Organic & Biomolecular Chemistry

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View Article Online
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Synthesis and evaluation of cyanine–styryl dyes with enhanced photostability for fluorescent DNA staining†

Cite this: DOI: 10.1039/c3ob41717d

Received 22nd August 2013,
Accepted 18th September 2013

DOI: 10.1039/c3ob41717d

www.rsc.org/obc

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The photostability of cyanine–styryl dyes of the indole–quinolinium type can be significantly improved by structural variations while the excellent optical properties including the bright fluorescence in the presence of DNA can be maintained or even improved, too.

The synthesis, chemistry and positive evaluation of new fluorescent dyes that show promising and interesting optical properties are important prerequisites for applications in the field of fluorescent bioanalytics and molecular cell imaging of nucleic acids.[1,2] The most important parameters to evaluate the potential of new dyes are brightness defined as extinction (ε) × quantum yield (Φ_F), photostability and fluorescence intensity enhancement in the presence of nucleic acids. Especially with respect to the latter property, cyanine derivatives are in the focus of research since they often exhibit drastically enhanced fluorescence intensity by binding to DNA or RNA.[3,4] They are also suitable to be assembled as DNA-templated aggregates.[5] Beside fluorescence intensity enhancement, cyanines exhibit high extinction coefficients, generally good synthetic accessibility, especially optical properties that can be tuned by organic synthesis, and good quantum yields.[3,4] It is important to mention here that electron-withdrawing groups, like fluoro substituents and cyano groups, are able to enhance the photostability of cyanine dyes.[6]

Thiazole orange probably represents one of the best known representative dyes of the cyanine class,[7] also known as SYBR Green,[8] TO-PRO and in its dimeric form TOTO.[9] Its binding properties as a combination of intercalation and groove binding[4,10] and the fluorescence enhancement in the presence of DNA has been investigated in detail.[11–13] Conjugates of thiazole orange with nucleic acids have been prepared and applied for molecular imaging,[14] including the so-called FIT probes based on PNA,[15] ECHO probes[16] and our DNA and RNA "traffic lights".[17,18] Most recently, a thiazole orange derivative that carries a cyano group at the methane bridge was presented as a twisted cyanine with superior photostability.[19] Alternatively, dyes of the cyanine–styryl-type represent very promising candidates for molecular imaging of nucleic acids with respect to their reported property as bright non-covalent binders to RNA.[20] We recently found out that one representative of these dyes, the cyanine–indole–quinolinium dye (CyIQ), exhibits not only an excellent brightness in the presence of nucleic acids but also a photostability that is significantly better than that of fluorescein, BODIPY and thiazole orange as covalently bound fluorescent DNA labels.[21] Herein, we present the synthesis and optical spectroscopy of a new set of cyanine–styryl dyes of the CyIQ type **1–12** with significantly improved brightness, photostability and fluorescence intensity enhancement in the presence of double-stranded DNA. These dyes represent important candidates for fluorescent DNA and RNA labelling and molecular imaging inside living cells.

The cyanine–styryl dyes of the CyIQ type consist of a quinolinium part that is bridged by a double bond to the indole part. This bridge can be attached either to the 2-position of the quinolinium moiety (like in **1**) or to the 4-position. Accordingly, we divide the discussion of the new synthetic dyes presented herein into two classes, **1–7** and **8–12** (Scheme 1), and compare them at the end. All dyes were alkylated by a propyl linker carrying a terminal hydroxyl group to provide the possibility for the later preparation of the corresponding DNA/RNA building blocks or for the postsynthetic attachment to DNA or RNA.[22] All synthetic dyes were analytically pure (see ESI†) and characterized by their absorption and fluorescence (Table 1).

Based on the structure of **1** six new cyanine dyes **2–7** were synthesized with the styryl bridge attached to the 2-position of the quinolinium part (Scheme 1). The structures of these dyes varied: (i) by the *N*-methyl group at the indole part (**2** *vs.* **1**, **6** *vs.* **5**), by an additional fused benzene ring (**3**/**7** *vs.* **1**) and by electron-withdrawing/-donating substituents at the quinolinium part (**4**/**5** *vs.* **1**, **6** *vs.* **2**). First, titration experiments

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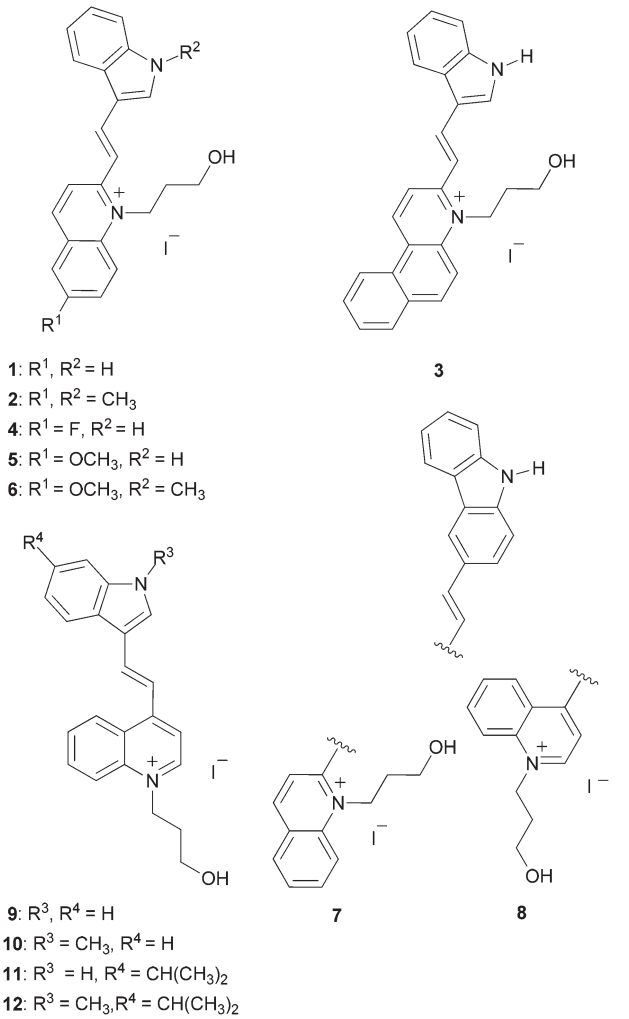
†Electronic supplementary information (ESI) available: Syntheses of dyes **1–12** including images of NMR, MS, IR and elemental analysis, details of titrations, photostability experiments, quantum yield/extinction coefficient measurements, analysis of photoproducts. See DOI: 10.1039/c3ob41717d



Scheme 1 Structures of dyes **1–12**.

were performed with a random sequence double-stranded DNA. Fig. 1 representatively shows the titration experiment of dye **5** with DNA which was followed by UV/Vis absorption and fluorescence spectroscopy. The addition of DNA gives a bathochromic shift (λ_{shift}, *ca.* 30 nm) of the main UV/Vis absorption peak of this dye which is a typical observation upon binding to DNA.[23] These absorption changes are: (i) due to the fact that dye aggregates in aqueous solution are broken up as a result of DNA binding, and/or (ii) the result of a geometric change (*e.g.* planarization) of the dye upon binding to DNA, and/or (iii) the result of the change of polarity in the DNA binding pocket and/or (iv) due to dipole–dipole interactions between the dye and the DNA bases. According to the measured fluorescence intensities of dye **5** a saturation plateau is reached after the addition of approximately 4 equiv. of DNA. These changes of optical properties have similarly been observed for all dyes, not only for the ones in this first set **1–7** but also for those in the second set **8–12** that will be described below. The binding constants were determined (see ESI†) and lie in the range of 1.5×10^4–4.4×10^4 M^{-1} (except dyes **7** and **8** which will be discussed below).

In order to elucidate the potential applicability for fluorescent DNA analytics and imaging, Stokes' shifts (λ_{Stokes}) and

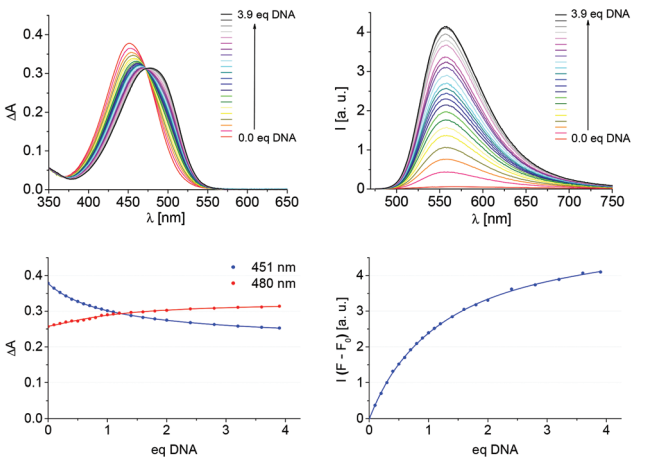


Fig. 1 Representative titration of dye **5** with DNA (10 μM **5**, 10 mM Na–P_i buffer, 250 mM NaCl, 2% ethanol, 0–40 μM dsDNA, λ_{exc} = 460 nm), followed by UV/Vis absorption (top left), change at 451 nm and 480 nm (bottom left), and by fluorescence (top right), emission intensity enhancement at 558 nm (bottom right). Other titrations are displayed in the ESI.†

Table 1 Optical properties of cyanine dyes **1–12**

Dye	λ_{max} [a] (nm)	ε (M^{-1} cm^{-1})	λ_{max} [b] (nm)	Φ_F [c] (%)	F/F_0 [d]	λ_{Stokes} [c] (cm^{-1})	B [c] (M^{-1} cm^{-1})	$t_{1/2}$ [e] (min)	K (M^{-1})
1	489	42 400	556	4.4	58	149	1900	90	1.7×10^4
2	493	42 700	560	6.6	58	149	2800	205	1.5×10^4
3	487	37 700	555	17.0	99	147	6400	13	4.3×10^4
4	486	38 000	568	1.5	36	122	600	50	1.8×10^4
5	480	39 400	558	5.7	73	128	2200	264	2.2×10^4
6	481	39 300	556	5.8	46	133	1700	425	2.0×10^4
7	473	35 700	625	7.1	5	66	2500	130	2.5×10^5
8	501	26 100	632	12.5	14	83	3300	134	2.5×10^5
9	509	30 800	590	12.4	34	123	3800	186	2.9×10^4
10	521	30 800	596	17.6	39	133	5400	331	3.0×10^4
11	515	27 500	598	12.7	55	120	3500	67	4.4×10^4
12	522	35 700	602	15.3	59	125	5500	241	3.9×10^4

[a] UV/Vis absorption. [b] Fluorescence. [c] In the presence of 4.0 equiv. of DNA. [d] F_0: fluorescence intensity without DNA, F: with 3.9 equiv. of DNA. [e] In the presence of 2.5 μM DNA.

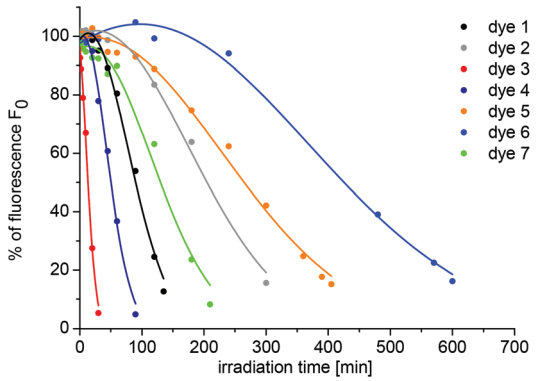
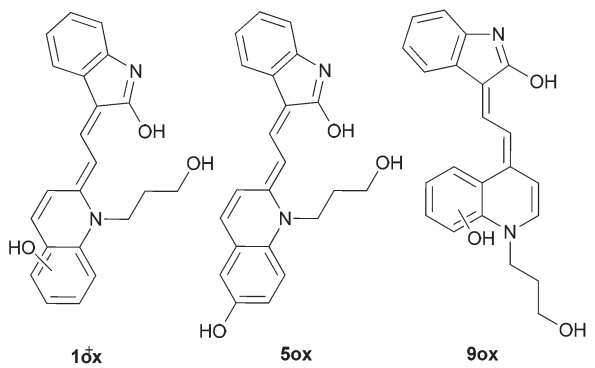


Fig. 2 Photostability of dyes **1–7** measured by the loss of fluorescence intensity in the presence of DNA (10 μM dye, 2.5 μM dsDNA, 10 mM Na–P_i buffer, 250 mM NaCl, 5% ethanol).

fluorescence intensity enhancements (F/F_0) were recorded as characteristic values for each dye. Quantum yields (Φ_F) and brightnesses (B) were measured in the presence of 4.0 equiv. of DNA to ensure that the majority of dye molecules are bound to DNA. The half-life times ($t_{1/2}$) quantify the photostabilities during irradiation with a 75 W Xe lamp equipped with a 305 nm cutoff filter to avoid excitation of the DNA components. The photodegradation of the dyes was recorded by the loss of fluorescence intensity in the presence of DNA (Fig. 2). Table 1 summarizes these values for dyes **1–7**.

Dye **2** carries an additional methyl group at the indole nitrogen. This small structural change does not alter the optical properties of the dye significantly but improves the photostability of the dye by a factor of more than 2. A similar result was obtained by comparison of the methoxysubstituted dyes **5** and **6**. In order to study this effect photoirradiated dye samples were carefully analyzed by HPLC and ESI-MS experiments. In case of the dyes **1** and **5** that are *not* methylated at the indole nitrogen the hydroxylated derivatives **1ox** and **5ox** were identified as primary photooxidation products (Scheme 2). The UV/Vis absorption of these products indicates that the conjugation in these uncharged compounds is maintained but the fluorescence is lost. Hence, these compounds must be oxidized at the indole or the quinolinium part. The hydroxylation at the quinolinium part will be commented below. It is more important to mention here that similar photooxidation at the indole part (see ESI†) was not obtained with the *N*-methylated dyes **2** and **6**. Instead, photoproducts with a ring-opened indole part were observed after longer irradiation times.[24] Obviously, the *N*-methyl group of **2** and **6** protects the indole part from hydroxylation and thereby from one of the primary photodegradation pathways at the indole.



Scheme 2 Photoproducts **1ox**, **5ox** and **9ox**.

Fusing a benzene ring to the quinolinium moiety in dye **3** enhances mainly the quantum yield to 17% due to the enlarged aromatic system and thereby the brightness of this dye, but lowers the photostability. On the other hand the additional benzene ring at the indole moiety of dye **7** (carbazole) does not deteriorate the photostability to such an extent, but yields a quite remarkable Stokes' shift that is enhanced from 147–149 cm^{-1} (**1–3**) to 66 cm^{-1} (**7**) and a significantly stronger binding to DNA (K = 2.5 × 10^5 M^{-1}). Obviously the charge transfer character of the excited state (bonded exciplex)[25] of this dye is more pronounced.

The work of Armitage *et al.* has shown that fluoro substituents at the aromatic parts of cyanine dyes enhance the photostability quite dramatically.[6] This has been rationalized by the electron-withdrawing effect on the conjugated bridge between the two aromatic parts of the dye that reduces bleaching by oxygen. Accordingly, dye **4** bears a fluoro substituent (electron-withdrawing) at the quinolinium part, whereas dyes **5** and **6** carry a methoxy group (electron-donating) at the same position. Surprisingly, dyes **5** and **6** but not dye **4** (!) exhibit significantly improved photostabilities (by a factor of 3 and 4) and nearly unchanged optical properties compared to CyIQ (**1**). Again, the comparison of the primary photoproducts gives ESI† for these unexpected results. Hydroxylation of the quinolinium part is a known photochemical degradation[26] and has been observed in **1ox** but not **5ox**. In the latter compound only the methoxy substituent has been cleaved to the hydroxyl group (see ESI†). Obviously, the electron-donating effect or the photodeprotective reactivity of the methoxy substituent in **5** (and **6**) prevents photooxidation of the quinolinium part and thereby improves photostability of these dyes.

The second set of dyes, **8–12**, bear the styryl bridge connected to the 4-position of the quinolinium part. The experiments that were performed to elucidate the optical and photochemical properties of these dyes were identical to the ones described above and are summarized in Fig. 3 and again in Table 1. Remarkably, especially the quantum yields and photostabilities of dyes **9** and **10** significantly improved in comparison to the corresponding dyes **1** and **2** in the previous set (**1–7**). This can be rationalized by the fact that the 4-position of **9** (and **10**) is protected by the styryl bridge and thus prevents photooxidation at this position (as evidenced in **1ox**). Photooxidation of **9** is observed at the 2-position (**9ox**, Scheme 2) but only after significantly longer irradiation times.

Starting with dye **9** as the regioisomer to **1** we varied the structure slightly in order to improve the optical properties and photostabilities. Methylation at the nitrogen of the indole group yields dye **10** with a remarkable quantum yield and astonishing photostability. Both values are the best in the

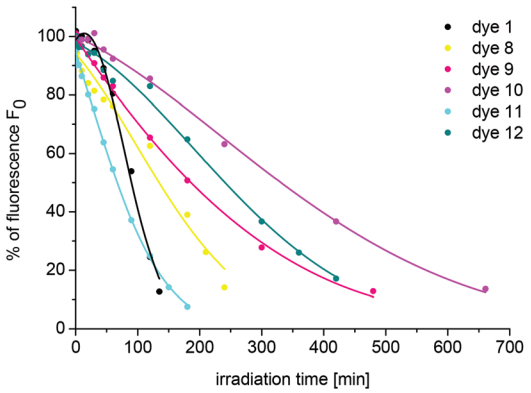


Fig. 3 Photostability of dyes **1**, **8–12** measured by the loss of fluorescence intensity in the presence of DNA (10 μM dye, 2.5 μM dsDNA, 10 mM Na–P_i buffer, 250 mM NaCl, 5% ethanol).

whole set of CyIQ dyes presented herein (**1–12**) and make this dye the most promising one for molecular imaging.

Fusing a benzene ring to the quinolinium moiety was not tried in the set, but an additional benzene ring at the indole moiety (carbazole) gives dye **8**. In comparison to dye **9** (and similar to dyes **7** *vs.* **1**), the photostability of dye **8** is deteriorated and the Stokes' shift is enhanced from 123 cm^{-1} to 83 cm^{-1}. Dye **8** (and **7** too) exhibits the strongest binding to DNA among all dyes presented herein (K = 2.5 × 10^5 M^{-1}). Finally, two additional dyes **11** and **12** were synthesized based on the commercial availability of indole precursors bearing an isopropyl group at the 6-position. Dye **11** is not methylated at the nitrogen of the indole part and shows similar optical properties as dye **9** but significantly reduced photostability. In contrast, the *N*-methylated dye **12** shows improved quantum yield, fluorescence enhancement in the presence of DNA and photostability. This makes this dye another promising candidate for fluorescent imaging.

In conclusion, we could show that the photostability of cyanine–styryl dyes of the indole–quinolinium type (CyIQ) can be improved significantly while the excellent optical properties including the bright fluorescence in the presence of DNA can be maintained or even improved, too. The attachment of the styryl bridge to the 4-position of the quinolinium part improves both optical properties and photostabilities. Moreover, methylation of the indole nitrogen in dyes **2**, **6**, **10** and **12** and/or the introduction of a methoxy substituent into the quinolinium part of dyes **5** and **6** yields astonishingly photostable fluorophores. These effects were rationalized by identification of the primary photoproducts. Finally, dye **10** was elucidated as the most promising candidate for fluorescent DNA/RNA imaging based on its excellent photostability, quantum yield in the presence of DNA and brightness.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (DFG) and KIT is gratefully acknowledged. We thank Ulrike Schwarz (Institute of Physical Chemistry at KIT) for ESI-MS measurements.

Notes and references

1 A. Waggoner, *Curr. Opin. Chem. Biol.*, 2006, **10**, 62–66; L. D. Lavis and R. T. Raines, *ACS Chem. Biol.*, 2008, **3**, 142–155; M. Y. Berezin and S. Achilefu, *Chem. Rev.*, 2010, **110**, 2641–2684; H. Kobayashi, M. Ogawa, R. Alford, P. L. Choyke and Y. Urano, *Chem. Rev.*, 2010, **110**, 2620–2640; L. M. Wysocki and L. D. Lavis, *Curr. Opin. Chem. Biol.*, 2011, **15**, 752–759; J.-S. Lee, M. Vendrell and Y.-T. Chang, *Curr. Opin. Chem. Biol.*, 2011, **15**, 760–767.
2 U. Asselyne, *Curr. Org. Chem.*, 2006, **10**, 491–518; D. M. Kolpashchikov, *Chem. Rev.*, 2010, **110**, 4709–4723; R. W. Sinkeldam, N. J. Greco and Y. Tor, *Chem. Rev.*, 2010, **110**, 2579–2619.
3 A. Mishra, R. K. Behera, B. K. Mishra and G. B. Behera, *Chem. Rev.*, 2000, **100**, 1973–2011; S. Dash, M. Panigrahi, S. Baliyarsingh, P. K. Behera, S. Patel and B. K. Mishra, *Curr. Org. Chem.*, 2011, **15**, 2673–2689; M. Levitus and S. Ranjit, *Q. Rev. Biophys.*, 2011, **44**, 123–151.
4 B. A. Armitage, *Top. Curr. Chem.*, 2005, **253**, 55–76.
5 K. C. Hannah and B. A. Armitage, *Acc. Chem. Res.*, 2004, **37**, 845–853.
6 N. I. Shank, K. J. Zanotti, F. Lanni, P. B. Berget and B. A. Armitage, *J. Am. Chem. Soc.*, 2009, **131**, 12960–12969.
7 H. S. Rye, S. Yue, D. E. Wemmer, M. A. Quesada, R. P. Haugland, R. A. Mathies and A. N. Glazer, *Nucleic Acids Res.*, 1992, **20**, 2803–2812.
8 A. I. Dragan, R. Pavlovic, J. B. McGivney, J. R. Casas-Finet, E. S. Bishop, R. J. Strouse, M. A. Schenerman and C. D. Geddes, *J. Fluoresc.*, 2012, **22**, 1189–1199.
9 H. P. Spielmann, D. E. Wemmer and J.-P. Jacobsen, *Biochemistry*, 1995, **34**, 8542–8533.
10 H. Ihmels and D. Otto, *Top. Curr. Chem.*, 2005, **258**, 161–204.
11 A. Biancardi, T. Biver, A. Marini, B. Mennucci and F. Secco, *Phys. Chem. Chem. Phys.*, 2011, **13**, 12595–12602.
12 G. L. Silva, V. Ediz, D. Yaron and B. A. Armitage, *J. Am. Chem. Soc.*, 2007, **129**, 5710–5718.
13 V. Karunakaran, J. L. P. Lustres, L. Zhao, N. P. Ernsting and O. Seitz, *J. Am. Chem. Soc.*, 2006, **128**, 2954–2962.
14 X. Wang and U. J. Krull, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 1725–1729; R. Lartia and U. Asselyne, *Chem.–Eur. J.*, 2006, **12**, 2270–2281; L. Bethge, I. Singh and O. Seitz, *Org. Biomol. Chem.*, 2010, **8**, 2439–2448.
15 O. Köhler, D. V. Jarikote and O. Seitz, *ChemBioChem*, 2005, **6**, 69–77; E. Socher, A. Knoll and O. Seitz, *Org. Biomol. Chem.*, 2012, **10**, 7363–7371; S. Kummer, A. Knoll, E. Socher, L. Bethge, A. Herrmann and O. Seitz, *Bioconjugate Chem.*, 2012, **23**, 2051–2060.
16 A. Okamoto, *Chem. Soc. Rev.*, 2011, **40**, 5815–5828.

17 C. Holzhauser and H.-A. Wagenknecht, *Angew. Chem., Int. Ed.*, 2011, **50**, 7268–7272; C. Holzhauser and H.-A. Wagenknecht, *ChemBioChem*, 2012, **13**, 1168–1138.
18 C. Holzhauser, R. Liebl, A. Goepferich, H.-A. Wagenknecht and M. Breunig, *ACS Chem. Biol.*, 2013, **8**, 890–894; C. Holzhauser and H.-A. Wagenknecht, *J. Org. Chem.*, 2013, **78**, 7373–7379.
19 N. I. Shank, H. H. Pham, A. S. Waggoner and B. A. Armitage, *J. Am. Chem. Soc.*, 2013, **135**, 242–251.
20 J. W. Lee, M. Jung, G. R. Rosania and Y.-T. Chang, *Chem. Commun.*, 2003, 1852–1853; Q. Li, Y. Kim, J. Namm, A. Kulkarni, G. R. Rosania, Y.-H. Ahn and Y.-T. Chang, *Chem. Biol.*, 2006, **13**, 615–623; Y. T. Chang and Q. Li, *Nat. Protocols*, 2006, **1**, 2922–2932.
21 M. M. Rubner, C. Holzhauser, P. R. Bohländer and H.-A. Wagenknecht, *Chem.–Eur. J.*, 2012, **18**, 1299–1302.
22 W. Schmucker and H.-A. Wagenknecht, *Synlett*, 2012, 2435–2448.
23 C. Prunkl, M. Pichlmaier, R. Winter, V. Kharlanov, W. Rettig and H.-A. Wagenknecht, *Chem.–Eur. J.*, 2010, **16**, 3392–3402.
24 F.-L. Benitez, J. L. Acero, F. J. Real, G. Roldan and E. Rodriguez, *Water Res.*, 2013, **47**, 870–880.
25 Y. Wang, O. Haze, J. P. Dinnocenzo, S. Farid, R. S. Farid and I. R. Gould, *J. Org. Chem.*, 2007, **72**, 6970–6981.
26 J. Jung, W. Li, A. Boyd, Y. Zhang, V. L. Colvin and W. W. Yu, *J. Hazard. Mater.*, 2012, **237–238**, 247–255.