Oligonucleotides, part 5+: synthesis and fluorescence studies of DNA oligomers $d(AT)_5$ containing adenines covalently linked at C-8 with dansyl fluorophore

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ABSTRACT

The synthesis of oligodeoxynucleotides $d(AT)_5$ in which specific adenines are linked at C-8 position with dansyl fluorophores via a variable polymethylene spacer chain are reported. This was achieved by a strategy involving prelabelling at the monomeric stage followed by solid phase assembly of oligonucleotides to obtain regiospecifically labeled oligonucleotides. Several mono and polydansyl $d(AT)_5$ derivatives in which the fluorophore is linked via ethylene, tetramethylene and hexamethylene spacer arms were synthesised for a systematic study of their fluorescence characteristics. It was observed that (i) enhancements in fluorescence intensity and emission quantum yields are seen due to multiple labelling, (ii) the magnitude of enhancements are related to labelling configuration and (iii) quenching efficiency is minimal with shorter and rigid spacer arms. The results may aid rational design of multiple fluorescent DNA probes for non-radioactive detection of nucleic acids.

INTRODUCTION

Fluorescent oligonucleotides have recently attracted wide attention as probes for detection of nucleic acid hybridization (1,2), DNA sequencing (3−6), nucleic acid-protein interactions (7) and applications in medical diagnostics (6). Although fluorescent probes offer a facile and direct method for detection, they are less sensitive than other non-radioactive probes (8,9). One novel way to improve the sensitivity of this technique would be to label DNA at multiple internal sites (4,9,10), which is possible if fluorophores are linked to base residues, instead of the commonly employed 3′/5′-terminus (11−17). So far, most of the strategies for labelling (4,8,11−17) involve synthesis of nucleic acids incorporated with modified pyrimidines carrying nucleophilic handles such as alkyl sulphydryls or amines, followed by a labelling reaction. The regiospecificity and degree of labelling in such a reaction is governed by the specificity of the fluorophoric reagent used and the reaction conditions. Though this method is satisfactory for monolabelling, incorporation of multiple labels leads to an inseparable mixture of oligonucleotides with components that differ in number of labels per oligonucleotide chain. Recently, the alternative method of pre-labelling before the oligonucleotide assembly by phosphoramidite method has been reported (18). However, in all these cases, the C-5 position of base uridine (2′-deoxyuridine) or the 4-NH_2 of cytidine have been used for covalent derivatization of nucleic acids with fluorophores or other reporter ligands.

We report here a strategy for chemical incorporation of fluorescent labels directly before the oligonucleotide synthesis to enable control over the position and number of labels in each DNA molecule and a systematic study of the consequent fluorescence properties. This is achieved by synthesis of protected nucleotides, *1*, which contain a dansyl fluorophore attached to adenine base residue at C-8 and their insertion into DNA sequences at desired positions by solid phase phosphotriester method. The deprotected and purified products show retention of fluorophore. By this method, a number of oligonucleotides corresponding to the series $d(AT)_5$, *10-14*, in which specific adenine residues are labeled with dansyl fluorophore through a variable polymethylene spacer chain have been prepared. Though the synthesis of adenosine derivatives linked to biotin at C-8 has been reported (19), to our knowledge, the present work is the first report of the use of C-8 position of a purine for covalent fluorescent derivatization followed by incorporation into an oligonucleotide chain. The results not only demonstrate the feasibility of covalent attachment of multiple fluorophores, but also define necessary requirements for achieving optimum enhancements in fluorescence by multiple fluorophore labelling.

RESULTS AND DISCUSSION

Synthesis and characterization of 8-amino-(ω-dansylaminoalkyl) adenosine nucleotides *1*

The C-8 position of adenine is easily amenable for bromination and hence suitable for linking a fluorophoric side chain. The strategy employed for synthesis of 8-amino-(ω-dansylaminoalkyl)-adenosine nucleotides *1* is shown in scheme I. The treatment of

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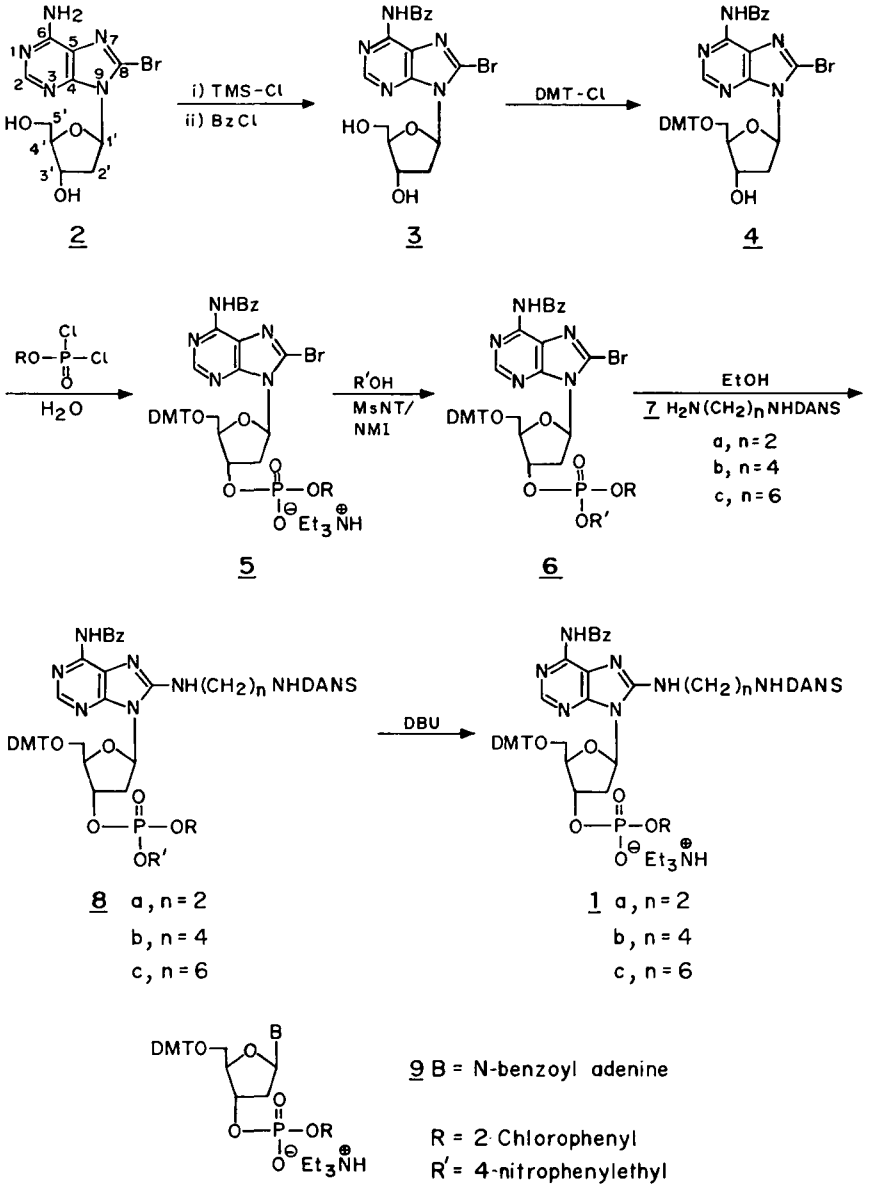

2'-deoxyadenosine with bromine water in sodium acetate buffer at pH 5.0 gave 8-bromo-2-deoxyadenosine (20). This was N-benzoylated by the transient protection method (21) to yield *3* and then protected at 5'-O position with 4,4'-dimethoxytrityl group to give *4*. Subsequent reaction of N,O-protected-8-bromo-2'-deoxyadenosine *4* with 2-chlorophenyl dichlorophosphate in pyridine containing molar equivalent H_2O (22,23) yielded the phosphodiester triethylammonium salt, *5*, which was converted to 8-bromoadenosine-3'-phosphotriester *6*, by condensation with 4-nitrophenylethanol in presence of 2-mesitylene sulphonyl chloride in pyridine (24). The 8-bromo adenylated phosphotriester *6* is the key intermediate in the present synthetic strategy. The dansyl fluorophore is introduced into *6* at C-8 by a facile aminoalkylation reaction with monodansylated diamines *7*. The latter was obtained from ω-diaminoalkanes (1,2-diaminoethane, 1,4-diaminobutane and 1,6-diamino hexane) by reaction of excess diamines with dansyl chlorides in dry dioxane (19) followed by silica gel chromatography. The reaction of excess *7* with *6* in refluxing ethanol for 6–8 hr. gave the fluoro conjugate *8* in about 70% yield. The removal of 4-nitrophenylethyl protecting group at phosphate residue was effected by treatment with 1,8-diazobicyclo-(5,4,0)-undec-7-ene to yield the dansylated deoxynucleotide monomers *1* needed for solid phase phosphotriester synthesis. No N-debenzoylation was observed at this stage due the presence of basic amine groups in *7*. It may be pointed out that the concentration of amine at the condensation stage is only about 1M whereas for debenzoylation higher base concentration (20M) is required.

The phosphodiester monomers *1* were purified and isolated as corresponding triethylammonium salts by silica gel chromatography and characterized by ^1H (table-1) and ^{31}P NMR spectroscopy. All derivatives *1* gave a single ^{31}P resonance indicating the homogeneity of these compounds with respect to phosphate content. Further, the ^{31}P chemical shift values were similar to those found in other similar phosphate derivatives. The phosphorylation reactions are best done before aminoalkylation step; otherwise minor side products arising due to N-phosphorylations were observed and these decrease the yield of the desired products. Moreover the present strategy consisting of aminoalkylation subsequent to phosphorylation involves a common precursor *6* from which direct access to several derivatives containing different spacer arms at C-8 is possible

Table. 1:^{1}H NMR Chemical Shifts*

No.	H1′*t*	H2′2″*m*	H3′*dd*	H4′*b*	H5′5″*b*	N(CH$_3$)$_2$*s*	NHCH$_2$	(CH$_2$)$_n$*b*
5	6.41	2.60	5.54	4.40	3.20	–	–	–
6	6.50	2.60	5.30	4.55	3.40	(2.93,PhCH$_2$;3.90,t,OCH$_2$)		
1a	6.45	2.50	5.20	4.30	3.44	2.75	2.90(t)	–
1b	6.51	2.50	5.21	4.38	3.40	2.80	2.90(q)	2.10
1c	6.45	2.65	5.20	4.34	3.40	2.80	3.00(m)	1.70

*Solvent: $CDCl_3$, Reference: Tetramethylsilane.

by a simple variation of the diamine component during the alkylation step.

Synthesis and characterization of fluorescent dansyl oligonucleotides d(AT)$_5$ *10-14(a-c)*

The oligonucleotides of the series d(AT)$_5$ were assembled by standard solid phase phosphotriester method (25) on T-CPG resin, involving chain growth in 3′-5′ direction. At predetermined stages during the synthesis, the dansylated adenosine nucleotides *1* were employed for coupling (see experimental) instead of the standard protected adenosine diesters *9*. The trityl analysis at each condensation step indicated that the coupling rates and efficiency were unaffected by the presence of additional side chain containing fluorophoric group. At the end of the assembly, the oligonucleotides were subjected to standard deprotection procedures, followed by gel filtration (sephadex G-15) to remove the non-nucleotidic contaminants. The void volume peak containing the crude oligonucleotide product was purified by FPLC over Mono Q (Pharmacia) anion exchange column followed by checking the purity on a Pep RPC (Pharmacia) column (figure 1). The purity of compounds as estimated from FPLC was over 95%. As expected, the dansylated oligonucleotides are more hydrophobic than unlabeled d(AT)$_5$ and so elute later in reverse phase separation. The various sequences synthesised are shown in table 2.

The UV absorbance of the products at 335nm (broad band) along with that due to DNA at 260nm (figure 2) and the fluorescence spectra with emission at 530nm on excitation at 335nm (figure 3) clearly indicated the retention of the dansyl groups after all deprotection reactions. The UV absorption spectra of the fluorophore labeled oligomer duplexes exhibit the characteristic band of both DNA and the label. For comparison purposes, the absorption spectra of the labeled and unlabeled DNA (figure 2, inset) duplexes and that of N-monodansyl hexanediamine are all presented. The UV absorption due to dansyl group in the labeled oligomers (ex,*11b* and *12a*) at 335nm is similar to that seen in the unlabeled oligomer. Due to the large differences in the extinction coefficients of dansyl absorption at 330nm and the DNA base absorption at 260nm and the relatively high molar equivalent concentrations of bases compared to dansyl group in the labeled oligomers, the dansyl UV band at 330nm is not well resolved from DNA at 260nm band and appears as a weak tail. Nevertheless, the dansyl presence in the oligomers *10-14* is evident from a comparison with the UV spectra of d(AT)$_5$, which shows a flat profile beyond 300nm.

Fluorescence studies of dansylated oligonucleotides

The fluorescence spectra of the various labeled oligomers are qualitatively similar to that of N-monodansyl hexanediamine (figure 3a), though the relative intensities of various derivatives showed differences. All sequences are self-complementary and expected to be present as duplexes under experimental conditions.

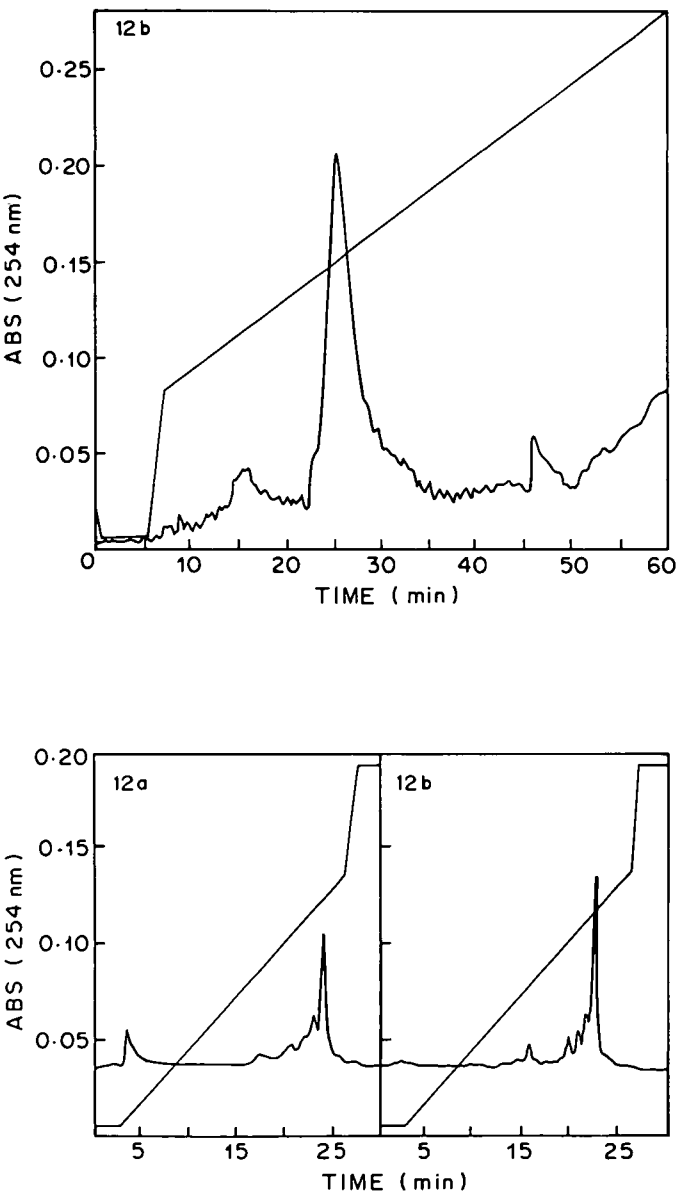

Figure 1. FPLC analysis of some labeled DNA oligomers. (a) and (b) Ion-exchange on Mono Q (5/5), *12a* and *12b* respectively. (c) Reverse phase on Pep RPC (5/5), *12b* . For FPLC conditions see experimental section.

The covalent attachment of DNA containing numerous functional groups, to a fluorescent label can provide many pathways for non-radioactive relaxation of the fluorophore's electronic excited state (10,26). In principle, two types of molecular associations may influence these processes, (i) fluorophore-fluorophore and

(ii) fluorophore-DNA; these may occur both at intermolecular and intramolecular levels, resulting in net loss of fluorescence in labeled oligomers compared to the label alone. In order to delineate the relative effects of these interactions and their dependence on inter-fluorophoric distances, the fluorescence of all compounds was recorded under a variety of conditions. The figure 3 and table-3 depict some of the typical results obtained. The decrease in fluorescence intensity is linear with the order of dilution with buffer (figure 3b). This suggests that in the concentration range studied intermolecular (interduplex) DNA-fluorophore and fluorophore-fluorophore associations do not contribute significantly to the quenching process.

To investigate the extent of intrastrand fluorophore-fluorophore interactions, the fluorescence intensities of labeled duplexes *11-14* were measured (figure 3c) after equimolar dilution with unlabeled $d(AT)_5$. The consequent fluorescence intensity changes were similar in both mono and didansyl labeled $d(AT)_5$ and resembled that seen in the buffer dilution experiments suggesting that interstrand interactions are not the major source for suppression of fluorescence. Finally, the effects due to intrastrand fluorophore-fluorophore quenching in didansyl derivatives is shown by data in figure 3d for compounds *12-14*. It is seen from this and the data in table-3 that the enhancements are maximum when two fluorophore labels are in the interior of the duplex helix (*12*) as compared to corresponding monodansyl and the positionally isomeric didansyl derivatives *13* and *14*. It is also seen in general that the fluorescence intensities are higher in oligomers with shorter spacer arms (n=2) as compared to those with longer arms (n=4 and 6).

Though the figures 3c and 3d represent the observed trends qualitatively, support for the above conclusions are borne out by data on emission quantum yields shown in table-3. The emission quantum yields of monodansyl derivatives (entry 1, 2 and 3) relative to the model N-monodansyl hexanediamine indicates about 50% fluorescence quenching due to probe-DNA interactions. The quenching efficiency is relatively less in n=2 derivatives as compared to n=6 (entry 1 and 3) and maximum sensitivity enhancements were observed for *12a*. A longer and flexible spacer arm may allow easy association of the fluorophore with adjacent base pairs or phosphate backbone causing rapid radiationless decay. It thus appears that to achieve higher sensitivity by attachment of multiple internal labels to DNA oligomers via bases, short and perhaps rigid spacer chains are preferable. A similar suggestion has been postulated in a recent study involving fluorophores linked to pyrimidine bases (10). As expected the relative emission quantum yields are uniformly higher for didansyl derivatives compared to monodansyl oligomers (entry 4 to 9). Further, upon 1:1 dilution with unlabeled $d(AT)_5$ there was only a slight increase in quantum yield for monodansyl conjugates (entry 1−3), whereas this was quite significant for didansyl conjugates (entry 4−9). However, the sensitivity enhancements seen in didansyl derivatives vary both as a function of length of spacer arm and the position of didansyl substitutions along the DNA chain. The maximum enhancements are seen for didansyl conjugates *12* (entry 4,5) in which fluorophores are located in the interior of the double helix rather than at the terminals as in *13* and *14* (entry 6−9).

It was recently reported that in case of acridine (17) and pyrene (10) linked oligomers, duplex formation with complementary strands led to ten fold decrease in emission quantum yields. Acridine and pyrene are well known intercalators and the observed enormous fluorescence quenching was attributed to rapid radiationless decay via excited electron transfer from the fluorophore to the base pairs in the intercalation mode. However, in the present work, changes of such magnitudes in emission quantum yields were absent, even upon dilution with unlabeled $d(AT)_5$. The slight increase in quantum yields seen on dilution with $d(AT)_5$ (entry 5−9, <10%) may be due to interfluorophore interaction rather than fluorophore-DNA interaction. From our results, it may therefore be surmised that dansyl group is not interacting with DNA through intercalation. This is substantiated by the fact that no gross differences among the melting profiles of dansylated and non-dansylated $d(AT)_5$ were observed. However, a direct T_m determination from the melting profiles was not possible due to the inherent nature of the oligomeric sequence $d(AT)_5$, which has a low T_m (10−15°C) with a very broad helix-coil transition (27,28). The

Table. 2: DNA sequences*

	1	2	3	4	5	6	7	8	9	10
10	A	T	A	T	A	T	A	T	A	T
11(a-c)	A	T	A	T	A	T	A	T	A	T
12(a-c)	A	T	A	T	A	T	A	T	A	T
13(a-c)	A	T	A	T	A	T	A	T	A	T
14(a-c)	A	T	A	T	A	T	A	T	A	T

*A represents adenines linked to dansyl fluorophore via spacer chains. (a) n=2, (b) n=4 and (c) n=6.

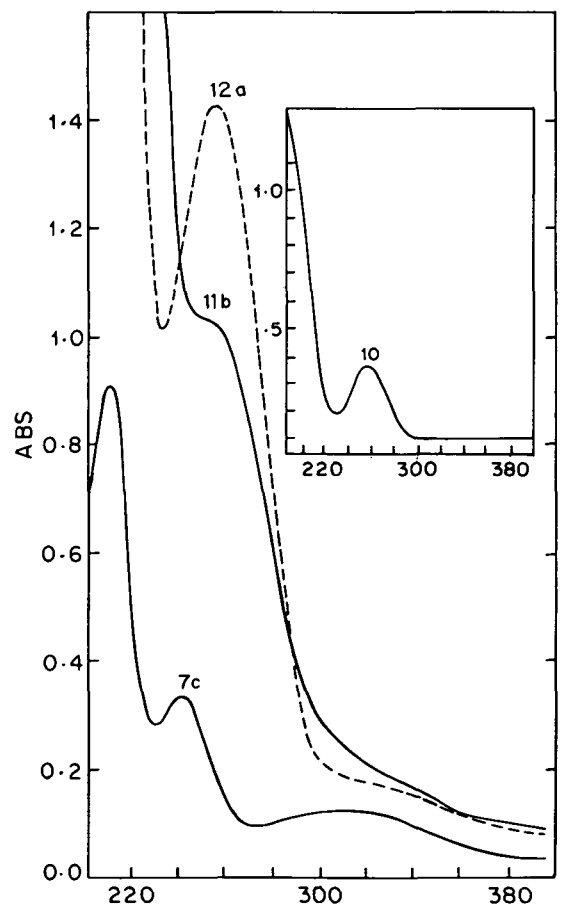

Figure 2. UV spectra of *7c*, *11b* and *12a* . Inset, *10*. Solvent: Buffer, pH 7.0, 10mM sodium phosphate, 0.1mM EDTA and 1M NaCl.

dansyl group may not remain entirely passive in duplex formation, particularly when present on terminal nucleosides as in *13* and *14*. Stacking effects at the ends of the duplex may be significant in these cases, leading to lower quantum efficiencies. In addition, the decrease in fluorescence in *13* and *14* may also be attributed to the DNA strand mobility associated with duplex ends. Though ligand intercalation and stacking effects stabilise the duplexes, they may not be advantageous when the ligand is employed as a fluorophore probe.

It was observed that the covalent attachment of a label to a site directly involved in base pairing can significantly perturb DNA hybridization (10). This was the situation with DNA oligomers in which cytidines are linked to fluorophores at N4 amino position leading to altered stability of duplexes. However, it was not the case in the present examples where consequent upon its attachment at C-8 of purines, the fluorophore may be expected to project into the major groove of DNA in B-form. Bulky groups on C-8 are known to stabilise bases in *syn* conformation, in which case $d(AT)_n$ sequences can still form stable duplexes via Hoogsteen base pairs (29) involving the labeled purines. To delineate the steric and electronic effects on the duplex stabilities upon covalent attachment of fluorophore at C-8 and understand the consequent fluorophore properties, study of mixed DNA sequences incorporating C-8 labeled adenines are under progress. It may be pointed out at this stage that the sensitivity of detection of target DNA by biotin-labeled oligonucleotide probes was strongly dependent upon position of biotin label (9). The oligonucleotides containing biotin labels near the ends of the hybridizing sequence were more effective probes than those having internal labels. The two detection methods are not directly comparable as the basic principles are different.

In this paper, we have demonstrated a viable strategy for regiospecific mono and multiple fluorescent labeling of oligonucleotides via pre-modified monomers. In order to avoid adverse effect of the label or the modified base on the desired hybridization characteristics of the probes, we have attached the label to C8 position of a purine residue. This region of purine which is not directly involved in base pairing, is exposed into the major groove of B DNA and expected to cause minimal interference with duplex formation. Our strategy of fluorophore incorporation prior to the assembly of oligonucleotides avoids the ambiguity in the regiospecificity and degree of labeling per oligonucleotide. The sulphonamide linkage present in this spacer arm linking the fluorophore moiety is stable to all synthetic and deprotection conditions. The strategy also enables easy variability of spacer chain and ultimately the fluorophore characteristics in a simple way from a common simple intermediate *6*. Though dansyl group has been chosen in the present studies, the method can be easily extended for incorporation of other high fluorogenic probes. More importantly, the derivatization via purines is chemically more flexible and easier than that of pyrimidines. A similar strategy can also be adapted for phosphoramidite method of DNA synthesis.

CONCLUSIONS

The salient features emerging from the present fluorescent studies are that (i) an increasing number of dansyl moieties on oligonucleotides enhance the fluorescence intensity and emission quantum yields, (ii) the magnitude of the sensitivity enhancement depends on the labelling configurations, with the labels in the interior of the duplex displaying greater enhancements as compared to those at the duplex ends and (iii) fluorophores linked through shorter spacer arms (n=2) are more efficient probes. The work demonstrates the feasibility of covalent attachment of several internal labels to synthesize DNA oligomers through adenine residues at C-8 position, for sensitivity enhancements. The attachment of fluorophores to C-8 of purine residues does not impair the hybridization process. The results obtained from the present study may aid rational design of high sensitivity fluorescent probes in which more efficient fluorogens are placed in a favorable configuration.

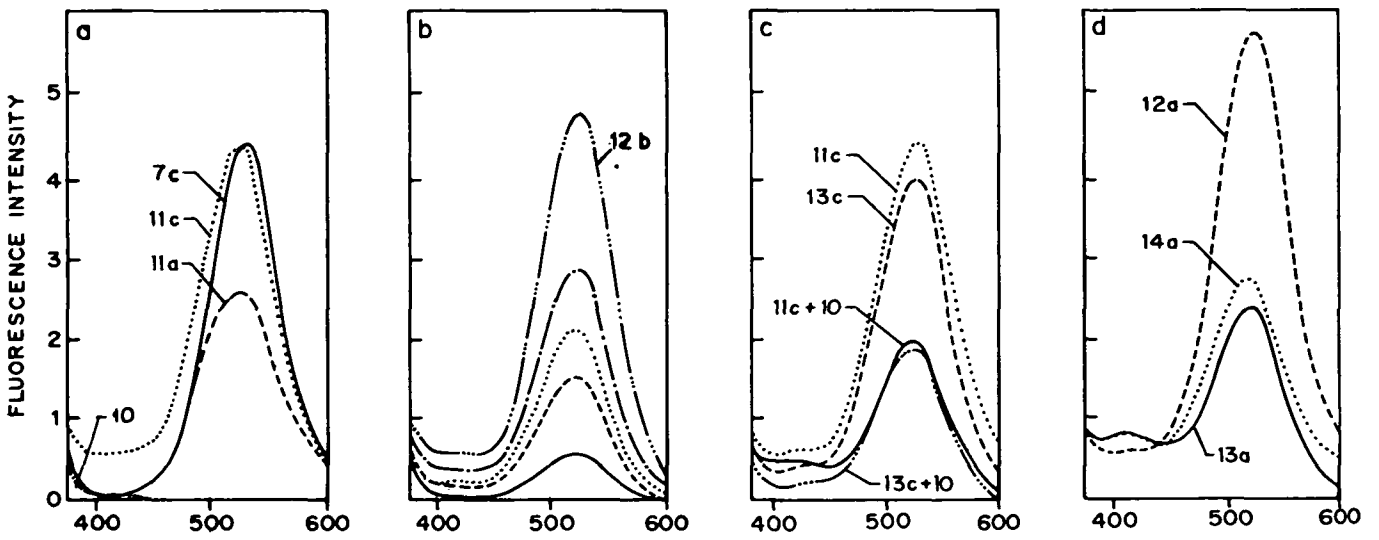

Figure 3. Fluorescence spectra of labeled oligomers, Excitation 335nm. The compound numbers are indicated in each spectra. (a) spectra of *7a*, *11a*, *11c* and *10* in buffer (b) spectra of *12b* on serial dilution with buffer, (c) spectra of hybrid duplexes of *11c and 13c* obtained by dilution with unlabeled $d(AT)_5$, *10*, in buffer and (d) spectra of didansyl derivatives *12a*, *13a* and *14a* in buffer. All samples were adjusted to similar concentrations by absorbance at 260nm. Buffer is same as used for UV spectroscopy.

Table. 3: Fluorescence properties of dansyl $d(AT)_5$

Entry	Compound	QY#	Relative QY$	Sensitivity enhancement+
1.	*11a*	0.15	0.61	1.0
		(0.17)*	(0.66)	(1.0)
2.	*11b*	0.12	0.48	1.0
		(0.14)	(0.55)	(1.0)
3.	*11c*	0.14	0.56	1.0
		(0.16)	(0.64)	(1.0)
4.	*12a*	0.18	0.71	1.16
		(0.50)	(2.00)	(3.03)
5.	*12b*	0.29	1.15	2.40
		(0.38)	(1.50)	(2.72)
6.	*13a*	0.14	0.55	0.90
		(0.14)	(0.58)	(0.88)
7.	*13c*	0.28	1.09	1.95
		(0.30)	(1.19)	(1.85)
8.	*14a*	0.14	0.56	0.92
		(0.15)	(0.58)	(0.88)
9.	*14b*	0.22	0.89	1.85
		(0.27)	(1.07)	(1.95)

#Quantum yield relative to quinine sulphate
$Relative quantum yield with respect to *7c*
+Calculated for polydansyl derivatives *12-14* by assuming quantum yield for corresponding monodansyl compounds *11a-c* as 1.
*Numbers in brackets indicate fluorescence values after equimolar dilution with $d(AT)_5$ in buffer.

EXPERIMENTAL PROCEDURES

Chemicals

8-bromo-2'-deoxyadenosine *2* and the nucleotide monomers *9* and *10* were prepared from corresponding 2-deoxynucleosides as previously described (19,23) and checked for homogeneity by standard procedures (TLC, ^1H and ^{31}P NMR). All 2'-deoxyribonucleosides, 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole [MSNT], dansyl chloride and 2-chlorophenyl dichlorophosphate were obtained from Aldrich, U.K. and 1-methyl imidazole was obtained from Fluka. Pyridine used in solid phase synthesis was distilled over KOH followed by calcium hydride. Dichloroethane (Fluka) was passed over neutral alumina and then distilled over phosphorus pentoxide.

Chromatography

Column chromatography was carried out on silica gel (Merck 7734). The TLC was performed on silica gel GF_{254} pre-coated on aluminum sheets (Merck 5554) and the solvent system used are (A) chloroform/ethanol/pyridine (9:1:0.05, v/v) and (B) ethyl acetate/ acetone/water/pyridine (5:10:1:0.05, v/v). Spots were visualized by spraying with 60% perchloric acid-ethanol solutions when DMTr compounds show orange spots, followed by heating when dark spots show up. The amino compounds were detected by ninhydrin spray.

Spectroscopy

^1H NMR spectra was recorded at 300 MHz on a Brucker MSL 300 Spectrometer, equipped with Aspect 3000 computer. The ^1H spectra are referenced to tetramethyl silane (TMS) as internal standard and the chemical shifts are in scale. The ^{31}P NMR (121MHz) reference was 85% H_3PO_4 (external). Qualitative UV-visible spectra were recorded on Hitachi spectrometer. Fluorescence spectra were recorded on Kontron SFM-25 spectrofluorimeter. All UV and fluorescence spectra were measured in buffer containing 10mM sodium phosphate, 0.1mM EDTA and 1M NaCl (pH 7.0).

N-Monodansyl ω-diaminoalkanes 7(a-c)

These were synthesised from dansyl chloride and w-diaminoalkanes according to reported procedures (18), purified by column chromatography over silica gel (30gms/gm) using isopropanol/ammonia/water (6:1:1); Yields: N,N'-didansyl-aminoalkanes (35%), N-monodansyl aminoalkanes, *7(a-c)* (30%) and unreacted (35%).

6N-Benzoyl-8-bromo-2'-deoxyadenosine *3*

8-Bromo-2'-deoxyadenosine (*2*, 3.31gm, 10mmol) was dried by coevaporation with dry pyridine. It was suspended in dry pyridine (100ml), cooled to 0°C and trimethylchlorosilane (6.5ml, 50mmol) was added dropwise while cooling and after 30min. while stirring benzoyl chloride (6ml, 50mmol) was similarly added. The reaction mixture was allowed to reach at room temperature over 2hr. The reaction was quenched by slow addition of cold water (20ml), followed by hydrolysis with concentrated ammonia (30%, 20ml). After 15 min. TLC indicated a single spot due to benzoyl derivative *3* and prolonged reaction times gave back the starting material. The reaction mixture was concentrated to an oil, redissolved in water and extracted with ether (2×30ml). The aqueous layer on standing overnight at 5°C gave *3* (2.1gm, 55% yield).

6N-Benzoyl-8-bromo-5'-O-(4,4'-dimethoxytrityl)-2-deoxyadenosine *4*

The N6-benzoyl-8-bromo-5'-dimethoxytrityl-2-deoxyadenosine *3* (5mmol, 2.1gm) was dried by coevaporating with dry pyridine and the solid was suspended in pyridine (10ml) followed by treatment with 4,4'-dimethoxytriphenylmethyl chloride (2.0gm, 6mmol). The mixture was stirred at room temperature for 20hr. in a sealed flask during which time pyridine hydrochloride separated out. Methanol (1ml) was added to reaction mixture and extracted with chloroform. The organic layer was washed with 1M aq. sodium bicarbonate and evaporated to dryness to obtain a gummy mass. This was chromotographed on a short silica column and eluted with dichloromethane/pyridine (99:1) containing increasing amounts of methanol. The product *4* eluted in 2−3% MeOH-CH_2Cl_2 (2.4gm).

6N-Benzoyl-8-bromo-5'O-(4,4'-dimethoxytrityl)-3'O-(chlorophenyl)-phosphodiester triethylammonium salts *5*

The compound *4* (3mmol) was dried by coevaporating with dry pyridine followed by dissolution in pyridine (25ml). 2-Cholorophenyl phosphorodichloridate (4.8ml, 30mmol) was separately added to pyridine (30ml) contained in glass reaction vessel fitted with sintered disc and stop cock. The solution was cooled to 0°C and water (30mmol) was added dropwise via syringe taking care not to increase the temperature beyond 10°C. Pyridine hydrochloride seperated out on this addition. The reaction mixture was stirred for 30min. and reagent was filtered into the flask containing the dried nucleoside *4*. The mixture was concenterated to half its volume and stirred for 10min. at room temperature when TLC indicated completion of reaction. Triethylammonium carbonate (1M, 10ml) was added and mixture extracted with chloroform. The organic layer was washed with 0.1M solution of TEAB, dried over sodium sulphate and concentrated to yield a gum which was purified over a column of silica gel. The product *5* was obtained as a foam (2gm), λ_{max} 280 (MeOH).

6N-Benzoyl-8-bromo-5'-O-(4,4'-dimethoxytrityl)-3'-O-(2-Cholrophenyl-4-nitro phenylethyl) phosphate *6*

The triethylammonium salt *5* (1.01gm, 1mmol) was treated with 4-nitrophenyl ethanol (0.3gm, 2mmol), 1-mesitylene-2-sulphonyl chloride (0.33gm, 1.5 mmol) and 1 methyl imidazole (0.12gm, 1.5mmol) in dry pyridine (10ml). The reaction followed by TLC was complete in 15min. and was quenched with aq. sodium bicarbonate solution. The phosphate triester *6* was extracted into chloroform and further purified by column chromotography over silica gel (1.1gm), λ_{max} 287 (MeOH).

6N-benzoyl-(ω-dansylamino-alkylamino)-3'-O-(2-chloro-phenyl) phosphodiester triethylammonium salt *1*

The 8-bromotriester *6* (0.3gm, 0.3mmol) was suspended in absolute ethanol (10ml) to which was added ω-monodansylamino alkylamine *7* (0.7mmol) in ethanol. The reaction mixture was refluxed for 6–8hr. and checked by TLC for disappearence of *6*. The solvent was evaporated and this residue was purified by column chromatography on silica gel to yield a foam of *8*. This product was treated with 1,8-diazobicyclo-(5,4,0)-undec-7-ene (DBU) in pyridine (3ml) for 3hr. The reaction workup using triethylammonium bicarbonate gave the product directly as triethylammonium salts *1* (0.25gm, 70%) λ_{max} 307 (MeOH). ^{31}P *NMR* (External reference: 80% H_3PO_4), *1a*, −6.10; *1b*, −5.96; *1c*, −6.02 ppm.

Solid phase synthesis of oligonucleotides

Oligonucleotides were assembled on a Cruachem PS100 Manual synthesiser using phosphotriester method. The support used was control pore glass (CPG) resin from Pierce functionalised with T through standard succimide linkage to have a loading value of about 20μm per gm. The coupling mixture stoichiometry for reactions on 0.9μm scale (50mg CPG resin) was nucleotide monomer (13μm), MSNT (65μm) and N-methyl imidazole (10μl) in pyridine (100μl). The coupling time was 15min. with the following purging cycle: (i) pyridine 2min., (ii) dichloroethane 2min., (iii) 2% dichloroacetic acid in dichloroethane 2min. (iv) dichloroethane, 2min. and (v) pyridine 2min., all at a flow rate of about 1ml/min . The yield per coupling as estimated from trityl analysis at each step was about 97%. At the end of the synthesis the resin was subjected to the following sequential deprotection steps: (i) 4-nitrobenzaldoxime (35mg) and 1,1,3,3-tetramethyl-guanidine (25μl) in dioxane-water (1:1, 0.5ml) at 30°C for 20hr. (ii) conc. ammonia (5ml) at 60°C, 17hr and (iii) 80% acetic acid (5ml, 30°C) for 30min. The residual product after the final step was passed over Sephadex G-15 column and eluted with methanol:water (2:8). The void volume peak was collected, lyophilised and further purified by FPLC.

Purification of oligonucleotides by FPLC

The compounds were purified on Pharmacia FPLC machine using anion exchange Mono Q column (5/5 analytical and 10/10 preparative) under the following conditions. *Buffer A*: 10mM sodium hydroxide (pH 12). *Buffer B*: 10mM aq. sodium hydroxide containing 1M NaCl, *Flow rate*: 0.5 ml/min for analytical and 1.5ml/min for preparative runs. *Gradient*: 0% to 100% B over 25min. The collected peaks were neutralized to pH7.0 with dil. HCl (1:1) and lyophilized. The product was recovered after desalting over Sephadex G10. The final purity as checked by reverse phase (Pep RPC) was over 95%.

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