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Solid-phase synthesis of asymmetric cyanine dyes

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Abstract—An efficient solid-phase synthesis of asymmetric cyanine dyes is described. Combinatorial synthesis and spectroscopic analysis of four dyes was carried out. The yields are quantitative after 20 h, but the reaction can be halted earlier, since it is shown that the starting material does not disturb the fluorescence measurements in these binding studies.

Cyanine dyes have been known for a long time and are used in a variety of applications,[1] such as photosensitisers for colour photography,[2] markers for flow cytometry,[3] studies and detection of nucleic acids[4] and as phototherapeutic agents.[5]

Asymmetric cyanine dyes consist of two different heteroaromatic fragments conjugated by a mono- or polymethine chain (Fig. 1). By varying the length of this chain, the photophysical properties of these dyes can be altered.

Additionally, the absorption and fluorescence characteristics of these dyes are sensitive to environmental conditions, e.g. the fluorescence quantum yield of certain cyanine dyes is drastically increased upon interaction with nucleic acids. This extraordinary increase in fluorescence has been assigned to restricted rotation between the two heteroaromatic fragments upon binding to nucleic acids.[6] Since replacement or addition of substitutents in the dye molecule may lead to better or new properties, the development of efficient ways to make cyanine dyes is of great value.

Recently, we presented a new probe technique for the detection of specific nucleic acid sequences in homogeneous solution.[7,8] The light-up probe consists of an asymmetric cyanine dye tethered to a peptide nucleic acid, PNA, sequence. When the PNA hybridises to its target DNA, the dye interacts with the nucleotides and becomes fluorescent. Light-up probes are synthesised by peptide solid-phase chemistry and the cyanine dye is coupled to the PNA nucleotides as the last step, by formation of an amide bond between the acid group of the linker-modified dye and the primary amine of the final base.[7]

The aim of this study was to synthesise asymmetric cyanine dyes using solid-phase chemistry. This approach would make the production of pure dyes on a

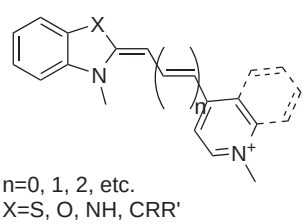

Figure 1. The general formula of the cyanine dye.

Abbreviations: **BO**, *N*-carboxyethyl-4-[3-(3-methyl-3*H*-benzothiazol-2-ylidene methyl)]pyridinium salt; **BO-3**, *N*-carboxyethyl-4-[3-(3-methyl-3*H*-benzothiazol-2-ylidene)propenyl]pyridinium salt; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Et_3N, triethylamine; Fmoc, fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; MBHA, *p*-methylbenzhydrylamine; MS, mass spectrometry; PNA, peptide nucleic acid; TFA, trifluoroacetic acid; **TO**, *N*-carboxyethyl-4-[3-(3-methyl-3*H*-benzothiazol-2-ylidene methyl)]quinolinium salt; **TO-3**, *N*-carboxyethyl-4-[3-(3-methyl-3*H*-benzothiazol-2-ylidene) propenyl]quinolinium salt; **TO-N′-10**, *N*-methyl-4-[3-(3-carboxydecyl-3*H*-benzothiazol-2-ylidene methyl)]quinolinium salt; Φ_F^{free}, fluorescence quantum yield for free dye; Φ_F^{bound}, fluorescence quantum yield for dye bound to calf thymus DNA.

Keywords: cyanine dye; solid-phase; combinatorial; DNA; light-up probe.

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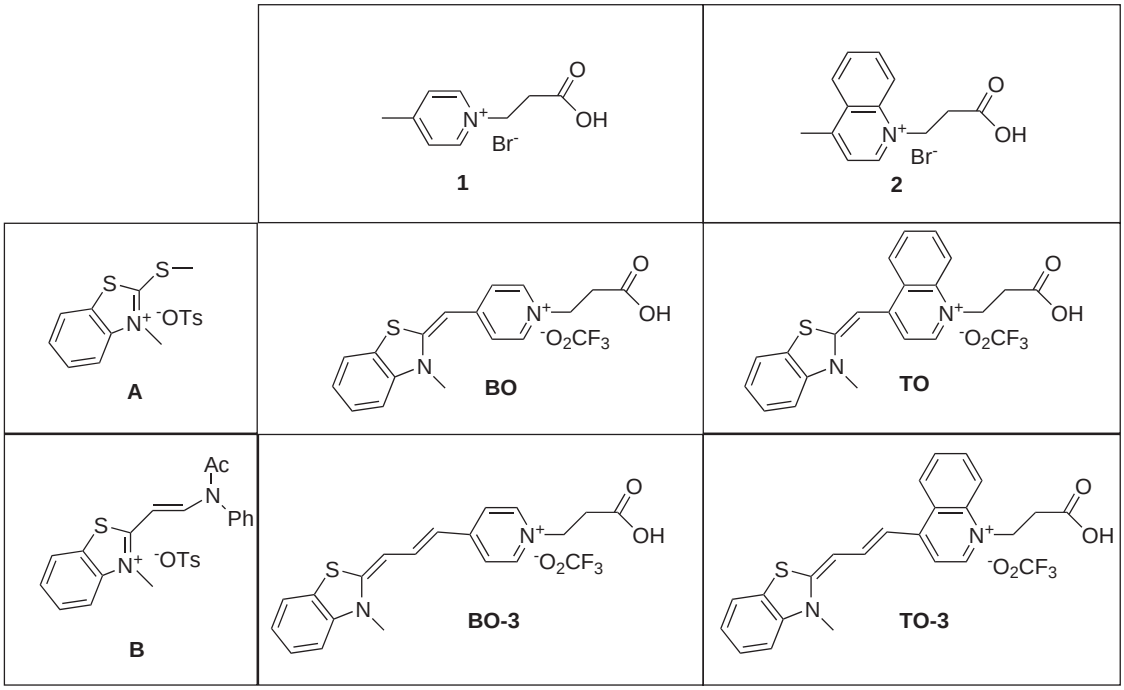


Scheme 1. Combinatorial synthesis of four cyanine dyes.

small scale easier, and a combinatorial methodology would enhance the efficiency of dye development. Additionally, the synthesis of light-up probes would be facilitated, by omitting the need for pre-synthesis and laborious purification of the linker-modified dyes. Cyanine dyes substituted with a carboxyl linker on the benzothiazole nitrogen can be problematic to handle, since some of these are sensitive to light and sometimes undergo intramolecular ring closure reactions when stored.[9]

Four differently coloured cyanine dyes were synthesised to illustrate the combinatorial possibilities of solid-phase dye synthesis. The starting materials were combined according to Scheme 1.

Compounds **1** and **2** were attached to the solid-phase resin,[†] and compounds **A** and **B** were subsequently condensed with the coupled picoline and lepidine moieties.[‡] The visual results of the reactions were four differently coloured products: **BO** (yellow), **TO** (orange), **BO-3** (purple) and **TO-3** (blue), where the colour changes upon addition of **A** and **B** were immediate. Following cleavage from the resin,[§] MS analysis[¶] showed the expected product masses, but also a fraction of the unreacted starting materials attached to the resin, i.e. the masses of the picoline and lepidine moieties.

The spectroscopic properties of the four crude dyes, free in solution as well as in the presence of calf thymus DNA, were investigated. Absorption spectra of the free dyes are presented in Fig. 2.

As shown in Table 1, all the four dyes synthesised here exhibit similar properties when interacting with DNA: the strong fluorescence enhancement associated with the restricted rotation upon intercalation.[6]

BO-3 is somewhat different compared to the other three dyes, by having a substantially higher fluorescence quantum yield when free in solution, Φ_F^{free}. Measurements conducted on purified **BO-3**, synthesised by solution phase techniques, gave the same high Φ_F^{free} which, accordingly, is the true dye fluorescence and not an increased fluorescence originating from any by-product or contamination. Therefore, **BO-3** is not useful in applications in which a large fluorescence increase associated with DNA binding is critical. The high Φ_F^{bound} for **BO-3** could, however, be beneficial in certain cases to reach a lower detection limit. The most appropriate

[†] De-protection of the Fmoc rink-amide MBHA polystyrene resin (50 mg, substitution level 0.55 mmol/g) was carried out with 25% piperidine in DMF for 30 min. The resin was split into two 25 mg portions and the acid-linker picoline and lepidine derivatives (Scheme 1, compounds **1** and **2**) were coupled to the resins in 4-fold molar excess to the substitution level, using the conventional reagents HBTU and DIEA in 50% DMF/pyridine (300 μl). Reactions were allowed to proceed for 2 h at ambient temperature and the resin was washed with DMF (2×2 min) after completion. Finally, the resins were split into two 10 mg portions each.

[‡] The benzothiazole compounds **A** and **B** (Scheme 1) were condensed (4-fold molar excess) with the resin-coupled **1** and **2** in the presence of Et_3N (5-fold molar excess) in DCM (300 μl) for 3 h at ambient temperature. The resins were finally washed with DCM (2 min) and MeOH (10 min).

[§] Products were cleaved by treatment with 95% TFA/water (300 μl) for 90 min, and subsequently evaporated.

[¶] Positive FAB-MS spectra were obtained on a VG ZabSpec instrument and measurements were carried out using a primary atom beam of Cs (19 keV) and glycerol as matrix.

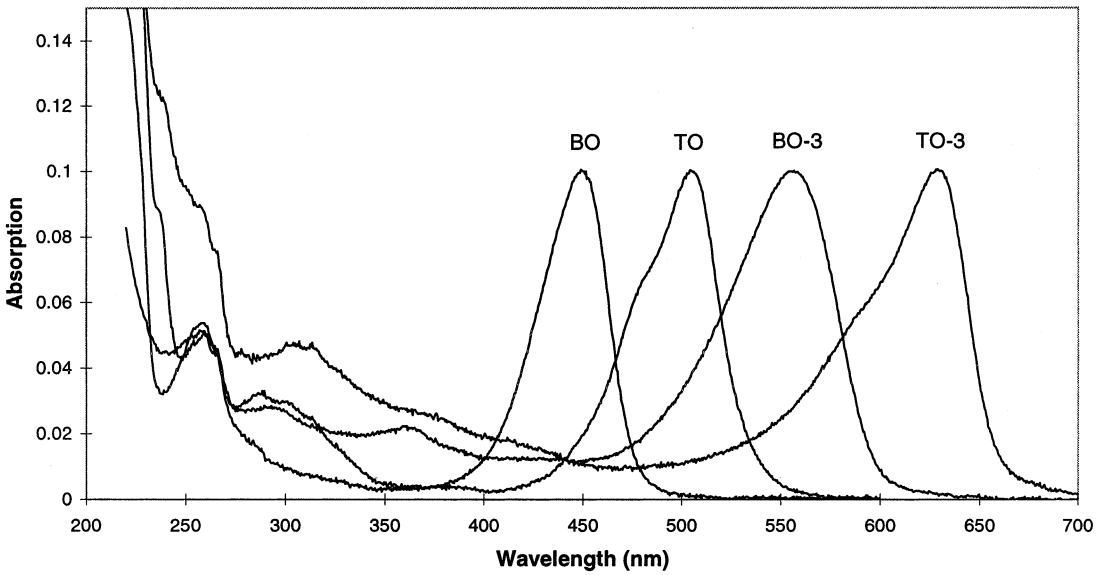


Figure 2. Normalised absorption spectra of the solid-phase synthesised dyes after cleavage from the resin. Spectra were recorded on a Cary 40 instrument, using a scan rate of 150 nm/min.

Table 1. Fluorescence properties of the combinatorially synthesised dyes

Dye	λ_{ex} (nm)[a]	λ_{em}^{free} (nm)	λ_{em}^{bound} (nm)[b]	Φ_F^{free}[c]	Φ_F^{bound}	$\Phi_F^{bound}/\Phi_F^{free}$
BO	420	480	488	0.00020	0.083	410
TO	470	515	532	0.00016	0.23	1400
BO-3	515	595	605	0.011	0.26	24
TO-3	590	650	658	0.00026	0.048	180

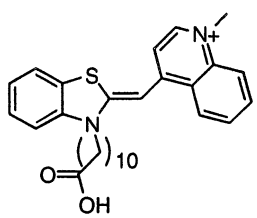
[a] Fluorescence spectra were recorded on an ISS PC1 instrument at ambient temperature using 4 nm bandwidths.
[b] The bound state characteristics were determined in the presence of calf thymus DNA at a dye:basepair ratio of 1:30.
[c] Fluorescence quantum yields, Φ_F, were determined relative to fluorescein in 0.1 M NaOH, assuming a Φ_F of 0.93.

dye for DNA probing is **TO**, with its high Φ_F^{bound} and high fluorescence increase upon DNA binding. For multiplexing purposes, **TO** can be used in combination with **BO** and/or **TO-3**. The determined spectroscopic properties of the solid-phase synthesised dyes are comparable to the properties of the corresponding commercially available PRO-variants of the dyes.[10] Small absorption and emission shifts are observed, which can be assigned to the different linker structures of the molecules.

Solid-phase synthesis of **TO-N′-10**,[‖] the dye commonly used in light-up probes,[7] was carried out to investigate the efficiency of the condensation step. Since the **TO-N′-10** dye has its carbon linker on the benzothiazole nitrogen, this synthesis was carried out by coupling of the linker-modified benzothiazole salt to the resin,[†] and subsequently condensing the quinolinium salt to it. The condensation efficiencies using the bases DIEA in 50% DMF–pyridine and Et_3N in DCM were compared. After 20 h, the Et_3N activated reaction had proceeded to 100%, while the one containing DIEA still contained 17% of the unreacted benzothiazole. The reaction time of the condensation, using Et_3N in DCM, was subsequently investigated. Aliquots of the resin were removed at certain time points during the reaction and subjected to cleavage[§] and MS analysis.[¶] The proceeding reaction is illustrated in Fig. 3, as a plot of the disappearance of benzothiazole reagent, versus reaction time of the experiment.

If the purpose of the dye synthesis is to produce a library for screening of the fluorescence properties of the dyes, and the assay time needs to be reduced, the reaction can be halted before it has gone to completion. This is because the small amounts of unreacted starting material remaining do not seem to affect or disturb the fluorescence measurements in this type of binding study.

[‖] *N*-Methyl-4-[3-(3-carboxydecyl-3*H*-benzothiazol-2-ylidene methyl)-quinolinium salt.



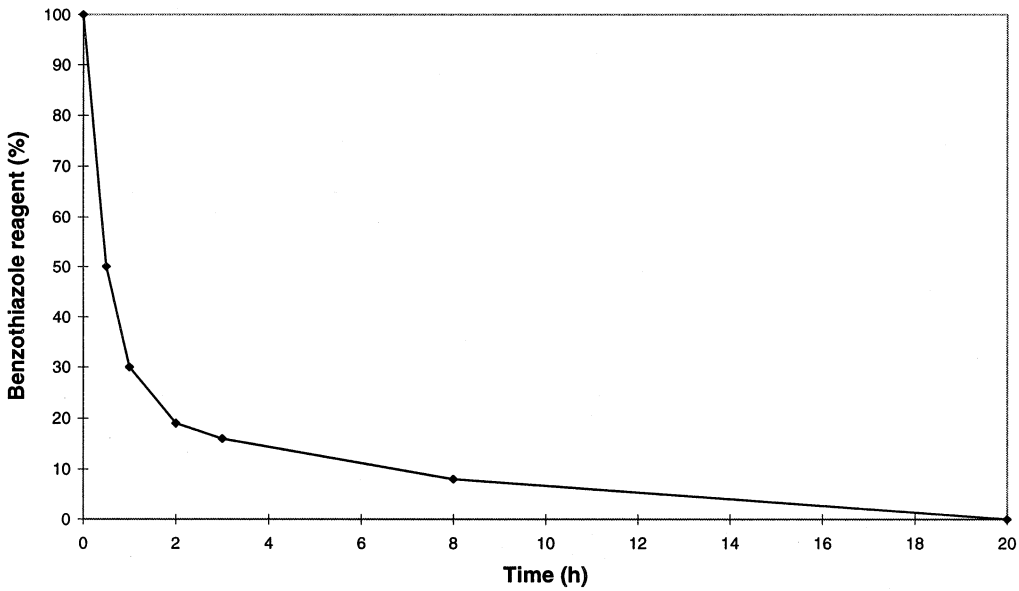


Figure 3. Time course of the condensation reaction for formation of **TO-N′-10**, illustrated as the percentage benzothiazole reagent remaining at certain time points after the reaction start. The reaction mixture composition was determined by MS analysis.¶

Finally, a light-up probe was synthesised, in which the **TO-N′-10** dye‖ was condensed on the PNA sequence as described above. Since light-up probes are purified by HPLC before use, probes containing only the benzothiazole moiety will be separated from the correct probes and thus, the condensation reaction time is not that critical. The light-up probe synthesised in this way has the same properties as the corresponding probe synthesised in the ordinary way, where the dye is coupled to the PNA bases as the last synthesis step.[7]

In summary, we herein show the synthesis of different asymmetric cyanine dyes, utilising solid-phase chemistry. The complete yields obtained when using a sufficient condensation time make purification of the dyes unnecessary. The combinatorial approach makes it easier to develop new dyes, and the small synthetic scale is convenient for screening purposes. Finally, this methodology facilitates synthesis of light-up probes, since the production of pure dyes prior to probe synthesis is unnecessary. The starting materials are easier to store, they are not sensitive to light or subject to ring closure, which has been a problem with pre-synthesised dyes.[9]

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