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Color coded triarylmethyl protecting groups useful for deoxypolynucleotide synthesis

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ABSTRACT

Triarylmethyl groups having different colors but similar chemical reactivities in acid were examined as potential aids for monitoring the stepwise addition of mononucleotides to a deoxyoligonucleotide. The successful application of these protecting groups to deoxyoligonucleotide synthesis on polymer supports was demonstrated.

INTRODUCTION

Characterization of synthetic oligonucleotides continues to be an important problem in the nucleic acid field. Currently available are procedures for analyzing the sequence and purity of completely deprotected (1) and protected (2) synthetic oligonucleotides. These procedures, however, are useful only if the oligonucleotide has been isolated and purified in some manner. This purification process is usually the time consuming step and essentially limits characterization to the final, synthetic product. Oligonucleotide synthesis, however, is a cyclic process requiring the repetitive addition of mononucleotides, dinucleotides, or larger oligonucleotides to the growing segment. It is therefore of interest to develop procedures for monitoring each intermediate reaction. One would like to know with a minimum investment of material and time if each condensation proceeds satisfactorily and if the correct mono-, di-, or oligonucleotide has been added to the growing segment. We have now developed a useful procedure for monitoring the progress of a deoxyoligonucleotide synthesis. Generally the procedure involves color-coding mononucleotides which consequently permits the operator to monitor the sequence and yield for each synthetic step. In this paper we outline the analytical method and illustrate how it can

be used to monitor the synthesis of deoxyoligonucleotides on a polymer support.

The basic strategy we have developed for the stepwise, polymer-supported synthesis of a deoxyoligonucleotide (3) is outlined in Figure 1. Thus the addition of one mononucleotide to the support bound nucleoside involves detritylation, condensation with the appropriate deoxynucleoside phosphoramidite (compounds 3a-d) using tetrazole as an activating agent, capping unreacted segments with acetic anhydride and 4-N,N-dimethylaminopyridine (DMAP), and oxidation of the phosphite to the phosphate. Repetition of this cycle with appropriate deoxynucleoside phosphoramidites leads to the synthesis of a sequence defined deoxyoligonucleotide. As can be seen by examining this reaction scheme, a di-p-anisylphenylmethyl (trivially known as dimethoxytrityl) group is removed during each synthetic cycle. More importantly this di-p-anisylphenylmethyl group is part of the mononucleotide (3a-d) most recently added to the support bound deoxyoligonucleotide. The di-p-anisylphenylmethyl group forms an orange color when removed from the nucleotide with either protic or Lewis acids. If four triarylmethyl protecting groups could be identified that have similar chemical reactivities but different colors

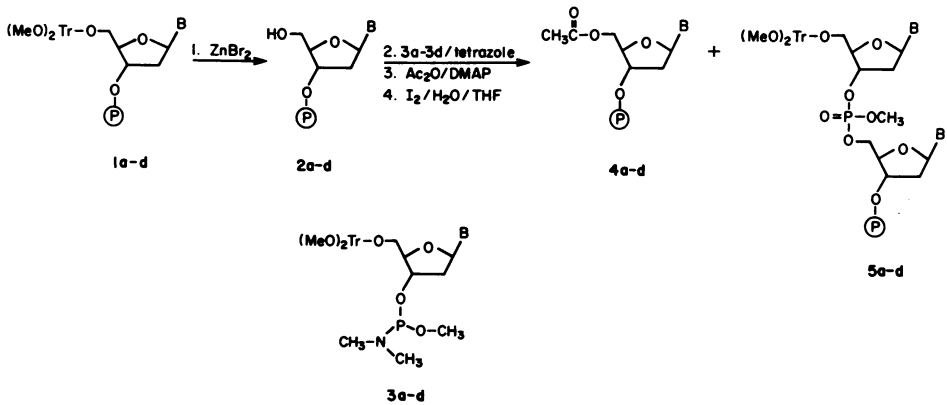

Fig. 1. Steps in the synthesis of a dinucleotide. B refers to thymine, N-benzoylcytosine, N-benzoyladenine, and N-isobutyrylguanine for 1a-d, respectively. A similar nomenclature applies to 2a-d, 3a-d, 4a-d and 5a-d. $(MeO)_2Tr$ refers to the di-p-anisylphenylmethyl group when 3d is used. For 3a, 3b, and 3c, trityl groups as defined in Table 4 were used.

in acid solution, then each condensation step could be individually monitored. For example, if the di-p-anisylphenylmethyl group were assigned only to the N-isobutyryldeoxyguanosine derivative of 3 (i.e. 3d), then an orange color during the subsequent acid cleavage step would indicate that N-isobutyryldeoxyguanosine had been added to the growing oligonucleotide during the preceeding condensation step. By measuring the color intensity, an approximate quantitation of the condensation yield would also be possible. A different color resulting from another triarylmethyl protecting group would indicate that one of the other mononucleotides had been added during the condensation. Thus by attaching different triarylmethyl protecting groups to the four mononucleotides, condensation reactions can be monitored as to yield and as a check on the nucleotide sequence. In this paper we present a series of triarylmethyl protecting groups that meet these criteria.

MATERIALS AND METHODS

4-Bromobiphenyl, o-bromoanisole, p-bromoanisole, 1-bromonaphthalene, 4-chlorobenzophenone, 4-fluorobenzophenone, methyl-2-furoate, methyl-1-naphthoate and 4-nitrobenzophenone were obtained from Aldrich. Bromobenzene, methyl benzoate and methyl 4-methyl benzoate were obtained from Pfaltz and Bauer.

The triarylcarbinols were initially synthesized in test tubes via a standard Grignard reaction. After neutralization, chromatography on thin layer plates, and exposure to HCl vapors, the colors for various triarylcarbinols were observed. Based on these colors, structural considerations, and the cost of different reagents, certain triarylcarbinols were synthesized in gram quantities by reacting the methyl ester of an aryl carboxylic acid with an arylmagnesium bromide (2.2 equivalents) or by reacting a diarylketone with an arylmagnesium bromide (1.1 equivalents). The reactions were stirred overnight at room temperature and analyzed by thin-layer chromatography (tlc). The reaction mixtures were quenched by addition of aqueous ammonium sulfate (10% w/v). The products were extracted into toluene, dried over sodium sulfate and concentrated *in vacuo* to a gum. The residues were redissolved in toluene (3 ml/mmol initial aryl carbonyl com-

pound) and converted to their corresponding chlorides by addition of acetyl chloride (2 equivalents). The appropriate trityl chlorides were isolated by crystallization. Based on the carbonyl compounds, yields were 44-55%.

The 5'-O-triarylmethyldeoxynucleosides were prepared using standard procedures (4) and converted to 3'-methoxy-N,N-dimethylaminophosphines as described by Beaucage and Caruthers (5). Phosphorus-31 NMR spectra were recorded on a Joel PFT-100 spectrophotometer with a Nicolet-1080 data system in CD_3CN as solvent with 85% phosphoric acid as an external standard. The visible spectral characteristics of various 5'-O-triarylmethyldeoxythymidine derivatives were examined in 0.08 M $ZnBr_2$ in nitromethane. Spectra were recorded on a Carey Model 21 scanning from 350 nm to 600 nm.

Detritylation rates for various 5'-O-triarylmethyldeoxynucleosides were estimated by analysis using tlc. A 2 μM solution of the 5'-O-triarylmethyldeoxynucleoside was treated with either 0.06 M $ZnBr_2$ in nitromethane or 0.9 M chloroacetic acid in nitromethane. Aliquots were removed from the reaction mixture, quenched with sodium bicarbonate, and analyzed by tlc (chloroform:methanol, 9:1).

Nucleoside phosphoramidites, labeled with the alternative triarylmethyl protecting groups, were used to synthesize four deoxyoligonucleotides according to published procedures (3,6).The deoxyoligonucleotides were purified on a Waters HPLC. The separations were completed isocratically on a μbondapak C_{18} column using varying percentages of acetonitrile and water. The collected peak was evaporated to dryness. The residue was dissolved in acetic acid:water (8:2). After 30 min the sample was concentrated to dryness and then dissolved in 5 mM Tris (pH = 7.6)/1 mM EDTA. Two dimension analysis of synthetic deoxyoligonucleotides was performed following a published procedure (1).

RESULTS AND DISCUSSION

Triarylmethyl protecting groups have been an integral part of deoxyoligonucleotide chemistry for some time. The triphenylmethyl group was introduced by Levene and Tipson in 1935 (7). More recently, Khorana's laboratory investigated the triphenyl-

methyl, the p-anisyldiphenylmethyl, the di-p-anisylphenylmethyl, and the tri-p-anisylmethyl groups for protecting the 5'-hydroxyl of nucleosides (8). The replacement of phenyl groups with anisyl groups increases the lability of the triarylmethyl blocking group toward acid cleavage. Additionally in acid solutions, these triarylcarbinols are yellow, yellow-orange, orange, and red,respectively. However neither the triphenylmethyl or tri-p-anisylphenylmethyl protecting groups are useful in deoxyoligonucleotide synthesis. The former is too stable toward protic acid hydrolysis (considerable depurination occurs before deprotection is complete) and only slowly hydrolyzes with $ZnBr_2$ when compared to the di-p-anisylphenylmethyl group (9). The latter is unstable during preparation of the nucleoside derivative and during its use in oligonucleotide synthesis. Letsinger and Finan have reported the synthesis of the 5'-O-1-naphthyldiphenylmethyl nucleoside (10). However the protic acid conditions suggested for its removal indicate that this group is much too stable for use with purine nucleosides. Thus of the triarylmethyl groups so far tested, only the p-anisyldiphenylmethyl and the di-p-anisylphenylmethyl groups have been used respectively in the diester and triester deoxyoligonucleotide synthesis procedures (11). A search for additional triarylmethyl protecting groups was therefore conducted. The objective was to identify several triarylmethylcarbinols that had reactivity toward Lewis and protic acids similar to the di-p-anisylphenylmethyl protecting group but with a wide range of colors in acid solution. Approximately 100 triarylcarbinols having the general formula given in Figure 2 were prepared. Most derivatives in acid exhibited colors ranging from yellow to various shades of orange or red. However certain other derivatives, having the functional groups listed in Figure 2, exhibited a wide variety of colors in acidic solutions (Table 1). As can be seen from this list, essentially every visible color was observed by selecting the appropriate aromatic functional groups.

Various triarylmethylcarbinols were tested as potential 5'-O-deoxynucleoside protecting groups. Selected 5'-O-triarylmethyldeoxynucleosides were synthesized and the hydrolysis rates in chloroacetic acid and $ZnBr_2$ were measured and compared to the

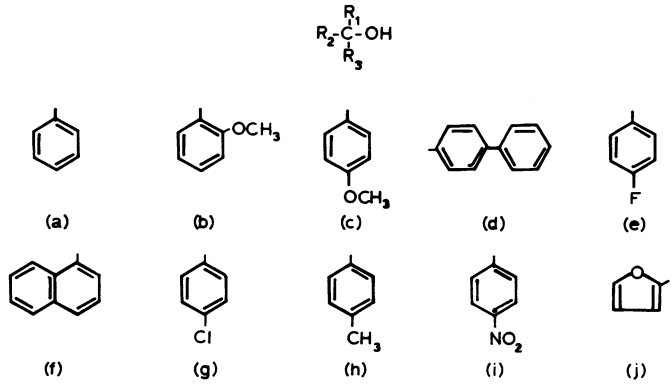

Fig. 2. General Formula for Triarylcarbinols. R_1, R_2 and R_3 represent various aromatic functional groups.

same deoxynucleoside containing a 5'-di-p-anisylphenylmethyl group. These results are recorded in Table 2. In the presence of $ZnBr_2$, several triarylmethyl groups forming different colors in acid solution are hydrolyzed at approximately the same rate. These results are similar to previous observations that the di-p-anisylphenylmethyl and p-anisyldiphenylmethyl protecting

Table 1. A List of Observed Colors for Various Triarylcarbinols in Acid Solution

Triarylcarbinol[1]	Color
R_1 = a; R_2 = c; R_3 = f	Red
R_1 = R_2 = b; R_3 = f	Blue
R_1 = a; R_2 = e; R_3 = f	Green
R_1 = a; R_2 = f; R_3 = g	Green
R_1 = R_2 = a; R_3 = h	Yellow
R_1 = a; R_2 = R_3 = b	Black
R_1 = R_2 = c; R_3 = f	Violet
R_1 = R_2 = d; R_3 = f	Dark Purple
R_1 = R_2 = b; R_3 = d	Purple
R_1 = R_2 = b; R_3 = j	Lavender[2]
R_1 = R_2 = a; R_3 = i	Red-violet
R_1 = R_2 = d; R_3 = j	Pink

[1]The aryl functional groups are defined by the structures and assigned letters as included in Figure 2.
[2]We thank M. Matteucci for discovering this triarylcarbinol.

Table 2. 5'-O-Triarylmethyldeoxynucleoside Hydrolysis Rates[1,2]

5'-O-Triarylmethyl Group	Deoxynucleoside	$t_{1/2}$[3] (sec)	$t_{1/2}$[4] (sec)
di-p-anisylphenylmethyl	T	45	150
p-fluorophenyl-1-naphthylphenylmethyl	T	480	3000
p-anisyl-1-naphthylphenylmethyl	T	180	300
di-o-anisyl-1-naphthylmethyl	T	90	300
di-o-anisylphenylmethyl	T	90	270
p-tolyldiphenylmethyl	T	240	1200
di-p-anisylphenylmethyl	C	120	75
di-o-anisyl-1-naphthylmethyl	C	150	420
di-p-anisylphenylmethyl	G	<20	60
di-o-anisylphenylmethyl	G	20	240
di-p-anisylphenylmethyl	A	<20	100
p-tolyldiphenylmethyl	A	180	1200

[1] The symbols T, C, G, and A refer to deoxythymidine, N-benzoyldeoxycytidine, N-isobutyryldeoxyguanosine and N-benzoyldeoxyadenosine, respectively.

[2] Reaction conditions were 0.06 M $ZnBr_2$ in nitromethane and 0.9 M chloroacetic acid in nitromethane. Time points were taken and reactions quenched in 100 µl saturated sodium bicarbonate and 50 µl n-butanol. Reaction products were analyzed by tlc.

[3] $ZnBr_2$ in nitromethane was used primarily to reduce the hydrolysis rate so that the kinetics could be determined more accurately. However a saturated solution of $ZnBr_2$ in nitromethane: methanol (95:5) is the solvent of choice during chemical synthesis since hydrolysis is complete within 5 min for all these 5'-O-triarylmethyl groups.

[4] In order to reduce the hydrolysis rate in protic acid, chloroacetic acid was used for these measurements. However trichloroacetic acid is routinely used as the protic acid of choice during chemical synthesis (11). With trichloroacetic acid (2% w/v) in dichloromethane, hydrolysis was extremely rapid with the reaction being complete within 30 sec for di-p-anisylphenylmethyldeoxynucleosides, p-anisyl-1-naphthylphenylmethyldeoxynucleosides, di-o-anisylphenylmethyldeoxynucleosides, and di-o-anisyl-1-naphthylmethyldeoxynucleosides. The p-tolyldiphenylmethyldeoxynucleosides and p-fluorophenyl-1-naphthylphenylmethyldeoxynucleosides required 75 sec and 120 sec, respectively, for complete hydrolysis.

groups are hydrolyzed at the same rate in a nitromethane solution of $ZnBr_2$ (9). The rates are more variable with protic acids. The absorption spectra of several triarylmethyl cations in acid were also measured and these results are recorded in Table 3.

Table 3. Visible Spectral Characteristics of Various 5'-O-Triarylmethyldeoxythymidine Derivatives in Acid[1]

Triarylmethyl Cation	Maximum(s) (nanometers)	Extinction Coefficient (Molar^{-1} cm^{-1})	Color
di-o-anisylphenylmethyl	423	9300	Black
	503	5200	
	586	3900	
p-tolyldiphenylmethyl	452	42000	Yellow
p-fluorophenyl-1-naphthylphenylmethyl	545	25000	Green
	455	28000	
di-o-anisyl-1-naphthylmethyl	586	15500	Blue
p-anisyl-1-naphthylphenylmethyl	577	9500	Red
	421	20500	
di-p-anisylphenylmethyl	498	72000	Orange

[1]The 5'-O-triarylmethyldeoxythymidine derivatives were dissolved in 0.08 M $ZnBr_2$ in nitromethane and the spectra recorded within 10 minutes.

Several of these triarylmethyl cations have multiple relative absorption maxima. These differences in absorbtion maxima should prove useful for synthesizing mixed probes where more than one trityl group must be monitored simultaneously. For example, if the p-anisyl-1-naphthylphenylmethyl and di-o-anisyl-1-naphthylmethyl were being used for monitoring the simultaneous addition of two deoxymononucleotides (i.e. T and C) to a probe, then the relative amounts of these mononucleotides could be monitored at 421 and 586 nm. Based on these studies, appropriate triarylmethyl groups were assigned to specific deoxynucleosides as summarized in Table 4. These 5'-O-triarylmethyldeoxynucleosides were converted to the 3'-methoxy-N,N-dimethylaminophosphoramidites with yields comparable to those previously reported for the 5'-O-di-p-anisylphenylmethyldeoxynucleosides (5). Thus additions of thymidine, deoxycytidine, deoxyadenosine, or deoxyguanosine were monitored by the formation of red, blue, yellow, and orange colors, respectively, during the detritylation step.

The utility of color coded deoxymononucleotides for monitoring deoxyoligonucleotide synthesis was tested by synthesizing

Table 4. Phosphorus-31 NMR Chemical Shift Data for 5'-O-Triaryl-methyldeoxynucleoside-3'-N,N-dimethylaminophosphines

Nucleotide	Color	NMR Chemical Shifts
5'-O-di-p-anisylphenylmethyl-N-isobutyryldeoxyguanosine-3'-N,N dimethylaminomethoxyphosphine	Orange	146.1, 146.3
5'-O-p-anisyl-1-naphthylphenyl-methyldeoxythymidine-3'-N,N dimethylaminomethoxyphosphine	Red	145.7, 146.4
5'-O-di-o-anisyl-1-naphthylmethyl-N-benzoyldeoxycytidine-3'-N,N dimethylaminomethoxyphosphine	Blue	145.4, 147.6
5'-O-p-tolyldiphenylmethyl-N-benzoyldeoxyadenosine-3'-N,N dimethylaminomethoxyphosphine	Yellow	146.1, 146.4

d(G-T-A-T-A-A-C-T-A-C-A-C), d(C-A-T-A-A-A-G-A-A-A-A-A), d(G-T-A-C-A-G-C-T-G-G-C-T) and d(C-C-C-T-T-T-C-T-T-A-A-A). The synthesis of d(G-T-A-C-A-G-C-T-G-G-C-T) began with deoxythymidine attached covalently to silica gel (12-14). The first step was condensation of the polymer-supported deoxynucleoside with 5'-O-di-o-anisyl-1-naphthyl-N-benzoyldeoxycytidine-3'-N,N-dimethylamino-methoxyphosphine. After acylation and oxidation, detritylation was completed using a saturated solution of $ZnBr_2$ in nitromethane:methanol (95:5). A blue color indicating the addition of N-benzoyldeoxycytidine was observed. The remaining nucleotides were added in a similar manner. During each subsequent detritylation step, orange, orange, red, blue, orange, yellow, blue, yellow, red and orange colors were observed. These colors confirmed that the deoxyoligonucleotide had been synthesized. The correct nucleotide sequence was re-confirmed by two dimension sequence analysis. The three remaining deoxyoligonucleotides were synthesized and characterized in the same manner. These deoxyoligonucleotides are currently being used in collaboration with A. Berk for site directed mutagenesis experiments on early adenovirus mRNA splicing and maturation (16). For deoxyoligonucleotide synthesis where protic acids are used to remove triarylmethyl groups

rather than $ZnBr_2$, the di-o-anisylphenylmethyl protecting group which registers a black color should replace the diphenyl-p-tolylmethyl as a 5'-N-benzoyldeoxyadenosine protecting group because of the slow hydrolysis rate for the latter triaryl protecting group ($t_{1/2}$ = 240 and 1200 sec, respectively). Extensive exposure to protic acids as required if the diphenyl-p-tolylmethyl were used with N-benzoylyldeoxyadenosine could lead to considerable depurination.

These results demonstrate that colored triarylmethyl protecting groups can be used to monitor the synthesis of a sequence defined DNA. Moreover because of the large difference in visible absorption spectra for the various triarylmethyl cations, these groups can be used to monitor the extent of reaction and also the relative extent of condensation when more than one mononucleotide is added during a specific synthesis step. The latter possibility should be extremely useful for the synthesis of mixed sequence probes. Several triarylmethyl groups having a large range of colors as listed in Table 2 have not been assigned to one of the four deoxyoligonucleotides. We anticipate that some of these triarylmethyl groups can be assigned to deoxymononucleotide analogs (deoxyuridine, deoxyinosine and others) for synthesizing modified deoxyoligonucleotides useful for various biochemical experiments (17).

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