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Synthesis of a benzotriazole azo dye phosphoramidite for labelling of oligonucleotides

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Abstract—The synthesis of a benzotriazole azo dye phosphoramidite and the subsequent use in solid phase synthesis of oligonucleotides is reported. The azo dye is shown as a surface enhanced resonance Raman label for oligonucleotides that is capable of immobilisation of the oligonucleotide on metal surfaces such as silver nanoparticles.

Rapid and sensitive detection of specific DNA sequences is a requirement in the post human genome era. The information produced from the human genome project has yielded a host of targets that can be used in a number of applications including diagnostics and pharmacogenomics.[1,2] To make use of this information new methodologies that can detect specific DNA sequences or defects rapidly, accurately and at low cost need to be developed. A number of promising approaches exist and they tend to rely on the addition of an external label to the oligonucleotide sequence used as a probe. The most prevalent techniques use fluorescence spectroscopy and favour the use of quenching molecules to provide systems that are homogeneous in nature.[3–5] An alternative technique is surface enhanced resonance Raman scattering (SERRS) which makes use of a coloured molecule adsorbed onto a suitable metal surface. The range of labels that can be used in SERRS far exceeds those possible in fluorescence but to date a specifically designed SERRS phosphoramidite for labelling of oligonucleotides has not been reported.

We report here the synthesis of a benzotriazole azo dye phosphoramidite for use in routine solid phase synthesis of oligonucleotides. Benzotriazole azo dyes have previously been shown to be excellent labels for SERRS due to the strong metal complexing ability of the benzotriazole.[6,7] We have reported the addition of benzotriazole dyes to an oligonucleotide via a manual coupling step after the oligonucleotide had been synthesised.[8] These approaches were suitable to allow evaluation of the dye labelled oligonucleotides for SERRS but are not suitable for routine automated synthesis.

A number of routes to the dye phosphoramidite were attempted and the preferred route is shown in Scheme 1. The dye chosen to work with was synthesised by diazotisation of 5-aminobenzotriazole and subsequent coupling to 3,5-dimethoxyphenol in bicarbonate buffer. An attempt was made to alkylate 3,5-dimethoxyphenol prior to the azo coupling with a suitable linker containing a primary alcohol. Unfortunately the dye formation occurred in poor yield making this approach less favoured.

Thus, the first step in the synthesis was formation of the benzotriazole azo dye (**1**). Although the starting material was purchased as 5-aminobenzotriazole subsequent characterisation of azo dyes has indicated that the most stable isomer is the 6-isomer of the dye.[9] Additionally, the most nucleophilic site in the dye is now the triazole amine and not the phenol as may be expected. This is due to the more extensive delocalisation of the dimethoxyphenol ring into the azo bond compared to the benzotriazole. Protection of the benzotriazole group was achieved by use of trityl chloride in pyridine with DMAP. Protecting groups that form via an amide such as benzoyl and pivaloyl were unstable when used with this dye due to the excellent leaving group nature of the benzotriazole azo. The trityl group was found to be stable and also has the added benefit of allowing 'trityl on' purification of the final oligonucleotide once the dye has been added at the 5′-terminus. Alkylation of the phenol in acetonitrile using 6-bromohexanol with DBU yielded the protected dye (**3**) with a linker suitable for phosphitylation. The final step in the synthesis

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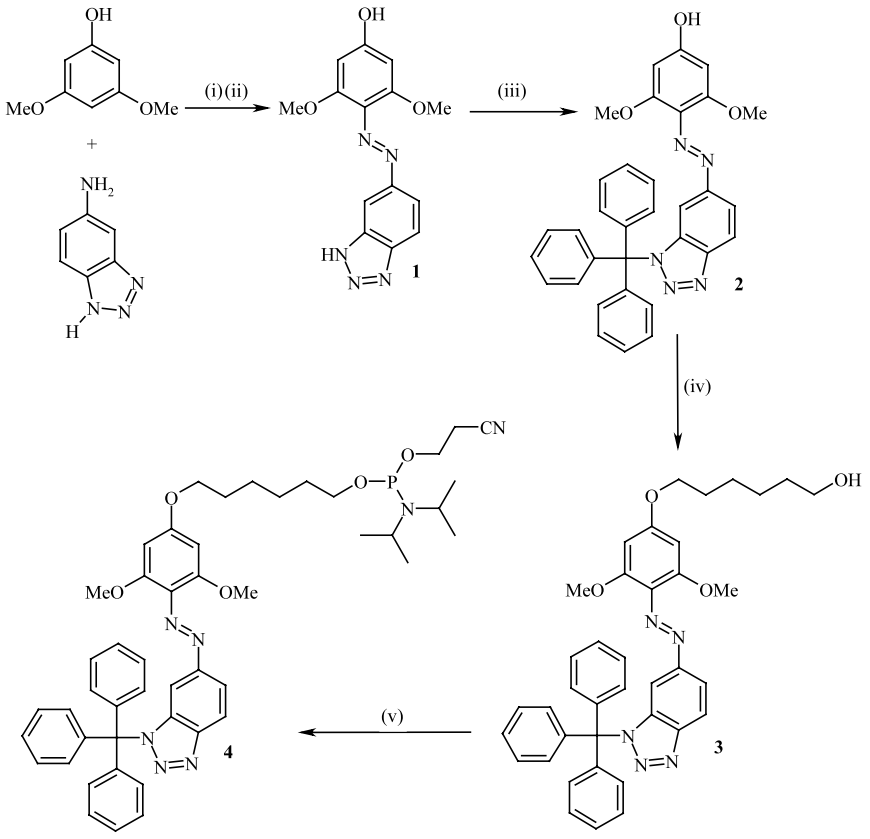

Scheme 1. *Reagents and conditions*: (i) HCl, $NaNO_2$; (ii) $NaHCO_3$ pH 8.0, 64%; (iii) TrCl, Py, DMAP, 69%; (iv) $Br(CH_2)_6OH$, DBU, MeCN, 73%; (v) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphine, THF, DIPEA, 96%.

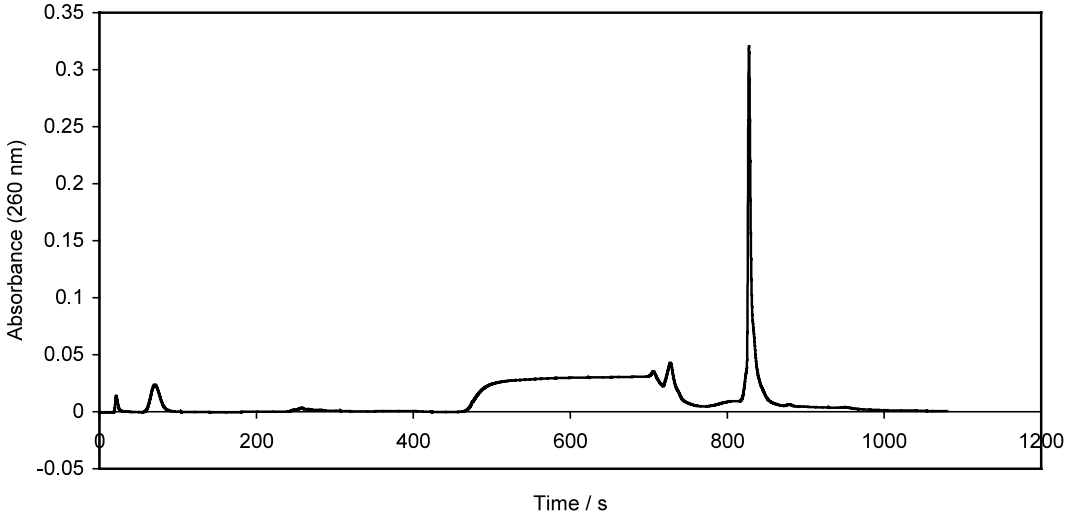

Figure 1. The HPLC trace of the benzotriazole dye labelled oligonucleotide 5′-BT Dye GAA TCA CGA ATA TCA ATT TGT AGC using an Oligo R3 column at 5 ml/min and monitoring at 260 nm. Buffers: A = 0.1 M triethylammonium acetate pH 7.0 B = 100% acetonitrile C = 5% TFA (aq) Gradient: 100% A, 3 min; 0–5% B in A, 3 min; 5% B in A, 3 min; 100% C, 4 min; 5% B in A, 2 min, 5–25% B in A, 6 min.

was the production of the reactive phosphite (**4**) by use of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in THF with DIPEA.

The phosphoramidite was then used on a solid phase synthesiser to produce an oligonucleotide using a coupling time of 15 min. The trityl group was left on the benzotriazole azo dye to aid the purification by HPLC after cleavage from the solid support and removal of the other protecting groups by ammonia. On column detritylation was achieved by use of 5% aqueous TFA on an Oligo R3 column and the coupling efficiency of

the phosphoramidite was estimated as being >90% (Fig. 1).

Subsequent characterisation of the oligonucleotide proved to be difficult due to the metal complexing nature of the dye. This meant that the oligonucleotide was not easily released from the plate used for MALDI and gave broad, indistinct peaks. The presence of the benzotriazole dye was used to provide surface enhanced resonance Raman scattering, SERRS using silver nanoparticles. SERRS is a type of Raman spectroscopy that is up to 10^{10} more sensitive than normal Raman spectroscopy and provides a molecular fingerprint of a coloured analyte adsorbed onto a suitable metal surface.[10] The metal surface used in this case was that of citrate reduced silver nanoparticles, which give excellent SERRS when aggregated. When examining oligonucleotides, using spermine further enhances the intensity and signal to noise ratio of the enhanced scattering.[11,12] The SERRS spectrum from the modified oligonucleotide is shown in Figure 2. The peaks arise from vibrations of the benzotriazole azo dye and are similar to those obtained for the dye that has not been conjugated to the oligonucleotide.

This class of labelled oligonucleotide has different characteristics to that of previously reported SERRS active oligonucleotides as it has a specific metal complexing group attached to the 5′-terminus. This allows immobilisation of the oligonucleotide onto the surface of silver metallic nanoparticles in a manner similar to that used by Mirkin for thiolated oligonucleotides and gold nanoparticles.[13,14] A number of experiments were conducted to optimise the conditions for the attachment and SERRS.

When using nanoparticles to provide SERRS, it is best that they are aggregated into discrete clusters as this changes the electric field and maximises the surface enhancement of the scattered light. A number of compounds can be used to aggregate the nanoparticles, however, in previous studies we found that spermine was optimal for labelled oligonucleotides. The spermine interacts with the phosphate backbone and allows any modification such as the benzotriazole dye to attach to the surface more easily. In addition, the spermine reduces the charge around the nanoparticles causing aggregation. The benzotriazole dye labelled oligonucleotide was no different to the other in that spermine gave better SERRS than sodium chloride or poly(L-lysine). However, we found that better signals were obtained when less spermine was used in contrast to the oligonucleotides labelled with fluorescent dyes.[15] In this case the overall concentration of spermine that gave the best SERRS was 5×10^{-6} M. This was attributed to the different mode of surface attachment. Another noteworthy point was that the highest intensity signals were obtained immediately after the addition of the spermine as the signal strength decreased dramatically with time.

The final aspect that was optimised was the time required to complex effectively to the silver nanoparti-

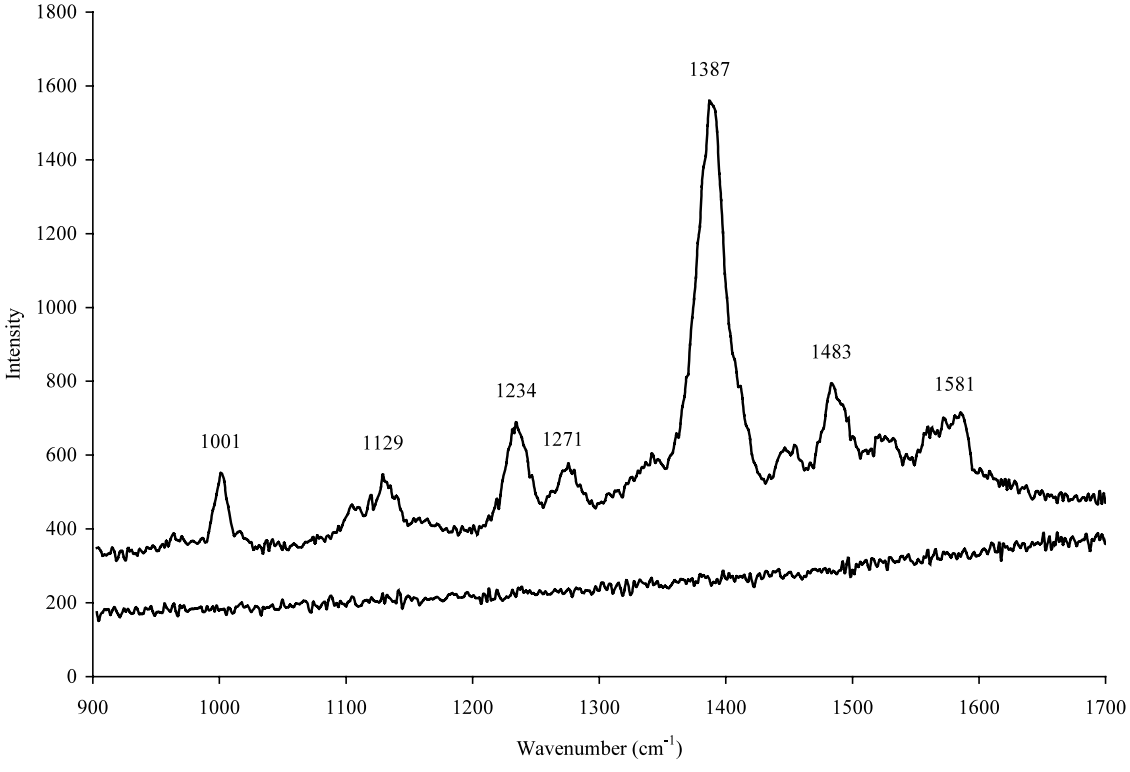

Figure 2. SERRS spectra of the benzotriazole azo dye modified oligonucleotide using silver nanoparticles to provide the enhancement. 514.5 nm excitation was used on a mixture of 450 ml of silver nanoparticles, 25 μl of a 4×10^{-6} M solution of oligo with 25 μl of 0.001 M spermine.

cles. A number of experiments were set-up and left for different lengths of time ranging from 0 min to 24 h. The SERRS signals were monitored in each case using the optimised aggregation conditions from above and the intensity of the main peak at 1387 cm^{-1} (azo stretch) was used as a guide to the amount of labelled oligonucleotide complexed to the silver surface. The signals started as being pretty weak but then reached a maximum after 1 h and diminished slightly (12%) over the next 6 h before remaining constant for the next 18 h. Thus, to ensure maximum attachment of benzotriazole dye labelled oligonucleotides to silver nanoparticles they must be left for a least 1 h prior to further manipulation or examination.

In conclusion we have synthesised a benzotriazole azo dye phosphoramidite that was used in the solid phase synthesis of an oligonucleotide to provide a 5′-labelled species. The labelled oligonucleotide was capable of attaching to silver nanoparticles and gave excellent SERRS signals with spermine demonstrating the surface attachment.

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