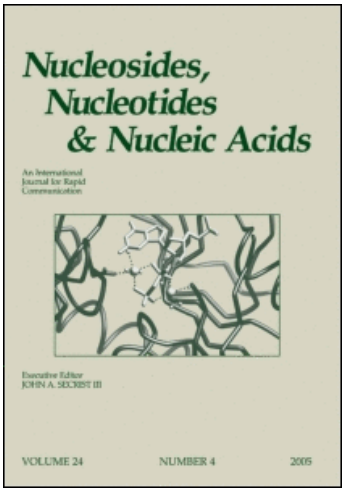

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Improved Methods for 3'-O-Succinylation of 2'-Deoxyribo- and Ribonucleosides and Their Covalent Anchoring on Polymer Supports for Oligonucleotide Synthesis

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To cite this Article Kumar, P. , Ghosh, N. N. , Sadana, K. L. , Garg, B. S. and Gupta, K. C.(1993) 'Improved Methods for 3'-O-Succinylation of 2'-Deoxyribo- and Ribonucleosides and Their Covalent Anchoring on Polymer Supports for Oligonucleotide Synthesis', Nucleosides, Nucleotides and Nucleic Acids, 12: 6, 565 — 584
To link to this Article: DOI: 10.1080/07328319308019012
URL: http://dx.doi.org/10.1080/07328319308019012

NUCLEOSIDES & NUCLEOTIDES, 12(6), 565-584 (1993)

IMPROVED METHODS FOR 3'-O-SUCCINYLATION OF 2'-DEOXYRIBO- AND RIBO-NUCLEOSIDES AND THEIR COVALENT ANCHORING ON POLYMER SUPPORTS FOR OLIGONUCLEOTIDE SYNTHESIS

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ABSTRACT: A more convenient and efficient method is described for the preparation of 3'-O-succinates of 2'-deoxyribo- and ribonucleosides, in a variety of solvents. Also, a new one-pot procedure has been developed for anchoring these succinates to polymer supports, suitable for solid phase synthesis of oligonucleotides.

In the last ten years, significant improvements have been made in the various aspects of solid phase synthesis of oligonucleoides (1-6). The nature of the solid support and the type of linkage for anchoring the first nucleoside to the support play a vital role in machine-aided synthesis of oligonucleotides via. phosphoramidite, phosphotriester or similar chemistries. The most widely used current methodology (3,7,8) utilises nucleosides anchored on long chain alkylamine-controlled pore glass (LCAA-CPG) by a succinyl linkage.

Several special linkages (9-12) are also known for synthesising normal and modified oligonucleotides with various functional groups (13-18) at their 3'-termini. Recently, Alul et al. (19) have used oxalyl linkage which is suitable for the synthesis of base sensitive O-alkylphosphotriesters. Still, the succinate linkage remains the most favoured for solid phase oligonucleotide synthesis.

Basically, two approaches (20-22) are in use for the preparation of key intermediates, 2'-deoxyribo- and ribonucleoside-3'-O-succinates, required for derivatisation of polymer supports. One involves the succinylation with a large excess of succinic anhydride followed by purification by chromatographic methods while the other one is based on complete consumption of succinic anhydride rather than free

nucleosides.The free nucleosides do not interfere in the following reaction. Obviously, the yield of succinates is in the range of 60-80%.

The covalent anchoring of these nucleoside-3'-O-succinates to the polymer supports involves the DCC mediated synthesis of p-nitrophenyl or pentachlorophenyl active esters of appropriately protected nucleoside-3'-O-succinates which subsequently react with the primary amino groups on the polymer supports to generate the fully functionalised polymer supports. But the method generally yields moderate loading of support-bound nucleoside (10-25 μmol / g LCAA-CPG). 1-(Dimethylaminopropyl)-3-ethylcarbodiimide (DEC) has also been used for direct covalent linking of either nucleoside-3'-O-succinates with LCAA-CPG (23) or 2'-deoxyribo-, ribo- and arabinonucleosides to alkylamidopropanoic acid-CPG (24) via. 2'- or 3'-hydroxyl groups. The time taken for this process at room temperature is about 1-2 days. Ogilvie et al. (25) have also described a direct functionalisation procedure for linking 3'-O-TBDMS-ribonucleoside-2'-O-succinates with LCAA-CPG in the presence of triethylamine in dry pyridine for 4 days at room temperature which results in the nucleoside loadings in the range of 18-20 μmol/g.

In this communication, we have tried to study the effect of various parameters such as concentration of succinic anhydride, 4-dimethylamino-pyridine (DMAP) (an acylating catalyst) and effect of temperature and solvent on time consuming succinylation reaction. According to our procedure, the succinylation reaction proceeds very fast when it is carried out in non-polar solvents in the presence of catalytic amount of DMAP. 2'-Deoxyribonucleosides and ribonucleosides are completly succinylated in just 10 and 55 min respectively using 1.5 equivalents of succinic anhydride and 0.5 equivalent of DMAP at 50°C in 1,2-dichloroethane.

The nucleoside-3'-O-succinates have been anchored to the polymer supports in one pot general method using alkyl and aryl diisocyanates. The method involves the reaction of nucleoside-3'-O-succinates with one equivalent of tolylene-2,4-diisocyanate or hexamethylene-1,6-diisocyanate in the presence of 4-dimethylaminopyridine (DMAP) in dry dichloromethane (DCM) to generate monoisocyanates which are directly used in the following reaction with the amino groups of the polymer supports in the presence of N-ethyldiisopropylamine to obtain a fully functionalised polymer support in 8-10h with excellent nucleoside loadings (38-40 μmol nucleoside / g LCAA-CPG). Other commonly used polymer supports have also been functionalised by this procedure.

RESULTS AND DISCUSSION :

3'-O-Succinylation of 2'-deoxyribo- and ribonucleosides

The 3'-O-succinylation of appropriately protected nucleosides following standard protocols is carried out by the reaction of succinic

anhydride in pyridine using a catalytic amount of an acylating catalyst, 4-dimethylaminopyridine (DMAP). The rate of succinylation under these conditions is very slow. Moreover, chromatographic purification of the final product is often required and this makes the synthesis of these intermediates a time consuming process.

This problem has been rectified by a systematic study of the effect of various parameters e.g temperature, solvent, concentration of succinic anhydride and 4-dimethylaminopyridine on the rate of succinylation. The role of DMAP as acylating catalyst is now well established (27,28). In the very first step, DMAP rapidly forms an N-acylpyridinium salt which gets stabilised by resonance. The pyridinium salt exists as loose ion-pairs in non-polar solvents which favours the nucleophilic attack at the acyl group. The second step is controlled by counter ion i.e. general base catalysis. Therefore, acid anhydrides are better acylating agents than acid chloride under similar conditions. In order to increase the rate of second step, a strong base such as triethylamine in stoichiometric amount is used which prevents the protonation of DMAP as well as detritylation of nucleoside by the acid generated during the progress of the reaction. We have used these conditions for the rapid 3'-O-succinylation of nucleosides and their covalent anchoring on polymer supports using alkyl and aryl diisocyanates.

The 3'-O-succinylation of 2'-deoxyribonucleosides was carried out by taking 1.5 equivalents of succinic anhydride, 0.5 equivalent of DMAP in 1,2-dichloroethane at 50°C. The reaction proceeded very fast and was completed in just 10 min.

The 3'-O-succinylation of ribonucleosides was completed in 55 min under identical conditions used for 2'-deoxynucleosides.

Effect of various parameters on rate of succinylation of nucleosides

The effect of various parameters, viz., solvent, temperature, concentration of succinic anhydride and DMAP on rate of succinylation of 2'-deoxyribo- and ribonucleosides have been studied in detail in order to arrive at the optimal conditions required for the succinylation reaction.

Fig. 1(a-d) shows the kinetic studies performed to determine the optimum conditions for the succinylation reaction. The data indicate, as expected, that the rate of succinylation reaction depends on the concentration of succinic anhydride. The optimum temperature for this reaction was found to be 50°C (Fig. 1c). Higher temperatures (>50°) are not recommended since this may cause detritylation and modification of nucleosides.

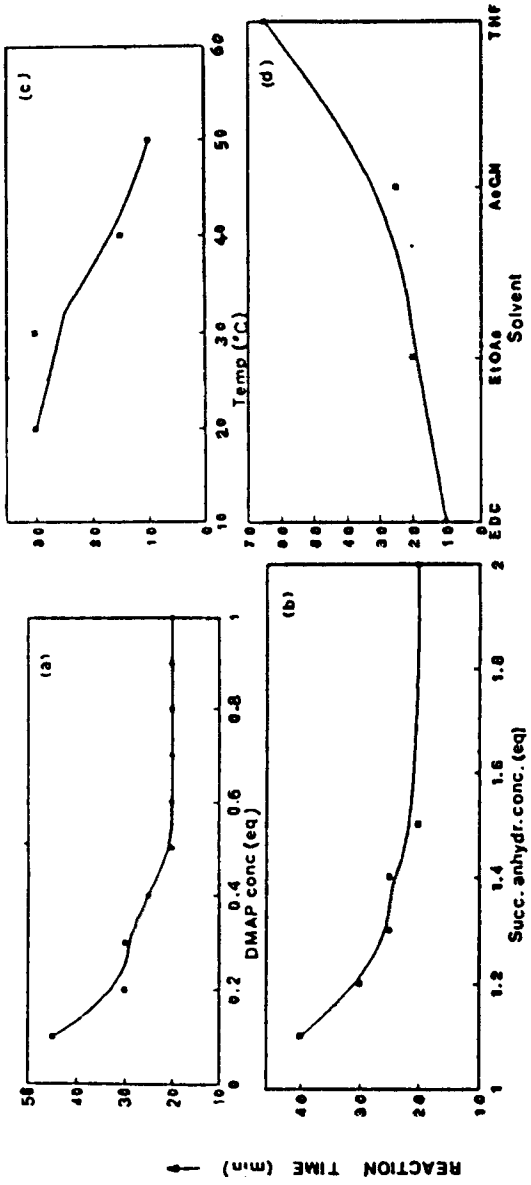

FIGURE 1. Time course of the succinylation reaction using (a) 1.5 eq. of succinic anhydride with varying amounts of DMAP in EDC; (b) 0.5 eq. of DMAP with varying amounts of succinic anhydride in EDC; (c) 1.5 eq. of succinic anhydride and 0.5 eq. of DMAP at different temperatures in EDC; (d) 1.5 eq. of succinic anhydride and 0.5 eq. of DMAP in different solvents at 50°C.

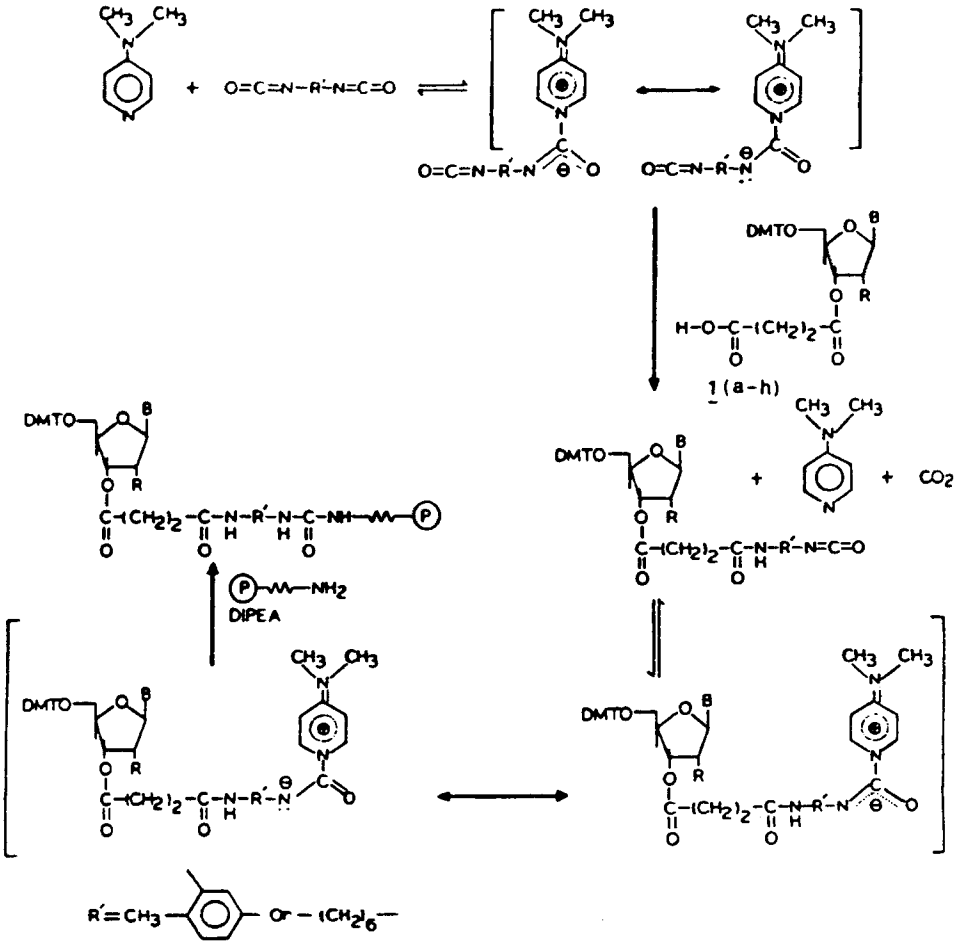

FIGURE 2. Reaction mechanism involved in the reaction of aryl or alkyldiisocyanate with appropriately protected 3'-O-succinylated nucleoside and amino group containing polymer support

The effect of solvent on rate of succinylation was studied by carrying out the reaction in tetrahydrofuran (THF), acetonitrile (AcCN), ethyl acetate (EtOAc) and 1,2-dichloroethane (EDC). The rate of reaction was found to be in the order EDC > EtOAc > AcCN > THF (Fig. 1d). EDC was selected as a solvent for succinylation reaction because first, being a higher boiling solvent, the reaction could be performed at higher temperature (50°C) and second, the reaction mixture could directly be subjected to aqueous work up without prior evaporation of the reaction mixture.

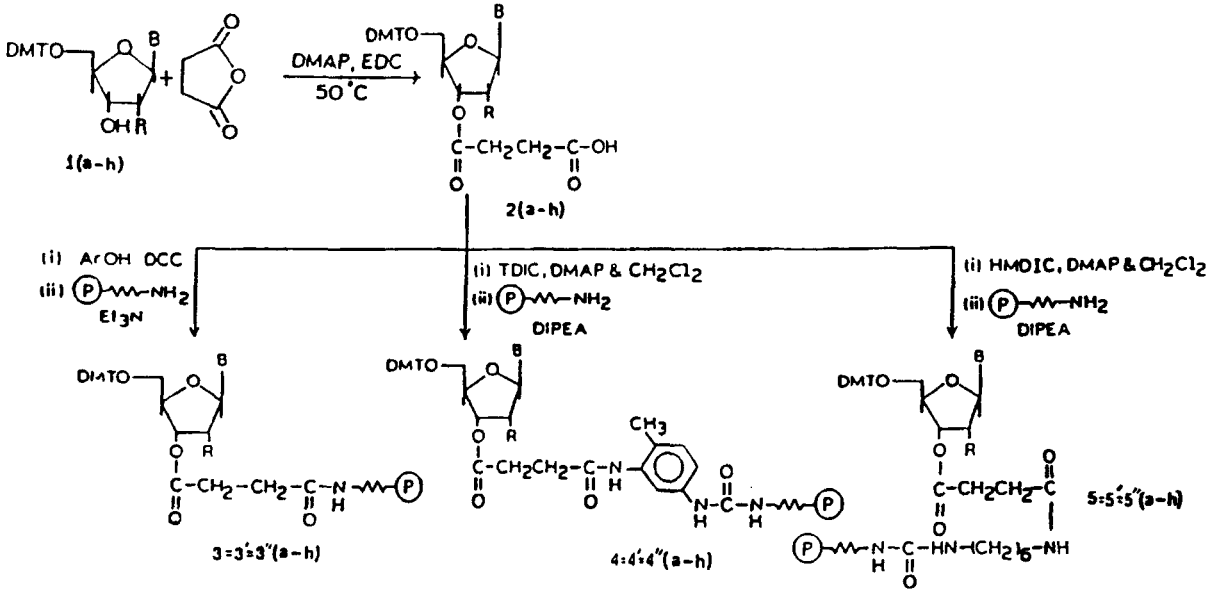

FIGURE 3. Reaction scheme for the functionalisation of polymer support. R= H or O-TBDMS, B = A^{bz}, C^{bz}, G^{ibu}, T or U. 3 = 4 = 5(a-h) = LCAA-CPG, 3'= 4'= 5'(a-h)= AP-CPG, 3"= 4"= 5"(a-h)= AM-Polystyrene; DCC, dicyclohexylcarbodiimide; Ar, p-nitrophenyl or pentachlorophenyl; TDIC, toluene-2,4-diisocyanate; HMDIC, hexamethylene-1,6-diisocyanate; DMAP, 4-dimethylaminopyridine and DIPEA, N-ethyldiisopropylamine.

TABLE-1

Nucleoside loadings on different supports functionalized by methods A and B

Appropriately protected nucleoside	Loading in μmol nucleoside/g support by different methods					
	LCAA-CPG		AP-CPG		AM-Polystyrene	
	(A)	(B)	(A)	(B)	(A)	(B)
1a) DMTdT	38.0	37.7	55.3	56.3	99.1	108.1
1b) DMTdA(bz)	38.3	39.2	51.5	52.4	103.8	105.8
1c) DMTdC(bz)	40.1	38.8	51.3	49.4	106.2	106.2
1d) DMTdG(ibu)	40.0	40.7	48.5	54.5	105.7	107.9
1e) DMTU-2'-TBDMS	38.5	37.5	53.5	51.4	93.9	96.4
1f) DMTrA(bz)-2'-TBDMS	38.9	38.8	50.4	52.0	89.7	92.1
1g) DMTrC(bz)-2'-TBDMS	37.8	41.4	49.8	52.4	93.1	90.0
1h) DMTrG(ibu)-2'-TBDMS	39.8	39.0	52.1	49.2	91.3	95.0

AP-CPG = 3-Aminopropylated controlled pore glass,
AM-Polystyrene = Aminomethylpolystyrene
A= Derivatisation using tolylene-2,4-diisocyanate
B= Derivatisation using hexamethylene-1,6-diisocyanate

Functionalisation of polymer supports using alkyl and aryl diisocyanates

In the present work, we have made use of a well known fast reaction (28) of isocyanates with carboxylic acids to form amides in the presence of DMAP in DCM /EDC. It is assumed that DMAP and the isocyanates are in equilibrium with a dipole which is converted by protonation into the more reactive N-carbamoyl pyridinium ion which reacts readily with carboxylates (with the loss of CO_2) to yield amides (Fig. 2). This finding prompted us to use both aryl and alkyl diisocyanates as as bifunctional reagents to anchor an appropriately protected 2'-deoxyribo- or 2'-TBDMS-ribonucleoside-3'-O-succinate on the amino groups of polymer supports as outlined in Fig. 3. The derivatisation of supports by the suggested methods can be performed in one pot.

The first step in our methodology involves the reaction of an appropriately protected 2'-deoxyribo- or 2'-TBDMS-ribonucleoside-3'-O-succinates with one equivalent of toluene-2,4-diisocyanate or hexamethylene-1,6-diisocyanate in the presence of one equivalent of 4-dimethylaminopyridine in dry dichloromethane to generate a monoisocyanate which was allowed to react without isolation with the amino groups of polymer supports in the presence of N-ethyldiisopropylamine.

Various fully functionalised polymer supports having excellent nucleoside loadings are given in Table-1. The unreacted isocyanate groups were hydrolysed by treating the support with aq. pyridine for 2h at room temperature followed by capping of the residual amino groups on the support. Fig. 4 shows the optimum time (6h) required for the coupling of the monoiso cyanate formed by the reaction of appropriately protected 2'-deoxyguanosine-3'-O-succi nate and TDIC / HMDIC to the amino groups on polymer supports 4/5 (LCAA-CPG).

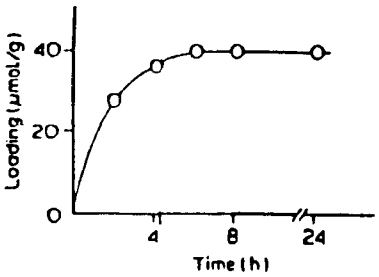

FIGURE 4. Time course of the reaction of monoisocyanate of DMTdG-(ibu)-3'-O-succinate with LCAA-CPG

Oligonucleotide synthesis

The authenticity of the derivatised supports, 3-aminopropylated-CPG or LCAA-CPG was established by synthesizing a number of identical oligodeoxynucleotide sequences using standard phosphoramidite chemistry (3,7). Oligomers were assembled on a 0.2μmol scale following the manufacturer's recommendations (29) and using β-cyanoethyl phosphoramidite monomers. The coupling efficiency based on the released 4,4'-dimethoxytrityl cation exceeded 98%, which is comparable to that of oligomers synthesised using standard supports. The synthesised oligomers were found to be identical in respect of their retention times (HPLC), UV spectra and isolated yields (determined by measuring absorbance at 260 nm). Fig. 5 shows the FPLC elution profile of a crude oligomer d(ATA TAT ATA TAT ATA) synthesised using polymer support 4'(b).

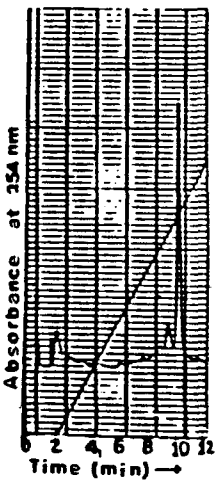

FIGURE 5. FPLC (31) profile of crude d(ATA TAT ATA TAT ATA)

In order to ascertain the efficiency of the derivatised polymer supports for the synthesis of long oligonucleotide sequences, two 45 mer oligos, viz., d (TCT GGT CCT CGT GGT CTC CCT GGC CCC CCT GGT GCA CCT GGT GGG) (X) and d(ACG AGG ACC AGA CCC ACC AGG TGC ACC AGG GGG GCC AGG GAG

ACC) (Y) were synthesised using the supports 4(c) and 4(b). The same sequences (X' and Y') were synthesised on standard LCAA-CPG under identical conditions. The coupling yields based on the released 4,4'-dimethoxytrityl cation were found to be > 98% in both the cases and comparable with the sequences prepared on the standard LCAA-CPG supports These sequences were found to be identical in all respect (coupling yields based on released DMTr cation and overall yields before and after purification on 12% polyacrylamide gel). The sequences were 5'-phosphorylated using (γ-^{32}P) ATP and T_4 Poly-nucleotide Kinase (30) and subjected to analysis on a polyacrylamide (20%) slab gel electrophoresis. The results are shown in Fig. 6.

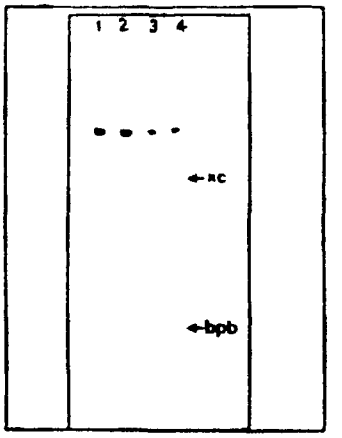

FIGURE 6. Autoradiograph (32) of 20 % polyacrylamide / 7M urea gel electrophoresis of 5'-phosphorylated oligomers X, Y, X' and Y'

Cleavage of nucleosides and oligonucleotides from the derivatised supports prepared by methods A and B

In order to ensure the cleavage of 2'-deoxyribo- and ribonucleosides from the derivatised supports using methods A and B, a small quantity of each of the derivatised supports and supports prepared by the known methods (3,7) in sealed vials were exposed to aq. ammonia (25 %) for 30 min at room temperature followed by loading determination by perchloric acid treatment (1). None of the supports showed any nucleoside loading on them, clearly indicating the complete cleavage. Moreover, in order to check the reactivity of the diisocyanates on nucleic acids, we have conducted an experiment in which all derivatised supports prepared by methods A and B were treated with aq. ammonia at room temperature for 10 min. The ammonia solution was decanted off and concentrated in Speed vac concentrator followed by extraction with diethylether. After removing the diethylether, the cleaved nucleoside was dissolved in methanol/ethanol and subjected to UV analysis (26). The UV analysis of the cleaved nucleosides were found to be comparable to the standard nucleosides which clearly indicates that no modification of

TABLE-2

Comparison of methods A and B with the existing methods for the functionalisation of LCAA-CPG with appropriately protected 2'-deoxynucleoside-3'-O-succinates

	Methods				
	TDIC (A)	HMDIC (B)	Pon et al.(23)	Damha et al.(24)	Active ester
Functional groups on support	$-NH_2$	$-NH_2$	$-NH_2$	-COOH	$-NH_2$
Starting nucleosidic material	R-3'-O-suc.	R-3'-O-suc.	R-3'-O-suc.	R-3'-OH	R-3'-O-suc.
Coupling time, h	6	6	24	16	30
Capping time, h	0.5	0.5	0.5	16	0.5
Loading (μmol/g LCAA-CPG)	38-40	38-40	30-65	30-40	10-25

R-3'-O-suc. = Appropriately protected 2'-deoxynucleoside-3'-O-succinates
R-3'-OH = Appropriately protected 2'-deoxynucleosides

the heterocyclic bases occured during the derivatisation of the polymer supports. The oligonucleotides were also cleaved in an identical manner from the supports.

Comparison of methods A and B with currently followed methods

Data in tables 2 and 3 can be used to evaluate the suggested methods for the functionalisation of LCAA-CPG in respect of coupling and capping times as well as nucleoside loadings. Methods A and B considerably save time as compared to the methods currently used.

Advantage of method B over other methods

Method B has a definite advantage over all other methods as the nucleoside is anchored on 3-aminopropylated-CPG through a 14-carbon long spacer arm which is comparable to 17-carbon long spacer arm available on functionalised LCAA-CPG. The efficiency of the support was confirmed by synthesising twice a 30-mer on a Pharmacia LKB Gene Assembler Plus using CPG support derivatised by method B and a LCAA-CPG support prepared by reported methods (3,7). The coupling efficiency based on the released 4,4'-dimethoxytrityl cation in both the cases averaged 98% and the oligonucleotides isolated from the two runs were identical in all respects.

TABLE-3

Comparison of methods A and B with the existing methods for the functionalisation of LCAA-CPG with appropriately protected 2'-O-TBDMS-ribonucleoside-3'-O-succinates

	Methods			
	TDIC (A)	HMDIC (B)	Ogilvie et al.(25)	Damha et al.(24)
Functional groups on support	$-NH_2$	$-NH_2$	$-NH_2$	-COOH
Starting nucleosidic material	R-3'-O-suc.	R-3'-O-suc.	R-3'-O-suc.	R-3'-OH
Coupling time, h	6	6	72	16-24
Capping time, h	0.5	0.5	0.5	16
Loading (μmol/g LCAA-CPG)	38-40	38-40	18-20	28-40

R-3'-O-suc. = Appropriately protected 2'-O-TBDMS-ribonucleoside-3'-O-succinates.
R-3'-OH = Appropriately protected 2'-O-TBDMS-ribonucleosides.

CONCLUSION

A rapid and economical method for 3'-O-succinylation of 2'-deoxyribo- and ribonucleosides has been developed. The method eliminates the need to carry out succinylation reaction overnight and time consuming chromatographic purification of the desired material. The succinylation reaction of 2'-deoxyribo- and ribonucleosides requires, respectively, 10 and 55 min. for completion. These nucleoside-3'-O-succinates have been anchored to the polymer supports in one pot method using commercially available reagents. The method obviates the need to prepare active ester of nucleoside-3'-O-succinates and the use of coupling reagents like DEC and DCC. The entire derivatisation process has been scaled up to derivatise several grams quantities of supports without much difficulty. The method B is more attractive as it is possible to use cheaper CPG as compared to LCAA-CPG.

EXPERIMENTAL

The melting points of the compounds were determined on a Kofler hot bench melting point apparatus and are uncorrected. Thin layer chromatography (tlc) was performed on silica gel 60 F-254 plates (Merck, Darmstadt, Germany) with the solvents [A (chloroform : MeOH : TEA ::

9:1:0.1) and B (CH_3CN : water :: 9:1)] and compounds were detected under short wavelength ultraviolet light followed by perchloric acid spray and heating at 100°C. Ultraviolet-visible spectra were recorded in methanol/ethanol on a Gilford Response II UV-VIS spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectral studies were performed on a Bruker FT-400 MHz and 125 MHz spectrometer respectively using tetramethylsilane (TMS) as an internal standard. Chemical shifts (δ) are reported in parts per million (ppm) and signals are quoted as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad and Ar, aromatic. Elemental analysis was carried out on Heraeus Rapid CHN analyser. Mass spectra were obtained with a Jeol JMS D 300 machine operating at 70ev. Oligonucleotide synthesis was performed on Pharmacia LKB. Gene Assembler Plus.

3-Aminopropylation of controlled pore glass (CPG) was carried out according to procedure described by Atkinson and Smith (7). 3-Aminopropyl-CPG, long chain alkylamine-controlled pore glass (LCAA-CPG) and aminomethyl-polystyrene resin were activated by 3 % trichloroacetic acid (TCA) treatment as described by Damha et al. (24).

5'-O-(4,4'-Dimethoxytrityl)-N-protected-2'-deoxynucleoside-3'-O-succinate :

To a solution of dry 5'-O-(4,4'-dimethoxytrityl)-N-protected-2'-deoxynucleoside 1(a-d) (0.5 mmol) in anhydrous 1,2-dichloroethane (1 ml), 4-dimethylaminopyridine (0.25 mmol, 30.5 mg), succinic anhydride (0.75 mmol, 75 mg) and triethylamine (0.5 mmol, 69.2 µl) were added. The reaction mixture was allowed to stir at 50°C for 10 minutes and cooled, showing the formation of a single, more polar product on the tlc. The solution was further diluted with 1,2-dichloroethane (15 ml), washed with ice-cold aqueous solution of 10 % citric acid (3 x10 ml) and water (2 x 10 ml) in a separating funnel. The organic layer was collected and dried over anhydrous sodium sulfate. The resulting solution was filtered, concentrated under reduced pressure and precipitated from ether : hexane (1:1, v/v). The solid material was filtered off and dried under vacuum at room temperature, giving the 5'-O-(4,4'-dimethoxytrityl)-N-protected-2'-deoxynucleoside-3'-O-succinates 2(a-d) in quantitative yields.

5'-O-(4,4'-Dimethoxytrityl)-thymidine-3'-O-succinate 2a

mp, 109-11°; mass spectrum (+FAB), m/z 644 (M^+-1); Rf (0.27, solvent A; 0.60,solvent B). ^{1}H-nmr ($CDCl_3$) δ : 1.38 (s, 3H, $-CH_3$), 2.46-2.69 (m, 6H,

$-CH_2-CH_2-$ & 5'-$H_{a,b}$), 3.45 (m, 2H, 2'-$H_{a,b}$), 3.78 (s, 6H, 2x $-OCH_3$), 4.16 (m, 1H, 4'-H), 5.44 (m, 1H, 3'-H), 6.40 (t, 1H, 1'-H), 6.51-7.32 (m, 13H, Ar-H) and 7.62 (s, 1H, 6-H). ^{13}C-nmr ($CDCl_3$) δ : 11.59 ($C-CH_3$), 30.57 & 30.21 ($\alpha,\beta-CH_2-$), 41.2 (C-2'), 55.27 ($-OCH_3$), 63.79 (C-5'), 75.4 (C-3'), 83.89 (C-4'), 84.45 (C-1'), 87.17 (trityl central C), 110.5 (C-5), 113.34 (methoxyphenyl C-3, 5), 128.03 & 128.19 (phenyl -CH), 130.11 (methoxyphenyl C-2,6), 135.32 (methoxyphenyl C-4), 135.59 (C-6), 144.1 (phenyl C), 150.5 (C-2), 158.78 (methoxyphenyl C-4), 163.4 (C-4), 172.7 (-OCO-). Anal. calcd. for $C_{35}H_{36}N_2O_{10}$, C(65.20); H(5.62); N(4.34), Found: C(64.81); H(5.58); N(4.47).

5'-O-(4,4'-Dimethoxytrityl)-N^4-benzoyl-2'-deoxycytidine-3'-O-succinate 2b

mp, 106-08°; mass spectrum (+FAB), m/z 734 (M^++1); Rf (0.25, solvent A; 0.62,solvent B). ^{1}H-nmr ($CDCl_3$) δ : 2.58-2.71 (m, 4H, $-CH_2-CH_2-$), 3.4 (m, 2H, 2'-$H_{a,b}$), 3.79 (s, 6H, 2x $-OCH_3$), 4.26 (m, 1H, 4'-H), 5.42 (m, 1H, 3'-H), 6.28 (t, 1H, 1'-H), 6.80-7.60 (m, 19H, Ar-H & H-5) and 7.92 (s, 1H, 6-H). ^{13}C-nmr ($CDCl_3$) δ : 31.23 & 31.6 (α,β $-CH_2-$), 40.82 (C-2'), 55.23 ($-OCH_3$), 63.75 (C-5'), 75.17 (C-3'), 84.52 (C-4'), 84.66 (C-1'), 87.5 (trityl central C), 98.0 (C-5), 113.22 (methoxyphenyl C-3,5), 127.91 & 128.14 (phenyl -CH), 127.86 (C-2,3,5,6 of bz), 130.04 (methoxyphenyl C-2,6 & C-4 of bz), 132.78 (C-1 of bz), 135.57 (methoxyphenyl C-1), 144.0 (phenyl C-1), 145.0 (C-6), 152.5 (C-2), 158.6 (methoxyphenyl C-4), 160.0 (C-4), 168.0 (-C=O), 172.81 (-OCO-). Anal. calcd. for $C_{41}H_{39}N_3O_{10}$, C(67.1); H(5.35); N(5.72), Found: C(66.98); H(5.29); N(5.59).

5'-O-(4,4'-Dimethoxytrityl)-N^6-benzoyl-2'-deoxyadenosine-3'-O-succinate 2c

mp, 104-05°; mass spectrum (+FAB), m/z 758 (M^+-1); Rf (0.27, solvent A; 0.65,solvent B). ^{1}H-nmr ($CDCl_3$) δ : 2.60-2.72 (m, 6H, $-CH_2-CH_2-$ & 5'-$H_{a,b}$), 3.40 (m, 2H, 2'-$H_{a,b}$), 3.78 (s, 6H, 2x $-OCH_3$), 4.34 (m, 1H, 4'-H), 5.50 (m, 1H, 3'-H), 6.50 (t, 1H, 1'-H), 6.77-7.60 (m, 18H, Ar-H) 8.2 (s, 1H, 2-H) and 8.72 (s, 1H, 8-H). ^{13}C-nmr ($CDCl_3$) δ : 31.23 & 31.6 (α,β $-CH_2-$), 40.82 (C-2'), 55.23 ($-OCH_3$), 63.75 (C-5'), 75.17 (C-3'), 84.65 (C-4'), 86.72 (C-1' & trityl central C), 113.23 (methoxyphenyl C-3,5), 126.98 (C-5), 127.91 & 128.14 (phenyl -CH), 128.85 (C-2,3,5,6 of bz), 130.06 (methoxyphenyl C-2,6 & C-4 of bz), 131.6 (C-1 of bz), 135.56 (methoxyphenyl C-1), 141.47 (C-8), 144.3 (phenyl C-1), 149.8, 150.51 & 152.0 (C-2,4,6), 158.60 (methoxyphenyl C-4), 167.98 (-C=O), 172.81 (-

OCO-). Anal. calcd. for $C_{42}H_{39}N_5O_9$, C(66.56); H(5.18); N(9.24), Found: C(66.54); H(5.17); N(9.28).

5'-O-(4,4'-Dimethoxytrityl)-N^2-isobutyryl-2'-deoxyguanosine-3'-O-succinate 2d

mp, 142-44°; mass spectrum (+FAB), m/z 740 (M^++1); Rf (0.20, solvent A; 0.58,solvent B). ^{1}H-nmr ($CDCl_3$) δ: 2.48-2.78 (m, 6H, $-CH_2-CH_2-$ & 5'-$H_{a,b}$), 3.35 (m, 2H, 2'-$H_{a,b}$), 3.78 (s, 6H, 2x $-OCH_3$), 4.22 (m, 1H, 4'-H), 5.42 (m, 1H, 3'-H), 6.10 (t, 1H, 1'-H), 6.74-7.40 (m, 13H, Ar-H) 7.78 (s, 1H, 8-H). ^{13}C-nmr ($CDCl_3$) δ: 18.89 & 18.95 (2x-CH_3 of isobutyryl), 31.2 & 31.4 (α,β $-CH_2-$), 35.92 (-CH of isobutyryl) 39.75 (C-2'), 55.24 ($-OCH_3$), 63.54 (C-5'), 75.04 (C-3'), 84.76 (C-4'), 85.05 (C-1'), 87.02 (trityl central C), 113.26 (methoxyphenyl C-3,5), 121.54 (C-5), 127.95 & 128.1 (phenyl -CH), 130.04 (methoxyphenyl C-2,6), 135.02 (methoxyphenyl C-1), 137.58 (C-8), 144.06 (phenyl C-1), 148.0 & 148.3 (C-2,4), 156.4 (C-6), 158.69 (methoxyphenyl C-4), 172.18 (-OCO-), 180.1 (-C=O). Anal. calcd. for $C_{39}H_{41}N_5O_{10}$, C(63.31); H(5.58); N(9.46), Found: C(62.78); H(5.55); N(9.38).

5'-O-(4,4'-Dimethoxytrityl)-N-protected-2'-TBDMS-ribonucleoside-3'-O-succinate :

To a solution of dry 5'-O-(4,4'-dimethoxytrityl)-N-protected-2'-TBDMS-ribonucleoside **1(e-h)** (0.5 mmol) in anhydrous 1,2-dichloroethane (1 ml) was added 4-dimethylaminopyridine (0.25 mmol, 30.5 mg), succinic anhydride (0.75 mmol, 75 mg) and triethylamine (0.5 mmol, 69.2 µl). The reaction mixture was agitated at 50°C for 55 minutes and after cooling, checked for completion by tlc. The reaction mixture was diluted and washed with ice-cold aqueous 10 % citric acid solution (3 x 10 ml) and water (2 x 10 ml). The organic phase was separated and dried over anhydrous Na_2SO_4. After filtration, the organic phase was concentrated (5-6 ml) and precipitated from ether : hexane (1:1, v/v). The precipiated material was filtered and dried under vacuum at room temperature, giving the 5'-O-(4,4'-dimethoxytrityl)-N-protected-2'-TBDMS-ribonucleoside-3'-O-succinates **2(e-h)** in quantitative yields.

5'-O-(4,4'-Dimethoxytrityl)-2'-TBDMS-uridine-3'-O-succinate 2e

mp, 107-08°; mass spectrum (+FAB), m/z 761 (M^++1); Rf (0.48, solvent A; 0.66,solvent B). ^{1}H-nmr ($CDCl_3$) δ: 0.06 (s, 3H, Si-CH_3), 0.07 (s, 3H, Si-CH_3), 0.84 (s, 9H, Si-t-But), 2.45-2.70 (m, 4H, $-CH_2-CH_2-$), 3.81 (s, 6H, 2x $-OCH_3$), 4.25 (m, 1H, 4'-H), 4.54 (m, 1H, 2'-H), 5.30 (dd, 1H, 3'-H), 5.92 (d,1H, 1'-H), 6.84-7.40 (m, 14H, Ar-H & 5-H) and 7.9 (d, 1H, 6-

H). Anal. calcd. for $C_{40}H_{48}N_2O_{11}$ Si, C(63.13); H(6.37); N(3.68), Found: C(62.52); H(6.27); N(3.57).

5'-O-(4,4'-Dimethoxytrityl)-N^4-benzoyl-2'-TBDMS-cytidine-3'-O-succinate 2f

mp, 105-06°; mass spectrum (+FAB), m/z 864 (M^+-1); Rf (0.60, solvent A; 0.65,solvent B). ^{1}H-nmr ($CDCl_3$) δ: 0.05 (s, 3H, Si-CH_3), 0.18 (s, 3H, Si-CH_3), 0.86 (s, 9H, Si-t-But), 2.57-2.67 (m, 6H, -CH_2-CH_2- & 5'-$H_{a,b}$), 3.80 (s, 6H, 2x -OCH_3), 4.39 (m, 1H, 4'-H), 4.54 (m, 1H, 2'-H), 5.15 (dd, 1H, 3'-H), 5.95 (d, 1H, 1'-H), 6.75-7.41 (m, 18H, Ar-H) and 7.92 (s, 1H, 6-H). Anal. calcd. for $C_{47}H_{54}N_3O_{11}Si$, C(65.25); H(6.29); N(4.85), Found: C(64.94); H(6.27); N(4.81).

5'-O-(4,4'-Dimethoxytrityl)-N^6-benzoyl-2'-TBDMS-adenosine-3'-O-succinate 2g

mp, 101-03°; mass spectrum (+FAB), m/z 888 (M^++1); Rf (0.59, solvent A; 0.67,solvent B). ^{1}H-nmr ($CDCl_3$) δ: -0.02 (s, 3H, Si-CH_3), 0.02 (s, 3H, Si-CH_3), 0.85 (s, 9H, Si-t-But), 2.60-2.78 (m, 4H, -CH_2-CH_2-), 3.04 (d, 2H, 5'-$H_{a,b}$), 3.78 (s, 6H, 2x -OCH_3), 4.32 (m, 1H, 2'-H), 5.15 (dd, 1H, 3'-H), 6.13 (d, 1H, 1'-H), 7.22-7.65 (m, 18H, Ar-H) 8.26 (s, 1H, 2-H) and 8.72 (s, 1H, 8-H). Anal. calcd. for $C_{48}H_{53}N_5O_{10}Si$, C(64.91); H(6.02); N(7.88), Found: C(64.82); H(6.05); N(7.77).

5'-O-(4,4'-Dimethoxytrityl)-N^2-isobutyryl-2'-TBDMS-guanosine-3'-O-succinate 2h

mp, 112-13°; mass spectrum (+FAB), m/z 870 (M^++1); Rf (0.41, solvent A; 0.63,solvent B). ^{1}H-nmr ($CDCl_3$) δ: -0.12 (s, 3H, Si-CH_3), -0.10 (s, 3H, Si-CH_3), 0.92 (s, 9H, Si-t-But), 2.62-2.75 (m, 4H, -CH_2-CH_2-), 3.05 (m, 2H, 5'-$H_{a,b}$), 3.81 (s, 6H, 2x -OCH_3), 4.25 (bd, 1H, 2'-H), 5.25 (dd, 1H, 3'-H), 5.95 (d, 1H, 1'-H), 6.72-7.61 (m, 13H, Ar-H), 7.85 (s, 1H, 8-H). Anal. calcd. for $C_{45}H_{56}N_5O_{11}$ Si, C(62.04); H(6.48); N(8.04), Found: C(62.06); H(6.51); N(7.89).

Effect of various parameters, viz., concentration of dimethylaminopyridine, succinic anhydride, temperature and solvent on the rate of succinylation of appropriately protected nucleosides were studied in detail as described below.

(a) **Effect of 4-dimethylaminopyridine concentration**

In order to arrive at the optimal concentration of DMAP for the succinylation reaction, an appropriately protected 2'-deoxynucleoside (0.5 mmol) was taken in six 25 ml round-bottomed flasks. Then, in each flask was added anhydrous 1,2-dichloroethane, succinic anhydride (0.75

mmol, 75 mg) and triethylamine (0.5 mmol, 69.2 µl). The flasks were charged with varying amounts, viz., 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 equivalent of DMAP and stirred at room temperature. The progress of the reaction in each flask was monitored by withdrawing a small aliquot at the interval of 5 minutes. The optimum quantity of DMAP required for the completion of the succinylation reaction was determined by the kinetic data obtained from the tlc analysis of the aliquots collected at different time intervals.

(b) Effect of succinic anhydride concentration

The optimum concentration of succinic anhydride required for the reaction was determined in the same manner as described above except that varying amounts, viz., 1.1, 1.2, 1.3, 1.4, 1.5 and 2.0 equivalents of succinic anhydride and 0.5 eq. of DMAP were used.

(c) **Effect of temperature**

In order to determine the effect of temperature, the reaction was carried out at four different temperatures. 5'-O-(4,4'-Dimethoxytrityl)-N-protected-2'-deoxyribonucleoside (0.5 mmol) was taken in four different 25 ml round-bottomed flasks and dried in the analogous manner as described above. Each reaction flask was charged with 1,2-dichloroethane, DMAP (0.25 mmol, 30.5 mg), succinic anhydride (0.75 mmol, 75 mg) and triethylamine (0.5 mmol, 69.2 µl). The reaction mixtures were stirred at four different temperatures, viz., 20°, 30°, 40° and 50°C, respectively. The progress of the reaction was monitored as described above.

(d) Effect of solvent on succinylation reaction

The effect of solvent on succinylation reaction was studied by performing succinylation reactions with 1.5 equivalent of succinic anhydride and 0.5 eq. of DMAP at 50°C in different solvents.

Functionalisation of polymer supports

Method A. Derivatisation of polymer supports using toluene-2,4-diisocyanate (TDIC)

Appropriately protected 2'-deoxyribo- 2(a-d) or ribonucleoside-3'-O-succinate 2(e-h) (0.2 mmol) was taken in a septum sealed vial fitted with a small needle and kept over phosphorous pentoxide under continuous vacuum for several hours. Vacuum was released with dry argon gas, the needle removed, and the solid was dissolved in dry dichloromethane (5 ml). DMAP (0.2 mmol, 24.4 mg) and TDIC (0.2 mmol, 28.8 µl) (CAUTION : SUSPECTED CARCINOGEN) were added to the vial under anhydrous conditions.

The reaction was allowed to proceed at room temperature. After 10 min, the vial was carefully opened under argon atmosphere and the desired polymer support 4 (1.0 g, ca. 0.1 mmol of amino groups) and N-ethyldiisopropylamine (0.2 mmol, 34.3 µl) were added. The mixture was agitated briefly to ensure thorough mixing and then left in the dark for 6h at room temperature with occasional shaking. The polymer support was then recovered on a sintered glass funnel, washed with dichloromethane (3x10 ml) and diethyl ether (3x10 ml). The polymer support was then suspended in pyridine / water (25 ml, 8:2 v/v) for 2h at room temperature to hydrolyse any unreacted isocyanate groups. The polymer support was washed with pyridine (3x10 ml), and the residual amino groups were capped with acetic anhydride / triethylamine / N-methylimidazole / dichloromethane (10 ml, 1 : 1 : 0.3 : 6, v/v) for 30 min at room temperature. The derivatised polymer support was recovered again on a sintered glass funnel and washed with dichloromethane and dry diethylether (10 ml of each). The support was first dried in air and then under vacuum and stored at -20^{o}C until required for use. Same procedure was followed for the derivatization of polymer supports 4' and 4''.

Method B. Derivatisation of polymer supports using hexamethylene-1,6-diisocyanate(HMDIC)

The derivatisation of polymer supports 5, 5' and 5'' using HMDIC was carried out in analogous manner as described in method A except that HMDIC (0.2 mmol, 32.19 µl) was used in place of TDIC. Other steps, viz., washings, capping etc. were followed as described in method A. See results and discussion for special merit of this method.

Determination of optimum time required for the derivatization of polymer supports

The general procedure can be illustrated by the derivatization of LCAA-CPG with appropriately protected 2'-deoxyguanisine-3'-O-succinate using TDIC / HMDIC. The 2'-deoxyguanosine-3'-O-succinate and LCAA-CPG were dried as described in method A. 2'-Deoxyguanosine-3'-O-succinate (1 mmol, 644 mg) was taken in 25 ml dry dichloromethane. DMAP (1 mmol, 122 mg) and TDIC (1 mmol, 144 µl) or HMDIC (1 mmol, 161 µl) were added to the reaction flask under the exclusion of moisture. After stirring the reaction mixture for 10 min at room temperature, the reaction vial was opened and LCAA-CPG (5g, ca. 0.5 mmol of amino groups) and N-ethyldiisopropylamine (1 mmol, 171.2 µl) were added and reaction was

allowed to continue at room temperature. Small aliquots of the LCAA-CPG support were withdrawn at different intervals of time (1h, 2h, 3h....24h) and after thorough washing their nucleoside loading was determined acccording to the reported method (7).

ACKNOWLEDGEMENTS

We thank Dr. S.V. Gangal for his comments and careful reading of the MSS. The financial assistance from the Department of Biotechnology, N. Delhi is gratefully acknowledged. One of us (P.K.) is thankful to the Council of Scientific and Industrial Research for a Senior Research Fellowship. Dr. K.L. Sadana wants to acknowledge the support of Department of Biotechnology, Government of India, NSERC, Ottawa, Canada and the University of Manitoba for financial assistance. Thanks are due to Dr. F.H. Hruska, Mr. K. Marat and Mr. W. Buchannon for help in obtaining NMR and MS spectra.

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REFERENCES & NOTES

1. Gait, M.J., Singh, M., Sheppard, R. C., Edge, M.D., Greene, A.R., Heathcliffe, G.R., Atkinson, T.C., Newton, C.R. and Markham, A.F. (1980) Nucl. Acids Res., 8, 1081-1096.
2. Beaucage, S.L. and Caruthers, M.H. (9881) Tetrahedron Lett., 22, 1859-1862.
3. Adams, S.P., Kavka, K.S., Wykes, E.J., Holder, S.B. and Galluppi, G.R. (1983) J. Am. Chem. Soc., 105, 661-663.
4. Sinha, N.D., Biernat, J., McManus, J. and Koster, H. (1984) Nucl. Acids Res., 12, 4539-4557.
5. Schulhof, J.C., Molko, D. and Teoule, R. (1987) Nucl. Acids Res., 15, 397-416.
6. Schulhof, J.C., Molko, D. and Teoule, R. (1988) Nucleic Acids Res., 16, 319-326.
7. Atkinson, T. and Smith, M. (1984) In Gait, M.J. (ed.) Oligonucleotide Synthesis a practical approach. IRL Press, Oxford, pp. 35-81.
8. Caruthers, M.H., Barone, A.D., Beaucage, S.L., Dodds, D.R., Fisher, E.F., McBride, L.J., Metteucci, M., Stabinsky, Z. and Tang, J.-Y. (1987) Meth. Enz., 154, 287-313.
9. Dobrynin, V.N., Filippova, S.A., Bystrov, N.S., Severtsova, I.V. and Kolosov, M.N. (1983) Bioorg. Khim., 9, 706-710.
10. Katzhendler, J., Cohen, S., Rahamin, E., Weiss, M., Ringel, I. and Deutsch, J. (1989) Tetrahedron, 45, 2777-2780.

11. Eritja, R., Robles, J., Fernandez, D., Albericio, F. Giralt, E. and Pedroso, E. (1991) Tetrahedron Lett., 32, 1511-1514.
12. Sproat, B.S. and Brown, D.M. (1985) Nucl. Acids Res.,13, 2979-2987.
13. Gough, G.R., Brunden,K.J. and Gilham, P.T. (1983) Tetrahedron Lett., 24, 5317-5320.
14. Kumar, P., Bose, N.K. and Gupta, K.C. (1991) Tetrahedron Lett., 32, 967-970.
15. Gupta, K.C., Sharma, P., Sathyanarayana, S. and Kumar, P. (1990) Tetrahedron Lett., 31, 5305-5308.
16. Gupta, K.C., Sharma, P., Kumar, P. and Sathyanarayana, S. (1991) Nucl. Acids Res., 19, 3019-3026.
17. Asseline, U. and Thuong, N.T. (1990) Tetrahedron Lett., 31, 81-84.
18. Markiewicz, W.T. and Wyrzykiewicz, T.K. (1989) Nucl. Acids Res., 17, 7149-7158.
19. Alul, R.H., Singman, C.N., Zhang, Guangrong and Letsinger, R.L. (1991) Nucl. Acids Res., 19, 1527-1532.
20. Sproat, B.S. and Gait, M.J. (1984) In Gait, M.J. (ed.) Oligonucleotide Synthesis a practical approach. IRL press, Oxford, pp. 83-115.
21. Gait, M.J., Matthes, H.W.D., Singh, M., Sproat, B.S. and Titmas, R.C. (1982) In Gassen, H.G. and Lang, A.(ed.) Chemical and Enzymatic Synthesis of Gene Fragments a laboratory manual. Verlag Chemie, Weinheim. Deerfield Beach. Florida. Basel, pp. 1-42.
22. Atkinson, T. and Smith, M. (1984) In Gait, M.J. (ed.) Oligonucleotide Synthesis a practical approach. IRL Press, Oxford, pp. 45-49.
23. Pon, R.T., Usman, N. and Ogilvie, K.K. (1988) BioTechiques, 6, 768-775.
24. (a) Damha, M.J., Giannaris, P.A. and Zabarylo, S.V. (1990) Nucl. Acids Res., 18, 3813-3821. (b) Damha, M.J., Ganeshan, K., Hudson, R.H.E. and Zabarylo, S.V. (1992) Nucl. Acids Res., 20, 6565-6573.
25. Usman, N., Ogilvie, K.K., Jiang, M.Y. and Cedegren, R.G. (1987) J. Am. Chem. Soc., 109, 7845-7854.
26. (a) The ultraviolet absorption characteristics of the released nucleosides were: DMTrdT (in methanol), λ_{max} = 268.5 and 234.0 nm, λ_{min} = 254.5 nm; DMTrdC^{bz} (in methanol), λ_{max} = 306.0, 259.5 and 236.0 nm and λ_{min} = 292.5, 251.0 and 223.5 nm; DMTrdA^{bz} (in ethanol), λ_{max} = 279.0 and 234 nm and λ_{min} = 260.0 and 223.5 nm; DMTrdG^{ibu} (in ethanol), λ_{max} = 273.0 and 235 nm and λ_{min} = 250.0

and 223.5 nm. (b) Kumar, P. and Gupta K.C. (1992) BioMed. Chem. Lett., 7, 727-730.

27. Scriven, E.F.V. (1983) Chem. Soc. Rev., 12 129-161.
28. Hofle, G., Steglich, W. and Vörbruggen, H. (1978) Angew. Chem. Int. Ed. Engl., 17, 569-583.
29. Gene Assembler Plus Mannual, Pharmacia LKB, Uppsala, Sweden.
30. Wu, R., Wu, N.-H., Hanna, Z., Georges, F. and Narang, S.A. (1984) In Gait, M.J. (ed.) Oligonucleotide Synthesis a practical approach. IRL Press, Oxford, pp. 135-151.
31. FPLC profile of crude 15-mer d(ATA TAT ATA TAT ATA) synthesised on support 4'(b). Column Mono Q HR 5/5. Buffers: A = 0.4M NaCl and 0.01M NaOH, pH 12; B = 1.5M NaCl and 0.01M NaOH, pH 12. Gradient : 0 % B for 2 min, 0-100% B in 23 min. Flow rate 1.0 ml/min, Auf 0.2.
32. Autoradiograph of 20 % polyacrylamide / 7M urea gel electrophoresis of 5'-phosphorylated d (TCT GGT CCT CGT GGT CTC CCT GGC CCC CCT GGT GCA CCT GGT GGG) (X), d(ACG AGG ACC AGA CCC ACC AGG TGC ACC AGG GGG GCC AGG GAG ACC) (Y), d(TCT GGT CCT CGT GGT CTC CCT GGC CCC CCT GGT GCA CCT GGT GGG) (X') and d(ACG AGG ACC AGA CCC ACC AGG TGC ACC AGG GGG GCC AGG GAG ACC) (Y'). Lane 1, 5'-phosphorylated X'; Lane 2, 5'-phosphorylated Y'; Lane 3, 5'-phosphorylated X; Lane 4 and 5'-phosphorylated Y.

Received 8/17/92
Accepted 4/22/93