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An efficient and versatile approach for the preparation of a rhodamine B ester bioprobe library

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ABSTRACT

A general approach for the preparation of a library consisting of reactive rhodamine B (RhB) bioprobes based on ester or thioester linkages is described. The synthesis of this library proceeds fast and efficiently in one reaction step. Pure RhB ester chromophores are readily obtained directly from the reaction mixture following a simple and straight forward workup procedure without further HPLC purification required. A variety of functional groups are attached to the RhB scaffold yielding the functional chromophores in moderate to high yields with particular focus on introducing bioorthogonal substituents suitable for protein and peptide labeling. The approach reported herein provides a concise and practical route to access a variety of reactive RhB fluorophores that could be applied for various bioconjugation chemistries.

1. Introduction

An emerging area of growth in dye chemistry has involved colorants that are utilized for distinct applications [1,2]. Such dyes are named functional dyes and their design is not merely directed toward color-tuning [1]. For instance, they have been used in biology to trace intracellular events [3], to determine ion concentrations [4,5] like sensing Cu^{2+} for live cell imaging [6], to develop photochromic switches [7] to estimate the shape of a molecule *via* fluorescence anisotropy study [8], and to investigate protein dynamics *via* fluorescence correlation spectroscopy (FCS) [9]. Additionally, fluorophore pairs have been investigated as a molecular ruler to measure the distance of biomolecule assembles *via* Förster resonance energy transfer (FRET) [10]. More recently, they have been applied to prepare fluorescent nanoparticles in nanoscience [11].

Rhodamine B (RhB) represents a low cost and widely used fluorophore known for its relatively high photostability and acceptable water-solubility [12]. It features a conjugated fluorescent xanthene ring and a free benzoic acid moiety for derivatization. These characteristics render RhBs attractive for various uses. However additional functional handles need to be introduced to broaden their application spectrum [13,14]. Although there are a few functionalized rhodamine dyes commercially available, they are usually extremely expensive (typically $>$ 40,000 Euro/g) and therefore organic synthesis is required to obtain the respective rhodamine derivative of choice in larger amounts.

However, there are several challenges associated when aiming at preparing RhB derivatives as bioprobes. Purification of functionalized RhB derivatives is tedious and often not feasible due to the high polarity of the tetraethyl-RhB scaffold. Additionally, RhBs are either prone to lactone formation under basic condition [15] or rapidly cyclize to form a non-fluorescent spirolactam when it reacts with primary amines or hydrazines [16]. These byproducts in a mixture with the unreacted RhB chromophore share very similar polarities and thus cannot be readily separated from the desired product by conventional column chromatography. Previously, a general approach for the preparation of RhB probes based on a tertiary amide linkage *via* an RhB-piperazine amide intermediate [17] has been reported. This three step synthetic route is generally applicable for the preparation of functionalized RhB chromophores but it often requires highly reactive reagents, e.g. trimethylaluminum as well as tedious purification steps.

Herein, we report an alternative and broadly applicable approach for the preparation of RhB probes featuring an ester bond

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linkage. In this way, no spirolactam byproducts are formed and the corresponding ester product could be purified *via* an optimized, simple workup procedure in combination with an optional column chromatography. A series of RhB ester probes has been synthesized and this general reaction scheme has been further broadened facilitating also the preparation of RhB thioesters. Although there have been few different RhB esters reported previously [18–20], the general and concise synthesis of pure RhB esters, in particular those carrying reactive groups suitable for biomolecule labeling, has yet to be developed. Herein, we report the systematic and straight forward synthesis of an RhB ester library bearing reactive functionalities that allow bioconjugation to native peptides and proteins as well as engineered proteins bearing bioorthogonal ethynyl or azido groups. In addition, optical properties and 2D-NMR of these compounds are discussed, providing new insights and structural information of this important class of chromophores.

2. Experimental

2.1. Chemicals and instruments

All chemical reagents were purchased from Sigma–Aldrich, Merck or Regent and were used without further purification unless otherwise mentioned. Anhydrous DMF was dried over freshly activated 3 Å molecular sieves. ^{1}H NMR, ^{13}C NMR, DEPT 135, HSQC and HMBC spectra were recorded on Bruker AC 300, AC 400, AMX 500 or DRX500 NMR spectrometers operating at 400 MHz for ^{1}H and 75.48 MHz for ^{13}C NMR at 25 °C. Chemical shifts were reported in ppm (δ scale) relative to the solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0), and coupling constant (J) values were reported in hertz (Hz). High-resolution mass spectra (HRMS) were recorded on a Finnigan MAT95XL-T mass spectrometer by direct infusion of the solution of each compound by using electrospray ionization (ESI) in the positive mode. Low resolution ESI-MS spectra were determined using a Finnigan LCQ quadrupole ion trap mass spectrometer. MALDI-ToF-MS spectra of protein samples were recorded on Autoflex MALDI-ToF (Bruker Daltonics) mass spectrometer using sinapinic acid solution as matrix. Purity studies by LC-MS (ESI) were achieved on a Shimadzu LC-20AD/SPD-20A/SIL-20AC/LCMS-2010EV instrument equipped with a C-18 or C-8 column using MeCN/H_2O as eluent. UV–Vis and fluorescence spectra were recorded by TECAN Microplate Reader at the concentration of 50 μM in PBS buffer (0.1 M, pH 7.2).

2.2. Synthesis and characterization

2.2.1. The general procedure for the preparation of RhB (thio)ester probes

In a typical reaction, RhB (479 mg, 1.0 mmol, 1.0 equiv.), the corresponding (thio)alcohol (1.1 mmol, 1.1 equiv.), EDC·HCl (*N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride salt) (211 mg, 1.1 mmol) and DMAP (4-dimethylaminopyridine) (24.4 mg, 0.2 mmol, 0.2 equiv.) were combined in a Schlenk tube equipped with a stir bar. DCM (5 mL) was injected and the reaction flask was wrapped with aluminum foil to exclude light. The resultant reaction mixture was stirred at RT (room temperature) under Ar for 4 h. DCM (10 mL) was added and the reaction mixture was washed with 10 mL of DI-H_2O. The aqueous layer was extracted four times by DCM and all organic layers were combined, washed with 0.1 M HCl (10 mL), brine (5 mL), dried over anhydrous Na_2SO_4, filtrated, concentrated and purified *via* silica gel chromatography (MeOH:$CHCl_3$ 1:5, $R_f \sim 0.5$) to afford the corresponding RhB (thio) esters in a moderate to good yield between 57 and 79%. The purity of each compound was assessed by LC-MS analysis at 254 nm.

2.2.2. RhB ethyl ester (**2**, from 479 mg of RhB)

351 mg dark violet solid was obtained as the product in a yield of 69%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.28 (ddd, $J_1 = 6.32$ Hz, $J_2 = 1.04$ Hz, $J_3 = 0.36$ Hz, 1H), 7.80 (td, $J_1 = 6.04$ Hz, $J_2 = 1.08$ Hz, 1H), 7.73 (td, $J_1 = 6.24$ Hz, $J_2 = 1.04$ Hz, 1H), 7.30 (td, $J_1 = 6.08$ Hz, $J_2 = 0.88$ Hz, 1H), 7.07 (d, $J = 7.6$ Hz, 2H), 6.90 (dd, $J_1 = 7.6$ Hz, $J_2 = 2.00$ Hz, 2H), 6.82 (d, $J = 2.00$ Hz, 2H), 4.07 (q, $J = 5.72$ Hz, 2H), 3.64 (q, $J = 5.76$ Hz, 8H), 1.32 (t, $J = 5.72$ Hz, 12H), 1.07 (t, $J = 5.72$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 165.00, 158.92, 157.72, 133.44, 132.94, 131.28, 131.26, 130.32, 130.14, 130.14, 114.20, 113.54, 96.34, 61.52, 46.14, 13.79, 12.63; LC-MS (ESI): t_R 98.1% purity (254 nm), $C_{30}H_{35}N_2O_3^+$ calcd. 471.21, found 471.14 $[M]^+$; UV–Vis (pH 7.2, 50 μM): $\lambda_{max} = 559$ nm, $\varepsilon = 8.6 \times 10^4\ M^{-1}\ cm^{-1}$; Fluorescence (pH 7.2, 50 μM): $\lambda_{max} = 591$ nm (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.34.

2.2.3. RhB propargyl ester (**3**, from 479 mg of RhB)

320 mg dark violet solid was obtained as the product in a yield of 63%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.26 (dd, $J_1 = 7.92$ Hz, $J_2 = 1.04$ Hz, 1H), 7.79 (td, $J_1 = 7.56$ Hz, $J_2 = 1.28$ Hz, 1H), 7.70 (td, $J_1 = 7.80$ Hz, $J_2 = 1.24$ Hz, 1H), 7.28 (dd, $J_1 = 7.56$ Hz, $J_2 = 1.04$ Hz, 1H), 7.01 (d, $J = 9.52$ Hz, 2H), 6.87 (dd, $J_1 = 9.48$ Hz, $J_2 = 2.40$ Hz, 2H), 6.76 (d, $J = 2.36$ Hz, 2H), 4.56 (d, $J = 2.44$ Hz, 2H), 3.60 (q, $J = 7.12$ Hz, 8H), 2.36 (t, $J = 2.44$ Hz, 1H), 1.27 (t, $J = 7.04$ Hz, 12H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 164.08, 158.09, 157.65, 155.43, 133.59, 133.36, 131.37, 131.05, 130.30, 130.18, 129.02, 114.21, 113.40, 96.21, 76.31, 75.40, 52.75, 46.04, 12.53; HRMS (ESI): $C_{31}H_{33}O_3N_2^+$, calcd. 481.2486, found 481.2471; EA: $C_{31}H_{33}O_3N_2Cl \cdot 2H_2O$, calcd. C 67.32%, H 6.74%, N 5.06%, Cl 6.41%, found C 67.46%, H 6.71%, N 5.14%, Cl 6.94%; LC-MS (ESI): 96.3% (254 nm); UV–Vis (pH 7.2, 50 μM): $\lambda_{max} = 560$ nm, $\varepsilon = 8.3 \times 10^4\ M^{-1}\ cm^{-1}$; Fluorescence (pH 7.2, 50 μM): $\lambda_{max} = 561$ nm (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.36.

2.2.4. RhB allyl ester (**4**, from 479 mg of RhB)

295 mg dark violet solid was obtained as the product in a yield of 57%; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.14 (d, $J = 7.76$ Hz, 1H), 7.67 (t, $J = 7.42$ Hz, 1H), 7.59 (t, $J = 7.64$ Hz, 1H), 7.16 (d, $J = 7.44$ Hz, 1H), 6.92 (d, $J = 9.48$ Hz, 2H), 6.77 (dd, $J_1 = 9.44$ Hz, 2H), 6.64 (d, 2H), 5.51 (m, 1H), 4.98 (m, 2H), 4.34 (d, $J = 5.64$ Hz, 2H), 3.50 (q, $J = 7.12$ Hz, 8H), 1.17 (t, $J = 6.96$ Hz, 12H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 164.25, 158.24, 157.29, 155.10, 133.11, 132.73, 130.88, 130.81, 130.66, 130.00, 129.78, 129.47, 118.60, 113.88, 113.07, 95.83, 77.21, 65.60, 45.74, 12.25; HRMS (ESI): $C_{31}H_{35}N_2O_3^+$, calcd. 483.2642, found 483.2638; LC-MS (ESI): 97.0% purity (254 nm); UV–Vis (pH 7.2, 50 μM): $\lambda_{max} = 559$ nm, $\varepsilon = 7.9 \times 10^4\ M^{-1}\ cm^{-1}$; Fluorescence (pH 7.2, 50 μM): $\lambda_{max} = 592$ nm (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.37.

2.2.5. RhB-NHS ester (**5**, from 479 mg of RhB)

330 mg dark violet solid was obtained as the product in a yield of 57%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.41 (dd, $J_1 = 7.92$ Hz, $J_2 = 0.88$ Hz, 1H), 7.97 (td, $J_1 = 7.64$ Hz, $J_2 = 1.24$ Hz, 1H), 7.82 (td, $J_1 = 7.18$ Hz, $J_2 = 1.40$ Hz, 1H), 7.47 (dd, $J_1 = 7.68$ Hz, $J_2 = 0.88$ Hz, 1H), 7.07 (d, $J = 9.4$ Hz, 2H), 6.87 (dd, $J_1 = 9.40$ Hz, $J_2 = 2.44$ Hz, 2H), 6.84 (d, $J = 2.40$ Hz, 2H), 3.69 (q, $J = 7.60$ Hz, 8H), 2.76 (s, br, 4H), 1.32 (t, $J = 7.12$ Hz, 12H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 168.57, 160.57, 157.60, 155.57, 155.43, 134.80, 134.23, 131.58, 130.83, 130.81, 130.57, 125.19, 114.29, 113.21, 96.30, 46.05, 25.48, 12.50; LC-MS (ESI): 96.2% purity (254 nm); UV–Vis (pH 7.2, 50 μM): $\lambda_{max} = 563$ nm, $\varepsilon = 8.1 \times 10^4\ M^{-1}\ cm^{-1}$; Fluorescence (pH 7.2, 50 μM): $\lambda_{max} = 595$ nm (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.31.

2.2.6. RhB 4-iodobenzyl ester (**6**, from 240 mg of RhB, 8 h)

400 mg reddish violet solid was obtained as the product in a yield of 58%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.25 (dd, $J_1 = 7.80$ Hz, $J_2 = 1.12$ Hz, 1H), 7.76 (td, $J_1 = 7.52$ Hz, $J_2 = 11.32$ Hz, 1H), 7.69 (td, $J_1 = 7.76$ Hz, $J_2 = 1.32$ Hz, 1H), 7.46 (dm, $J_1 = 8.28$ Hz, 2H), 7.23