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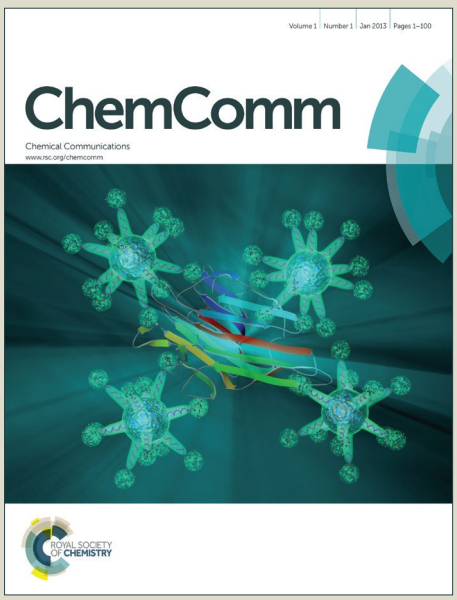

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Synthesis of a far-red fluorophore and its use as an esterase probe in living cells

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We report the synthesis of a new far-red fluorophore, 1,3-dichloro-7-hydroxy-2*H*-spiro[acridine-9,1'-cyclohexane]-2',5'-diene-2,4'-dione (DSACO), which was modified to make two esterase probes: DSACO-2-AME and DSACO-7-AME. Both probes act as "turn-on" substrates for esterases and lipases. DSACO-2-AME exhibited efficient esterase-activated fluorescence inside living cells and is a stable, far-red alternative for the widely-used fluorescein diacetate.

Advances in understanding biology at the molecular level are aided by the use of chemical probes, including small-molecule fluorophores. For detecting and imaging enzyme activities, fluorogenic, or "turn-on," probes are particularly powerful tools. These molecules provide a bright fluorescent signal upon enzyme-mediated hydrolysis and can be targeted to proteases, sulfatases, esterases, and other enzymes.[1–6] Most fluorogenic probes utilize the blue-fluorescent coumarins (e.g., 4-methylumbelliferone) and green-fluorescent xanthenes (e.g., fluorescein), net neutral fluorescent molecules with high quantum yields. These two fluorophore families are easy to synthesize and derivatize, but they fluoresce below 550 nm, where cellular absorption, light scattering, and autofluorescence are high.[7] Phenoxazines, such as resorufin ($\lambda_{abs}/\lambda_{em}$ = 571/578 nm, ϕ = 0.74; Figure 1), are further red-shifted and can be converted into fluorogenic probes.[8–10] However, they can undergo both oxidation and reduction in cells, which radically alters their fluorescent properties and complicates analyses.[4]

Probes that fluoresce in the red and far-red (i.e., above 600 nm) are desirable for cellular imaging. Cyanines, which include Alexa Fluor 647, Cy5, and Cy7, are a widely used family of fluorophores that emit in the far-red spectral region. They are resistant to photobleaching, a favorable imaging property, but their charged nature limits their ability to cross cell membranes.[6]

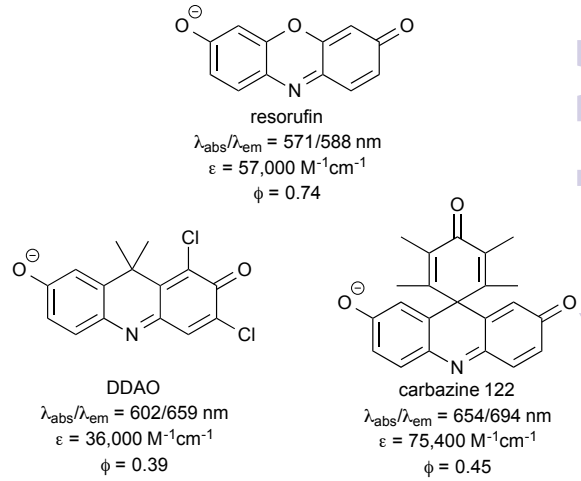
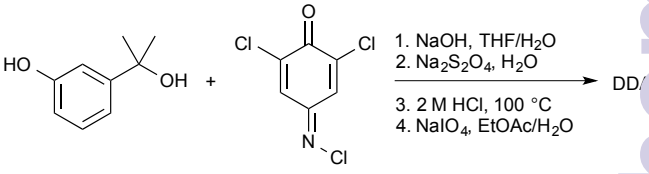


Figure 1. Examples of phenoxazine and carbazine fluorophores. Resorufin[10] and DDAO were analyzed in aqueous buffer, while the spectral properties of carbazine-122 were analyzed in basic ethanol.[11,12]

Carbazines are a versatile alternative to the far-red cyanines. The prototypical example of this important sub-class is 1,3-dichloro-7-hydroxy-9,9-dimethylacridin-2(9*H*)-one (DDAO, $\lambda_{abs}/\lambda_{em}$ 602/659 nm, ϕ = 0.39; Figure 1), a commercially available fluorophore. DDAO has been converted into fluorogenic substrates for sulfatases,[13] esterases,[10] and β-galactosidases.[14,15] Regrettably, the synthesis of DDAO is hampered by the geminal dimethyl substituents at the central carbon. This necessitates a multi-step procedure using costly starting materials (Scheme 1).[14] A single-step synthesis of carbazines would make this auspicious dye class available for widespread use.

Herein, we report the synthesis and spectral characterization of DSACO, a readily synthesized carbazine fluorophore. We demonstrate the value of this dye by masking it with an esterase-cleavable acetoxymethyl ether (AME) group. The new fluorogenic substrates were used in an enzyme screen and for live-cell



Scheme 1. Synthesis of DDAO, a representative member of the carbazine dye class.

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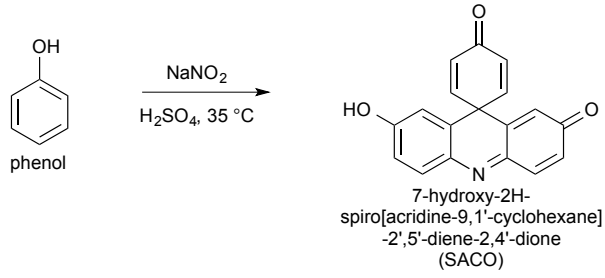
fluorescence imaging.

We chose to focus on a subclass of carbazines with a spirocycle in place of the germinal dimethyl at the central carbon atom of DDAO. Publications indicated that these compounds were formed as by-products in the Liebermann test for phenols.[16,17] Only limited spectral data exists for these compounds,[11,16,18,19] but carbazine-122 ($\lambda_{abs}/\lambda_{em}$ = 654/694 nm, ϕ = 0.45; Figure 1)[11] was formerly sold as a laser dye by Lambda Physik AG and Exciton, Inc.[12] This led us to conclude that these molecules retain the fluorescent properties of simpler carbazines (i.e., DDAO).

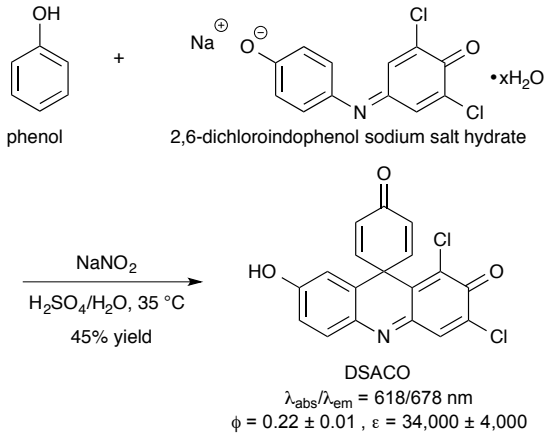
Our efforts to synthesize these spiro-acridines commenced with an analysis of the original synthesis of the parent compound 7-hydroxy-2H-spiro[acridine-9,1'-cyclohexane]-2',5'-diene-2,4'-dione (SACO) (Scheme 2).[16] Hill *et al.* synthesized SACO in a single step from a Liebermann reaction between a phenol and an indophenol.[16,19] Despite varying temperature, time, concentrations, and water content, we were unable to improve upon their reported 12% yield. Additionally, our isolated material was contaminated with decomposition products.

In an effort to determine the origin of the low yield, we separately synthesized the indophenol intermediate by reacting phenol and 4-aminophenol under oxidizing conditions.[20] Attempts to purify this compound resulted in decomposition, as did exposing the crude material to the highly acidic conditions needed for spirocycle formation. We hypothesized that this same instability was consuming the indophenol intermediate formed *in situ* in Hill's synthesis. We then employed the more substituted and stable[21] 2,6-dichloroindophenol in place of the unstable intermediate. Unexpectedly, this did not lead to an increase in the yield. Examination of the literature revealed an alternative reaction mechanism whereby the indophenol reacts not with phenol directly, but with *p*-nitrosophenol.[17, 19] The formation of this intermediate occurs via nitrosation of phenol by sodium nitrite. Thus, we altered Hill's synthesis to include super-stoichiometric sodium nitrite and obtained DSACO in a 45% yield (Scheme 3).

We determined the spectral properties of DSACO and compared them to DDAO (for spectra, see Supporting Information Figure S1). DSACO exhibited a broad absorbance peak with a maximum at 618 nm. The emission maximum (λ_{em} = 678 nm) was 19 nm red-shifted compared to DDAO. The pK_a of DSACO was 5.0 (Figure S2), which enables the highly fluorescent deprotonated form to be used in assays across a broad pH range. In aqueous buffer, the extinction coefficient (ε) of DSACO was 34,000 ± 4,000 $M^{-1}cm^{-1}$, which is similar to that of DDAO (ε = 36,000 ± 4,000 $M^{-1}cm^{-1}$). The quantum



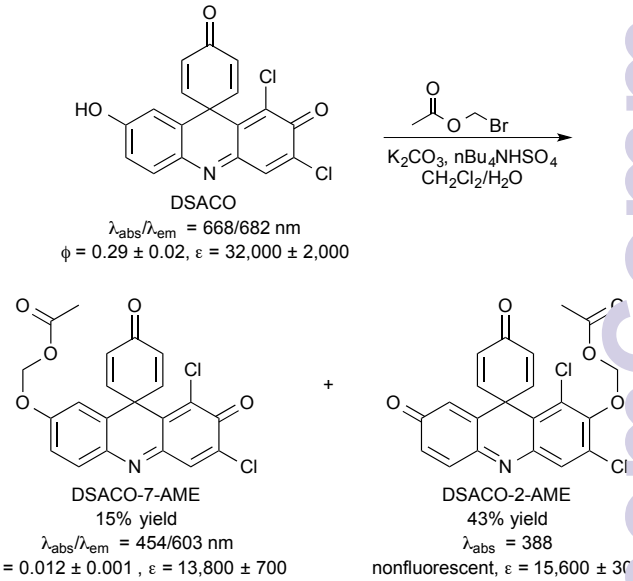
Scheme 2. Hill's 1970 synthesis of a spirocyclic carbazine.



Scheme 3. Synthesis of DSACO, a halogenated spirocyclic carbazine. Spectral data were measured in aqueous buffer (pH 7.3).

yield (ϕ) of DSACO in aqueous buffer was 0.22 ± 0.01, which is lower than DDAO's (ϕ = 0.39 ± 0.04). Nonetheless, the carbazine quantum yields are comparable to the far-red cyanines, including Cy5 (ϕ = 0.27),[22] Alexa Fluor 647 (ϕ = 0.33),[23] and indocyanine green (ϕ = 0.13).[24]

In stark contrast to most cyanines, the fluorescence of the carbazine dyes can be suppressed by alkylation of the phenolic oxyanion. As a proof-of-concept, we masked DSACO's fluorescence by installing an AME group. We synthesized the 2- and 7-masked DSACO-AMEs using biphasic conditions originally developed for resorufin (Scheme 4).[9] The regioisomers were separable by column chromatography and their structures were assigned by 2D-NMR experiments. We spectrally characterized these fluorogenic probes in ethanol and compared them to DSACO. The AME derivatives had blue-shifted absorbance spectra compared to deprotonated DSACO, with maxima at 388 nm (DSACO-2-AME) and 454 nm (DSACO-7-AME) (Figure 2). The absorbance and emission spectra of the masked compounds in aqueous buffer (10 mM HEPES, pH 7.3) were not substantially different than the spectra in ethanol. Measurement of the extinction coefficients and quantum yields was



Scheme 4. Synthesis of acetoxymethyl ether masked regioisomers of DSAC spectral data were measured in ethanol.

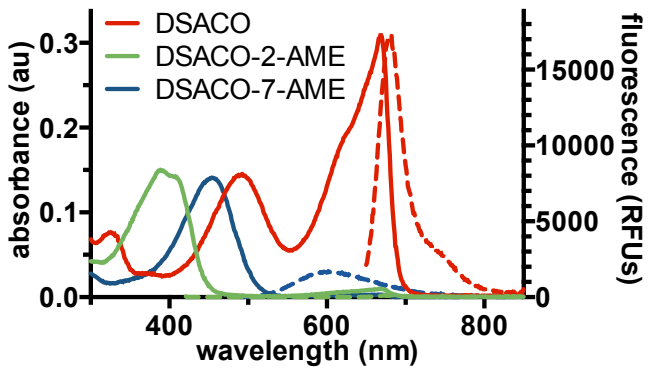


Figure 2. The absorbance and emission spectra of DSACO, DSACO-2-AME, and DSACO-7-AME in ethanol. Samples for absorbance measurements were prepared at 10 μM (solid lines). Samples for fluorescence emission were prepared at 1 μM (dashed lines).

not possible in an aqueous medium due to solubility issues at the necessary concentrations. The extinction coefficients of both DSACO-2-AME (ε = 15,600 ± 300 $M^{-1}cm^{-1}$) and DSACO-7-AME (ε = 13,800 ± 700 $M^{-1}cm^{-1}$) were less than half that of DSACO (ε = 32,000 ± 2,000 $M^{-1}cm^{-1}$). Analogous to DDAO-derived esterase probes,[10] only substitution at the C2 position resulted in complete fluorescence quenching. DSACO-7-AME retained very little fluorescence (ϕ = 0.012 ± 0.001), and its emission maximum (λ_{em} = 603 nm) was 80 nm blue-shifted compared to DSACO. Combined, these features make both AME-masked probes turn-on substrates.

We validated DSACO-2-AME and DSACO-7-AME as enzyme substrates with a selection of esterases and lipases (Figure 3). Our panel included porcine liver esterase (PLE), *B. subtilis* esterase, *S. cerevisiae* esterase, and nine lipases [from *Aspergillus*, *C. antarctica*, *C. rugosa*, *M. miehei*, *P. cepacia*, *P. fluorescens*, *R. arrhizus*, *R. niveus*, and porcine pancreas (PPL)]. After a 10 minute incubation at 37 °C in HEPES buffer (pH 7.3), both probes displayed significant reactivity with PLE and *B. subtilis* esterase, while *S. cerevisiae* and heat-killed PLE remained dark (Figure 3A). Less reactivity was observed in the presence of the lipases. However, both probes gave statistically significant responses with seven of the nine lipases screened. Neither probe was unmasked by PPL. While *M. miehei* did not produce a statistically significant signal with DSACO-2-AME, this lipase activated DSACO-7-AME. Conversely, DSACO-7-AME was not hydrolysed by *R. niveus* lipase, but DSACO-2-AME was.

In screening the panel of enzymes, we found that DSACO-2-AME was generally a better esterase substrate than DSACO-7-AME. This was further explored in a detection-limit study with PLE (Figure S3). Using a 10 μM solution of probe, 10 pg of PLE was detected using fluorescein diacetate (FDA), a commercially-available esterase probe that produces a strong fluorescent signal ($\lambda_{ex/em}$ = 490/525 nm, ϕ = 0.89). Under the same conditions, DSACO-2-AME could detect as little as 30 pg of PLE. In contrast, DSACO-7-AME could only detect 200 pg of PLE. These results demonstrate that our far-red DSACO-2-AME probe is a reasonable alternative to FDA.

Kinetic characterization confirmed that DSACO-2-AME is a favorable PLE substrate. With 50 ng/mL of enzyme in HEPES buffer (pH 7.3) containing 20% acetonitrile, the probe exhibited a Michaelis constant (K_M) of 110 ± 19 μM and a maximum velocity (V_{max}) of 1.01 ± 0.12 pmol/s. The catalytic constant (k_{cat}) and

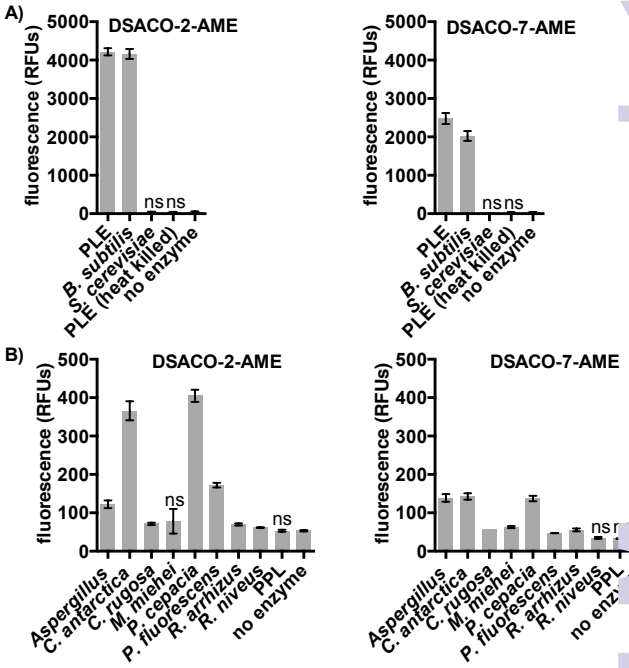


Figure 3. AME-masked DSACO derivatives are hydrolyzed by a variety of esterases (A) and lipases (B). All unlabeled responses are statistically significant ($p < 0.01$). Responses labeled "ns" are not significant compared to the no enzyme control. Error bars represent one standard deviation; n = 3.

specificity constant (k_{cat}/K_M) were determined to be 11 s^{-1} and 1.[illegible] 10^5 $M^{-1}s^{-1}$, respectively.

Non-specific fluorogenic probe hydrolysis can be a significa[illegible] problem in aqueous buffer or media. For example, FDA is unstabl[illegible] in PBS with a half-life ($t_{1/2}$) of 8 h, and it is even less stable in th[illegible] presence of serum.[10] However, AME-masked probes are general[illegible] more stable than acetate masked ones.[9,25,26] Therefore, w[illegible] evaluated the stability of the new probes both in aqueous buff[illegible] and in serum-containing media. In PBS (pH 7.4), DSACO-2-AM[illegible] displayed a $t_{1/2}$ of 27 hours, while DSACO-7-AME was more stable with $t_{1/2}$ = 83 hours. As expected, both probes underwent hydrol[illegible] in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (DSACO-2-AME $t_{1/2}$ = 0.4 and DSACO-7-AME $t_{1/2}$ = 1.5 h), but both were more stable than FDA ($t_{1/2}$ = 0.3 h).[10] In DME[illegible] without serum, the half-lives were closer to those observed in PBS (DSACO-2-AME $t_{1/2}$ = 25 h and DSACO-7-AME $t_{1/2}$ = 64 h). The high stability of the AME-masked probes is a powerful advantage f[illegible] live-cell imaging.

In addition to transforming the parent fluorophore into a fluorogenic probe, masking DSACO's phenolate anion enables liv[illegible]-cell fluorescence imaging. One example of this is the use of FDA as a cell-permeable esterase probe. In this fashion, intracellular este[illegible]ase activity revealed by FDA has been used as a proxy for cell viab[illegible]y, but spontaneous unmasking of FDA produces high background fluorescence.[27,28] We anticipated that our DSACO-AME probe[illegible] might offer a far-red live-cell imaging alternative, which w[illegible] investigated by imaging Rat-1 fibroblasts by confocal fluorescence microscopy. Cells were incubated for 20 minutes with each pro[illegible] followed by a single quick wash with PBS (Figure 4). DSACO-7-AM[illegible] gave limited and inconsistent staining in live cells, possibly due to [illegible]s low solubility or poor cellular uptake. For images of DSACO-7-AME

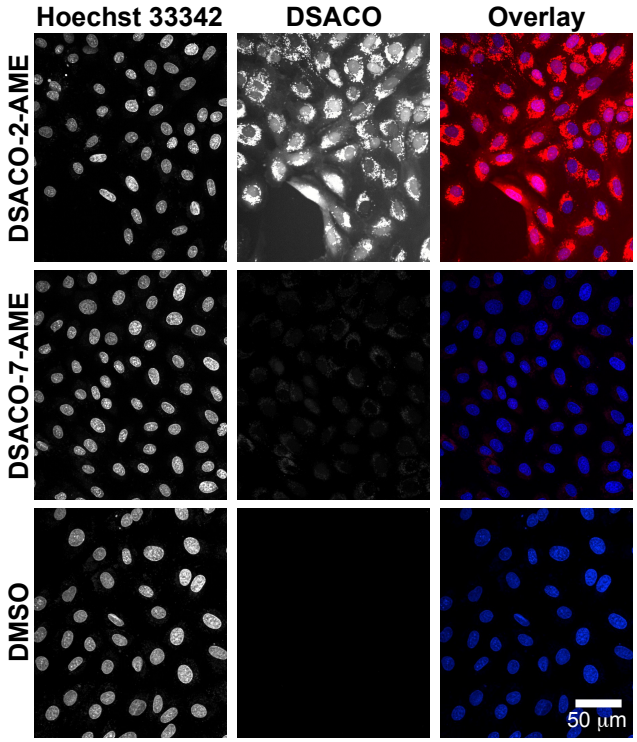


Figure 4. Live cell imaging of esterase activity in Rat1-fibroblast cells via confocal fluorescence microscopy. The cells were incubated with either 10 μM DSACO-2-AME or DSACO-7-AME for 20 min, washed once, and then imaged to detect DSACO fluorescence (λ_{ex} = 640 nm). Cells were counter-stained with Hoechst 33342 (λ_{ex} = 405 nm). Scale bar represents 50 μm.

staining compared to the DMSO control, see Figures 4 and S4. In contrast, cells treated with DSACO-2-AME were brightly fluorescent, presumably due to cleavage of the masking group by intracellular esterases. The fluorescence signal was primarily located in the cytosol and was retained for hours. Our imaging results demonstrate the usefulness of DSACO-2-AME as a reporter of esterase activity in living cells and as a live-cell stain.

We confirmed that our compounds do not affect cell viability. Cells incubated with DSACO, DSACO-2-AME, or DSACO-7-AME for 1 h or 24 h were as viable as mock-treated or FDA-treated cells (Figure S5).

In summary, we optimized the synthesis of DSACO, a readily accessible far-red fluorophore of the carbazine class. We then synthesized two spectrally distinct fluorogenic esterase probes: DSACO-2-AME and DSACO-7-AME. Both regioisomers were hydrolyzed by a variety of esterases and lipases *in vitro*. DSACO-2-AME labeled the interior of living cells, resulting in a bright fluorescent signal in the far-red channel. We anticipate that the synthetic accessibility and favorable spectral properties of DSACO will make custom-masked far-red fluorogenic probes more widely available.

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References

1 S. A. Hilderbrand, in *Live Cell Imaging: Methods and Protocols*, ed. D. B. Papkovsky, Springer, New York, NY, 2010, ch. 2, pp. 17–45.
2 A. Baruch, D. A. Jeffery and M. Bogyo, *Trends Cell Biol.*, 2004, **14**, 29–35.
3 K. E. Beatty, *Mol. Biosyst.*, 2011, **7**, 2360.
4 L. D. Lavis and R. T. Raines, *ACS Chem. Biol.*, 2014, **9**, 855–866.
5 X. Li, X. Gao, W. Shi and H. Ma, *Chem. Rev.*, 2014, **114**, 590–659.
6 J. B. Grimm, B. P. English, J. Chen, J. P. Slaughter, Z. Zhang, A. Revyakin, R. Patel, J. J. Macklin, D. Normanno, R. H. Singer, T. Lionnet and L. D. Lavis, *Nat. Methods*, 2015, **12**, 244–250.
7 R. Weissleder and V. Ntziachristos, *Nat. Med.*, 2003, **9**, 123–128.
8 G. G. Guilbault and D. N. Kramer, *Anal. Chem.*, 1965, **37**, 120–123.
9 L. D. Lavis, T.-Y. Chao and R. T. Raines, *Chem. Sci.*, 2011, **2**, 521–530.
10 K. R. Tallman and K. E. Beatty, *ChemBioChem*, 2015, **16**, 70–75.
11 R. Sens and K. H. Drexhage, *J. Lumin.*, 1981, **24**, 709–712.
12 U. Brackmann, *Lambdachrome Laser Dyes*, Lambda Physik AG, Goettingen, Germany, 3rd edn, 2000.
13 K. E. Beatty, M. Williams, B. L. Carlson, B. M. Swarts, R. M. Warren, P. D. van Helden and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 12911–12916.
14 P. F. Corey, R. W. Trimmer and W. G. Biddlecom, *Angew. Chem. Int. Ed. Engl.*, 1991, **30**, 1646–1648.
15 C.-H. Tung, Q. Zeng, K. Shah, D.-E. Kim, D. Schellingerhout and R. Weissleder, *Cancer Res.*, 2004, **64**, 1579–1583.
16 R. Hill, G. R. Bedford and B. R. Webster, *J. Chem. Soc. C*, 1970, 2462–2466.
17 M. Fraenkl, D. Svobodová and J. Gasparič, *Microchim. Acta*, 1986, **90**, 367–386.
18 R. Hill, A. R. Crofts, R. C. Prince, E. H. Evans, N. E. Good and D. A. Walker, *New Phytol.*, 1976, 1–9.
19 USPTO, 5,567,624, 1996, 23 pp.
20 H. H. Tucker, *J. Soc. Cosmet. Chem.*, 1971, **22**, 379–398.
21 F. Brody and M. S. Burns, *J. Soc. Cosmet. Chem.*, 1968, **22**, 361–379.
22 R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis and A. S. Waggoner, *Bioconjugate Chem.*, 1993, **4**, 105–111.
23 I. Johnson and M. T. Z. Spence, Eds., *The Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies*, Life Technologies, Inc., Eugene, OR, 11th edn, 2010.
24 K. Rurack and M. Spieles, *Anal. Chem.*, 2011, **83**, 1232–1242.
25 E. Leroy, N. Bensel and J.-L. Reymond, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2105–2108.
26 Y. Yang, P. Babiak and J.-L. Reymond, *Helv. Chim. Acta*, 2006, **89**, 404–415.
27 B. Rotman and B. W. Papermaster, *Proc. Natl. Acad. Sci. U. S. A.*, 1966, **55**, 134.
28 S. A. Weston and C. R. Parish, *J. Immunol. Methods*, 1990, **133**, 87–97.