Dyes and Pigments 94 (2012) 296–303

Contents lists available at SciVerse ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

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

Xi Chen [a,b], Qianzhen Wu [a], Lars Henschke [a], Günther Weber [a], Tanja Weil [a,b,c,*]

[a] Institute of Organic Chemistry III, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany
[b] Department of Chemistry, National University of Singapore, 3 Science Drive 3, 117543 Singapore, Singapore
[c] Max-Planck-Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany

ARTICLE INFO

Article history:
Received 10 November 2011
Received in revised form
9 January 2012
Accepted 11 January 2012
Available online 20 January 2012

Keywords:
Rhodamine
Bioprobe
Bioorthogonal groups
Protein labeling
2D-NMR
"Click" chemistry

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

* Corresponding author. Institute of Organic Chemistry III, University of Ulm, Albert-Einstein-Allee 11, 89069 Ulm, Germany. Tel.: +49 731 5022870; fax: +49 731 50 22883.
E-mail address: tanja.weil@uni-ulm.de (T. Weil).

0143-7208/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dyepig.2012.01.005

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

(dd, J_1 = 7.56 Hz, J_2 = 1.08 Hz, 1H), 6.99 (d, J = 9.48 Hz, 2H), 6.84 (dd, J_1 = 9.52 Hz, J_2 = 2.40 Hz, 2H), 6.68 (d, J = 2.44 Hz, 2H), 6.67 (d, J = 8.2 Hz, 2H), 4.87 (s, 2H), 3.63 (qd, J_1 = 7.24 Hz, J_2 = 1.64 Hz, 8H), 1.30 (t, J = 7.12 Hz, 12H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 164.78, 158.03, 157.40, 155.35, 137.43, 134.04, 133.10, 133.07, 131.34, 131.11, 130.33, 130.10, 130.06, 129.60, 114.18, 113.30, 96.06, 94.14, 66.56, 46.13, 12.61; HRMS (ESI): $C_{35}H_{36}IN_2O_3^+$, calcd. 659.1765, found 659.1757; LC-MS (ESI): 95.8% purity (254 nm); UV–Vis (pH 7.2, 50 μM): λ_{max} = 561 nm, $\varepsilon = 6.7 \times 10^4$ M^{-1} cm^{-1}; Fluorescence (pH 7.4): λ_{max} = 590 nm (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.42.

*2.2.7. RhB 2-azidoethyl ester (**7**, from 144 mg of RhB)*

96.8 mg dark violet solid was obtained as the product in a yield of 60%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.30 (dd, J_1 = 7.6 Hz, 1H), 7.82 (td, J_1 = 7.6 Hz, J_2 = 1.1 Hz, 1H), 7.75 (td, J_1 = 8.2 Hz, J_2 = 1.3 Hz, 1H), 7.31 (dd, J_1 = 7.6 Hz, 1H), 7.07 (d, J = 9.5 Hz, 2H), 6.91 (dd, J_1 = 9.5 Hz, J_2 = 2.6 Hz, 2H), 6.82 (d, J = 2.5 Hz, 2H), 4.16 (t, J = 4.04 Hz, 2H), 3.65 (q, J = 6.56 Hz, 8H), 3.36 (t, J = 3.52 Hz, 2H), 1.32 (t, J = 6.56 Hz, 12H); ^{13}C NMR (500 MHz, $CDCl_3$): δ 164.74, 158.36, 157.79, 155.60, 133.63, 133.34, 131.49, 131.22, 130.46, 130.29, 129.43, 114.34, 113.55, 96.30, 63.72, 49.53, 46.15, 12.62; HRMS (ESI): $C_{30}H_{34}N_5O_3^+$, calcd. 512.2656, found 512.2639; LC-MS (ESI): 97.0% (254 nm); UV–Vis (pH 7.2, 50 μM): λ_{max} = 559 nm, $\varepsilon = 8.3 \times 10^4$ M^{-1} cm^{-1}; Fluorescence (pH 7.2, 50 μM): λ_{max} = 591 nm (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.44.

*2.2.8. RhB N-Boc aminoethyl ester (**8**, from 479 mg of RhB)*

410 mg dark violet solid was obtained as the product in a yield of 66%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.31 (d, J = 7.68 Hz, 1H), 7.76 (td, J_1 = 8.64 Hz, J_2 = 1.16 Hz, 1H), 7.69 (td, J_1 = 7.76 Hz, J_2 = 1.36 Hz, 1H), 7.25 (d, J = 7.72 Hz, 1H), 7.03 (d, J = 9.48 Hz, 2H), 6.87 (dd, J_1 = 9.52 Hz, J_2 = 2.40 Hz, 2H), 6.74 (d, J = 2.36 Hz, 2H), 5.17 (s, br, 1H, NH), 4.07 (t, J = 5.52 Hz, 2H), 3.60 (qd, J_1 = 7.34 Hz, J_2 = 1.40 Hz, 8H), 3.24 (m, 2H), 1.36 (s, 9H), 1.29 (t, J = 7.14 Hz, 12H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 164.69, 157.61, 155.46, 133.03, 132.99, 131.53, 131.24, 130.34, 130.01, 129.62, 114.21, 113.42, 96.20, 77.21, 64.71, 50.33, 46.03, 28.26, 12.54; HRMS (ESI): $C_{35}H_{44}N_3O_5^+$, calcd. 586.3276, found 586.3270; LC-MS (ESI): 95.6% purity (254 nm); UV–Vis (pH 7.2, 50 μM): λ_{max} = 560 nm $\varepsilon = 8.2 \times 10^4$ M^{-1} cm^{-1}; Fluorescence (pH 7.2, 50 μM): λ_{max} = 591 (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.37.

*2.2.9. RhB 3-tert-butoxycarbonylethyl ester (**10**, from 479 mg of RhB)*

437 mg violet solid was obtained as the product in a yield of 72%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.23 (d, J = 7.88 Hz, 1H), 7.81 (td, J_1 = 7.56 Hz, J_2 = 1.16 Hz, 1H), 7.71 (td, J_1 = 7.68 Hz, J_2 = 1.2 Hz, 1H), 7.29 (d, J = 7.56 Hz, 1H), 7.04 (d, J = 9.48 Hz, 2.00 Hz), 6.89 (dd, J_1 = 9.48 Hz, J_2 = 2.36 Hz, 2H), 6.79 (d, J = 2.36 Hz, 2H), 4.26 (t, J = 6.40 Hz, 2H), 3.63 (q, J = 7.12 Hz, 8H), 2.46 (t, J = 6.32 Hz, 2H), 1.39 (s, 9H), 1.31 (t, J = 7.04 Hz, 12H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 169.39, 164.60, 158.66, 157.69, 155.49, 133.71, 133.21, 131.32, 131.19, 130.34, 130.28, 129.53, 114.23, 113.47, 96.30, 81.18, 61.19, 46.11, 34.73, 27.99, 12.62; HRMS (ESI): $C_{35}H_{43}N_2O_5^+$ calcd. 571.3167, found 571.3163; LC-MS (ESI): 95.2% purity (254 nm); UV–Vis (pH 7.2, 50 μM): λ_{max} = 560 nm, $\varepsilon = 8.1 \times 10^4$ M^{-1} cm^{-1}; Fluorescence (pH 7.2, 50 μM): λ_{max} = 591 nm (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.44.

*2.2.10. RhB benzyl thioester (**12**)*

RhB (479 mg, 1.0 mmol), benzyl mercaptan (136.4 mg, 1.1 mmol), EDC·HCl (211 mg, 1.1 mmol) and DMAP (24.4 mg, 0.2 mmol) were combined in a Schlenk flask equipped with a stir bar. DCM (5 mL) was injected under Ar atmosphere and the reaction flask was wrapped with aluminum foil to exclude light. The resultant solution was stirred under Ar at RT for 4 h. The reaction mixture was partitioned in DCM/H_2O and the organic layer was separated. The aqueous layer was extracted four additional times by DCM. All organic layers were combined, washed with 0.1 M HCl (10 mL), brine, dried over anhydrous Na_2SO_4, filtered, concentrated and purified *via* silica gel chromatography (MeOH:$CHCl_3$ 1:5, R_f 0.5) to yield 463 mg dark violet crystal as the product in a yield of 79%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.14 (d, J = 7.88 Hz, 1H), 7.81 (td, J_1 = 7.56 Hz, J_2 = 1.16 Hz, 1H), 7.72 (td, J_1 = 7.72 Hz, J_2 = 1.12 Hz, 1H), 7.33 (d, J = 7.52 Hz, 1H), 7.14–12 (m, 3H), 7.08–03 (m, 4H), 6.85–81 (m, 4H), 4.03 (s, 2H), 3.64 (q, J = 7.12 Hz, 8H), 1.33 (t, J = 7.0 Hz, 12H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 190.99, 157.62, 157.61, 155.48, 137.27, 136.68, 132.96, 131.16, 130.56, 130.53, 129.07, 128.63, 128.40, 127.23, 114.15, 113.48, 96.33, 46.10, 33.62, 12.63; HRMS (ESI): $C_{35}H_{37}N_2O_2S^+$ calcd. 549.2571, found 549.2566; LC-MS (ESI): 95.2 purity (254 nm); UV–Vis (pH 7.2, 50 μM): λ_{max} = 564 nm $\varepsilon = 6.4 \times 10^4$ M^{-1} cm^{-1}; Fluorescence (pH 7.2, 50 μM): λ_{max} = 595 (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.42.

*2.2.11. RhB 2-aminoethyl ester trifluoroacetic acid salt (**9**)*

RhB *N*-Boc aminoethyl ester (190 mg, 31.5 mmol) was dissolved in DCM (5 mL). TFA (2.5 mL) was injected dropwise to this stirring solution. The resultant solution was stirred in the dark at RT for 30 min. DCM and TFA were removed under high vacuum to yield the red solid as the product in a quantitative yield. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.88 (m, br, 3H), 8.45 (d, J = 7.32 Hz, 1H), 8.16 (s, br, 2H), 7.75 (t, J = 7.32 Hz, 1H), 7.70 (t, J = 7.20 Hz, 1H), 7.23 (d, J = 6.72 Hz, 1H), 7.07 (t, J = 9.44 Hz, 2H), 6.80 (dd, J_1 = 9.44 Hz, J_2 = 2.04 Hz, 2H), 6.76 (d, J = 2.12 Hz, 2H), 4.32 (t, br, 2H), 3.56 (q, J = 6.36 Hz 8H), 3.27 (s, br, 2H), 1.29 (t, J = 6.92 Hz, 12H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 164.40, 159.20, 157.75, 155.55, 133.75, 133.10, 132.05, 131.30, 130.54, 129.76, 128.76, 114.02, 113.48, 96.13, 61.45, 45.87, 38.90, 12.32; HRMS (ESI): $C_{30}H_{36}N_3O_3^+$, calcd. 486.2751, found 486.2749; LC-MS (ESI): 95.5% purity; UV–Vis (pH 7.2, 50 μM): λ_{max} = 560 nm, $\varepsilon = 7.9 \times 10^4$ M^{-1} cm^{-1}; Fluorescence (pH 7.2, 50 μM): λ_{max} = 590 (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.45.

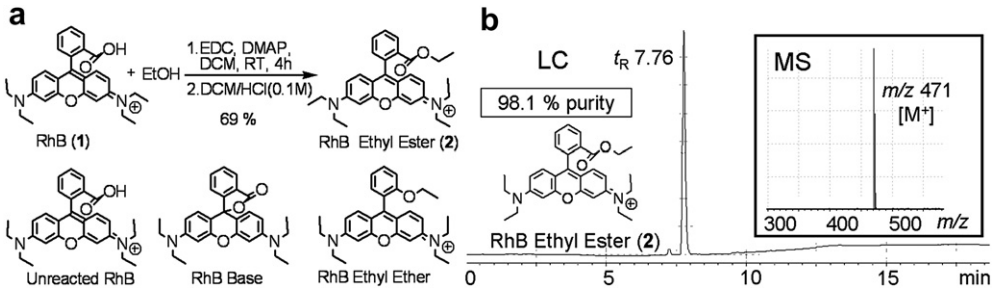
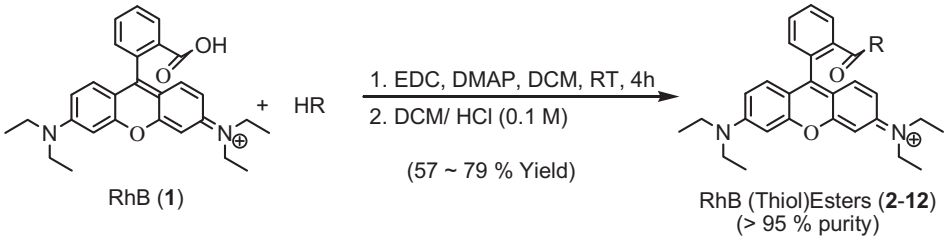
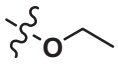
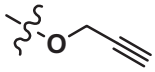
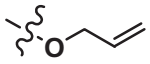
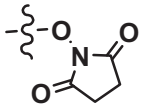
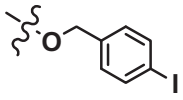
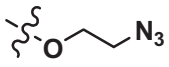
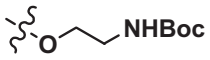
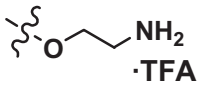
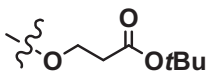
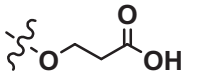
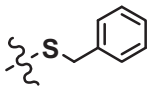
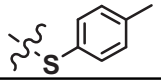


Fig. 1. a) Model reaction between RhB (**1**) and ethanol to afford the RhB ethyl ester (**2**) for evaluating RhB derivatization *via* Steglich esterification (above); three byproducts are formed according to LC-MS, including a decarbonyl etherification byproduct, RhB ethyl ether (below). (b) The LC-MS chromatograph of the final RhB ethyl ester (**2**) reveals a purity of 98.1% (m/z = 471).

Table 1
Summary of the yields, maximal absorption (λ_{abs}) and emission (λ_{em}) wavelengths, molar extinction coefficients (ε) and quantum yields (Φ_F) of RhB and its (thio)ester derivatives. Within this chromophore library, functional RhB ester **6**, **8**, **9**, **10**, **11** and **12** represent novel compounds that have not been reported elsewhere.



Compound	*R*	Yield/%	Purity/%	λ_{ab}/nm (ε/k M^{-1} cm^{-1})	λ_{em}/nm	Φ_F
1	**RhB**	–	–	554 (85)	584	0.32 [21]
2		69	98.1	559 (86)	591	0.34
3		63	96.3	560 (83)	591	0.36
4		57	97.0	559 (79)	592	0.37
5		57	96.2	563 (81)	595	0.31
6		58	95.8	561 (67)	590	0.42
7		60	97.0	559 (83)	591	0.44
8		66	95.6	560 (82)	591	0.37
9		99	95.5	560 (79)	590	0.45
10		72	95.2	560 (81)	591	0.44
11		99	95.4	559 (84)	591	0.36
12		79	95.5	564 (64)	595	0.42
13		0	–	–	–	–

*2.2.12. RhB 2-carboxyethyl ester (**11**)*

RhB 2-tert-butoxycarbonylethyl ester (422 mg, 0.695 mmol) was dissolved in DCM (5 mL) and cooled down to 0 °C by an ice-water bath. TFA (5 mL) was added and the resultant solution was wrapped with aluminum foil and stirred at RT overnight. DCM and TFA were removed under high vacuum. The resultant residue was dissolved in DMSO and freeze dried in order to remove traces of TFA. 379 mg of a dark violet solid was obtained in quantitative yields. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.28 (dd, J_1 = 7.00 Hz, J_2 = 1.88 Hz, 1H), 7.73–7.71 (m, 2H), 7.23 (dd, J_1 = 6.88 Hz, J_2 = 1.52 Hz, 1H), 7.04 (d, J = 9.4 Hz, 2H), 6.91 (d, 2H), 6.81 (dd, J_1 = 9.1 Hz, 2H), 4.19 (t, J = 5.24 Hz, 2H), 3.60 (q, J = 7.0 Hz, 8H),

2.27 (t, $J = 5.16$ Hz, 2H), 1.32 (t, $J = 6.84$ Hz, 12H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 171.52, 165.41, 157.93, 157.80, 155.55, 132.94, 132.49, 131.69, 130.99, 130.50, 130.20, 129.85, 113.99, 113.50, 96.90, 61.18, 45.99, 32.83, 12.51; HRMS (ESI): $C_{31}H_{35}N_2O_5^+$ calcd. 515.2451, found 515.2537; LC-MS (ESI): 95.4% purity (254 nm); UV–Vis (pH 7.2, 50 μM): $\lambda_{max} = 559$ nm, $\varepsilon = 8.4 \times 10^4$ M^{-1} cm^{-1}; Fluorescence (pH 7.2, 50 μM): $\lambda_{max} = 591$ nm (ex. 530 nm); Φ_F (pH 7.2, 50 μM) = 0.36.

3. Results and discussions

3.1. Development of the preparation and purification procedure of RhB esters

Ethanol was chosen to react with RhB in the model reaction. The coupling was mediated by EDC under the catalysis of DMAP in DCM at RT. After 4 h, the LC-MS revealed the formation of the target RhB ethyl ester (**2**) with *m*/*z* 471.15 [M^+] together with impurities (Fig. 1a). Column chromatography failed to efficaciously remove the impurities. Apart from 1-ethyl-3-(3-dimethylaminopropyl)urea (EDU) and DMAP, two major impurities were isolated and identified as the RhB precursor and RhB base (the lactone form of RhB, Fig. 1a) based on their mass spectra and ^{1}H NMR/^{13}C NMR spectra. Unfortunately, these impurities could not be suppressed by altering the reaction conditions, e.g. extending the reaction time during synthesis.

Since these two impurities together with EDU and DMAP represent amines derivatives, we envisioned that they could be removed simply by washing with acid solution, e.g. aq. HCl. As the RhB ethyl ester also contains a cationic amine which could be removed by adding aq. HCl, different organic phases in combination with varying concentrations of aq. HCl solution were screened. Finally, the combination of DCM as organic phase in combination with 0.1 M HCl was identified to be ideally suited to separate the reaction product from the educts and byproducts. After dissolving the reaction mixture in DCM followed by washing with 0.1 M HCl, most of the RhB, RhB base, EDU and DMAP impurities were removed in one purification step only, while most of the RhB ethyl ester (**2**) product remained in the organic layer. The application of other organic solvents such as EtOAc instead of DCM proceeded less efficiently since the RhB ethyl ester (**2**) is lost during washing. Additionally, applying more concentrated HCl caused undesired loss of the RhB ethyl ester (**2**) while usage of more diluted HCl solution did not remove the impurities efficiently. After removal of DCM under high vacuum, the RhB ethyl ester product (**2**) was obtained with a purity above 90%. An optional column chromatography (MeOH:$CHCl_3$ 1:5, R_f 0.5) allows to further increase the purity to 98.1% (t_R 7.76 min, *m*/*z* 471) according to LC-MS (Fig. 1b) and the pure RhB ethyl ester (**2**) was isolated in an acceptable yield of 69%.

3.2. Synthesis of an RhB ester-based bioprobe library

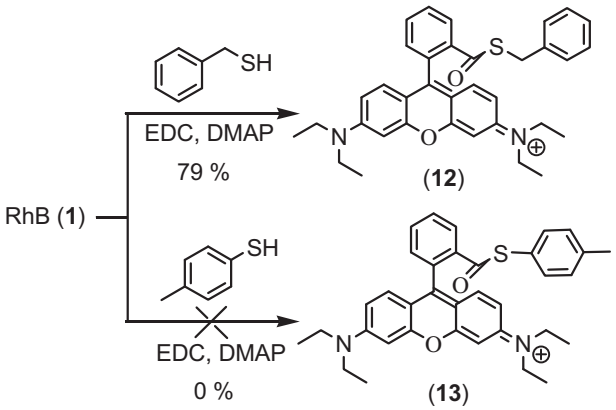
After establishing the preparation and purification protocol, this approach was applied to prepare a variety of RhB ester probes, including an RhB benzyl thioester (Table 1, **12**), which is very valuable for native chemical ligation (NCL) reactions to proteins and peptides. Acceptable yields between 57 and 79% were achieved for different chromophore derivatives in high purities according to LC-MS (Table 1).

The preparation of RhB 2-aminoethyl ester (Table 1, **9**) and RhB 2-carboxyethyl ester chromophores (Table 1, **11**) requires a different reaction protocol since they carry reactive and ionizable groups. Therefore, a protection/deprotection strategy is applied. Briefly, their protected precursors, RhB *N*-Boc-2-aminoethyl ester and RhB 2-*tert*-butoxycarbonylethyl ester (Table 1, **8** and **10**) were synthesized in high purities using the established protocol. After treatment with TFA and vacuum drying, the unprotected bioprobes were obtained in a straight forward fashion without further purification steps required. Noteworthy, even though RhB benzyl thioesters such as (**12**) could be achieved easily, all attempts to synthesize RhB 4-methylphenyl thioester (**13**) failed (Scheme 1).

These reactive RhB ester chromophores summarized in Table 1 represent attractive probes for targeting natural (probe **5**, **9**, **11** and **12**) as well as unnatural peptides and proteins bearing e.g. unnatural amino acids (probe **3**, **4**, **6** and **7**, Fig. 2). In particular, RhB propargyl ester (**3**, Table 1) is attractive for Huisgen 1,3-Dipolar cycloaddition reactions (click labeling) [22] to e.g. proteins bearing the unnatural amino acid azidohomoalanine (Aha) [23] and Sonogashira labeling to proteins carrying iodo groups [24]. RhB allyl ester (**4**, Table 1) allows labeling *via* olefin metathesis [25] and the amine-reactive RhB-NHS ester (**5**, Table 1) reacts with lysine residues [18]. RhB 4-iodobenzyl ester (**6**, Table 1) could be applied for Sonogashira reactions [26] and RhB 2-azidoethyl ester (**7**, Table 1) offers click labeling [22] or labeling *via* Staudinger ligation [27]. Additionally, RhB benzyl thioester (**12**, Table 1) offers the potential for native chemical ligation (NCL) labeling at N-terminal cysteines [28]. Furthermore, there are two RhB derivatives (**9** and **11**, Table 1) carrying amino or carboxylic group suitable for bioconjugation reactions on proteins *via* EDC-mediated or dimethylglycine (DMG) ester-catalyzed [29] modifications. These reactions usually proceed smoothly on proteins [22–29] and labeling of proteins using these synthesized RhB ester probes is not within the focus of this work. However, a representative protein labeling experiment is given in the following section to underline the suitability of these chromophores for such purposes.

3.3. Reactivity of RhB propargyl ester (*4*) for labeling azido-RNase A (*14*)

One of the most widely used bioorthogonal labeling reactions for protein modifications represents the Huisgen 1,3-dipolar cycloaddition reaction, also known as click reaction. As a representative demonstration of the reactivity of the synthesized rhodamine bioprobes, RhB propargyl ester (**4**) is reacted with the azido-containing protein, bis-Aha-RNase A (**14**) under the catalysis of Cu^+ at physiological pH in aqueous solution (Fig. 3a). Bis-Aha-RNase A (**14**) with two surface-exposed L-azidohomoalanine (L-Aha) groups (Fig. 3b) at the position of Met 1 and Met 41 has been obtained by selective pressure incorporation [30] using methionine auxotroph *Escherichia coli* strains by replacing the natural amino acid L-methionine by L-Aha. The full sequence of bis-Aha-RNase A (**14**) is given in the Supporting Information (Figure S1). According to MALDI-ToF-MS analysis, bis-Aha-RNase A (**14**) displays a M.W. of around 15.8 kDa.



Scheme 1. Esterification of RhB using 4-thiocresol and benzyl mercaptan.

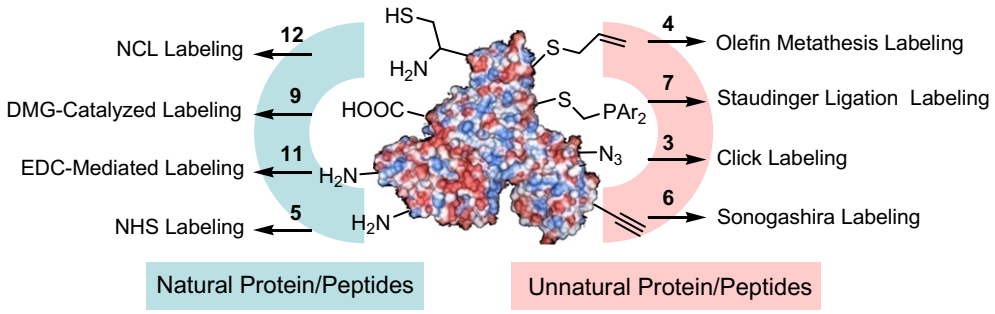


Fig. 2. The potential of labeling native proteins (left) or proteins decorated with unnatural residues applying the synthesized RhB ester probes.

In order to further demonstrate that RhB propargyl ester (**4**) is an efficient click labeling probe for modifying azido proteins, probe (**4**) is reacted with bis-Aha-RNase A (**14**) in PBS buffer in the presence of Cu^{+} catalyst (**15**). Gel-electrophoresis of three different solutions has been performed: (1) the click reaction mixture (Fig. 3-d1), (2) a reaction mixture consisting of **14** and **4** but in the absence of the Cu^{+} catalyst (**15**) (Fig. 3-d2) as control to prove the absence of any non-specific interactions between the azido protein (**14**) and **4**, as well as (3) pure bis-Aha-RNase A (**14**). Fig. 3-d4 reveals the protein ladder. According to Fig. 3-d1 (above), a highly fluorescent band is observed at ~16 kDa whereas pure bis-Aha-RNase A (**14**) and the control reaction gave only non-fluorescent gel bands. Therefore, this study successfully demonstrates the reactivity of the representative chromophore, RhB propargyl ester (**4**), as an attractive bioprobe for labeling azido-functionalized proteins.

3.4. Optical and NMR characteristics of RhB (thio)ester probes

Most synthesized RhB esters, regardless of the introduced functional group, display similar maximum absorption wavelengths (λ_{max}) and molar extinction coefficients (ε) of around 559–560 nm and 8.0–8.5 $\times$ 10^4 M^{-1} cm^{-1}, respectively (Fig. 4a) with a red shift of 5–6 nm relative to RhB (**1**) (black dotted line, λ_{max} 554 nm). Interestingly, RhB-NHS ester (**5**, bright green) and RhB benzyl thioester (**12**, blue) reveal an obviously larger red shift of 9 nm (λ_{max} 563 nm) and 10 nm (λ_{max} 564 nm), respectively, originating from the conjugated electron-donating effects of the NHS group or the sulfur atom. RhB 4-iodobenzyl ester (**6**, bright blue) and RhB benzyl thioester (**12**, blue) bear comparatively lower extinction coefficients below 7 $\times$ 10^4 M^{-1} cm^{-1}, which might be due to the presence of aromatic groups on RhB. Furthermore, RhB benzyl thioester (**12**, blue curve) exhibits a characteristic shoulder peak at 607 nm which is absent in all other RhB ester derivatives.

For most of RhB esters, a similar maximal fluorescence emission wavelength at around 591 nm (Fig. 4b) is recorded, which is 7 nm shifted bathochromically compared with RhB (**1**, dotted black line). Interestingly, the emission maxima of RhB-NHS ester (**5**, blue) and RhB benzyl thioester (**12**, bright blue) reveal a bathochromic shift of about 11 nm compared with RhB and their emission maxima are found at 595 nm. A similar stroke shift of about 31 nm was recorded for all RhB (thio)esters, including RhB, suggesting that this feature

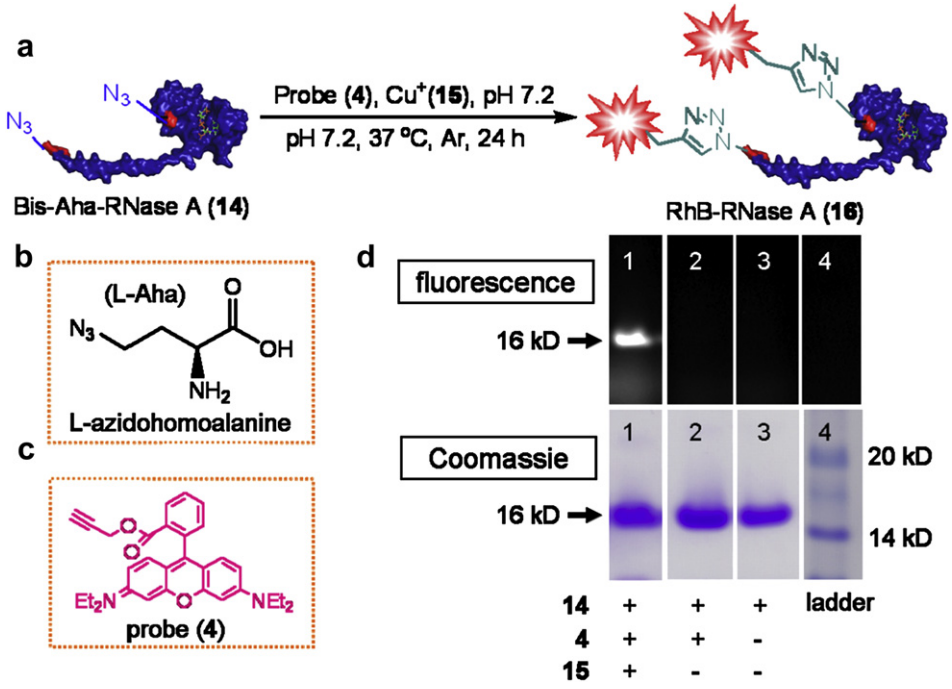


Fig. 3. a) The reaction scheme of labeling of bis-Aha-RNase A (**14**) with RhB propargyl ester (**4**) *via* click reaction; (b) the chemical structure of the unnatural amino acid, L-azidohomoalanine (L-Aha); (c) the chemical structure of probe (**4**); (d) the gel-electrophoresis image (above: fluorescence; below: Coomassie stain) of the click reaction solution (lane 1), the control reaction solution in the absence of the Cu^{+} catalyst (**15**, lane 2), pure bis-Aha-RNase A (**14**) (lane 3) and the protein ladder (lane 4).

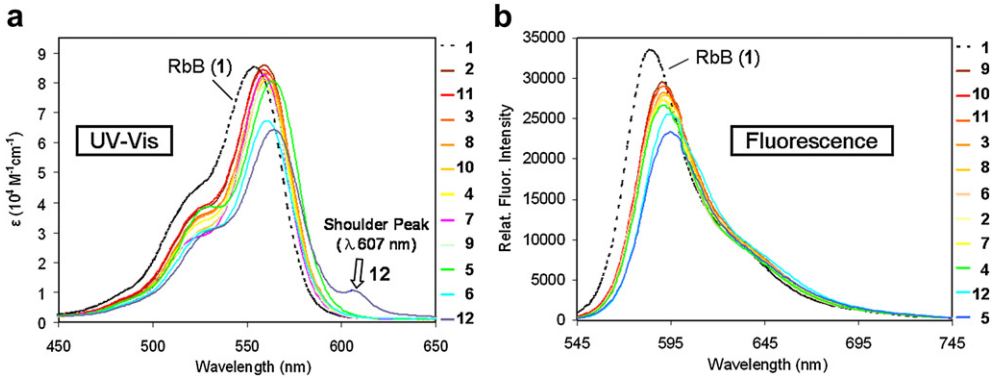


Fig. 4. a) The UV absorption spectra of RhB and RhB (thio)esters (pH 7.2, 50 μM); (b) The fluorescence emission spectra of RhB and RhB (thio)esters (pH 7.2, 50 μM, excitation at 530 nm).

of RhB is independent of the respective functional group. Finally, the quantum yield (Φ_F) of each ester derivative in PBS buffer (pH 7.2, 50 μM) was also calculated (Table 1) by comparison with the reported Φ_F value of the zwitter-ion form of RhB ($\Phi_F = 0.32$) [21].

Previously, most reported optical data (e.g. λ_{abs}, λ_{em}, ε_{max}, and Φ_F) of RhB ester derivatives were determined in organic solvents, e.g. EtOH or THF [31]. In this study, all the data were collected at physiological pH in PBS buffer and therefore, they will be invaluable for the selection of an ideal chromophore for either *in vivo* or in *vitro* biomolecule labeling.

Since RhB esters carry multiple tertiary and quaternary carbons, a full interpretation of typical RhB ester necessitates combined ^{1}H NMR, ^{13}C NMR, DEPT 135, HSQC and HMBC (Figure S2) spectra analysis which is exemplified for RhB propargyl ester (**3**). This interpretation will serve as a general guideline to interpret all other RhB esters since they all share a very similar pattern in the NMR spectra. All RhB esters reveal a characteristic cluster of seven carbon signals in the region of δ 129–134 ppm, which will be referred to as the *RhB Ester Carbon Heptet* (Figure S2a). Noteworthy, the chemical shifts of the proton signals of the RhB esters vary with chromophore concentration whereas their chemical shifts in the ^{13}C NMR are almost independent of the concentration. Therefore, RhB esters could be rather characterized based on their ^{13}C NMR spectra instead of their ^{1}H-NMR spectra.

4. Conclusions

In summary, a straight forward synthetic route toward functionalized RhB probes carrying an ester or thioester bond linkage is described. In the standard protocol, the approach proceeds in one reaction step only within 4 h and the purification only requires DCM/HCl (0.1 M) workup combined with an optional column chromatography. A library of RhB probes has been prepared in moderated to good yields with reproducible purities of greater than 95% according to LC-MS. These chromophores represent highly promising bioprobes for natural or unnatural protein modifications *via* e.g. click labeling (**3**), Staudinger ligation labeling (**7**), olefin metathesis labeling (**4**), Sonogashira labeling (**6**), NHS labeling (**5**), NCL (native chemical ligation) labeling (**12**), DMG (dimethylglycine) ester-catalyzed labeling (**9**), EDC-mediated labeling (**5**). As a proof of the reactivity of the RhB ester bioprobes, the representative RhB propargyl ester (**4**) has been reacted successfully with the azido-functionalized unnatural protein, bis-Aha-RNase A (**14**) *via* click labeling. NMR spectra characteristics have been discussed and all optical properties have been collected at physiological pH allowing the selection of an ideal chromophore for protein labeling approaches.

Acknowledgment

We thank NUS Cross Faculty Grant (Grant No. WBS-R-143-000-393-123) and the Deutsche Forschungsgemeinschaft (SFB 625, P3246 029 DFG) for the financial support of this work and J. Wilhelmi for developing a protocol for the preparation of bis-Aha-RNase A (**14**).

Appendix. Supplementary information

Supplementary data related to this article can be found online at doi:10.1016/j.dyepig.2012.01.005.

References

[1] Weil T, Vosch T, Hofken J, Peneva K, Müllen K. The rylene colorant family—tailored nanoemitters for photonics research and applications. Angew Chem Int Ed 2010;49(48):9068–93.
[2] Sameiro M, Gonçalves T. Fluorescent labeling of biomolecules with organic probes. Chem Rev 2009;109:190–212.
[3] For a general review, see Mason WT. Fluorescent and luminescent probes for biological activity. San Diego: Academic Press; 1999.
[4] Kim HN, Lee MH, Kim HJ, Kim JS, Yoon J. A new trend in rhodamine-based chemosensors: applications of spirolactam ring-opening to sensing ions. Chem Soc Rev 2008;37:1465–72.
[5] Yuan YH, Tian MZ, Feng F, Meng SM, Bai YF. Rhodamine-based fluorescent probes for cations. Prog Chem 2010;22(10):1929–39.
[6] Huang L, Hou F-P, Xi P, Bai D, Xu M, Li Z, et al. A rhodamine-based "turn-on" fluorescent chemodosimeter for Cu^{2+} and its application in living cell imaging. J Inorg Biochem 2011;105(6):800–5.
[7] Tian H, Yang S. Recent progresses on diarylethene based photochromic switches. Chem Soc Rev 2004;33:85–97.
[8] Lackowicz JR. Principles of fluorescence spectroscopy. 3rd ed. New York: Springer; 2006. 353–354 and 361–364.
[9] Bagshaw CR, Cherny D. Blinking fluorophores: what do they tell us about protein dynamics? Biochem Soc T 2006;34(5).
[10] Michalet X, Weiss S, Jäger M. Single-molecule fluorescence studies of protein folding and conformational dynamics. Chem Rev 2006;106:1785–813.
[11] Zhang J, Li JX, Razavi FS, Mumin AM. One-pot synthesis and characterization of rhodamine derivative-loaded magnetic core-shell nanoparticles. J Nanopart Res 2011;13:1909–16.
[12] Amat-Guerri F, Costela A, Figuera JM, Florido F, Sastre R. Laser action from rhodamine 6G-dopped poly (2-hydroxyethylmethacrylate)matrices with different crosslinking degrees. Chem Phys Lett 1993;209(4):352–6.
[13] Beja M, Afonso CAM, Martinho JMG. Synthesis and applications of rhodamine derivatives as fluorescent probes. Chem Soc Rev 2009;38:2410–33.
[14] Yan F, Chen L, Yan X, Ge F, Duan E. Synthesis and applications of rhodamine fluorescent dyes. Prog Chem 2006;18(2/3):252–61.
[15] Wang XQ, Song MY, Long YC. Synthesis, characterization, and crystal structure of the lactone form of rhodamine B. J Solid State Chem 2001;156:325–30.
[16] Dujols V, Ford F, Czarnik AW. A long-wavelength fluorescent chemodosimeter selective for Cu(II) ion in water. J Am Chem Soc 1997;119:7386–7.
[17] Nguyen T, Francis MB. Practical synthetic route to functionalized rhodamine dyes. Org Lett 2003;5(18):3245–8.

[18] Meng QH, Yu MJ, Zhang HF, Ren JC, Huang DY. Synthesis and application of *N*-hydroxysuccinimidyl rhodamine B ester as an amine-reactive fluorescent probe. Dyes Pigm 2007;73:254–60.
[19] Ross JA, Ross BP, Cosgrove KL, Rubinsztein-Dunlop H, McGeary RP. Rhodamine B pentyl ester and its methyl, ethyl, propyl, and butyl homologues. Molbank; 2006:M515.
[20] Afonso CAM, Santhakumar V, Lough A, Batey RA. An expedient synthesis of cationic rhodamine fluorescent probes suitable for conjugation to amino acids and peptides. Synthesis 2003;17:2647–54.
[21] López Arbeloa F, Ruiz Ojeda P, López Arbeloa I. Fluorescence self-quenching of the molecular forms of rhodamine B in aqueous and ethanolic solutions. J Lumin 1989;44:105–12.
[22] Deiters A, Cropp TA, Mukherji M, Chin JW, Anderson JC, Schultz PG. Adding amino acids with novel reactivity to the genetic code of *Saccharomyces cerevisiae*. J Am Chem Soc 2003;125:11782–3.
[23] Kiick KL, Saxon E, Bertozzi CR, Tirrell DA. Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. Proc Natl Acad Sci 2002;99:12–24.
[24] Li N, Lim RKV, Edwardraja S, Lin Q. Copper-free Sonogashira cross-coupling for functionalization of alkyne-encoded proteins in aqueous medium and in bacterial cells. J Am Chem Soc 2011;133:15316–9.
[25] Lin YA, Chalker JM, Davis BG. Olefin cross-metathesis on proteins: investigation of allylic chalcogen effects and guiding principles in metathesis. J Am Chem Soc 2010;132:16805–11.
[26] Pfeiffer H, Rojas A, Niesel J, Schatzschneider U. Sonogashira and "Click" reactions for the *N*-terminal and side-chain functionalization of peptides with $[Mn(CO)_3(tpm)]^+$-based CO releasing molecules (tpm = tris(pyrazolyl) methane). Dalton Trans; 2009:4292–8.
[27] Tam A, Soellner MB, Raines RT. Water-soluble phosphinothiols for traceless Staudinger ligation and integration with expressed protein ligation. J Am Chem Soc 2007;129:11421–30.
[28] Busch GK, Tate EW, Gaffney PRJ, Rosivatz E, Woscholski R, Leatherbarrow RJ. Specific N-terminal protein labeling: use of FMDV $3C^{pro}$ protease and native chemical ligation. Chem Comm 2008;29:3369–71.
[29] Nakanishi S, Tanaka H, Hioki K, Yamada K, Kunishima M. Labeling study of avidin by modular method for affinity labeling (MoAL). Chem Comm 2010;20: 7050–3.
[30] Johnson JA, Lu YY, van Deventer JA, Tirrell DA. Residue-specific incorporation of non-canonical amino acids into proteins: recent developments and applications. Curr Opin Chem Biol 2010;14:774–80.
[31] Ramos SS, Vihena AF, Santos L, Almeida P. ^1H and ^{13}C NMR spectra of commercial rhodamine ester derivatives. Magn Reson Chem 2000;38:475–8.