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From Spirolactam Mixtures to Regioisomerically Pure 5- and 6-Rhodamines: A Chemodosimeter-Inspired Strategy

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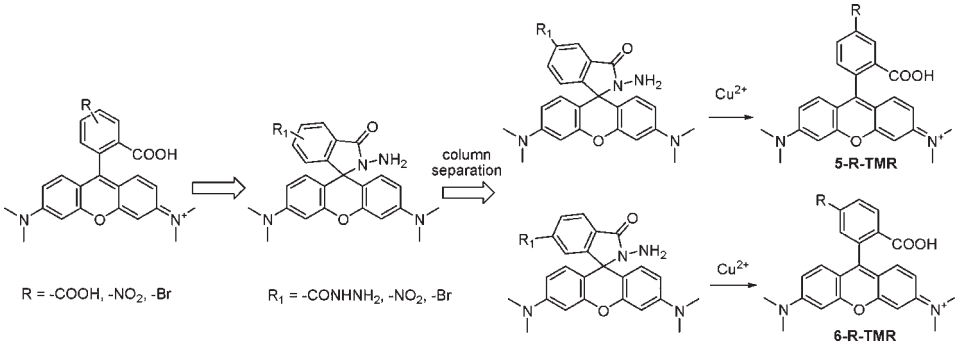
ABSTRACT



Inspired by the ring-open reaction of rhodamine spriolactams as typical chemodosimeters, a general strategy is proposed to conveniently and efficiently synthesize isomerically pure 5- and 6-R-tetramethylrhodamine on a larger scale.

Rhodamine dyes, bearing a linker on the 5 or 6 position of the benzoic acid moiety, are well-known as fluorescent labels to covalently attach to many biomolecules (oligonucleotides and proteins).[1−3] Using one of the pure 5 or 6 regioisomerical rhodamines is highly recommended in some advanced biomedical fields where use of their mixture might be problematic.[4] For example, in DNA sequencing, an oligonucleotide primer attaching to a mixture of 5/6-R-rhodamine isomers will cause significant spreading of PAGE (PolyAcrylamide Gel Electrophoresis) bands and give rise to multiple bands of identically sized oligonucleotides, which leads to false sequence determination.[5] In the typical synthesis of rhodamines, mixtures of 5/6 isomers are generated simultaneously by the

Scheme 1. Concept of the Chemodosimeter Based on Rhodamine-Spirolactam: Metal-Induced Ring-Opening Reaction

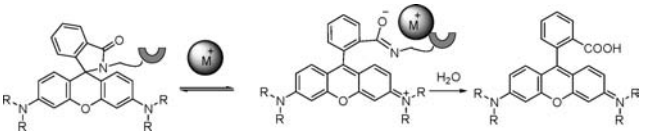


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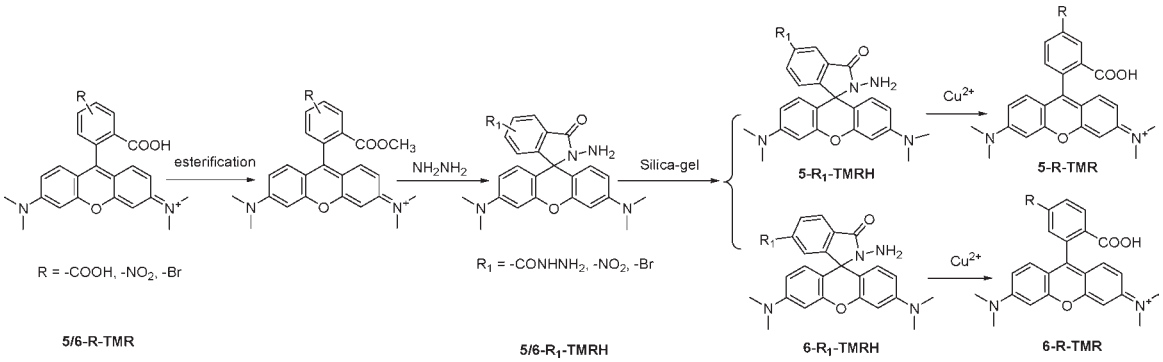
Table 1. Approach to Regioisomerically Pure 5-R-TMR and 6-R-TMR



5/6-R-TMR	yields	purity and ratio of 5/6 isomer[a]	5/6-R_1-TMRH	R_f[b]	yield[c]	purity[d]	5/6-R-TMR	total yield[e]	purity[f]
5/6-Br-TMR	74%	80% 3:2	5-Br-TMRH	0.21	53%	>95%	5-Br-TMR	39%	98%
			6-Br-TMRH	0.12	45%	>95%	6-Br-TMR	31%	99%
5/6-CO-TMR	80%	97% 4:5	5-CN-TMRH	0.36	48%	>95%	5-CO-TMR	37%	99%
			6-CN-TMRH	0.48	41%	>95%	6-CO-TMR	33%	99%
5/6-NO-TMR	85%	97% 1:2	5-NO-TMRH	0.32	44%	>95%	5-NO-TMR	38%	98%
			6-NO-TMRH	0.21	55%	>95%	6-NO-TMR	45%	98%

[a] Determined by High Performance Liquid Chromatography (HLPC). [b] Eluent systems could be found in the Supporting Information. [c] Isolated yields. [d] Determined by ^{1}H NMR [e] Isolated yields [f] Determined by High Performance Liquid Chromatography (HLPC)

condensation of a monofunctionalized phthalic anhydride with *m*-aminophenols.[6] To date, column chromatography is the main method for obtaining pure 5/6-R-rhodamine isomers. However, owing to the extreme resemblance between the two isomers and the characteristic of rhodamine dyes as being cationic, the separation of them through this method is cumbersome.[7] Alternatively, a stepwise synthesis has been used to produce a pure rhodamine isomer, for each of the 5/6-R-rhodamine isomers, but the total yields are not satisfactory.[8,9] Hence, any regioisomerically pure 5- or 6-R-rhodamine from a commercial source is extremely expensive ($37 000−40 000/g).

To this end, we proposed an efficient and general strategy to separate the mixture of 5/6-R-tetramethyl rhodamine isomers (5/6-R-TMR), which was inspired from a chemodosimeter of rhodamine spirolactams, as shown in Scheme 1. These spirolactams widely utilized as fluorescent sensors occur by a ring-opening process and further hydrolysis into rhodamine dyes induced by metal ions and other analytes.[10] Based on our previous studies,[11] we found that neutral spirolactams had lower polarity and better solubility in less polar solvent, compared with cationic rhodamines, and could be easily purified through conventional silica-gel column chromatography. Thus, as shown in Table 1, our strategy involved converting 5/6-R-TMR into the corresponding rhodamine spirolactam, namely 5/6-R-rhodamine hydrazine (5/6-R-TMRH), and then purifying two isomers of 5/6-R-TMRH through silica-gel column chromatography. Finally, the two regioisomerical 5/6-R-TMR would be obtained by hydrolysis of the Cu^{2+} ion, respectively. To confirm the universality of our method, three tetramethylrhodamine dyes 5/6-Br-TMR, 5/6-CO-TMR, and 5/6-NO-TMR, important precursors for rhodamine labeling reagents, were chosen in our experiments.

According to our strategy, three pairs of rhodamine dyes have been produced and successfully separated to obtain a pure isomer with high yields. The related data were compiled in Table 1. Herein, 5/6-Br-TMR was used as an example to illustrate the protocol of our approach. A mixture of 1 equiv of 4-bromophthalic anhydride and 2 equiv of *N,N*-dimethylaminophenol, as starting materials, was heated at 180 °C. During this process there are two sequential Friedel−Crafts type electrophilic aromatic

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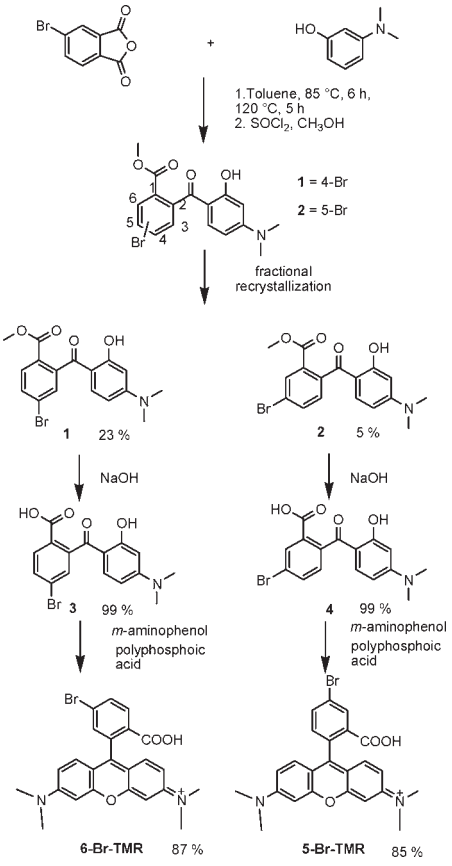
substitution reactions for the formation of the xanthene skeleton. Unfortunately, according to the method reported previously,[12] large amounts of benzophenone derivatives were obtained and they failed ultimately to convert into rhodamine dyes, even despite prolonging the reaction time. To improve the yield of rhodamine, dry DMF containing polyphosphoric acid as a catalyst was particularly adopted to promote intramolecular Friedel–Crafts reactions. A mixture of 5/6-Br-TMR was obtained in 74% yield, and the ratio of two isomers was approximately 3:2, analyzed by HLPC (Supporting Information). Then the mixture was esterified with methanol in the presence of $SOCl_2$. The products of rhodamine ester, without further purification, were directly converted into rhodamine hydrazide in high yield with hydrazine hydrate ($NH_2NH_2 \cdot H_2O$) at room temperature. Next, the mixture of 5/6-R-TMRH was separated by column chromatography (silica gel 200–300 mesh, 15 cm column, petroleum ether/ethyl acetate 3:1, v/v). Separation by chromatography afforded 53% isolated yields for 5-Br-TMRH with R_f 0.32 and 45% isolated yields for 6-Br-TMRH with R_f 0.21. Cu(II) has been known to promote the hydrolysis of acylhydrizine and usually used as a deprotection reagent in organic synthesis.[13] Czarnik reported that $Cu(OAc)_2$ could enhance the hydrolysis of rhodamine hydrazine into rhodamine B in acetronitrile.[10a] In our experiment, extensive optimization was performed to develop conditions for the hydrolysis of TMRH in high yield. Finally, 1 equiv of $CuCl_2$ exhibited a high efficiency of TMRH hydrolysis in a solvent mixture of methanol and tetrahydrofuran (1:9, v/v), and in order to remove Cu(II) from this system, a sodium hydroxide solvent was added. After purification by a short silica gel pad, we obtained a 31% total yield for 5-Br-TMR and 39% total yield for 6-Br-TMR, respectively. Depending on the same strategy, we also obtained 38% and 45% isolated yields for 5-NO-TMR and 6-NO-TMR or 37% and 33% isolated yields for 5-CO-TMR and 6-CO-TMR, respectively. Moreover, the purities of these rhodamine dyes were more than 98%, determined by HLPC, shown in Figures S3–S5.

In previous reports, isomerically pure 5/6-NO-TMR and 5/6-CO-TMR had been synthesized by a stepwise method aided by fractional crystallization of the intermediate.[8,9] However, in the previous stepwise synthesis, the total yields of the corresponding pure regioisomers were far lower than those we obtained through the spirolactam-based strategy. The reported yields of 5- and 6-CO-TMR were just 15% and 10%; nevertheless, in our experiment they were obtained in 37% and 33% yields, respectively. Herein, for further comparison, we also synthesized pure 5- and 6-Br-TMR, through a similar two-step method previously adopted for 5/6-NO-TMR and 5/6-CO-TMR. As shown in Scheme 2, a mixture of 4-bromophthalic anhydride and an equimolar quantity of *N,N*-dimethylaminophenol was used as starting materials. A mixture of intermediate 1 and 2 was easily obtained by silica gel column chromatography after the esterification reaction. Fractional crystallization was successfully applied to isolate isomer 1 with a 23% isolated yield and isomer 2 with a 5% yield. Isomers 1 and 2 were hydrolyzed in 10% NaOH to produce the corresponding carboxylic acid, respectively, which then reacted with another equimolar quantity of *m*-aminophenol catalyzed by polyphosphoric acid.

Scheme 2. Stepwise Synthesis of 5-Br-TMR and 6-Br-TMR



The regioisomerically pure 5-Br-TMR and 6-Br-TMR were obtained in 4% and 20% total yields, respectively. As described above, the yields of 5-Br-TMR and 6-Br-TMR were 31% and 39% according to our strategy. Thus, we again confirmed that our new spirolactam-based strategy for 5/6- substituted TMR was not only more straightforward but also more efficient compared to the stepwise method.

The complete structural analyses of 5-Br-TMR and 6-Br-TMR were deduced using the 1D and 2D spectrum of isomer 1, shown in Figure 1a: in the HMBC spectrum of isomer 1, the downfield signal at 196.7 ppm corresponded to the carbonyl group at the C-13 position, which showed strong remote coupling effects of adjacent proton 3 (δ 7.52 ppm) and 12 (δ 6.88 ppm); the singlet signal of proton 3 indicated that the bromo group was located at the 4 position. Proton 6 was split by adjacent proton 5, and it appeared downfield as a doublet (δ 7.89 ppm). Different from isomer 1, proton 6 of isomer 2 displayed a single peak at a lower field due to the bromo group locating at the 5 position, shown in Figure 1b. Based on the successful structural analysis of isomers 1 and 2, the structure of 5-Br-TMR and 6-Br-TMR can be conveniently determined, shown in Figure 2. The deshielding effect of the

electron-withdrawing carboxyl group made the chemical shifts of the phenylcarboxyl moiety appear at a lower field, compared with xanthenes. For 5-Br-TMR, due to the bromo group at the 5 position and the strong deshielding effect of the carboxyl group, the singlet signal at δ 8.30 ppm was assigned for proton H_a. Two separated doublet signals at δ 8.06 and 7.42 ppm were assigned for two adjacent aryl protons H_b and H_c, respectively, with typical values of coupling constants (J = 8.2 Hz). Moreover, there was a weak vicinal coupling constant between proton H_a and H_c (J = 1.6 Hz). Compared with 5-Br-TMR, 6-Br-TMR exhibited different proton chemical shifts. In the ^{1}H NMR spectrum of 6-Br-TMR, doublet signals at δ 8.13 and 8.02 corresponded to proton $H_{a'}$ and $H_{b'}$, attributed to the bromo group at the 6 position, with a large vicinal coupling constant (J = 8.4 Hz). The single signal at δ 7.81 ppm was assigned for $H_{c'}$ and showed a weak correlation with proton $H_{b'}$. Different from 5-Br-TMR, the signal of the xanthenes moiety of 6-Br-TMR did not appear as multiplets, but rather as a broad single peak. The chemical shift of the aliphatic protons ($N(CH_3)_2$) are equivalent, and there was no difference between 5-Br-TMR and 6-Br-TMR. Similar to 5-Br-TMR and 6-Br-TMR, the structural analysis of 5/6-CO-TMR and 5/6-NO-TMR can be easily confirmed from the chemical shift and coupling patterns for the aromatic proton in the downfield region of the ^{1}H NMR spectrum. The NMR spectra of 5/6-CO-TMR and 5/6-NO-TMR are described in the Supporting Information.

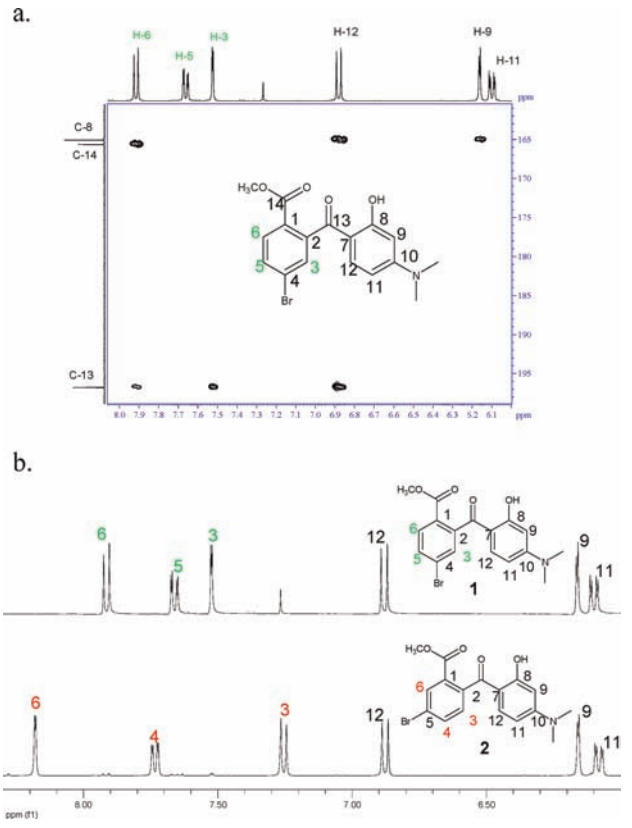


Figure 1. (a) HMBC spectrum of isomer 1. (b) Low-field section of ^{1}H NMR of two isomers 1 (top) and 2 (bottom).

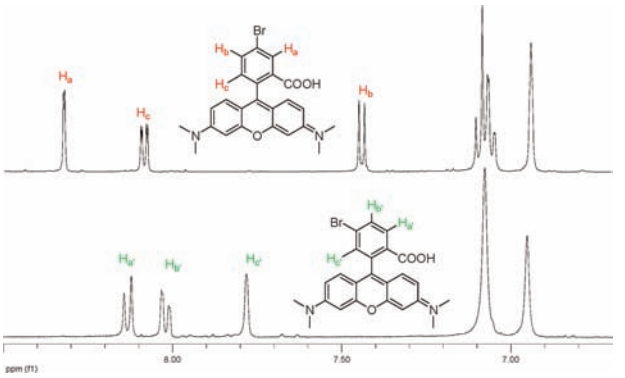


Figure 2. Low-field section of ^{1}H NMR of 5-Br-TMR (top) and 6-Br-TMR (bottom).

In summary, inspired by the design concept of a rhodamine spirolactams-based chemodosimeter, we proposed a novel approach to prepare a regioisomerical 5-R-TMR and 6-R-TMR, e.g. 5/6-Br-TMR, 5/6-CO-TMR, and 5/6-NO-TMR, in high yields and high purities. This approach is more concise and more efficient, compared with stepwise methods previously established. Moreover, it is worth noting that this approach is also suitable for preparation of regioisomerically pure 5- and 6- rhodamine dyes on a larger scale. In a typical experiment, we successfully separated a mixture of 5/6-NO-TMR on an 8.0 g scale. The availability of a facile and inexpensive route to 5-R-TMR and 6-R-TMR should cut down the cost of rhodamine-labeling reagents and enable their adoption for a broad range of applications in biomedical research.

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Supporting Information Available. Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.