Synthesis Characterization and Biological Activities of 4-Hydroxycoumarin Derivatives

Juliana Chineze Obi[1]
Tagbo E. Ezenwa[1]
Ezigbo Vera[1]

[1]Chukwuemeka Odumegwu Ojokwu University, Uli, Nigeria

Abstract. Three varieties of 4-hydroxycoumarin derivatives (1a-c) were synthesized by reacting an appropriate acetophenone with diethyl carbonate in the presence of sodium hydride, at reflux for 3 hours. The products were obtained in good yields (62-67%). These 4-hydroxycourin derivatives serve as precursors for the synthesis of symmetrical analogues of dicoumarol (4a) which subsequently undergo C-C reductive cleavage using sodium cyanoborohydride ($NaBH_3CN$) to give asymmetrical dicoumarol (2a-e). The enzyme assay investigation revealed that compounds (2a-e) exhibit moderate to good inhibitory potency towards NAD(P)H:oxidoreductase quinine 1 (NQO1) enzyme. The purity and structural identities of all the synthesized compounds were confirmed by melting point, mass spectrometry, IR, ^{13}C and ^{1}H NMR spectroscopy.

Key words: synthesis, 4-hydroxy coumarin derivatives, symmetrical dicoumarol, asymmetrical dicoumarol, enzyme assay.

Introduction

Coumarins and flavonoids belong to a group of compounds known as benzopyrones which consist of a benzene ring fused to a pyrone ring. The benzopyrones are further categorized into *α*-benzopyrones (**3a**) and *γ*-benzopyrones (**3b**). Coumarins substituted in the pyrone ring such as 4-hydroxycoumarin (**3c**), 7-hydroxycoumarin (**3d**) and their derivatives like warfarin (**3e**) belong to the family of *α*-benzopyrones, whereas the flavonoids such as quercetin (**3f**) belong to the *γ*-benzopyrones (Fig. 1).

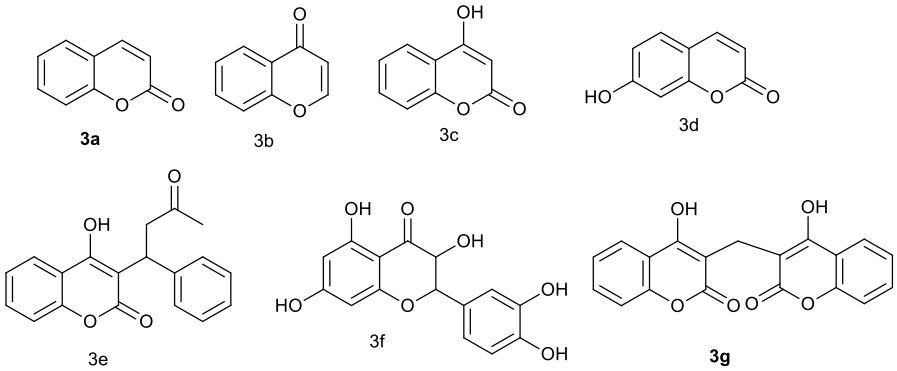

Fig. 1. Structures of derivatives of α-benzopyrones (3a-e), γ-benzopyrone (3f) and dicoumarol (3g)

Coumarins are of great importance to synthetic organic chemists due to their biological properties (Borges et al., 2005: 887), (Musa et al.,2008: 2664) such as anticoagulant (Overmunn et al., 1944: 5-7), anti-oxidant, anti-bacterial, antiviral (Chohan et al., 2006: 741-743), anti-tumor (Abdel Latif et al., 2016: 116-119) and anti-coagulant activities (Manolov et al., 2006: 882-890). They are consequently highly utilized for the synthesis of a variety of therapeutic agents (Kostova, 2005: 29-46), (Jung and Park, 2009: 4790-4803). For example, research carried out by Weber and co-workers revealed that

coumarin and some of its metabolites display anti-tumor properties towards many human cell lines (Jung and Park, 2009: 4790-4803). Similarly, compounds derived from the benzopyrones have proven to be potent inhibitors of the proliferation of several carcinoma cell lines: for example, 4-hydroxycoumarin (**3c**) and 7-hydroxycoumarin (**3d**) inhibited cell growth in a gastric carcinoma cell line (Weber et al., 1998: 193-206). In a related research, Velasco-Velazquez reported the *invitro* effects of 4-hydroxycoumarin in murine melanoma cell lines (B16-F10) and non-malignant fibroblastic cell lines (B82) (Velasco-Velazquz, 2003:179-186). It was observed that 4-hydroxycoumarin disordered the actins cytoskeleton of B16-F10 cells without any significant effect on the fibroblasts. Vitamin K_3, being an NQO1enzyme co-substrate has a structural relationship with coumarin which makes it possible for dicoumarol (**3g**) to bind with NQO1 (IC_{50} = 2.6 nM) (Ernester, 1967: 309-317), (Nolan et al., 2009: 7144).

The previous synthesis of asymmetrical dicoumarol analogues (**3h**) was carried out using commercially available 4-hydroxycoumarin (**3i**) which was condensed in the presence of different substituted aromatic aldehyde under reflux (Fig. 2). These compounds (**3h**) exhibited potent inhibitory activity towards NQO1 enzyme (Obi and Ezenwa, 2018: 1-7). NQO1 is a detoxifying enzyme, which protect cells against toxic metabolites. Most of the current researches on NQO1 suggested that the enzyme plays a key role in cancer chemoprevention (Siegel et al., 2004: 1238), (Li, 1995: 8846). Research have also revealed that there is over expression of NQO1 enzyme in some tumor tissues compared with the corresponding normal tissues such as breast, colon, liver and lung (Schlager and Powis, 1990: 403-409). Findings such as these have lead to the chemotherapeutic targeting of NQO1 in some cancer treatments (Harada et al., 2003: 205).

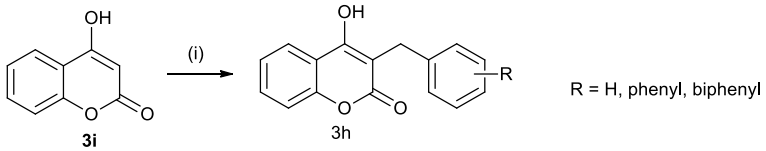

Fig. 2. Synthesis of asymmetrical analogues of dicoumarol using 4-hydroxycoumarin. Reagents and conditions: (i) 4-hydroxycoumarin (1 eq), aromatic aldehyde (1 eq), ethanol, 80 ^{o}C, 24 hrs

In view of this, structural modifications of 4-hydroxycoumarin was carried and then utilized for the new synthesis of asymmetrical analogues of dicoumarol (**2a-e**). The targeted compounds are listed in (Fig. 3) below:

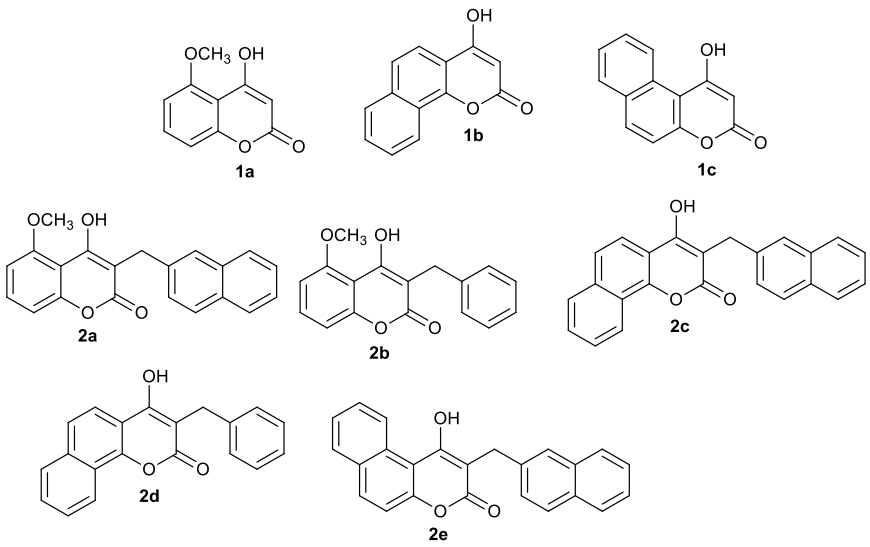

Fig. 3. Structures of targetted 4-hydroxycoumarin derivatives (1a-c) and asymmetrical analogues of dicoumarol (2a-e)

Synthesis and Discussion

The 4-hydroxycoumarin derivatives (**1a-c**) were synthesized by reacting the appropriate acetophenone with diethyl carbonate in the presences of sodium hydride (NaH) as depicted in (Fig. 3). A plausible mechanism for the reaction is illustrated in (Fig. 4). The 'hydride' ion from sodium hydride abstracts the *α*-hydrogen, of the acetophenone to give the enolate anion. The second step involves the nucleophilic attack of the electrophilic carbon of the carbonyl of diethyl carbonate to form an intermediate β-keto ester. The intermediate undergoes rapid keto-enol tautomerisation to give the target compound (**1a**).

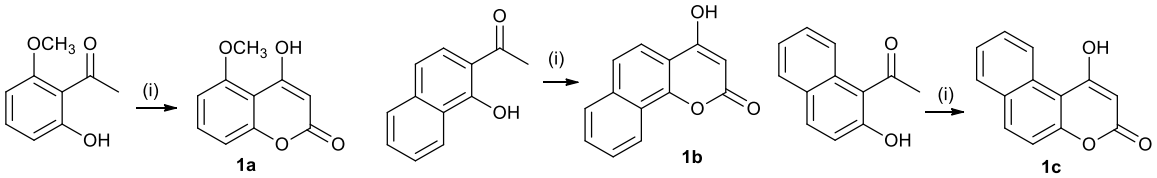

Fig. 4. Reaction scheme for the synthesis of 4-hydroxycoumarin derivatives of (1a-c). Reagents and conditions: (i) diethyl carbonate, sodium hydride, at reflux, 3hr

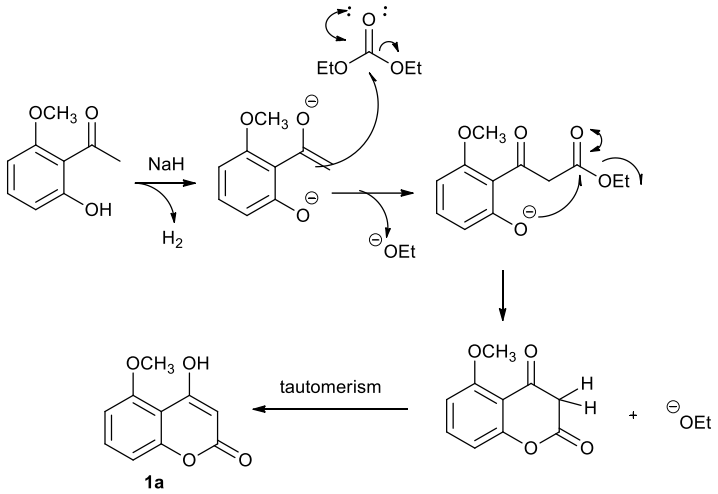

Fig. 5. Base-mediated cyclisation reaction of 2-hydroxy-6-methoxy acetophenone to give (1a). The mechanism is applicable to compounds (1b) and (1c)

The structural identities of all the cyclised compounds (**1a-c**) were confirmed by IR, ^{13}C and ^{1}H NMR spectroscopy which revealed the presence of only one product as revealed by the ^{1}H NMR spectrum (Figure 6). The infra-red absorption spectra of the synthesized compounds in the solid state resembled that of a carboxylic acid with a broad O-H stretch between 3200-3400 cm^{-1} and a C=O stretching frequency between 1650-1700 cm^{-1}. The presence of a vinylogous carboxylic acid makes the compounds moderately strong acids.

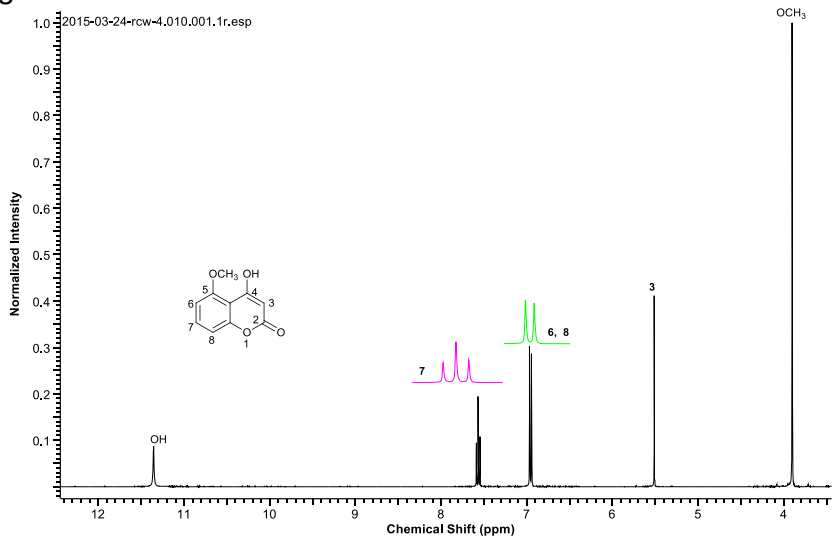

Fig. 6. ^{1}H NMR spectrum of compound (1a) in DMSO-d_6; 3, 6, 7 and 8 represent protons and their multiplicity

Synthesis of asymmetrical analogues of dicoumarol (2a-e)

The pharmacological and biochemical properties and the therapeutic use of simple coumarin depend upon the pattern of simple substitution technique. The 4-hydroxycoumarin has served as an important precursor for the design and synthesis of more active analogues of dicoumarol. The C-3 position of 4-hydroxycoumarin and its

derivatives as depicted in (Fig. 7), is reactive as a nucleophile as it is the central carbon atom of an enolic 1,3-dicarbonyl compound and it is this feature that facilitates the synthesis of symmetrical analogues of dicoumarol (**4a**). A plausible mechanism for this reaction is shown in (Fig. 8). The first step involves the nucleophilic addition of 4-hydroxycoumarin derivative to the polarized carbonyl bond of the aldehyde to form aldol adduct, followed by tautomerism. *α,β*-Unsaturated carbonyl compound is then formed by loss of water. The next step is the nucleophilic conjugate addition of a second molecule of 4-hydroxycoumarin derivative, which subsequently undergoes keto-enol tautomerism to give symmetrical dicoumarol analogues (**4a**).

These symmetrical compounds (**4a**) were transformed into asymmetrical analogues (**2a-e**) with regeneration of 1 mole of 4-hydroxycoumarin derivatives by C-C cleavage using sodium cyanoborohydride ($NaBH_3CV$) as illustrated in Fig. 9.

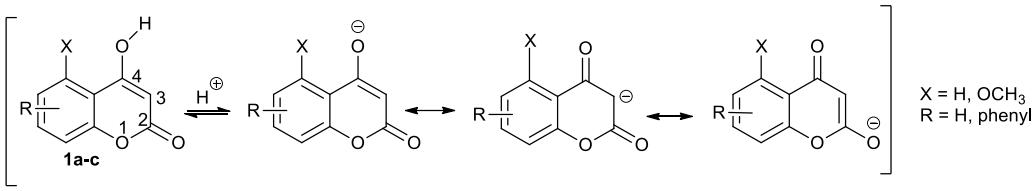

Fig. 7. Resonance forms of ionized 4-hydroxycoumarin and its derivatives

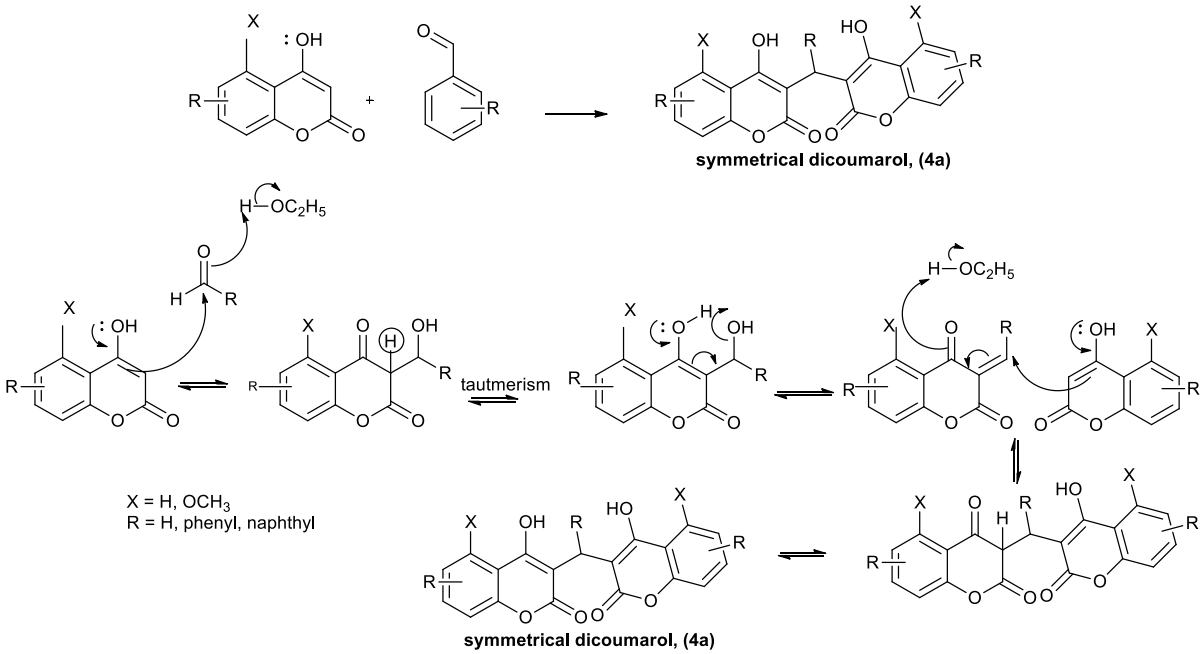

Fig. 8. General mechanism for the condensation and dimerization of 4-hydroxycoumarin derivatives

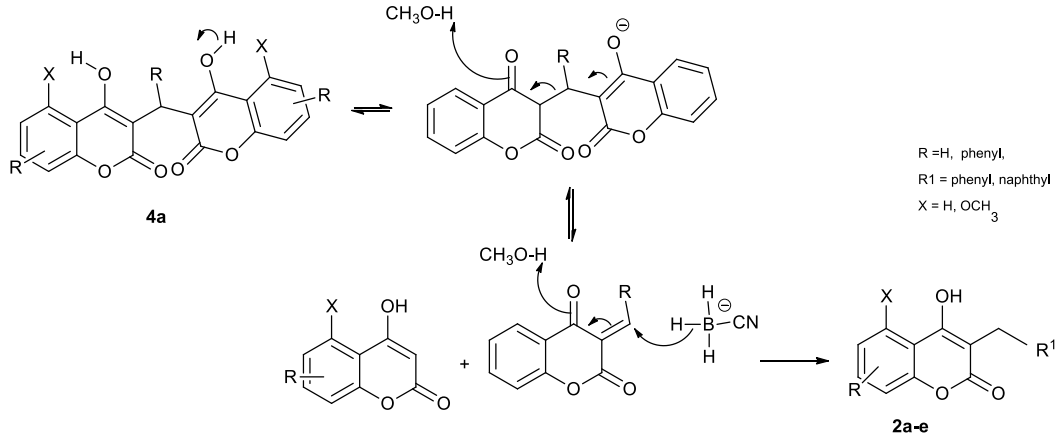

Fig. 9. Propose mechanism for reductive C-C cleavage of symmetrical dicoumarol (4a) to give asymmetrical dicoumarol (2a-e) using $NaBH_3CN$

Experimental Part

All the reagents used were obtained from commercial sources (Sigma-Aldrich Co., Alfa Aesar amd Fisher Scientific). 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 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 reference to the solvent residual peak. The coupling constant (*J*) are given in Hz. The abbreviations used are s-singlet, d-doublet, t-triplet, dd-doublet of doublets, m-multiplet. Proton assignments were assisted by DEPT, ^{1}HCOSY and HMQC.

Synthesis of 4-hydroxy-5-methoxy-2H-chromen-2-one (1a)

2-Hydroxy-6-methoxyacetophone (500 mg, 3.01 mmol) dissolved in diethyl carbonate (3 mL) was added to a suspension of sodium hydride (60% dispersion in mineral oil, 600 mg, 15.0 mmol) in diethyl carbonate (3 mL) and heated at 100 ^{o}C for 3 hours. The reaction mixture was left to cool to 0 ^{o}C in an ice bath and it was then quenched by dropwise addition of water until effervescence stopped. The aqueous layer was washed with diethyl ether (3 x 10mL). Concentrated hydrochloric acid was added dropwise to the aqueous layer to adjust the pH to 4 and the resulting precipitate was collected by filteration, washed with water and left to dry overnight at 90 ^{o}C. The titled compound (**1a**) was isolated as an off-white solid (360 mg, 62%): Mpt 155-157 ^{o}C; v_{max}/cm^{-1} 3260 (w, OH), 1705 (s, C=O), 1640; δ_H(400MHz, DMSO-d_6) 3.89 (3H, s), 5,50 (1H, s), 6.95 (2H, d, *J*=8.6), 7.56 (1H, t, *J*=8.4x2), 11.35 (1H, s); δ_C (100 MHz; DMSO-$_6$) 56.5, 90.8, 105.0, 106.7, 109.2, 133.0, 155.2, 157.3, 161.4, 167.2; *m/z* (+ES) 215.1 ([M+Na]$^+$, 100%); Found 215.0329; $C_{10}H_8O_4Na$ ([M + Na]$^+$) requires 215.0320.

Synthesis of 4-hydroxy-2H-benzo[h]chromen -2-one (1b)

Using the procedure described for the synthesis of compound (**1a**), 1-hydroxy-2-acetophenone (1.5 g, 8.06 mmol), diethyl carbonate (20 mL) and sodium hydride (1.8 g, 45.0 mmol), gave the title compound (**1b**) as an off white solid (1.14 g, 67%): Mp 285-287 ^{o}C. v_{max}/cm^{-1} 3410 (br, s, OH), 1644 (s, C=O), 1640;δ_H(400MHz, DMSO-d_6) 5.70 (1H, s), 7.70-7.74 (2H, m), 7.82-7.84 (2H, m), 8.03 (1H, m), 8.34-8.36 (1H, m), 12.67 (1H, br, s); δ_C (100 MHz; DMSO-d_6) 90.6, 111.1, 118.9, 121.7, 122.2, 123.6, 127.3, 128.1, 128.8, 134.8, 150.7, 161.8, 166.6; (C=O); *m/z* (-ES) 211.1 ([M-H]$^-$, 100%); Found 211.0392; $C_{13}H_7O_3$ ([M - H]$^-$) requires 211.0395.

Synthesis of 1-hydroxy-3H-benzo[f]chromen -3-one (1c)

Using the procedure described for the synthesis of compound (**1b**), 2-hydroxy-1-acetophenone (1.5 g, 8.06 mmol), diethyl carbonate (20 mL) and sodium hydride (1.8 g, 45.0 mmol), gave the title compound (**1c**) as an off white solid (1.14 g, 67%): Mp 285-287 ^{o}C. ν_{max}/cm^{-1} 3376 (br, w, OH), 1650 (s, C=O), 1640; δ_H(400MHz, DMSO-d_6) 5.76 (1H, s), 7.53 (1H, d, J 8.6), 7.60 (1H, ddd, J 8.6, 7.0, 1.5), 7.70 (1H, ddd, J 8.6, 7.0, 1.5), 8.04(1H, dd, J 8.6, 1.5), 8.21 (1H, d, J 8.6), 9.29 (1H, dd, J 8.6, 1.5), 12.93 (1H, br, s); δ_C (100 MHz; DMSO-d_6) 91.1, 108.6, 117.2, 125.6, 126.0, 128.3, 128.8, 128.9, 130.3, 134.2, 154.9, 161.3, 169.4;*m/z* (-ES) 211.1 ([M-H]$^-$, 100%); Found 211.0392; $C_{13}H_7O_3$ ([M - H]$^-$) requires 211.0395.

General procedures for the synthesis of symmetrical analogues of dicoumarol (4a). Method A

The appropriate 4-hydroxycoumarin derivative was reacted with aromatic aldehyde (37% aqueous solution stabilized with 12% methanol). Ethanol was added to give a solution of 0.25M concentration with respect to 4-hydroxycoumarin derivative. The reaction mixture was subjected to microwave irradiation at 80 ^{o}C for 4 hours. The resultant mixture was allowed to cool to room temperature and the precipitate formed was collected by filtration, washed with ethanol and dried.

General procedures for the synthesis of asymmetrical analogues of dicoumarol (2a-e). Mehtod B

Sodium cyanoborohydride (5 eq), was added to a suspension of symmetrical analogues dicoumarol (**4a**) (1 eq), in methanol (20 mL) and the reaction mixture was heated under reflux for 18 hours at 70 ^{o}C under nitrogen. The resulting solution was concentrated under vacuo in a ventilated fume hood. Saturated NH_4Cl solution (15 mL) was added to the residue and organic material was extracted into ethyl acetate (4x20 mL). The combine organic extracts were washed with saturated NH_4Cl solution (3x20 mL), brine (20 mL), dried over $MgSO_4$ and concentrated in vacuo to give the crude product. The crude material was purified by flash column chromatography on silica gel (petroleum: ethyl acetate).

Synthesis of (2a). Using method B, compound (**2a**) was obtained as a white solid (59% yield): Mp 157 ^{o}C; ν_{max}/cm^{-1} 3293 (s, OH), 1702 (s, C=O), 1637 (s, C=C); δ_H(400MHz, DMSO-d_6) 3.99 (2H, s), 4.06 (3H, s), 7.07 (2H, d), 7.47-7.53 (3H, m), 7.63 (1H, t), 7.78 (1H, s), 7.85-7.88 (3H, m); *m/z* (+ES) 355.1 ([M] + Na]$^+$, 100%); Found 355.0961; $C_{21}H_{16}O_4$ ([M + Na]$^+$) requires 355.0946.

Synthesis of (2b). Using method B, compound (**2b**) was obtained as a creamed coloured solid (52% yield): Mp 170-172 ^{o}C; ν_{max}/cm^{-1} 3320 (br, w, OH), 1686 (s, C=O), 1643 (s, C=C); δ_H(400MHz, DMSO-d_6) 3.91 (2H, s), 4.06 (3H, s), 4.06 (3H, s), 6.78 (1H, dd), 7.01 (1H, dd), 7.16-7.20 (1H, m), 7.25-7.28 (2H, m), 7.40-7.44 (3H, m), 9.73 (1H, s); *m/z* (-ES) 283.1 ([M-H]$^-$, 100%); Found 305.0796; $C_{17}H_{14}O_6Na$ ([M + Na]$^+$) requires 305.0790.

Synthesis of (2c). Using method B, compound was obtained as off-white solid (58% yield): Mp 276-278 ^{o}C; ν_{max}/cm^{-1} 3160 (br, w, OH), 1650 (s, C=O), 1607 (s, C=C); δ_H(400MHz, DMSO-d_6) 4.14 (2H, s), 7.41-7.51 (3H, m), 7.70-7.75 (3H, m), 7.82-7.89 (4H, m), 8.05 (2H, dd), 8.38 (1H, dd), 11.92 (1H, br, s); *m/z* (+ES) 375.1 ([M+Na]$^+$, 100%); Found 375.1002; $C_{24}H_{16}O_3Na$ ([M + Na]$^+$) requires 375.0997.

Synthesis of (2d). Using method B, compound (**2d**) was obtained as off-white solid (56% yield): Mp 260-262 ^{o}C; ν_{max}/cm^{-1} 3028 (br, w, OH), 1606 (s, C=O), 1557 (s, C=C); δ_H(400MHz, DMSO-d_6) 3.95 (2H, s), 7.15-7.18 (1H, m), 7.24-7.29 (4H, m), 7.70-7.73 (2H, m), 7.88 (1H, d), 8.01 (1H, d), 8.06 (1H, dd), 8.34-8.37 (1H, m); *m/z* (+ES) 325.1 ([M+Na]$^+$, 100%); Found 325.0851; $C_{20}H_{14}O_3Na$ ([M + Na]$^+$) requires 325.0841.

Synthesis of (2e). Using method B, compound (**2e**) was obtained as off-white solid (58% yield): Mp 270-272 ^{o}C; δ_H(400MHz, DMSO-d_6) 4.21 (2H, s), 7.40-7.49 (3H, m), 7.54-7.62 (2H, m), 7.68-7.71 (2H, m), 7.81-7.85 (3H, m), 8.05 (1H, d), 8.06 (1H, dd), 8.81 (1H, d), 9.44 (1H, d), 11.99 (1H, s); *m/z* (-ES) 351.3 ([M-H]$^-$, 100%); Found 351.1020; $C_{24}H_{15}O_3$ ([M - H]$^-$), requires 351.1021.

Enzyme assay

Serial dilution of the stock solutions of the synthetic asymmetrical dicoumarol (2a-e) (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 IC_{50} values were measured using nonlinear fitting as implemented in the program Excel (GraphPad Prism 5). Each measurement was made in triplicate and the experiments were repeated three times.[13] The concentration - response plots obtained demonstrate moderate to good inhibitory potency towards NQO1 enzyme (Fig. 10).

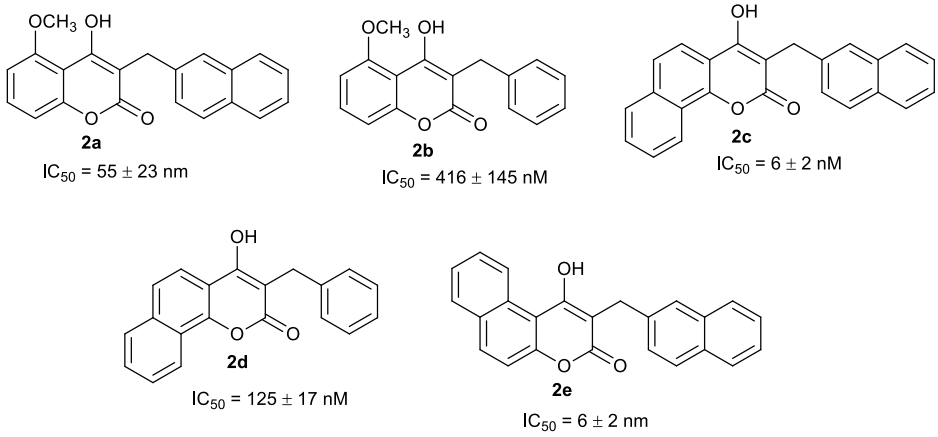

Fig. 10. IC_{50} values of asymmetrical dicoumarol (2a-e)

Conclusion

In summary, all the synthesized 4-hydroxycoumarin derivatives (**1a-c**) were obtained in good yields (62-67%). The reaction was carried out at higher temperature (110 ^{o}C) in order to obtain complete conversion of the reactants. These derivatives was converted to symmetrical dicoumarol (4a) which in turn undergo C-C reductive cleavage to give asymmetrical analogues of dicoumarol (**2a-e**) using $NaBH_3CN$. The enzyme essay conducted on compound (2a-e) revealed moderate to good inhibitory potency towards NQO1 enzyme activities.

Acknowledgements

The authors would like to thank the School of Chemistry, University of Manchester for providing the necessary and refined equipments. This research work was supported in part by TETfund Nigeria.

References

Abdel Latif, N. A., Batran, R. Z., Khedr, M. A., Abdalla, M. M. (2016). 3-Substituted-4-hydroxycoumarin as a new scaffold with potent CDK inhibition and promising anticancer effect: Synthesis, molecular modeling and QSAR studies. Bioorg. Chem., 10, 116-119.

Borges, F., Roleira, F., Milhazes, N., Santana, L., Uriarte, E. (2005). Simple coumarins and analogues in medicinal chemistry: occurrence, synthesis and biological activity. Cur. Med. Chem, 12, 887-916.

Chohan, Z. H., Shaikh, A. U., Rauf, A., Supuran, C. T. (2006). Antibacterial, antifungal and cytotoxic properties of novel N-substituted sulfonamides from 4-hydroxycoumarin. J. Enzyme. Inbid. Med. Chem., 21, 741-748.

Ernester, L. (1967). DT Diaphorase. Methods. Enzymol., 10, 309-317.

Harada, S., Tachikawa, H., Kawanishi, Y. (2003). A possible association between an insertion/deletion polymorphism of the NQO2 gene and schizophrenia. Genet., 13, 205-209.

Jung, J.-Ch., Park, O.-S., (2009). Synthetic Approaches and Biological Activities of 4-Hydroxycoumarin Derivatives *Molecules*, 14, 4790-4803.

Kostova, I. (2005). Synthetic and Natural Coumarins as Cytotoxic Agents. Cur. Med. Chem., 5, 29-46.

Li, R., Bianchet, M. A., Talalay, P., Amzel, L. M. (1995). The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. Proc. Natl. Acad. Sci., 92, 8846-8850.

Manolov, I., Maichle-Moessmer, C., Nicolova, I., Danche, N. (2006). Synthesis and anticoagulant activities of substituted 2,4-diketochromans, biscoumarins, and chromanocoumarins. Eur. J. Med. Chem., 41, 882-890.

Musa, M. A., Cooperwood, J. S., Khan, M. O. (2008). A review of coumarin derivatives in pharmacotherapy of breast cancer. Cur. Med. Chem., 15, 2664-2679.

Nolan, K. A., Doncaster, J. R., Dunstan, M. S., Scott, K. A., Frenkel, A. D., Siegel, D., Rose, D., Barnes, J., Levy, C., Leys, D., Whitehead, R. C., Stratford, I. J., Bryce, R. A. (2009). Synthesis and biological evaluation of coumarin-based inhibitors of NAD(P)H: quinone oxidoreductase-1 (NQO1). J. Med. Chem., 52, 7144-7154.

Obi, J. C., Ezenwa, T. E. (2018). Synthesis of Analogues of Dicoumarol and Their Biological Evaluation. Int. J. Chem, 10, 1-7.

Overmunn, R.S., Stahmann, M. A., Henbner, C. F., Sullivan, W. R., Spero, L., Doherty, D. G., Ikawa, M., Graf, L., Roseman, S., Link, K. P. (1944). A review of coumarin derivatives in pharmacotherapy of breast cancer. J. Biol. Chem., 153, 5-24.

Schlager, J.J., Powis, G. (1990). The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. J. Cancer, 45, 403-409.

Siegel, D., Gustafson, D. L., Dehn, D. L., Han, J. Y., Boonchong, P., Berliner, L. J., Ross, D. (2004). NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger. Mol. Pharmacol., 65, 1238-1247.

Velasco-Velazquez, M. A., Agramonte-Hevia, J., Barrera, D., Jimenez-Orozco, A., Garcia-Mondragon, M. J., Mendoza-Patino, N. (2003). 4-Hydroxycoumarin disorganizes the actin cytoskeleton in B16-F10 melanoma cells but not in B82 fibroblasts, decreasing their adhesion to extracellular matrix proteins and motility. Caner Letts., 198, 179-186.

Weber, U. S., Steffen, B., Siegers, C. P. (1998). Antitumor-activities of coumarin, 7-hydroxy-coumarin and its glucuronide in several human tumor cell lines. Res. Commun. Mol. Pathol. Pharmacol., 99, 193-206.