Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 4330

www.rsc.org/obc

COMMUNICATION

New insights into the water-solubilisation of fluorophores by post-synthetic "click" and Sonogashira reactions†

Cédrik Massif,[a] Sébastien Dautrey,[a] Alexandre Haefele,[b] Raymond Ziessel,[b] Pierre-Yves Renard[a,c,d] and Anthony Romieu*[a,c,d]

Received 27th February 2012, Accepted 17th April 2012
DOI: 10.1039/c2ob25428j

New synthetic methodologies for the efficient chemical conversion of hydrophobic fluorescent dyes into bioconjugable and water-soluble derivatives are described. The combined use of an original sulfonated terminal alkyne and a metal-mediated reaction, namely the copper-catalysed Huisgen 1,3-dipolar cycloaddition ("click" reaction) or the Sonogashira cross-coupling, is the cornerstone of these novel post-synthetic sulfonation approaches.

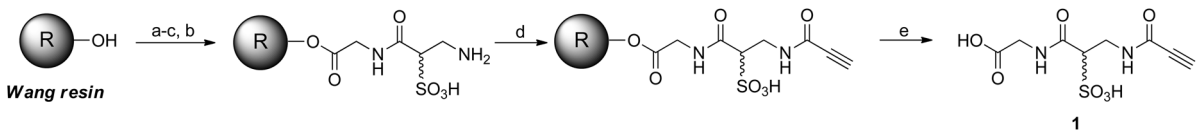
Introduction

It is now well established that fluorescent organic dyes are the preferred markers for the massive number of bioanalytical and biomedical applications whose practical implementation involves biomolecular labelling.[1] Among the numerous requirements for an ideal fluorescent bio-label, high brightness, water-solubility, resistance to self-aggregation and bio-orthogonal reactivity are the most important items currently intensively explored. To address these non-trivial issues, the most popular strategies are based on the introduction of hydrophilic moieties (positively or negatively charged groups, PEG-type chains and biopolymers) and a carboxylic acid function (easily convertible in active esters) in various positions of the polycyclic aromatic scaffolds.[2] Due to its unique solubilising properties and chemical inertness (especially in the context of bioconjugation reactions), sulfonate ($-SO_3^-$) is probably the most popular and widely used water-solubilising group to synthesise hydrophilic derivatives of various fluorescent architectures.

For the majority of fluorophores belonging to the BODIPY (and aza analogues), coumarine, cyanine, oxazine and xanthene families, the common sulfonation reactions include: (1) electrophilic aromatic substitution with H_2SO_4 (70–100%), oleum or $ClSO_3H$,[3,4] (2) alkylation reaction with a β-haloethanesulfonic acid or a sultone derivative,[5–7] and (3) Schotten–Baumann amidification reaction with an (α-sulfo-β-alanine)-based peptide (β-Ala(SO_3H)-based peptide).[8] This latter one was developed by our group and proved efficient to produce water-soluble and bioconjugable of a wide range of fluorescent organic dyes and supramolecular compounds.[2,7,9] Some of them, especially rhodamines and red-emitting BODIPY dyes, are viable alternatives to commercial Alexa Fluor® and CyDye™.[10] In order to get more rapidly these valuable fluorescent markers, we have recently reported the implementation of a solid-phase version of this Schotten–Baumann reaction using *N*-Fmoc-β-Ala(SO_3H) as the key building block.[11] Despite these significant achievements, our synthetic methodology is still limited to fluorophores possessing a carboxylic acid function. To expand the scope of our "post-synthetic" and mild sulfonation methodology to a wider range of fluorescent architectures that do not necessarily contain functional groups able to readily react with nucleophilic sulfonated linkers, there is an overriding need for designing alternative reactions allowing the grafting of sulfonated peptide-based linkers. Since the C- and N-terminal sides of β-Ala(SO_3H) polypeptides can be easily derivatised with a terminal alkyne moiety through peptide coupling reactions with propargylamine and propiolic acid respectively, it seemed obvious to us that we should explore chemical transformations involving a terminal alkyne as a versatile reaction partner. Thus, we have decided to explore the potential of popular transition metal-mediated reactions, namely copper-catalysed azide–alkyne 1,3-dipolar cycloaddition (CuAAC, also named Huisgen–Sharpless–Meldal or "click" reaction)[12] and palladium-catalysed Sonogashira reaction,[13] to achieve this ambitious goal. To the best of our knowledge, Sonogashira cross-coupling has never been used to impart water-solubility of fluorescent organic dyes. However, we can mention the preliminary works from the Burgess group published in 2007 and devoted to the spectral dispersion and water solubilisation of the tetramethyl-BODIPY system *via* Heck-type coupling

[a]*Université de Rouen, Laboratory COBRA UMR 6014 and FR 3038, IRCOF, 1 Rue Tesnière, 76821 Mont St Aignan Cedex, France. E-mail: anthony.romieu@univ-rouen.fr; Fax: + 33 (0)2 35 52 29 71; Tel: + 33 (0)2 35 52 24 27*
[b]*Laboratoire de Chimie Moléculaire et Spectroscopies Avancées (LCOSA), Ecole Européenne de Chimie, Polymères et Matériaux, 25 rue Becquerel, 67087 Strasbourg Cedex 02, France. E-mail: ziessel@unistra.fr; Fax: (+) 33 3-68-85-27-61*
[c]*INSA de Rouen, Avenue de l'Université, 76800 St Etienne du Rouvray, France*
[d]*CNRS Délégation Normandie, 14 Rue Alfred Kastler, 14052 Caen Cedex, France*

†Electronic supplementary information (ESI) available: Detailed synthetic procedures and characterisation/spectral data for compounds **1**, **5–12**, **14** & **17–20**. See DOI: 10.1039/c2ob25428j



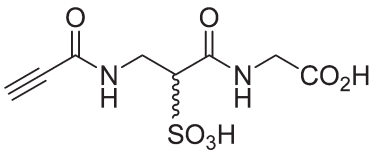
Scheme 1 *Reagents and conditions*: (a) Fmoc-Gly-OH (4 equiv.), DIC (2 equiv.), DMAP (0.15 equiv.), CH_2Cl_2–NMP (6 : 4), rt, overnight; (b) piperidine–NMP (2 : 8), rt, 10 min; (c) Fmoc-β-Ala(SO_3H)-OH (4 equiv.), BOP (4 equiv.), DIEA (12 equiv.), NMP–DMF (55 : 45), rt, overnight; (d) propiolic acid (5 equiv.), DIC (5 equiv.), DMAP (0.3 equiv.), CH_2Cl_2–NMP (6 : 4), rt, overnight; (e) TFA–CH_2Cl_2 (1 : 1), 4 °C to rt, 1 h followed by azeotropic evaporation, RP-HPLC purification and lyophilisation, 45% overall yield.

(C–H activation) involving an activated alkene (*i.e.*, a Michael acceptor bearing –CO_2H or –SO_3H group).[14] Despite interesting spectral properties in aqueous buffers, the corresponding derivatised BODIPYs were obtained in very poor yields and no further improvements of this synthetic methodology have been reported to date. Conversely, "click" reaction was used intensively in the context of fluorophores aimed at developing valuable new approaches for challenging biological labelling and bio-sensing applications. Thus, valuable new concepts such as "clickable"[15] or "click-on"[16–18] fluorophores/dyes and "click" fluorogenic reactions[19] have recently emerged. Interestingly, CuAAC has also been implemented for the introduction of bioconjugable or water-solubilising moieties onto hydrophobic fluorescent cores, especially those belonging to the (aza-)BODIPY, cyanine or squaraine rotaxane families.[20] For instance, Shi *et al.* have reported a colorimetric and fluorescent probe for Cu^{2+} and Hg^{2+} ions based on a distyryl BODIPY where solubility in water was readily obtained through a classical "click" reaction between a tetrakis-alkyne BODIPY precursor and four units of a triethylene glycol substituted azido derivative.[21]

Herein, we report the transposition and practical application of these well-known Cu(I)- and Pd(0)-catalysed reactions to the synthesis of fluorophores whose bioconjugation ability and hydrophilic character are brought by the introduction of a sulfonated peptide-based linker. A set of structurally different fluorescent organic dyes, including napththalene, 7-hydroxycoumarine, fluorescein, rhodamine 6G (R6G) and BODIPY derivatives which cover a broad spectral range from the UV-C to the visible green region, has been derivatised.[22] The choice of these polycyclic aromatic compounds was based on the fact that the required azido or iodo derivatives are synthetically easily available through literature procedures. The optical properties of the resulting water-soluble fluorophores were then evaluated under physiological conditions to demonstrate their potential utility as biolabelling reagents.

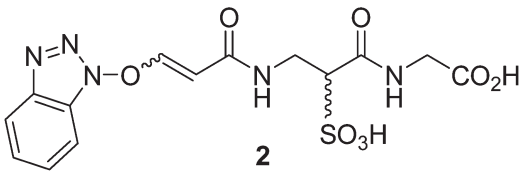
Results and discussion

The first step of the present study was devoted to the design of a convenient synthesis of a terminal alkyne derived from β-Ala (SO_3H). Since the *N*-Fmoc derivative of this unusual amino acid is fully compatible with manual or automated solid-phase peptide synthesis (SPPS) protocols,[11] we have considered the "on-resin" derivatisation of the C-terminal side of (β-Ala (SO_3H))-containing short peptides with propiolic acid. As we previously showed, the use of the Wang resin requires the incorporation of a glycine spacer prior to the loading of *N*-Fmoc-β-Ala(SO_3H), to get good immobilisation yields. Thus, we have chosen to prepare the sulfonated terminal alkyne **1**, according to a solid-phase strategy.



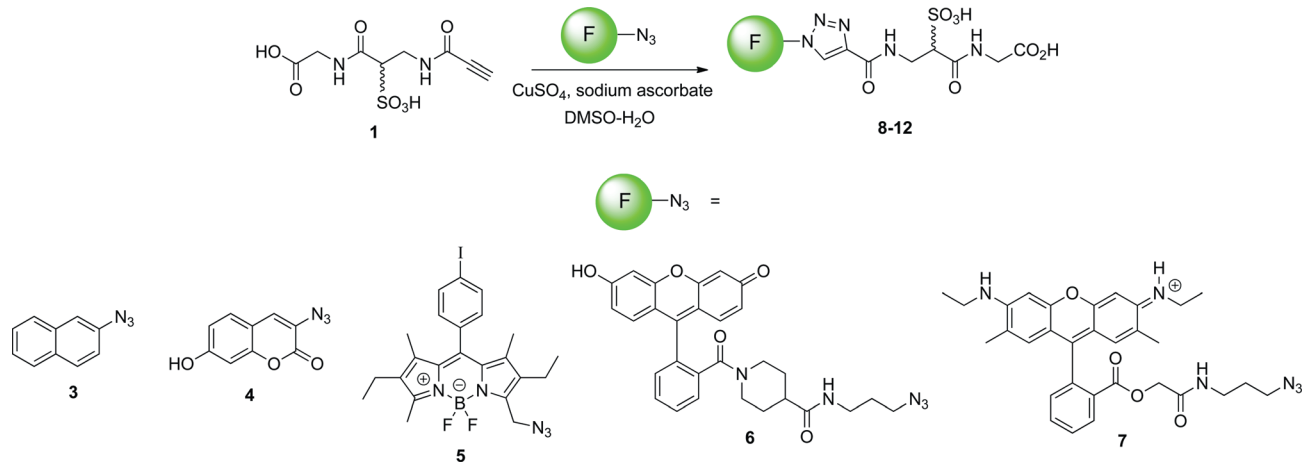
1

The experimental conditions from our previous published study[11] were used to couple the first two amino acids (glycine and β-Ala(SO_3H)) but the amidification of propiolic acid required further optimisation (Scheme 1). Indeed, we have observed that it is not relevant to couple this carboxylic acid under standard conditions involving either *N*,*N*′-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) or a phosphonium (or an uronium)-based peptide coupling reagent (*e.g.*, BOP or TSTU/HOBt),[23] due to a Michael-type reaction of the released HOBt with the activated alkyne bond, leading to the quantitative formation of the vinyloxy-benzotriazole derivative **2**. To avoid this side-reaction, it is possible to use a non-activated alkynated carboxylic acid analogue such as 4-pentynoic acid but this latter will lead to a too long and flexible linker, which is sometimes deleterious for conjugation to biomolecules/biopolymers or spectral properties in aqueous environments by favoring the surfactant behavior of the resulting derivatised fluorophores.

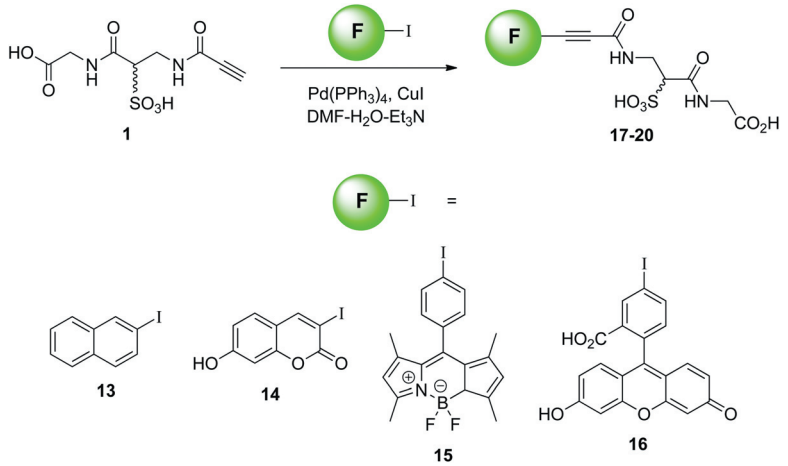


2

Since we assumed that this non-desired reaction would occur with all coupling reagents bearing a masked α-nucleophile (*e.g.*, *N*-hydroxysuccinimide (NHS), 1-hydroxy-7-azabenzotriazole (HOAt)…), DIC was used alone but no amidification of propiolic acid was observed. Nevertheless, the reaction did take place when a catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP) was added to the coupling mixture. The same conditions have been recently reported by Cravero *et al.* who used resin-bound propiolic acid and cyclohexanediones in tandem Michael–Michael cyclisations.[24] The magnetic stirring of the heterogeneous solid–liquid reaction mixture also has a strong influence because when the propiolic acid coupling is performed on a large scale of resin (~800 mg, 0.75 mmol), it is essential to



Scheme 2 *Reagents and conditions for the CuAAC reaction*: "Clickable" fluorophore (1 equiv.), sulfonated terminal alkyne **1** (1.2 equiv.), $CuSO_4$ (0.05 equiv.), sodium ascorbate (0.2 equiv.), DMSO–H_2O (1 : 1), rt, overnight followed by RP-HPLC purification and lyophilisation.



Scheme 3 *Reagents and conditions for the Sonogashira reaction*: Iodo-fluorophore (1 equiv.), sulfonated terminal alkyne **1** (1.2 equiv.), $Pd(PPh_3)_4$ (0.1 equiv.), CuI (0.2 equiv.), DMF–H_2O–Et_3N (2 : 1 : 1), rt, 3 h followed by RP-HPLC purification and lyophilisation.

divide the batch of resin into three equal parts and to conduct the reaction in three different flasks, each having a magnetic stirrer, otherwise the reaction does not work even if a large excess of acid is used. After cleavage from the resin using TFA–CH_2Cl_2 (1 : 1) and a rapid purification by reversed-phase HPLC (RP-HPLC), compound **1** was obtained in a pure form with a satisfactory 45% overall yield.

The availability of this unusual alkyne led us to consider its reactivity toward various fluorescent aryl-azido derivatives **3–7** (for the synthesis of these "clickable" fluorophores, see ESI†) under the standard conditions currently used to perform CuAAC reaction (*i.e.*, $CuSO_4$ and sodium ascorbate as the catalytic system). A mixture of DMSO–H_2O (1 : 1) was used as solvent except for BODIPY dye **5** which was found to be soluble only in CH_2Cl_2–DMSO–H_2O (1 : 1 : 1) (Scheme 2). This latter azido-BODIPY was prepared from the non-isolable bromomethyl derivative and sodium azide in DMF, itself prepared from the methyl-BODIPY *via* a reaction involving *N*-bromosuccinimide (NBS) in CH_2Cl_2 at rt (see ESI†).[25] All reactions were generally found to be complete within 12 h, and the corresponding monosulfonated fluorophores **8–12** were recovered in a pure form by semi-preparative RP-HPLC, with isolated yields ranging from 35 to 55%. Their structures were confirmed by detailed measurements, including ESI mass spectrometry and NMR analyses (see ESI†).

The practical implementation of the Sonogashira reaction with the fluorescent aryl-iodo derivatives **13–16** (for the synthesis of these iodo-fluorophores, see ESI†) required minor modifications of conditions currently employed for such Pd(0)-catalysed cross-coupling (*i.e.*, $Pd(Ph_3)_4$ and CuI as the catalytic system). These modifications were only focused on the reaction solvent (Scheme 3). A mixture of DMF–H_2O–Et_3N (2 : 1 : 1) was the best compromise to solubilise both the hydrophobic dye and the hydrophilic sulfonated alkyne **1**. For all aryl-iodo derivatives, complete conversion was observed within 3 h of vigorous stirring at room temperature. Conversely, it is important to note that the same reactions performed with the aryl-bromo analogues were always incomplete even upon further additions of alkyne **1** and catalyst. By analogy with the tetrazole-based fluorophores, the resulting monosulfonated fluorescent derivatives **17–20** were

Table 1 Spectral properties of water-soluble fluorophores **8–12** and **17–20** in PBS at 25 °C

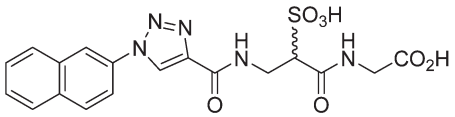
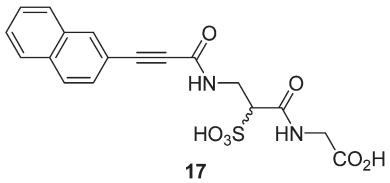
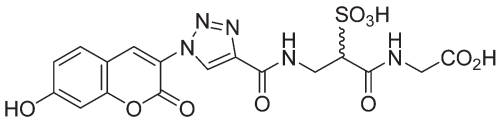
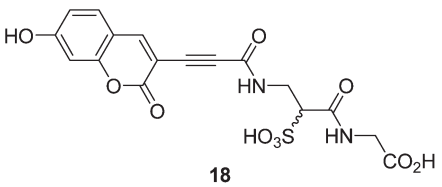
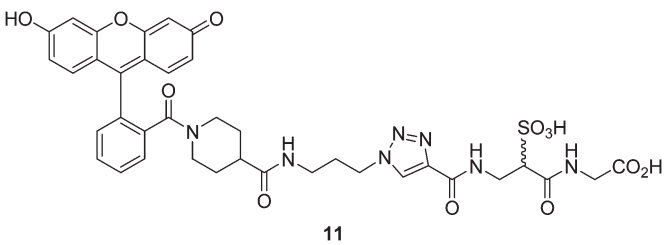
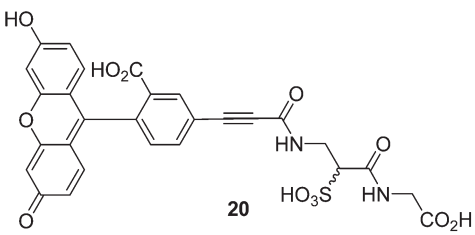
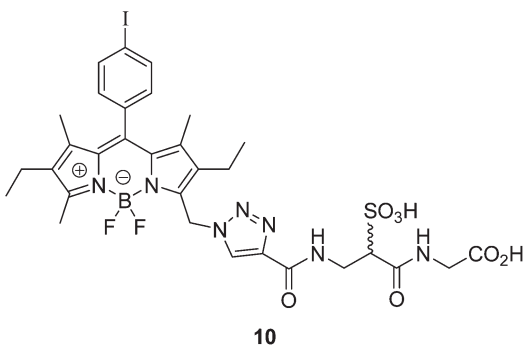
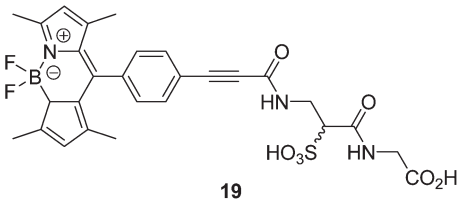
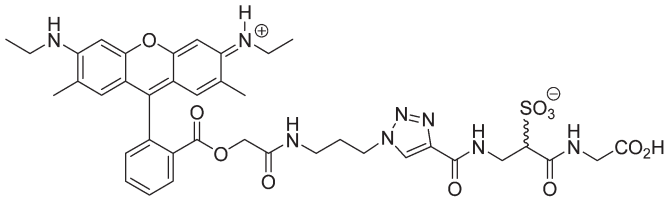
Compound	λ_{max}, abs/nm	ε/dm^3 mol^{-1} cm^{-1}	λ_{max}, em/nm	Φ_F [a]
 **8**	230	28 100	373	0.56 (0.23)[b]
 **17**	244	33 190	387	0.02 (0.23)[b]
 **9**	393	17 650	471	0.78 (0.76)[b]
 **18**	422	28 300	463	0.37 (0.76)[b]
 **11**	497	48 350	521	0.81 (0.90)[b]
 **20**	497	66 700	526	0.60 (0.90)[b]
 **10**	517	—[c]	537	0.25 (0.78)

Table 1 *(Contd.)*

Compound	λ_{max}, abs/nm	ε/dm^3 mol^{-1} cm^{-1}	λ_{max}, em/nm	Φ_F [a]
 **19**	497	—[c]	510	0.31 (0.69)
 **12**	530	80 200	551	0.86 (0.88)

[a] See ESI† for the experimental details related to these measurements (standards, λ ex/nm…). [b] Quantum yield of the non-sulfonated parent fluorophore: naphthalene in C_6H_{12},[29] 7-hydroxycoumarine in PBS,[30] fluorescein in 0.1 N NaOH,[31] 8-(4-iodophenyl)-BODIPYs in benzene[32] and R6G in ethanol.[31] [c] Quantity isolated was too small for a highly accurate measurement.

isolated by semi-preparative RP-HPLC (yield 40–90%) and fully-characterised through various spectroscopic data (see ESI†).

As expected, these novel monosulfonated derivatives were found to be perfectly soluble in water and related aq. buffers in the concentration range (1.0 μM to 1.0 mM) suitable for bio-labelling applications, except the R6G **12** which requires the use of DMSO as a co-solvent to prepare a homogeneous 1.0 mM stock solution (DMSO–H_2O, 2 : 8). The photophysical properties of these novel water-soluble fluorophores **8–12** and **17–20** were then determined under simulated physiological conditions (*i.e.*, phosphate buffered saline (PBS), pH 7.5) by using UV-vis spectroscopy and spectrofluorimetric analysis (Table 1 and see Fig. 1 and ESI† for the corresponding absorption/excitation/emission spectra). These results confirmed that the newly synthesised monosulfonated derivatives behaved in the similar manner as compared to the parent hydrophobic dyes. Indeed, molar absorption coefficients (ε) are comparable, and quantum yields (Φ_F) are good except for alkynated naphthalene **17**. For this latter compound, the dramatic reduction in fluorescence efficiency may be attributed to the presence of an electron-deficient alkyne at the 2-position of the naphthalene ring, as already observed for other fluorescent dyes such as benzothiazole, coumarine and naphthalimide derivatives.[18] As a general rule and in perfect agreement with the fluorescence behavior of "click-on" fluorogenic dyes recently reported in the literature,[16,18] a "click" reaction which adds a conjugated triazole ring onto the parent fluorophore ring leads to a red-shift of absorption/emission maxima and a higher quantum yield (see water-soluble naphthalene and coumarine **8** and **9**, entries 1 and 3 in Table 1). For the other water-soluble fluorophores **10–12** and **19–20**, the grafting of the sulfonated alkyne **1** did not overwhelm the overall electronic structure of their fluorescent scaffolds, and molecular motions of the resulting water-solubilising chain would cause radiationless deactivation and partly account for the decrease of quantum yield in aq. environment. Finally, to confirm the absence of aggregation behavior in aq. solution, the excitation spectrum of each triazole- or alkyne-containing dye was recorded and found to perfectly match with the absorption spectrum in all cases (see Fig. 1 for **8–11** and ESI† for **12** and **17–20**).

Conclusions and future work

In this account, we have described the solid-phase synthesis of an original sulfonated terminal alkyne and its potential utility in the water-solubilisation of a variety of fluorescent dyes through "click" and Sonogashira reactions. The versatility of this synthetic approach has been demonstrated with naphthalene, coumarine, BODIPY, fluorescein and rhodamine dyes. Furthermore, this is the first report focused on the implementation of popular transition-metal catalysed reactions to graft sulfonated moieties onto hydrophobic molecular architectures. This "post-synthetic" sulfonation methodology might also be auspicious to enhance the water solubility of redox active multi-component systems such as "cascatelle" dyes[26] or through-bond energy transfer cassettes,[27] or fullerene-based nanomaterials for biomedical applications.[28] For all these molecular materials carboxylic acid functionalities are not easily available.

Acknowledgements

This work was supported by the Agence Nationale de la Recherche (Programme Blanc 2009, ANR-09-BLAN-0081-01) especially for a PhD grant to Cédrik Massif, (Programme PIRIBio 2009, ANR "CLICKMASSLINK") for a postdoctoral fellowship to Sébastien Dautrey, and La Région Haute-Normandie *via* the CRUNCh program (CPER 2007–2013). We thank Dr. Cyrille Sabot (CNRS researcher, Bioorganic Chemistry Team, Université de Rouen) and Nicolas Maindron (Ph.D., Bioorganic Chemistry Team, Université de Rouen) for achieving NMR analyses on Bruker AVANCE I 400 spectrometer

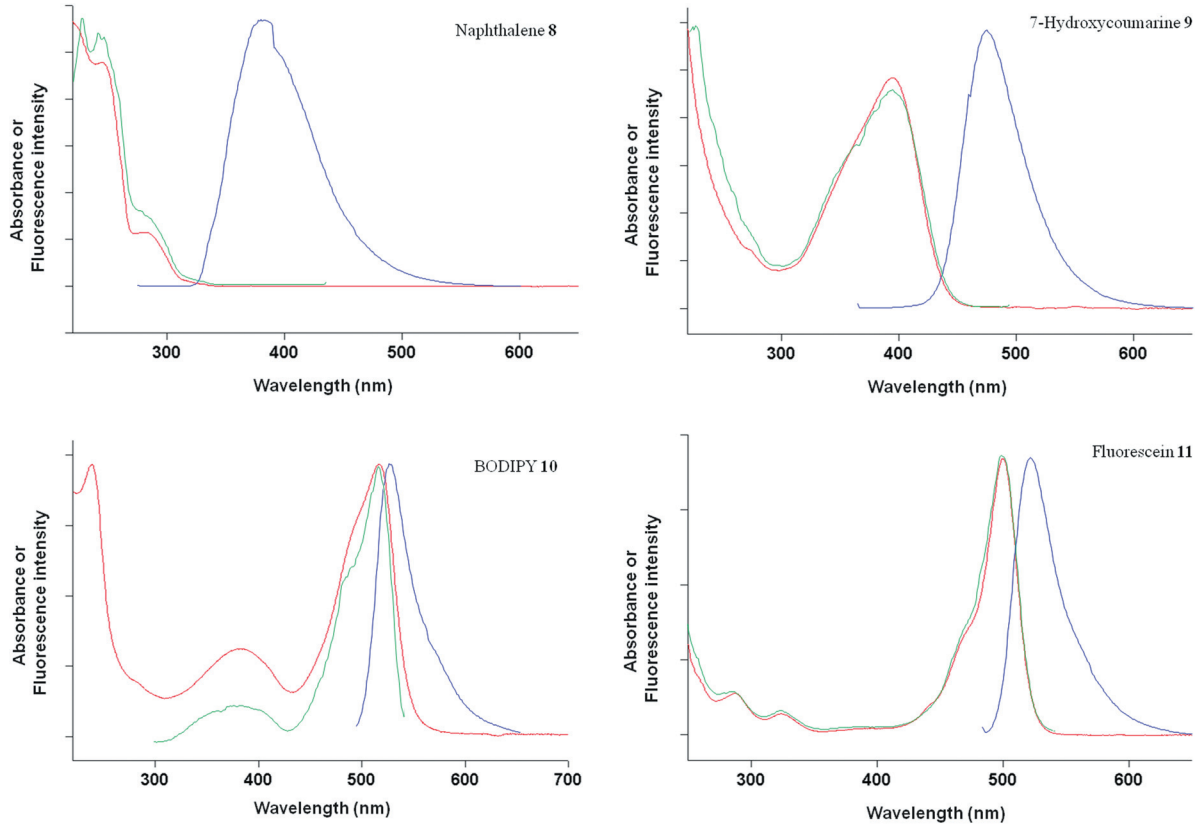


Fig. 1 Absorption (red), excitation (green) and emission (blue) spectra of monosulfonated triazole-based fluorophores **8–11** recorded in PBS at 25 °C.

and for IR measurements respectively. We also warmly thank Dr Gilles Ulrich, Dr Antoinette De Nicola and Sandra Rihn from the LCOSA for helpful discussions.

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