A general synthetic route to isomerically pure functionalized rhodamine dyes
Rhodamines are a family of fluorescent dyes based on a xanthene core scaffold (figure 1). They have broad reaching applications in imaging and bioanalysis due to their photostability, high absorption coefficients and excellent fluorescence quantum yields [1]. Tetramethylrhodamine (TMR) has been used extensively in protein, oligonucleotide labelling, and DNA sequencing, amongst other areas [2–4].

Despite the widespread utility of TMR and related functionalized rhodamines in biochemical processes, the preparation and isolation of these dyes remains a significant synthetic challenge. As yet, a general isomer-selective synthesis has not been described. The standard synthetic route currently employed produces two isomers that are known to display different properties. These include differing electrophoretic mobility, reactivity and even cell toxicity in some cases [5–7].

TMR and other commercially available rhodamines are generally prepared via two sequential Friedel Crafts type reactions of a phthalic anhydride with two equivalents of a meta-aminophenol, which forms the xanthene core [8–10]. However, when applied to the synthesis of functionalized rhodamines—which contain a functional handle required for labelling of chemical and biological entities—this route leads to a mixture of isomeric rhodamines (scheme 1).

The functional handle is introduced at the beginning of the synthesis via use of a functionalized anhydride starting material (for carboxytetramethylrhodamine, carboxyphthalic anhydride, 3 is used). However, as both carbonyls of the anhydride can participate in the Friedel Crafts reaction, this generates regioisomeric intermediates 5, and eventually two isomers of the desired rhodamine dye 6, which are difficult to separate.

Numerous methods for isomer separation have been reported [11–14], including fractional crystallisation and column chromatography, both of which present individual difficulties. As a result, a general and simple method by which commonly used single isomer functionalized rhodamines can be produced remains a significant but important challenge to synthetic chemists.

Recently, Lavis et al reported an elegant synthetic strategy where a variety of single isomer rhodamines bearing a 5-carboxylic acid linker were synthesized via conversion of isomerically pure fluoresceins into rhodamines using palladium mediated C-N cross coupling (scheme 2) [15].

Whilst useful, this method does not allow the generation of X-rhodamine and Q-rhodamine, where the xanthene anilines are substituted with fused cyclic rings. The use of palladium cross-coupling could also
preclude the incorporation of a halogen functionality onto the pendent aromatic ring. The elevated temperatures employed in this reaction also render the use of volatile amines such as dimethylamine (used to generate tetramethylrhodamine) impractical on a large scale.

Herein, we report a simple and robust synthetic route to commonly used single isomer functionalized rhodamines bearing a range of groups on the pendent ring, allowing for conjugation to other entities by a range of chemical methods.

Our investigation began by assessing alternative starting materials, key to preventing isomer generation during rhodamine synthesis. Replacement of the commonly used anhydride with a compound which bears a single point for aminophenols to react was targeted. In the same manner that aminophenols can be condensed with benzaldehydes to give rosamines (scheme 3(a)) [16, 17], and sulfoxanthene dyes can be obtained from formylbenzene-1,3-disulfonic acid [18], it was envisaged that functionalized phthalaldehydic acids (or 2-formyl benzoic acids) could be potential replacements for anhydrides in the synthesis of rhodamine dyes (scheme 3(b)). The use of phthalaldehydic acids ensures the generation of only one isomer, while keeping the ortho-carboxylic acid present in rhodamines which is absent in rosamines.

Phthalaldehydic acids can be generated from phthalides which are commercially available with a range of pre-installed functional groups in the 5- and 6-position. This conversion is well documented in the literature [19, 20] and involves two simple chemical manipulations which allow access to the desired acids; bromination of the 3-position of the phthalide, followed by subjection of the brominated species to hot water, which gives the desired aldehyde and ortho-carboxylic acid functionality.

Phthalides bearing a variety of useful functional groups in the 5- and 6-position were transformed into the corresponding phthalaldehydic acids (table 1). The process was amenable to all substituents tested, with bromination of the phthalides proceeding in 61–97% yield. This was achieved by heating in the presence of N-bromosuccinimide (NBS) and a radical initiator 1,1′-azobis(cyclohexanecarbonitrile) (ACHN) under UV irradiation. Subsequent opening of the lactone ring by heating at 90 °C in H2O proceeded smoothly within a few hours in near quantitative yield (85–98%) for all reactions.
Next, the phthalaldehydic acids were reacted with 3 equivalents of 3-dimethylaminophenol to generate the corresponding tetramethylrhodamines (table 2). Solubility of the reagents proved to be an issue in solvents such as methanesulfonic acid, 60% hydrochloric acid and 60% sulfuric acid, however propionic acid proved suitable and was therefore utilized for this reaction. The reagents were heated in propionic acid with catalytic p-toluene sulfonic acid to form diphenol intermediate. The intermediate was oxidized in situ using chloranil to yield the desired functionalized TMRs as single isomers, which could be easily isolated using silica gel chromatography.

The consistent yields observed for table 2, entries 1–3 (50–54%) suggest that changes in linkage groups at the 6-position on the pendent ring are well tolerated in this reaction. The scope of the reaction also extends to include substituents at the 5-position of TMR, demonstrated by the isolation of 5-NO2-TMR in 58% yield (entry 4, table 2).

Carboxylic acid functionalized tetramethylrhodamine (TAMRA) is commonly used for tethering biological or chemical entities via amide bond formation, and so the production of isomerically pure derivatives of this was pursued next. Both 6-CN-TMR and 6-CO2Me-TMR were assessed to ascertain the best method for generation of 6-CO2H-TMR (or 6-TAMRA).

Hydrolysis of the nitrile group of 6-CN-TMR by refluxing in 85% H2SO4 proved to be slow (incomplete after 36 h) and side products began to form, causing concern for the integrity of the dye under harsh conditions. Conversely, hydrolysis of the methyl ester group of 6-CO2Me-TMR proceeded to completion cleanly.
within one hour at room temperature using sodium hydroxide (scheme 4).

Acidification of the reaction mixture caused 6-TAMRA 21 to precipitate out of the aqueous solution, allowing it to be filtered and isolated in excellent yield (89%). The synthesis of 6-TAMRA 21 was proven to be amenable to gram scale amounts, with yields comparable to that of the smaller scale on which development reactions were typically carried out (see SI for further details).

The scope of the process was further investigated by assessing other aminophenols in order to access additional rhodamine family members (scheme 5). 3-Diethylaminophenol 23 was employed under the conditions shown above with phthalaldehydic acid 22. This method successfully produced rhodamine-B 24 in 70% yield. 8-Hydroxyjulolidine 25 was also evaluated in the system, giving X-rhodamine analogue 26 in 47% yield.

Azide functionalized TAMRA is a highly useful reagent for labeling alkyne-containing biological molecules in an orthogonal fashion through the well-known copper-catalyzed azide—alkyne cycloaddition (CuAAC), click chemistry [21–26]. This fluorescent probe can be prepared through functionalization of 6-TAMRA 21 with 3-azidopropylamine 27 [27], via amide coupling to give 6-N3-TMR 28.

To ensure optimal conversion of 6-TAMRA 21 to azide bearing rhodamine 28, a systematic survey of coupling reagents was undertaken (table 3). Reactions were carried out in the presence of 2 equivalents of DIPEA and 5 equivalents of 3-azidopropylamine 27. Reactions were monitored at 1, 2, 4, 8 and 24 h intervals via HPLC. HATU was determined to give the highest conversion (entry 1).

When carrying out the amide coupling with HATU, 6-N3-TMR 28 was isolated in 40% yield when purified by semi-preparative HPLC.

In conclusion, a robust and scalable synthesis for the generation of highly valuable single isomer functionalized rhodamines has been developed. This approach allows great flexibility in rhodamine synthesis and shows broad scope, enabling access to a range of functionalized rhodamines from phthalides without the need for arduous isomer separation. It is expected that this work will allow easy generation of novel single isomer rhodamine compounds, useful in exciting new areas of fluorescence technology, e.g. the generation of improved dyes for super resolution microscopy [28, 29].
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Associated content

Supporting information
Synthetic procedures and characterization of compounds. This material is available free of charge via the Internet at stacks.iop.org/MAF/3/045002/mmedia.

Notes
The authors declare no competing interests exist.

Dedication
This article is submitted in memory of Dr Angus Brown.

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