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Coloration Technology

SHORT COMMUNICATION

Towards structurally new cyanine dyes—investigating the photophysical and potential antifungal properties of linear substituted heptamethine dyes

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Abstract

The synthesis of a range of new linear substituted heptamethine dyes has been designed and described. The photophysical properties of all the dyes were investigated, with many exhibiting improved fluorescent quantum yields when compared with indocyanine green. Finally, growth inhibition studies were performed in the fission yeast *Saccharomyces pombe*, which suggests potential antifungals activity in the μM range.

1 | INTRODUCTION

Near infrared (NIR) imaging of tissues to visualise and investigate *in vivo* molecular targets using fluorophores with absorption and fluorescence spectra within the 700–900 nm NIR range has several advantages over fluorophores that have spectra in the visible region. These include lower absorption by tissue chromophores, including haemoglobin, less scattering within the tissue and lower levels of tissue autofluorescence with lower toxicity and phototoxicity, allowing imaging in tissues to over 1 cm depth.[1,2] The heptamethine cyanine dye, indocyanine green (ICG) is a good example of such a dye. ICG has been approved by the FDA (Food and Drug Administration) for the visualisation of lymph nodes of patients with breast cancer and melanoma.[3] Although ICG exhibits a NIR absorption/emission profile and has low toxicity, its clinical application is limited due to several failings, one such being low fluorescence quantum yield.[4] ICG and IR820 shown in Figure 1, are notable in having absorption and emission spectra considerably further to the NIR region than most other potential fluorophores such as fluorescein and BODIPY based probes.[5] With this in mind, there is a need to develop new NIR fluorophores with improved photophysical performance compared to ICG for medical applications. In recent years, our group have been investigating synthetic procedures which would allow us to access a range of linear substituted heptamethine dyes with improved photophysical properties based on the Zincke reaction.[6] Herein, we report a flexible synthesis of these types of dyes using an *in situ* cascade reaction strategy as outlined in Scheme 1 along

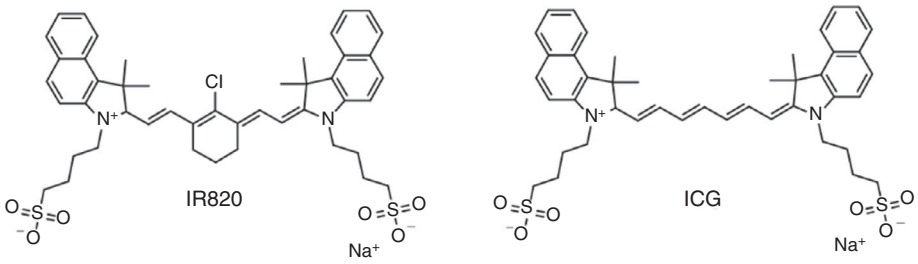
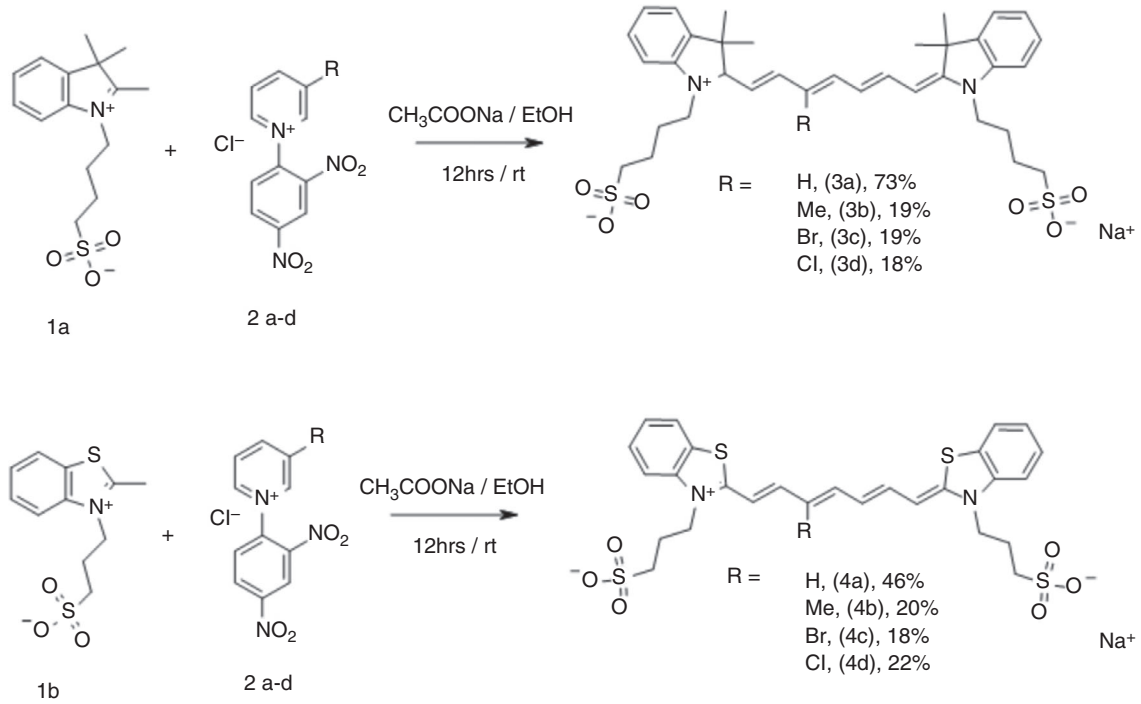


FIGURE 1 The structure of IR820 and ICG.



SCHEME 1 Synthetic route to the target dyes **3a**–**3d** and **4a**–**4d**.

with the photophysical properties and the growth inhibition studies with the compounds produced. The impact of this research is timely, highlighting the synthetic development of cost-effective molecular probes with enhanced photophysical and toxicity characteristics when compared against the current clinical standard ICG and IR820.

2 | RESULTS AND DISCUSSION

The synthesis of both sets of functionalised linear heptamethine cyanine dyes (**3a–3d** and **4a–4d**) was straightforward and required no harsh conditions as shown in Scheme 1. *N*-Alkylation of 2,3,3-trimethylindolenine with 1,4-butanesultone (**1a**) and 2-methylbenzothiazole with 1,3-propanesultone (**1b**) was accomplished in high yields. The change in one carbon addition on the *N*-alkyl chain for the 2,3,3-trimethylindolenine highlights the flexibility of the system. The synthesis of the substituted *N*-(2,4-dinitrophenyl)-pyridinium chloride (Zincke salts), **2a–2d**, was accomplished in using 1-chloro-2,4-dinitrobenzene and the relevant meta-substituted pyridines in refluxing acetone. All Zincke salts precipitated directly from the reaction mixture, without need of purification and were isolated at the pump in high yields. Both sets of linear cyanine dyes (**3a–3d** and **4a–4d**) were produced through an *in situ* cascade reaction in one pot. This was accomplished via the Zincke ring opening of the substituted *N*-(2,4-dinitrophenyl)-pyridinium chloride with the N-alkylated substituted indolene salts under basic conditions.[7] The whole reaction taking place at room temperature over a period of 12 h, with a strong green colour becoming prevalent from the start of the reaction. The crude dyes were all purified by column chromatography using silica gel to obtain the pure compounds. The yields of the substituted dyes (**3b–3d** and **4b–4d**) are shown in Table 1 and are all comparable (19%–22%). Aniline is usually required to facilitate the ring opening of Zincke salts.[8] However, aniline was not employed in this instance to emphasise the reactivity of the substituted

TABLE 1 Photophysical data for dyes within methanol solution.

Compounds					Fluorescence studies				Growth inhibition studies	
Code	X	Y	Z	Yield (%)	Absorption (nm)	Emission (nm)[a]	Stokes shift (nm)	Fluorescence quantum yield[b]	Minimum growth inhibition (μM)	Log *P*
3a	$(CH_3)_2$	$CH_2(CH_2)_3SO_3^-$	H	73	747	775	28	0.13	15.3	−1.995
3b	$(CH_3)_2$	$CH_2(CH_2)_3SO_3^-$	CH_3	19	784	814	30	0.07	9.4	0.361
3c	$(CH_3)_2$	$CH_2(CH_2)_3SO_3^-$	Br	19	743	769	26	0.09	8.5	0.279
3d	$(CH_3)_2$	$CH_2(CH_2)_3SO_3^-$	Cl	18	747	774	27	0.12	9.1	0.148
4a	S	$CH_2(CH_2)_2SO_3^-$	H	46	762	789	27	0.15	15.9	−3.542
4b	S	$CH_2(CH_2)_2SO_3^-$	CH_3	20	761	784	23	0.15	10.1	−1.185
4c	S	$CH_2(CH_2)_2SO_3^-$	Br	18	753	779	26	0.13	9.2	−1.267
4d	S	$CH_2(CH_2)_2SO_3^-$	Cl	22	758	783	25	0.16	9.8	−1.398
5a	Figure 2			46	782	802	20	0.085	17.2	−1.780
5b	Figure 2			15	798	815	17	0.066	18.4	−2.422
ICG[c]	$(CH_3)_2$	$CH_2(CH_2)_3SO_3^-$	H	N/A	785	814	29	0.072	16.6	1.591

[a]Excitation at 785 nm.

[b]Quantum yields ± 10% measured as described in Okoh et al,[7] λ_{max} ± 1 nm. Indocyanine green (ICG) has the additional benzo unit on the core. Minimum inhibitory concentration (MIC) of synthesised compounds tested in *Saccharomyces pombe*. Cells were inoculated at a concentration of 3×10^4/mL. Culture media tested were in yeast extract (YE) broth for *S. pombe*. Growth of yeast was determined visually after 24 h incubation at 30°C. The MIC of the compounds were determined to be well before yeast growth was first seen. The experiment was repeated twice for reproducibility.

[c]Yield of ICG not applicable (N/A) as this was purchased for Merck.

Zincke salts in this article. It should be mentioned that the greater yields seen for **3a**, **4a**, **5a**, and **5b** were attributed to the use of aniline to ring open the unsubstituted Zincke salts. Furthermore, the use of aniline to ring open **2b–2d**, also lead to purification issues requiring further column separations for substituted dyes (**3b–3d** and **4b–4d**). For all dyes, proton nuclear magnetic resonance (^{1}H NMR) confirmed the structure with high-resolution mass spectrometry (HRMS) confirming the molecular weight. Full information on synthetic proceedures and analysis of all dyes within this paper can be found within the supporting information.

The photophysical properties of both the substituted linear hepthamethine dyes used in this study are summarised in Table 1, with all stock solutions being prepared in methanol. The fluorescence quantum yield of 0.13 for ICG in dimethyl sulphoxide (DMSO) solution was used as the quantum yield standard. The absorbance and fluorescence spectra of each of the dyes were measured sequentially to reduce photobleaching and solubility issues. The fluorescence quantum yields of the dyes were calculated using the relative method, that is from plots for standard and cyanine dyes of their individual integrated fluorescence peak areas versus fraction of light absorbed at the excitation wavelength as described in Okoh et al.[9] In this study, emphasis was focused on the effects of structural diversity around the heptamethine backbone of the synthesised NIR heptamethine cyanine dyes on their photophysical properties.

All dyes (**3a–3d** and **4a–4d**) exhibited absorption spectra maxima in the NIR region between 743 and 762 nm, which is in line with other non-substituted heptamethine dyes. The fused benzyl rings on ICG leads to an increased bathochromic shift by approximately 20–40 nm into the red, explaining the difference in its absorption and emission. It is worth noting that the Stokes shift for all compounds is comparable with ICG. As reported in a previous article the replacement of a 3,3-dimethylindolenine ring with a benzathiazole also shifts the absorption and fluorescence maxima deeper into the red as shown by comparing all the dyes and a good example is **3a** (747 nm, 775 nm) with **4a** (762 nm, 789 nm), respectively.[9] In addition to this, dyes **4a–4d** (bearing the benzothiazole ring) all showed the highest quantum yield when compared with dyes **3a–3d,** this can possibly be attributed to the sulphur heterocyclic atom (on the benzothiazole) being sp2 hybridised and thus helping maintain planarity, whereas the dimethyl substituted sp3 carbon atom would distort 'pucker' the ring.[10–12]

Finally, we wanted to determine how the photophysical properties of these compounds compared against rigid heptamethine dyes **5a** and **5b** (Figure 2) as shown in Figure 3. Unsurprisingly, the linear heptamethine dyes (**3a–3d** and **4a–3d**) had superior Stokes shifts and fluorescence quantum yields compared with the rigid heptamethine dyes and this is in line with previous studies.[9] Its important to note that atoms of larger mass have increased spin orbit coupling, which promotes the efficiency of electron excitation from a S0 to S1 state.[13] Thus, the presence of a Cl atom on the central heptamethine dyes (**3d** and **4d**) should increase fluorescence quantum yield. This is supported by the data provided in Table 1. Moreover, replacement of the methylene moiety by sulphur in the indolyl ring of these compounds results in bathochromic shifts of the absorption and fluorescence spectra. It is possible for the sulphur atom in **4a–4d** and **5b** to be sp2 hybridised leading to extended electron delocalisation and conjugation with the sp2 hybridised methine carbon atoms within the polyene. This is expected to lower the highest occupied molecular orbital–lowest unoccupied molecular orbital (HOMO–LUMO) energy gap that is responsible for these transitions.[14,15] This effect appears to be slightly greater in the more rigid structures **5a** and **5b** (16 nm shift in the absorption maximum from **5a** to **5b** on sulphur substitution) than when comparing **3d** and **5b** for which then bathochromic shift is only 11 nm. A full investigation of this effect would require more extensive measurements of fluorescence lifetimes and solvent effects.

Having determined that many of the compounds prepared have significantly higher fluorescence quantum yield compared with ICG, **5a** and **5b**, we tested them in the fission yeast *Saccharomyces pombe*, as a model organism for human cells and thus provide an estimate of compound toxicity. These compounds caused significant growth inhibition of *S. pombe* cells suggesting that they have a role as an antifungal agent which is consistent with published data.[11] The minimum inhibitory concentration (MIC) values for these compounds are shown in Table 1 and it is interesting to note that for compounds

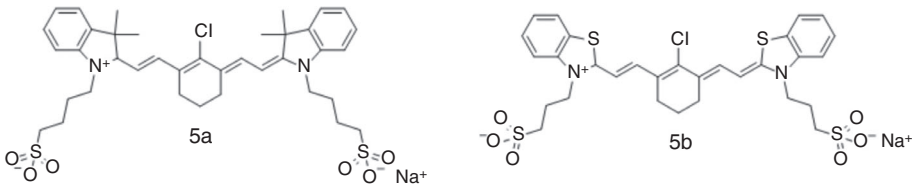


FIGURE 2 The rigid heptamethine dyes **5a** and **5b**.

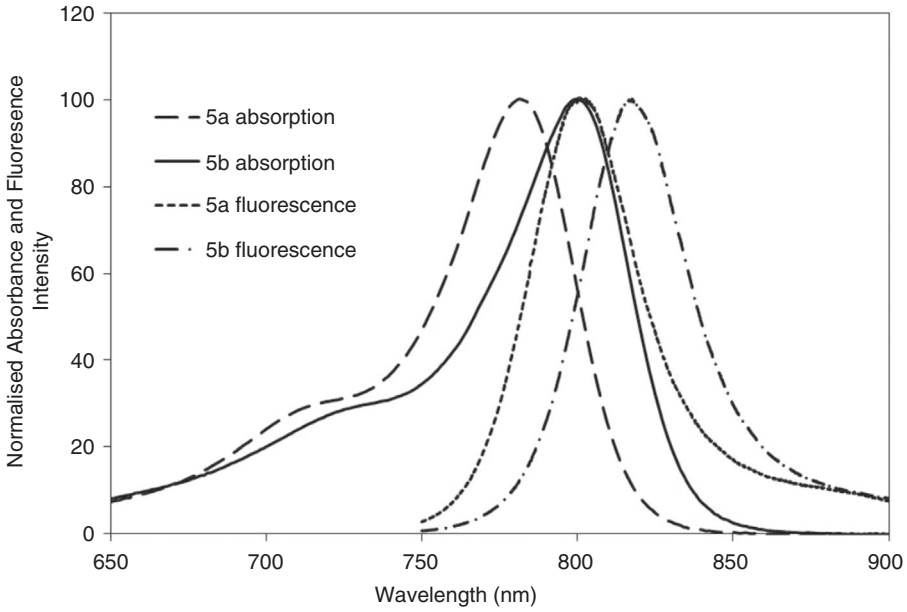


FIGURE 3 Photophysical properties of **5a** and **5b**.

3b–3d and **4b–4d** the MIC values are less than 11 μM. Due to its polyene structure, classical antifungal against amphotericin B was used as control bioassay for which we determined a MIC of 0.53 μM. We hypothesise that these compounds could have two possible modes of action, due to structural comparisons with amphotericin B. One possibility is pore formation within the yeast membrane via mycosamine-mediated interaction with ergosterol and this is based on the mode of action of amphotericin B which is a polyene antifungal.[15] A second possible mechanism might be attributed to the negative charge on the yeast cell surfaces which could lead to an interaction between the yeast cell surface and the quaternary N-alkylated sub-units on these compounds.[16] It is important to highlight that for each of the compounds listed in Table 1, the MIC falls below 20 μM indicating that these compounds are potent against *S. pombe*. It is interesting to note that ICG shows a slightly higher MIC at 16.60 μM compared to compounds **3b–3d** and **4b–4d.** Although it could be suggested that the increased lipophilicity caused by extra fused aromatic ring has an affect on growth inhibition, it is important to note that this is in line with the other non-substituted compounds **3a** and **4a,** which have similar MICs. The compounds are currently being extensively screened against the more pathogenic fungi, *Candida albicans*.

3 | CONCLUSION

In summary, we report the synthesis, photophysical properties and growth inhibition properties of novel linear substituted heptamethine dyes. The synthetic route to these dyes is simple and provides the opportunity to develop a plethora of structural alternatives, suitable for further modification. A good example of such would be the incorporation of a boronic acid motif for Suzuki chemistry. The photophysical properties of all compounds prepared in this study show an increase in fluorescence quantum yield when compared against the standard ICG. Moreover, compounds **3a**, **3d** and **4c** all show a < 1.5-fold increase with **4a**, **4b**, and **4d** all showing a ≥ 2.0-fold excess. In all cases the dyes developed in this study show enhanced photophysical properties when compared against the rigid heptamethine dyes **5a** and **5b**. Further structural enhancements using the methodology could see structural alternative developed which include the attachment of sugar units towards tumour targeting or photodynamic agents such as methylene blue, presenting an opportunity to develop some interesting light activated biocides.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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