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(α-Pyridyl) methyl phosphoro-bis-triazolide as a new phosphorylating reagent for internucleotide bond formation*

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Abstract, (α-Pyridyl)methyl phosphoro-bis-triazolide has been found to be a reagent of choice for phosphate protection in oligodeoxyribonucleotide synthesis. The reagent has been used successfully to phosphorylate all the four 5'-and N-protected deoxynucleosides. The resulting 3'-phosphorylated derivatives were found to be fairly stable as either triethyl ammonium salts or cyanoethyl derivatives. The phosphorylated derivatives were used in the preparation of the dimers T_pT and $d(A_pT)$ in solution phase and a tetramer, TTTT, and a hexamer, d(ATATAT), on solid phase using glass support. The method gave excellent yields. Considerably reduced condensation time (6-9 min) and practically no cleavage of the internucleotide bond during the removal of the group are the advantages.

Keywords. Phosphorylation; internucleolide-bond.

Introduction

Despite tremendous progress in oligonucleotide synthesis, there still remain many unsolved problems. Improvements in oligonucleotide synthesis are being continuously introduced, including the use of better protecting groups at different nucleophilic sites, mainly amino and phosphate groups. A number of groups have been used in this laboratory for exocyclic amino protection (Mishra and Mishra, 1986; Singh and Mishra, 1987; Mishra, K., Dikshit, A., Singh, R. K. and Chaddha, M., unpublished results). The phosphate protecting group must remain intact throughout the assembly of the oligonucleotide chain and therefore has to be selected with great care. It should not be sensitive to acid or base catalyzed hydrolysis. The reagent itself as well as the phosphorylated nucleoside should be stable, easily purifiable, storable for prolonged periods and simultaneously reactive enough to react with another monomer nucleoside without much activation. Thus, there are severe problems of selectivity and compatibility in the use of these protecting groups.

Several groups have been reported for the protection of the phosphate moiety, specially for use in the formation of.internucleotide bond by the triester approach (Crämer *et al.,* 1963; Reese and Saffhil, 1968; Letsinger and Mungall, 1970; van Boom *et al.,* 1976).

The phosphorodichloridates used earlier for phosphorylation led to the formation of 3'-3' and 5'-5' symmetrical dimers. In order to get rid of this problem, aryl

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Abbreviations used: 5′-O-DMTrT, 5′-O-Dimethoxytrityl-thymidine; 5′-O-DMTr-N^6bz-2′-dA, 5′-O-dimethoxytrityl-N^6-benzoyl-2'-deoxyadenosine; 5′-O-DMTr-N^4-bz-2'-dC, 5′-0-dimelhoxytrityl-N^4-benzoyl-2′-deoxycytidinc; 5'-O-DMTr-N^2-ibu-2′-dG, 5′-O-dimethoxytrityl-N2-isobutyryl-2'-deoxyguanosine; Melm, 1-methyl imidazole; TPSCl, triisopropylbenzenesulphonyl chloride; MSNT, mesitylenesulphonyl-nitrotriazole; LCAA-CPG, long chain alkylamine controlled pore glass; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography: THF. tetrahydrofuran.

phosphoroditriazolides were introduced as an alternative to dichloridates (Katagiri *et al.,* 1975).

We have now phosphorylated all the 4 suitably protected deoxynucleosides, *viz.,* 5′-O-dimethoxytrityl-thymidine (5′-O-DMTrT), 5′-O-dimethoxytrityl-N^6-benzoyl-2′-deoxyadenosine (5′-O-DMTr-N^6bz-2′-dA), 5′-0-dimethoxytrityl-N4-benzoyl-2′-deoxycytidine (5′-O-DMTr-N^4 bz-2′-dC), 5′-0-dimethoxytrityl-N^2-isobutyryl-2′-deoxyguanosine (5′-O-DMTr-N^2ibu-2′-dG) with the bistriazolide of 2-methylpyridyl phosphorodichloridate. These derivatives have been obtained in the form of their triethylammonium salts or cyanoethyl derivatives. The latter are sufficiently stable to be stored at 0°C for prolonged periods.

One of the major problems encountered during the removal of the phosphate protecting groups so far reported, has been the cleavage, to different degrees, of internucleotide bond (Cusack *et al.,* 1973). During the removal of (α-pyridyl) methyl group by the attack of oximate anion, practically no cleavage has been observed.

Materials and methods

The deoxynucleosides were purchased from Yoshitomi Pharmaceutical Co., Japan, The base protection and tritylation was carried out as reported by Schaller *et al.* (1963). Dimethoxytrityl chloride, triazole, 1,1,3,3-tetramethyl guanidine, 4-nitrobenzaldoxime, 1-methylimidazole (Melm), triisopropylbenzenesulphonyl chloride (TPSC1), mesitylenesulphonylnitrotriazole (MSNT) and long chain alkylamine controlled pore glass (LCAA-CPG) were purchased from Fluka, Buchs, Switzerland, Sigma Chemical Co., St. Louis, Missouri, USA, Biosearch, London, UK and Cruachem Chemical Co., Livingston, Scotland.

Solvents were duly purified prior to use. Thin-layer chrimatography (TLC) was carried out on silica gel G (E. Merck, Germany) plates and the plates sprayed with Ischerwood reagent, iodine and H_2SO_4 for location and differentiation of spots. Solid-phase synthesis was performed on a DNA double bench synthesiser (Omnifit Kit). Ultraviolet absorption was measured on a Hitachi 220 S spectrophotometer. High pressure liquid chromatography (HPLC) was carried out on LKB ultrapac (ODS Column, 9·4 × 250 mm). *β*-Cyanoethanol was prepared in the laboratory by reaction of 2-chlorcethanol and KCN.

Preparation of 2-methylpyridine-N-oxide

The N-oxide of 2-methylpyridine was prepared by a method similar to that for pyridine-N-oxide (Vozza, 1962). The product, 2-methylpyridine-N-oxide was distilled at reduced pressure (138–140°C/15 mm Hg), yield 90 ml (91%).

Preparation of 2-acetoxymethylpyridine (Oae *et al.,* 1962)

A mixture of acetic anhydride (100 ml) and 2-methyl pyridine-N-oxide (80 ml) was heated gently to 140°C and heating was continued for a further 5 min. After removing acetic acid and acetic anhydride from the reaction mixture, 2-acetoxymethyl pyridine was distilled at reduced pressure (90–92°C/5 mm Hg), yield 63·5 ml (79%).

Preparation of 2-hydroxymethyl pyridine

2-Acetoxymethyl pyridine (60 ml) was refluxed with methanolic KOH (70 g in 250 ml) CH_3OH) (Ford and Swan, 1965). After hydrolysis the reaction mixture was dried in

vacuo and the residue was extracted with $CHC1_3$. The desired product was distilled at reduced pressure (111–112°C/15 mm Hg). UV: λ_{max} (CH_2Cl_2) 285 nm; yield 57 ml (95%).

Preparation of (α *pyridyl*)*methyl phosphorodichloridate*

2-Hydroxymethyl pyridine (93 ml; 1 mol) was treated with $POC1_3$ (360 ml; 4 mol) and the reaction mixture was heated for 3 h in the presence of $A1C1_3$ (catalyst). The product was distilled at reduced pressure 120°C/12 mm Hg), yield 47 ml (50%).

The product was confirmed by its alkaline hydrolysis. After hydrolysis, starting alcohol, 2-hydroxymethylpyridine, was recovered, R_f 0·90 (C_6H_6/ CH_3O H; 8·5:1·5, v/v), identical with the authentic sample.

Preparation of (α *pyridyl*)*methyl phosphoro-bis-triazolide*

1,2,4-Triazole (13·35 g, 200 mmol) was dissolved in THF (500 ml). To this was added (α-pyridyl)methyl phosphorodichloridate (12 ml, 75 mmol) and triethylamine (24·40 ml, 175 mmol). After shaking the reaction mixture for a few min, a copious white precipitate of triethylammonium chloride appeared. The precipitate was filtered off and the filtrate was directly used for the phosphorylation of 5′- and N-protected deoxynucleosides.

Preparation of triethylammonium-5′-O-DMTrT-3′-O-(α *pyridyl*)*methyl phosphate*

5'-O-DMTrT (545 mg, 1 mmol) was taken in dry THF (15 ml) and (α-pyridyl)methyl phosphoro-bis-triazolide (1·5 mmol) and 1-methylimidazole was added to the reaction flask. Reaction was complete in 20 min as checked by TLC. The reaction was quenched by adding aqueous triethylammonium bicarbonate (200 ml, 1 M, pH ≈ 8) and then extracted with CH_2C1_2 (2 × 15 ml). The organic layer was dried over sodium sulphate and then evaporated *in vacuo* to a gum.

The gum was subjected to silica gel column chromatography and elution was carried out with CH_2C1_2/CH_3OH in increasing polarity with 1% $(C_2H_5)_3N$. Fractions showing absorption at 270 nm were pooled and evaporated to dryness *in vacuo*. The residue was dissolved in CH_2C1_2 (2 ml) containing 1% $(C_2H_5)_3N$, and the product was precipitated by dropwise addition of this solution to vigorously stirred dry pentane (50 ml). The white precipitate was collected by centrifugation at 4°C and washed with pentane. The pure product was dried over KOH and stored in a sealed container at low temperature, R_f 0·40 (CH_2C1_2/CH_3OH; 9:1, v/v), UV: λ_{max} (CH_2C1_2) 270 nm, yield 730 mg (90%).

Confirmation of the phosphorylated unit was carried out by total deprotection when T_P (nucleotide) was obtained in place of T (nucleoside) as confirmed by direct comparison with authentic samples chromatographically and spectroscopically.

Similarly, phosphorylated derivatives of 5′-O-DMTr-N^6 bz-2′-dA (R_f 0·58, CH_2C1_2/CH_3OH; 9:1, v/v, λ_{max}280 nm), 5′-O-DMTr-N^4 bz-2′-dC (R_f 0·50, CH_2C1_2/ CH_3OH; 9:1, v/v, λ_{max}305 and 261 nm), 5′-O-DMTr-N^2 ibu-2′dG (R_f 0·56, CH_2C1_2/ CH_3OH; 9:1, v/v, λ_{max}278 and 262 nm) were also prepared.

Preparation of 5′-O-DMTrT-3′-O-(α. pyridyl)methyl β-cyanoethyl phosphate

To 5'-O-DMTrT (545 mg; 1 mmol) taken in dry THF, was added freshly prepared (α-pyridyl)methyl phosphoro-bis-triazolide (15 mmol) and 1-methyl imidazole used as a

catalyst for phosphorylation reaction. The reaction mixture was stirred for 20 min at room temperature under anhydrous conditions. Completion of reaction was checked by TLC (CH_2C1_2/CH_3OH; 9·5:0·5, v/v). After completion of the reaction, *β*-cyano-ethanol (20 mmol) was added to the flask and the mixture kept for 1 h under vigorous stirring. The reaction mixture was evaporated to a gum *in vacuo.* This was then dissolved in CH_2C1_2, washed with 0·1 M NaH_2PO_4 (3 × 20 ml) and then with water (2 × 20 ml). The organic part was dried over Na2SO4, evaporated to a gum and subjected to silica gel column chromatography eluting it with CH_2Cl_2/CH_3OH in the presence of 1% $(C_2H_5)_3N$. Fractions were monitored at 270 nm and pooled appropriately. The solution was evaporated to dryness *in vacuo* dissolved in CH_2Cl_2, (5 ml). The product was precipitated with a mixture of dry ether/petroleum ether (50 ml, 2:3, v/v), R_f 0·60 (CH_2C1_2/CH_3OH; 9·5:0·5, v/v), UV: λ_{max} (CH_2Cl_2) 270 nm, yield 624 mg (84%).

Similarly, cyanoethylated derivatives of the other protected nucleosides were also prepared.

Removal of (α-*pyridyl*)*methyl group*

Triethylammonium salt of 5'-O-DMTrT-3'-O-(a-pyridyl)methyl phosphate (0·1 mmol) was treated with 1,1,3,3-tetramethylguanidinium salt of 4-nitrobenaldoxime (0·3 mmol). Reaction was followed by TLC. Complete deprotection was achieved in 20 min.

Preparation of dimers, T_PT and d(A_PT)

The dinucleotides T_pT and d(A_pT) were prepared by condensing appropriate units, and co-evaporated to dryness *in vacuo* with the respective solvents, under varied reaction conditions (table 1). In condensation reactions, 1·5-fold molar excess of P-component over 5′-OH component and 3-fold molar excess of TPSC1 and MeIm each relative to P-component were used. After the appropriate time, water (5 ml) was added to the ice-cooled reaction mixture. The reaction mixture was extracted with CH_2C1_2, washed with Na HCO_3 (0·1 M), dried over sodium sulphate and evaporated to a gum *in vacuo.* The dimers were purified by silica gel column (20 × 2 cm)

Table 1. Preparation and characterisation of dimers T_pT and d(A_pT).

3-Phosphate unit	Units 5′-OH unit	Solvent	Condensing reagent	Condensation time (min)	R_f^*	Yield (%)
5′-O-DMTr-T-3′-O [(α-Pyridyl)-methyl]-phosphate	T-3′-OAc	Pyr	TPSCl	30	0·3	80
5′-O-DMTr-N-bz-dA-3′-O [(α-pyridyl)methyl]-phosphate	T-3′-O-Ac	Pyr	TPSCl + MeIm	9	0·33	84
		CH_3CN	TPSCl + MeIm	6	0·33	89

*Solvent CH_2Cl_2/MeOH; 9:1, v/v.

chromatography (figure 1) eluting with CH_2C1_2/CH_3OH in increasing polarity in the presence of 1% triethylamine. Fractions were monitored at 270 and 280 nm, respectively, and fractions containing the desired product were pooled and evaporated to dryness *in vacuo.*

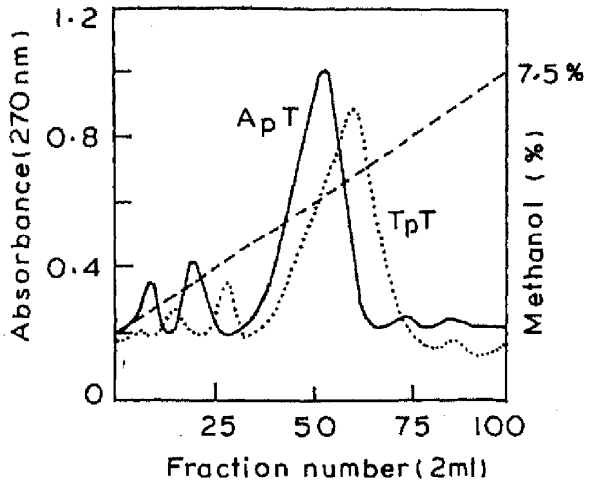


Figure 1. Column chromatography of fully protected dinucleotidcs.

The dry residue obtained above was treated with l,1,3,3-tetramethylguanidinium salt of 4-nitrobenzaldoxime (0·23 mmol) in dioxane/water (1:1, v/v) for 20 min. The reaction mixture was evaporated to dryness and treated with ammonia (40%, 2 ml) for 4 h to remove acetyl and benzoyl groups. The mixture was again evaporated to dryness *in vacuo* and treated with CH_3COOH (80%, 2 ml) at room temperature for 30 rnin. The reaction mixture was evaporated to dryness and the residue was subjected to reversed phase silica gel column (10 × 2 cm) chromatography (figure 2) eluting with acetone/water in increasing polarity. The dimers were obtained in good yields as determined by trityl estimation (table 1).

In order to confirm the nature of the dimers, an aliquout from each was subjected to hydrolysis with cone. NH_3 at 150°C for 24 h. The products, T_P and T, in the case of T_pT and A_P and T in the case of $d(A_pT)$, were confirmed chromatographically and spectroscopically by direct comparison with authentic samples.

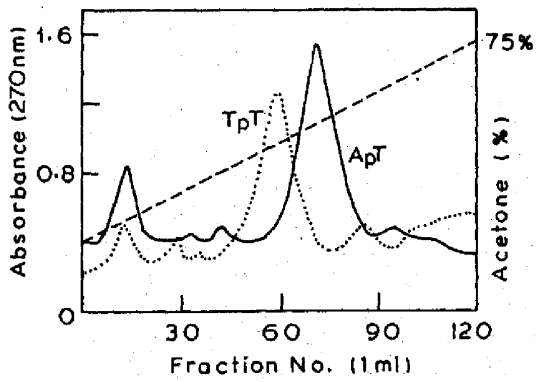


Figure 2. Reversed phase column chromatography of deprotected dinucleotides.

Preparation of 5'-O-DMTrT-3'-O-succinate

To 5'-O-TrT (545 mg,1 mmol) dissolved in pyridine (5 ml), triethylamine (0·4 ml) and succinic anhydride (ll0 mg, 1·1 mmol) were added. The mixture was kept at room temperture for 12 h and then applied to a Dowex-50 (pyridinium form) column (10 × 2 cm) and eluted with pyridine/water (1:4, v/v). The eluate was evaporated to dryness *in vacuo,* dissolved in CH_2Cl_2 (0·5 ml) and purified on a silica gel column (6 × 2 cm). Elution was first carried out with CH_2Cl_2 followed by C_2H_5OH/CH_2Cl_2 (3:97, v/v). Trityl and sugar positive fractions of latter elution were pooled and the product precipitated with ether/pentane (3:2, v/v). *Rf* 0·26, (CH_2Cl_2/CH_3OH; 9:1, v/v), yield 476·6 mg (74%).

Preparation of tetramer TTTT and hexamer d(ATATAT) on solid support

The compound 5′-O-DMTrT-3′-O-succinate was linked to the solid support LCAA-CPG (Gough *et al.,* 1981). Loading was found to be 35 μmol g^{-1} as estimated by trityl analysis (Gait *et al.,* 1980). This derivatised support was used for the synthesis of tetramer and hexamer.

Functionalised support was taken in both columns (100 mg each) of the DNA bench synthesiser and the wash cycle run in the following order:

	Min
3% TCA in CH_3CN-CH_2Cl_2 (7:3, v/v)	3
CH_3CN-CH_2Cl_2 (7:3, v/v)	2
Coupling mix in CH_3CN-CH_2Cl_2 (7:3, v/v)	9
CH_3CN-CH_2Cl_2 (7:3, v/v)	2
CH_3CN-Ac_2O-MeIm (17:2:1)	3
CH_3CN-CH_2Cl_2 (7:3, v/v)	2

The cycles of wash and addition of incoming nucleotide unit (28 μmol) were carried out until the required chain length of oligomers was obtained. After the final coupling reaction, the support was washed with CH_2C1_2, MeOH and ether. The support was taken out of the column and dried.

Deprotection and isolation of oligonucleotides

The tetramer and hexamer linked to the support were treated with 0·5 M solution of l,l,3,3-tetramethylguanidinium-4-nitrobenzaldoxime in dioxane-water (1:1, v/v) for 16 h at room temperature. The support was filtered and the filtrates evaporated to dryness *in vacuo.* The residue was taken in 40% ammonia (5 ml). The flasks were sealed carefully and put in a thermostat bath at 60°C for 5 h. The reaction mixture was then evaporated to dryness *in vacuo.*

The above mass was taken in 0·1 M triethylammonium acetate and was analysed on reversed phase HPLC using C_{18} column. A gradient system of 20–30% acetonitrile in 0·1 M triethylammonium acetate over 15 min, was used for tritylated oligomers. The tritylated oligonucleotides were easily identified and isolated (figure 3). Fractions containing the desired sequences were concentrated and treated with 80% acetic acid (5 ml) to remove trityl group. After complete deprotection (30 min), the solution was concentrated and extracted with ether thoroughly. The fully deprotected oligonucleotides were analysed and isolated by reversed Phase HPLC

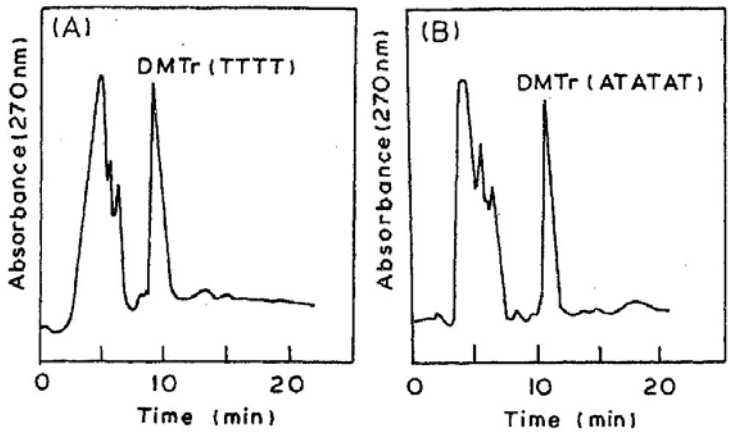


Figure 3. HPLC profiles of dimethoxytritylated tetramer (A) and hexarner (B).

through C_{18} column using a gradient of 10-15 % acetonitrile over 15 min at the rate of 1 ml min^{-1} (figure 4).

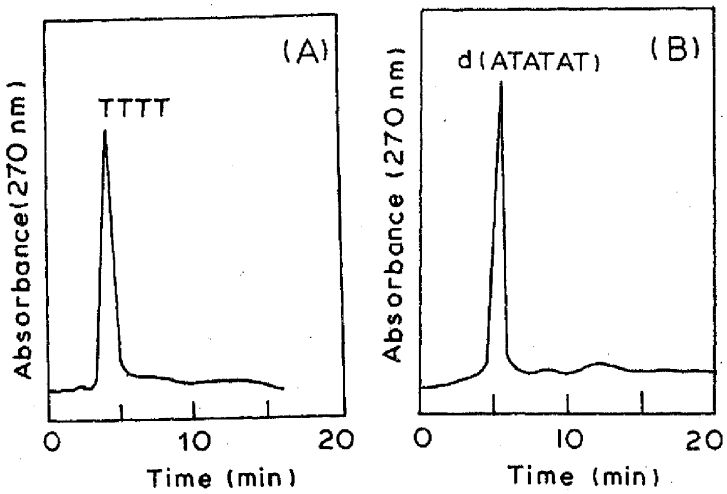
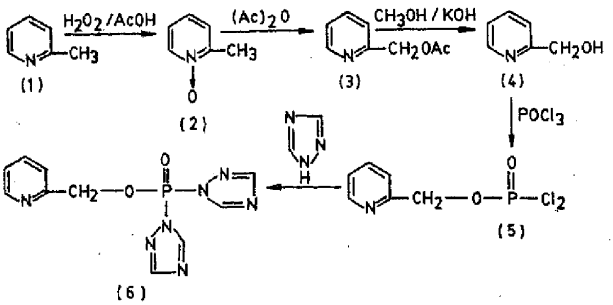


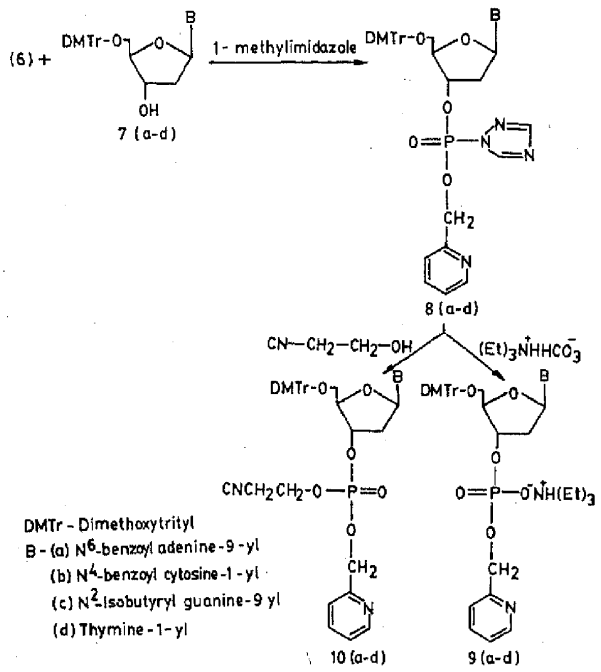
Figure 4. HPLC profiles of tetramer (A) and hexamer (B).

Results and discussion

The reagent (α-pyridyl) methyl phosphoro-bis-triazolide (scheme 1) has been found to be a very promising phosphorylating agent, for all the 4 suitably protected deoxynucleosides. The phosphorylated derivatives (7, a–d) were obtained in approxi mately 90% yield and were isolated in the form of either triethylammonium salts or cyanoethyl derivatives (scheme 2). The reactivity of the reagent was evident from the high yields obtained which are comparable to those with *o*- and *p*-chlorophenyl phosphoro-bis-triazolides. The cyanoethyl derivatives were comparatively very stable and could be stored at 0°C for prolonged periods without any decomposition. The problem of formation of symmetrical dimers during phosphorylation was also



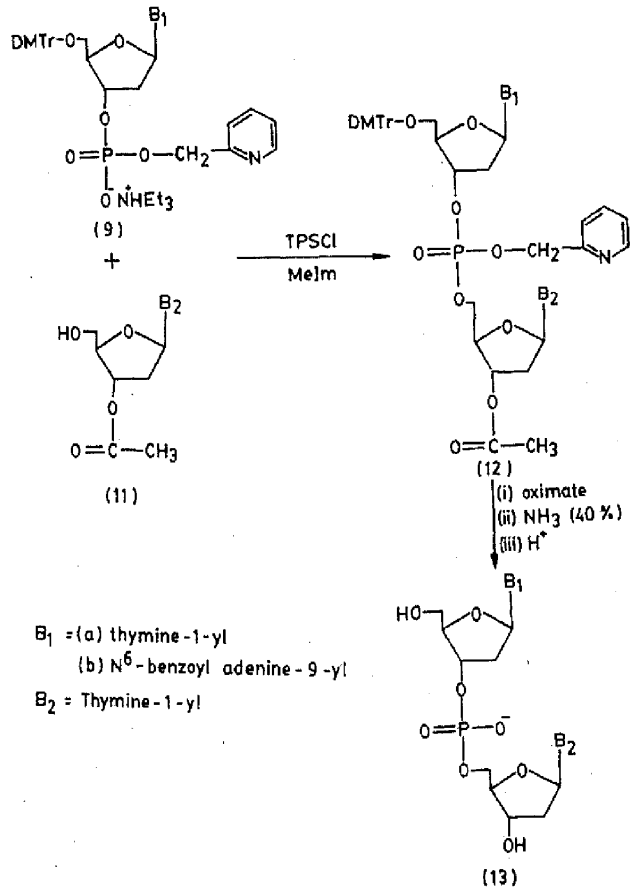
Scheme 1.



Scheme 2

looked into and no detectable dimerisation was observed using 1·5-fold molar excess of reagent and dry THF as solvent. This observation also supports the earlier reports (Broka *et al.,* 1980).

In order to prove the efficiency of the phosphate protecting group, two dimers, T_pT and $d(A_pT)$ have been prepared in solution phase (scheme 3) using different conditions for the condensation reaction (table 1). In the preparation of T_pT, condensation time was reduced to 30 min using TPSC1 as condensation reagent (*cf.* 2–3 h in the case of *o*- and *p*-chlorophenyl derivatives; Broka *et al.,* 1980). During the preparation of the dimer, $d(A_pT)$, varied conditions for condensation were attempted and significant improvement was noticed. The condensation time was found to be 6 min using acetonitrile as solvent and 9 min using pyridine; 1-methylimidazole was used as catalyst. In all the cases good yields (80–90%) of the dimers were obtained as suggested by trityl analysis (Gait *et al.*, 1980).

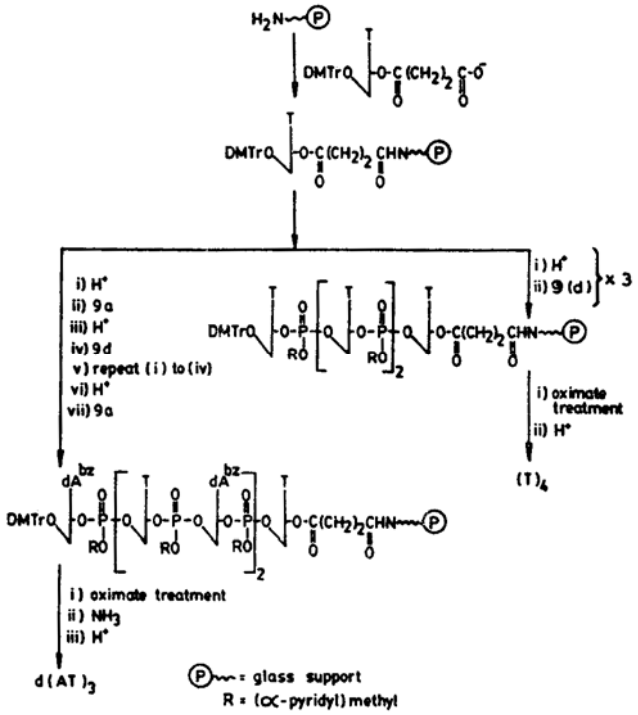


Scheme 3.

The reagent was further checked for versatility of use in oligonucleotide synthesis using solid phase methodology. Two oligomers, TTTT and d(ATATAT) were prepared (scheme 4) in good yields (79 and 68%, respectively)·using LCAA-CPG as the solid support. Synthesis was achieved by successive addition of fully protected nucleotide derivatives. At each step, 8-fold excess of P-component over the nucleoside capacity on the support was used. The coupling reagent MSNT and the catalyst Melm were used in 3-fold and 6-fold molar excess respectively to P-component. In both cases, one-solvent procedure for rapid solid phase synthesis was used (Efimov *et al.,* 1982).

The dimethoxytrityl group was removed after each coupling step using 3% trichloroacetic acid in a mixture of acetonitrile and dichloromethane. Trityl·analysis (Gait *et al.,* 1980) at each step suggested nearly a constant yield of approximately 9 4 % (as in the case of shorter sequences). The repeated acid treatment did not cause any detectable depurination in the case of the hexamer as no peak corresponding to the base adenine was observed during HPLC purification.

The constant coupling yield suggested minimum of failure sequences or internucleotide chain scission. The percentage yield of di- and trimer in the case of tetramer and di-, tri-, tetra- and pentamer in the case of hexamer as suggested by HPLC analysis of the mixture after the removal of the group were found to be



Scheme 4.

congruous with the loss at each coupling step in their preparation (as proved by trityl estimation). These results suggest the complete absence of internucleotide bond cleavage or very insignificant cleavage, if any, during the removal of the group.

Thus the remarkable advantages of the reagent are its easy introduction with good yields, better stability of the derivatives, practically no cleavage of the internucleotide bond during its removal and reduced coupling time with higher yields.

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References

Broka, C, Hozumi, T., Arentzen, R. and Itakura, K. (1980) *Nucleic Acids Res.,* **8,** 5461.
Cramer, F., Wittmann, R., Daneck, K. and Weimann, G. (1963) *Angew. Chem.,* **75,** 92.
Cusack, N. J., Reese, C. B. and van Boom, J. H. (1973) *Tetrahedron Lett.,* 2209.
Efimov, V. A., Reverdatto, S. V. and Chakhamakhcheva, O. G. (1982) *Nucleic Acids Res.,* **10,** 6675.
Ford, P. W. and Swan, J. M. (1965) *Aust. J. Chem.,* **18,** 867.
Gait, M. I, Singh, M. and Sheppard, R. C. (1980) *Nucleic Acids Res.,* **8,** 1081.
Gough, G. R., Brunden, M. J. and Gilham, P. T. (1981) *Tetrahedron Lett,* 4177.
Katagiri, N., Itakura, K. and Narang, S. A. (1975) *J. Am. Chem. Soc,* **97,** 7332.:
Letsinger, R. L and Mungall, W. S. (1970) J. *Org. Chem.,* **35,** 3800.
Mishra, R. K. and Misra, K. (1986) *Nucleic Acids Res.,* **14,** 6197.

Oae, S., Kitao, T. and Kitaoka, Y. (1962) *J. Am. Chem. Soc,* **84,** 3359.
Reese, C. B. and Saffhil, R. (1968) *Chem. Commun.,* **2,** 767.
Schaller, H., Weimann, G., Lerch, B. and Khorana, H. G. (1963) *J. Am. Chem. Soc,* **85,** 3821.
Singh, R. K. and Misra, K. (1987) *Indian J. Chem., (in* press).
van Boom, J. H., Burgers, P. M. J. and van Deursen, P. H. (1976) *Tetrahedron Lett.,* 869.
Vozza, F. J. (1962) *J. Org. Chem.,* **27,** 3835.