Pergamon

Tetrahedron, Vol. 53, No. 1, pp. 321-330, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(96)00988-X

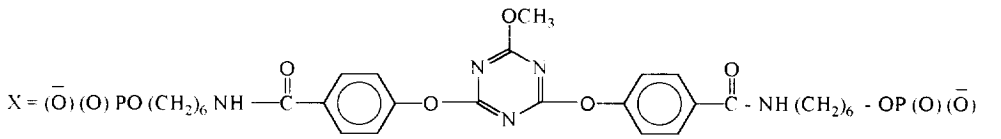
Binding of Oligonucleotides by use of Nonnucleotide Linkers

Bhanudas. P. Kavitake, Suchita. V. Patil and Manikrao. M. Salunkhe*

Department of Chemistry, The Institue of Science, 15, Madam Cama Road,

Mumbai- 400 032, INDIA.

Abstract **: A linker containing a 2,4-bis(4-carboxyphenoxy)-6-methoxy-S-triazine amide group (X) or thiol group (Y) is shown to be an effective structural element for organizing oligonucleotide chains in solutions. Aliphatic nonnucleotide linker Y is found to be more effective than aromatic linker X, in stabilizing oligonucleotides triplexes.** Copyright © 1996 Elsevier Science Ltd



$X = (\bar{O})(O)PO(CH_2)_6NH$ — C(=O) — C$_6$H$_4$ — O — C$_3$N$_3$(OCH$_3$) — O — C$_6$H$_4$ — C(=O) — $NH(CH_2)_6 - OP(O)(\bar{O})$

$Y = (\bar{O})(O)PO(CH_2)_6 - S\text{-}S - (CH_2)_6 - OP(O)(\bar{O})$

Introduction :

There has been growing interest in recent years in the development of synthetically altered nucleic acid structures, as diagnostic tools in the study of gene expression and as potential therapeutic agents[1]. Synthetic modification of the DNA and RNA structure can lead to potentially useful properties such as increased lifetime in biological media, improved ability to be taken up into cells, or improved hybridization properties[2]. Oligonucleotides containing palindromic segments linked by short runs of noncomplementary nucleotides[3] or by a hexaethylene glycol bridge[4]; a bisamide linker[5], dodecyl chains[6], octaethylene glycol bridge[7] exhibit high Tm values. The enhanced stability of these intramolecular complexes relative to duplex structures with equivalent base pairs, may be attributed to the high effective local concentration of the complementary intramolecular segments.

Results and Discussion :

In exploring structural features that could prove useful as elements in organising oligonucleotide frameworks, we have prepared and examined a number of oligonucleotide derivatives in which the linker X or Y serves as a bridge between 3' terminal oxygen atom of one oligomer and the 5' terminal oxygen atom of another.

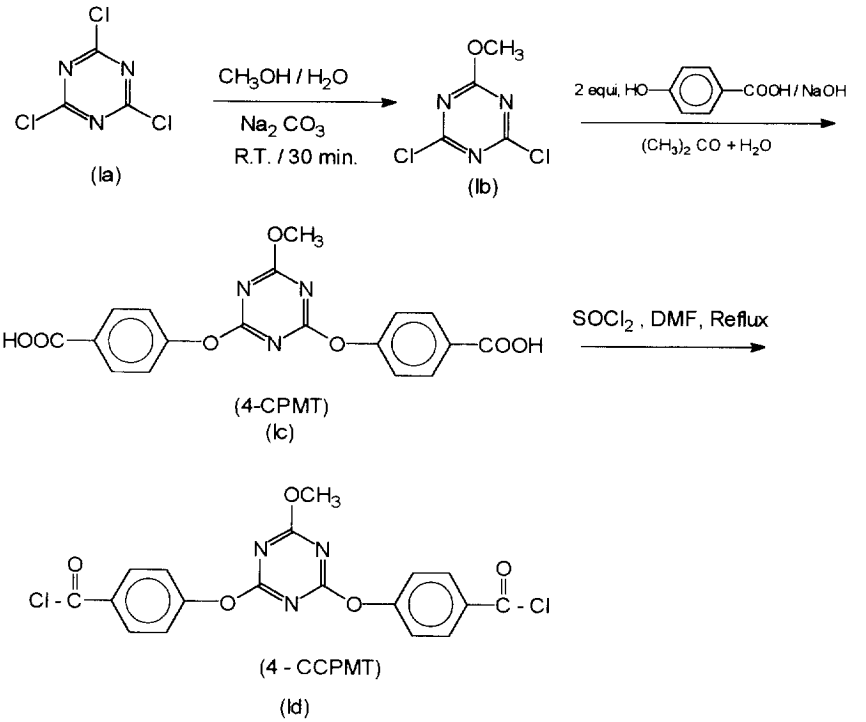
Several features make the linker, X, an attractive candidate for study. The remarkable stability of the S-triazine ring can be explained by the electronic configuration which resembles that of benzene to a certain extent. The resonance energy of benzene is 36 kcal/mole whereas that of the S-triazine ring is 41.2 kcals/mole. The ring system is stabilized by delocalization of its π-electrons which are spread over six ring atoms; and this is responsible for its high aromatic character and thermal stability. The essential difference exists in electronic configuration between S-triazine and benzene as a consequence of the greater electronegativity of the nitrogen atoms as compared to that of the carbon atoms.

Formulae for the modified oligonucleotides selected for study are indicated in Table - 1. These compounds were designed to test the effect of the linker in systems potentially capable of folding into a bimolecular hairpin triplex structure in combination with appropriate complementary targets. S-triazine containing two acid chlorides namely 2,4-bis (4-chlorocarbonylphenoxy)-6-methoxy-S-triazine (4-CCPMT) **1d**, was synthesized from cyanuric chloride[8] **1a**. The monoprotected diol, **2d** was synthesized from 6-aminohexanol and 2,4-bis(4-chlorocarbonylphenoxy)-6-methoxy-S-triazine, (4-CCPMT), via intermediates **2a** - **2c**. This path utilizing several protection-deprotection steps proved more satisfactory than more direct routes, where difficulties with insoluble intermediates or polymers were encountered. The alcohol was then converted to hydrogen phosphonate by standard phosphitylation procedure. These reagents could be readily utilized in conventional protocols for synthesizing oligoncleotides on solid supports. The synthon **2e** served satisfactorily for incorporating the 2,4-bis (4-carboxyphenoxy)-6-methoxy-S-triazine amide linker, into oligonucleotides. **[Chart - 1 & 2]**

The role that a nucleotide bridging element can play in organizing oligonucleotide tracks in solution was studied with oligomers containing the linker X or Y. The bridged system is related to one described by Durand et al.[8] in which a hexaethylene glycol unit connecting two monomer strands led to an enhancement in Tm values relative to a duplex formed from nonlinked monomer strands. Such bridges, which resemble single stranded oligonucleotide loops[9] in facilitating formation of hairpin structures but differ markedly from an oligonucleotide loops in structure and biochemical properties, offer new opportunities in the design and utilization of oligonucleotide derivatives.

Interaction of d (TTTTTT) with d (AAAAAA) is very weak (Exp. I, Tm $< 2°C$)[6]. In sharp contrast, the linked oligomer d(TTTTT-Y-TTTTTT) forms a stable complex (Tm 22.5°C Table 1) with d (AAAAAA) under the same conditions (Exp. II). This experiment suggests that a target as small as six nucleotide units can be recognised in dilute solutions. A similar type of stabilization of

CHART - 1



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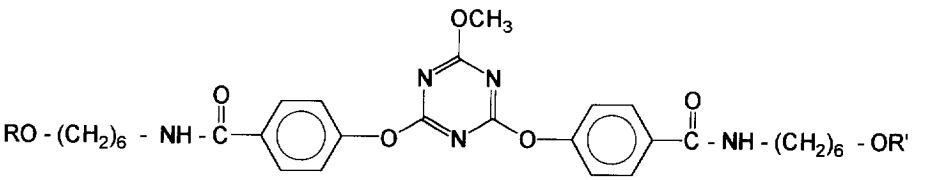


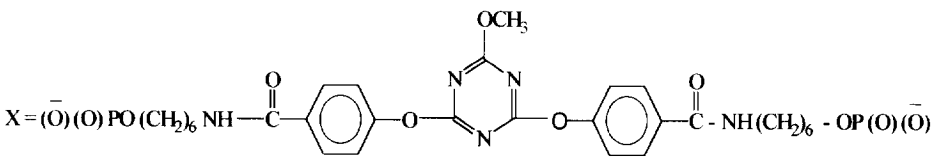
2a	R = - t Bu Si (Me_2) -	R' = - t Bu Si(Me_2)
2b	R = H	R' = - t Bu Si(Me_2)
2c	R = DMT	R' = - t Bu Si(Me_2)
2d	R = DMT	R' = H
2e	R = DMT	R' = P (H) (O) ($\bar{O}$)

Table No. I

Thermal Dissociation of Oligomer Complexes

Exp. No.	Oligonucleotides	Tm °C	
		0.1 M NaCl	1 M NaCl
I	d (TTTTTT) + d (TTTTTT) + d (AAAAAA)	-	<2
II	d (TTTTTT - Y - TTTTTT) + d (AAAAAA)	12.5	22.5
III	d (TTTTTTTTTTT) + d (TTTTTTTTTTT) + d (AAAAAAAAAAA)	25.0	42.5
IV	d (TTTTTTTTTTT) + d (TTTTTTTTTTT) + d (AAGATAGAACA)	15.0	30.0
V	d (TTTTTTTTTTT - Y - TTTTTTTTTTT) + d (AAAAAAAAAAA)	52.0	72.5
VI	d (TTTTTTTTTTT - Y - TTTTTTTTTTT) + d (AAGATAGAACA)	32.0	47.5
VII	d ((TTTTTTTTTTT - X - TTTTTTTTTTT) + d (AAAAAAAAAAAA)	48.0	68.0
VII	d (TTTTTTTTTTT - X - TTTTTTTTTTT) + d (AAGATAGAACA)	35.0	50.0

Tris HCl buffer, 10 mM pH = 7.0.
The total nucleotide concentration was = 1.0 A620 units/ml at 25°C.



Y=(O)(O)PO(CH2)6 - S-S - (CH2)6 - OP(O)(O)

complex between the oligomer d (T_{11}-Y-T_{11}) with d A_{11} (Exp V Tm 72.5°C) is observed as compared to the complex formed between (2 equv) with dA_{11} (1 equiv) (Exp III, Tm 42.5°C). Here the interaction is comparatively more than that observed in the case of experiment I. The proper base pairing is important in the system as shown by the experiment with d (T_{11}-Y-T_{11}) and d (AAGATAGAACA) which incorporated four mismatches, resulting in a decrease of Tm by 25°C (Exp VI, Tm 47.5°C). Similarly the complex formed between dT_{11} (2 equiv) and d (AAGATAGAACA) (1 equiv) with four mismatches leads to destabilization by 12.5°C (Exp IV, Tm 30°C). Also in the complex between the oligomer d (T_{11} X T_{11}) containing linker 'X' with dA_{11} (Exp. VIII, Tm 68°C) stabilization is observed, while the complex formed between d (T_{11} X T_{11}) and d (AAGATAGAACA) with four mismatches leads to the destabilization by 18°C (Exp VIII, Tm 50°C).

In conclusion the aliphatic non-nucleotide linker 'Y' was more effective than linker X.

Experimental :

Materials : Cyanuric chloride (Fluka) was recrystallised from petroleum ether.

Protected nucleosides and nucleoside H-phosphonates were purchased from Glen Research Corpn USA. Acetonitrile and pyridine (Aldrich chemical company, USA) were refluxed with calcium hydride for 6 hours and then distilled and stored in airtight bottles over 4Å molecular sieves.

Thiol modifier (6-S-S namely [1-O-Dimethoxytritylhexyl-disulphide, 1'-[(2-cyanoethyl) - (N-N-disopropyl)] phosphoramidite (DMT - O - $(CH_2)_6$ -S-S-$(CH_2)_6$-O-P (O CH_2 CH_2CN) $N(ipr)_2$ was purchased from Glen Research Laboratory USA.

TLC : TLC was carried out using Kodak 13181 silica gel with Fluorescent indicator precoated plates.

HPLC : HPLC analysis was performed with a C-18 reverse phase column (Nucleosil 10 μm) 250 x 4.6 mm or sp 8800 liquid chromatography (spectra physics) with a gradient of Acetonitrile in 0.03 M triethylammonium acetate, pH = 7, 1 % increase in acetonitrile per minute.

NMR : NMR spectra were characterised in $CDCl_3$ on MSL 300 spectrometer. ^{31}PNMR spectrum was obtained by using a varian XLA 400 FT NMR spectrometer.

U.V : Spectra were recorded on shimadzu 160 A spectrophotometer.

M.S : FAB mass spectra were obtained on a VG - 70 - 250 SE mass spectrometer/IIMS 30 double beam mass spectrometer.

Binding study : The shimadzu 160 A spectrophotometer attached with a TCC controller (TCC 240 A) system was used for the binding study and the melting curve determination.

The thermal dissociation data for the folded conformers and complexes formed by the bridged oligonucleotides are summarised in Table - 1, along with comparative data for comodified oligonucleotide.

Preparation of 2,4-Dichloro-6-methoxy-S-triazine (1b) :

In a single necked 500 ml R.B. flash containing a magnetic stirring bar fitted with a reflux condenser were added methanol (50 ml), water (10 ml), sodium bicarbonate (8.49, 0.1 mole) and cyanuric chloride (9.2 g, 0.05 mol) at room temperature. The reaction was stirred at 30°C until the evolution of CO_2 gas had nearly ceased (30 min.). Then water (100 ml) was added to the flask. The separated crystalline solid was filtered and washed with water several times. It was recrystallised with petroleum ether (60 - 80°C) and dried under vaccum.

Yield = 4.6g (47 %), M.P = 88-89° [Lit[10] M.P. 88-89°C], MS, m/e = 180, 182 & 184, Mcalcd = 180.

Preparation of 2,4-Bis (4-carboxyphenoxy)-6-methoxy-S-triazine-(4-CPMT) (1c) :

In a 500 ml three necked R.B.flask fitted with a dropping funnel, a thermowell and a stirring arrangement, were added 2,4-dichloro-6-methoxy-S-triazine (4.6 g, 0.025 mol.) and acetone (50 ml). The solution was cooled to 5°C by using ice. Then a solution of sodium hydroxide (4.0 g, 0.1 mol) and p-hydroxy benzoic acid (6.9 g, 0.05 mol) in 50 ml. of distilled water was added dropwise over a period of 30 min. with constant stirring. The reaction was continued further at the same temp for 3 hours. At the end of the reaction the contents of the flask were poured into acidified (10 %) ice cold water. The solid product which separated was filtered and washed several times with hot water and then dried under reduced pressure at 100°C for 5.6 hr. Yield - 6.5 g (95%), M.P. - 270°C, (Lit[11] M.P. 270 - 272 °C), MS, m/e = 383, Mcalcd = 383.

Synthesis of 2,4-Bis (4-chlorocarbonylphenoxy)-6-methoxy-S-triazine (4-CCPMT) (1d) :

In a 250 ml R.B. flask fitted with a magnetic stirring bar and reflux condenser with a guard tube, were added 4-CPMT (3.83 g, 0.01 mol) and thionyl chloride (40 ml). The mixture was heated at reflux for 24 hrs and then 1-2 drops of dry dimethyl formamide were added. Heating was continued for a further 2 hours. The excess thionyl chloride was removed under reduced pressure at 40 - 50°C. The crude product obtained was dissolved in hot dry benzene and the solution was

filtered hot. Upon cooling solution to room temp, 4-CCPMT separated from solution as a crystalline solid. Yield - 8.1 g (90 %), M.P - 151°C [Lit[12] M.P. 151°C], MS, m/e = 420, 422 & 424, Mcalcd = 420.

Synthesis of N-N'-Bis[6-(tert-butyldimethylsiloxy)hexyl 4-CPMT amide (2a) :

A solution of 6-aminohexanol (2.127 g, 18.17 m mol) and imidazole (2.48 g, 36.34 m mol) in DMF (20 ml) was cooled to O°C and treated with tert-butyldimethylsilyl chloride (5.81 g, 39.94 m mol). The mixture was allowed to warm to room temp. stirred overnight, concentrated under vaccum, and partitioned between water and CH_2Cl_2. The organic layer was dried (Na_2SO_4) and evaporated to give a thick oil (4.50 g) Rf (TLC ; $CHCl_3$ / MeOH, 90/10, v/v) 0.49. The presence of the free amino group was shown by a +ve ninhydrin test. To a portion of this material (4.30 g, 18 mmol) dissolved in benzene / ether (1/1, v/v, 120 ml) was added ice (40 g), K_2CO_3 (1.23 g, 9 mmol) and a cold solution of 4-CCPMT (1.9 g, 4.5 mmol) in benzene/ether (1/1, v/v, 60 ml). The reaction mixture was stirred overnight. The organic layer was dried and evaporated. Purification of the product (3.075 g) by chromatography [EtoH/C_6H_6, 30/70, v/v eluent] afforded the oily compound. Yield = 2.7 g (87 %), Rf (TLC, EtoAc/C_6H_6) 0.52; FAB MS M + = 810, M cal = 809, 2a, ^{1}H-NMR = 0.03 (s, $2Me_2Si$), 0.86 (s, 2t, BuSi), 1.25 - 1.55 (m, 2 $(CH_2)_4$); 3.30 - 3.60 (m, $2CH_2N$, $2CH_2O$), 3.90 (s, - OCH_3), 6.60 (t, 2NH), 7.15 (d, 4H, Ar-H, meta to -CO grp), 7.79 (d, 4H, Ar-H, ortho to - CO grp.)

Synthesis of N-[6-(tert-butyldimethylsiloxy)hexyl]-N'-(6-hydroxyhexyl) 4-CPMT amide (2b):

A solution of compound **2a** (2.68g, 3.3 mol) and Bu_4NF (2.48 m mol) in THF (60 ml) was stirred for 2.5 hours the solvent was removed at reduced pressure, and the residue was taken up in CH_2Cl_2 and washed with water. Chromatography afforded, after elution of some starting bis-silyl derivative (1.2 g), the desired monosilyl compound **2b** as an oil. Yield = 0.775 g (42 %), R_f (TLC, EtoAc/C_6H_6) = 0.231 FAB MS, M + 1 = 696, Mcalcd = 695. 2b ^{1}H-NMR-0.05 (s,Me_2Si), 0.86 (s,t-BaSi), 1.29 - 1.60 (m, $2(CH_2)_4$), 3.25 - 3.65 (m, $2CH_2N$, $2CH_2O$), 4.01 (s, OCH_3), 6.45 (t, 2NH), 7.15 (d, 4H, Ar-H, meta to -CO grp), 7.80 (d, 4H, Ar-H, Ortho to -CO grp).

Synthesis of N-[6-(tert-butyldimethylsiloxy) hexyl]-N-[(6-(dimethoxytrityl)oxy) hexyll 4-**CPMT amide (2c) :**

A mixture of compound **2b** (0.760 g, 1.01 mmol) and 4,4' dimethoxytrityl chloride in

pyridine (15 ml) was stirred overnight with a catalytic amount of 4-(dimethylamino) pyridine, evaporated at reduced pressure and partitioned between water and CH_2Cl_2. The solid obtained. from the organic layer was taken up in EtOAc and purified by column chromatography. Elution with EtOAc/C_6H_6/Et_3N (30/68/2 v/v/v, to elute dimethoxytritanol), followed by EtOAc/C_6H_6/Et_3N (30/68/2 v/v/v) afforded the DMT derivative as an oil. Yield : 0.370 g (65 %), Rf (TLC, EtoAc/C_6H_6/Et_3N 30/68/2, v/v/v) 0.44, FAB MS, M + 1 = 997, Mcalcd = 996 positive DMT test. 2C ^{1}H-NMR 0.5 (s, Me_2Si), 0.87 (S, t-BuSi), 1.29 - 160 (m, 2 $(CH_2)_4$, 3.05 (t, CH_2OC), 3.30 (m, $2CH_2N$), 3.62 (CH_2OSi) ; 3.86 (s, 3MeO), 5.60 (m, 2NH), 6.82 (d, 4H, 3H and 5H of anisyl), 7.20 - 7.40 (m, rest of DMT) ; 7.45 (d, 4H, Ar-H meta to CO grp) ; 8.15 (d, 4H, Ar-H, ortho to -CO grp).

Synthesis of N-[6-(dimethoxytrityl)oxy)hexyl]-N'-(6-hydroxyhexyl) 4-CPMT amide (2d) :

A portion of compound **2c** (0.185 g, 0.176 mol) was stirred with Bu_4NF (0.23 nmol) in dry THF (10ml) for 5 hrs and the mixture was concentrated under reduced pressure and chromatographed to give compound **2d** as an oily substance. Yield - 0.090 g (60 %), Rf (TLC, EtOAc/C_6H_6/Et_3N 30/68/2) 0.45, FAB MS M + 1 = 883, Mcaled = 882, 2d 1.28 - 1.60 (m, $2(CH_2)_4$, 3.05 (t, CH_2OC), 3.30 (m, $2CH_2N$), 3.65 (m, CH_2 - Hydroxyl), 3.83 (s, 3MeO), 6.2 (t, NH), 6.3 (t, NH), 6.8 (d, 4H, 3H, 5H of anisyl) ; 7.20 - 7.40 (m - rest of DMT), 7.4 (d, 4H, Ar-H, m to -CO grp) ; 8.15 (d, 4H, Ar-H ortho to -CO grp).

Synthesis of an hydrogen phosphonate derivative of compound (2e) :

To imidazole (0.080 g, 1.20 mmol) in MeCN (5 ml) at ice temperature (0° C) was successively added, with stirring, PCl_3, Et_3N and compound **2d**. After removing the ice bath, stirring for 4 hours, addition of water (2ml) and stirring for 30 min, the mixture was evaporated under reduced pressure, co-evaporated with pyridine/Et_3N and partitioned between $CHCl_3$ and water. Purification was by chromatography ($CHCl_3$/MeOH/Et_3N 90/7/3 v/v/v) and yielded the desired triethylammonium hydrogen phosphonate. The ^{31}P NMR spectrum give signal at 2.35 ppm (Jp -$_H$ 761.6 Hz), Yield - 0.060 g, Rf (TLC, $CHCl_3$/MeOH/Et_3N 90/7/3 v/v/v) 0.76.

Procedure for synthesis of Oligonucleotides :

Synthesis of oligodeoxynucleotides : Oligodeoxynucleotides were synthesized on the 1.0 μ mol scale with an automatic DNA synthesiser (Pharmacia GA plus) using the phosphoramidite

protocol provided by the manufacturer.

Synthesis of a Oligonucleotide linker :

Oligonucleotides were synthesized on CPG support using nucleoside cyanoethyl phosphoramidite reagents for addition of nucleoside units and hydrogen phosphonate reagent for addition of linker. Conventional protocols for synthesis with phosphoramidite reagents[13] and Hydrogen phosphonate reagents[14] were followed, starting with 1.0 μmol of loaded nucleoside, couplings were carried out manually by syringe technique[15].

The oligonucleotides, worked up in a conventional manner and isolated by reverse phase HPLC, were more than 95 % pure by analysis by HPLC.

Acknowledgement :

The financial assistance from the Dept. of Biotechnology, New Delhi is gratefully acknowledged and the authorities of Shivaji University Kolhapur for providing research facilities. We are thankful to Dr.Ganesh, Dr. A.A.Natu and Co-Workers NCL, Pune for encouraging discussions and for assistance with NMR experiments.

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(*Received in UK* 4 *September* 1996; *accepted* 24 *October* 1996)