Catch and release microwave mediated synthesis of cyanine dyes†

Maria Lopalco,[a] Eftychia N. Koini,[b] Jin Ku Cho[a] and Mark Bradley*[a]

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Unsymmetrical functionalised cyanine dyes, covering the whole colour range, were readily synthesised (in 100 mg amounts) by a combination of microwave and solid-phase methodologies.

Fluorescent probes represent a versatile tool for visualising specific molecular targets and events *in vitro* and increasingly *in vivo*.[1–3] Multicolour fluorescence detection has proved to be useful for multiplexed assays on a variety of microarray formats[4,5] and for the simultaneous investigation of multiple biological processes in living cells.[6] As a consequence, an increasingly large number of fluorescent probes, available in different colours, with the ability to be straightforwardly conjugated to biomolecules, are required.[7]

The cyanine dyes (Fig. 1), a class of highly fluorescent compounds, have all the key requirements necessary for highly sensitive multicolour detection, with wavelengths which are tunable, by synthesis, across the visible spectrum. They also display excellent photophysical properties, including high extinction coefficients and quantum yields, while having fluorescence wavelengths remote from the natural autofluorescence of biomolecules.[8–10] They are thus routinely used as fluorescent probes in a wide-range of applications such as DNA sequencing,[11] cellular analysis,[12] flow cytometry[13] and *in vivo* imaging.[14,15]

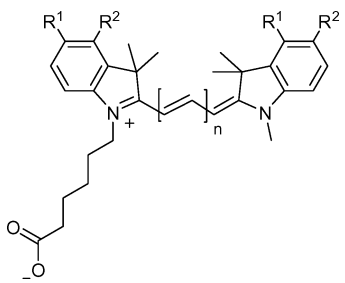


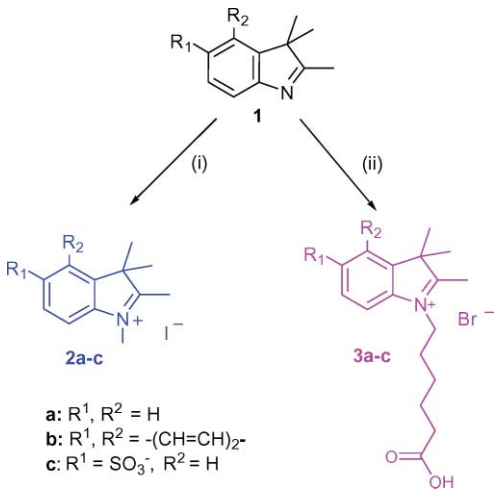
Fig. 1 Structure of cyanine dye. R^1, R^2 = H, -(CH=CH)$_2$-, R^1 = SO_3^-, R^2 = H; n = 1, 2, 3.

Conventional synthetic methods for the preparation of unsymmetrical cyanine dyes are based on the condensation of quaternised indolenine derivatives with vinylogous homologues of diphenyl formamidine and typically affording mixtures of the desired compound along with unreacted hemicyanine and undesired symmetrical analogues.[16]

To avoid time-consuming purifications and allow enhanced access to a broad range of cyanine dyes, solid-phase synthesis methods[17–19] have been reported. In particular, Mason *et al.*[20] reported a highly versatile strategy based on the attack of a heterocyclic carbon nucleophile onto a polyene-chain precursor immobilised onto a solid support to generate unsymmetrical trimethine and pentamethine dyes.

However, there is still a great demand for the development of a facile synthetic method for the preparation of sulfonated and non-sulfonated cyanine dyes, available in different colours (*e.g.* far-red profiles for *in vivo* imaging) and amenable to biomolecule conjugation (*e.g.* with an appendant carboxyl group or equivalent).

Herein, we report a common synthetic pathway for the preparation of a wide colour-range of hydrophobic and hydrophilic cyanine dyes suitable for bioconjugation, using both microwave and solid-phase methodologies, the combination of which offers a practical approach to the rapid and practical preparation of a variety of Cy-fluorophores. The general synthetic procedure (Schemes 2 and 3) shows the parallel synthesis of the range of cyanine dyes with the starting point being the *N*-alkylation of indolenines (Scheme 1).



Scheme 1 Microwave-mediated alkylation of indolenines. (i) methyl iodide, acetonitrile, 150 °C, 30 min to 1 h, microwave; (ii) 6-bromohexanoic acid, acetonitrile, 150 °C, 1 to 3 h, microwave.

The heterocyclic quaternary ammonium salts **2** and **3** are usually obtained by heating the corresponding heteroaromatic base **1** with an excess of alkylating agent in an aprotic solvent[16,21] over several days. In the first simplification of the synthesis it was possible to reduce the reaction time by microwave heating the reaction mixture at 150 °C in acetonitrile (Scheme 1) to give rapid access to a broad range of desired heterocyclic quaternary ammonium salts

[a]*School of Chemistry, The University of Edinburgh, West Mains Road, Edinburgh, United Kingdom EH9 3JJ. E-mail: mark.bradley@ed.ac.uk*
[b]*National Hellenic Research Foundation, 48 Vas. Constantinou Avenue, 11635 Athens, Greece*

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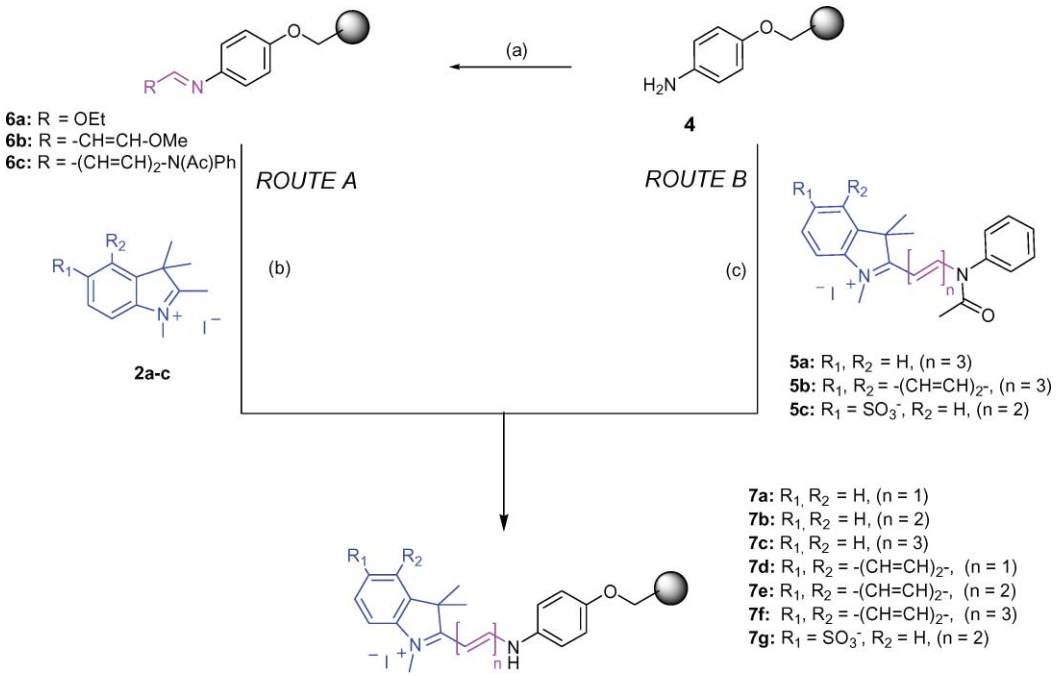
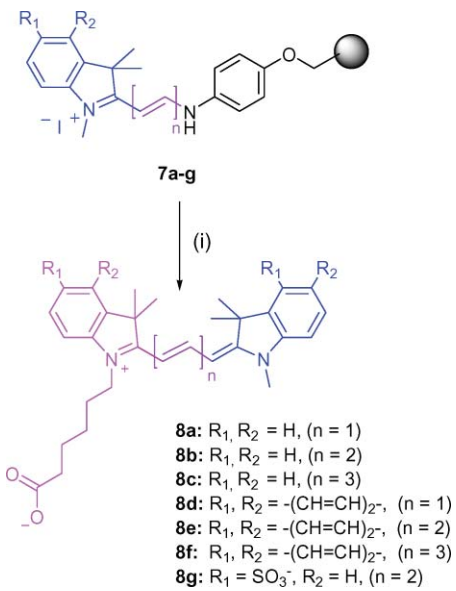


Scheme 2 Solid phase synthesis of the cyanine dyes: (a) triethylorthoformate for **6a**, malonaldehyde *bis* dimethyl-acetal for **6b**, $BF_3{\cdot}Et_2O$, DCM, 6 h; glutaconaldehyde (2-pentenedial) dianilide hydrochloride, Ac_2O, DIEA, and pyridine for **6c**; (b) DMF, 120 °C, 15 min, microwave, for trimethine and pentamethine dyes; Ac_2O, DIEA, and pyridine, 1.5 h; (c) DCM, 1 h.



Scheme 3 Cleavage of the cyanine dyes from the resin. (i) Ac_2O, (DIEA), and pyridine, 2 h.

in good yields (66–95%), including sulfonated (**2c**, **3c**) and/or carboxylated (**3a–c**) variants.

To synthesise cyanine dyes the alkylated indolenines (**2a–c**) were reacted with the resin bound polymethine imines (**6a–c**) (Scheme 2, route A) as originally reported by Mason *et al.*,[20] although again using microwave irradiation (15 mins, 120 °C). Though microwave heating accelerates the attack of a second molecule of the indolenium salt **2** onto the polymer-bound imine **6**, the symmetrical impurity obtained is cleaved from the resin and

Table 1 Properties of dyes synthesised on solid phase

Dye	$\lambda_{max,\,abs}$ (nm)	$\lambda_{max,\,em}$ (nm)[a]	Yield[b]
8a	547	561	68%
8b	640	660	84%
8c	743	765	86%
8d	587	603	49%
8e	678	704	92%
8f	781	808	51%
8g	647	667	94%

[a] Measured in MeOH (except for compound **8g** which was dissolved in H_2O). [b] Yield of isolated product in the final step (based on the amount of heterocycle used in the cleavage step).

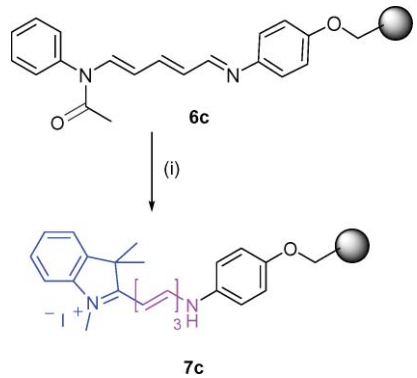
it was observed that microwave heating in this step gave rise to higher yields and purities of the final compound. Cleavage from the solid support was then accomplished using the carboxylated indolium salt (**3a–c**) to give functionalised cyanine dyes suitable for conjugation with biomolecules (Scheme 3). The unsymmetrical trimethine and pentamethine cyanine dyes were obtained in good yield (Table 1) and purity (85–100% by ELSD detection) and in preparative scale (up to 100 mg).

Further studies were carried out to extend this protocol to the synthesis of heptamethine dyes, as they have attracted great interest as fluorescent probes for *in vivo* imaging.[15,22,23]

In an initial approach the monofunctionalised heptamethine dye **8c** was obtained following the route A. Glutaconaldehyde dianilide hydrochloride was reacted with the supported aniline **4**, in the presence of Ac_2O, to form the polymer-bound polymethine **6c**.

The reaction of the indolenium salt **2a** with **6c** produced the desired supported hemicyanine **7c** *via* loss of *N*-phenylacetamide,

but resulted also in the release of the hemicyanine in solution by cleavage from the resin, with a consequent decrease in the yield of the hemicyanine **7c** and of the final compound **8c** (12%) (Scheme 4).



Scheme 4 Formation of hemicyanine **7c**. (i) **2a**, Ac_2O, DIEA, pyridine, 1.5 h.

This led to the development of an alternative catch-and-release-strategy (Scheme 2, route B) based on the formation of the hemicyanine intermediates **5a,b** in solution and the captive loading onto the solid support (**4**) to form **7c,f**. The loading of the hemicyanine **5a** onto the resin in this manner was carried out in DCM for 1 h, with an 86% yield as determined by nitrogen elemental analysis. The desired dyes were then cleaved from the resin by nucleophilic attack of **3a,b** onto the intermediates **7c,f** in pyridine:Ac_2O (10:1) to afford the unsymmetrical heptamethine dyes **8c** and **8f** in good yield (Table 1) and purity (>90%), as determined by HPLC analysis and ^{1}H NMR (Fig. 2).

The catch-and-release protocol described above has proven to be effective also for the synthesis of sulfoindocyanine dyes. The presence of sulfonate groups on the ring systems improves water solubility, prevents aggregation in water and reduces non-specific binding to biomolecules and cellular constituents.[8] However, purification by chromatography of these dyes is difficult.

To overcome this limitation, Jiang *et al.*[19] recently suggested the use of poly(ethylene glycol) as a soluble support, which has its own handling problems and low loading. In addition, Mason *et al.*[20] described the synthesis of sulfonated cyanine dyes on polystyrene resin; however they reported that the sulfonated heterocycles did not react during the formation of the hemicyanine intermediate on solid phase but they did react in the dye formation step allowing the synthesis of mono-sulfonated cyanine dyes.

Microwave heating enabled the solid-phase synthesis of the pentamethine sulfoindocyanine dye **8g** from polystyrene resin (route A), although in low yield (10%) and purity.

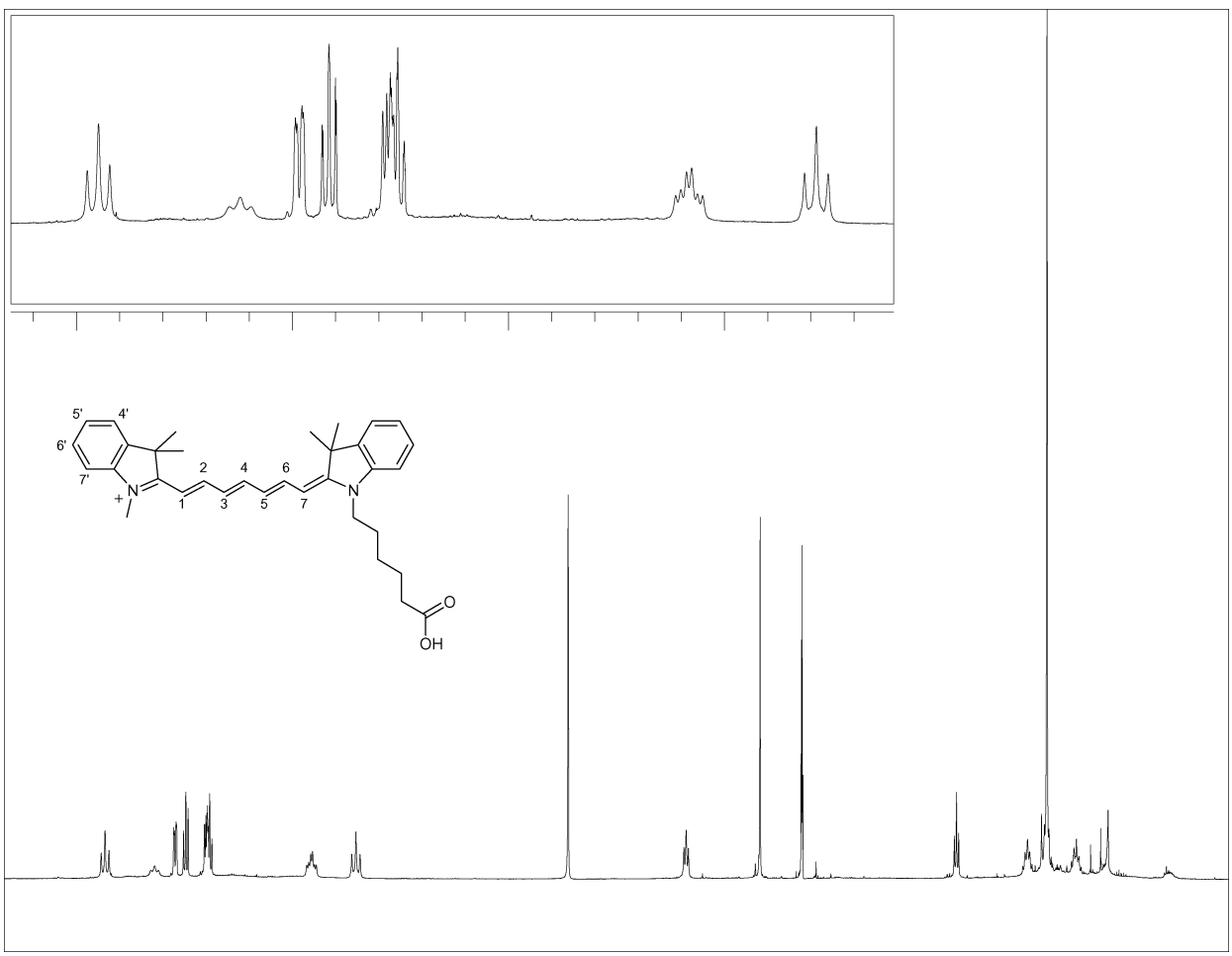


Fig. 2 ^{1}H NMR of the crude heptamethine dye **8c**.

The catch-and-release method (route B) proved to be far more effective, resulting in the formation of **8g** in excellent yield and purity (94%), without the need for further purification by chromatography (Fig. 3).

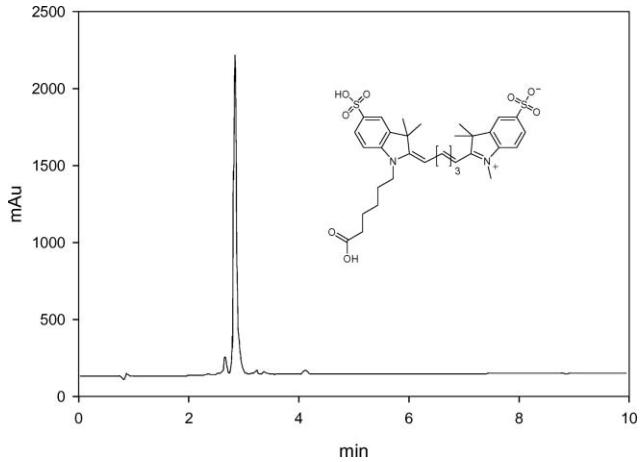


Fig. 3 HPLC chromatogram of the crude sulfonated dye **8g**.

In conclusion, two practical approaches to the solid phase synthesis of unsymmetrical cyanine dyes suitable for bioconjugation have been reported. The method proposed is straightforward and enables, in a few steps, the synthesis of cyanine dyes spanning the whole colour range. Through a catch-and-release strategy we have also been able to synthesise, on solid phase, heptamethine and water soluble cyanine dyes in high yield and purity without the need for chromatographic purification‡, thus providing an easy route to the preparation of these invaluable fluorescent probes in amounts sufficient to satisfy the most hungry of biological appetites.

Acknowledgements

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Notes and references

‡ Purification by column chromatography of the dyes obtained from the supported imidates was carried out to provide their detailed characterisation but may not be necessary for application of the dyes as labeling reagents.

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