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A versatile access to new halogenated 7-azidocoumarins for photoaffinity labeling: Synthesis and photophysical properties

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ABSTRACT

A versatile methodology for the synthesis of 6/8-halogenated 7-aminocoumarins from the corresponding 7-hydroxy analogs using Pd-catalyzed amination reaction as the key step is presented. Further readily conversion into 7-azidocoumarins was performed and the resulting aryl azides proved higher stability and reactivity than the corresponding non-halogenated parent compound. These new compounds may thus constitute attractive scaffolds for designing novel photoaffinity reagents for various challenging bio-labeling applications.

1. Introduction

The prominence of coumarins in natural products and biologically active molecules (e.g., wedelolactone, combretastatins or novobiocin) has promoted considerable efforts toward their synthesis [1–3]. Moreover, their unique photophysical properties (especially their broad Stokes' shift associated to high quantum yields under physiological conditions) make them important units for the development of various blue- or violet-fluorescent probes [4]. 7-Hydroxycoumarins have been widely used in the preparation of fluorescent dye-protein conjugates and are the fluorescent reporter molecules commonly found in various fluorogenic enzyme substrates [5]. However their further development as fluorescent probes has been hampered both by their sensitivity to strong nucleophiles and alkaline media and by the strong pH-dependence of their fluorescence [6]. Thus, the development of new phenolic coumarin scaffolds possessing improved photophysical and photochemical properties has received a lot of attention during the past two decades [7,8]. Some improvements were achieved in lowering the pKa of their phenolic proton, and thus shift the useful fluorescence range toward neutral pH. In this aim, chlorination and/or fluorination of the 6- and 8-positions of 7-hydroxycoumarins were studied [7,9] and two fluorescent dyes exhibiting excellent photophysical properties and named Marina Blue™ (i.e., 6,8-difluoro-7-hydroxy-4-methylcoumarin) and Pacific Blue™ (i.e., 3-carboxy-6,8-difluoro-7-hydroxycoumarin) are now commercially available from Invitrogen. The introduction of a dialkylaminomethyl (or a methyleneiminodiacetic acid) group in position 8 of the coumarin chromophore readily achieved by a Mannich reaction [8,10], was also successful at increasing emission intensities in the physiological pH range. Interestingly, 7-dialkylaminocoumarin derivatives have also been considered as less pH-sensitive 7-hydroxycoumarin surrogates [11]. However, they are less easy to prepare and so there are actually only limited examples of their chemical modification aimed at improving their spectral properties. Recently, we became interested in the study of small size fluorophores that can be used for the photoaffinity labeling of modified proteins in physiological conditions. Thus, pro-fluorescent 7-azidocoumarinic analogs bearing a bio-conjugatable and water-solubilizing moiety in the 4-position, were firstly considered. These fluorogenic labels derived from the core structure of commercially available photoaffinity labeling

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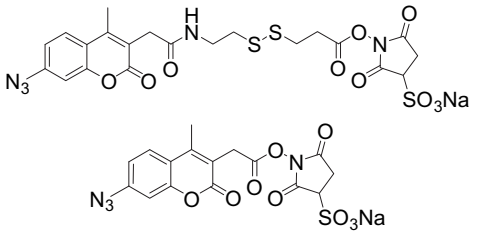


Fig. 1. Commercially available photoaffinity labeling 7-azidocoumarins: Sulfo-SAED (top) and Sulfo-SAMCA (bottom).

reagents Sulfo-SAED and Sulfo-SAMCA (Fig. 1). Indeed, these latter 7-azidocoumarinic derivatives are currently used for the protein modification, but suffer from a low water-solubility and a poor photostability [12]. Those 7-azidocoumarins are of particular interest since they display a low fluorescence whereas once the nitrene has been trapped and inserted either in a C–H or N–H bond, the resulting 7-amino or 7-hydrazinocoumarin is highly fluorescent, thus allowing a direct quantification of the labeling efficiency (Fig. 2) [13]. However, to our knowledge, no general synthetic routes have been developed for introducing chemical modifications aimed at modulating the physicochemical properties and photoreactivity of these unusual pro-fluorescent aryl azide derivatives.

In this article, we report an original and efficient synthetic scheme for preparing functionalized 7-azidocoumarin pro-fluorophores which fulfill all requirements for further designing efficient photoaffinity labeling reagents.

2. Experimental

2.1. Chemicals and instruments

Flash column chromatography purifications were performed on Geduran® Si 60 silica gel (40–63 μm) from Merck. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminum sheets. The spots were visualized by illumination with UV lamp ($\lambda = 254$ nm or 365 nm) and/or staining with a phosphomolybdic acid or $KMnO_4$ solution. All solvents were dried following standard procedures (CH_2Cl_2: distillation over P_2O_5, CH_3CN: distillation over CaH_2, DIEA: distillation over CaH_2 and storage over BaO, DME: distillation over CaH_2, DMF: distillation over BaO and storage over 4 Å molecular sieves, MeOH: distillation over Mg and storage over 4 Å molecular sieves, THF: distillation over Na°/benzophenone, toluene: distillation over Na°). Phosphate Buffered Saline (PBS, pH 7.5) was prepared using deionized water purified with a Milli-Q system (purified to 18.2 MΩ cm). The photoreactions at 380 nm were performed with a Rayonet reactor RPR-100 (Southern New England Ultra Violet company) equipped with 8 lamps (8 × 15 W). ^1H, ^{13}C and ^{19}F NMR spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) using the solvent peak for calibration [14]. *J* values are expressed in Hz. Infrared (IR) spectra were recorded as KBr pellets using a Perkin Elmer FT-IR Paragon 500 spectrometer with frequencies given in reciprocal centimeters (cm^{-1}). The elemental analyses were carried out with a Flash 2000 Organic Elemental Analyzer (Thermo Scientific). Mass spectra were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray (ESI) or APCI probe. UV–visible spectra were obtained on a Varian Cary 50 scan spectrophotometer. Fluorescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer. The absorption spectra of 7-OH/7-NH_2 coumarins were recorded (220–500 nm) in PBS (0.1 M phosphate + 0.15 M NaCl, pH 7.5) (concentration: 1.0–10.0 μM) at 25 °C. Emission spectra were recorded under the same conditions after excitation at 370 nm (excitation and emission filters: auto, excitation and emission slit = 5 nm) in PBS. Relative quantum yields were measured in PBS at 25 °C by a relative method using 7-hydroxycoumarin ($\Phi_F = 0.76$ in PBS) as a standard [15]. The following equation was used to determine the relative fluorescence quantum yield:

$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\Phi_F(s)$$

Where *A* is the absorbance (in the range 0.01–0.1 A.U.), *F* is the area under the emission curve, *n* is the refractive index of the solvents (at 25 °C) used in measurements ($n = 1.337$ for PBS), and the subscripts s and x represent standard and unknown, respectively.

For the detailed synthetic procedures of compounds **1**–**4**, **9**–**11**, **19**–**20** and starting 2-fluororesorcinol, see Supplementary material.

2.2. Synthesis of the 7-hydroxycoumarins and triflate derivatives

2.2.1. Methyl 2-(7-hydroxy-2-oxo-2H-chromen-4-yl)acetate (*12*)

Resorcinol (0.5 g, 4.54 mmol, 1 equiv), dimethyl 1,3-acetonedicarboxylate (0.79 g, 4.54 mmol, 1 equiv) and samarium(III) nitrate hexahydrate (0.40 g, 0.91 mmol, 0.2 equiv) were heated at 80 °C overnight under an argon atmosphere. The reaction was checked for completion by TLC (cyclohexane–EtOAc, 5:5, v/v).Thereafter, the resulting dark red oil was dissolved in EtOAc (50 mL) and washed with deionized water (2 × 50 mL) and brine (50 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The crude was purified by flash column chromatography (cyclohexane–EtOAc, 6:4, v/v) to give the expected coumarin derivative as a white solid (0.66 g, yield 62%). R_f (cyclohexane–EtOAc, 5:5, v/v) = 0.3; ^1H NMR (300 MHz, acetone-d_6): $\delta = 9.48$ (1H, s br, O*H*), 7.57 (1H, d, CH_{coum}, $J = 8.7$ Hz), 6.85 (1H, dd, CH_{coum}, $J = 8.7$ and 2.5 Hz), 6.77 (1H, d, CH_{coum}, $J = 2.5$ Hz), 6.22 (1H, s, $CH_{coum}CO$), 3.91 (2H, s, CH_2), 3.69 (3H, s, CH_3); ^{13}C NMR (75.5 MHz, acetone-d_6): δ = 170.2, 161.9, 160.8, 156.6, 149.9, 127.4, 113.7, 113.6, 112.8, 103.5, 52.6, 37.9; MS (ESI, negative mode): *m/z* 233.20 $[M - H]^-$, calcd mass for $C_{12}H_{10}O_5$ 234.21.

2.2.2. Methyl 2-(8-fluoro-7-hydroxy-2-oxo-2H-chromen-4-yl) acetate (*13*)

To a solution of 2-fluororesorcinol (3.95 g, 30.8 mmol, 1 equiv) in methanesulfonic acid (56 mL) at 0 °C was added dropwise dimethyl 1,3-acetonedicarboxylate (5.0 mL, 37.0 mmol, 1.2 equiv). The resulting brown viscous solution was kept at 0 °C with vigorous stirring for further 30 min then allowed to warm up at rt and stirred for further 7 days. The reaction was checked for completion by TLC (cyclohexane–EtOAc, 5:5, v/v). Thereafter, the reaction mixture was added dropwise under vigorous stirring to pre-cooled deionized water (500 mL) and then stirred for further 30 min at rt. The precipitating product (as a yellow-beige bulky solid) was then

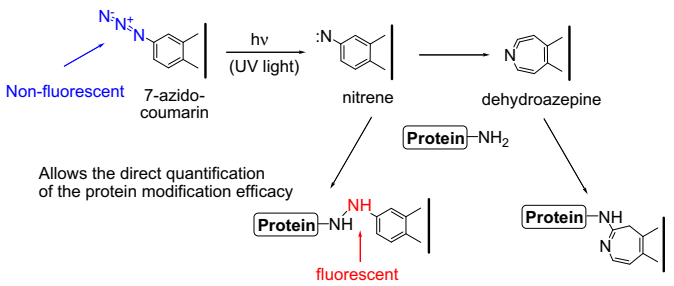


Fig. 2. Photoactivation of 7-azidocoumarins for protein labeling purposes.

recovered by filtration, and washed sequentially with MeOH, Et_2O, CH_2Cl_2 and acetone. The precipitate thus obtained represents a first crop of the desired product (3.02 g) which could be further used without any other purification. The washing organic filtrate was also concentrated and further submitted to successive recrystallization (in mixtures of Et_2O, CH_2Cl_2 and acetone) and flash column chromatography (cyclohexane–EtOAc with a step gradient from 7:3 to 4:6) but the desired coumarin could not be separated from the dimeric structure also formed in the reaction (*vide infra*). The expected product was thus obtained as pale yellow solid (3.02 g, yield 39%). R_f (cyclohexane–EtOAc, 5:5, v/v) = 0.3; ^{1}H NMR (300 MHz, acetone-d_6): δ = 9.50–10.10 (1H, s br, O*H*), 7.39 (1H, dd, CH_{coum} γ F, J = 1.9 and 8.9 Hz), 7.00 (1H, dd, CH_{coum} β F, J = 8.9 and 7.9 Hz), 6.31 (1H, s, $CH_{coum}CO$), 3.94 (2H, s, $Ar_{coum}CH_2CO$), 3.69 (3H, s, OCH_3); ^{13}C NMR (75 MHz, acetone-d_6): δ = 170.1, 159.6, 150.0, 149.0 (d, J = 10 Hz), 144.4 (d, J = 9 Hz), 139.6 (d, J = 244 Hz), 121.2 (d, J = 4 Hz), 114.3, 114.2, 113.8, 52.7, 37.9; ^{19}F NMR (282.5 MHz, acetone-d_6): δ = 17.02; MS (ESI, negative mode): *m/z* 251.40 $[M - H]^-$, calcd mass for $C_{12}H_9FO_5$ 252.20; elemental analysis (%) calcd: C, 57.15; H, 3.60; found: C, 58.36; H, 4.10.

2.2.3. Side product – dimeric structure (**15**)

Pale green solid. R_f (cyclohexane–EtOAc, 5:5, v/v) = 0.4; ^{1}H NMR (300 MHz, acetone-d_6): δ = 9.10–9.60 (2H, s br, 2O*H*), 6.82 (2H, dd overlayed, $2CH_{coum}$ β F, J = 8.3 and 8.3 Hz), 6.71 (2H, dd, CH_{coum} γ F, J = 1.3 and 8.3 Hz), 3.38 (2H, d, 2(0.5CH_2CO), J = 15.5 Hz), 3.14 (2H, d, 2(0.5CH_2CO), J = 15.5 Hz); ^{13}C NMR (75.5 MHz, acetone-d_6): δ = 165.4 (2C), 147.0 (2C, d, J = 10 Hz), 141.2 (2C, d, J = 9 Hz), 140.7 (2C, d, J = 245 Hz), 120.9 (2C, d, J = 4 Hz), 118.7 (2C, d, J = 1 Hz), 114.0 (2C, d, J = 2 Hz), 40.6 (2C), 30.6. ^{19}F NMR (282.5 MHz, acetone-d_6): δ = 20.11; MS (ESI, negative mode): *m/z* 347.13 $[M - H]^-$, 694.80 $[2M - H]^-$, calcd mass for $C_{17}H_{10}F_2O_6$ 348.26.

2.2.4. Methyl 2-(6-chloro-7-hydroxy-2-oxo-2H-chromen-4-yl) acetate (**14**)

To a solution of 4-chlororesorcinol (4.0 g, 27.7 mmol, 1 equiv) in methanesulfonic acid (56 mL) at 0 °C was added dropwise dimethyl 1,3-acetonedicarboxylate (4.8 mL, 33.2 mmol, 1.2 equiv) over 30 min under vigorous stirring. The resulting brown viscous solution was kept at 0 °C for further 30 min then allowed warm up at rt and stirred for further 5 days. The reaction was checked for completion by TLC (cyclohexane–EtOAc, 5:5, v/v). Thereafter, the reaction mixture was added dropwise under vigorous stirring to pre-cooled deionized water and then stirred for further 30 min at rt. The precipitating product (as a yellow-beige bulky solid) was then recovered by filtration, and washed sequentially with MeOH, Et_2O, CH_2Cl_2 and acetone. The precipitate thus obtained represents a first crop of the desired product (1.53 g). The washing organic filtrate was also concentrated and further submitted to successive recrystallizations (in mixtures of Et_2O, CH_2Cl_2 and acetone) and flash column chromatography (cyclohexane–EtOAc with a step gradient from 7:3 to 4:6, v/v) to recover a second crop of pure desired product (1.54 g). The expected product was thus obtained as a pale pink solid (3.07 g, 11.44 mmol, yield 41%). R_f (cyclohexane–EtOAc, 5:5, v/v) = 0.5; R_f (CH_2Cl_2–Et_2O, 5:0.5, v/v) = 0.3; ^{1}H NMR (300 MHz, acetone-d_6): δ = 7.54 (1H, s, CH_{coum} α Cl), 6.95 (1H, s, CH_{coum} γ Cl), 6.30 (1H, s, $CH_{coum}CO$), 3.97 (2H, s, CH_2), 3.71 (3H, s, CH_3); ^{13}C NMR (75.5 MHz, acetone-d_6): δ = 170.1, 160.3, 157.0, 154.8, 149.2, 127.1, 118.2, 114.9, 113.7, 104.7, 52.7, 37.7; MS (ESI, negative mode): *m/z* 267.46 and 269.39 $[M - H]^-$, calcd mass for $C_{12}H_9ClO_5$ 268.66; elemental analysis (%) calcd: C, 53.65; H, 3.38; found: C, 53.89; H, 3.39.

2.2.5. Side-product – dimeric structure (**16**)

Pale orange solid; R_f (CH_2Cl_2–Et_2O, 5:0.5, v/v) = 0.5; ^{1}H NMR (300 MHz, acetone-d_6): δ = 9.90–10.10 (2H, s br, 2O*H*), 7.01 (2H, s, $2CH_{coum}$ α Cl), 6.86 (2H, s, $2CH_{coum}$ β Cl), 3.33 (2H, d, 2(0.5CH_2CO), J = 15.5 Hz), 3.09 (2H, d, 2(0.5CH_2CO), J = 15.5 Hz). ^{13}C NMR (75.5 MHz, acetone-d_6): δ = 166.0 (2C), 155.0 (2C), 151.5 (2C), 127.5 (2C), 118.4 (2C), 117.5 (2C), 106.4 (2C), 40.55 (2C), 39.1 (1C, C-4); MS (ESI, negative mode): *m/z* 379.13 and 381.13 $[M - H]^-$, 758.73 and 762.63 $[2M - H]^-$, calcd mass for $C_{17}H_{10}Cl_2O_6$ 381.17.

2.2.6. Methyl 2-(8-fluoro-2-oxo-7-(trifluoromethylsulfonyloxy)-2H-chromen-4-yl)acetate (**17**)

To a suspension of phenol **13** (2.2 g, 8.7 mmol, 1 equiv) and *N*-phenyl-bis(trifluoromethanesulfonimide) (3.43 g, 9.6 mmol, 1.1 equiv) in dry CH_3CN (100 mL), was added DIEA (2.05 mL, 12.2 mmol, 1.4 equiv) and the resulting yellow solution was stirred at rt overnight. The reaction was checked for completion by TLC (cyclohexane–EtOAc, 6:4, v/v). Thereafter, the reaction mixture was concentrated and the obtained crude was dissolved in EtOAc (200 mL) and washed with deionized water (200 mL) and brine (150 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified by flash column chromatography (cyclohexane–EtOAc with a step gradient from 7:3 to 6:4, v/v) to give the desired product as a pale yellow solid (3.24 g, yield 97%). R_f (cyclohexane–EtOAc, 6:4, v/v) = 0.3; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.46 (1H, dd, CH_{coum} γ F, J = 9.1 and 2.1 Hz), 7.29 (1H, dd, CH_{coum} β F, J = 9.1 and 6.2 Hz), 6.50 (1H, s, $CH_{coum}CO$), 3.81 (2H, s, CH_2CO), 3.76 (3H, s, OCH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 168.5, 157.6, 147.0 (d, J = 2 Hz), 143.3 (d, J = 9 Hz), 142.1 (d, J = 258 Hz), 138.5 (d, J = 11 Hz), 120.8 (1C, C-9), 120.0 (d, J = 5 Hz), 118.8, 118.7 (d, J = 319 Hz), 118.2, 53.1, 38.0; ^{19}F NMR (282.5 MHz, $CDCl_3$): δ = −72.93 (3F, d, CF_3, J = 5.2 Hz), −144.56 (1F, qv, F, J = 5.2 Hz); MS (ESI, negative mode): *m/z* 383.00 and 385.07 $[M - H]^-$, calcd mass for $C_{13}H_8F_4O_7S$ 384.26; elemental analysis (%) calcd: C, 40.64; H, 2.10; S, 8.34; found: C, 40.75; H, 2.29; S, 8.50.

2.2.7. Methyl 2-(6-chloro-2-oxo-7-(trifluoromethylsulfonyloxy)-2H-chromen-4-yl)acetate (**18**)

To a suspension of phenol **14** (0.23 g, 0.86 mmol, 1 equiv) and *N*-phenyl-bis(trifluoromethanesulfonimide) (0.34 g, 0.95 mmol, 1.1 equiv) in dry CH_3CN (20 mL), was added DIEA (0.2 mL, 1.21 mmol, 1.4 equiv) and the resulting yellow solution was stirred at rt overnight. The reaction was checked for completion by TLC (cyclohexane–EtOAc, 6:4, v/v). Thereafter, the reaction mixture was concentrated and the resulting residue was dissolved in EtOAc (100 mL) and washed with deionized water (100 mL) and brine (100 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified by flash column chromatography (cyclohexane–EtOAc with a step gradient from 7:3 to 6:4, v/v) to give the desired product as a white solid (0.36 g, yield 94%). R_f (cyclohexane–EtOAc, 7:3, v/v) = 0.3; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.70 (1H, s, CH_{coum} α Cl), 7.33 (1H, s, CH_{coum} β Cl), 6.46 (1H, s, $CH_{coum}CO$), 3.74 (5H, s, CH_2, CH_3); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 168.6, 158.9, 152.4, 146.9, 146.4, 134.3, 126.8, 119.8, 119.0, 118.6 (d, J = 319 Hz), 112.4, 53.1, 37.7; ^{19}F NMR (282.5 MHz, $CDCl_3$): δ = −73.09 (3F, CF_3); MS (ESI, negative mode): *m/z* 399.12 and 403.11 $[M - H]^-$, calcd mass for $C_{13}H_8ClF_3O_7S$ 400.71; elemental analysis (%) calcd: C, 38.97; H, 2.01; S, 8.00; found: C, 39.16; H, 2.24; S, 8.11.

2.3. Paladium-catalyzed amination reactions

2.3.1. Methyl 2-(7-amino-8-fluoro-2-oxo-2H-chromen-4-yl)acetate (**5**)

Previous to use, DME was degassed for at least 30 min under an argon flow. To a suspension of $Pd_2(dba)_3$ (48 mg, 0.052 mmol, 0.1 equiv) and Xantphos (60 mg, 0.104 mmol, 0.2 equiv) in DME (5 mL)

was added a solution of aryl triflate **17** (200 mg, 0.52 mmol, 1 equiv) in DME (15 mL) and the resulting violet-dark red suspension was further stirred at rt for 10min. Then, benzophenone imine (105 μL, 0.62 mmol, 1.2 equiv) and anhydrous K_2CO_3 (101 mg, 0.73 mmol, 1.4 equiv) were sequentially added and the suspension was further degassed under an argon flow for 15 min before heating at 70–75 °C. The reaction was checked for completion by TLC (cyclohexane–EtOAc, 7:3, v/v and CH_2Cl_2–Et_2O, 18:0.5, v/v) and was stopped after 4 h. Thereafter, the reaction mixture was cooled to rt, filtered on a Celite® pad which was further washed with EtOAc (3 × 50 mL) and Et_2O (3 × 50 mL) and the filtrate was concentrated. The crude thus obtained was pre-purified by flash column chromatography (cyclohexane–EtOAc with a step gradient from 7:3 to 6:4, v/v). The yellow oil thus obtained (113 mg) was then submitted to an acid treatment (1.0 M aq. HCl-THF, 1:1, v/v, 12 mL) in order to hydrolyze the imine moiety and release the free aniline. The reaction evolution was monitored by TLC (cyclohexane–EtOAc, 5:5, v/v). After stirring at rt for 2 h, the reaction mixture was concentrated and the remaining aqueous phase was extracted with EtOAc (3 × 50 mL), then basified (pH 8–9, using sat. aq. $NaHCO_3$) and re-extracted with EtOAc (3 × 50 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated. The crude was purified by flash column chromatography (cyclohexane–EtOAc with a step gradient from 6:4 to 5:5, v/v) to give the desired product as a pale yellow solid (31 mg, yield 25%). R_f (cyclohexane–EtOAc, 5:5, v/v) = 0.4; ^{1}H NMR (300 MHz, acetone-d_6): δ = 7.25 (1H, dd, CH_{coum} γ F, J = 8.9 and 1.7 Hz), 6.81 (1H, dd overlayed peaks, CH_{coum} β F, J = 8.9 and 8.9 Hz), 6.12 (1H, s, $CH_{coum}CO$), 5.65 (2H, s br, NH_2), 3.86 (2H, s, CH_2), 3.68 (3H, s, CH_3); ^{13}C NMR (75.5 MHz, acetone-d_6): δ = 170.2, 160.0, 150.3 (d, J = 3 Hz), 144.1 (d, J = 9 Hz), 141.1 (d, J = 10 Hz), 138.1 (d, J = 239 Hz), 121.5 (d, J = 4 Hz), 112.5 (d, J = 3 Hz), 111.8, 110.6, 52.6, 37.9; ^{19}F NMR (282.5 MHz, acetone-d_6): δ = 16.12; MS (ESI, positive mode): m/z 252.29 $[M + H]^+$, calcd mass for $C_{12}H_{10}FNO_4$ 251.22; elemental analysis (%) calcd: C, 57.37; H, 4.01; N, 5.58; found: C, 58.48; H, 4.36; N, 5.68.

2.3.2. Methyl 2-(7-amino-6-chloro-2-oxo-2H-chromen-4-yl) acetate (**6**)

This 7-aminocoumarin was prepared and purified by the same procedure used for **5**, and obtained as a pale yellow solid (96 mg, yield 48% from aryl triflate **18** (300 mg, 0.76 mmol)). R_f (cyclohexane–EtOAc, 5:5, v/v) = 0.5; ^{1}H NMR (300 MHz, acetone-d_6): δ = 7.60 (1H, s, CH_{coum} α Cl), 6.75 (1H, s, CH_{coum} β Cl), 6.12 (1H, s, $CH_{coum}CO$), 5.87 (2H, s br, NH_2), 3.89 (2H, s, CH_2), 3.70 (3H, s, CH_3); ^{13}C NMR (75.5 MHz, acetone-d_6): δ = 170.2, 160.7, 155.3, 149.3, 149.0, 126.5, 115.5, 112.5, 110.8, 101.7, 52.6, 37.7; MS (ESI, positive mode): m/z 268.20 and 270.13 $[M + H]^+$, calcd mass for $C_{12}H_{10}ClNO_4$ 267.67; m.p. (°C): 208–210 with sublimation.

2.4. Synthesis and photolysis of 7-azidocoumarins

2.4.1. Methyl 2-(7-azido-8-fluoro-2-oxo-2H-chromen-4-yl)acetate (**7**)

7-Aminocoumarin **5** (40 mg, 0.16 mmol, 1 equiv) was dissolved in 17% aq. HCl (5 mL). After cooling to 0–5 °C, $NaNO_2$ (13 mg, 0.19 mmol, 1.2 equiv) was added in several portions. The resulting mixture was stirred at 0–5 °C for 1 h. Maintaining the same temperature, NaN_3 (12 mg, 0.19 mmol, 1.2 equiv) was added in several portions and the mixture was stirred for 5 h. The reaction mixture was allowed to warm up at rt. and left overnight under stirring. The precipitated product was rapidly filtered, washed with ice-cold water (2 × 5 mL) and dried under vacuum to yield the expected product as a pale yellow solid (37 mg, yield 87%). The product was stored at −20 °C under dark. R_f (cyclohexane–EtOAc, 6:4, v/v) = 0.6; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.31 (1H, dd, CH_{coum} γ F, J = 8.7 and 1.9 Hz), 6.97 (1H, dd, CH_{coum} β F, J = 8.7 and 7.2 Hz), 6.37 (1H, s, $CH_{coum}CO$), 3.75 (2H, s, $Ar_{coum}CH_2CO$), 3.74 (3H, s, OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 168.9, 158.6, 147.5 (d, J = 3 Hz), 143.2 (d, J = 9 Hz), 142.3 (d, J = 255 Hz), 131.6 (d, J = 8 Hz), 120.0 (d, J = 5 Hz), 117.2, 116.6, 115.9, 53.0, 38.1; ^{19}F NMR (282.5 MHz, $CDCl_3$): δ = −145.61; MS (ESI, positive mode): m/z 278.01$[M + H]^+$; MS (ESI, negative mode): m/z 276.20 $[M - H]^-$, calcd mass for $C_{12}H_8FN_3O_4$ 277.21.

2.4.2. Methyl 2-(7-azido-6-chloro-2-oxo-2H-chromen-4-yl)acetate (**8**)

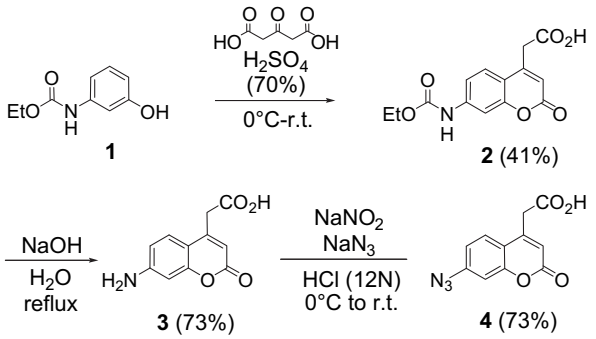
This compound was prepared and purified by the same procedure used for **7**, and obtained as a pale yellow solid (59 mg, yield 91% from 7-aminocoumarin **6** (60 mg, 0.22 mmol)). R_f (cyclohexane–EtOAc, 6:4, v/v) = 0.6; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.57 (1H, s, CH_{coum} α Cl), 7.14 (1H, s, CH_{coum} β Cl), 6.37 (1H, s, $CH_{coum}CO$), 3.77 (3H, s, CH_3), 3.73 (2H, s, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 168.8, 159.4, 153.1, 146.7, 141.3, 126.4, 121.3, 117.0, 116.8, 108.0, 53.1, 37.8; MS (ESI, negative mode): m/z 292.13 and 294.13 $[M - H]^-$, calcd mass for $C_{12}H_8ClN_3O_4$ 293.67.

2.4.3. 8-Fluoro-4-(2-oxo-2-(pyrrolidin-1-yl)ethyl)-7-(pyrrolidin-1-ylamino)-2H-chromen-2-one (**21**)

7-Azidocoumarin **7** (36 mg, 0.13 mmol, 1 equiv) was dissolved in deaerated $CHCl_3$ (5 mL) and kept under an inert atmosphere. Then, pyrrolidine (108 μL, 1.30 mmol, 10 equiv) was added and the resulting reaction mixture was irradiated at 380 nm for 6 h. Thereafter, this mixture was concentrated under vacuum and then the crude photo-irradiated mixture was resolved by preparative TLC (using EtOAc as eluent) to give the hydrazine **21** as an orange solid (9 mg, yield 19%). R_f (EtOAc) = 0.2; ^{1}H NMR (300 MHz, acetone-d_6): δ = 7.30 (1H, d, CH_{coum} γ F, J = 8.7 Hz), 6.77 (1H, dd overlayed peaks, CH_{coum} β F, J = 8.7 and 8.7 Hz), 6.02 (1H, s, $CH_{coum}CO$), 5.59 (1H, s br, N*H*), 3.62 (4H, t, $2CONCH_2$, J = 6.8 Hz), 3.38 (4H, t, $2NHNCH_2$, J = 6.8 Hz), 3.30 (2H, s, CH_2CO), 1.99 (4H, dt overlayed peaks, $2CONCH_2CH_2$, J = 6.8 and 6.8 Hz), 1.85 (4H, dt overlayed peaks, $2NHNCH_2CH_2$, J = 6.6 and 6.6 Hz); ^{13}C NMR (75 MHz, acetone-d_6): δ = 166.9, 160.2, 152.2 (d, J = 3 Hz), 144.0 (d, J = 9 Hz), 140.7 (d, J = 10 Hz), 138.0 (d, J = 238 Hz), 121.9 (d, J = 3 Hz), 112.3 (d, J = 4 Hz), 110.8 (2C), 49.6, 47.4 (2C), 46.5 (2C), 26.8 (2C), 25.0 (2C); ^{19}F NMR (282.5 MHz, acetone-d_6): δ = 15.74; MS (ESI, positive mode): m/z 360.27 $[M + H]^+$, calcd mass for $C_{19}H_{22}FN_3O_3$ 359.40.

2.4.4. 6-Chloro-4-(2-oxo-2-(pyrrolidin-1-yl)ethyl)-7-(pyrrolidin-1-ylamino)-2H-chromen-2-one (**22**)

This compound was prepared and purified (preparative TLC using cyclohexane–EtOAc, 3:7, v/v, as eluent) by the same procedure used for **21**, and obtained as an orange solid (11 mg, yield 15% from 7-azidocoumarin **8** (59 mg, 0.20 mmol)). R_f (cyclohexane–EtOAc, 2:8,



Scheme 1. Synthesis of 7-azidocoumarin-4-acetic acid **4**.

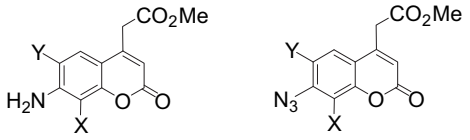
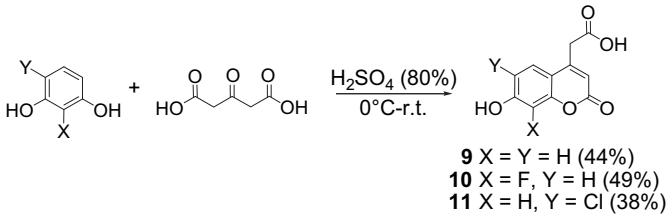


Fig. 3. Targeted coumarins studied in this article.



Scheme 3. Synthesis of 7-hydroxycoumarin-4-acetic acids **9**–**11** through standard Pechmann condensation.

v/v) = 0.28; ^{1}H NMR (300 MHz, acetone-d_6): δ = 7.66 (1H, s, CH_{coum} α Cl), 6.72 (1H, s, CH_{coum} β Cl), 6.02 (1H, s, $CH_{coum}CO$), 5.80 (1H, s br, NH), 3.85 (2H, s, CH_2), 3.63 (4H, t, $2CONCH_2$, J = 6.6 Hz), 3.39 (4H, t, $2NHNCH_2$, J = 6.6 Hz), 1.99 (4H, dt overlayed peaks, $2CONCH_2CH_2$, J = 6.6 and 6.6 Hz), 1.86 (4H, dt supperposed, $2NHNCH_2CH_2$, J = 6.6 and 6.6 Hz); ^{13}C NMR (75.5 MHz, acetone-d_6): δ = 166.8, 160.9, 155.2, 151.3, 148.7, 127.0, 115.2, 111.9, 111.6, 101.5, 47.4 (2C), 46.5 (2C), 38.9, 26.9 (2C), 25.0 (2C); MS (ESI, positive mode): m/z 376.13 $[M + H]^+$, calcd mass for $C_{19}H_{22}ClN_3O_3$ 375.86.

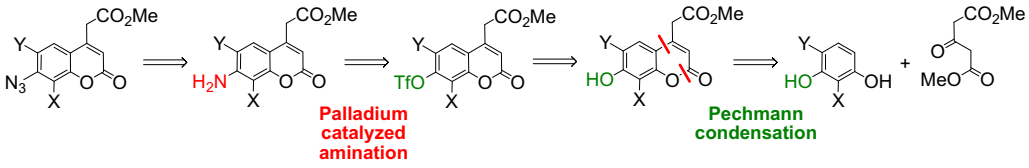
3. Results and discussion

3.1. Synthesis of 7-amino/azido halogeno coumarins 5–8

Firstly, we undertook the synthesis of water-soluble 7-azido derivative **4** obtained in 4 steps with a 21% overall yield through standard protocols (Scheme 1) [16]. In our hands, this derivative exhibited a high chemical instability and once irradiated led to a poor cross-linking efficiency. This is probably due to the too short lifetime of the singlet nitrene intermediate. The only side-product we were able to observe was the non-fluorescent azepine intermediate coming from the fast aromatic ring expansion (Fig. 2). Moreover, once synthesized, even when kept under an inert argon atmosphere at 4 °C or −20 °C in the dark, 7-azidocoumarin **4** was completely degraded within a few days, and could thus not be used as a valuable photoaffinity labeling tool [17].

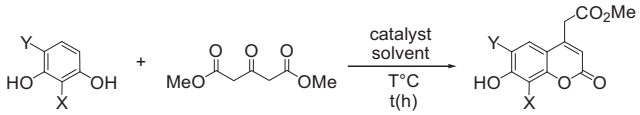
Consequently, we became interested in the synthesis and evaluation of the photophysical properties of original coumarinic analogs functionalized in the 4- and 7-positions for introducing chemical diversity on the coumarin core: a carboxylic acid function which increases the water-solubility and could be used as a bioconjugatable linker (introduced as an acetic acid pendant arm), and a photoreactive, yet stabilized azido group in position 7 (readily obtainable from the corresponding 7-amino precursor). As already discussed in the literature, stabilization of the singlet nitrene intermediate could be obtained by lowering the electron density within the coumarin aromatic ring [18]. Thus, the introduction of an electron-withdrawing halogen atom adjacent to the 7-azido group appears to be the most appropriate strategy to achieve this goal (Fig. 3). Since the corresponding 7-aminocoumarin derivatives **5** and **6** could not be readily obtained through standard Pechmann or Perkin condensations, we designed a new synthetic route for these fluorescent anilines and their subsequent conversion to the targeted 7-azido halogen coumarins **7** and **8**. As presented in Scheme 2, our retro-synthetic pathway involves two key steps: (1) a Pd-catalyzed amination reaction to convert the 7-OH group of 7-hydroxycoumarins into an unsubstituted primary aniline group [19], and (2) a Pechmann reaction that enables the formation of the 7-hydroxy halogenated coumarin units from the corresponding halogenated resorcinols and correctly substituted β-keto-diesters [20].

Thus, we first focused on optimizing the synthesis of key methyl 7-hydroxycoumarin-4-acetates bearing either a chlorine atom in the 6-position or a fluorine atom in the 8-position. The parent 4-acetic acid derivatives **10** and **11** were obtained from the β-ketoglutaric acid and the corresponding resorcinol (4-chlororesorcinol or 2-fluororesorcinol) through a Pechmann condensation performed under standard acidic conditions [16] (Scheme 3). However, the subsequent conversion of their carboxylic acid to methyl ester in the presence of the free 7-OH group proved to be difficult to achieve because of the similar reactivity of these two functions. Consequently, the Pechmann reaction between dimethyl β-ketoglutarate and the halogenated resorcinol was then considered and different acid catalysts were tested [21] (Table 1). If non-halogenated 7-hydroxycoumarin derivative **12** was obtained in a reasonable yield through samarium nitrate catalysis (entry 1), any attempt to obtain the 8-fluorinated derivative by using a similar Lewis acid catalysis failed (entries 2 and 3). Finally, we found that the use of methanesulfonic acid both as acid source and solvent [7,22] allowed us to isolate the desired 7-hydroxycoumarin methyl esters **13** and **14** in satisfying yields (entries 4 and 5) despite of the formation of ca. 10–15% amount of a dimeric side-product (Fig. 4), which drastically complicated the purification process [23]. Thereafter, phenols **13** and **14** were triflated to provide the activated intermediates **17** and **18** in almost quantitative yields (Scheme 4). The use of $PhN(SO_2CF_3)_2$ [24] was preferred to standard conditions (triflic anhydride/pyridine) because it gives higher yields. The triflates **17** and **18** were next coupled with amines under the catalysis of palladium–phosphine complex, widely known as Buchwald–Hartwig amination reaction [25]. Despite the large number of publications describing Pd-catalyzed cross-coupling reactions on the reactive 3-position of various coumarins [26], a few of them concern amination of such substrates [3]. Furthermore, it is known that reactions involving electron-deficient aryl triflates often give low yields owing to premature triflate hydrolysis which would be important in our case considering the electronegativity of the halogen atom introduced in 6- or 8-position. Thus, the choice of the reaction conditions (catalyst, base and temperature) is essential to get high coupling yields. These key amination conditions were optimized for the reaction of **17** and **18** with benzylamine through the screening of various Pd sources



Scheme 2. Retro-synthetic plan for the preparation of 7-azidocoumarins **7** and **8**.

Table 1
Optimization of the synthesis of methyl 7-hydroxycoumarin-4-acetates.



Entry	Coumarin	X	Y	Catalyst	Solvent	*T* (°C)	*t*	Isolated yield (%)
1	**12**	H	H	$Sm(NO_3)_2.6H_2O$	None	80–85	18 h	62
2	**13**	F	H	$Sm(NO_3)_2.6H_2O$	None	80–85	24 h	10[a]
3	**13**	F	H	$ZnCl_2$	MeOH	70	24 h	–[b]
4	**13**	F	H	CH_3SO_3H	CH_3SO_3H	0-rt	7 d	39[c]
5	**14**	H	Cl	CH_3SO_3H	CH_3SO_3H	0-rt	5 d	41[c]

[a] Significant degradation of the reaction mixture was observed.
[b] No reaction evolution.
[c] Formation of dimeric side-product **15** or **16** which makes purification difficult.

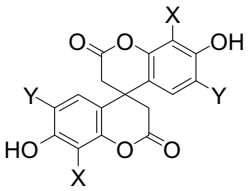
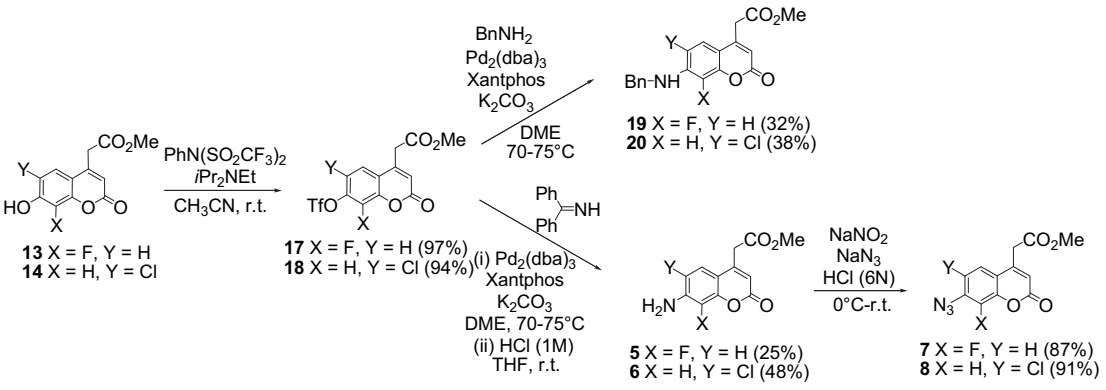


Fig. 4. Proposed structures for the dimeric side-products **15** (X = F, Y = H) and **16** (X = H, Y = Cl) isolated from the CH_3SO_3H-catalyzed Pechmann reaction.

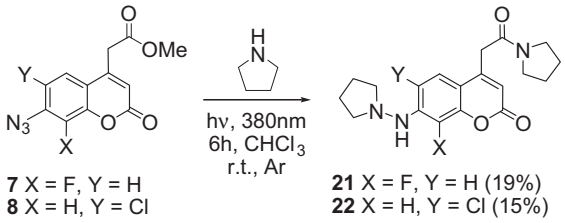
($Pd(OAc)_2$ or $Pd_2(dba)_3$), ligands (BINAP or Xantphos), solvents (THF or DME) and bases (Cs_2CO_3, K_2CO_3 or *t*BuONa). Because coumarins are sensitive substrates in alkaline media and may result in the cleavage of the lactone ring, we carefully examined the alkaline conditions for such cross-coupling reaction. Satisfying yields were obtained using the following conditions: $Pd_2(dba)_3$-Xantphos (5 and 10 mol % respectively), K_2CO_3 as base, in DME, at 70–75 °C. Since all our attempts to remove the benzyl group of **19** and **20** through Pd- or Pt-catalyzed hydrogenolysis have led to the corresponding 7-aminocoumarins in poor yields, we next extended these coupling reaction conditions to benzophenone imine which acts as a surrogate of ammonia [27]. A final acidic hydrolysis using a mixture of THF and aq. 1.0 M HCl at room temperature provided the targeted 7-aminocoumarins **5** and **6** in 25% and 48% yields (for the two steps) respectively. As a rather general rule, lower yields of isolated product were obtained in the case of Pd-catalyzed amination reaction involving the 8-fluoro substituted coumarins instead of their 6-chloro congeners, probably due to the higher electronegativity of the fluorine atom leading to a more electron-deficient substrates whose lactone moiety is more prone to alkaline hydrolysis. These 7-aminocoumarins **5** and **6** were then readily converted to the corresponding azide derivatives **7** and **8** through a nitrous acid deamination reaction in the presence of NaN_3 [28]. The corresponding aryl azides were characterized by NMR and ESI-MS, and all data are in agreement with the assigned structures.

3.2. Photoreactivity and photophysical properties of halogenated 7-azidocoumarins 7 and 8

Contrary to 7-azidocoumarin **4** which had to be used within a few hours after chromatographic purification, we were pleased to observe that both **7** and **8** proved to be full stable over a prolonged period of time when kept in the dark at rt [29]. In order to evaluate their potential as photoactivatable latent fluorescent probes, aryl azides **4**, **7** and **8** were submitted to a photoactivation reaction under UV-A irradiation (380 nm) and in the presence of a secondary amine (pyrrolidine) acting as the nucleophilic trapping agent [30] (Scheme 5). The reaction was performed in degassed chloroform since halogenated solvents are inert in such photolysis reactions. If aryl azide **4** only gave decomposition products, and traces of non-fluorescent azepines, for aryl azides **7** and **8**, two main products were obtained [31], the free aniline as a side-product corresponding to other pathways of intermediate singlet nitrene stabilization, and an hydrazine derivative which corresponds to the targeted N–H insertion product. Compounds **21** and **22** were isolated in ca. 15–20% yield and their structures confirmed by NMR and ESI-MS analyses. The photophysical properties of **21** and **22**



Scheme 4. Synthesis of 7-NH_2/7-N_3 coumarins through Pd-catalyzed Buchwald–Hartwig amination reaction.



7 X = F, Y = H
8 X = H, Y = Cl

21 X = F, Y = H (19%)
22 X = H, Y = Cl (15%)

Scheme 5. UV-A irradiation of 7-azidocoumarins **7** and **8**.

Table 2
Photophysical properties of synthesized 7-OH/7-NH_2 coumarins in physiological conditions (PBS).[a]

Dye	$\lambda_{max,\ abs}$ (nm)	$\lambda_{max,\ em}$ (nm)	Stokes shift (nm)	ε (M^{-1} cm^{-1})	Φ_F[b]
12	327	457	130	7 860	0.76
13	368	474	106	15 030	0.63
14	373	461	88	17 700	0.62
3	348	450	102	12 500	0.82
5	346	465	119	10 300	0.79
6	355	452	97	12 600	0.71
21	345	461	116	13 200	0.79
22	353	450	97	13 400	0.75

[a] Stock solutions of coumarins were prepared in DMSO (1 mg/mL) and subsequent dilutions with PBS (100 mM phosphate buffer, 150 mM NaCl, pH 7.5) were done for spectral measurements. Final DMSO concentration was assumed to be always below 0.1%.

[b] Determined at 25 °C by using 7-hydroxycoumarin ($\Phi_F = 0.76$, Ex. $\lambda = 370$ nm) as standard [15].

also confirmed that the N–H insertion reaction has led to the expected fluorescence unveiling of the newly developed 7-azidocoumarins (Table 2) [32]. Thus, the presence of a halogen atom in α position to the azido group stabilizes the singlet nitrene intermediate allowing the formation of the highly fluorescent N–H insertion derivatives. These data are in agreement with the behavior of other photoreactive cross-linkers such as perfluorinated phenyl azides or azido fluoroquinolones previously reported in the literature [30,33].

In addition to these preliminary photochemical reactions, we have also evaluated under simulated physiological conditions (i.e., phosphate buffered saline (PBS), pH 7.5), the photophysical properties of most of coumarins synthesized in this work (Table 2). Indeed, this comprehensive study is a possible way to discover new blue fluorescent dyes suitable for various bio-labeling applications (especially after the release of their carboxylic acid function which will be used as the bioconjugatable moiety). As expected, since the pKa of their phenol is significantly decreased by the α-halogen 7-hydroxycoumarins, 8-fluoro and 6-chloro derivatives **13** and **14**, exhibit a slightly higher brightness ($\varepsilon \times \Phi_F$) accompanied by a significant red shift of both absorption and emission maxima. Conversely, all 7-amino and 7-hydrazinocoumarin derivatives **5**, **6**, **21** and **22** proved to have absorption and fluorescence properties nearly identical to those of 7-amino-4-carboxymethylcoumarin **3**. Thus, the introduction of either halogen atom in the α position has little effect on fluorescence properties, but significantly increased the stability of the corresponding 7-azido pro-fluorophore (vide supra).

4. Conclusion and future work

In summary, a novel synthetic route for 7-aminocoumarin fluorophores using Pd-catalyzed Buchwald–Hartwig amination reaction has been developed to prepare original 6/8-halogenated and 4-functionalized 7-aminocoumarin derivatives. Further conversion to the corresponding 7-azidocoumarins has enabled us to get fully stable latent blue fluorescent dyes. These new compounds are promising candidates for designing novel photoreactive cross-linkers based on the coumarin core structure and useful in the context of UV-induced bio-labeling reactions. Further work is in progress aimed at converting **7** and **8** into water-soluble bioconjugatable derivatives through the derivatization of their acetic acid arm with a sulfonated amino acid moiety [34]. Furthermore, we believe that these original aryl azide reagents have a strong potential either to rapidly design and synthesize novel classes of "clicked" fluorophores [35] or in the context of fluorogenic "click" reactions aimed at detecting various analytes such as metal ions [36].

Acknowledgments

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dyepig.2011.05.008.

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