

## Designing, Synthesis and Characterization of Fluorescent Fluorescein Hydrazides and Antibacterial Studies against Sinusitis Isolates and Hemolytic Activities

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**Summary:** Fluorescein and its various derivatives show remarkable biological and bio-analytical applications because of its solubility, high degree of inertness and high fluorescent activity. To broaden the spectrum of its applications, we synthesized novel fluorescein-based hydrazides (FHJ-0.2 - FHJ-0.6) using different substituted aryl hydrazine. The newly designed and synthesized products have been monitored by thin layer chromatography and characterized by <sup>1</sup>HNMR, IR and MS data. To find the application of these hydrazides, the biological screening is considered and screened by antimicrobial and hemolytic activity studies. Time-kill assay revealed that none of these hydrazides are appreciable antimicrobial agent but all of these show promising hemolytic results. Results are compared with Triton X-100 (positive control) with 89.54% hemolytic activity. Compared to fluorescein (FHJ-0.1) activity (3.07%), FHJ-0.3(3.54%), FHJ-0.2 (4.37%), FHJ-0.2 (4.37%) and FHJ-0.6 (5.87%), presented better anti- hemolytic activity showing that changing the substituent and position affects the activity remarkably. These fluorescein based hydrazides may prove to be useful for the treatment of hemolytic disorders.

**Keywords:** Fluorescein derivatives, Hydrazides, Fluorescent, Anti-Hemolytic activity, Antimicrobial activity, Hemolytic disorders.

### Introduction

Heterocyclic compound due to its synthetic and effective biological significance attracted much attention and revealed various pharmaceutically appealing drug candidates containing anti-viral, anti-fungal, anti-migraine, and anti-anxiety compounds [1–9]. The medicinal and pesticide chemical science gives a prime place to heterocyclic compounds because these reveal a wide range spectrum of bioactivities including anti-cancer, [10] anti-inflammatory, [11, 12] antimicrobial, [13-17] anti-depressant, [18] and selective enzymes inhibitory activities [19] etc.

Fluorescein is one of those aromatic and heterocyclic species that have so-called fluorophore. The chromophore of these fluorescent species is responsible for its color. The phenomenon of auto fluorescence is present due to endogenous fluorophores of cellular organelles such as

mitochondria and lysosomes [20]. Therefore fluorescence is characterized by polycyclic aromatic combination that are extensively useful as a brand of manmade bio-molecule [21, 22].

Fluorescent dyes since the late 1800s have been in practice in biotechnological presentations [23, 24] of all the generally accessible fluorescent dyes, fluorescein as well as its various derivatives are most suitable due to its extraordinary solubility in biological medium and remarkable fluorescence at physiological range of pH. [25] Fluorescein being highly fluorescent is used as tracer in a several biological, medicinal and bio-analytical applications because it shows absorption at 492 nm and emission at 517 nm with 0.92 quantum yield at pH > 8 in water. [26, 27] It occurs in two forms: an open structure which is highly fluorescent and a non-fluorescent closed structure and

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both of them existed in equilibrium in aqueous medium [4].

During 1990, extraordinary biotechnological and medicinal practice stood expanded in the emergent period of research of this ground. [25, 28-33] the zone of fluorescent compounds, fluorescein owed to tremendous photo-physical properties quite a key center of research and getting importance to date.

Fluorescein has a variety of applications such as fluorescent tracers in microscopy, in forensics to identify latent blood stains, and have miscellaneous physicochemical, photo-physical and biological assay. [34]. the suitability of fluorescein to drug development is due to its biological activities such as antibacterial and forensic in the growing field.

Monitoring of pH has always been a crucial parameter to detect acidic metabolite in various biological applications including medicine, environment and various industrial processes [35]. This has been carried out by adopting optical approach using fluorescence based indicators. Fluorescein is proved to be a highly sensitive notorious fluorescent probe to investigate pH in biomedical applications. [36]

Recently fluorescein based pH nanosensors are developed that exhibited promising applications to investigate pH in the cell. The spacer (polyether) employed to bind fluorescein to carbon nanotubes (CNTs) not only enhanced its solubility in biological medium but also reduced fluorescence quenching due to the proximity between fluorophore and CTN. These fluorescein labeled nanosensors are tested in the pH range of 4.5–8. [37].

In fluorescent angiography, it is widely being used in the surgery of brain tumors and to diagnose retinal vascular disorders such as intraocular inflammation, intraocular tumors, muscular degeneration and diabetic retinopathy. In open heart surgery, fluorescein is used to localize latent defects of muscles and ventricular septum. It is used to identify residual defect, if any, after surgery. [38]

Fluorescein based guided surgery to resect breast cancer and brain metastasis is considered safe, simple and practical as compared to standard surgery as its visibility of tumor is significantly enhanced. It offered a great advantage when a small tumor was navigated and helped in improving the quality of patients' life after cerebral metastasis. [39]

The caged carboxy fluorescein (the OH group at 3' and 6' are protected to esters) acts as an indicator to check the water quality. This procedure not only quenches the fluorescence but also promotes its passive diffusion into the bacterium, if present any. The carboxy-fluorescein gets activated by the hydrolase enzyme, thus shows ON response to living but not dead bacteria. [40]

Fluorescein based bio-sensors play a crucial role to visualize the biological responses by converting it into electronic, electrochemical, acoustic and optical signals. So a number of fluorescein based probes have been developed until now to increase their sensing abilities for various bio-analytical application to selective and sensitive detection of  $\text{Cu}^{+2}$ ,  $\text{Fe}^{+2,3}$ ,  $\text{Zn}^{+2}$ ,  $\text{Pb}^{+2}$ ,  $\text{Hg}^{+2}$ ,  $\text{Au}^{+3}$ , and  $\text{Ag}^{+1}$  [41]

In flow cytometry, hydroxyl terminated polymer labeled with fluorescein is used as tracer to investigate polymer surface interaction in a number of chemical and biological systems. [42] In plant sciences, vasculature of plant is being studied using fluorescein because it is unable to cross the plasma membrane but can move easily in the xylem to track the water movement. [43]

Currently, we are investigating fluorescein for its biological activities and the C-N bond formation in the lactone moiety of fluorescein by developing an efficient method is an interesting subject in synthetic organic chemistry. Therefore, the conversion of lactone moiety into lactam by the reaction with different substituted phenyl hydrazines to give new fluorescein hydrazide was subjected to hemolytic and antimicrobial activities studies.

## Experimental

### Instruments and Chemicals

All the chemicals required for the accomplishment of research work were of analytical grade and used as such without applying any procedure of purification. Fluorescein was purchased from Merck (Germany) while different substituted phenyl hydrazine was purchased from Sigma Aldrich (USA). The solvents such as ethanol, methanol and DCM were also bought from Sigma Aldrich. To check the extent of reaction and detection of the product, thin layer chromatography is done after every 30 minutes. Silica-coated TLC plates are used and envisaged under a UV lamp at 254 nm and 366 nm. Solvent system used in thin layer chromatography is DCM and methanol (9:1 ratio). The  $^1\text{H}$ NMR and  $^{13}\text{C}$ NMR spectra were recorded at 500 MHz spectrometers.

## Results and Discussion

The IR spectrogram was taken for all of the compounds, and we are going to discuss the IR, NMR, and mass spectrum of FHJ-0.2 and FHJ-0.5. The different functional groups in both of these compounds are identified by IR spectroscopy. In case of FHJ-0.2, an intense peak at  $1630\text{ cm}^{-1}$  confirmed the C=O stretching of amide. The characteristics N-H stretching at  $3280\text{ cm}^{-1}$ , C-N stretching at  $1170\text{ cm}^{-1}$ , and the peak of C-Cl aromatic stretching at  $1110\text{ cm}^{-1}$  showed the formation of FHJ-0.2. Similarly an intense peak in case of FHJ-0.5 at  $1481\text{ cm}^{-1}$  confirmed the aromatic C=C bond. The characteristics peak of C-N stretching at  $1242\text{ cm}^{-1}$ , peak of N-H stretching at  $3280\text{ cm}^{-1}$  and peak of C-H stretching at  $2827\text{ cm}^{-1}$  confirmed the formation of FHJ-0.5.

The structure of FHJ-0.2 is elucidated by subjecting it to  $^1\text{H}$ NMR in  $\text{CD}_3\text{OD}$  at the frequency of 500 MHz. The protons of aromatic appeared in the range of 7.997-6.546 while N-H proton appeared as singlet at 6.563 ppm. A doublet appeared at 7.99 ppm is due to protons at position 5 and 10 with coupling constant of 7.5 Hz. Protons at position 2 and 3 appeared as multiplet. A triplet of 2' and 3' position proton appeared at 7.70 with J value 7.5 Hz for ortho coupling and 7 Hz for meta coupling. A doublet of doublet at 6.768 Hz is due to a proton at position 4 with J value of 2 Hz. The protons at 7 and 8 position appeared as singlet due to the symmetrical nature of fluorophore.

Similarly  $^1\text{H}$ NMR of FHJ-0.5 confirms its structure in which the protons (H-1') of methyl appeared as a doublet in the range of 1.071-1.077 ppm with a coupling constant of  $1'J=7\text{ Hz}$ . The protons of aromatic rings appeared within the range of 6.337-9.98 ppm. The protons at position 1 are highly de-shielded due to electron withdrawing (EW) effect of a carbonyl functional group and highly electronegative nitrogen atom; thus, appeared at 7.98 ppm in the form of the doublet with 1 J value of 7 Hz. Three protons at positions 2, 3, 4 appeared in the multiplet form at 6.56 ppm with coupling constants of 2 Hz and 2.5 Hz. A singlet of two protons (H-7 and H-8) is appeared at 6.53 ppm. Both the protons at positions 5, 10 appeared in the form of doublet ( $J=8.5\text{ Hz}$ ) at the same Chemical shift of 6.35 ppm due to symmetry in the fluorophore of fluorescein. Likewise, the doublet of doublet is given by protons at positions 6 and 9 at 6.47 ppm ( $J=1.5\text{ Hz}$ ,  $J=8.5\text{ Hz}$ ). The doublets that appeared at 7.17 ppm with coupling constant of  $J=7.5\text{ Hz}$  and 6.79 ppm with coupling constant of  $J=8.5\text{ Hz}$  are assigned to the protons of positions 3' and 4', respectively.

The mass of FHJ-0.5 is confirmed by mass spectrometry which exhibited that the peak of molecular ion is at  $m/z$  464. It showed mass fragments of  $m/z$  of 449, 360, 316, 287, 271 and 259. The fragment with an  $m/z$  of 449 is formed when the molecular ion lost a methyl free radical. The fragment with  $m/z$  value of 259 is generated from the moiety of  $m/z$  of 287 when the later lost CO. The two fragments with the same  $m/z$  value of 271 are formed when the fragment with  $m/z$  287 lost an oxygen free radical. The fragment with  $m/z$  of 449 is stabilized by the formation of tropylium ion and resulted in the formation of a fragment with  $m/z$  of 360. The mass fragment having  $m/z$  of 360 has undergone McLafferty rearrangement with the formation of two different fragments with  $m/z$  values of 316 and 331. The mass spectra of FHJ-0.2 and FHJ-0.5 are relevant to some extent with slight similarities and differences. The fragment with  $m/z$  of 360 is absent in FHJ-0.2. The base peak in FHJ-0.2 has  $m/z$  of 456 which is also the molecular ion peak while the mass spectrum of FHJ-0.5 has different base peak ( $m/z = 287$ ) and molecular ions peak ( $m/z = 464$ ). The fragments with  $m/z$  value of 316, 287 and 271 are common to both FHJ-0.2 and FHJ-0.5.

The mass spectrum of FHJ-0.2 exhibited the molecular ion peak (base peak) at  $m/z$  456. It showed fragments with  $m/z$  value of 421, 391, 330, 316, 287, 271 and 259. The fragment having  $m/z$  of 421 is formed when chlorine free radical is lost from molecular ion. The two fragments with same  $m/z$  value of 271 are formed when fragment with  $m/z$  287 lost free radical of oxygen. The fragment with  $m/z$  value of 287 generated fragment with  $m/z$  of 259 by losing CO. Proposed Structure of FHJ-0.2 and FHJ-0.5 are given in Fig 3 and 4 respectively.

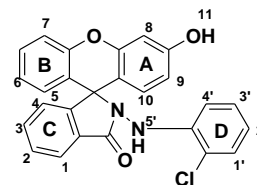


Fig. 3: Proposed Structure of FHJ-0.2.

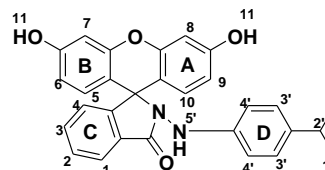


Fig. 4: Proposed Structure of FHJ-0.5.

### Antibacterial Activity

The antibacterial activity of each of the synthesized compounds was assessed by the disc diffusion method as described in the material and methods. The results indicate that none of the fluorescein hydrazides have shown the antibacterial property. The compound with the bromo group shows a certain result but not an impressive one. The presence of different substituents at various positions of fluorescein hydrazides did not prove to be antibacterial as compared to standard. The results are indicative of an absence of good antimicrobial properties of synthesized compounds. The results are given in Fig 1.

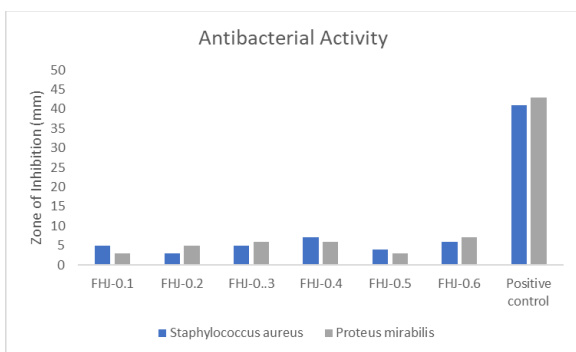


Fig. 1: Antibacterial Activity of Compounds.

### Hemolytic activity

The results of hemolytic activity were depicted in Fig 2. The results showed good anti-hemolytic activity for all samples. Results are compared with Triton X-100 (positive control) with 89.54% hemolytic activity. From the results, it appeared that maximum anti-hemolytic activity was present for FHJ-0.4 (1.27%). The maximum hemolytic activity was shown by compound FHJ-0.6 (5.87%), while FHJ-0.2 (4.37%), FHJ-0.3 (3.54%) & FHJ-0.5 (2.69%). From the obtained results it is evident that substitution of different functionalities viz halo, alkyl, etc. at different positions of fluorescein hydrazide affect hemolysis. Compared with the activity result of fluorescein FHJ-0.1 (3.07%), it is obvious that hydrazide of fluorescein with bromo substituent at meta position shows excellent anti hemolysis and is safer for drug use. Although the other substituents also show good anti-hemolysis results but changing the substituent and position affects the activity

remarkably. In all the cases, meta-substituted compounds give good results while ortho and para substitution did not prove to be so effective. Among all the hydrazides of fluorescein bromo and isopropyl groups give remarkable results. Hence it can be concluded that alkyl and bromo substituted fluorescein hydrazides prove to be good candidates for drug use. The results are given in Fig 2.

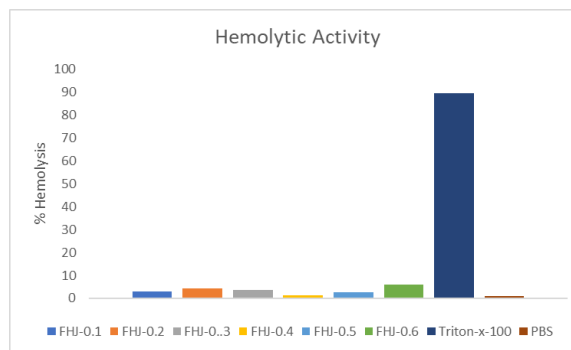
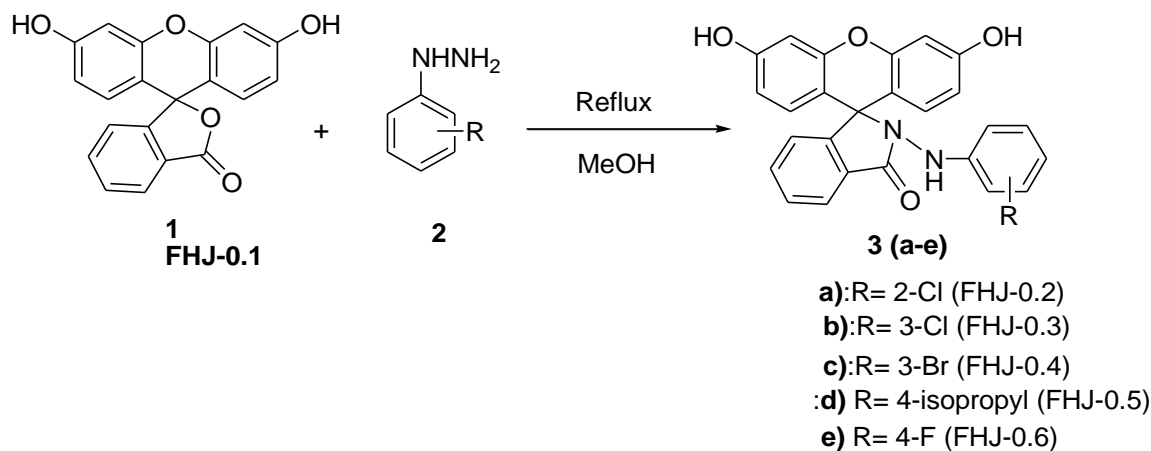


Fig. 2. Hemolytic activity of compounds.

### Preparation and characterization

#### Synthesis of N-fluorescein-lactam-phenyl hydrazides (B)

The different hydrazides of fluorescein (FHJ-0.2-FHJ-0.6) are synthesized by refluxing stoichiometric amount (1g or 3.04 mmol) of fluorescein with excess (4 mmol) of corresponding substituted phenyl hydrazine hydrochloride (1h-5h). The reflux is carried out with continuous stirring at 70 °C in ethanol as a solvent for FHJ-0.5 while in case of FHJ-0.2, FHJ-0.4, FHJ-0.4, FHJ-0.6 methanol is used. Methanol is found to be an excellent solvent as compared to ethanol giving a better yield. The reaction progress is monitored by thin layer chromatography using commercial TLC plates (silica-coated). Methanol and DCM (1: 9) is used as a solvent system in TLC. After completion of reaction, the reaction mixture is cooled to room temperature and kept overnight. 200ml of distilled water is then added, the expected products being insoluble precipitated out with high yield. The precipitate of each is further purified by repeated washing with distilled water. General Scheme of Preparation of fluorescein aryl hydrazides is given in Scheme 1



Scheme-1: General Scheme of Preparation of fluorescein aryl hydrazides.

**Characterization Data**

2-((2-chlorophenyl)amino)-3',6'-  
dihydroxyspiro[isoindoline-1-9'-xanthen]-3-one  
(FHJ-0.2)

Fade orange precipitate; Yield 88%; Rf 0.62;  
Molecular formula  $C_{26}H_{17}ClN_2O_4$ ; Molar mass  
456.88g/mol; mp>300°C; IR (KBr)  $cm^{-1}$  3640, 3280,  
1630, 1170, 1100;  $^1H$ NMR (500 MHz, DMSO- $d_6$ )  
 $\delta$  10.18 (s, 1H), 9.90 (s, 2H), 8.00 (d, 1H,  $J$  7.6Hz), 7.92  
(d, 1H,  $J$  7.5Hz), 7.80 (t, 1H,  $J$  7.4Hz), 7.72 (t, 2H,  $J$   
7.25Hz), 7.66 (t, 1H,  $J$  7.45Hz), 7.27 (d, 2H,  $J$  1.45Hz),  
7.15 (d, 1H,  $J$  9.2Hz), 7.13 (d, 1H,  $J$  7Hz), 6.56 (s, 2H),  
6.40 (d, 2H,  $J$  1.5Hz);  $^{13}C$ NMR (125 MHz, DMSO- $d_6$ )  
65.73, 102.61, 109.80, 110.06, 112.51, 113.09,  
114.45, 117.76, 120.71, 123.38, 126.63, 127.57,  
129.81, 130.53, 134.26, 136.05, 143.30, 150.38,  
152.94, 153.53, 158.95, 159.9, 165.85, 169.20; EMIS  
(relative intensity, %)  $m/z$ , 456 ( $M^+$ ) 421, 391, 330,  
316, 287, 271, 259.

2-((3-chlorophenyl)amino)-3',6'-  
dihydroxyspiro[isoindoline-1-9'-xanthen]-3-one  
(FHJ-0.3)

Dark red precipitate; Yield 92%; Rf 0.62;  
Molecular formula  $C_{26}H_{17}ClN_2O_4$ ; Molar mass 456.88  
g/mol; mp>300°C; IR (KBr)  $cm^{-1}$  3680, 1640, 1630,  
1180 (C-N *str*), 1050, 1480;  $^1H$ NMR (500 MHz,  
DMSO- $d_6$ )  $\delta$  8.00 (d, 1H,  $J$  3.4Hz), 7.91 (d, 1H,  $J$   
7.8Hz), 7.85 (t, 2H,  $J$  7.8Hz), 7.71 (t, 1H,  $J$  7.4Hz), 7.28  
(d, 2H,  $J$  5.15Hz), 7.14 (d, 2H,  $J$  2.1Hz), 6.93 (t, 1H,  $J$   
8.05Hz), 6.58 (s, 2H), 6.41 (d, 2H,  $J$  2.2Hz);  $^{13}C$ NMR  
(125 MHz, DMSO- $d_6$ )  $\delta$  65.91, 83.70, 102.75, 110.17,  
112.55, 113.11, 118.63, 123.33, 124.83, 129.56,

130.53, 133.49, 134.16, 136.02, 150.12, 152.92,  
153.59, 159.98, 165.54, 169.20.

2-((3-bromophenyl)amino)-3',6'-  
dihydroxyspiro[isoindoline-1-9'-xanthen]-3-one  
(FHJ-0.4)

Orange red precipitate; Yield 82%; Rf 0.62;  
Molecular formula  $C_{26}H_{17}BrN_2O_4$ ; Molar mass  
501.33 g/mol; mp 253°C; IR (KBr)  $cm^{-1}$  3650, 3270,  
1635, 1150, 1070, 1475;  $^1H$ NMR (500 MHz, DMSO-  
 $d_6$ )  $\delta$  10.19 (s, 2H), 9.93 (s, 1H), 8.21 (d, 1H,  $J$  7.45Hz),  
7.79 (t, 2H,  $J$  7.2Hz), 7.65 (t, 1H,  $J$  7.6Hz), 7.47 (d,  
2H,  $J$  7.5Hz), 6.87 (t, 1H,  $J$  7.7Hz) 6.72 (s, 2H);  
 $^{13}C$ NMR (125 MHz, DMSO- $d_6$ )  $\delta$  65.53, 83.59,  
102.76, 110.16, 111.92, 112.57, 113.10, 115.17,  
121.52, 123.35, 124.83, 126.66, 129.57, 130.49,  
134.16, 136.01, 150.11, 152.36, 152.95, 153.59,  
159.03, 159.96, 165.55, 169.21.

2-((4-isopropylphenyl)amino)-3',6'-  
dihydroxyspiro[isoindoline-1-9'-xanthen]-3-one  
(FHJ-0.5)

Fade orange precipitate; Yield 78%; Rf 0.67;  
Molecular formula  $C_{29}H_{24}N_2O_4$ ; Molar mass  
464.51g/mol; mp 275-278°C; IR (KBr)  $cm^{-1}$   
3280, 1734, 1242, 2827, 1481;  $^1H$ NMR (500 MHz,  
 $CDCl_3$ )  $\delta$  10.18 (s, 2H), 9.89 (s, 1H), 8.00 (d, 1H,  
 $J$  7.65Hz); 7.87 (d, 1H,  $J$  = 7.5Hz), 7.78 (m, 2H), 7.27  
(d, 2H,  $J$  7.65Hz), 7.18 (d, 1H,  $J$  7.6Hz), 6.78 (d, 1H,  
 $J$  8.15Hz), 6.71 (s, 2H), 6.38 (d, 2H,  $J$  8.2Hz), 1.23  
(septet, 1H), 1.02 (d, 6H,  $J$  6.9Hz);  $^{13}C$ NMR (125 MHz,  
DMSO- $d_6$ )  $\delta$  24.56, 32.95, 65.42, 83.57, 102.74,  
110.39, 113.19, 123.10, 125.06, 126.06, 130.66,  
133.93, 136.03, 139.16, 146.38, 150.71, 152.94,

153.46, 158.82, 159.96, 165.88, 169.20; EMIS m/z (relative intensity, %) m/z, 464 (M<sup>+</sup>), 449, 360, 331, 316, 287, 271, 259.

2-((4-fluorophenyl)amino)-3',6'-dihydroxyspiro[isindoline-1-9'-xanthen]-3-one (FHJ-0.6)

Reddish-orange precipitate; Yield: 84%; Rf 0.62; Molecular formula C<sub>26</sub>H<sub>17</sub>FN<sub>2</sub>O<sub>4</sub>; Molar mass 440.42 g/mol; mp 203°C; IR (KBr), cm<sup>-1</sup> 3650, 1600, 1100, 1150, 1475; <sup>1</sup>HNMR (500 MHz, DMSO-d<sub>6</sub>) 8.29 (d, 1H, J 7.6Hz), 7.99 (d, 2H, J 6Hz), 7.87 (t, 2H, J 2.5Hz), 7.70 (t, 1H, J 7.4Hz), 7.44 (d, 2H, J 2.2Hz), 7.39 (d, 3H, J 9.2Hz) 7.27 (d, 2H, J 7.6Hz), 6.49 (s, 2H); <sup>13</sup>CNMR (125 MHz, DMSO-d<sub>6</sub>) δ 53.02, 65.45, 102.85, 110.27, 117.13, 123.20, 126.68, 129.54, 136.00, 142.82, 144.68, 150.40, 153.52, 155.30, 160.09, 165.76, 169.19, 171.84

### Biological studies

#### Antibacterial Activity

Each sample was tested individually against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Proteus mirabilis*) strains of bacteria. Identification and purification were verified by Institute of Microbiology, University of Agriculture Faisalabad, Pakistan. The strains were cultured on nutrient agar (Oxoid, Great Britten) overnight at 37 °C. Method of disc diffusion was used to determine the anti bacterial activity.

Briefly, there was suspension of 100 µL of microorganisms under test, that contains 10<sup>7</sup> colony-forming units (CFU)/mL of bacteria cells. Each filter disc was individually impregnated with solution of the compound and kept on the agar plates (already been inoculated with the tested microorganisms). Discs containing no samples were employed as a negative control. Rifampicin (30µg/dish) was used as a positive reference with a view to compare the sensitivity of isolate in analyzed microbial species. After 2 h at 4 °C, plates were incubated for 18 h at 37 °C for bacterial strains. Antibacterial activity has been evaluated by measuring the diameter in millimeter of the zone where growth had been inhibited (zone reader) for the organisms and comparing them to the control (44-46).

#### Hemolytic activity

To study the hemolytic activity, method employed by Rubab *etal.* (2017) and Shahzadi *etal.* (2019) was used. Fresh heparinized bovine blood (3 mL) was obtained from the Department of Clinical

Medicine, University of Agriculture Faisalabad, Pakistan. The sample of blood was centrifuged at 1000xg for 5 minutes, cells were separated, washed with 5 ml of sterile isotonic buffered saline (PBS) having pH of 7.4. Each assay contained erythrocytes of 10<sup>8</sup> cells per mL of sample. Hundred µL of each compound was mixed with human (10<sup>8</sup>cells/mL) separately. Samples were kept in incubation at 37°C for half an hour and agitated after 15 min. After incubation, samples were kept of ice (for 5mint), Centrifuged at 1000xg for 5 min. Supernatant (100 µL) were obtained from each tube and 10 times diluted with PBS (chilled at 4°C). Triton X-100 (0.1% v/v) (as positive control) and phosphate buffer saline (PBS- as negative control) were undergone through the same process. The absorbance at 576 nm using µQuant (Biotek, USA) was noted and the % hemolysis of RBCs for each sample was calculate

### Conclusion

All the synthesized compounds were confirmed by spectroscopic techniques. In the biological activities of synthesized hydrazides, no potent antibacterial activity was observed for the prepared compounds. Depending on activity results it is found that the following compounds can be used for hemolytic disorders. Moreover, this work also gives route that there is a need to design further fluorescein hydrazides with alkyl substituents and halo groups at meta-position for anti-hemolytic activities.

### References

1. C. Wioleta, S. Joanna and K. R. Anna, Antifungal styryloquinolines as *Candida albicans* efux pump inhibitors: styryloquinolines are ABC transporter inhibitors, *Molecules.*, **25**, 345 (2020).
2. A. H. Ameen, A. H. Sami and A. Z. Magdi, Synthesis of novel benzodifuranyl; 1,3,5-triazines; 1,3,5-oxadiazepines; and thiazolopyrimidines derived from visnaginone and khellinone as anti-inflammatory and analgesic agents, *Molecules.*, **25**, 220 (2020).
3. L. A. S. Mousa, M. Reem, M. Amjad, F. K. Omar, M. Majed and K. H. Masadeh, Synthesis, characterization, antimicrobial activity, and genotoxicity assessment of two heterocyclic compounds containing 1,2,3-selena- or 1,2,3-thiadiazole rings, *Molecules.*, **24**, 4082 (2019).
4. S. S. Sayran, A. S. Siham and A. M. Israa, Biological activity Study for some heterocyclic compounds and their impact on the grampositive and negative bacteria, *Energy Procedia.*, **157**, 296 (2019).

5. R. Dua, S. Shrivastava, S. K. Sonwane and S. K. Srivastava, Pharmacological significance of synthetic heterocycles scaffold, *Adv. Biol. Res.*, **53**, 120 (2011).
6. A. Gomtsyan, Heterocycles in drugs and drug discovery, *Chem. Heterocycl. Compd.*, **48**, 7 (2012).
7. N. I. Paphitou, Antimicrobial resistance: action to combat the rising microbial challenges, *Int. J. Antimicrob. Agents.*, **42**, 8 (2013).
8. I. M. Gould, M. Z. David, S. Esposito, J. Garau, G. Lina, T. Mazzei and G. Peters, New insights into meticillin-resistant *Staphylococcus aureus* (MRSA) pathogenesis, treatment, and resistance, *Int. J. Antimicrob. Agents.*, **39**, 96 (2012).
9. M. Singh and N. Raghav, Biological activities of hydrazones, *Int. J. Pharm. Sci.*, **3**, 26 (2011).
10. A. M. Isloor, B. Kalluraya and P. Shetty, Regioselective reaction: synthesis, characterization and pharmacological studies of some new Mannich bases derived from 1,2,4-triazoles, *Eur. J. Med. Chem.*, **44**, 3784 (2009).
11. I. V. Magedov, M. Manpadi, S. V. Slambrouck, F. A. Steelant, E. Rozhkova, N. M. Przhheval'skii, S. Rogelj and A. Kornienko, Discovery and investigation of antiproliferative and apoptosis-inducing properties of new heterocyclic podophyllotoxin analogues accessible by a one-step multicomponent synthesis, *J. Med. Chem.*, **50**, 5183 (2007).
12. G. Szabo, J. Fischer, A. Kis-Varga and K. Gyires, New celecoxib derivatives as anti-inflammatory agents, *J. Med. Chem.*, **51**, 142 (2007).
13. U. Theuretzbacher, Global antibacterial resistance; The never-ending story, *J. Global Antimicrob. Resist.*, **1**, 63 (2013).
14. P. S. Nareshvarma, S. Prasanthi and G. Supriya, Synthesis and in vitro study of some fused 1,2,4-triazole derivatives as antimycobacterial agents, *J. Saudi. Chem. Soc.*, **20**, 411 (2016).
15. T. J. Sultivan, J. J. Truglio, M. E. Boyne, P. Novichenok, X. Zhang, C. F. Stratton, H. J. Li, T. Kaur, A. Amin, F. Johnson, R. A. Stayden, C. Kisker and P. J. Tonge, High-affinity InhA inhibitors with activity against drug-resistant strains of *Mycobacterium tuberculosis*, *ACS. Chem. Biol.*, **1**, 43 (2007).
16. A. M. Gilbert, A. Failli, J. Shumsky, Y. Yang, A. Severin, G. Singh, W. Hu, D. Keeney, P. J. Petersen and A. H. Katz, Pyrazolidine-3,5-diones and 5-hydroxy-1H-pyrazol-3(2H)-ones, inhibitors of UDP-N-acetylenolpyruvyl glucosamine reductase, *J. Med. Chem.*, **49**, 6027 (2006).
17. O. Prakash, R. Kumar and V. Parkash, Synthesis and antifungal activity of some new 3-hydroxy-2-(1-phenyl-3-aryl-4-pyrazolyl) chromones, *Eur. J. Med. Chem.*, **43**, 435 (2008).
18. N. Benaamane, B. Nedjar-Kolli, Y. Bentarzi, L. Hammal, A. Geronikaki, P. Eleftheriou and A. Languin, Synthesis and in silico biological activity evaluation of new N-substituted pyrazolooxazin-2-one systems, *Bioorg. Med. Chem.*, **16**, 3059 (2008).
19. A. Sener, M. K. Sener, I. Bildmci, R. Kasimogullari and Y. Akcamur, Studies on the reactions of cyclic oxalyl compounds with hydrazines or hydrazones II: synthesis and reactions of 4-benzoyl-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3-carboxylic acid, *J. Heterocycl. Chem.*, **39**, 869 (2002).
20. M. Monici, Cell and tissue autofluorescence research and diagnostic applications, *Biotechnol. Ann. Rev.*, **11**, 227 (2005).
21. M. K. Smalley and S. K. Silvermann, Fluorescence of covalently attached pyrene as a general RNA folding probe, *Nucl. Acids. Res.*, **34**, 152 (2006).
22. A. Loudet and K. Burgess, BODIPY dyes and their derivatives: synthesis and spectroscopic properties, *Chem. Rev.*, **107**, 4891 (2007).
23. R. Stewart, *The Immunoassay Handbook*, Stockton Press, New York, NY (1994).
24. F. H. Kasten, In *Fluorescent and Luminescent Probes for Biological Activity* (Ed: W. T. Mason), Wiley Interscience, New York, Ch.2 NY (1983).
25. R. P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed., Molecular Probes, Eugene, p. 132 (1996).
26. P. Smart and I. Laidlaw, An evaluation of some fluorescent dyes for water tracing, *Water resources research.*, **13**, 15 (1977).
27. D. Guibault, General aspects of luminescence spectroscopy, *Practical fluorescence.*, **2**, 143 (1990).
28. G. W. Gordon, G. Berry, H. L. Xiao, B. Levine and B. Herman, Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy, *J. Biophys.*, **74**, 2702 (1998).
29. G. Weber, Fluorescence in biophysics: accomplishments and deficiencies, *Methods. Enzymol.*, **1**, 278 (1997).
30. P. R. Banks and D. M. Paquette, Comparison of three common amine reactive fluorescent probes used for conjugation to biomolecules by capillary zone electrophoresis, *Bioconj. Chem.*, **6**, 447 (1995).
31. V. Dujols, F. Ford and A.W. Czarnik, A Hlong-wavelength fluorescent chemodosimeter selective for Cu(II) ion in water, *J. Am. Chem. Soc.*, **119**, 7386 (1997).

32. M. Adamczyk, J. Grote and J. Moore, Chemoenzymatic Synthesis of 3'-O-(Carboxyalkyl)fluorescein Labels, *Bioconj. Chem.*, **10**, 544 (1999).
33. M. Adamczyk, J. R. Fishpaugh and P. G. Mattingly, Resin-supported labeling reagents, *Bioorg. Med. Chem. Lett.*, **9**, 217 (1999).
34. N. Sandhu, S. K. Pandey, R. K. Tittal, K. Kumar, Fluorescein dye derivative: Synthesis, characterization, quantum chemical and promising antimicrobial activity studies, *J. of Heterocycl. Chem.*, **24**, 231 (2021).
35. O. S. Wolfbeis, Fiber-optic chemical sensors and biosensors, *Ana. Chem.*, **80**(12), 4269 (2008).
36. L. Guern, F. Mussard, V. Gaucher, A. Rottman and M. D. Prim, Fluorescein Derivatives as Fluorescent Probes for pH Monitoring along Recent Biological Applications, *Int. J. of Molecul. Sci.*, **21**, 9217 (2020).
37. G. Ghini, C. Trono, A. Giannetti, G. Puleo, L. Luconi, J. Amadou and F. Baldini, Carbon nanotubes modified with fluorescein derivatives for pH nanosensing, *Sensors and Actuators B: Chemical.*, **179**, 163 (2013).
38. T. Mathew, S. Kundan, M. I. Abdulsamad, S. Menon, B.S. Dharan, and K. Jayakumar, Multiple muscular ventricular septal defects: use of fluorescein dye to identify residual defects. *The Annals of thoracic surgery*, **97**, 27-28 (2014).
39. S. Y. Xiao, J. Zhang, Z. Zhu, Y. Li, W. Zhong and J. Chen, Application of fluorescein sodium in breast cancer brain-metastasis surgery, *Cancer management and research*, **10**, 4325 (2018).
40. C. Guilini, C. Baehr, E. Schaeffer, P. Gizzi, F. Rufi, J. Haiech and J. L. Galzi, New fluorescein precursors for live bacteria detection. *Analytical chemistry*, **87**, 8858 (2015).
41. M. Rajasekar, Recent development in fluorescein derivatives, *Journal of Molecular Structure.*, **12**, 9085 (2020).
42. F. Ahmed, P. Alexandridis and S. Neelamegham, Synthesis and application of fluorescein-labeled pluronic block copolymers to the study of polymer-surface interactions, *Langmuir*, **17**, 537 (2001).
43. S. Pfautsch, j. Renard, M.G. Tjoelker and A. Salih, Phloem as capacitor: radial transfer of water into xylem of tree stems occurs via symplastic transport in ray parenchyma, *Plant physiology.*, **167**, 963 (2015).