CONTENTS

INTRODUCTION

Fluorophores are very useful and sensitive labels for biomolecules, and fluorescent labels are by far the most common labels used for synthetic oligonucleotides. Many different fluorophores can be readily attached to oligonucleotides and they form the basis of the detection systems used in DNA sequencing, forensic science and genetic analysis. The principles of fluorescence and the synthesis of fluorescent oligonucleotides are described below.

The major *applications* of fluorescent and fluorogenic oligonucleotides are discussed in the section on sequencing, forensic analysis and genetic analysis.

PRINCIPLES OF FLUORESCENCE

Prior to excitation, a fluorescent molecule is in the lowest vibrational level of the electronic ground state S_0. Excitation by the absorption of light of an appropriate wavelength produces a change to an excited vibrational level of the first excited singlet state S_1. After a very short time period (10^{-12} s) the lowest vibrational level of S_1 is reached by a series of non-radiative energy transfer processes known as internal conversion, in which the molecule collides with surrounding solvent molecules and loses energy as heat. The system remains at this level for a significantly longer period (10^{-9} s) before a transition occurs to the S_0 state with the emission of a photon. This process is known as fluorescence (Figure 1).

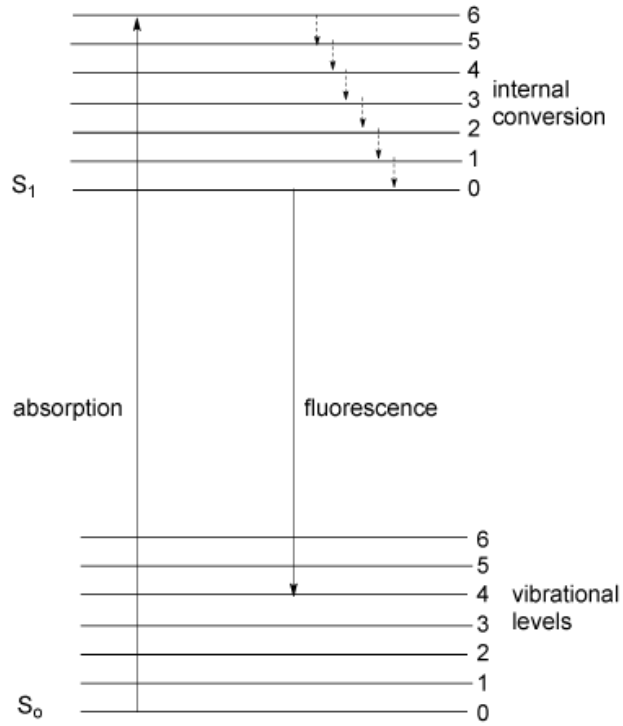

Figure 1 | **Jablonski diagram, showing the energy levels of a fluorescent molecule in solution**

Eventually photobleaching occurs and the molecule becomes non-fluorescent. Fluorescence emission is of lower energy than absorption and is therefore observed at a higher wavelength. The difference in frequency between excitation and emission is called the Stokes shift. A large Stokes shift facilitates the measurement of fluorescence and leads to a lower background signal.

COMMON FLUOROPHORES

Many different fluorescent dyes are available. All these molecules are organic dyes that emit in the visible region of the electromagnetic spectrum. They can be conveniently excited by laser light (Table 1).

Table 1 | Common fluorescent dyes; their associated wavelengths of absorption (excitation) and emission, and colours

Dye	Absorption wavelength / nm	Emission wavelength / nm	Colour
FAM	495	520	Green/yellow
TET	525	550	Orange/yellow
HEX	535	565	Pink
JOE	520	548	Pink/Green
Cyanine-3	550	570	Dark pink
TAMRA	550	575	Red
ROX	580	605	Purple
Cyanine-3.5	580	595	Purple/blue
Cyanine-5	650	670	Blue
Cyanine-5.5	675	695	Blue

Some of the most commonly employed fluorescent dyes are 6-carboxyfluorescein (FAM), its tetra- and hexachlorinated analogues (TET and HEX) (Figure 7), and carboxy-X-rhodamine (ROX,). However, their properties are not ideal; they have broad emission spectra, are non-fluorescent at low pH, and are susceptible to photobleaching. These inadequacies led to the development of alternatives such as the cyanine Dyes (Cyanine Dyes) which have the general structure shown in Figure 2. Their spectral properties can be tuned by the appropriate choice of heterocyclic nuclei (X

and Y) and the length of the polymethine chain (*n*) to provide fluorophores that emit in the range 500 to 750 nm. The groups R_1 − R_4 can be varied to provide the desired aqueous solubility.

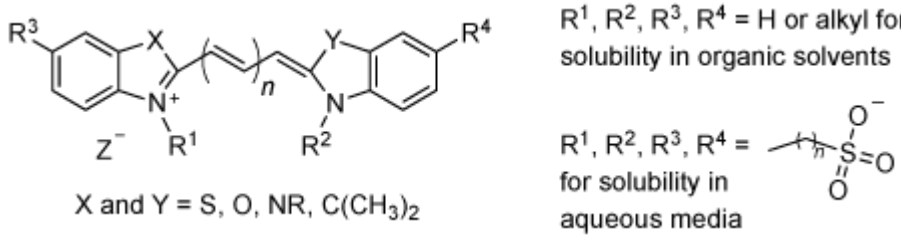

Figure 2 | **Cyanine dyes**General structure of the cyanine dyes. The spectral properties of the Cyanine dyes can be tuned by altering the heterocyclic nuclei (X and Y) and the length of the polymethine chain (*n*). The groups R_1 − R_4 can be varied to provide aqueous solubility.

For further information see FAM (fluorescein), HEX, JOE, ROX, TAMRA, TET, Texas Red® and others and Cyanine dyes. Other important fluorescent dyes include BODIPY® dyes, Alexa® dyes, ATTO dyes and Yakima Yellow.

FLUORESCENT BASE ANALOGUES

Standard nucleobases (A, G, C, T and U) are not fluorescent under standard conditions. Fluorescent base analogues are structural analogues of the standard bases that are fluorescent, while still forming hydrogen bonds with standard bases.

Fluorescent labels are commonly attached to the double helix at the end of a linker, which places the fluorophore relatively far from the DNA bases. This is useful in many circumstances, but sometimes it is necessary to incorporate a fluorophore closer to the DNA or RNA double helix, without perturbing the helix. As fluorescent base analogues are located rigidly in the double helix, their movement is restricted. This restricted movement results in a more predictable orientation of the fluorophore, an advantage in many applications such as fluorescence resonance energy transfer (FRET) and fluorescence anisotropy.

2-Aminopurine

2-Aminopurine (Figure 3) is the original, and most frequently used, fluorescent base analogue. It is highly fluorescent, and readily available.

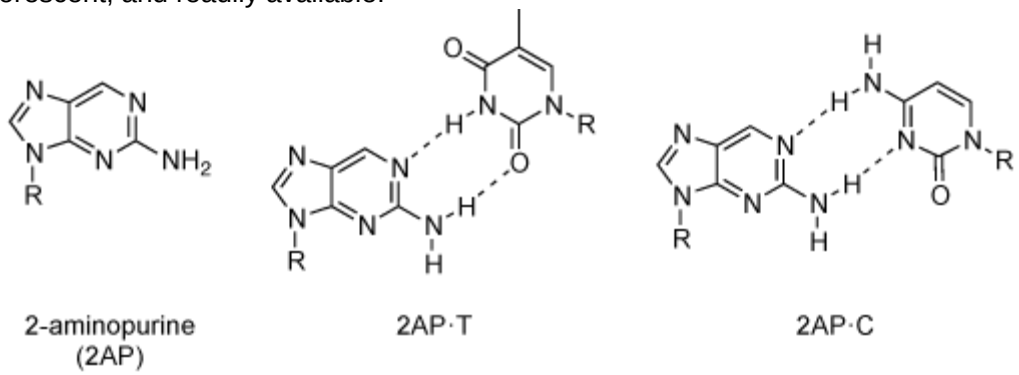

Figure 3 | **2-Aminopurine**Structure of 2-aminopurine (2AP), the first fluorescent base analogue, and structures of base pairs between 2-aminopurine and thymine (T) and cytosine (C).

Like almost all fluorescent base analogues, 2-aminiopurine has a quantum yield that is heavily dependent on its environment. The DNA duplex quenches the fluorescence of more fluorescent base analogues, limiting their usefulness. This effect can be pronounced: 2-aminopurine has a good quantum yield of 0.68 in water, but this decreases to less than 0.02 in single-stranded DNA and less than 0.01 in double-stranded DNA.

Tricyclic cytosine analogues

The tricyclic cytosine analogues (Figure 4), intended for use in antisense therapy but recently discovered to be strongly fluorescent, are the only fluorescent base analogues to have quantum

yields that are not affected appreciably by the environment. The quantum yield of the tricyclic cytosine analogue 1,3-diaza-2-oxophenothiazine (tC) does not vary much whether it is in monomeric form, in single- and double-stranded DNA; and whatever bases surround it.

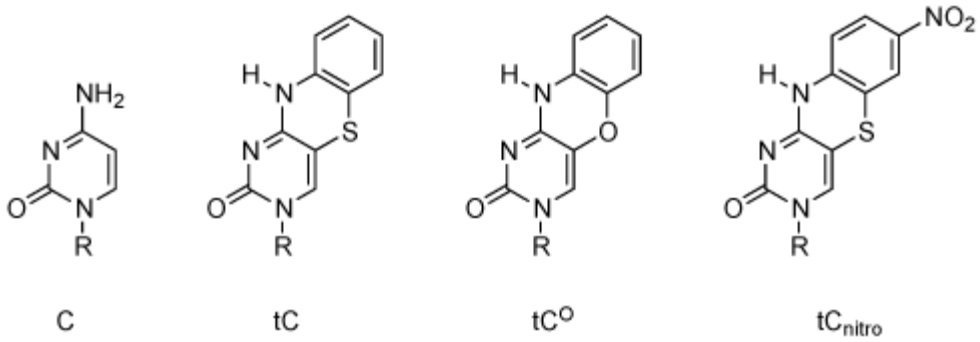

Figure 4 | **Tricyclic cytosine fluorescent base analogues**Structures of cytosine (C), and the tricyclic cytosine analogues 1,3-diaza-2-oxophenothiazine (tC), 1,3-diaza-2-oxophenoxazine (tC^o) and 7-nitro-1,3-diaza-2-oxophenothiazine (tC_{nitro}) − the only fluorescent base analogues with quantum yields not appreciably affected by environment.

Like cytosine, the tricyclic cytosine analogues form hydrogen-bonding interactions with guanine (Figure 5), but not with adenine. If cytosine is replaced with tC in a DNA duplex, the DNA adopts the normal B-form, and only small distortions in the helix are observed around the base analogue: tC is therefore an excellent fluorescent base analogue.

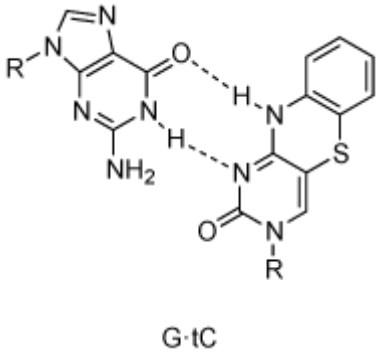

Figure 5 | **Structure of the G·tC base pair**

Relative to cytosine, tC has makes increased base stacking interactions in the DNA duplex, which means that duplexes containing tC are slightly more stable than analogous unmodified DNA.

The oxygen derivative of tC, tC^o (Figure 4), is also an excellent base analogue and, with an extinction coefficient more than double that of tC (Table 2), tC^o is the brightest fluorescent base analogue. The 7-nitro analogue of tC, tC_{nitro} (Figure 4), is not fluorescent under standard conditions, but is used as a quencher, in combination with tC or tC^o in a FRET pair.

Table 2 | Physical properties of fluorescent base analogues

Name	λ_{max} / nm (absorption)	λ_{max} / nm (emission)	*E* at λ_{max}	Φ (solution)	Φ (duplex)	τ / ns (solution)	τ / ns (duplex)
2-aminopyridine	303	371	3 600	0.68	< 0.01	-	-
tC	395	505	4000	0.20	0.16–0.21	3.7	6.3
tC^o	360	465	9000	0.30	0.22	3.4	4.1
tC_{nitro}	440	-	5400	-	-	-	-

E: extinction coefficient; Φ = quantum yield; τ = fluorescence lifetime. Data from Wilhelmsson, Q. Rev. of Biophys. 43, 2 (2010), 159–183 and other literature sources cited therein, and www.glenresearch.com

The tricyclic cytosine analogues tC, tC^o, and tC_{nitro} can be incorporated into oligonucleotides during solid-phase synthesis via their commercially-available phosphoramidites (Figure 6).

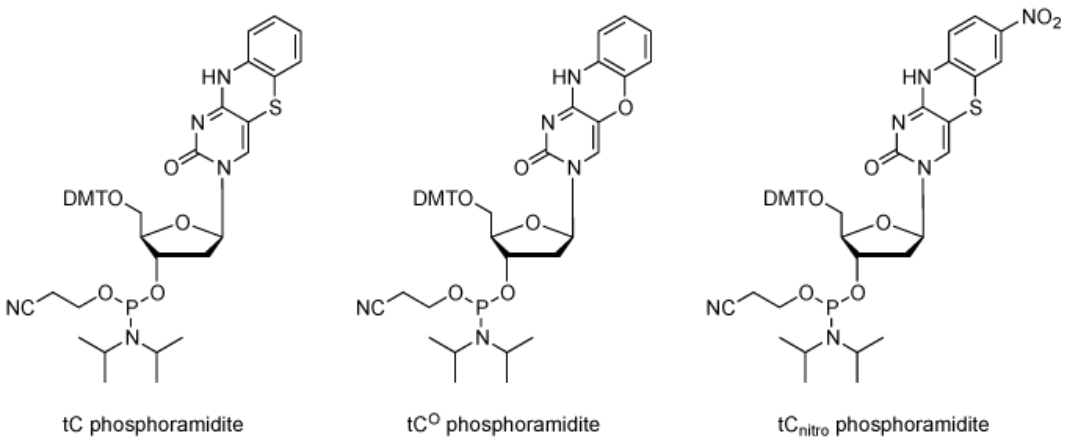

Figure 6 | **Tricyclic cytosine analogue phosphoramidites**Structures of tricyclic cytosine analogue phosphoramidites, for incorporation of the tricyclic cytosine analogues tC, tC^{O}and tC$_{nitro}$ into oligonucleotides during phosphoramidite oligo synthesis.

SYNTHESIS OF FLUORESCENT OLIGONUCLEOTIDES

Synthesis of oligonucleotides with the 5′-fluorescent dyes

FAM, HEX, TET and ROX

The synthesis of 5′-ROX and TAMRA oligonucleotides by post-synthetic labelling using 5′-amino-modified oligonucleotides and active esters of ROX and TAMRA is discussed in the article on chemically modified oligonucleotides. This method is used when the dye is unstable to oligonucleotide synthesis/deprotection conditions.
It is more convenient and efficient to add the fluorescent dye during solid-phase synthesis and a number of dye phosphoramidites have been synthesized to facilitate this. Fluorescein and its tetra- and hexachlorinated derivatives TET and HEX are sufficiently stable to survive oligonucleotide deprotection so they are added to oligonucleotides as phosphoramidite monomers (Figure 7).

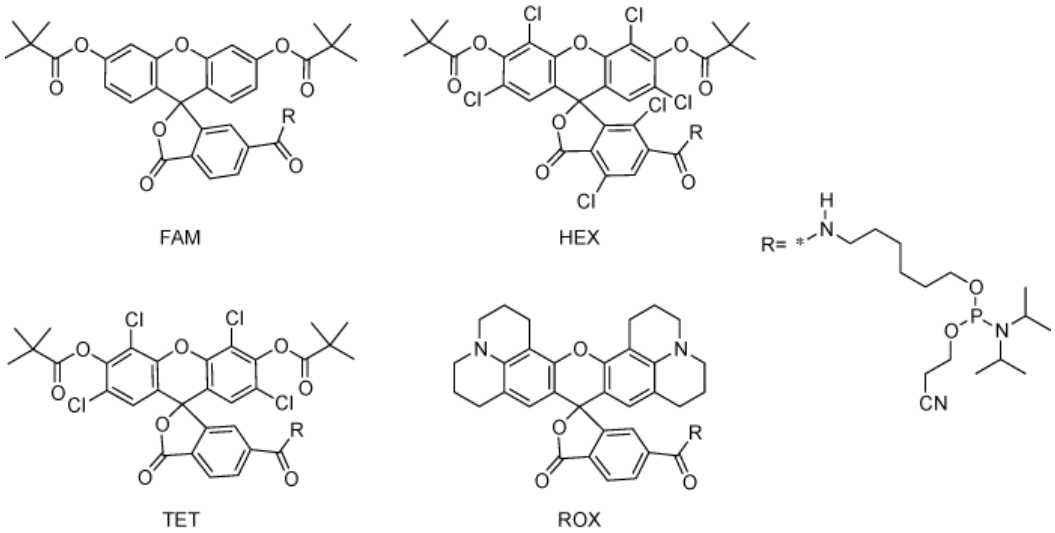

Figure 7 | **Fluorescein phosphoramidite monomer, and chlorinated derivatives**Structures of the fluorescein (FAM) phosphoramidite monomer, and its chlorinated derivatives HEX, TET and ROX.

The phenolic hydroxyl groups are protected as pivaloyl esters to prevent side-reactions during oligonucleotide synthesis, and a six-carbon spacer is inserted between the dye and the phosphoramidite group to distance the dye from the DNA and minimize the possibility of fluorescence quenching induced by the proximity of the dye to DNA bases.

Cyanine dyes

The Cyanine dyes are particularly useful when high quantum yields and resistance to photobleaching are important, and Cyanine dye analogues are available that cover a wide range of the visible absorption and emission spectrum. Some Cyanine dyes are available as phosphoramidites (Figure 8) for addition to the 5′-end of oligonucleotides during solid-phase synthesis. These monomers can be incorporated within the nucleotide sequence of oligonucleotides as they have a DMT-protected hydroxyl group and a phosphoramidite group. However, this would destabilize any duplex formed between the oligonucleotide and a complementary strand, as the monomers do not possess a heterocyclic base, and therefore have no means of base pairing. Cyanine dye phosphoramidites are therefore typically added to the 5′-terminus of oligonucleotides, although 3′-labelling is also carried out for special applications.

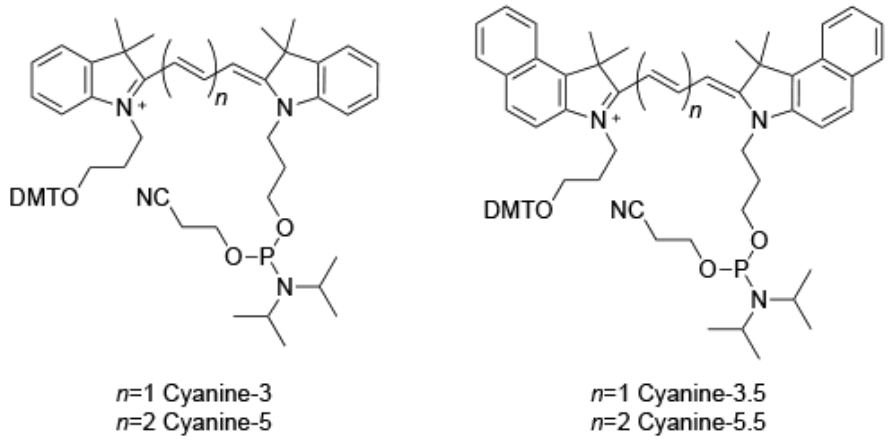

Figure 8 | **Cyanine dye phosphoramidite monomers**Structures of cyanine dye phosphoramidite monomers.
Cyanine dyes are not stable to the standard conditions of oligonucleotide deprotection (concentrated aqueous ammonia for 5 hours at 55 °C) but do withstand brief treatment (1 hour at 55 °C) with this reagent. Therefore it is necessary to use the more labile "ultramild" dmf-protected dG monomer rather than the more common isobutyryl dG phosphoramidite in the synthesis of Cyanine dye-labelled oligonucleotides (see Deprotection of heterocyclic bases).

Synthesis of oligonucleotides with internal fluorescent dyes

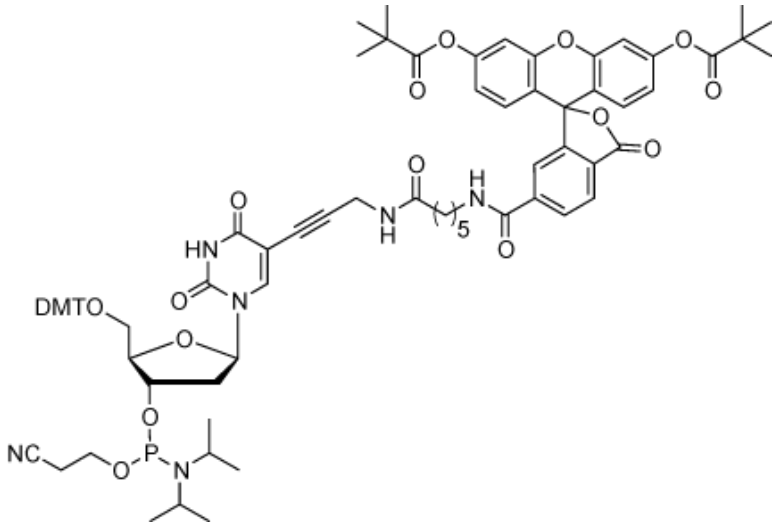

Figure 9 | **Fluorescein phosphoramidite monomer**Structure of the monomer used to label oligonucleotides with fluorescein at thymidine sites.
2′-Deoxyuridine is converted to its 5-iodo derivative by treatment with iodine monochloride, and the 5′-DMT group is then added. In a parallel reaction sequence an aminoalkyl side chain is built up by reacting propargylamine with TFA-protected 6-aminohexanoic acid. This is coupled to the 5-position of 5′-DMT-5-iodo-2′-deoxyuridine in a Sonogashira reaction using tetrakistriphenylphosphine palladium (0), copper iodide and triethylamine in DMF. The product of this reaction is a very useful

intermediate with a protected amino group attached via a linker to the 5-position of thymidine. The trifluoroacetyl protecting group can be removed by treatment with ammonia and a range of labels can be added as active esters or as carboxylic acids using a diimide coupling reagent (Figure 10). The monomer in Figure 9 can be made by this method. It is used to add internal labels to oligonucleotides at thymidine sites. The fluorescein and linker moieties do not affect the ability of the labelled thymine to form base pairs.

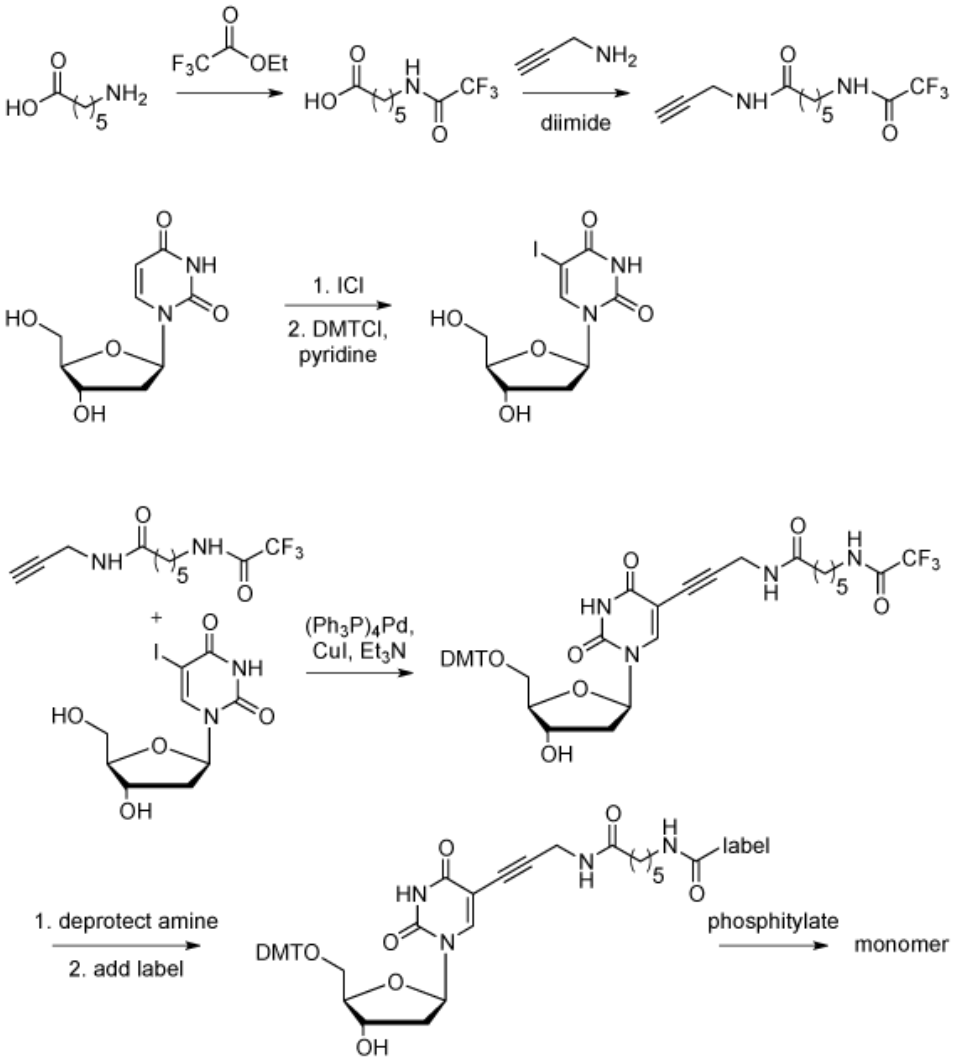

Figure 10 | **Fluorescently labelled phosphoramidite monomer synthesis**Scheme showing the attachment of a fluorescent label to a nucleoside phosphoramidite.