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Synthesis and cytotoxicity study of novel 3-(triazolyl)coumarins based fluorescent scaffolds

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ABSTRACT: Recently a choice of fluorescent bioimaging probes have been developed as medical diagnostic tools. Herein, we have introduced a series of coumarin-based target specific probes for cancer theranostic application which play a dual role in the field of both diagnosis and therapy. A fluorogenic version of 1, 3-dipolar cycloaddition between azides and alkynes (DBCO) has been introduced to develop the triazolylcoumarin based fluorescent scaffolds. These scaffolds were screened for their anticancer activity against breast cancer (MCF7) & human Epitheloid Cervix Carcinoma (HeLa) cell line. It was established that traizolylcoumarins (**5c** & **5d**) are having electronegative substitution in the benzene ring displayed most effective anticancer profile in both the cell lines. Compound **5a** & **5d** exhibited maximum quantum yield and strong cellular uptake in the MCF-7 cell line.

Keywords: *triazolylcoumarin, anticancer activity, theranostic application, fluorescence study, Fluorescence imaging*

The term "theranostic" signifies a specific combination of the diagnostic and therapeutic capabilities into a single agent (called "companion diagnostic"). This includes the use of diagnostic tests to identify a particular disease, select a treatment regimen for it and monitor the patient compliance. The diagnostic tests are used to identify a biomarker which allows the use of the new drug.[1] Theranostic promises significantly in the area of personalized medicine which customizes healthcare to individual patients and for better patient compliance. The efficient process of theranostic development is quite complex as it requires a stepwise phasing from development to remuneration. All the stages of the system should be harmonized to process simultaneous availability and accessibility of the drugs. In spite of this, it is essential to simplify the rules and regulations to create a better modification of theranostic towards the robustness of invention.[2] The execution of theranostic is challenging since it possess considerable economic disputes for industrial organisations as well as the process and timelines for drug and test development are different from one another.

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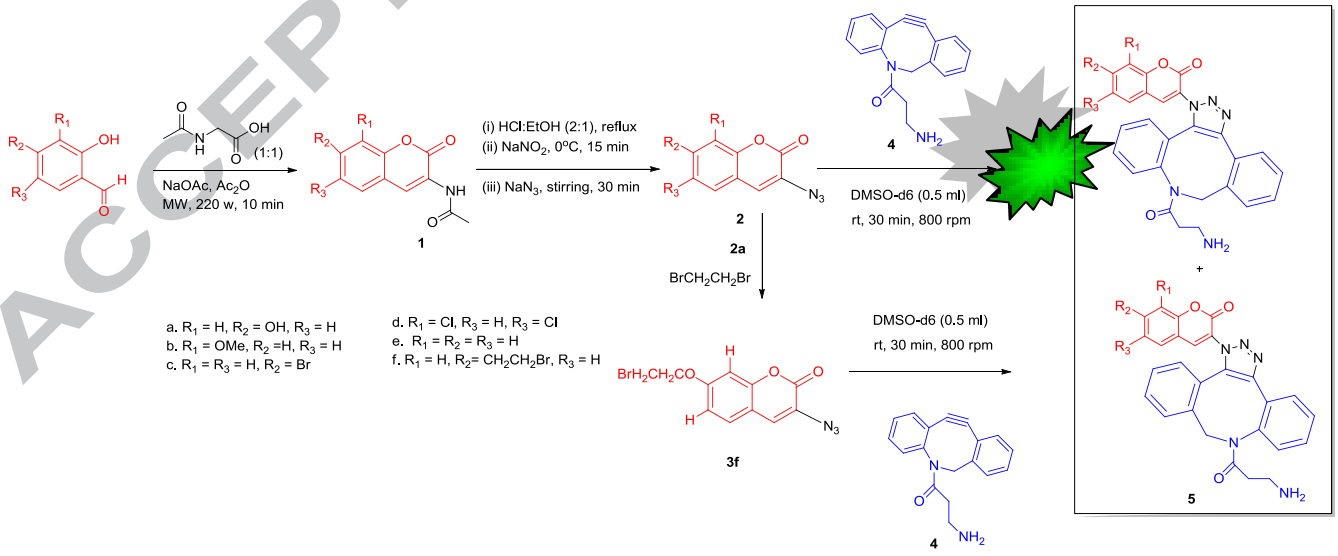
"Click chemistry" is a selective, quantitative, cost effective and pH-insensitive green approach,[3,4] and hence this approach is useful for the synthesis of a variety of bio conjugates including peptides,[5] proteins,[6] polysaccharides,[7] and even entire viruses[8] and cells.[9] However, the conventional "Click Chemistry" requires Cu(I) catalyst for 1,3-dipolar cycloaddition between azides and terminal alkynes which is toxic to most organisms[10] and thus, prevents its use in many biological systems. The novel Copper-free Click Chemistry is based on the reaction of a cyclooctyne (DBCO) moiety with an azide-labelled reaction partner, known as strain-promoted alkyne azide cycloaddition (SPAAC).[11] SPAAC has several benefits over the conventional click technique as it is extremely fast at room temperature, biocompatible, bioorthogonal along with highly specific and stable in nature.[12]

3-Azido coumarin, a profluorophore, reacts with terminal alkynes to generate highly fluorescent triazole compounds which are very useful for biological applications, especially for *in vivo* labelling.[13] Furthermore, substitutions at the 3- and 7-positions of coumarin dyes are known to have a strong impact on their fluorescence properties.[14] Coumarin derivatives have also been developed as an anti-tumour agents and their metabolite 7-hydroxycoumarin was tested in several human tumour cell lines.[15] However, the biological actions of coumarins are greatly enhanced by attaching the triazole rings with these moieties. These unique templates have been associated with anti-viral, anti-fungal, anti-tumour, anti-bacterial, anti-inflammatory and also CNS activities.[16] These scaffolds were also reported as anti-microbial, anti-inflammatory, anti-tubercular, anti-HIV, anti-malarial, cardiovascular and diuretic agents.[17-20] In light of these research works going on, we have successfully developed a series of azido coumarins which on further treatment with DBCO provide a class of fluorescent triazoles for theranostic application.

Our approach was to develop a new class of coumarin-based fluorogenic probes having cytotoxicity against various cancer cell lines by the application of 1,3-dipolar cycloaddition of azides and alkynes. Initially, 3-acetamido coumarin analogues (**1a-e**) were synthesized by using a choice of substituted salicylaldehyde and *N*-acetyl glycine in presence of acetic anhydride under microwave conditions. Microwave is an efficient tools for the construction of carbon-carbon building blocks with high reaction rate and yield.[21] These coumarins were then refluxed with HCl/Ethanol (1:1) mixture for 2 h and further treated with sodium nitrite followed by sodium azide to get the desired 3-azido coumarin derivatives (**2a-e**) (**scheme 1**).[22] Subsequently, Compound **2a** was treated with dibromoethane for the formation of desired

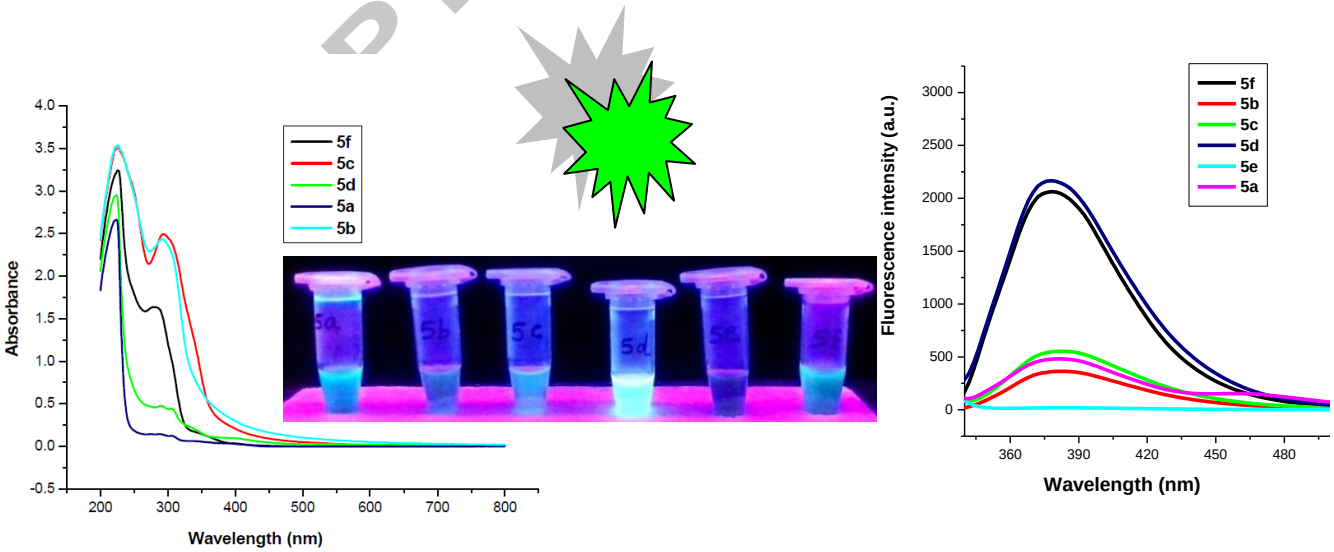
bromoalkoxyazidocoumarin (**3f**). Briefly, compound **2a** was dissolved in acetone, K_2CO_3 (1:10) was added to the reaction mixture and the resulting solution was refluxed for 30 min at 80°C. Thereafter, dibromoethane (1:12) was added to it and the mixture was refluxed for 5 hours. Progress of the reaction was monitored by TLC in hexane/ethyl acetate (3:1) solvent system. After complete conversion of the starting materials, the solution was filtered and the filtrate was evaporated to dryness to get the desired product (**3f**). The structure of compound **3f** was confirmed by ^{1}H-NMR spectroscopy and ESI-MS. The characteristic peaks of two CH_2 exhibited at σ 3.65 & 4.35 as two distinct triplets. Subsequently, DBCO (**4**) was treated with 3-azidocoumarin analogues (**2a-e**, **3f**) in 0.5 ml of DMSO-*d6* at ambient temperature for 30 min. The progress of the reaction was monitored by TLC. The reaction resulted in the formation of two distinct regioisomers (1,4-triazole and 1,5-triazole regioisomer), which is a well known phenomenon for copper free click reaction. But in all the cases we could not isolate the regioisomer even after HPLC purification.[23] The structure of triazolylcoumarins (**5a**) was further confirmed by ^{1}H NMR, ^{13}C NMR and Mass spectroscopy. The characteristic peaks of three CH_2 and one NH_2 were observed in the range of σ 1.86-1.88, 2.62, 3.18 and 5.04-5.07 ppm respectively. In the ^{13}C NMR spectra characteristic peaks of tertiary amide & ketone was observed at σ 169.8 and 159.0 ppm respectively. Likewise, three CH_2 peaks were appeared in the range of σ 33-54 ppm. The quantitative formation of all the 3-(triazolyl)coumarins derivatives (**5a-f**) were supported by NMR & Mass spectroscopy.[24]



Scheme 1: Plausible pathway for the synthesis of 3-(triazolyl)coumarin **(5a-f)**

The fluorescence spectra of compounds **5a-f** were determined at concentrations of $3x10^{-6}$mol L^{-1} in water. Emission spectra of compounds **5a-f** are presented in **Figure 1**. The fluorescence excitation wavelengths (λex/nm) at 315, 296, 315, 325, 325 & 300 nm were used for compounds **5a**, **5b**, **5c**, **5d**, **5e** and **5f** respectively. It was also observed that all the compounds are significantly fluorescent in water (**Figure 1**, **Table 1**). The characteristic fluorescence of compound **5d** in DMSO-*d6* (reaction solvent) was depicted in **Figure 2**.

We have calculated the emission of quantum yield (**Φ**) of the fluorescence active compounds in water (**Table 1**). The **ΦR** was calculated by using below formula, **Φ = (ΦR * IS * ODR * ηs) / (IR * ODS * ηR)** Where **ΦR** = emission of quantum yield of reference, **IS** and **IR** = integral area of reference and sample respectively, **ODS** and **ODR** = excited absorbance of sample and reference respectively, **ηS** and **ηR** = refractive index of sample solvent and reference solvent respectively. Here, we have used Quinine Sulphate as a standard for calculating emission of quantum yield (**Table 1**). We have used 0.5 M H_2SO_4 & water as solvent for the reference and synthesized compounds respectively. From **Table 1** we found that compound **5a** & **5d** exhibited best quantum yields (~ 0.20) among all the synthesized scaffolds.



(a) (b) (c)

Figure 1. UV & Fluorescence emission spectra of 3-(triazolyl)coumarin **(5a-f)** in water

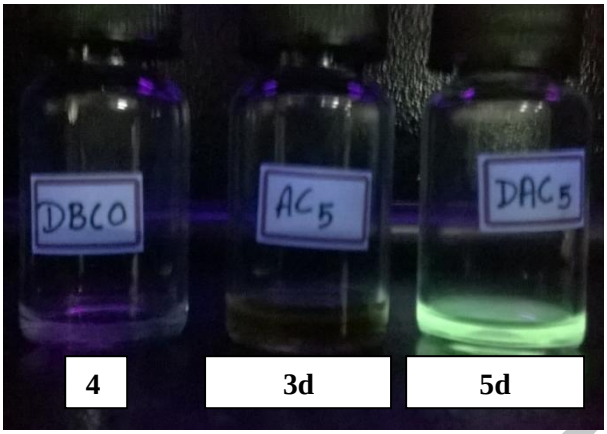


Figure 2 Fluorescence images of Starrting material (**4, 3d**) & product (**5d**) in DMSO-*d6* under UV chamber (λ)

Table 1. Spectroscopic data for 3-(triazolyl)coumarin **(5a-f)** at 298 K in water.

Entry	λ_{max}[a] (abs,nm)	λ_{max}[b] (em,nm)	Stokes Shift (nm)	OD[c]	I[d]	Φ[e]
5a	315	382	67	0.12	79195	0.16
5b	296	382	86	2.4	46928	0.00478
5c	315	382	67	2.46	68596	0.006
5d	325	378	53	0.24	182975	0.20
5e	325	384	59	1.68	7867	0.0011
5f	300	378	78	1.3	168450	0.04
Quinine Sulphate	350	452	102	0.029	63905	0.57

[a]abs = absorbance; [b]em = emission; [c]OD = excited absorbance; [d]I = integral area; [e]Φ = emission of quantum yield

The cytotoxicity of all the synthesized compounds (**2a–e** & **5a-f**) (**Table 2**) was evaluated using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay beside a panel of cell lines such as human epithelioid cervix carcinoma (HeLa) and human breast carcinoma cell line (MCF-7) in triplicates. These cells were incubated with compounds (**2a–e** & **5a-f**) along with Cisplatin and Doxorubicin as standard drugs at concentration 1–100 µM for 24

h. Most of these triazolocoumarin (**5a-f**) scaffolds exhibited comparable anticancer activity with cisplatin. However, these compounds showed less potency than doxorubicin. Likewise, azidocoumarin analogues (**2a-e**) & DBCO (**4**) were not showing any potency in both the cell lines. DMSO was used as control where it was also not showing any inhibition of cell. In MCF-7 cells, the IC_{50} values for triazolylcoumarin derivatives were in the range of 9–21μM (**Table 2**). Similarly, in HeLa cells, the IC_{50} values were found in the range of 17–26 μM. In the literature, it was found that substitution of strong electronegative atom such as chloro/bromo at the para position of the aromatic ring increases the lipophilicity of molecules which is accountable for enhanced cytotoxicity in MTT model.[25] In our present work, it was observed that compounds **5c** & **5d** having halogen substitution in the benzene ring shows very significant cytotoxicity in both the cells whereas corresponding azidocoumarin derivatives (**2c** & **2d**) did not show any potency. Moreover, starting DBCO moiety was also not showing any potency in both the cancer cell lines. Therefore, the cyclooctyne fused triazole moiety might be the key template for high potency in cancer cell lines. The structure–activity relationship studies revealed that the introduction of electronegative chlorine & bromine atom in the benzene ring (compound **5c** & **5d**) enhanced the cytotoxicity. While, the anticancer efficacy is being highly reduced once introduction of electron releasing groups (OH, OMe, alkyl) in the triazolylcoumarin rings (**5a**, **5b**, **5f**). Herein, we have used normal human fetal lung fibroblast (MRC-5) as a model of healthy cell to estimate the selective potency of these scaffolds in cancer cell lines with respect to normal cell line. It was observed that most of the 3-(triazolyl)coumarins are 3-11 fold more selective in cancer cell line than normal fibroblast. Among all these scaffolds, compound **5d** displayed best potency & selectivity profile in all the reported cancer cell lines. While, cisplatin was not exhibited any selectivity in those cancer cell lines.

Table: 2 Preliminary MTT cytotoxicity screening of synthesized triazolylcoumarin derivatives (**5a-f**), azidocoumarins (**2a-2e**) and DBCO (**4**) at 24 h of drug exposure

Compounds	**IC50 (μM)**[a]			**SF**[b]	
	Cell lines				
	HeLa	MCF-7	MRC-5	HeLa	MCF-7
5a	21.12±0.91	18.4±0.87	90.2±0.87	4.27	4.90
5b	26.21±0.64	21.87±1.67	92.8±1.54	3.54	4.24
5c	18.24±1.15	10.65±0.68	100.2±1.88	5.49	9.40
5d	17.5±1.22	9.83±0.69	185.22±1.65	10.58	18.84
5e	19.8±0.88	12.66±1.11	110±1.43	5.55	8.68
5f	19.5±0.66	18.51±0.90	120.87±1.88	6.19	6.52
2a	41.62±0.81	38.4±0.97	62.7±1.44	1.50	1.63
2b	36.41±0.54	31.54±2.17	58.66±1.89	1.61	1.85
2c	28.34±1.15	23.65±0.68	70.4±1.66	2.48	2.97
2d	27.6±1.22	18.63±1.69	67.2±0.88	2.43	3.60
2e	29.6±1.88	26.66±1.11	70.2±1.11	2.37	2.63
4	32.06±1.22	28±1.22	60.4±1.11	1.88	2.15
DMSO	-	-	-	-	-
CisPlatin	18±0.5	10±0.6	20.2±1.2	1.12	2.02
Doxorubicin	5.2±0.92	3.83±0.49	70.6±1.55	13.57	18.43

[a]IC_{50} is the concentration at which 50% of cells were undergoing cytotoxic cell death due to synthesized compound treatment. [c]SF(selectivity factor) = ratio of IC_{50} for MRC-5/IC_{50} for all the cancer cell lines. MRC-5 fibroblasts are typically chosen as models for health cells to evaluate the selectivity of chemotherapeutic drugs.

We performed cellular imaging experiment using MCF-7 cell line. MCF-7 cells were incubated for 2 hour at 37° C with compound **5a** & **5d** (20 μM). We observed strong green fluorescence in live cells using excitation and absorbance filters 320 and 400 nm, respectively (**Figure 3**). In Figure 3, a strong cellular uptake of compound **5a** & **5d** was observed. Hence, we concluded that compound **5a** & **5d** might be applicable as cancer theranostic for its high quantum yield as well as high potency & selectivity in cancer cell lines.

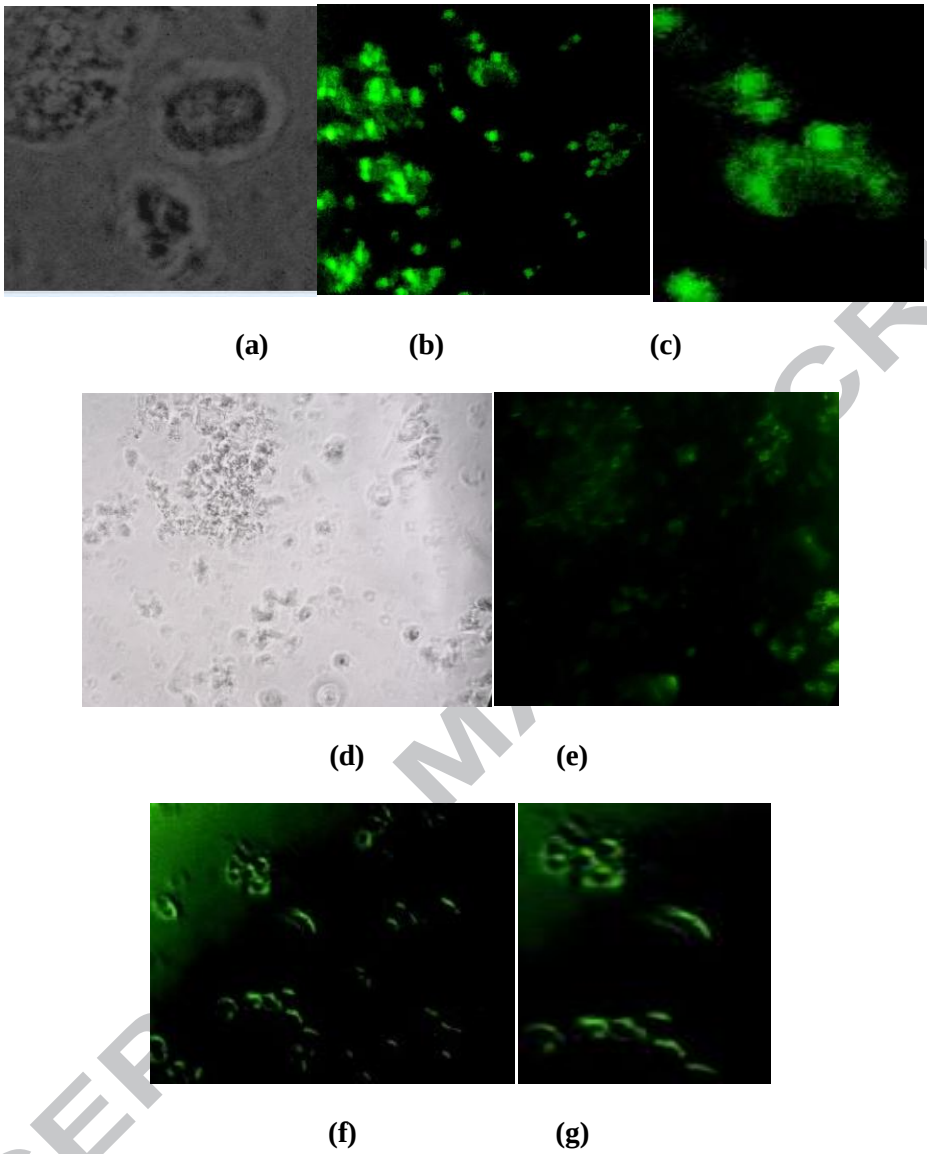


Figure 2. Fluorescence microscopy images and phase photos of live cells: (a) Phase photo of MCF-7 cells without the drug (b) Fluorescent image of MCF-7 with compound **5d** (20 µM in PBS buffer); incubation time 2 h (c) Fluorescent image of MCF-7 with compound **5d** (20 µM in PBS buffer); incubation time 2 h, zoom scan (d) Phase photo of MCF-7 cells with compound **5d**; 8 h incubation (e) Fluorescent image of MCF-7 with compound **5d** (20 µM in PBS buffer); incubation time 8 h (f) Fluorescent image of MCF-7 with compound **5a** (20 µM in PBS buffer); incubation time 2 h (g) Fluorescent image of MCF-7 with compound **5a** (20 µM in PBS buffer); incubation time 2 h, zoom scan

Conclusion

In review, a class of 3-(triazolyl)coumarins derivatives was synthesized using a simplest & effective technique. This technique allows a great compact of synthetic flexibility and offers the opportunity of synthesizing newer 3-(triazolyl)coumarins analogues for anticancer screening. Interestingly, 3-(triazolyl)coumarins (**5d**) is having two electronegative chlorine substitutions in the benzene ring shows most potency in both the cancer cell lines. Compound **5d** might be utilized for cancer theranostic because of its high quantum yield, high potency and strong cellular uptake.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/....

†Equal contribution

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24. Preparation of dibenzo triazolo[4,5-d] azocinyl chromen-2-one (5a-f): 3-Azido coumarin derivatives (**2a-e, 3f**) were shaken with DBCO (**4**) (1:1 mole ratio) in 0.5 ml of DMSO-d^6 at room temperature for 30 minutes at 1000 rpm. The reaction was completed within 30 minutes which was confirmed by TLC. Consequently, NMR was performed with the solution. The Solvent has been evaporated and a mixture of regioisomers (1:1) was formed with high yield.

3-(8-(3-aminopropanoyl)-8,9-dihydro-3H-dibenzo[b,f][1,2,3]triazolo[4,5-d]azocin-3-yl)-7-hydroxy-2H-chromen-2-one (**5a**): Yield: 95%; mp: 128-130°C; R_f (2% MeOH in EA): 0.36; ^{1}H NMR (400 MHz, DMSO-d^6): σ 1.86-1.88 (m, 2H, CH_2), 2.62 (brs, 2H, CH_2), 3.18 (s, 2H, CH_2), 5.04-5.07 (m, 2H, NH_2), 7.23-7.40 (m, 7H, ArH), 7.48-7.51 (m, 3H, ArH), 7.63-7.65 (m, 2H, ArH); ^{13}C NMR (125 MHz, DMSO-d^6): σ 33.4 (CH_2), 35.8 (CH_2), 54.9 (CH_2), 101.8 (CH), 107. 9 (CH), 109.0 (CH), 113.0 (CH), 114.3 (CH), 121.5 (CH), 122.3 (CH), 125.2 (CH), 125.7 (C), 126.9 (CH), 127.8 (C), 128.1 (CH), 128.4 (C), 128.8 (C), 129.1 (CH), 129.6 (CH), 130.3 (C), 132.4 (CH), 148.2 (C), 149.3 (C), 150.9 (C), 156.2 (-COH), 159.0 (-OCO), 169.8 (-NCO); ESI-MS (MeOH): 480 $[M+H]^+$.

3-(8-(3-aminopropanoyl)-8,9-dihydro-3H-dibenzo[b,f][1,2,3]triazolo[4,5-d]azocin-3-yl)-6-bromo-2H-chromen-2-one (**5c**): Yield: 97%; mp: 138-140°C; R_f (2% MeOH in EA): 0.48; ^{1}H NMR (400 MHz, DMSO-d^6): σ 1.84-1.88 (m, 2H, CH_2), 2.61-2.66 (m, 2H, CH_2), 3.63 (s, 2H, CH_2), 5.0-5.05 (m, 2H, NH_2), 7.03 (d, 1H, J = 8.8 Hz, ArH), 7.25 (brs, 1H, ArH), 7.28-7.30 (m, 1H, ArH), 7.32-7.38 (m, 3H, ArH), 7.44-7.49 (m, 4H, ArH), 7.61-7.63 (m, 2H, ArH); ^{13}C NMR (125 MHz, DMSO-d^6): σ 35.9 (CH_2), 36.9 (CH_2), 54.8 (CH_2), 103.7 (CH), 108.1 (CH), 114.2 (CH), 115.7 (CH), 116.6 (C), 121.4 (C), 122.3 (C), 122.4 (C), 123.8 (C), 125.2 (CH), 126.7 (CH), 127.7 (CH), 128.1 (CH), 128.2 (CH),

128.8 (C), 128.9 (CH), 129.3 (C), 129.6 (CH), 132.4 (CH), 144.6 (C), 148.3 (C), 151.4 (C), 154.0 (-OCO), 170.5 (-NCO); ESI-MS (MeOH): 542 $[M+H]^+$.

3-(8-(3-aminopropanoyl)-8,9-dihydro-3H-dibenzo[b,f][1,2,3]triazolo[4,5-d]azocin-3-yl)-6,8-dichloro-2H-chromen-2-one (**5d**): Yield: 90%; mp: 129-131°C; R_f (2% MeOH in EA): 0.44; ^{1}H NMR (400 MHz, DMSO-d^6): σ 1.86-1.93 (m, 2H, CH_2), 2.68-2.76 (m, 2H, CH_2), 3.20 (s, 2H, CH_2), 5.01-5.06 (m, 2H, NH_2), 6.99 (s, 1H, ArH), 7.28-7.30 (m, 1H, ArH), 7.32-7.39 (m, 3H, ArH), 7.45-7.49 (m, 3H, ArH), 7.57 (s, 1H, ArH), 7.61-7.62 (m, 2H, ArH); ^{13}C NMR (125 MHz, DMSO-d^6): σ 31.6 (CH_2), 35.1 (CH_2), 54.8 (CH_2), 107.7 (CH), 112.7 (CH), 114.3 (CH), 120.1 (C), 121.5 (C), 122.3 (C), 124.1 (C), 125.3 (CH), 126.2 (C), 126.8 (C), 126.9 (CH), 127.8 (CH), 127.9 (C), 128.2 (CH), 128.4 (CH), 128.6 (CH), 129.2 (C), 129.4 (CH), 132.3 (CH), 143.0 (C), 148.0 (C), 150.1 (C), 157.4 (-OCO), 169.4 (-NCO); ESI-MS (MeOH): 532 $[M+H]^+$

3-(8-(3-aminopropanoyl)-8,9-dihydro-3H-dibenzo[b,f][1,2,3]triazolo[4,5-d]azocin-3-yl)-2H-chromen-2-one (**5e**) Yield: 94%; mp: 108-110°C; R_f (2% MeOH in EA): 0.40; ^{1}H NMR (400 MHz, DMSO-d^6): σ 1.83-1.90 (m, 2H, CH_2), 3.05-3.10 (m, 2H, CH_2), 3.66-3.68 (m, 2H, CH_2), 5.05-5.08 (m, 2H, NH_2), 6.68-6.74 (m, 2H, ArH), 6.97 (t, 1H, J = 6.0 Hz), 7.10-7.36 (m, 2H, ArH), 7.38-7.46 (m, 5H, ArH), 7.47-7.51 (m, 2H, ArH), 7.64 (t, 1H, J = 7.2 Hz, ArH); ESI-MS (MeOH): 464 $[M+H]^+$

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Graphical Abstract

Synthesis and cytotoxicity study of novel 3-(triazolyl) coumarin based fluorescent scaffolds

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