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ARTICLE

Evaluation of Some Salicylaldehyde-derived Baylis-Hillman Adducts and Coumarin Derivatives as Potential Antisickling Compounds

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Abstract

Some salicylaldehyde-derived Baylis-Hillman adducts and 3-(chloromethyl)coumarins have been synthesised and evaluated *in vitro* for their antisickling activities. The compounds were screened for inhibitory and reversal activity against mutated haemoglobin (HbSS) in red blood cells at four different concentrations (4 mg/mL, 2 mg/mL, 1 mg/mL and 0.5 mg/mL) as a measure of their antisickling potentials. Among the synthesized compounds, 6-chloro-3-(chloromethyl)coumarin **4d** showed the highest inhibitory activity (83.75±1.90%), followed by 6-chlorocoumarin-3-methylsulfinic acid **5d** (80.90 ±0.91%) and the least was *tert*-butyl-3-hydroxy-3-(2-hydroxyphenyl)-2-methylenepropanoate **3a (**33.33±1.86%**)**. The results obtained from the reversal antisickling experiment showed that the percentage of sickle cells able to revert to the normal biconcave shape was dose dependent. Compound **5d** had the highest reversal activity (66.49±1.39%) followed by 6-bromo-3-(chloromethyl)coumarin **4c** (59.66±2.95) and **4d** (55.50±1.95%) at 4 mg/mL. Compound **4c** had higher reversal activity than the standard *p*-hydroxybenzoic acid at 2 mg/mL, 1 mg/mL and at 0.5 mg/mL. The 3-substituted coumarins **4a-d**, and **5d** had higher inhibitory antisickling activities than their Baylis-Hillman precursors **3a-d**. Effect of **4a-d** and **5d** on the rate of polymerization of sickle cell heamoglobin was further studied spectrophotomerically using hemolysate of HbSS. The considerable inhibitory and reversal activities of these compounds make them good candidates for further antisickling studies.

Keywords: Antisickling Activity; Baylis-Hillman Adducts; Coumarins; HbSS Red Blood; Polymerization.

1.0 Introduction

Sickle cell disease (SCD) is one of the commonest haemoglobinopathies affecting the human population with considerable morbidity and mortality (Ashley-Koch *et al*., 2000; Makani *et al*., 2013). This disease remains a major genetic disorder of the blood affecting tropical areas with its highest prevalence in Sub-Sahara Africa (Imaga *et al*., 2013). Sickle cell disease results from a point mutation in the gene coding on the sixth position of β-subunit of the haemoglobin leading to a single amino acid substitution, where valine substitutes glutamic acid (Ashley-Koch *et al*., 2000). This condition impairs the tertiary structure of the mutated haemoglobin protein (HbSS) and in turn loses some of its functional properties. Under low oxygen tension, HbSS polymerizes distorting the biconcave-shaped red blood cells into sickled shape and this modification makes the red blood cells fragile and less flexible thereby causing many clinical features that HbSS carriers suffer (Mehanna, 2001; Buchanan *et al*., 2004).

Several studies have revealed the antisickling potential of calcium channel blocker, nifedipine (Ma *et al*., 2012); fetal haemoglobin inducers, sodium dimethylbutyrate (Kutlar, 2012) and hydroxyurea (Charache *et al*., 1995; Steinberg, 1999). Among these compounds, hydroxyurea is the only agent in clinical use for the treatment of SCD. However, the limitations of hydroxyurea include lower extremity ulceration, xerosis, myelosuppression and possible carcinogenicity (Saraceno *et al*., 2008; Haywood *et al*., 2011).

In recent times, haematopoietic stem cell transplant (HSCT) appears to be the only curative treatment for sickle cell disease available (Bernaudin *et al*., 2007; Panepinto *et al*., 2007). However, lack of suitably matched donors and the chronic use of immunosuppressive drugs with serious side effects have limited the use of HSCT for the treatment of SCD (Bernaudin *et al*., 2007; Hoban *et al*., 2016). Hence, there is need to continue search for other effective therapies that could be used in the management of the disease.

Meanwhile, simple Morita-Baylis-Hillman adducts have become an important class of bioactive compounds with diverse biological activities such as anticancer (Dadwal *et al*., 2006), antimalarial (Narender *et al*., 2005), molluscidal (Vasconcellos *et al*., 2006) amongst others. In addition, coumarins form an important pharmacophore in medicinal chemistry and they have been found to exhibit a variety of biological activities usually associated with low toxicity. This has raised considerable interest because of their potential beneficial effects on human health (Hoult and Paya, 1996; Kennedy and Thornes, 1997). Coumarins possess pharmacological activities such as anti-HIV (Ma *et al*., 2008), anticoagulant (Kidane *et al*., 2004), antibacterial (Appendino *et al*., 2004), antioxidant (Kontogiorgis and Hadjipavlou, 2004), antimalarial (Yang *et al*., 1992), calcium channel blocking activity among other broad spectrum biological activities (He *et al*., 2007; Asif, 2015), but are yet to be implicated in the management of sickle cell disease.

In continuation of our interest in exploring the biological applications of coumarins (Olomola *et al*., 2013; Olomola *et al*., 2014), we herein report the antisickling potentials of some salicylaldehyde derived Baylis-Hillman adducts and their 3-substituted coumarins.

2.0 Materials and Methods

2.1 General

Salicyaldehyde, 3-ethoxysalicyaldehyde, 5-bromosalicyaldehyde, 5-chlorosalicyaldehyde, diazabicyclo [2.2.2] octane, *tert*-butylacrylate, vanillic acid and *para*-hydroxybenzoic acid, thiourea, glacial acetic acid, concentrated HCl, Phosphate buffered saline (PBS) pH 7.0 were purchased from Sigma Aldrich and used without further purification. Ethylacetate, chloroform, n-hexane and methanol were purchased from BDH chemical, formalin (Fluka), sodium metabisulphite (Hopkins and Williams). The solvents were distilled prior to use. Melting points were determined by open capillaries and uncorrected using a Gallenkamp melting point device. IR spectra were recorded neat on a Pelmer Elmer spectrum 100 FT-IR spectrometer. NMR spectra were recorded on a Bruker 400 MHz AVANCE spectrometer and were referenced using solvent signals (δ_H: 7.26 ppm for residual $CHCl_3$; δ_C: 77.0 ppm for $CDCl_3$). Column chromatography was carried out using Merck silica gel 60-120 mesh size as stationary phase and mixture of n-hexane: ethyl acetate as eluant. Antisickling experiments were carried out in the Tissue Culture Laboratory in the Department of Drug Research and Production Unit, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife. Sickle cell blood samples were viewed using a binocular microscope (Feica) and the cells were counted using a manual counter. Incubation of sickled red blood cells was carried out in a thermostated water bath (Tecam). Photomicrograph of prepared slides of HbS blood cells were taken using ScopeImage 9.0 (H1C) camera.

2.1.1 Collection of blood

Venous blood collected into ethylenediaminetetraacetic acid bottles from confirmed sickle cell anemia patients between ages 12-30 years in steady state, who attend regular Haematology clinic at the Obafemi Awolowo University Teaching Hospital, Ile-Ife, Nigeria was used. Ethical clearance with reference number IRB/IEC/0004553 was given by the Ethical and Research committee of the Obafemi Awolowo University Teaching Hospital Complex for this project

2.2 Chemistry

2.2.1 Synthesis of Baylis-Hillman adducts 3a-d and 3-(chloromethyl) coumarins 4a-d

In this present study, salicylaldehyde derivatives **1a-d** and *tert*-butyl acrylate **2** (Scheme1) were used as starting materials for the preparation of Baylis-Hillman adducts **(3a-d)** using literature methods (Kaye *et al*., 2003**).** The Baylis-Hillman adducts were further cyclized to 3-(chloromethyl)coumarins **4a-d** using acid catalysis (Kaye *et al*., 2003).

2.2.2 *tert*-Butyl-3-hydroxy-3-(2-hydroxyphenyl)-2-methylene propanoate [*see spectra 3a, Supporting Document*]

White solid, melting point: 108-110 ^oC (lit. 108-110^oC) (Kaye *et al*., 2003). IR spectrum: 3194 cm^{-1} (aliphatic O-H); 1688 cm^{-1} (vinylic C=O of ester); 1635 cm^{-1} (olefinic C=C stretching); 1150 cm^{-1} and 1020 cm^{-1} (C-O of ester). ^1H NMR: δ 1.56 (9H, s, $C[CH_3]_3$); δ 4.45 (1H, d , J = 4.0 Hz, aliphatic OH); δ 5.55 and 6.29(2H, 2 x s, $C=CH_2$, diastereotopic protons); δ 5.75 (1H, d, J = 4.0 Hz, CH-OH); δ 6.86-7.31 (4H, m, Ar-H) and δ 8.21 (1H, s, Ar-O-H). ^{13}C NMR: δ 28.0 ($C[CH_3]_3$); δ 82.6 ($O-C[CH_3]_3$); δ 73.6 (CH-OH) ; δ 117.5, 119.9, 127.9 and 129.5 (protonated ArC); δ 127.0 (ArCq), δ 124.2 and δ 141.0 ($C=CH_2$); δ 156.0 (ArC-OH) and δ 166.8 (C=O).

2.2.3 *tert*-Butyl-3-(3-ethoxy-2-hydroxyphenyl)-3-hydroxy-2-methylene propanoate [*see spectra 3b, Supporting Document*]

Yellow oil; IR spectrum: 3452 cm^{-1} (O-H); 1705 cm^{-1} (vinylic C=O of ester); 1142 cm^{-1} and 1028 cm^{-1} (C-O stretching of ester). ^{1}H NMR δ 1.90 (12H, overlapping s and t, C[CH_3]$_3$) and CH_2CH_3); δ 4.20 (1H, s, broad CH-OH, hydroxyl proton); δ 4.60 (2H, q, OCH_2CH_3), δ 6.30 (1H, s, methine proton, CH-OH); δ 6.16- 6.70 (2H, 2xs, C=CH_2, diastereotopic protons); δ 7.00 (1H,b, Ar-OH) and δ 7.27-7.30 (3H, m, overlapping, Ar-H). ^{13}C NMR δ 14.9(-OCH_2*C*H_3); δ 27.9 (C[*C*H_3]$_3$), δ 64.6 (O*C*H_2CH_3), δ 69.3 (CH-OH), δ 81.6 (O-*C*[CH_3]$_3$); δ 111.4, 119.5 and 119.4 (Ar-C), 125.1 (C=CH_2), δ 126.6 (Ar-C), δ 142.2 (C=CH_2); δ 143.7 (ArC-OH); δ 146.1 (Ar-*C*-OCH_2CH_3) and δ 166.1 (C=O).

2.2.4*tert*-Butyl-3-(5-bromo-2-hydroxyphenyl)-3-hydroxy-2-methylene propanoate [*see spectra 3c, Supporting Document*]

White solid, melting point: 182-185 ^{o}C (lit. 186-188 ^{o}C) (Kaye *et al*., 2003). IR spectrum: 3287 cm^{-1} (O-H); 1680 cm^{-1} (vinylic C=O); 1630 cm^{-1} (olefinic C=C stretch);1142 and 1024 cm^{-1} (C-O stretching of ester). ^{1}H NMR: δ 1.31 (9H, s, C[CH_3]$_3$); δ 5.51 (1H, d, OH); δ 5.64 (2H, overlapping, methine proton 1H, d, CH-OH, hydroxyl proton and 1H, s, C=CH_2) and δ 5.64 and 6.05 (2H, 2 x s, C=CH_2, diastereotopic protons); δ 6.77 (1H, d, *J* = 12 Hz, Ar-H), δ 7.18- 7.20 (2H, m, Ar-H) and δ 9.75 (1H, s, Ar-O-H). ^{13}C NMR: δ 28.0 (C[*C*H_3]$_3$), δ 65.0 (CH-OH); δ 80.6 (O-*C*[CH_3]$_3$), δ 117.7, 130.3, and 131.0 (protonated ArC), 123.5 and 132.4 (C=CH_2), δ 110.1 and 145.4 (ArCq); δ 154.3 (Ar-C-OH) and δ 166.8 (C=O).

2.2.5 *tert*-Butyl-3-(5-chloro-2-hydroxyphenyl)-3-hydroxy-2-methylene propanoate [*see spectra 3d, Supporting Document*]

White solid, melting point: 178-180^{o}C (lit. 185-187 ^{o}C) (Rashamuse *et al*.,2009). IR spectrum: 3457 cm^{-1} (O-H), 1738 cm^{-1} (vinylic C=O); 1633 cm^{-1} (olefinic C=C stretch), 1229 cm^{-1} and 1146 cm^{-1} (C-O of ester). ^{1}H NMR: δ 1.32 (9H, s, C[CH_3]$_3$), δ 5.67 (1H, s, methine proton, CH-OH); δ 5.64 and 6.05 (2H, 2 x s, C=CH_2, diastereotopic protons), δ 6.80 (1H,d, J = 8 Hz, Ar-H) and δ 7.06-7.11 (2H,m, Ar-H). ^{13}C NMR: δ 28.4 (C[*C*H_3]$_3$), 81.0 (O-*C*[CH_3]$_3$), δ 65.5 (CH-OH) 117.5, 127.8 and 128.4 (ArC), δ 122.9 and 145.8 (ArCq); δ 123.8 and 132.3 (C=CH_2); 154.3 (ArC-OH) and δ 165. 9 (C=O).

2.2.6 3-Chloromethylcoumarin [*see spectra 4a, Supporting Document*]

Purple solid, melting point: 119-121 ^{o}C (lit.108-110 ^{o}C) (Kaye and Musa, 2002). IR spectrum: 1739 cm^{-1} (C=O); 1630 cm^{-1} (olefinic C=C stretch); 2970 cm^{-1} (sp3 C-H); 1228 and 1217 cm^{-1} (C-O of ester). ^{1}H NMR: δ 4.49 (2H, s, CH_2); δ 7.19-7.31 (2H, m. Ar-H); δ 7.45-7.49 (2H, m, Ar-H) and δ 7.81 (1H, s, methine proton, CH). ^{13}C NMR: δ 41.1 (CH_2); δ 116.7, 128.1, 132.1 and 141.1 (protonated ArC); δ 124.7 (overlapping, C=CH and ArC_q); δ 153.5 (C=Cq), δ 118.8 (ArC_q)and δ 160.13 (C=O).

2.2.7 3-(Chloromethyl)-8-ethoxycoumarin [*see spectra 4b, Supporting Document*]

Brown solid, melting point: 118-120 ^{o}C (lit. 122-124 ^{o}C) (Kaye and Musa, 2002). IR spectrum: 1737 cm^{-1} (C=O); 1632 cm^{-1} (olefinic C=C); 2970 cm^{-1} (sp^3 C-H), 1226 and 1217 cm^{-1} (C-O of ester). ^{1}H NMR: δ 1.49 (3H, t, CH_2C*H*$_3$); δ 4.19 (2H, q, C*H*$_2CH_3$); δ 4.55 (2H, s, CH_2Cl); δ 7.06 (1H, d, *J* = 8 Hz, Ar-H); δ 7.08 (1H, d, *J* = 8 Hz, Ar-H), δ 7.25 (1H, dd, Ar-H) and δ 7.84 (1H, s, methine proton, CH). ^{13}C NMR: δ 15.2 (CH_3); δ 41.8 (CH_2Cl); δ 65.5 (O-CH_2), δ 115.7, 119.8, and 141.6 (protonated ArC), δ 120.1 and 125.6 (ArC_q) 124.9 (protonated C=CH); δ 144.0 (ArC-OEt); δ 147.0 (C=CH_2)and δ 160.1 (C=O).

2.2.8 6-Bromo-3-(chloromethyl)coumarin [*see spectra 4c, Supporting Document*]

White solid, melting point: 113-115 ^{o}C (lit. 116-118 ^{o}C) (Kaye and Musa, 2002). IR spectrum: 1737 cm^{-1} (C=O); 1627 cm^{-1} (olefinic C=C); 2970 cm^{-1} (sp^3 C-H stretch); 1228 and 1217 cm^{-1} (C-O of ester). ^{1}H NMR: δ 4.53 (2H, s, CH_2Cl), δ 7.25(1H, d, J=8Hz, Ar-H); δ 7.61-7.66 (2H, m, Ar-H); δ 7.80(1H, s, methine proton, CH). ^{13}C NMR: δ 41.2 (CH_2Cl); δ 118.8, 135.7 and 139.9 (protonated ArC), 117.7 and 120.7 (ArCq); δ 126.8 (ArC-Br); δ 130.8 (protonated C=CH); δ 152.76 (C=CH_2)and δ 159.76 (C=O).

2.2.9 6-Chloro-3-(chloromethyl)coumarin [*see spectra 4d, Supporting Document*]

White solid, melting point: 112-114 ^{o}C (lit. 114-116 ^{o}C) (Kaye and Musa, 2002). IR spectrum: 1717 cm^{-1} (C=O); 1634 cm^{-1} (olefinic C=C); 3031 cm^{-1} (sp^2 C-H stretch); 1186 and 1160 cm^{-1} (C-O of ester). ^{1}H NMR: δ 4.54 (2H, s, CH_2Cl); δ 7.30 (1H, m, Ar-H); δ 7.48-7.51 (2H, m Ar-H); δ 7.81 (1H, s, methine proton, CH). ^{13}C NMR: δ 41.3 (CH_2Cl); δ 118.6, 132.4 and 140.1 (protonated ArC), δ 120.2 and 152.2 (ArC_q); δ 126.7 (ArC-Cl), δ 127.7 (C=CH); δ 152.2 (C=CH_2)and δ 159.9 (C=O).

2.2.10 Synthesis of 6-chlorocoumarin-3-methylsulfinic acid [*see spectra 5d, Supporting Document*]

6-Chloro-3-(chloromethyl)coumarin **4d** (0.5 g, 1.63 mmol) was added to thiourea (0.31 g, 4.08 mmol), the mixture was dissolved in 5 mL of water-ethanol mixture (1:1). After stirring at room temperature for 48 h, the reaction mixture was concentrated *in vacuo* and purified using flash chromatography [on silica gel; elution with hexane-EtOAc (5:1)] to afford *6-chlorocoumarin-3-methylsulfinic acid* **5d** as a white solid (0.3 g, 54.5%); m.p. 172-174 °C.

The IR spectra of compound **5d** showed some diagnostic bands at 3372 cm^{-1} (OH), 1603 cm^{-1} (C=O),1074 cm^{-1} (S=O), 731 cm^{-1} (C-S); The NMR spectra showed δ_H (400 MHz, $CDCl_3$) δ 2.64 (1H, broad s, OH), 4.63 (2H, s, CH_2SO_2H), 7.29 (1H, m, Ar-H), 7.48 (2H, m, Ar-H) and 7.70 (1H, s, Ar-H); δ_C (100 MHz, $CDCl_3$) δ 60.8 (CH_2SO_2H); 118.1, 131.3 and 137.2 (protonated ArC), 120.2 and 129.93 (ArC_q), 129.3 (ArC-Cl), 127.1 (C=CH), 151.5 (C=CH_2)and 160.6 (C=O).

2.3 Biological evaluation

Compounds **3a-d**, **4a-d** and **5d** were accurately weighed and because they are insoluble in water, were dissolved in 1% Tween 80 to prepare the stock concentration 4 mg/mL of each compound. Lower concentrations such as 2 mg/mL, 1 mg/mL and 0.5 mg/mL were prepared by serial dilution. Portions (0.1 mL) of whole blood samples already collected in EDTA bottles were used for the sickle cell reversal and inhibition experiments.

2.3.1 Sickle cell reversion and inhibitory assay:

The reversal and inhibitory antisickling assays were carried out *in vitro* according to the method of Egunyomi and co-workers (Egunyomi *et al*., 2009). The inhibitory assay involves pre-incubating HbSS blood cells with a known concentration of the test compound in the presence of 2% sodium metabisulphite solution, with vanillic acid as positive controland phosphate buffered saline (PBS) of pH 7.0 as negative control. Red blood cells were fixed in 40% formalin until ready for counting. Slides were prepared from the fixed cells after centrifugation. An aliquot of the fixed cells was taken with the use of capillary tube, dropped on a microscope

slide and carefully covered with a cover slip. A drop of each reaction mixture was then smeared on the slide and then viewed under microscope (× 400). Each slide is divided into five fields and the number of sickled and unsickled cells was then counted per field. The percentage sickled red blood cells was calculated as:

$$\% \text{ sickled cells} = \frac{\text{no.of sickle cells}}{\text{total no.of sickle cells}} \times 100 \quad (1)$$

The percentage inhibition/reversal was thereafter calculated from the percent sickled cells using the formula:

$$\% \text{ inhibition} = \frac{\% \text{ sickled cells (C)} - \% \text{ sickled cells (S)}}{\% \text{ sickled cells in negative (C)}} \times 100 \quad (2)$$

where C is control and S is sample.

3.3.2 Data treatment

Values were expressed as mean ± standard error for triplicate analysis. The significant differences were analysed using the Student-Newman-Keuls Multiple Comparison test (*t*-test) and Analysis of Variance (ANOVA), values $p < 0.05$ were considered to be significant.

2.3.3 Effect on rate of polymerization

HbSS erythrocytes were isolated from the blood samples by centrifugation at 4000 rpm for 5 min. Following careful siphoning of the plasma using a Pasteur pipette, the erythrocytes were repeatedly washed with 0.9% normal saline until a clear supernatant was obtained. The clear supernatant was removed leaving the HbSS erythrocytes as hemolysate for the polymerization experiment. The method of Iwuand co-workers (Iwu *et al*., 1988) was used for the determination of polymerization rates. 4.4 mL of 2% sodium metabisulphite, 0.5 mL normal saline (0.9% NaCl) and 0.1 mL haemoglobin SS were pipetted into a cuvette, shaken and absorbance is taken at 700 nm (T70 UV/VIS Unicamsectronic Spectrophotometer) 2 minutes interval for 30 minutes. This represents the control. For the test compounds, 4.4 mL 2% NaS_20_5, 0.5 mL of each test compound and 0.1 mL HbSS solution were assayed as above. The rates of polymerization were calculated. Tests were carried out in triplicates and mean values were used for determination of the relative % polymerization. Percentage polymerization rates were plotted against various concentrations using Origin 8.0 software package.

Percentage polymerization was calculated according to Chikezie and co-workers (Chikezie *et al*., 2010), thus:

$$\% \text{ Polymerization} = \frac{A_{t/c} \times 100}{A_{c30^{th}\,min}} \quad (3)$$

$A_{t/c}$ = Absorbance of test/control assay at time *t* mins

$A_{c30^{th}min}$ = Absorbance of control assay at the 30th min

3.0 Results and Discussion

3.1 Synthesis of Baylis-Hillman adducts and 3-substituted coumarins

Various Baylis-Hillman adducts **3a-d** were synthesized following literature methods (Kaye *et al*., 2003), using salicylaldehyde derivatives as electrophiles and *tert*-butyl acrylate as the activated alkene in the presence of diazabicyclo[2.2.2]octane (DABCO). The Baylis-Hillman adducts obtained were cyclized using HCl and AcOH to afford the coumarins **4a-d** in very good yields. The Baylis-Hillman adducts **3a-d** and 3-chloromethyl(coumarins) **4a-d** were characterized and their spectra data are in accordance with literature (Kaye *et al*., 2003). In an attempt to synthesize a hydroxyurea analogue, 6-chloro-3-(chloromethyl)coumarin (**4d**) was treated with thiourea in the hope of obtaining a nucleophilic substitution product but rather spectroscopic evidence suggests the formation of *chlorocoumarin-3-sulfinic acid* **5d** (Scheme 1).

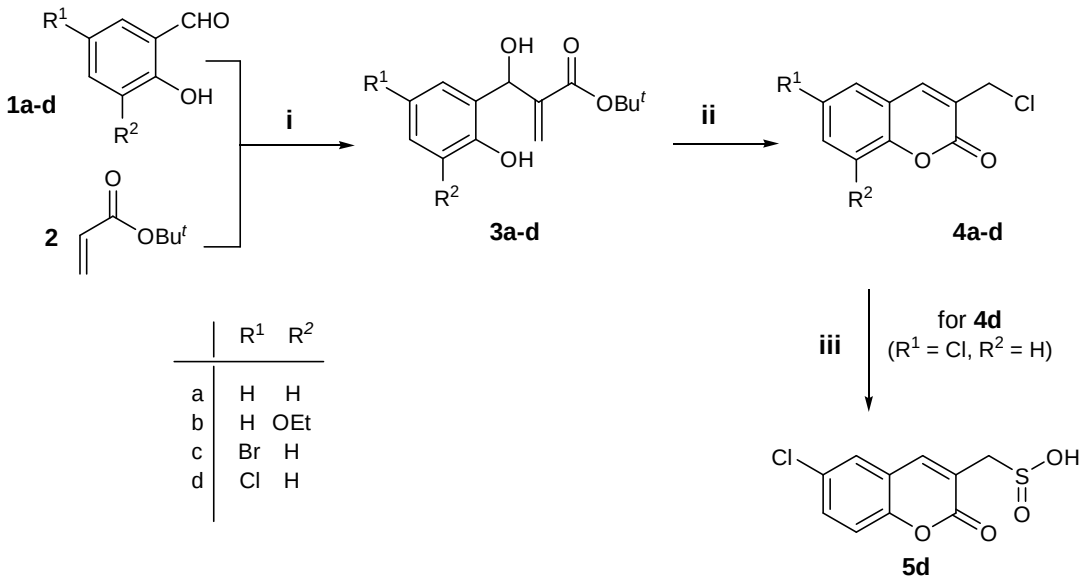
3.2 Biological activity

In the inhibitory antisickling assay, incubation of the test compounds with HbSS red blood cell recorded an increase in inhibitory activity which was found to be concentration dependent (Table 1). Figure 1 shows typical sodium metabisulphite-induced sickled HbSS red blood cellswhile Figure 2 shows the inhibitory effect of compound **4d** on HbSS red blood cells *in vitro*.

Among the synthesized compounds, **4d** showed the highest inhibitory activity (83.75 ± 1.90%), though lower than the standard, vanillic acid (97%) at the same concentration, followed by compound **5d** (80.90 ± 0.91%) and the least was **3a (**33.33 ± 1.86%**)**. Compound **3c** showed its highest inhibitory activity (81.33 ± 5.13%) at a lower concentration, 2 mg/mL, which is comparable to vanillic acid at the same concentration, although vanillic acid gave significantly higher ($p< 0.05$) inhibitory activity than all the test compounds (96.71 ± 0.91%) at 4mg/mL.. Compound **4c** had the red blood cells clumped up at 4 mg/mL (possibly suggesting that the compound may be too toxic to the cells at this concentration) but exhibited significant percentage inhibition (69%) at 2 mg/mL. Meanwhile, compounds **3b** and **3d** appeared to measure the same percentage inhibition across the concentration gradients as there were no significant differences in the measurements at $p< 0.05$.

Compounds **3b**, **3c**, **4a-d** and **5d** inhibited sodium metabisulphite-induced sickling within the experimental time and it could be that these compounds exhibited inhibitory activities by the inhibition of Ca^{2+}-activated K^+ channel. Under hypoxia condition, this channel had been identified to facilitate the loss of water from the erythrocyte membrane with subsequent increase in the intracellular concentration of HbSS leading to polymerization and its associated vaso-occlusive crises (Archer *et al*., 2015).

The reversal activities of the compounds were also found to be concentration dependent and there were significant differences along the concentration gradient for each group (Table 2). Compound **5d** had the highest reversal activity (66.49 ± 1.39%) followed by **4c** (59.66 ± 2.95) and **4d** (55.50 ± 1.95%) at 4 mg/mL. Compound **4c** had higher reversal activity than the standard PHBA at 2 mg/mL, 1 mg/mL and at 0.5 mg/mL. The results obtained from the sickling reversal antisickling experiment showed that the percentage of sickle cells able to revert to the normal biconcave shape was dose dependent. Upon re-oxygenation, sickled red blood cells could be restored to the normal shape (Oder, 2016). Hence, it could be suggested that the synthesized compounds bound allosterically to the HbSS red blood cells under hypoxia conditions to give a more stable conformer.



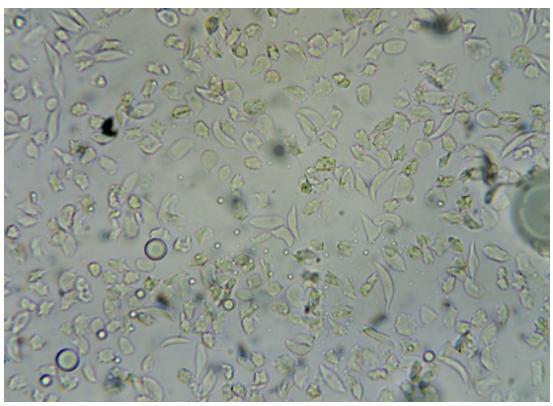
Scheme 1. Reagents *and conditions: (i) DABCO, $CHCl_3$, r.t.; (ii) HCl, AcOH, reflux; (iii) Thiourea, EtOH/H_2O (1:1), r.t.*

Table 1: % Inhibitory Activity of Synthesized Compounds on Sodium Metabisulphite-Induced Sickling.

Compounds	4 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL
3a	33.33 ± 1.86[b]	28.83 ± 1.60[b]	26.83 ± 2.57[b]	17.60 ± 2.34[a]
3b	58.67 ± 3.77[b]	57.33 ± 2.93[b]	48.58 ± 3.31[b]	51.25 ± 2.48[b]
3c	67.83 ± 3.36[a]	81.33 ± 5.13[b]	80.50 ± 3.58[b]	37.42 ± 3.86[a]
3d	41.42 ± 6.03[b]	49.67 ± 4.11[b]	51.25 ± 3.46[b]	50.50 ± 4.24[b]
4a	76.00 ± 2.59[a]	40.00 ± 4.24[b]	30.25 ± 5.51[b]	30.25 ± 4.83[b]
4b	70.00 ± 2.59[a]	46.83 ± 3.16[b]	43.41 ± 3.93[b]	4.83 ± 3.12[a]
4c	Clumped together	69.00 ± 2.49[a]	54. 58 ± 4.60[b]	46.58 ± 4.18[b]
4d	83.75 ± 1.90[a]	53.75 ± 2.06[b]	46.25 ± 2.32[b]	20.17 ± 3.94[a]
5d	80.90 ± 0.91[a]	69.16 ± 3.11[a]	35.42 ± 0.51[a]	22.73 ± 1.34[a]
Vanillic acid	96.71 ± 0.91[a]	81.69 ±3.90[a]	59.25 ± 1.90[a]	48.88 ± 2.08[a]

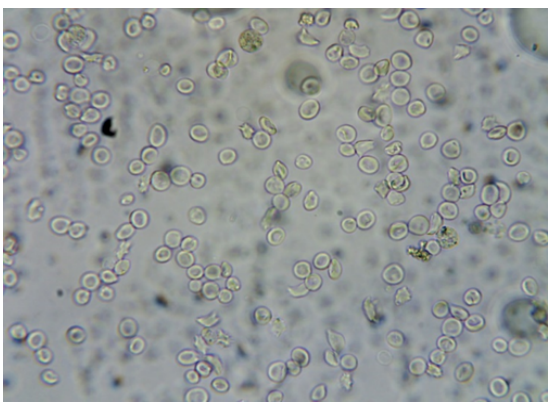
Data are expressed as mean ± standard error of mean (n =3) for each group.

At a particular concentration, variation in the triplicate measurements for each sample is expressed as 'a' (significant), or 'b' (not significant) at $p< 0.05$.



Mag. x400

Figure 1: *Photomicrograph showing the morphology of HbSS blood cells after deoxygenation with sodium metabisulphite*



Mag. x400

Figure 2: *Photomicrograph showing the morphology of HbSS blood cells treated with 4mg/mL of compound **4d***

It can be deduced from the inhibitory and reversal model that compound **5d** was the most active followed by **4d**. The three other compounds **4a,4b** and **4c** were also active. These five compounds were further tested using spectrophotometric method to determine their rate of inhibition of polymerization.The results obtained from rate of polymerization experiment (Figures3a, 3b, 3c and 3d) below showed the effect of 3-substituted coumarins on HbSS hemolysate with a decrease in the sickling rate which was concentration dependent. Within the experimental time t = 30 minutes,there were varying percentages of sickling at different concentrations.4 mg/mL of compound **4d** produced a rapid decrease in the rate of polymerization from 34.19 ± 0.1% at time t = 0 to 5.95 ± 0.5% at t = 30 minutes while 4 mg/mL of compound **5d** gave 6.87 ± 0.1% and **4c** gave a value of 5.86 ± 0.01% at the same concentration compared to the negative control which gave 100 ± 0.2% polymerization with percentage differences in high significance at $p< 0.05$ (Figure 3a).4 mg/mL of compound **4d** produced a rapid decrease in the rate of polymerization from 34.19 ± 0.1% at time t = 0 to 5.95 ± 0.5% at t = 30 minutes while 4 mg/mL of compound **5d** gave 6.87 ± 0.1% and **4c** gave a value of 5.86 ± 0.01% at the same concentration compared to the negative control which gave 100 ± 0.2% polymerization with percentage differences in high significance at $p< 0.05$ (Figure 3a). Furthermore, the test compounds reduce the rate of polymerization significantly at 2 mg/mL with 18.94 ± 0.2% for compound **4d**, 22.29 ± 1.7% for compound **5d**, 26.12% ± 0.00% for compound **4c** and 21.55 ± 0.1% for compound **4b** (Figure 3b). The compounds exhibited significant inhibitory antisickling activity at 1 mg/mL and 0.5 mg/mL but at a slower rate (Figures 3c, 3d).

4.0 Conclusion

The inhibitions of the aggregation of deoxyHbS by the synthesized compounds are noteworthy and could serve as antisickling agents with mode of action similar to that of hydroxyurea. The antisickling results showed that the test compounds have comparable activities with the standard drugs, *p*-hydroxybenzoic acid and vanillic acid. The 3-substituted coumarins **4a-d**, and **5d** had higher inhibitory antisickling activities than their Baylis-Hillman precursors **3a-d.** Thus, this work will be very useful for further studies in terms of effects of the synthesized compounds on the oxygen binding properties of HbSS red blood cells to improve their biological and pharmacological activities. The *in vitro* antisickling experiments described in this study were carried out in the absence of oxygen. Clinically, sickling occurs significantly under milder conditions with the partial pressure of oxygen of HbSS around 5.5 kPa (Nelson and Cox, 2008), this would suggest that lower concentrations of the synthesized compounds could be effective in inhibiting sickling *in vivo*. Also, attempted nucleophilic substitution reactions of 3-(chloromethyl)coumarin substrates with other semicarbazide derivatives is currently being undertaken in our laboratories.

Table 2: % Reversal Activity of Synthesized Compounds on Sodium Metabisulphite-Induced Sickling.

Compounds	4 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL
3a	12.33 ± 5.16[b]	7.42 ± 4.92[b]	7.08 ± 3.53 [b]	2.75 ± 0.48 [b]
3b	22.42 ± 4.97 [a]	13.08 ± 3.67 [b]	9.17 ± 3.84 % [b]	3.75 ± 0.37[a]
3c	19.33 ± 4.87[b]	16.36 ± 4.47 [b]	12.83 ± 3.18 [b]	9.08 ± 2.94[b]
3d	53.33 ± 4.38	55.33± 1.90[a]	37.94 ± 2.70 [a]	21.86 ± 4.30 [a]
4a	45.00 ± 4.89[a]	4.50 ± 1.37[b]	3.25 ± 1.42 [b]	0.10 ± 0.12[a]
4b	55.50 ± 1.98 [a]	43.29 ± 1.86 [a]	33.91 ± 2.11 [a]	24.46 ± 2.14 [a]
4c	59.66 ± 2.95 [b]	57.91 ± 3.30 [b]	55.33 ± 4.60 [b]	56.58 ± 1.72 [b]
4d	55.50 ± 1.95 [a]	43.30 ± 1.86 [a]	33.91 ± 2.11 [a]	24.40 ± 2.14 [a]
5d	66.49 ± 1.39 [a]	46.61 ± 2.20 [a]	10.86 ± 1.62 [a]	5.38 ± 1.36 [a]
PHBA	78.97 ± 1.89 [a]	55.32 ± 1.90 [a]	37.94 ± 2.70 [a]	21.86 ± 4.30[a]

Data are expressed as mean ± standard error of mean (n =3) for each group.
At a particular concentration, variation in the triplicate measurements for each sample is expressed as 'a' (significant), or 'b' (not significant) at $p< 0.05$.
PHBA = para-hydroxybenzoic acid.

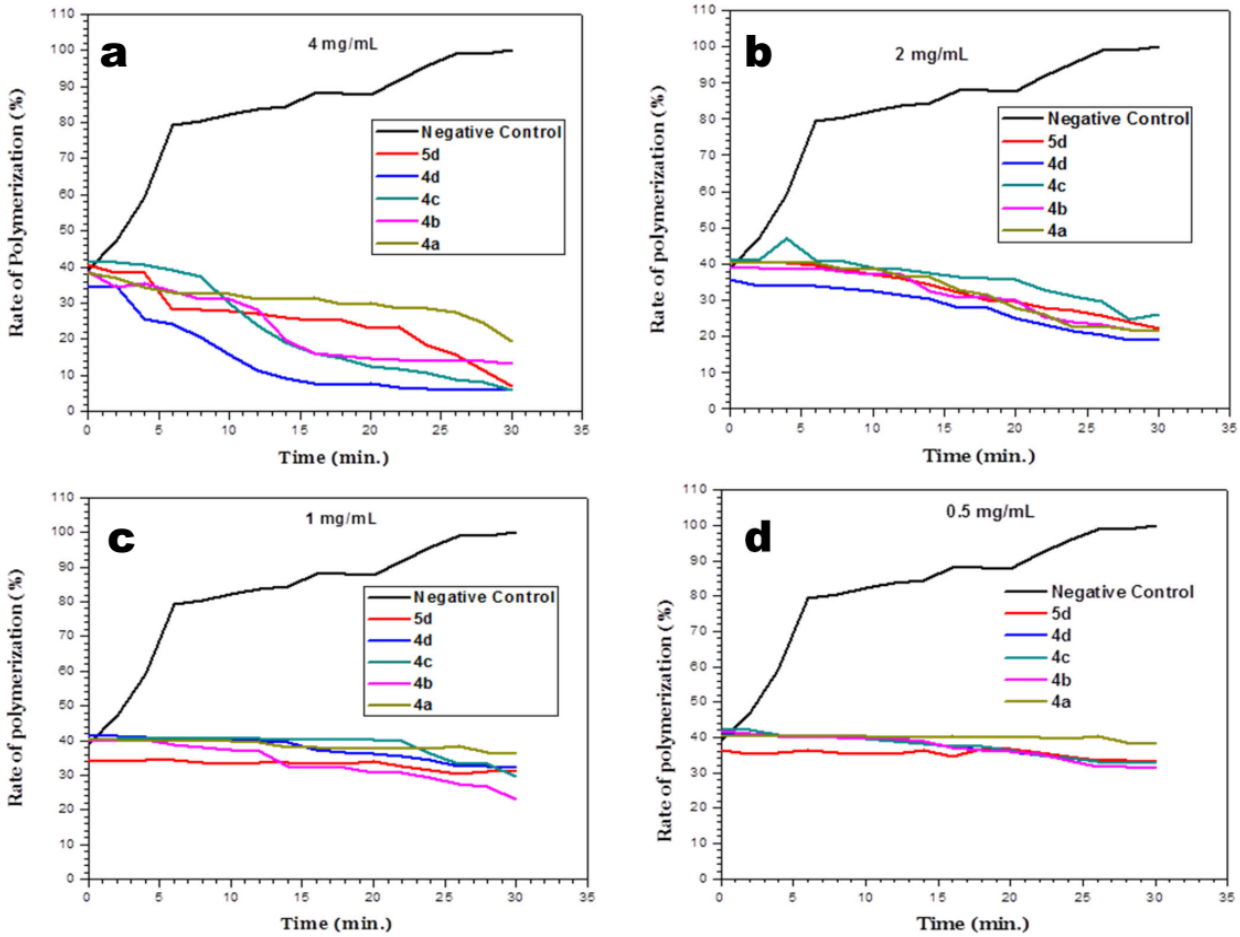


Figure 3: *Rate of HbSS polymerization in the presence of* ***(a)*** *4 mg/mL of test compounds* ***(b)*** *2 mg/mL of test compounds* ***(c)*** *1 mg/mL of test compounds* ***(d)*** *0.5 mg/mL of test compounds.*

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Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of Interest

Authors declare no conflict of interest.

Authors Contribution

Conception: TOO; Design: TOO and JMA; Execution: TOS and MCC Interpretation: TOO, TOS and MCC; Writing the paper: TOO, TOS, MCC and JMA

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