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Solid phase combinatorial synthesis of a xanthone library using click chemistry and its application to an embryonic stem cell probe†

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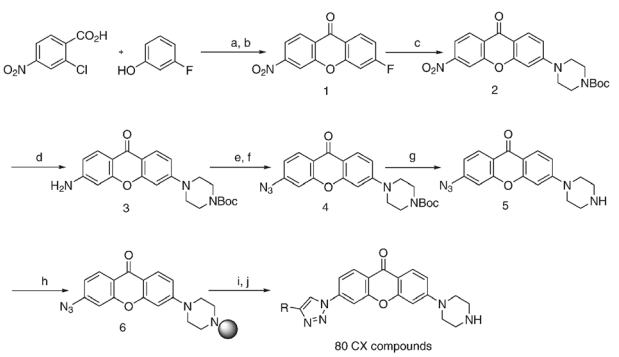
We report the first solid phase synthesis of a xanthone library CX and its application to embryonic stem cell probe development. The CX library was further derivatised with an activated ester resin to provide an acetylated CX (CXAC) library. Screening of these libraries led to the discovery of a novel fluorescent mESC probe, CDb8.

Fluorescent probe molecules have been widely used in bioimaging and medicinal applications for several decades due to their high sensitivity and easy visibility. As an alternative to a traditional knowledge-based Target Oriented Approach (TOA),[1] recently several research groups including our group introduced a Diversity Oriented Fluorescence Library Approach (DOFLA) for rapid discovery of novel fluorescent probes and sensors. Several DOFL scaffolds including coumarin,[2a,b] dapoxyl,[2c] styryl,[2d] hemicyanine,[2e] rosamine,[3a] and BODIPY[3b] have been developed and their potential application to imaging probe development demonstrated. DOFLA has a unique advantage over TOA especially when information about the target analyte is not available. Recently, we reported the discovery of the first embryonic stem cell (ESC) probe **CDy1** (Compound of Designation yellow 1, $\lambda_{ex}/\lambda_{em}$ = 535/570 nm)[4] through DOFLA, incorporating a high-throughput screening of the rosamine library in ESC and mouse embryonic fibroblast (MEF). While powerful, the yellow region of the fluorescence by **CDy1** has a significant color overlapping problem when multi-color staining was tried together with standard YFP (yellow fluorescent protein). To overcome this color limitation, we expanded our library to blue/green or red/near IR light emitting scaffolds. The xanthone scaffold was chosen for the blue library due to its superior photophysical properties such as high photostability, large stock shift (120 nm) and moderate quantum yield. The structural similarity of the xanthone to rosamine library, which **CDy1** belongs to, was also considered for the library design. Herein, we report the first solid phase synthesis of a click xanthone library (CX), and its acetyl derivatives, a CXAC library and the identification of a novel blue fluorescent compound, **CDb8**, which selectively stains mESC compared to MEF and other differentiated cells.

Compared to the relatively well studied fluorescent scaffolds, such as BODIPY, rosamine, coumarin, cyanine and styryl,[5] the xanthone scaffold is still in its infancy for library synthesis and sensor application. Copper catalysed Huisgen's 1,3-dipolar cycloaddition reaction between an azide and a terminal alkyne has become one of the most popular click reactions because of its superior regioselectivity, simple reaction conditions, biocompatibility, and excellent yield.[6] The simplicity of this reaction has made it an attractive choice for construction of various chemical libraries. Although a simple synthesis of a xanthone library has been reported using solution phase click chemistry,[7] the biological application of the library compounds may require careful purification of the product from the potentially toxic copper catalyst or other impurities. In order to overcome these problems, we adopted a solid phase chemistry for the combinatorial diversification of the xanthone core.



Scheme 1 Synthesis of CX derivatives. *Reagents and conditions*: (a) K_2CO_3, Cu, DMF, 130 °C, 12 h; (b) H_2SO_4, 90 °C, 1 h; (c) 1-Boc piperazine, DMSO, 90 °C, 12 h; (d) NH_2–NH_2·H_2O, Pd/C, EtOH, 90 °C, 4 h; (e) H_2O/AcOH (1 : 1), $NaNO_2$, 0 °C, 1 h; (f) NaN_3, 0 °C to r.t., 1 h; (g) 10% TFA in DCM, r.t., 2 h; (h) DMF/DCM, DIEA, Trityl-Cl, r.t., 12 h; (i) DMF/piperidine (4 : 1), CuI, ascorbic acid, RCCH, r.t., 16 h; (j) 2% TFA in DCM, r.t., 10 min.

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The general synthetic strategy of xanthone azide (Scheme 1) involves the reaction between 2-chloro-4-nitro benzoic acid and 3-fluorophenol followed by a cyclization in the presence of concentrated sulfuric acid to give 3-fluoro-6-nitro xanthone, **1**. The 3-fluoro group was then replaced by tertiary-butoxycarbonyl (Boc) protected piperazine and the nitro group was reduced to aniline with hydrazine and Pd/C in ethanol to provide intermediate **3**. Treatment of **3** with sodium nitrite in water–acetic acid (1 : 1) at 0 °C followed by reaction with sodium azide afforded 6-azido-3-piperazine substituted xanthone, **4**. The reaction proceeded quantitatively, and subsequent deprotection of the Boc group with trifluoroacetic acid afforded the desired xanthone azide, **5**. The xanthone azide showed no fluorescence, maybe due to the quenching effect of electron rich α-nitrogen. Once the click chemistry is performed on the xanthone azide, the fluorescence was recovered. Thus, the intermediate **5** was loaded onto the solid support and a broad chemical diversity was introduced by 90 aliphatic and aromatic terminal alkynes (Chart 1) in the presence of Cu(I). The formation of the heterocyclic triazole moiety following Huisgen's 1,3-dipolar cycloaddition was highly efficient and for most of the cases, the reaction was completed within 12 hours at room temperature. After acidic cleavage, the entire library compounds were characterized by HPLC-MS without further purification and 80 relatively pure compounds (CX library, average purity is 93%, measured at 254 nm (Table S1, ESI†)) were collected for further study. Most of the library compounds showed a blue range of fluorescence as

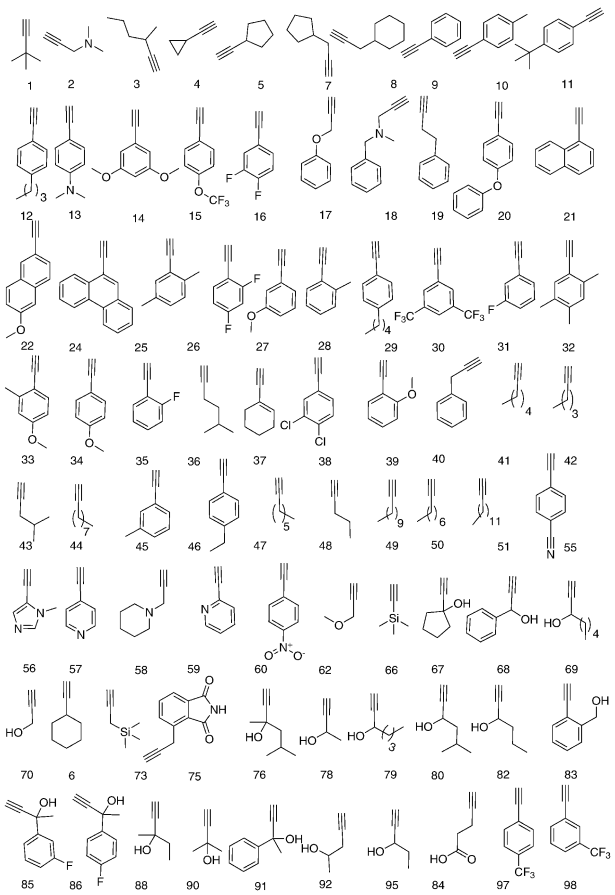
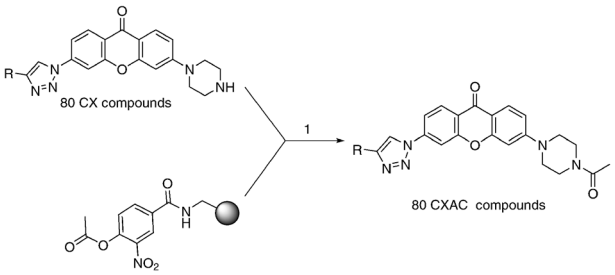


Chart 1 Alkyne building blocks for a CX library.



Scheme 2 Synthesis of CXAC derivatives. *Reagents and conditions*: **1**·DCM/ACN (7 : 1), $NaHCO_3$, r.t., 2 h.

expected (excitation ranges from 360 to 370 nm and emission ranges from 480 to 495 nm). The quantum yield of each molecule varied from 0.02 to 0.16 reflecting diverse structural and electronic characteristics.

The piperazine tag of the CX compounds can be easily used for further diversification. To fully enjoy this possibility, we synthesized an acetyl version of CX (CXAC) compounds using solid phase activated ester chemistry. Firstly, active ester resins were prepared by treating the nitrophenol resin with acetyl chloride. The reaction of the resulting resin with the highly reactive piperazine moiety of CX compounds in the presence of mild base $NaHCO_3$ afforded the corresponding CXAC compounds (Scheme 2). The reaction completed within 2 hours and the products were obtained after a simple filtration with an average purity of above 90% without further purification. This simple but powerful chemistry can be applied to a broad range of acyl group derivatization leaving the potential of further diversification of CX.[8] The resulting CXAC compounds have an average excitation at 365 nm and emission at around 495 nm (Table S2, ESI†).

For the discovery of a stem cell selective blue probe, the 160 compounds of the CX and CXAC libraries were screened against mESC, MEF and co-culture of the two cells using high throughput cell imaging screening. For the primary screening, the dye (1 μM) was incubated with cells and many of the

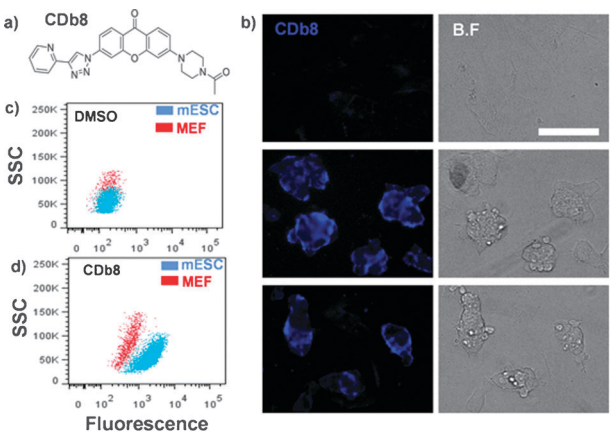


Fig. 1 Selective staining of mESC by **CDb8**. (a) Chemical structure of **CDb8**; (b) mESC was selectively stained by **CDb8** at 1 μM for 1 h. Upper panel: mouse embryonic fibroblasts (MEF), middle: mouse embryonic stem cells (mESC), lower panel: mESC on MEF feeder. (c) Flow cytometry analysis of DMSO control cells. (d) Flow cytometry analysis of **CDb8** stained cells. The cells are loaded after 1 h incubation at 1 μM. B.F: bright field, scale bar: 100 μm.

compounds smoothly stained cells to different extent. The compounds were minimally toxic to the cells over several days of incubation (Fig. S1, ESI†), demonstrating the suitability of the library compounds for biological application, free from the toxic copper catalyst. Dyes staining mESC stronger than MEF were selected as primary hits (Fig. 1b) and further tested by flow cytometry to confirm the selective staining of mESC. CXAC-59 was selected as the best dye out of total 160 compounds in terms of selective staining of mESC and separation of the two cell populations from flow cytometry (Fig. 1d). As the first blue fluorescent compound which selectively stains mESC, we dubbed the compound **CDb8** (Compound of Designation blue 8) (Fig. 1a). We also investigated the selectivity of **CDb8** in differentiated mESC and observed that the differentiated cells were not stained by **CDb8** (Fig. 1b; Fig. S2, ESI†).

In conclusion, we have successfully synthesized the first solid phase combinatorial xanthone library (CX) using click chemistry. The secondary derivatization of CX afforded a further library, CXAC with high yield and purity, demonstrating the robust acylation platform for new library generation. These xanthone library compounds have demonstrated their biocompatibility in terms of easy cell penetration and low toxicity. One mESC selective compound **CDb8** was identified as a blue color imaging and flow cytometry probe for embryonic stem cells. Detailed biological applications of **CDb8** will be reported in due course.

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