^+$, 100%), 349.3 ([M+H]$^+$, 63%).

Synthesis of 4-hydroxy-3((2-hydroxynaphthalen-1-yl)methyl)-2H-benzo[h]chromen-2-one (1c)

Using method B: Reaction of compound (**4c**) (150 mg, 0.41 mmol) and sodium borohydride (93.03 mg, 2.46 mmol) in methanol (12 mL) gave the title compound (**1c**) as a white solid (80 mg, 53%): Mp 282-284 ^oC; v_{max}/cm^{-1} 2999 (br,w, O-H), 1657 (s, C=O), 1596 (s, C=C); δ_H (400 MHz; DMSO-*d*6) 4.30 (2H, s), 7,24 (1H, d), 7.29 (1H, ddd), 7.47 (1H, ddd), 7.66-7.74 (3H, m), 7.77-7.81 (2H, m), 7.89 (1H, d), 7.98-8.02 (1H, m), 8.31-8.35 (1H, m), 8.44 (1H, d); *m/z* (+ES) 369.1 ([M+H]$^+$, 100%); (Found 369.1120; C24H17O4 ([M+H]$^+$), requires 369.1103).

Synthesis of 4-hydroxy-3-(2-hydroxybenzyl)-2H-chromen-2-one (1d)

Using method B: Reaction of $LiBH_4$ (54 mg, 2.48 mmol) and compound (**4d**) (380 mg, 1.43 mmol) in THF (5 mL) gave the title compound (**1d**) as a white solid (70 mg, 18%): Mp 236-238 ^oC; v_{max}/cm^{-1} 2940 (br, w, O-H), 1653 (s, C=O), 1600 (s, C=C); 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); *m/z* (+ES) 269.1 ([M+H]$^+$, 100%); (Found 291.0628; $C_{16}H_{12}O_4Na$ ([M+Na]$^+$), requires 291.0633).

Synthesis of 4-hydroxy-3-(2-hydroxyl)-5-methoxy-2H-chromen-2-one (1e)

Using method B: Reaction of compound (**4e**) (40 mg, 0.14 mmol) and sodium borohydride (10 mg, 0.27 mmol) in methanol (2 mL) gave the title compound (**1e**) as white solid (20 mg, 50%): Mp 206-208 ^oC; v_{max}/cm^{-1} 3286 (w, OH), 1661 (s, C=O), 1635 (s, C=C); δ_H (400 MHz; $CDCl_3$) 3.79 (2H, s), 4.03 (3H, s), 6.73-6.77 (2H, 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); *m/z* (+ES) 297.1 ([M-H]$^-$, 100%); (+ES) (Found 299.0905; $C_{17}H_{15}O_5$ ([M+H]$^+$), requires 299.0919).

Synthesis of 4-hydroxy-3((2-hydroxynaphthalen-1-yl)methyl)-2H-benzo[h]chromen-2-one (1f)

Using method B: Reaction of compound (**4e**) (150 mg, 0.41 mmol) and sodium borohydride (93.03 mg, 2.46 mmol) in methanol (12 mL) gave the title compound (**1f**) as a white solid (80 mg, 53%): Mp 284 ^oC; v_{max}/cm^{-1}2999 (br,w, O-H), 1657 (s, C=O), 1596 (s, C=C); δ_H (400 MHz; DMSO-d_6) 4.30 (2H, s), 7.24 (1H, d), 7.29 (1H, ddd), 7.47 (1H, ddd), 7.66-7.74 (3H, m), 7.77-7.81 (2H, m), 7.89 (1H, d), 7.98-8.02 (1H, m), 8.31-8.35

(1H, m), 8.44 (1H, d); *m/z* (+ES) 369.1 ($[M+H]^+$, 100%); (Found 369.1120; $C_{24}H_{17}O_4$ ($[M+H]^+$), requires 369.1103).

Conclusion

In conclusion, all the synthesized unsymmetrical analogues of dicoumarol (**1a-f**) exhibit moderate to good inhibitory potency towards the NQO1 enzyme with the exception of compounds (**1d and 1e)** as a result of the hydrophobic nature of the enzyme active site.

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