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| (54) Title: RAPID SOLID PHASE SYNTHESIS OF OLIGONUCLEOTIDES USING PHOSPHORUS OXYCHLORIDE ACTIVATION |
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<tr>
<th>(57) Abstract</th>
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A method for the synthesis of oligonucleotides which comprises the steps of packing a flow reactor with supported nucleoside comprised of a first nucleoside attached at its 5'-position of an insoluble solid support, activating said support-nucleoside by treatment with phosphorus oxychloride in the presence of a basic catalyst to provide a 3'-dichlorophosphoryl group on said first nucleoside, coupling a second similar or dissimilar nucleoside, unprotected at the 3'-and 5'-position, to said first nucleoside by condensation with said dichlorophosphoryl group and repeating said activation and coupling step until the desired oligonucleotide sequence is obtained. The oligonucleotide may then be recovered by cleavage from the support and purified, if required.
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RAPID SOLID PHASE SYNTHESIS OF
OLIGONUCLEOTIDES USING PHOSPHORUS
OXYCHLORIDE ACTIVATION

Technical Field

This invention relates to the synthesis of oligonucleotides. More particularly, the invention is
directed to the solid-phase synthesis of oligonucleotides using phosphorous oxychloride activation.

Background Art

Ribo- and deoxyribo oligonucleotides are of extreme
interest and importance currently because of the
explosive development of the recombinant DNA field, for
which "libraries" of codon and other oligonucleotide
sequences are required. The synthesis of these mole-
cules is also of interest simply because of the great
challenge they present to the synthetic organic chemist.
However, despite the fact that the synthesis of several
small nucleic acids has now been achieved, implying
that the chemical methodology has been developed fully,
procedures are still extremely tedious and rarely pro-
duce good yields of oligonucleotides. In fact, the
recent synthesis of the yeast alanine t-RNA gene by
Khorana and co-workers required 20 man-years! Even the
application of the solid-phase approach has failed to
result in generally applicable methods which result in
the rapid synthesis of high yields of oligonucleotides.
This can, for the most part, be traced to the complex-
ity of the chemistry of nucleosides and nucleotides.
Internucleotide bond formation involves the phosphory-
lation of sugar hydroxyl groups which have low reactiv-
ities, especially the secondary alcohol at the 3'-
position. Coupling yields are, therefore, invariably
relatively low (rarely more than 80-90%), resulting in
very low recoveries of oligonucleotides. For example,
the maximum theoretical yield of a pentanucleotide is only 32\% if the coupling yield is only 80\% at each coupling. However, because of the possibility of numerous side reactions which can occur on deprotection or because of the sensitivity of glycosidic linkages or the heterocyclic base in the molecule, yields are often reduced even further. Syntheses, therefore, result in complicated mixtures which contain "deletion" and truncated sequences which are invariably difficult and tedious to separate.

Because of these problems, a plethora of protecting groups has been developed for temporarily blocking functional groups during the formation of the internucleotide phosphodiester bond. A number of synthetic approaches have also emerged. The first of these, the so-called phosphodiester method, originally developed by Khorana and co-workers, utilizes a 3'-protected nucleoside 5'-phosphate (II) which is activated (for example with dicyclohexylcarbodiimide) and coupled with a 5'-protected nucleoside (I)

\[
\text{RO-}H_2C_6\text{O}_{\text{Base}} + \text{HO-P-OCH}_2\text{O}_{\text{Base'}}
\]

(I) \hspace{5cm} (II)
DCC = dicyclohexylcarbodiimide
R and R' = protecting groups

The protecting groups (R and R') must be stable to the synthetic conditions but must be selectively removable in order to extend the oligonucleotide chain. In the ribooligonucleotide series, the added problems of 2'-3' phosphate interchange and consequent need for protection exist. This scheme is also further complicated by the possibility of side reactions associated with the reactivity of the phosphate oxygen atom [asterisked in (III)] which remains unprotected throughout the synthesis. For this reason, the so-called "phosphotriester approach" was developed by Letsinger in which this phosphate oxygen atom is also blocked by a suitable protecting group throughout the synthesis.

In an even more recent approach, the so-called "phosphite method", also developed by Letsinger, nucleosides are activated as their phosphites (IV) (i.e. phosphorus in oxidation state +3 rather than phosphorus in oxidation state +5 as in phosphates) and the phosphite oxidized to the phosphate after the coupling reaction, i.e.:
Although the above description refers to prior art syntheses of 2'-deoxyribooligonucleotides, through appropriate protection of the 2'-hydroxyl group, the same approaches have been applied to the synthesis of ribooligonucleotides.

The approaches described above are, for the most part, effective in producing low to moderate yields of oligonucleotides but all require the synthesis of protected nucleoside derivatives which can be both time-consuming and expensive. There is, therefore, a need for a reproducible method which rapidly leads to the highest possible yields of oligonucleotides. Furthermore, the method should, if possible, use relatively inexpensive, unprotected nucleosides as starting materials.
Disclosure of the Invention

Accordingly, it is an object of the invention to provide a reproducible method which rapidly provides high yields of oligonucleotides.

Another object of the invention is a method for the preparation of oligonucleotides that enables the use of unprotected nucleosides in the synthesis.

Yet another object of the invention is to provide an inexpensive method for synthesizing oligonucleotides which avoids the need for deprotection steps and the possibility of side reactions so that yields are substantially improved.

A further object of the invention is to provide a solid-phase method for the facile, large scale production of oligonucleotides.

These requirements are satisfied by a novel, solid-phase synthesis that is based on the use of phosphorus oxychloride for activation and unprotected nucleosides as the "building blocks" for oligonucleotide synthesis.

In accordance with the present invention oligonucleotides are synthesized by a method comprising the steps of activating a support-nucleoside comprised of a first nucleoside attached at its 5'-position to an insoluble solid support by treatment with phosphorus oxychloride in the presence of a basic catalyst to provide a 3'-dichlorophosphoryl group on said first nucleoside, coupling a second similar or dissimilar nucleoside, unprotected at the 3'- and 5'-position, to said first nucleoside by condensation of said dichlorophosphoryl group with said second nucleoside at the 5'-position of said second nucleoside, and repeating said activation and coupling steps until the desired oligonucleotide sequence is obtained.
The oligonucleotide sequence or chain may then be recovered by cleaving the linkage between the support and oligonucleotide formed. The oligonucleotide is then purified, if required.

The synthesis of the invention is advantageously carried out in a flow reactor. In a preferred aspect of the invention, a pressurized flow reactor is used and all phases of the synthesis are preferably carried out under a pressure of at least atmospheric plus 25 psi or 0.0003625 dyne/cm² (i.e. at least 40 psi or 0.00058 dyne/cm²). Generally the pressures fall in the range of at least 100 psi or 0.00145 dyne/cm² up to 1000 psi or 0.0145 dyne/cm², although pressures up to 10,000 psi or 0.145 dyne/cm² or more may be used. This reactor maximizes the efficiency of all of the operations in the synthetic cycle through the application of the mass action effect, which forces reactions to completion, thereby maximizing both rates and yields in coupling reactions. The flow reactor also minimizes side reactions through efficient removal of excess reagents and also by-products of reactions. Scale-up of syntheses is also more facile than in conventional, shaken reactors.

In an embodiment of the invention, the support-nucleoside is formed in the flow reactor as the initial step of the synthesis. According to this embodiment of the invention, the flow reactor is packed with an insoluble solid support containing substituent groups reactive with a nucleoside to form a stable linkage with said nucleoside, passing in continuous flow a first nucleoside through said packed reactor to couple said first nucleoside to said support by said linkage at the 5'-position of said nucleoside and thereby form a support-nucleoside, activating said support-nucleoside by passing phosphorus oxychloride in the presence of a
basic catalyst through said reactor to provide a 3'-
dichlorophosphoryl group on said first nucleoside,
coupling a second similar or dissimilar nucleoside,
unprotected at the 3' or 5' position, to said nucleoside
by condensation of said dichlorophosphoryl group with
said second nucleoside at the 5'-position of said
second nucleoside, and repeating said activation and
coupling steps until the desired oligonucleotide
sequence is obtained.

Modes for Carrying Out the Invention

In the synthesis of the present invention, an
insoluble solid support or matrix, advantageously in
bead form, such as any of the conventional solid-phase
polymeric substrates conventionally employed for the
synthesis of polynucleotides or polypeptides can be
utilized. Typical of such polymeric resins are cross-
linked polystyrene resins, polyacrylamide derivatives,
silica gel, porous glass, clays, celite, crosslinked
dextran, and similar insoluble solid supports which
either naturally contain reactive sites for coupling
with nucleosides by condensation with the 5'-positioned
hydroxy groups of the nucleosides or which can be pro-
vided with such reactive sites. In practise, rigid,
non-swollen resins are preferred such as those of the
macroporous type or silica-based.

Typical of the reactive sites either naturally
present or provided the solid support are groups con-
densible with nucleosides such as carboxyl groups,
alkyl, aralkyl or aryl halide groups, alcohol groups,
amine groups and the like. The preferred reactive
sites are carboxyl groups. A variety of methods for
deriving these carboxyl group-containing insoluble
solid supports (generally identified below as \( \mathbb{P} \))
are known in the art and frequently involve the
inclusion of spacer groups to extend the distance of the carboxyl group from the support or polymer backbone for the purpose of facilitating the coupling reaction to the nucleoside. For example, polystyrene (VI) may be derivatized readily to first form an aminomethylated derivative (VII), i.e.

\[
\begin{align*}
\text{P} & \text{-} \text{HOCH}_2\text{-}R \\
& \xrightarrow{1. \text{CF}_3\text{SO}_3\text{H}} \\
& \xrightarrow{2. \text{H}_2\text{NNH}_2} \\
\text{P} & \text{-} \text{CH}_2\text{NH}_2 \\
& \text{R=NH} \\
\text{O}
\end{align*}
\]

This resin (VII) may then be further derivatized with spacer groups terminated in carboxyl groups, i.e.

\[
\begin{align*}
\text{VII} & + \text{HO}_2\text{C} \xrightarrow{1. \text{DCC}} \text{CH}_2\text{CO}_2\text{Me} \\
& \xrightarrow{2. \text{OH}} \\
\text{P} & \text{-} \text{CH}_2\text{NH} \text{-CO-} \text{(CH}_2\text{H}_2\text{CO}_2\text{H}} \\
\text{O}
\end{align*}
\]

Alternatively, polystyrene may be derivatized directly to a carboxyl-containing support (XI) by Friedel-Crafts acylation using activated dicarboxylic acids such as succinic anhydride:

\[
\begin{align*}
\text{P} & \text{-} \text{AlCl}_3 \\
& \xrightarrow{\text{P}} \text{CO} \text{-CH}_2 \text{CO}_2\text{H} \\
\text{O}
\end{align*}
\]

Spacer groups may be added to this resin through use of \(\omega\)-amino acids, i.e.

\[
\begin{align*}
\text{IX} & + \text{H}_2\text{N-} \text{(CH}_2\text{H}_2\text{n CO}_2\text{Me}} \\
& \xrightarrow{1. \text{DCC}} \\
& \xrightarrow{2. \text{OH}} \\
\text{P} & \text{-} \text{CO-} \text{(CH}_2\text{H}_2\text{n CO-NH-} \text{CH}_2\text{H}_2\text{n CO}_2\text{H}} \\
\text{O}
\end{align*}
\]
In the case of macroporous polyacrylic acid, the resin may be further derivatized to the polyacrylamide derivative in order to remove the carboxyl groups from proximity to the polymer backbone, through use of \( \omega \)-amino acids, i.e.

\[
\begin{align*}
\text{P} - \text{CO}_2\text{H} & \xrightarrow{\text{SOCl}_2} \text{P} - \text{COCl} & 1. \text{H}_2\text{N} - \text{CH}_2\text{CH}_2\text{CO}_2\text{Me} \\
& \quad \xrightarrow{2. \text{OH}^-} \text{P} - \text{CO-NH-} (\text{CH}_2)_n \text{CO}_2\text{H} \\
\end{align*}
\]

(X)

Supports of the silica-type (silica gel, porous gel, porous glass, etc.) are first derivatized by treatment with triethoxyaminopropyl silane to provide amine-functionalized supports (XI), which may be transformed to carboxyl-containing support (XII) by similar methods to those described above for polystyrene, i.e.

\[
\begin{align*}
\text{Si} - \text{OH} + (\text{EtO})_3\text{Si} - \text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2 & \rightarrow \text{Si} - \text{O-Si} - \text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2 \\
\text{(XI)} & \\
& \quad \xrightarrow{1. \text{HO}_2\text{C} - \text{CH}_2\text{CH}_2\text{CO}_2\text{Me/DCC}} \\
& \quad \xrightarrow{2. \text{OH}^-} \\
\text{Si} - \text{O-Si} - \text{CH}_2\text{CH}_2\text{NH-CO-} \text{CH}_2\text{CH}_2\text{CO}_2\text{H} & \\
\text{(XII)} & \\
\end{align*}
\]

Using these or similar supports containing carboxyl groups, the first nucleoside in the desired sequence is attached to the support via an ester linkage to the 5'-hydroxyl group, i.e.

\[
\begin{align*}
\text{P} - \text{CO}_2\text{H} & \xrightarrow{\text{DCC/DMAP} \text{ Nucleoside}} \text{P} - \text{CO}_2\text{CH}_2\text{Base} \\
\text{DMAP} = 4\text{-dimethylaminopyridine} & \\
\text{MTr} = \text{Monomethoxytrityl} & \\
\text{OR (XIII) R=H, MTr} & \\
\end{align*}
\]
It is probably not necessary to protect the 3'-hydroxyl function in the nucleoside because of the much greater reactivity of the 5'-hydroxyl group. However, if the 3'-hydroxyl is temporarily protected as the monomethoxytrityl derivative, cleavage of this group under mild, acidic conditions (e.g. 1% to 5% trifluoroacetic acid in benzene) permits rapid quantitation of the extent of incorporation of the nucleoside on the support by spectrophotometric techniques. In the case of adenine, quanine and cytosine-containing nucleosides, "minimal protection" of amine functions on the bases improves yields through elimination of the possibility of amine phosphorylation.

The resin-nucleoside (XIII) (R = H) is packed into the column reactor and activated by treatment with phosphorus oxychloride in the presence of a basic catalyst, i.e.

\[
\begin{array}{c}
\text{POCl}_3/\text{base} \\
\text{TMP}
\end{array}
\xrightarrow{(XII)}
\begin{array}{c}
\begin{array}{c}
\text{CO}_2\text{-CH}_2
\end{array}
\end{array}
\begin{array}{c}
\text{Base}
\end{array}
\begin{array}{c}
\begin{array}{c}
\text{Cl}
\end{array}
\end{array}
\begin{array}{c}
\begin{array}{c}
\text{P}
\end{array}
\end{array}
\begin{array}{c}
\begin{array}{c}
\text{Cl}
\end{array}
\end{array}
\begin{array}{c}
\begin{array}{c}
\text{O}
\end{array}
\end{array}
\end{array}
\begin{array}{c}
\text{TMP} = \text{trimethyl phosphate}
\end{array}
\begin{array}{c}
\text{(XIV)}
\end{array}
\]

The phosphorus oxychloride and tertiary base are advantageously made up in the form of an activating solution employing a suitable solvent for both the base and POCl\(_3\). The preferred solvent for this reaction is trimethyl phosphate and the preferred basic catalyst is imidazole and its derivatives, such as 1-methylimidazole, a tertiary base such as diethylaniline, and the like, which have been found to promote the most rapid phosphorylation.

Coupling with a second similar or dissimilar nucleoside is effected by passing a solution of the nucleoside, a tertiary base and catalytic amounts of water in trimethyl
phosphate through the column, i.e.

\[ \text{Nucleoside/base/H}_2\text{O} \xrightarrow{\text{TMP}} \]

Once again, unprotected nucleosides may be used because of the vast differences in reactivity between the 5' - and 3' -hydroxyl groups. (Additional specificity may be provided by the addition of trace amounts of water to the reaction mixture). Couplings are rapid (typically 5-30 minutes for the phosphorylation step and 10-60 minutes for the phosphodiester formation step) and yields are also high (up to 90-95% per coupling). The synthesis is continued by repeating the phosphorylation and coupling steps until the desired oligonucleotide sequence is obtained. The product is finally cleaved from the polymeric support by any of the standard methods used in the art. The method selected in any given case will be dependant principally on the particular linkage being cleaved. For instance, in the case of an ester linkage, cleavage is obtained by hydrolysis. Hydrolysis can be accomplished by one of several techniques, including saponification with alkali, treatment with ammonia or hydrazine, etc. Purification of the final product, if required, may be carried out by standard, ion exchange techniques using, e.g. DEAE-Sephadex, QAE-Sephadex, etc.

Although the above description refers to the synthesis of 2'-deoxyribonucleotides, through appropriate choice of protecting groups for the 2'-hydroxyl group well
known to one skilled in the art, the same method can be employed for the synthesis of ribooligonucleotides.

The nucleosides useful in the production of oligonucleotides include any of the 3'- and 5'-hydroxyl group containing nucleosides. Illustrative of these complex compounds are thymidine, uridine, deoxyuridine, cytidine, deoxycytidine, adenosine, deoxyadenosine, guanosine, deoxyguanosine and the like.

As aforementioned, in the preferred embodiment of the invention, rapid, large scale production of oligonucleotides can be obtained by conducting the entire series of coupling reactions in a pressurized flow reactor. Elevated reactor pressures can be generated by use of commercial pressurizing equipment and methods.

For example, any of the commercially available reciprocating pumps capable of generating the required pressures can be used and the reactants and reagents pumped directly into and through the reactor.

Alternatively, the reactants and reagents may be pumped through the reactor by means of pressurization with an inert gas such as nitrogen and the pressure in the reactor regulated by controlling the volume of inert gas released to transfer the reactants and reagents into and through the reactor.

A simple method of delivering reactants and reagents under the high pressure required for this method involves the use of a conventional high pressure liquid chromatography apparatus. The columns of such a system can serve as reactors for the synthesis and the high performance pump generally found in such an apparatus may be easily adapted to pump reagents and reactants through the column reactor. However, such an apparatus is limited to small scale synthesis since the maximum possible flow rates in such a system are too low (generally less than 10 ml per minute) for larger scale synthesis.
The following examples are included to further illustrate the present invention. In all of the examples, the nitrogen-pressurized flow system described in Example 2 of United States Patent No. 4,192,798, incorporated herein by reference, was employed.

**Example 1: Preparation of Adipoyl-aminomethyl Polystyrene**

To a stirred suspension of macroporous polystyrene (Amberlite XAD-2) in 200 ml of 1:1 trifluoroacetic acid: methylene chloride is added 0.45 ml (5 mmol) hydroxy-methylphthalamide. After 5 hrs the resin is filtered and washed with 1:1 trifluoroacetic acid: methylene chloride, methylene chloride and ethanol. The resin is suspended in 250 ml of ethanol containing 12 ml hydrazine and refluxed for 24 hr. The resin is filtered and washed with hot ethanol. The resin is dried in vacuum, then resuspended in 100 ml of dimethylacetamide. Adipic acid monomethyl ester, 10 mmol (1.6 g) is added, and the suspension cooled to 0°C. A solution of dicyclohexylcarbodiimide, 10 mmol (2.1 g), in 10 ml dimethylacetamide is added and after 1 hr at 0-5°C, the suspension is shaken for 24 hr at room temperature. Benzoyl chloride, 1 ml, and pyridine, 4 ml, are then added to block any unreacted amino groups. After 4 hr, the resin is filtered and washed with dimethylacetamide, methanol and methylene chloride. The resin is dried, then saponified by shaking with 100 ml of 1:1 N sodium hydroxide-dioxane for 24 hr at room temperature. The resin is filtered and washed with N hydrochloric acid, water, and methanol. After drying a sample of the resin is titrated and shown to have 0.3 mmol/g of free carboxyl groups.

**Example 2: Preparation of Poly (acrylamidoundecarboxylic acid)**

To a mechanically stirred suspension of 10 g dried macroporous polyacrylic acid (Bio-Rex 70-H⁻) (10 mmol/g of carboxyl groups) in 100 ml methylene chloride is added
150 mmol (31 g) of phosphorous pentachloride. After shaking 6 hrs at room temperature, the resin is filtered and washed with methylene chloride. After drying in vacuum, the resin is resuspended in tetrahydrofuran, (100 ml). Triethylamine, 2.8 ml (20 mmol) is added followed by aminoundecanoic acid methyl ester hydrochloride, 2.5 g (10 mmol). After 24 hrs at room temperature, 20 ml (200 mmol) n-butylamine is added to block unreacted acid chloride functions. After 6 hrs, the resin is filtered and washed with tetrahydrofuran, methanol and water. The resin is saponified for 24 hrs with Na hydroxide-dioxane as in Example 1. Titration of the resultant poly (acrylamidoundecanoic acid) resin shows 1-2 mmol/g of carboxyl groups.

Example 3: Attachment of thymidine to Adipoyl-aminomethyl Resin

To a suspension of 1 g adipoyl-aminomethyl polystyrene (carboxyl substitution 0.3 mmol/g) in 10 ml dimethylacetamide, 0.51 g (1 mmol) of 3'-methoxytritylthymidine and 0.12 g (1 mmol) of dimethylaminopyridine is added. The suspension is maintained at 0-5°C for 1 hr, then shaken at room temperature for 24 hrs. The suspension is recooled to 0°C and an additional 0.2 g of dicyclohexylcarbodiimide plus 0.1 ml (1 mmol) nbutylamine are added. After 24 hrs of shaking, the resin is filtered and washed with dimethylacetamide, methanol and methylene chloride. The dry 3'-methoxytrityl-5'-thymidyl-adipoylaminomethyl resin is weighed, then treated with 50 ml of 5% trifluoroacetic acid in benzene for 30 min at room temperature. The resin suspension is filtered and washed with 5% trifluoroacetic acid in benzene until the wash is nearly colorless. The filtrate is taken up to a known volume and an aliquot
diluted for visible spectrum. The substitution is calculated based on the known extinction of the peak at 480 nm for methoxytritylcarbonium ion. The 5'-thymidyl-adipopolyaminomethyl polystyrene resin is washed with benzene, methanol and methylene chloride, then dried in vacuum.

Example 4: Preparation of Thymidylyl (3'-5') thymidine on Adipoyl-aminomethyl Polystyrene

5'-Thymidyl-adipoylaminomethyl polystyrene (0.5 g) (substitution 0.1 mmol/g) is packed in a stainless steel column with dioxane wash at 1000 psi or 0.0145 dyne/cm². The resin is washed with 100 ml trimethylphosphate, then a freshly prepared solution of 100 ml 0.5M phosphorous oxychloride - 0.5M imidazole in trimethylphosphate passed through the column for 20 min at room temperature. The column is washed with trimethylphosphosphate until a negative chloride test with silver nitrate is obtained for the effluent (∼100 ml required). A solution of 100 ml 0.1 M thymidine - 0.1 M imidazole in trimethylphosphate is then passed through the column over 60 min at room temperature. The column is finally washed with trimethylphosphosphate until the effluent is UV-transparent. The column is unpacked and the resin washed on a funnel with water, then resuspended in 20 ml of N sodium hydroxide. The suspension is shaken for 24 hrs at room temperature. The resin is filtered and washed with several portions of water. The total filtrate is neutralized with Dowex 50 pyridinium, 20 cc. The suspension is filtered and the filtrate evaporated to dryness. The residue is redissolved in water and applied to a column of QAE-Sephadex bicarbonate. The column is eluted with a gradient of ammonium bicarbonate. Effluent is monitored at 26.7 nm. The peak for dinucleotide was collected and lyophilized to give 50-80% yield of product. Purity is
confirmed by HPLC on a Whatmann 10/25 SAX anion exchange column or a Unimetrics Lichrosorb RP-18 column.

Example 5: Preparation of thymidylyl (3'-5')-thymidylyl-thymidine on Adipoylaminomethyl Polystyrene Resin

The 5'-thymidylyl-(3'-5')-thymidine-adipoylaminomethyl resin of Example 4 is submitted to one more cycle of 3'-phosphorylation followed by coupling to free thymidine. The oligonucleotide is then cleaved with sodium hydroxide and purified as in Example 4.

Example 6: Attachment of N-benzoyldeoxycytidine to Poly (acyloylaminoundecanoic acid) Resin

3'-Methoxytrityl-N-benzoyldeoxycytidine is attached to poly (acyloylaminoundecanoic acid) resin and the resultant resin detritylated by the procedure given in Example 3.

Example 7: Preparation of deoxycytidylyl-(3'-5')-thymidylyl-(3'-5')-deoxycytidine on Poly (acyloylaminoundecanoic acid)

5'-deoxycytidylyl-poly (acyloylaminoundecanoic acid) resin from Example 6 is converted to 3'-dichlorophosphoryl resin and subsequently coupled to free thymidine as in Example 4. The dinucleotide resin is submitted to a further cycle of phosphorylation followed by coupling to free N-benzoyldeoxycytidine. The oligonucleotide is cleaved from the resin and simultaneously deprotected of N-benzoyl groups by treatment with N NaOH for 24 hrs. Purification is accomplished on QAE-Sphendex bicarbonate resin as for Example 4.
IT IS CLAIMED:

1. A method for the synthesis of oligonucleotides which comprises the steps of activating a support-nucleoside comprised of a first nucleoside attached at its 5'-position to an insoluble solid support by treatment with phosphorus oxychloride in the presence of a basic catalyst to provide a 3'-dichlorophosphoryl group on said first nucleoside, coupling a second similar or dissimilar nucleoside, unprotected at the 3'- and 5'-position, to said first nucleoside by condensation of said dichlorophosphoryl group with said second nucleoside at the 5'-position of said second nucleoside, and repeating said activation and coupling steps until the desired oligonucleotide sequence is obtained.

2. A method according to Claim 1 wherein the oligonucleotide sequence is recovered by cleaving it from said support.

3. A method according to Claim 1 wherein the synthesis is conducted in a flow reactor.

4. A method according to Claim 3 wherein the flow reactor is pressurized.

5. A method according to Claim 4 wherein the pressure in the reactor is at least 40 psi or 0.00058 dyne/cm².

6. A method according to Claim 1 wherein the first nucleoside is attached at the 5'-position at the insoluble support via an ester linkage.

7. A method according to Claim 1 wherein the support is crosslinked polystyrene containing reactive groups condensable with a nucleoside.

8. A method according to Claim 1 wherein the support is a crosslinked polyacrylamide containing reactive condensable with a nucleoside.
9. A method according to Claim 1 wherein the support is a silica based support containing reactive groups condensable with a nucleoside.

10. A method for the synthesis of oligonucleotides which comprises the steps of packing a flow reactor with an insoluble solid support containing substituent groups reactive with a nucleoside to form a stable linkage with said nucleoside, passing in continuous flow a first nucleoside through said packed reactor to couple said first nucleoside to said support by said linkage at the 5'-position of said nucleoside and thereby form a support-nucleoside, activating said support-nucleoside by passing phosphorus oxychloride in the presence of a basic catalyst through said reactor to provide a 3'-dichlorophosphoryl group on said first nucleoside, coupling a second similar or dissimilar nucleoside, unprotected at the 3'-' and 5'-position, to said first nucleoside by condensation of said dichlorophosphoryl group with said second nucleoside at the 5'-position of said nucleoside, and repeating said activation and coupling step until the desired oligonucleotide sequence is obtained.

11. A method according to Claim 10 wherein the oligonucleotide sequence is recovered by cleaving it from said support.

12. A method according to Claim 10 wherein the flow reactor is pressurized.

13. A method according to Claim 12 wherein the pressure in the reactor is at least 40 psi or 0.00058 dyne/cm².

14. A method according to Claim 10 wherein the first nucleoside is attached at the 5'-position to the insoluble support via an ester linkage.
15. A method according to Claim 10 wherein the support is crosslinked polystyrene containing reactive groups condensible with a nucleoside.

16. A method according to Claim 10 wherein the support is a crosslinked polyacrylamide containing reactive groups condensible with a nucleoside.

17. A method according to Claim 10 wherein the support is a silica based support containing reactive groups condensible with a nucleoside.

18. A method according to Claim 14 wherein the oligonucleotide is recovered by hydrolyzing the ester linkage between the support and oligonucleotide sequence to cleave said sequence from the support.
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)

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

Int. Cl. C07H 21/02, 21/04
US. Cl. 536/27,28,29

II. FIELDS SEARCHED

Minimum Documentation Searched

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<th>Classification System</th>
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<td>US.</td>
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Documentation Searched other than Minimum Documentation to the extent that upon Documents are Included in the Fields Searched

Chemical Abstracts...Oligonucleotides
Volumes 66 to 95.

III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No.</th>
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<tbody>
<tr>
<td>X</td>
<td>US, A, 3,466,273, Published 09 September 1969, Sowa et al.</td>
<td>1-18</td>
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<tr>
<td>X</td>
<td>US, A, 3,407,190, Published 22 October 1968, Honjo et al.</td>
<td>1-18</td>
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<tr>
<td>X</td>
<td>US, A, 3,288,780, Published 29 November 1966, Tsuchiya et al.</td>
<td>1-18</td>
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  T: later document published on or 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

IV. CERTIFICATION

Date of the Actual Completion of the International Search 10 June 1982
Date of Mailing of this International Search Report 17 JUN 1982

International Searching Authority ISA/US
Signature of Authorized Officer

Form PCT/ISA/210 (second sheet) (October 1977)