Synthesis of New Xanthene Derivatives

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Summary

Title: Synthesis of New Xanthene Derivatives

Xanthene dyes, including fluorescein, are a well-known class of fluorescent dyes, which have widespread applications in natural sciences. The synthesis of xanthene derivatives *via* acid catalyzed condensation of substituted phenols with phthalic anhydride, to afford the asymmetric derivatives, is well established. The high temperature, harsh reaction conditions and often low yields make this method less convenient. The synthesis of xanthene dyes by direct modification of the fluorophore moiety is a great option to circumvent the above mentioned drawbacks.

Our new synthetic strategy for the preparation of novel asymmetric xanthene dyes *via* direct conversion of hydroxyl groups on 3'- and 6'-positions into leaving groups by mesylation is reported. It was discovered that 3',6'-di-mesylated fluorescein underwent a nucleophilic aromatic substitution with sulfur nucleophiles and afforded new asymmetric xanthene sulfides.

The impact of substituents possessing an electron-withdrawing character such as chlorines and bromines was investigated with the aim to improve the aromatic substitution on the electron-rich fluorescein structure. It was observed that the incorporation of these groups did not considerably affect the substitution reaction and the yields were comparable with the unsubstituted fluorescein.

This strategy provided novel fluorescent probes with the linker suitable to further modifications. The modifications of the linker delivered fluorescein derivatives that could be used as fluorescent labels in peptides, oligonucleotides and for cell imaging.

The hydroxyl group on the linker was modified to achieve potent bioconjugate functionality such as azide. The new fluorescent azides were obtained in a 4-step synthesis, namely 2-(6-(2-azidoethylthio)-3-oxo-3H-xanthen-9-yl)benzoic acid with an overall yield of 13%, its 2',7'-dichloro derivative with an overall yield of 10% and its 2',4',5'-tribromo derivative with an overall yield of 1%, respectively.

An asymmetric xanthene sulfide with an amino functionality placed on the aliphatic linker, namely 2-(6-((2-aminoethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid, was obtained in a 3-step synthesis with an overall yield of 33%.

The impact of the substitution with sulfur nucleophiles on the 6'-position of the xanthene moiety on its fluorescent characteristics was investigated. In comparison with fluorescein new asymmetric xanthene sulfides afforded lower extinction coefficients and fluorescent quantum yields. On the other hand, the substitution with a sulfur nucleophile significantly improved the photostability of xanthene dyes. It was shown that after 10 hours of continuous excitation, the asymmetric sulfur-containing xanthene fluorophores exhibited 58-94% of their initial fluorescent intensities. This observation suggested that the novel dyes were 1-2 orders of magnitude more stable than fluorescein.

The azido-modified xanthenes were “clicked” *via* Cu(I)-catalyzed azide-alkyne cycloaddition with an oligonucleotide, which contained the terminal alkyne residue.

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Zusammenfassung

Titel: Synthesis of New Xanthene Derivatives

Xanthen Farbstoffe, insbesondere Fluoreszein, sind sehr bekannt als Fluoreszenzfarbstoffe und haben eine Vielzahl von Anwendungen. Die Synthese von Xanthen Derivaten findet *via* eine säurekatalysierte Kondensation substituierter Phenole mit Phthalsäureanhydrid statt. Die hohen Temperaturen und extremen Bedingungen, unter denen diese Reaktion abläuft, führen oft zu schlechten Ausbeuten. Daher bietet eine direkte Modifikation der Fluorophore eine hervorragende Alternative.

In dieser Doktorarbeit konnten neue Synthesewege zur Herstellung neuer asymmetrischer Xanthen Farbstoffe mittels direkter Überführung der Hydroxyl Gruppen an 3'- und 6'-Position in Abgangsgruppen durch Mesylierung eingeführt werden. Des Weiteren konnte gezeigt werden, dass 3',6'-di-mesyliertes Fluoreszein eine nukleophile aromatischen Substitution mit Schwefel Nukleophilen eingeht, wobei neue asymmetrische Xanthen Sulfide erhalten wurden.

Inwieweit elektronenziehende Substituenten, wie zum Beispiel Chlorid oder Bromid, die aromatische Substitution beeinflussen, wurde im Rahmen dieser Arbeit eingehend untersucht. Dabei wurde beobachtet, dass die Einführung dieser Gruppen keine signifikante Auswirkung auf die Substitutionsreaktion hat und die Ausbeuten vergleichbar waren mit der Reaktion an unsubstituiertem Fluoreszein.

Um weitere Modifikationen einzuführen, wurden die Schwefel Nukleophile mit einem aliphatischen Linker verknüpft. Über diesen Linker wurden so Hydroxylgruppen eingeführt, die wiederum mit gängigen Biokonjugaten wie z.B. Aziden modifiziert wurden.

Die Synthese eines neuen fluoreszenten Azids, 2-(6-(2-Azidoethylthio)-3-oxo-3H-xanthen-9-yl) Benzoesäure, konnte in nur 4 Stufen mit einer Gesamtausbeute von 13% realisiert werden. Auf dem gleichen Weg konnten das 2',7'-Dichloro- und 2',4',5'-Tribromo-Derivat in Ausbeuten von 10% und 1% gewonnen werden.

In lediglich drei Stufen wurde ein asymmetrisches Xanthen Sulfid, welches als funktionelle Gruppe ein Amin am aliphatischen Linker trug, mit einer Gesamtausbeute von 33% synthetisiert: 2-(6-((2-Aminoethyl)thio)-3-oxo-3H-xanthen-9-yl) Benzoesäure.

Der Einfluss der Substitution mit Schwefelnukleophilen in der 6'-Position von Xanthen auf das Fluoreszenzverhalten wurde eingehend untersucht und es konnte gezeigt werden, dass asymmetrische Xanthene, die Sulfide als funktionelle Gruppen tragen, einen niedrigeren Extinktionskoeffizent und eine niedrigere Quantenausbeute aufweisen als Fluoreszein selbst. Allerdings wiesen diese neuen modifizierten Xanthene eine erheblich bessere Photostabilität als herkömmliches Fluoreszein auf. Selbst nach kontinuierlicher Anregung über 10 Stunden waren noch 58-94% der ursprünglichen Floureszenz vorhanden. Diese Beobachtung führt zu der Fesstellung, dass die Stabilität dieser neuen Farbstoffe 1-2 Größenordnungen höher liegt als die des Fluoreszeins.

Weiterhin konnten die mit einem Azid modifizierten Xanthene in einer „Click“ Reaktion durch eine Kupfer(I) katalysierte Azid-Alkin Cycloaddition mit einem Oligonukleotid umgesetzt werden, welches einen terminalen Alkinrest enthielt.

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1 Introduction

1.1 Phenomena of Fluorescence

Photoluminescence is the emission of light from substances after the absorption of light or other electromagnetic radiation. Depending on the nature of electronically excited states, photoluminescence can be divided into two categories: fluorescence and phosphorescence.[1]
The visible emission was first reported by Herschel in 1845, who studied chinine in aqueous solution.[2] In 1852 G. G. Stoke described the ability of fluorspar and uranium glass to change invisible light beyond the violet end of the visible spectrum into blue light. He showed that this phenomenon was due to the absorption and then emission of light by these substances and assigned the term "fluorescence".[3]
Absorption of light appears very fast (approximately a femtosecond) in discrete amounts termed quanta. Absorption of light can lead to excitation of the fluorophore from the ground state to an excited state. The emission of a photon through fluorescence or phosphorescence is also measured in terms of quanta. The energy in a quantum is expressed by **Planck's Law** (Equation 1):

$$\mathbf{E = h\nu = hc/\lambda} \quad \textbf{(Equation 1)}$$

where **E** is the energy, **h** is Planck's constant, **ν** and **λ** are the frequency and wavelength of the incoming photon respectively, and **c** is the speed of light.
The absorption of a photon of energy by a fluorophore takes place due to an interaction of the light wave with electrons in the molecule. This can only occur with the light of specific wavelengths, known as absorption bands. Excitation of a molecule by absorption normally occurs without a change in electron spin-pairing, which means that the excited state is a singlet.[4]
In general, fluorescence experiments are carried out with radiation having wavelengths ranging from the ultraviolet to the visible regions of the electromagnetic spectrum (250 to 700 nanometers).
The processes that occur between absorption and emission of light are illustrated in the Jablonski diagram (Figure 1).

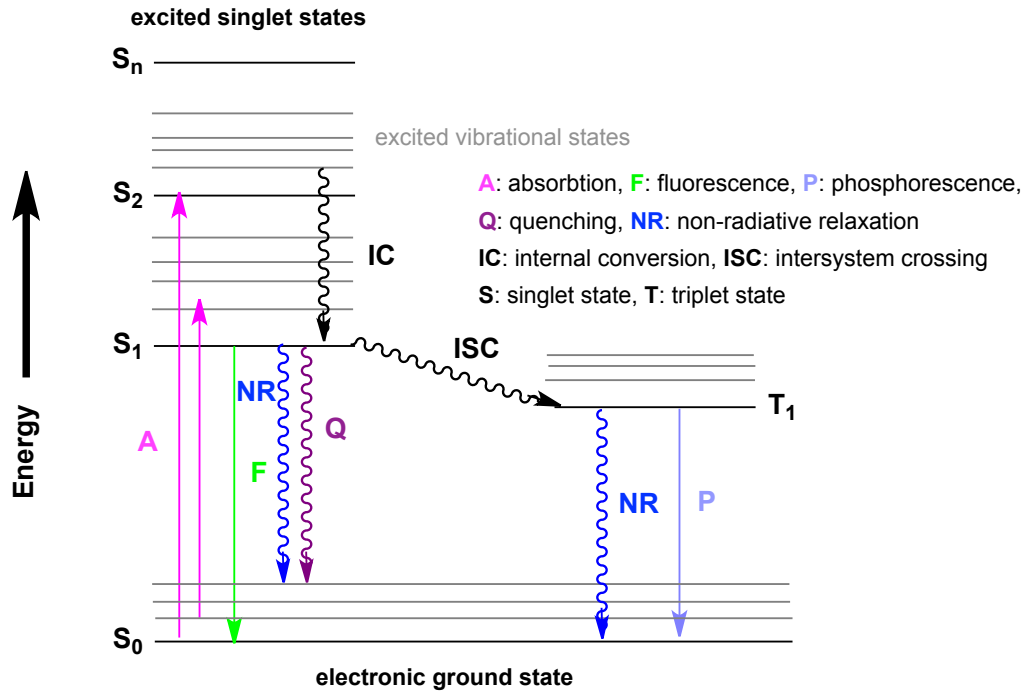


Figure 1. Jablonski diagram.

Fluorescence is the result of a three-stage process. [5, 6, 7]

In the first stage, excitation is taking place. A photon of energy necessary for fluorophore excitation ($h\nu_{ex}$) supplied by an external source, fluorescent lamp or a laser is absorbed and excited to higher vibrational levels of the first electronic single state S_1 or second S_2.

In the second stage, immediately following absorption of a photon, relaxation to the lowest vibrational energy level of the first excited state, S_1 will occur. This process is known as internal conversion or vibrational relaxation (loss of energy in the absence of light emission) and generally occurs in a picosecond or less. The excess vibrational energy is converted into heat, which is absorbed by neighboring solvent molecules upon colliding with the excited state fluorophore. An excited molecule exists in the lowest excited singlet state S_1 for periods of nanoseconds.

In the third stage, a photon of energy is emitted, returning the fluorophore to its ground state S_0. The process is known as fluorescence. The closely spaced vibrational energy levels of the ground state, when coupled with normal thermal motion, produce a wide range of photon energies during emission. As a result, fluorescence is normally observed as emission at a certain wavelength.

Except of photon emission, the decay of the excited state can occur in a non-radiative (NR) fashion. This NR quenching of fluorophore excited state can occur through variety of processes, like bond rotation or vibration, molecular collision, and photoinduced electron

transfer (PeT).[8] The excited state can also undergo forbidden intersystem crossing (ITC) to the triplet excited state (T_1) and subsequent relaxation by either photon emission, widely known as phosphorescence or again NR decay (Figure 1). Another important pathway for a decay of excited state is FRET to an acceptor molecule. This process is distance-dependent and can be used as a measure of proximity of labeled entities.[9]

Due to energy dissipation during the excited-state lifetime, the energy of this photon ($h\nu_{em}$) is lower, and therefore of longer wavelength, than the energy of excitation photon ($h\nu_{ex}$). The difference in energy represented by $h\nu_{ex}$ - $h\nu_{em}$ is called the Stokes shift. In the Figure 2 are illustrated excitation and emission spectra of a fluorophore. The excitation of a fluorophore at different wavelengths does not change the emission profile, but produce variations in the emission intensity. Stokes shift represents the difference between the excitation band maxima and emission band maxima.[6]

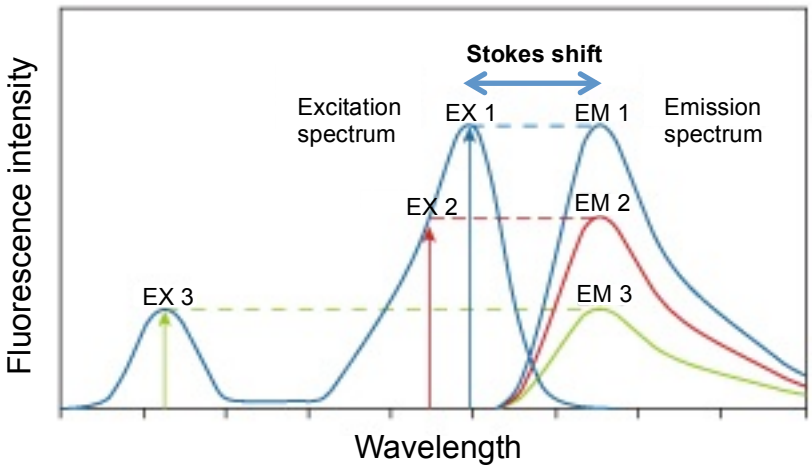


Figure 2. Excitation of a fluorophore at three different wavelengths (EX 1, EX 2, EX 3) and their corresponding fluorescent emission intensities (EM 1, EM 2, EM 3).

Fluorescent properties of functional fluorophore can be described by the extinction coefficient and the quantum yield. The molar absorption coefficient, also known as the molar extinction coefficient (**ε**) defines the absorptivity of a given molecule at the maximum absorption wavelength (λ_{max}). According to **Beer-Lambert law** (Equation 2):

A* = *ε c l **(Equation 2)**

the actual absorbance, **A**, of a sample is dependent on the pathlength, **ℓ**, and the concentration, **c**, of the species. The SI units for ε in practice are usually given as M^{-1} cm^{-1}.

Another important property of a fluorophore is its quantum yield (Φ), which is the ratio of photons emitted to those absorbed.[5] In other words, the quantum yield gives the probability of the excited state being deactivated by fluorescence rather than by another, non-radiative mechanism such as already mentioned internal conversion and vibrational relaxation or intersystem crossing (Figure 1).

Photobleaching occurs when a fluorophore looses its ability to fluoresce, its covalent structure is damaged. The absorption of light usually leads to photochemical reactions, which can upon repetition result in the destruction of fluorescence. This phenomenon usually appears after many excitation and emission cycles and depends on the composition of a fluorophore. Some fluorophores bleached quickly after emitting a few photons, others can repeat the excitation and emission cycles thousands or millions of times before the highly reactive excited state molecule is photobleached.

1.2 Classes of fluorescent Dyes

Fluorescence typically occurs in aromatic molecules and heterocycles, called fluorophores or fluorescent dyes. Fluorescent molecules always contain a chromophore, the part of a molecule responsible for its color. Chromophores usually appear in conjugated π-systems and metal complexes. Fluorophores differ from each other in fluorescent characteristics such as the maximum absorption wavelength (λ_{max}), the maximum emission wavelength (λ_{em}), extinction coefficient (ε) and quantum yield (Φ).

Many naturally occurring chromophores exhibit measurable fluorescence, but only some of them produce durable fluorescence suitable for analytics. These include aromatic amino acids, nicotinamide cofactors, flavins, porphyrins, pyridoxal derivatives, nucleosides and so on. These endogenous fluorophores are responsible for autofluorescence of biological structures such as mitochondria or lysosomes.[10]

Polycyclic aromatic compounds represent a class of fluorescent dyes widely applied as useful synthetic biomolecule labels. Here belong large number of naphthalene derivatives such as the amine reactive 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl) chloride or 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS) used widely in FRET-based experiments.[11] 4-amino-3,6-disulfonylnaphthalimides called “Lucifer yellow” are effective polar traces.[6]

Pyrene derivatives are used to measure important biomolecular processes such as protein conformation[12] and to report RNA folding.[13]

Perylene derivatives constitute a class of fluorophores that exhibit very high quantum yields.[14] One of the first discovered fluorophores, quinine is still employed as fluorescent standard.[15] The 6-methoxyquinoline moiety upon the alkylation can be quenched by halide ions in solution and can therefore be used as fluorescent ion indicator.[16]

Indoles and imidazoles are also known for their fluorescent properties. 4',6'-diamidino-2-phenylindole (DAPI) binds in the minor groove of DNA. This binding is accompanied by a large increase in fluorescence; therefore this molecule is often used to stain DNA by cellular imaging. The dibenzimidazoles known as Hoechst dyes just like DAPI, bind in the minor groove of DNA. They are used as nucleic acid stains for fluorescent microscopy and flow cytometry.[6]

The boron difluoride dipyrromethene known as BODIPY dye has been used for fluorescent labeling thanks to its tunable wavelength through appropriate substitution.[17] This nature of BODIPY dyes allows their utilization as surrogates for traditional dyes such as fluorescein, tetramethylrhodamine, *etc*.

Another important class of fluorescent markers are cyanine dyes, which consist of a dye system with a polymethine chain between two nitrogens (R_2N-$(CH=CH)_n$-$CH=N^+R_2$).[7] Both nitrogens are each independently part of a heteroaromatic moiety, such as pyrrole, imidazole, thiazole, pyridine, indole, *etc*. Cyanine dyes in modern bioresearch, also known as the CyeDye flurophores are those bearing a sulfoindocyanine structure[18], and are named according to the number of carbon atoms between the dihydroindole units, for example Cy3, Cy5, Cy7. They exhibit different spectral characteristics according to this length.[19] CyDyes are often used as labels in nucleic acid and protein labeling,[6] DNA staining[20], dead-cell staining,[21] *etc*. Active derivatives, for example succinimidyl esters are attached to the nitrogen through linkers.[18]

Coumarins refer to a broad class of natural products, pharmaceuticals, and fluorophores. Molecular probes built on the coumarin scaffold are applied as efficient biomolecular labels. The modification with reactive groups is usually attached at the 3 or 4 position of coumarin.[6] Common examples are 7-hydroxy-4-methylcoumarin and 7-amino-4-methyl coumarin (AMC) excitable with UV light. The substitution on the amino group or other substitution such as fluorination[22] or sulfonation[23] can improve the solubility in aqueous solution and also lead to lower pH sensitivity.[6] Coumarins are often used for assembling enzyme substrates.

Recently, a new derivative of coumarin, 7-azido-4-(bromomethyl)coumarin (N3BC), selective for uridine, was used as alkylating agent for derivatisation of RNA.[24] The azido function of

N3BC is further used for derivatization by click-chemistry, so the azidocoumarin-modified RNA can be employed in structure-function studies.

Some of the above mentioned synthetic fluorophores are shown in Figure 3.

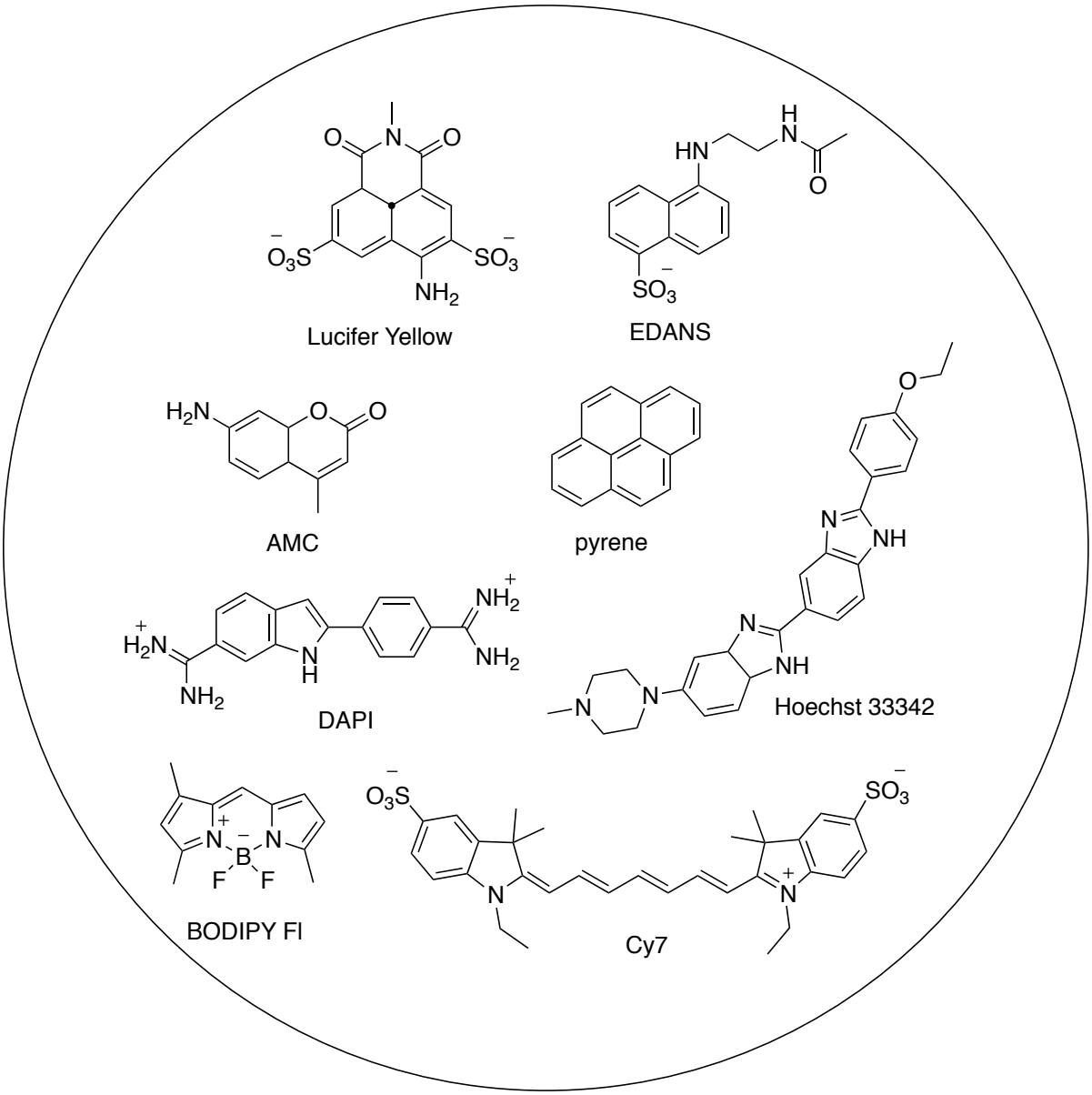


Figure 3. Fluorophoric moieties of some fluorescent probes.

1.3 Xanthene Dyes

Fluorescent dyes, which contain a xanthene, three-membered ring structure in their molecules, are known as xanthene dyes. Xanthenes are structurally related to xanthones, which represent the central core of many naturally occurring compounds. Derivatives of xanthones exhibit

diverse pharmacological activities. The biological activities of synthetic and naturally occurring xanthone derivatives depends on the various substituents and their position.[25, 26] Fluorescein and rhodamine belong to the most known xanthene fluorophores. Structurally related to xanthene is coumarin chromophore. 7-Amino-4-methyl coumarin can be seen as a structural part of rhodol fluorophore, the hybrid structure of fluorescein and rhodamine. 3,6-Dihydroxyxanthenone and its derivatives can be used as building blocks in the synthesis of new xanthene dyes such as Tokyo Green. The structure relation between the xanthene dyes and coumarins and xanthone are illustrated in figure 4.

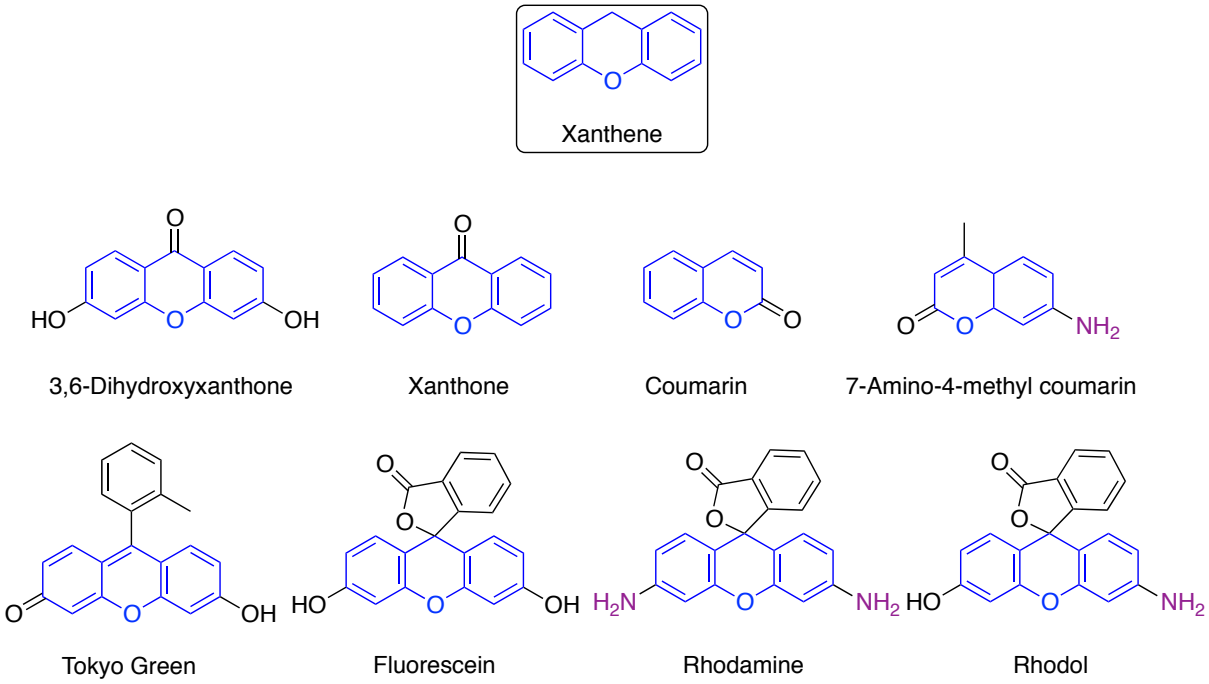


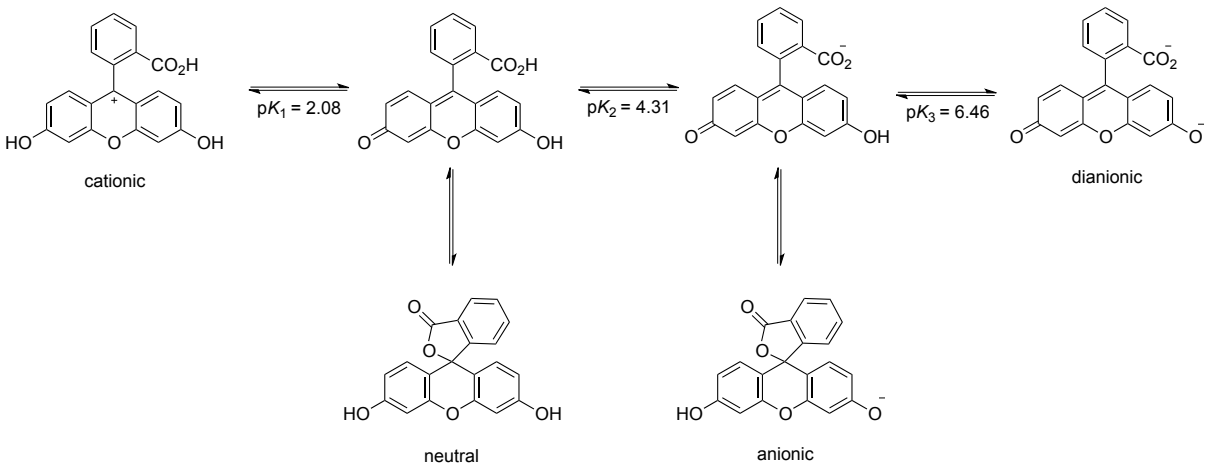
Figure 4. Structure relation between xanthene dyes and other fluorophores.

1.3.1 Fluorescein

In 1871 the German chemist, Nobel prize laureate, discoverer of plant dye indigo Adolf von Baeyer[27] investigated the synthesis of phenolphthalein by condensation of phthalic anhydride with phenol under acidic conditions. That same year performed the $ZnCl_2$-catalyzed synthesis of resorcinophthalein from phthalic anhydride and 1,3-dihydroxybenzene, known as resorcinol.[28] This compound was assigned as fluorescein. Despite its antiquity, fluorescein remains one of the most widely utilized xanthene fluorophores in modern biochemical, biological and medicinal research.

1.3.1.1 Spectral Properties of Fluorescein

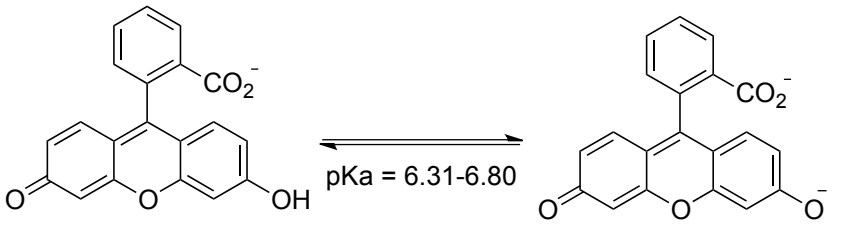
Fluorescence of fluorescein is based on the formation of conjugated system in the xanthene moiety. Fluorescein exists in equilibrium between a "closed" lactone and an "open" quinoid form and has multiple ionization equilibria[29, 30] (Scheme 1). This leads to pH dependent absorption and emission over the range of 5 to 9. Under acidic pH (pH 2-4) the fluorescein moiety exists mostly in a "closed" lactone form. Under milder acidic pH (pH 5-7) a considerable population of fluorescein is still in the protonated, non-fluorescent form and in the less fluorescent monoanionic form. Under physiological conditions (pH 7.4) fluorescein is predominantly a highly hydrophilic and fluorescent dianion.[31]



Scheme 1. pH dependence of fluorescein equilibria.

The most biologically relevant molecular forms are the monoanion and dianion, principal ground-state species that interchange with a p*K*a ~ 6.4. Scheme 2 shows these two forms of fluorescein in the range of 6.31 to 6.80 phenolic p*K*a values and corresponding spectral properties in aqueous solution.[6] Generally the monoanionic form has lower absorbance and the maxima are blue-shifted relative to the dianionic form. The quantum yield of the monoanion is significantly lower than the one of the dianion. The dianionic form has strong visible absorbance and potent fluorescent emission.

To overcome this pH sensitivity the structure of fluorescein can be further modified. For example 2',7'-dichlorofluorescein is less basic (p*K*a = 4.6) than fluorescein (p*K*a = 6.4), maintains fluorescein-like wavelengths and most important exhibits increased photostability relative to fluorescein.[32, 33, 34]



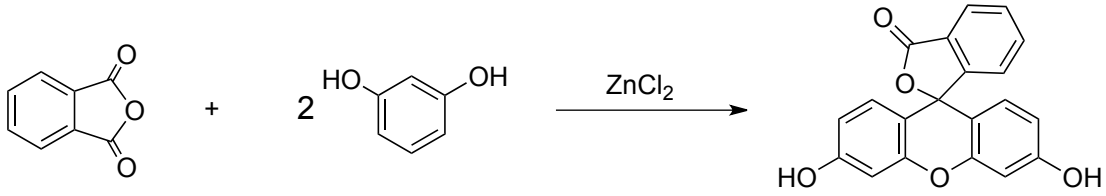
λ_{max} = 453, 472 nm
ε = 29 000-32 600 M^{-1} cm^{-1}
Φ = 0.36-0.37

λ_{max} = 490 nm
ε = 76 900-87 600 M^{-1} cm^{-1}
Φ = 0.92-0.95

Scheme 2. Monoanionic and dianionic forms of fluorescein.

1.3.1.2 Synthesis of Fluorescein

Generally fluorescein can be synthesized by 2 processes first of which is the above mentioned Friedel Craft's reaction of phthalic anhydride and resorcinol, using $ZnCl_2$ as a catalyst (Scheme 3) or using methanesulfonic acid. The traditional $ZnCl_2$ catalyzed synthesis of fluorescein has been performed in concentrated HCl at high temperatures usually 170-180 °C. It was not suitable for small-scale reactions because considerable amounts of the starting materials were lost during sublimation. It was found that methanesulfonic acid served as both a suitable solvent and acid catalyst in the reaction, affording better yields under milder conditions.[35]

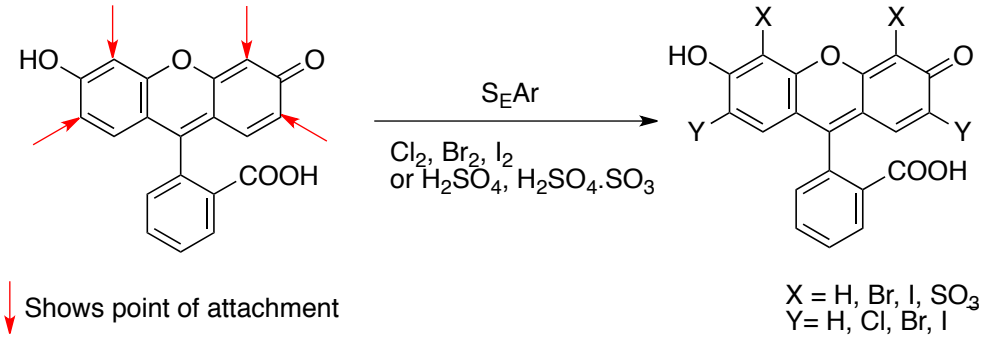


1 **2** **3**

Scheme 3. Synthesis of fluorescein.

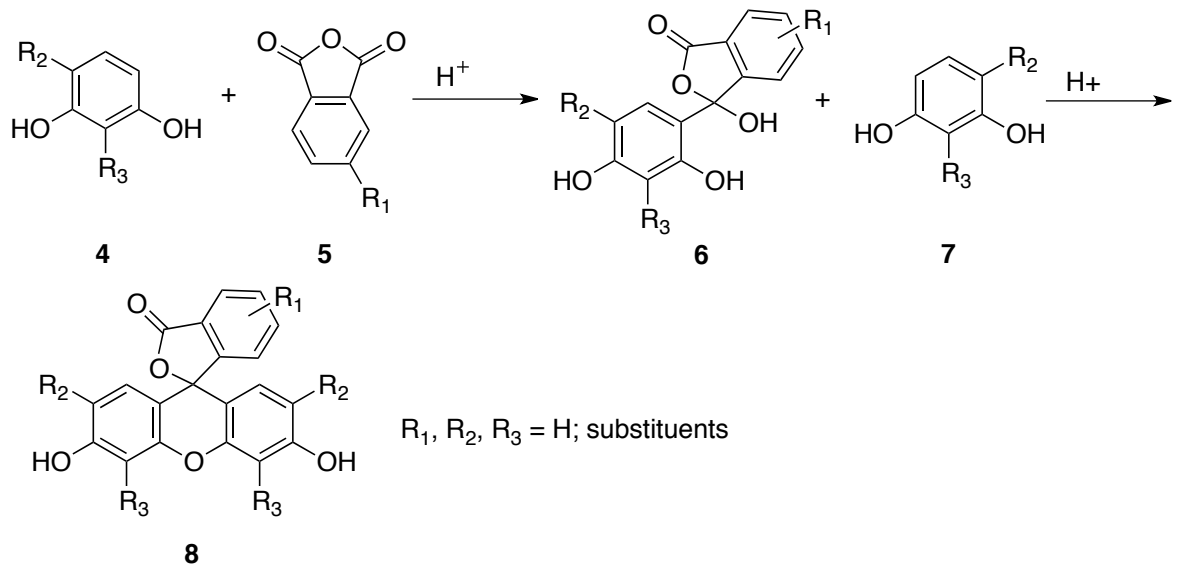
There are several active positions on fluorescein that are readily available for direct electrophilic aromatic substitution (S_EAr) such as halogenation with bromine, chlorine and iodine to produce 2',4',5',7'-tetrabromofluorescein (eosin)[36], 2',4',5',7'-tetraiodofluoroscein (erythrosin)[36], 2',7'-dichlorofluorescein…*etc*[37] or sulfonation with H_2SO_4, H_2SO_4 .SO_3 to obtain 4'- monosulfonated fluorescein or 4',5'-disulfonated fluorescein[38] (Scheme 4). Iodination of 4,5,6,7-tetrachlorofluorescein offers 2',4',5',7'-tetraiodo-4,5,6,7-tetrachloro-fluorescein (rose bengal).[39, 40] Halogenated derivatives are obtained with different purities

regarding the substitution pattern. Often difficult purification of substituted fluoresceins is usually simplified by acetylation of free hydroxyl groups for easier recrystallization, column chromatography or HPLC.[41] The acetyl groups are hydrolyzed back to obtain free dye in moderate to good purity.



Scheme 4. Electrophilic aromatic substitution on fluorescein.

Fluorescein derivatives are mostly prepared *via* acid-catalyzed condensation reaction of substituted phenols with substituted phthalic anhydride to yield the product **8** (Scheme 5). Usually desirable substituents are incorporated into the structure of fluorescein before condensation. Substituted building blocks have to be prepared separately followed by several synthetic steps. Also use of phthalic anhydrides bearing a substituent (R_1 in **5**) for bioconjugation yields products as unmanageable mixtures of 5- and 6-substituted regioisomers, which are difficult to separate. Many of them are commercially available as mixtures.[6]



Scheme 5. General synthesis of fluorescein derivatives.

Sun *et al.* reported the synthesis of fluorinated fluoresceins (Oregon Green dyes) by the reaction of fluororesorcinols with phthalic anhydride and its derivatives.[29] They described a novel regiospecific synthesis of fluororesorcinols *via* (polyfluoro)-nitrobenzenes. The nitro group directed substitutions at *ortho* and *para* positions afforded the first straightforward synthesis of substituted building blocks (2-fluroresorcinol, 4-fluororesorcinol…*etc*.) Afterwards the nitro group was in two steps converted into hydroxyl group. This method allows controlled substitution of the building blocks and mainly controlled substitution pattern on fluorescein.[42]

1.3.1.3 **Reactive Groups on Fluorescein**

Over the years fluorescein and its unique chemistry took a long run and a variety of derivatives were prepared and used as fluorescent detection reagents. Fluorescein and its derivatives recorded wide spread application in biological experiments in which they were covalently attached to materials such as peptides, proteins (antibodies), nucleotides, oligonucleotides, drugs, hormones, lipids, and other biomolecules.

Fluorescein and its derivatives are usually modified with an active group on the phenyl ring, which forms a stable covalent bond with the active group of the molecule of interest. Common commercially available classes of fluorescent dyes are those reactive with amines. In particular, the carboxylic acid moiety and its active forms such as succinimidyl ester, sulfosuccinimdyl ester, tetrafluorophenyl ester (TFP), and sulfotetrafluorophenyl ester (STP) are often used for conjugation with amines *via* an amide bond (Figure 5). The advantage of sulfonated reagents is their higher water solubility, which allows the conjugation reaction even in water. The carboxylic acids can be turned into acid chlorides and anhydrides and used to modify aromatic amines and alcohols.[6] Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) are widely used fluorescent probes for reactions with amines forming thiourea products.

Another class of fluorescein dyes for labeling of biomolecules include thiol reactive fluorescein maleimides, iodoacetamides and methyl bromides.[6] Thiol containing biomolecules are labeled with the fluorescein moiety constructing a thioether functional group. Iodoacetamide and maleimide derivatives are difficult to prepare in pure form and the commercially available batches usually contain variable mixtures of 5- and 6- isomers.[6]

Fluorescein azides are applied in the Cu^{I}-catalyzed Huisgen 1,3-dipolar cycloaddition reaction with terminal alkyne labeled biomolecules to afford 1,4-regioisomers of 1,2,3-triazole.[43, 44] This type of bond formation is widely known as the "click" reaction. The above mentioned fluorescein derivatives are illustrated in figure 5.

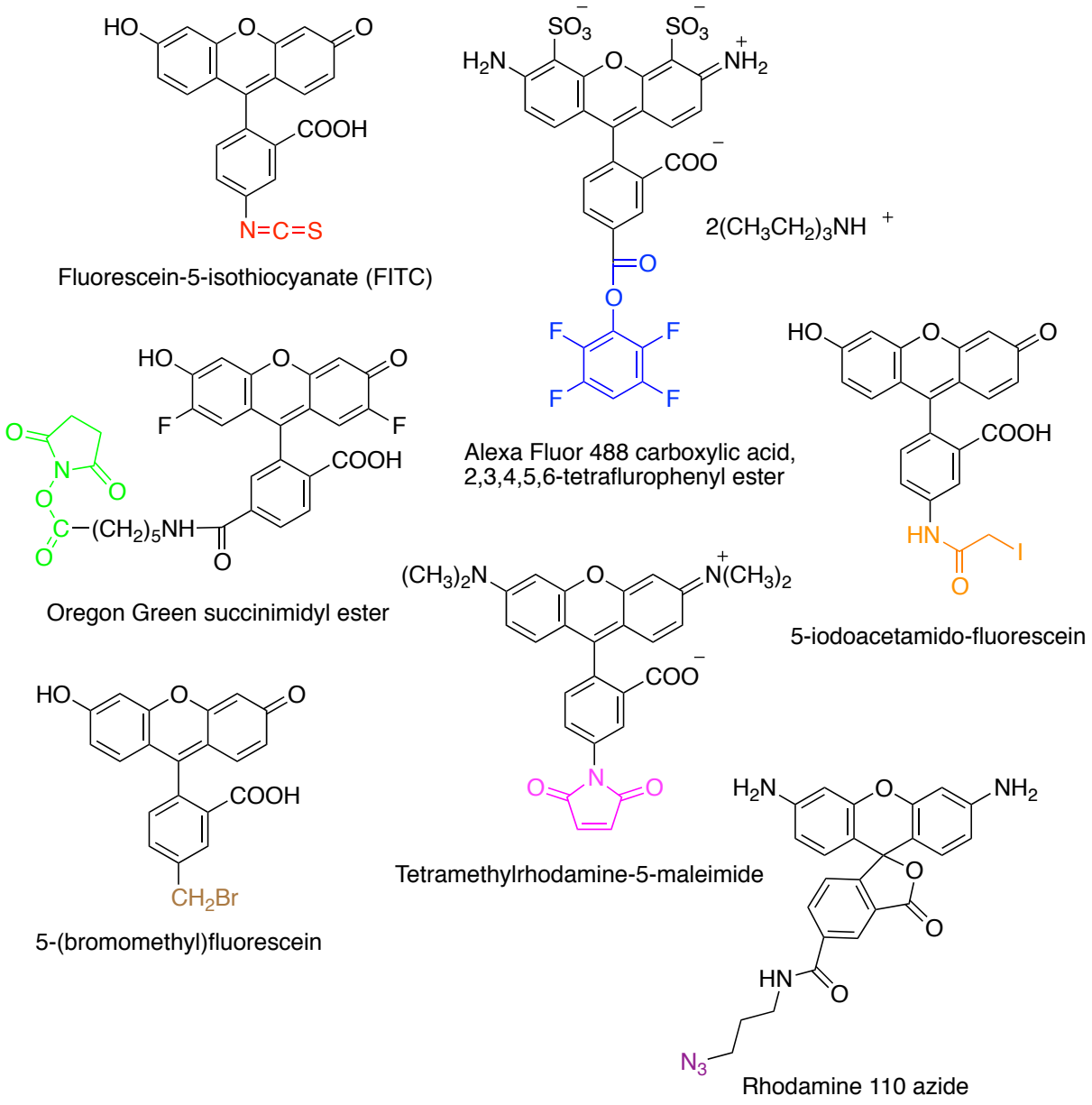


Figure 5. Selected fluorescein dyes with different reactive groups.

1.3.2 Tokyo Green Dyes and their Derivatives

Urano *et al.* were the first to introduce the methyl group instead of carboxylic group into the structure of fluorescein.[45] From the structure studies, it was proven, that the carboxylic group of traditional fluorescein dyes played no role in the fluorescent properties of the fluorescein

molecule (Figure 6). Tokyo Green dyes were synthesized by a Grignard reaction of xanthone diTBDMS ether with phenylmagnesium bromide.

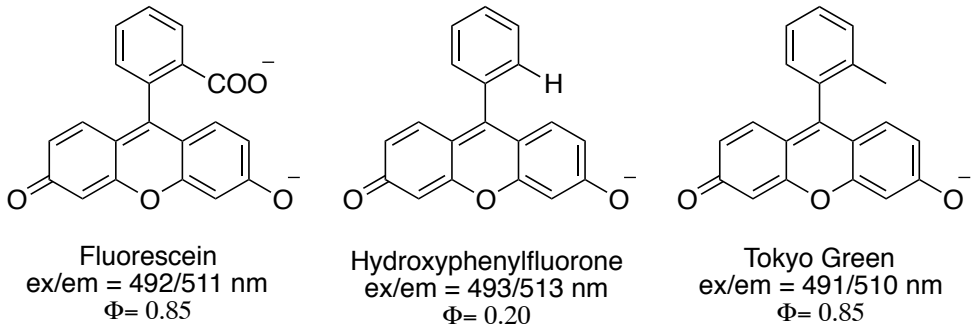


Figure 6. Replacement of carboxylic group with other substituents in the structure of fluorescein.

Following this discovery two new compounds based on the Tokyo Green structure were prepared. Pennsylvania Green as a 2',7'-difluro analogue of Tokyo Green[46] and Singapore Green[47] as a structural hybrid of Tokyo Green and Rhodamine 110 were introduced. Pennsylvania Green was proven to be the more photostable analogue of Tokyo Green.

1.3.3 Rhodols and Rhodamines

Isologues of fluorescein, rhodols and rhodamines have a widespread application as laser dyes, tracer agents, and biological probes.[6] Different *N*-alkyl substitution patterns on the rhodamine correspond to different spectral characteristics. Attachment of alkyl moieties to the nitrogen core of rhodamine can tune absorption and fluorescent emission, which is here dependent on the number and type of alkyl groups. The simplest member of this class of fluorescein dyes, rhodamine 110 (Rh_{110}), exhibits fluorescein-like spectral properties with λ_{max} = 496 nm, λ_{em} = 517 nm, $\varepsilon = 7.4 \times 10^4$ M^{-1} cm^{-1}, and $\Phi = 0.92$ in aqueous solution.[48] Substitution to tetramethylrhodamine (TMR) gives longer excitation and emission wavelengths (λ_{max} / λ_{em}; 540/565 nm), but a lower quantum yield ($\Phi = 0.68$).[7] In general, quantum yields of rhodols and rhodamines decrease with increasing carbon number and the bulk of the substituents.[49, 50] On the other hand, there is an exception where the julolidine ring incorporation into the rhodamine structure, Sulforhodamine (Rh_{101}), shows an increase in quantum yields and exhibit longer excitation and emission wavelengths.[6] The above mentioned rhodamines are illustrated in figure 7.

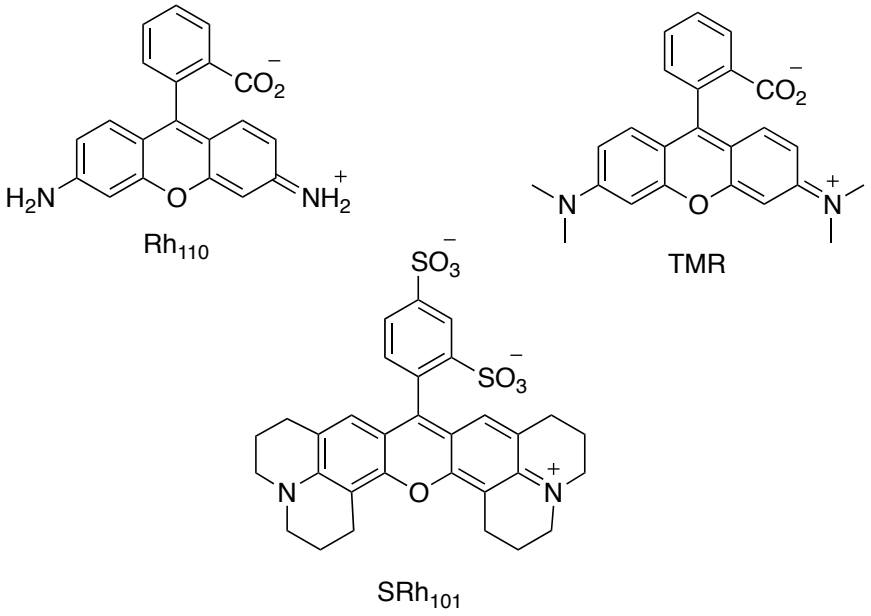
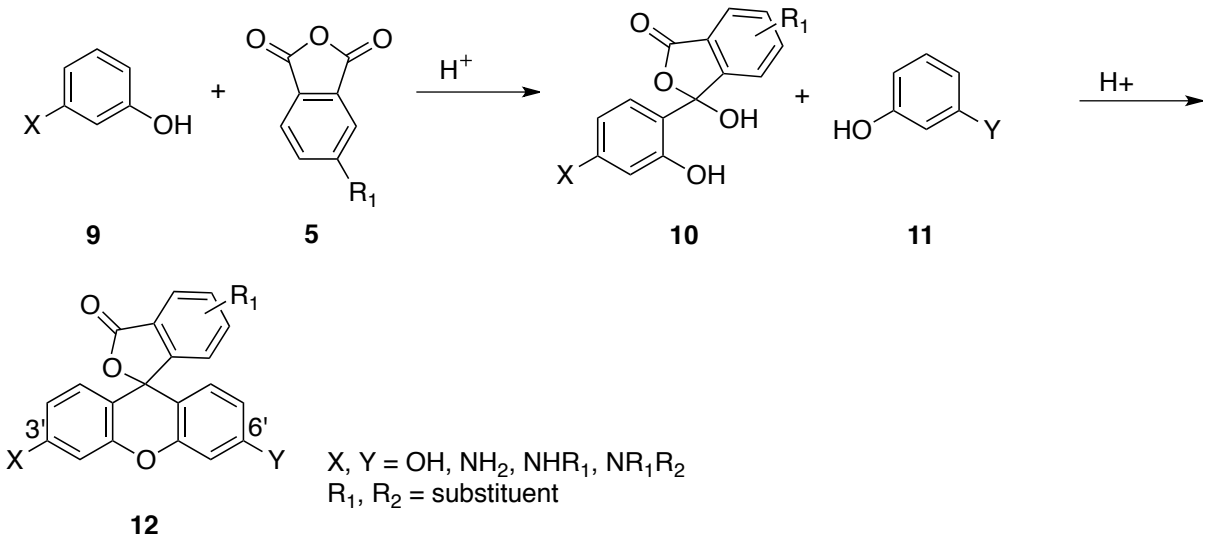


Figure 7. Structures of selected rhodamines.

Rhodols, rhodamines and their derivatives are usually prepared through acid-catalyzed condensation of an aminophenol (**9**) with a phthalic anhydride (**5**) (Scheme 6).[50] The use of phthalic anhydrides bearing a substituent (R_2) for bioconjugation yields products as intractable mixtures of 5- and 6-substituted regioisomers. Therefore functionalized commercially available rhodamines are often sold as regioisomeric mixtures.[6]

Old Route

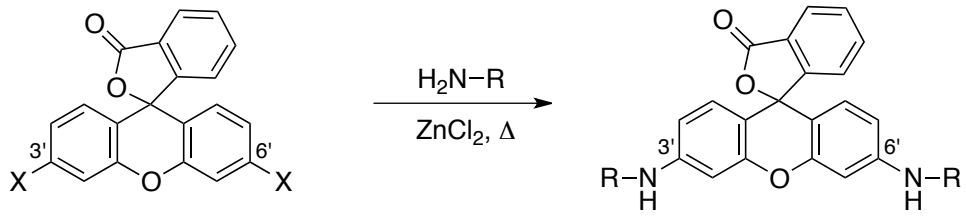


Scheme 6. General synthesis of rhodols and rhodamines.

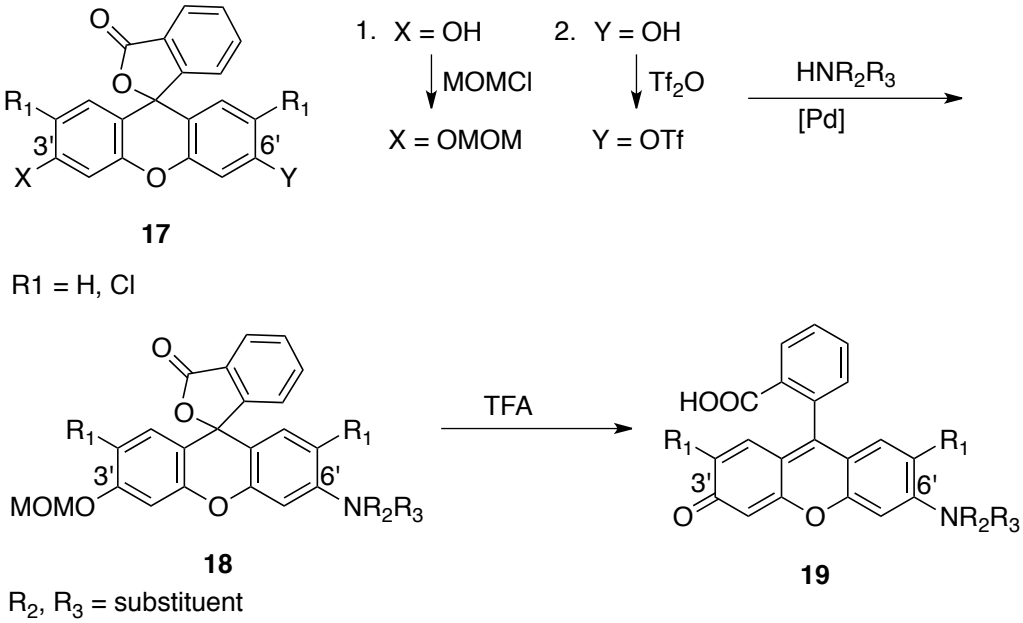
Synthesis of rhodamines by $ZnCl_2$-catalyzed direct substitution of 3',6'-dichlorofluoresceins with amines was reported (Scheme 7).[51, 52, 53] This reaction is accompanied by harsh

temperature conditions and difficult work-up. Woodroofe *et al.* described synthesis of rhodamine from 3',6'-dibromofluoresceins applying two strategies: the above mentioned $ZnCl_2$-catalyzed substitution with pyrrolidone and Pd-catalyzed Buchwald-Hartwig amination reaction.[54] Recently, a similar strategy for synthesis of rhodols was reported by Peng *et al.* in 2010.[49] This new route consists of the mono-protection of the 3'-position of fluorescein by MOM, followed by the triflation of the 6'-position. This triflated intermediate was coupled with different amines under the catalysis of a palladium-phosphine complex, widely known as the Buchwald-Hartwig amination reaction (Scheme 8).[55] In 2011 Grimm *et al.* used the above mentioned route for the preparation of rhodamines and *N,N*-diacetylated rhodamines (Scheme 9).[56] Just like Peng *et al.* this group used triflation on the 3'- and also 6'-position to obtain fluorescein ditriflates. These intermediates were directly converted to not only *N*-alkyl rhodamines but also *N*-aryl and *N*-acyl derivatives. This method allows the synthesis of regioisomerically pure 5- and 6-substituted rhodamine dyes.

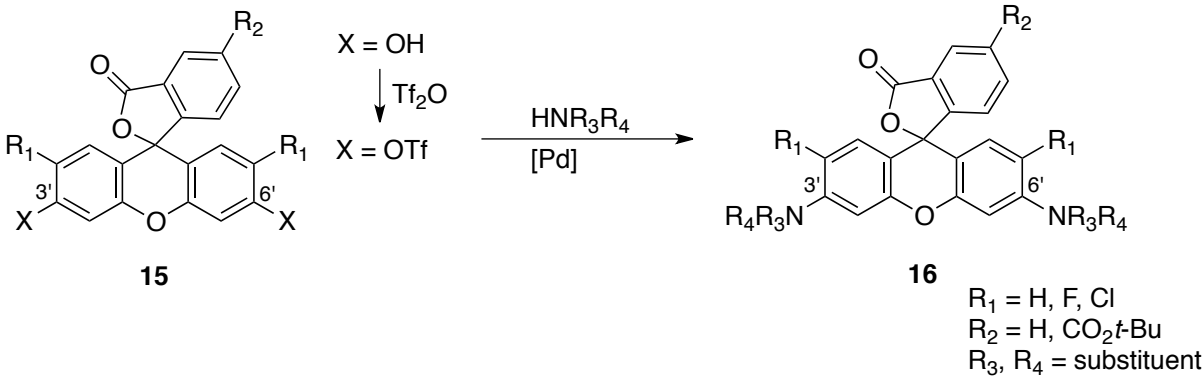
New Routes



Scheme 7. Synthesis of rhodamines *via* direct nucleophilic substitution from halogenated fluoresceins.



Scheme 8. Synthesis of rhodol fluorophores *via* Buchwald-Hartwig amination reaction.



Scheme 9. Synthesis of rhodamines *via* Buchwald-Hartwig amination reaction.

Rhodamine dyes are often used for FRET based experiments frequently pairing with other fluorescein dyes.[6] Dye constructs containing both fluorescein and rhodamine moieties were utilized for DNA sequencing. The fluorescein donor of these fluorophores can be excited by a single-wavelength light source, and the emission is determined by the rhodamine derivative that serves as the FRET acceptor. [57]

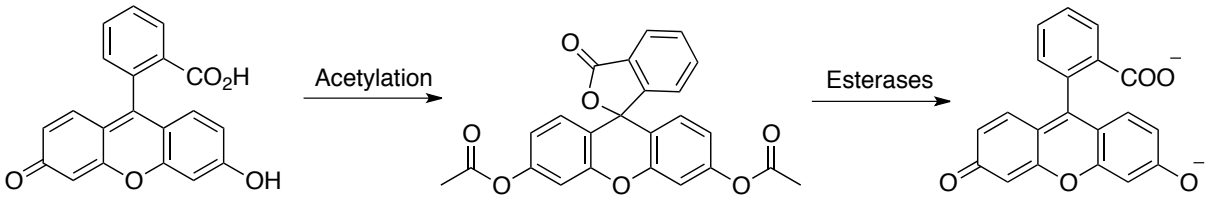
Recently Hirsch *et al.* developed new fluorescent FRET dye pairs Atto488/Atto590 both rhodamine dyes for sensing the integrity of duplex siRNA, which allows evaluation of the degradation status of an siRNA cell population by live cell imaging.[58]

1.4 Leuco Dyes

Fluoresceins and rhodamines can be chemically modified to form colorless, non-fluorescent leuco dyes by substitution of both hydroxyl groups of fluorescein or both amino groups of rhodamine. Substitutions such as acylation or alkylation lock the molecule into the non-fluorescent lactone form, thus they can serve as the basis for a variety of fluorogenic substrates for esterases, phosphatases, glycosylases, and other enzymes.[59,]

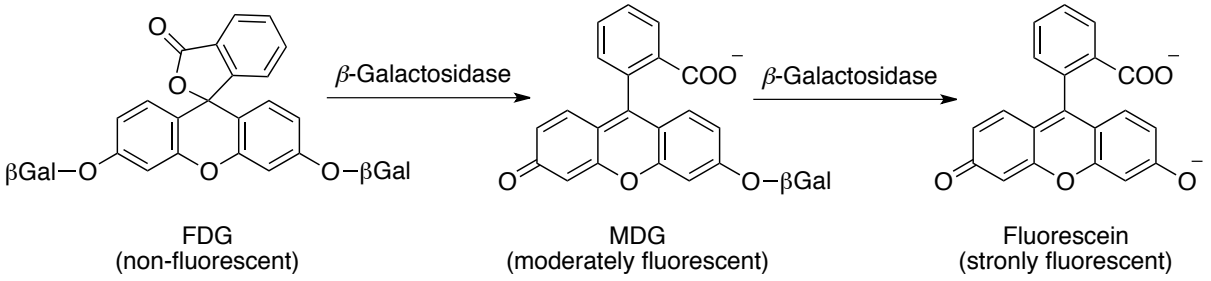
The acetylated forms of fluorescein and its derivatives are employed as cell viability indicators. The di-acetylated form is converted into its fluorescent analogue upon nonspecific esterase hydrolysis (Scheme 10).[60]

For monitoring enzyme activity, leuco dyes incorporate two moieties, each of which serves as a substrate for the enzyme. These substrates are hydrolytically cleaved off by the enzyme, first to the mono-substituted analogue and then to the free fluorescent dye, as shown in Scheme 11.



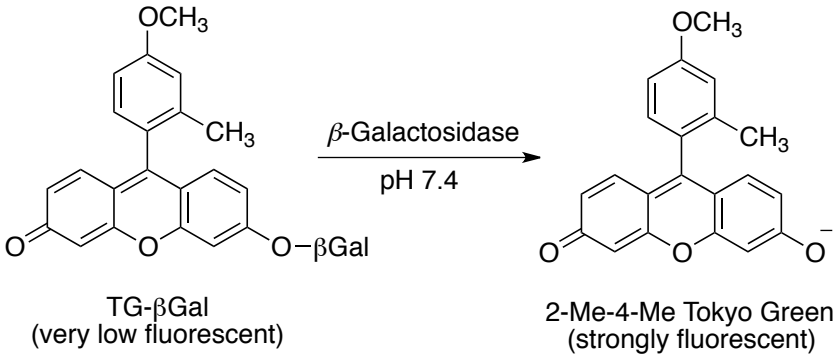
Scheme 10. Esterase hydrolysis of di-acetylated fluorescein.

The activity of *β*-galactosidase is usually detected with fluorescein di-*β*-galactopyranoside (FDG) (Scheme 11).[61] This method was also applied for detection of glycosidase enzyme activity in living cells. Not only the activity of a working enzyme, but also its transition to a deactivated state, is of great interest.



Scheme 11. Reaction of fluorescein di-*β*-galactopyranoside (FDG), fluorescent probe for *β*-galactosidase

Tokyo Green dye coupled with *β*-galactoside (2-Me-4-OMe Tokyo Green (TG- *β*Gal)) was used as a novel fluorescent probe to image *β*–galactosidase activity in living cells. In comparison with di-*O*-*β*-galactoside fluorescein probe, this new probe, due to its hydrophilicity is membrane-permeable, and enables a one-step hydrolysis, offering a higher fluorescence increase and higher sensitivity (Scheme 12).[46]



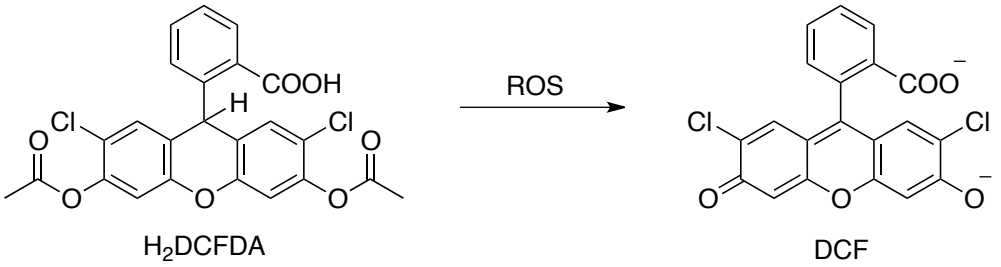
Scheme 12. Reaction of TG- *β*Gal with *β*-galactosidase.

Singapore Green, a peptide coupled rhodol-like molecule allows the indirect measurement of peptidase activity as an increase of the fluorescent intensity in a microarray-based protease substrate profiling and live-cell imaging experiments.[47]

De Cremer *et al.* reported the analysis of the α-chymotrypsin (from bovine pancreas) dynamics at the single-enzyme level by monitoring the α-chymotrypsin catalyzed hydrolysis and formation of fluorescent rhodamine 110 from the profluorescent rhodamine 110 bis(suc-Ala-Ala-Pro-Phe) substrate.[62]

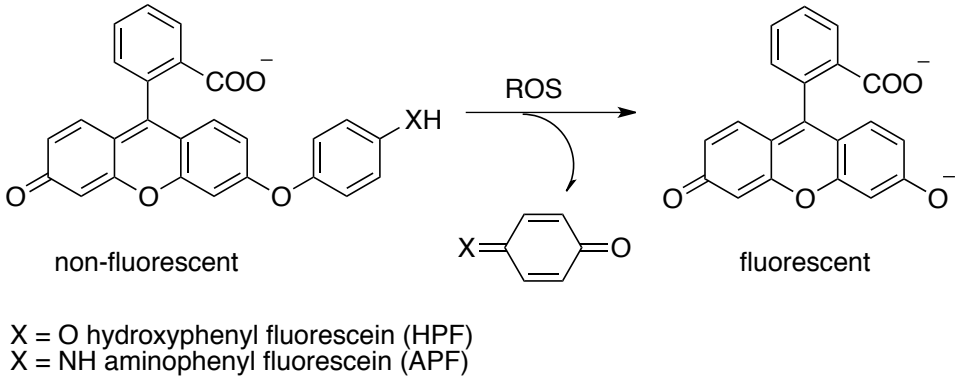
Commercially available leuco dyes for detection of reactive oxygen species (ROS) are sometimes reduced "dihydro" derivatives because of their better stability towards autooxidation (Scheme 13).[63, 64] These derivatives are readily oxidized back to the parent dye by some reactive oxygen species and thus can serve as fluorogenic probes for detecting oxidative activity in cells and tissue.[6]

The oxidation of non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate[63] (H_2DCFDA) (Scheme 13) to the highly fluorescent 2',7'-dichlorofluorescein (DCF) is commonly used to detect the generation of reactive oxygen intermediates in neutrophils and macrophages.[65] Cell-permeable, non-fluorescent H_2DCFDA becomes fluorescent in the presence of a wide variety of intracellular reactive oxygen species (ROS), including peroxyl ($ROO^{\cdot}$), hydroxyl ($OH^{\cdot}$) radicals and the peroxinitrite anion ($ONOO^{-}$).[6]



Scheme 13. Oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA).

3'-(*p*-aminophenyl) fluorescein (APF) and 3'-(*p*-hydroxyphenyl) fluorescein (HPF), prepared by Setsukinai *et al*. are commonly used ROS indicators.[66] In comparison with H_2DCFDA they have great resistance towards light-induced oxidation and limited reactivity with different ROS. Non-fluorescent APF and HPF react mostly with the hydroxyl radical or peroxonitrite anion ROS and yield a bright green-fluorescent product (Scheme 14).



Scheme 14. Detection of ROS with APF and HPF.

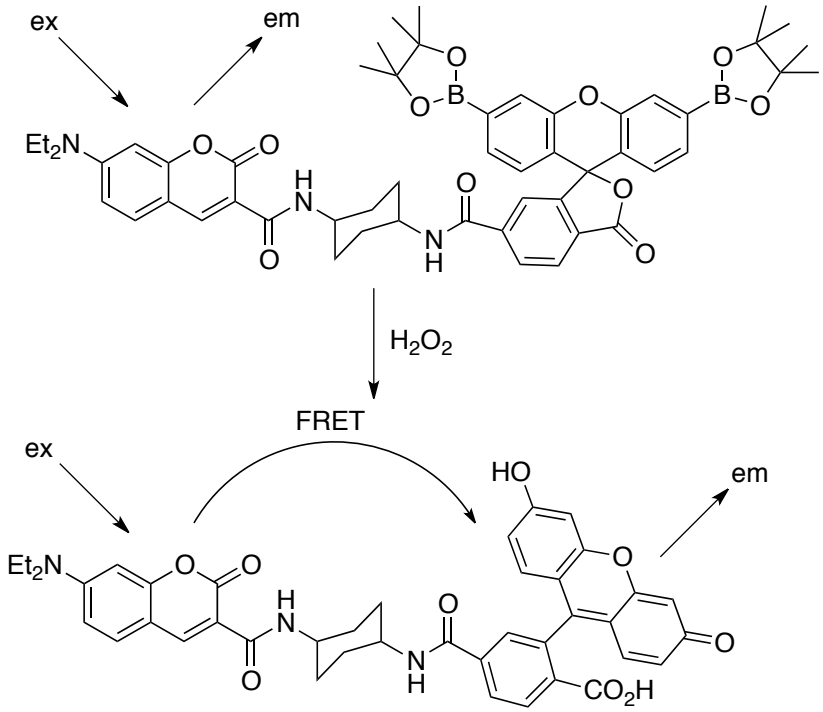
Leuco dyes for selective detecting and quantifying H_2O_2 production based on emission increase in living biological samples have been reported.[67, 68] This strategy relies on the highly selective H_2O_2-mediated transformation of arylboronates to phenols.[69] The 3',6'-diiodo or 3',6'-dibromo fluoresceins underwent palladium-catalyzed transmetalation under Miyaura conditions with bis(pinacolato)diboron and thus transformed into diboronates of fluorescein. The boronic ester groups at the 3' and 6' positions of the xanthene scaffold locked fluorescein in a "closed", non-fluorescent form. Upon treatment with H_2O_2, hydrolytic deprotection of the boronates generated the "open" fluorescein product showed by Dickinson *et al.* in an *in-vitro* assay to monitor the H_2O_2 production in the mitochondria of living cells.[68]

In the same fashion, the ratiometric detection of H_2O_2 based on FRET was introduced, consisting of two fluorophore cassette, comprised of a coumarin donor and a boronate-protected fluorescein acceptor linked by a rigid spacer (Scheme 15).[70] In the absence of H_2O_2

only coumarin fluorescence was observed and FRET was suppressed. Upon selective reaction with H_2O_2, the fluorescein "open" form was generated. The excitation of the donor coumarin chromophore resulted in increased green fluorescent acceptor emission by FRET.

Srikum *et al.* also employed H_2O_2-mediated deprotection of boronate esters to phenols.

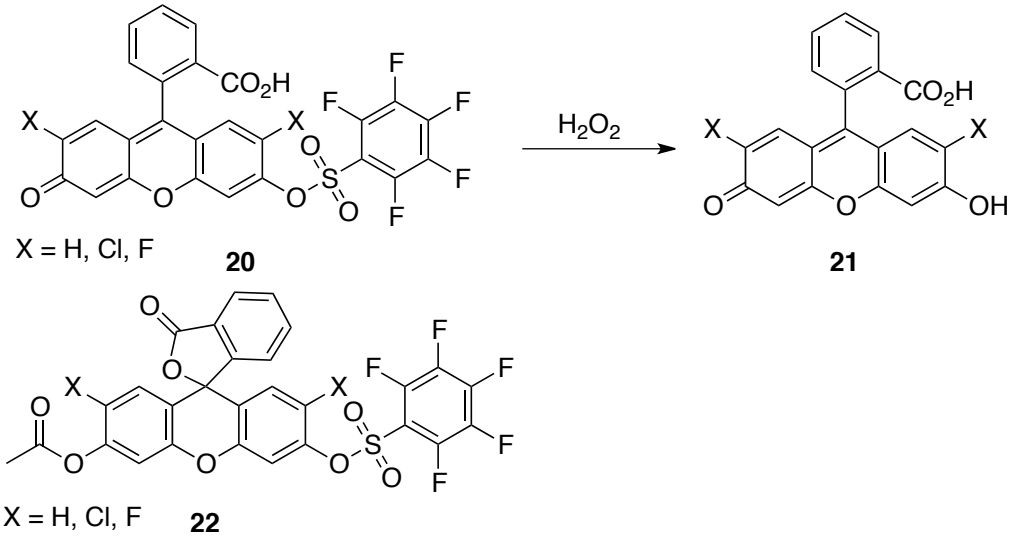
They created subcellular-targetable H_2O_2 fluorescent probes by combining the SNAP-tag technology for site-specific protein labeling with small molecules on the surface or interior of living cells with the use of boronate-capped Tokyo Green dyes.[71]



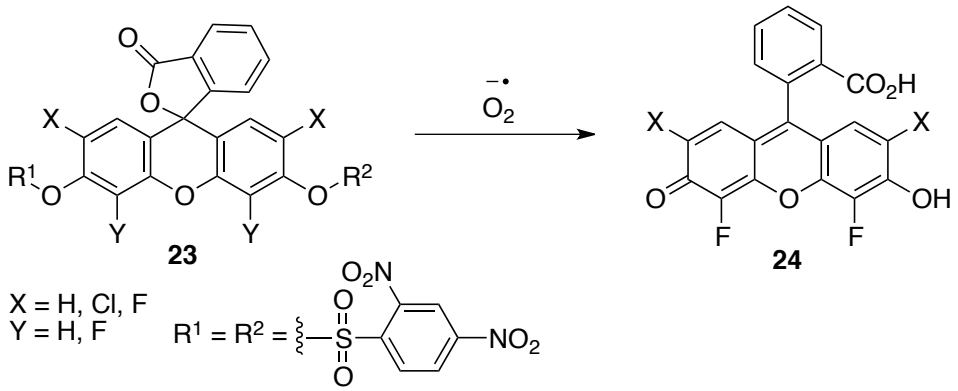
Scheme 15. Activation of coumarin donor and a boronate-protected fluorescein acceptor reporter.

Lippert *et al.* reported a pair of new reaction-based fluorescent probes for the selective imaging of H_2S in living cells that exploit the H_2S-mediated reduction of fluorescein 3'-azides to fluorescent amines.[72]

Maeda *et al.* developed probes based on H_2O_2 mediated deprotection of monopentafluorobenzenesulfonyl fluoresceins by a non-oxidative mechanism (Scheme 16).[73] The corresponding bis-substituted derivatives did not react with H_2O_2 at all. It was demonstrated that the acetylated **22** permeate the cells of green algae, is then converted into **20** again by cellular esterases and can be further deprotected by cellular H_2O_2.[74] The di-substituted fluorescein (**23**) was applied as selective probe for detection of $O_2^{-\cdot}$ based on a nonredox mechanism (Scheme 17).[75]



Scheme 16. Reaction of selective H_2O_2 probe.



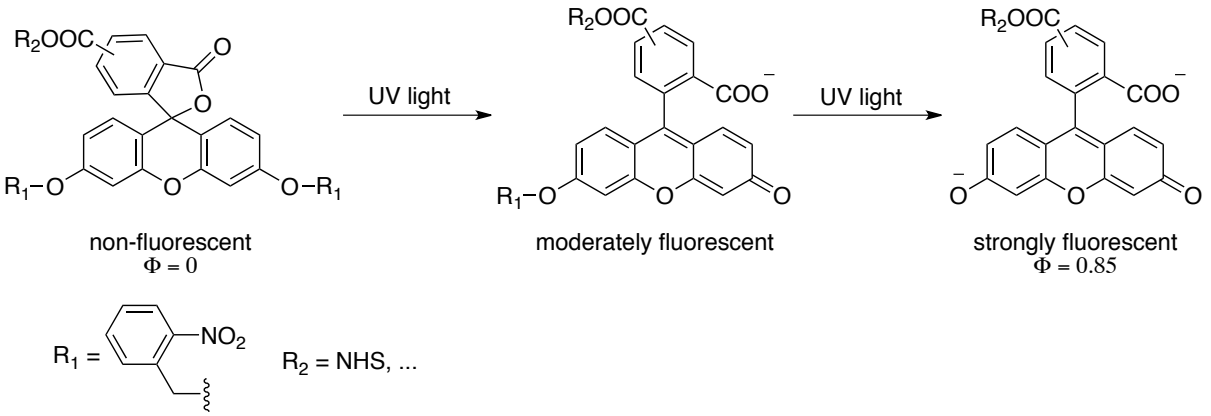
Scheme 17. Deprotection of fluorescent probe selectively mediated by $O_2^{-\bullet}$.

1.5 Caged fluorescent Dyes

The cellular dynamics of living cells can be investigated by using small organic molecules such as fluorescent dyes. One approach is to use caged (masked) fluorescent dye. Caged dyes contain photochemically labile protective groups, which keep the fluorophore in a non-fluorescent state. The photosensitive masking group or "molecular cage" can be cleaved off by irradiation with UV light, producing dye fluorescence and releasing bioactive molecules.[76, 77] Among many caged fluorophores, fluoresceins and rhodamines are the most popular dyes. Rhodamines and fluoresceins exist in equilibrium between their brightly fluorescent "open" quinoid structure and a colorless, "closed" lactone. This equilibrium can be controlled in a light-dependent manner using several strategies.[78, 79] Electron withdrawing 2-nitrobenzyl groups or derivatives with an alkyl or a carboxyl group in the α-position to the phenyl ring (at

the CH_2 group) are often used as masking groups. They are attached to the aniline nitrogens of rhodamines or phenolic oxygens of fluorescein. However, the use of these dyes is limited because of their rather complex synthesis and the unwanted photobyproducts, which may show cytotoxicity.[80]

Caged fluorescein is in the bis-caged lactone form, full activation requires deprotection of two photoremovable protecting groups (Scheme 18).[81]



Scheme 18. Uncaging scheme of caged fluorescein.

Kobayashi *et al.* reported novel caged Tokyo Greens, where only one photolabile group from 2-nitrobenzyl and its derivatives was used for caging. Mononitrobenzyl-TG is almost non-fluorescent in comparison with mono-substituted fluorescein. In a biological application, fluorescence labeling of a single living cell, caged fluorescein requires longer irradiation to remove both protective groups. Instead of, upon the brief irradiation with the UV light, TG showed a large fluorescent enhancement thanks to only one photoremovable group.[81]

Ito *et al*. utilized a concept of caged fluorescent probes based on azido-masked fluoresceins for sensing nucleic acids in living cells. Rhodamine 6'-azide incorporated in oligonucleotides can be activated only by reducing agent such as dithio-1,4-threitol or triphenylphosphine.[82] The fluorescent signal appeared after reduction of the azido group. In a similar manner azidomethyl fluorescein was utilized, where the fluorescence occurred upon Staudinger reduction (Figure 8).[83]

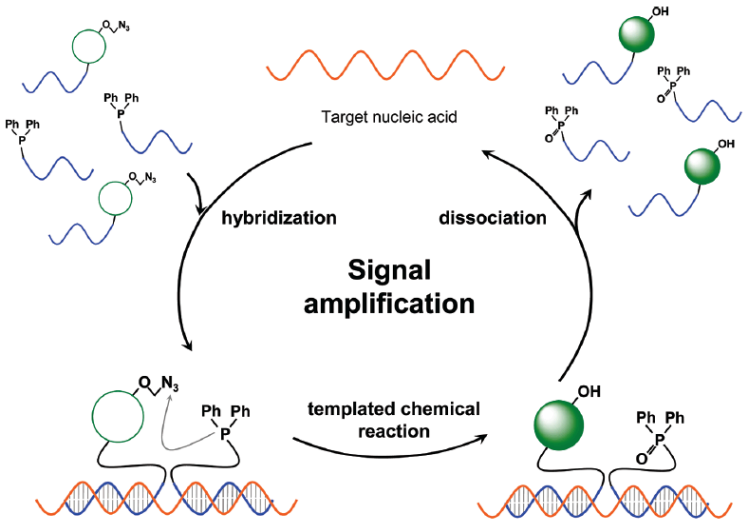
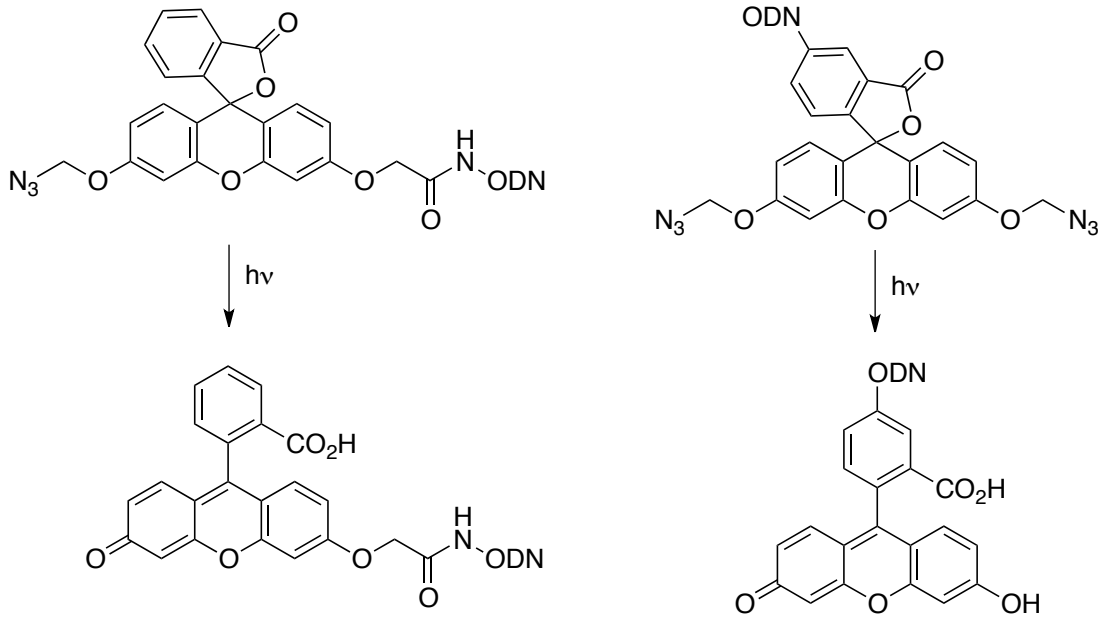


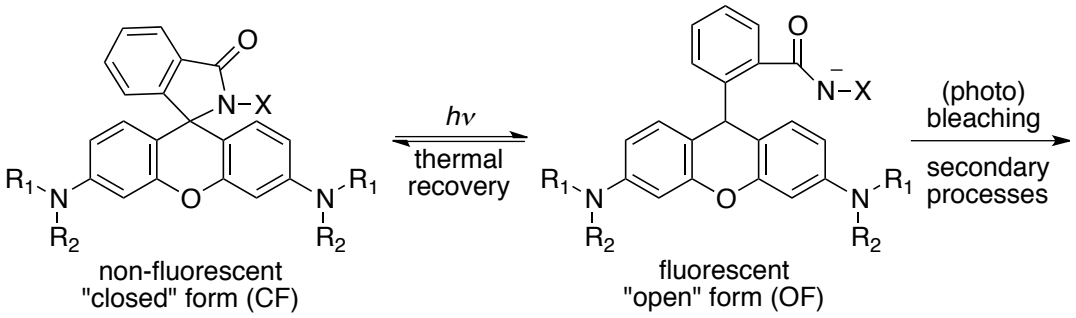
Figure 8. Two strands of DNA or RNA bind onto target oligonucleotides, one probe carrying reducible fluorogenic compound while the others carrying reducing agent.[83]

Further caged fluorescein derivatives with monoazidomethyl and bis-azidomethyl protective groups can be uncaged upon UV-irradiation transformed the azido group into a nitrene group, which can abstract a proton from a water to form an amino-hemiacetal. This group is quickly hydrolyzed in aqueous environment to produce unmasked phenol group of fluorescein and the probe emits fluorescence (Scheme 19).[84]



Scheme 19. Uncaging scheme of mono-azidomethyl fluorescein and bis-azidomethyl fluorescein-labeled oligodeoxynucleotide.

Except of dyes including standard caged 3',6'-positions, a novel class of caged compounds, rhodamine spiroamides (RSAs) was introduced and applied in single-molecule switching microscopy (SMS).[79] The photochromic reaction of RSA was reported for the first time in the 1970s by Knauer and Gleiter.[74] The photoinduced ring-opening reaction generates the rhodamine chromophore, which typically absorbs in the green region and emits in the red. The closed spiro form is transparent across the whole visible range providing a huge contrast between the signals of the two states. The open isomer returns thermally to the "closed" form (CF) with the lifetime of a few milliseconds (Scheme 20). Markers in the "open" form (OF) are bleached by a strong pulse of the same excitation laser after being imaged, to reduce imaging time.[85]



Scheme 20. Photochromic (caged) RSAs in the colorless and colored form.

Dyes designed in this manner were employed in the imaging of nanoparticles and (co)-localization studies of various biological objects (Figure 9).[85, 86, 87]

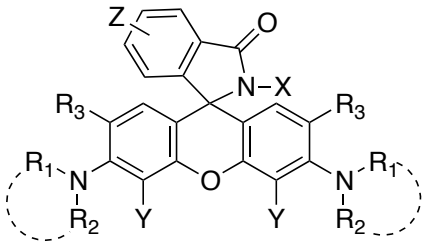
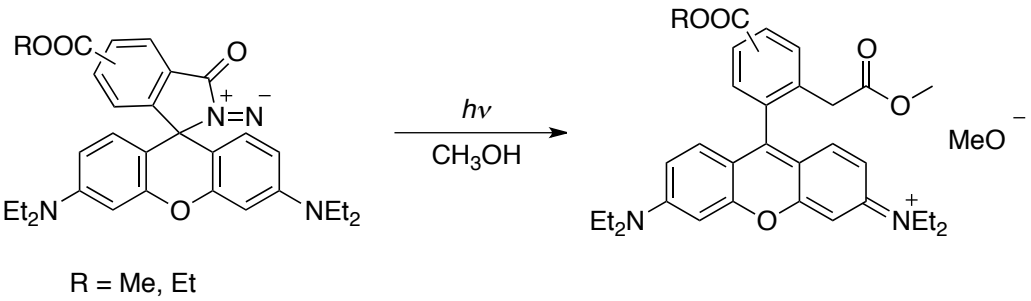


Figure 9. RSAs for multicolor single-molecule photoactivation experiments.

Rhodamine NN dyes, which have a 2-diazoketone (COCNN) caging group incorporated into a spiro-9H-xanthene fragment were reported by Belov *et al* (Scheme 21).[78] Rhodamine NN dyes conjugated with biomolecules undergo rapid uncaging under regular irradiation conditions ($\lambda \leq 420$ nm). These new dyes were used in multicolor microscopy for monochromatic multilabel imaging, where three cellular structures labeled with different dyes were imaged using only green light for excitation and a single detection channel.



R = Me, Et

Scheme 21. Uncaging of TMR-NN derivative.

2 Objectives and Scope

The objective of this thesis was the development of a new synthetic strategy for the preparation of xanthene derivatives. It was discovered that 3',6'-positions of fluorescein and its derivatives undergo nucleophilic aromatic substitution with nucleophiles. A new synthetic method for the preparation of novel asymmetric xanthene dyes *via* direct conversion of hydroxyl groups on 3'- and 6'-positions into leaving groups by sulfonylation was reported.

The synthesis of various sulfonated fluoresceins, mostly di-mesylated derivatives, offered a convenient avenue for attaching sulfur nucleophiles equipped with a linker suitable for further modifications. The modifications of the linker enabled to prepare xanthene derivatives that can be used as fluorescent markers in biological experiments, including labeling of nucleotides, oligonucleotides, peptides, proteins, drugs, and other biomolecules.

Particular emphasis was placed on the investigation of the impact of substituents with electron-withdrawing character such as Br, Cl and SO_3H groups on 4',5'- or 2',7'-positions of fluorescein on nucleophilic aromatic substitution. It would be expected that these groups improve the substitution reaction on electron-rich fluorescein.

The photophysical properties of new xanthene dyes were characterized. It was observed that substitution with sulfur nucleophiles significantly improved photostability of the xanthene dyes in comparison with fluorescein and 2',7'-dichlorofluorescein.

3 Results and Discussions

3.1 Overview

First mono- and di-sulfonylation of fluorescein, which were successfully adapted to various derivatives, were discussed. These sulfonated derivatives were prepared with the intent to undergo nucleophilic aromatic substitution.

Tokyo Green was synthesized with the aim to investigate the sulfonylation reaction on the 3',6'-positions and further substitution reactions. An azido derivative attached on the phenyl of Tokyo Green was prepared for the comparison with new azido xanthene derivatives.

The substitution reaction on the 3',6'-positions of fluorescein, based on Williamson ether synthesis with propargyl alcohol, was performed to obtain 6'-(prop-2-yn-1-yloxy)fluorescein. It was discovered that the 6'-prop-2-yn-1-yloxy group underwent nucleophilic aromatic substitution with methanolate.

The sulfonated 3',6'-positions of fluorescein were tested on nucleophilic aromatic substitution with *N*-nucleophiles and offered low yields. These observations suggested that this kind of substitution reaction was possible.

The nucleophilic aromatic substitution reaction of different 3',6'-sulfonated fluorescein and its derivatives was further investigated with *S*-nucleophiles. New asymmetric xanthene sulfides were prepared. These xanthene dyes possessed a linker, which could be further modified.

The modification of a sulfur linker to obtain new xanthene azides and a xanthene amine was described.

The photophysical properties of new asymmetric xanthene dyes were characterized and discussed.

To prove the capability of the synthesized xanthene azides, they were “clicked” with oligonucleotides bearing a terminal alkyne group.

3.2 Synthesis of Sulfonate Esters of Fluoresceins

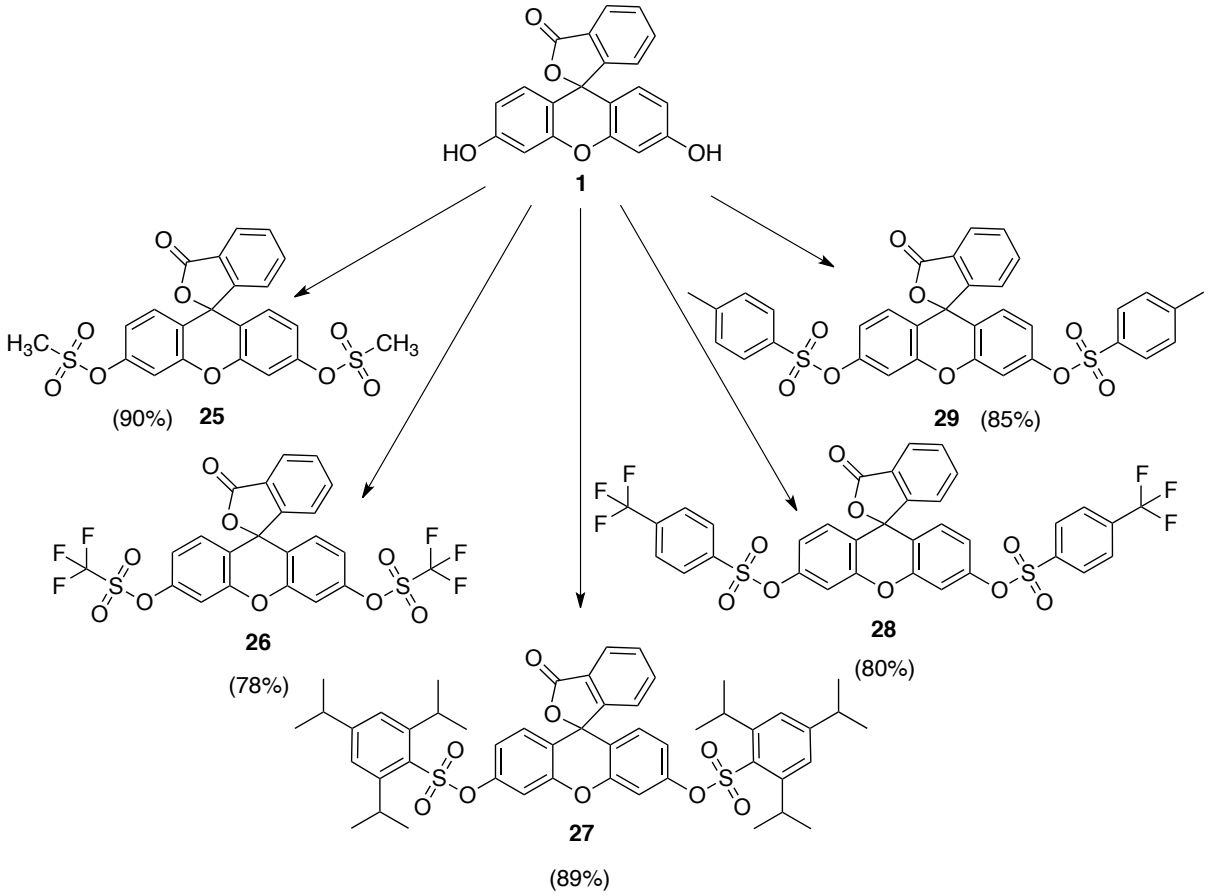
3.2.1 Di-sulfonylation of Fluorescein

Various approaches for the conversion of alcohols to sulfonates are known and used in organic chemistry. Generally substitution of the hydroxyl groups by sulfonylation is carried

out with sulfonyl chlorides or anhydrides in a basic medium. An old approach with pyridine as solvent provides basic conditions, which are necessary for the reaction to take place. Sulfonylation of the 3',6'-positions applied to fluorescein and its derivatives have been reported previously. Sulfonylation in pyridine applied to fluorescein was introduced for the first time in 2006[88] and the same procedure was further used.[89, 90, 56] Another method to obtain sulfonate esters of fluorescein employed Et_3N or DIPEA in CH_2Cl_2.[75, 91]

Our synthetic strategy was based on work of Seidu-Larry[89], where pyridine was used as a necessary base and solvent for the mesylation reaction. The reaction conditions for di-sulfonylation involve 4 equivalents of sulfonated reagents, particularly methanesulfonyl chloride, 4-toluenesulfonyl chloride, trifluoromethanesulfonic anhydride, trifluorotoluenesulfonyl chloride and 2,4,6-triisopropylbenzenesulfonyl chloride (Scheme 22). Trifluromethanesulfonyl anhydride had to be used instead of trifluoromethanesulfonic chloride to achieve ditriflation.[56] Even with higher amounts of equivalents of trifluoromethanesulfonic chloride the ditriflation was not proceeding. The only product, which was delivered, was the mono-triflated form of fluorescein.

To avoid the Py·HCl by-product[92] formation and thus the concomitant loss of the sulfonates, the reaction was carried out at 0 °C during the addition of sulfonated reagents. The temperature of reaction was maintained at 0 °C for an additional 10-30 min. The overall reaction time was usually 4-6 h and the yield of the di-sulfonylation reaction was 78-95%. This type of reaction did not need an extensive work-up, just evaporation of pyridine to dryness, and the purification was then accomplished by column chromatography on silica gel, where unpolar di-sulfonate esters products were simply eluted from silica gel using pure CH_2Cl_2, making this a routine procedure for purification of di-sulfonated fluoresceins. Ditriflation of fluorescein was carried out according to recently published literature[56] with the work-up involving dilution with water, followed by extraction into CH_2Cl_2 to remove pyridine. Since removal of pyridine after the reaction seems rather easy, this unnecessary step could be avoided.



Scheme 22. Disulfonylation of fluorescein; Reagents and conditions: sulfonyl chloride or anhydride (4.0 equiv), pyridine, 0 °C for 30 min, rt for 4-6 h.

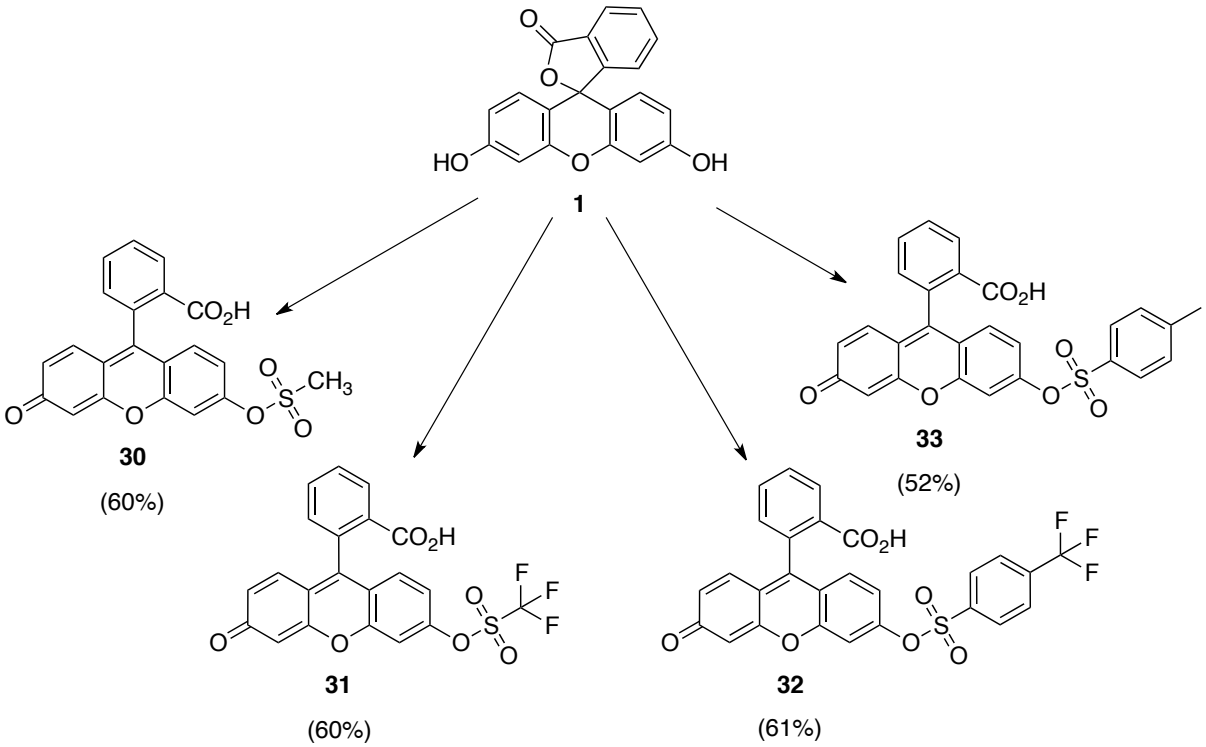
The tosylation reaction was performed successfully not only in pyridine, but also in THF-water using NaOH as base. This newly invented method for tosylation was adopted on two aromatic alcohol groups of fluorescein from a literature used for tosylation of oligoethylene glycols.[93] The yield of the reaction decreased from 85% to 70%. This reaction showed that toluenesulfonyl chloride was stable to hydrolysis in the basic pH and di-tosylated fluorescein (**29**) was not hydrolyzed under the same conditions either, rendering this synthetic path universal for tosylation of fluorescein.

The sulfonylation reactions were repeated employing commercially available pyridine without drying. Only a minor decrease in yields was obtained.

3.2.2 Mono-sulfonylation of Fluorescein

Mono-sulfonated fluoresceins were prepared in the same manner, in pyridine, using 0.9 equivalent of sulfonated reagents (Scheme 23). To ensure full conversion the reaction time

was elongated to overnight. Incomplete conversion of starting material, a formation of disulfonated by-product made the purification more demanding. Mono-sulfonylation delivered products with the yields between 52-61%.



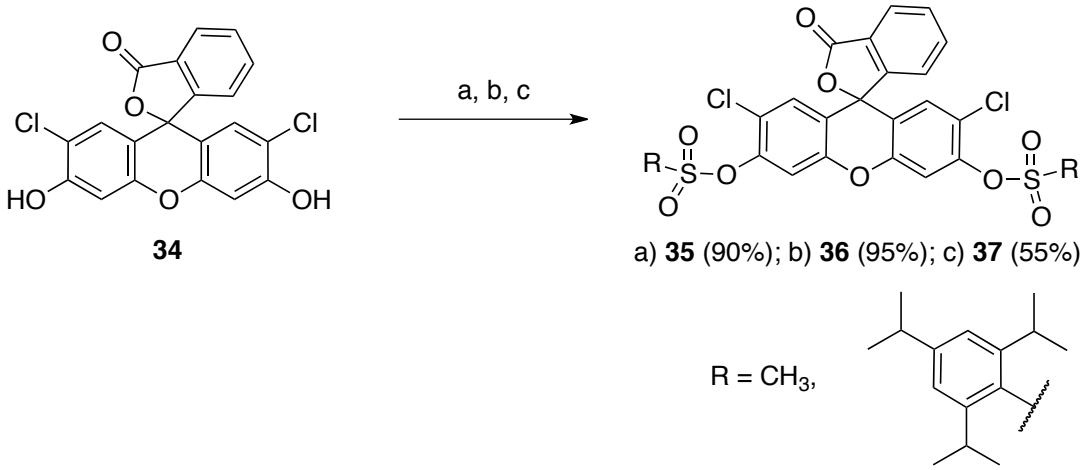
Scheme 23. Mono-sulfonylation of fluorescein; Reagents and conditions: sulfonyl chloride (0.9 equiv), pyridine, 0 °C for 30 min, rt for 6 h to overnight.

3.2.3 Sulfonylation of Derivatives of Fluorescein

Fluorescein derivatives with chlorine and bromine were chosen according to their electron-withdrawing character. The electron-rich character of fluorescein can be tuned by incorporation of these groups and should have a positive effect on the following substitution reaction.

Sulfonylation of fluorescein derivatives was carried out according to previously optimize conditions. Mesylation of 2',7'-dichlorofluorescein was performed with 4 equivalents of methanesulfonyl chloride in pyridine during 4 h (Scheme 24). After purification 82% of di-mesylated 2',7'-dichlorofluorescein were obtained, demonstrating a comparable good yield to di-mesylation of fluorescein (90%). Ditriisopropylbenzenesulfonate 2',7'-dichlorofluorescein was prepared from the reaction of 2',7'-dichlorofluorescein with 4 equivalents of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) in pyridine for 4 h (Scheme 24). The product

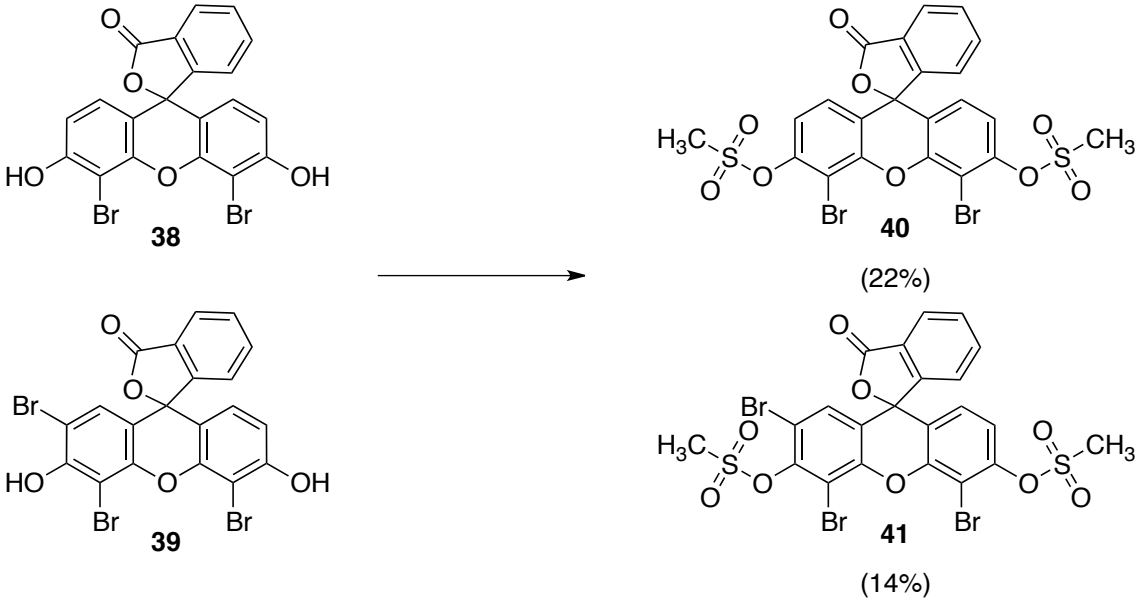
was obtained with the yield of 95%, which was also comparable to the yield of 89% delivered in the same reaction with fluorescein.



Scheme 24. Sufonylation of 2',7'-dichlorofluorescein; Reagents and conditions for di-substitution: a) methanesulfonyl chloride (4.0 equiv), b) 2,4,6-triisopropylbenzenesulfonyl chloride (4.0 equiv), pyridine, 0 °C for 30 min, rt for 4 h; Reagents and conditions for mono-mesylation: c) methanesulfonyl chloride (0.9 equiv), pyridine, 0 °C for 30 min, rt for 6 h.

Mono-mesylation of 2',7'-dichlorofluorescein was carried out with 0.9 equivalent of methanesulfonyl chloride in pyridine. After 6 h of reaction and purification by chromatography the product was obtained with the yield of 55% (Scheme 24).

Commercially available 4',5'-di-bromofluorescein, obtained from different commercial sources, contained the mixture of 4'-monobromofluorescein, 4',5'-dibromofluorescein (**38**) and 4',5',7'-tribromofluorescein (**39**) derivatives. This mixture of starting material was inseparable. The challenging column chromatography delivered only traces of pure 4',5'-dibromofluorescein. An option could be an acetylation step, followed by the column chromatography and the cleavage of acetylated groups to simplify the purification of these difficult mixtures, but overall it can be expected that the yield of starting material should be decreased significantly.[29] It was discovered that an additional synthetic step could circumvent this problem. For this reason, the crude brominated starting material was used for mesylation with standard 4 equivalents of methanesulfonyl chloride in pyridine (Scheme 25), thus could be later separated in the forms of di-mesylated products. The reaction took 5 h. The purification was achieved with gradient column chromatography and di-mesylated di-brominated **40** and di-mesylated tri-brominated fluoresceins **41** were isolated with the yields of 22% and 14%, respectively.



Scheme 25. Mesylation of brominated fluorescein; Reagents and conditions: methanesulfonyl chloride (4.0 equiv), pyridine, 0 °C for 30 min, rt for 5 h.

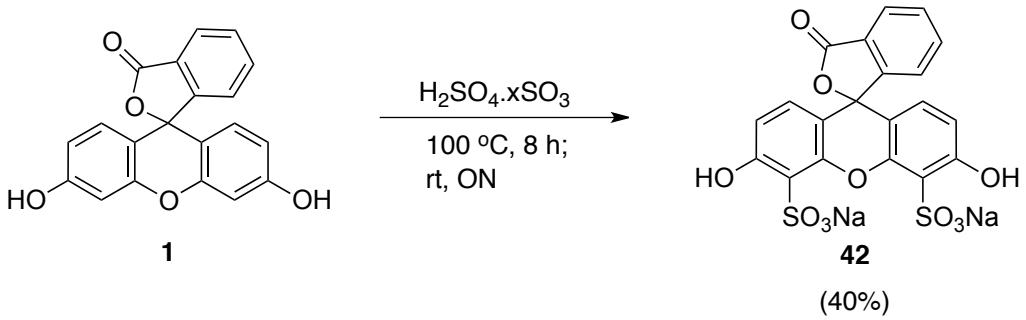
3.3 Sulfonation of Fluorescein

The direct sulfonation of fluorescein was reported by Roskulyak *et al*.[38] Usually sulfo groups are introduced into the structure of fluorescein indirectly by substitution of building blocks like phenols, followed by their condensation reaction with phthalic anhydride to deliver the desired sulfonated fluoresceins. Sulfonation based on the direct electrophilic substitution on fluorescein simplified the process of the synthesis and offered an interesting starting material for further modifications. Sulfonation is known to shift the absorption and the emission bands up to ≈5 nm. The spectral properties are usually not really altered by these groups.[94] On the other hand, introduction of the sulfonic acid residue can improve solubility in aqueous media and increase photostability and fluorescence quantum yields.[42]

3.3.1 Di-sulfonation of Fluorescein on 4',5'-Positions

Di-sulfonation[38] was carried out in oleum (30%SO_3 in H_2SO_4), heated at 100 °C for 8 h and stirred at rt overnight (Scheme 26). The synthesis delivered di-sulfonated product **42** selectively on 4',5'-positions of fluorescein, which was proven by ^{1}H NMR. **42** was prepared

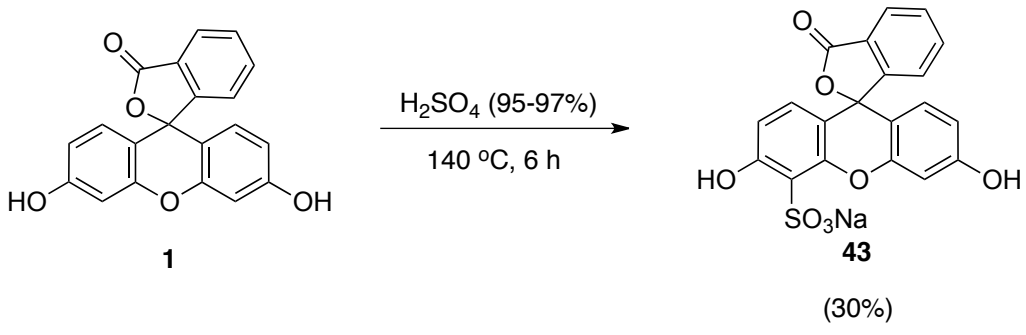
as a di-sodium salt and purified by simple crystallization from water, which offered a yellow solid with 40% yield.



Scheme 26. Di-sulfonation of fluorescein.

3.3.2 Mono-sulfonation of Fluorescein on 4'- or 5'-Position

Mono-sulfonation of fluorescein was performed according to literature[38] in concentrated H_2SO_4, heating the reaction mixture for 6 h at 140 °C (Scheme 27). The reaction took place selectively on the 4'-position according to ^1H NMR and delivered only one product. After the work-up, the column chromatography was applied to separate the product, mono-sulfonated fluorescein from unreacted fluorescein and afforded a yellow solid with 30% yield. Upon the storage at -24 °C in the freezer for one month mono-sulfonated fluorescein decomposed to red fluorescein. It was discovered that mono-sulfonated fluorescein **43** prepared as a sodium salt, was its more stable form towards the undesirable decomposition.

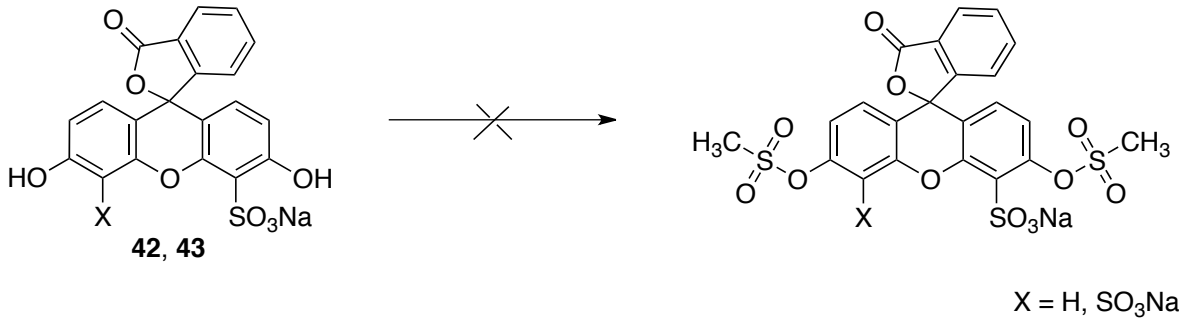


Scheme 27. Mono-sulfonation of fluorescein.

3.4 Synthesis of 3',6'-Di-mesylated-4',5'-Di-sulfonated Fluorescein

The sulfonated derivatives, in particular **42** and **43** were synthesized with the aim to obtain derivatives that would undergo the sulfonylation reaction and deliver products for additional substitution reactions.

The sulfonylation of sulfonated fluoresceins was performed using methanesulfonyl chloride in different reaction conditions (Scheme 28). The mesylation was carried out under various conditions with 4 equivalents of methanesulfonyl chloride in dry pyridine, with 4 equivalents of methanesulfonyl chloride, 4 equivalents of DIPEA in dry DMF, 4 equivalents of methanesulfonyl chloride, 4 equivalents of Et_3N in dry DMF and finally with 4 equivalents of methanesulfonyl chloride using the stronger non-nucleophilic base DBU (6 equivalents) in dry DMF. Traditional CH_2Cl_2 used for sulfonylation had to be replaced with DMF because of the insolubility of polar sulfonated fluoresceins in unpolar organic solvents. Upon no reaction proceeding, the reaction times were elongated and the mixtures were heated to 50 °C. The mesylation of di-sulfonated or mono-sulfonated fluoresceins did not proceed.



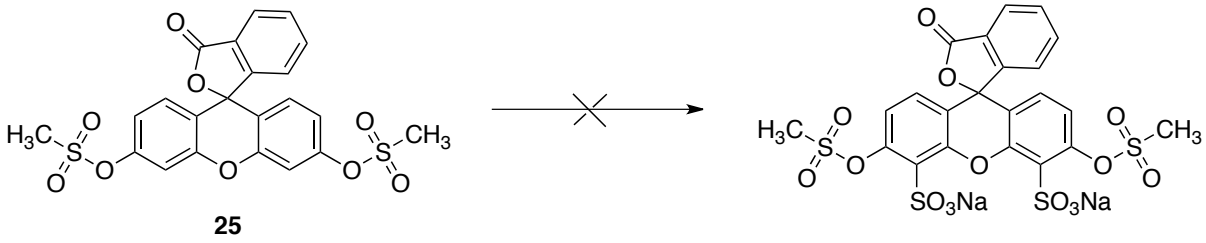
Scheme 28. Mesylation of 4'-sulfonated fluorescein (**42**) and 4',5'-disulfonated fluorescein (**43**).

Sulfonyl chloride is probably not stable enough in the acidic solution of sulfonated fluoresceins in DMF. It is known that the sulfonylation reaction requires basic conditions to stabilize sulfonyl chloride, and to protect it against its hydrolysis and formation of CH_3SO_3H.[95]

Another attempt to obtain mesylated di-sulfonated or mono-sulfonated fluoresceins based on sulfonation of di-mesylated fluorescein (**25**) was implemented. The same reaction conditions employed in sulfonation of fluorescein were used for **25**. Since mono-sulfonated fluorescein is less stable than its di-sulfonated derivative, di-mesylated fluorescein was first heated in

oleum at 100 °C for 8 h and was further stirred at rt overnight (Scheme 29). The mesyl groups should be stable under these reaction conditions. The work-up required the dilution of the reaction mixture in water and precipitation from a saturated solution of NaCl. These conditions should not accelerate cleavage of the mesylated groups on the 3',6'-positions of fluorescein. The crude mixture was analyzed by mass spectrometry and proved the existence of a mixture of mono-mesylated and di-mesylated mono-sulfonated and di-sulfonated fluorescein together with mono- and di-sulfonated fluoresceins. There were several attempts to purify this troublesome mixture by chromatography on silica gel. The only pure delivered product, which was analyzed by NMR, clearly indicated the existence of 4',5'-disulfonated fluorescein. The attempt to modify di-sulfonated fluorescein and mono-sulfonated fluorescein by mesylation was not successful.

In the literature Mitronova *et al.* discovered, that the sulfonation of the xanthene moiety of some rhodols and rhodamines was compatible with 30% SO_3 in H_2SO_4 at 0 °C for 2-3 days.[42] They described the purification as tedious, making use of reverse-phase chromatographic separation by preparative HPLC. In our conditions the separation was done by simple column chromatography on silica gel and was complicated due to the instability of the mesylated groups and the unselective sulfonation of fluorescein.



25

Scheme 29. Sulfonation of 3',6'-di-mesylated fluorescein (**25**).

3.5 Synthesis of Tokyo Green and its Derivatives

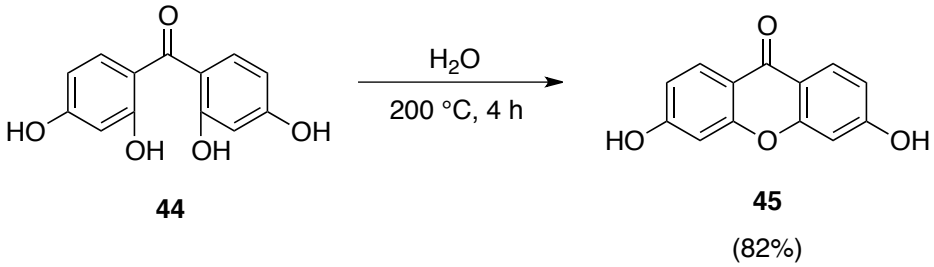
Tokyo Green was first developed by Urano *et al.* to demonstrate, that the carboxylic group of fluorescein plays no role in its fluorescent properties by exchanging it for methyl group.[45]

Tokyo Green is very similar to fluorescein in fluorescent properties, which are almost identical and both are having excellent quantum yields. Also its pH sensitivity especially decrease in fluorescence in acidic medium shows relevance of formation of anionic form. Interestingly fluorescein can be in two anionic forms mono-anionic and di-anionic, depends on pH. Mono-anionic form is known to be less fluorescent and the di-anionic form represents

the high absorption maxima and high quantum yields of fluorescein. Tokyo Green can adopt only mono-anionic form and still keep the fluorescent properties comparable to fluorescein.

3.5.1 Synthesis and Protection of 3,6-Dihydroxy-9H-xanthen-9-one

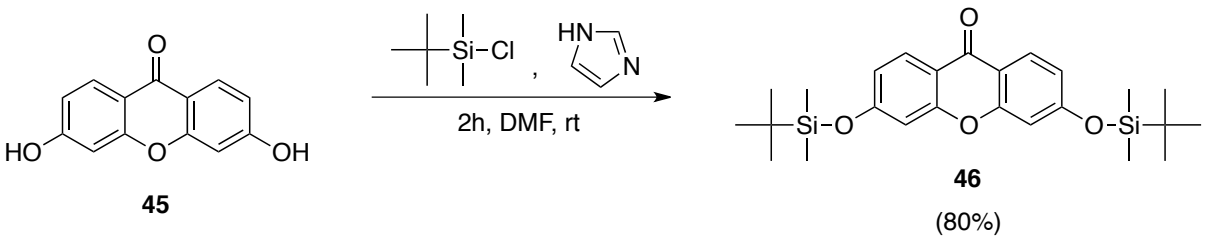
3,6-dihydroxyxanthen-9-one (**45**) represents important building block for the synthesis of Tokyo Green. The commercially available starting material 2,2',4,4'-tetrahydroxybenzophenone (**44**) was heated in water at 200 °C for 4 h (Scheme 30).[37] This reaction was accomplished in special pressure tubes, which upon good sealing can be heated in the classical heating oven for 200 °C. **45** was obtained on cooling. The re-crystallization delivered the pure product with the yield of 82%, which was comparable with the literature yield of 90%.



44 **45** (82%)

Scheme 30. Synthesis of 3,6-dihydroxyxanthenone (**45**).

45 has to be protected on the 3,6-positions, to be used for the synthesis of Tokyo Green. The protection was accomplished in DMF with *tert*-butylchlorodimethyl silane (TBDMS-Cl) (6 equivalents) and imidazole (10 equivalents) at rt for 2 h (Scheme 31).[96] The product was recrystallized from ethanol to give the di-protected product **46** with the yield of 80%.

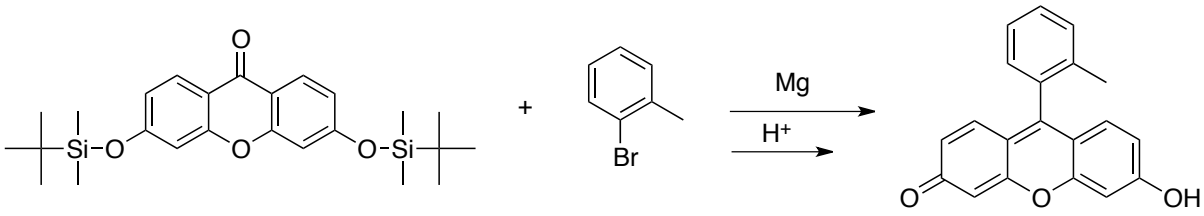


45 **46** (80%)

Scheme 31. Synthesis of 3,6-bisTBDMS-xanthenone (**46**).

3.5.2 Synthesis of 6-Hydroxy-9-(o-tolyl)-3H-xanthen-3-one (Tokyo Green)

Tokyo Green was synthesized based on optimized protocol from literature by Grignard reaction of 2-bromotoluene (**47**) with **46** (Scheme 32).[45] After the activation of magnesium pellets in dry Et_2O with the help of activating agent 1,2-dibromoethane, **47** (5 equivalents) was added and the mixture was stirred and frequently heated with a heat gun for 2 h. After no heat formation was observed, complete conversion of **47** into *o*-tolylmagnesium bromide was assumed to take place. Subsequently the mixture was cooled to 0 °C and **46** in dry THF was added. The mixture was stirred at 0 °C for 10 min. The color of reaction mixture changed several times, ending with brown color. The reaction is claimed to take 10 min[45] up to 16 h[46]. Color change did not prove to be a good indicator for the completeness of the reaction. After 2 h of reaction time and no more color change of the reaction mixture, the deprotection of 3,6-positions was initiated with HCl. The unreacted xanthenone was still detected in the mixture. For this reason the time of Grignard reaction was elongated at rt overnight. Afterwards xanthenone was fully converted and the yield of the reaction increased from 40% to 70%. The column chromatography afforded highly fluorescent product **48** with the yield of 70%.



46 **47** **48** (70%)

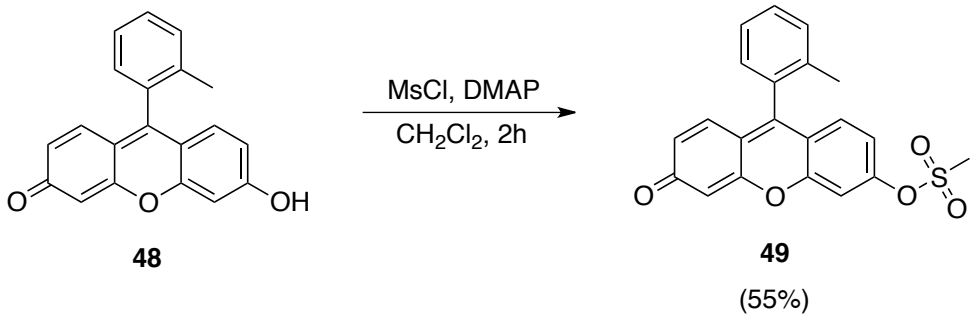
Scheme 32. Synthesis of Tokyo Green; Reagents and conditions: activation of 2-bromotoluene (5 equiv), Mg, 1,2-dibromoethane, Et_2O, THF, Ar, heating, 2 h; addition of 3,6-bis(tert-butyldimethylsilyl)oxy)xanthenone (1 equiv), THF, Ar, 0 °C, 10 min, rt overnight.

3.5.3 Mesylation of Tokyo Green

Mesylation of Tokyo Green **48** was performed with the aim to synthesize di-mesylated product and to obtain better leaving groups for further substitution on the 3',6'-positions.

Tokyo Green had very low solubility in pyridine and also in the mixture of pyridine/CH_2Cl_2, 1:1. The mesylation reaction was optimized and carried out with methanesulfonyl chloride (4 equivalents) in CH_2Cl_2 using DMAP (6 equivalents). Tokyo Green was after 2 h completely

converted and **49** occurred (Scheme 33). This type of reaction clearly delivered mono-substituted product. After the work-up the mono-mesylated product **49** was obtained with the yield of 55%.



48 **49**

(55%)

Scheme 33. Mono-mesylation of Tokyo Green.

There was an assumption that the di-mesylated product was forming. According to TLC (CH_2Cl_2/CH_3OH, 9:1, 95:5) some non-fluorescent spots with higher R_F value were also present. Upon several attempts for optimization, the reaction was repeated with the same conditions with methanesulfonyl chloride (4 equivalents), DMAP (6 equivalents) in CH_2Cl_2. After 4 h of the reaction time additional 4 equivalents of methanesulfonyl chloride were added and the reaction was stirred at rt overnight. The precipitate occurred, which after adding some drops of acetic acid disappeared. Interestingly, according to TLC the mono-mesylated product diminished and non-fluorescent spots appeared more visible. The isolation of pure product *via* column chromatography failed.

Another attempt to synthesize di-mesylated Tokyo Green was carried out in pyridine. Tokyo Green dissolved in pyridine after an addition of methanesulfonyl chloride (4 equivalents). After two hours of the reaction time additional 4 equivalents of methanesulfonyl chloride were added and the reaction was stirred at rt overnight. The precipitate occurred. The reaction was proceeding almost identical as with DMAP in CH_2Cl_2. The resulting mixture after the removal of pyridine was very well soluble in water and polar protic solvents.

Tokyo Green is normally soluble in polar organic solvents and water. Upon the incorporation of one mesyl group into the structure, Tokyo Green became soluble in nonpolar organic solvents such as chloroform. A new substance putative pyridinium salt of Tokyo Green was non-fluorescent with a good solubility in water. The mixture was re-purified and further analyzed. ESI-MS showed mass 539.18 with the highest intensity (Figure 10), which corresponds to the molecular mass of di-mesylated Tokyo Green (460.52 g/mol) plus molecular mass of pyridine (79.1 g/mol). ^{1}H NMR was performed in various solvents. The

peaks corresponding to Tokyo Green and pyridine were present. The visualization of methyl and mesyl peaks was troublesome and solvent dependent.

In order to obtain mesyl groups on the both sides, Tokyo Green might undergo formation of inner cation so the putative di-mesylated Tokyo Green might form.

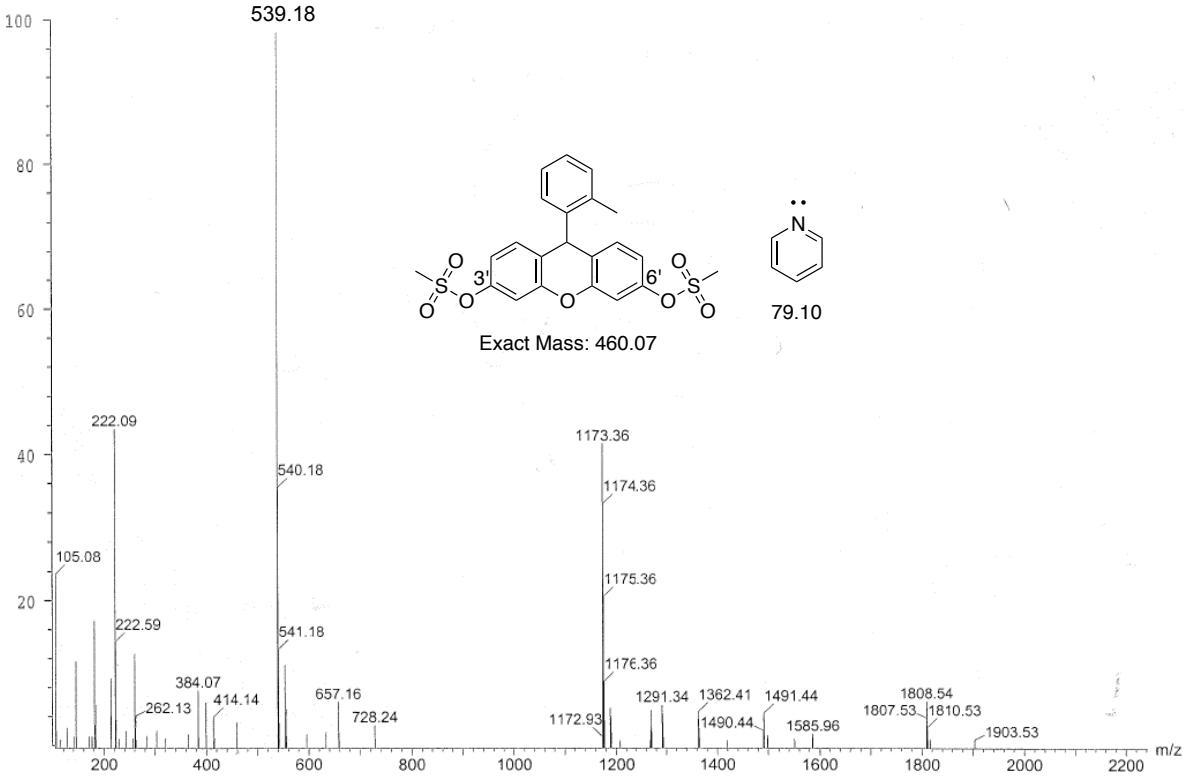


Figure 10. MS-FD of putative pyridinium salt of Tokyo Green.

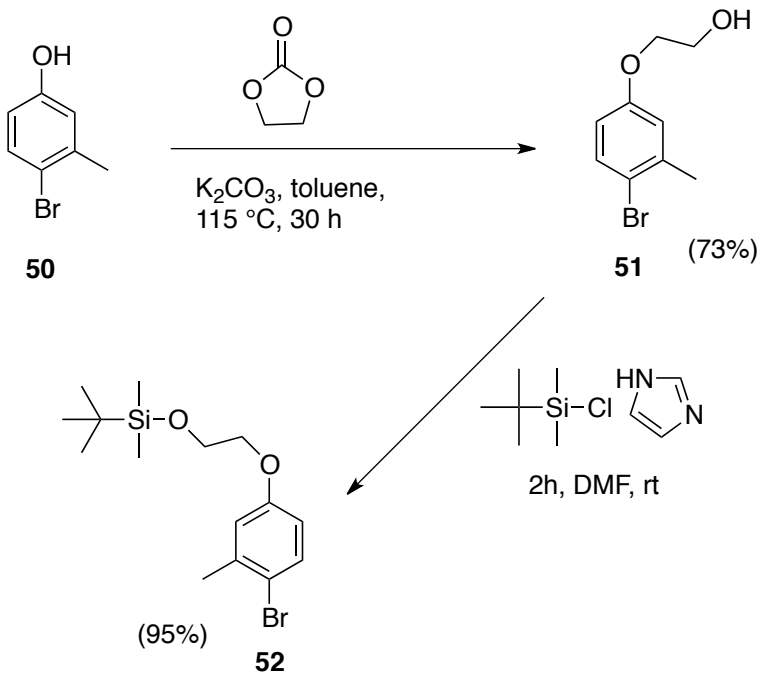
3.5.4 Synthesis of Azido Tokyo Green

4-Azidoethoxy modified Tokyo Green was synthesized according to literature.[97] This Tokyo Green fluorophore has the azido group attached on benzene moiety through ethoxy linker.

Indeed on the onset of the synthesis making this azido dye was intended for future comparison with 3',6'-modified Tokyo Green. Our aim was to prepare substituted Tokyo Green with azido functionality attached to the linker on the 6'-position, which could be compared to Tokyo Green with 4-azidoethoxy group on phenyl moiety. Since di-mesylation of Tokyo Green delivered presumable product as pyridinium salt and its mono-mesylated product appeared to be unreactive towards nucleophilic substitution reaction, Tokyo Green with azido-linker attached to the 6'-position was not synthesized.

The building block for the synthesis of azido Tokyo Green, 2-(4-bromo-3-methylphenoxy) ethanol (**51**) was prepared by reaction of 3-methyl-4-bromophenol (**50**) (1 equivalent) with ethylene carbonate (4 equivalents) and K_2CO_3 (2 equivalents) in dry toluene (Scheme 35). The mixture was refluxed at 115 °C for 48 h. The reaction time was elongated from 24 h used in literature, because the conversion of starting material during the period of 24 h was not finished. Even after 48 h there was starting material remaining. The product **51** was obtained after purification with the yield of 73% (Scheme 34).

The protection of free hydroxyl group took place with TBDMS-Cl (1.4 equivalents), imidazole (3 equivalents) in DMF in 2h (Scheme 34). The chromatography afforded the product **52** with the yield of 94%.

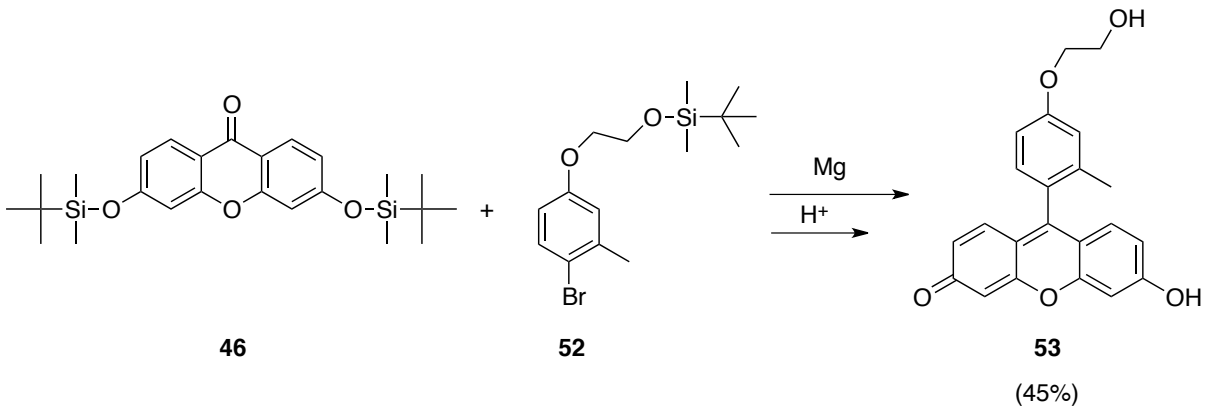


Scheme 34. Synthesis of (2-(4-bromo-3-methylphenoxy)ethoxy)(*tert*-butyl)dimethylsilane (**52**).

(4-(2-hydroxyethoxy) Tokyo Green **53** was prepared by Grignard reaction of **52** with **46** (Scheme 35).[97]

Mg in dry Et_2O was activated with 1,2-dibromoethane and after **52** (1.8 equivalents) in dry Et_2O was added. The activation was performed similar to 2-bromotoluene, which was already mentioned. After additional stirring at room temperature for 30 min, the mixture was cooled to 0 °C and **46** (1 equivalent) in dry THF was added in a dropwise manner. The reaction color changed for several times within 2 h as it was already noticed in the synthesis of Tokyo Green. The mixture was stirred for an additional 4 h. After standard work-up procedure

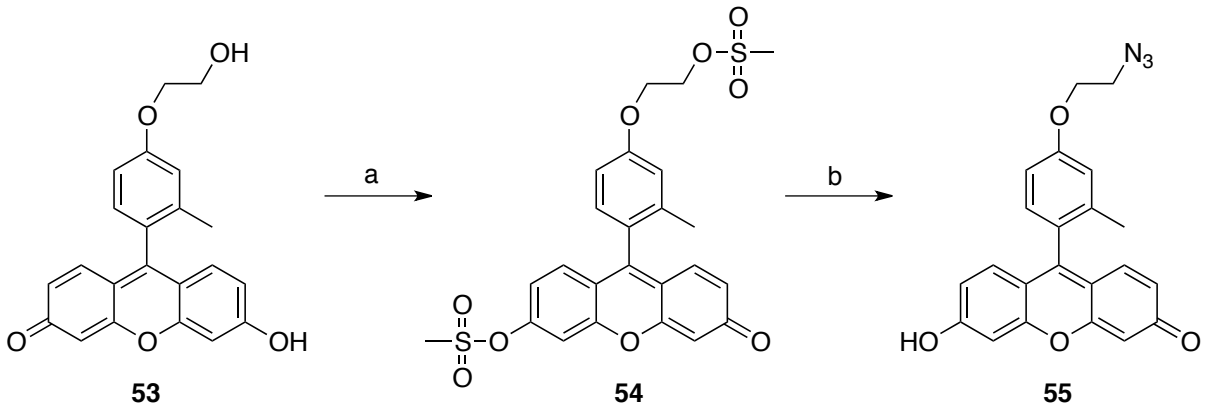
optimized with Tokyo Green, the deprotection of the TBDMS groups with HCl took place, accompanied with the development of fluorescence. After work-up and column chromatography **53** was obtained with the yield of 45%, which was lower than 82% mentioned in literature.[97]



Scheme 35. Synthesis of (4-(2-hydroxyethoxy) Tokyo Green; Reagents and conditions: activation of **52** (1.8 equiv), Mg, 1,2-dibromoethane, Et_2O, THF, Ar, heating, 1 h, 30 min at rt; addition of **46** (1 equiv), THF, Ar, 0 °C, 10 min, rt, 4 h.

(4-(2-hydroxyethoxy) Tokyo Green **53** was synthesized with the aim to be transformed into azido Tokyo Green. Azido functionalized Tokyo Green can be used for labeling of oligonucleotides with terminal alkyne group by Cu(I)-catalyzed 1,3 dipolar cycloaddition.
Before the azido functionality could be introduced into the Tokyo Green, the hydroxyl group on phenyl moiety was mesylated. The mesylation reaction was carried out using methanesulfonyl chloride (4 equivalents) and DMAP (4 equivalents) in dry CH_2Cl_2 (Scheme 36).[97] According to literature procedure the mixture was stirred overnight. Theoretically there are three possibilities, where the mesyl group could be attached. Those are hydroxyl group on the xanthene moiety, the carbonyl group on the 3'-position of xanthene moiety and desired hydroxyl group on the aliphatic chain. In the literature the substitution was reported on hydroxyl group on the 6'-position of xanthene only or on hydroxyl group on the 6'-position and hydroxyl group on aliphatic chain. The literature procedure was further modified. Since purification of this mixture is rather difficult, and the mesyl group on the 6'-position should not have any impact on the followed azidation reaction, additional 4 equivalents of methanesulfonyl chloride was applied to obtain mostly di-mesylated product **54** (Scheme 36). The product after preparative TLC was delivered with the yield of 35%.

In the next step **54** reacted with NaN_3 (6 equivalents) in dry DMF at 60 °C for 46 h. The reaction time was elongated in comparison with literature. The product **55** after preparative TLC and lyophilization was obtained with the yield of 23% (Lit 27%).[97]



Scheme 36. Synthesis of azido Tokyo Green **55**; Reagents and conditions: a) MsCl (4 equiv), DMAP (4 equiv), dry CH_2Cl_2, Ar, 0 °C, 30 min; rt, 24 h, add. MsCl (4 equiv), 0 °C, 30 min; rt, 24 h b) NaN_3 (6 equiv), dry DMF, Ar, 60 °C, 46 h.

3.6 Substitution on 3',6'-Positions of Fluorescein

Not so much literature engaged in direct substitution on the 3',6'-positions of fluorescein is known.

The most common avenue to introduce substituents is based on Williamson ether synthesis, where the fluorescein hydroxyl groups are usually treated with K_2CO_3, NaOH or other inorganic bases to generate nucleophiles, which can react with alkyl halides and form fluorescein ethers.[98, 81, 84, 71, 91]

The development of new synthetic routes to achieve different substituents on the 3',6'-positions of xanthene residue is still not completely covered.

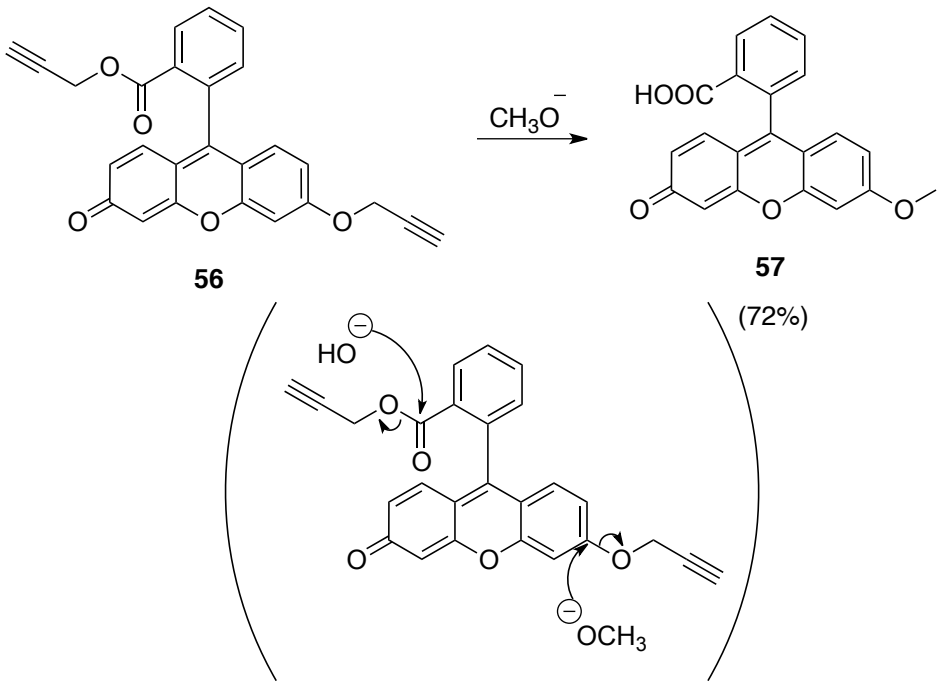
3.6.1 Synthesis of 6'-(Prop-2-yn-1-yloxy)fluorescein

Fluorescein with the terminal alkynyl group was synthesized by the above mentioned Williamson ether synthesis (Scheme 38). Fluorescein was treated in acetone with K_2CO_3 (2 equivalents) to deprotonate hydroxyl groups. The propargyl bromide (2 equivalents) was added and the mixture was refluxed overnight. This type of reaction was not selective, but

gave a rise to different products such as allyl ether ester and the allyl diether of fluorescein, monoallyl ether and monoallyl ester of fluorescein. The unreacted fluorescein was also present. By pouring the reaction mixture into water, propargyl ether ester of fluorescein and fluorescein precipitated. After re-crystallization propargyl ether ester of fluorescein **56** was obtained with the yield of 15%. (Lit. 42%)[98]

The reactive alkynyl group was attached not only to 6'-position of xanthene, but also to the carboxyl group of the phenyl moiety. For the later applications concerning click chemistry it could be tedious to distinguish between the groups.

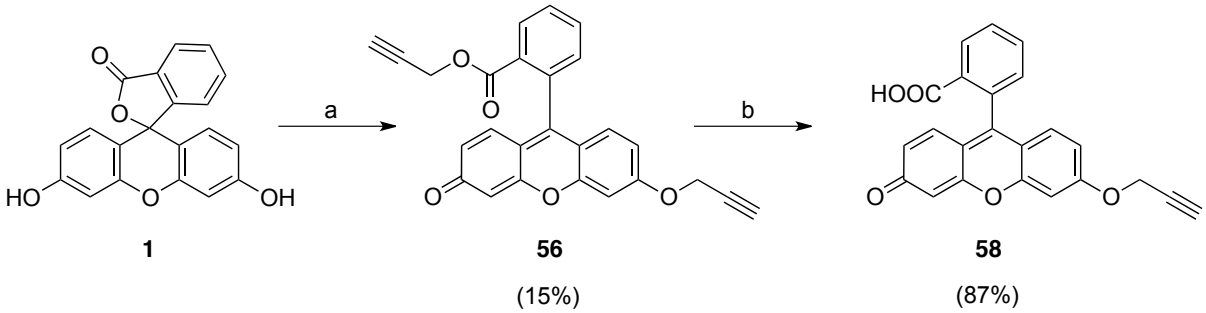
For this reason two attempts to hydrolyze ester bond were performed. The first method was simple hydrolysis reaction of propargyl ether ester of fluorescein with NaOH (5 equivalents) in methanol. The reaction mixture was refluxed for 2 h. After the work-up the by-product methyl monoether **57** was delivered with the yield of 72%. Interestingly the carboxylic group was hydrolyzed but a new methyl mono-ether bond was formed. These observations showed that the aromatic substitution reaction by methanolate on the 6'-position was taking place (Scheme 37).



Scheme 37. Hydrolysis of ester group; Reagents and conditions: NaOH (5 equiv), methanol, reflux, 2 h.

The second method for hydrolysis was carried out with NaOH (4 equivalents) in propargyl alcohol to not only cleave ester bond but also to obtain again propargyl ether. 5 h of reflux of

the reaction mixture afforded the product, 6'-(prop-2-yn-1-yloxy)fluorescein (**5**8) with the yield of 87%.



Scheme 38. Synthesis of 6'-(prop-2-yn-1-yloxy)fluorescein (**58**); Reagents and conditions: a) K_2CO_3 (2 equiv), propargyl bromide (2 equiv), aceton, reflux, ON; b) NaOH (4 equiv), propargyl alcohol, reflux, 5 h.

This compound found applications in "click" chemistry. In particular **58** was applied in azide-alkyne click derivatization with azidocoumarin-modified RNA.[24]

3.6.2 Synthesis of Mono-piperidyl and Di-piperidyl Fluorescein

Development of a new synthetic strategy to generate rhodol and rhodamines by a direct substitution on 3',6'-positions was the subject of a few studies in recent years. Since the standard procedure for preparation of rhodols and rhodamines involves old fashioned chemistry based on acid-catalyzed condensation of an 3-aminophenol with a phthalic anhydride, there was a big demand to generate these fluorophores in a more convenient way. Additionally only a limited number of 3-amino-phenols is compatible with this century old procedure.

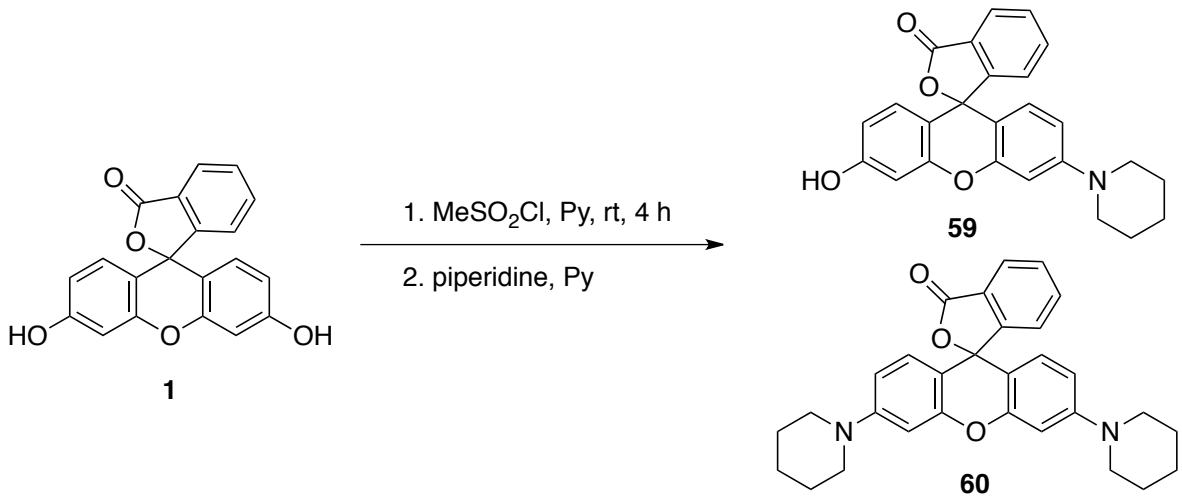
Most of the literature involving substitution on the 3',6'-positions of fluorescein by amines is patented. Basically there are 2 known routes for direct conversion of fluorescein into rhodols and rhodamines. One of the methods is based on $ZnCl_2$-catalyzed direct substitution of 3',6'-halogenated fluoresceins with amines[51, 52, 53] and the second method recently published involves Buchwald-Hartwig amination of mono- or di-triflated fluorescein under the catalysis of palladium-phosphine complex.[49, 56, 55]

Harsh reaction conditions, often resulting in lower yields and therefore high cost of these dyes prompted us to study the mechanism of direct substitution on 3',6'-positions by *N*-nucleophiles in more detail.

The synthetic approach was based on the work of Seidu-Larry.[89] Development of 3',6'-dimesylated fluorescein probe offered possible new ways for incorporation of different substituents on these positions. It was claimed, that 3',6'-di-mesylated fluorescein undergo direct nucleophilic substitution with different amines.

Fluorescein was reacted in pyridine with 0.9 or 4 equivalents of methanesulfonyl chloride depended on the goal of substitution. Mono-mesylated or di-mesylated fluoresceins were generated. After the mesylation reaction was finished, no work-up was applied, but directly the next step was carried out by simply cooling of the reaction mixture to 0 °C and adding an excess of amine. The reactions were performed under an argon atmosphere.

This synthetic method was applied to synthesize mono-piperidyl **59** and di-piperidyl fluorescein **60**. The reaction was carried out with 0.9 or 4 equivalents of methanesulfonyl chloride respectively to obtain **59** or **60** (Scheme 39). In both cases unmanageable mixtures of fluorescein, mono-mesylated fluorescein, di-mesylated fluorescein, and desired mono- or di-piperidyl products were delivered. The column chromatography did not afford pure product, and therefore preparative TLC was employed. Preparative TLC proved existence of both products, but the yield was not established. The products were characterized by MS and NMR.



Scheme 39. Synthesis of piperidyl fluorescein.

As an attempt to simplify the purification of the product, the synthetic approach was altered by a purification step after the mesylation step.

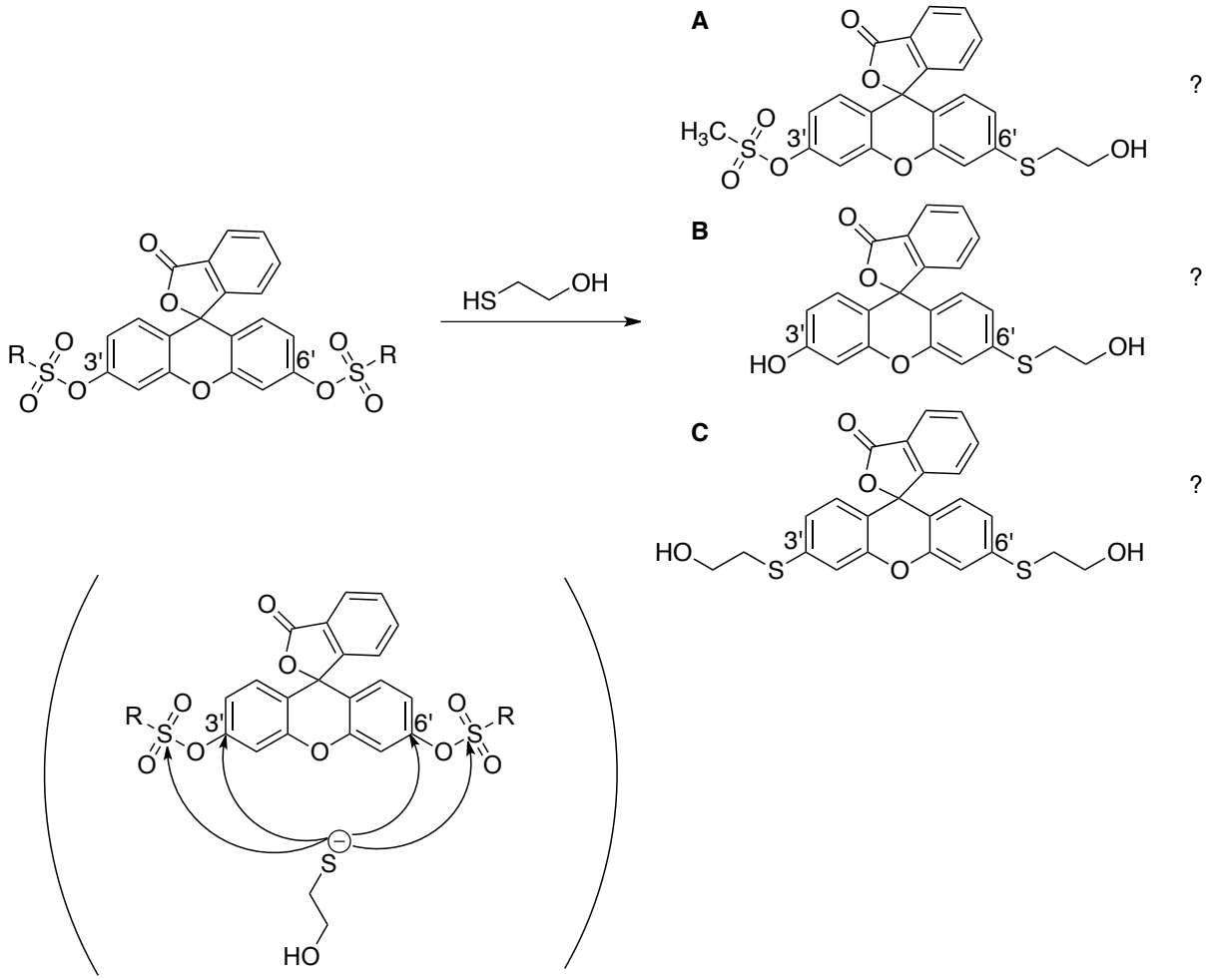
The starting material, di-mesylated fluorescein was suspended in pyridine and upon cooling to 0 °C an excess of amine was added. The reaction was stirred overnight, but no product formation was observed. The mixture was again cooled to 0 °C and an excess of amine was

added. The reaction mixture was stirred for several days and except for decomposition and formation of fluorescein no generation of product was observed. The amines piperidine and jeffamine were used in this study. The reaction was repeated in dry DMF with the same negative results.

It was observed that the reaction was proceeding only in one flask, where the mesylation step was followed by adding at least a 10-fold excess of amine. This type of two-step reaction in one flask delivered difficult mixtures. It is known that sulfonyl groups are generally sensitive to basic conditions and can be spontaneously cleaved off. Handling these conditions in leading to formation of preferable product was not successful. The yield of this reaction was for the above mentioned reasons dramatically decreased.

3.6.3 The Reaction of di-sulfonated Fluorescein with an *S*-nucleophile and Optimization of Reaction Conditions

Conversion of fluorescein and its derivatives into their di-sulfonated reactive intermediates offered interesting targets for nucleophilic aromatic substitution (S_NAr) with different nucleophiles. An initial trial with amines gave low yields but strongly suggested that this kind of substitution is possible. Nucleophilic aromatic substitution on an electron rich fluorescein and its derivatives was not reported so far. It was decided to investigate this type of reaction further with other nucleophiles, such as thiols. Expected pathways of substitution and a formation of potential products are illustrated in scheme 40.



Scheme 40. The substitution of 3',6'-di-sulfonated fluorescein with the *S*-nucleophile (2-mercaptoethanol) and potential products, which could be formed. The structure in brackets illustrates possible attacks of *S*-nucleophile.

S-nucleophiles, particularly 2-mercaptoethanol were tested for the reaction with di-mesylated fluorescein in pyridine and DMF. According to the procedure applied in the reaction with *N*-nucleophiles, di-mesylated fluorescein was dissolved in pyridine or in DMF, the mixture was cooled to 0 °C and 4 equivalents of 2-mercaptoethanol in a small amount of the corresponding solvent were added. After the stirring of the mixtures over the weekend neither product, nor decomposition of starting material appeared.

Various bases were tested for deprotonation and activation of the *S*-nucleophile in 2-mercaptoethanol (Table 1). 4 equivalents of mercaptoethanol were used with the aim to obtain di-substitution. The attempt to use regular organic bases such as Et_3N and DIPEA did not deliver any product, but only the decomposition of di-mesylated fluorescein tok place. The reaction with DMAP was also not successful. Strong, non-nucleophilic bases with bulky, sterically demanding substituents such as DBU and LiHMDS showed formation of a new

product immediately. LiHMDS was not applied further because a high level of decomposition of di-mesylated fluorescein into fluorescein was observed. Inorganic bases such as NaOH and K_2CO_3 showed similar results as with 1 equivalent of DBU and the product started to form immediately if 2-3 equivalents of base were employed. It was observed that the reaction with 1 equivalent of DBU delivered mostly product and the deprotection of starting material was slower. In comparison with DBU, the reaction with inorganic bases required higher amounts of their equivalents and thus decomposition of starting material was accelerated. Since there was no real advantage of inorganic bases such as K_2CO_3 and NaOH to DBU the use of them was not studied further.

Table 1. Optimization of reaction conditions of 3',6'-dimesylated fluorescein with 2-mercaptoethanol. Green check mark means that the formation of the product was observed and the red cross indicates only cleavage of starting material.

2-Mercaptoethanol	Base	Product
4 equiv	Et_3N (1-4 equiv)	✖
4 equiv	DIPEA (1-4 equiv)	✖
4 equiv	K_2CO_3 (1-3 equiv)	✓
4 equiv	NaOH (1-4 equiv)	✓
4 equiv	DMAP (1-4 equiv)	✖
4 equiv	DBU (1-2 equiv)	✓
4 equiv	LiHMDS (1-2 equiv)	✓

The nucleophilic substitution of di-mesylated fluorescein with 2-mercaptoethanol by the use of DBU was further optimized to get better conversion of starting material. Using less equivalents of 2-mercaptoethanol had a negative impact on the speed of the reaction and the yield decreased too. It was observed that with using 0.5 equivalents of DBU the yield was reduced. On the other hand, the use of 2 and more equivalents of DBU accelerated the cleavage of mesylated groups and the formation of fluorescein. For this reason 1 equivalent DBU was applied with 4 equivalents of 2-mercaptoethanol separately in a small amount of dry DMF and the mixture was stirred for10-30 min. Di-mesylated fluorescein dissolved in DMF was cooled to 0 °C and the separately prepared mixture was added dropwise. Within 30 min the product started to form and within 4 hours fluorescein and mono-mesylated fluorescein developed as a result of decomposition of starting material.

The substitution reaction was proceeding selectively only with an *S*-nucleophile. This was proven later by a modification of free hydroxyl group on the 6'-(ethyl)thio linker and also by substitution with Boc-cysteamine.

2-mercaptoethanol possesses two potential functional groups, which can both react as nucleophiles. Deprotonation and activation of the nucleophile can in principle take place on both groups, however even when using more equivalents of the base no indication of an *O*-nucleophilic attack was observed. It is known that the thiolate ion (RS^-) is a soft base, possessing low basicity and a good nucleophilicity and generally it is a better nucleophile than alcoholate (RO^-), and therefore the substitution is less favorable.

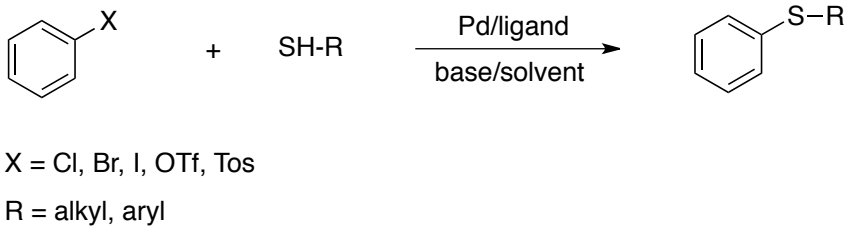
A substitution of fluorescein on the 3'- or 6'-position with an *S*-nucleophile has so far not been reported and consequently substituted xanthene dyes with sulfides on the 3'- or 6'-position are not described in the literature either. This is in big contrast with the literature that covers the syntheses of rhodols and rhodamines, starting from the old fashioned acid-catalyzed condensation of different 3-aminophenols with phthalic anhydride, followed by the direct substitution on the 3'- or 6'-position of fluorescein with *N*-nucleophiles such as the $ZnCl_2$-catalyzed reaction of 3',6'-dichloro or 3',6'-dibromo fluorescein with primary and secondary amines[52, 54] or recently published Pd-catalyzed reactions of 3'-monotriflate or 3',6'-ditriflates of fluorescein with different aliphatic and aromatic amines.[49, 56]

From the synthetic point of view there are two methods for forming an aryl-sulfur bond. Classically the formation of these bonds demands harsh reaction conditions. The development of new carbon-sulfur bonds by a nucleophilic aromatic substitution reaction *via* an addition-elimination mechanism requires electron-deficient aryl halides as leaving groups.

A second method for formation of aryl sulfides described in a literature employs transition-metal catalyst such as Pd (0), Ni (0), and Cu (I) to promote substitution also on electron rich aryl halides.[99, 100, 101] The most reproducible results were obtained by Pd- and Cu-catalyzed cross-coupling of thiols with aryl halides. Metal-catalyzed reactions usually demand suitable ligand and base. The bases often used are K_2CO_3, Na_2CO_3, CsF, KF, K_3PO_4, *i*-Pr_2Net, NaO*t*-Bu or KO*t*-Bu.

Since the preparation of aryl halides was not so straightforward, aryl triflates offered better leaving groups and convenient synthesis from their phenol precursors (Scheme 41).[102] Even aryl tosylate was applied in the reaction with thiolate.[103] It was reported, that the Pd-catalyzed carbon-sulfur bond formation reaction required, in addition of the catalyst, a phosphine ligand such as Xantphos or BINAP, a suitable base and solvent, high reaction temperatures and

usually reflux.[104] The choice of the base is often critical and each reaction might be unique. Also various palladium catalysts and biphosphine ligands were employed to improve oxidative addition of aryl halides and sulfonates and promote reductive elimination of aryl thiols.[103]

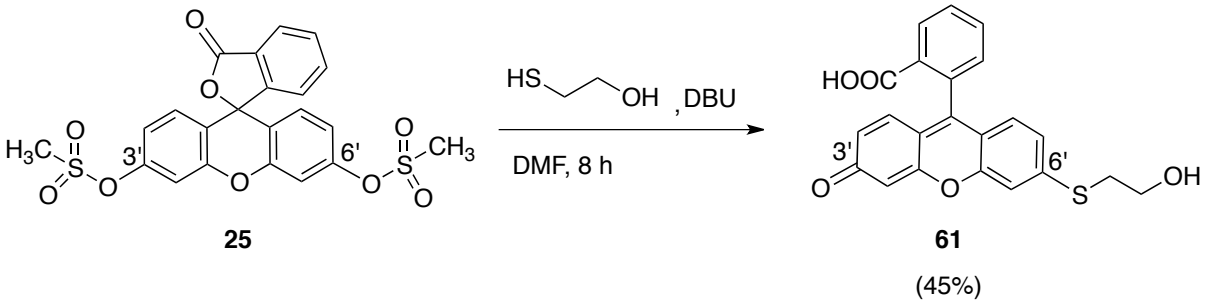


Scheme 41. Pd-catalyzed carbon-sulfur bond formation of aryl thiols.

In 1980 Ono *et al.* introduced the synthesis of sulfides by simply stirring a mixture of alkyl or aryl thiols, DBU, and alkyl halides in benzene for 1-5 h at room temperature. The products were prepared mostly with excellent yields.[105] DBU was also applied as a base for Pd- or Cu-catalyzed reactions, but its applications did not gain wide employment.

Our synthetic strategy to obtain new xanthene derivatives modified on the 3'- or 6'-position with the linker suitable for further modifications was based on the direct substitution of fluorescein sulfonates and its derivatives with *S*-nucleophiles. DBU showed to be an effective base to accelerate this type of reaction. This approach offered convenient, mild and catalyst free reaction conditions and most of all a completely new type of derivatives.

The reaction of di-mesylated fluorescein with 4 equivalents of 2-mercaptoethanol and 1 equivalent of DBU in DMF delivered only one mono-substituted product, particularly 6'-(2-hydroxyethyl)thio xanthene **61** (MK-43) with the yield of 45% (Scheme 42).



25 **61** (45%)

Scheme 42. Synthesis of **61** (MK-43); Reagents and conditions: 2-mercaptoethanol (4 equiv), DBU (1 equiv), dry DMF, Ar, 0 °C, 30 min; rt, 8 h.

In the reaction of di-mesylated fluorescein the product started to form in 30 min and within the reaction time di-mesylated fluorescein was decomposing into mono-mesylated fluorescein and fluorescein. The complete conversion of starting material was not reached even by adding more equivalents of reagents.

Different di-sulfonyl groups of fluorescein were examined as leaving groups with 2-meraptoethanol activated with DBU. Four types of leaving groups with different electron densities were tested for this substitution reaction.

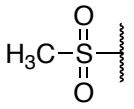
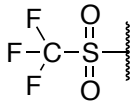
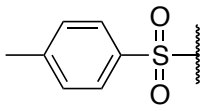
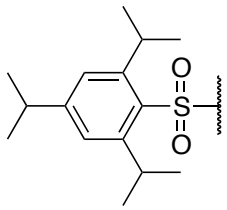
Substitution of di-tosylated fluorescein, di-TPS fluorescein and di-triflate of fluorescein were monitored parallel towards di-mesylated fluorescein.

The reaction with di-tosylated fluorescein proceeded a lot slower than with di-mesylated fluorescein and after 4 h only traces of the product were present. To obtain a better conversion the reaction was elongated overnight, but the conversion remained low, and therefore the yield was not determined.

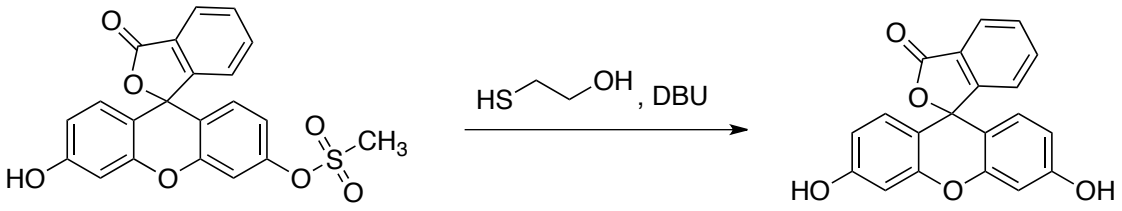
Due to their electron-donating isopropyl groups triisopropylbenzenesulfonyl groups proved to be better leaving groups than the tosyl groups, possessing only one methyl group. The substitution reaction with di-TPS fluorescein delivered slightly higher yields than with di-mesylated fluorescein. Di-triflate of fluorescein, possessing three fluoro groups on the leaving group, showed similar results like di-mesylated fluorescein.

The effects of different sulfonyl leaving groups of fluorescein on the substitution with 2-mercaptoethanol were not significant. The aromatic substitution on fluorescein took place with various sulfonyl groups (Table 2).

Table 2. Comparison of 3',6'-disulfonates of fluorescein.

Leaving group	Reaction time (h)	Yield (%)
	4-8	40-50
	4-8	40-50
	> 4-8	Not determined
	4-8	45-50

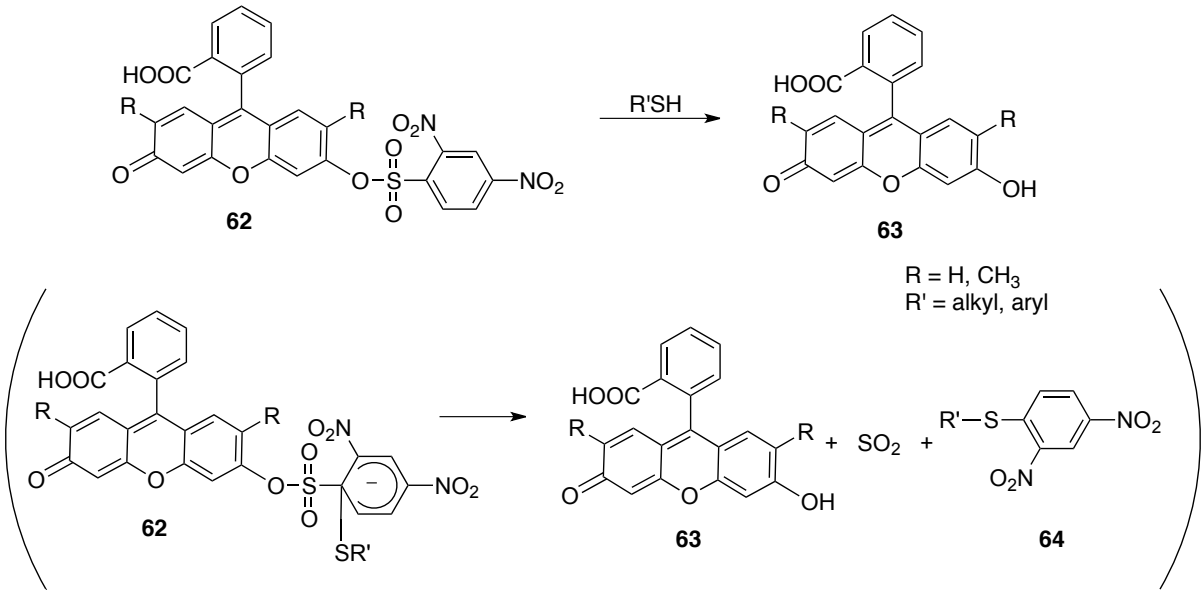
The reaction of mono-sulfonated fluoresceins **30** with 2-mercaptoethanol did not afford any product of substitution. The sulfonyl group was cleaved off and gave rise to fluorescein (Scheme 43). For this reason mono-sulfonated fluorescein and its derivatives were not applied in the synthesis with alkyl thiols.



30 **1**

Scheme 43. Substitution on mono-mesylated fluorescein.

So far substitution on sulfonated fluorescein was reported by *Maeda et al*.[106] Mono-sulfonated fluorescein, 3'- (2,4-dinitrobenzenesulfonyl)-2',7'-dimethyl-fluorescein (**62**) was utilized as a selective thiol probe based on nucleophilic aromatic substitution on the benzene moiety of 2,4-dinitrobenzenesulfonyl group (Scheme 44). **62** was treated with various thiols and after 10 minutes mono-sulfonated fluorescein was fully consumed and (2,4-dinitrophenyl) alkyl- or aryl-sulfide **64** was formed. This type of reaction was tested for the determination of different thiols in aqueous media, through the switching "on" of fluorescence. **62** was also used for thiol- and selenol- determination enzyme assays.[107]



Scheme 44. Probe for thiol-quantification enzyme assays based on nucleophilic aromatic substitution.

The substitution reaction by thiols was taking place selectively on the benzene moiety of the sulfonyl group (96%). No by-products other than PhSSPh (4%) were observed. The fluorescent probe was designed with the 3'-benzenesulfonyl fragment containing two electron-withdrawing nitro groups and with two methyl groups on fluorescein to make the xanthene moiety even more electron richer and thus promote the substitution by thiolate anions on position 1 of the electron poor 2,4-dinitrobenzenesulfonyl group. A substitution on the 3'- or 6'-position or an attack on the sulfur of sulfonyl moiety has not been reported.

Opposing to this, our synthetic strategy was based on a direct conversion of hydroxyl groups on the 3',6'-positions into better leaving groups by sulfonylation (Scheme 45). The chosen sulfonyl groups were mesyl, tosyl, triflate and TPS groups. They contained moderate electron donating sulfonyl groups and thus facilitated the aromatic substitution on the xanthene fragment. No substitution on tosylate or TPS was observed.

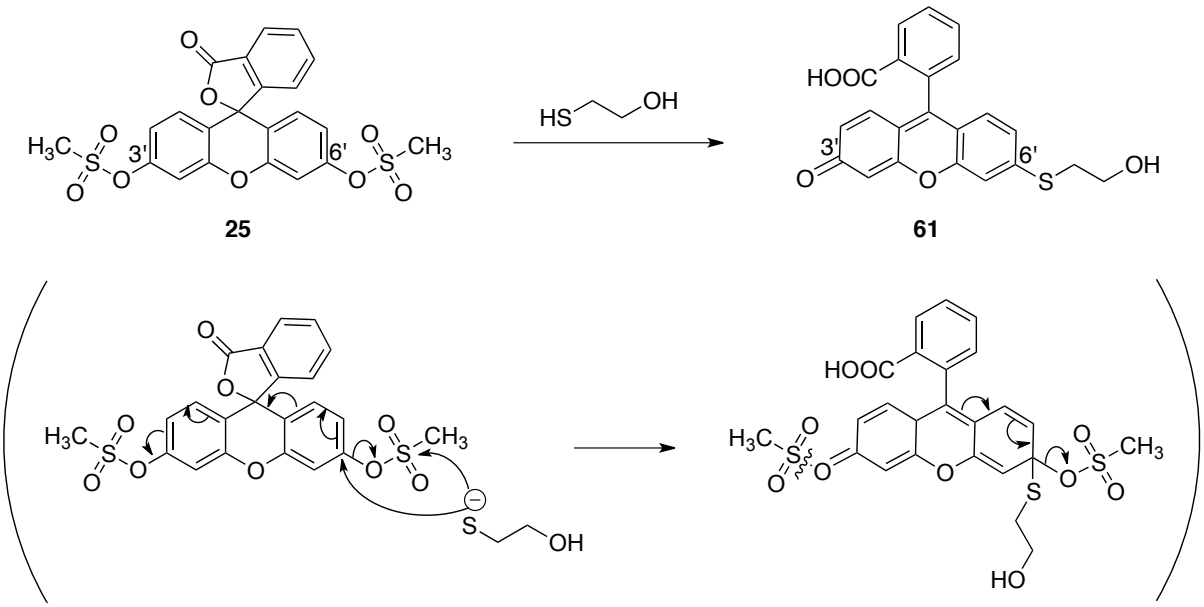
Di-sulfonated fluorescein was required for some sort of addition-elimination mechanism, which was taking place with an *S*-nucleophile on the 6'-position of the xanthene fluorophore (Scheme 45). The standard mechanism of the nucleophilic aromatic substitution involves an addition of a nucleophile followed by elimination of the leaving group – the addition-elimination mechanism.[108]

It is highly expected that the second mesyl group plays an important role in the mechanism of a nucleophilic substitution and it is cleaved off during the reaction. The generation of the

conjugated system upon the attack with the *S*-nucleophile causes formation of a ketone on the 3'-position and might pull out the mesyl group (Scheme 45).

An *S*-nucleophile can attack the xanthene moiety on the 6'-position or on the sulfur of the methanesulfonyl group. The nucleophilic attack on sulfur could lead to a cleavage of the methanesulfonyl group and therefore to the formation of by-products, such as mono-mesylated fluorescein and fluorescein. The formation of these by-products was observed.

On the other hand the formation of a methanesulfonothioate as a by-product of the attack of *S*-nucleophile on the sulfur of the methanesulfonyl group was not detected.



Scheme 45. Proposed mechanism of aromatic nucleophilic substitution of di-mesylated fluorescein with *S*-nucleophile.

3.6.4 Reactions of Derivatives of Fluorescein with 2-Mercaptoethanol

An impact of substituents such as chlorine and bromine on substitution with *S*-nucleophile was investigated. Chlorine and bromine were chosen for their electron-withdrawing properties with the aim to improve a mechanism of the nucleophilic substitution on fluorescein.

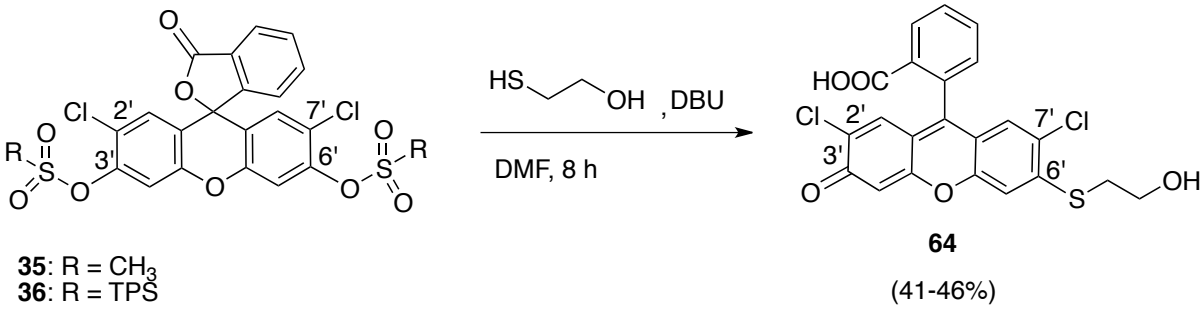
According to the optimized procedure with 3',6'-di-mesylated fluorescein, di-mesylated 2',7'-dichlorofluorescein, 4',5'-dibromofluorescein and 2',4',5'-tribromofluorescein were used. The reactions of these mesylated derivatives were monitored towards standard 3',6'-di-mesylated fluorescein. The optimized procedure consisted of dilution of di-mesylated starting material in dry DMF under an argon atmosphere and cooling to 0 °C. 4 equivalents of mercaptoethanol in dry DMF were agitated with 1 equivalent of DBU for 30 min and afterwards added dropwise

to the di-mesylated fluorescein derivatives. Then the reaction was allowed to warm up to room temperature. These reaction conditions were used to obtain asymmetric 6'-(2-hydroxyethyl)thio-xanthene derivatives.

The reactions of di-mesylated fluorescein and di-mesylated 2',7'-dichlorofluorescein **35** with 2-mercaptoethanol were monitored in parallel. The formation of the product was first observed at 30 min. The rate of hydrolysis in the form of cleavage of the methanesulfonyl groups was visibly increased in the reaction with **35**. The reaction and also the cleavage of leaving groups were accelerated due to the electron-withdrawing effects of chlorine groups on nucleophilic substitution with 2-mercaptoethanol. The reaction time sufficient to obtain product remained between 4-8 h. Elongating of the reaction time to overnight or adding extra equivalents of deprotonated 2-mercaptoethanol did not increase the conversion of starting material neither the final yield. The product **64** was obtained with the yield of 41% (Scheme 46).

The incorporation of chlorine groups did not enhance the conversion of starting material. The yield was comparable with the substitution of di-mesylated fluorescein.

When 3',6'-di-TPS-2',7'-dichlorofluorescein **36** was applied, the same pattern but slightly higher yield of the product **64** (46%) was delivered (Scheme 46).



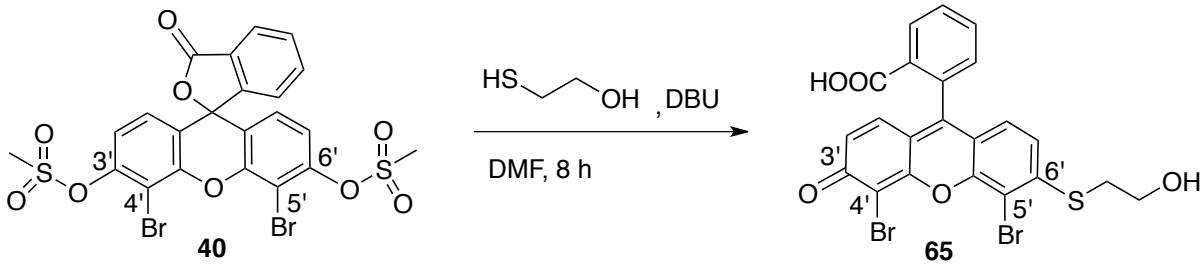
35: R = CH_3
36: R = TPS

64
(41-46%)

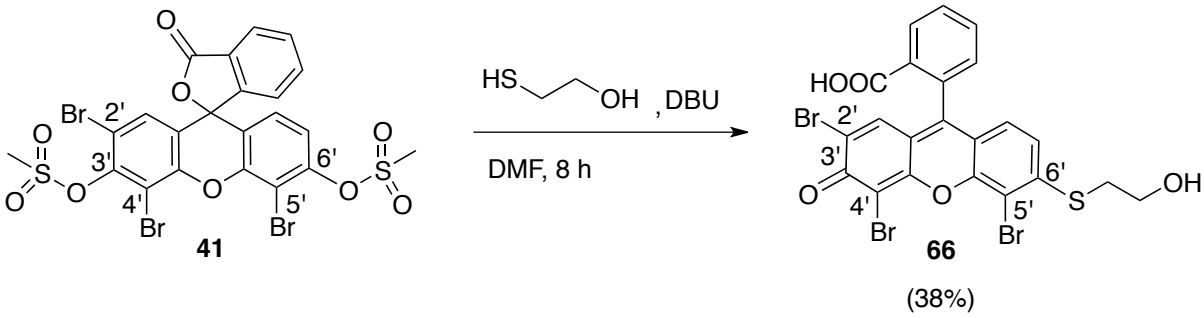
Scheme 46. Synthesis of 2',7'-dichloro-6'-(2-hydroxyethyl)thio-xanthene derivative **64** (MK-67); Reagents and conditions: 2-mercaptoethanol (4 equiv), DBU (1 equiv), dry DMF, Ar, 0 °C, 30 min; rt, 8 h.

Due to their electron-withdrawing character bromine substituents, just like chlorines, were intended to improve nucleophilic aromatic substitution on fluorescein. 3',6'-di-mesylated 4',5'-dibromofluorescein **40** and 2',4',5'-tribromofluorescein **41** reacted with 2-mercaptoethanol according to the standardized procedure in a similar manner as 2',7'-dichlorofluorescein. Bromine groups, due to their electron-withdrawing effects, accelerated the reaction but also

the cleavage of mesylated groups and formation of unreactive mono-mesylated by-product. The reaction time was about 8 h up to overnight. The reaction afforded dibromo product **65** (MK-74-1) with the yield of 53% (Scheme 47) and tribromo product **66** (MK-74-2) with the yield of 38% (Scheme 48). The yields of substitution were comparable with non-substituted fluorescein.



Scheme 47. Synthesis of 4',5'-dibromo-6'-(2-hydroxyethyl)thio-xanthene derivative **65** (MK-74-1); Reagents and conditions: 2-mercaptoethanol (4 equiv), DBU (1 equiv), dry DMF, Ar, 0 °C, 30 min; rt, 8 h up to ON.



Scheme 48. Synthesis of 2',4',5'-tribromo-6'-(2-hydroxyethyl)thio-xanthene derivative **66** (MK-74-2); Reagents and conditions: 2-mercaptoethanol (4 equiv), DBU (1 equiv), dry DMF, Ar, 0 °C, 30 min; rt, 8 h up to ON.

The structure of **65** (MK-74-1) possessing two bromine groups on the 4',5'-positions was confirmed by ^{1}H NMR. Four protons on the xanthene moiety of **65** namely H-2', H-7', H-1' and H-8' were split into doublets with corresponding ortho coupling constants, ^{3}J = 8.8 Hz, 8.4 Hz. H-2', H-7' were shifted to the higher frequencies due to the neighboring electron-accepting substituents (Figure 11).

In ^{1}H NMR 2',4',5'-tribromo derivative **66** (MK-74-2) had two protons on the xanthene moiety, which split into two doublets with corresponding ortho coupling constants, ^{3}J = 8.6

Hz and one proton with corresponding singlet (Figure 12). The ^{1}H NMR spectrum was obtained in CD_3OD.

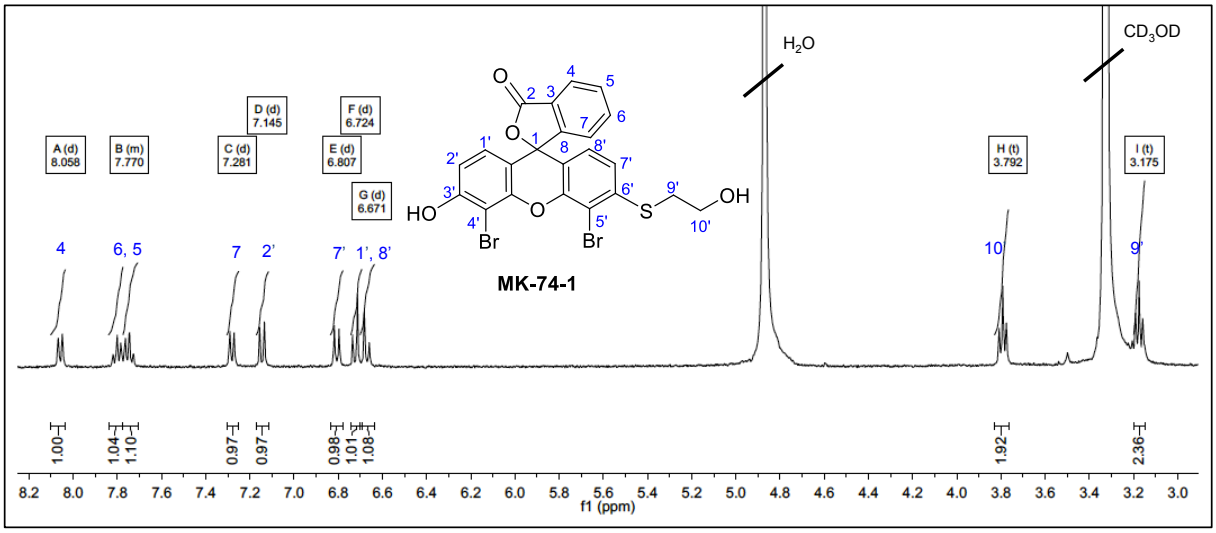


Figure 11. ^{1}H NMR of MK-74-1.

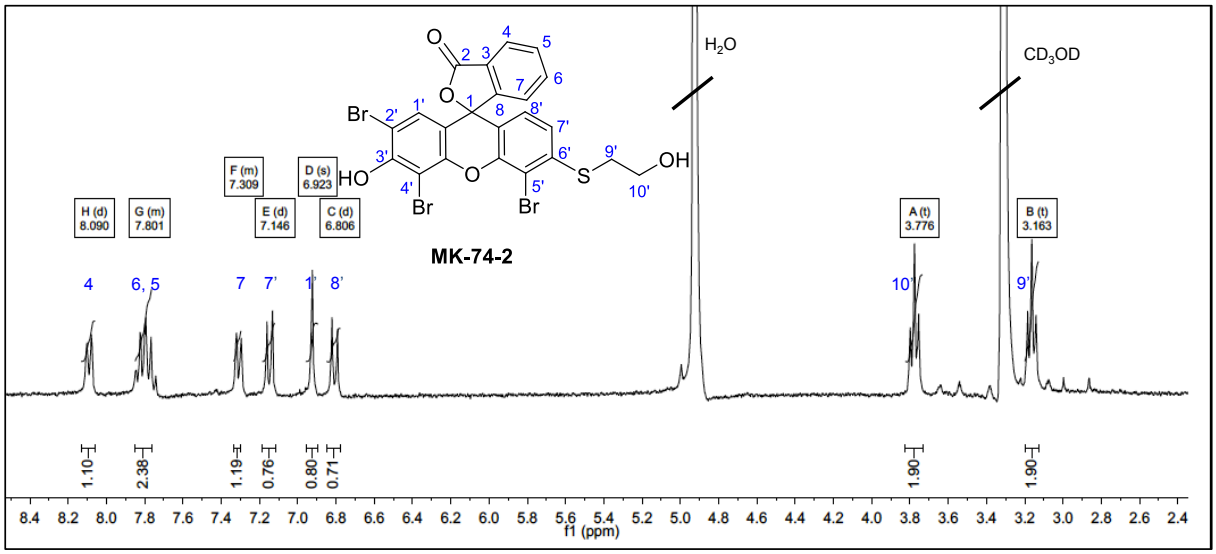
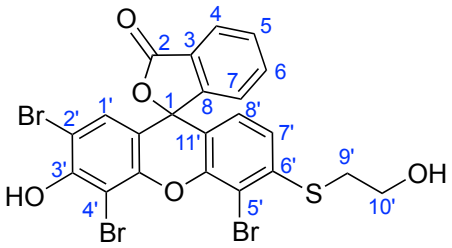


Figure 12. ^{1}H NMR of MK-74-2.

To distinguish whether the substitution reaction took place on the 3' or 6'-position of the xanthene moiety in MK-74-2, two-dimensional carbon-proton shift correlation *via* long-range C-H coupling was applied. In particular, the heteronuclear multiple bond correlation (HMBC), which indicates long-range C-H couplings mostly 2- or 3-bond couplings ($^{2}J_{CH}$ and $^{3}J_{CH}$) and $^{1}J_{CH}$ coupling is usually suppressed, was employed (Figure 13). In HMBC, a ^{1}H NMR is displayed in the f2 dimension and a 13C NMR in the f1 dimension.

It was observed that three protons namely H-1', H-7' and H-8' corresponding to two doublets and one singlet in CD_3OD (Figure 11) were displayed as two doublets in DMSO (Figure 12). In the HMBC spectrum, grey lines show correlations from proton H-7' to carbons C-11' and C-5' (3-bond couplings). Green lines show correlations from two protons H-1' and H-8' to carbons C-3' and C-6' (all are 3-bond couplings). These results confirmed that the substitution with 2-mercaptoethanol proceeded on the 6'-position of the xanthene moiety.



MK-74-2

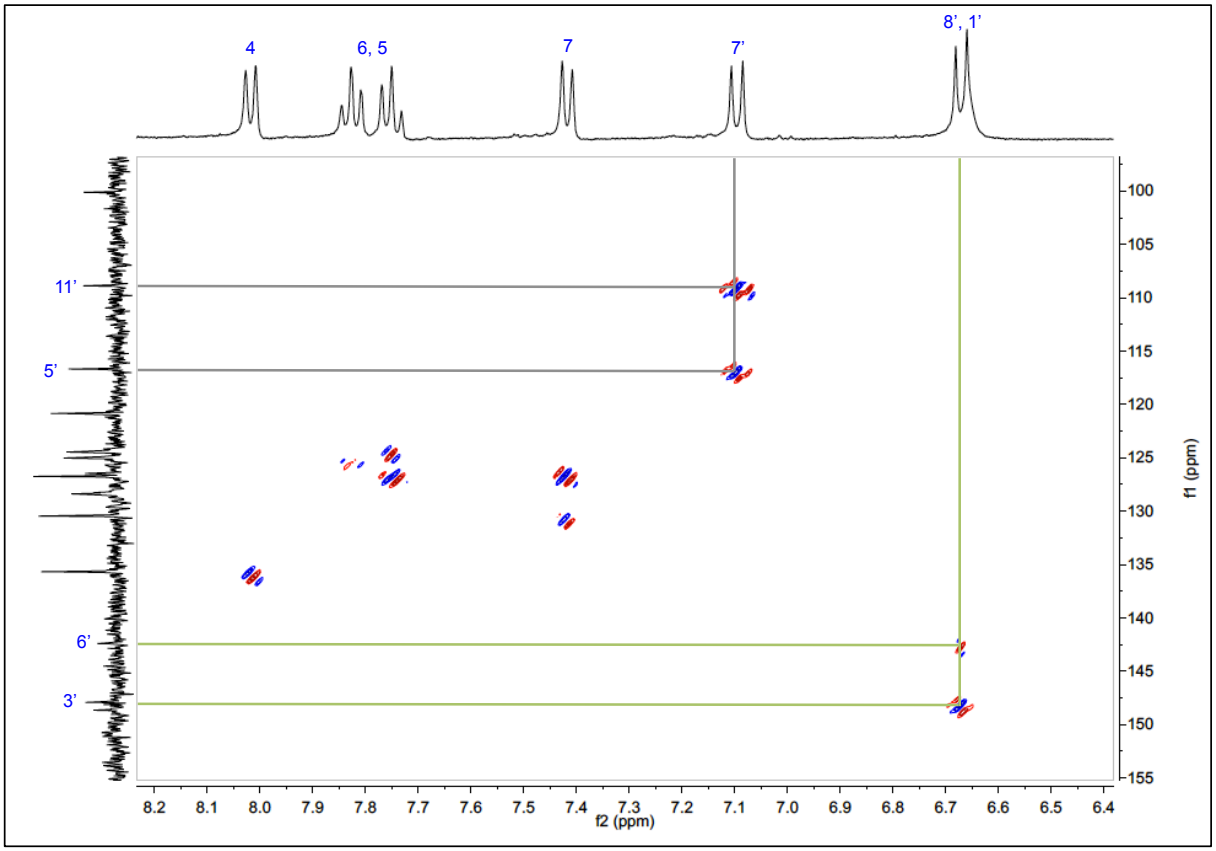


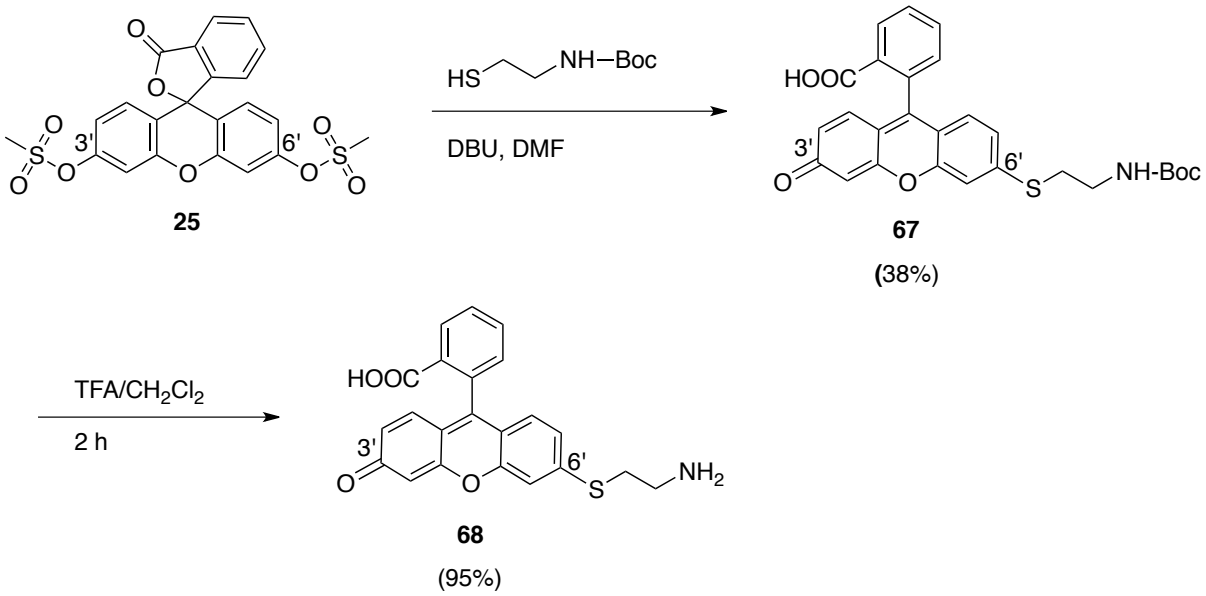
Figure 13. HMBC experiment of MK-74-2.

3.6.5 Reaction of 3',6'-dimesylated Fluorescein with Boc-cysteamine

To illustrate the usefulness of the reaction of *S*-nucleophiles with fluorescein and the formation of new xanthene sulfides, another *S*-nucleophile with a linker bearing a Boc-

protected amino group was employed. The standard method, based on the activation of the nucleophile in Boc-cysteamine with DBU in dry DMF, followed by the addition of the mixture into cooled di-mesylated fluorescein **25** in DMF and stirring at rt overnight, delivered the product **67** with the yield of 38%. Removal of the Boc-protective group, followed by precipitation delivered the product **68** with the yield of 95% (Scheme 49).

A new xanthene derivative **68** was prepared in a 3-step synthesis with the overall yield of 33%.



25 **67** (38%) **68** (95%)

Scheme 49. Synthesis of 6-(2-aminoethyl)thio-3-oxo-3H-xanthen-9-yl)benzoic acid **68** (MK-69); Reagents and conditions: a) Boc-cysteamine (4 equiv), DBU (1 equiv), dry DMF, Ar, 0 °C, 30 min; rt, ON; b) 1:5 CH_2Cl_2/TFA, 2h, rt.

Fluorescein, bearing free amino group on the aliphatic linker can be used for coupling reactions with carboxylic acid, aldehyde or ketone containing biomolecules. Further modification of the aliphatic amino group, for example with *N,N'*-disuccinimidyl carbonate can afford succinimidyl ester and turn this compound into an amine-reactive fluorescent derivative.

3.7 Modification of 6'-(2-Hydroxyethyl)thio-linker on Xanthene Derivatives

In order to exploit the well-developed reaction with 2-mercaptoethanol, we made use of the free aliphatic hydroxyl group, which was transformed into a standard bioconjugate group.

Our aim was to synthesize asymmetric xanthene dyes, bearing an azido functionality, which could be utilized for the azide-alkyne "click" chemistry with terminal alkyne labeled oligonucleotides.

Previous attempts to directly substitute sulfonated fluoresceins with NaN_3 were not successful. The azidation of di-mesylated fluorescein was not confirmed and only mono-mesylated fluorescein and fluorescein as by-products of cleavage of starting material developed. The azidation reaction of mono-mesylated fluorescein delivered only fluorescein as a matter of decomposition.

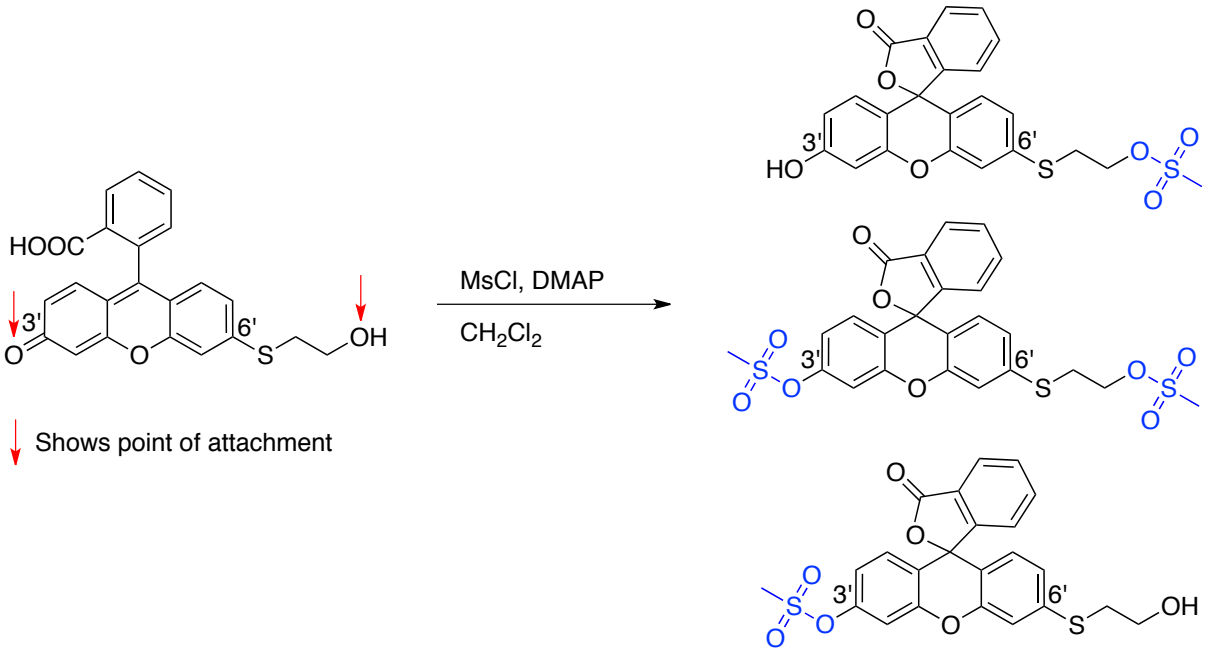
The azide placed directly on the 3'- or 6'-position was reported in literature[82] , where *t*-Boc-rhodamine 110 with the free amino group was transformed to the diazo derivative by the treatment with $NaNO_2$, and the diazo group was replaced with an azido group by addition of NaN_3.

Since our method for the synthesis of aromatic azides of fluorescein did not proceed, we made use of the substitution with an *S*-nucleophile to obtain a group for further transformation into an azide.

3.7.1 Preparation of 6'-(2-Azidoethyl)thio-xanthene Derivatives

New asymmetric xanthene derivatives, carrying a 6'-(2-hydroxyethyl)thio-linker were reacted with methanesulfonyl chloride to obtain a better leaving group to undergo the azidation reaction.

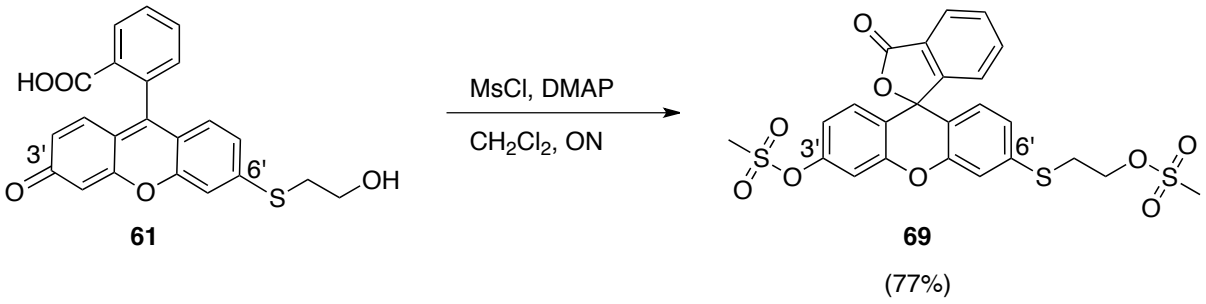
There were two possible places, where the sulfonylation could take place and those were the 3'-hydroxy group and 2-hydroxyethyl group of xanthene fluorophore (Scheme 50). The course to direct the mesylation mostly on 2-hydroxyethyl group was not achievable. Since the mesyl group on the 3'-position should be inert towards reaction with NaN_3, the reaction conditions were tuned to obtain mesyl groups on both positions. The mesylation reaction was carried out according to optimized conditions using 4 equivalents of methanesylfonyl chloride and 6 equivalents of DMAP in CH_2Cl_2 to yield mostly di-mesylated product.



Scheme 50. Possible incorporation of mesyl group.

61 (MK-43) and 6 equivalents of DMAP were suspended in dry CH_2Cl_2 and stirred for 30 min, the reaction mixture was cooled to 0 °C and 4 equivalents of methanesulfonyl chloride in dry CH_2Cl_2 were added. The mixture was stirred at rt overnight.

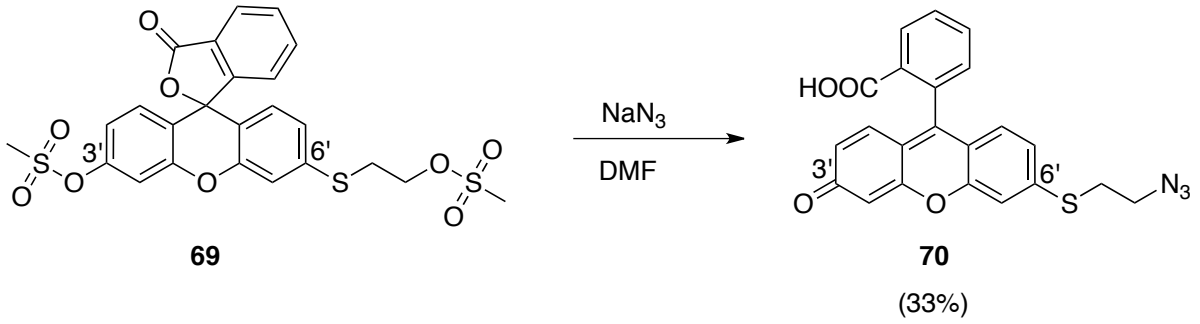
In comparison with the standard mesylation reaction of fluorescein, the reaction time was elongated to facilitate formation of the methanesulfonyl group also on the 2-ethylthio linker. The di-mesylated product **69** was obtained with the yield of 77% (Scheme 51).



Scheme 51. Mesylation of **61** (MK-43); Reagents and conditions: a) MsCl (4 equiv), DMAP (6 equiv), dry DCM, Ar, 0 °C, 30 min; rt, ON.

The azidation reaction was carried out according to literature used for the synthesis of azido Tokyo Green[97] with 5 equivalents of NaN_3 in dry DMF with an elongated reaction time (Scheme 52). The reaction mixture was heated to 60 °C and stirred for 52 h. The purification by column chromatography followed by preparative TLC delivered the product **70** (MK-61)

with the yield of 33%. The azido functionality was attached only on the aliphatic chain and the 3'-methanesulfonyl group was cleaved off. The azido xanthene derivative **70** (MK-61) was prepared in a 4-step synthesis with the total yield of 13.4%.



69 **70**

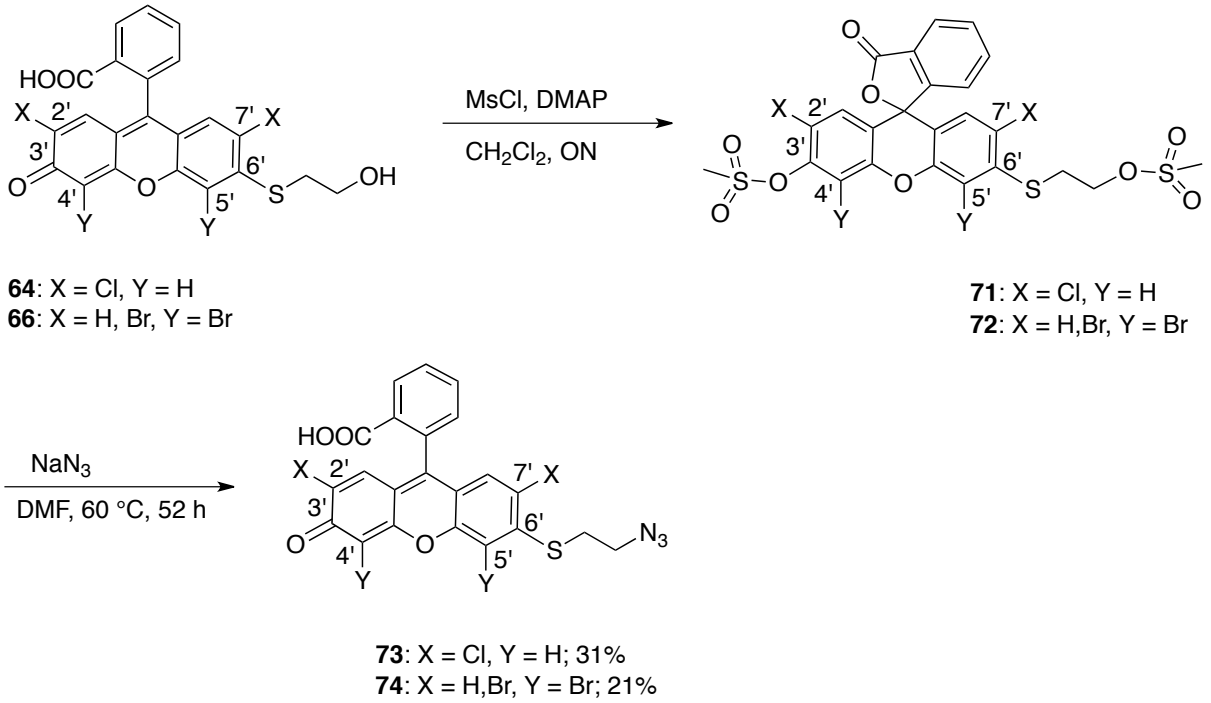
(33%)

Scheme 52. Synthesis of 6'-((2-azidoethyl)thio)-xanthene derivative**70** (MK-61); Reagents and conditions: a) NaN_3 (5 equiv), dry DMF, Ar, 60 °C, 52 h.

The mesylation of **64** (MK-67) (Scheme 53) was carried out according to the above mentioned optimized procedure. TLC showed mostly one non-fluorescent product and therefore the crude product was used in the next reaction step without further purification.

The azidation step (Scheme 53) was performed according to the above mentioned procedure applied with **70.** After 52 h of stirring and heating at 60 °C, starting material and 2 new fluorescent spots, corresponding to **64** and the product were present. The previous starting material was generated, which means the mesylation did not fully occur on the aliphatic hydroxyl group. This observation suggested that the mesylation proceed faster on the aromatic moiety as compared to the aliphatic chain. The product **73** (MK-75) was obtained with the yield of 31% and with the total yield of a 4-step synthesis of 10.4%.

The synthesis of the tribrominated xanthene azide **74** (MK-83) was carried out with **66** (MK-74-2) according to the optimized procedure for mesylation and azidation (Scheme 53). The azido product was obtained **74** with the yield of 19%. The total yield of a 4-step synthesis was 1%. The total yield was decreased due to the first synthetic step consisting of the mesylation reaction of 4',5',7'-tribromofluorescein obtained from different commercial sources in the mixture of 4',5'-di-bromofluorescein, 4'-monobromofluorescein and 4',5',7'-tribromo fluorescein derivatives and therefore demanding purification.



64: X = Cl, Y = H
66: X = H, Br, Y = Br

71: X = Cl, Y = H
72: X = H,Br, Y = Br

73: X = Cl, Y = H; 31%
74: X = H,Br, Y = Br; 21%

Scheme 53. Synthesis of 6'-(2-azidoethyl)thio-xanthene derivatives **73** (MK-75), **74** (MK-83).

It is known that organic azides, particularly incorporated onto the aliphatic chains, are exceptionally stable toward the common reactive chemicals which range from dioxygen and water to the aqueous solutions of highly functionalized organic molecules, which make up living cells and therefore the new xanthene azides could be stable fluorescent targets in biolabeling applications.[109]

3.8 Fluorescent Properties of New Xanthene Dyes

An impact of the substitution with *S*-nucleophiles on the 6'-position of the xanthene moiety on its fluorescent characteristics such as absorption (A), extinction coefficient (ε) at the maximum absorption wavelength (λ_{max}), excitation (λ_{ex}), emission (λ_{em}), quantum yield (Φ) and photostability was investigated and the obtained data are included in the tables 3 and 4.
New asymmetric xanthene dyes, whose photophysical properties were characterized, are included in figure 14.

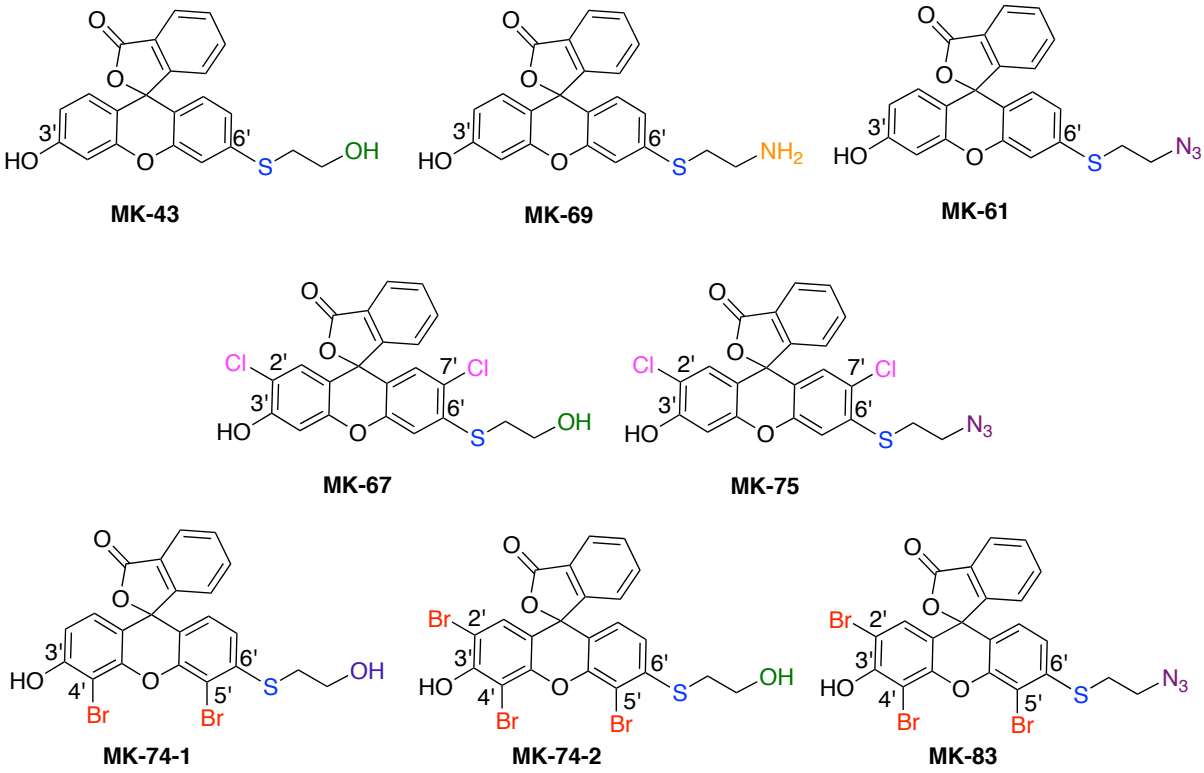


Figure 14. Structures of new asymmetric xanthene dyes.

Table 3. Spectral data of new xanthene dyes in Et_3NHOAc buffer, pH 7.3.

Compound	λ_{max} (nm)	ε at λ_{max} (M^{-1} cm^{-1})	λ_{ex} (nm)	λ_{em} (nm)	Φ
MK-43	467, 494	26 000	465, 491	527, 558	0.22
MK-69	464, 489	20 200	465, 490	528	0.20
MK-61	466, 493	28 500	470, 490	527, 558	0.24
MK-67	473, 503	26 500	477, 503	533	0.17
MK-75	473, 502	10 200	348, 501	533	0.16
MK-74-1	480	11 600	486	570	-
MK-74-2	484, 509	7 400	488, 511	564	-
MK-83	484, 509	7 500	490, 510	566	-

Table 4. Spectral data of new xanthene dyes in 1 mM NaOH.

Compound	λ_{max} (nm)	ε at λ_{max} (M^{-1} cm^{-1})	λ_{ex} (nm)	λ_{em} (nm)	Φ
MK-43	466, 495	34 000	465, 495	526, 560	0.22
MK-69	466, 495	25 000	465, 496	526, 562	0.20
MK-61	465, 495	38 000	497	527, 556	0.24
MK-67	471, 502	27 000	472, 502	531, 568	0.17
MK-75	470, 502	12 000	474, 504	533, 565	0.16
MK-74-1	473	13 100	514	538	-
MK-74-2	481, 511	4 500	516	554, 588	-
MK-83	480, 511	6 300	485, 511	554, 584	-

3.8.1 Determination of Absorption Maxima and Extinction Coefficients

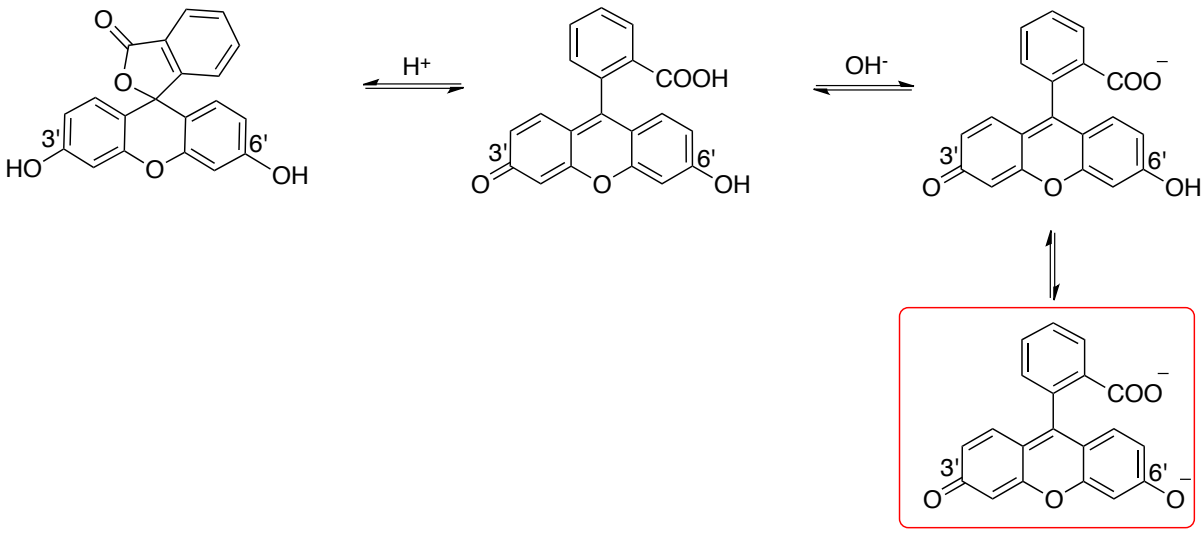
The extinction coefficient, also known as the molar absorption coefficient defines how strongly a substance of a certain concentration absorbs light at a given wavelength. The extinction coefficients of fluorescent dyes are determined at the maximum absorption wavelength (λ_{max}).

UV absorbance of the dyes was measured in acidic medium (0.1 M HCl), in neutral medium (NH_4OAc 0.1 M), in basic medium (0.1 M NaOH) and in Et_3NHOAc buffer (10 mM), pH 7.3 at 25 °C. For the measurements in acidic and neutral medium 50 μM solutions of dyes were used, which was 5 times more than the concentrations used in buffer and basic medium (10 μM). The absorption in acidic medium was usually under 0.05, which is extremely low. One exception to this general observation was MK-61, which showed the highest absorption maxima. In neutral medium the absorption maxima of dyes were increased but usually did not reach absorption of 0.1. The measurements of dye solutions in buffer pH 7.3 and in basic medium exhibited potent absorption over 0.1.

Fluorescein-based dyes and xanthene dyes can exist in aqueous solutions in cationic, neutral, anionic and dianionic forms, making them strongly pH dependent.[31] Our observations confirmed the general trend, where the dyes were fully protonated under acidic pH, therefore exhibited very low absorption. Under neutral pH the absorption of dyes slightly increased but still remained very low. Increase of pH from neutral to 7.3 in buffer produced significantly higher absorption. This contributed to the fact that some quantities of the dyes underwent from the "closed" lactone form to the "open" form, and the conjugated system formed. It is known that under physiological conditions (pH 7.4) a considerable population is still in the

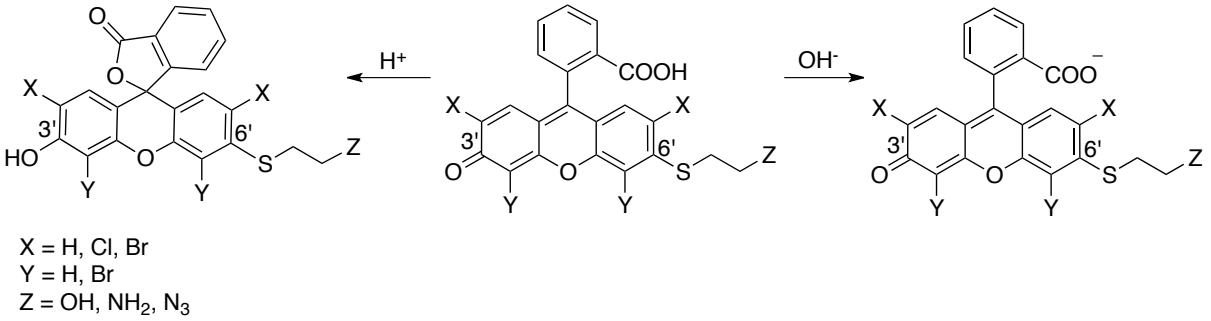
protonated, non-fluorescent form.[29] In basic medium (pH ≈ 8) the modest enhancement of absorption of the examined dyes was detected. Measuring the dyes at higher pH (9-10) might increase their absorptions, but from the application point of view it is more or less useless. The dyes were synthesized for conjugation with biomolecules at physiological pH.

Under alkaline conditions unsubstituted fluorescein is usually deprotonated. The chromophore system, consisting of benzene rings, is extended by additional n-molecular orbitals. The photon of energy that is needed for an electron to be excited from highest occupied molecular orbital (HOMO) to lowest unoccupied molecular orbital (LUMO) decreases and the wavelength is shifted to a range of longer wavelengths. The dianionic form causes extension of a mesomeric system and increase of the wavelength called bathochromic shift. The protonation causes reduction of the mesomeric system and movement to lower wavelengths known as a hypsochromic shift. The monoanionic form usually occurs in equilibrium with the dianionic form. The increase of pH leads predominantly to the formation of the highly fluorescent dianion. This phenomena is in the shorten version illustrated in scheme 54.



Scheme 54. Impact of pH on the structure of fluorescein.

Xanthene dyes containing an ethylthio-linker can presumably form conjugated systems at the physiological pH 7.4 and the free carboxylic acid can form a carboxylate anion (Scheme 55).



X = H, Cl, Br
Y = H, Br
Z = OH, NH_2, N_3

Scheme 55. Impact of pH on the structure of new xanthene derivatives.

The photophysical properties of new xanthene derivatives possessing the 6'-(2-hydroxyethyl)thio-linker, 6'-(2-azidoethyl)thio-linker and 6'-(2-aminoethyl)thio-linker in basic medium (0.1 M NaOH in ethanol, pH ≈ 8) and in Et_3NHOAc buffer (10 mM), pH 7.3 were characterized.

The extinction coefficients were calculated from the absorption at λ_{max}. Since every substance had two absorption maxima, an extinction coefficient was calculated for the λ_{max}, which is the wavelength of maximum absorption. This corresponds to the first value in the column for λ_{max} in tables 3 and 4. Frequently two absorption maxima were observed, which were very similar and separated by about 30 nm. Xanthene dyes displayed slightly higher extinction coefficients in a basic medium. In comparison with unsubstituted fluorescein, the extinction coefficients of new fluorophores were lower. Fluorescein under physiological conditions exists in the equilibrium between its less fluorescent monoanionic (ε = 29 000-32 600 M^{-1} cm^{-1}) and strongly fluorescent dianionic (ε = 76 900-87 600 M^{-1} cm^{-1}) form.[32] Substitution of the hydroxyl group on the 6'-position of the xanthene moiety of fluorescein with *S*-nucleophile causes that n-electrons of the heteroatom are no longer available to the chromophore system. This might be a reason for decrease of extinction coefficients of the new fluorophores (Table 3 and 4).

Interestingly, the absorption maxima at different pH-values displayed almost no shifts towards shorter or longer wavelengths. This might contribute to the fact that the formation of anion on the xanthene moiety is not possible and the increase of absorbance is only pH dependent, where the conjugated system is formed and the carboxylate anion simply extends the mesomeric system.

The extinction coefficients of di-brominated and tri-brominated fluorophores were significantly lower than other dyes, substituted with an ethylthio-linker. The effect of halogenation of new xanthene dyes has produced bathochromic shifts of λ_{max} ≈ 5 nm in chlorinated dyes and ≈15 nm in brominated dyes. This phenomena is known from the

literature, where the number of halogenated groups can be quantitatively determined from the bathochromic shifts of the substituted dyes.[41]

3.8.2 Fluorescent Excitation and Emission Spectra

Fluorescent excitation and emission spectra were measured in basic medium and in Et_3NHOAc buffer (pH 7.3). Just like the absorption spectra, the excitation spectra have two maxima in both the buffer and in basic medium. The emission spectra, as mirror images of the excitation spectra shifted to the higher wavelengths due to the Stoke's shift, also possessed two emission maxima. The second maximum in emission spectra was difficult to recognize in the buffer system (Table 3 and 4).

Fluorescein and its derivatives displayed excitation maxima 490-500 nm and emission maxima 510-520 nm from physiological pH (7.4) to basic pH (9). Upon the incorporation of an ethylthio linker on the 6'-position of the xanthene moiety, these new asymmetric dyes - particularly MK-43, MK-69, MK-61- exhibited at pH 7.3 and pH ≈ 8 in excitation spectra an extra double-peak 465 nm and 490-495 nm. The emission spectra contained also a double-peak ≈ 525 and 560 nm.

The emission and excitation spectra of the chlorinated xanthene dyes (MK-67, MK75) were red shifted by about ≈5 nm in comparison with MK-43, MK-69, MK-61, which did not contain any halogen groups. The excitation maxima were at pH 7.3 and pH ≈ 8, approximately 472-477 nm and 502-504 nm and the emission maxima 533 nm and 565-568 nm (Table 3 and 4).

The asymmetric di-brominated and tri-brominated xanthene dyes, namely MK-74-1, MK-74-2, MK-83, were ≈15 nm red shifted in comparison with non-halogenated xanthene derivatives. The corresponding excitation and emission maxima are listed in tables 3 and 4.

The halogenation of the xanthene dyes caused a bathochromic shift towards higher wavelengths.

3.8.3 Determination of Quantum Yields

The fluorescent quantum yield (Φ) defines the ratio of photons absorbed to photons emitted through fluorescence rather than by another non-radiative mechanism as it was already laid out in the introduction 1.1. The maximum fluorescent quantum yield is 1.0 (100%), but even substances with $\Phi = 0.1$ are considered quite fluorescent.[5]

The quantum yields of xanthene dyes were determined by using diluted samples (A < 0.1) in 0.1 M NaOH. These values were obtained by the comparison of the integrated area of the emission spectrum of the samples with that of fluorescein in 0.1 M NaOH, which has a quantum efficiency of 0.95 ± 0.03.[32] The concentration of the sample was adjusted to match the absorbance of the fluorescein reference at the excitation wavelength (in details described in 5.9.4 Determination of Quantum Yields).

The quantum yields of new asymmetric xanthene dyes were for non-chlorinated fluorophores (MK-69, MK-43, MK-61) 0.2-0.24 and for chlorinated fluorophores (MK-67, MK-75) 0.17 and 0.16. The quantum yields for brominated fluorophores were not determined because at a given concentrations the fluorescent emissions were extremely low. It is known that the substitution of aromatic molecules by bromine results in a higher population of triplet state and the derivatives undergo intersystem crossing after light absorption.[110, 111] This can lead to fluorescence quenching also known as the internal heavy atom effect.

There is no literature known which would study an impact of the substitution on the 3'- or 6'-position of the xanthene moiety of fluorescein on its quantum yield. It is mostly because commercially available xanthene dyes have the bioconjugate group attached on the benzene moiety, and thus the 3',6'-positions stay free to form conjugated system and the highly fluorescent dianion. Sometimes the introduction of biomolecule on the benzene moiety of fluorescein dye can lead to diminish of fluorescence or even complete quenching. This might be because of fluorescent quenching based on PeT (photoinduced electron transfer), where the benzene moiety functions as electron donor and the xanthene moiety as electron acceptor. Introducing spacer between the fluorophore and the biomolecule to which it is conjugated, can potentially reduced the quenching.[6] Our new synthetic dyes should display the same fluorescent properties upon the incorporation into the biomolecule due to the standard bioconjugate group attach to the linker on the 6'-position. Since there are no modifications on the benzene moiety, PeT should play no significant role on their quantum yields.

A decrease of quantum yields upon the substitution on the 3' or 6'-position is disputable. The substitution causes that the higly fluorescent dianionic form cannot be obtained. This might be one of the reasons for diminishing the quantum yields.

On the other hand, methyl ether ester of fluorescein, where both hydroxyl groups are modified, is known to be highly fluorescent.[112]

Since these new fluorescent dyes are asymmetric they can be compared with rhodols. It is known that rhodols quantum yields are usually decreasing with the length of the linker

incorporated on the nitrogen atom. Decrease in quantum yields was observed also with fluorescein ethers.[83, 98]

These observations suggest that the effect of substitution on the quantum yield is rather complicated and different aspects should be considered.

3.8.4 Photobleaching Studies

Photobleaching is a dynamic process by which a fluorophore undergoes a photoinduced chemical destruction upon exposure to light and thus loses its ability to fluoresce.

Fluorescein is known for its relatively high rate of photobleaching. The photobleaching of fluorescein makes quantitative measurements with this fluorophore problematic. Fluorescein's photobleaching limits present significant disadvantages for applications requiring ultrasensitive detection, such as DNA sequencing, fluorescence *in situ* hybridization and localization of low-abundance receptors.[6]

These limitations have encouraged us in the development of alternative fluorophores.

One option to improve the photostability of fluorescein is the incorporation of chlorine or fluorine groups onto the xanthene moiety, which almost does not alter the photophysical properties. Usually the photostability increases with the number of these groups.[29]

Rhodols and rhodamines possessing on 3'- or 3',6'-amino functionalities exhibit increased photostability in comparison with unsubstituted fluorescein.[50, 49, 56]

The photostability of new asymmetric xanthene fluorophores equipped with the ethylthio linker on the 3'-position was analyzed upon permanent excitation at broad excitation bandwidth, 488 ± 5 nm and monitoring the emission spectra at 30 seconds intervals for 2 h in the cuvette. The known sensitivity of fluorescein to bleaching became apparent, as it suffered 92% loss of fluorescence within 2 h meaning it exhibited 8% of initial intensity. The more photostable derivative 2',7'-dichlorofluorescein exhibited 28% of initial intensity after 2 h of exposure. New xanthene dyes displayed significantly improved photostability in comparison with fluorescein and 2',7'-dichlorofluorescein, which are 81% for MK-43, 96% for MK-69 and 95% for MK-67 of the initial intensity after 2 h of excitation. The azido-modified fluorophores exhibited 78% of the initial intensity for MK-61 and 85% for MK-75.

The photostability of the new fluorescent dyes is illustrated in the figures 15 and 16.

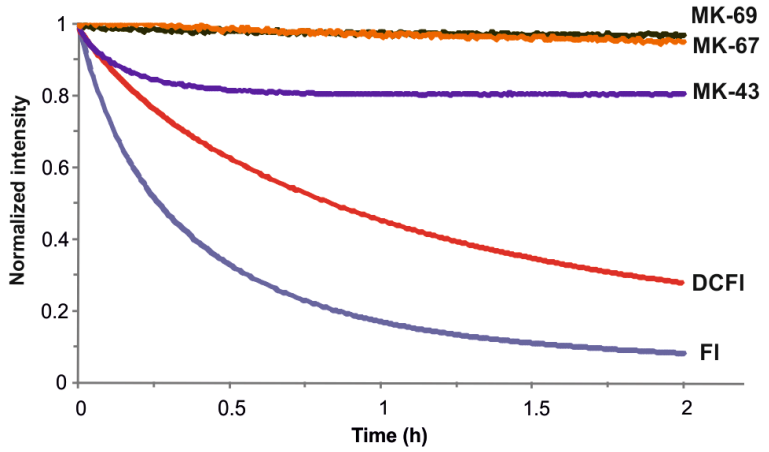


Figure 15. Photobleaching of new xanthene dyes (MK-43, MK-67, MK-69), fluorescein (Fl) and 2',7'-dichlorofluorescein (DCFl) in the cuvette upon excitation at 488 ± 5 nm for 2 h.

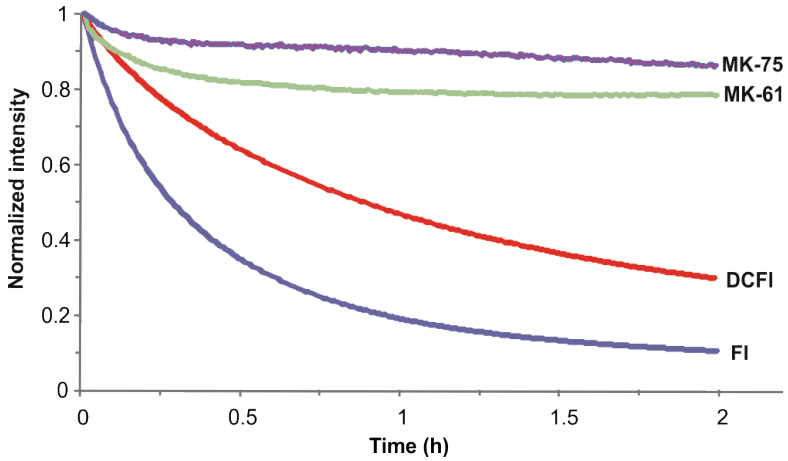


Figure 16. Photobleaching of azido-modifed xanthene dyes (MK-61, MK-75), fluorescein (Fl) and 2',7'-dichlorofluorescein (DCFl) in the cuvette upon excitation at 488 ± 5 nm for 2 h.

In measurements over a period of 10 h, the samples MK-43 showed 78% of its initial intensity, MK-69 94% of its initial intensity, MK-67 76% of its initial intensity and MK-61 58% and MK-75 75% of their initial intensities, respectively (Figure 17).

Half-life of fluorescein was 16 min and 2',7'-dichlorofluorescein was 50 min. According to the bleaching studies within 10 h we could estimate the half-life of novel asymmetric dyes. The half-life of MK-43 was evaluated at 23 hours, MK-61 at 12 hours, MK-67 at 21 hours, MK-69 at 83 hours, MK-75 at 20 hours. The fluorophores were compared to fluorescein. These observations suggested that MK-43 was 86x more stable, MK-61 45x, MK-67 79x, MK-69 311x, and MK-75 75x more stable. Because the bleaching of these compounds did not proceed by simple exponential decay, these factors may include relatively large errors.

However these results demonstrate superior photostability of the xanthene moiety upon substitution with an *S*-nucleophile. Since fluorescein derivatives photobleach relatively fast upon the repeated illumination with the strong excitation source used in fluorescence microscopy, the presented dyes could be applied as potential fluorescent labels instead. They could be particularly employed in fluorescence imaging in living cells.

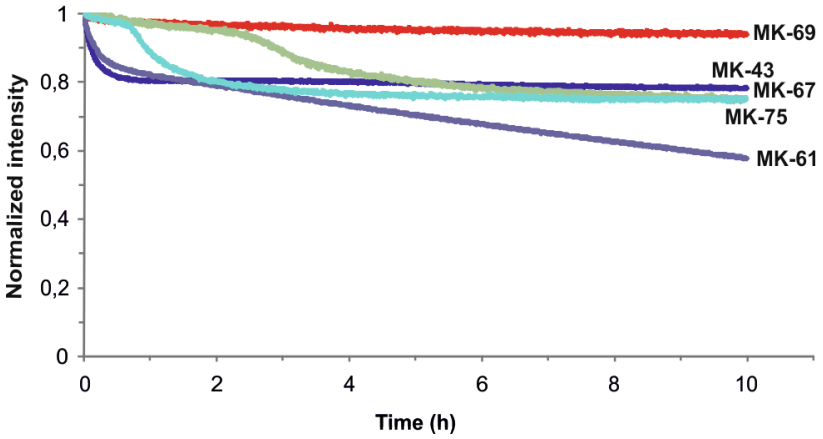


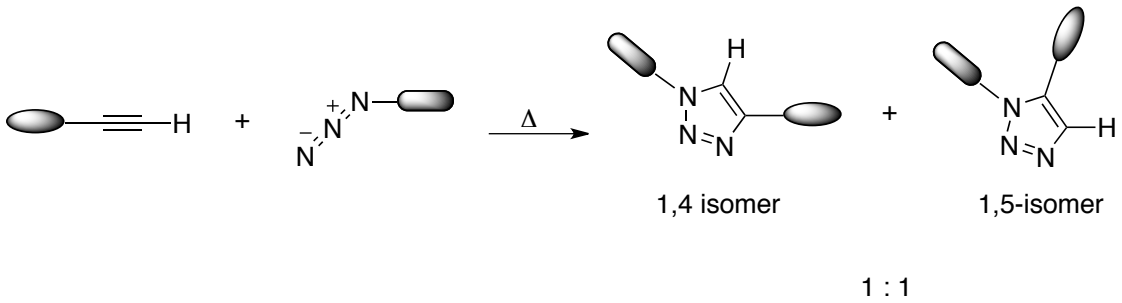
Figure 17. Photobleaching of new xanthene dyes in the cuvette upon excitation at 488 ± 5 nm for 10 h.

3.9 Azide-Alkyne "Click" Chemistry

3.9.1 Scientific Background

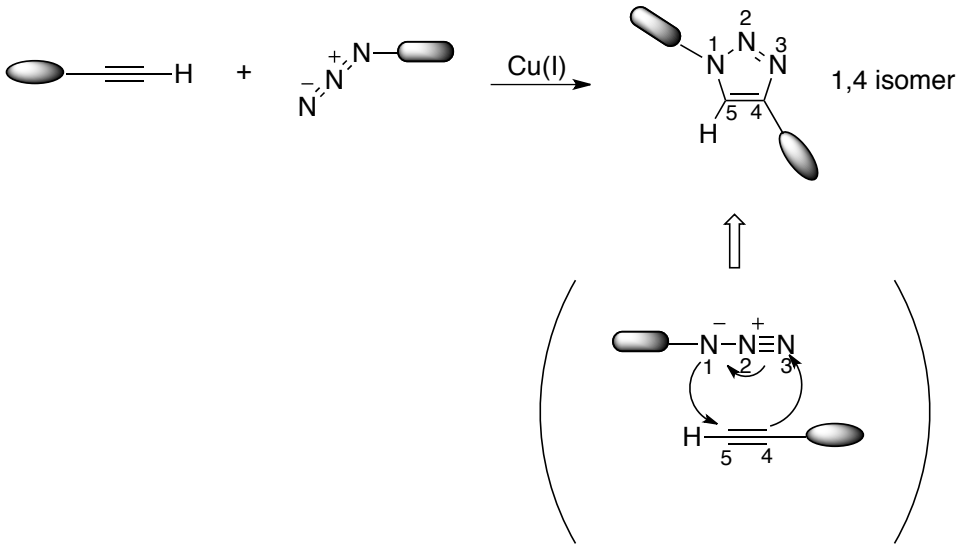
"Click" chemistry was first described by L. Pauling in 1933 and brought to the life by K. B. Sharpless in 2001.[113, 44] The concept of "click" chemistry is based on the rapid and efficient assembly of molecules, enables to create a new bond and guarantees reliable syntheses with high yields and purity.

The Huisgen 1,3-dipolar cycloaddition of azides and alkynes is a great representative of such a powerful reaction, which offers high yields and tolerance of oxygen and water (Scheme 56).[44, 114]



Scheme 56. Cycloaddition between azides and acetylenes to form triazoles.

Adding Cu(I)-catalyst into this reaction, which is formed by the reduction of Cu^{II} salts furnished selectively 1,4-disubstituted triazole product (Scheme 57).[115]



Scheme 57. Cu(I)-catalyzed-azide-alkyne-cycloadditon (CuAAC).

This Cu(I)-catalyzed Huisgen-Sharpless-Meldal azide-alkyne 1,3-dipolar cycloaddition known also as the "click" reaction achieved unique importance because of its significant characteristics such as regiospecificity, delivering only 1,4-substituted triazoles, high yields, high purity, shorter reaction times, aqueous conditions, room temperature, functional group tolerance and low cost. For these reasons the azide-alkyne "click" reaction has gained wide applications in organic synthesis, molecular biology and materials science.[116, 117, 118]

3.9.2 "Click" Reaction of the Azido-Xanthene Dyes with Alkynyl Labeled Oligonucleotide

Azide-alkyne "click" chemistry represents a straightforward strategy for labeling of oligonucleotides.

The newly synthesized azido-modified xanthene dyes were tested for selective conjugation with a commercial oligonucleotide, bearing the terminal alkyne residue. New synthesized azido-functionalized xanthene dyes, namely MK-57, MK-61, MK-75, MK-83 reacted *via* Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) with the commercially available DNA primer, 5'-hexynyl CGC GCG AAG CTT AAT ACG ACT CAC TAT. The reactions took place in water/phosphate buffer 1:2. The Cu(I)-catalyst was generated by reduction of $CuSO_4$. $5H_2O$ using sodium ascorbate. THPTA (tris(hydroxypropyl)triazolylmethyl-amine) was applied as stabilizing ligand of the Cu(I)-catalyst. After the reaction the labeled oligonucleotides were de-salted and precipitated according to an optimized procedure. They were purified and analyzed by PAGE electrophoresis to prove the clickable capability of azido functionalities of the new xanthene dyes and formation of triazole bonds with the oligonucleotides. Scanning the gel with blue laser (λ_{ex} = 488 nm) confirmed the existence of all "clicked" products. As a control, the commercially available azido-modified dye, Atto488, was also "clicked" with the 5'-hexynyl oligonucleotide. The free dyes were also visible upon irradiation with the blue laser, but they migrated differently usually a bit lower than the coupled dyes, which could contribute to their small sizes. The free oligonucleotides were not visible under these scanning settings. To visualize the free oligonucleotides, the gel was stained with a GelRed solution, which afterwards showed the stained oligonucleotides. Stains-All was used as a final staining. The "click" ability of the examined azido xanthene dyes was proven. MK-61 was also "clicked" with the terminal alkyne at position 12 of 4-propargylcarbamoyl-phenyl-modified 28mer DNA-oligonucleotide.[119]

These new azido dyes are ready to be used for a "click" reaction with other acetylene-modified nucleosides and to be incorporated into living cells during cellular transcription and translation.

4 Conclusions and Outlook

A new synthetic strategy for the preparation of novel asymmetric xanthene dyes *via* direct conversion of hydroxyl groups on the 3'- and 6'-positions into leaving groups by sulfonylation was developed. In particular, sulfonyl groups with moderate electron donating groups were applied. The mesyl groups proved to be sufficient to trigger the substitution reaction and therefore they were further utilized as leaving groups of choice.

It was discovered that 3',6'-dimesylated fluorescein underwent catalyst-free nucleophilic aromatic substitution with sulfur nucleophiles. This reaction proceeded within 4-8 hours on fluorescein as electron rich aromatic system and without a need to apply high temperatures or expensive catalysts.

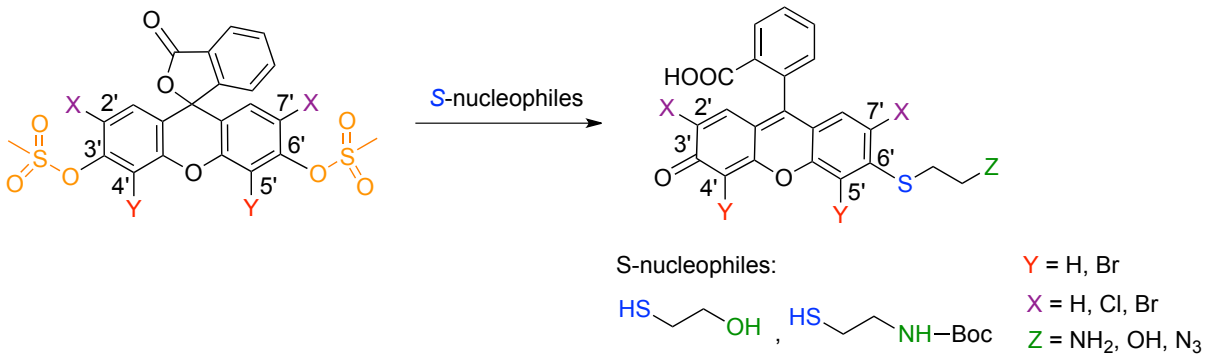
Not only 3',6'-mesylated fluorescein, but also 3',6'-mesylated 2',7'-dichloro, 4',5'-dibromo or 4',5',7'-tribromo derivatives of fluorescein were used as starting materials for the preparation of novel fluorescent dyes. The impact of these substituents with an electron withdrawing character on substitution with *S*-nucleophiles was investigated. It was observed that these groups not only accelerate the substitution reaction but also the cleavage of sulfonyl groups. This compensation did not affect the yields of the reactions, which remained comparable to unsubstituted fluorescein.

The mild reaction conditions, using an *S*-nucleophile, and DBU as base activated the displacement of the mesylate and gave rise to asymmetric xanthene derivatives, bearing 6'-(2-ethyl)thio linkers.

These xanthene derivatives have not yet been reported. The invented strategy provides novel fluorescent probes with the linker, which upon further modification can be utilized for conjugation with biomolecules. Fluorescent probes can be used for labeling of peptides, oligonucleotides and for cell imaging.

New azido xanthene derivatives were synthesized and successfully tested in bioconjugation reactions *via* “click” reactions with terminal alkyne-labeled oligonucleotides. Using protected cysteamine as an *S*-nucleophile offered xanthene derivative with a free amino group after the deprotection, which may be employed in a fluorescent labeling with the carboxylic acid, aldehyde or ketone containing biomolecules. Modification of the aliphatic amino group with *N,N*'-disuccinimidyl carbonate can afford succinimidyl ester and turn this compound into an amine-reactive fluorescent derivative.

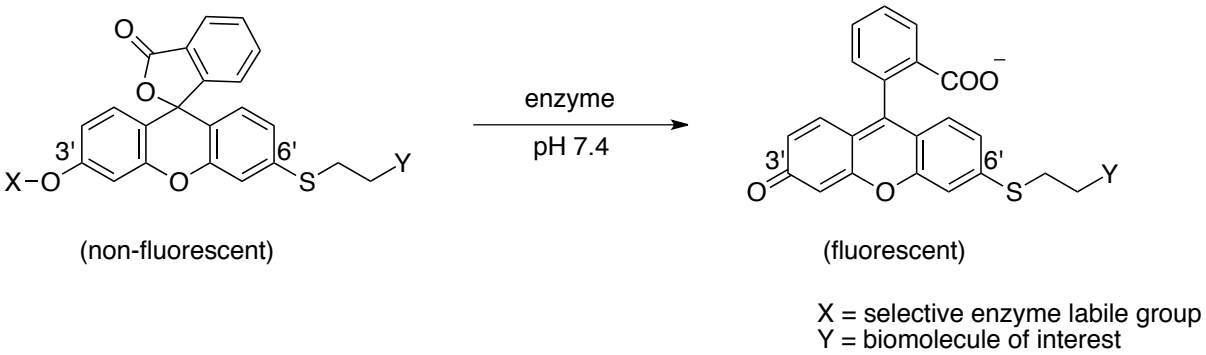
Scheme 58 displays the nucleophilic aromatic substitution of di-mesylated fluorescein and its derivatives with different *S*-nucleophiles. New functional groups are incorporated onto the *S*-linkers.



Scheme 58. Nucleophilic aromatic substitution of fluorescein and its derivatives with thiols.

The photophysical properties of previously unknown asymmetric xanthene dyes bearing *S*-linkers were characterized. Substitution on the 6'-position of xanthene residues afforded lower extinction coefficients and lower quantum yields of the corresponding dyes in comparison with non-substituted fluorescein. On the other hand, this type of modification delivered xanthene dyes with superior photostability. Even after 10 hours of continuous excitation, the asymmetric sulfur-containing dyes still possessed 58-94% of their initial fluorescent intensities. In comparison fluorescein and 2',7'-dichlorofluorescein lose most of their fluorescent intensity within 2-3 h. This observation suggested that they were 1-2 orders of magnitude more stable than fluorescein. The novel dyes can be applied in labeling of biomolecules and their long-term fluorescent imaging in cells.

The asymmetric fluorophores still possess the free 3'-hydroxyl group, which can be further substituted. One option would be the preparation of leuco dyes, where the xathene dyes are locked in the non-fluorescent lactone form by the incorporation of additional acyl or alkyl groups. The fluorophores can be linked with biomolecules of interest and still remain in their non-fluorescent form. Upon the incorporation into cells after contact with a selective enzyme the enzyme sensitive groups would be cleaved off and the fluorescence of bioconjugates would be developed (Scheme 59). This method might be interesting for tracing certain biomolecules within the living cells and enable determination of the localization within the cells. The advantage of this system is that it consists of only a one-step hydrolysis and therefore can make better detection sensitivity possible in comparison with standard fluorescein.



Scheme 59. Leuco-dye strategy for development of potential new fluorogenic probes.

This leuco-dye strategy, based on different modifications of ethylthio linkers and the installation of selective enzyme groups on the fluorophore may allow the construction of a wide range of useful fluorogenic probes.

5 Experimantal Section

5.1 General Information

Chemical reagents were obtained from various commercial suppliers mostly from Sigma Aldrich Chemie, Acros Organics (Steinheim, Germany), Merck KgaA (Darmstadt, Germany), Fischer Scientific GmbH, (Nidderau Germany), Fluka Chemie AG (Deisenhofen, Germany), and Alfa Aeser GmbH & Co KG (Karlsruhe, Germany) were usually used without further purification. Deuterized solvents were purchased from Deutero (Kastellaun, Germany).

Thin layer chromatography (TLC): Pre-coated silica gel plates Polygram Sil G/UV_{254} (40 x 80 mm) from Macherey-Nagel, Düren. Compounds were made visible by using UV light at λ = 254 nm and/or λ = 365 nm.

Preparative thin layer chromatography (pTLC): Pre-coated siliga gel glass plates, Silica gel 60 F_{254} (layer thickness 1 mm), Merck KGaA, Darmstadt (Germany)

Column chromatography (CC): Silica gel 60 (230-400 mesh) from Fluka, Silica gel 60 (230-400 mesh), Merck KGaA, Darmstadt (Germany)

NMR-spectroscopy: Varian 300 MHz (^{1}H: 300 MHz, ^{13}C: 75 MHz) and Bruker 500 MHz (^{1}H: 500 MHz, ^{13}C: 125 MHz), IPMB, University of Heidelberg; Bruker AC 300 MHz, Institute of Pharmacy and Biochemistry, University of Mainz; Bruker 400 MHz (^{1}H: 400 MHz, ^{13}C: 100 MHz) Institute for Inorganic and Analytical Chemistry, University of Mainz.

^{1}H and ^{13}C NMR spectra were calibrated to TMS on the basis of the relative chemical shift of the solvent as an internal standard. Chemical shifts are in ppm and abbreviations used are as follows: s = singlet, d = doublet, t = triplet, m= multiplet. Proton signals on xanthene moiety or on the benzene ring of fluorescein are often signed as doublets, because of the lower frequency of the NMR machine. In reality, they should be split into a doublet of doublets or doublet of triplets.

Mass spectrometry: FAB and EI mass spectra were recorded on a JEOL JMS-700 sector field mass spectrometer. MALDI-TOF (positive mode) mass spectra were recorded on a Bruker BIFLEX III spectrometer with matrix 2,4,6-trihydroxyacetophenone, $(NH_4)_2$-citrate. HR-ESI mass spectra were recorded on a Bruker MicroTOF-Q II spectrometer. FD-MS spectra on a Finnigan MAT 95 and ESI-MS spectra on a Micromass LCT at the Institute of Organic Chemistry, University of Mainz.

IR spectroscopy: Nicolet Avatar 330 FT-IR, Thermo electron corporation, Institute of

Pharmacy and Biochemistry, University Mainz

Melting point temperature: temperature < 200 °C, Apparatur nach Dr. Tottoli, Buchi, Flawil; temperature > 200 °C, Electrothermal IA 9200, Institute of Pharmacy and Biochemistry, University of Mainz.

5.2 Instruments and Special Materials

Analytical Balance	Sartorius (Germany)
Centrifuge 1-15 PK	Sigma (Osterode am Harz, Germany)
Electrophoresis Chamber	CBS Scientific
NAP columns, Sephadex G-25	GE Healthcare (Amersham Biosciences)
DyeEx 2.0 Spin Kit 250	Qiagen, Hilden (Germany)
Jasco FP-6500 fluorimeter	Jasco (Groß-Umstadt, Germany)
Jasco V-6500 spectrophotometer	Jasco (Groß-Umstadt, Germany)
UV cuvettes, SUPRASIL quartz glass cuvettes, 10 mm and 3 mm pathlength	Hellma (Müllheim, Germany)
UV-Lamp 254 nm	Herolab Molekulare Trenntechnik (Germany)
NanoDrop ND-2000	Peqlab (Erlangen, Germany)
Eppendorf tubes, silanized	Roth (Germany)
NMR spectrometer	Bruker AC-300 and AM-400 Mercury Plus 300, Varian VNMR S 500
pH-Meter	Mettler Toledo FE20/EL20
Pipettes	Abimed P2, P20, P100, P1000
Rotary evaporator	RV 06-ML
Freeze dryer	Alpha 2-4 LD plus, Christ (Germany)
Silica gel 60, (0.063-0.200 nm)	Merck (Darmstadt, Germany)
Silica gel plates Polygram® Sil G/UV254	Macherey-Nagel (Germany)
Thermomixer comfort	Eppendorf (Hamburg, Germany)
Typhoon 9400 variable mode imager	GE Healthcare (München, Germany)
ZipTip	Millipore (Schwalbach, Germany)
Mass Spectrometer:	
- MALDI-TOF	Bruker BIFLEX III
- FAB and EI	JEOL JMS-700
- ESI	LIFDI JEOL JMS-700, Micromass LCT

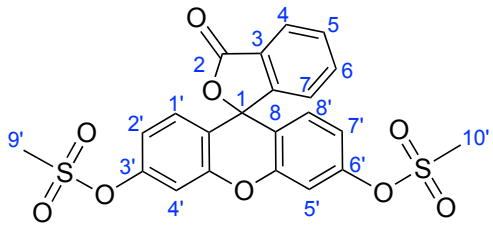
-ESI FT-ICR	Bruker MicroTOF-QII
-FD	Finnigan MAT 95
Rotiphorese sequencing gel buffer concentrate	Roth (Germany)
Rotiphorese sequencing gel concentrate	Roth (Germany)
Rotiphorese sequencing gel diluents	Roth (Germany)
Rotiphorese 10x TBE buffer	Roth (Germany)
Ultrapure Water Purification System	Mili-Q, Milipore
Spin filters	Nanosep® MF Centrifugal devices, 0.2 µm, Roth (Germany)
TEMED	Roth (Germany)
HPLC	Agilent 1100, Merck-Hitachi
HPLC column	Lichrosorb 125 mm, RP-C18

Buffers and Staining Solutions:

$LiClO_4$ for precipitation	2% (m/m) in acetone
PAGE loading buffer, colorless	1x TBE, 90% (v/v) formamide
PAGE loading buffer	1x TBE, 90% (v/v) formamide, 0.1% bromophenol blue, 0.1% xylene cyanol
TBE buffer	100 mM Tris (pH 8.3), 90 mM boric acid, 1 mM EDTA
GelRed	Biotium (Hayward, CA, USA)
StainsAll	Sigma Aldrich (Germany)

5.3 Sulfonylation of Fluorescein and its Derivatives

5.3.1 Synthesis of 3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]- 3',6'-diyl dimethanesulfonate (25)



Chemical Formula: $C_{22}H_{16}O_9S_2$
Molecular Weight: 488.49

Fluorescein (0.68 g, 2 mmol) was dissolved under an argon atmosphere in 10 mL of dry pyridine. The mixture was cooled to 0 °C and stirred vigorously. Methanesulfonyl chloride (620 µL, 8 mmol, 4 equiv) was dilluted in 5 mL of dry pyridine and added dropwise to the cooled solution of fluorescein. The ice bath was removed and the mixture was stirred for 4 h at rt. TLC on silica gel (CH_2Cl_2/CH_3OH, 100:1) showed almost complete conversion of the starting material and the presence of a new product. The solvent was evaporated *in vacuo*. The mixture was separated by column chromatography over silica gel, where the product was eluted with dichloromethane and unreacted fluorescein remained on the column. The solvent was evaporated and the resulting product was dried *in vacuo*. The product was obtained as a white solid with the yield of 90% (0.88 g, 1.8 mmol), which was comparable to literature.[89]

TLC (silica gel, CH_2Cl_2/CH_3OH, 100:1) $R_f = 0.89$

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.09 (d, ^3J = 8.3 Hz, 1H, H-4), 7.80 (m, 2H, H-5, H-6), 7.52 (d, ^4J = 2.6 Hz, 2H, H-4', H-5'), 7.43 (d, ^3J = 8.3 Hz, 1H, H-7), 7.17 (dd, ^3J = 9.6, ^4J = 2.6 Hz, 2H, H-2', H-7'), 7.01 (d, ^3J = 9.6 Hz, 2H, H-1', H-8'), 3.48 (s, 6H, H-9', H-10') ppm

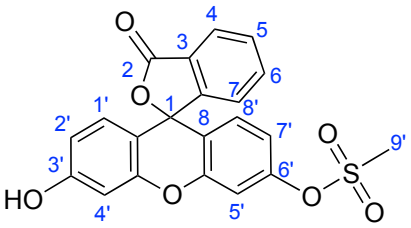
^{13}C NMR (75 MHz, DMSO-d_6) δ 168.5 (C=O), 152.3, 151.1 (2C), 150.5 (2C), 136.4, 131.0, 130.2 (3C), 125.5, 124.4, 119.2 (2C), 118.0 (2C), 111.2 (2C), 80.5, 37.9 (2C, C-9', C-10') ppm

MALDI-TOF-MS: 489.23 $[M + H]^+$

FT-IR $\tilde{V}$ (cm^{-1}) 3039 ν (C-H, arom.), 2937 ν (CH_3, aliph.), 1772 ν (C=O, lactone), 1607 ν (C=C, arom.), 1488, 1423, 1353 ν (S=O), 1233, 1133, 1106

m.p. 263-265 °C

5.3.2 Synthesis of 3'-hydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl methanesulfonate (30)



Chemical Formula: $C_{21}H_{14}O_7S$
Molecular Weight: 410.40

Fluorescein (1 g, 3 mmol) was dissolved in dry pyridine (13 mL) under an argon atmosphere. The mixture was cooled to 0° C and stirred vigorously. Methanesulfonyl chloride (210 µL, 2.7 mmol, 0.9 equiv), diluted in 2 mL of dry pyridine was added dropwise over 10 min to the cooled reaction mixture. The cooling was removed after 15 min and the mixture was stirred for an additional 3-4 h at rt. The reaction was monitored by TLC (CH_2Cl_2/CH_3OH, 95:5) and after 4 hours the new product was formed, but starting material still remained. Also some insignificant amount of di-mesylated by-product appeared. The reaction mixture was stirred over night. The solvent was evaporated *in vacuo*. The mixture was separated by column chromatography over silica gel using the gradient eluent mixture from 100% CH_2Cl_2 up to 95:5 CH_2Cl_2/CH_3OH. The solvent was evaporated *in vacuo* and the resulting product was dried under reduced pressure. The product was obtained as a slightly yellow solid with the yield of 60% (0.74 g, 1.8 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_F = 0.83

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.04 (d, ^3J = 8 Hz, 1H, H-3), 7.83 – 7.74 (m, 2H, H-4, H-5), 7.45 (d, ^4J = 3 Hz, 1H, H-5'), 7.34 (d, ^3J = 8 Hz, 1H, H-6), 7.10 (dd, ^3J = 9.6, ^4J = 3 Hz, 1H, H-7'), 6.91 (d, ^3J = 9.6 Hz, 1H, H-8'), 6.74 (dd, ^4J = 1.8 Hz, 1H, H-4'), 6.61 (m, 2H, H-1', H-2'), 3.46 (s, 3H, H-9') ppm

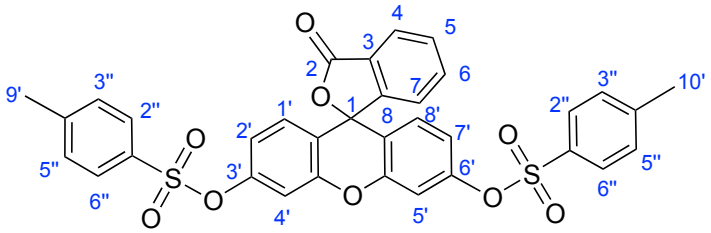
^{13}C NMR (125 MHz, DMSO-d_6) δ 168.4 (C=O), 159.7, 152.1, 151.3, 151.2, 130.2, 129.8 (3C), 128.9, 125.6, 124.9, 118.2, 118.1 (2C), 113.1, 110.7, 108.9, 102.4, 81.6, 37.4 (C-9') ppm

MALDI-TOF-MS: 411.1 $[M + H]^+$

FT-IR $\tilde{V}$ (cm^{-1}) 3624 3525 ν (OH), 3342 ν (OH), 3035 ν (C-H, arom.), 2933 ν (CH_3-, aliph.), 1728 ν (C=O, lactone), 1610 ν (C=C, arom.), 1429, 1369 ν (S=O), 1333 1168, 1107

m.p. 163-166 °C

5.3.3 Synthesis of 3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl bis(4-methylbenzene sulfonate) (29)



Chemical Formula: $C_{34}H_{24}O_9S_2$
Molecular Weight: 640.68

1st synthetic method:

Fluorescein (0.68 g, 2 mmol) was dissolved under an argon atmosphere in 15 mL of dry pyridine. The mixture was cooled to 0° C and vigorously stirred. 4-toluenesulfonyl chloride (1.6 g, 8 mmol, 4 equiv) was diluted in 5 mL of dry pyridine and added dropwise to the cooled solution of fluorescein. The cooling was removed after 15 min and the mixture was stirred for 5 h. TLC on silica gel (CH_2Cl_2/ CH_3OH, 80:1) showed almost complete conversion of the starting material and the presence of a new product. The solvent was evaporated *in vacuo* and the crude reaction mixture was dried under reduced pressure. The mixture was separated by column chromatography over silica gel, where the new product was eluted with dichloromethane and unreacted starting material stayed on the column. The solvent was evaporated and the resulting product was dried *in vacuo*. The product was obtained as a white solid with the yield of 85% (1.1 g, 1.7 mmol).

2nd synthetic method:

To a stirring solution of fluorescein (1 g, 3 mmol) in 5 mL of THF cooled to 0 °C was added NaOH (480 mg, 12 mmol, 4.0 equiv) as solution in 5 mL of water. 4-toluenesulfonyl chloride (2.3 mg, 12 mmol, 4.0 equiv) in 5 mL of THF was added dropwise over 2 h with continuous cooling. The solution was cooled additional 2 h. The mixture was monitored by TLC on silica gel (CH_2Cl_2/ CH_3OH, 95:5). After 4 h the conversion of starting material was still not complete. The cooling was removed and the mixture was stirred over night. The white precipitate was formed in the reaction mixture. It was filtrated off and washed thoroughly with water and then re-crystallized twice from methanol. The product was obtained as a white solid with the yield of 70% (1.34 g, 2.09 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 100:1) $R_f = 0.89$

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.03 (d, ^{3}J = 8.3 Hz, 1H, H-4), 7.80 (pd, J = 9.6 Hz, 4H, H-2", H-6"), 7.74 (dd, ^{3}J = 8.3 Hz, ^{4}J =2 Hz, 2H, H-5, H-6), 7.48 (pd, ^{3}J = 9.6 Hz, ^{4}J = 0.6 Hz, 4H, H-3", H-5"), 7.33 (d, ^{3}J = 8.3 Hz, 1H, H-7), 7.17 (d, ^{4}J = 3 Hz, 2H, H-4', H-5'), 6.90 (d, ^{3}J = 9.6 Hz, 2H, H-1', H-8'), 6.83 (dd, ^{3}J = 9.6 Hz ^{4}J = 3 Hz, 2H, H-2', H-7'), 2.41 (s, 6H, H-9', H-10') ppm

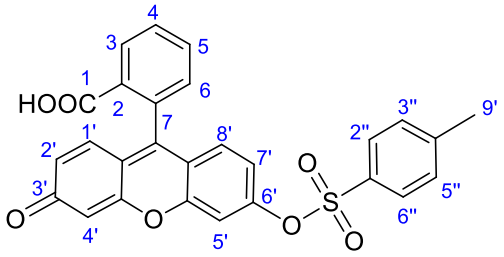
^{13}C NMR (100 MHz, DMSO-d_6) δ 168.3 (C=O), 152, 150.5 (2C), 150.1 (2C), 146.1 (2C), 136.1, 131.2 (2C) 130.5 (4C), 130.0 (2C), 128.3 (4C), 126.4, 125.0 (2C), 124.0, 118.5 (2C), 117.6 (2C), 110.7 (2C), 79.7, 21.1 (2C, C-9', C-10') ppm

FD-MS: m/z (%): 641.6 (100)

FT-IR $\tilde{V}$ (cm^{-1}) 1750 ν (C=O, lactone), 1596 ν (C=C, arom.), 1491, 1432, 1371 ν (S=O), 1235, 1142, 1092

m.p. 172-174 °C

5.3.4 Synthesis of 3'-hydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl 4-methylbenzenesulfonate (33)



Chemical Formula: $C_{27}H_{18}O_7S$
Molecular Weight: 486,49

Fluorescein (0.68 g, 2 mmol) was dissolved under an argon atmosphere in 15 mL of dry pyridine. The mixture was cooled to 0° C stirred vigorously. 4-toluenesulfonyl chloride (0.35 g, 1.8 mmol, 0.9 equiv) was diluted in 5 mL of dry pyridine and added dropwise to the cooled solution of fluorescein. The ice bath was removed after 15 min and the mixture was stirred for 6 h at rt. TLC (CH_2Cl_2/CH_3OH, 98:2) showed the presence of the starting material and the formation of a new product. The solvent was evaporated *in vacuo*. The crude mixture was separated by gradient flash column chromatography over silica gel, using 100% CH_2Cl_2 to 99:1CH_2Cl_2/CH_3OH, where the resulting product of yellow color was separated from di-tosylated by-product and unreacted fluorescein. The product was obtained as a yellow solid with the yield of 52% (505 mg, 1.04 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) $R_f = 0.74$

^1H NMR (400 MHz, DMSO-d_6) δ 10.23 (s, 1H, COOH), 8.04 – 7.98 (m, 1H, H-3), 7.85 – 7.69 (m, 4H, H-2", H-6", H-4, H-5), 7.49 (pd, ^3J = 8.4 Hz, 2H, H-3", H-5"), 7.30 (d, ^3J = 7.6 Hz, 1H, H-6), 7.13 (d, ^4J = 2.2 Hz, 1H, H-4'), 6.83 (d, ^3J = 8.8 Hz, 1H, H-1'), 6.79 (dd, ^3J = 8.8 Hz, ^3J = 2.2 Hz, 1H, H-2'), 6.70 (m, 1H, H-5'), 6.59 (m, 2H, H-7', H-8'), 2.42 (s, 3H, H-9') ppm

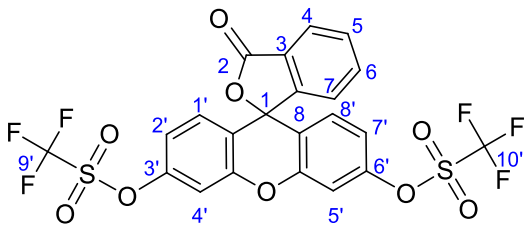
^{13}C NMR (100 MHz, DMSO-d_6) δ 168.5 (C=O), 159.8 (COOH), 152.2, 151.3, 151.1, 149.9, 146.1, 135.9, 131.2, 130.4, 129.6 (3C), 129.1, 128.3 (2C), 125.6, 124.9, 124.0, 118.2, 117.9, 113.3, 110.6, 108.9, 102.3, 81.5, 21.3 (C-9') ppm

FAB-MS: 487.1 $[M + H]^+$

FT-IR $\tilde{V}$ (cm^{-1}) 1766 ν (C=O, chinone), 1734 ν (COOH), 1607 ν (C=C, arom.), 1425, 1371 ν (S=O), 1245, 1192, 1106

m.p. 118-120 °C

5.3.5 Synthesis of 3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl bis(trifluoromethanesulfonate) (56)



Chemical Formula: $C_{22}H_{10}F_6O_9S_2$
Molecular Weight: 596,43

Fluorescein (1 g, 3 mmol) was suspended in CH_2Cl_2 (15 mL) and cooled to 0 °C. Pyridine (1.93 mL, 24 mmol, 8.0 equiv) and trifluoromethanesulfonic anhydride (2.08 mL, 12 mmol, 4.0 equiv) were added, and the ice bath was removed. The reaction was stirred at room temperature for 4 h. It was subsequently diluted with water (15 ml) and extracted twice with CH_2Cl_2 (2x15 ml). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. Purification by column chromatography on silica gel (CH_2Cl_2/CH_3OH, 99:1) afforded the product as a white solid with the yield of 78% (1.4 g, 2.34 mmol). (Lit. 82%)[56]

TLC (silica gel, CH_2Cl_2) $R_f = 0.58$

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.08 (d, ^{3}J = 7.5 Hz, 1H, H-4), 7.80 – 7.64 (m, 2H, H-5, H-6), 7.30 (d, ^{4}J = 2.3 Hz, 2H, H-4', H-5'), 7.20 (d, ^{3}J = 7.5 Hz, 1H, H-7), 7.03 (dd, ^{3}J = 8.8 Hz, ^{4}J = 2.3 Hz, 2H, H-2', H-7'), 6.96 (d, ^{3}J = 8.8 Hz, 2H, H-1', H-8') ppm

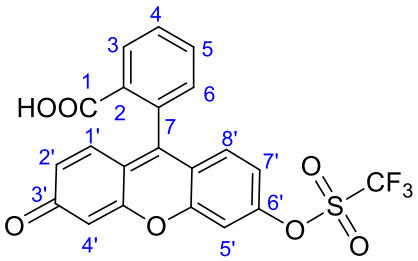
^{13}C NMR (75 MHz, $CDCl_3$) δ 168.6 (C=O, C-2), 152.3, 151.5 (2C), 150.4 (2C), 136.0, 130.8, 130.1 (3C), 125.9, 125.8, 123.9, 120.9, 119.5, 117.8 (2C), 116.7, 110.8 (2C), 80.2 ppm

FD-MS: m/z (%): 596.3 (100)

FT-IR $\tilde{V}$ (cm^{-1}) 3095 ν (C-H, arom.), 1755 ν (C=O, lactone), 1605 ν (C=C, arom.), 1485, 1425 ν (S=O), 1197, 1159, 1104, 984, 866

m.p. 109-111 °C

5.3.6 Synthesis of 3'-hydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl trifluoromethanesulfonate (31)



Chemical Formula: $C_{21}H_{11}F_3O_7S$
Molecular Weight: 464,37

Fluorescein (0.68 g, 2 mmol) was dissolved under an argon atmosphere in 15 ml of dry pyridine. The mixture was cooled to 0° C and trifluoromethanesulfonyl chloride (0.190 mL, 1.8 mmol, 0.9 equiv) was added dropwise while stirring vigorously. The ice bath was removed. In 1 hour the color changed from orange-red to slightly yellow. The mixture was stirred over night. TLC (CH_2Cl_2/CH_3OH, 95:5) showed unreacted starting material and the product. The solvent was evaporated *in vacuo* and the crude reaction mixture was dried under reduced pressure for several hours. The crude mixture was purified by gradient column chromatography over silica gel with CH_2Cl_2 100% to CH_2Cl_2/CH_3OH, 95:5. The solution with product was evaporated and dried *in vacuo*. The product was obtained as a yelow solid with the yield of 60% (0.557 g, 1.2 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 80:1) R_f = 0.47

^{1}H NMR (300 MHz, DMSO-d_6) δ 10.29 (s, 1H, COOH), 8.04 (d, ^{3}J = 8.3 Hz, 1H, H-3), 7.90 – 7.72 (m, 2H, H-4, H-5), 7.71 (d, ^{4}J = 3 Hz, 1H, H-4'), 7.38 (d, ^{3}J = 8.3 Hz, 1H, H-6), 7.24

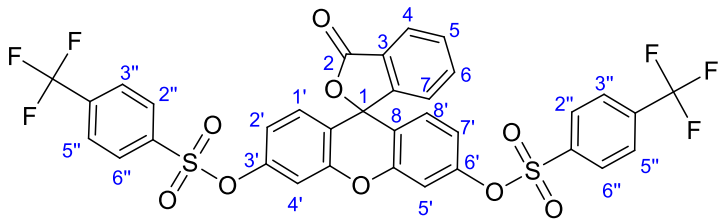
(dd, ^{3}J = 8.8 Hz, ^{4}J = 3 Hz, 1H, H-2'), 7.02 (d, ^{3}J = 8.8 Hz, 1H, H-1'), 6.75 (m, 1H, H-5'), 6.62 (m, 2H, H-7', H-8') ppm

^{13}C NMR (100 MHz, DMSO-d_6) δ 168.1 (C=O, C-3'), 159.5 (COOH, C-1), 151.9, 151.1, 150.9, 149.3, 135.7, 130.4 (3C), 129.1, 125.3, 124.7, 123.9, 119.7, 117.1, 113.3, 110.3, 108.5, 102.2, 81.0 ppm

FD-MS: m/z (%): 464.5 (100)

FT-IR $\tilde{V}$ (cm^{-1}) 3376 ν (OH), 1734 ν (COOH), 1611 ν (C=C, arom.), 1493, 1419 ν (S=O), 1202, 1135, 1105

5.3.7 Synthesis of 3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diylbis(4-(trifluoromethyl) benzenesulfonate) (28)



Chemical Formula: $C_{34}H_{18}F_6O_9S_2$
Molecular Weight: 748,62

Fluorescein (0.68 g, 2 mmol) was dissolved under an argon atmosphere in 15 mL of dry pyridine. The mixture was cooled to 0 °C while stirring vigorously. Trifluorotoluenesulfonyl chloride (2 g, 8 mmol, 4 equiv) was diluted in 5 mL of dry pyridine and added dropwise to the cooled solution of fluorescein. The ice bath was removed after 10 min and the mixture was stirred for 6 h. TLC (CH_2Cl_2/CH_3OH, 98:2) showed almost complete conversion of starting material and the presence of a new product. The solvent was evaporated *in vacuo*. The mixture was separated by column chromatography over silica gel, where the product was eluted with CH_2Cl_2 and traces of unreacted starting material remained on the column. The solvent was evaporated and the product was dried *in vacuo*. The product was obtained as a white solid with the yield of 80% (1.18 g, 1.6 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.9

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.16 (pd, ^{3}J = 9.3 Hz, 4H, H-3", H-5"), 8.07 (pd, ^{3}J = 9.3 Hz, 5H, H-2", H-6", H-4), 7.83 – 7.70 (m, 2H, H-5, H-6), 7.36 (d, ^{3}J = 7.6 Hz, 1H, H-7), 7.26 (d, ^{4}J = 2.3 Hz, 2H, H-4', H-5'), 6.97 – 6.85 (m, 4H, H-7', H-8', H-1', H-2') ppm

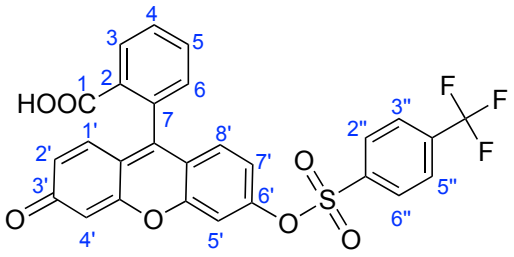
^{13}C NMR (100 MHz, DMSO-d_6) δ 168.0 (C=O), 151.8, 150.3 (2C), 149.6 (2C), 137.8 (2C), 135.9, 134.5, 134.1, 130.5, 129.9 (2C), 129.2 (6C), 126.9 (2C), 124.9, 124.8, 124.3, 123.8, 121.5, 118.4 (2C), 117.9 (2C), 110.7 (2C), 79.8 (C-1) ppm

FD-MS: m/z (%): 748.6 (100)

FT-IR $\tilde{V}$ (cm^{-1}) 3106 ν (C-H, arom.), 1765 ν (C=O, lactone), 1607 ν (C=C, arom.), 1488, 1379 ν (S=O), 1319, 1192, 1133

m.p. 172-175 °C

5.3.8 Synthesis of 2-(3-oxo-6-(((4-(trifluoromethyl)phenyl)sulfonyl)oxy)-3H-xanthen-9-yl)benzoic acid (32)



Chemical Formula: $C_{27}H_{15}F_3O_7S$
Molecular Weight: 540,46

Fluorescein (0.68 g, 2 mmol) was diluted under an argon atmosphere in 15 mL of dry pyridine. While stirring vigorously the mixture was cooled to 0° C and trifluorotoluenesulfonyl chloride (0.44 g, 1.8 mmol, 0.9 equiv), disolved in 5 mL of dry pyridine, was added dropwise. After 15 min the ice bath was removed and the mixture was stirred over an additional 5 h. TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) showed the presence of the starting material, but also a new spot. The pyridine was evaporated *in vacuo* and the crude reaction mixture was further dried under reduced pressure. The resulting mixture was separated by column chromatography over silica gel using CH_2Cl_2/CH_3OH, 98:2, where the yellow product was separated from the unreacted red fluorescein. The solvent was evaporated *in vacuo*. The product was obtained as a yellow solid with the yield of 61% (0.66 g, 1.25 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 98:2) R_f = 0.75

^{1}H NMR (300 MHz, DMSO-d_6) δ 10.26 (s, 1H, COOH), δ 8.17 (pd, ^3J = 9.6 Hz, 2H, H-3", H-5"), 8.08 (pd, ^3J = 9.6 Hz, 2H, H-2", H-6"), 8.01 (d, ^3J = 8 Hz, 1H, H-3), 7.76 (m, ^3J = 8 Hz, 2H, H-4, H-5), 7.31 (d, ^3J = 8 Hz, 1H, H-6), 7.24 (d, ^4J = 2 Hz, 1H, H-4'), 6.84 (m, 2H, H-1', H-2'), 6.70 (m, 1H, H-5'), 6.59 (m, 2H, H-7', H-8') ppm

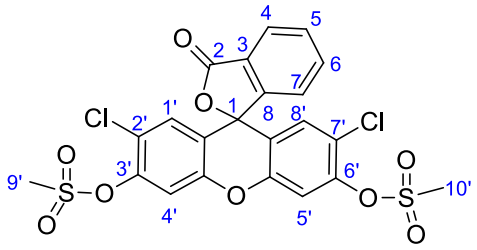
^{13}C NMR (100 MHz, DMSO-*d6*) δ 168.5 (C=O, C-3'), 159.8 (COOH, C-1), 152.2, 151.2 (2C), 149.6, 138.1, 135.9, 130.5, 129.9, 129.5 (4C), 129.2, 127.3, 125.6, 124.8 (2C), 123.9, 118.5, 117.9, 113.3, 110.77, 108.8, 102.2, 81.4 ppm

MS-FAB: 541.1 $[M + H]^+$

FT-IR $\tilde{V}$ (cm^{-1}) 3112 ν (C-H, arom.), 1766 ν (C=O, chinone), 1748 ν (COOH), 1613 ν (C=C, arom.), 1495, 1444, 1321 ν (S=O), 1241, 1196, 1109

m.p. 120-122 °C

5.3.9 Synthesis of 2',7'-dichloro-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl Dimethanesulfonate (35)



Chemical Formula: $C_{22}H_{14}Cl_2O_9S_2$
Molecular Weight: 557,38

2',7'-dichlorofluorescein (1 g, 2.5 mmol) was suspended in 20 mL of dry pyridine under an argon atmosphere and cooled to 0 °C. Methanesulfonyl chloride (770 µL, 10 mol, 4.0 equiv) in 5 mL of dry pyridine was added dropwise. The cooling was removed after 10 min and the mixture was allowed to warm up to rt. According to TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) after 3 h, no starting material was present. The mixture was stirred additional 1 h. The solvent, pyridine, was evaporated *in vacuo*. The residue was dried several hours under reduced pressure. The mixture was separated by column chromatography on silica gel, using pure CH_2Cl_2. The product was obtained as a white solid with the yield of 82.4% (1.145 g, 2.05 mmol)

TLC (silica gel, CH_2Cl_2/CH_3OH, 99:1) R_f = 0.59

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.07 (d, ^3J = 7.6 Hz, 1H, H-4), 7.82 (m, 2H, H-5, H-6), 7.72 (s, 2H, H-4', H-5'), 7.47 (d, ^3J = 7.6 Hz, 1H, H-7), 7.18 (s, 2H, H-1', H-8'), 3.61 (s, 6H, H-9', H-10') ppm

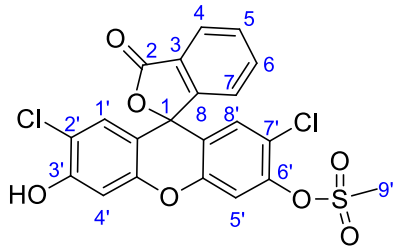
^{13}C NMR (100 MHz, DMSO-d_6) δ 167.9 (C=O), 151.1, 149.2 (2C), 146.2 (2C), 136.2, 130.9, 129.4 (2C), 125.7, 125.3, 124.1, 122.4 (2C), 118.8 (2C), 113.1 (2C), 79.3, 39.5 (2C, C-9', C-10') ppm

FD-MS: m/z (%): 556.1 (100), 557 (28), 558 (82)

FT-IR $\tilde{V}$ (cm^{-1}) 3015 ν (C-H, arom.), 2931 ν (CH_3, aliph.), 1768 ν (C=O, lactone), 1608 ν (C=C, arom.), 1476, 1407, 1370 ν (S=O), 1187, 1159, 1077, 965

m.p. 263 °C

5.3.10 Synthesis of 2-(2,7-dichloro-6-((methylsulfonyl)oxy)-3-oxo-3H-xanthen-9-yl)benzoic acid (37)



Chemical Formula: $C_{21}H_{12}Cl_2O_7S$
Molecular Weight: 479,29

2',7'-dichlorofluorescein (500 mg, 1.2 mmol) was dissolved under an argon atmosphere in 13 mL of dry pyridine. The mixture was cooled to 0 °C while stirring vigorously. Methanesulfonyl chloride (84 µL, 1.08 mmol, 0.9 equiv) diluted in 2 mL of dry pyridine was added dropwise. The cooling was removed and the mixture was stirred at rt for 6 h. TLC (CH_2Cl_2/CH_3OH, 98:2) showed presence of the starting material and formation of a new product. The solvent was evaporated *in vacuo*. The mixture was separated by column chromatography on silica gel using a gradient from CH_2Cl_2 to CH_2Cl_2/CH_3OH (98:2), where the product was separated from by-product and unreacted 2',7'-dichlorofluorescein. The product was obtained as a slightly yellow solid with the yield of 55% (315 mg, 0.66 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.73

^{1}H NMR (400 MHz, CD_3OD) δ 8.10 (d, ^3J = 7.6 Hz, 1H, H-4), 7.85 (t, ^3J = 7.6 Hz, 1H, H-6), 7.79 (t, ^3J = 7.6 Hz, 1H, H-5), 7.54 (s, 1H, H-5'), 7.31 (d, ^3J = 7.6 Hz, 1H, H-7), 6.95 (s, 1H, H-4'), 6.91 (s, 1H, H-8'), 6.69 (s, 1H, H-1'), 5.51 (s, 1H, OH), 3.40 (s, 3H, 3H-9') ppm

^{13}C NMR (75 MHz, CD_3OD) δ 170.6 (C=O), 157.5, 153.5, 151.9, 148.1, 137.4 (2C), 132.2, 130.8 (2C), 129.8, 126.7, 125.4, 123.6, 120.9, 119.4, 114.6 (2C), 111.9, 105.3, 39.5 (C-9') ppm

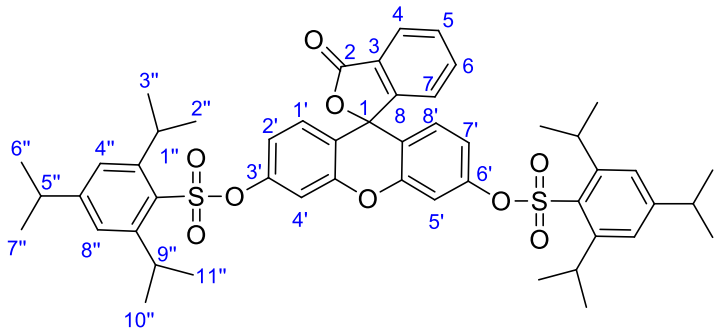
FD-MS: m/z (%): 478.2 (100), 479.2 (50), 480.3 (88), 481.2 (31)

FT-IR $\tilde{V}$ (cm^{-1}) 3265 ν (OH), 3023 ν (C-H, arom.), 2940 ν (CH_3, aliph.), 1727 ν (C=O, lactone), 1603 ν (C=C, arom.), 1481, 1409, ν 1374 (S=O), 1251, 1177

m.p. 190-193 °C

5.3.11 General Procedure for Formation of Fluorescein and 2',7'-Dichlorofluorescein ditriisopropylbenzenesulfonates

5.3.11.1 3-Oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl bis(2,4,6-triisopropylbenzene sulfonate) (27)



Chemical Formula: $C_{50}H_{56}O_9S_2$
Molecular Weight: 865,10

Fluorescein (1 g, 3.01 mmol) was suspended in 30 mL of dry pyridine under an argon atmosphere and cooled to 0 °C. 2,4,6-triisopropylbenzenesulfonyl chloride (3.63 g, 0.012 mol, 4.0 equiv) dissolved in 5 ml of dry pyridine was added to the mixture in a dropwise manner. The cooling was removed after 30 min and the mixture was stirred at rt for 4 h. The solvent was evaporated *in vacuo*. The residue was dried under reduced pressure and then separated by column chromatography on silica gel using pure CH_2Cl_2. The product was obtained as a white fluffy solid with 89% yield (2.32 g, 2.68 mmol).

TLC (silica gel, CH_2Cl_2) R_f = 0.53

^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.02 (d, ^3J = 7.6 Hz, 1H, H-4), 7.73 (m, 2H, H-5, H-6), 7.36 (s, 4H, H-4", H-8"), 7.17 (d, ^3J = 7.6 Hz, 1H, H-7), 6.99 – 6.91 (m, 4H, H-4', H-5', H-2', H-7'), 6.80 (d, J = 8.8 Hz, 2H, H-1', H-8'), 3.86 (m, 4H, 2H-1", 2H-9"), 2.98 (m, 2H, H-5"), 1.22 (d, J = 6.8 Hz, 12H, H-6", H-7"), 1.15 – 1.06 (m, 24H, H-2", H- 3", H-10", H-11") ppm

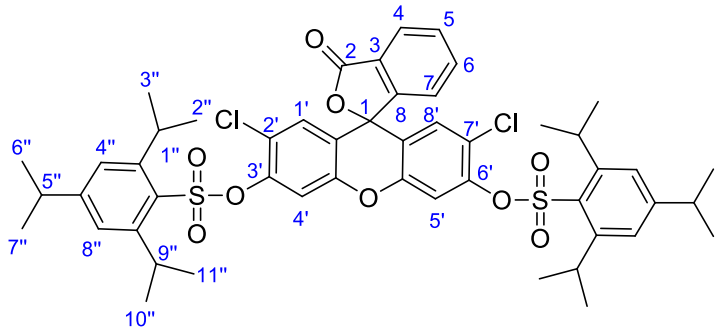
^{13}C NMR (75 MHz DMSO-*d6*) δ168.3 (C=O), 155.1 (2C), 152.3, 150.8 (4C), 150.3 (2C), 149.8 (2C), 136.0, 130.7, 130.1 (2C), 128.3 (2C), 125.2, 124.8, 124.3 (4C), 123.7, 118.9 (2C), 117.7 (2C), 110.6 (2C), 79.9, 33.5 (2C), 29.4 (4C), 24.1 (8C), 23.2 (4C) ppm

FD-MS: m/z (%): 865.0 (100), 866 (81), 867 (39.25)

FT-IR $\tilde{V}$ (cm^{-1}) 3016 ν (C-H, arom.), 2932 ν (CH_3, aliph.), 1768 ν (C=O, lactone), 1608 ν (C=C, arom.), 1477, 1409, 1370 (S=O), 1186, 1160

m.p. 95-98 °C

5.3.11.2 2',7'-Dichloro-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl bis(2,4,6-triisopropylbenzenesulfonate) (36)



Chemical Formula: $C_{50}H_{54}Cl_2O_9S_2$
Molecular Weight: 933,99

This compound was prepared from 2',7'–dichlorofluorescein (1 g, 2.5 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (3.02 g, 9.97 mmol, 4.0 equiv) according to the general procedure as described above. The product was obtained as a white fluffy solid with 95% yield (2.216 g, 2.37 mmol).

TLC (silica gel, CH_2Cl_2) R_f = 0.74

^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.04 (d, ^3J = 7.2 Hz, 1H, H-4), 7.83 – 7.72 (m, 2H, H-5, H-6), 7.41 (s, 4H, 2H-4", 2H-8"), 7.28 (d, ^3J = 7.2 Hz, 1H, H-7), 7.20 (s, 2H, H-4', H-5'), 6.84 (s, 2H, H-1', H-8'), 3.89 (p, J = 6.8 Hz, 4H, H-1", H-9"), 3.00 (p, ^3J = 6.8 Hz, 2H, H-5"), 1.25 (d, ^3J = 6.8 Hz, 12H, H-6", H-7"), 1.16 (m, 24H, H-2", H- 3", H-10", H-11") ppm

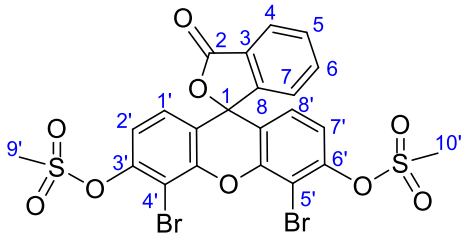
^{13}C NMR (100 MHz, DMSO-*d6*) δ 167.9 (C=O), 155.4 (2C), 151.26, 150.7 (4C), 148.6 (2C), 146.0 (2C), 136.1, 130.9, 129.7 (2C), 129 (2C), 125.7, 125.0, 124.4 (4C), 123.6, 122.8 (2C), 118.8 (2C), 112.0 (2C), 78.8, 33.5 (2C), 29.7 (4C), 24.1 (8C), 23.2 (4C) ppm

FD-MS: m/z (%): 932.9 (100), 933.8 (63.2), 934.8 (72.8), 935.8 (47.2)

FT-IR $\tilde{V}$ (cm^{-1}) 3016 ν (C-H, arom.), 2962 ν (CH_3, aliph.), 2931 ν (CH, aliph.), 1769 ν (C=O, lactone), 1608 ν (C=C, arom.), 1477, 1408, 1370 ν (S=O), 1186, 1158

m.p. 115-118 °C

5.3.12 Synthesis of 4',5'-dibromo-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (40) and 2',4',5'-tribromo-3-oxo-3H-spiro [isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (41)



Chemical Formula: $C_{22}H_{14}Br_2O_9S_2$
Molecular Weight: 646,28

4',5'-dibromofluorescein and 2',4',5'-tribromofluorescein (2 g, 4.08 mmol) both commercially available under 4',5'-dibromofluorescein were dissolved under an argon atmosphere in 30 mL of dry pyridine. The reaction mixture was cooled to 0 °C and stirred vigorously. Methanesulfonyl chloride (1.26 mL, 16.3 mmol, 4.0 equiv) in 5 mL of dry pyridine was added dropwise. The cooling was removed after 30 min and the reaction mixture was stirred at room temperature for 5 h. TLC on silica gel (CH_2Cl_2/CH_3OH, 99:1) showed complete conversion of starting materials. Pyridine was evaporated *in vacuo* and the residue was dried under reduced pressure. Purification by gradient column chromatography over silica gel using CH_2Cl_2, CH_2Cl_2/CH_3OH (99:1) as elution mixture allowed separation of two products namely di-mesylated dibrominated derivative and di-mesylated tribrominated derivative as white solids with the yield of 22% (590 mg, 0.91 mmol) and 14% (410 mg, 0.56 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 99:1) R_f = 0.69

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10 (d, ^3J = 7.6 Hz, 1H, H-4), 7.97 – 7.67 (m, 2H, H-5, H-6), 7.55 (d, ^3J = 7.6 Hz, 1H, H-7), 7.36 (d, ^3J = 8.8 Hz, 2H, H-2', H-7'), 7.03 (d, ^3J = 8.8 Hz, 2H, H-1', H-8'), 3.59 (s, 6H, H-9', H-10') ppm

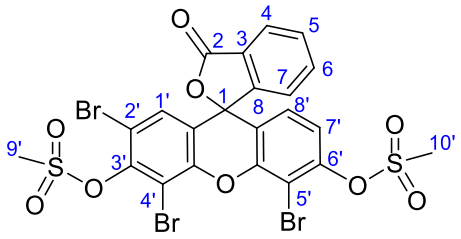
^{13}C NMR (100 MHz, DMSO-d_6) δ 168 (C=O), 151.3, 148.4 (2C), 148.2 (2C), 136.1, 131.3, 128.2 (2C), 125.3, 125.1, 124.5, 119.8 (2C), 118.9 (2C), 106.1 (2C), 80.4, 55.0 (2C, C-9', C-10') ppm

FD-MS: m/z (%): 646.5 (100), 647.5 (28), 648.4 (68), 644.5 (44)

FT-IR $\tilde{V}$ (cm^{-1}) 3039 ν (C-H, arom.), 2941 ν (CH_3, aliph.), 1750 ν (C=O, lactone), 1585 ν (C=C, arom.), 1413, 1360 ν (S=O)

m.p. 274-276 °C

2',4',5'-tribromo-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (41)



Chemical Formula: $C_{22}H_{13}Br_3O_9S_2$
Molecular Weight: 725,18

TLC (silica gel, CH_2Cl_2/CH_3OH, 99:1) $R_f = 0.77$

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, ^3J = 7.2 Hz, 1H, H-4), 7.81 – 7.65 (m, 2H, H-5, H-6), 7.25 (d, ^3J = 8.8 Hz, 1H, H-7'), 7.20 (d, ^3J = 7.2 Hz, 1H, H-7), 7.10 (s, 1H, H-1'), 6.87 (d, ^3J = 8.8 Hz, 1H, H-8'), 3.57 (s, 3H, H-10'), 3.33 (s, 3H, H-9') ppm

^{13}C NMR (100 MHz, $CDCl_3$) δ 151.47, 148.56, 136.2 (2C), 131.2 (2C), 130.9 (2C), 127.4 (2C), 126.1 (2C), 125.5, 124.0 (2C), 121.2, 120.2 (2C), 119.2, 113.4, 42.2, 39.5 ppm

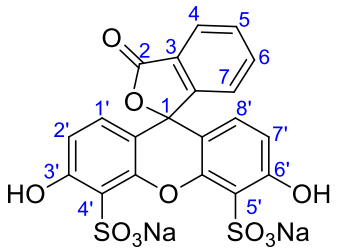
FD-MS: m/z (%): 726.3 (100), 725.3 (38), 724.3 (99), 727.3 (26)

FT-IR $\tilde{V}$ (cm^{-1}) ν 3039 ν (C-H, arom.), 2937 ν (CH_3, aliph.), 1764 ν (C=O, lactone), 1591 ν (C=C, arom.), 1418, 1367 ν (S=O), 1178, 1048, 832

m.p. 276-280 °C

5.4 Sulfonation of Fluorescein

5.4.1 Synthesis of sodium 3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-4',5'-disulfonate (42)



Chemical Formula: $C_{20}H_{10}Na_2O_{11}S_2$
Molecular Weight: 536,40

Fluorescein (5 g, 15 mmol) in 10 mL of oleum ($H_2SO_4.xSO_3$) was heated in an oil bath at 100 °C for 8 h.[38] Then reaction mixture was allowed to cool down to rt and was stirred over night. The mixture was poured in small portions onto 50 ml of ice. The addition of a saturated solution of NaCl resulted in the formation of the diSO_3NaFl as precipitate. The filtrate was recrystallized from a small amount of water. The disodium salt was obtained with good solubility in water and weak solubility in ethanol. The column chromatography on silica gel was performed with CH_2Cl_2/CH_3OH, 8:2. The product was obtained as a yellow solid with the yield of 40% (3.22 g, 6 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 8:2) R_f = 0.47

^{1}H NMR (300 MHz, D_2O) δ 8.13 (d, ^3J = 7.5 Hz, 1H, H-4), 7.82 (m, 2H, H-5, H-6), 7.33 (d, ^3J = 7.5 Hz, 1H, H-7), 7.05 (d, ^3J = 8.9 Hz, 2H, H-2', H-7'), 6.86 (d, ^3J = 8.9 Hz, 2H, H-1', H-8') ppm

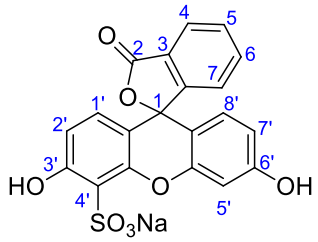
^{13}C NMR (100 MHz, DMSO-*d6*) δ 168.8 (C=O, C-2), 157.2 (2C), 152.7, 149.6 (2C), 130.8, 130.5 (2C), 126.7, 125.3 (2C), 124.6, 117.3 (2C), 114.9 (2C), 109.5 (2C), 99.9 ppm

MALDI-TOF-MS: 537.8 $[M + H]^+$

FT-IR $\tilde{V}$ (cm^{-1}) 3497 ν (OH), 1715 ν (C=O, lactone), 1585 ν (C=C, arom.), 1413, 1150, 1029

m.p. 258-260 °C

5.4.2 Synthesis of sodium 9-(2-carboxyphenyl)-6-hydroxy-3-oxo-3H-xanthene-5-sulfonate (43)



Chemical Formula: $C_{20}H_{11}NaO_8S$
Molecular Weight: 434,35

Fluorescein (5 g, 15 mmol) was heated at 140 °C in 10 mL of concentrated H_2SO_4 (95-97%) and stirred for 6 h.[38] The reaction mixture was cooled down and slowly poured in small portions onto 150 ml of stirring ice. The yellow-green precipitate occurred and was filtrated off. 3% NH_3 in water solution (200 mL) was added to dissolve the precipitate. The solution was filtrated off. CH_3COOH was added to an ammonia solution up to pH 6 as attempt to precipitate fluorescein but no precipitation occurred. The access of CH_3COOH was added and precipitate of HSO_3Fl was filtrated off and dried. Not only HSO_3Fl but also fluorescein was present. Therefore column chromatography on silica gel was performed with CH_2Cl_2/CH_3OH, 9:1 to elute fluorescein and afterwards 8:2 and finally 7:3 to obtain yellow solid with the yield of 30% (2 g, 4.6 mmol). After 1 month of storage of product at -24 °C in freezer the sulfo group was cleaved off and only fluorescein was presented. Therefore after the reaction proceeded and after adding of access of CH_3COOH, the precipitate should be treated with saturated NaCl to obtain sodium salt. The salt was proven to be stable form of mono-sulfo analogue of fluorescein.

TLC (silica gel, CH_2Cl_2/CH_3OH, 8:2) $R_f = 0.22$

^1H NMR (300 MHz, CD_3OD) δ 8.03 (d, ^3J = 7.4 Hz, 1H, H-4), 7.77 (m, 2H, H-5, H-6), 7.27 (d, ^3J = 7.4 Hz, 1H, H-7), 7.18 (s, 1H, H-5'), 6.81 (s, 1H, H-2'), 6.73 (s, 1H, H-1'), 6.58 (m, 2H, H-1', H-2') ppm

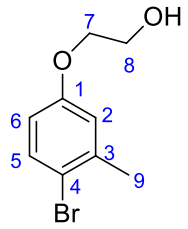
MALDI-TOF-MS: 413, 435, 457, 479 $[M + H]^+ + Na$

FT-IR $\tilde{V}$ (cm^{-1}) 3211 ν (OH), 1736 ν (C=O, lactone), 1585 ν (C=C, arom.), 1463, 1176, 1070

m.p. > 330 °C

5.5 Synthesis of Tokyo Green, its Building Blocks and Derivatives

5.5.1 Synthesis of 2-(4-bromo-3-methylphenoxy)ethanol (51)



Chemical Formula: $C_9H_{11}BrO_2$
Molecular Weight: 231,09

3-methyl-4-bromophenol (2 g, 10.7 mmol), ethylene carbonate (3.8 g, 42.8 mmol, 4.0 equiv) and K_2CO_3 (3 g, 21.4 mmol, 2.0 equiv) were dissolved in 55 mL of dry toluene under an argon atmosphere. The mixture was heated at 115 °C under reflux and stirred under an argon atmosphere for 45 h.[97] The crude reaction mixture was poured into water (100 mL) and extracted with EtOAc (3x120 mL). The organic layers were combined, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. According to TLC on silica gel (nHex/EtOAc, 1:1) the starting material was still presented but also a certain amount of product was formed. The column chromatography on silica gel was performed, using nHex/EtOAc, 2:1 as solvent system. The product a white semi-solid was obtained with the yield of 73% (1.69 g, 7.3 mmol).

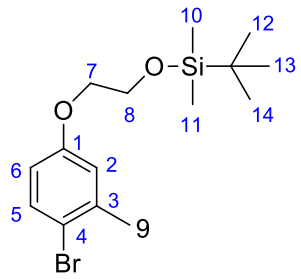
TLC (silica gel, nHex/EtOAc, 1:1) $R_f = 0.46$

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41 (d, ^3J = 9.6 Hz, 1H, H-5), 6.81 (d, ^4J = 3.3 Hz, 1H, H-2), 6.63 (dd, ^3J = 9.6, ^4J = 3.3 Hz, 1H, H-6), 4.04 (m, 2H, H-7), 3.95 (m, 2H, H-8), 2.36 (s, 3H, H-9) ppm

^{13}C NMR (75 MHz, $CDCl_3$) δ 157.6 (C-1), 137.8 (C-4), 132.7 (C-3), 117.1(C-6), 115.5 (C-2), 111.5 (C-5), 69.3 (C-7), 61.3 (C-8), 22.7 (C-9) ppm

FD-MS: m/z (%): 231.9 (100), 230.0 (97), 231.0 (9.9)

5.5.2 Synthesis of (2-(4-bromo-3-methylphenoxy)ethoxy)(*tert*-butyl)dimethylsilane (52)



Chemical Formula: $C_{15}H_{25}BrO_2Si$
Molecular Weight: 345,35

To a stirred solution of 2-(4-bromo-3-methylphenoxy)ethanol (1.69 g, 7.39 mmol) in DMF (40 ml) were added TBDMS-Cl (1.54 g, 10.24 mmol, 1.4 equiv) and imidazole (1.49 g, 21.94 mmol, 3.0 equiv).[97] The reaction was finished after stirring at room temperature for 2 h. According to TLC (nHex/EtOAc, 2:1) only a new product was present. DMF was evaporated *in vacuo*. The column chromatography on silica gel (nHex/EtOAc, 2:1) afforded the product as a colorless oil with the yield of 94% (2.37 g, 6.9 mmol).

TLC (silica gel, nHex/EtOAc, 1:1) $R_f = 0.80$

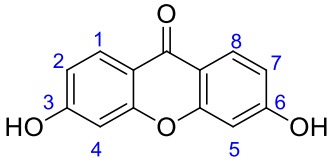
^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 (d, ^3J = 9.6 Hz, 1H, H-5), 6.80 (d, ^4J = 3.0 Hz, 1H, H-2), 6.62 (dd, ^3J = 9.6, ^4J = 3.0 Hz, 1H, H-6), 4.10 – 3.89 (m, 4H, H-7, H-8), 2.35 (s, 3H, H-9), 0.90 (s, 9H, H-12, H-13, H-14), 0.09 (s, 6H, H-10, H-11) ppm

^{13}C NMR (100 MHz, $CDCl_3$) δ 158.5 (C-1), 139.0, 132.7, 117.4, 115.7, 113.7, 77.2, 69.8, 62.1, 26.3 (3C, C-12, C-13, C-14), 23.4, 18.6, -4.94 (2C, C-10, C-11) ppm

FD-MS: m/z (%): 344.5 (100), 345.5 (16), 346.5 (90)

FT-IR $\tilde{V}$ (cm^{-1}) 2953 ν (CH_3, aliph.), 2928 (CH_3, aliph.), 2856 ν (CH_2, aliph.), 1594 ν (C=C, arom.), 1573, 1473, 1241, 1116, 831, 776

5.5.3 Synthesis of 3,6-dihydroxy-9H-xanthen-9-one (45)



Chemical Formula: $C_{13}H_8O_4$
Molecular Weight: 228,20

2,2',4,4'-tetrahydroxybenzophenone (10 g, 0.04 mol) was divided into 5 pressure tubes (2 g and 20 mL of distilled water in each). The reaction tubes were heated in an autoclave-like device at 200 °C for 4 h. After cooling the crude products were combined, filtered off and mixed with 100 ml of water. The resulting suspension was refluxed for 15 min and then filtered at 60 °C. The crystallization was repeated twice, because the starting material was still present after the first purification according to the TLC (nHex/EtOAc, 1:1). After the second re-crystallization, the product 3,6-dihydroxy-9*H*-xanthen-9-one was obtained as a light brown solid with the yield of 82% (7.67 g, 33.6 mmol).[37]

TLC (silica gel, nHex/EtOAc, 1:2) $R_f = 0.26$

^{1}H NMR (300 MHz, DMSO-d_6) δ 10.84 (s, 2H, H-4, H-5), 7.98 (d, ^3J = 8.7 Hz, 2H, H-1, H-8), 6.99 – 6.73 (m, 4H, H-2, H-7) ppm

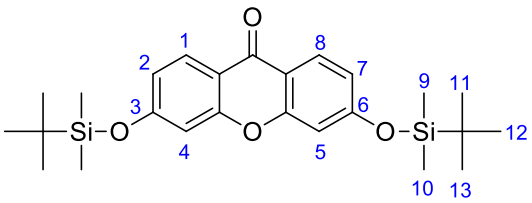
^{13}C NMR (100 MHz, DMSO-d_6) δ 173.9 (C=O), 163.5 (2C, C-3, C-6), 157.6 (2C, CO), 127.9 (2C, C-1, C-8), 114.2 (2C, C-2, C-7), 113.8 (2C), 102.1 (2C, C-4, C-5) ppm

FD-MS: m/z (%): 228.4 (100)

FT-IR $\tilde{V}$ (cm^{-1}) 3371 ν (OH), 3118 ν (C-H, arom.), 1609 (C=O), 1576 ν (C=C, arom.), 1453, 1254, 1170

m.p. > 330 °C

5.5.4 Synthesis of 3,6-bis((tert-butyldimethylsilyl)oxy)-9H-xanthen-9-one (46)



Chemical Formula: $C_{25}H_{36}O_4Si_2$
Molecular Weight: 456,72

To a stirred solution of 3,6-dihydroxy-9*H*-xanthen-9-one (3 g, 13.1 mmol) in DMF (80 ml) were added *tert*-butylchlorodimethylsilane (11.9 g, 78.9 mmol, 6 equiv) and imidazole (8.9 g, 131.5 mmol, 10 equiv).[97] The reaction was finished after stirring at room temperature for 2 h. The crude mixture was diluted with toluene, washed extensively 3 times with water and dried over Na_2SO_4. The solvent was evaporated *in vacuo* and a light brown solid occurred. The solid was recrystallized from ethanol to give the di-protected product as a white needle-like solid. The yield was 80% (4.8 g, 0.01 mol).

TLC (silica gel, nHex/EtOAc, 1:2) R_f = 0.88

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (d, ^3J = 9.1 Hz, 2H, H-1, H-8), 6.88 – 6.82 (m, 4H, H-2, H-4, H-5, H-7), 1.01 (s, 18H, H-11, H-12, H-13), 0.28 (s, 12H, H-9, H-10) ppm

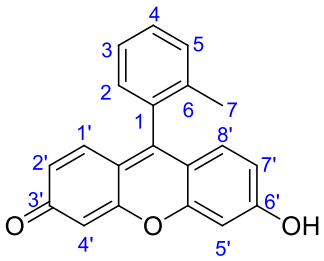
^{13}C NMR (100 MHz, $CDCl_3$) δ 175.9 (C=O), 161.7 (2C, C-3, C-6), 157.8 (2C, CO), 128.5 (2C, C-4, C-5), 117.7 (2C), 116.5 (2C), 107.5 (2C), 25.7 (6C, 2C-11, 2C-12, 2C-13), 18.6 (2C), -3.99 (4C, C-9, C-10) ppm

FD-MS: m/z (%): 456.6 (100)

FT-IR $\tilde{V}$ (cm^{-1}) 2955 ν (CH_3, aliph.), 2927 ν (CH_3, aliph.), 2892 ν (CH_3, aliph.), 2856 ν (CH_3, aliph.), 1656 ν (C=O), 1609 ν (C=C, arom.), 1439, 1254, 1181, 839

m.p. 151 °C

5.5.5 Synthesis of 6-hydroxy-9-(o-tolyl)-3H-xanthen-3-one (48)



Chemical Formula: $C_{20}H_{14}O_3$
Molecular Weight: 302,32

Following procedure was modified from literature.[45] In a dried 3-neck flask equip with condenser, magnesium pellets (530 mg, 22 mmol, 10.0 equiv) previously dried for 2 h at 160 °C, were suspended in 2 mL of dry Et_2O. The mixture was stirred under an argon atmosphere. 1,2-dibromoethane (200 µl, 2.2 mmol, 1.0 equiv) was added and after 10 min of stirring, 5 mL of 2-bromotoluene (794 µL, 11 mmol, 5 equiv) in dry Et_2O was added and the mixture was stirred for 2 h. The activation was proceeding according to the subsequent formation of heat and the turbid color. When no more heat formation was observed the solution of formed *o*-tolylmagnesium bromide was cooled to 0 °C and 3,6-bis((tert-butyldimethylsilyl)oxy)-9H-xanthen-9-one (1 g, 2.19 mmol) in 8 mL of dry THF was added. The mixture was stirred at 0 °C for 10 min. The color of reaction mixture changed from turbid to purple to brown and clear brown within 1 h. The reaction was stirred at rt overnight (16 h), quenched with a little amount of CH_3OH (the mixture already turned fluorescent), followed by filtration of Mg. 2 M HCl (20 mL) was added and stirred for 1 h while monitored by TLC on silica gel

(CH_2Cl_2/CH_3OH, 9:1) every 15 min. The mixture was extracted several times with EtOAc until the water phase lost fluorescence. The organic layers were collected and dried over Na_2SO_4. The solvent was evaporated *in vacuo*. The column chromatography on silica gel (CH_2Cl_2/CH_3OH, 95:5) was performed and afforded the product as an orange-red solid with the yield of 70% (460 mg, 1.52 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 9:1) R_f = 0.34

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.51 – 7.41 (m, 2H, H-2, H-3), 7.38 (td, ^3J = 7.2 Hz, ^4J =1.6 Hz, 1H, H-4), 7.21 (d, ^3J = 7.2 Hz, 1H, H-5), 6.63 (d, ^3J = 9.3 Hz, 2H, H-1', H-8'), 6.31 (dd, ^3J = 9.3 Hz, ^4J = 2.0 Hz, 2H, H-2', H-7'), 6.24 (d, ^4J = 2.0 Hz, 2H, H-4', H-5'), 2.02 (s, 3H, H-7) ppm

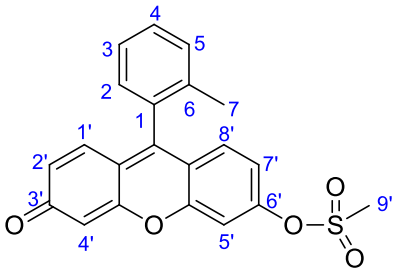
^{13}C NMR (75 MHz, DMSO-d_6) δ 161.0 (C=O), 155.8, 154.0, 153.4, 126.5 (4C), 111.5 (4C), 109.1, 107.8 (2C), 98.8 (4C), 18.3 (C-7) ppm

FD-MS: m/z (%): 303.5 (100), 302.5 (42)

FT-IR $\tilde{V}$ (cm^{-1}) 3068 ν (C-H, arom.), 2921 ν (CH_3, aliph.), 1733 ν (C=O), 1568 ν (C=C, arom.), 1417, 1250, 1156

m.p. > 330 °C

5.5.6 Synthesis of 3-oxo-9-(o-tolyl)-3H-xanthen-6-yl methanesulfonate (49)



Chemical Formula: $C_{21}H_{16}O_5S$
Molecular Weight: 380,41

Tokyo Green (50 mg, 0.165 mmol) was dissolved in 5 mL of dry DCM under an argon atmosphere. DMAP (122 mg, 0.99 mmol, 6.0 equiv) was added and the mixture was stirred for 30 min. The reaction mixture was cooled to 0 °C and methanesulfonyl chloride (51 µl, 0.66 mmol, 6.0 equiv) in dry CH_2Cl_2 (0.5 ml) was added in a dropwise manner. After adding methanesulfonyl chloride, the orange fluorescent clear solution became turbid.

The suspension was monitored by TLC on silica gel (CH_2Cl_2/CH_3OH, 9:1, 95:5). After 2 h the starting material was converted and the reaction was quenched with small amount of acetic acid.

The crude reaction mixture was diluted with 10 mL of CH_2Cl_2, adding 10 mL of water and extracting with 2x10 mL of CH_2Cl_2. The organic phase was collected and dried over Na_2SO_4. The solvent was evaporated *in vacuo* and the crude product was purified by chromatography on silica gel using gradient 100% CH_2Cl_2 to CH_2Cl_2/MeOH 98:2. The TLC on silica gel (CH_2Cl_2/CH_3OH, 9:1) showed after longer irradiation under UV light additional spot with higher R_F value. The product was re-purified by column chromatography on silica gel using elution mixture petrolether/EtOAc 2:1. The product was obtained as a light brown solid with the yield of 55% (34 mg, 0.09 mmol).

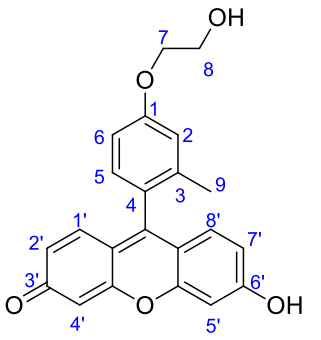
TLC (silica gel, CH_2Cl_2/CH_3OH, 9:1) $R_f = 0.8$

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49 (m, 2H, H-2, H-3), 7.41 (m, 2H, H-4, H-5), 7.16 (m, 3H, H-1', H-2', H-4'), (d, 7.05 (d, $^3J = 9.6$ Hz, 1H, H-8'), 6.74 (dd, $^3J = 9.6$ Hz, $^4J = 2.0$ Hz, 1H, H-7'), 6.69 (s, 1H, H-5'), 3.28 (s, 3H, H-9'), 2.08 (s, 3H, H-7) ppm

^{13}C NMR (75 MHz, $CDCl_3$) δ 160.9 (C=O), 159.0, 155.2, 154.0, 134.2, 131.0, 130.1, 128.5 (4C), 124.6 (2C), 121.0, 112.3, 109.1, 107.8 (2C), 99.1, 97.3, 18.6 (C-7) ppm

FD-MS: m/z (%): 380.5 (100)

5.5.7 Synthesis of 6-hydroxy-9-(4-(2-hydroxyethoxy)-2-methylphenyl)-3*H*-xanthen-3-one (53)



Chemical Formula: $C_{22}H_{18}O_5$
Molecular Weight: 362,38

In a flame-dried 3-neck flask under fitted with condenser dried Mg (1 g, 40 mmol) was suspended in a minute quantity of dry Et_2O under an argon atmosphere. 1,2-dibromoethane (200 µL, 2.2 mmol, 1.0 equiv) was added. The activation took approximately 10 min,

followed by the dropwise addition of (2-(4-bromo-3-methylphenoxy)ethoxy)(*tert*-butyl)dimethylsilane (1.41 g, 4.09 mmol, 1.8 equiv) in dry Et_2O (6 ml). The formation of heat occurred and the flask warmed up. The gas formation was maintained by interval warming with a heat gun. The activation took place approximately 1 h. When no more heat formation was observed, the mixture was stirred for 30 min at room temperature then cooled to 0 °C and 3,6-bis((tert-butyldimethylsilyl)oxy)-9H-xanthen-9-one (1 g, 2.19 mmol) in dry THF (5 mL) was added dropwise. The reaction color changed from blurry to purple within 5 min. The reaction color then changed from dark purple to dark green within 2 h. The mixture was stirred for an additional 4 h. The reaction was quenched with CH_3OH (stirred for 5 min), the color became slightly green fluorescent and the remaining Mg was filtered off. The deprotection of the TBDMS groups, using 20 mL of 2 M HCl took place and the mixture became orange. After 10 min TLC on silica gel (CH_2Cl_2/CH_3OH, 9:1) showed only one fluorescent spot. H_2O (5 mL) was added and the mixture was extracted several times with 40 ml of EtOAc. The organic layers were combined and dried over Na_2SO_4. The solvent was evaporated *in vacuo*. The crude mixture was purified by gradient column chromatography on silica gel, using CH_2Cl_2/CH_3OH, 98:2, 95:5, 9:1. The product, modified TokyoGreen, was obtained as an orange-red solid with the yield of 45% (360 mg, 0.99 mmol).[97]

TLC (silica gel, CH_2Cl_2/CH_3OH, 9:1) R_f = 0.44

^{1}H NMR (400 MHz, CD_3OD) δ 7.15 (dd, ^3J = 9.6 Hz, ^4J = 3.8 Hz, 3H, H-2, H-5, H-6), 7.08 (d, ^4J = 2.2 Hz, 1H, H-4'), 7.03 (dd, ^3J = 8.4 Hz, ^4J = 2.2 Hz, 1H, H-2'), 6.74 (m, 4H, H-1', H-5', H-7', H-8'), 4.17 (t, ^3J = 4.4, 2H, H-7), 3.94 (t, ^3J = 4.4, 2H, H-8), 2.03 (s, 3H, H-9) ppm

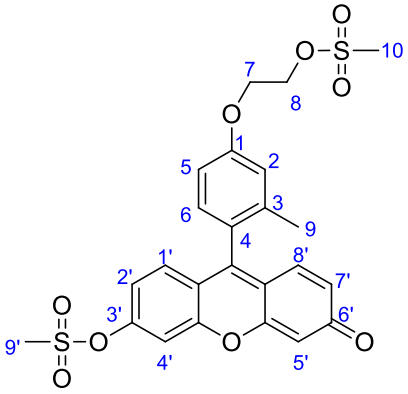
^{13}C NMR (100 MHz, CD_3OD) δ 161.6, 159.3 (2C), 156.2 (2C), 139.0, 132.6 (2C), 131.5 (2C), 125.8 (2C), 117.7 (2C), 116.8, 113.4 (2C), 104.4 (2C), 70.8 (C-7), 61.6 (C-8), 19.9 (C-9) ppm

FD-MS: m/z (%): 362.5 (100), 363.5 (53)

FT-IR $\tilde{V}$ (cm^{-1}) 3064 ν (C-H, arom.), 2921 ν (CH_3, aliph.), 2859 ν (CH_2, aliph.), 1593 ν (C=C, arom.), 1455, 1380, 1239, 1201

m.p. 247-250 °C

5.5.8 Synthesis of 9-(2-methyl-4-(2-((methylsulfonyl)oxy)ethoxy)phenyl)-3-oxo-3H-xanthen-6-yl methanesulfonate (54)



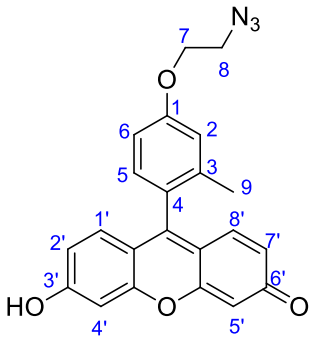
Chemical Formula: $C_{24}H_{22}O_9S_2$
Molecular Weight: 518,56

In a 2-neck flask 6-hydroxy-9-(4-(2-hydroxyethoxy)-2-methylphenyl)-3*H*-xanthen-3-one (70 mg, 0.19 mmol) was suspended in dry CH_2Cl_2 (7 mL).[97] Under an argon atmosphere, the suspension was cooled to 0 °C and DMAP (93 mg, 0.76 mmol, 4.0 equiv) was added. After 5 min methanesulfonyl chloride (60 µL, 0.76 mmol, 4.0 equiv) in 1 mL of dry CH_2Cl_2 was added. The suspension was stirred for 30 min at 0 °C and then warmed to room temperature and stirred for 24 h to obtain a clear solution. According to TLC on silica gel (CH_2Cl_2/CH_3OH, 9:1) no starting material was present and some new fluorescent spots appeared. The mixture was cooled to 0 °C and additional methanesulfonyl chloride (60 µL, 0.77 mmol, 4.0 equiv) in 0.5 mL of dry CH_2Cl_2 was added. After additional 24 h according to TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5), 3 spots were present. The solvent was evaporated and the crude mixture without any extensive work-up was purified by preparative TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5). The di-mesylated product was obtained as orange oil with the yield of 35% (35 mg, 0.067 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 9:1) $R_f = 0.7$

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 (d, ^3J = 8.6 Hz, 1H, H-5), 7.15 (m, 3H, H-2, H-6, H-5'), 6.99 (m, 4H, H-2', H-4', H-7', H-8'), (d, J = 16.9 Hz, 1H, H-1'), 4.57 (m, 2H, H-7), 4.24 (m, 2H, H-8), 3.19 (s, 3H, H-9'), 3.10 (s, 3H, H-10) ppm

5.5.9 Synthesis of 9-(4-(2-azidoethoxy)-2-methylphenyl)-6-hydroxy-3H-xanthen-3-one (MK-57) (55)



Chemical Formula: $C_{22}H_{17}N_3O_4$
Molecular Weight: 387,39

(30 mg, 0.068 mmol) of mesylated starting material and NaN_3 (22 mg, 0.338 mmol, 5.0 equiv) were dissolved under an argon atmosphere in 7 mL of dry DMF. The mixture was warmed to 60 °C and stirred for 46 h.[97] DMF was evaporated and the preparative TLC on silica gel, CH_2Cl_2/CH_3OH, 95:5 was performed. The product was removed from the TLC plate and extracted from silica gel with CH_2Cl_2/CH_3OH, 9:1. Lyophilization from dioxane/water 3:1 afforded the product as an orange powder with the yield of 23% (6 mg, 0.015 mmol).

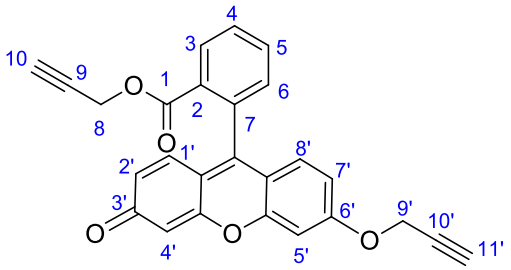
TLC (silica gel, CH_2Cl_2/CH_3OH, 9:1) $R_f = 0.52$

^{1}H NMR (400 MHz, CD_3OD) δ 7.16 (d, ^3J = 8.3 Hz, 1H, H-5), 7.11 (d, ^3J = 9.6 Hz, 2H, H-1', H-8'), 7.08 (d, ^4J = 2.5 Hz, 1H, H-2), 7.05 (dd, ^3J = 8.3 Hz, ^4J = 2.5 Hz, 1H, H-6), 6.71 (d, ^4J = 2.0 Hz, 2H, H-4', H-5'), 6.68 (m, 2H, H-5', H-7'), 4.30 (t, ^3J = 4.6 Hz, 2H, H-7), 3.60 (t, ^3J = 4.6 Hz, 2H, H-8), 2.03 (s, 3H, H-9) ppm

FT-IR $\tilde{V}$ (cm^{-1}) 3374 ν (OH), 2922 ν (CH_3, aliph.), 2853 ν (CH_2, aliph.), 2108 ν (N_3), 1699 ν (C=O), 1600 ν (C=C, arom.), 1503, 1397, 1294, 1074

5.6 Synthesis of Propargyl Ether Fluorescein

5.6.1 Synthesis of prop-2-yn-1-yl 2-(3-oxo-6-(prop-2-yn-1-yloxy)-3H-xanthen-9-yl)benzoate (56)



Chemical Formula: $C_{26}H_{16}O_5$
Molecular Weight: 408,40

Fluorescein (5 g, 0.015 mol) and K_2CO_3 (4.14 g, 0.03 mol, 2.0 equiv) were suspended under an argon atmosphere in acetone (50 mL). The mixture was stirred for approximately 30 min. During this period of time fluorescein did not dissolve. The propargyl bromide (2.67 mL, 0.03 mol, 2.0 equiv) in 10 mL of acetone was added to the reaction mixture. The mixture was refluxed overnight at 56 °C (boiling point of acetone). The color of the reaction mixture changed from dark red to orange. The mixture was poured onto 100 mL of cold water. The allyl ether ester and the allyl diether of fluorescein precipitated, while the unreacted fluorescein but also a small amount of monoallyl ether and monoallyl ester dissolved, giving a rise to a deep red solution, which exhibited a green fluorescence. The orange precipitate was filtrated off, washed with water and dried under reduced pressure. Upon re-crystallizing the crude material from tetrachloromethane, the product propargyl ether ester of fluorescein was obtained as an orange solid with the yield of 15% (900 mg, 2.2 mmol). (Lit. 42%)[98]

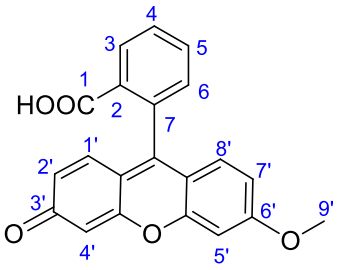
TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.64

^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.22 (d, ^3J = 7.5 Hz, 1H, H-3), 7.91 (td, ^3J = 7.5 Hz, ^4J =1.0 Hz, 1H, H-5), 7.82 (td, ^3J = 7.5 Hz, ^4J =1.0 Hz, 1H, H-4), 7.54 (d, ^3J = 7.5 Hz, 1H, H-6), 7.32 (d, ^4J = 2.0 Hz, 1H, H-4'), 7.00 – 6.91 (m, 2H, H-1', H-2'), 6.88 (d, ^3J = 9.7 Hz, 1H, H-7'), 6.49 (d, ^3J = 9.7 Hz, 1H, H-8'), 6.38 (s, 1H, H-5'), 5.02 (d, ^4J = 2.5 Hz, 2H, H-8), 4.65 (d, ^4J = 2.5 Hz, 2H, H-9'), 3.70 (t, ^4J = 2.5 Hz, 1H, H-10), 3.40 (t, ^4J = 2.5 Hz, 1H, H-11') ppm

^{13}C NMR (125 MHz, CD_3OD) δ 162.2, 158.5, 133.7, 133.5, 130.9, 130.7, 130.7 (2C), 130.3, 129.2, 128.9 (2C), 128.7, 117.2, 115.1, 114.5, 108.7, 104.4, 101.6 (2C), 79.3 (2C, C-10', C-9), 77.9 (2C, C-11', C-10), 56.5 (C-8), 52.7 (C-9') ppm

MALDI-TOF-MS: 409.3 $[M + H]^+$

5.6.2 Synthesis of 2-(6-methoxy-3-oxo-3H-xanthen-9-yl)benzoic acid (57)



Chemical Formula: $C_{21}H_{14}O_5$
Molecular Weight: 346,33

Hydrolysis of ester bond

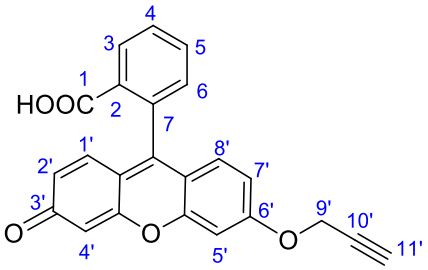
Propargyl ether ester of fluorescein (900 mg, 2.59 mmol) was dissolved in 8 mL of MeOH. NaOH (500 mg, 12.5 mmol, 5.0 equiv) was added and the mixture was refluxed for 2 h. The reaction was monitored by TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5). After 2 h only traces of starting product, small amount of fluorescein and new product were present. A part of the CH_3OH was distilled off and the solution was poured into 20 mL of water, filtered to remove traces of insoluble impurities and acidified with hydrochloric acid. A yellow precipitate formed immediately. The precipitate was washed with water and dried under reduced pressure. The residue was purified by chromatography on silica gel with CH_2Cl_2/CH_3OH, 98:2 as elution mixture. The by-product, fluorescein methyl monoether was obtained as yellow solid with the yield of 72% (650 mg, 1.88 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.8

^{1}H NMR (500 MHz, DMSO-*d6*) δ 10.16 (s, 1H, COOH), 8.00 (dt, ^3J = 8 Hz, ^4J = 1 Hz, 1H, H-3), 7.79 (td, ^3J = 7.5 Hz, ^4J =1.5 Hz, 1H, H-5), 7.72 (td, ^3J = 7.5 Hz, ^4J =1.5 Hz, 1H, H-4), 7.27 (dt, ^3J = 8 Hz, ^4J = 1Hz, H-6), 6.94 (d, ^4J = 2.5 Hz, 1H, H-4'), 6.74 – 6.67 (m, 2H, H-1', H-2'), 6.65 (d, ^3J = 8.8 Hz, 1H, H-8'), 6.60 – 6.54 (m, 2H, H-5', H-7'), 3.81 (s, 3H, H-9') ppm

MALDI-TOF-MS: 347.0 $[M + H]^+$

5.6.3 Synthesis of 2-(3-oxo-6-(prop-2-yn-1-yloxy)-3H-xanthen-9-yl)benzoic acid (58)



Chemical Formula: $C_{23}H_{14}O_5$
Molecular Weight: 370,35

Hydrolysis of ester bond using propargyl alcohol

The propargyl ether ester of fluorescein (230 mg, 0.56 mmol) was dissolved in 2 mL of propargyl alcohol. NaOH (100 mg, 2.5 mmol, 4.0 equiv) was added and the mixture was heated at 75 °C for 5 h. The reaction was monitored by TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) over 5 h. Afterwards propargyl alcohol was distilled off, the solution was poured into 4 ml of water, filtered to remove traces of insoluble impurities and acidified with HCl. A yellow precipitate formed immediately. The precipitate was washed with water and dried under the reduced pressure. The residue was purified by chromatography on silica gel with CH_2Cl_2/CH_3OH, 98:2 as elution mixture. The product fluorescein propargyl monoether was obtained as a yellow solid with the yield of 87% (180 mg, 0.486 mmol).[98]

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.76

^{1}H NMR (400 MHz, CD_3OD) δ 8.00 (d, ^3J = 7.6 Hz, 1H, H-3), 7.76 (td, ^3J = 7.6 Hz, ^4J =1.2 Hz, 1H, H-5), 7.72 – 7.64 (td, ^3J = 7.6 Hz, ^4J =1.2 Hz, 1H, H-4), 7.20 (d, ^3J = 7.6 Hz, 1H, H-6), 6.93 (d, ^4J = 2.3 Hz, 1H, H-4'), 6.83 – 6.64 (m, 3H, H-1', H-2', H-8'), 6.64 – 6.47 (m, 2H, H-5', H-7'), 4.78 (d, ^4J = 2.4 Hz, 2H, H-9'), 2.99 (t, ^4J = 2.4 Hz, 1H, H-11') ppm

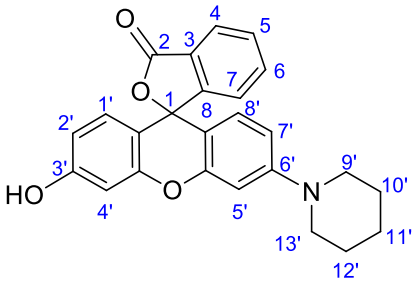
^{13}C NMR (100 MHz, CD_3OD) δ 171.5, 161.2, 160.9, 154.3, 153.9, 153.8, 136.6, 131.1, 130.1, 130.1 (2C), 128.1, 125.8, 125.3, 113.7, 113.2 (2C), 111.2, 103.6, 103.1, 79.2 (C-10'), 77.3 (C-11'), 56.9 (C-9') ppm

MALDI-TOF-MS: 371.0 $[M + H]^+$

FT-IR $\tilde{V}$ (cm^{-1}) 3303 ν (C-H, alkyne), 2161 ν (C≡C), 1733 ν (C=O), 1609 ν (C=C, arom.), 1502, 1433, 1179

5.7 Synthesis of Mono-piperidyl and Bis-piperidyl Xanthene Derivatives

5.7.1 Synthesis of 3'-hydroxy-6'-(piperidin-1-yl)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (59) and 3',6'-di(piperidin-1-yl)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (60)



Chemical Formula: $C_{25}H_{21}NO_4$
Molecular Weight: 399,44

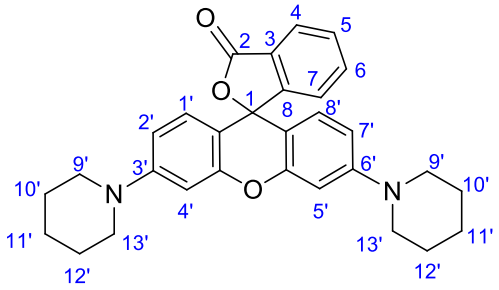
Fluorescein (100 mg, 0.3 mmol) was dissolved under an argon atmosphere in pyridine (4 mL). The mixture was stirred for 10 min until fluorescein was completely dissolved. Then the mixture was cooled to 0 °C and methanesulfonyl chloride (21 µL, 0.27 mmol, 0.9 equiv) in pyridine (1 ml) was added slowly in a dropwise manner. The reaction was stirred for 30 min and then allowed to warm to rt. The reaction was monitored by TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) to prove formation of mono-mesylated fluorescein. In addition to this intermediate product also starting material and di-mesylated by-product were also presented. The mixture was stirred for 4 h and then cooled to 0 °C. The piperidine in excess (296 µL, 3 mmol, 10.0 equiv) was added to the solution and stirred at 0 °C for 30 min.[89] Then the cooling was removed and the mixture was stirred over night. According to TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) several fluorescent and non-fluorescent spots were presented. The solvent was evaporated *in vacuo* and the residue was dried under reduced pressure. The purification by preparative TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5, 3% CH_3COOH) delivered mono-piperidyl product as an orange solid with insignificant yield. Fluorescein, mono-mesylated fluorescein, di-mesylated fluorescein and red solid di-piperidyl of fluorescein were presented as by-products.

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5, 3% CH_3COOH) R_f = 0.34

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (m, 1H, H-4), 7,62-7.72 (m, 2H, H-5, H-6), 7.32 (m, 1H, H-7), 6.63 (m, 2H, H-4', H-5'), 6.42-6.57 (m, 4H, H-2', H-7', H-1', H-8'), 3.27 (m, 4H, H-9', H-13'), 1.56 (m, 2H, H-11'), 1.23 (m, 4H, H-10', H-12') ppm

MALDI-TOF-MS: 400.58 $[M + H]^+$

3',6'-Di(piperidin-1-yl)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (60)



Chemical Formula: $C_{30}H_{30}N_2O_3$
Molecular Weight: 466,57

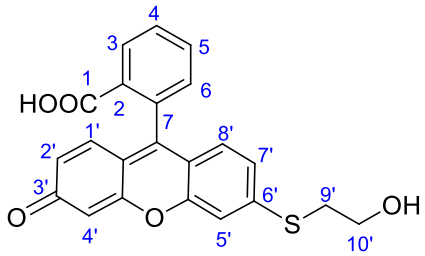
TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5, 3% CH_3COOH) $R_f = 0.2$

^1H NMR (300 MHz, CD_3OD-*d4*) δ 8.02 (dt, $^3J = 8$ Hz, $^4J = 1.3$ Hz, 1H, H-4), 7.72 (td, $^3J = 8.0$ Hz, $^4J = 1.3$ Hz, 1H, H-6), 7.61 (td, $^3J = 8$ Hz, $^4J = 1.3$ Hz, 1H, H-5), 7.40 (dt, $^3J = 8$ Hz, $^4J = 1.3$ Hz, 1H, H-7), 6.72 (d, $^3J = 9.6$ Hz, 2H, H-1', H-8'), 6.58 (d, $^4J = 2.7$ Hz, 2H, H-4', H-5'), 6.55 (dd, $^3J = 9.6$ Hz, $^4J = 2.7$ Hz, 2H, H-2', H-7'), 3.37 (m, 8H, H-9', H-13'), 1.42 (m, 4H, H-11'), 1.28 (m, 8H, H-10', H-12') ppm

MALDI-TOF-MS: 467.6 $[M + H]^+$

5.8 Synthesis of 6'-Ethylthio-xanthene Dyes and Related Derivatives

5.8.1 Synthesis of 2-(6-((2-hydroxyethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (MK-43) (61)



Chemical Formula: $C_{22}H_{16}O_5S$
Molecular Weight: 392,42

3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (2 g, 4.1 mmol) was dissolved under an argon atmosphere in 24 mL of dry DMF. DBU (0.61 ml, 4.1 mmol, 1.0 equiv) was added into 2-mercaptoethanol (1.15 ml, 16.4 mmol, 4.0 equiv) in 2 mL of dry DMF and the mixture was stirred for 30 min. The reaction mixture of starting material was cooled to 0 °C and the mixture with activated 2-mercaptoethanol was added dropwise. Then the reaction was allowed to warm up to room temperature. The pH of the reaction mixture after adding the reagents was 8. The reaction was monitored by TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) over a period of 4 h. After 4 hours the starting material was not completely converted but fluorescein started to appear on TLC. The mixture was stirred additionally for 4 h, and then neutralized with acetic acid and the solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel using the gradient elution mixture from 100% CH_2Cl_2 up to 95:5 CH_2Cl_2/CH_3OH. The product was obtained as a slightly yellow solid with the yield of 45% (0.720 g, 1.8 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) $R_f = 0.33$

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (s, 1H, COOH), 8.01 (d, ^3J = 7.6 Hz, 1H, H-3), 7.79 (t, ^3J = 7.6 Hz, 1H, H-5), 7.72 (t, ^3J = 7.6 Hz, 1H, H-4), 7.34 – 7.23 (m, 2H, H-6, H-4'), 7.02 (dd, ^3J = 8.4 Hz, ^4J = 1.6 Hz, 1H, H-2'), 6.71 (s, 1H, H-5'), 6.65 (d, ^3J = 8.4 Hz, 1H, H-1'), 6.59 (m, 2H, H-7', H-8'), 3.61 (t, ^3J = 6.8 Hz, 2H, H-10'), 3.13 (t, ^3J = 6.8 Hz, 2H, H-9') ppm

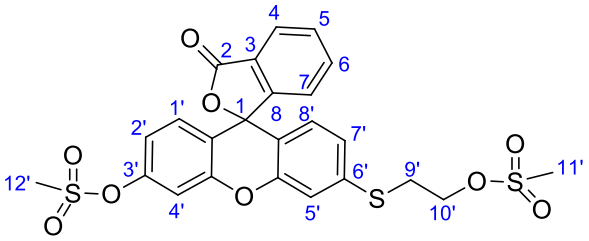
^{13}C NMR (100 MHz, DMSO-d_6) δ 168.62 (COOH), 159.6 (C=O), 152.3, 151.5, 151.0, 140.8, 135.7, 130.2, 129.1, 128.2, 125.8, 124.7, 124.0, 122.7, 115.5, 113.8, 112.9, 109.2, 102.3, 82.3, 39.5 (C-10'), 34.0 (C-9') ppm

FD-MS: m/z (%): 392.1 (100)

FT-IR $\tilde{\nu}$ (cm^{-1}) 3520 ν (OH), 3260 ν (OH), 1731 ν (C=O), 1603 ν (C=C, arom.), 1405, 1235, 1113, 844

m.p. 232-235 °C

5.8.2 Synthesis of 2-(3'-(methylsulfonyloxy)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-ylthio)ethyl methanesulfonate (MK-60) (69)



Chemical Formula: $C_{24}H_{20}O_9S_3$
Molecular Weight: 548,61

2-(6-((2-hydroxyethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (200 mg, 0.51 mmol) and DMAP (374 mg, 3.06 mmol, 6.0 equiv) were suspended in 10 mL of dry CH_2Cl_2 under an argon atmosphere. The turbid mixture was stirred for 30 min. The reaction mixture was cooled to 0 °C and methanesulfonyl chloride (158 µL, 2.04 mmol, 4.0 equiv) in 1 mL of dry CH_2Cl_2 was added dropwise. The suspension was stirred for 30 min at 0 °C, warmed to room temperature and then stirred for 14 h. Additional 10 mL of CH_2Cl_2 was added and the solution was washed with 15 mL of water. The water phase was extracted twice with CH_2Cl_2 (15 mL). The organic phases were combined, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was separated by gradient column chromatography over silica gel (100% CH_2Cl_2 up to CH_2Cl_2/CH_3OH, 99:1). (Silica gel was first deactivated with 3% of Et_3N in CH_2Cl_2). The product was obtained as a white-beige fluffy solid with the yield of 77.5% (217 mg, 0.39 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.89

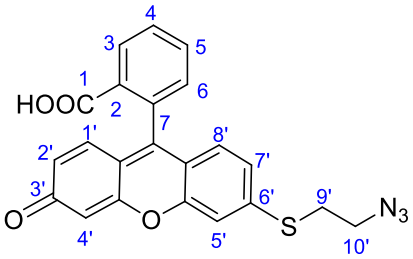
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 – 8.02 (m, 1H, H-4), 7.73 – 7.63 (m, 2H, H-5, H-6), 7.29 (d, ^4J = 2.4 Hz, 2H, H-4', H-5'), 7.17 (m, 1H, H-7), 7.04 (dd, ^3J = 8.4 Hz, ^4J = 1.9 Hz, 1H, H-2'), 7.00 (dd, ^3J = 8.7 Hz, ^4J = 2.4 Hz, 1H, H-7'), 6.88 (d, ^3J = 8.7 Hz, 1H, H-8'), 6.77 (d, ^3J = 8.4 Hz, 1H, H-1'), 4.37 (t, ^3J = 7.2 Hz, 2H, H-10'), 3.31 (t, ^3J = 7.2 Hz, 2H, H-9'), 3.20 (s, 3H, H-12'), 3.02 (s, 3H, H-11') ppm

^{13}C NMR (100 MHz, $CDCl_3$) δ 169.1 (C=O), 152.7, 151.8, 151.3, 150.3, 138.7, 135.7, 130.5, 129.8 (2C), 128.8, 126.2, 125.6, 124.6, 124.0, 118.4, 117.9, 117.1, 116.7, 111.2, 67.3 (C-10'), 38.0 (C-11'), 37.9 (C-12'), 32.0 (C-9') ppm

ESI-MS: m/z (%): 549.06 (100)

FT-IR $\tilde{V}$ (cm^{-1}) 1764 ν (C=O, lactone), 1598 ν (C=C, arom.), 1405, 1352 ν (S=O), 1171, 1106, 967

5.8.3 Synthesis of 2-(6-(2-azidoethylthio)-3-oxo-3H-xanthen-9-yl)benzoic acid (MK-61) (70)



Chemical Formula: $C_{22}H_{15}N_3O_4S$
Molecular Weight: 417,44

Under an argon atmosphere, 2-(3'-(methylsulfonyloxy)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-ylthio)ethyl methanesulfonate (76 mg, 0.138 mmol) and sodium azide (45 mg, 0.69 mmol, 5.0 equiv) were dissolved in 10 mL of dry DMF. The reaction mixture was warmed to 60 °C and stirred for 52 h. According to TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) the starting material was not completely converted, but 2 new fluorescent spots were present. Silica gel for chromatography was deactivated using 3% Et_3N in CH_2Cl_2. Column chromatography on silica gel using a gradient elution system of 100% CH_2Cl_2 up to CH_2Cl_2/CH_3OH, 97:3 did not deliver pure product. Preparative TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) was performed. The product 2-(6-(2-azidoethylthio)-3-oxo-3H-xanthen-9-yl)benzoic acid was obtained as an orange fluffy solid with the yield of 33% (19 mg, 0.0455 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.77

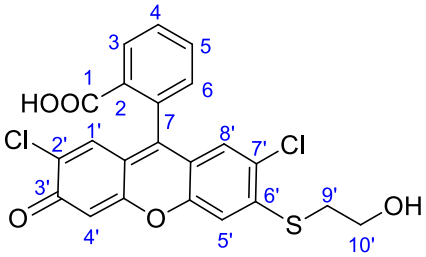
^{1}H NMR (400 MHz, CD_3OD) δ 8.04 (dd, ^3J = 7.6 Hz, ^4J = 0.8 Hz, 1H, H-3), 7.79 (td, ^3J = 7.6 Hz, 1.2 Hz, 1H, H-5), 7.73 (td, ^3J = 7.6 Hz, ^4J = 1.2 Hz, 1H, H-4), 7.33 (d, ^4J = 1.9 Hz, 1H, H-4'), 7.23 (d, ^3J = 7.6 Hz, 1H, H-6), 7.07 (dd, ^3J = 8.4 Hz, ^4J = 1.9 Hz, 1H, H-2'), 6.73 (m, 2H, H-1', H-5'), 6.62 (d, ^3J = 8.8 Hz, 1H, H-8'), 6.57 (dd, ^3J = 8.8, Hz, ^4J = 2.4 Hz, 1H, H-7'), 3.54 (t, ^3J = 6.6 Hz, 2H, H-10'), 3.24 (t, ^3J = 6.6 Hz, 2H, H-9') ppm

^{13}C NMR (100 MHz, CD_3OD) δ 171.3 (COOH), 154.4, 153.6, 153.0, 140.8, 136.7, 131.3, 130.1, 129.6 (2C), 127.8, 125.9, 125.2, 124.8, 118.1, 117.1, 113.8, 110.9, 103.6, 51.3, 49.0 (C-10'), 33.3 (C-9') ppm

ESI-MS: m/z (%): 418.10 (100)

FT-IR $\tilde{V}$ (cm^{-1}) ν 3258 (OH), 2933 (CH_2, aliph.), 2102 ν (N_3), 1730 ν (C=O), 1630, 1601 ν (C=C, arom.), 1457, 1405, 1221, 1112

5.8.4 Synthesis of 2-(2,7-dichloro-6-((2-hydroxyethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (MK-67) (64)



Chemical Formula: $C_{22}H_{14}Cl_2O_5S$
Molecular Weight: 461,31

2',7'-dichloro-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (500 mg, 0.897 mmol) was dissolved under an argon atmosphere in 20 mL of dry DMF. Separately DBU (134 µL, 0.897 mmol, 1.0 equiv) was added into mercaptoethanol (575 µL, 3.59 mmol, 4.0 equiv) in 2 mL of dry DMF and the mixture was stirred for 30 min. The reaction mixture of starting material was cooled to 0 °C and the separately prepared mixture was added dropwise. Then the reaction was allowed to warm up to room temperature. The reaction was monitored by TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5) within time period of 4 h. After 4 h the starting material was still presented in large amount therefore the mixture was stirred over night. After quenching of the reaction with 50 µl of conc. acetic acid, the solvent DMF was evaporated *in vacuo*. The crude mixture was co-distillated from CCl_4 to remove access of mercaptoethanol. The residue was dried under reduced pressure and purified by gradient column chromatography over silica gel using CH_2Cl_2/CH_3OH, 99:1 to remove di-mesylated starting material plus mono-mesylated by-product (both products of hydrolysis). An increase of CH_3OH from 1% to 2-4% delivered an orange solid product with the yield of 41% (140 mg, 0.303 mmol).

The exact reaction with the same reaction conditions using 3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diylbis(2,4,6-triisopropylbenzenesulfonate) (840 mg, 0.897 mmol) delivered an orange solid product with the yield of 46% (190 mg, 0.412 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.37

^{1}H NMR (400 MHz, DMSO-*d6*) δ 11.13 (s, 1H, COOH), 8.03 (d, 1H, ^3J = 7.6 Hz, H-3), 7.82 (td, ^3J = 7.6 Hz, ^4J = 1.2 Hz, 1H, H-5), 7.76 (td, ^3J = 7.6 Hz, ^4J = 1.2 Hz, 1H, H-4), 7.41 (s, 1H, H-4'), 7.36 (d, ^3J = 7.6 Hz, 1H, H-6), 6.95 (s, 1H, H-5'), 6.79 (s, 1H, H-1'), 6.70 (s, 1H, H-8'), 5.11 (t, ^3J = 5.6 Hz, 1H, OH), 3.69 (q, ^3J = 6.4 Hz, 2H, H-10'), 3.22 (t, ^3J = 6.4 Hz, 2H, H-9') ppm

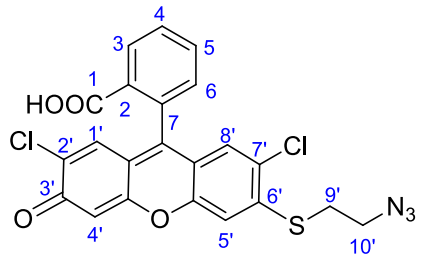
^{13}C (75 MHz, DMSO-*d6*) δ 168.2 (C=O), 155.3 (C=O), 151.4, 149.9, 149.6, 140.7, 136.0, 130.7, 128.3, 127.6, 125.7, 124.9, 123.8, 116.5 (2C, C-2', C-7'), 115.9, 114.00, 110.2, 103.7, 80.9, 59.1 (C-10'), 33.8 (C-9') ppm

FD-MS: m/z (%): 460.5 (100), 461.5 (29.5), 462.5 (57.5)

FT-IR $\tilde{V}$ (cm^{-1}) 3536 ν (OH), 3160 ν (OH), 2923 ν (CH_2, aliph.), 1767 ν (C=O), 1593 ν (C=C, arom.), 1439, 1387, 1284, 860

m.p. 274-276 °C

5.8.5 Synthesis of 2-(6-((2-azidoethyl)thio)-2,7-dichloro-3-oxo-3H-xanthen-9-yl)benzoic acid (MK-75) (73)



Chemical Formula: $C_{22}H_{13}Cl_2N_3O_4S$
Molecular Weight: 486,33

In a 2-neck flask (100 mL) under an argon atmosphere 2-(2,7-dichloro-6-((2-hydroxyethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (100 mg, 0.217 mmol) was suspended in 8 mL of dry CH_2Cl_2. DMAP (160 mg, 1.3 mmol, 6.0 equiv) was added and the suspension was stirred for 30 min. The turbid mixture was cooled to 0 °C and methanesulfonyl chloride (67 µL, 0.87 mmol, 4.0 equiv) in 2 mL of dry CH_2Cl_2 was added in a dropwise manner. The suspension was stirred for 30 min at 0 °C, warmed to room temperature and then stirred for 20 h. Additional 10 mL of CH_2Cl_2 was added and the reaction mixture was extracted with 10 mL of water. The organic phase was dry over anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo*. This mixture was used further without any purification. The residue was dissolved in 10 mL of dry DMF and NaN_3 (70 mg, 1.09 mmol, 5.0 equiv) was added. The reaction mixture was heated up to 60 °C and stirred for 52 h. Then the solvent was evaporated *in vacuo* and the residue was dried under the reduced pressure. The crude reaction mixture was purified by column chromatography over silica gel using CH_2Cl_2/CH_3OH, 99:1. The product was obtained as an orange solid with the yield of 31% (20 mg, 0.04 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) $R_f = 0.76$

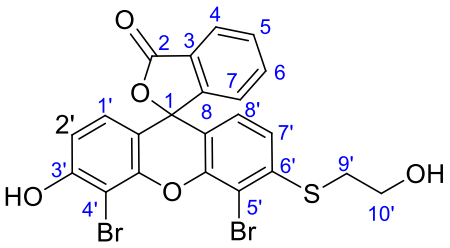
^1H NMR (400 MHz, DMSO-*d6*) δ 8.03 (d, ^3J = 7.6 Hz, 1H, H-3), 7.82 (td, ^3J = 7.6 Hz, ^4J = 1.2 Hz, 1H, H-5), 7.76 (td, ^3J = 7.6 Hz, ^4J = 1.2 Hz, 1H, H-4), 7.46 (s, 1H, H-4'), 7.36 (d, ^3J = 7.6 Hz, 1H, H-6), 6.95 (s, 1H, H-5'), 6.83 (s, 1H, H-1'), 6.70 (s, 1H, H-8'), 3.90 (t, ^3J = 7.2 Hz, 2H, H-10'), 3.58 (t, ^3J = 7.2 Hz, 2H, H-9') ppm

^{13}C NMR (75 MHz, DMSO-*d6*) δ 168.2, 155.4, 151.4, 149.8, 149.7, 138.9, 136.0, 130.7, 128.3, 127.9, 125.7, 125.5, 125.3, 123.9, 116.6 (2C, C-2', C-7'), 114.9, 110.1, 103.7, 80.8, 42.4 (C-10'), 33.1 (C-9') ppm

FD-MS: m/z (%): 485.4 (94), 486.4 (13), 487.4 (100), 488.4 (29)

FT-IR $\tilde{V}$ (cm^{-1}) 3412 ν (OH), 2922 ν (CH_2, aliph.), 2105 ν (N_3), 1768 ν (C=O, lactone), 1601 ν (C=C, arom.), 1481, 1384, 1184

5.8.6 Synthesis of 4',5'-dibromo-3'-hydroxy-6'-((2-hydroxyethyl)thio)-3H-spiro [isobenzofuran-1,9'-xanthen]-3-one (MK-74-1) (65)



Chemical Formula: $C_{22}H_{14}Br_2O_5S$
Molecular Weight: 550,22

4',5'-dibromo-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (200 mg, 0.309 mmol) was dissolved in 9 mL of dry DMF under an argon atmosphere and stirred vigorously. The mixture of 2-mercaptoethanol (87 µL, 1.236 mmol, 4.0 equiv) and DBU (46.2 µL, 0.309 mmol, 1.0 equiv) were added to 2 mL of dry DMF and agitated for 30 min. The initial solution was cooled to 0 °C and the separately prepared mixture was added slowly in a dropwise manner. The cooling was removed after 10 min and the solution was stirred for 20 h. DMF was evaporated *in vacuo* and the residue was dried under reduced pressure. Purification by column chromatography over silica gel using the gradient elution mixture from CH_2Cl_2 to CH_2Cl_2/CH_3OH, 98:2 delivered the product as an orange solid with the yield of 53% (90 mg, 0.163 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) $R_f = 0.65$

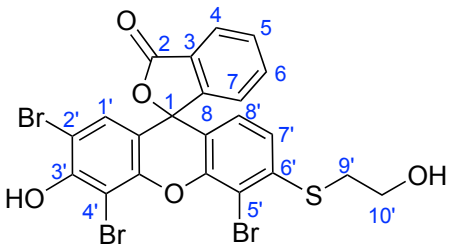
^{1}H NMR (400 MHz, CD_3OD) δ 8.06 (d, ^{3}J = 7.2 Hz, 1H, H-4), 7.83 – 7.70 (td, ^{3}J = 7.2 Hz, ^{4}J = 1.2 Hz, 2H, H-5, H-6), 7.28 (d, ^{3}J = 7.2 Hz, 1H, H-7), 7.15 (d, ^{3}J = 8.8 Hz, 1H, H-2'), 6.81 (d, ^{3}J = 8.4 Hz, 1H, H-7'), 6.72 (d, ^{3}J = 8.8 Hz, 1H, H-1'), 6.67 (d, ^{3}J = 8.8 Hz, 1H, H-8'), 3.79 (t, ^{3}J = 6.6 Hz, 2H, H-10'), 3.17 (t, ^{3}J = 6.6 Hz, 2H, H-9') ppm

^{13}C NMR (75 MHz, CD_3OD) δ 168.4 (C=O), 157, 151.6, 148.5, 142.6, 135.9, 130.8 (2C), 127.5, 127.2 (2C), 125.7, 124.9, 124.3, 121.2, 116.3 (2C), 113, 110.6, 97.6, 59.1, 34.4 ppm

FD-MS: m/z (%): 548.1 (100), 550.2 (50)

FT-IR $\tilde{V}$ (cm^{-1}) 3500-3100 ν (OH), 2930 ν (CH_2, aliph.), 1748 ν (C=O, lactone), 1599 ν (C=C, arom.), 1435, 1399, 1286, 1107

5.8.7 Synthesis of 2',4',5'-tribromo-3'-hydroxy-6'-((2-hydroxyethyl)thio)-3H-spiro [isobenzofuran-1,9'-xanthen]-3-one (MK-74-2) (66)



Chemical Formula: $C_{22}H_{13}Br_3O_5S$
Molecular Weight: 629,11

2',4',5'-tribromo-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (1 g, 1.58 mmol) was dissolved in 20 mL of dry DMF and stirred vigorously. The reaction was performed under an argon atmosphere. The mixture of mercaptoethanol (435 µl, 6.2 mmol, 4.0 equiv) and DBU (231 µl, 1.55 mmol, 1.0 equiv) were added to 5 mL of dry DMF and agitated for 30 min. The initial solution was cooled to 0 °C and the separately prepared mixture was added slowly in a dropwise manner. The cooling was removed and the solution was stirred at rt for 8 h. DMF was evaporated *in vacuo* and crude reaction mixture was dried several hours under reduced pressure. The residue was purified by column chromatography over silica gel using a gradient elution mixture from CH_2Cl_2 to CH_2Cl_2/CH_3OH, 98:2. The product was obtained as a red solid with the yield of 39% (380 mg, 0.6 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.55

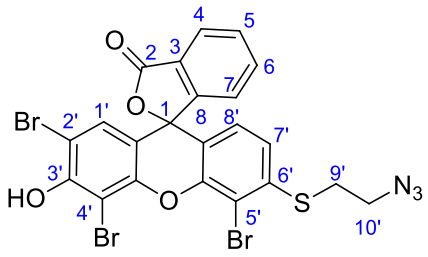
^{1}H NMR NMR (300 MHz, DMSO-*d6*) δ 8.04 (d, ^{3}J = 7.5 Hz, 1H, H-4), 7.80 (m, 2H, H-5, H-6), 7.45 (d, ^{3}J = 7.5 Hz, 1H, H-7), 7.13 (d, ^{3}J = 8.6 Hz, 1H, H-7'), 6.85 (s, 1H, H-1'), 6.72 (d, ^{3}J = 8.6 Hz, 1H, H-8'), 3.63 (t, ^{3}J = 6.3 Hz, 2H, H-10'), 3.10 (t, ^{3}J = 6.3 Hz, 2H, H-9') ppm

^{13}C NMR (75 MHz, DMSO-*d6*) δ 168.3 (2C), 148.7, 147.9, 142.4, 135.7 (2C), 130.5 (2C), 128.4, 126.7 (2C), 126.5, 125.0, 124.5, 120.9 (2C), 116.7, 108.9, 100.1, 59.1 (C-10'), 34.4 (C-9') ppm

FD-MS: m/z (%): 629.1 (100), 629.99 (42), 631.0 (39), 633.0 (11)

FT-IR $\tilde{V}$ (cm^{-1}) 3391 ν (OH), 3105 ν (arom.), 2941 ν (CH_2, aliph.), 1708 ν (C=O, lactone), 1593 ν (C=C, arom.), 1429, 1397, 1282

5.8.8 Synthesis of 6'-((2-azidoethyl)thio)-2',4',5'-tribromo-3'-hydroxy-3H-spiro [isobenzofuran-1,9'-xanthen]-3-one (MK-83) (74)



Chemical Formula: $C_{22}H_{12}Br_3N_3O_4S$
Molecular Weight: 654,13

2',4',5'-tribromo-3'-hydroxy-6'-((2-hydroxyethyl)thio)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (100 mg, 0.158 mmol) was suspended in 5 mL of dry CH_2Cl_2 in a 2-neck-rb flask (100 mL) under an argon atmosphere. DMAP (133 mg, 1.09 mmol, 6.0 equiv) was added and the suspension was stirred for 30 min. The turbid mixture was cooled to 0 °C and methanesulfonyl chloride (56 µL, 0.72 mmol, 4.0 equiv) in 1 mL of dry CH_2Cl_2 was added dropwise. The suspension was stirred for 30 min at 0 °C, warmed to room temperature and then stirred for 21 h. Additional 15 mL of CH_2Cl_2 was added and the reaction mixture was extracted with 15 mL of water. The organic phase was dry over anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo*. This mixture was used without further purification. The residue was dissolved in 8 mL of dry DMF and NaN_3 (58 mg, 0.9 mmol, 5.0 equiv) was added. The reaction mixture was warmed to 60 °C and stirred for 52 h. The solvent was evaporated *in vacuo* and the residue was dried under reduced pressure. The crude reaction mixture was purified by gradient column chromatography over silica gel with CH_2Cl_2 to

CH_2Cl_2/CH_3OH, 98:2. The product was obtained with as red solid with the yield of 19% (20 mg, 0.03 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 98:2) $R_f = 0.81$

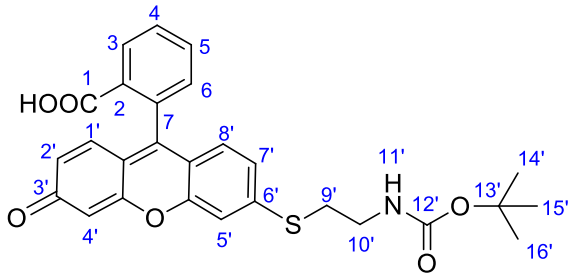
^1H NMR (400 MHz, CD_3OD) δ 8.10 (d, $^3J = 7.6$ Hz, 1H, H-4), 7.92 – 7.68 (m, 2H, H-5, H-6), 7.32 (d, $^3J = 7.6$ Hz, 1H, H-7), 7.19 (d, $^3J = 8.4$ Hz, 1H, H-7'), 6.94 (s, 1H, H-1'), 6.85 (d, $^3J = 8.4$ Hz, 1H, H-8'), 3.60 (t, $^3J = 6.8$ Hz, 2H, H-10'), 3.30 – 3.22 (t, $^3J = 6.8$ Hz, 2H, H-9') ppm

^{13}C NMR (75 MHz, CD_3OD) δ 170.5 (C=O), 150.3 (C-3'), 149.7, 143.6, 136.7, 131.8 (2C), 131.1, 130.4, 128.3, 128 (2C), 126.7, 125.6, 123.1 (2C), 118.5, 114, 111.6, 77.1, 51 (C-10'), 32.7 (C-9') ppm

FD-MS: m/z (%): 653.2 (100), 654.3 (39), 655.2 (97)

FT-IR $\tilde{V}$ (cm^{-1}) 3077 ν (C-H, arom.), 2928 ν (CH_2, aliph.), 1760 ν (C=O, lactone), 2101 ν (N_3), 1600 ν (C=C, arom.), 1403, 1193

5.8.9 Synthesis of 2-(6-((2-((*tert*-butoxycarbonyl)amino)ethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (67)



Chemical Formula: $C_{27}H_{25}NO_6S$
Molecular Weight: 491,56

3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate (1 g, 2 mmol) was dissolved in 30 mL of dry DMF. The mixture of Boc-cysteamine (1.38 mL, 8.2 mmol, 4.0 equiv) and DBU (306 µL, 2 mmol, 1.0 equiv) were added to 5 mL of dry DMF and agitated for 30 min. The initial solution was cooled to 0 °C and the separately prepared mixture was added slowly in a dropwise manner. The color of reaction mixture changed from yellow to red-orange clear solution. After 30 min the cooling was removed and the solution was stirred at rt over night. DMF was evaporated *in vacuo* and the reaction mixture was dried under reduced pressure. The residue was purified by the gradient column chromatography over silica gel with CH_2Cl_2 to CH_2Cl_2/CH_3OH, 99:1-97:3. The product was obtained as a yellow solid with the yield of 38% (373 mg, 0.76 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 95:5) R_f = 0.67

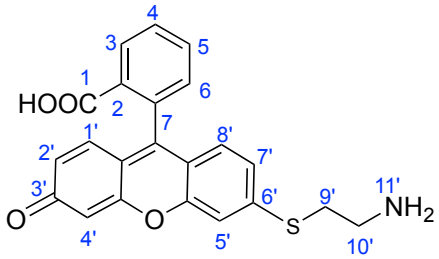
^{1}H NMR (300 MHz, DMSO-*d6*) δ 8.02 (d, ^3J = 7.2 Hz, 1H, H-3), 7.83 – 7.43 (m, 2H, H-4, H-5), 7.34 (d, ^4J = 2Hz, 1H, H-4'), 7.27 (d, ^3J = 7.2 Hz, 1H, H-6), 7.1 (t, ^3J = 5.4 Hz, 1H, N-H), 7.02 (dd, ^3J = 8.4, ^4J =2.0 Hz, 1H, H-2'), 6.68 (d, ^3J = 8.4 Hz, 1H, H-1'), 6.61 (pd, ^3J = 8.7 Hz, 2H, H-7', H-5'), 6.52 (d, ^3J = 8.7 Hz, H-8'), 3.15 (t, ^3J = 6.3 Hz, 2H, H-10'), 3.08 (t, ^3J = 6.3 Hz, 2H, H-9'), 1.37 (s, 9H, H-14', H-15', H-16') ppm

^{13}C NMR (75 MHz, DMSO-*d6*) δ 168.5, 155.5, 152.3, 151.2, 140.5, 134.9, 130.1 (2C), 129.3 (2C), 128.3 (2C), 125.3, 124.5, 122.6, 116.1, 113.9, 109.6, 102.4 (2C), 99.5, 77.9, 39.5 (C-10'), 30.9 (C-9'), 28.2 (3C, C-14', C-15', C-16') ppm

FD-MS: m/z (%): 491.7 (100)

FT-IR $\tilde{V}$ (cm^{-1}) 3325 ν (OH), 2970 ν (CH_3, aliph.), 2925 ν (CH_2, aliph.), 1760 ν (C=O, lactone), 1658 δ (N-H), 1597 ν (C=C, arom.), 1560, 1462, 1242, 1106

5.8.10 Synthesis of 2-(6-((2-aminoethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (MK-69) (68)



Chemical Formula: $C_{22}H_{17}NO_4S$
Molecular Weight: 391,44

2-(6-((2-((tert-butoxycarbonyl)amino)ethyl)thio)-3-oxo-3H-xanthen-9-yl)benzoic acid (50 mg, 0.102 mmol) was taken up in 2.5 mL of CH_2Cl_2 and TFA (0.5 mL) to cleave Boc-protective group. The reaction mixture was stirred at room temperature for 2 h monitored by TLC on silica gel (CH_2Cl_2/CH_3OH, 95:5). After 2 h starting material was fully converted and a spot staying on the start of TLC plate presumable new product (not moving because of formation of TFA salt) was presented. Toluene (3 mL) was added; the reaction mixture was concentrated to dryness and then azeotroped with CH_3OH three times. The red residue was triturated with CH_2Cl_2 and filtered off. Then washed with CH_2Cl_2 and ether and dried *in vacuo*. The lyophilization from water afforded product as an orange-gold solid with the yield of 95% (38 mg, 0.097 mmol).

TLC (silica gel, CH_2Cl_2/CH_3OH, 9:1) $R_f = 0.17$

^{1}H NMR (300 MHz, DMSO-*d6*) δ 10.30 (s, 1H, H-1), 8.03 (d, 1H, ^3J = 7.3 Hz, H-3), 7.87 (s, 2H, NH_2), 7.84 – 7.64 (m, 2H, H-4, H-5), 7.41 (d, ^4J = 1.9 Hz, 1H, H-4'), 7.30 (d, ^3J = 7.3 Hz, 1H, H-6), 7.06 (dd, ^3J = 8.4 Hz, ^4J =1.2 Hz, 1H, H-7'), 6.70 (d, ^3J = 8.1 Hz, 2H, H-1', H-8'), 6.60 (m, 2H, H-2', H-5'), 3.27 (t, ^3J = 6.3 Hz, 2H, H-10'), 3.02 (t, ^3J = 6.3 Hz, 2H, H-9') ppm

^{13}C NMR (75 MHz, DMSO-*d6*) δ 168.6, 159.8, 152.3, 151.50, 151.2, 138.4, 135.8, 130.3, 129.2, 128.6, 125.9, 124.8, 124.0, 123.3, 116.4, 114.6, 113.1, 109.1, 102.2, 82.1, 37.9 (C-10'), 28.4 (C-9') ppm

FD-MS: m/z (%): 392.4 (100)

FT-IR $\tilde{V}$ (cm^{-1}) 3056 ν (C-H, arom.), 1736 ν (C=O), 1666 δ (N-H), 1597 ν (C=C, arom.), 1401, 1196, 1106

5.9 Fluorescent Characteristics

Photophysical properties of new xanthene dyes such as absorption, extinction coefficient at the maximum absorption wavelength (λ_{max}), excitation (λ_{ex}), emission (λ_{em}), quantum yield (Φ), and photostability were characterized.

Stock solutions of the fluorophores were prepared by accurate weighing and dissolving 2-8 mg of the fluorophore in ethanol (HPLC grade) to obtain a concentration of 5 mM. New xanthene dyes, which photophysical properties were characterized, are included in figure 18.

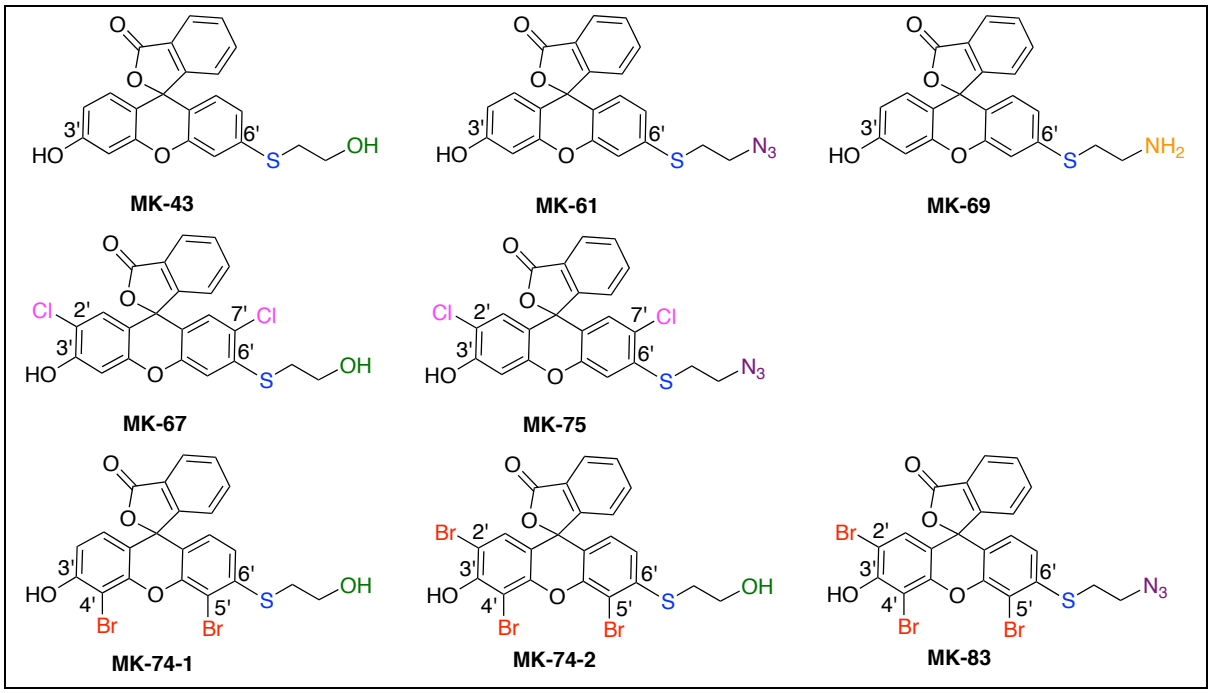
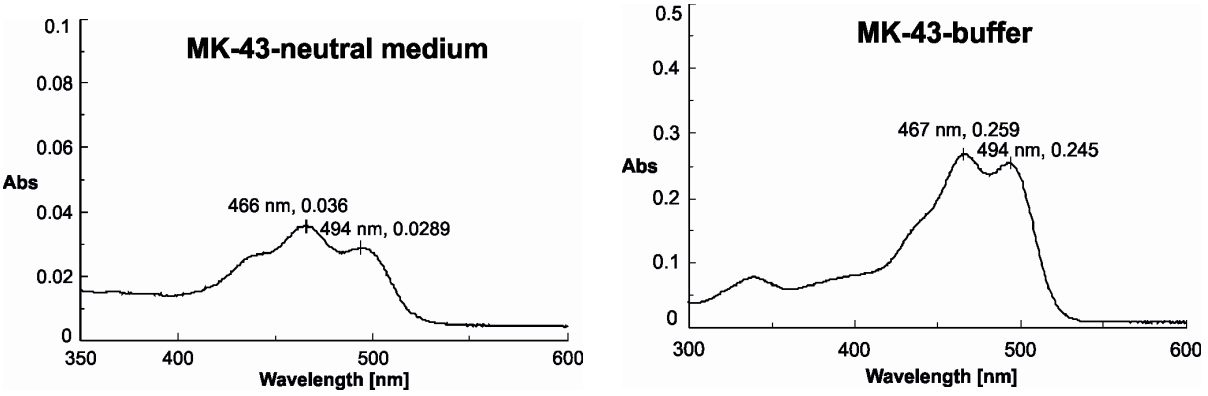
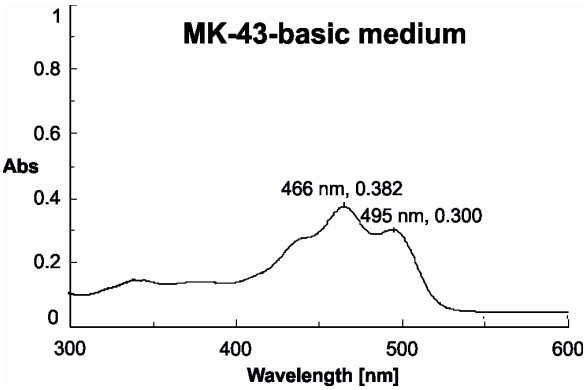


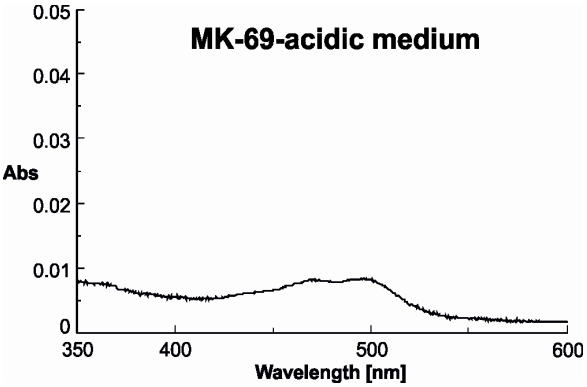
Figure 18. Structures of new xanthene dyes.

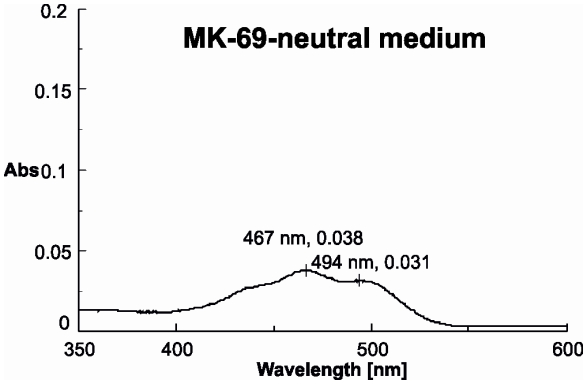
5.9.1 Recording of Absorption of Fluorescent Probes in Acidic, neutral, Basic Medium and in a Buffer

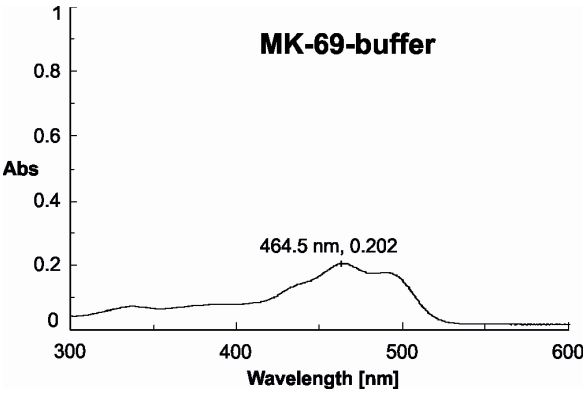
Absorption spectra were measured in Suprasil quartz glass cuvettes (Hellma, Müllheim, Germany) with 0.1 cm path length in acidic medium (0.1 M HCl), in neutral medium (NH_4OAc 0.1 M), in basic medium (0.1 M NaOH) and in 10 mM Et_3NHOAc buffer (pH 7.3) at 25 °C. The samples were prepared as stock solutions in ethanol and diluted such that the ethanol concentration did not exceed 1% v/v. Absorption spectra were recorded on a Jasco V-650 spectrophotometer (Jasco, Groß-Umstadt, Germany) to observe the impact of pH on the absorption of new dyes and to determine the maximum absorption wavelength (λ_{max}). Absorption spectra were recorded from 300 to 600 nm. For measurement in basic medium and Et_3NHOAc buffer, pH 7.3, 10 µM solutions of dyes were prepared. For measurements in acidic and neutral medium, 50 µM solutions of dyes were used. MK-43, MK-67, MK-69 at 50 µM concentrations in acidic medium showed almost no absorption. The brominated probes (MK-74-1, MK-74-2 and MK-83) exhibited very low absorption in acidic and neutral medium. The impact of pH on absorption at given concentration of xanthene dyes is illustrated in the corresponding graphs in figure 19. The absorbance in acidic and neutral medium (A < 0.1) is usually not pictured. The dye samples produced two absorption maxima of similar values within the difference of 30 nm.

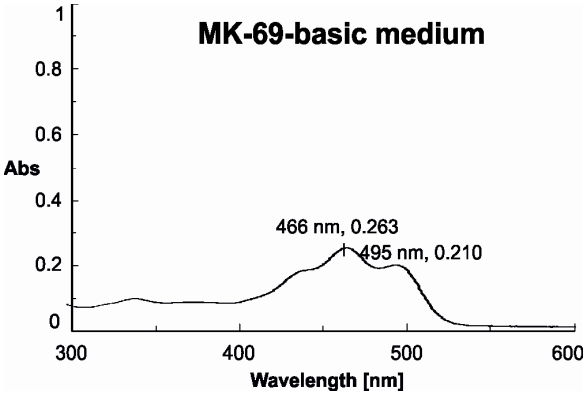


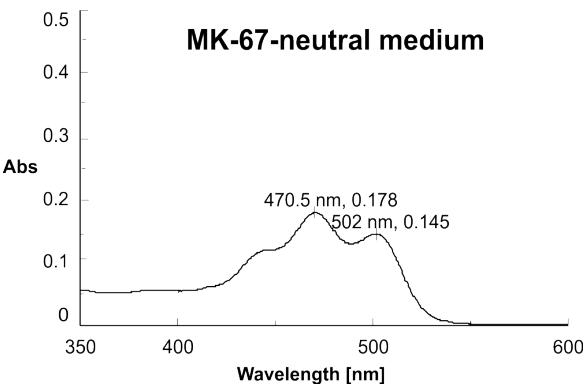

MK-43-basic medium
Abs
Wavelength [nm]
466 nm, 0.382
495 nm, 0.300

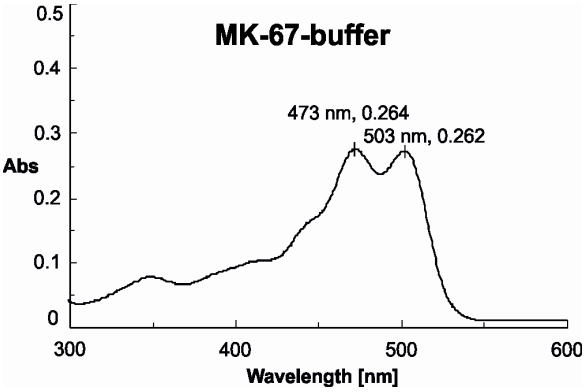

MK-69-acidic medium
Abs
Wavelength [nm]

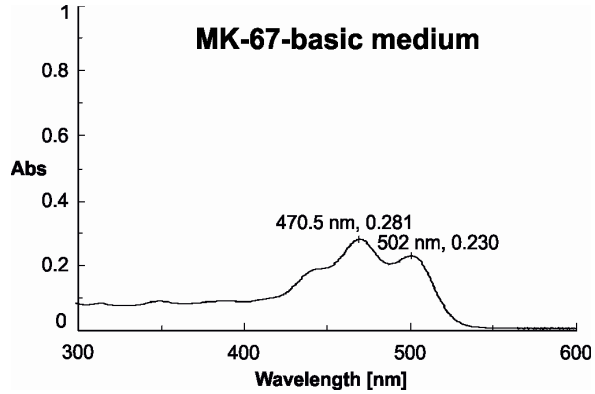

MK-69-neutral medium
Abs
Wavelength [nm]
467 nm, 0.038
494 nm, 0.031

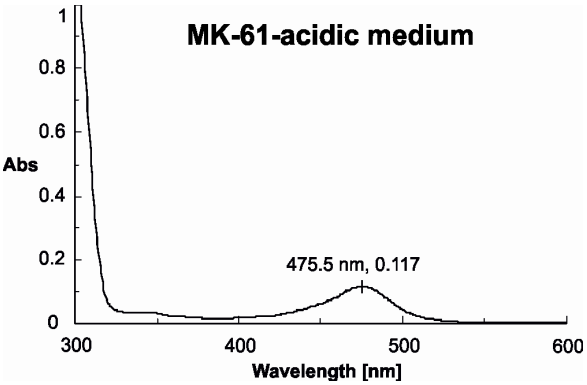

MK-69-buffer
Abs
Wavelength [nm]
464.5 nm, 0.202

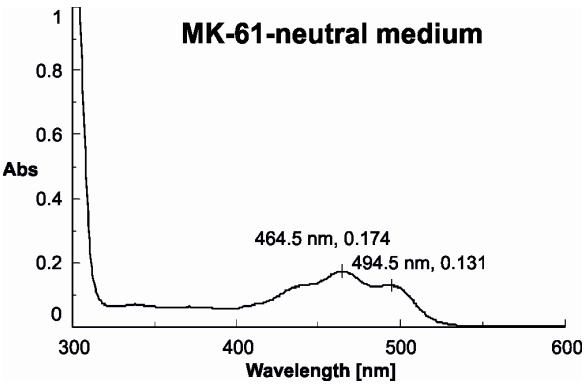

MK-69-basic medium
Abs
Wavelength [nm]
466 nm, 0.263
495 nm, 0.210

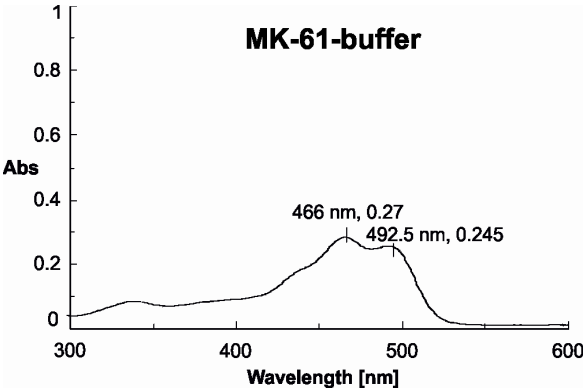

MK-67-neutral medium
Abs
Wavelength [nm]
470.5 nm, 0.178
502 nm, 0.145

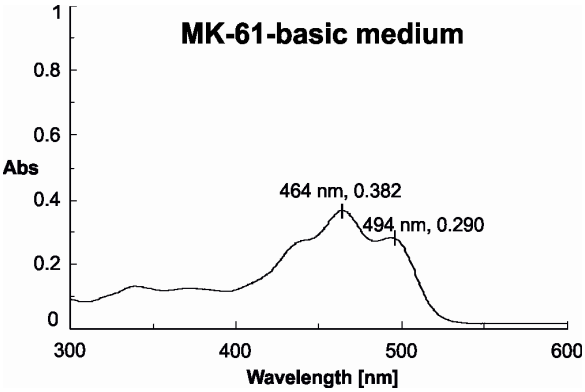

MK-67-buffer
Abs
Wavelength [nm]
473 nm, 0.264
503 nm, 0.262

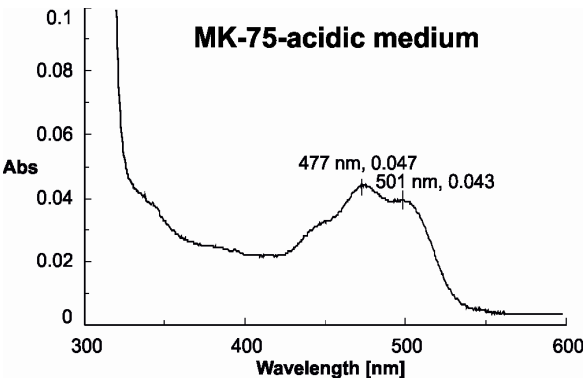

MK-67-basic medium
Abs
Wavelength [nm]
470.5 nm, 0.281
502 nm, 0.230

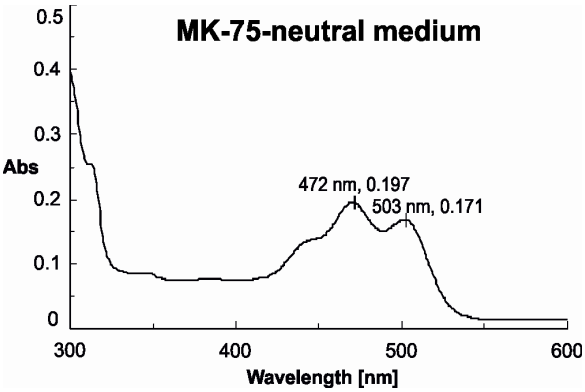

MK-61-acidic medium
Abs
Wavelength [nm]
475.5 nm, 0.117


MK-61-neutral medium
Abs
Wavelength [nm]
464.5 nm, 0.174
494.5 nm, 0.131


MK-61-buffer
Abs
Wavelength [nm]
466 nm, 0.27
492.5 nm, 0.245


MK-61-basic medium
Abs
Wavelength [nm]
464 nm, 0.382
494 nm, 0.290


MK-75-acidic medium
Abs
Wavelength [nm]
477 nm, 0.047
501 nm, 0.043


MK-75-neutral medium
Abs
Wavelength [nm]
472 nm, 0.197
503 nm, 0.171

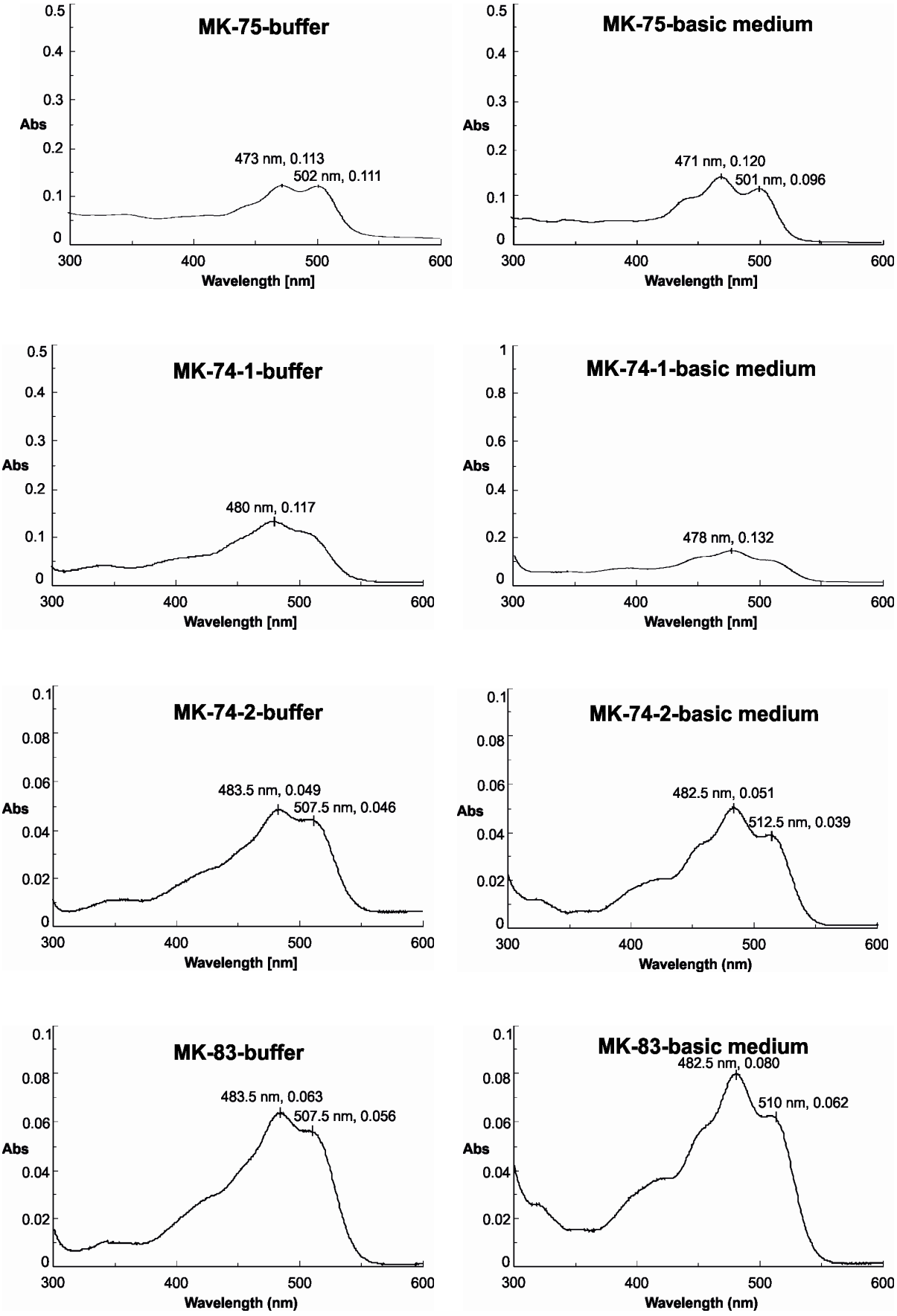
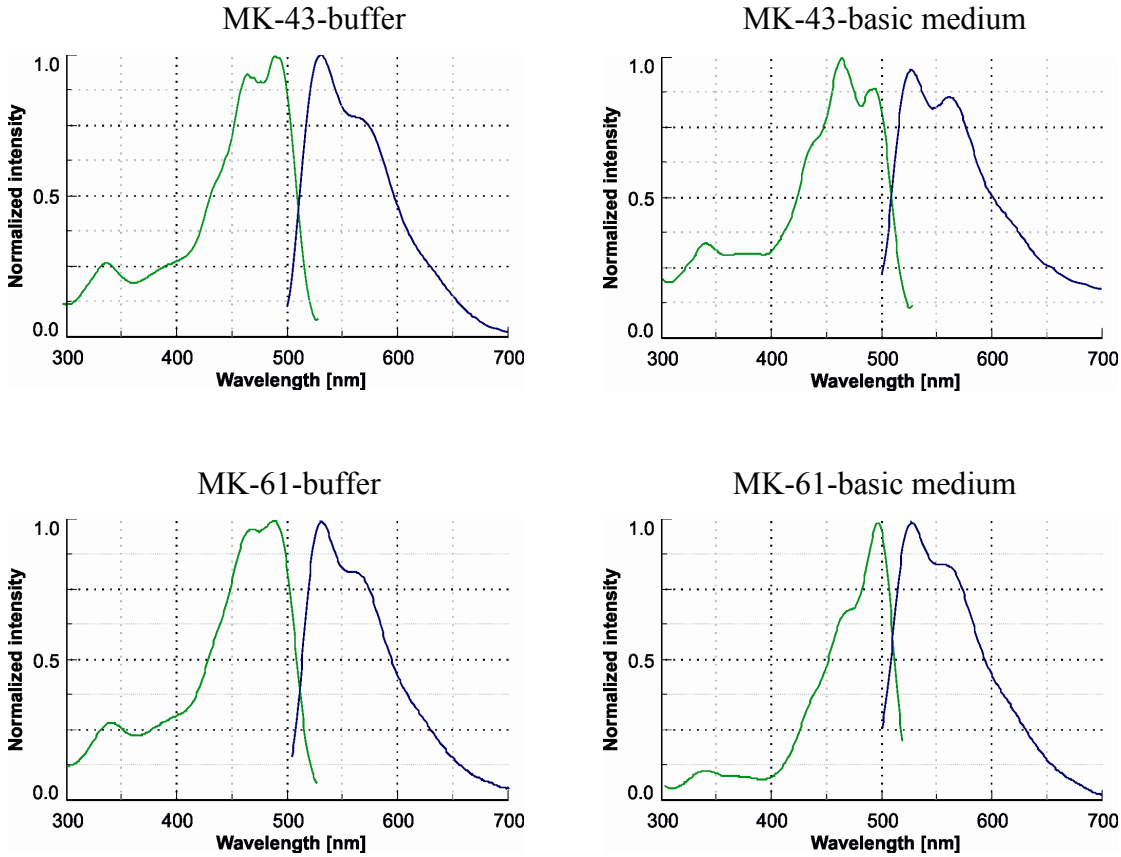


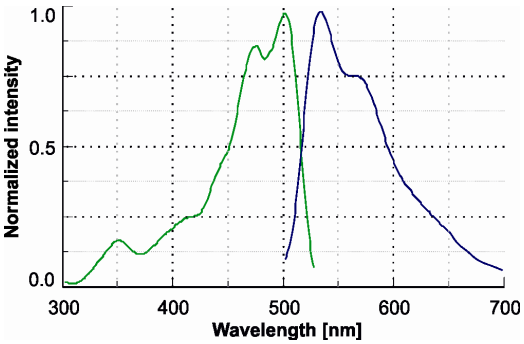
Figure 19. Absorption graphs of new xanthene dyes under different conditions to determine their maximal absorptions. Global and local absorption maxima are indicated in the graphs.

5.9.2 Fluorescence Excitation and Emission Scans

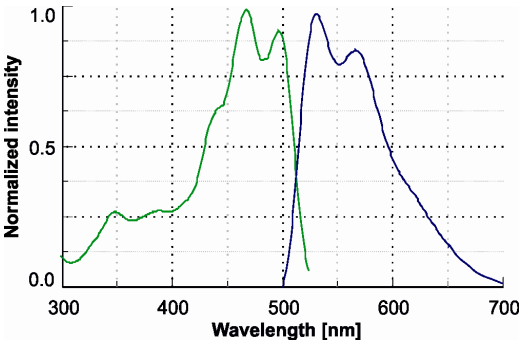
Fluorescent measurements were performed on Jasco FP-6500 fluorimeter (Jasco Groß-Umstadt, Germany) equipped with a Peltier element for heating and cooling. Fluorescent probes were diluted to final concentration of 5 µM in basic medium (0.1 M NaOH) and in Et_3NHOAc buffer (10 mM, pH 7.3) and were analyzed in Suprasil quartz glass cuvettes with 3 mm path length. Excitation and emission scans were performed at 25 °C with the following parameters: 3 nm bandwidth of excitation and emission, data pitch of 1 nm, response time of 0.5 s, scanning speed of 1000 nm min, and spectral correction of excitation and emission. The normalized excitation and emission spectra of the xanthene dyes are illustrated in figure 20.



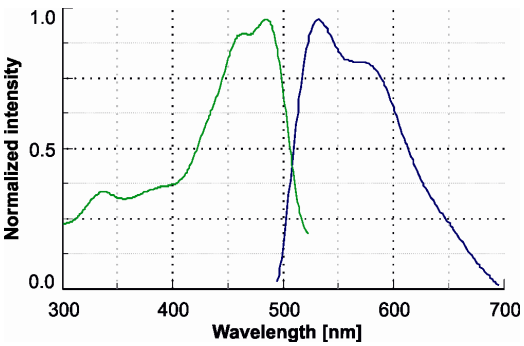
MK-67-buffer



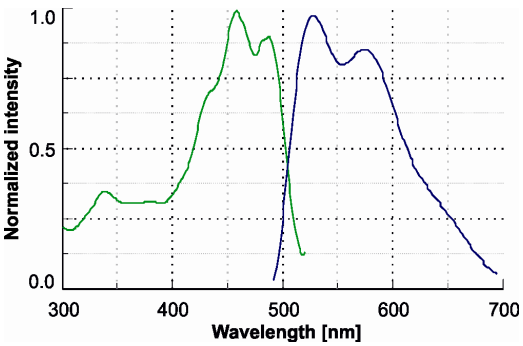
MK-67-basic medium



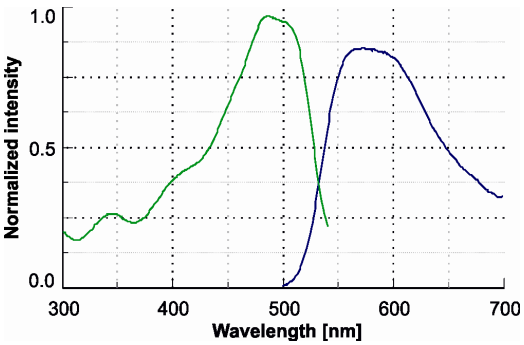
MK-69-buffer



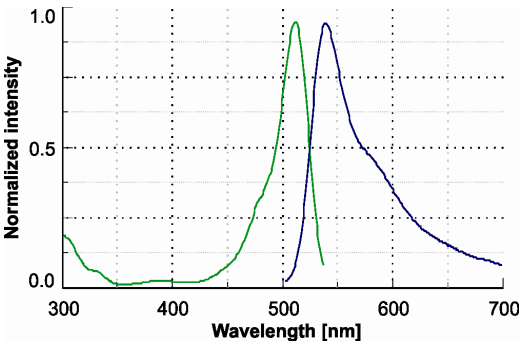
MK-69-basic medium



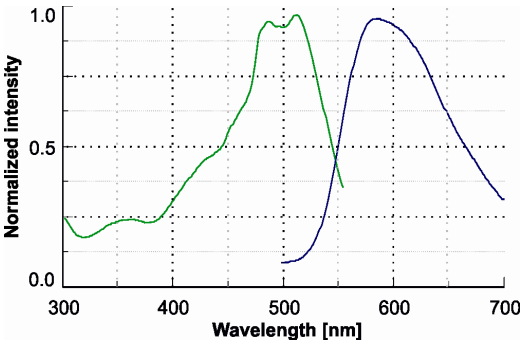
MK-74-1-buffer



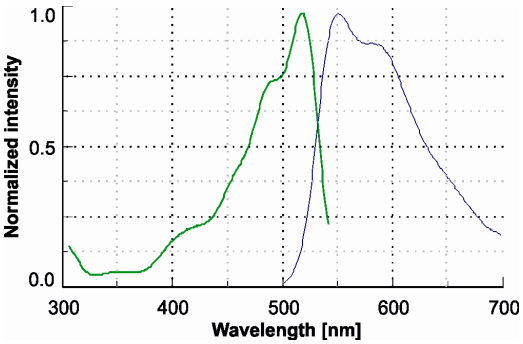
MK-74-1-basic medium



MK-74-2-buffer



MK-74-2-basic medium



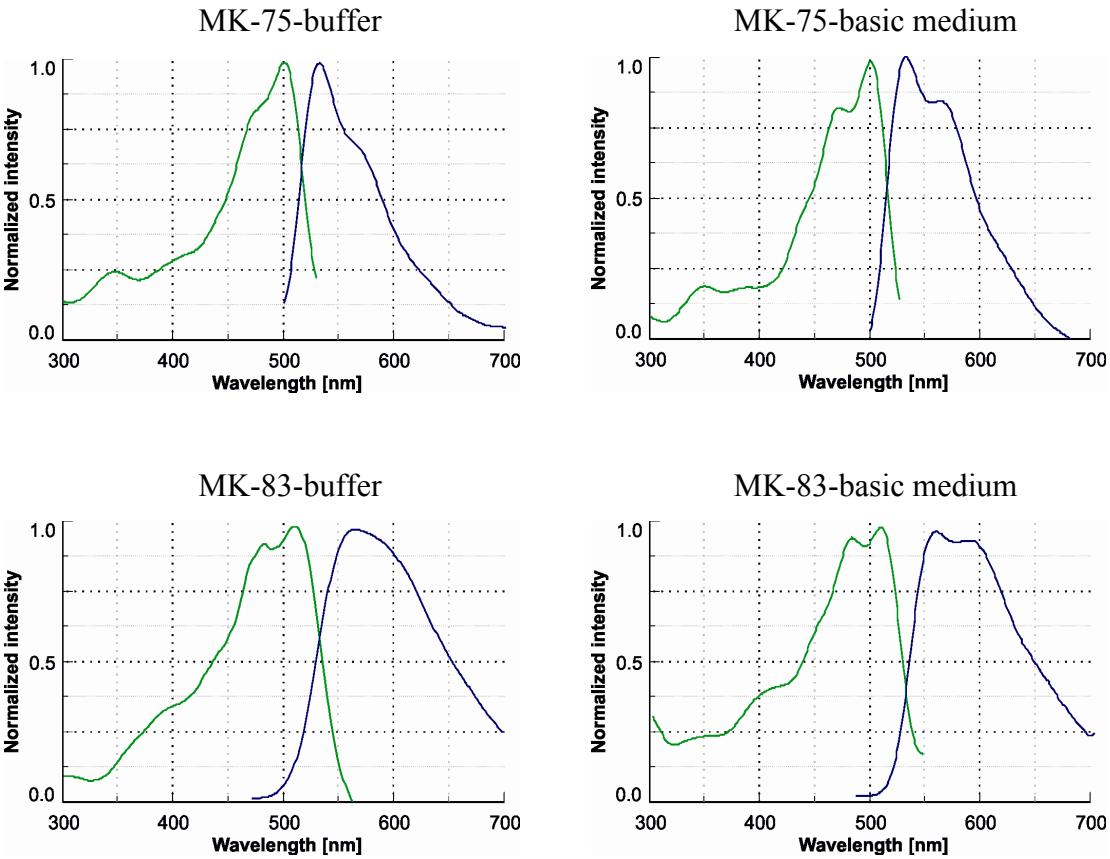


Figure 20. Excitation and emission spectra of selected dyes in buffer and basic medium.

5.9.3 Determination of Extinction Coefficients

The Beer-Lambert law ($A = \varepsilon \cdot c \cdot l$) was used to determine the extinction coefficients of samples by measuring the absorbance of solutions at four known concentrations. The absorbances (y-axis) were plotted towards the corresponding concentrations (x-axis) and the extinction coefficients were calculated by linear regression using Beer's law (Figure 21, 22). The absorption spectra were measured in 1-cm path length cuvettes, in 10 mM Et_3NHOAc buffer pH 7.3 and in 1 mM NaOH. The samples were prepared as stock solutions in ethanol and diluted such as the ethanol concentration did not exceed 1% v/v. Extinction coefficients were determined from the absorptions with higher values. Extinction coefficients of xanthene dyes in buffer pH 7.3 and basic medium are included in table 5.

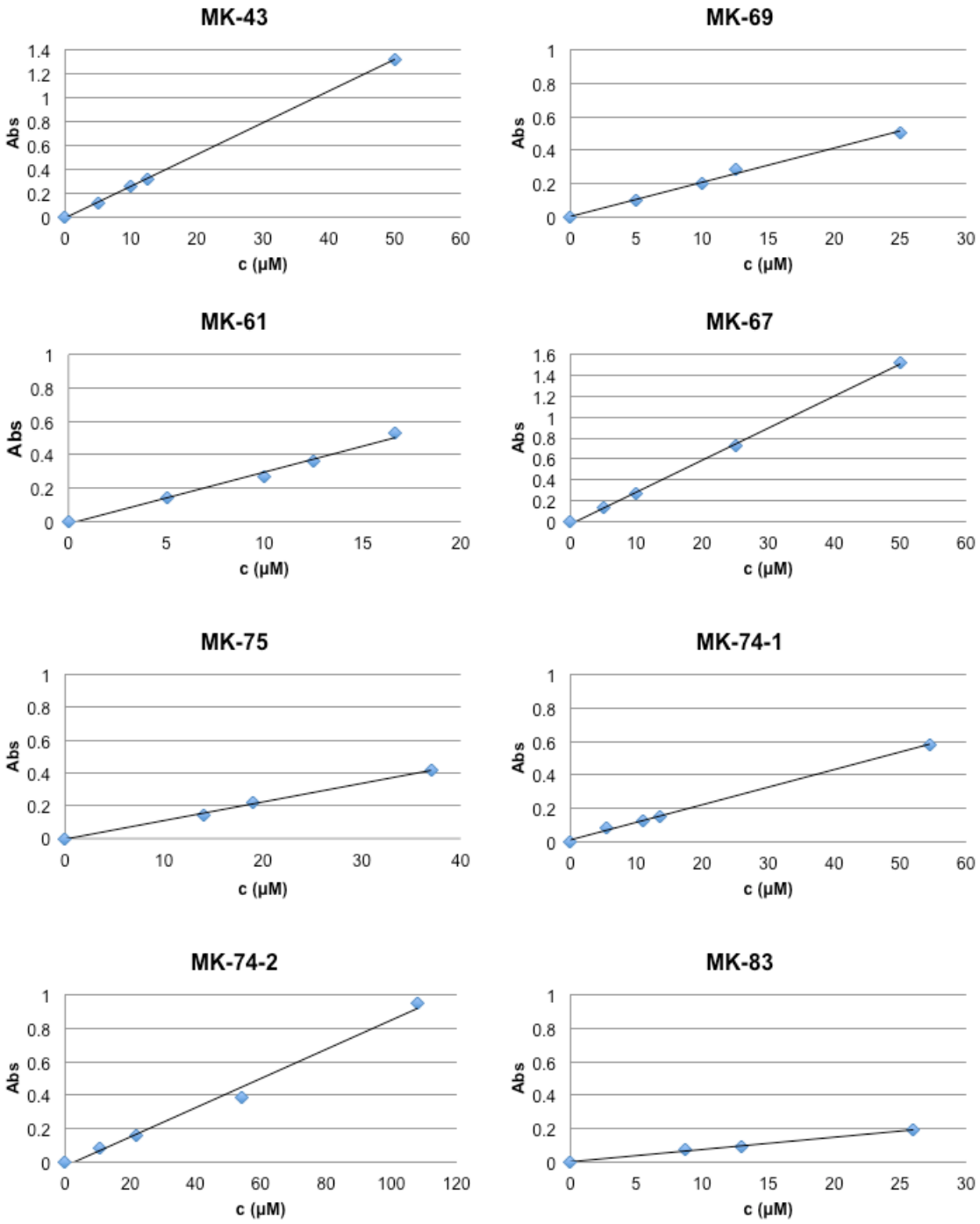


Figure 21. Absorption of fluorescent dyes in a serial dilution at λ_{max} used for calculation of the extinction coefficients in 10 mM Et_3NHAc buffer (pH 7.3).

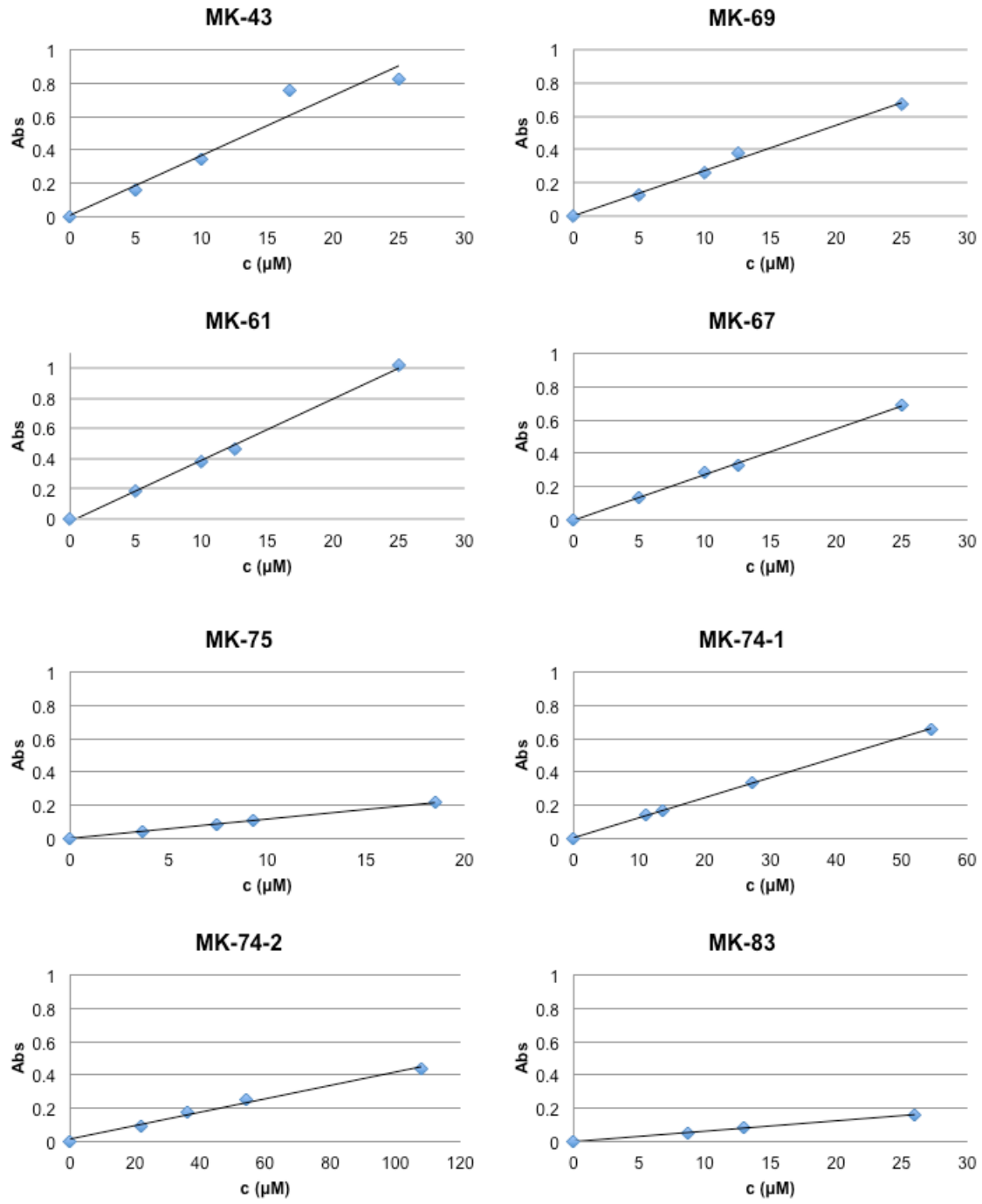


Figure 22. Absorption of fluorescent dyes in a serial dilution at λ_{max} used for calculation of the extinction coefficients in 1 mM NaOH.

Table 5. Extinction coefficients of xanthene dyes at λ_{max} in 10 mM Et_3NHOAc buffer pH 7.3 and in 1 mM NaOH

Dye	ε at λ_{max} (M^{-1} cm^{-1}) in buffer pH 7.3	ε at λ_{max} (M^{-1} cm^{-1}) in 1 mM NaOH
MK-43	26 000	34 000
MK-69	20 200	25 000
MK-61	28 500	38 000
MK-67	26 500	27 000
MK-75	10 200	12 000
MK-74-1	11 600	13 100
MK-74-2	7400	4500
MK-83	7500	6300

5.9.4 Determination of Quantum Yields

Absorption spectra were measured as described earlier in this work. The quantum yields were determined by using diluted samples (A < 0.1) in 0.1 M NaOH in water. Emission scans were performed at 25 °C with the following parameters: 3 nm bandwidth of excitation and emission, data pitch of 1 nm, response of 0.5 s, scanning speed of 1000 nm min, and spectral correction of multiplier and illumination lamp. Samples were excited at 488 nm and the emission was recorded from at least 5 nm above the excitation wavelength to 700 nm (emission profile). These quantum yields were obtained by comparison of the integrated area of the emission spectra ($\int F_{em,\ sample}$) of the samples with the integrated area of emission spectra of standard sample ($\int F_{em,\ standard}$), fluorescein in 0.1 M NaOH in water, which has a quantum efficiency 0.95 ± 0.03.[32, 56] The integrated area of the emission spectrum was calculated in the section between 500 - 600 nm. The quantum yield of a sample was related to that of the standard, and determined by the equation 3[49]

$$\Phi_{sample} = (A_{standard}/A_{sample})\ (\int F_{em,\ sample}/\int F_{em,\ standard})\ (\eta_{sample}/\eta_{standard})^2\ \Phi_{standard} \quad \textbf{(Equation 3)}$$

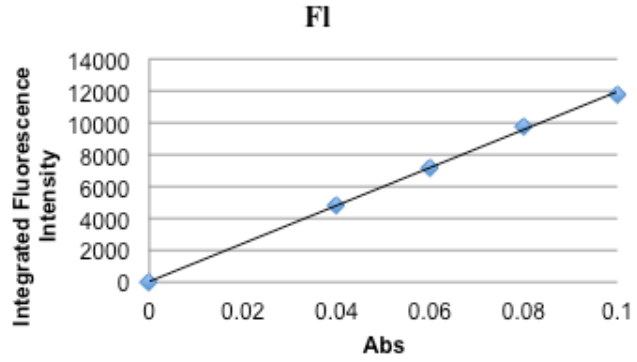
wherein Φ is the fluorescent quantum yield, A is the absorbance at the excitation wavelength, $\int F$ is the area under the emission curve, η is the refractive index of the solvent.

The concentrations of the test samples were adjusted to match the absorbance of the standard at the excitation wavelength so that the absorbance ratio is equal to 1. The refractive index

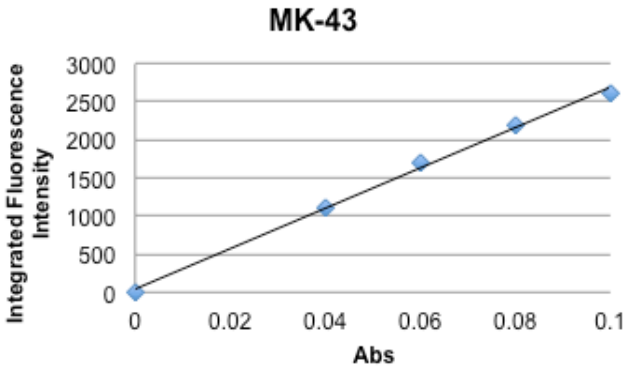
ratio is also equal 1 since water was used for both the standard and the sample solutions. Under these conditions the quantum yield (Φ) was calculated with the following equation 4[32]

$$\Phi_{sample} = \Phi_{standard} \left(\int F_{em,\,sample} / \int F_{em,\,standard}\right) \quad \textbf{(Equation 4)}$$

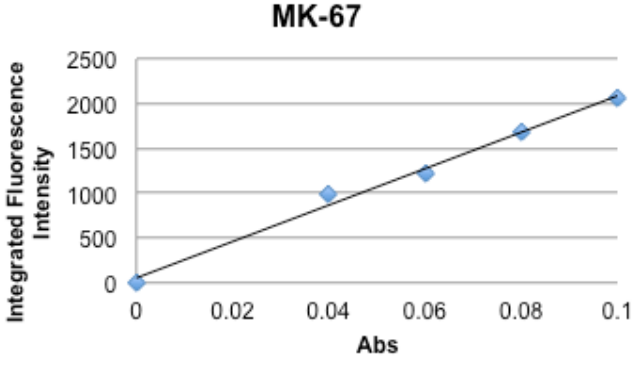
Integrated fluorescent intensity of four different concentrations was plotted against corresponding absorbance at this concentration (Figure 23). The quantum yields of brominated fluorescein dyes MK-74-1, MK-74-2, MK-83 were not determined because their fluorescence at such a low concentrations was too weak to be detected. The determined quantum yields are included in table 6.



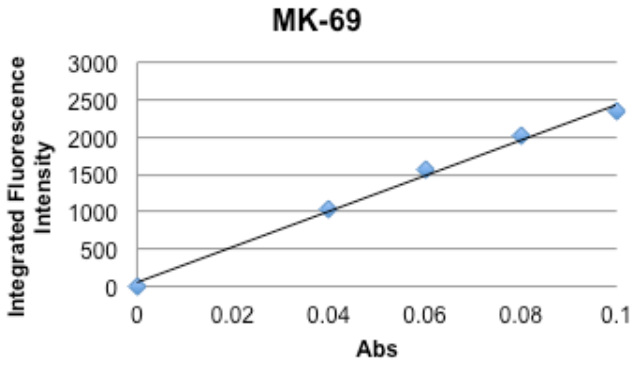
Abs	$\int F_{em,\,standard}$
0,04	4811
0,06	7220
0,08	9770
0,1	11809



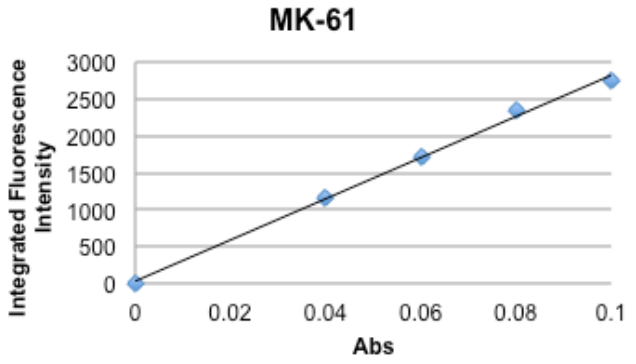
Abs	$\int F_{em,\,MK\text{-}43}$
0,04	1109
0,06	1711
0,08	2187
0,1	2619



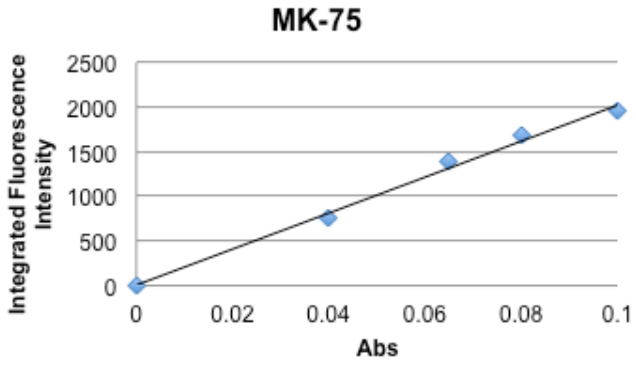
Abs	$\int F_{em,\,MK\text{-}67}$
0,04	988
0,06	1221
0,08	1688
0,1	2064



Abs	$\int F_{em,\ MK\text{-}69}$
0,04	1024
0,06	1564
0,08	2008
0,1	2338



Abs	$\int F_{em,\ MK\text{-}61}$
0,04	1165
0,06	1711
0,08	2357
0,1	2742



Abs	$\int F_{em,\ MK\text{-}75}$
0,04	764
0,065	1383
0,08	1673
0,1	1950

Figure 23. Linear plot of standard and samples (integrated fluorescent intensity vs. absorbance) and their corresponding tables.

Table 6. Quantum yields of xanthene dyes obtained by the comparison of the integrated area of the emission spectrum ($\int F_{em}$) of the samples with fluorescein in 0.1 M NaOH (Φ = 0.95).[3232]

Dye	Φ
MK-43	0.22
MK-61	0.24
MK-69	0.20
MK-67	0.17
MK-75	0.16

5.9.5 Bleaching Studies

Photobleaching of new fluorescent xanthene dyes was performed in 1.5 mm path length Suprasil quartz glass cuvette on a Jasco FP-6500 fluorimeter. For the analysis of photostability of dyes, samples were diluted to the concentration of 1 μM in Et_3NHOAc buffer (pH 7.3). Additionally, the parameters were adjusted to 10 nm bandwidth excitation to guarantee sufficient bleaching. Samples were constantly excited for 2 h at 488 nm recording the emission spectra in 30 seconds intervals. The photostability of dyes was analyzed towards fluorescein (Fl) and 2',7'-dichlorofluorescein (DCFl). For long-term analysis, the emission spectrums were recorded under the same settings for 10 h (Figure 25).

The photostability of fluorescein and 2',7'-dichlorofluorescein was analyzed for 2 h by 488 ± 5 nm excitation. Fluorescein showed 8% of initial intensity and 2',7'-dichlorofluorescein 28% of initial intensity after the bleaching study (Figure 24).

Percentage of initial intensities of xanthene dyes upon continuous excitation for 2 h and for 10 h is included in table 7.

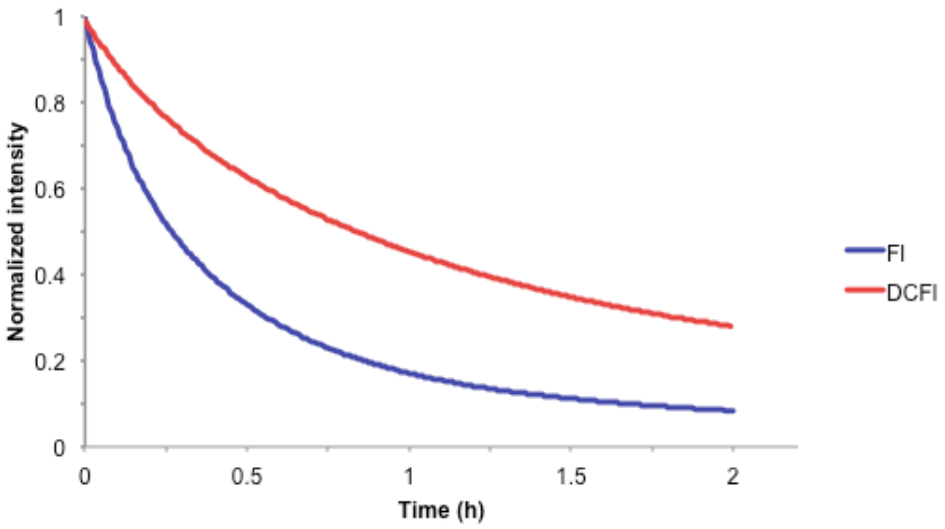
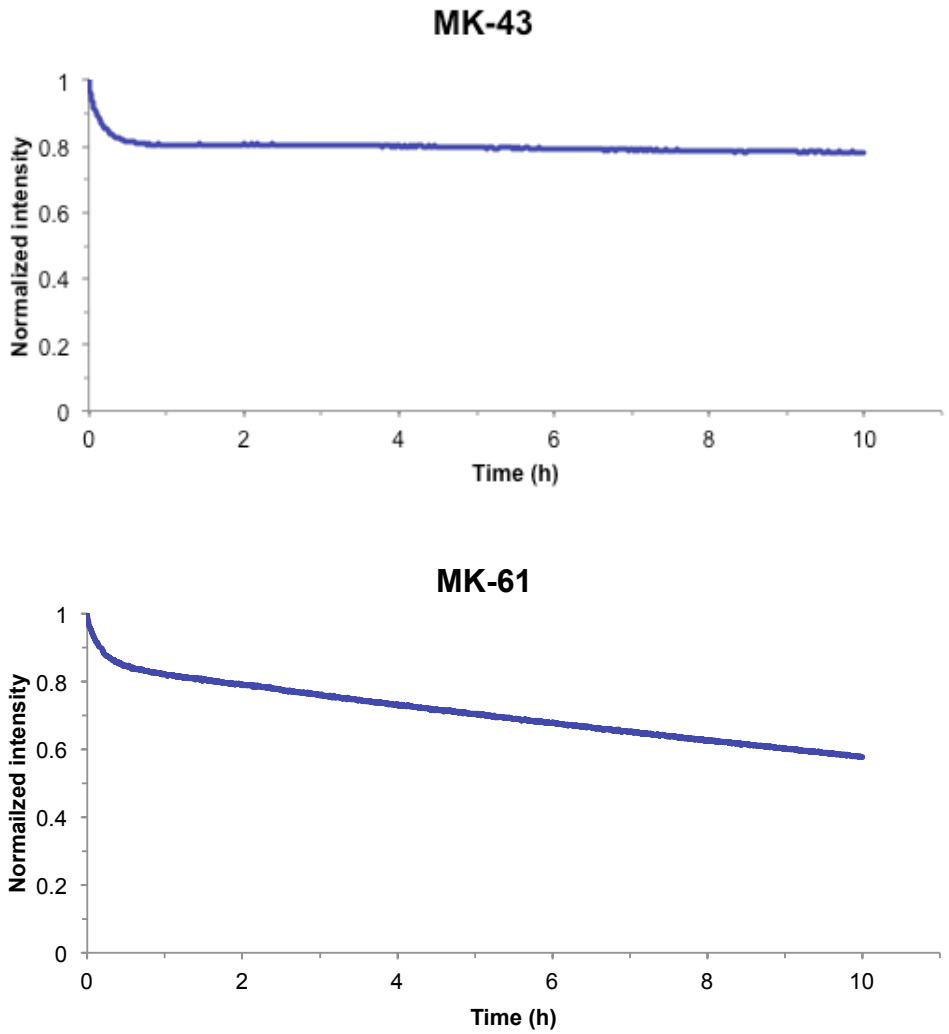


Figure 24. Photostability of fluorescein (Fl) and 2',7'-dichlorofluorescein (DCFl) in the cuvette upon 488 ± 5 nm excitation for 2 h.



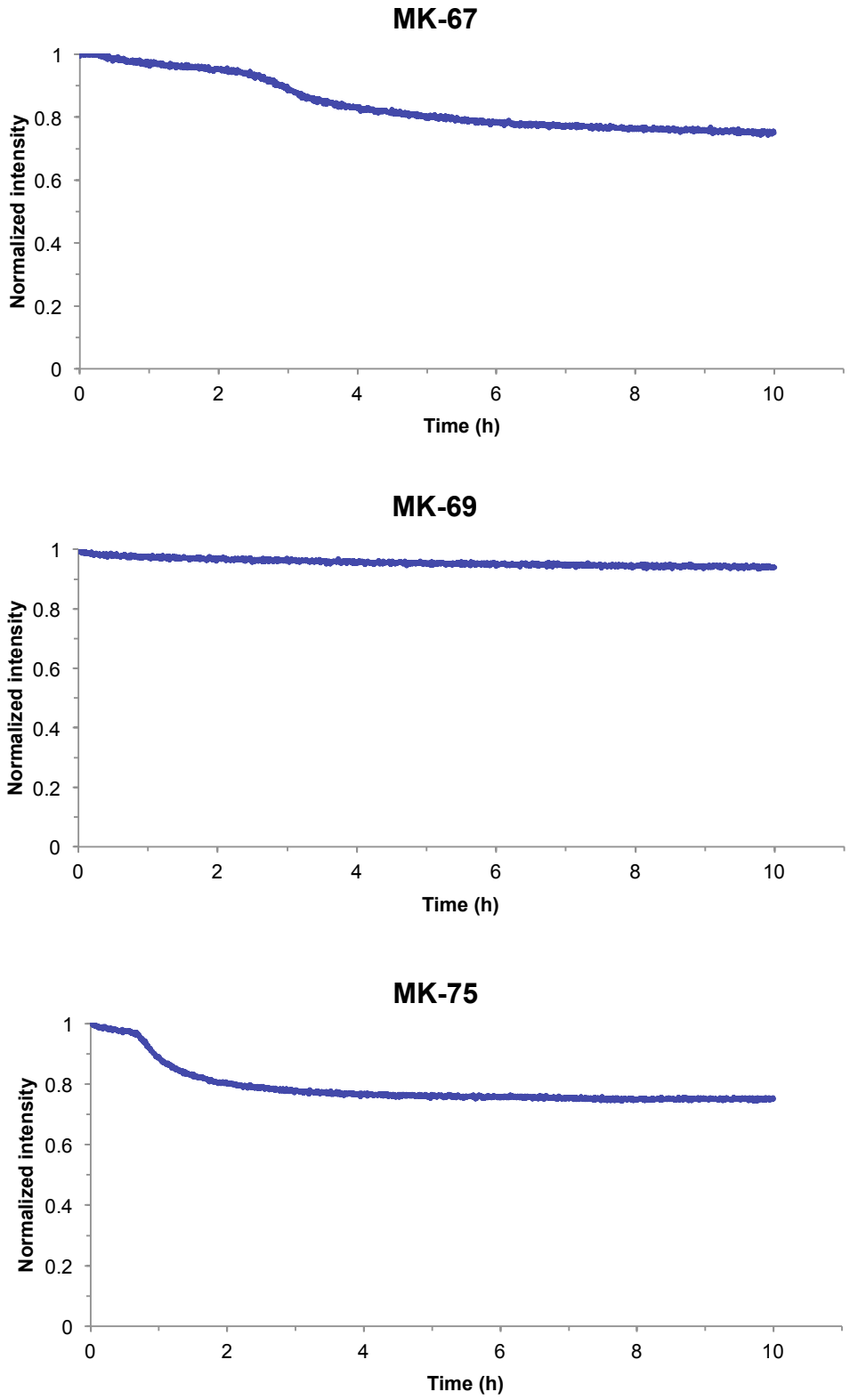


Figure 25. Photostability of xanthene dyes. Normalized emission upon permanent excitation 488 ± 5 nm over 10 h.

Table 7. Percentage of initial intensities of xanthene dyes upon continuous excitation for 2 h and for 10 h. Fl and DCFl were almost photobleached after 2 h experiments and therefore 10 h resulted in complete bleaching.

Dye	% of initial intensity (2 h)	% of initial intensity (10 h)
MK-43	81	78
MK-61	78	58
MK-69	96	94
MK-67	95	76
MK-75	85	75
Fl	8	-
DCFl	28	-

5.10 Labeling of Alkynyl Oligonucleotide with Azido-functionalized Xanthene Dyes

5.10.1 "Click" reaction

New synthesized azido-functionalized xanthene dyes (MK-57, MK-61, MK-75, MK-83) were tested for labeling of oligonucleotides. The dyes were "clicked" *via* Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC)[44] with the commercially available DNA primer, which contains a terminal alkyne, 5'-hexynyl CGC GCG AAG CTT AAT ACG ACT CAC TAT A (Glen Research No. 10-1908-90, IBA). The click reaction with each dye took place under light protection in a benchtop thermomixer (550 rpm) for 3 h at 25 °C. The reactions were performed in a mixture of 1:2 water and phosphate buffer (pH 8). The Cu(I)-catalyst was generated from $CuSO_4$. $5H_2O$, using sodium ascorbate as a reducing agent. THPTA (tris(hydroxypropyl)triazolylmethyl-amine) was applied as stabilizing ligand of the Cu(I)-species. The protocol for the "click" reactions in analytical and preparative scales with new xanthene azides are included in tables 8 and 9.

Table 8. General protocol for fluorescent labeling of oligonucleotide *via* "click" reaction.

Compound	c_{stock} (mM)	c (mM)	V (µL)
Buffer NaH_2PO_4	500	100	4
THPTA	50	2.5	1
Na Ascorbate	50	5	2
$CuSO_4 . 5H_2O$	5	0.5	2
dye	1	0.05	1
oligo	0.1	0.01	2
H_2O			8
			20

Table 9. General protocol for fluorescent labeling of oligonucleotide in preparative scale *via* the "click" reaction.

Compound	c_{stock} (mM)	c (mM)	V (µL)
Buffer NaH_2PO_4	500	100	20
THPTA	50	2.5	5
Na Ascorbate	50	5	10
$CuSO_4 . 5H_2O$	5	0.5	10
dye	1	0.05	5
oligo	0.1	0.01	10
H_2O			40
			100

5.10.2 Precipitation of Nucleic Acids

The method of choice for precipitation of nucleic acids depends on the size and the desired purity of the nucleic acids. Standard ways of precipitation of nucleic acids are with ethanol, isopropanol and lithium perchlorate.

5.10.2.1 Precipitation with Ethanol and Lithium Perchlorate

After a reaction time of 3 hours the reaction solutions were treated on NAP-5 column to remove salts and unreacted dyes. In the same manner DyeEx spin column were used to remove unreacted material and most of all the unreacted dye. It was discovered that using

DyeEx columns led to a significant loss of oligonucleotide.

The precipitation with lithium perchlorate and ethanol, respectively were applied. Depending on the scale of the reaction, 2% of $LiClO_4$ in acetone (10 fold excess) was added for precipitation of the "clicked" oligonucleotide and the mixture was centrifuged at rt at 18 000 x g for 1 h. The supernatant was aspirated 2% and the pellet of the fluorescent labeled oligonucleotide was washed with 1 mL of pure acetone. The solution was centrifuged again at 18 000 x g for 10 min. Acetone was removed and the pellet was dissolved in 10 µL or 100 µL of water, depending on the scale of the reaction. Samples that were prepared in this manner were used for gel electrophoretic analysis and further purification.

For the ethanol precipitation the solution was treated on a NAP-5 column, the sample was inverted with a 10-fold excess of cold ethanol and incubated at - 24 °C for 2 h. The sample was centrifuged at 16 000 x g at -4 °C for 1 h, after which a tiny pellet was visible. In comparison with the $LiClO_4$ precipitation the pellet was significantly smaller. It is known that for rather short nucleic acids best yields have been observed by precipitation with lithium perchlorate.

5.10.3 Determining the Concentration of Nucleic Acids

5.10.3.1 Photometric Determination

Concentrations of fluorescent-labeled oligonucleotides were determined by UV measurements on a Nanodrop ND-2000 (PeqLab, Erlangen). By measuring the concentrations of labeled oligonucleotides on a Nanodrop ND-2000, the efficiency of the above mentioned precipitations and also different purification using NAP-5 column or DyEx was compared to optimize the best work-up conditions.

5.10.4 Gel Electrophoretic Method

Separation of nucleic acids *via* gel electrophoretic methods is based on the different migration of molecules with different charges and masses in an electrical field. The migration depends on the electric field and the connectivity of the gel matrix. Agarose and polyacrylamide are commonly used as gel matrix.

5.10.4.1 Denaturing Polyacrylamide gel Electrophoresis (PAGE)

PAGE has been used for detection and purification of the 5'-hexynyl oligonucleotide "clicked" with the azido dyes. The denaturing conditions of the PAGE analysis are mild and conserve secondary and tertiary structures of nucleic acids. Oligonucleotides have been separated on 20% polyacrylamide gels. The gel solution has been prepared with Rotiphorese® Ready-to-Use Gel Solutions, containing sequencing gel concentrate, sequencing gel diluent and sequencing gel buffer. After the addition of ammonium persulfate (APS (1% (w/v)), was added as a radical donator and *N,N,N′,N′*-tetramethylethylenediamine (TEMED (0.1% (v/v)) as catalyst for the formation of free radicals in the presence of ammonium persulfate and is thus used as an enhancer for the polymerization. Vertical gels have been produced between two glass plates. The layer thickness was 1.0 mm for analytical and also for preparative gels.
Loading buffer (TBE-buffer) containing two dyes xylenecyanol and bromophenol blue assisted in monitoring the migration behavior of the nucleic acids. For the analysis of fluorescent-labeled oligonucleotides bromophenol blue was allowed to run out of the gel or cut off in the scan.

5.10.5 Detection of Fluorescent-labeled Nucleic Acids

5.10.5.1 Fluorescent detection

Visualization of PAGE analyses was done by imaging with a Typhoon 9400 (GE Healthcare, Munich) and UV shadowing and imaging by an AlphaImager (Alpha Innotech Corporation, San Leandro). Typhoon 9400 provides 457/488/532/633 nm excitation, with the following lasers:
red 10 mW Helium neon laser 632.8 nm,
green 20 mW solid state doubled frequency SYAG laser 532 nm,
blue 30 mW argon ion laser 488 nm (20 mW), 547 nm (4 mW).
The wavelength for imaging was tailored to the fluorescence spectra (excitation and emission) of the fluorescein dyes. The blue laser was particularly used.[120]

5.10.5.2 **UV-shadowing**

UV-shadowing was used as one of the detection methods applied in gel electrophoresis. UV-shadowing allows the detection of nucleic acids without any treatment; however, large amounts (> 1.0 nmol) of nucleic acids are necessary as compared to above mentioned methods. The gels were covered in plastic and placed on fluorescent TLC plates. Exposed to UV light (254 nm), the oligonucleotides quenched the fluorescence and were visible as dark shadows. Documentation of the readout can be carried out with the AlphaImager.

5.10.5.3 **Staining**

The fluorescent nucleic acid gel stain, GelRed was used for the staining of the nucleic acids instead of the highly toxic ethidium bromide. This was particularly used to visualize free, unreacted oligonucleotides. PAGE gels were stained by agitating them in the GelRed solution for 10 min.

PAGE gels were also stained by agitating them in a solution of Stains-All under the light protection overnight.

The fact that the excitation maxima for the dye-nucleic acid complex is around 495 nm and the emission maxima at ~537 nm made the Typhoon 9400 the perfect readout.

The gels were usually at first scanned on Typhoon 9400 using the blue laser to visualize the oligonucleotides that were “clicked” with the dyes. Afterwards the gels were stained with a GelRed solution to visualize free, “unclicked” oligonucleotides. Stains-All was used as a final staining to image the general content of the gel.

5.10.6 PAGE Electrophoretic Results

The oligonucleotide with a terminal alkyne was “clicked” with the new fluorescent xanthene dyes MK-57, MK-61, MK-75, MK-83 and with the commercially available fluorescent azide, Atto488, as control. In figure 26 are “click” products visualized with the blue laser (488 nm), as was scanned on the Typhoon 9400.

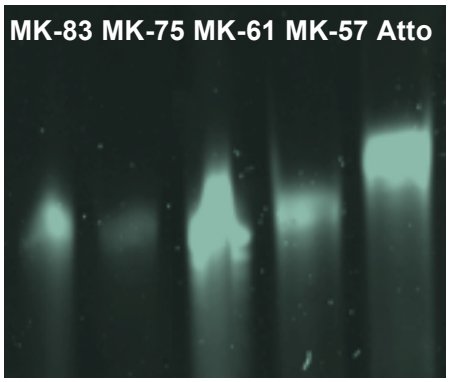


Figure 26. PAGE electrophoresis of „click“ products after scanning on Typhoon 9400 upon excitation with the blue laser (488 nm).

In figure 27 is illustrated an overlay of images, particularly an image from the scan with blue laser, an image from staining with GelRed and Stains-All. In the columns 1 and 2 are placed free oligonucleotide 5'-hexynyl CGC GCG AAG CTT AAT ACG ACT CAC TAT A, in the third column an oligonucleotide with the same sequence, however lacking terminal alkyne group, was loaded. MK-57 “clicked” with the alkynyl oligonucleotide was loaded in the column 4. Column 5 contains free dye, MK-57. Columns 6 and 7 carry MK-61 “clicked” with the alkyne containing oligonucleotide and MK-61 dye, respectively.

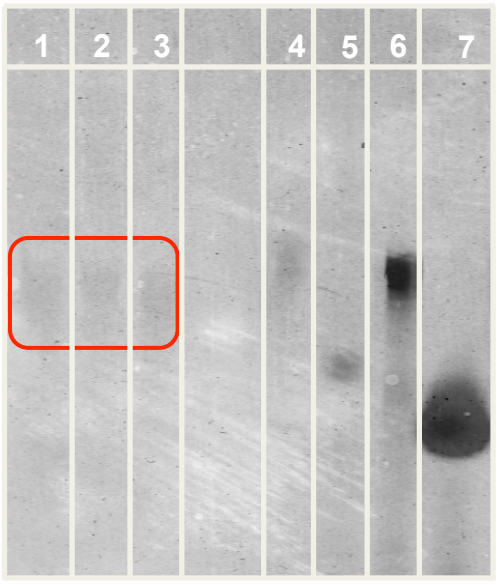


Figure 27. PAGE electrophoresis of free oligonucleotides, selected „click“ products and corresponding fluorescent dyes via an overlay of images, in particular an image from the scan with the blue laser, an image from the staining with GelRed and Stains-All.

6 List of Abbreviations

A, Abs	Absorbance
AMC	7-amino-4-methyl coumarin
APF	3'-(*p*-aminophenyl) fluorescein
BINAP	2,2'-Bis(diphenylphosphino)-1,1'-binaphthyl
Boc	*Tert*-butyloxycarbonyl
BODIPY	Boron difluoride dipyrromethene
c	Concentration
c	Speed of light
CuAAC	Copper(I)-catalyzed azide-alkyne cycloaddition
Cy7	Cyanine 7
CyDyes	Cyanine dyes
d	Doublet
DAPI	4',6'-diamidino-2-phenylindole
DBU	1,8-diazabicycloundec-7-ene
DCF	2',7'-dichlorofluorescein
DCM	Dichloromethane
DIPEA	*N,N*-diisopropylethylamine
diSO_3NaFl	Sodium 3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-4',5'-disulfonate
DMAP	4-Dimethylaminopyridine
DMF	*N,N'*-Dimethylformamide
DMSO	Dimethyl sulfoxide
E	Energy
EDANS	5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid
em	Emission
equiv	Equivalent
Et	Ethyl
EtOAc	Ethyl acetate
ex	Excitation
FDG	Fluorescein di-β-galactopyranoside
FITC	Fluorescein isothiocyanate

FRET	Fluorescence resonance energy transfer
g	Relative centrifuge force
h	Hour(s)
h	Planck's constant
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HPF	3'-(*p*-hydroxyphenyl) fluorescein
HPLC	High pressure liquid chromatography
HSO_3Fl	3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-4'-sulfonic acid
hν	Energy of photon
$h\nu_{em}$	Energy of the emitted photon
$h\nu_{ex}$	Energy of the excitated photon
IR	Infrared spectroscopy
ITC	Intersystem crossing
J	Coupling constant
L	liter
LiHMDS	Lithium hexamethyldisilazide
m	Multiplet
M	Molar concentration
MeOH	Methanol
mg	Miligram
min	Minute
mL	milliliter
MOM	Methoxymethyl group
MS	Mass spectrometry
MsCl	Methanesulfonyl chloride
N3BC	7-azido-4-(bromomethyl)coumarin
nHex	*n*-Hexane
NMR	Nucleic magnetic resonance
NR	nonradiative
ON	Overnight
PeT	Photoinduced electron transfer
pK_a	Acid dissociation constant, negative logarithm
ppm	Parts per million

q	Quadruplet
R_f	Retention factor
Rh_{110}	Rhodamine
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSA	Rhodamine spiroamide
rt	Room temperature
rt	Room temperature
s	Singlet
S_1	First electronic single state
S_2	Second electronic single state
S_EAr	Electrophilic aromatic substitution
SMS	Single-molecule switching microscopy
STP	Sulfotetrafluorophenyl ester
STP	sulfotetrafluorophenyl ester
t	Triplet
T_1	Triplet excited state
TBDMS-Cl	Tert-butyldimethylsilyl chloride
TEMED	*N*,*N*,*N*',*N*'-Tetramethyl-ethane-1,2-diamine
TFP	Tetrafluorophenyl ester
TG	Tokyo Green
THPTA	Tris(hydroxypropyl)triazolylmethyl-amine
TLC	Thin layer chromatography
TMR	Tetramethylrhodamine
TMR-NN	*N,N,N',N'*-tetramethylrhodamine diazoketone
TPS	2,4,6-triisopropylbenzenesulfonyl
TPSCl	2,4,6-triisopropylbenzenesulfonyl chloride
TRITC	tetramethylrhodamine isothiocyanate
UV	Ultraviolet
Xantphos	4,5-Bis(diphenylphosphino)-9,9-dimethylxanthene
δ	Chemical shift
ε	Molar Extinction coefficient
λ	Wavelenght
λ_{em}	Wavelenght of maximum emission

λ_{max}	Wavelenght of maximum absorption
μL	microliter
ν	Frequency
Φ	Quantum yield

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