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Synthesis and cell imaging applications of fluorescent mono/di/tri-heterocyclyl-2,6-dicyanoanilines

Mahesh M. Pisal [a], Ritesh A. Annadate [a], Meghana C. Athalye [b], Deepak Kumar [c], Subhash P. Chavan [a], Dhiman Sarkar [b], Hanumant B. Borate [a,*]

[a] Division of Organic Chemistry, CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pashan, Pune 411 008, India
[b] Combichem Bio-resource Centre, CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pashan, Pune 411 008, India
[c] Physical Chemistry Division, CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pashan, Pune 411 008, India

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ABSTRACT

Synthesis of 3,4,5-triheterocyclyl-2,6-dicyanoanilines, starting from heterocyclic aldehydes and 1,2-diheterocycle-substituted ethanones, is described. 2,6-Dicyanoanilines with one or two heterocyclic substituents have also been synthesized. It was found that some of these molecules have selective cell-staining properties useful for cell imaging applications. The compounds **1**g, **10f** and **11** were found to stain cytoplasm of the cells in contact but not the nucleus while the compound **12** showed affinity to apoptotic cells resulting in blue fluorescence. The cell imaging results with compound **12** were similar to Annexin V-FITC, a known reagent containing recombinant Annexin V conjugated to green-fluorescent FITC dye, used for detection of apoptotic cells. These compounds were found to be non-cytotoxic and have potential application as cell imaging agents.

Substituted 2,6-dicyanoanilines are important compounds studied[1a] as such or as intermediates in diverse fields like optical materials, dyes, textile printing, heat resistant polymers, chiral stationary phases in chromatography *etc*. They also constitute molecular skeleton of a number of compounds, with an array of structural variations, some of which have been studied for potential medicinal use. The synthetic methods to prepare these moieties and their applications have been reviewed recently[1a] and the continued interest in these compounds has resulted in further publications.[1b,1c,2a–c] We desired to study the synthesis and optical/biological properties of dicyanoanilines with one, two or three heterocyclic substituents. The literature survey revealed that there are many references for 3-heterocyclyl-2,6-dicyanoanilines,[1a] a few references for 3,5-diheterocyclyl-2,6-dicyanoanilines[2] and a very few references for 4-heterocyclyl-2,6-dicyanoanilines[3] but there are no references for 3,4-diheterocyclyl-2,6-dicyanoanilines or 3,4,5-triheterocyclyl-2,6-dicyanoanilines. We report herein synthesis of various hitherto unknown 3,4-diheterocyclyl-2,6-dicyanoanilines and 3,4,5-triheterocyclyl-2,6-dicyanoanilines, their fluorescence properties and cell imaging applications. Though there are a large number of publications about cell imaging,[4] to our knowledge, this is the first report describing cell imaging potential of 2,6-dicyanoanilines and these preliminary results will lead to new applications of dicyanoanilines for cell imaging.

Various hitherto unknown 3,4,5-triheterocyclyl-2,6-dicyanoanilines were prepared from 1-benzyl-1,2,3[1*H*]-triazole-4- carboxaldehyde **8**, malononitrile and required 1,2-diheterocyclyl ethanone as exemplified by the synthesis of 2-amino-4-(1-benzyl-1*H*-1,2,3-triazol-4-yl)-5-(4-oxo-6-propylthieno[2,3-*d*]pyrimidin-3(4*H*)-yl)-6-(thiophen-2-yl)isophthalonitrile (**1a**) shown in Scheme 1.

Initially, thienopyrimidinone was chosen as one of the heterocycles because thienopyrimidinones[5] with various substituents can be synthesized easily from substituted 2-aminothiophene-3-carboxylates which in turn can be prepared by Gewald synthesis.[6] Thus, ketone **6a** was prepared from 2-bromoacetylthiophene[7] **3a** and 6-propylthieno[2,3-*d*]pyrimidin-4(3*H*)-one **5a**[5] in the presence of triethyl amine in ethyl acetate and reacted[8] with 1-benzyl-1,2,3[1*H*]-triazole-4-carboxaldehyde **8**[9] and malononitrile in DMF in the presence of morpholine to obtain the 3,4,5-triheterocyclyl-2,6-dicyanoaniline **1a** in 43% isolated yield. The 3,4,5-triheterocyclyl-2,6-dicyanoanilines and 3,4-diheterocyclyl-5-aryl-2,6-dicyanoanilines **1a–j** thus prepared, using aldehyde **8** and appropriate ketones, are shown in Table 1.

Structures of these compounds were assigned based on ^{1}H NMR, ^{13}C NMR, IR and HRMS data.[10] The structures of representative

* Corresponding author.
E-mail address: hb.borate@ncl.res.in (H.B. Borate).

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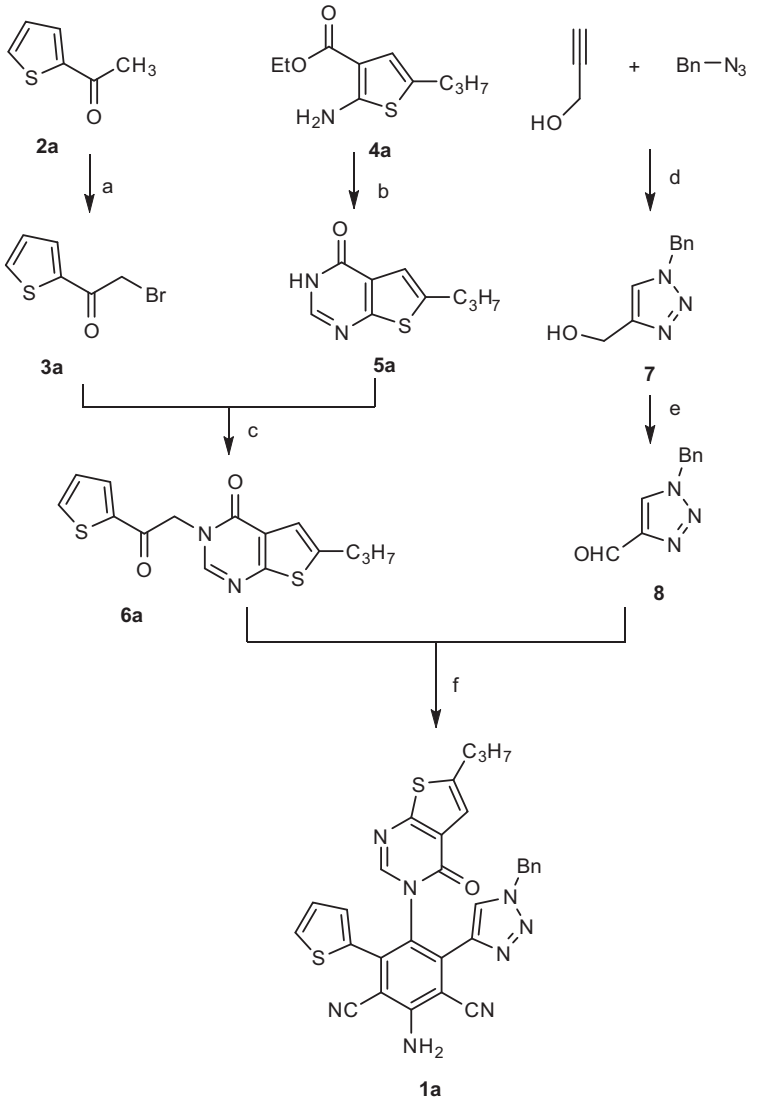
0960-894X/



Scheme 1. Reagents and conditions: (a) Br_2, ether, 0 °C, 1.5 h, 72%; (b) Formamide, ammonium acetate, 145 °C, 10 h, 75%; (c) Triethyl amine, acetone, 0 °C, 1 h, RT, 3 h, 86%; (d) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, *t*-butanol-water, RT, 12 h, 94%; (e) MnO_2, ethyl acetate, RT, 2 h, 92%; (f) Malononitrile, morpholine, DMF, 80 °C, 12 h, 43%.

compounds **1c** and **1e** were further confirmed by X-ray crystallography.[10]

Using a similar strategy, various hitherto unknown 3-heterocyclyl-2,6-dicyanoanilines **9**, **10a–f**, **11** and **12** were prepared by reacting aldehyde **8** with appropriate aldehyde/ketone (Table 2). The dicyanoanilines **1e** and **1f** were methylated[2a] by subjecting them to NaH-MeI in THF to get the corresponding *N,N*-dimethylated compounds 5-(1-benzyl-1H-1,2,3-triazol-4-yl)-3-(dimethylamino)-2′,4′-difluoro-6-(1H-1,2,4-triazol-1-yl)-[1,1′-biphenyl]-2,4-dicarbonitrile (**13e**) and 5-(1-benzyl-1H-1,2,3-triazol-4-yl)-3-(dimethylamino)-4′-fluoro-6-(1H-1,2,4-triazol-1-yl)-[1,1′-biphenyl]-2,4-dicarbonitrile (**13f**).[10] Similarly, compounds **10a–f** were methylated to obtain the corresponding *N,N*-dimethylated compounds **14a–f** (Table 2).

There is a possibility to modify the structures of the compounds in the present work, in order to tune the optical properties, as there is a lot of flexibility in the synthetic schemes described.

These compounds were studied for their optical properties as they were colored in solution (Please see Supplementary data Table S2).

The UV–visible absorption spectra, of 2.5×10^{-5} M solutions in acetonitrile, for representative 2,6-dicyanoaniline compounds **1e**, **1g**, **10f**, **11** and **12** and N,*N*-dimethylated compounds **13e** and **14f** are shown in Fig. 1**A** while fluorescence spectra for these compounds are shown in Fig. 1**B**.

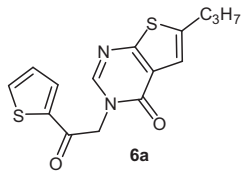
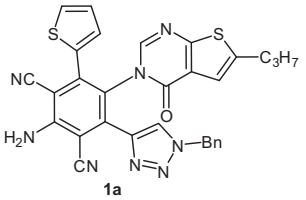
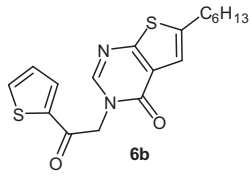
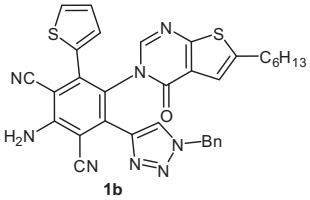
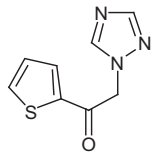
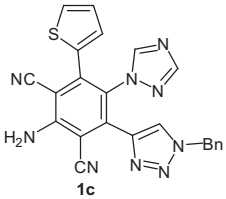
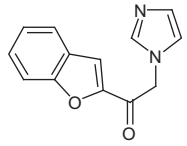
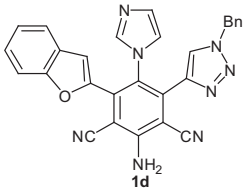
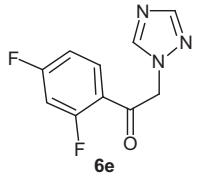
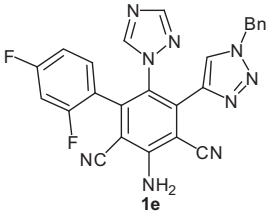
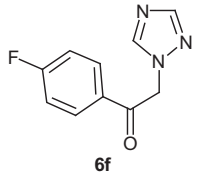
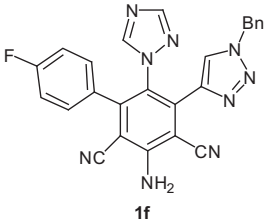
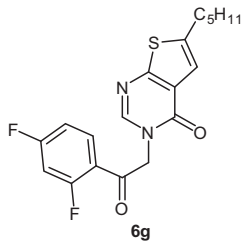
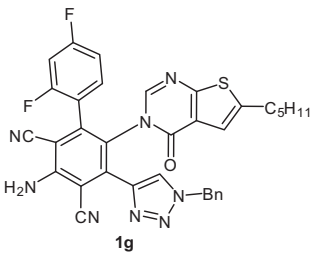
It was observed that there was a red shift in absorption maximum of the amino group after methylation due to enhanced electron-donating ability e.g. the λ_{max} value for amino compound **1e** was 367 nm while that for its corresponding *N,N*-dimethylated derivative **13e** was 395 nm. It was observed that the Stokes shift for the amino compound **1e** was 47 nm while the stokes shift for the corresponding *N,N*-dimethylated derivative **13e** was 74 nm indicating that the Stokes shift is increased after *N,N*-dimethylation of amino group. There was change in color of fluorescence in acetonitrile solutions of same concentration of these compounds. The compounds studied in the present work are soluble in DMSO, DMF, THF, chloroform, dichloromethane, ethyl acetate, acetone, acetonitrile, methanol and ethanol while they are insoluble in water and pet ether. The HOMO-LUMO gaps estimated from DFT calculations[11] were found to be in good agreement with the experimental values derived from uv-visible spectra (Please see Supplementary data).

The fluorescent molecules have a wide application in biological research where they have been developed as valuable tools to

study basic physiological processes within biological systems and there are a number of reports wherein they have been explored for cell imaging studies.[4]

Apoptosis is a process of programmed cell death in which the cell shrinks, nuclear envelope disassembles and DNA breaks into fragments.[12] The cell surface is altered which serves as a signal for the neighboring macrophages to phagocytose it.[13,14] Phosphatidylserine (PS) which is actively held on the interior (cytosolic) side of the lipid bilayer is externalized which serves as a cell surface signal for apoptosis.[15,16] Annexin V is a Ca^{2+} dependent

Table 1
The 3,4,5-triheterocyclyl-2,6-dicyanoanilines and 3,4-diheterocyclyl-5-aryl-2,6-dicyanoanilines prepared.

Entry no	Ketone used	Product obtained	Yield %
1	 **6a**	 **1a**	43
2	 **6b**	 **1b**	45
3	 **6c**	 **1c**	44
4	 **6d**	 **1d**	38
5	 **6e**	 **1e**	49
6	 **6f**	 **1f**	52
7	 **6g**	 **1g**	31

(*continued on next page*)

Table 1 (*continued*)

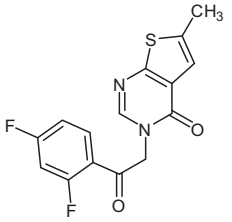
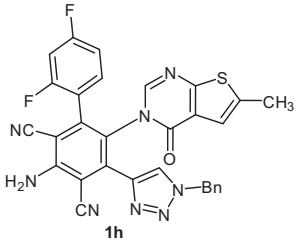
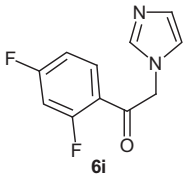
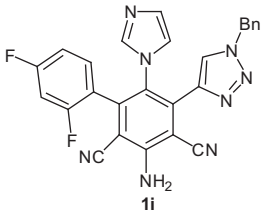
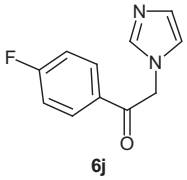
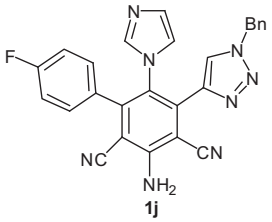
Entry no	Ketone used	Product obtained	Yield %
8	 **6h**	 **1h**	37
9	 **6i**	 **1i**	43
10	 **6j**	 **1j**	47

Table 2
The novel 3-heterocyclyl-2,6-dicyanoanilines and 3,4-diheterocyclyl-2,6-dicyanoanilines prepared.

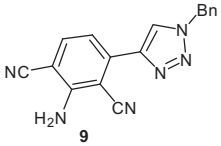
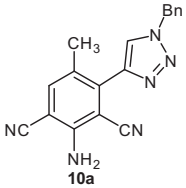
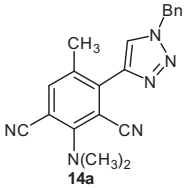
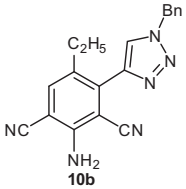
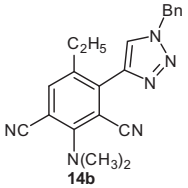
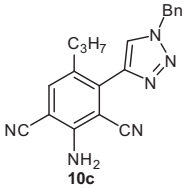
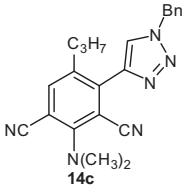
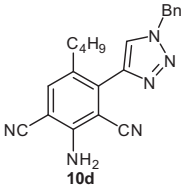
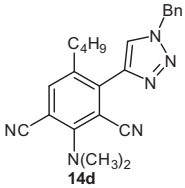
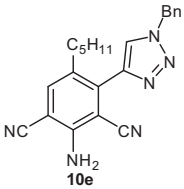
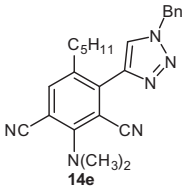
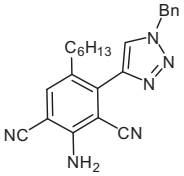
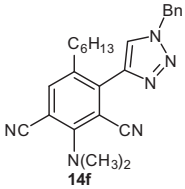
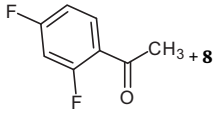
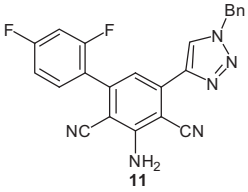
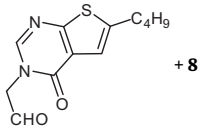
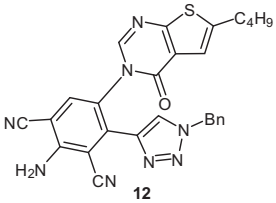
Entry no	Aldehyde/ketone used	Product obtained	Isolated Yield %	Corresponding methylated product prepared	Isolated Yield %
1	Acetaldehyde + **8**	 **9**	50	ND	–
2	Propionaldehyde + **8**	 **10a**	61	 **14a**	75
3	Butyraldehyde + **8**	 **10b**	64	 **14b**	73
4	Valeraldehyde + **8**	 **10c**	59	 **14c**	70

Table 2 (*continued*)

Entry no	Aldehyde/ketone used	Product obtained	Isolated Yield %	Corresponding methylated product prepared	Isolated Yield %
5	Hexanal + **8**	 **10d**	60	 **14d**	72
6	Heptanal + **8**	 **10e**	61	 **14e**	69
7	Octanal + **8**	 **10f**	63	 **14f**	77
8		 **11**	55	ND	–
9		 **12**	65	ND	–

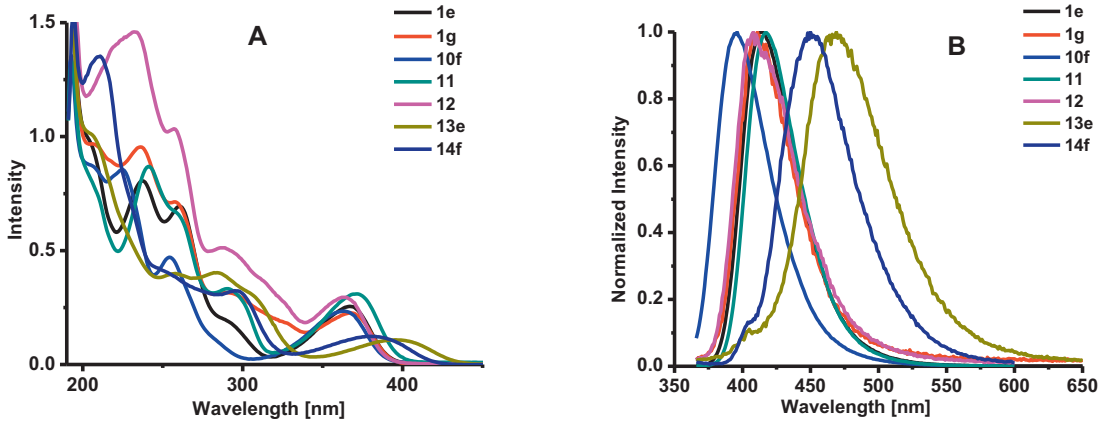


Fig. 1. (A) UV–visible absorption and (B) fluorescence spectra of compounds **1e**, **1g**, **10f**, **11**, **12**, **13e** and **14f**.

phospholipid-binding protein with high affinity for PS. Annexin V conjugated with a fluorophore, fluorescein isothiocyanate (FITC), is widely used to detect this PS externalization which indicates apoptosis.[17] This has a profound application in identifying apoptotic cells in a cell population. In some biological studies it becomes necessary to identify such cell populations in order to interpret cell death inducing mechanisms if necessary. Propidium iodide is another widely used dye which intercalates with the

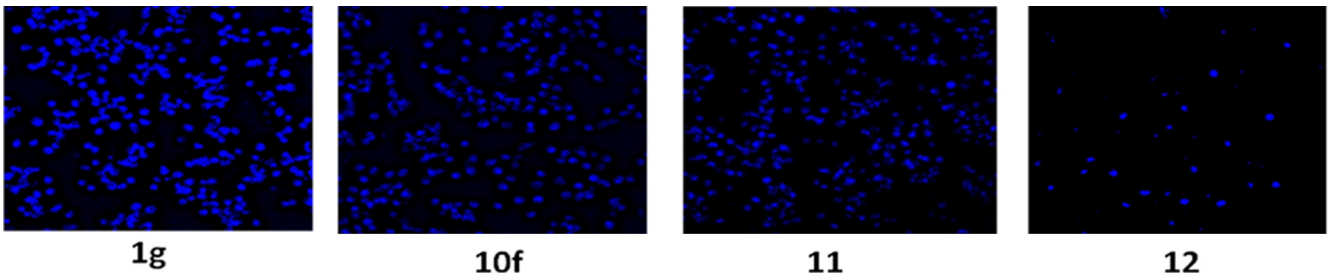


Fig. 2. Cell images captured after staining THP-1 cells with compounds **1g**, **10f**, **11** and **12** (blue fluorescence). Imaging assay buffer: Pre-warmed (37 °C) phosphate-buffered saline (PBS, pH 7.2) containing compounds at 30 μg/mL final concentrations. Incubation time: 20 min, Temperature: 37 °C in CO_2 incubator.

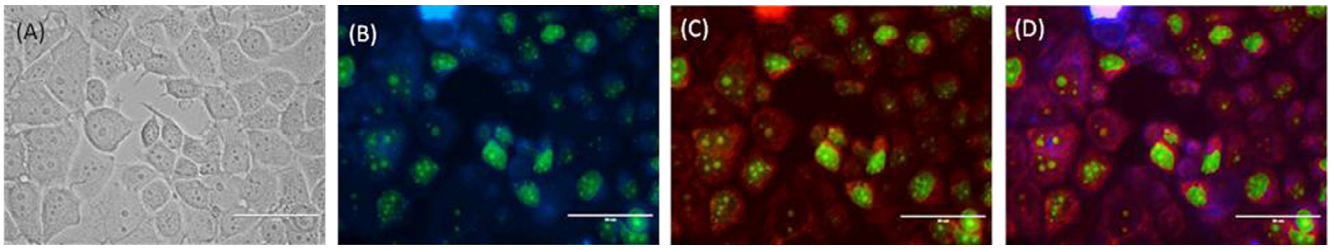


Fig. 3. MCF-7 cells stained with the compound **1g**, SYTO 9 and Nile red. The cells were stained with compound **1g** (100 μg/mL, 0.16 mM, blue fluorescence), SYTO 9 which stains nucleus (green fluorescence) and Nile red which stains cell membrane (red fluorescence) observed at 60X magnification. (A) Cells seen in bright field; (B) Overlay image of blue and green channels, Blue – compound **1g**, Green – SYTO 9 (stains nucleus); (C) Overlay image of green and red channels: Red – Nile red (stains cell membrane), Green – SYTO 9 (stains nucleus); (D) Overlay image of (B) and (C). Scale on image measures 50 μm. Imaging assay buffer: Pre-warmed (37 °C) phosphate-buffered saline (PBS, pH 7.2) containing compound **1g** (100 μg/mL, 0.16 mM), SYTO 9 and Nile red. Incubation time: 20 min, Temperature: 37 °C in CO_2 incubator.

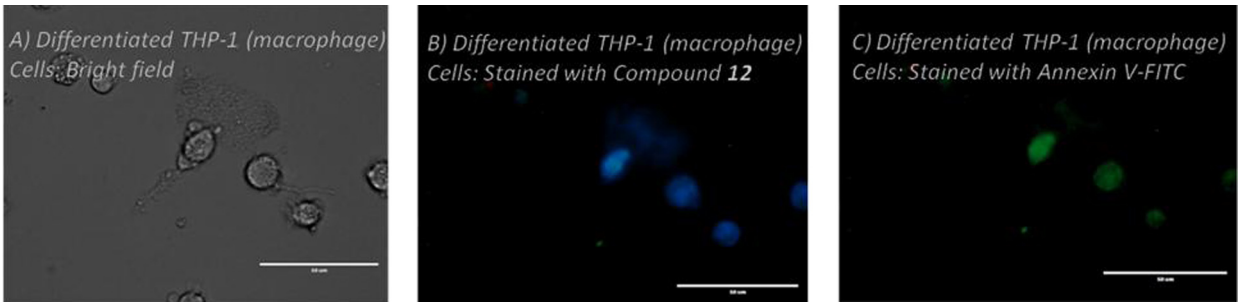


Fig. 4. Differentiated THP-1 cells (A) In bright field; (B) Stained with compound **12**; (C) Stained with Annexin V-FITC. Imaging assay buffer: 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4, compound **12** (final concentration 100 μg/mL, 0.2 mM), Annexin V-FITC conjugate (5 μL v/v). Incubation time: 15 min, Temperature: 37 °C in CO_2 incubator

DNA. It is specifically used to detect dead cell population in research[18] and is often used along with Annexin-V-FITC.

The fluorescent molecules can be tagged or conjugated to specific moieties and generate fluorescent signals which thus serve as detectable or measurable signals. These have applications in microscopy, fluorimetry, flow cytometry, *etc*. The molecules synthesized in the present work show fluorescent properties which were explored for cell imaging studies. Cell imaging analysis was carried out on fluorescent compounds prepared in the present work and 4 compounds (**1g**, **10f**, **11** and **12**) were found to stain eukaryotic cells. Three cell lines (differentiated THP-1, MCF-7 and MDA-MB-231) were tested for cell staining using known staining agents [SYTO 9 which stains nucleus (green fluorescence), Nile red which stains cell membrane (red fluorescence), Annexin-V-FITC which detects apoptotic cells (green fluorescence) and Propidium iodide which stains dead cells (red fluorescence)].

Cell images captured, using High Content Screening System (ArrayScanXTI, Thermo Scientific) at 20X magnification, after staining differentiated THP-1 cells with 30 μg/mL solutions of compounds **1g**, **10f**, **11** and **12** are shown in Fig. 2. Differentiated THP-1 cells were observed to retain these fluorescent compounds.

The compounds **1g**, **10f** and **11** were found to stain all the cells in contact while compound **12** was found to stain only some cells out of total cells. The compounds **1g**, **10f**, **11** and **12** were studied further for staining MCF-7 and MDA-MB-231 cells. The representative cell-imaging results for the compound **1g** for MCF-7 cells are shown in Fig. 3.

The detailed cell images for staining of differentiated THP-1, MCF-7 and MDA-MB-231 cell lines with the compounds **1g**, **10f** and **11** are given in the Supplementary Information. The compounds **1g**, **10f** and **11** were found to specifically stain only the cytoplasm of the cells but not the nucleus. This was verified by using known counter stains like SYTO 9 (for nucleus) which gives green fluorescence and Nile red (lipid molecules and cell membrane) which gives red fluorescence.

However, compound **12** was found to stain selectively some cells out of total cells and the patterns were similar to Annexin V-FITC as shown in Fig. 4.

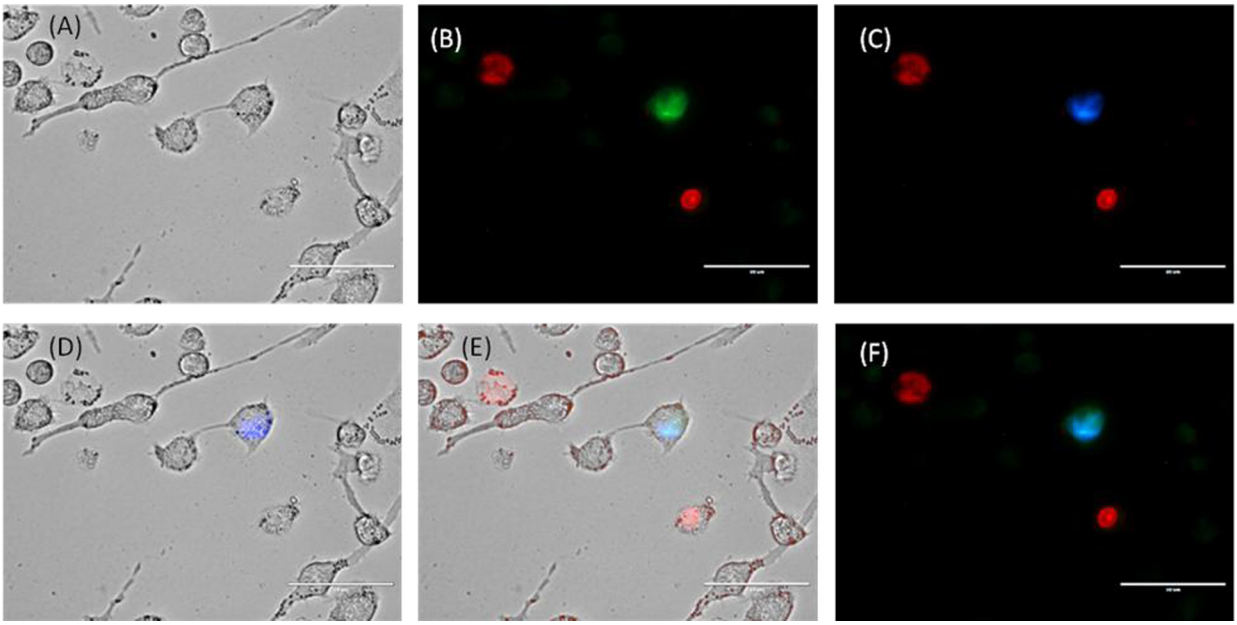


Fig. 5. Differentiated THP-1 cells stained with compound **12**, Annexin-V-FITC and propidium iodide. The cells were stained with compound **12** (100 μg/mL, 0.2 mM, blue fluorescence), Annexin-V-FITC which detects apoptotic cells (green fluorescence) and propidium iodide (PI) which stains dead cells (red fluorescence) observed at 60X magnification. (A) Cells seen in bright field; (B) Overlay image of green and red channels: Green – Annexin-V-FITC (apoptosis), Red – PI (dead); (C) Overlay image of blue and red channels: Blue – compound **12**, Red – PI (dead); (D) Overlay image of bright field with blue channel – Blue - compound **12** (E) - Overlay image of (A), (B) and (C); (F) - Overlay image of (B) and (C). Imaging assay buffer: 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4, Annexin V-FITC conjugate (5 μL v/v) and PI (10 μg/mL) along with compound **12** at final concentration of 100 μg/mL, 0.2 mM. Incubation time: 15 min, Temperature: 37 °C in CO_2 incubator.

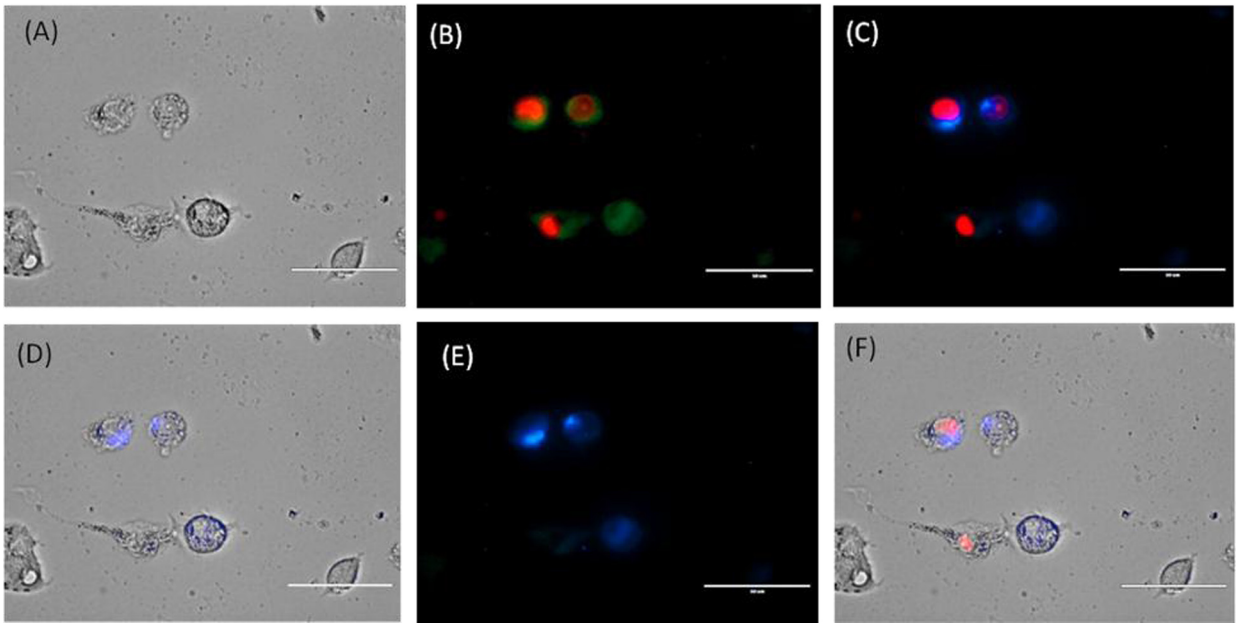


Fig. 6. Differentiated THP-1 cells with induced apoptosis using Paclitaxel, stained with the compound **12**, Annexin-V-FITC and propidium iodide. Differentiated THP-1 (macrophage) cells with induced apoptosis using Paclitaxel IC90 concentration, were stained with the compound **12** (100 μg/mL, 0.2 mM, blue fluorescence), Annexin-V-FITC (green fluorescence for apoptotic cells) and propidium iodide (PI) (red fluorescence for dead cells) observed at 60X magnification. (A) Cells seen in bright field; (B) Overlay image of green and red channels, Green – Annexin-V-FITC (stains apoptotic cells), Red – PI (stains dead cells); (C) Overlay image of blue and red channels: Blue – compound **12**, Red – PI (stains dead cells); (D) Overlay image of bright field with blue channel– compound **12**; (E) Blue – compound **12**; (F) Overlay image of (A) and (C). Imaging assay buffer: 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4, Annexin V-FITC conjugate (5 μL v/v) and PI (10 μg/mL) along with compound **12** at final concentration of 100 μg/mL, 0.2 mM. Incubation time: 15 min, Temperature: 37 °C in CO_2 incubator.

This differential staining was studied further using known markers and inducers. In order to study the differential staining property of **12**, the cells were seeded and apoptosis / cell death was induced using known anti-cancer, cytotoxic compounds namely Paclitaxel and Doxorubicin at their IC50 and IC90 concentrations. Known markers like Annexin V-FITC for apoptosis and

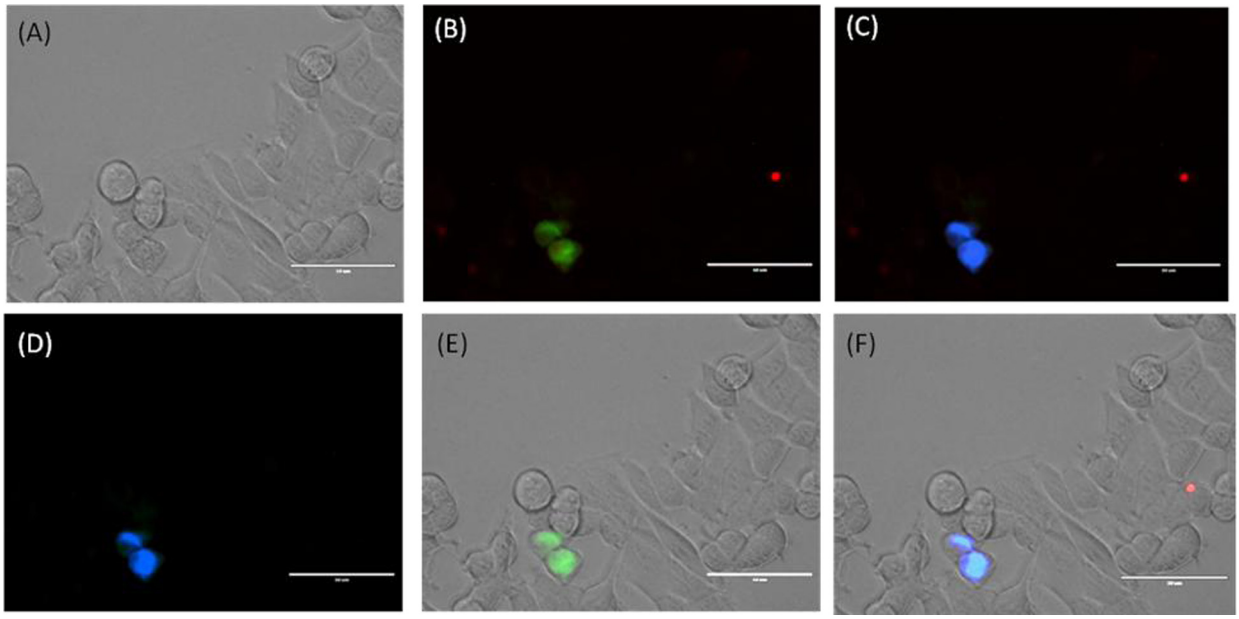


Fig. 7. MCF-7 cells stained with the compound **12**, Annexin-V-FITC (green fluorescence) and propidium iodide. The MCF-7 cells were stained with the compound **12** (100 μg/mL, 0.2 mM, blue fluorescence), Annexin-V-FITC (green fluorescence) and propidium iodide (red fluorescence) observed at 60X magnification. (A) Cells seen in bright field; (B) Overlay image of green and red channels: Green – Annexin-V-FITC (apoptosis), Red – PI (dead cells); (C) Overlay image of blue and red channels: Blue – the compound **12**, Red – PI (dead cells); (D) Blue – the compound **12**; (E) - Overlay image of (A) and (B); (F) Overlay image of (A) and (C). Scale on image measures 50 μm. Imaging assay buffer: 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4, Annexin V-FITC conjugate (5 μL v/v) and PI (10 μg/mL) along with compound **12** at final concentration of 100 μg/mL, 0.2 mM. Incubation time: 15 min, Temperature: 37 °C in CO_2 incubator.

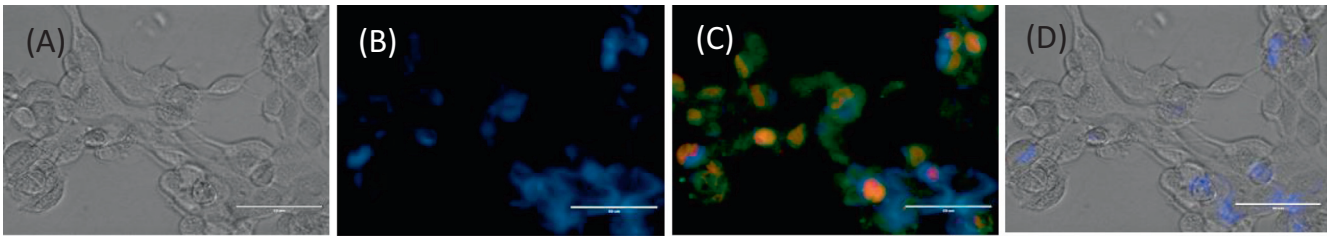


Fig. 8. MCF-7 cells with induced apoptosis using Doxorubicin, stained with the compound **12**, Annexin-V-FITC and propidium iodide. MCF-7 cells with induced apoptosis using Doxorubicin IC50 concentration were stained with the compound **12** (100 μg/mL, 0.2 mM, blue fluorescence), Annexin-V-FITC (green fluorescence) and propidium iodide (red fluorescence) observed at 60X magnification. (A) Cells seen in bright field; (B) Blue – compound **12** (C) Overlay image of green, red and blue channels: Green – Annexin-V-FITC (apoptosis), Red – (dead cells) and Blue – compound **12**; (D) - Overlay image of (A) and (B). Scale on image measures 50 μm. Imaging assay buffer: 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4, Annexin V-FITC conjugate (5 μL v/v) and PI (10 μg/mL) along with compound **12** at final concentration of 100 μg/mL, 0.2 mM. Incubation time: 15 min, Temperature: 37 °C in CO_2 incubator.

propidium iodide for dead cells were used along with the compound **12**. Standard staining protocol for apoptosis/necrosis using Annexin V-FITC/PI was carried out with addition of this new compound **12**. Healthy cells were used as negative control. The cell images for differentiated THP-1 (macrophage) cells stained with the compound **12** (100 μg/mL, 0.2 mM, blue fluorescence), Annexin-V-FITC which detects apoptotic cells (green fluorescence) and Propidium iodide (PI) which stains dead cells (red fluorescence) are shown in Fig. 5.

It was observed that the staining of the differentiated THP-1 (macrophage) cells with the compound **12** corresponds to that with Annexin-V-FITC which is known to stain the apoptotic cells. The compound **12** does not stain dead cells. The results were further confirmed by inducing apoptosis in the THP-1 (macrophage) cells with Paclitaxel and repeating the study (Fig. 6).

The results of imaging in case of MCF-7 cells stained with the compound **12**, Annexin-V-FITC and Propidium iodide are shown in Fig. 7 which also shows that the staining pattern of the compound **12** is similar to Annexin-V-FITC (which shows green fluorescence for apoptotic cells).

Further, apoptosis was induced in MCF-7 cells by treating them with Doxorubicin and the cells were stained with the compound **12**, Annexin-V-FITC and Propidium iodide and the results are shown in Fig. 8.

The imaging properties of the compound **12** were further studied with MDA-MB-231 cells. The apoptosis was induced with Doxorubicin and the cells were stained with the compound **12** (100 μg/mL, 0.2 mM, blue fluorescence), Annexin-V-FITC (green fluorescence) and Propidium iodide (PI) (red fluorescence) as before (Fig. 9).

All these results indicated that the staining patterns of the compound **12** (which shows blue fluorescence) are similar to Annexin-V-FITC which shows green fluorescence for apoptotic cells and the compound **12** has potential as staining agent for imaging of apoptotic cells.

Toxicity of the compounds **1g**, **10f**, **11** and **12** against THP-1 cells was studied[19] and the data are given in Supplementary data.

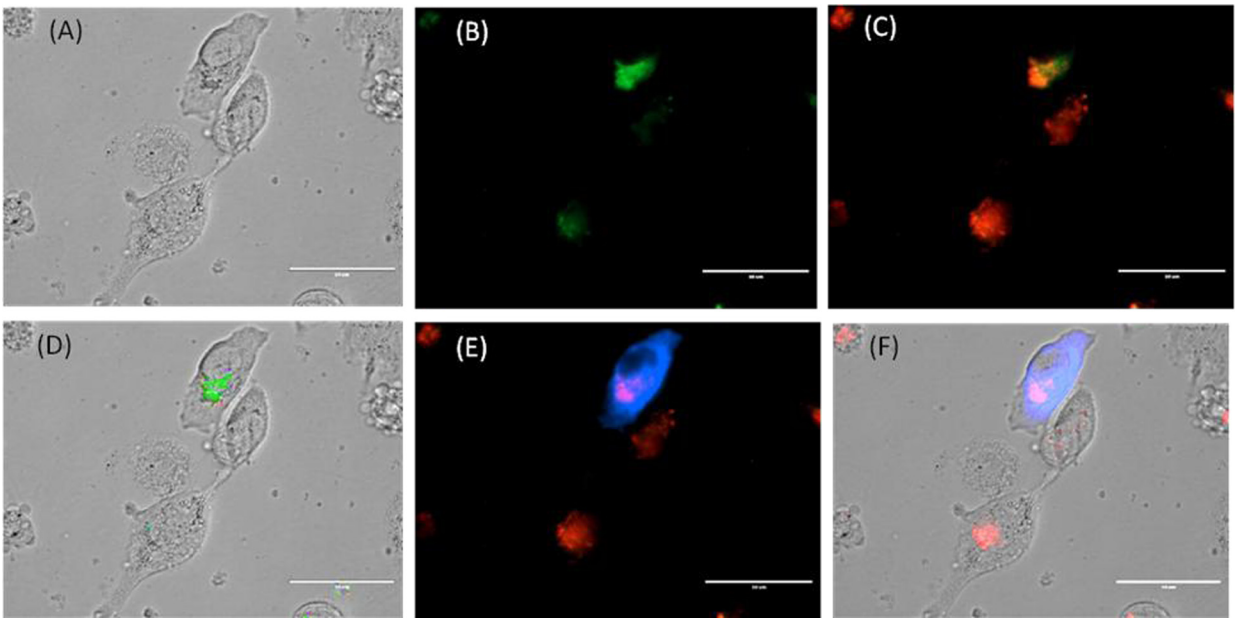


Fig. 9. MDA-MB-231 cells with induced apoptosis using Doxorubicin, stained with the compound **12**, Annexin-V-FITC and propidium iodide. MDA-MB-231 cells with induced apoptosis using Doxorubicin IC90 concentration, were stained with the compound **12** (100 μg/mL, 0.2 mM blue fluorescence), Annexin-V-FITC (green fluorescence) and propidium iodide (red fluorescence) observed at 60X magnification. (A) Cells seen in bright field; (B) Green – Annexin-V-FITC (apoptosis); (C) Overlay image of green and red channels: Green – Annexin-V-FITC (apoptosis), Red – PI (dead cells); (D) - Overlay image of (A) and (B); (E) Bright field with blue – compound **12** and red – PI (dead cells); (F)-Overlay image of (A) and (E). Scale on image measures 50 μm. Imaging assay buffer: 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4, Annexin V-FITC conjugate (5 μL v/v) and PI (10 μg/mL) along with compound **12** at final concentration of 100 μg/mL, 0.2 mM. Incubation time: 15 min, Temperature: 37 °C in CO_2 incubator.

Since the IC_{50} values of compounds are >100 μg/mL, these compounds can be considered as non-cytotoxic.

In conclusion, synthesis of 3,4,5-triheterocyclyl/3,4-diheterocyclyl/3-heterocyclyl-2,6-dicyanoanilines, starting from 1-benzyl-1,2,3[1*H*]-triazole-4- carboxaldehyde **8**, malononitrile and required aldehydes/ketones was achieved. The optical properties of the synthesized compounds were studied which indicated that fluorescence properties of this class of compounds can be modified. The compounds were further studied from the point of their application for cell imaging wherein it was found that compounds **1g**, **10f** and **11** stain cytoplasm of the cells in contact but not the nucleus while the compound **12** showed selective affinity to apoptotic cells resulting in blue fluorescence. The cell imaging results with compound **12** were compared with Annexin V-FITC, a known reagent containing recombinant Annexin V conjugated to green-fluorescent FITC dye, used for detection of apoptotic cells. These encouraging results indicate the significant potential of these compounds as valuable tools for cell imaging.

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A. Supplementary material

Copies of ^{1}H and ^{13}C NMR spectra of all new compounds, X-ray crystallographic data for compounds **1c** and **1e**, optimized geometry and HOMO-LUMO levels of compounds **1g**, **10f**, **11** and **12** and detailed cell imaging data for compounds **1g**, **10f**, **11** and **12**. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.12.074.

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