Recent Progress on Synthesis of Fluorescein Probes

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Abstract: After its first synthesis in 1871, fluorescein and its derivatives have been used as a powerful tool for molecular and cellular studies in biology, molecular biology, interactions between ligands and macromolecules, for drug discovery and environmental research. In recent 20 years, research attention has been paid particularly to synthesis and separation of fluorescein derivatives having various linker groups. This mini-review briefly summarizes synthesis of fluorescein derivatives for improving their chemical, fluorescent and biological properties and conjugation suitability.

Keywords: Fluorescein, fluorescent probe, fluorescent dye, fluorescence spectroscopy.

I. INTRODUCTION

Fluorescent detection technique has played a significant role on the advancement of modern medicine and molecular biology and has achieved rapid development. Fluorescent detection has the advantages of being very sensitive, selective, rapid, safe, reproducible and suitable for high-throughput screening applications. It has been widely used in determining ion concentrations in cell, protein structures and functions, protein-protein interactions, drug-receptor interactions, DNA sequencing, immunoassay, and quantitative analysis of small molecules such as toxins and drugs [1-2]. Fluorescent probe *in vivo* labeling for various applications in cell biology has been an emerging area of research in the recent few years [3-5]. Fluorescein (**1**) has a rigid coplanar tricyclic structure, a high molar absorptivity (88,000 $cm^{-1}M^{-1}$ at pH 9) and high fluorescence quantum yield (0.92 at pH> 8) [6-9]. It has been derived into versatile fluorescent reagents comprising necessary, diverse reactive functional groups. Therefore, the fluorescein dyes have become one of the most significant and practical classes of fluorescent probes [9].

Even though wide applications of fluorescein, synthesis of its new analogues is required for the improvement of some properties such as the instability of fluorescein conjugates, irreversible photobleaching by a powerful laser to cause rapid decline of fluorescence signal, and strong fluorescence pH dependence in aqueous solutions [8, 10,11]. In addition, the commercial fluorescein dyes are often very expensive and the protocols for labeling with bioactive molecules often require further optimization, particularly for those bearing multiple reactive functionalities. Therefore, new scale-up synthesis routes and new fluorescein dye syntheses are of a current research interest. Numerous studies of fluorescein dyes currently reported were to synthesize new fluorescein dyes, simplify synthetic routes, investigate practical separation methods of regioisomers such as 5(6)-carboxyfluoresceins, and to introduce special terminal functional groups for convenient coupling with biomolecules.

Fluorescein (**1**) was first synthesized by von Bayer in 1871 with resorcinol (**2**) and phthalic anhydride (**3**) via Friedel-Crafts acylation/cyclodehydration (Fig. (**1**)) (Scheme **1**) [12]. Fluorescein itself does not have a coupling group, and thus, a research focus has been the syntheses of its derivatives with diverse linkers at different positions such as 5(6)-carboxy-, amino-, and isothiocyanato-fluoresceins (Fig. (**1**)). It is to note that many well-known, widely used halogenated xanthene dye salts such as eosin, phloxine B, chloroflouresceins, erythrosin B, and rose bengal are commercially available and used as coloring additives in food, cosmetics and drugs as well as insecticides [13-15], which are not reviewed here.

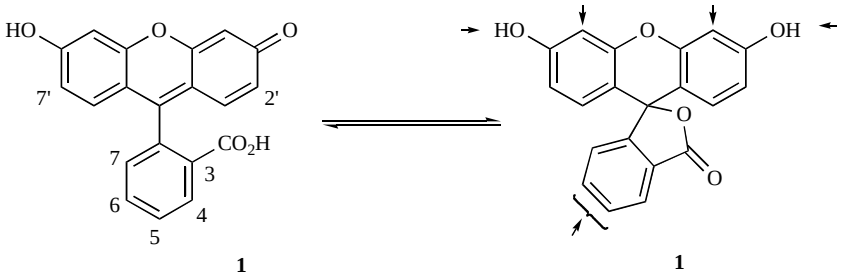
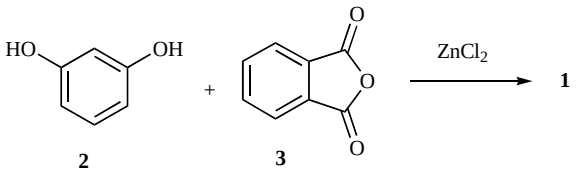


Fig. (1). Structure of fluorescein and common sites of modification and linker attachment. Arrows indicate sites commonly used for linker attachment while the other positions have been often modified with halogens or other substituents to improve the chemical and fluorescence properties of the dye.



Scheme 1. Synthesis of fluorescein.

II. SYNTHESIS OF FLUORESCEIN DERIVATIVES

1. 5(6)-Carboxyfluoresceins

5(6)-Carboxyfluoresceins are commonly activated with dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) followed by conjugation with target biomolecules (Scheme **2**) [16-18]. 5(6)-Carboxyfluorescein succinimidyl esters (**5a** and **5b**) are reported to satisfactorily monitor the internal pH in *Escherichia coli*

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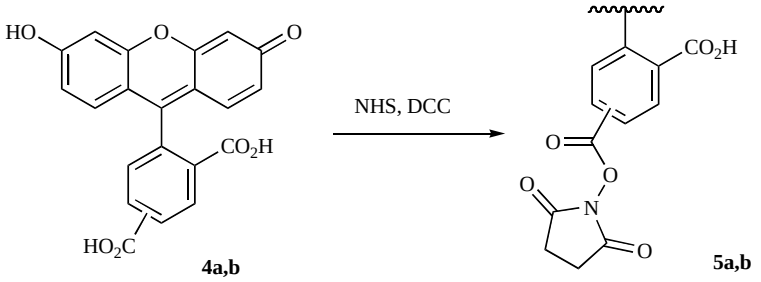
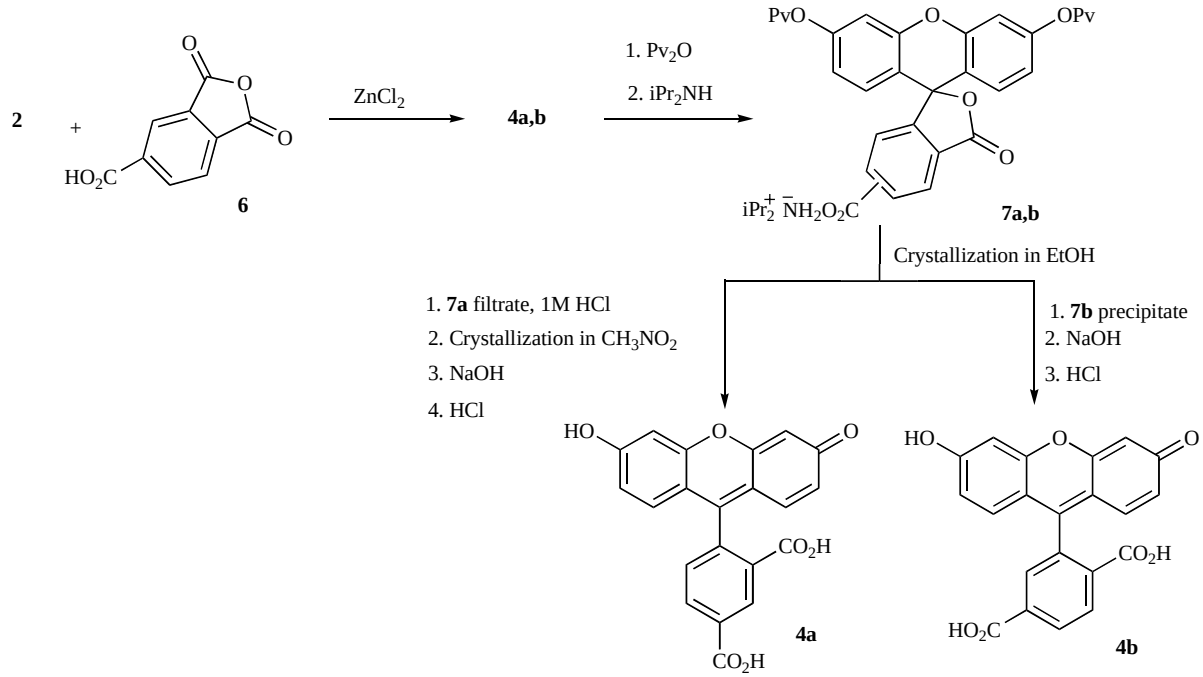
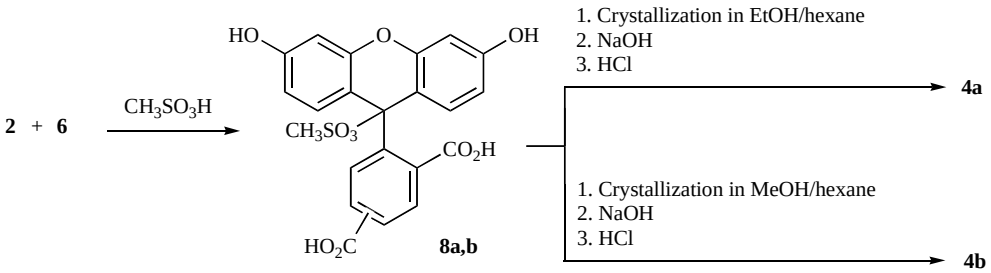


Scheme 2. Synthesis of 5(6)-carboxyfluorescein succinimidyl esters. NHS, N-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide.



Scheme 3. Synthesis and derivatization-based separation of 5- and 6-carboxyfluorescein. Pv_2O, pivalic anhydride; iPr_2NH, diisopropylamine.

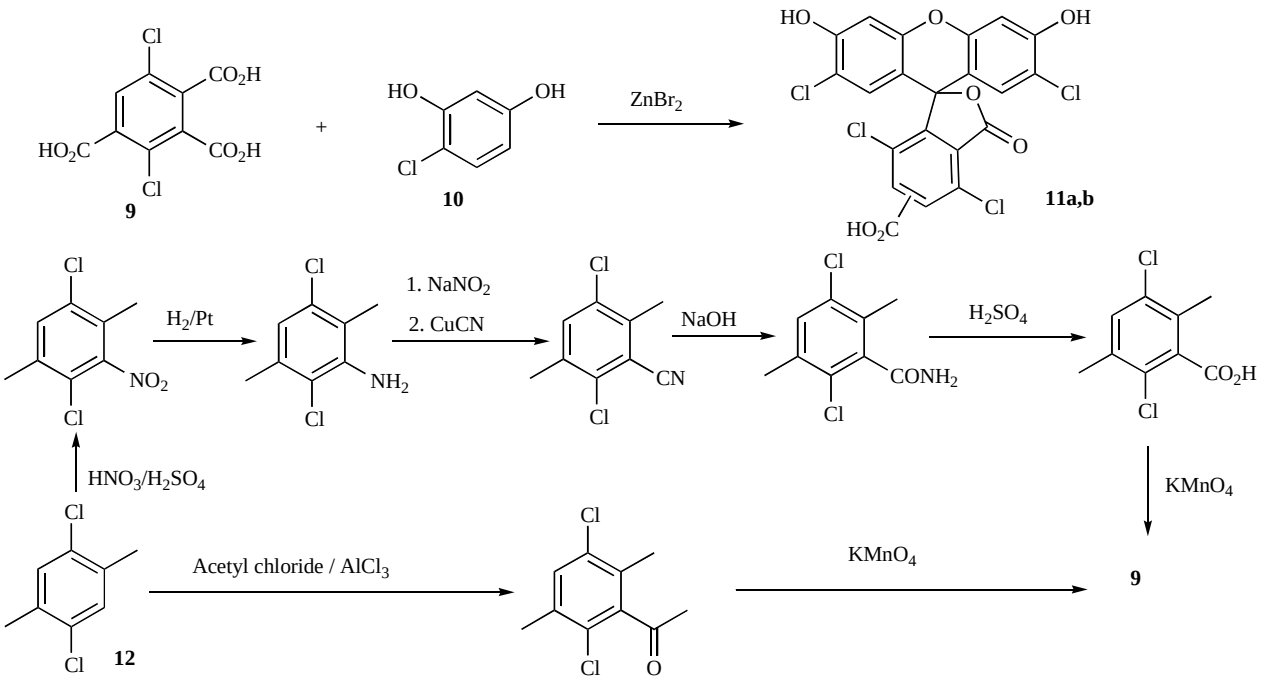


Scheme 4. Methanesulfonic acid catalyzed synthesis and separation of 5- and 6-carboxyfluoresceins.

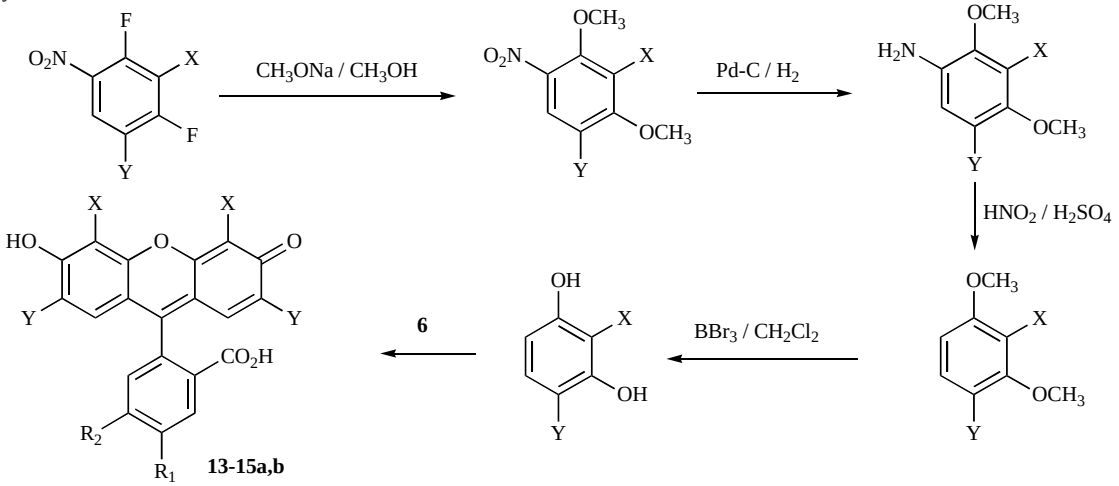
cells due to their extreme sensitivity to pH changes [19]. The **5a** and **5b** diacetates, which can be easily hydrolyzed to **5a** and **5b** correspondingly by esterases, are also used to determine cellular pH changes, particularly when they are under stress conditions [19]. The length of the linker of **4a** has been often extended to meet different application requirements, of which 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester that is commercially available is a good example [9].

5(6)-Carboxyfluoresceins (**4a,b**) are typically formed by Friedel-Crafts acylation and cyclodehydration of **2** and 4-carboxyphthalic anhydride (**6**) in presence of zinc chloride and derivatized with pivalic anhydride (Pv_2O)and diisopropylamine (iPr_2NH) to form the corresponding diester salts (**7a,b**) which 6-carboxyfluorescein diester salt (**7b**) is precipitated out from the mixture in absolute ethanol (Scheme **3**). The free form 5-carboxyfluorescein dipivalate (**7a**) is then crystallized out of the mixture with nitromethane. After hydrolysis of the dipivalates (**7a,b**), the final yields of **4a** and **4b** are 20% and 21%, respectively [20]. In addition to the irreproducible separation problem of **7a** and **7b** [21], we have used pivaloyl chloride instead of its anhydride, **7b** was readily obtained, but the crystallization of 5-carboxyfluorescein dipivalate failed.

Ueno *et al.* [22] reported another practical method to prepare **4a** and **4b**, in which methanesulfonic acid was used as a dehydration solvent and catalyst (Scheme **4**). The products were a mixture of 5(6)-carboxyfluorescein methanesulfonates. Of the two regioi-



Scheme 5. Synthesis of 4,7,2',7'-tetrachloro-5(6)-carboxyfluorescein from a reaction between chlororesorcinol and 3,6-dichloro-4-carboxyphthalic acid which the latter was synthesized in two different routes.



13. X = F, Y = H;
14. X = H, Y = F;
15. X = Y = F

a. $R_1 = CO_2H$, $R_2 = H$;
b. $R_1 = H$, $R_2 = CO_2H$

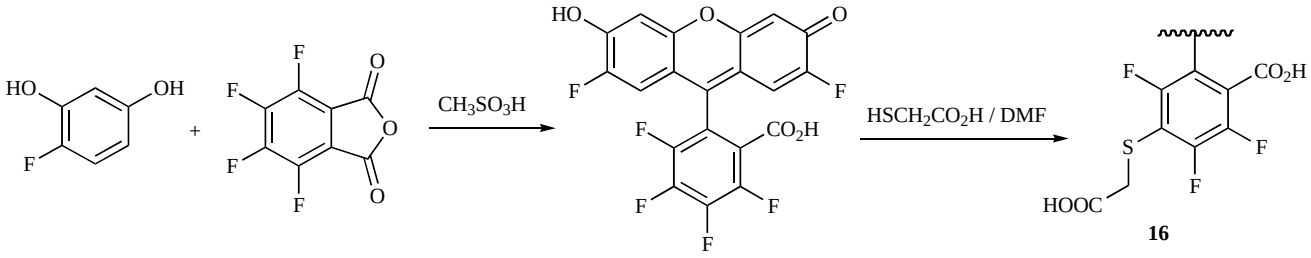
Scheme 6. Synthesis of fluorinated 5-(6-)carboxyfluoresceins.

somers, 5-carboxyfluorescein methanesulfonate (**8a**) preferentially crystallizes out in a mixture of ethanol and hexane while 6-carboxyfluorescein methanesulfonate (**8b**) in a mixture of methanol and hexane. The regioisomerically pure **8a** and **8b** were then converted easily to **4a** and **4b**, respectively, with NaOH and HCl treatments. This method has shortened the synthesis steps and, thus, increased the yield to 40%, and has been carried out in a multi-gram scale.

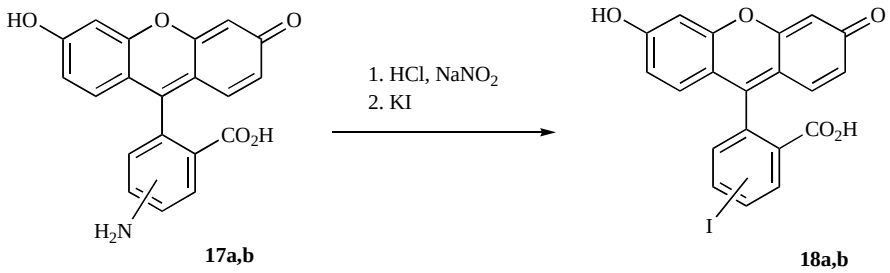
2. Halogenated 5- and 6-carboxyfluoresceins

Halogenations of carboxyfluorescein can diversify its chemical, fluorescence and bioactivity properties for various applications. Although carboxyfluorescein is an excellent fluorescent dye, it displays limited fluorescence diversity with different bioactive molecules [23]. Several studies were conducted to incorporate halide atoms into **4a** and **4b** to increase and diversify the maximum excitation (λ_{ex}) and emission (λ_{em}) wavelengths for detecting multiple target substances and to narrow the emission and absorption band widths for high selectivity. Khanna and Ullman [24] synthesized 4,7,2',7'-tetrachloro-5(6)-carboxyfluorescein (**11a**,**b**) from a reaction between 3,6-dichloro-4-carboxyphthalic acid (**9**) and chlororesorcinol (**10**), the former was synthesized from 2,5-dicloro-1,4-xylene (**12**) in six steps (Scheme **5**). However, this approach gave a complex mixture of products which was difficult to purify [25]. Lyttle *et al.* [25] simplified the synthesis of **9** via acylation of **12** and subsequent oxidation (Scheme **5**). This method gave a 21% yield of **11a** and **11b**.

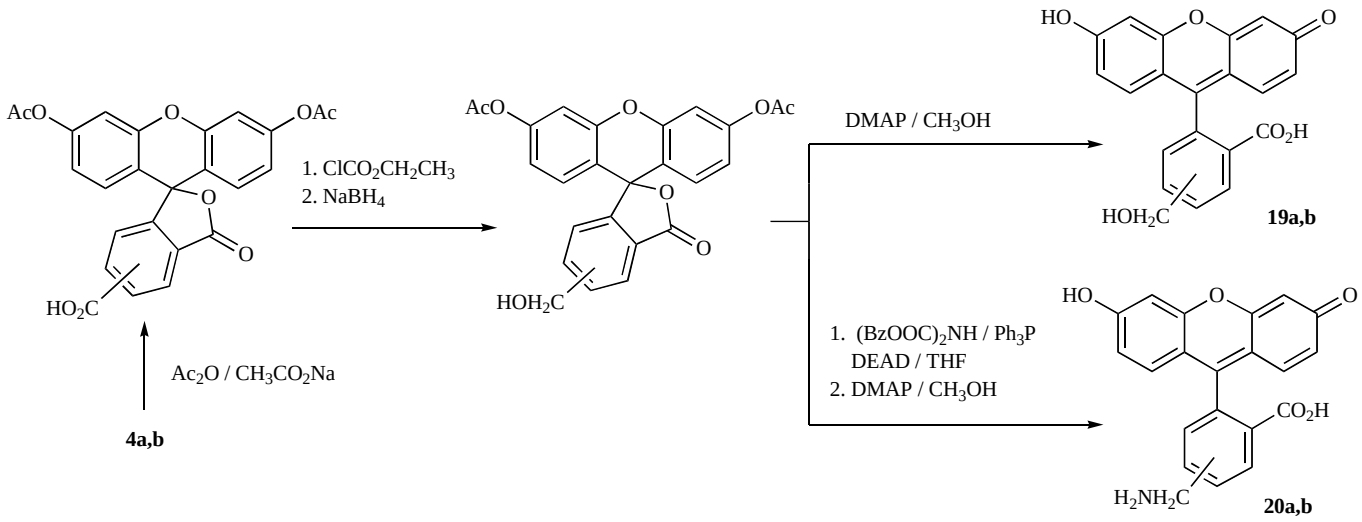
Being a similar size with hydrogen, fluorine is often used to replace hydrogen on a molecule to alter chemical properties due to the high electronegativity of fluorine [26]. Sun *et al.* [7] incorporated fluorine atoms into resorcinol (**2**) via diazotization and subsequently synthesized fluorinated 5(6)-carboxyfluoresceins (**13-15a,b**) (Scheme **6**) that showed higher photostability and lower pKa



Scheme 7. Synthesis of 6-carboxymethylthio-4,5,7,2',7'-pentafluorofluorescein.



Scheme 8. Synthesis of 5- and 6- iodofluoresceins.



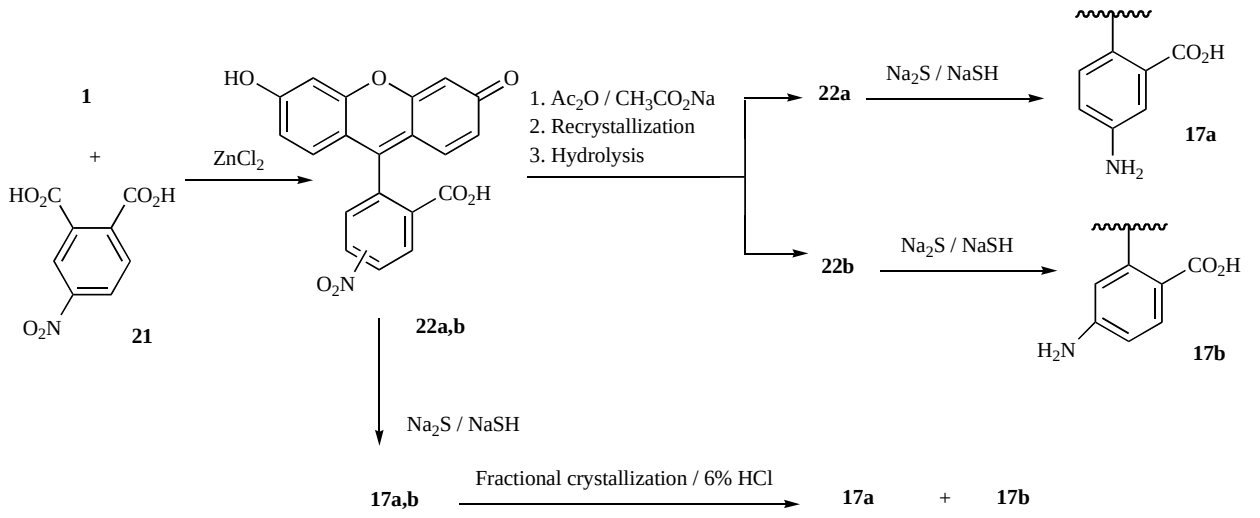
Scheme 9. Synthesis of 5(6)-hydroxymethylfluorescein and 5(6)-aminomethylfluorescein. Ac_2O, acetic anhydride; DMAP, 4-dimethylaminopyridine; DEAD, diethyl azodicarboxylate.

than the corresponding carboxyfluoresceins. For example, 5(6)-carboxy-2',7'-difluorofluoresceins are good replacements for carboxyfluorescein and isothiocyanatofluorescein [7, 9]. Gee *et al.* [27] reacted fluororesorcinol with tetrafluorophthalic anhydride in methanesulfonic acid to give 4,5,6,7,2',7'-hexafluorofluorescein that further reacts with 2-mercaptoacetic acid to obtain the final product **16** (Scheme **7**).

Jiao *et al.* [28] described the synthesis of 5(6)-iodofluoresceins (**18a,b**) by diazotization of 5(6)-aminofluorescein (**17a,b**, the synthesis is discussed in section 4**)** followed by treatment with potassium iodide while the bromo-analogues were obtained by using cuprous bromide (Scheme **8**). In general, the polarity of halogens is less than that of carboxyl and nitro groups, regioiosmeric halofluoresceins and their diesters are more difficult to be separated via fractional crystallization. 5(6)-Bromofluorescein diacetates can be readily synthesized from **1** and 4-bromophthalic anhydride followed by treatment of acetic anhydride in which 5-bromofluorescein diacetates can be crystallized out easily [28].

3. 5(6)-Hydroxy(amino)methylfluorescein

Adamczyk *et al.* and Mattingly [29, 30] reacted **4a**,**b** with acetic anhydride to form 5(6)-carboxyfluorescein diacetates, and then reacted it with ethyl chloroformate followed by sodium borohydride reduction to result 5(6)-hydroxymethylfluorescein diacetates (Scheme **9**). The regioisomeric diacetates, separated by flash chromatography, were treated with 4-dimethylaminopyridine (DMAP) in methanol to give the corresponding hydroxymethylfluoresceins (**19a** and **19b**) or treated with dibenzyl imidodicarbonate ($(BzOOC)_2NH$), triphenylphosphine (Ph_3P) and diethyl azodicarboxylate (DEAD) in THF followed by 4-dimethylaminopyridine in methanol to form the corresponding aminomethylfluorescein (**20a** and **20b**). The dyes **20a** and **20b** are widely used in immunohistochemical analyses while 4'-aminomethylfluorescein decomposes easily in a retro-Mannich reaction or deamination and, thus, is unstable in aqueous solution during long-term storage [29,31].



Scheme 10. Synthesis and separation of 5(6)-aminofluorescein. Ac_2O, acetic anhydride.

4. 5(6)-Aminofluorescein

In addition to **20a** and **20b**, 5(6)-aminofluoresceins (**17a,b**) are commonly used in elucidation of protein and membrane structures, analysis of nucleic acids and nucleotides, lipid transport and metabolism due to the convenient coupling of the amino group with an activated carboxylic group of the target molecules [32]. Kremer *et al.* and Sigmund and Pfleiderer [33, 34] reacted **1** with 4-nitrophthalic acid (**21**), separated the regioisomers via fractional crystallization, and then reduced the nitro group to yield **17a** and **17b** (Scheme **10**). Pan *et al.* [35] improved the separation **17a** and **17b**, for which **22a** and **22b** in a mixture were reduced with sodium sulfide and sodium hydrosulfide to obtain a mixture of **17a** and **17b** which both were separated via fractional crystallization in dilute HCl (Scheme **10**). The amino group on **17a** and **17b** offers convenient functional group replacement and linker length extension as exemplified with the dye **23** in Fig. (**2**) [9, 27].

5. 5(6)-Azidofluorescein

Azide is one of the most commonly used photoactivatable groups in photoaffinity labeling reagents [36-38]. Rotman [39] described synthesis of azidofluorescein, **17a** in DMF was acidified and diazotized with n-amylnitrite at 4 ºC followed by a reaction with sodium azide to give 5-azidofluorescein (**24**) (Scheme **11**). Acylation of **24** generates 5-azidofluorescein diacetate (**25**) in an overall yield of 42%, which **25** can infiltrate into cells, and be transformed into 5-nitrenefluorescein to react with intracellular proteins upon exposure to light at a wavelength greater than 3000 nm for the protein detection [39].

6. 5(6)-Isothiocyanatofluorescein

Isothiocyanate couples well with a reactive amino group in a protein and peptide chain under mild conditions. Isothiocyanatofluorescein (**26**) (Fig. (**3**)) is widely used in labeling different biomolecules such as immunoglobulins, lectins, proteins, peptides, nucleic acids, oligo-and polysaccharides [40]. The derivatives of **26** are also extensively utilized in biological analyses [41-43]. It, although being a versatile fluorescent probe, has some problems such as small Stoke shift that causes quenching phenomenon, large fluorescence dependence upon pH, and high light-sensitivity [31].

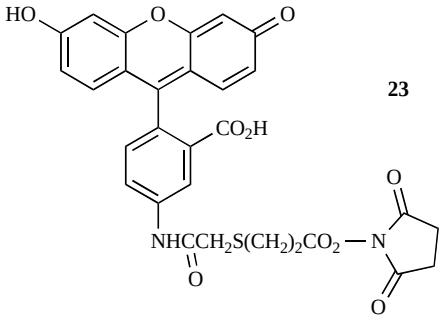


Fig. (2). Structure of 3-(fluorescein-5-carbamoylmethylthio)propionic acid, succinimidyl ester.

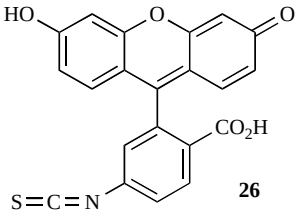
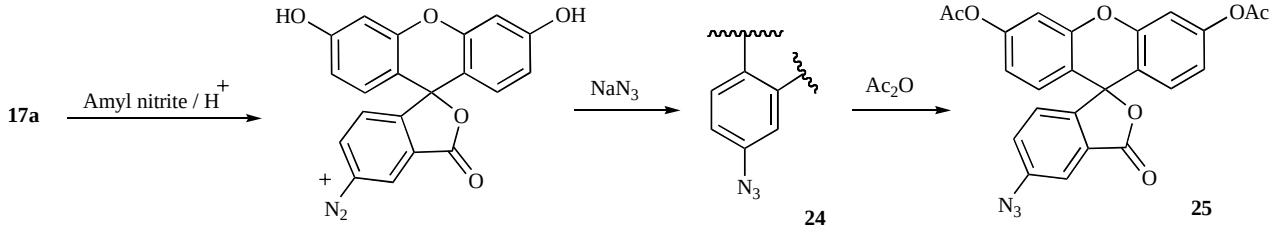
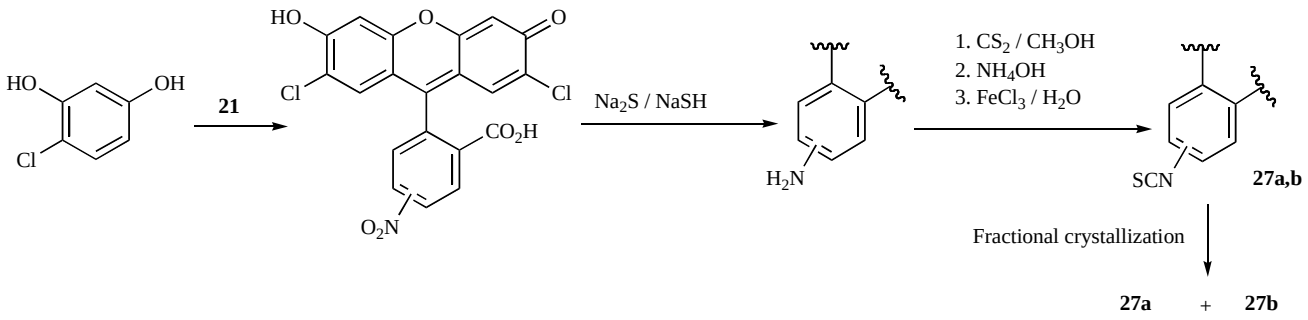


Fig. (3). Structure of 6-isothiocyanatofluorescein.

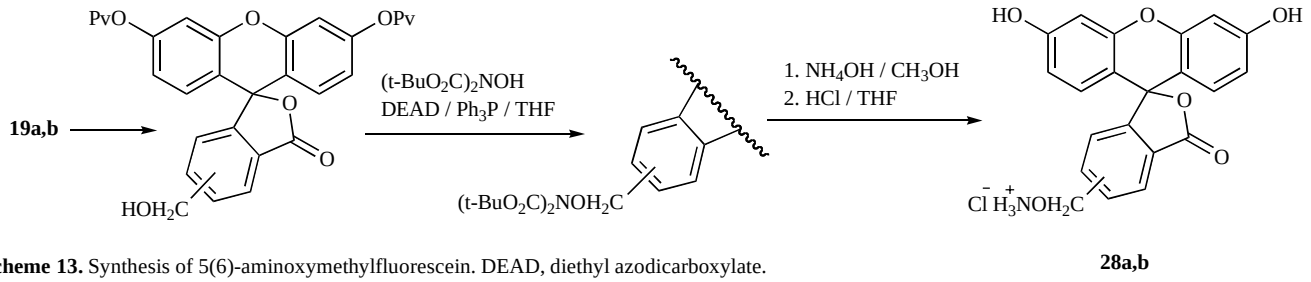
To improve the properties of **26**, Pan *et al.* [44] prepared 5(6)-isothiocyanato-2',7'- dichlorofluoresceins (**27a,b**) by a reaction of



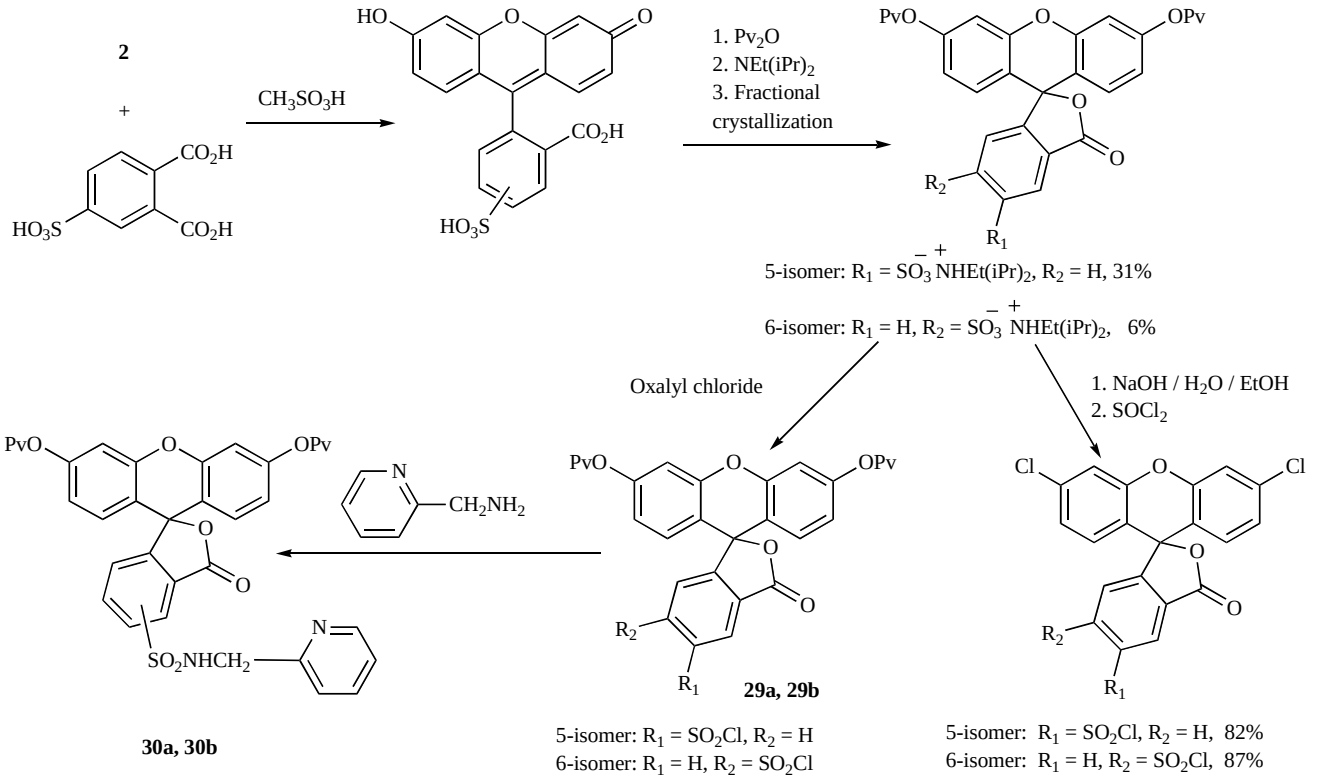
Scheme 11. Synthesis of 5-azidofluorescein and 5-azidofluorescein diacetate. Ac_2O, acetic anhydride.



Scheme 12. Synthesis of 2',7'-dichloro-5(6)-isothiocyanatofluoresceins.



Scheme 13. Synthesis of 5(6)-aminoxymethylfluorescein. DEAD, diethyl azodicarboxylate.



Scheme 14. Synthesis of fluorescein-5(6)-sulfonyl chlorides and fluorescein-5(6)-sulfonamindes. Pv_2O, pivalic anhydride; $NEt(iPr)_2$, diisopropylethylamine.

4-chlorocresorcinol with **21** in presence of zinc chloride, reduction with sodium sulfide and sodium hydrosulfide, and treatment with carbon disulfide in methanol, ammonium hydroxide, aqueous ferric chloride solution, and then purification via crystallization in acetone and flash chromatography (Scheme **12**). The compounds **27a** and **27b** show excellent labeling characteristics. For example, their λ_{ex} and λ_{em} wavelengths are approximately 10 nm longer than those of **26**, which minimizes background interference and improves measurement sensitivity. The high fluorescent emission at pH 7-11 extends its application range in comparison with **26** emitting fluorescence at pH 9-11 [44].

7. 5(6)-Aminoxymethylfluorescein

Adamczyk *et al.* [45] reacted **19a** or **19b** with N,N-bis-*tert*-butoxycarbonylhydroxylamine under Mitsunobu conditions and undertook subsequent treatments with an excess of ammonium hydroxide in methanol at ambient temperature and refluxing the mixture in aqueous HCl and THF to obtain O-(5-fluorescein-methyl)hydroxylamine hydrochloride (**28a**) and its 6-regioisomer (**28b**) (Scheme **13**). Due to the specific reaction between the amino group of **28a,b** and abasic sites of damaged nucleic acids, **28a** and **28b** have been used as a fluorescent reagent to detect damaged

nucleic acid [45]. They have been also used to label oxosteroid analogues for clinical immunoassays via an oxime linkage [46].

8. 5(6)-Sulfofluorescein

Studies have shown that sulfonamides can be used as Zn^{2+} sensors [47]. Woodroofe *et al.* [48] designed and prepared fluorescein-5(6)-sulfonamides for a study on a metal-induced change in fluorescence. Reaction of **2** and 4-sulfophthalic acid and subsequent esterification and ammoniation yielded a mixture of dipivaloyl-5(6)-fluorescein sulfonate salts that were separated with fractional crystallization. The subsequent basic hydrolysis and chlorination with thionyl chloride lead to the non-fluorescent 3',6'-dichlorofluoran instead of the anticipated target compounds (Scheme **14**). Using oxalyl chloride rather than thionyl chloride afforded the fluorescein sulfonyl chloride (**29a, 29b**) that reacted with an amine to give the corresponding fluorescein-sulfonamides such as **30a** and **30b** [48] or a bifunctional reagent such as diamine to extend the linker via a sulfonamide bond.

9. O-alkylated Fluorescein

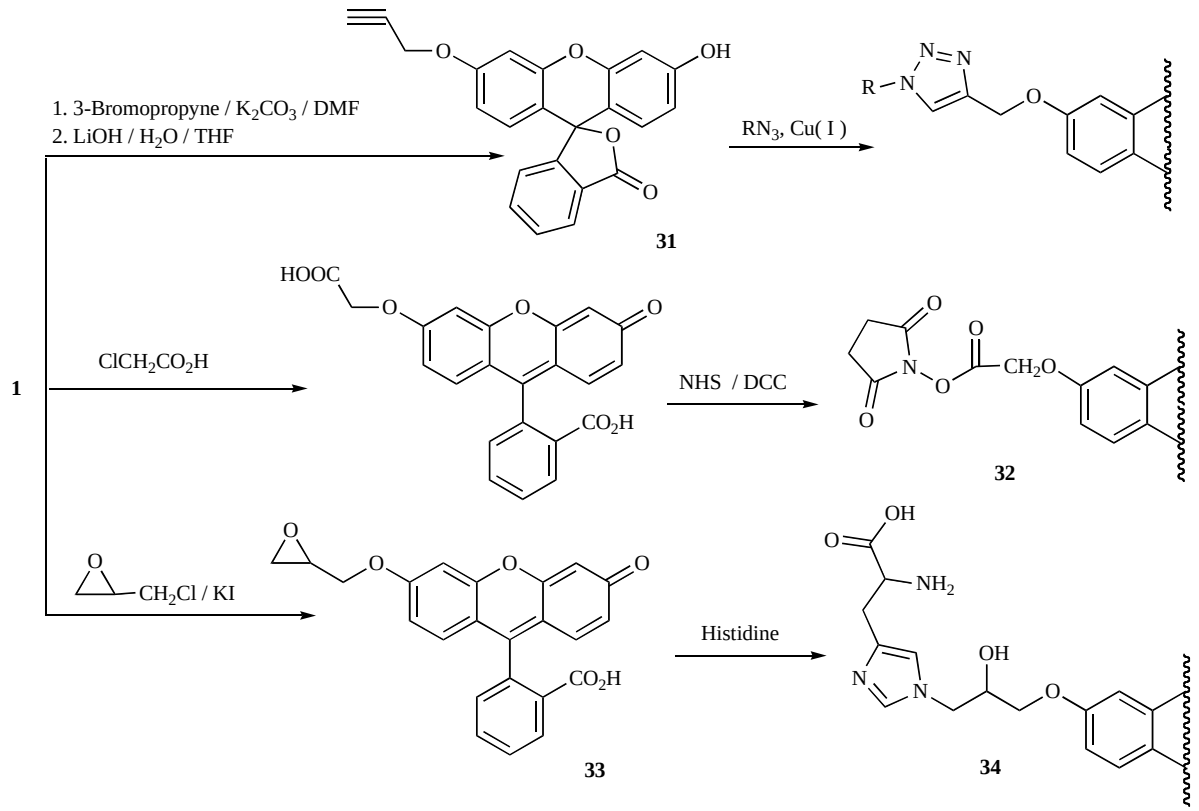
A novel class of fluorescent labeling reagents has appeared recently along with the emerging 'click' chemistry, a type of reactions by which chemicals are coupled with the target molecules through a convenient formation of a heterocyclic ring in high yields and selectivity [49]. In addition to the 5- and 6- positions on fluorescein for coupling, the 3'- and 6'-positions have been studied as a handle for linkage. In 2007, Zhang and Gao [50] employed this strategy to develop an economical protocol by which O-propargylfluorescein (**31**) can be linked covalently with bioactive molecules bearing an azide group via a Cu(I)-catalyzed Huisgen reaction (Scheme **15**).

Wang *et al.* [51] synthesized N-hydroxy-succinimidyl-fluorescein-O-acetate (**32**) (Scheme **15**), which can react specifically with a primary amine. This dye can be used for the analysis of aliphatic amines in the environment, which are difficult to be detected with conventional methods [51].

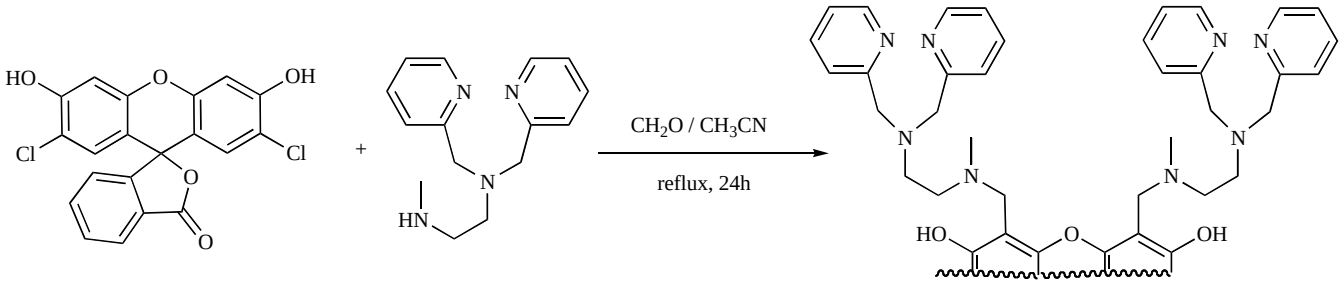
Li *et al.* [52] prepared O-glycidylfluorescein (**33**) by etherification of fluorescein with 2-chloromethyloxirane (Scheme **15**). The dye **33** dissolved in basic aqueous solution or ethanol has long λ_{em} and high fluorescence quantum yield. Its epoxy group reacts easily and specifically with imidazole ring and, thus, **33** has been successfully developed as a selective tracer of histidine by formation of **34** [52].

10. 4'(5')-Alkylated Fluorescein

There have been three strategies to introduce a liker on fluorescein probes. One is that the linker is incorporated into the starting chemical prior to the dye synthesis. The second is that the linker is attached to an existing group on the fluorescein dye to make it suitable for a specific conjugation requirement. A new strategy that has been recently reported is the use of the reactive 4' and 5'-protons on fluorescein to introduce a linker. For example, 2',7'-dichlorofluorescein condensed with formaldehyde and N-(2-methylaminoethyl)- bis(2-pyridylmethyl)amine via Mannich reaction to result in the dye **35** (Scheme **16**), which was developed to selectively examine PPi (pyrophosphate) and ATP in 100% aqueous solution in the presence of Mn^{2+} [53]. Lippard and Lim [54] have reacted 2'-chloro-5'-formylfluorescein with 2-substituted-8-aminoquinolines to form novel fluorescein probes (**36**) for tracking nitric oxide in cells (Scheme **17**). Such a class of fluorescein probes has been applied in biochemistry [55-57] and environmental analysis of toxic metals such as copper(II) [58]. Sarin (O-isopropyl-methylphosphono fluoridate) and soman (O-pinacolyl-methylphosphonofluoridate) are two well known potent warfare agents. Numerous studies have focused on their decontamination and detection over the last five decades [59-61]. Ansyln and



Scheme 15. Synthesis of O-propargylfluorescein, N-hydroxysuccinimidyl-fluorescein-O-acetate and O-glycidylfluorescein and their conjugation reactions. NHS, N-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide.



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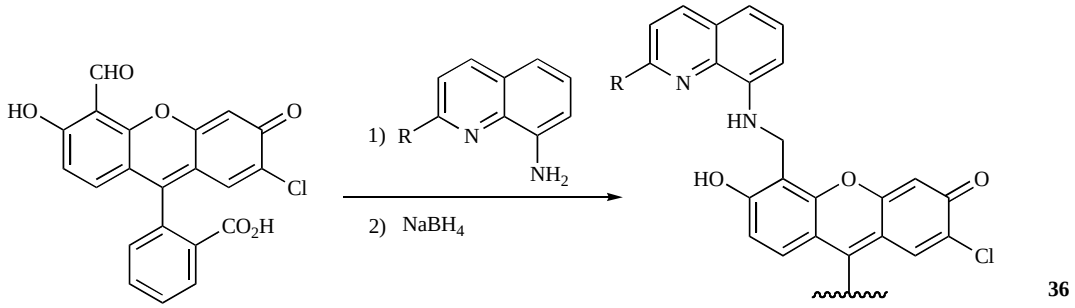
Scheme 16. Synthesis of 2',7'-dichloro-4',5'-bis-({[2-(bis-pyridin-2-ylmethylamino)-ethyl]- methylamino}-methyl)fluorescein.



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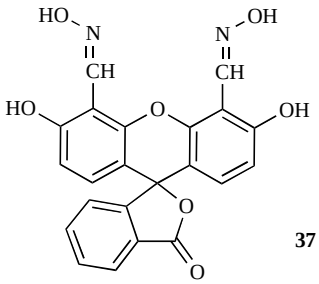
R = CO_2CH_3, CO_2H, CH_2OH, CH_3, H

Scheme 17. Synthesis of 5'-(2-substituted-8-aminoquinolino)fluorescein.

Wallace [62] reported recently that the probe (**37**), which was prepared from fluorescein-4',5'-dialdehyde with hydroxyamine in Fig. (**4**), can readily detect chemical warfare agents containing phosphoryl fluoride.



37

Fig. (4). Structure of fluorescein-4',5'-dialdoxime.

III. CONCLUSIONS

The fluorescent probes play important roles in the life and environmental sciences. Having met various chemical and biological property requirements, fluorescein-based probes as a common class of fluorescent reagents have proven to be a sensitive, specific analytical tool in life sciences and chemical analyses as well as replacement of radioisotope uses. Since the synthesis of fluorescein in 1871, many novel fluorescein-based dyes have been synthesized via utilization of new starting chemicals bearing a linker, modification of an existing group to a linker and introduction of a linker at different positions. Chemical, fluorescent and biological properties of fluorescein-based dyes are often improved via attachment of halogens and other substituents with unique properties such as chelation with metals. As the exciting applications of fluorescein probes continue to grow, design and synthesis of novel fluorescein probes will certainly attract more attention, in addition to their high yield synthesis and efficient separation.

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