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COMMUNICATION

N-Fmoc-α-sulfo-β-alanine: a versatile building block for the water solubilisation of chromophores and fluorophores by solid-phase strategy†

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An easy and efficient solid-phase synthesis strategy to obtain rapidly water-soluble chromophores/fluorophores in highly pure form has been developed. This first successful use of *N*-Fmoc-α-sulfo-β-alanine as a SPPS building block opens the way to the future development of promising direct "on-resin" peptide labelling and water-solubilising methods.

Introduction

Over the last decade, fluorescence reporter technologies (especially fluorescent bio-probes, polypeptide tags and reporter proteins) have become very powerful and useful tools in life science research, especially in the context of challenging bio-analytical and medical applications.[1] Thus, the covalent labelling of natural biological molecules or related biopolymers with fluorescent organic dyes (*i.e.*, fluorescent bio-labelling) has progressively emerged as a convenient alternative to radioisotope labelling and is now widely adopted to generate molecular probes of biological interest.[2] Among the myriad of commercial and/or published fluorophores, some of them exhibit interesting photophysical properties (yet mainly in organic solvents) but few of them fulfil all the requirements for their routine use as bio-labelling reagents: good water solubility, resistance to aggregation, high fluorescence emission in aq. media and the presence of a functional group readily and selectively derivatisable under mild bio-compatible conditions.

In recent years, some academic groups and private biotech companies have revisited the chemistry of well-known fluorescent cores such as BODIPY,[3,4] coumarin,[5] cyanine,[6] oxazine[7] and xanthene dyes,[8] to synthesise new analogues with these latter valuable properties. In this context, we have recently reported a straightforward method to enhance the water solubility of organic dyes by a post-synthetic chemical derivatisation of their carboxylic acid functionality (or equivalent) with a poly-sulfonated peptide-based linker (*i.e.*, α-sulfo-β-alanine di- or tripeptide) through a Schotten–Baumann reaction performed in aq. or organic media.[9–11] Despite the efficiency of this "solution-phase" synthetic strategy, the laborious and time-consuming handling and purification of highly polar sulfonated intermediates prevents the rapid generation of libraries of bio-conjugatable dyes in high purity. This major drawback could be circumvented by using a more practical approach based on a solid-phase methodology. Indeed, Cardenas-Maestre and Sanchez-Martin have recently reported the synthesis of naphthofluorescein derivatives bearing various bio-conjugatable moieties (*i.e.*, amino, carboxylic acid and sulfhydryl groups) in high yield and purity, through an efficient solid-phase strategy involving the use of 2-chlorotrityl resin.[12] Furthermore, the "catch-and-release" method is now well established as the preferred method for the synthesis of unsymmetrical cyanine dyes in good yields.[13] However, a solid-phase synthesis approach has never been applied to the derivatisation of fluorophores with water-solubilising moieties whereas this latter methodology would be helpful to get rapidly these valuable fluorescent markers in high yield and with a purity which does not necessarily require chromatographic purification.

Herein, we report the implementation of this practical approach for the water solubilisation of inexpensive commercially available chromophores/fluorophores (*i.e.*, anthracene, benzophenone, 7-hydroxycoumarin and naphthalene) and more functionalised "in house" fluorescent organic dyes (*i.e.*, rhodamine 6G (R6G) and non-symmetrical pentamethine cyanine dye analogues)[14] covering a broad spectral range from the UV to the near-infrared (NIR) region. Furthermore, the choice of these polycyclic aromatic compounds has been also guided by their well-known stability under harsh anhydrous acidic conditions (*i.e.*, TFA) currently used for releasing products from standard resins involved in solid-phase peptide synthesis (SPPS). The successful use of *N*-Fmoc protected α-sulfo-β-alanine **1** both in manual and automated SPPS constitutes the cornerstone of this synthetic strategy.[15] The optical properties of the resulting water-soluble fluorophores were then evaluated under physiological conditions to demonstrate their potential utility as bio-labelling reagents.

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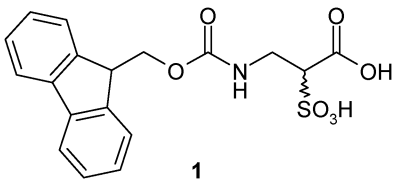
† Electronic supplementary information (ESI) available: Detailed synthetic procedures and characterisation/spectral data for compounds **1–14** and **10-BSA** conjugate. See DOI: 10.1039/c1ob05730h



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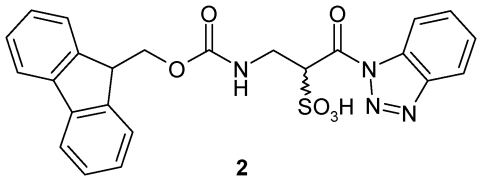
Results and discussion

Since SPPS protocols require the use of protected amino acid building blocks in large excess (generally, a 4- to 10-fold molar excess is used), we first revisited the synthesis of **1** previously reported by us,[9] in order to produce this *N*-Fmoc building block in large scale (10 g scale synthesis) and in a very cheap and simple manner. Thus, we found that the use of 9-fluorenylmethyl chloroformate (Fmoc-Cl) instead of the succinimidyl carbonate derivative (FmocOSu) enables us to get **1** in quantitative yield and high purity (>95%) from the corresponding α-sulfo-β-alanine through simple washing with CH_3CN and subsequent recrystallisation in water and lyophilisation of the crude product. Next, the anchoring of this unusual *N*-Fmoc sulfonated amino acid to the Wang resin was studied (Scheme 1).[16] Surprisingly, use of standard esterification conditions (*i.e.*, DIC/DMAP) allowing the *in situ* formation of the corresponding symmetrical anhydride of **1** completely failed.[17] Three other immobilisation methods, namely: (1) BOP/DIEA (*in situ* formation of HOBt active esters of **1**),[18] (2) Boc_2O/pyridine/DMAP (*in situ* formation of mixed carbonic carboxylic anhydride of **1**)[19] and (3) S_N2 reaction of the Wang chloride resin with **1** in the presence of KI/DIEA,[20] were attempted but failed too except for the S_N2-alkylation which gave the resin-bound product in a very poor and unusable yield (5%). At this stage of the study, we do not have definitive relevant explanation(s) for these surprising negative results because *N*-Fmoc β-amino acids such as β-alanine are known to behave like the corresponding α-amino acids in SPPS.[21] However, we suspect that the active *N*- or *O*-acyl derivatives of **1** (*i.e.*, HOBt esters or anhydrides) are unreactive toward the 4-alkoxybenzyl alcohol moiety of the Wang resin (effect of the free sulfonate group?). Such hypothesis was confirmed by a solution-phase BOP/DIEA-mediated coupling reaction between **1** and 4-methoxybenzyl alcohol, which gave only the benzotriazole ester (Bt) **2** as the main isolated product. Indeed, this latter compound is formally the reduced derivative of HOBt esters and its formation is known to be favoured by the absence of strong enough nucleophiles in the coupling reaction mixture.[22]



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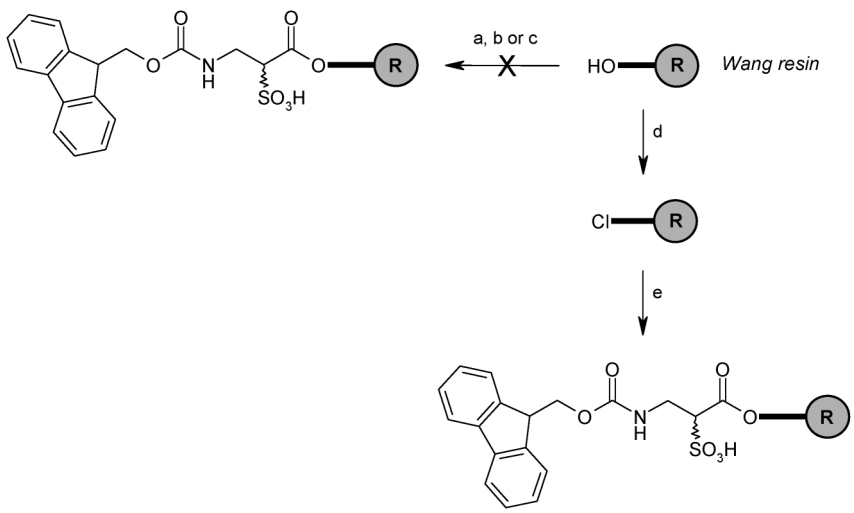
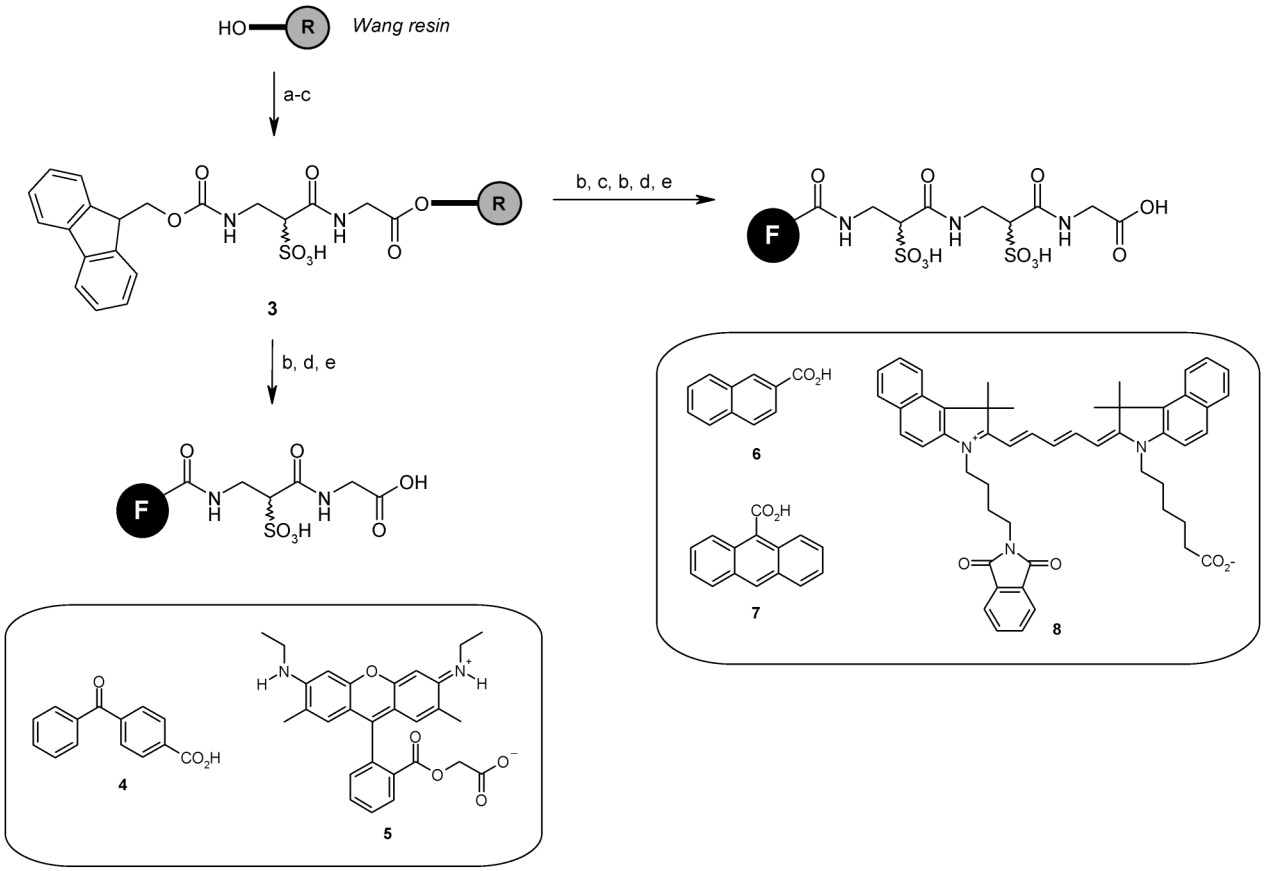
Concerning the disappointing result obtained with the S_N2 approach, this hypothesis (*vide supra*) cannot be invoked because the nucleophilic character of the carboxylate anion of **1** is the key factor influencing the rate of this latter reaction. Thus, the alternative hypothesis that the free sulfonate group could have a deleterious effect on the stability of the ester linkage connecting the sulfonated amino acid to the solid support seems also judicious. To overcome this major synthetic difficulty, we decided to first derivatise the Wang resin with a glycine spacer by using the standard symmetrical anhydride method (*i.e.*, DIC/DMAP), and after removal of the Fmoc group, make an efficient peptide coupling reaction with *N*-Fmoc α-sulfo-β-alanine **1** to give resin **3** (Scheme 2). BOP reagent in the presence of DIEA was found to be effective in this reaction performed either automatically or manually. Following Fmoc removal with 20% piperidine in NMP, benzophenone-4-carboxylic acid **4** and R6G carboxylic acid **5** readily reacted with aminosulfonate-resin through BOP/DIEA activation.[23] The same protocol was used for the solid-phase immobilisation of other fluorophores but to counterbalance their higher hydrophobic character, the resin bearing $(\alpha\text{-sulfo-}\beta\text{-alaninyl})_2$-glycinyl spacer arm was preferred (Scheme 2). It is interesting to note that these coupling reactions, especially those involving sterically hindered dyes such as **5** and **8**, are more efficient



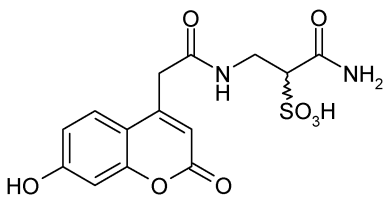
Scheme 1 *Reagents and conditions*: (a) **1** (4 equiv.), DIC (2 equiv.), DMAP (0.15 equiv.), NMP, rt, overnight; (b) **1** (4 equiv.), BOP (4 equiv.), DIEA (12 equiv.), NMP, rt, overnight; (c) **1** (5 equiv.), Boc_2O (5 equiv.), pyridine (10 equiv.), DMAP (0.3 equiv.), DMF, rt, 19 h; (d) $SOCl_2$ (5 equiv.), CH_2Cl_2, 4 °C, 45 min; (e) **1** (3 equiv.), KI (3 equiv.), DIEA (6 equiv.), DMF, rt, 24 h, 5% (overall yield for the steps d–e).



Scheme 2 *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) **1** (4 equiv.), BOP (4 equiv.), DIEA (12 equiv.), NMP–DMF (55 : 45), rt, 30 min; (d) chromophore or fluorophore **4**, **5**, **6**, **7** or **8** (5 equiv.), BOP (5 equiv.), DIEA (20 equiv.), NMP, rt, overnight; (e) TFA–CH_2Cl_2 (1 : 1), 4 °C to rt, 1 h followed by azeotropic evaporation, lyophilisation and RP-HPLC purification.

when the carboxylic acid is converted into the corresponding HOBt esters rather than the conventional *N*-hydroxysuccinimidyl (NHS) ester (*in situ* formation with TSTU/DIEA)[24] After cleavage from the resin using TFA–CH_2Cl_2 (1 : 1) and subsequent azeotropic evaporation and lyophilisation, the desired sulfonated chromophores/fluorophores **9–13** were obtained in satisfactory yields (10–50% isolated overall yield from the underivatised Wang resin and after RP-HPLC purification and lyophilisation) and relatively good purity (70–80%), suitable for applications of these dyes as bio-labelling reagents. However, additional reversed-phase HPLC (RP-HPLC) purifications were carried out to provide these compounds in a pure form (purity >95%) suitable for both detailed characterisation (in particular NMR and mass spectrometry data, see ESI†) and accurate measurement of their optical properties.

In order to expand the scope of this water-solubilising method to fluorogenic phenol based dyes for which the presence of a free carboxylic acid moiety on the fluorophore core is not required for bioconjugation, and with applications in mind based on the pro-fluorescence concept,[25] we have also investigated the derivatisation of Rink Amide MBHA resin and its subsequent use in the sulfonation of 7-hydroxycoumarin-4-acetic acid.[26] Thus, the same peptide coupling conditions used for the immobilisation of fluorophores **4–8** were applied to 7-hydroxycoumarin-4-acetic acid, and TFA cleavage provided the desired water-soluble derivative **14** in good overall yield (41%) and a high purity (see ESI†).



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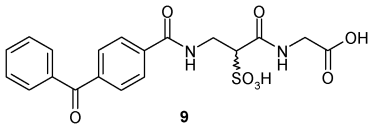
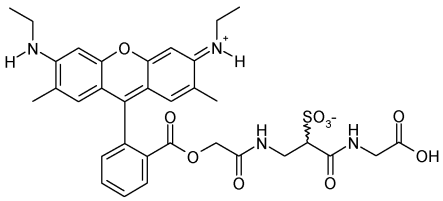
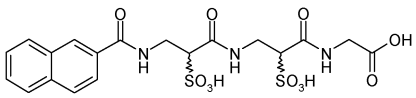
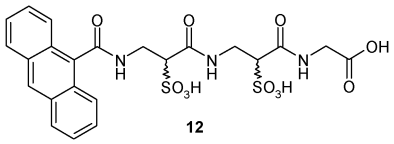
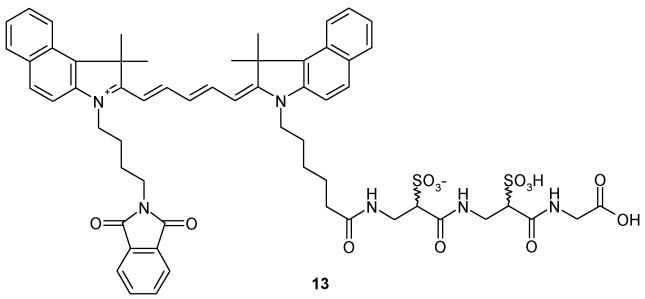
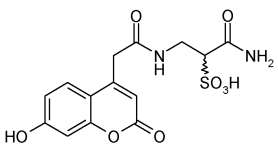
As expected, these novel sulfonated dyes were found to be perfectly soluble in water and related aq. buffers in the concentration range (1.0 μM to 10 mM) suitable for bio-labelling applications, except the *N*-protected Cy 5.5 amino acid **13** which requires the use of CH_3CN as a co-solvent to prepare a homogeneous 1.0 mM stock solution (CH_3CH–H_2O, 2 : 1). All compounds bearing a glycine tether are potentially useful for covalent labelling of biological material through the formation of carboxamide linkages. As an illustrative example, water-soluble R6G **10** was converted into the corresponding NHS ester whose the ability to readily react with NH_2 groups of lysine residues of proteins was demonstrated through the fluorescent labelling of bovine serum albumin (BSA). The attached fluorophore to protein molar (*F*/*P*) ratio and

Table 1 Spectral properties of water-soluble chromophores/fluorophores **9–14** in PBS at 25 °C

Compound	λ_{max}, abs/nm	ε/dm^3 mol^{-1} cm^{-1}	λ_{max}, em/nm	Φ_F[a]
 **9**	263	23 250	—[b]	—[b]
 **10**	528	65 000	554	0.70
 **11**	235.5, 281.5	39 900, 5 500	368	0.23
 **12**	254[c]	124 400	395, 416	0.12
 **13**	632, 680 698[d]	65 860, 89 700 157 500[d]	700 712[d]	0.05 0.21[d]
 **14**	338, 364	7800, 6800	467	0.77

[a] See ESI for the experimental details related to these measurements (standards, λ ex/nm, ...). [b] Non fluorescent. [c] Other maxima were observed at longer wavelengths but their ε are very low (see ESI). [d] In PBS + 5% BSA.

quantum yield of the resulting fluorescent bio-conjugate were found to be 0.5 and 0.24 respectively (see ESI† for the experimental protocol and the corresponding spectra). Furthermore, water-soluble and non-fluorescent chromophores such as benzophenone **9** are highly valuable (but seldom reported in the literature), because these aryl ketones are key photoreactive components of activity-based probes currently used in the identification of enzyme activities in complex proteomes.[27] The photophysical properties of these novel water-soluble chromophore/fluorophores **9–14** 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 ESI† for the corresponding spectra). These results confirm that the newly synthesised sulfonated derivatives behaved similarly to the parent hydrophobic dyes. Indeed, molar absorption coefficients (ε) are comparable, and quantum yields in aq. buffers (Φ_F) are good especially for the water-soluble coumarin and rhodamine **10** and **14**. Due to its amphiphilic character, cyanine **13** shows a tendency to aggregate in aq. solution, which caused a severe quenching of its fluorescence (Table 1, entry 5). The observed blue shift in the absorption maximum at 632 nm is in keeping with the formation of H-dimers. This aggregation is persistent even

at concentrations as low as 1 μM in PBS. The free fluorescent monomer of **13** is obtained in PBS using 5% (w/v) BSA, an additive often used in buffers for mimicking body fluids. The strong enhancement in emission in conjunction with the red shift in the absorption maximum in the presence of BSA observed for **13** points to dye–BSA interactions. Such a beneficial effect of dye–protein interactions on quantum yield has been already reported for series of BODIPY, cyanine and rhodamine labels.[4,11,28]

Conclusions and future work

In conclusion, we have developed an easy and efficient method for the sulfonation of inexpensive commercially available or readily synthetically accessible chromophore and/or fluorescent cores, in order to get rapidly and with high efficacy novel water-soluble and bio-conjugatable optical markers. Thus, the full-compatibility of new commercial *N*-Fmoc α-sulfo-β-alanine building block **1** with standard SPPS protocols was demonstrated for the first time and we found that the use of a protecting group for the sulfonic acid moiety is not required. The exemplification of this promising strategy to more sophisticated and fragile fluorophores by using an hyper acid-labile resin such as 2-chlorotrityl, SASRIN™ or Sieber resin is currently under investigation. Further efforts are also devoted to the synthesis and SPPS applications of enzyme-labile sulfonate esters[29] derived from *N*-Fmoc α-sulfo-β-alanine in order to produce cell-permeable (fluorescently-labelled) peptides of biological interest.

Acknowledgements

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