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RESEARCH ARTICLE

Novel Fluorescent Benzimidazoles: Synthesis, Characterization, Crystal Structure and Evaluation of Their Anticancer Properties

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Abstract: ***Background***: The benzimidazole core structure is an interesting platform for drug discovery since it possess a wide spectrum of pharmacological activities such as antiviral, anti-inflammatory and anticancer. Previously the antiproliferative effect of novel substituted benzimidazole derivative was demonstrated based on the ethyl 1-(2-hydroxyethyl)-2-phenyl-1H-benzo[d]imidazole-5-carboxylate scaffold through the inhibition of sirtuin activity. This work aimed to further explore the previous work for identifying novel fluorescent benzimidazoles which possess anti proliferative activities based on the reported scaffold.

Methods: Compounds were synthesized based on a multistep but facile protocol. Structure of the compounds was elucidated using NMR, FT-IR, LC-MS, elemental analysis and unambiguously confirmed through crystal X-ray diffraction. Molar extinction coefficient of the autofluorescence compounds were determined using UV spectroscopy while cancer cell growth inhibitory activity was carried out using MTS assay.

Results: Four novel benzimidazole derivatives were successfully synthesized in this study. All four compounds were found to emit blue fluorescence when light-irradiated with molar extinction coefficient ranging from 21000 to 29000 $(\text{mol L}^{-1})^{-1}\text{cm}^{-1}$. Two of the synthesized compounds showed good anti proliferative activity against four cancer cell lines tested in this study.

Conclusion: Four novel benzimidazole derivatives presented in this study were synthesized using multistep protocol starting from 4-fluoro-3-nitrobenzoic acid. Their structures have been elucidated using multiple techniques such as NMR, FT-IR, LC-MS, elemental analysis and X-ray crystallography where possible. They were found to have high autofluorescence and two of them were able to inhibit the growth of cancer cells tested in this study.

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INTRODUCTION

The benzimidazole core structure is an interesting platform for drug discovery [1] since it possess a wide spectrum of pharmacological activities such as antiviral [2], anti-inflammatory [3, 4] and anticancer [5]. The applications of benzimidazoles arewell-documented. Furthermore, their structures and physical properties are well established and characterized through techniques such as X-ray diffraction [6, 7]. Their potential as cancer therapeutics is especially very exciting. Previously the antiproliferative effect of novel substituted benzimidazole derivative was demonstrated based on the ethyl 1-(2-hydroxyethyl)-2-phenyl-1H-benzo[d]imidazole-5-carboxylate scaffold through the inhibition of sirtuin activity [8].

Encouraged by the previous findings, this study further explored the previous work for identifying novel fluorescent compounds which possess anti proliferative activities based on reported scaffold. To achieve this, the side chain substitution of the novel compounds were carefully chosen

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(electron donating/basic terminal) to ensure their high fluorescence properties. Highly fluorescent compounds which possess biological activities are currently being touted as theranostic modulators [9]. These compounds may prove useful in biomedical area especially in cell imaging. Furthermore, fluorescent molecules may have the potential in sensitizing cancer cells to radiotherapy through the *in situ* creation of reactive oxygen species [10].

Herein, this study reports the synthesis, structure, physicochemical and antiproliferative activities of these novel compounds. Structure of the compounds was elucidated through various techniques such as NMR, FT-IR, elemental as well as LC-MS analysis. Crystal structures were also determined where possible. In addition, their physicochemical attributes such as ultraviolet and fluorescent properties were also described in detail.

MATERIALS AND METHODS

All general chemicals were supplied by Sigma-Aldrich (U.S.A) and Merck Chemicals (Germany). Purity of the compounds was checked on the thin layer chromatography (TLC) plates (silica gel G) in the solvent system chloroform-methanol (9:1). The spots were located under short (254nm)/ long (365nm) UV light. Elemental analyses were performed on Perkin Elmer 2400 Series II CHN Elemental Analyzer. ^{1}H NMR was performed on Bruker Avance 500 spectrometer in either $CDCl_3$ or CD_3OD. Mass spectra were recorded on Varian 320-MS TQ LC/MS in positive electrospray ionization (ESI) mode. FT-IR was performed on Perkin Elmer Frontier Spectrometer. Crystal structure analysis was carried out using BrukerAPEX Duo CCD area-detector diffractometer. The fluorescence emission spectra were analyzed using Agilent Cary Eclipse fluorescence spectrophotometer with emission slit of 5 nm width. UV spectra were analyzed using Agilent 8453 UV-VIS spectrophotometer.

Synthesis of Compounds

Synthesis of the interested compounds was outlined as stated. Briefly, 4-fluoro-3-nitro benzoic acid (5 g, 27 mmol) was esterified in the presence of catalytic sulfuric acid (2 mL) in ethanol (50 mL) by refluxing for 8 hours to afford the ethylbenzoate **1** in 75% yield. The ethylbenzoate **1** (0.5 g, 2.34 mmol), was then treated with ethanolamine (0.15 mL, 2.58 mmol) and DIPEA (0.49 mL, 2.78 mmol) in dry dichloromethane (10 mL) at room temperature yielded ethyl 4-(2-hydroxyethylamino)-3-nitrobenzoate **2** (89%), which was then reduced to the diamine **3** using ammonium formate (0.19 g, 3 mmol) and 10% Pd/C (0.05 g) by refluxing for 3 hours. Ethyl 4-(2-hydroxyethylamino)-3-aminobenzoate **3** (0.22 g, 1 mmol) was then refluxed with various substituted bisulfite adducts (1.5 mmol) of aromatic aldehydes **4** in DMF overnight to afford **5a** (76%) and **5b** (73%). Compound **5a** and **5b** were then hydrolyzed in basic ethanol to yield **6a** (49%) and **6b** (55%) respectively.

Ethyl 2-(4-(diethylamino)phenyl)-1-(2-hydroxyethyl)-1H-benzo[d]imidazole-5-carboxylate(5a)

Cream colored powder; Yield: 76%. mp: 144-146°C. IR υ/cm^{-1}: 3120 (O-H stretch, H bonded), 3068, 2974 (C-H stretch), 1710 (C=O), 1606 & 1488 (C-C stretch, aromatic), 1381, 1294 (C-N stretch), 1207 (C-O stretch); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.15 (6H, t, J = 7.0 Hz, -N($CH_2\underline{CH_3}$)$_2$), 1.45 (3H, t, J = 7.0 Hz, $\underline{CH_3}CH_2O$-), 3.30 (4H, q, J = 7.0 Hz, -N($\underline{CH_2}CH_3$)$_2$), 4.18 (2H, t, J = 5.0 H, -N$\underline{CH_2}$-), 4.43 (2H, t, J = 5.0 Hz, -$\underline{CH_2}$OH), 4.62 (2H, q, J = 7.0 Hz, $CH_3\underline{CH_2}$O-), 6.64 (1H, d, J = 9.0 Hz, H arom.), 7.20-7.40 (2H, d, J = 9.0 Hz, H arom.) 7.50-7.70 (2H, d, J = 9.0 Hz, H arom.), 8.01 (1H, dd, J = 1.5 Hz, 9.0 Hz, H arom.), 8.15 (1H, s, H arom.). ^{13}C NMR (125 MHz, $CDCl_3$): 12.54 (-N($CH_2\underline{C}H_3$)$_2$) , 14.39 ($\underline{C}H_3CH_2O$-), 40.15 (-N($\underline{C}H_2CH_3$)$_2$), 47.00 (C8), 60.79 (C9), 60.91 ($CH_3\underline{C}H_2O$-), 109.09 (C6), 111.21 (C12), 121.54 (C3), 122.70 (C4), 124.49 (C5), 125.00 (C10), 130.44 (C11), 131.57 (C7), 138.77 (C2), 147.15 (C13), 156.12 (C1), 167.04 (C=O). ESI-MS: m/z 382.2 $[M+H]^+$. Anal. Calc for $C_{22}H_{27}N_3O_3$: C, 69.27%; H, 7.13%; N, 11.02%. Found : C, 69.06%; H, 7.11%; N, 11.24%.

Ethyl 1-(2-hydroxyethyl)-2-(4-morpholinophenyl)-1H-benzo[d]imidazole-5-carboxylate (5b)

Brown crystal; Yield 73%. mp: 181-183°C. IR υ/cm^{-1}: 3191 (O-H stretch, H bonded), 2956, 2919 (C-H stretch), 1693 (C=O), 1610 & 1500 (C-C stretch, aromatic), 1390, 1276 (C-N stretch), 1238 (C-O stretch); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.34 (3H, t, J = 7.0 Hz, $\underline{CH_3}CH_2O$-), 3.23 (4H, t, J = 5.0, -N($\underline{CH_2}$)$_2$(CH_2)$_2$O-), 3.78 (4H, t, J = 5.0 Hz, -N(CH_2)$_2$($\underline{CH_2}$)$_2$O-), 3.96 (2H, t, J = 5.0 Hz, -N$\underline{CH_2}$-), 4.34 (2H, q, J = 7.0 Hz, -$\underline{CH_2}$OH), 4.40 (2H, t, J = 5.0 Hz, $CH_3\underline{CH_2}$O-), 6.93 (2H, d, J = 9.0 Hz, H arom.), 7.58 (1H, d, J = 9.0 Hz, H arom.), 7.73 (2H, d, J = 9.0 Hz, H arom.), 8.01 (1H, dd, J = 1.5 Hz, 9.0 Hz, H arom.), 8.35 (1H, s, H arom.). ^{13}C NMR (125 MHz, $CDCl_3$): 14.42 ($\underline{C}H_3CH_2O$-), 41.03 (-N($\underline{C}H_2$)$_2$(CH_2)$_2$O-), 47.17 (C8), 59.95 (C9), 60.77 ($CH_3\underline{C}H_2O$-), 66.19 (-N(CH_2)$_2$($\underline{C}H_2$)$_2$O-), 111.13 (C6), 112.18 (C12), 114.55 (C3), 118.67 (C4), 125.20 (C5), 125.93 (C10), 126.96 (C11), 131.28 (C7), 137.40 (C2), 153.44 (C13), 154.86 (C1), 166.92 (C=O). ESI-MS: m/z 396.2 $[M+H]^+$. Anal. Calc. for $C_{22}H_{25}N_3O_4$: C, 66.82%; H, 6.37%; N, 10.63%. Found : C, 66.92; H, 6.30; N, 10.60%.

2-(4-(diethylamino)phenyl)-1-(2-hydroxyethyl)-1H-benzo[d]imidazole-5-carboxylic acid (6a)

Light brown powder; Yield: 49%.mp: 156-158°C.^{1}H NMR (500 MHz, CD_3OD): δ 1.14 (6H, t, J = 7.0 Hz, -N($CH_2\underline{CH_3}$)$_2$), 3.13 (4H, q, J = 7.0 Hz, -N($\underline{CH_2}CH_3$)$_2$), 4.12 (2H, t, J = 5.0 Hz, -N$\underline{CH_2}$-), 4.50 (2H, t, J = 5.0 Hz, -$\underline{CH_2}$OH), 6.84 (2H, d, J = 9.0 Hz, H arom.), 7.57 (1H, d, J = 9.0 Hz, H arom.) 7.92 (1H, dd, J = 1.5 Hz, 9.0 Hz, H arom.), 7.93 (2H, d, J = 9.0 Hz, H arom.), 8.22 (1H, s, H arom.). ^{13}C NMR (125 MHz, CD_3OD): 12.60 (-N($CH_2\underline{C}H_3$)$_2$), 41.02 (-N($\underline{C}H_2CH_3$)$_2$), 47.18 (C8), 61.55 (C9), 112.29 (C6), 115.18 (C12), 118.92 (C3), 121.10 (C4), 123.89 (C5), 125.43 (C10), 130.50 (C11), 134.23 (C7), 142.45 (C2), 144.71 (C13), 154.86 (C1), 170.04 (C=O). ESI-MS: m/z 354.2 $[M+H]^+$. Anal. Calc for $C_{20}H_{23}N_3O_3$: C, 67.97%; H, 6.56%; N, 11.89%. Found : C, 68.07%; H, 6.82%; N, 11.66%.

1-(2-hydroxyethyl)-2-(4-morpholinophenyl)-1H-benzo[d]imidazole-5-carboxylic acid (6b)

Light brown powder; Yield 55%. mp: 200-202°C. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.21 (4H, t, J = 5.0, -N($\underline{CH_2}$)$_2$(CH_2)$_2$O-), 3.86 (4H, t, J = 5.0 Hz, -N(CH_2)$_2$($\underline{CH_2}$)$_2$O-),

4.20-4.40 (4H, m), 6.50-6.70 (2H, d, J = 9.0 Hz, H arom.), 7.21 (1H, d, J = 9.0 Hz, H arom.), 7.33 (1H, d, J = 9.0 Hz, H arom.), 7.41 (1H, d, J = 9.0 Hz, H arom.), 7.72 (1H, dd, J = 1.5 Hz, 9.0 Hz, H arom.), 7.76 (1H, s, H arom.). ^{13}C NMR (125 MHz, $CDCl_3$): 40.10 (-N($\underline{C}H_2$)$_2$(CH_2)$_2$O-), 47.09 (C8), 60.81 (C9), 66.87 (-N(CH_2)$_2$($\underline{C}H_2$)$_2$O-), 110.12 (C6), 112.26 (C12), 114.46 (C3), 115.05 (C4), 122.78 (C5), 124.31 (C10), 127.05 (C11), 129.48 (C7), 135.63 (C2), 152.21 (C13), 153.07 (C1), 167.34 (C=O). ESI-MS: m/z 368.2 $[M+H]^+$. Anal. Calc. for $C_{20}H_{21}N_3O_4$: C, 65.38%; H, 5.76%; N, 11.44%. Found : C, 65.09; H, 5.90; N, 11.51%.

Single-crystal X-Ray Structure Determination

X-ray data of **5b** was collected by using Bruker APEX DUO CCD area detector diffractometer [11] with Mo Kα radiation (λ = 0.71073 Å). The crystal was placed in the cold stream of an Oxford Cryosystems Cobra open-flow nitrogen cryostat [12] operating at 100 (1) K. SAINT and SADABS programs were employed to perform data reduction and absorption correction. The structure of **5b** was determined using direct methods and further refined by full-matrix least squares tehniques using F^2 using SHELXTL package [13]. The hydroxyl O atom was located using difference Fourier map and refined freely [d(O3−H1O3) = 0.84(2) Å]. The co-crystallized water molecule was partially occupied with the site occupancies of 0.15 and 0.30 for O and H atoms, respectively, since the O atom, O1W, resided on a two-fold axis. The hydrate H atom with low residual density peak was located using difference Fourier map, fixed at the original position with AFIX 3 constraint and refined freely [d(O1W−H1W1) = 0.8509Å]. The remaining C-bound H atoms were calculated geometrically and refined with U_{iso}(methine or methylene H) = 1.2 U_{eq}(C)and U_{iso}(methyl H) = 1.5 U_{eq}(C). A riding model (AFIX 137) was applied to methyl group.

Molar Extinction Coefficient

Molar extinction coefficient, ε, of the compounds was determined using a UV spectroscopy method. A series of dilution (100, 50, 20, 10, 5, 1 μM) was made by dissolving the test compound in DMSO. These solutions were analyzed by UV spectroscopy and their absorbance at their respective λ_{max} was plotted against the concentration to generate a calibration curve. The molar extinction coefficient was calculated from the gradient of the optimal line.

Cell Proliferation Assay

All cell lines were obtained from the American Type Culture Collection (Rockville, MD). The cells were seeded in 96-well plates at a density of 5×10^3 per well. The cells were treated with interested compounds and allowed to adhere for 72 hours. Then, the proliferative activity was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI) to monitor the number of viable cells according to the manufacturer's instructions. Briefly, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, the inner salt solution was added at 20 μL/well, and after 1 hour of incubation at 37°C in a humidified 5% CO_2 atmosphere, the conversion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, the inner salt to formazan was measured in a plate reader at 490 nm. All experiments were done in triplicate, and the proliferation rate was calculated as the ratio of the absorbance under each experimental condition to that of the control.

RESULTS AND DISCUSSION

Four novel benzimidazoles were designed and synthesized according to Fig. (**1**) as shown below. The first step involved the esterification of the starting material 4-fluoro-3-nitro benzoic acid. Following this, the F atom was replaced with ethanolamine through nucleophilic substitution. The third step of the synthesis involved reduction of the nitro group to the amino group to prepare the intermediate for cyclization. Finally, the last step involved formation of the benzimidazole core with various sodium bisulfite adducts attached with either a diethylamino (**5a** and **6a**) or morpholinyl (**5b** and **6b**) substitution at the *para* position of the benzene ring.

Structures of the newly prepared compounds (Fig. **2**) were determined by NMR, FT-IR, elemental as well as LC-MS analysis. The detailed examination of ^{1}H NMR was based on the analysis of the number of protons and the peak splitting (H-H coupling constants) as well as the chemical shifts. The diethylamino group from **5a**/**6a** was confirmed by the presence of a triplet at δ 1.15/1.14 ppm (6H, -N($CH_2\underline{CH_3}$)$_2$) and a quartet at δ 3.30/3.13 ppm (4H, -N($\underline{CH_2}CH_3$)$_2$). The morpholinyl group from **5b**/**6b** was confirmed by the presence of a pair of triplets at δ 3.23/3.21 ppm and 3.78/3.86 ppm. The absence of ester group from the ^{1}H NMR spectrum of **6a** and **6b** indicated that both compounds have been successfully hydrolyzed.

Infrared spectrum of **5b** also demonstrated the presence of O-H stretching band at ~ 3200-3100 cm^{-1} region, implying that the OH group was H bonded. This observation was further confirmed by the single-crystal X-ray analysis. The single crystal X-ray structure of **5b** was successfully obtained when the crude compound was recrystallized from ethyl acetate. Detailed analysis of the crystal structure revealed that the asymmetric unit of **5b** (See supplementary data) consists of a discrete ethyl 1-(2-hydroxyethyl)-2-(4-morpholinophenyl)-1*H*-benzo[*d*]imidazole-5-carboxylate-molecule and 15% partially occupied co-crystallized water molecules. The benzimidazole ring (N1/C1-C6/N2/C7) made a dihedral angle of 42.94(4)° with adjacent phenyl ring (C13-C18). The morpholine ring (N3/C19/C20/O4/C21/C22) adopts a chair conformation with puckering parameters Q = 0.5576(17) Å, θ= 2.00(16)° and φ = 343(6)° [14]. In the crystal of **5b**, strong intermolecular O—H···N hydrogen bond joined benzimidazole-derived molecules into face-to-face dimers which were stabilized by π···π interactions, involving the centroids of phenyl and imidazole rings in the benzimidazole group [centroid-to-centroid distances: 3.5591(8) Å, 3.5941(8) Å, and 3.6625(8) Å, symmetry code: $-x+1$, y, $-z+1/2$]. O(water)—H···O hydrogen bond was observed in between two dimers along the crystallographic *a*-axis. Weak non-classical C—H···O hydrogen bonds linked dimers into a three-dimensional network (See supplementary data).

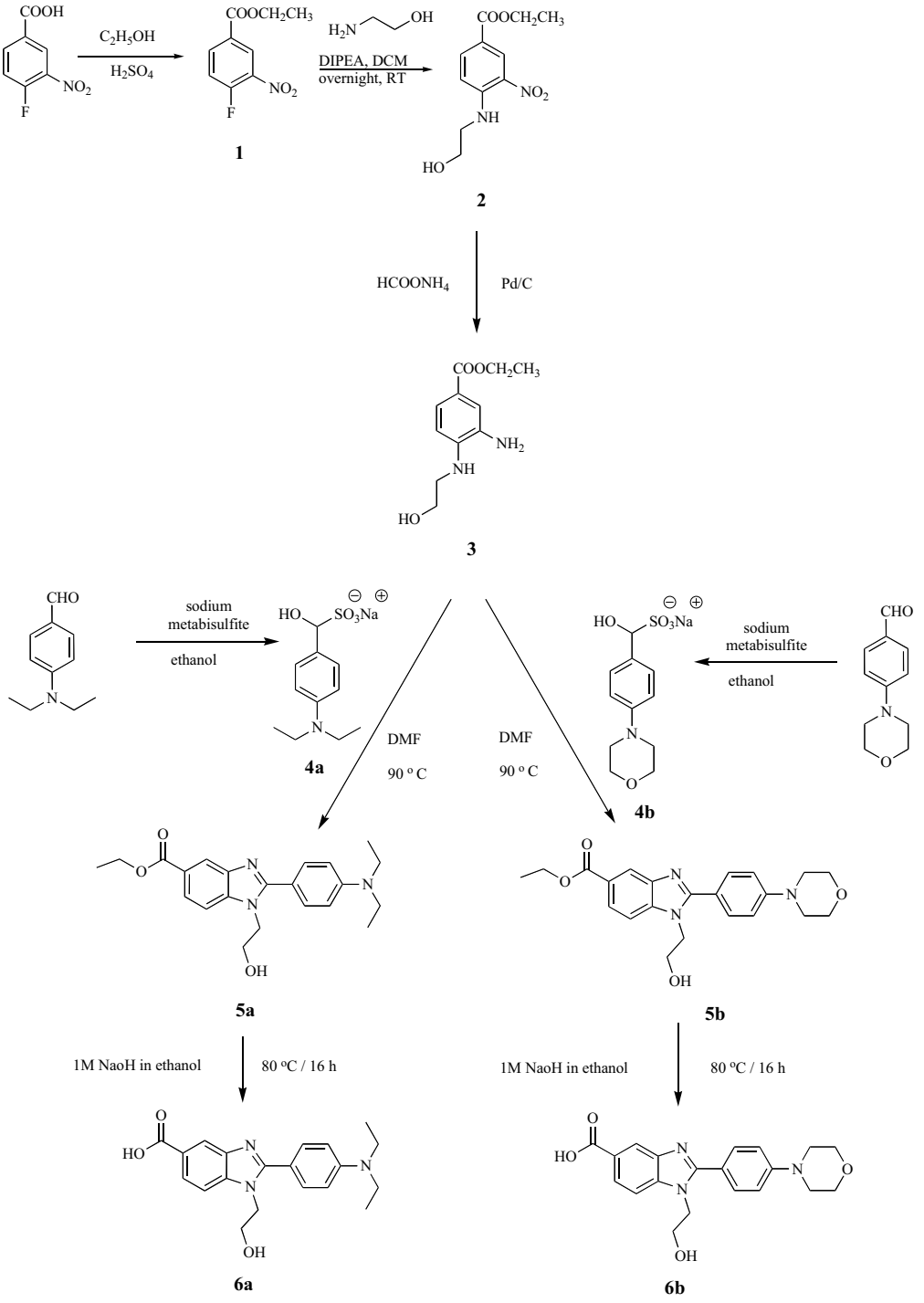

Fig. (1). Synthesis protocol of titled compounds.

Elemental analysis showed that the observed C/H/N values were within ± 0.4% of the calculated values. On top of that, direct infusion mass spectrum of **5a, 5b, 6a** and **6b** showed the major molecular mass at m/z 382.2 $[M+H]^+$, 396.2 $[M+H]^+$, 354.2 $[M+H]^+$ and 368.2 $[M+H]^+$ respectively, which corresponds to their calculated mass.

The antitumor activity of benzimidazole analogs based on the ethyl 1-(2-hydroxyethyl)-2-phenyl-1H-benzo[d] imidazole-5-carboxylate scaffold was previously shown. Thus, the newly synthesized compounds were screened for their anti-proliferative effect against MDA-MB-468, MCF7, CCRF-CEM and HT29 cancer cells. Interestingly, both **5a** and **5b** demonstrated good cytotoxic effect against all four cancer cell lines with MCF7 cancer cells being the most susceptible towards their effect (Table **1**).

However, both **6a** and **6b** showed markedly weaker inhibitory effect compared to their ester analogues. One of the possible reasons is that the ionization of the carboxylic acid group renders them more difficult to diffuse across the lipophilic cell membrane. This suggests that capping the end

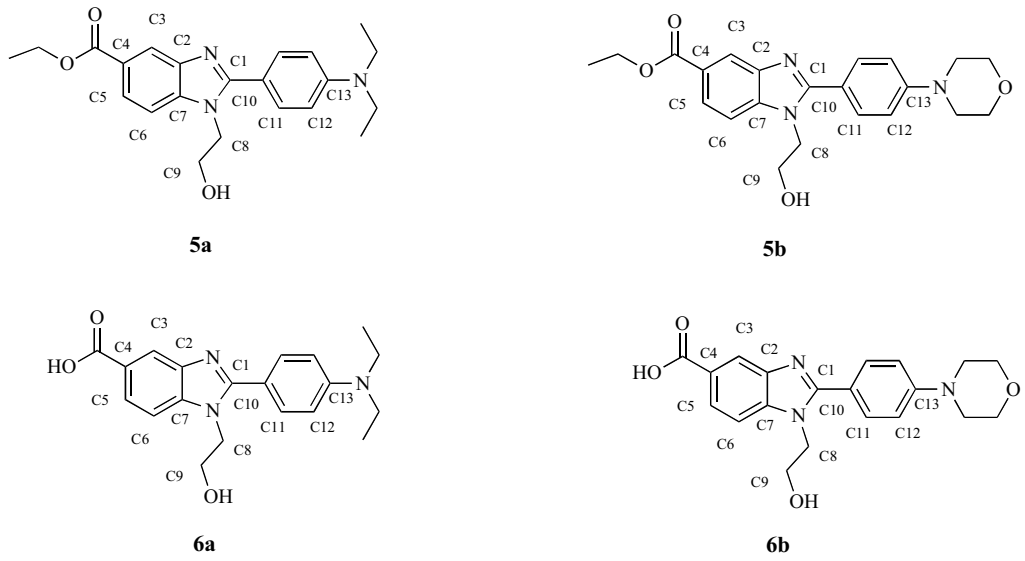

Fig. (2). Structure of synthesized compounds (**5a, 5b, 6a** and **6b**).

Table 1. Inhibitory activity of the newly synthesized compounds against different cancer cell lines.

Compound	IC_{50} (μM)			
	MDA-MB-468	**MCF-7**	**CCRF-CEM**	**HT29**
5a	40.1 ± 1.8	21.2 ± 1.7	42.8 ± 1.1	29.3 ± 1.3
5b	46.7 ± 1.5	30.9 ± 1.1	49.5 ± 1.0	35.5 ± 1.2
6a	> 50	> 50	> 50	> 50
6b	> 50	> 50	> 50	> 50
Doxorubicin	6.6 ± 0.5	8.0 ± 0.5	10.6 ± 0.4	3.7 ± 0.5

Table 2. Fluorescence properties of the novel benzimidazole derivatives.

Compound	Wavelength /cm^{-1}	Molar Extinction Coeffiecient, ε /$(mol\ L^{-1})^{-1}cm^{-1}$
5a	λ_{ex} = 337 nm; λ_{em} = 431 nm	24000 (± 15%)
5b	λ_{ex} = 322 nm; λ_{em} = 429 nm	29000 (± 15%)
6a	λ_{ex} = 347 nm; λ_{em} = 435 nm	21000 (± 15%)
6b	λ_{ex} = 339 nm; λ_{em} = 434 nm	23000 (± 15%)

of a charged terminal may facilitate the permeability of the compound into cells.

One interesting aspect of the newly synthesized compounds is the discovery of their fluorescence attribute. All four compounds were found to emit blue fluorescence when light-irradiated (Table **2**). They were found to be highly fluorescent with molar extinction coefficient ranging from 21000 to 29000 $(mol\ L^{-1})^{-1}cm^{-1}$. The autofluorescent properties of the compounds may prove useful in monitoring morphological and phenotype changes in cancer cells as well as other imaging studies.

CONCLUSION

In conclusion,the novel compounds presented in this study were synthesized using multistep protocol starting from 4-fluoro-3-nitrobenzoic acid. Their structures have been elucidated using multiple techniques such as NMR, FT-IR, LC-MS, elemental analysis and X-ray crystallography where possible. An interesting aspect of this study was the discovery that all the four synthesized compounds (**5a, 5b, 6a** and **6b**) possessed high autofluorescence attributes. They were found to emit blue fluorescence with molar extinction coefficient ranging from 21000 to 29000 $(mol\ L^{-1})^{-1}cm^{-1}$.

Moreover, compound **5a** and **5b** were found to be active against four cancer cell lines tested in this study, with MCF7 breast cancer cells the most susceptible to their effect. Highly fluorescent compounds which possessed anti-cancer activities such as **5a** and **5b** should be further explored to increase their potency. Further work in this area is currently going on in the laboratory.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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