Separation of the 5- and 6-Carboxy Regioisomers of ROX and JOE Dyes with Examples of *N*-(3-Azidopropyl)amide Synthesis

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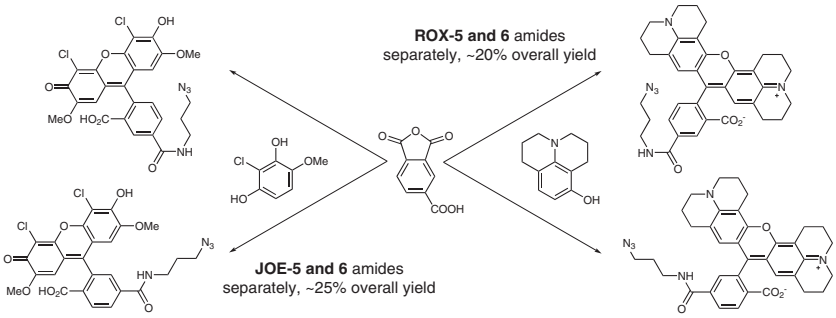

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Abstract Despite the widespread applications of various rhodamine and fluorescein 5- and 6-carboxy derivatives, the preparation of their pure regioisomers, in particular cases, remains a complex task. In the present paper we propose optimized approaches to the synthesis and separation of these isomers of ROX and JOE dyes, and also demonstrate their applicability in the synthesis of the corresponding *N*-(3-azidopropyl)amides.

Key words fluorescent dyes, fluorescein, rhodamine, JOE, ROX, click-chemistry, azide

Various rhodamine and fluorescein derivatives, as well as other xanthene dyes, have long attracted the interest of researchers, and such compounds are actively used for fluorescent labeling in biological research.[1] Their 5- and 6-carboxy derivatives are typically used for binding with target objects or for other functionalization (Figure 1).

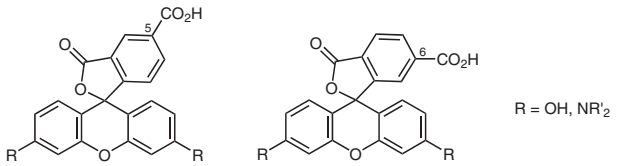


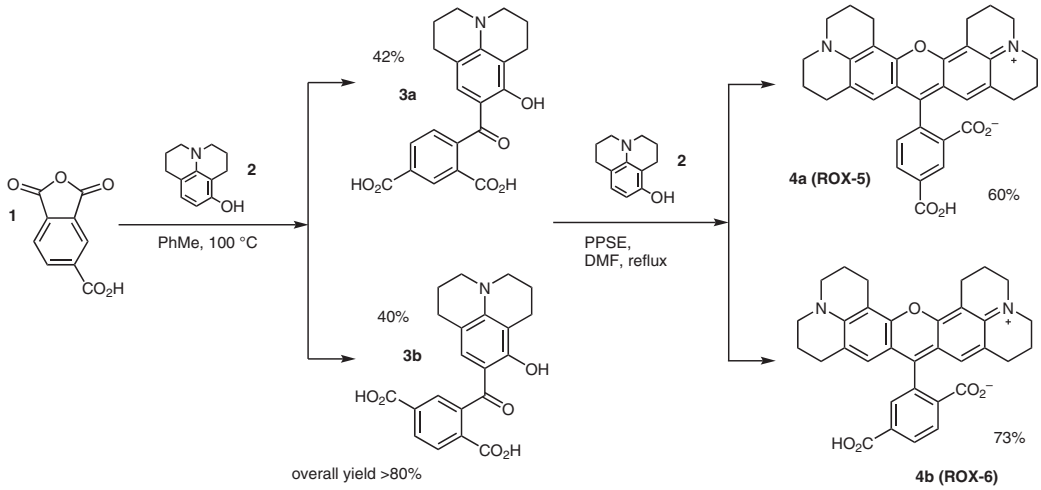
Figure 1 The 5- and 6-carboxy derivatives of xanthene dyes

Despite the widespread applications of these carboxy derivatives, it is surprisingly difficult to obtain these compounds as pure regioisomers. The separation of these isomers is well enough developed for unsubstituted fluorescein[2] and several rhodamines,[3] but for other derivatives, quite complex procedures are used. In particular, such separation (as indeed the synthesis itself) is difficult for one of the most redshifted amino derivatives – ROX (rhodamine 101 or X-rhodamine), as well as for a novel 4′,5′-dichloro-2′,7′-dimethoxyfluorescein – JOE, which has recently been actively used as an analogue of an even more inaccessible hexachlorofluorescein (HEX).[4] The most typical separation methods for obtaining of 5- and 6-carboxy-X-rhodamines are the use of difficult to obtain phthalic mono-aldehydes[5] or chromatographic separation of the final products on large columns using gradient eluting systems,[5a,6] which lead to large losses due to oxidation processes. Typically, isomers of various halogenated carboxyfluoresceins can be separated via fractional crystallization of their lactone diester diethylammonium salts,[4a,7] which is accompanied by a noticeable material loss (at least for one of the isomers), while an efficient yielding column separation of JOE isomers has been reported only for one example of the pentafluorophenyl esters.[4b]

In the present paper we propose optimized approaches to the synthesis and separation of the 5- and 6-carboxy isomers of ROX and JOE dyes, and also demonstrate their applicability in the synthesis of the corresponding *N*-(3-azidopropyl)amides, which are widely used for fluorescent labeling of alkyne-modified oligonucleotides and other targets, which are applied in various fields of biology.[4c,8]

Surprisingly while these derivatives are widely commercially available, their synthetic pathways are still not described in the scientific literature, except for one of the ROX isomers.[9]

The key finding that enabled us to improve the yield significantly and readily separate the 5- and 6-carboxy-X-rhodamines was a stepwise synthesis (Scheme 1).



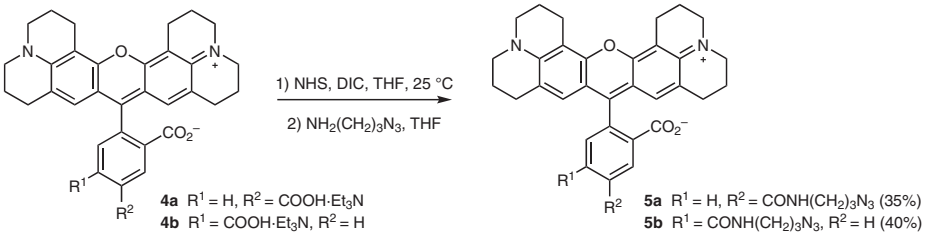
Scheme 1 Preparation of 5- and 6-carboxy-X-rhodamines **4**

Usually, the condensation of aminophenol **2**[10] with 4-carboxyphthalic anhydride **1** is carried out under the action of strong acids and heating (for example, by heating to reflux in propanoic or butanoic acid in the presence of H_2SO_4 or PPSA[5,6]), leading directly to the formation of xanthene products. However, the intermediate ketones **3** can be obtained in the absence of strong acids and with a different ratio of the starting materials. It is known from the literature that the isomers have quite different properties, which has previously allowed their separation by recrystallization in the case of dimethylaminophenol,[3] allowing isomer **3a** to be isolated after condensation of compounds **1** and **2** in acetic acid.[11] Replacing the solvent with toluene allowed us to synthesize in good yield both compounds **3a** and **3b**, which possessed markedly different properties: substance **3b** was amorphous and readily soluble in diethyl ether, whereas isomer **3a** was crystalline and practically insoluble. In this regard, after the polymeric by-products were removed using short flash chromatography, the majority of compound **3a** was isolated from the mixture by simple trituration with ether. Moreover, the remaining mixture of substances **3a** and **3b** was separated by straightforward chromatography on silica gel, eluting with $CHCl_3$/MeOH/Et_3N.

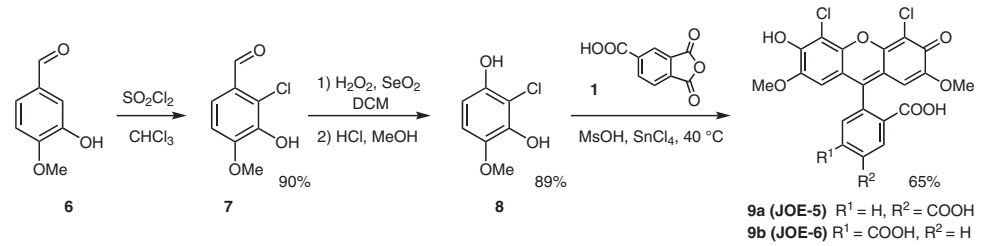
The subsequent condensation to give the corresponding carboxy-X-rhodamines was carried out with an additional equivalent of aminophenol **2** in *N*,*N*-dimethylformamide (DMF) using trimethylsilyl polyphosphate as a dehydrating agent, itself readily obtained from hexamethyldisiloxane and P_2O_5. The resulting isomers of carboxy-X-rhodamines were obtained in good purity (more than 80% by NMR analysis); however, flash chromatography ($CHCl_3$/MeOH/Et_3N) allowed them to be obtained in a purer form and to be converted into the stable triethylammonium salts.

The final conversion of these derivatives into *N*-(3-azidopropyl)-amides **5** is also most efficiently carried out in a stepwise manner, with isolation and crude purification of the intermediate *N*-hydroxysuccinimide ester, which makes it possible to obtain product **5**, not only in good yield, but also in high purity (Scheme 2).

The synthesis of 5- and 6-carboxy derivatives of JOE started from 4-methoxyresorcinol **8**, which is not commercially available but can be synthesized from isovanillin **6**. This synthesis was optimized by Tsybulsky et al.[4b] but it may be noted that gaseous chlorine used in the first step can be conveniently replaced with sulfuryl chloride (Scheme 3).



Scheme 2 Synthesis of 3-azidopropylaminocarbonyl-X-rhodamines **5**



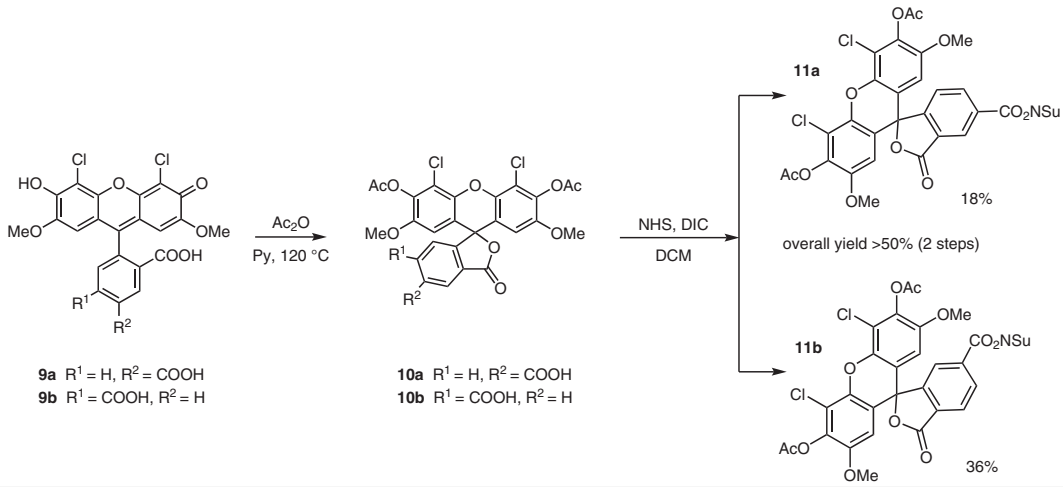
Scheme 3 Synthesis of 5- and 6-carboxy-JOE dyes **9**

Several methods for condensing the resulting resorcinol **8** with 4-carboxyphthalic anhydride **1** are presented in the literature.[4] However, we confirmed that simply gentle heating in the presence of tin chloride, as proposed by Tsybulsky et al.[4b] reproducibly leads to the formation of compound **9**. It should be noted that the isomers can be separated even at this stage using a chromatographic system of NH_3 in EtOH on silica (R_f = 0.30 and 0.45 in EtOH/NH_3(sat, aq), 9:1).

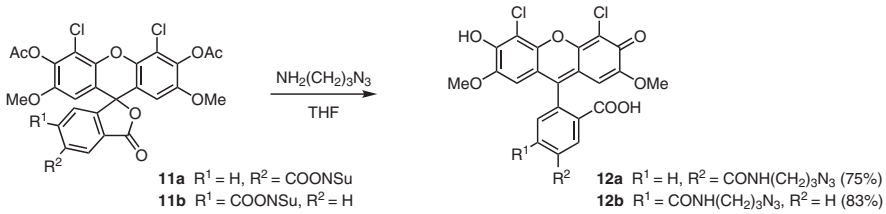
However, even more convenient is the sequence previously proposed for fluorescein[2b] associated with the use of acetylated *N*-succinimidyl esters **11** (Scheme 4), which are well-separated chromatographically on silica using toluene/EtOAc mixtures.

The esters obtained this way can be readily converted into amides by the action of various primary amines, leading directly to the desired amides of JOE; distinguishing this method from the approach using a cyclohexanecarbonyl protective group,[4b] removal of which requires additional effort.

Of course, such an approach leads to the loss of two equivalents of the amine used, but this is not critical against the background of the total cost of the whole synthesis. As an example, this transformation was carried out by us to furnish the corresponding *N*-(3-azidopropyl)-amides **12**, which were obtained with yields of more than 75% each (Scheme 5).



Scheme 4 Synthesis and separation of 5- and 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein derivatives **11**



Scheme 5 Synthesis of 3-azidopropyl-JOE derivatives **12**

Commercially available reagents were used without additional purification. E. Merck Kieselgel 60 was used for column chromatography. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 glass-backed plates (MERCK). Visualization was effected by UV light (254 or 312 nm) and staining with $KMnO_4$.

NMR spectra were recorded with a 700 MHz Bruker Avance III NMR at 293 K, a 800 MHz Bruker Avance III NMR at 333 K, and a Bruker Fourier 300. Chemical shifts are reported relative to residual peaks of $CDCl_3$ (δ = 7.27 ppm for ^{1}H and δ = 77.0 ppm for ^{13}C), D_2O (δ = 4.79 ppm for ^{1}H) or DMSO-d_6 (δ = 2.51 ppm for ^{1}H and δ = 39.5 ppm for ^{13}C). Melting points were measured with an SMP 30 apparatus. High-resolution mass spectra (HRMS) spectra were recorded with an LTQ Orbitrap XL (ThermoFisher Scientific, USA) equipped with a dual-nebulizer ESI source.

Procedures

Trimethylsilyl Polyphosphate (PPSE) in Solution

A mixture of phosphorus pentoxide (20 g, 70 mmol), hexamethyldisiloxane (50 mL, 247 mmol) and chloroform (100 mL) was heated at reflux for 1 h until the solution was clear, then the mixture allowed to cool to r.t. The reagent solution may be stored at 4 °C for 3 months.

(8-Hydroxyjulolidine-9-carbonyl)phthalic Acids 3

To a stirred solution of 8-hydroxyjulolidine **2** (1.89 g, 10 mmol) in toluene (30 mL), 4-carboxyphthalic anhydride **1** (2.3 g, 12 mmol) was added at 60 °C and the reaction mixture was stirred for 48 h at 100 °C. After cooling to r.t., the solvent was decanted and the residue was dissolved in $CHCl_3$/MeOH (60:40) and filtered through a thin layer of silica, collecting slightly colored fractions. The resulting mixture of isomers **3a** and **3b** was heated to reflux in Et_2O (75 mL), cooled to r.t., filtered, and washed with Et_2O (25 mL) to give pure **3a** as a yellow crystalline solid (1.1 g). The mother liquor was evaporated and the residual mixture of **3a** and **3b** was separated by column chromatography ($CHCl_3$/MeOH/Et_3NEt_3N, 75:20:5) to afford compounds **3a** and **3b** as their triethylammonium salts. The obtained salts were dissolved in EtOAc (300 mL) and washed with HCl (3%, 5 × 100 mL). The aqueous phase was extracted with EtOAc (3 × 50 mL) and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated.

4-(8-Hydroxyjulolidine-9-carbonyl)isophthalic Acid (3a)

Overall yield: 1.6 g (42%); yellow solid; m.p. >210 °C (decomp.); R_f = 0.44 ($CHCl_3$/MeOH/Et_3N, 70:25:5).

^{1}H NMR (700 MHz, DMSO-d_6): δ = 13.30 (br. s., 2 H, COOH), 12.83 (s, 1 H, OH), 8.11 (dd, J = 8.1, 1.3 Hz, 1 H, Ar), 8.03 (d, J = 8.1 Hz, 1 H, Ar), 7.78 (d, J = 1.3 Hz, 1 H, Ar), 6.41 (s, 1 H, Ar), 3.23–3.27 (m, 4 H, CH_2), 2.59 (t, J = 6.4 Hz, 2 H, CH_2), 2.42 (t, J = 6.0 Hz, 2 H, CH_2), 1.83–1.87 (m, 2 H, CH_2), 1.73–1.78 (m, 2 H, CH_2).

^{13}C NMR (176 MHz, DMSO-d_6): δ = 19.5, 20.0, 21.0, 26.6, 48.9, 49.4, 104.6, 108.1, 112.7, 128.2, 129.5, 129.9, 130.3, 133.5, 133.7, 140.1, 149.0, 159.8, 166.1, 166.5, 196.5.

HRMS: m/z [M + H]$^+$ calcd for $C_{21}H_{19}NO_6$: 382.1285; found: 382.1296.

2-(8-Hydroxyjulolidine-9-carbonyl)terephthalic Acid (3b)

Yield: 1.5 g (40%); yellow foam; R_f = 0.25 ($CHCl_3$/MeOH/Et_3N, 70:25:5).

^{1}H NMR (700 MHz, DMSO-d_6): δ = 12.97 (br. s., 2 H, COOH), 12.80 (s, 1 H, OH), 8.46 (d, J = 1.3 Hz, 1 H, Ar), 8.18 (dd, J = 7.7, 1.5 Hz, 1 H, Ar), 7.47 (d, J = 7.9 Hz, 1 H, Ar), 6.38 (s, 1 H, Ar), 3.23–3.27 (m, 4 H, CH_2), 2.59 (t, J = 6.4 Hz, 2 H, CH_2), 2.41 (t, J = 6.0 Hz, 2 H, CH_2), 1.82–1.88 (m, 2 H, CH_2), 1.72–1.79 (m, 2 H, CH_2).

^{13}C NMR (176 MHz, DMSO-d_6): δ = 19.5, 20.0, 21.0, 26.6, 48.9, 49.4, 104.6, 108.1, 112.7, 128.3, 129.5, 130.1, 130.7, 131.5, 132.5, 143.8, 149.0, 159.7, 166.2, 171.9, 196.8.

HRMS: m/z [M + H]$^+$ calcd for $C_{21}H_{19}NO_6$: 382.1285; found: 382.1281.

5(6)-Carboxy-X-rhodamines 4a and 4b[6]

The product from the previous stage (**3b**; 1.15 g, 3 mmol) and 8-hydroxyjulolidine (0.83 g, 4.4 mmol) were dissolved in DMF (25 mL). Trimethylsilylpolyphosphate solution in chloroform (7 mL) (see above) was added, and mixture was heated to reflux for 3 h. The solvent was evaporated, the residue was dissolved in NaOH (5%, 26.5 mL) and the mixture stirred at r.t. overnight. The solution was then diluted with HCl (36%, 3.6 mL), and the solid was filtered off, washed with water (20 mL), and air-dried. The product was purified by flash chromatography (gradient of MeOH in $CHCl_3$/Et_3N from 10:85:5 to 40:55:5).

5-Carboxy-X-rhodamines (4a·Et_3N)[6]

Yield: 1.15 g (60%); purple solid; m.p. >250 °C (decomp.); R_f = 0.34 ($CHCl_3$/MeOH/Et_3N, 70:25:5).

^{1}H NMR (700 MHz, DMSO-d_6): δ = 8.38 (s, 1 H, Ar), 8.18 (d, J = 8.1 Hz, 1 H, Ar), 7.17 (d, J = 7.9 Hz, 1 H, Ar), 6.15 (s, 2 H, Ar), 3.23 (t, J = 5.4 Hz, 4 H, CH_2), 3.19 (br. s., 4 H, CH_2), 2.86–2.89 (m, 4 H, CH_2), 2.75–2.81 (m, 6 H, CH_2), 2.45–2.50 (m, 4 H, CH_2), 1.94–1.98 (m, 4 H, CH_2), 1.76–1.80 (m, 4 H, CH_2), 1.07 (t, J = 7.2 Hz, 9 H, CH_3).

6-Carboxy-X-rhodamines (4b·Et_3N)[6]

Yield: 1.4 g (73%); purple solid; m.p. >250 °C (decomp.); R_f = 0.16 ($CHCl_3$/MeOH/Et_3N, 70:25:5).

^{1}H NMR (700 MHz, DMSO-d_6): δ = 8.07 (d, J = 7.8 Hz, 1 H, Ar), 7.88 (d, J = 7.9 Hz, 1 H, Ar), 7.48 (s, 1 H, Ar), 6.12 (s, 2 H, Ar), 3.21 (t, J = 5.5 Hz, 4 H, CH_2), 3.17 (t, J = 5.2 Hz, 4 H, CH_2), 2.88 (t, J = 6.5 Hz, 4 H, CH_2), 2.68 (br. s., 6 H, CH_2), 2.45–2.49 (m, 4 H, CH_2), 1.95–1.99 (m, 4 H, CH_2), 1.77–1.81 (m, 4 H, CH_2), 1.02 (t, J = 7.2 Hz, 9 H, CH_3).

5(6)-(3-Azidopropylaminocarbonyl)-X-rhodamine 5a and 5b

To a stirred solution of the product from the previous stage (**4b**; 1 g, 1.6 mmol) in THF (150 mL), DIC (2 mmol) and NHS (2 mmol) were added, and the mixture was stirred at r.t. for 5 days. The solution was evaporated and the product was purified by flash column chromatography ($CHCl_3$/MeOH, 80:20; R_f ca. 0.25). The product was dissolved in THF (50 mL), 3-azidopropylamine (2 mmol) was added, and the mixture was stirred at r.t. for 1 h. Then, AcOH (0.1 mL) was added and the mixture was stirred 30 min. The solution was evaporated and the product was purified by column chromatography ($CHCl_3$/MeOH/Et_3N, 85:10:5).

5-(3-Azidopropylaminocarbonyl)-X-rhodamine (5a)

Yield: 340 mg (35%); purple solid; m.p. >250 °C (decomp.); R_f = 0.33 ($CHCl_3$/MeOH/Et_3N, 85:10:5).

^{1}H NMR (700 MHz, DMSO-d_6): δ = 8.83 (t, J = 5.5 Hz, 1 H, NH), 8.43 (s, 1 H, Ar), 8.15 (dd, J = 8.0, 1.2 Hz, 1 H, Ar), 7.29 (d, J = 7.9 Hz, 1 H, Ar), 6.13 (s, 1 H, Ar), 3.45 (t, J = 6.8 Hz, 2 H, CH_2), 3.36–3.41 (m, 2 H, CH_2),

3.24 (br. s., 4 H, CH_2), 3.15–3.22 (m, 4 H, CH_2), 2.84–2.92 (m, 4 H, CH_2), 2.43–2.50 (m, 4 H, CH_2), 1.94–2.00 (m, 4 H, CH_2), 1.85–1.77 (m, 6 H, CH_2).

^{13}C NMR (176 MHz, DMSO-d_6): δ = 20.2, 20.3, 21.0, 26.7, 28.3, 36.7, 48.5, 48.8, 49.3, 106.1, 106.4, 115.6, 118.3, 124.3, 124.5, 125.3, 129.5, 130.1, 132.8, 135.6, 145.5, 148.3, 165.1, 167.9.

HRMS: *m*/*z* [M + H]$^+$ calcd for $C_{36}H_{37}N_6O_4$: 617.2871; found: 617.2878.

6-(3-Azidopropylaminocarbonyl)-X-rhodamine (5b)[9]

Yield: 390 mg (40%); purple solid; m.p. >250 °C (decomp); R_f = 0.25 ($CHCl_3$/MeOH/Et_3N, 85:10:5).[9]

^{1}H NMR (800 MHz, DMSO-d_6): δ = 8.68 (t, *J* = 5.5 Hz, 1 H, NH), 8.11 (d, *J* = 8.8 Hz, 1 H, Ar), 8.02 (d, *J* = 7.6 Hz, 1 H, Ar), 7.60 (s, 1 H, Ar), 6.11 (s, 2 H, Ar), 3.37 (t, *J* = 6.7 Hz, 2 H, CH_2), 3.26–3.29 (m, 2 H, CH_2), 3.21–3.24 (m, 4 H, CH_2), 3.16–3.19 (m, 4 H, CH_2), 2.85–2.91 (m, 4 H, CH_2), 2.44–2.49 (m, 4 H, CH_2), 1.94–1.99 (m, 4 H, CH_2), 1.78–1.80 (m, 4 H, CH_2), 1.74 (quin, *J* = 6.9 Hz, 2 H, CH_2).

2-Chloro-3-hydroxy-4-methoxybenzaldehyde (7)[4a,4b]

To a solution of isovanillin **6** (25 g, 165 mmol) in anhydrous $CHCl_3$ (300 mL, EtOH stabilizer must have been removed), SO_2Cl_2 (45 mL, 200 mmol) was added dropwise and the mixture was stirred at r.t. for 24 h. The solid was filtered off, washed with $CHCl_3$, and air-dried to afford **7**.

Yield: 27 g (90%); white solid; m.p. 202–204 °C (Lit.[4a] 203–205 °C).

^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.19 (s, 1 H, CHO), 9.83 (br. s., 1 H, OH), 7.41 (d, *J* = 8.7 Hz, 1 H, Ar), 7.12 (d, *J* = 8.6 Hz, 1 H, Ar), 3.93 (s, 3 H, CH_3).

2-Chloro-4-methoxyresorcinol (8)[4a,4b]

To a suspension of 2-chloro-3-hydroxy-4-methoxybenzaldehyde **7** (4.66 g, 0.025 mol) in CH_2Cl_2 (80 mL), SeO_2 (222 mg, 0.002 mol) and 35% aq H_2O_2 (5 mL, 0.05 mol) were added. The mixture was stirred 48 h at r.t., then the organic layer was separated, washed with 10% $NaHSO_3$ (20 mL), dried over Na_2SO_4, filtered, and evaporated. The resulting solid was dissolved in methanolic HCl [obtained by addition of AcCl (2 mL) to MeOH (40 mL)], then the solution was stirred for 60 min at r.t. and evaporated. The resulting oil was purified by flash chromatography (pure CH_2Cl_2) to afford **8**.

Yield: 3.9 g (89%); white solid; m.p. 75–77 °C (Lit.[4b] 76–77 °C).

^{1}H NMR (300 MHz, DMSO-d_6): δ = 9.37 (s, 1 H, OH), 9.12 (s, 1 H, OH), 6.73 (d, *J* = 8.9 Hz, 1 H, Ar), 6.35 (d, *J* = 8.9 Hz, 1 H, Ar), 3.71 (s, 3 H, CH_3).

4′,5′-Dichloro-2′,7′-dimethoxy-5(6)-carboxyfluorescein (9)[4a,4b]

To a solution of 2-chloro-4-methoxyresorcinol **8** (4.05 g, 23 mmol) and 4-carboxyphthalic anhydride **1** (2.23 g, 11.5 mmol) in methanesulfonic acid (12 mL) was added $SnCl_4$ (1.4 mL, 12 mmol) and the mixture was stirred at 40 °C for 6 h. After cooling to r.t., the reaction mixture was poured into ice-water (200 mL). The solid was filtered off, dissolved in NaOH (15%, 50 mL), and the solution was acidified to pH 2 with HCl (17%). The precipitate formed was collected and air-dried. The product was purified by column chromatography (gradient of NH_3 in EtOH from 10:90 to 25:75). The obtained mixture of isomers was dissolved in water and the solution was acidified to pH 2 with concentrated HCl. The precipitate was collected and air-dried to afford a mixture **9a** and **9b**.

Yield: 3.9 g (65%); red solid.

^{1}H NMR [700 MHz, D_2O+NH_3 (traces)]: δ = 8.25 (s, 1 H, Ar), 8.16–8.23 (m, 2 H, Ar), 8.10 (d, *J* = 8.3 Hz, 1 H, Ar), 7.88 (d, *J* = 7.9 Hz, 1 H, Ar), 7.58 (br. s., 1 H, Ar), 6.43 (s, 2 H, Ar), 6.27 (br. s., 2 H, Ar), 3.65 (s, 6 H, CH_3), 3.59 (br. s., 6 H, CH_3).

3′,6′-Diacetoxy-4′,5′-dichloro-2′,7′-dimethoxy-5(6)-carboxyfluorescein (10)

The product of the previous stage (mixture of **9a** and **9b**) (2 g, 4 mmol) and pyridine (3 mL) in acetic anhydride (28 mL) was stirred for 2 h at 120 °C. After cooling to r.t. the solvent was evaporated, the residue was dissolved in EtOAc (250 mL), and the solution was washed with brine (4 × 50 mL) and dried over Na_2SO_4. After filtration, the solvent was evaporated and the product obtained was used in the next stage without additional purification.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.48 (d, *J* = 0.7 Hz, 1 H, Ar), 8.26–8.34 (m, 2 H, Ar), 8.16–8.20 (m, 1 H, Ar), 7.98 (s, 1 H, Ar), 7.64 (d, *J* = 8.1 Hz, 1 H, Ar), 6.46 (s, 2 H, Ar), 6.42 (s, 2 H, Ar), 3.56–3.61 (m, 12 H, CH_3), 2.38 (s, 12 H, CH_3).

5(6)-Carboxy-3′,6′-diacetoxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein *N*-Oxysuccinimide Ester (11a and 11b)

The product of the previous stage (mixture of **10a** and **10b**) was dissolved in CH_2Cl_2 (100 mL). To the solution were added NHS (0.85 g, 6.4 mmol) and DIC (0.88 g, 7 mmol) and the reaction mixture was stirred for 24 h at r.t. The solvent was evaporated and the product was purified by flash chromatography (toluene/EtOAc, 50:50). The mixture of isomers thus obtained was separated by column chromatography (gradient of EtOAc in toluene from 20:80 to 40:60).

5-Carboxy-3′,6′-diacetoxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein *N*-Oxysuccinimide Ester (11a)

Yield: 490 mg (18%); purple solid; m.p. 200 °C (decomp.); R_f = 0.28 (toluene/EtOAc, 1:1).

^{1}H NMR (700 MHz, $CDCl_3$): δ = 8.83 (s, 1 H, Ar), 8.44 (dd, *J* = 8.0, 1.4 Hz, 1 H, Ar), 7.42 (d, *J* = 8.1 Hz, 1 H, Ar), 6.19 (s, 2 H, Ar), 3.65 (s, 6 H, CH_3), 2.96 (br. s., 4 H, CH_2), 2.40 (s, 6 H, CH_3).

^{13}C NMR (176 MHz, $CDCl_3$): δ = 20.2, 25.7, 56.6, 106.7, 115.2, 118.2, 125.3, 126.1, 128.0, 128.2, 129.0, 137.2, 139.6, 141.4, 149.1, 160.3, 167.0, 167.3, 168.8.

HRMS: *m*/*z* [M + H]$^+$ calcd for $C_{31}H_{21}Cl_2NO_{13}$: 686.0463; found: 686.0471.

6-Carboxy-3′,6′-diacetoxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein *N*-Oxysuccinimide Ester (11b)

Yield: 990 mg (36%); purple solid; m.p. 195 °C (decomp.); R_f = 0.48 (toluene/EtOAc, 1:1).

^{1}H NMR (700 MHz, $CDCl_3$): δ = 8.41 (dd, *J* = 8.0, 1.2 Hz, 1 H, Ar), 8.20 (d, *J* = 8.1 Hz, 1 H, Ar), 7.95 (s, 1 H, Ar), 6.20 (s, 2 H, Ar), 3.65 (s, 6 H, CH_3), 2.90 (br. s., 4 H, CH_2), 2.39 (s, 6 H, CH_3).

^{13}C NMR (176 MHz, $CDCl_3$): δ = 20.2, 25.6, 56.5, 106.7, 115.2, 118.2, 125.3, 126.1, 128.2, 129.0, 129.9, 132.0, 132.4, 139.6, 141.4, 149.1, 160.3, 167.2, 167.4, 168.6.

HRMS: *m*/*z* [M + H]$^+$ calcd for $C_{31}H_{21}Cl_2NO_{13}$: 686.0463; found: 686.0470.

5(6)-(3-Azidopropylaminocarbonyl)-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (12a and 12b)

The product of the previous stage (**11b**; 280 mg, 0.4 mmol) was dissolved in THF (20 mL), 3-azidopropylamine (320 mg, 3.2 mmol) was added, and the mixture was stirred at r.t. for 8 h. The solution was evaporated, the residue was dissolved in H_2O (3 mL), and the solution was acidified to pH 2 with concentrated HCl and stirred at r.t. for 30 min. The solid was filtered off, washed with water (0.5 mL) and air-dried. The product was purified by column chromatography (gradient of MeOH in $CHCl_3/Et_3N$ from 20:75:5 to 30:65:5). The product obtained was dissolved in H_2O (3 mL), and the solution was acidified to pH 2 with concentrated HCl and stirred at r.t. for 30 min. The solid produced was filtered off, washed with water (0.5 mL) and air-dried.

5-(3-Azidopropylaminocarbonyl)-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (12a)

Yield: 180 mg (75%); purple solid; m.p. 260 °C (decomp.); R_f = 0.45 ($CHCl_3$/MeOH/Et_3N, 65:30:5).

^{1}H NMR [700 MHz, D_2O+NH_3 (traces)]: δ = 8.16 (d, *J* = 1.8 Hz, 1 H, Ar), 7.99 (dd, *J* = 7.9, 1.8 Hz, 1 H, Ar), 7.33 (d, *J* = 7.9 Hz, 1 H, Ar), 6.22 (s, 2 H, Ar), 3.61 (s, 6 H, CH_3), 3.56 (t, *J* = 6.7 Hz, 2 H, CH_2), 3.53 (t, *J* = 6.6 Hz, 2 H, CH_2), 1.98 (quin, *J* = 6.7 Hz, 2 H, CH_2).

^{13}C NMR [176 MHz, D_2O+NH_3 (traces)]: δ = 27.8, 37.6, 49.0, 55.4, 102.7, 107.9, 110.4, 127.2, 127.7, 131.2, 134.6, 135.0, 140.6, 149.5, 151.4, 152.7, 166.9, 169.7, 173.7.

HRMS: *m*/*z* [M + H]$^+$ calcd for $C_{26}H_{21}Cl_2N_4O_8$: 587.0731; found: 587.0734.

6-(3-Azidopropylaminocarbonyl)-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (12b)

Yield: 200 mg (83%); purple solid; m.p. 250 °C (decomp.); R_f = 0.33 ($CHCl_3$/MeOH/Et_3N, 65:30:5).

^{1}H NMR [700 MHz, D_2O+NH_3 (traces)]: δ = 8.38 (d, *J* = 1.1 Hz, 1 H, Ar), 8.02 (dd, *J* = 8.2, 1.6 Hz, 1 H, Ar), 7.89 (d, *J* = 8.1 Hz, 1 H, Ar), 6.30 (s, 2 H, Ar), 3.61 (s, 6 H, CH_3), 3.58 (t, *J* = 6.6 Hz, 2 H, CH_2), 3.54 (t, *J* = 6.7 Hz, 2 H, CH_2), 1.99 (quin, *J* = 6.6 Hz, 2 H, CH_2).

^{13}C NMR [176 MHz, D_2O+NH_3 (traces)]: δ = 27.9, 37.6, 49.0, 55.4, 103.2, 107.6, 110.8, 128.2, 128.7, 130.0, 131.7, 134.7, 142.9, 149.5, 151.2, 152.8, 166.7, 169.8, 174.0.

HRMS: *m*/*z* [M + H]$^+$ calcd for $C_{26}H_{21}Cl_2N_4O_8$: 587.0731; found: 587.0744.

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0037-1610216.

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