Naphthaloyl group: a new selective amino protecting group for deoxynucleosides in oligonucleotide synthesis

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Received November 18, 1987[2]

ARCHANA DIKSHIT, MANJULA CHADDHA, R. K. SINGH, and KRISHNA MISRA. Can. J. Chem. **66**, 2989 (1988).

The naphthaloyl group has been found to be a selective amino protecting group for deoxycytidine, deoxyadenosine, and deoxyguanosine in oligodeoxyribonucleotide synthesis. All three protected monomers obtained (78–85%), being six-membered cyclic imides, were fairly stable. These protected monomers were used successfully for the preparation of dimers (phosphodiester approach) and tetramers (phosphotriester approach) in solution as well as solid phase, respectively. The group acted as a purification tool due to its high lipophilicity. No adverse effect has been observed either on the glycosidic bond (depurination) or the internucleotidic bond during its removal. The monomeric units were characterized by UV, NMR, and elemental analyses whereas the tetramers were characterized by enzymatic hydrolyses with snake venom phosphodiesterase followed by alkaline phosphatase.

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On a trouvé que le groupe naphtaloyle constitue un groupe protecteur sélectif de la fonction amine de la déoxycytidine, de la déoxyadénosine et de la déoxyguanosine dans la synthèse de l'oligodéoxyribonucléotide. Les trois monomères protégés obtenus (78–85%) sont des imides cycliques à six chainons assez stables. On a utilisé avantageusement ces trois monomères protégés dans la préparation des dimères (approche phosphodiester) et des tétramères (approche phosphotriesters) en solution aussi bien qu'en phase solide respectivement. La grande lipophilicité de ce groupe en fait un outil de purification. On n'a pas observé d'effets contraires ni sur la liaison glycosidique (dépurination) ni sur la liaison internucléotidique lors de la déprotection. On a caractérisé les unités monomères par ultraviolet, par résonance magnétique nucléaire et par analyse élémentaire tandis que l'hydrolyse enzymatique par le phosphodiestérase du venin de serpent suivie d'une phosphatase alcaline ont permis de caractériser les tétramères.

[Traduit par la revue]

Introduction

The importance of synthetic oligodeoxyribonucleotides in molecular biology (1–5) has long attracted attention to the refining of chemical synthetic methods. The most important aspect of chemical syntheses of oligodeoxyribonucleotides is the selective protection of different nucleophilic sites of the monomer building blocks, and amino protection is one of them. The amino protecting groups remain in place throughout the course of the synthesis and, therefore, are extremely important. Several groups have been used for the protection of the amino function (5–10) but selective protection of the amino group, despite repeated attempts, is still a challenging and important problem. Notable success has been achieved only in the case of deoxycytidine (11–14). We have now successfully used naphthaloyl as a new selective amino protecting group for the protection of all three deoxynucleosides, i.e., dA, dG, and dC. The protected monomers are found to be compatible with conditions for the synthesis of oligodeoxyribonucleotides, as evidenced by good yields of dimers and tetramers synthesized in solution phase and solid phase, respectively.

Experimental

Nucleosides, nucleotides, dimethoxytrityl chloride, 5′-*O*-dimethoxytritylthymidine-3′-*O*-chlorophenyl phosphate (triethylammonium salt), mesitylenesulfonyl-3-nitro-1,2,4-triazole, triisopropylbenzene sulfonyl chloride (TPSCl), 1,1,3,3-tetramethylguanidine, 4-nitrobenzaldoxime, 1-methylimidazole, DCC, Fmoc-glycine, ninhydrin, and acenaphthene were purchased from Fluka, Sigma, and Cruachem Chemical Co. The enzymes, snake venom phosphodiesterase (EC 3.1.4.1., *Crotalus durissus*) and alkaline phosphatase (EC 3.1.3.1., *Escherichia coli*), were purchased from Sigma Chemical Co. Naphthalic anhydride was prepared by dichromate oxidation of acenaphthene (15). Composite polydimethylacrylamide/kieselguhr support was used for solid phase synthesis, which was carried out on a dual column DNA bench synthesizer (OMNIFIT Ltd., Cambridge). HPLC separations were made on an LKB Ultrapac TSK DEAE-35W column (7.5 × 150 mm) and UV absorption was measured on a Hitachi 220 S spectrophotometer. Elemental analyses were carried out using a Carlo Erba 1106 analyser.

All the tlc analyses were done on silica gel G (Merck) using solvent system *a* (CH_2Cl_2/CH_3OH ; 9:1,v/v) or *b* (CH_2Cl_2/CH_3OH ; 9.5:0.5, v/v).

General preparation of N-*napthaloyldeoxynucleosides (N-nph-dNS)*

Each deoxynucleoside (1 mmol) was dried by evaporation *in vacuo* with pyridine (3 mL), twice. It was then suspended in dry pyridine (10 mL) and naphthalic anhydride (1 mmol) was added. Triethylamine (0.02 mL) was used as a catalyst. The flask was sealed and shaken in the dark at 70°C for 7 h, the mixture was evaporated to a gum *in vacuo*, poured into water (10 mL), and extracted with dichloromethane (4×5 mL). The organic layer was backwashed with water, dried over Na_2SO_4, and filtered. This organic part yielded 55, 55, and 50% of the N-protected derivatives of dC, dA, and dG, respectively. The aqueous layer was repeatedly coevaporated to dryness with pyridine, the residue reacted with naphthalic anhydride (1 mmol), and, after an appropriate time, it was extracted with dichloromethane. The combined dichloromethane solution was evaporated to dryness *in vacuo* and the residue was subjected to silica gel column chromatography. Elution was done with CH_2Cl_2/ CH_3OH of increasing polarity (Scheme 1). Data obtained are given in Table 1.

Conditions for removal of naphthaloyl group

Conditions for the removal of the naphthaloyl group were worked out by treating the N-protected derivatives with 40% ammonia at 30, 50, and 70°C. Reaction was quenched after 1, 2, 3, 4, 5, and 6 h duration. After hydrolysis, the mixtures were analysed for deprotected 2′-deoxynucleosides by preparative tlc and subsequent estimation by UV spectroscopy. Ammonia (40%) at 50°C for 4 h showed the best result and was taken to be the optimal condition for removal of the group, although the initial rate of hydrolysis varied in each case (Table 2).

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[2]Revision received August 3, 1988.

Printed in Canada / Imprimé au Canada

TABLE 1. Yield and characterization of naphthaloyl protected dC, dA, and dG

Compound	Yield (%)	R_f	UV (CH_2Cl_2) λ_{max} (nm)	UV (CH_2Cl_2) λ_{min} (nm)	^1H NMR ($CDCl_3$) (ppm)	Elemental analysis
N^4-nph-dC	85	0.30 (*a*)	290 302 310	285	8.25 (d,2H), 8.68 (d,2H), 7.92 (t,2H), 7.32 (d,1H), 7.54 (d,1H), 3.20–3.48 (sugar protons, 4H), 5.18 (m,1H)*	$C_{21}H_{17}N_3O_6$ (407) Calcd.: C61.94, H4.17, N10.32 Found: C61.88, H4.19, N10.35
N^6-nph-dA	82	0.35 (*b*)	270–272	258	8.25 (d,2H), 8.68 (d,2H), 7.92 (t, 2H), 9.0 (s,1H), 5.20 (m,1H,N-CH), 4.2–4.4 (sugar protons), 5.82 (m,1H)*	$C_{22}H_{17}O_5N_5$ (431) Calcd.: C61.25, H3.94, N16.24 Found: C61.39, H3.91, N16.30
N^2-nph-dG	78	0.31 (*b*)	258–262	250	8.25 (d,2H), 8.68 (d,2H), 7.92 (t,2H), 9.81 (s,1H, N-H), 5.20 (m,1H, N-CH), 3.30–3.6 (sugar protons 4H), 5.08 (m,1H)*	$C_{22}H_{17}N_5O_6$ (447) Calcd.: C59.05, H3.83, N15.65 Found: C59.11, H3.79, N15.62

a and *b* refer to solvent systems (see text), and * indicates anomeric protons.

TABLE 2. Percentage deprotection with 40% ammonia

Temperature (°C)	Time (h)	N^4-nph-dC	N^6-nph-dA	N^2-nph-dG
30	1	9.0	7.5	7.0
	2	17.2	16.1	15.3
	3	23.4	21.3	20.3
	4	30.1	27.2	28.0
	5	32.8	32.6	30.6
	6	42.0	41.6	40.0
50	1	10.8	10.2	9.8
	2	45.0	45.0	41.0
	3	83.2	80.6	79.1
	4	100	100	100
	5	100	100	100
	6	100	100	100
70	1	15.0	14.2	13.6
	2	60.1	58.2	56.8
	3	93.0	92.4	90.0
	4	100	100	100
	5	100	100	100
	6	100	100	100

General preparation of 5′-O-dimethoxytrityl-N-naphthaloyl-deoxynucleosides (5′-O-DMTr-N-nph-dNS)

The corresponding trityl derivatives were prepared by treating dried N-nph-dNS (1 mmol) with DMTrCl (1.2 mmol) in pyridine (10 mL) at room temperature (25–30°C) (Table 3). After completion of the reaction (as checked by tlc), the clear solution was evaporated to a gum *in vacuo*. Ice-cooled water (10 mL) was added and it was extracted with ethyl acetate (3×10 mL). The organic layer was dried over Na_2SO_4, concentrated, and applied to an alumina column, eluting with CH_2Cl_2/CH_3OH of increasing polarity. Yield, R_f, and λ_{max} of different derivatives are shown in Table 3.

General method for preparation of N,3′-diacetyldeoxyadenosine-5′-phosphate (pdA-N,3′-O-Ac_2)

Deoxyadenosine-5′-phosphate (1 mmol) was dried by evaporation *in vacuo* three times with dry pyridine (2 mL). The residue was placed in dry pyridine (5 mL), acetic anhydride (5 mL) added, and the solution stirred at room temperature. After 3 h, the reaction was checked for completion, by tlc, and methanol (5 mL) was added. The reaction mixture was evaporated to a gum. The gum was dissolved in a minimum amount of water, which was washed with ether. The aqueous solution was evaporated to a gum. The process of evaporation was repeated twice with 10% aqueous pyridine. The residue thus obtained was dissolved in dry pyridine, concentrated, and added dropwise to dry ether (5 times the volume of concentrated pyridine solution); the white precipitate was filtered, washed with ether, and dried under vacuum (yield, 90%; λ_{max} 272 nm).

TABLE 3. Preparation and characterization of 5′N-protected units

	Reaction time (h)	Yield (%)	R_f	λ_{max} (nm)
5′-O-DMTr-N^4-nph-dC	3	90	0.42 (*a*)	292,303,312
5′-O-DMTr-N^6-nph-dA	2	94	0.47 (*b*)	271–273
5′-O-DMTr-N^2-nph-dG	1.5	95	0.45 (*b*)	262–264

a and *b* refer to solvent systems (see text).

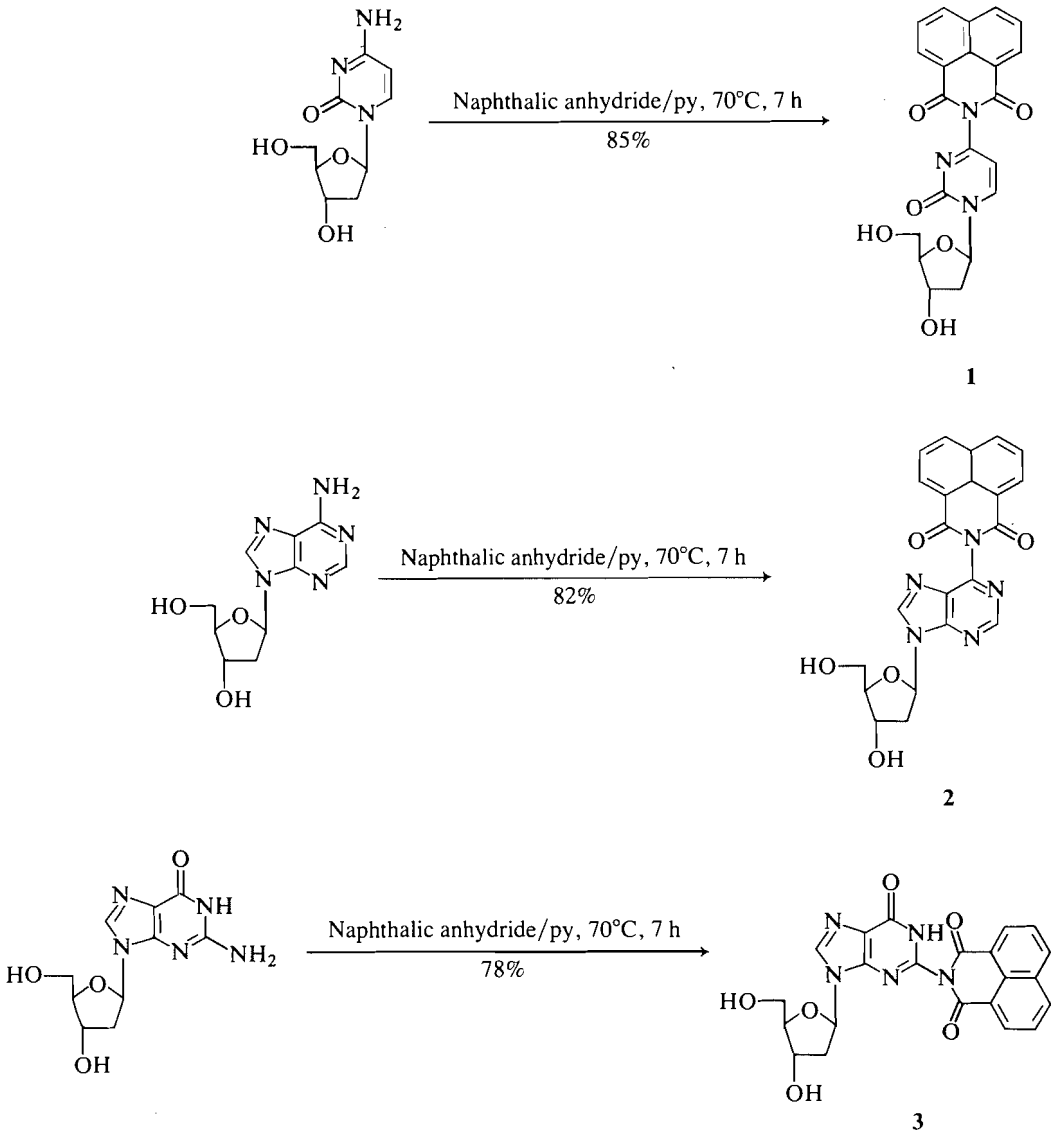
General procedure for dimer preparation

All three 5′-O-DMTr-N-naphthaloyldeoxynucleosides (1 mmol) and N,3′-diacetylated-5′-phosphates of dA (2 mmol) were evaporated *in vacuo* three times with dry pyridine and suspended in pyridine (5 mL). Triisopropyl benzenesulfonyl chloride (4 mmol) was added and the mixture stirred. After 4 h at room temperature (22–25°C), water (5 mL) was added while cooling the flask in an ice-bath (Scheme 2).

Purification of dimers

Purification of dimers was carried out on a DEAE cellulose (HCO_3^-) ion-exchange column. Elution was done by tris-HCl buffer (pH 8.3, 0.1 M) using a linear gradient of NaCl (0.05–0.15 M) and columns were monitored at 300, 270, and 260 nm in the case of CpA, ApA, and GpA, respectively. The desired fractions were pooled and evaporated to dryness *in vacuo*.

Dry residue dissolved in concentrated ammonia (40%) was kept sealed at 50°C for 4 h. It was then evaporated carefully, *in vacuo*, to dryness. Acetic acid (80%) was added to the flask and left at 25–30°C for 30 min. Acid was evaporated *in vacuo* and the residue dissolved in a minimum amount of water, diluted to twice the initial volume, and extracted four times with ether. The aqueous layer was lyophilized and stored under dry conditions. The dimers were obtained in 62–68% yield.



SCHEME 1. Preparation of cyclic imides of deoxynucleosides (dC, dA, and dG).

Preparation of 5'-O-DMTr-N-nph-dNS-3'-O-succinate

To 5'-O-DMTr-N-nph-dNS (0.5 mmol) dissolved in pyridine (2.5 mL) were added triethylamine (0.2 mL) and succinic anhydride (0.55 mmol). The reaction mixture was worked up as usual (14) to give the final product. Yield, 70–75%.

Preparation and purification of tetramers d(TTTC), d(TTTA), and d(TTTG)

5'-O-DMTr-N-nph-dNS-3'-O-succinate was linked to the support (kieselguhr/polydimethylacrylamide, 500 mg) with the help of the spacer, i.e., ethylenediamine and two glycine units, using Gait's procedure (16). Loading was estimated to be 68, 65, and 57 μmol g^{-1} in the case of dC, dA, and dG, respectively.

This functionalized resin (100 mg each) was placed in the column of the DNA bench synthesizer and allowed to stand in DMF for 5 min. Synthesis, deprotection, and purification of the tetramers were carried out by the usual procedure (14) (Fig. 1). Yield of d(TTTC), d(TTTA), and d(TTTG) was 72.6, 70.0, and 66.4%, respectively (Scheme 3).

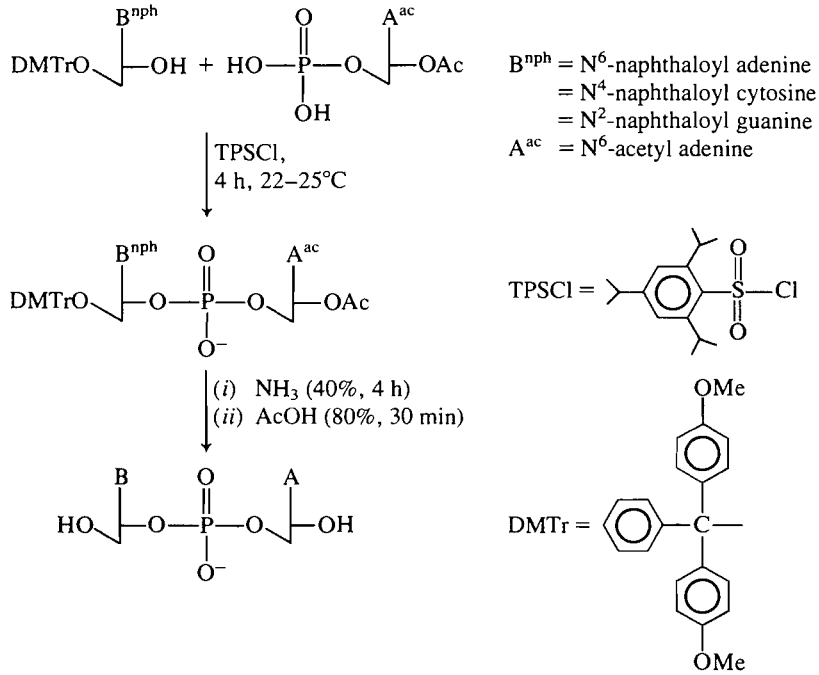
Enzymatic hydrolysis of tetramers

Each tetramer (0.4 A_{260} units) dissolved in 0.1 M Tris-HCl buffer, pH 8.5 (0.5 mL), was first digested with snake venom phosphodiesterase (5 μg, 37°C, 2 h) and then incubated with alkaline phosphatase (5 μg, 37°C, 1 h). The nucleosides thus obtained were separated (Fig. 2) using 0.1 M triethylammonium acetate, pH 7.0 (A), and acetonitrile (B). A gradient elution using 6% B over 20 min at a flow rate of 0.5 mL min^{-1} was achieved. After separation, quantification was made at 260 nm on the basis of peak areas and extinction coefficients of the nucleosides (E_{260} : dT, 8800; dC, 7300; dA, 15 400; and dG, 11 700).

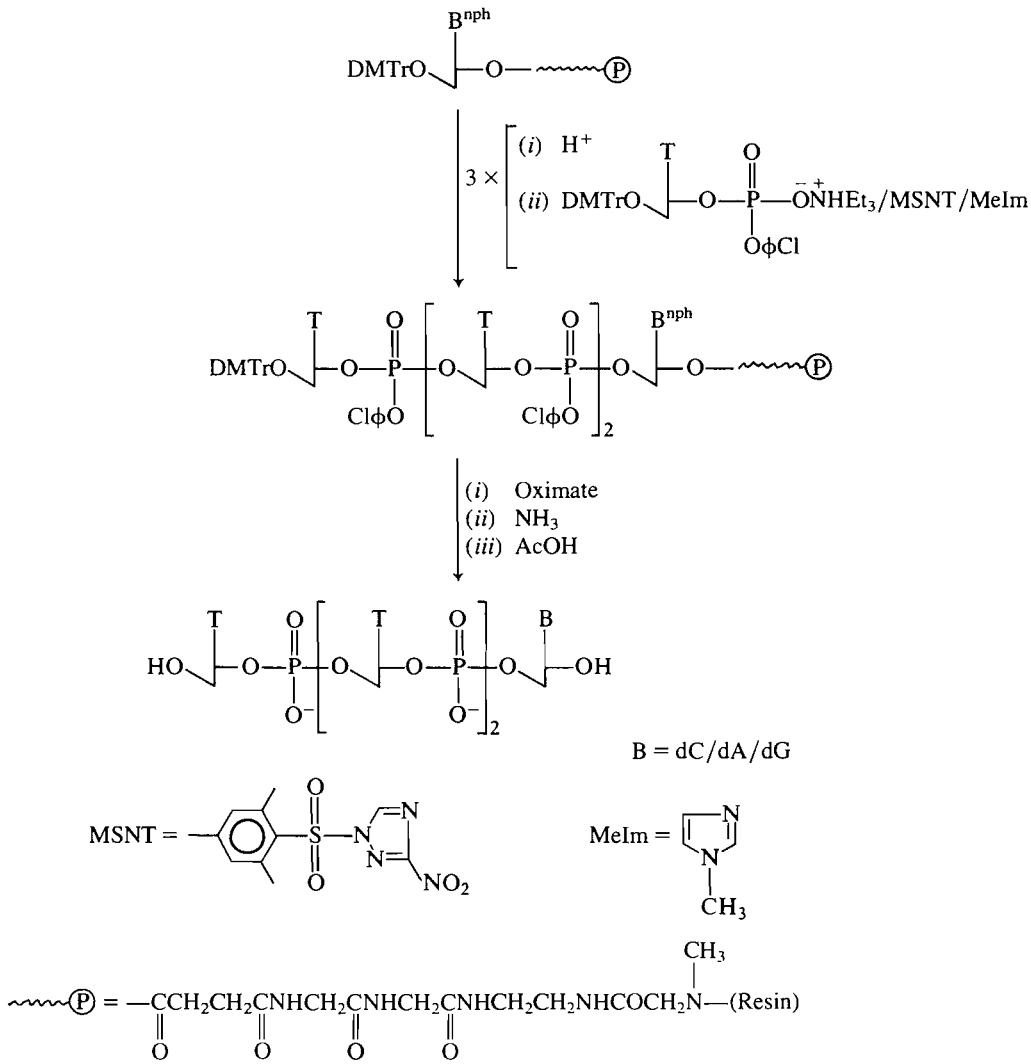
Results and discussion

The naphthaloyl group has been used for protection of the exocyclic amino function of all three deoxynucleosides, i.e., dA, dG, and dC, when cyclic imides were obtained (Scheme 1). These cyclic imides have been well characterized by elemental analyses, and by their UV and NMR spectra. There was no indication of any free -NH or -COOH group.

The absolute selectivity observed during protection under controlled experimental conditions for all three deoxynucleosides is of utmost importance. It eliminates the two-step procedure of protection whereby, first, a fully protected derivative is formed that on subsequent and selective hydrolysis gives the desired product (Khorana's procedure) (17), and it also avoids the need for prior silylation (Jones' procedure) (18). This selectivity may be explained as most probably due to the formation of stable cyclic imides, which drives the reaction predominantly in this direction.



SCHEME 2. Preparation of dimer in solution phase.



SCHEME 3. Synthesis of tetramer on solid support.

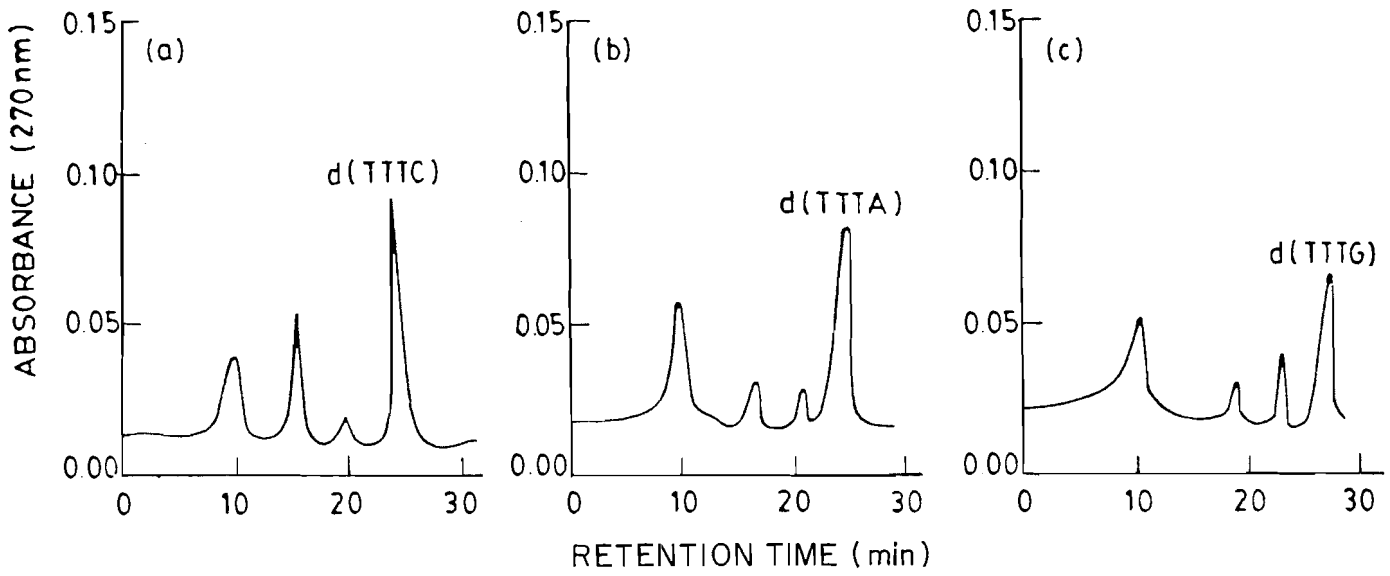


FIG. 1. Ion-exchange HPLC of tetramers, gradient 0–70% buffer B, 40 min, temperature 25°C.

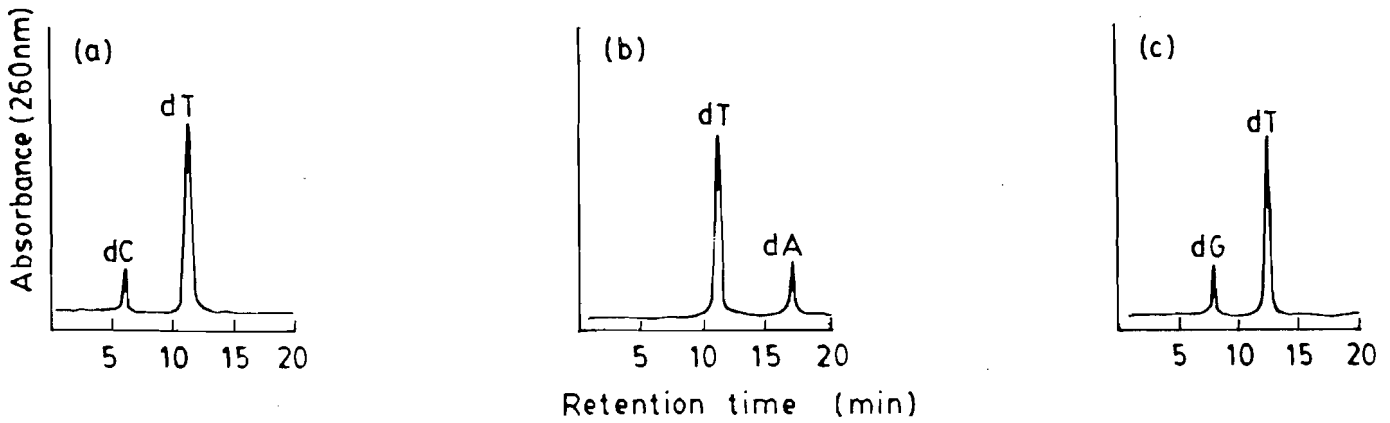


FIG. 2. Reversed phase HPLC of enzymatically hydrolyzed tetramers, 6% buffer B over 20 min, flow rate 0.5 mL min^{-1}.

A literature survey reveals that, so far, acid chlorides have been employed for acylation of the exocyclic amino function of deoxynucleosides. Because of the slow reaction between anhydride and amino function (cf. chloride and amino function), more selectivity can be observed. Thus, the use of anhydride is certainly advantageous, due also to its stable nature and easy storage. Furthermore, use of acid chlorides is well known to yield isomeric imides, while anhydrides are reported to exclude the formation of any such isomers (19). In this respect the use of the naphthaloyl group is more advantageous and convenient as compared to the phthaloyl group.

Other advantages envisaged for this group are its bulkier nature and higher lipophilicity. These are important considerations for N-acyl protecting groups. The naphthaloyl group has found extensive use in preparation of smaller blocks via solution phase methodology where column purification of those fully protected blocks after each condensation step is necessary. Because of high lipophilicity, the group helps in purification. It has been observed that phosphodiester oligomers have a tendency to become less soluble in organic solvents with increasing chain length due to polar effects (20). This can make experimental procedure difficult in connection with solvent extractions and adsorption chromatographic methods. However, naphthaloyl derivatives are highly soluble in organic solvents due to the hydrophobic nature of the group and are, therefore, well suited for adsorption chromatography as well as for purification of protected oligonucleotides by reversed phase HPLC (21). Thus, the group acts as a purification handle.

The protected monomer units have been found sufficiently stable. In the case of adenosine, the imidazole ring opened when the phthaloyl group was used for amino protection, under the reaction conditions of oligonucleotide synthesis (7), and thus many problems were created. But in the present case no ring opening was observed. This, in all probability, was due to the formation of a more stable six-membered ring in the cyclic imides in place of a five-membered ring as in the case of phthaloyl derivatives.

Conditions for removal of the naphthaloyl group are comparatively milder and speed of reaction is faster. It can be removed by concentrated ammonia (40%) at 50°C in 4 h (cf. the benzoyl group, 40% ammonia, 50°C, 5 h), although the initial rate of hydrolysis is different in the case of different nucleosides.

To adjudge the efficiency of the group in preparation of small blocks and oligonucleotides by solution and solid phase methods, different dimers and tetramers have been prepared by the phosphodiester and triester approach, respectively. An overall yield of 62–68% in the case of dimers and 66–72% in the case of tetramers was obtained, which is almost comparable

to the yields reported in the literature, thereby indicating the suitability of the group in both approaches, viz., solution and solid phase.

To confirm the structure of the tetramers, enzymatic hydrolysis with snake venom phosphodiesterase followed by alkaline phosphatase was carried out. After HPLC analysis, the cleavage pattern of the tetramers clearly indicated the presence of deoxynucleosides in a 3:1 ratio in each case (Fig. 2). The peak assignment, based on the retention time, was confirmed by direct comparison with authentic samples of deoxynucleosides. HPLC analyses clearly showed the absence of any base modification.

Acknowledgement

The authors are grateful to the C.S.I.R., Government of India, for financial assistance.

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