PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
C07H 21/00 // C12Q 1/68	A1	WO 90/03382
		(43) International Publication Date: 5 April 1990 (05.04.90)

(21) International Application Number: PCT/GB89/01114

(22) International Filing Date: 21 September 1989 (21.09.89)

(30) Priority data:
8822228.6 21 September 1988 (21.09.88) GB

(71) Applicant *(for all designated States except US):* ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* SOUTHERN, Edwin, M. [GB/GB]; 12 Scholl Road, Kidlington, Oxford OX1 3QU (GB). MASKOS, Uwe [DE/GB]; 25 Wellington Square, Oxford OX1 2JH (GB).

(74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, 5 Quality Court, Chancery Lane, London WC2A 1HZ (GB).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SUPPORT-BOUND OLIGONUCLEOTIDES

(57) Abstract

A method of preparing an oligonucleotide bound to a support, such as porous glass or glass beads, involves attaching a nucleoside 3′-reagent through the phosphate group by a covalent phosphodiester link to aliphatic hydroxyl groups carried by the support. The oligonucleotide is then synthesised on the supported nucleoside, and protecting groups removed from the synthetic oligonucleotide chain under standard conditions which do not cleave the oligonucleotide from the support. Suitable reagents include nucleoside phosphoramidite and nucleoside hydrogen phosphonate.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Fasso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic			SN	Senegal
CG	Congo	KR	Republic of Korea	SU	Soviet Union
CH	Switzerland	LI	Liechtenstein	TD	Chad
CM	Cameroon	LK	Sri Lanka	TG	Togo
DE	Germany, Federal Republic of	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

SUPPORT-BOUND OLIGONUCLEOTIDES

There are several potential applications for oligonucleotides bound to solid supports. They could be used to test for the presence of mutations in complex DNAs - for example for disease loci in Humans. They could be used to select specific nucleic acids from the complex mixtures; for example specific mRNAs from a whole cell population. They will be useful in the invention described in International Application PCT/GB89/00460 filed 2 May 1989.

Several papers describe methods for attaching nucleic acids to solid matrices (1-6). These methods suffer from two problems: they require complex and often inefficient steps; the nucleotide is linked through the bases as well as the ends. Linkage through the bases interferes with subsequent use of the bound polynucleotide in hybridisation reactions.

Methods for synthesising oligonucleotides on solid supports are well established (7-14). The linkage between the oligonucleotide and the support is labile to the the final reagent used to remove blocking groups in the bases, and so this step in the process also removes the oligonucleotide from the solid support. Oligonucleotides would remain tethered to the support if a stable link were used. Sproat and Brown (1985) have shown that a urethane link is more stable than the usual succinate link, however, it requires a complex synthesis, and is not completely stable to the final deprotection step.

Crea and Horn (1980) used a ribonucleotide, linked through the 5'-hydroxyl group to cellulose, to initiate oligonucleotide synthesis. The link between the first and second residues of the resulting chain is labile to the final deprotection step.

Arnold and Berg (1985) describe a polymeric support with a covalently bonded primer for oligonucleotide synthesis, wherein the primer is cleaved by selective oxidation without oxidizing other bonds of the oligonucleotide.

It is an object of this invention to provide a new link which is easy to synthesise and completely stable to standard deprotection steps.

In one aspect the invention provides a method of making a derivatised support suitable for oligonucleotide synthesis, which method comprises attaching a nucleoside reagent to a support carrying hydroxyl groups by a covalent phosphodiester link which is stable to conditions used for removing protective groups from oligonucleotide chains.

In another aspect the invention provides a method of preparing an oligonucleotide bound to a support by

a) attaching a nucleoside reagent to a support,

b) synthesising on the supported nucleoside an oligonucleotide chain including protecting groups, and

c) removing the protecting groups from the oligonucleotide chain,

characterised in that the support carries hydroxyl groups, whereby in step a) the nucleoside becomes attached to the support by a covalent phosphodiester link which is stable to the conditions used in step c).

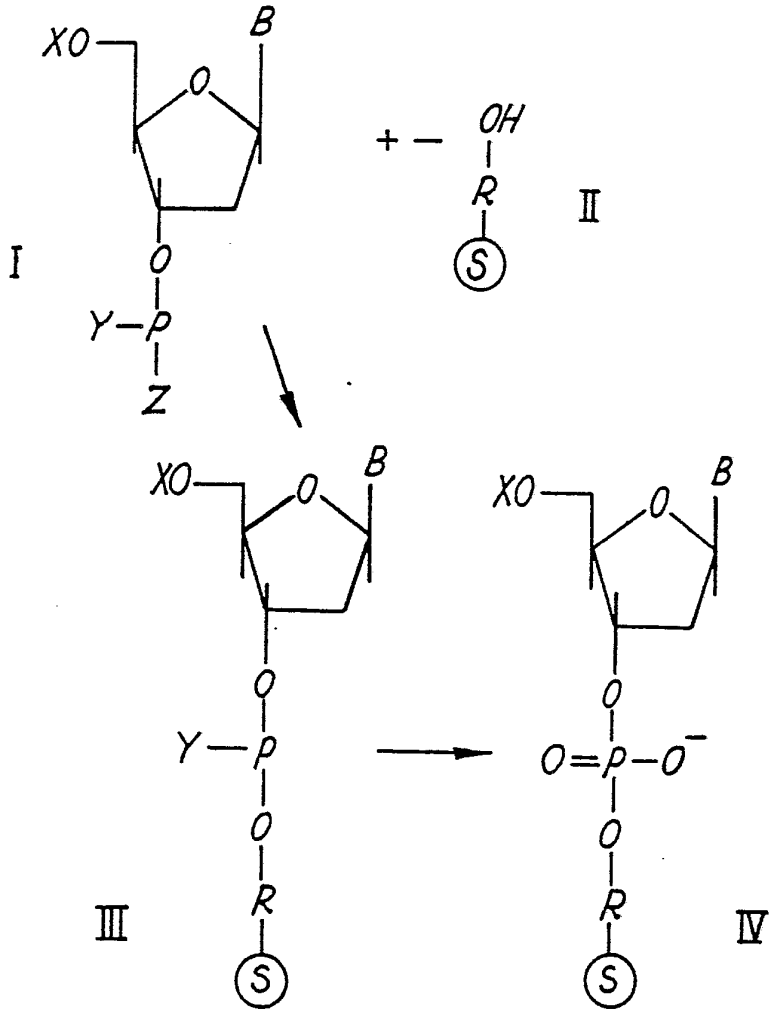
In a further aspect the invention provides a derivatised support suitable for oligonucleotide synthesis comprising a nucleoside linked to a support by means of a covalent phos-phodiester link of the structure -O-PY-O-, where Y is a protected or unprotected oxygen atom.

In yet another aspect the invention provides a support-bound oligonucleotide wherein the oligo-

nucleotide is bound to the support through a terminal phosphate group by a covalent phosphodiester link of the structure $-O-PO_2-O-(C_nH_{2n})-$ where n is ≥ 3.

The nature of the support is not critical to the invention. It may be massive or particulate and may for example be of derivatised silica gel or Kieselguhr-polydimethyl-acrylamide, or controlled-pore glass, or a plain glass surface. What is essential is that it carry aliphatic hydroxyl groups, and these in a form which are accessible for reaction with a nucleoside reagent. These hydroxyl groups may form part of hydroxy alkyl groups, such as may be formed by derivatising controlled-pore glass or other support with a long chain alkyl alcohol. Alternatively, the support may be derivatised with a long chain alkylamine, and the amine groups converted to hydroxyl groups in situ. Alternatively, the hydroxyl groups may be part of a polymeric structure, which either constitutes the solid support or is derivatised onto a solid support.

The nature of the nucleoside reagent is not critical to the invention. Reagents commonly used in oligonucleotide synthesis may be used here. Preferably, the reagent is a phosphoramidite (7 and 8). The reagents and the product formed are indicated in the following reaction scheme.


XO
B
O
OH
R
S
I
II
Y—P
Z
O=P—O⁻
III
IV

In this scheme, I represents the nucleoside 3'-phosphite reagent, II represents the derivatised support, III represents the covalent link initially formed by reaction between the two, and IV represents the final product.

B designates the base appropriate to the nucleoside concerned.

X may be a blocking group, such as a dimethoxytrityl group, whose nature is not critical to the invention; or may (particularly in IV) represent an oligonucleotide chain.

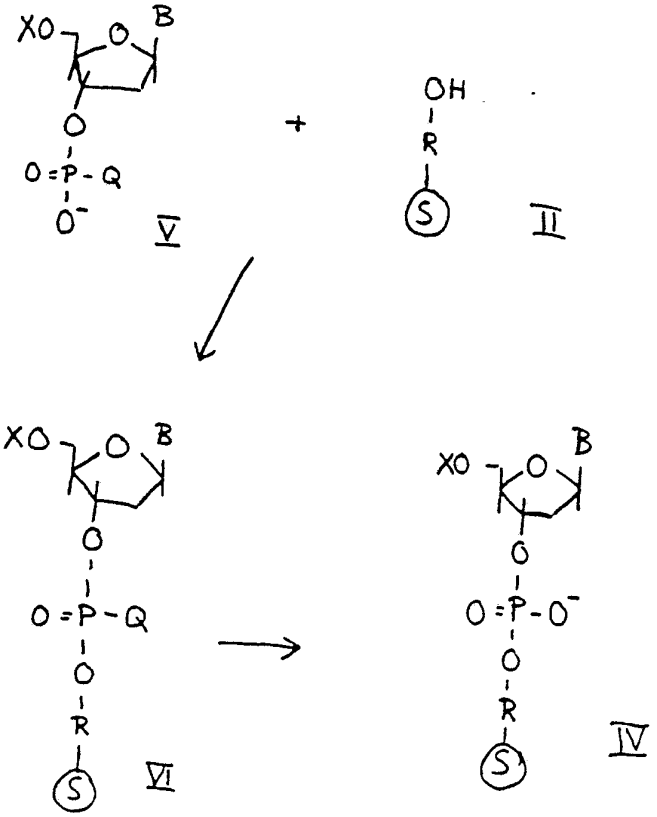
Y is a protected oxygen atom, generally an alkoxy group such a methoxy or beta-cyanoethoxy.

Z may be a di-(C1 to C4 alkyl)amine, or alternatively Cl or tetrazolyl.

R is aliphatic and may be an alkyl group.

S represents the solid support.

Alternatively, the nucleoside 3'- reagent may be a phosphonate or hydrogen phosphonate (12). The reagent and the product formed are indicated in the following reaction scheme.

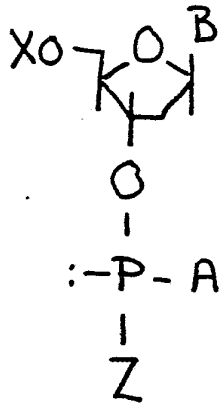


In this scheme, V represents the nucleoside phosphonate or hydrogen phosphonate reagent, II represents the derivatised support, VII represents the covalent link initially formed between the two, and IV represents the final product.

B, X, R and S are as defined above

Q is either hydrogen or an organic group that plays no part in the attachment and synthesis steps of the invention.

Alternatively again, the nucleoside 3'- reagent may be a phosphonamidite having the stucture VII where A is alkyl and B, X and Z are as defined as above. In a preferred example, A is methyl and Z is di-isopropylamine



VII

Automatic oligonucleotide synthesisers are commercially available. In prior techniques, it has been necessary to put into the synthesiser a solid support which has been pre-derivatised with the first nucleotide of the proposed oligonucleotide chain. This invention makes it possible to put into the synthesiser a solid support which carries aliphatic hydroxyl groups (e.g. hydroxyalkyl groups). The nucleoside 3'- reagent is then attached to the support in the synthesiser as the first nucleotide of the proposed oligonucleotide chain.

In the above reaction schemes, conversion of III to IV or of VI to IV involves an oxidation step. This may be affected using e.g. iodine or sulphur under standard conditions, either before or more usually after oligonucleotide synthesis.

Nucleoside 3'- reagents are more developed than are nucleoside 5'- reagents for solid state oligonucleotide synthesis. Nevertheless, nucleoside 5'-reagents are expected to form covalent 5'-phosphodiester links with solid supports carrying aliphatic hydroxyl (e.g. hydroxyalkyl) groups, and to serve as a basis for solid state oligonucleotide synthesis, in exactly the same way as their 3'- counterparts. This invention envisages the use of nucleoside 5'- reagents as alternatives to nucleoside 3'- reagents.

The next step of the method involves synthesising on the supported nucleoside an oligonucleotide chain. Techniques for doing this are well known, and indeed automatic microprocessor-controlled machines are available to do the job. These techniques invariably involve the provision of protective groups to avoid unwanted side reactions, and a final step in the synthesis of any oligonucleotide involves removal of protecting groups. It is this step that has previously resulted in solubilisation of the oligonucleotide. This step may typically involve removal of the methyl group from the phosphotriester groups e.g. by using thiophenoxide; removal of protecting groups from N atoms of the nucleotide bases, e.g. by means of ammonia at elevated temperature; and removal of a protective group from the 5'-position of the last nucleotide to have been added to the oligonucleotide chain, e.g. by means of dichloro acetic acid. The described covalent link between the initial nucleoside and the support is stable to these and other conventional deprotection

steps.

The following examples illustrate the invention.

Example 1

Ballotini glass beads (20g, 90-130 μm diam., Jencons), were suspended in a mixture of xylene (40ml), glycidoxypropyltrimethoxysilane, and a trace of diisopropylethylamine at 90°C overnight with stirring, then washed thoroughly with methanol, ether and air-dried. These derivatised beads (6g) were heated with stirring in hexaethyleneglycol containing a catalytic amount of concentrated sulphuric acid, overnight in an atmosphere of argon, at 80°C, to yield alkyl hydroxyl derivatised beads. After washing with methanol and ether, the beads were dried under vacuum and stored under argon at -20°C.

A small amount of the hydroxyalkyl derivatised beads was put into the reaction vessel of an automatic oligonucleotide synthesiser (Applied Biosystems), programmed to synthesise the sequence of the left cohesive end of bacteriophage lambda. The first nucleotide was a 3'-phosphoramidite (I) in which Y was beta-cyanoethoxy and Z was di-isopropylamine. This became covalently attached to the derivatised beads.

Each step in the synthesis can be monitored by measuring the amounts of trityl group removed, in the spectrophotometer. By this test, the stepwise yield was 96-99%. Thus both yield and purity were high, and we calculate that the 140mg of beads holds more than 4 nmol of the oligonucleotide.

The product was deprotected by the standard treatment with hot ammonia and washed thoroughly with distilled water. It was then used in a hybridisation with the complementary oligonucleotide cosL which had been labelled at the 5' end with 32P.

To 1mg of beads derivatised with the left end of

phage lambda (cosL) was added the complementary oligonucleotide, radioactively labelled (13,000 cpm ^{32}P in 20 µl, 100 mM NaCl, 1mM EDTA). The mixture was incubated for 2 hours at 30°C, the radioactive solution removed and the beads washed thoroughly with ice cold buffer. The oligonucleotide which remained attached to the beads (ca. 3,000 cpm, 23%) could be quantitatively removed by elution with distilled water.

A control "hybridisation" under identical conditions, with a non-complementary oligonucleotide showed 0.02% of non-specific binding to the beads.

These experiments show that the synthesis is straightforward and that the beads can be used successfully in hybridisation tests.

The glass beads are ideal for packing columns to provide an affinity matrix with many desirable properties.

The following Table 1 summarises physical parameters of beads derivatised by the general technique described in Example 1.

TABLE 1

Properties of derivatised ballotini beads

Bead size (microns)	90 - 130	3 - 6
surface area per bead (mm^2)	0.045	0.00011
volume per bead (mm^3)	$9x10^{-4}$	$1.1x10^{-7}$
density (g/cm^3)	2.5	2.5
mass per bead (mg)	0.0023	$2.8x10^{-7}$
number of beads per (mg)	435	$3.5x10^{6}$
surface area per mg (mm^2)	19.5	385
oligonucleotide loading per mg (pmol)	~80	~80
oligonucleotide loading per bead (fmol)	184	0.02
values calculated for radius (microns)	60	3

Example 2

Two glass plates were clamped together with a narrow gap between them. Derivatisation was effected by means of the same reagents, which were injected into the narrow gap, and under the same conditions as Example 1.

Oligonucleotide synthesis was performed by hand under standard conditions using the derivatised glass plate as a solid support. The first nucleotide was a 3'- hydrogen phosphate, used in the form of the triethylammonium salt. The yield and purity of both the first and subsequent steps of the oligonucleotide synthesis were high.

The following Examples demonstrate several ways of hybridizing labelled DNA fragments and oligonucleotides to the derivatised beads.

Example 3

Hybridisation to glass beads attached to sticks.

A plastic stick 2cm long was dipped into molten polypropylene and then brought into contact with a pile of derivatised glass beads and allowed to cool. Approximately 100 - 200 beads adhered to the stick. This method of holding the beads greatly facilitates hybridisation as will be shown in a number of typical experiments:

A stick with approximately 100 glass beads derivatised with the sequence 3' AGG TCG CCG CCC 5' was dipped into 30 ul of a solution containing 0.1 M NaCl and 80 fmol of the complementary oligonucleotide, labelled at the 5' end to an activity of 30,000 cpm.

After 30 min at 30^{o}C the stick was removed from the tube, rinsed and the bound material eluted by dipping the stick into 0.1M NaCl at 50^{o}C. The amount of oligonucleotide hybridised was then determined by scintillation counting.

Typically 4% of the input olignucleotide could be picked up this way; 0.1% were bound nonspecifically and could not be removed. Thus the binding capacity of a single bead is approximately 0.03 fmol oligonucleotide.

Larger proportions of the initial oligonucleotide could be picked up by decreasing the temperature and increasing the length of the hybridisation. Thus in a similar experiment 5.3% was hybridised at 30°C after 55 minutes with 0.05% bound nonspecifically, and 13% after 16 hours at 30°C with 0.2% binding nonspecifically.

As a control, noncomplementary oligonucleotide 5' GGG CGG CGA CCT 3' showed only 0.2% binding after 14 hours.

In summary, experiments with derivatised glass beads attached to plastic sticks have proved to be very easy and shown the high specificity of hybridisation to the beads.

Example 4

Batch hybridisation.

The amount of radioactive material hybridising to the beads could be increased further, and the non-specific binding decreased by carrying out hybridisation with beads typically 1 mg in 0.5 ml centrifuge tubes. After hybridisation the tubes were spun, the supernatant removed and the beads washed. Washing at a temperature higher than T_m resulted in complete melting of the hybrids so that the bound material could be measured by Cerenkov counting. In this way we determined the dependence of rate of hybridisation and elution on salt concentration and temperature as follows:

To each of 5 tubes as added an approximately equal number of beads and complementary oligonucleotide (30,000 cmp) in 50 µl of 0.1M NaCl. Hybridisation was carried out at 30°C overnight to maximise the amount of hybrid. The solution was removed and the beads washed

twice with ice-cold 0.1M NaCl. Elution was for increasingly longer times at a different temperature for each tube. After each interval the supernatant was removed, the beads were washed twice with ice-cold NaCl solution (100 µl 0.1M), and eluted with prewarmed NaCl solution (100 µl 0.1M).

Table 2 details the percentage of bound oligonucleotide eluted in the course of time. There is a clear dependence of elution rate on temperature. For example, three times as much material eluted at 65°C than at 30°C, within 5 minutes. Not suprisingly, even at 30°C which is well below T_m, there is a non-negligible rate.

In another experiment the concentration of input oligonucleotide was varied 50 fold. Hybridisation for 2 hours at 30°C in 0.1M NaCl, 1mM EDTA in 1.5ml centrifuge tubes was followed by removal of the supernatant, three washes with 0.1M NaCl at 0°C and the amount of radioactivity associated with the beads determined by Cerenkov counting. There is an almost linear relationship between concentration and amount of hybrid (i.e. rate of hybridisation, Table 3), which suggests that the hybridisation is a pseudo first order reaction.

The highest concentration of oligonucleotide was 160 fmol (corresponding to 13,000 cmp) in 20 µl. Only 0.03% bound nonspecifically and could not be eluted.

Furthermore, only 0.02% non-complementary oligonucleotide bound to the beads in a similar experiment (12 out of 65,000 cpm), a further indication that this method of isolating DNA fragments is very specific and clean.

TABLE 2

Effect of temperature on elution rate

Elution time	5'	20'	50'	110'	330'	
temperature	% eluted					% remaining
30°C	23	38	54	71	85	0
36°C	34	61	78	90	96	3
44°C	42	54	67	87	97	2
65°C	74	89	96	98	98	1.4

TABLE 3

Effect of oligonucleotide concentration on hybridisation rate

Relative concentration	50	20	10	2	1
% hybridised	23	14	5.5	0.8	0.6
relative rate	38	23	9	1.5	1

Example 5

Isolation of longer DNA fragments:

The isolation of longer DNA fragments was demonstrated in the following experiments:

Total λ DNA (5 µg in 35 µl restriction enzyme buffer) was digested with 15 units of the restriction endonuclease Hinf 1 and the resulting 143 restriction fragments dephosphorylated with calf intestinal phosphatase (1 unit). After one hour at 37°C 4 µl of EGTA was added, the mixture incubated for another 45 minutes at 65°C, phenol extracted and ethanol precipitated.

The mixture was dissolved in 50 µl kinase-labelling buffer (8 µl 10 x PNK buffer, 1 µl 0.1 M DTT, 4 µl PNK enzyme, 30 µl distilled water, 15 µCi γ-^{32}P.ATP), incubated for one hour at 37°C, made up to 100 µl and spun down a Sephadex G25 column to remove non-

incorporated nucleotides.

An aliquot of the ballotini glass beads (100 µm, Jencons) was derivatised with the sequence of the right cohesive end of bacteriophage lamba, viz. 3' - CCC GCC GCT GGA 5', deprotected in ammonia, washed, and a small amount put at the bottom of a U-shaped capillary. This bottom part was kept at 40°C in a controllable temperature block, the upper left and right arms of the capillary were kept at 67°C by jacketing them with a plastic syringe and pumping hot water through it. The hybridisation solution was added (75 µl 0.1M NaCl containing 10 pmol 5' ends, 33 fmol left λ cohesive ends complementary to the oligonucleotide on the beads, total radioactivity ca. 700,000 cpm).

A pump was attached to one arm of the capillary and the hybridising solution cycled back and forth between the parts of the capillary that were kept at 40°C and 67°C respectively. Hybridisation of the left end to the beads would occur at 40°C, and at 67°C the two sticky lambda ends that reannealed in solution would be denatured.

After 4 hours the hybridisation solution was removed, the beads washed extensively, then eluted in hot TE. An aliquot of the solution was loaded onto a 5% polyacrylamide gel. Autoradiography revealed only one band of the correct size in the washes and elution lanes. Altogther 1000 cpm (ca. 1.5 fmol) of the left end were bound by the beads which corresponded to 5% of the theoretical amount.

In summary, this experiment demonstrates the highly specific isolation of a long DNA fragment from a complex mixture.

Example 6

Column Chromatography.

Another easy and convenient way to isolate oligo-

nucleotide and to test the hybridisation behaviour of the novel support is by column chromatography.

A glass capillary (diameter 1.0 mm) was drawn out at one end so as to yield a very narrow pointed opening. This was then plugged by filling in crushed glass particles from the other end and sintering them in the flame of a Bunsen burner so that they adhered to the glass. The inside of the capillary was silanised by passing through a solution of dichlorodimethyl-silane in trichloroethane and washing with ethanol. Approximately 40 mg of the glass beads were layered on the glass frit and the top of the column connected to a syringe that could be driven by an infusion pump. In this way radioactive hybridisation solution and washing solutions could be applied to the column at different rates, typically in the range of 3 - 10 µl/min.

In a typical experiment, 0.2 pmol of labelled oligonucleotide (34,000 cpm) in 1 ml of a solution of 0.1M NaCl, 0.1% SDS in TE pH 7.5 was applied to the column at a rate of 3 µl/min. The jacketed column was kept at 35°C. 90 µl fractions were collected in micro-centrifuge tubes and the amount of radioactivity determined by Cerenkov counting. A 0.1M NaCl washing solution was applied in the same way and collected. Raising the temperature in the jacket allowed us to recover the oligonucleotide.

Thus it was determined that 70% of the oligo-nucleotide bound to the glass beads and could be eluted at a higher temperature with only 0.1% of the material remaining on the support.

A control experiment with non-complementary oligo-nucleotide (mismatch at position 7) showed a remaining 400 cpm out of 80,000 applied after washing at 35°C. At 40°C only 140 cpm = 0.2% remained.

The percentage of accessible oligonucleotide on the

support was determined. 0.13 pmol kinase-labelled and 5 pmol unlabelled oligonucleotide were applied to 40 mg beads. 10% of the material (ca. 500 fmol) hybridised to the support that contained a total of ca.3 nmol oligonucleotide, measured from the detriylation during synthesis.

From this result we calculate that one in 6000 oligonucleotides on the support hybridised under conditions used (35°C, low salt) where negligible binding of mismatched oligonucleotides occurs. The melting was very sharp again, with most of the oligonucleotides eluted in two 90 µl fractions at 48°C.

These experiments suggest that the derivatised beads will be useful in the chromatographic separation of nucleic acids.

REFERENCES

1. Langdale J A and Malcolm A D (1985)
A rapid method of gene detection using DNA bound to Sephacryl. Gene 1985, 36(3), 201-210.

2. Seed, B (1982)
Diazotizable anylamine cellulose paper for the coupling and hybridization of nucleic acids.
Nucl. Acids Res. 10, 1799 - 1810.

3. Allfrey and Inoue (1978)
Affinity chromatography of DNA-binding proteins on DNA covalently attached to solid supports.
Methods Cell Biol. 17, 253 - 270.

4. Banemann, H; Westhoff, P and Herrmann G (1982)
Immobilisation of Denatured DNA to Macroporous Supports: 1 Efficiency of different coupling procedures. Nucl. Acids Res. 10, 7163-7180.

5. Astell C R and Smith M (1972)
Synthesis and Properties of Oligonucleotide-Cellulose Columns.
Biochemistry 11, 4114-4120.

6. Gilham P.T., Biochemistry, 7, No 8, 1968, 2809-13.

7. Matteucci M D and Caruthers M H
J.Am. Chem.Soc. 1981, 103, 3185-3191.

8. Beaucage S L and Caruthers M H
Tetrahedron Letters, Vol 22, No.20, pp 1859-1862, 1981.

9. Adams S P et al, J.Am. Chem.Soc. 1983, 105, 661-663

10. Sproat D S and Brown D M
Nucleic Acids Research, Vol 13, No.8, 1985, 2979-2987.

11. Crea R. and Horn T, Nucleic Acids Research, 8, No 10, 1980, 2331-48.

12. Andrus A. et al., Tetrahedron Letters, Vol 29, No. 8, pp 861-4, 1988.

13. Applied Biosystems User Bulletin, Issue No. 43, Oct 1 1987, "Methyl phosphonamidite reagents and the synthesis and purification of methyl phosphonate analogs of DNA".

14. Miller P.S. et al., Nucleic Acids Research, 11, pages 6225-6242, 1983.

15. Arnold L.J. and Berg R.P., Molecular Biosystems Inc PCT Application WO 85/01051.

CLAIMS

1. A method of making a derivatised support suitable for oligo-nucleotide synthesis, which method comprises attaching a nucleoside reagent to a support carrying hydroxyl groups by a covalent phosphodiester link which is stable to conditions used for removing protective groups from oligonucleotide chains.

2. A method of preparing an oligonucleotide bound to a support by

a) attaching a nucleoside reagent to a support,

b) synthesising on the supported nucleoside an oligo-nucleotide chain including protecting groups, and

c) removing the protecting groups from the oligo-nucleotide chain,

characterised in that the support carries hydroxyl groups, whereby in step a) the nucleoside becomes attached to the support by a covalent phosphodiester link which is stable to the conditions used in step c).

3. A method as claimed in claim 1 or claim 2, wherein the support is of porous glass or glass beads.

4. A method as claimed in any one of claims 1 to 3, wherein the support carries hydroxyalkyl groups.

5. A method as claimed in any one of claims 1 to 4, wherein the nucleoside reagent is a nucleoside 3'-phosphoramidite.

6. A method as claimed in any one of claims 1 to 4, wherein the nucleoside reagent is a nucleoside 3'-phosphite.

7. A method as claimed in any one of claims 1 to 4, wherein the nucleoside reagent is a nucleoside 3'-phosphonate or 3'- hydrogen phosphonate.

8. A method as claimed in any one of claims 1 to 4, wherein the nucleoside reagent is a nucleoside 3'-methylphosphonamidite.

9. A method as claimed in any one of claims 2 to 8,

wherein step c) involves removing protecting groups from phosphotriester groups and from N atoms of the nucleotide bases and from the 5'-position on the last nucleotide of the chain.

10. A derivatised support suitable for oligonucleotide synthesis comprising a nucleoside linked to a support by means of a covalent phosphodiester link of the structure -O-PY-O-, where Y is a protected or unprotected oxygen atom.

11. A derivatised support as claimed in claim 10, wherein the support is of porous glass or glass beads.

12. A support-bound oligonucleotide wherein the oligonucleotide is bound to the support through a terminal phosphate group by a covalent phosphodiester link of the structure $-O-PO_2-O-(C_nH_{2n})-$ where n is > 3.

13. A support-bound oligonucleotide as claimed in claim 12, wherein the support is of porous glass or glass beads.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/01114

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 H 21/00 // C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC5	C 07 H; C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A1, 85/01051 (MOLECULAR BIOSYSTEMS, INC.) 14 March 1985, see page 12, line 7 - line 27; page 16; abstract; figures 1-7	1-13
A	EP, A1, 0261283 (APPLIED BIOSYSTEMS, INC.) 30 March 1988, see the whole document	1-13
A	EP, A1, 0241363 (COMMISSARIAT A L'ENERGIE ATOMIQUE) 14 October 1987, see the whole document	1-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
7th December 1989	22. 02. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	L. ROSSI

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A1, 0090789 (MONSANTO COMPANY) 5 October 1983, see the whole document	1-13
A	DE, A1, 3446635 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 27 June 1985, see the whole document	1-13

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 89/01114

SA 31382

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 08/11/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 85/01051	14/03/85	AU-D-	33196/84	29/03/85
		EP-A-	0155950	02/10/85
		JP-T-	60502155	12/12/85
		AU-A-	575586	04/08/88
EP-A1- 0261283	30/03/88	WO-A-	88/02004	24/03/88
EP-A1- 0241363	14/10/87	FR-A-B-	2596761	09/10/87
		JP-A-	62294692	22/12/87
EP-A1- 0090789	05/10/83	JP-A-	58180500	21/10/83
DE-A1- 3446635	27/06/85	SE-A-	8406484	21/06/85
		FR-A-B-	2556726	21/06/85
		GB-A-B-	2153356	21/08/85
		JP-A-	60197698	07/10/85
		CA-A-	1244786	15/11/88
		US-A-	4849513	18/07/89

EPO FORM P0479