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THE EFFECT OF SPACER, LINKAGE AND SOLID SUPPORT ON THE SYNTHESIS OF OLIGONUCLEOTIDES

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ABSTRACT

The effects of spacers on the synthesis of various chain- length oligonucleotides was tested. Fifteen different spacers were prepared on solid support systems and from the data obtained, it was inferred that: a) the length of the spacer is important for increasing purity and yield of the final product; b) there is a high probability that folded conformations in the spacer may increase the non-homogeneity of the product and; c) spacers made up of a fully-extended conformation mediate a high purity. and only suports with a large pore size produced excellent results.

INTRODUCTION

Several factors appear to play an important role in the synthesis of oligonucleotides on a solid support system. This includes: a) the type of solid support[1-8] used, b) the length and type of spacer[7-13] used and, c) the type of linkage between the spacer and the first nucleotide attached[7,11,14-20].

Recently we reported that the synthesis of oligonucleotides on aminoalkyl ureide-type spacers made up of only 12-21 atoms in length, produced poor results relatives to end product purity, homogeneity and yield. Purer products were obtained on spacers with lengths of at least 24 atoms.

We have interpreted this observation to be the result of conformational changes that take place in the spacers (an increase in their extended form), as a function of length. In order to gain a better understanding about the possible relationships that exist between the structural characteristics of spacers, such as rigidity, hydrogen bonding and/or internal dipole-dipole interactions and the efficiency of synthesis, we prepared several spacers (fifteen) for the synthesis of various chain-length oligonucleotides. The rationale behind the construction of these spacers was initiated by the idea that an increase in internal hydrogen bonding or in interactions should increase the probability of folded conformations and, consequently, decrease the yield and purity of the final product. Alternatively, spacers with a low probability for multiple structural orientations should produce a better yield and a purer product. The designed spacers were,

therefore, constructed to have an increasing distances between the carbonyl and the NH groups of the adjecent amido or (-CNH---NHC-),uriedo function in the repeating sequence of the spacer polymer.

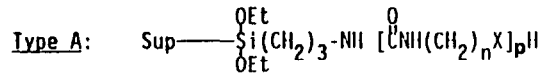
This includes polyglycine (type F) and poly-ureid spacers (type A), formed from 1,2-diaminoethane; 1,3-diaminopropane; 1,6-diaminoexane and; 1,12-diaminodecane. Another type of interaction derives from the dipole-dipole interaction. It is assumed that, due to these depole-dipole interactions, the polyethylene glycol spacer (type E) adopts mainly an extended form and will therefore, mediate a highly pure product.

The present study also includes the use of ureid spacers that have been derived from 1,4-phenylenediamine. Although these systems appear rigid, they may adopt a folded conformation, stabilized by π-interactions.

The bleeding of a product from the support during synthesis is assumed to be dependent upon the type of nucleotide-spacer bond selected. Most of the conventional methods that are used, consist of an ester-type linkage between the 3'OH group of the initial nucleotide and the spacer. The use of amide, sulfide and phosphate bonds has also been reported.[7,20,21] It is, therefore, of interest to search for other covalent bond attachments for nucleotides to the functional solid support. Carbonate and carbamate groups were checked for this possibility.

Much of our interest is also concerned with the synthesis of long-fragment oligonucleotides; 100mer. In this respect, we compared the applicability of three solid supports as matrix for the desired synthesis: Fractosil 500, 1000 and 2500.

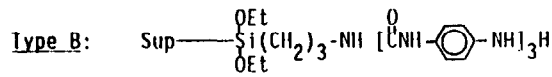
Structure of Spacers



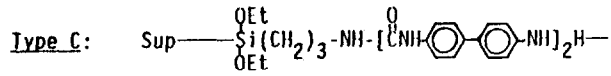
Type A: Sup—Si(OEt)(OEt)-$(CH_2)_3$-NH-[C(=O)NH$(CH_2)_n$X]$_p$H

Sup = CPG 500; Fractosil 500, 1000, 2500;

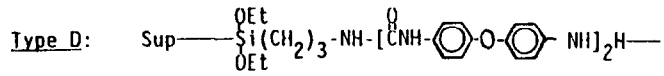
Spacers code and length 3,2,2,2,2 (n=2, P=4, X=NH); 3,2,2,2 (n=2, P=3, X=NH) 3,3,3,3,3 (n=3, P=4, X=NH); 3,3,3,3 (n=3, P=3, X=NH); 3,3,3 (n=3, P-2, X=NH) 3,6,6,6 (n=6, P=3, X=NH,X=O); 3,12,12 (n=12, P=2, X=NH, X=O); 3,12 (n=12,X=NH)



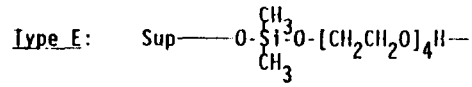
Type B: Sup—Si(OEt)(OEt)-$(CH_2)_3$-NH-[C(=O)NH-C$_6$H$_4$-NH]$_3$H

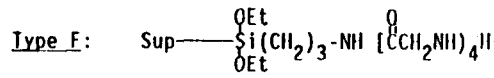


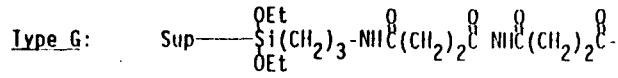
Type C: Sup—Si(OEt)(OEt)-$(CH_2)_3$-NH-[C(=O)NH-C$_6$H$_4$-C$_6$H$_4$-NH]$_2$H—



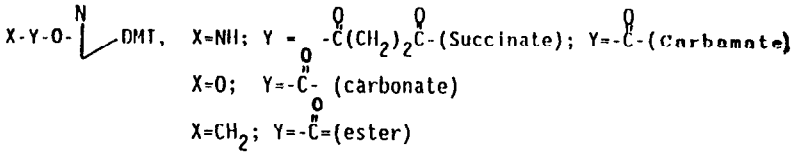
Type D: Sup—Si(OEt)(OEt)-$(CH_2)_3$-NH-[C(=O)NH-C$_6$H$_4$-O-C$_6$H$_4$-NH]$_2$H—







Bond linkage to X

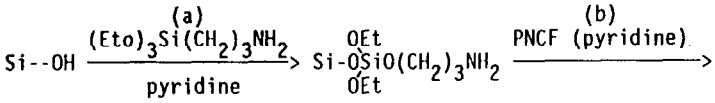


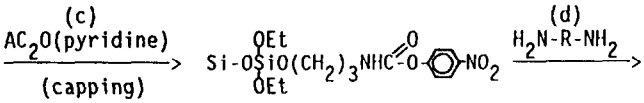
RESULTS AND DISCUSSION

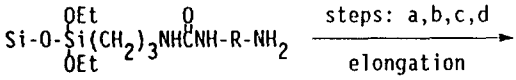
Spacers synthesis

Synthesis of the spacers was carried out according to general procedures outlined in Scheme 1.

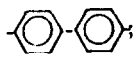
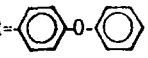
Scheme 1

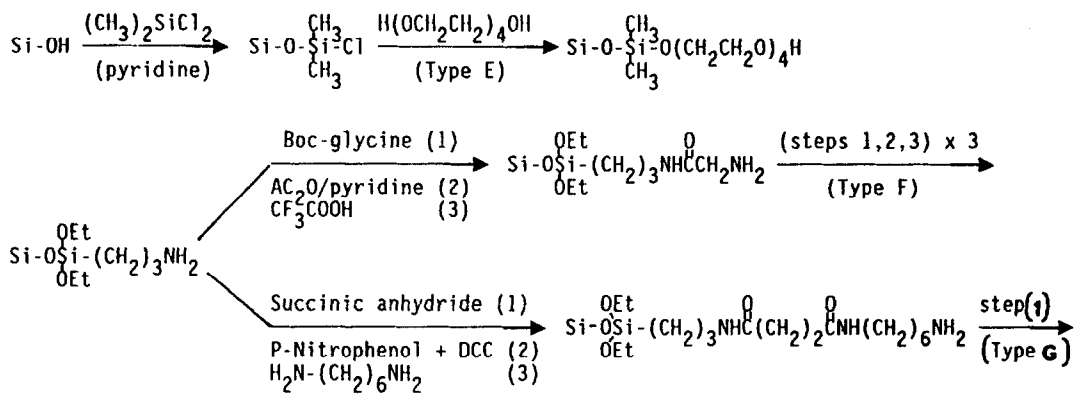






PNCF=p-nitrophenylchloroformate. Type A: R=$-(CH_2)_n-$ n=2,3,6,12;

Type B: R=  ; Type C: R=  ; Type D: R= 



In the case of spacers of type A,B,C,D chain elongation was achieved by treating the free amino end group on the solid support with p-nitrophenylchloroformate[22] (PNCF) in pyridine, followed by capping and addition of diamine. The yield of each elongation step was determined spectrophotometrically by the p-nitrophenol liberation or by the picric acid method.

The amide spacer F was prepared by condensing the amino-propyl silica with activated Boc glycine by DCC and hydroxybenztriazole in CH_2Cl_2. The non reacting aminopropyl group was than caped with acetic anhydride in pyridine and the Boc protecting group was removed by TFA. Spacer G was prepared[12] by reacting the aminopropyl silica with succinic anhydride followed by p-nitrophenol + dicyclohexylcarbodiimide (DCC) and then by 1,6-diaminohexane.

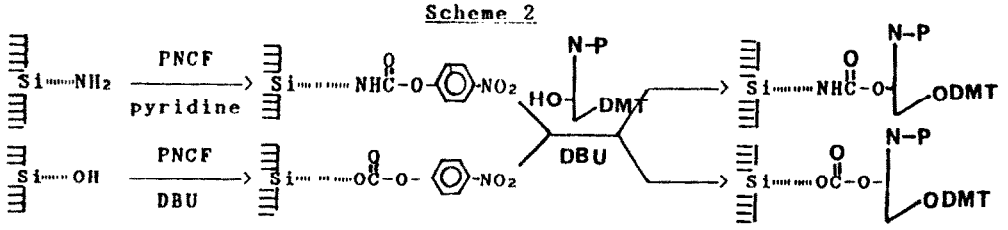
The synthesis of spacer E was carried out by treating a pretreated silica (Fractosil) with dimethyldichlorosilane and then with tetraethyleneglycol in DBU. The spacer E bears a terminal hydroxyl group as an anchor for the first nucleotide attachment. It is possible to create the hydroxy end group also in the other spacers described here by treating their terminal end group with $NaNO_2$ in H_2SO_4 (diluted 1:4 in cold water) for 10 min.

Linkages

Succinate linked supports were prepared according to the commonly used method; by reacting the free 3'-hydroxyl group of the protected 2'-deoxyribonucleoside with succinic anhydride.

The preparation of carbonate and carbamate type linkages is illustrated in scheme 2. Initially, the NH_2 or OH end group of the spacer was treated with PNCF (in pyridine) or PNCF (in DBU) respectively. After washing with CH_2Cl_2, the appropriate linkage (carbamate or carbonate to 2-deoxyribonucleoside) was carried out by substituting the p-nitrophenol moiety on the spacer by the 3'-OH of a protected nucleoside in DBU solution.

Scheme 2



Synthesis of oligonucleotides

Using the above outlined supports, we have synthesized twenty three polynucleotides. The oligonucleotides were assembled using an "Applied Biosystems" synthesizer model 380B according to the phosphite method; starting with 0.5μmole of immobilized 2'-deoxynucleoside. The steps utilized for each cycle (including capping and time interval) are those already described in the literature[23].

Table 1 summarizes the various lengths and compositions of the polynucleotides synthesized in the present study. Table 2 presents, for each oligonucleotide, the type of support used, the spacer structure and length being used, together with the nucleoside linkage, initial loading, total yield and calculated avarage yield for each condensation cycle. After ^{32}P labellling, purity and homogenity of the product was checked by examining the autoradiogram pattern obtained by polyacrylamide gel electrophoresis.

Table 1. Lengths and compositions of polynucleotides synthesized

TA1	CPG(500) 3,2,2,2-CM-3'-AAG GTA CTT AAG $(T)_{15}$-5'	27mer
TA2	CPG(500) 3,2,2,2-CM-3'-ACA AGT AGT GCT GAC GAC GTA GAG-5'	24mer
TA3	CPG(500)3,3,3,3,3-CM-3-AAC TTA AGC TTA GGC AGC TGT TCG AAA A5'	28mer
TA4	CPG(500)3,3,3,3-CM-3'-ACT GCA GCC GCG TCA ATT GCT ACCC C-5'	25mer
TA5	CPG(500)3,3,3-CM-3'-TGT CCC AGC TGG ATC CGC TTT AGA-5'	24mer
TA6	Frac(500)3,6,6,6-Suc-3'-GCT TAT AAA TAT GCT GCG TCG-5'	21mer
TA7	Frac(500)3,6,6,6-CM-3'-ACT GAC GCC GGG TGA ATT GTT ACC C-5'	25mer
TA8	Frac(500)3,6,6,6-CM-3'-AAC TTA AGC TTA GGC AGC TGT TCG AAA A	28mer
TA9	Frac(2500)3,6,6,6-CM-3'-GTA CGG GAT ACA ATT GAC CCG CGA ACA GTA ATG GTG GCG GCG CAG GGG TCG ACT ATA GAG TGG GAC CAG CTG CGC CAC CAC ACA CCA CAT CTA CAA GC——5'	98mer
TA10	Frac(2500)3,6,6,6-Suc AS TA9 1-94	94mer
TA11	Frac(1000)3,6,6,6-Suc AS TA9 1-76	76mer
TA12	Frac(1000)3,6,6,6-CM AS TA9 1-53	53mer
TA13	Frac(500)3,6,6,6-CM AS TA9 1-41	91mer
TA14	CPG(500)3,12,12-Suc 3'-TGC AGT AGT GCT GAC GAC GTA GAG-5'	24mer
TA15	CPG(500)3,12,12-CN 3'-GTC GAC TCT AAA GCG GAA CCA GCT-5'	24mer
TA16	CPG(500)3,12,12-Suc 3'-TGC AGT AGT GCT GAC GAC GTA GAG-5'	24mer
TA17	CPG(500)3,12-CM-3' ACA AGT AAT GGT GGC GGC GCA GGG-5'	24mer
TB	CPG(500)-CM-3'-GTA CGG GTA GCA ATT GAC GCG GC-5'	23mer
TC	CPG(500)-CM-3'-TGT CCC AGC TGC ATC CGC TTT AGA-5'	24mer
TD	CPG(500)-CM-3'-AAC AGT AAT GGT GGC GGC GCA GGG	24mer
TE	CPG(500)-CN-3'-AAC TTA AGC TTA GGC AGC TGT TCG AAA A-5'	28mer
TF	CPG(500)-CM-3'-ACT GTT CGC GGG TCA ATT GTT ACC C-5'	25mer
TG	Si(2500)-AS TA9 1-51	51mer

The abbreviations TA,TB,TC etc..denote the spacer's type: Type A, Type B, Type C etc.

Table 2. Coupling yields and type of supports, spacers and linkages employed in the synthesis of various oligonucleotides.

Type of spacers	Type of support	Spacer structure	Spacer length	linkage	oligonucleotide length	yield %	loading μmol/g	calculated yield/cycle
TA1	CPG 500	3,2,2,2,2	25	CM	27 mer	50	14	97.46
TA2	CPG 500	3,2,2,2	20	CM	24 mer	30	23.7	95.10
TA3	CPG 500	3,3,3,3,3	29	CM	28 mer	60	14.2	98.19
TA4	CPG 500	3,3,3,3	23	CM	25 mer	40	14.5	96.40
TA5	CPG 500	3,3,3	17	CM	24 mer	30	14	95.10
TA6	Frac 500	3,6,6,6	32	Suc	21 mer	77	14	98.76
TA7	Frac 500	3,6,6,6	32	CN	25 mer	77	9.7	98.95
TA8	Frac 500	3,6,6,6	32	CM	28 mer	73		98.88
TA9	Frac 2500	3,6,6,6	32	CM	98 mer	50	5μmol	99.29
TA10	Frac 2500	3,6,6,6	32	Suc	94 mer	50	14	99.86
TA11	Frac 1000	3,6,6,6	32	Suc	76 mer	48		99.04
TA12	Frac 1000	3,6,6,6	32	CM	53 mer	60	5.5	99.04
TA13	Frac 500	3,6,6,6	32	CM	41 mer	36	12	97.53
TA14	CPG 500	3,12,12	35	Suc	24 mer	85	13.3	99.32
TA15	CPG 500	3,12,12	35	CN	24 mer	90	10.5	99.56
TA16	CPG 500	3,12,12	35	CM	24 mer	88	12.6	99.46
TA17	CPG 500	3,12	20	CM	24 mer	13	16.6	91.85
TB	CPG 500	Phenyl		CM	23 mer	95	7	99.77
TC	CPG 500	Biphenyl		CM	24 mer	8.8	15.8	90.37
						35	24	95.72
TD	CPG 500	Biphenyl ether		CM	24 mer	13.6	15	92.02
						19	20	93.31
TE	CPG 500	Ethylene glycol	14	CM	28 mer	91	15.5	99.66
TF	CPG 500	glycine	17	CM	25 mer	12	15	91.86
TG	Frac 2500	Succinic acid +Hexane-diamine	21	CM	51 mer	56	10	98.86

Figs 1-4 are the autoradiograms of TF, TA4, TA1, TA3, TA6 (Fig. 1); TA5, TA2, TA17, TB, TC, TD (Fig. 2); of TA7, TA14, TA15, TE, TA8, TA16 (Fig. 3) and TG, TA11, TA10, TA12, TA9 (Fig. 4).

The effect of spacers on the synthesis

Various spacers were examined in order to determine whether a correlation between their structural features, such as chain composition, distance between functional groups or chain lengths and the yield or homogenity of the final product. By comparing the results obtained by using spacers of identical nucleoside linkage, it is inferred (from Table 1) that; (a) Increasing the chain length of the spacer (by a repeating segment), increases, accordingly, product yield. In the case of TA5 (CPG-3,3,3), TA4 (CPG-3,3,3,3) and, TA3 (CPG-3,3,3,3,3), the final yields (as determined by dimethoxytrityl cation optical density) are 30, 40 and, 60% respectively. The corresponding average yields for each condensation step are: 95.1, 96.4 and 98.1% respectively. A similar tendency was also observed with TA2 (CPG-3,2,2,2) and TA1 (CPG-3,2,2,2,2), where the respective yields are: 30% (95.1% per cycle) and 50% (97.46% per cycle) and with TA17 (CPG-3,12) and TA16

(CPG-3,12,12) where the yields are 13% (91.85% per cycle) and 88% (99.46% per cycle) respectively. (b) Increasing the number of intervening atoms between the carbonyl groups on the spacer increases the yield (compare TA3 with and TA1; TA4 with TA2 and TA16 with TA8). With respect to this observation, the polyglycine spacer (TF) is, indeed, less efficient for the synthesis process (compare TF with TA5 and TA2). (c) of the aromatic spacers TB, TC and TD, only TB induced high of product yield.

Since the homogenity of the products does not necessarily correlate with the total coupling yield, band distributions on sizing gels was also examined. Figs 1,2 and 3 demonstrate that in each set of spacers: TA5 (lane A, Fig. 2); TA4 (lane B, Fig. 1); TA3 (lane D, Fig. 1); TA2 (lane B, Fig. 2); TA1 (lane C, Fig. 1); TA8 (lane Fig. 3); TA17 (lane C, Fig. 2) and; TA16 (lane B, Fig. 3); the purity of the product is significantly increased when the spacer length is exceeded by a length of 25 atoms. Although TA3 and TA1 only produced a total yield of 60% (98.19% per cycle) and 50% (97.76% per cycle) respectively, their autoradiogram patterns exhibited an isolated strong band of the target compound. The decrease in total yield is probably due to the formation of very small

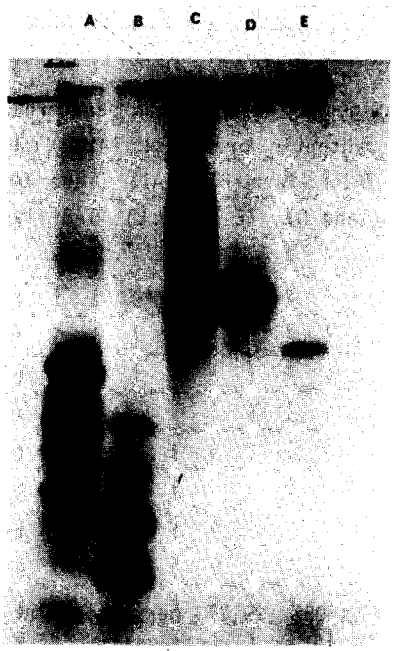


Fig. 1. Sizing gel of the synthetic oligonucleotides: TF (lane A) TA4 (lane B); TA1 (lane C); TA3 (lane D); TA6 (lane E).

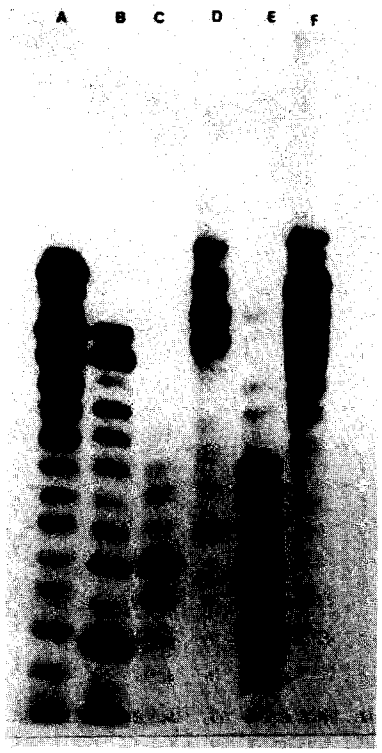


Fig. 2. Sizing gel of the synthetic products: TA5 (lane A) TA2 (lane B); TA17 (lane C); TB (lane D); TC (lane E). TD (lane F).

oligomers which migrate in front of the gel. As the spacer becomes more hydrophobic and the number of carbonyl groups decreases, (TA8 and TA16), there is a significant increase in both the total yield and the relative amount of the final product. The undesired effect of crowding carbonyl groups on product homogenity is displayed when using the glycine type spacer (TF, Fig. 1 lane A). This spacer mediates a highly non homogenious mixture consisting of various length of various oligomers. As well, the total yield after 25 cycles falls to the low value of 12% (91.86% per cycle).

The aromatic type models (Type B,C and D) are assumed to be relatively rigid spacers and thus probably more effective in the synthesis of oligonucleotides. However the experimental results obtained with these spacers were poor. This is inferred from the autoradiogram of some synthetic oligomers (Fig. 2 lanes, D,E,F) which displayed a large amount of concomitant undesired fragments.

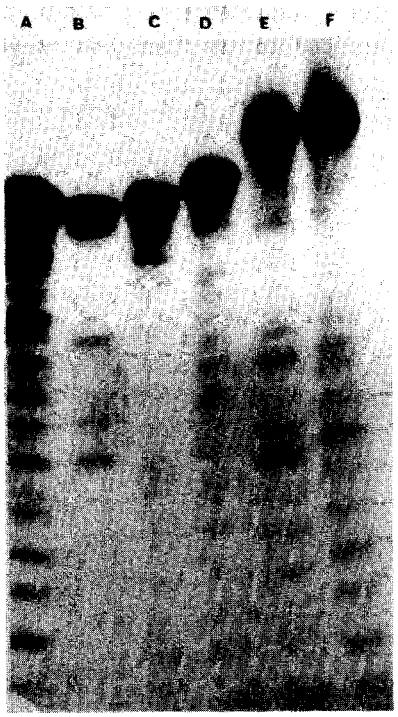


Fig. 3. Autoradiogam parttern of the synthetic products: TA14 (lane A); TA16 (lane B); TA15 (lane C); TA7 (lane D), TE (lane E); TA8 (lane F).

In the case of TB, even though the overall yield is 95% (99.77% per cycle) the final reaction mixture is highly non- homogenious; being made up of a substantially high level of various fragments. It is worthy to note that the tetraethyleneglycol spacer (Type E) is only 15 atoms long, but despite this it still mediates both a high coupling yield (99.66% per cycle) and a highly homogeneous product.

Fig. 4. Sizing gel of the synthetic oligonucleotides; TG (lane A) TA11 (lane B); TA10 (lane C); TA12 (lane D); TA9 (lane E).

The above results are in accord with the general view proposed in the introduction that: (a) the length of the spacer (more that 25 atoms) is important for increasing, substaintialy, product purity; (b) increasing the probability of folded conformations in spacers (by hydrogen bonding, π-interactions), accordingly increases product distribution and; (c) spacers consisting of a fully extended conformation (independent of spacer length Type E-tetraethyleneglycol) mediate high yields and purity.

Type of linkage

Three types of linkages with the solid phase were tested for their effeciency in the synthesis of oligonucleotides: a) a succinate linkage (succ); b) a carbamate linkage (CM) and; c) a carbonate linkage (CB). Fig. 3 exhibits the autoradiogram patern of several oligonuleotides prepared on long chain spacers (35 atoms and 32 atoms), linked during the synthesis by the aforementioned bonds. From Table 1 and Fig. 3, it is implied that there is no significant difference between the yields and purity of products synthesized via the various types of bond linkages.

TA14 (Succ, 24-mer), TA16 (CM, 24-mer) and TA15 (CN, 24-mer) produce both high total yields 85% (99.3% per cycle), 88% (99.46% per cycle) and 95% (99.56% per cycle) respectively) and high band densities of the final products on the autoradiograms.

The results with TA6 (Succ, 21-mer, Fig. 1) TA7 (CB, 25-mer, Fig. 3) and TA8 (CM, 28-mer, Fig. 3) are similar. These produce a total yield of 77% (98.76% per cycle), 77% (98.95 per cycle) and 73% (98.88 cycle per cycle) respectively. The effect of the succinate and the carbamate bond on the synthesis of oligonucleotides can also be compared in the case of very long oligomers such as TA9 (CM, 98-mer, Fig. 4) vs. TA10 (Succ, 94-mer, Fig. 4) and TA11 (Succ, 76-mer, Fig. 4) vs. TA12 (CM, 53-mer, Fig. 4).

Although the succinate bond is assumed to be more labile than the other linkages used, from the results obtained it is impossible to point out any significant advantages of using one of the above outlined types of bond linkages.

Synthesis of long oligonucleotides

Long fragments of oligonucleotides, made up of 41-mer, 51-mer, 53-mer, 76-mer, 94-mer and 98-mer, were prepared on one of the following solid supports: Fractosil 500, Fractosil 1000 and Fractosil 2500. In general the spacer used was of Type A (3,6,6,6), except in one case where type G was used. Bond linkages between the spacers and the oligonucleotides were either of the succinate (Succ) or the carbamate (CM) type.

From Table 1, it is inferred that silica pore size plays an important role in the preparation of long oligonucleotide fragments. The last effective carrier was found to be Fractosil 500 (Pore size≈420A°) where the total coupling yield after synthesis a fragment of 41-mer droped to 36% (97.53% per cycle). The effectiveness in synthesis increases when Fractosil 1000 (Pore size 1400A°) was used (TA-11 (76-mer) and TA12 (53-mer)). The average coupling yield per cycle for the above sequences was very high and reached a value of 99.04%. Better results were obtained by using Fractosil 2500 (Pore size 2500A). TA9 (98-mer) and TA10(94-mer) were synthesized on the latter support with an overall yield of 50%. This corresponds to an average coupling yield of 99.29 and 99.26% per condensation step respectively. With a shorter spacer of only 21 atoms in length, the synthesis (TG oligomer) was less favorable (98.86% per cycle). The results imply that a solid support containing large pore size and bound to a long alkyl chain arm is a highly effective combination for the synthesis of long oligomers. An average coupling of 99.29%, as observed in the synthesis of 98-mers, may lead to a total yield of 11.8% when an oligomer of 300-mer is constructed. Therefore, it appears to us that with some minor modifications of the support, the total synthesis of an entire gene of 700-800-mer is a feasible task.

Fig. 4 exhibits the autoradiogram patterns of TG (51-mer), TA11 (76-mer), TA10 (94-mer), TA12 (53-mer) and TA9 (98-mer). In all of the lanes, the end product appears as a distinct dense band. However, in other cases (e.g., TA11 and T9), the amount of coupled side products (lower segments) becomes significant. The crude product of TA10 (94-mer) is highly homogeneous.

In order to obtain more information about the surface structure and property of the various solid phases used here, some additional measurements were carried out: (a) the quantitative determination of support functionalization by 3-aminopropyl- triethoxy silane. This will thus allow us the capability of comparing the amount of free silanol groups on the surface of the different support systems. b) the quantitative determination of dyes adsorbed on the surface of the support. This may give us an indication of the degree of porosity of the surface and of the relative free area available for binding. Due to the substantially high molecular volume of the dyes utilized, it appears that, in contrast to large surface porosity, the presence of a large amount of small pores will be associated with a decrease in dye binding. and (c) the determination of surface structure by Scaning Electron Microscopy (SEM). The dyes employed were: 1-(4-butylamino)aminoanthraquinone (1-C4); 1,8-bis(4-butylamino)aminoanthraquinone (1,8-C4). Pararoseaniline (PRA) and Rosebengal (RSB). The results are summarized in Table 3.

Table 3: Functionality and Dye Adsorbtion on CPG and Fractosil

Solid Suport	Pore Size	Amino Propyl loading µg/g	Dye Adsorption µg/g			
			1-C4	1,8-C4	PRA	RSB
CPG-500	75A°	214		15.4	8.5	17.6
Fractosil-500	430A°	180	19.6	20.8	9.0	17.9
Fractosil-1000	1400A°	125	16.2	27.2	22.6	18.6
Fractosil-2500	2500A°	110	17.6	35.2	39.6	18.7

From Table 3, it is inferred that despite of the great differences in the surface area of the various types of Fractosil and CPG (50,20 and 8m^2/g for Fractosil 500, 1000 and 2500 respectively) used, the difference in the amount of dye absorbed is very small. Supports made up of narrow pore diameters (high surface area), bind equal or even less amounts of dye than supports made up of wide pore diameters. This implies that dyes of the size used here are restricted in their penetration into small pores.

On the other hand, the number of aminopropyl groups covalently attached to the silanol groups on the solid phase is higher in supports made up of small pore size. Thus, it appears that part of the aminopropyl groups are buried inside the pores and that the approach of a bulky reagent is thus hindered. This view can account for: (a) the favorable results obtained in the synthesis of oligonucleotides when long spacers were used. Inside the pores, the interior amino terminal groups can be stretched out when attached to long spacers and, subsequentlly this should increase their exposure to the coupling reagents and; (b) The favourable results obtained with silica made up of a wide pore size. This again implies that the approach of reagents to a surface of narrow pore size is more perturbed than in the case of one with a wide pore size.

In this context, it is worthy to note that the surface cage effect of several reactions are reported in the literature.[24] Furthermore, it appears to us that some additional factors related to surface structure and organization also play a role in the

efficiency of synthesis. In order to examine this scaning electron microscopic measurements were carried out. Fig. 5 displays the surface pattern of CPG-500 (pictures 1-3), Fractosil 500 (pictures 4-6), Fractosil 1000 (pictures 7-9) and, Fractosil 2500 (pictures 10-12). In the case of CPG-500 a smooth surface profile is apparently observed. Fractosil 500 display a relatively poor etching on the crystals surface, while Fractosil 1000 shows a heavily pitted surface pattern due to an extensive etching process.

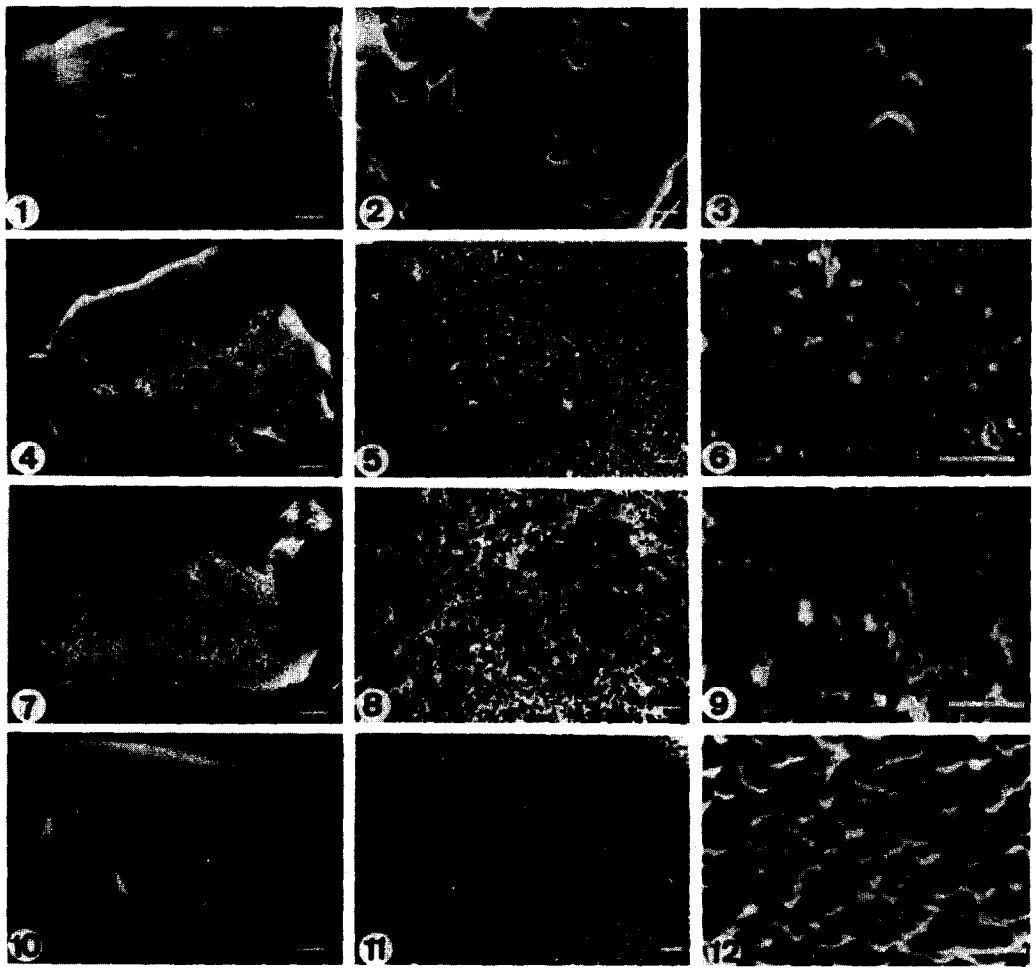


Fig. 5. Scaning electron micrigraphes of CGP-500 (pictures 1-3), Fractosil 500 (pictures 4-6) Fractosil 1000(pictures 7-9) and Fractosil 2500 (pictures 10-12). Bar = 10 micron in pictures 1,4,7,10 and 1 micron in pictures 2,3,,6,8,9,11,12. (lane E).

In the case of Fractosil 2500, the crystal surface is associate with a regular porosity and a delicate network of spaces can be seen. The electron micrographs imply that, as the pore size of the Fractosil increases, the surface structure of the pores become more organized. This probably affects the shape and the structure of the oligonucleotide strands being formed and, thus, the coupling yield and homogenity of the product formed.

EXPERIMENTAL

Synthesis of Spacers

Type A-D spacers were prepared as follows: To 1.5g of aminosilica (or CPG) in 5ml of dry dioxane and 1ml of triethylamine,a solution of 1.2g(5.96mmole) of p-nitrophenylchloroformate (PNCF) in 5ml dioxane was added in the cold. The mixture was stirred at room temperature for 3-4h. After filtration, the remaining solid material was washed with methylene chloride and the unreacted amino groups were capped with acetic anhydride in pyridine 5ml (1:1) for 2h. The support was then separated, washed with methylenechloride followed by ether and dried. Thirty of the solid was submitted for the loading test carried out by the addition of 0.2M NaOH (25ml). The nitrophenol liberated was monitored at 400 nm (ϵ=17,000)[22]. The p-nitrophenylurethane formed on the support was shaken overnight at room temperature with an appropriate diamine in dry dioxane (5ml). The support was filtered, washed with methanol followed by methylene chloride and then (if necessary) subjected to another cycle: (a) PNCF, (b) capping, (c) diamine.

Type E spacer was prepared by adding dimethyldichloro- silane (4.26g, 33mmol) in 10ml of pyridine to 2g of CPG. The suspension was shaken at room temperature for 2h and the supernatant decanted. The CPG was then treated with freshly distilled (4ml) tetraethyleneglycol and agitated for 24h at room temperature. The solid was separated, washed with dioxane followed by ether and then dryed in air. Loading determination was carried out by adding 5% DMTCl (Dimethoxytrityl chloride) solution in pyridine to 50mg of the derivatized support. After shaking for 1 h, the suspension was filtered, washed with methylenechloride and ether. Twenty of the solid were transfered to a 25ml volumetric flask and 2% DCA (dichloroacetic acid) solution in CH_2Cl_2 was added. The colour liberated was measured at 500nm (ϵ=80000).

Type F spacer was prepared as follows: Boc-glycine (350mg, 2mmol) in 2ml of methylene chloride and DCC (Dicyclohexyl carbodiimide, 452mg, 2,2mmol) in 2ml of the same solvent were mixed for 30min at room temperature. The dicyclohexyl urea (DCU) was filtered and 200mg of amino-CPG was added. After agitating for 24h and filtration and washing with methanol, the non-reacted free amine was capped with acetic anhydride in pyridine (6ml of 1:1 solution) and washed with methylene chloride. The protecting group (BOC) was removed by the addition of a 6ml solution of TFA (Trifluoroacetic acid) and methylenechloride (1:1 ratio) for 15 min. The suspension was filtered and washed with methanol, dichloromethane and ether. The solid was dryed in air and subjected to a second cycle. The Type G spacer was prepared according to a procedure already described in detail.[12]

Conversion of terminal NH_2 to OH

A solution of 2g (0.26mol) $NaNO_2$ in 40ml cold water (~5°C) was added to a mixture of 5.5ml concentrated H_2SO_4, 7.5ml H_2O 10g crushed ice and 100mg of functional support (3,6,6,6-NH_2) a added. After 10min of shaking, the reaction mixture was filtered and a new solution of 5.5ml H_2SO_4 and 7.5ml H_2O was added. The mixture was then shaken for another 10min.

The solid support was then separated and washed several times with 5% sodium bicarbonate solution until the filtrate became neutral. The support was further washed with water, methanol and ether and dried in air. The remaining amine sites were capped with 5ml acetic anhydride/pyridine solution (1:1) which was added to a suspension of the support in 4ml of pyridine/trimethylchlorosilane (3:1). After 2h, the support was washed with methanol (several times) and once with methylenechloride.

Linkage of the oligonucleotide to the support

(a) Carbamate linkage. Three hundred mg of silica or (CPG) bearing a long amino-alkylchain were converted to p-nitrophenylcarbamate by adding PNCF according to the procedure detailed in the synthesis of spacers. After filtration, 300mg of the protected nucleoside in 5ml of dry dioxane and 0.5ml of 1.8-Diaza-bicyclo[5.40]undec-7-ene (DBU) were added and shaken at room temperature for 24h.

The solid was separated, washed with methanol followed by methylene chloride and then capped with 5ml of acetic anhydride in pyridine (1:1) for 2h. The capped product was washed again with the above solvents, sucked and dried.

(b) Carbonate linkage. To 300mg of functional silica (or CPG), with a long alkyl chain bearing free terminal hydroxyl groups, a solution of 5ml dioxane, 0.5ml DBU and 0.5ml of triethylamine was added. The solution was chilled (5-10°C) and 0.3g of p-nitrophenylchloroformate (PNCF) in 1 ml dioxane was added dropwise with constant stirring. After 4 h at room temperature, the solid was then collected, washed with methylenechloride and dried in air. Thirty mg of the solid were submitted to the loading test. The attachment of a nucleotide was carried out according to the same procedure as described above for the carbamate linkage.

(c) Succinate linkage. The preparation of a succinate linkage was carried out by the procedure described by Gait.[25]

Dye adsorption on solid support

Four dyes were used for the adsorbtion studies: Pararosaniline (PRA) MW=305.38 and Rose Bengal (RSB) MW=1017.64 were commercially available. 1-(4-butylamino) aminoanthraquinone(1-C4) MW=294 and 1,8-bis(4-butylamino) anthraquinones (1,8-C4) MW=380 were synthesized in our laboratory.[26]

The following stock solutions mg/100ml DMF were prepared: (a) 1-C4, 50mg, (b) 1,8-C4, 30mg, (c) PRA, 50mg and, (d) RSB, 150mg. From each stock soltuion, 300μl were transferred to a 10ml volumetric flask and filled to 10ml with 15% DMF-H_2O solution. The diluted

solutions were used for a calibration curves by plotting their U.V. absorbances vs. their molar concentrations at 280mm, 250nm, 250mm and 250nm for PRA, RSB, 1-C4 and 1,8-C4 respectively. To 100mg of solid support (Fractosil 500, Fractosil 1000, Fractosil 2500 or CPG-500), 5ml of one of the above stock dye solutions was added and agitated for 24h at room temperature. The support was then filtered, washed with 0.5ml ether and the liquid phase was removed by vacuum suction. Thirty mg of the treated support were added to 3ml of DMF and the suspension stirred for 18h. After filtration, 1.5ml of the filtrate was diluted by water to a volume of 10ml and the U.V. absorbance of the solution determined at the apropriate wave length. The amount of dye liberated from the support was calculated by using the calibration curves.

Electron Microscopy

Glass coverslips with a 12mm in diameter were immeresed in Freon 43, sonicated for 5 min and then treated with 0.1% of an aqueous solution of polylysine hydrobromide (Polysciences Inc.) as described by Mazia et al[27]. The coverslips were mounted on stubs. Crystal samples of CPG were lightly dispersed on the coverslips and allowed to dry for 30 min. This leaves crystals adhered to coverslip. Stubs were introduced into a sputter coater apparatus (Polaron ES 100) and gold coating was carried out at 25mA for 1 min. The specimens were examined under aPhilips 505 SEM operated at accelerated voltage of 20kv.

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