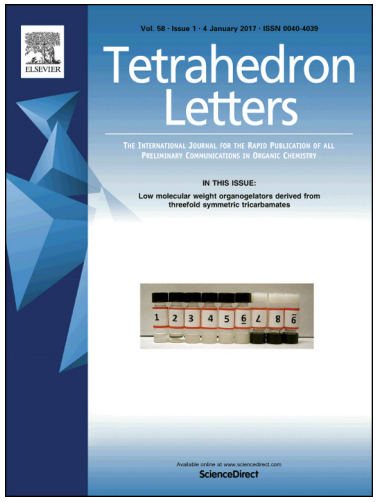

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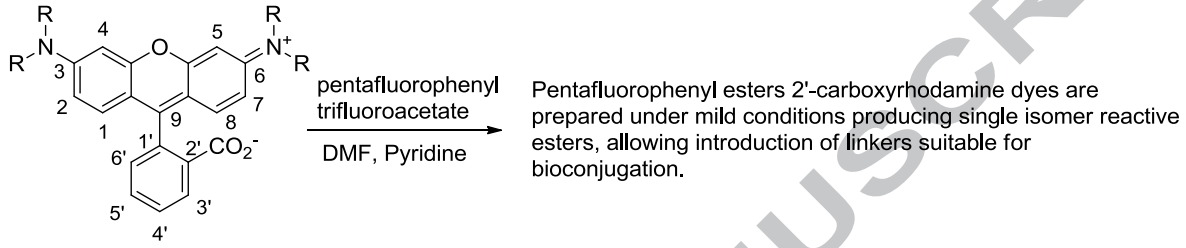

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Unexpected reactivity of the 2'-carboxyl functionality in rhodamine dyes

Richard A. Haack[a,*], Susan Gayda[b], Richard J. Himmelsbach[a] and Sergey Y. Tetin[b]

[a]*Organic Chemistry Process Design, Abbott Diagnostics Division, Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Il 60064-6016, USA*
[b] *Molecular Binding Characterization, Abbott Diagnostics Division, Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Il 60064-6016, USA*

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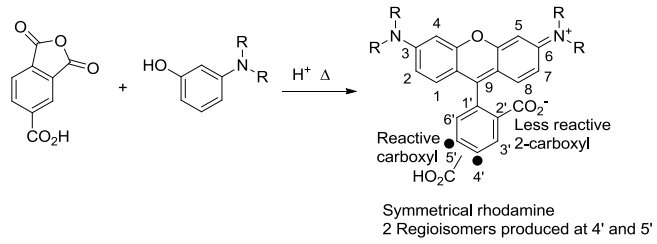
ABSTRACT

Contrary to previous literature reports, the reactivity of the 2' carboxylic acid in rhodamine dyes was found to be much more reactive than anticipated. Typically, the 4'- or 5'-carboxy functionality in dicaboxyrhodamines is targeted for bioconjugation use due to the supposed unreactivity of the 2' carboxylic acid. Reactive esters of 2'-carboxyrhodamine dyes lacking the 4'(5')-carboxy substitutions permit a simplified synthesis of single isomer dyes but possess similar reactivities useful for labeling studies.

* Corresponding author. Tel.: +0-000-000-0000; fax: +0-000-000-0000; e-mail: author@university.edu

Introduction

Xanthene dyes (fluoresceins, rhodols and rhodamines) containing a reactive linker functionality for bioconjugation are used extensively as labels, tags and probes to study biological systems.[1] In the xanthene dye family, the spectroscopic properties of rhodamine dyes' (RDs) and their conjugates' absorption/emission properties show less pH sensitivity and possess greater photostability compared to their fluorescein relatives.[2] The classic synthesis of reactive symmetrical RDs for use in conjugation chemistry employs reaction of trimellitic anhydride and an aminophenol that generates two regioisomeric dicarboxylic acids that are difficult to separate (Scheme 1).



Scheme 1. Classic synthesis of symmetrical 4'(5') regioisomeric RDs. Other numbering systems are also employed in the literature.[3-5]

Typically, the 4'- or 5'-carboxy functionality in dicarboxyrhodamines (or a mixture of both)[6] is targeted for bioconjugation since the 2' carboxylic acid is generally observed as difficult to activate, possessing low chemical reactivity due to steric hindrance of the xanthene system".[5,7,8] Activation of the 2'-carboxyl in these systems usually requires harsh reagents such as phosphorus oxychloride, which are often incompatible with other functionality in the molecule.[5,7,9] Single isomer RDs are preferable for biological studies because the 4' or 5' dicarboxy isomeric dyes often possess differing labeling specificities and photophysical properties when conjugated to biomolecules.[10] Also, purifications and spectroscopic characterizations during chemical synthesis of the dye and dye/linker combinations are greatly simplified when operating with a single isomer. Another reason the 2' position is undesirable for direct conjugation is that reaction with a primary amine (*e.g.* lysine residue in a protein) produces a secondary amide, which cyclizes under neutral to basic conditions to produce a non-fluorescent spiroamide product (Figure 1).[5,8]

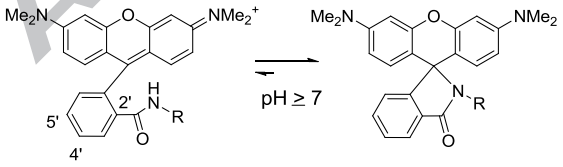
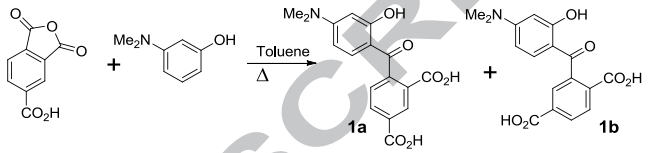


Figure 1. Non-fluorescent spirolactam formation from 2'-carboxyamido RDs derived from a primary amine.

Experimental Findings

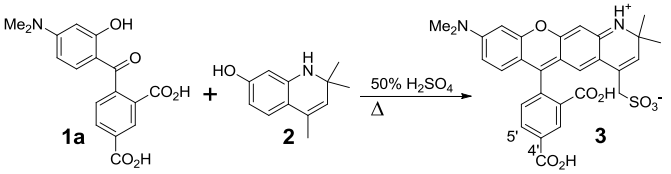
High definition immunoassay (HDIA)[11] and single molecule detection[12] research projects in our laboratories required the preparation of large quantities of photophysically customizable, photo-efficient RDs. Due to the challenging separations required during their synthesis, isomerically pure, 4'- or 5'-caboxyphenyl RDs are limited, extremely expensive[13] and many are sold only as a mixture of the two regioisomers.[5] Therefore, we began a program in our laboratories to prepare RDs tailored to our specific needs.

Initially, we utilized the method described by Shmanai *et al*[4] which condenses trimellitic anhydride and 3-(dimethylamino)phenol in toluene in the absence of strong acid catalysts to produce a mixture of two regioisomeric benzophenones (**1a** and **1b**). These are easily separable on a large scale by crystallization (Scheme 2).



Scheme 2. Condensation of trimellitic anhydride under non-acidic conditions.[4]

Either isolated benzophenone reacts with a variety of aminophenols under acidic conditions producing "unsymmetrical" single isomer RDs. Structural features of the aminophenol used in the reaction alter the spectroscopic and brightness properties of the resulting dye, which allows tailoring/tuning of the RD's with desired properties.[3,14] For example, benzophenone **1a** reacts with 2,2,4-trimethyl-1,2-dihydroquinolin-7-ol (**2**)[15] in 50% sulfuric acid at 100 °C with concomitant sulfonation of the allylic methyl group occurring under these reaction conditions[7] yielding 4'-carboxy-RD **3** (Scheme 3).



Scheme 3. Condensation of benzophenone **1a** with aminophenol **2**. (**1a** + 1.2 eq. **2**, 50% H_2SO_4, 125 °C, 2 hr, reverse phase HPLC purification, Yield **3** 10.4%)

During our initial attempt to prepare the 4'-mono-pentafluorophenyl active ester of RD **3** utilizing pentafluorophenyl trifluoroacetate/pyridine/DMF[16] (Scheme 4 left), UPLC-MS analysis showed the formation of a 1:1 mixture of two products with the same mass corresponding to two isomeric mono pentafluorophenyl esters, a small amount of the diester product and unreacted starting material **3** within the first 10 minutes of the reaction (Figure 2 top). After 1 hour, the reaction mixture contained a 1:1 mixture of unreacted RD **3** and the diester product **4** as well as a "steady state" 1:1 mixture of two monoester products (Figure 2 middle). After 24 hours, both the starting material and the two monoester products were consumed and diester **4** was the major product (Figure 2 bottom).

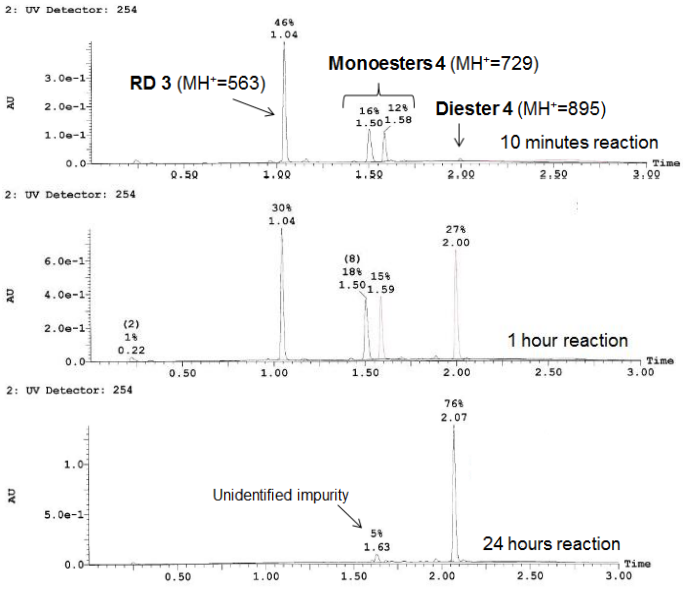
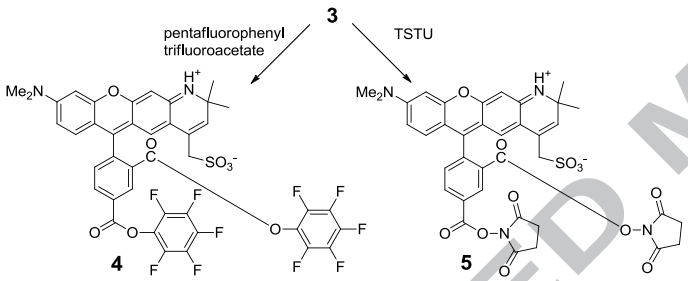


Figure 2. Pentafluorophenyl active ester formation after 10 minutes (top), 1 hour (middle) and 24 hours (bottom). Reaction monitored by UPLC-MS[17]



Scheme 4. Unexpected diester products **4** and **5** produced from **3** utilizing pentafluorophenyl trifluoroacetate and TSTU respectively.

The reactivity profile for the 2' and 4' carboxyl groups was further explored by using *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)-uronium tetrafluoroborate (TSTU)[5] to generate the corresponding NHS diester **5** (Scheme 4 right). The unexpected facile activation of the 2'carboxyl group of RD **3** lies in contrast to literature reports indicating that the 2' carboxylic acid is unreactive due to steric hindrance of the bulky xanthene ring system and its activation requires highly reactive reagents.[5,8] Additionally, it has been reported that either the 4' or 5' carboxyl groups may be easily converted to active esters and conjugated in the presence of the unreactive 2'-carboxylic acid.[8] Also, the low reactivity of the 2' carboxylic acid in RD systems is exemplified in a publication describing activation of a compound similar to RD **3** but containing only a 2' carboxylic acid (Figure 3), This RD required the highly reactive reagent, *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium-PF_6^- (HATU) and required overnight reaction run at 40-50 °C.[7]

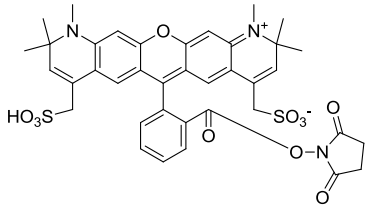


Figure 3. Literature example of a 2'-carboxyrhodamine

Bioconjugation studies typically require a single point of attachment of the RD making the reactive diesters unsuitable for this purpose since the 2' and 4'(5') esters react non-selectively with amines. For example, the pentafluorophenyl diester **4** shows no chemoselectivity with amines[18,19] providing a mixture of both monoamide products as well as the diamide product **6a/6b/7** (Figure 4) Thus, we further examined the 2'-carboxyl reactivity of RDs and fluoresceins.

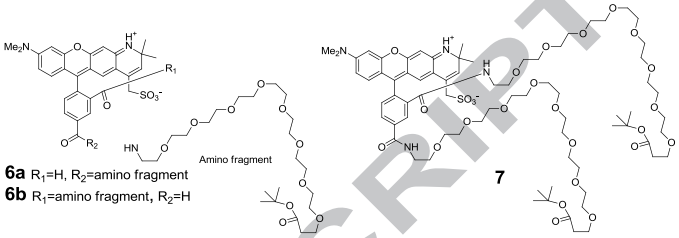
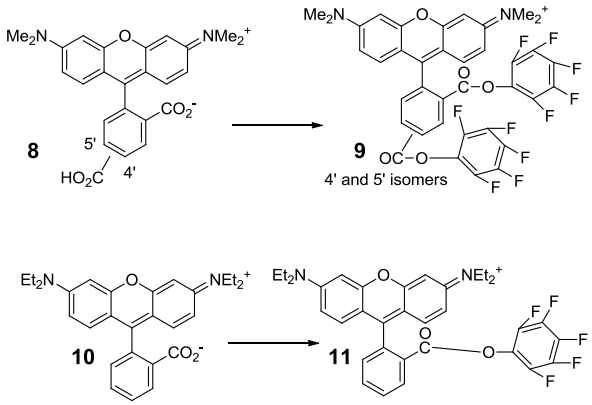


Figure 4. Example of mono and diamide products obtained from the dipentafluorophenyl ester **4**.

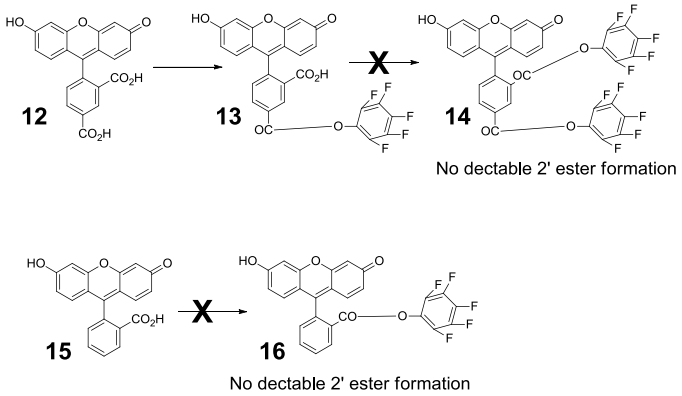
Confirmation of Reactivity

To ascertain the scope of the 2' carboxyl group's reactivity, we examined the 2'-monocarboxylic acid in both rhodamine and fluorescein dyes. 4'(5')-carboxytetramethyl rhodamine (**8**) (mixture of 4' and 5' position regioisomers) and rhodamine B (**10**) were treated with pentafluorophenyl trifluoroacetate in pyridine/DMF at room temperature. UPLC-MS analysis of each reaction indicated that after only 10 minutes of contact, the diester **9** and the 2' ester **11** had formed quantitatively (Scheme 5).



Scheme 5. Pentafluorophenyl active ester formation 4'(5')-carboxyrhodamine **8** (top) and rhodamine B **10** (bottom).

We then subjected 4'-carboxyfluorescein (**12**) and fluorescein (**15**) to the same conditions (Scheme 6). UPLC-MS analysis of the 4'-carboxyfluorescein reaction showed only a single mono-pentafluorophenyl ester, presumably 4'-**13**, without a trace of diester **14** and fluorescein gave no detectable 2'-ester **16**. These results clearly contrast the reactivity differences of the 2'-carboxy functionality in the fluorescein and rhodamine dye families.



Scheme 6. Pentafluorophenyl active ester formation 4'-carboxyfluorescein (top) and fluorescein (bottom).

Discussion

These results demonstrate the high reactivity of the 2'-carboxyl group of RDs and the inertness of the 2'-carboxyl group of fluorescein dyes. This suggests that in polar aprotic organic solvents, fluoresceins probably exist in the closed, spirolactone/zwitterionic form, rendering their 2'-carboxyl inert (Figure 5).[20-22] Contrarily, RDs exist in the open form through a broad pH (3-14) range in polar organic solvents.[23,24] This explanation is consistent with our observation that when either fluorescein, **12** or **15,** is dissolved in the reaction solvents (DMF containing pyridine), both give a pale straw colored and weakly fluorescent solution indicating the non-fluorescent spirolactone form predominates under the reaction conditions. However, upon acidification with 1N hydrochloric acid, these DMF solutions become bright yellow and are highly fluorescent when irradiated with a handheld long wave UV lamp (365 nm) indicating the presence of the fluorescent open form under these non-reaction conditions. In contrast, rhodamines **8** and **10**, under the same conditions give bright red, highly fluorescent solutions which remain unchanged upon acidification, indicating the fluorescent open form predominates under the reaction conditions.

Also, although the low reactivity of the 2'-carboxyl in the RD series has been attributed to steric hinderance,[8] molecular models show that the phenyl bearing the 2' carboxyl is essentially perpendicular to the xanthene system due to the congestion of the 1,8-peri hydrogens.[25,26] Also, X-ray studies have shown that the spirolactone or inner salt of the carboxylate/xanthene cation exists in the crystal state (Figure 5).[27,28] This situates the 2'-carboxyl above the plane of the xanthene system (Figure 5).

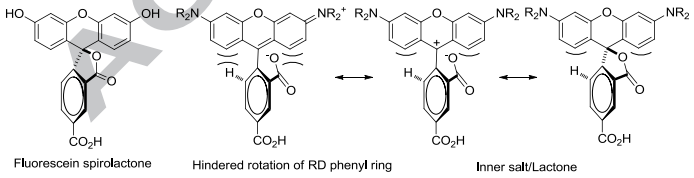


Figure 5. Fluorescein spirolactone (left) and RD (right).

Proton NMR confirmed the perpendicularity of the phenyl of RD **3** as its NMR spectrum clearly shows the hindered rotation of the phenyl ring as the two hydrogens of the sulfonylmethyl group are non-equivalent due to the chirality of the hindered system. An AB quartet centered at ~3.61 ppm (Figure 6) is observed showing the non-equivalency. This splitting pattern has been observed in a structurally similar, symmetrical RD-disulfonic acid as well.[7]

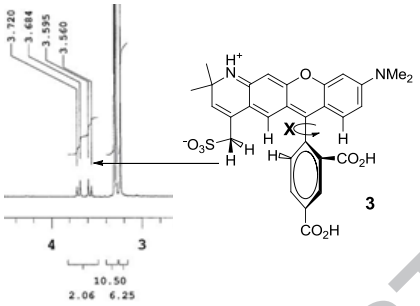
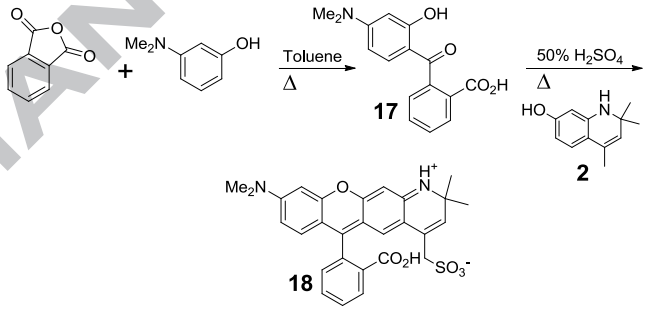


Figure 6. Nonequivalent sulfonylmethy ^{1}H signals of RD **3**.[29]

It is apparent that the 2'carboxyl of RDs is not as sterically hindered as previously claimed due to its positioning above the xanthene plane. The 2'-position appears to be as reactive as the 4' or 5' positions, making it a suitable point for conjugation in fluorescent studies.

For example, we prepared an unsymmetrical RD with phthalic anhydride to produce benzophenone **17**[14] which lacks the 4'(5')-carboxyl moiety, alleviating the need to separate regioisomers. The benzophenone **17** was condensed with hydroxy-dihydroquinoline **2** in 50% sulfuric acid at 125 °C, providing RD **18** (Scheme 7).



Scheme 7. Synthesis of unsymmetrical-RD **18** lacking the 4'(5')-carboxyl (same reaction conditions as in Schemes 2 and 3 above. Yield **18** 11%).

RD **3** and RD **18** possess photo-physical properties similar to the commercially available RD, Alexa Fluor 546.(Figure 7).[30]

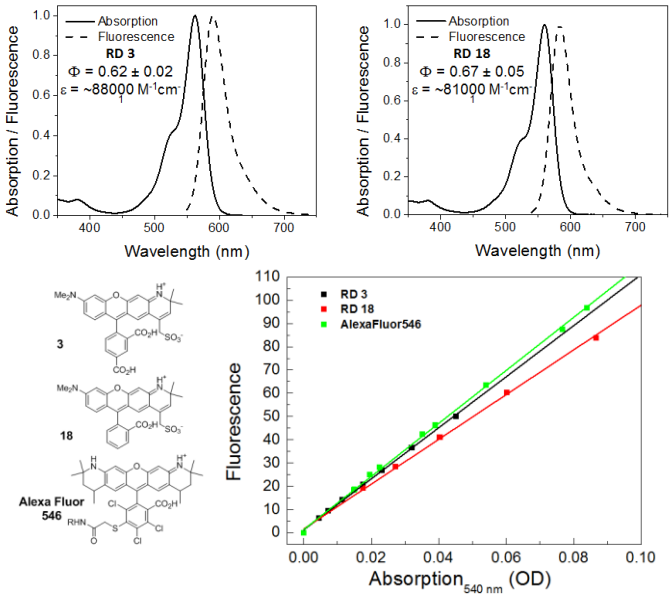
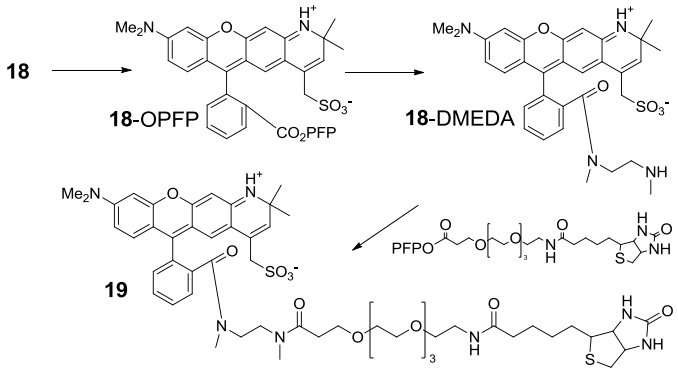


Figure 7. Absorption/emission properties of RD **3** and RD **18** (top). Fluorescence intensity compared to Alexa Fluor 546 (bottom).

For fluorescent bioconjugation studies, a secondary amine must be used to react with the 2' position to prevent spirolactamization.[5,8] Secondary amines such as piperazine, N-methyl-6-aminocaproic acid, N,N'-dimethylethylenediamine (DMEDA) have been employed by us and others[8] for this purpose. For example, we prepared a biotinylated-RD **19** containing a PEG linker. Reaction of RD **18**-pentafluorophenyl ester with excess DMEDA followed by reaction of **18**-DMEDA with a biotinylated PEG pentafluorophenyl ester provided highly fluorescent PEG-linked biotinylated-RD **19** (Scheme 8). The dye possesses essentially identical absorption/emission properties as that of the core RD **18** and the 2'-tertiary amide linkage makes it insensitive to pH changes.[5,8] The uses and properties of this dye will be presented elsewhere.



Scheme 8. Preparation of PEG-linked biotin-RD **19**: (*i*. RD **9**, pentafluorophenyl trifluoacetate, pyridine, DMF, room temperature; *ii*. excess DMEDA; *iii*. pegylated biotin acid[19], pentafluorophenyl trifluoacetate, pyridine, DMF; *iv*. product of step *i*. + product of step *iv*., DIEA, DMF) 75% overall yield from **18**.

Conclusion

Whilst the origin of the notion that the 2'-caboxyl group of rhodamine dyes is difficult to activate due to steric hindrance of the bulky xanthene remains unclear, it may be a carryover which stems from the 2'-carboxyl's inertness in the fluorescein series. We have found that, contrary to many discussions published on its reactivity, the 2'-carboxyl group of the rhodamine dye system can be easily activated with mild activating agents clearly demonstrating its availability for reaction equal to that of the less hindered 4' or 5' positions.

We demonstrated that the need for a "reactive" 4' or 5'-carboxyl group is unnecessary as an attachment point for conjugation. Synthesis of RDs lacking the 4' or 5' carboxylic acids and utilization of the mildly activated 2' position as an attachment point greatly simplifies synthesis and purification of RDs and eliminates the need for tedious isomer separations when single isomers are required.

Acknowledgments

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References and notes

* All new compounds exhibited analytical data (NMR and HRMS) consistent with their assigned structures. The yields reported are for chromatographically pure samples.

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(30) The absorption spectra were measured on Cary UV-Vis spectrophotometer. All fluorophores were diluted in PBS to various concentrations. Corresponding fluorescence emission spectrum of each sample was excited at 540nm and measured on Florolog spectrofluorometer. Total fluorescence intensity of each sample was plotted as a function of its absorption value at 540nm.

Highlights

- Unexpected reactivity of the 2’-carboxyl functionality in rhodamine dyes was found.
- Pentafluorophenyl esters of 2'-carboxyrhodamine are mildly prepared.
- A “reactive” 4’(5’)-carboxyl is unnecessary as an attachment point for conjugation.
- Rhodamine dyes lacking the 4’(5’) carboxyl produces a single isomer.
- No tedious isomer separations when single isomers are required.