

COMMONWEALTH OF AUSTRALIA
PATENTS ACT 1952

DECLARATION IN SUPPORT OF A
CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a patent for an invention entitled: "DEFINED SEQUENCE SINGLE STRAND OLIGONUCLEOTIDES INCORPORATING REPORTER GROUPS, PROCESS FOR THE CHEMICAL SYNTHESIS THEREOF, AND NUCLEOSIDES USEFUL IN SUCH SYNTHESIS"

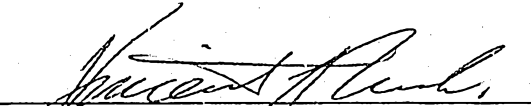
I Vincent A. Frank, Executive Vice President,
Molecular Biosystems, Inc., a Delaware corporation,
of 11180 Roselle Street, Suite A
San Diego, California 92121
United States of America

do solemnly and sincerely declare as follows:

1. I am authorised by MOLECULAR BIOSYSTEMS, INC. the applicant for the patent to make this declaration on its behalf.
2. The basic applications as defined by Section 141 of the Act were made in United States of America on 22 February, 1983, by JERRY L. RUTH and an International Application in the United States of America on 22 February, 1983 by MOLECULAR BIOSYSTEMS, INC.
3. JERRY L. RUTH of 2433 Levante Street, Carlsbad, California 92008, United States of America is the actual inventor of the invention and the facts upon which the applicant is entitled to make the application are as follows:
The said applicant is the assignee of the actual inventor

4. The basic applications referred to in paragraph 2 of this Declaration were the first applications made in a Convention country in respect of the invention(s) the subject of the application

DECLARED at San Diego this 19th day of November, 1984.



Vincent A. Frank

TO: COMMISSIONER OF PATENTS
AUSTRALIA

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(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 596068

(54) Title
DEFINED SEQUENCE SINGLE STRAND OLIGONUCLEOTIDES INCORPORATING
REPORTER GROUPS, PROCESS FOR THE CHEMICAL SYNTHESIS THEREOF, AND
NUCLEOSIDES USEFUL IN SUCH SYNTHESIS

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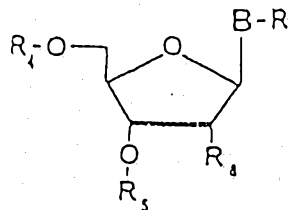
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JERRY L. RUTH

(74) Attorney or Agent
SPRUSON & FERGUSON

(57) Claim

1. A substantially pure single-stranded oligonucleotide comprising a preselected sequence of not more than approximately 200 nucleotides wherein at least one of said nucleotides is at a pre-selected position in said sequence and includes a base having a covalently attached linker arm attached to the base at a sterically tolerant site and having two or more carbon atoms if attached to a pyrimidine and one or more carbon atoms if attached to a purine, said linker arm containing or capable of binding at least one reporter group or a solid support.

8. A reactive nucleotide monomer useful as an intermediate in the chemical synthesis of a substantially pure single-stranded oligonucleotide comprising the structure:



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wherein B is a pyrimidine or purine base;

R is a linker arm attached to B at a sterically tolerant site and containing or capable of binding at least one reporter group or a solid support wherein said linker arm has two or more carbon atoms if attached to a pyrimidine and one or more carbon atoms if attached to a purine;

and provided that

when R_4 is a masking group then

R_5 is a reactive phosphorus-containing group or H; and

when R_5 is a masking group then

R_4 is a reactive phosphorus-containing group or H; and

R_8 is a H or masked hydroxyl group.

PCT

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US84/00279 (22) International Filing Date: 22 February 1984 (22.02.84) (31) Priority Application Numbers: PCT/US83/00254 468,498 (32) Priority Dates: 22 February 1983 (22.02.83) 22 February 1983 (22.02.83) (33) Priority Countries: AU US (60) Parent Application or Grant (63) Related by Continuation US 468,498 (CIP) Filed on 22 February 1983 (22.02.83) (71) Applicant (for all designated States except US): MO- LECULAR BIOSYSTEMS, INC. [US/US]; 11180 Ro- selle Street, Suite A, San Diego, CA 92121 (US).		(72) Inventor; and (75) Inventor/Applicant (for US only) : RUTH, Jerry, L. [US/ US]; 2433 Levante Street, Carlsbad, CA 92008 (US). (74) Agent: SCHWALBACH, Joseph, C.; 1010 Second Ave- nue, Suite 1607, San Diego, CA 92101 (US). (81) Designated States: AU, CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), JP, NL (European patent), NO, 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. A.O.J.P. 11 OCT 1984 AUSTRALIAN 10 SEP 1984 PATENT OFFICE	
(54) Title: DEFINED SEQUENCE SINGLE STRAND OLIGONUCLEOTIDES INCORPORATING REPORTER GROUPS, PROCESS FOR THE CHEMICAL SYNTHESIS THEREOF, AND NUCLEOSIDES USEFUL IN SUCH SYNTHESIS			
(57) Abstract Defined sequence single strand oligonucleotides which have a length of fewer than 200 units, incorporate modified nucleotide units which are pyrimidine or purine-based, and which may have readily detectable reporter groups attached to substituents on the modified nucleotide units thereof. Each reporter group is attached to the base of the respective nucleotide unit at a sterically tolerant site thereon, exemplified by the C-5 position of pyrimidine-based nucleotides and the C-8 position of purine-based nucleotides. Such oligonucleotides are useful in identification, localization, isolation and/or detection of complementary nucleic acid sequences of interest in cellular or cell-free systems. Also disclosed is a process for the chemical synthesis of single strand oligonucleotides having a predetermined number of modified pyrimidine and/or purine-based nucleotide units in predetermined sequence, to the bases of which readily detectable reporter groups are attached at sterically tolerant sites through substituents thereon, either prior to or after incorporation of such units into the oligonucleotide chain. Novel nucleosides useful in the chemical synthesis of labeled, defined sequence single strand oligonucleotides are also disclosed.			
This document contains the amendments allowed under Section 83 (2) by the Super- vising Examiner on and is correct for printing			

Description

Defined Sequence Single Strand Oligonucleotides Incorporating Reporter Groups, Process for the Chemical Synthesis Thereof, and Nucleosides Useful in Such Synthesis

5 Technical Field

The present invention relates to defined sequence single strand oligonucleotides having a length of fewer than 200 base units and which contain one or more nucleotide units to the base of which is attached a substituent group which has bound thereto one or more detectable reporter
10 groups; such oligonucleotides being useful in the identification, localization and detection of complementary nucleic acid sequences of interest in cellulose or cell-free systems.

Background Art

The enzymatic production of labelled, double stranded
15 deoxypolynucleotides has been accomplished with prior art techniques involving the incorporation of radioisotopes into double-strand DNA by the nick translation protocol of P. Rigby et al, J. Mol. Biol. 113: 237-251 (1977), or the gap-filling reaction described by G. Bourguignon et al, Journal of Virology, 20: 290-306 (1976). Specifically, a nick is
20 introduced via DNase, and then translated along the DNA strand using a DNA polymerase. During the nick translation procedure, DNA polymerase from E. coli (Pol I) will, in the presence of added deoxynucleoside triphosphates, condense nucleotides to the 3' hydroxyl terminus in a single strand nick region of double-stranded DNA. Simultaneously the enzyme will remove
25 nucleotides from



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the 5' end of the nick. If one or more of the added triphosphates is labelled, for example, with α - ^{32}P -phosphate, the label will be incorporated into the new strand by Pol I. Following the gap-filling procedure, recessed ends left after restriction endonuclease cutting can be filled in with Klenow fragments from Pol I or T4 DNA Polymerase.

Both the nick translation and the gap-filling procedures will yield double-stranded labelled DNA. The length of the product depends upon how much DNase I is added to the reaction. This is usually optimized so that thirty percent of the label is incorporated and the strands are 400 to 800 nucleotide units in length. The product length is unpredictably heterogeneous within the 400 to 800 unit range. In order to conserve enzyme as well as labelled nucleotide, only one microgram of DNA is usually labelled in each reaction vessel.

Double-stranded polynucleotides which incorporate pyrimidine bases modified by carbon chains at the C-5 position have been prepared enzymatically in a similar manner. This has been done by enzymatic elongation of a homopolymeric primer-template as reported by J. Sagi et al., Biochem. Biosphys. Acta. 606: 196-201 (1980). or by nick translation/gap filling by DNA polymerases using 2'-deoxyuridine 5'-triphosphate covalently linked to biotin as reported by P. Langer et al, Proc. Nat. Acad. Sci. USA 78:6633-6637 (1981), the biotin being capable of acting as a recognition site for avidin.

Enzymatic methods described by Rigby, et al, Bourguignon, et al. and Langer, et al, result in products having similar physical characteristics. Such enzymatically-prepared polynucleotides are 400-800 units in length,



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require double-stranded polynucleotides as starting materials, produce double-stranded polynucleotides in all cases, and do not allow labeling at pre-selected sites.

5 In addition, all enzymatic methods modify both strands of the polynucleotide. and such product strands cannot be isolated from one another. During such processes, enzymes replace either all units with modified units or, when provided with a mixture of modified
10 and naturally-occurring nucleoside triphosphates, randomly insert modified units. Furthermore, the enzymatic process described by Langer, et al, is incapable of producing polynucleotides incorporating such reporter groups as fluorescent, luminescent, or antigenic reporter
15 groups. None of this art is capable of producing oligonucleotides of defined length, defined sequence or single-stranded character, either with or without reporter groups. Moreover, by the prior art methods, modified bases having reporter groups attached thereto cannot be incorporated
20 in a polynucleotide at preselected sites.

Double-stranded polynucleotides which incorporate adenine bases modified at the C-8 position have also been prepared enzymatically. This has been done by incorporating 8-aminohexylamino-ATP (a ribonucleotide)
25 onto DNA fragments, as reported by C. Vincent et al, Nucl. Acids Res. 10:6787-6796 (1982). The method is limited in scope, however, allowing only 3'-end labeling with triphosphates of adenine ribonucleotides. No modified pyrimidine nucleosides can be
30 incorporated. Furthermore, as with other enzymatic methods, both strands of a double-stranded polynucleotide are labeled and no short (<100 units) oligonucleotides of defined sequence can be produced.



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The prior art enzymatic methods referred to above require the chemical synthesis of a substituted nucleoside 5'-triphosphate, and demand subsequent enzymatic recognition and incorporation of the unnatural substrate into nicked double-stranded DNA. Such methods are incapable of producing polynucleotides of any pre-selected length or sequence, and the polymerases and DNases used therein are expensive. Moreover, these methods are time consuming, inefficient, demand substantial enzymatic activity and are limited to double-stranded DNA. Only small amounts, i.e., micrograms, of ill-defined polynucleotides, usually restriction fragments, are produced, and these must be tediously isolated from natural sources. Moreover, the scope of modifications obtainable in the polynucleotide product is severely limited, since the DNA polymerase cannot recognize or incorporate potentially useful reporter groups such as fluorescein or dinitrophenyl.

Attachment of one fluorescent molecule to the 3' end of long polyribonucleotide molecules (RNA) for limited biological application is disclosed by J.G.J. Bauman et al, J. Histochem. Cytochem. 29:238 (1981). This approach also used very small amounts (microgram quantities) of RNA tediously isolated from natural sources using enzymatic methodology, and cannot be applied to DNA since both 2' and 3' hydroxyls are required therefor. The much greater chemical instability of RNA relative to DNA also minimizes the scope of application of the polyribonucleotide produced by this method.

The non-enzymatic synthesis of defined sequence oligonucleotides incorporating naturally-occurring nucleic acid bases has been reported or reviewed by S.A. Narang et al, Meth. Enzymol 65:610 (1980), R. Letsinger, J. Org. Chem. 45:2715 (1980), M. Matteucci et al, J. Amer. Chem.



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Soc. 103:3185 (1982), and G. Alvarado-Urbina et al, Science 214:270 (1981). Such synthesis usually involved chain extension by coupling an activated nucleotide monomer and a free hydroxyl-bearing terminal unit of a growing nucleotide chain. The coupling is effected through a phosphorus-containing group, as in the phosphate triester method reviewed by Narang et al, or one of the phosphite triester methods. Of the latter, those of Letsinger et al and Alvarado-Urbina et al use phosphochloridite chemistry, and that of Matteucci et al uses phosphoamidite chemistry.

The aforementioned chemical synthesis of oligonucleotides has incorporated only unmodified or naturally-occurring nucleic acid bases, and the end product there of is in short fragments and resembles unmodified or naturally-occurring RNA or DNA. It is also worthy of note that the end product of such synthesis does not incorporate any labels or reporter groups.

Direct modification of homopolymeric polynucleotides has been reported in systems of polyuridylic acid [Bigge, et al, J. Carb., Nucleosides, Nucleotides 8:259 (1981)]. The reported procedure is of limited scope and is not productive of useful products. Treatments described cause extensive polynucleotide cleavage and degradation, result in product irreversibly contaminated with metal ions, and are capable of modifying only cytosine residues of a DNA polynucleotide. The method is incapable of producing defined sequence oligonucleotides of specific lengths, cannot modify thymine or purine bases, and cannot modify at previously selected sites.



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From the foregoing it will be apparent that labelled, defined sequence polynucleotides have been produced heretofor only by enzymatic methods which have the disadvantages pointed out herein, particularly those relating to time, cost, product length, sequence and yield. Moreover, such methodology is productive of only double-stranded products. Such double-stranded polynucleotides can be denatured by alkali or heat to cause spontaneous short-term separation of strands in solution. However, the individual single strands cannot be physically isolated from each other, and removal of the denaturing conditions results in rapid renaturation to double-stranded form. Since conditions productive of hybridization are also productive of renaturation, subjecting the denatured polynucleotide to hybridization conditions results in return of the polynucleotide to its original double-stranded configuration in which hybridization of either of the strands to a target polynucleotide is limited by competition from the other strand.



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Modification of nucleosides has been undertaken, for example, in the synthesis of antiviral C-5 substituted pyrimidine nucleosides disclosed by Bergstrom, et al, J. Amer. Chem. Soc. 98:1587-1589 (1976); Ruth, et al, J. Org. Chem. 43:2870-2876 (1978); and Bergstrom, et al, U. S. Patent Nos. 4,247,544 and 4,267,171, or in the synthesis of C-8 substituted adenine derivatives disclosed by Zappelli, et al, U. S. Patent Nos. 4,336,188 and 4,199,498. These nucleosides, and others reported by Langer, et al, and D. Ward, European Patent Application No. 0063879, are not useful in the process of the present invention. Such reported nucleosides are highly reactive at undesired sites, and, if used in oligonucleotide synthesis by chemical methods, would result in undesired side products, uncontrollable synthesis, and no-desired product. Moreover, such reported nucleosides do not contain proper sites in the substituent group, cannot be modified by the attachment of reporter groups, nor do they contain masked reactive functionalities. No such nucleosides are useful in the process of the present invention.

No chemical synthesis of defined sequence oligonucleotides incorporating modified bases of any kind, either with or without reporter groups, has been disclosed in the prior art.

There is an urgent need for high quality labelled, defined sequence single strand oligonucleotides which do not involve hazardous and unstable radioisotopes, and satisfaction of this need is a principal object of



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the present invention. This object is accomplished by a chemical, i.e., nonenzymatic, process which provides a predictable, superior product in high yield. More particularly, the process of the present invention
5 accomplishes the chemical incorporation into defined sequence oligonucleotides of nucleotides modified with a wide variety of selected detectable reporter groups, such oligonucleotides being useful, for example, for the identification, localization, isolation and/or
10 quantitation of complementary sequences of interest.

Another object of the invention is to provide novel nucleosides useful in the chemical synthesis of labelled, defined sequence single strand oligonucleotides.

15 The process of the present invention accomplishes the de novo chemical synthesis of labelled, defined sequence oligonucleotides and is superior to prior art enzymatic methods in a number of respects. More specifically the process of the present invention makes
20 possible the synthesis of labelled, defined sequence single-stranded oligonucleotides of homogeneous predictably defined length, preferably having fewer than 200 base units, in contrast to the production of heterogeneous unpredictable populations of 400 to
25 10,000 base units of double-stranded character produced by prior art enzymatic methods.

The yield of product produced by the process of the present invention is of the order of hundreds to tens of thousands of micrograms, in contrast to the
30 yield of a few micrograms provided by the prior art enzymatic methods. Moreover, the product oligonucleotides of the present invention are single-stranded, rather than the double-stranded products of enzymatic



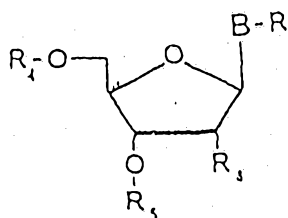
methods. The single strand configuration of the product oligonucleotides avoids the competition from a complementary strand inherent in rehybridization of double-stranded polynucleotides.

Disclosure of the Invention

5 According to a first embodiment of this invention there is provided a substantially pure single-stranded oligonucleotide comprising a preselected sequence of not more than approximately 200 nucleotides wherein at least one of said nucleotides is at a pre-selected position in said sequence and includes a base having a covalently attached linker arm attached to the
10 base at a sterically tolerant site and having two or more carbon atoms if attached to a pyrimidine and one or more carbon atoms if attached to a purine, said linker arm containing or capable of binding at least one reporter group or a solid support.

15 According to a second embodiment of this invention there is provided a process for the chemical synthesis of a substantially pure single-stranded oligonucleotide comprising a preselected sequence of not more than approximately 200 nucleotides wherein at least one of said nucleotides is at a preselected position in said sequence and contains a base having a covalently attached linker arm attached to the base a
20 sterically tolerant site and having two or more carbon atoms if attached to a pyrimidine and one or more carbon atoms if attached to a purine, said linker arm containing or capable of binding at least one reporter group or a solid support or a moiety capable of binding at least one reporter group or a solid support, the process comprising the stepwise addition of
25 selected reactive nucleotides to a substantially pure single-stranded oligonucleotide having less than approximately 200 nucleotides in a preselected sequence, wherein said nucleotide or said oligonucleotide contains a reactive phosphorus-containing group attached to a 3' or a 5' hydroxyl group and wherein at least one of said reactive nucleotides includes a base having a covalently attached linker arm containing or capable of binding at least one reporter group or a solid support.

30 According to a third embodiment of this invention there is provided a reactive nucleotide monomer useful as an intermediate in the chemical synthesis of a substantially pure single-stranded oligonucleotide comprising the structure:



wherein B is a pyrimidine or purine base;

R is a linker arm attached to B at a sterically tolerant site and containing or capable of binding at least one reporter group or a solid support wherein said linker arm has two or more carbon atoms if attached to a pyrimidine and one or more carbon atoms if attached to a purine;

and provided that

when R_4 is a masking group then

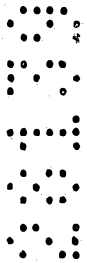
R_5 is a reactive phosphorus-containing group or H; and

when R_5 is a masking group then

R_4 is a reactive phosphorus-containing group or H; and

R_8 is a H or masked hydroxyl group.

The oligonucleotide produced by the process of the present invention may include one or more pyrimidine-based or purine-based units which may be ribonucleotides or deoxyribonucleotides, and prior to the synthesis thereof, the reporter groups are preselected, as are the particular nucleotide units to which the reporter groups are attached.



Best Mode of Carrying Out the Invention

~~The chemical process by which the defined sequence~~
single strand oligonucleotides of the present invention
are preferably synthesized comprises coupling an acti-
5 vated nucleotide monomer and a free hydroxy-bearing
terminal unit of growing nucleotide chain, at least
one of said monomer and terminal unit having its base
modified at a sterically tolerant site by attachment
thereto of a substituent group capable of binding one
10 ~~or more detectable reporter groups.~~

~~The substituent~~ ^{Preferably, the linker arms or} groups of the present invention
which are capable of binding reporter groups can be
generally characterized as those which exhibit nucleo-
philic properties with respect to such reporter groups.
15 Exemplary of such substituent groups are those which
contain primary or aromatic amines, carboxylic acids,
hydroxyls and the like.

The bases of the nucleotide monomer and terminal
unit are selected to provide the predetermined sequence
20 of nucleotide units desired in the end product oligo-
nucleotide. Such bases can take the form of the purines
adenine (A), guanine (G), or hypoxanthine (H), or of the
pyrimidines uracil (U), cytosine (C), or thymine (T).
Such bases may also take the form of any other base which
25 ~~can be isolated~~ ^{is} from natural sources.

A sterically tolerant site on the nucleotide unit can
be defined as a position on the nucleic acid base of the
unit at which modification of the unit can be effected
by attachment thereto of the substituent group without
causing significant interference with hybridization of
the product oligonucleotide and complementary nucleic
acid components, and without sterically preventing the
substituent group from binding one or more reporter
groups. Sterically tolerant sites are found at the C-8



position of purines and at the C-5 position of pyrimidines. Since the oligonucleotides of the present invention are particularly useful as hybridization probes, modifications which attach substituent and/or reporter groups should not be at sites on the pyrimidine or purine bases which are necessary for specific hybridization. Sites which should not be modified include N¹ and N⁶ of adenine bases; N¹, N², and O⁶ of guanine bases; N³ and N⁴ of cytosine bases. Generally, substitution at any heteroatom (N or O) should be avoided.

A reporter group ^{is} ~~can be~~ defined as a chemical group which may be aromatic and/or polycyclic and which has a physical, ^{or other} ~~or chemical~~ characteristic which can be readily measured or detected by appropriate physical or chemical detector systems or procedures. Reporter groups which are useful in oligonucleotides of the present invention are readily detectable. Ready detectability may be provided by such characteristics as color change, luminescence, fluorescence, or radioactivity; or it may be provided by the ability of the reporter group to serve as a ligand recognition site. Such groups are termed functionally colorimetric, luminescent, fluorescent, radioactive or ligand recognition groups. Among such groups are those suitable for ready detection by conventional detection techniques, such as colorimetric, spectrophotometric, fluorometric or radioactive detection, as well as those which are capable of participating in the formation of specific ligand-ligand complexes which contain groups detectable by such conventional detection procedures.

A reporter group as used herein may be characterized as a label which has physical, chemical or other



characteristics which can be readily measured or detected by the use of appropriate measurement or detection procedures. A reporter group as defined herein also includes ligand recognition groups which are capable
5 of initiating one or more ligand-ligand interactions which ultimately provide a reaction product or a complex having physical, chemical or other characteristics which can be readily measured or detected by the use of appropriate measurement or detection procedures.

10 Exemplary of the measurable or detectable characteristics which such groups, reaction products or complexes may exhibit or induce are a color change, luminescence, fluorescence or radioactivity. Such characteristics can be measured or detected by the use
15 of conventional colorimetric, spectrophotometric, fluorometric or radioactivity sensing instrumentation.

The interactions which usefully can be initiated by the reporter group defined herein include appropriately specific and selective ligand-ligand interactions
20 productive of groups or complexes which are readily detectable, for example, by colorimetric, spectrophotometric, fluorometric, or radioactive detection procedures. Such interactions may take the form of protein-ligand, enzyme-substrate, antibody-antigen, carbohydrate-
25 lectin, protein-cofactor, protein-effector, nucleic acid-nucleic acid or nucleic acid-ligand interactions. Exemplary of such ligand-ligand interactions are dinitrophenyl-dinitrophenyl antibody, bio:in-avidin, oligonucleotide-complementary oligonucleotide, DNA-DNA,
30 RNA-DNA and NADH-dehydrogenase. Either one of each such ligand-ligand pairs may serve as a ligand recognition type reporter group. Other useful interactions will suggest themselves to those skilled in the art.



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In the process of the present invention, a selected reporter group or groups can optionally be attached to the nucleotide monomer before coupling of the monomer to the terminal unit of the nucleotide chain, or it can be attached to the product oligonucleotide after formation thereof. The sequence of the nucleotide units in the product oligonucleotide is preselected to provide such oligonucleotide with precise specificity for its ultimate use. The oligonucleotides of the present invention are useful tools in recombinant DNA and other protocols involving nucleic acid rehybridization techniques. Among such uses are identification, localization, isolation and/or quantitation of complementary sequences of interest in cellular or cell-free systems. More specifically, such uses may include diagnostic applications or any fundamental biological event involving hybridization of nucleic acid components, or purification of complementary sequences by affinity chromatography when the product oligonucleotide is attached to a solid support through the modifications at a sterically tolerant site, with or without subsequent detection.

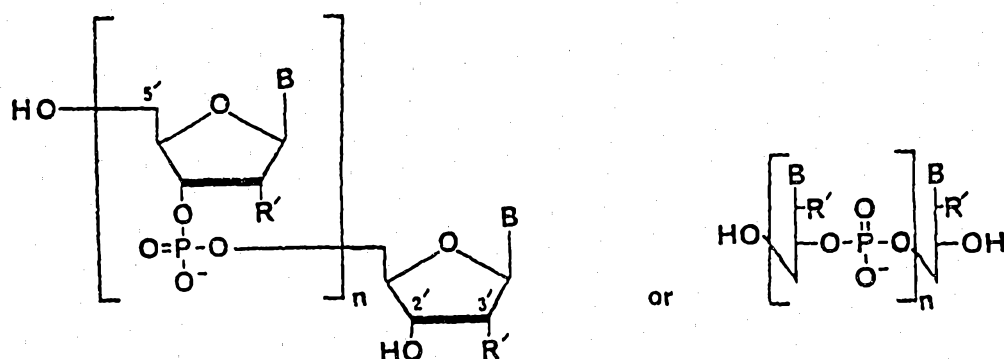
The nucleotide units in the product oligonucleotide may be purine or pyrimidine-based and may comprise units having naturally-occurring bases intermixed with units having modified bases. Such units may be ribonucleotides or deoxyribonucleotides. The coupling step preferably involves coupling of a monomer unit activated at the 3' position with a free 5' hydroxyl of the terminal unit of the growing nucleotide chain. Alternatively such coupling can involve coupling of a monomer unit activated at the 5' position with a free 3' hydroxyl of the terminal unit of the nucleotide chain. The



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terminal unit may be the initial or only unit in the growing nucleotide chain at the time of coupling thereto of the nucleotide monomer, or it may be the terminal one of a plurality of nucleotide units.

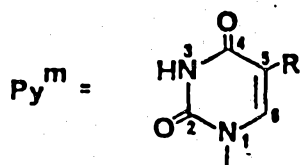
- 5 The process of the present invention produces defined sequence oligonucleotides of the following generic formula:



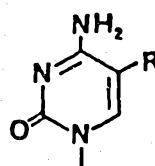
FORMULA I

- Wherein n is 1 to about 199, preferably about 5 to about 60, and most preferably about 10 to about 40, R' is hydrogen or hydroxy, and B is any of the naturally-occurring purine or pyrimidine bases adenine, guanine, cytosine, uracil, thymine, or any other naturally-occurring base, the nucleotide units having naturally-occurring bases being independently intermixed with one or more nucleotide units having modified bases (B^m). The modified pyrimidine bases (Py^m) are substituted at the C-5 position, and typical examples thereof are the uracil and cytosine bases illustrated by the following generic formulas:
- 20





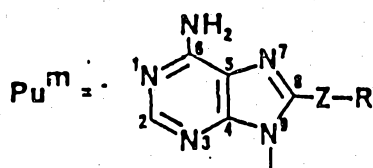
or



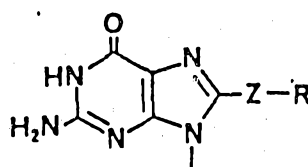
modified uracil base

modified cytosine base

The modified purine bases (Pu^m) are substituted at the C-8 position, and typical examples thereof are the modified adenine and guanine bases illustrated by the following generic formulas:



or



modified adenine base

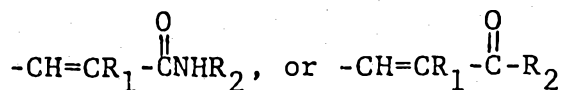
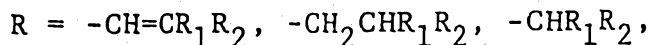
modified guanine base

The substituent group ^{or linker arm} R is characterized by its ability to bind one or more reporter groups. In the modified pyrimidine bases the substituent group R comprises two or more carbon atoms, whereas in the modified purine bases R comprises one or more carbon atoms. In this context,

10



R preferably takes the form of one of the following functionalized carbon chains:

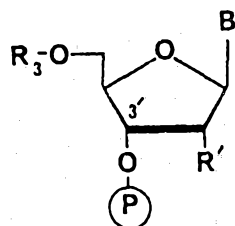


- 5 wherein R_1 is hydrogen or alkyl; R_2 is alkyl, alkenyl, aryl, or functionalized alkyl, alkenyl, aryl wherein functional groups include one or more amines, amides, nitriles, carboxylic acids and esters, hydroxy, dinitrophenyl, aminobenzenesulfonates, or the like; and Z
- 10 is a polyvalent heteroatom such as nitrogen, oxygen or sulfur. In addition, R_2 may be attached to a solid support, or to one or more reporter groups which function, for example, as a colorimetric, fluorescent, luminescent, radioactive, or ligand recognition group.
- 15 Functionally fluorescent groups include fluoresceins, rhodamines, and the like or adducts thereof; functionally luminescent groups include luminols, acridines, luciferins, dioxetanes, dioxamides, and the like or adducts thereof. Ligand recognition groups include
- 20 vitamins (such as biotin or adducts thereof, including iminobiotin and desthiobiotin), antigens such as dinitrophenols, carbohydrates and other functional groups or adducts of such groups which can be recognized by ligand-like interactions with proteins, or from which such ligand-
- 25 like interactions can be elicited. Another oligonucleotide capable of interaction with nucleic acids is illustrative of a group from which a ligand-like interaction can be elicited. Ligand recognition groups may also serve as
- 30 functionally colorimetric reporter groups when recognition results in dye formation. For example, when dinitrophenyl is used as a reporter group, known detection systems using an antidinitrophenyl antibody coupled to peroxidase can be used as a detection system, resulting in a color change. Functionally radioactive groups
- 35 incorporate a radioactive element in the chosen reporter group.

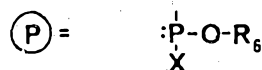


When reference is made herein to the use of purine or pyrimidine bases, such expressions are intended to include analogs of such bases. Among such analogs are the analogs of purine bases, such as the deazaadenosines (tubercidins, formycins, and the like), and the analogs of pyrimidine bases, such as deazauracil, deazacytosine, azauracils, azacytosines, and the like.

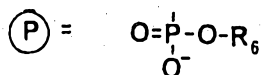
Oligonucleotides of Formula I are best prepared by chemical synthesis from monomer nucleotide analog units of the formula:



FORMULA II



or



where:

R_6 = methyl, chloro-phenyl

X = chloro, dialkyl-amino, morpholino

wherein R_3 is trityl (triphenylmethyl), dimethoxytrityl, or other appropriate masking group for the 5'-hydroxyl; B and R' are masked, if appropriate; and $\textcircled{\text{P}}$ represents a phosphorus-containing group suitable for internucleotide bond formation during chain extension in synthesis of a product oligonucleotide. The phosphorus-containing groups $\textcircled{\text{P}}$ suitable for internucleotide bond formation are preferably alkyl phosphomonochloridites or alkyl phosphomonoamidites. Alternatively phosphate triesters may be employed for this purpose. The monomer unit may



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alternatively have R_3 attached at the 3' hydroxyl and (P) attached at the 5'-hydroxyl.

Generally, the term "masking group" or "blocking group" is a functional expression referring to the chemical modification or "blocking" of an integral functional group by attachment of a second moiety to disguise the chemical reactivity of the functional group and prevent it from reacting in an undesired manner. Such modification is reversible, and allows subsequent conversion back to the original functional group by suitable treatment. In many cases, such masking formally interconverts structural functionality, e.g., a primary amine masked by acetylation becomes a substituted amide which can be later converted back to the primary amine by appropriate hydrolysis.

The compounds of Formula I include the acceptable conjugate acid salts thereof. Conjugate acids which may be used to prepare such salts are those containing nonreactive cations and include, for example, nitrogen-containing bases such as ammonium salts, mono-, di-, tri- or tetra-substituted amine salts, and the like, or suitable metal salts such as those of sodium, potassium, and the like.

The process steps of the present invention will now be generally described and illustrated diagrammatically. Thereafter, the invention will be illustrated more specifically and detailed examples thereof provided. Since the invention relates to oligonucleotides incorporating both pyrimidine-based and purine-based nucleotide units, the use of both pyrimidine and purine-based compounds in the synthetic process will be illustrated. The specific pyrimidine and purine-based compounds illustrated are only exemplary of the respective pyrimidine and purine classes, and it is to be understood that any other member



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of the respective class can be substituted therefore in the process and the product oligonucleotide, whenever suitable or desired. While deoxyribonucleotide compounds are shown for the most part, it is understood that ribonucleotide compounds are also contemplated by the invention and can be substituted for the deoxyribonucleotide compounds wherever ribonucleotide compounds are desired in the product oligonucleotide.

- 10 One of the more important aspects of the invention is the provision of a new class of nucleosides which are essential as intermediates in the process for synthesizing the new oligonucleotides. Such nucleosides each have a base which is modified by a substituent
- 15 group comprising a functionalized carbon chain and one or more amides, the nitrogen of the amides being attached to a sterically tolerant site on the base through the carbon chain. In the case of pyrimidine-based nucleosides, the carbon chain is attached at the C-5 position,
- 20 and in the case of the purine-based nucleosides, the carbon chain is attached at the C-8 position through a polyvalent heteroatom, such as nitrogen, oxygen or sulfur. In addition, such nucleosides are chemically blocked at the 5' position (or the 3' position) with
- 25 a group, such as dimethoxytrityl, appropriate for the chemical synthesis of oligonucleotides.



In the new class of nucleosides the substituent group may be chosen from $-\text{CH}_2\text{CHR}_1\text{C}_n\text{H}_{2n}\text{Y}$, $-\text{CH}=\text{CR}_1\text{C}_n\text{H}_{2n}\text{Y}$,

$\text{CHR}_1\text{C}_n\text{H}_{2n}\text{Y}$, $-\text{CH}=\text{CR}_1-\overset{\text{O}}{\parallel}\text{C}-\text{NHC}_n\text{H}_{2n}\text{Y}$, or $-\text{CH}=\text{CR}_1-\overset{\text{O}}{\parallel}\text{CC}_n\text{H}_{2n}\text{Y}$

wherein R_1 is hydrogen or C_{1-6} lower alkyl, n is

5 0 to 20 and Y is one or more amido, substituted amido, substituted amino or substituted aminoalkylphenyl groups.

More specifically, Y may include one or more $-\text{NH}\overset{\text{O}}{\parallel}\text{CCX}_3$ wherein X is hydrogen, fluorine or chlorine. Synthesis of these nucleosides, as well as of the masked forms thereof, is described hereinafter in Examples I, II, IV, VI, VII, VIII, X, XII, and XIII. Preferred nucleosides

incorporate the substituent group $-\text{CH}=\text{CH}\overset{\text{O}}{\parallel}\text{C}-\text{NHC}_n\text{H}_{2n}\text{Y}$ at the C-5 of pyrimidine nucleosides wherein $n = 3$ to 12

15 and Y is $-\text{NH}\overset{\text{O}}{\parallel}\text{CCX}_3$. Most preferred are such nucleosides wherein the pyrimidine base is uracil.

The process of the present invention may be initiated by the preparation of the selected nucleoside. Generally, the most preferred nucleosides are best prepared in the following manner. 5-(Methyl 3-acrylyl)-2'-deoxyuridine is prepared from 2'-deoxyuridine by the method of Bergstrom and Ruth [J. Amer. Chem. Soc. 96:1587 (1976)]. The nucleoside is then treated with 1.05 equivalents of dimethoxytrityl chloride in pyridine for 4 hours to block the 5' hydroxyl with dimethoxytrityl (DMT). The resulting product is purified by silica chromatography eluting a gradient of 0-10% methanol in chloroform containing 2% triethylamine. The purified 5'-DMT-5-(methyl 3-acrylyl)-2'-deoxyuridine is treated with 30 1 N KOH for 24 hrs. at ambient temperature to hydrolyze the methyl ester.

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The resulting 5'-DMT-5-(3-acrylyl)-2'-deoxyuridine is treated with excess dicyclohexylcarbodiimide and hydroxybenztriazole in pyridine. After 4 hours, a 2-5 fold excess of 1,7-diaminoheptane is added, and the reaction stirred overnight. After 12-20 hours, a 10-20 fold excess of trifluoroacetic anhydride is added, and the reaction stirred at room temperature for 4 hours. The product is purified by silica chromatography eluting a gradient of 0-10% methanol in chloroform containing 2% triethylamine, followed by exclusion chromatography using Sephadex LH-20 eluting 1% triethylamine in methanol. Appropriate fractions are combined to yield 5'-DMT-5-[N-(7-trifluoroacetyl-aminoheptyl)-1-acrylamido]-2'-deoxyuridine; such product is appropriate for oligonucleotide synthesis by the phosphochloridite procedure described in Examples XV and XVIII. Alternatively, such a compound can be prepared by the combination of methods described in Examples II and III. Replacing diaminoheptane in this process with other diaminoalkanes (e.g., diaminopropane, diaminohexane, diaminododecane) is productive of other compounds of varying substituent length wherein $n = 3, 6, \text{ or } 12$ and

$R = -CH=CH-\overset{\overset{O}{\parallel}}{C}-NHC_nH_{2n}-\overset{\overset{O}{\parallel}}{C}NHCCX_3$. Two such nucleosides, one pyrimidine (uracil)-based and the other purine (adenine)-based, are shown at the top of the diagram below illustrating the process. Reactive sites on the bases of the nucleosides are then masked, as shown in Reaction 1, by attachment of, for example, a benzoyl group (Bz) to the amine at the



6 position of the adenine-based nucleoside. Such masking is generally described in "Synthetic Procedures in Nucleic Acid Chemistry", Vol. 1, W. Zorbach and R. Tipson eds. (Wiley - Interscience, N.Y., 1968).

- 5 Unprotected amines on the substituent group are masked, for example, by attachment thereto of trifluoroacetyl groups (Ac), as also shown in Reaction 1.

The selected 3' or 5' hydroxyl of the nucleoside is then masked by attachment thereto of a dimethoxytrityl (DMT) group. In Reaction 2 illustrated below, the 5'-hydroxyl is masked, leaving the 3' hydroxyl free or available for reaction. Alternatively, the 3' hydroxyl could be masked, leaving the 5' hydroxyl free.

The nucleoside is then converted to an activated nucleotide monomer, preferably by attachment to its 3' hydroxyl of a phosphorus-containing group which includes an activating moiety. When the modified nucleoside is properly blocked, modifications of the procedures described by Letsinger, et al, Matteucci, et al, or as reviewed by Narang, et al can be utilized for oligonucleotide synthesis. The use of phosphochloridite chemistry such as that disclosed by Letsinger, et al, is detailed in Examples XVI-XVIII. In order to use phosphoramidite chemistry, a modification of the procedure of Matteucci, et al, is used, phosphitylating the protected modified nucleoside with methyl chloro (N, N-diisopropyl)phosphoramidite or methyl chlorophosphomorpholidite, as in the improved procedure of Dorper, et al [Nucleic Acids Res. 11:2575(1983)]. Al-



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ternatively, the protected modified nucleoside can be phosphorylated with 1.2 eq. chlorophenyl dichlorophosphate in trimethylphosphate at room temperature followed by quenching with water to give the 3'-chloro-5 phenyl phosphate adduct of the modified nucleoside, such adducts being useful in a modification of the phosphotriester approach as illustratively reviewed by Narang, et al. The diagram illustrates in Reaction 3 the synthesis of activated monomer nucleotide units of Formula 10 II by attachment to the nucleoside 3' hydroxyl of a phosphomonochloridite group in which the chlorine functions as an activating moiety.

Coupling or condensation of the selected activated nucleotide monomer, i.e. the uracil-based monomer or 15 the adenine-based monomer, to the terminal unit of a growing nucleotide chain is illustrated in Reaction 4 in the diagram. The nucleotide chain is shown as including in its right hand end a nucleoside unit having a naturally occurring base and having a solid support 20 or masking group R_4 attached to its 3' hydroxyl. The illustrated chain also includes one or more (n') nucleotide units having naturally-occurring bases, said units being coupled to the 5' hydroxyl of the nucleoside unit, the terminal one of the nucleotide units having a free 25 hydroxyl at the 5' position. In the coupling reaction



the chlorine of the monomer reacts with the hydrogen of the free hydroxyl of the terminal unit and is displaced, so that the oxygen of the terminal unit couples to the phosphorus of the monomer as shown, and the monomer
5 thereby becomes the new terminal unit of the nucleotide chain.

The DMT 5' blocking group is then removed to permit further extension of the nucleotide chain by sequential coupling thereto of additional activated nucleotide
10 monomer units. The nucleotide units added to the chain can be preselected and may have either naturally-occurring or modified bases. The diagram shows in Reaction 4a the further extension of the chain by the addition of one or more (n'') nucleotide units having naturally-
15 occurring bases.

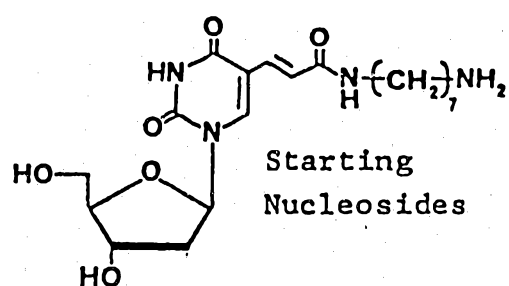
When an oligonucleotide of the selected length and sequence has been synthesized, the DMT group is removed from the terminal unit thereof, and the masked reactive groups are unmasked. Examples of modified uracil and
20 adenine bases with their reactive groups unmasked are also shown diagrammatically at Reaction 5. If the initial nucleotide unit of the chain is bound to a solid support R_4 , the chain is then removed from such solid support. The appropriate order of unmasking can be pre-
25 selected.

Reporter groups R_5 appropriate for the

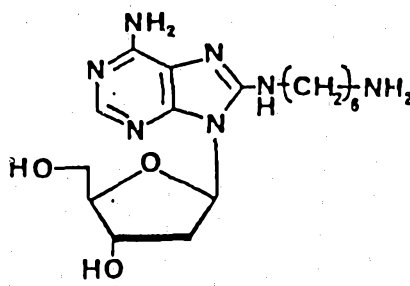


intended use of the product oligonucleotide can then be bound to such substituent groups as exemplified in Reaction 6, which illustrates the respective bases with reporter groups R_5 bound to the respective 5 substituent groups thereof.

Illustration of Synthetic Process

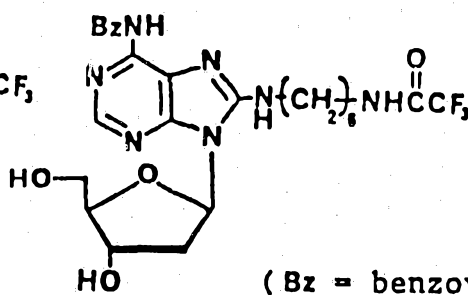
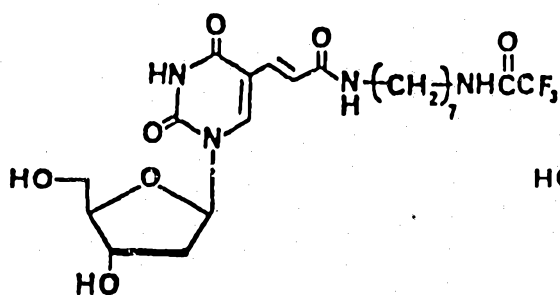


uracil-based



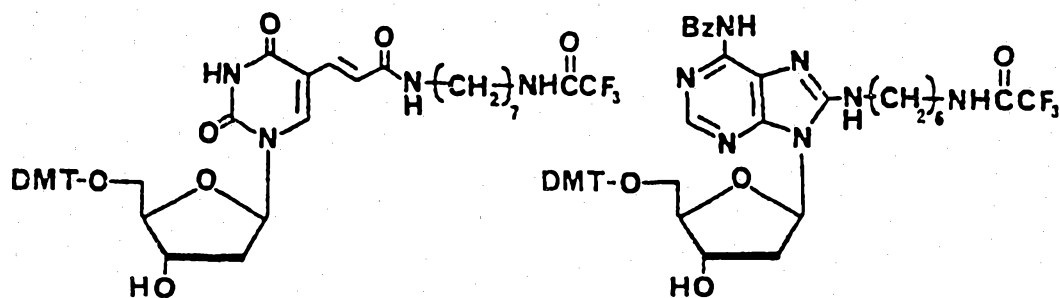
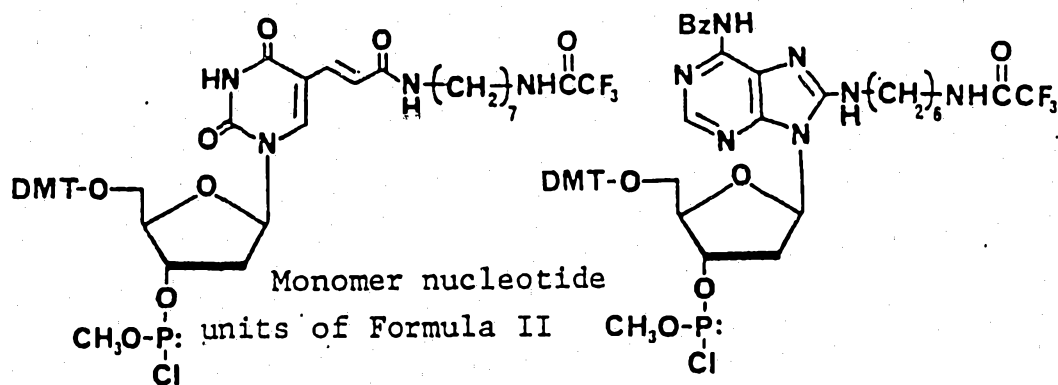
adenine-based

Reaction 1:
Masking of reactive sites
on nucleoside bases

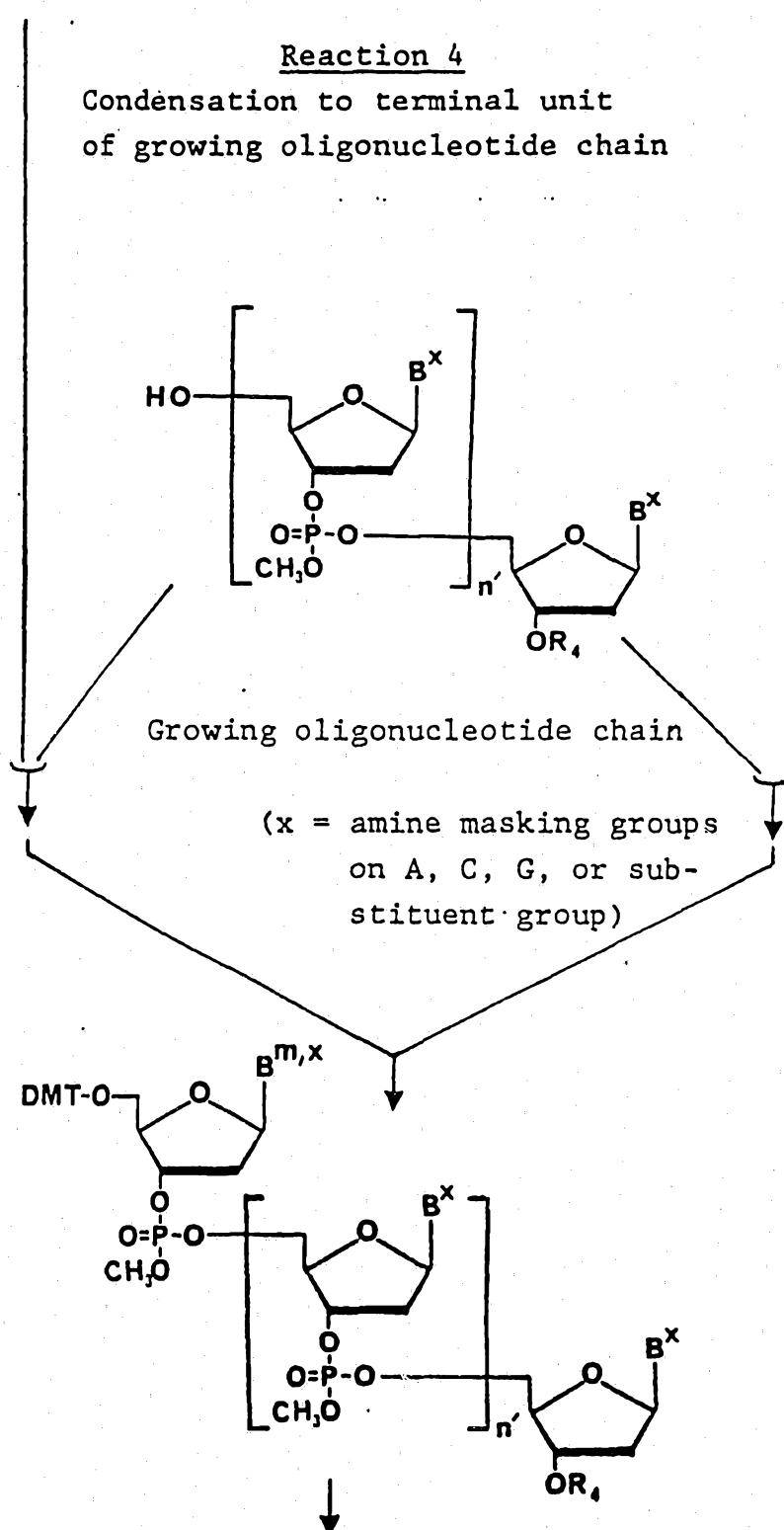


(Bz = benzoyl)

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Reaction 2Masking of 5'-hydroxyl
(DMT = dimethoxytrityl)Reaction 3Activation to phospho-
chloridite

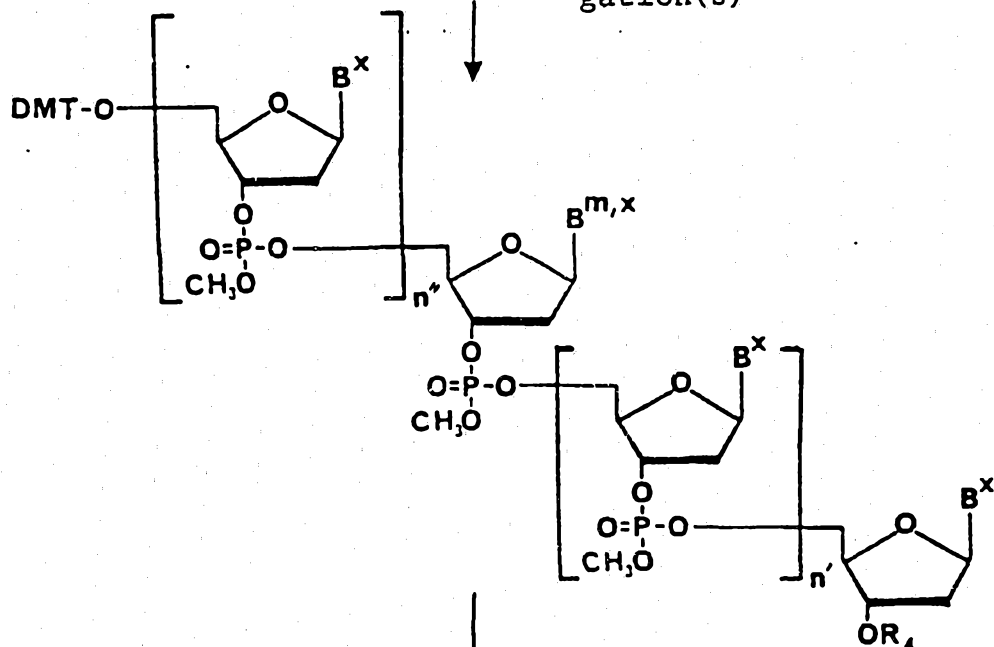
27-



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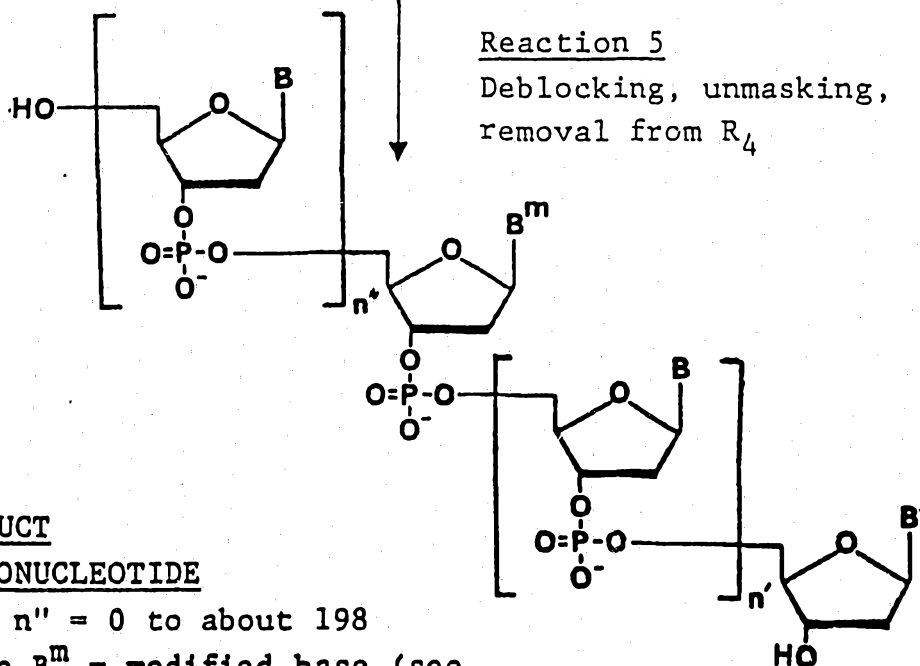
Reaction 4a

Further chain elongation(s)



Reaction 5

Deblocking, unmasking,
removal from R_4



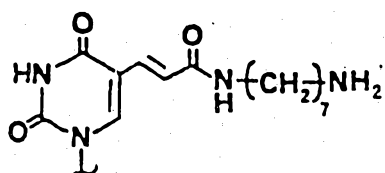
PRODUCT
OLIGONUCLEOTIDE

$n' + n'' = 0$ to about 198
where B^m = modified base (see
pg 29)

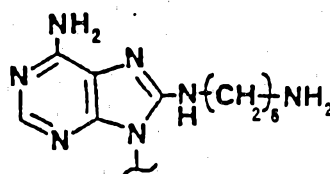


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where, for example, B^m in product oligonucleotide (pg 28) is:



uracil base
(modified)



adenine base
(modified)

To the product oligonucleotide a variety of useful reporter groups (R₅) may be attached. For example:

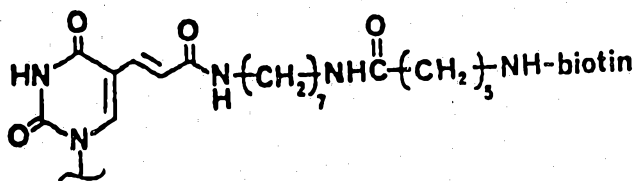
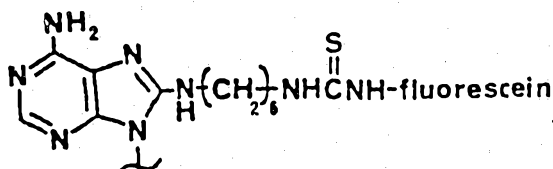
Product oligonucleotide
with above modified
uracil base

Product oligonucleotide
with above modified
adenine base

N-hydroxy-
succinimidyl
6-biotinyl-
aminocaproic
acid

Reaction 6
Attachment of
reporter groups (R₅)

FITC



PRODUCT OLIGONUCLEOTIDES WITH REPORTER GROUPS (where
R₅ = biotin or fluorescein)

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Having discussed the process of the present invention in general terms and illustrated the same diagrammatically, each of the reactions referred to will now be discussed more specifically.

5 With reference to Reaction 1, masking of chemically reactive amines such as N⁴ of cytosine, N⁶ of adenine, N² of guanine, and alkyl or aryl amines of the modified bases with suitable masking groups can be conveniently accomplished in suitable solvents such as alcohols, pyri-
10 dines, lutidines, chloroform, and the like, by reaction of the nucleosides with an excess of appropriate acid anhydrides for about 1 to 24 hours at temperatures in the range of 0°C to 110°C, generally 20°C to 80°C. Appropriate acid anhydrides include acetic anhydride,
15 trifluoroacetic anhydride, benzoyl anhydride, anisoyl anhydride, and the like. Preferred are acetyl, trifluoroacetyl, benzoyl, and isobutyryl anhydride.

Masking of the 5'-hydroxy in Reaction 2 can be conveniently effected by reaction of the nucleosides
20 with a slight excess of appropriate acid-labile masking reagents, such as tritylchlorides, monomethoxytrityl chloride, dimethoxytrityl chloride (DMTCl), trimethoxytrityl chloride and the like. Preferred is dimethoxytrityl chloride. Typical reactions are carried out in
25 suitable solvents, such as pyridine, lutidines, trialkylamines, and the like, at temperatures in the range of -20°C to 120°C, generally 20°C to 100°C, for about 1 to 48 hours. The preferred reaction utilizes 1.1 equivalents of DMTCl in pyridine at room temperature
30 for 2 hours.

It is generally preferred that the respective products of each reaction described hereinabove be separated and/or isolated prior to use as a starting material



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for a subsequent reaction. Separation and isolation can be effected by any suitable purification procedure such as, for example, evaporation, filtration, crystallization, column chromatography, thin layer chromatography, etc. Specific illustrations of typical separation and isolation procedures can be had by reference to the appropriate examples described hereinbelow; however, other equivalent separation procedures can, of course, also be used. Also, it should be appreciated that, where typical reaction conditions (e.g., temperatures, mole ratios, reaction times) have been given, conditions both above and below the typical ranges can also be used, though generally less conveniently.

Activation to the phosphite analog illustrated in Reaction 3 can be most conveniently effected by treatment of the nucleoside compounds with suitable phosphitylating agents in appropriate solvents at temperatures in the range of -90°C to 60°C for 1 minute to 2 hours. Suitable phosphitylating agents include methylphosphodichloridite, o-chlorophenylphosphodichloridite, p-chlorophenylphosphodichloridite, methylphospho(dialkylamino)monochloridite, and the like. Appropriate solvents include pyridine, lutidines, acetonitrile, tetrahydrofuran, dioxane, chloroform and the like containing 0-20% appropriate base (generally 1-5 vol %) such as lutidines, collidines, trialkylamines and the like. Preferred phosphitylating agents are methylphosphodichloridite, o-chlorophenylphosphodichloridite, and methylphospho(di-isopropylamino)-monochloridite. Preferred phosphitylating conditions are with 0.9 equivalents of methyl phosphodichloridite in pyridine or acetonitrile containing 5% 2,6-lutidine for 5 to 10 minutes at room temperature or below.



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The chemical incorporation of the modified nucleotide analog monomers into a growing nucleotide chain to produce defined sequence nucleotides is illustrated in Reactions 4 and 4a. Typical condensations are in appropriate solvents at temperatures in the range of -20°C to 50°C, preferably at ambient temperature, for about 1 to 60 minutes. Appropriate solvent mixtures include pyridine, lutidines, acetonitrile, tetrahydrofuran, dioxane, chloroform and the like containing 0-20% appropriate base (generally 1 to 5 volume %) such as lutidines, collidines, trialkylamines and the like. The growing chain may be soluble, insoluble, or attached to a suitable solid support by appropriate chemical methods known in the art. Preferred is attachment to a solid support. Furthermore, the growing chain may or may not have previously incorporated one or more modified nucleoside analogs.

After condensation of the activated monomer to the growing chain, in Reaction 4, the initial product is treated with suitable reagents to accomplish oxidation of the intermediate phosphitetriester, optional capping to block unreacted 5'-hydroxyls on the oligonucleotide chain, and removal of the 5'-DMT group. Oxidation of the phosphite triester can be accomplished by treatment with 0.1-5 w/vol % iodine in suitable solvents, for example, tetrahydrofuran/water/lutidine mixtures. Chemical capping of unreacted 5'-hydroxyls can be accomplished by acetylation or acylation with, for example, acetic anhydride and 4-dimethylaminopyridine in tetrahydrofuran/lutidine mixtures. Removal of the 5'-blocking group, usually DMT, is most conveniently effected by treatment with mild organic acids in nonprotic solvents, such as mild acids including, for example, 1-5 vol % dichloroacetic or trichloroacetic acid in chloroform or



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dichloromethane. The growing nucleotide chain, after removal of DMT, can now serve as acceptor for subsequent elongation by sequential reaction with activated monomers to eventually produce the oligonucleotide of 5 desired length and sequence, as shown in Reaction 4a.

After an oligonucleotide of desired sequence is produced, Reaction 5 is accomplished to provide the product oligonucleotide. To this end, thiophenol treatment is used to remove methyl masking groups from phosphate triesters, and suitable aqueous alkali or ammonia treatment is used to remove benzoyl, acetyl, isobutyl, trifluoroacetyl, or other groups from the protected amines and/or to remove the product from the solid support. Removal of DMT from the oligonucleotide product 15 is accomplished by the appropriate treatment with a mild acid, such as aqueous acetic acid at ambient temperature to 40°C for 10 to 60 minutes. Such reactions may be accomplished before or during final purifications. Final purification is accomplished by appropriate methods, such 20 as polyacrylamide gel electrophoresis, high pressure liquid chromatography (HPLC), reverse phase or anion exchange on DEAE-cellulose, or combinations of these methods.

The process described herein for synthesis of oligonucleotides can utilize modified deoxyribonucleosides 25 (where R' is H) or modified ribonucleosides (where R' is hydroxyl). When ribonucleosides are used, the 2'-hydroxyl is masked by an appropriate masking group such as, for example, that afforded by silylethers. Other ribose



analogs, including arabinose and 3'-deoxyribose, can also be accommodated in the process to produce the desired oligonucleotide.

The substituent group modifying a nucleotide
5 base must be capable of binding one or more reporter
groups either prior to or after the chain extension
coupling reaction. In the latter case, selected product
oligonucleotides are reacted with suitable agents to
attach such reporter groups. For example, when modified
10 bases are incorporated into the oligonucleotide and R_2
of the substituent group contains one or more primary
amines, coupling with amine-reactive groups such as
isocyanate, isothiocyanate, active carboxylic acid
conjugates, epoxides or active aromatic compounds
15 using suitable conditions is productive of amide, urea,
thiourea, amine or aromatic amine linkages. For example,
an oligonucleotide which contains an uracil or adenine
base modified by a substituent group having a primary
amine, as shown in the Reaction 5 diagram, can be
20 reacted with a suitable reagent, such as fluorescein
isothiocyanate or N-hydroxysuccinimidyl 6-biotinylamino-
caproic acid, to provide a reporter group R_5 (fluorescein
or biotin, respectively) bound to the substituent group
as shown in Reaction 6. Other reporter groups which
25 can be attached in similar manner include a wide
variety of organic moieties such as fluoresceins,
rhodamines, acridinium salts, dinitrophenyls,
benzenesulfonyls, luminols, luciferins, biotins,
vitamins, carboxyhydrates and the like. Suitably



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active reporter groups are available commercially, or can be synthesized, for example, by processes of the type generally described in "Bioluminescence and Chemiluminescence" [M. DeLuca and W. McElroy, eds., 5 Acad. Press, New York (1981)], by D. Roswell, et al., or H. Schroeder, et al. [Meth. Enzymol. LXII, 1978], and references cited therein.

Typically, attachment of reporter groups is conveniently accomplished in predominately aqueous 10 solvents by reaction of the substituent groups of modified bases wherein $R_2 = C_n H_{2n} NH_2$ with excess of the selected reporter group at temperatures in the range of about $-20^\circ C$ to $50^\circ C$ (preferably $20^\circ C$ to $40^\circ C$) for 1 to 24 hours. Suitable solvents are 15 an aqueous buffer and 0-50% organic solvents such as lower alcohols, tetrahydrofuran, dimethylformamide, pyridine, and the like. Preferred reporter group reactants include fluorescein isothiocyanates, dinitrophenylisothiocyanates, fluorodinitrobenzene, 20 N-hydroxysuccinimidylbiotin, N-hydroxysuccinimidyl dinitrobenzoate, isothiocyanates such as aminobutyl ethyl isoluminol isothiocyanate and the like, active esters of carboxyfluorescein, rhodamine, biotin adducts, dioxetanes, dioxamides, carboxyacridines, 25 carbohydrates and the like.

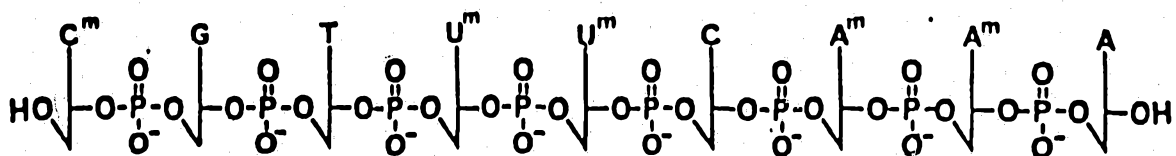
Additionally, when the product oligonucleotide includes modified bases wherein R contains one or more carboxylic acids, mild condensations with, for example, primary alkylamines is productive of amide linkages. 30 Typically, this is conveniently effected in predominately aqueous solvents by reaction of the oligonucleotide with excess reporter group which contains a primary amine in the presence of suitable condensing



agents, such as water-soluble carbodiimides, at temperatures in the range of about -20° C to 50° C (preferably 20°C to 40°C) for 6 to 72 hours. Preferred reporter groups of this class include (amino-alkyl)-amino-naphthalene-1,2-dicarboxylic acid hydrazides, amino-fluoresceins, aminorhodamines, aminoalkyl luminols, aminoalkylaminobenzenesulfonyl adducts, amino sugars, and the like. Furthermore, the chemical synthesis of the initial oligonucleotide product may be accomplished with modified nucleotide monomers wherein, prior to the coupling reaction, such reporter groups are attached to the substituent group. If any such reporter groups would adversely affect the coupling reaction, they are appropriately masked to forestall any such adverse effect. On the other hand, certain other reporter groups are substantially unreactive with respect to the coupling reaction and therefore do not require masking. For example, nitrophenyl adducts may be attached to the substituent group prior to the coupling reaction, and without masking, may be present on the modified nucleotide monomer during the coupling reaction without adverse effect.

Reporter groups useful in the method of this invention generally include aromatic, polyaromatic, cyclic, and polycyclic organic moieties which are further functionalized by inclusion of heteroatoms such as nitrogen, oxygen, sulfur and the like.

Product oligonucleotides can include more than one type of modification or more than one modified base. An illustrative example of an oligonucleotide of this type is one of the structure:



wherein C^m is 5-(3-aminopropyl) cytosine, U^m is 5-[N-(4-aminobutyl)-1-acrylamido]uracil, and A^m is 8-[6-2,4-dinitrophenyl)-aminohexyl]aminoadenine.

This product is further modified by reaction with
5 fluorescein isothiocyanate to provide a fluorescein reporter group on C^m and U^m .

Such a product oligonucleotide illustrates the variety of the selection of modified and unmodified nucleotide units in a product oligonucleotide made
10 possible by the process of the present invention. More specifically, such oligonucleotide illustrates the use of more than one type of nucleotide unit having its base modified by substituent groups having bound thereto reporter groups providing the same or
15 different types of reporter groups function. Also illustrated are units whose bases are modified by substituent groups to which reporter groups are bound after the coupling reaction, i.e., C^m and U^m , whereas A^m is illustrative of a unit whose base is modified by
20 a substituent group to which a dinitrophenyl reporter group was attached prior to the coupling reaction. Such oligonucleotide additionally illustrates that it can include more than one nucleotide unit of the same type, and that it can include units having unmodified
25 bases intermixed with units having modified bases.

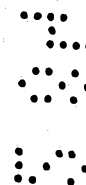
Instead of attaching reporter groups to the primary amines of the substituent groups as illustrated in Reaction 6, such amine or other group can alternatively be coupled to suitably activated solid
30 supports. This produces a defined sequence oligonucleotide which is covalently bound to such supports through the modified bases. Such solid supports are useful in the detection and isolation of complementary



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nucleic acid components. Alternatively, the modified nucleoside monomers can be coupled to solid supports prior to the chain extension coupling Reaction 4, to thereby provide solid supports for such monomers
5 during the coupling reaction.

The following specific examples are provided to enable those skilled in the art to practice the invention. The examples should not be considered limitations upon the scope of the invention, but merely as
10 being illustrative and representative thereof. To aid in structural clarification, references are made to the reactions illustrated in the aforementioned process diagram.



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EXAMPLE I

This example illustrates the synthesis of a modified nucleoside precursor 5-(3-trifluoroacetylaminopropenyl)-2'-deoxyuridine.

- 5 5-Chloromercuri-2'-deoxyuridine (3.6 g, 7.8 mmol) is suspended in 200 ml methanol. N-Allyltrifluoroacetamide (6.8 ml, 55 mmol) is added, followed by addition of 41 ml of 0.2 N lithium tetrachloropalladate in methanol. After 18 hours stirring at room temperature,
- 10 the reaction is gravity filtered to remove the black solid palladium, and the yellow methanolic filtrate is treated with five 200 mg portions of sodium borohydride, then concentrated under reduced pressure to solid residue. The residue is purified by flash column chromatography on silica gel eluting 15 vol % methanol in chloroform. Appropriately pure fractions of product are combined and concentrated under reduced pressure to give crystalline 5-(3-trifluoroacetylaminopropenyl)-2'-deoxyuridine (2.4 g). UV $\lambda_{\max}$ 291 nm (ϵ 7800), $\lambda_{\min}$
- 15 266 nm, (ϵ 4400); TLC (silica eluting 15 vol % methanol in chloroform) R_f = 0.4.
- 20

EXAMPLE II

This example illustrates the synthesis of a modified nucleoside precursor 5-[N-(trifluoroacetylminoheptyl)-1-acrylamido]-2'-deoxyuridine.

- 25 5-Chloromercuri-2'-deoxyuridine (3.6 g, 7.8 mmol) is suspended in 200 ml methanol. N-(7-trifluoroacetylaminohexyl)-acrylamide (55 mmol) is added, followed by addition of 41 ml of 0.2 N lithium tetrachloropalladate in
- 30 methanol. After 18 hours stirring at room temperature, the reaction is gravity filtered to remove the black solid palladium, and the yellow methanolic filtrate is treated with five 200 mg portions of sodium borohydride,



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then concentrated under reduced pressure to solid residue. The residue is purified by flash column chromatography on silica gel eluting 10 vol % methanol in chloroform. Appropriately pure fractions of product are
5 combined and concentrated under reduced pressure to give crystalline 5-[N-(7-trifluoroacetylaminohexyl)-1-acrylamido]-2'-deoxyuridine (2.8 g). UV $\lambda_{\max}$ 302 nm (ϵ 18000), $\lambda_{\min}$ 230 nm, 280 nm; TLC (silica eluting 15 vol % methanol in chloroform) R_f = 0.3.

10

EXAMPLE III

This example illustrates masking of 5'-hydroxyl to produce 5'-dimethoxytrityl-5-(3-trifluoroacetylaminopropenyl)-2'-deoxyuridine as illustrated in Reaction 2.

15

5-(3-trifluoroacetylaminopropenyl)-2'-deoxyuridine (2.4 g) is thoroughly evaporated twice from pyridine, then stirred in 40 ml pyridine. Dimethoxytrityl(DMT)chloride(2.3 g, 6.6 mmol) is added, and the mixture stirred at room temperature for four hours.

20

After thin layer chromatography (TLC) on silica eluting 10 vol % methanol in chloroform indicates reaction is complete, the reaction is concentrated to a solid residue.

25

This residue is purified by column chromatography on silica eluting chloroform until all faster running impurities have eluted, then bringing off product with 5 vol % methanol in chloroform. The residue is then concentrated to give 5'-dimethoxytrityl-5-(3-trifluoroacetylaminopropenyl)-2'-deoxyuridine as a white fluffy solid (4 g). Product decomposes upon heating; UV $\lambda_{\max}$

30

291 nm, $\lambda_{\min}$ 266 nm; TLC R_f 0.6 on silica eluting 10 vol % methanol in chloroform.



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EXAMPLE IV

This example illustrates hydrogenation of exocyclic double bond and 5'-hydroxyl masking to produce 5'-dimethoxytrityl-5-(3-trifluoroacetylaminopropyl)-2'-deoxyuridine.

Repeating the nucleoside precursor synthesis and 5'-hydroxyl masking procedures of Examples I and III, but, prior to the addition of the DMT chloride, subjecting the purified 5-(3-trifluoroacetylaminopropenyl)-2'-deoxyuridine to two atmospheres of hydrogen while stirring at room temperature in methanol over 10% palladium-on-carbon catalyst is productive of 5'-dimethoxytrityl-5-(3-trifluoroacetylaminopropyl)-2'-deoxyuridine.

15 Examples V to VIII illustrate the synthesis of additional modified uracil nucleosides, and subsequent masking of 5'-hydroxyls as represented by Reaction 2.

EXAMPLE V

Repeating the nucleoside precursor synthesis and 20 5' hydroxyl masking procedures of Examples I and III, but replacing N-allyltrifluoroacetamide with compounds numbered 1 through 8 below is productive of the compounds numbered 1' through 8' below, respectively; i.e. substitution of.

- 25 1 N-(3-butenyl)trichloroacetamide
2 N-(5-hexenyl)trifluoroacetamide



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- 3 N-(2-methyl-2-propenyl)trifluoroacetamide
4 N-(4-ethenylphenylmethyl)trifluoroacetamide
5 N-(1-methyl-3-butenyl)trifluoroacetamide
6 N-(12-trichloroaminododecyl)acrylamide
5 7 N-(pertrifluoroacetylpolyllysyl)acrylamide
8 N-(3-trifluoroacetylamidopropyl)acrylamide
 is productive of
1' 5'-dimethoxytrityl-5-(4-trichloroacetylaminobuten-1-yl)-2'-deoxyuridine
 10 2' 5'-dimethoxytrityl-5-(6-trifluoroacetylaminohexen-1-yl)-2'-deoxyuridine
3' 5'-dimethoxytrityl-5-(3-trifluoroacetyl-amino-2-methyl-propen-1-yl)-2'-deoxyuridine
4' 5'-dimethoxytrityl-5-[2-(4-trifluoroacetylaminomethyl-phenyl)ethen-1-yl]-2'-deoxyuridine
 15 5' 5'-dimethoxyltrityl-5-(4-trifluoroacetyl-amino-4-methyl-buten-1-yl)-2'-deoxyuridine
6' 5'-dimethoxytrityl-5-[N-(12-trichloroacetylaminododecyl)-1-acrylamido]-2'-deoxyuridine
 20 7' 5'-dimethoxytrityl-5-[N-(pertrifluoroacetylpolyllysyl)-1-acrylamido]-2'-deoxyuridine
8' 5'-dimethoxytrityl-5-[N-(3-trichloroacetyl-amino-propyl)-acrylamido]-2'-deoxyuridine

EXAMPLE VI

- 25 Repeating the 5' hydroxyl masking procedure of Example III but replacing 5-(3-trifluoroacetylaminopropenyl)-2'-deoxyuridine with the 5-substituted-2'-deoxyuridines numbered 9 through 18 below is productive of the products numbered 9' through 18' below, respectively;
 30 i.e. substituting



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- 9 5-(propen-1-yl)-2'-deoxyuridine
10 5-(carbmethoxyethyl)-2'-deoxyuridine
11 5-(3-carbmethoxyprop-1-yl)-2'-deoxyuridine
12 5-(4-carbmethoxy-2-methylbuten-1-yl)-2'-deoxyuridine
5 13 5-(3-cyanopropen-1-yl)-2'-deoxyuridine
14 5-(4-cyano-2-methylbuten-1-yl)-2'-deoxyuridine
15 5-[2-(4-carbmethoxyphenyl)ethen-1-yl]-2'-deoxyuridine
16 5-(4-acetoxybuten-1-yl)-2'-deoxyuridine
17 5-(4-acetoxybut-1-yl)-2'-deoxyuridine
10 18 5-[4-(2,4-dinitrophenyl)butyl]-2'-deoxyuridine

is productive of the following 5'-dimethoxytrityl-5-alkyl-2'-deoxyuridines

- 9' 5'-dimethoxytrityl-5-(propen-1-yl)-2'-deoxyuridine
10' 5'-dimethoxytrityl-5-(2-carbmethoxyethyl)-2'-deoxy-
15 uridine
11' 5'-dimethoxytrityl-5-(3-carbmethoxyprop-1-yl)-2'-
deoxyuridine
12' 5'-dimethoxytrityl-5-(4-carbmethoxy-2-methylbuten-
1-yl)-2'-deoxyuridine
20 13' 5'-dimethoxytrityl-5-(3-cyanopropen-1-yl)-2'-deoxy-
uridine
14' 5'-dimethoxytrityl-5-(4-cyano-2-methylbuten-1-yl)-
2'-deoxyuridine
15' 5'-dimethoxytrityl-5-[2-(4-carbmethoxyphenyl)ethen-
25 1-yl]-2'-deoxyuridine
16' 5'-dimethoxytrityl-5-(4-acetoxybuten-1-yl)-2'-
deoxyuridine
17' 5'-dimethoxytrityl-5-(4-acetoxybut-1-yl)-2'-deoxy-
uridine
30 18' 5'-dimethoxytrityl-5-[4-(2,4-dinitrophenyl)butyl]-
2'-deoxyuridine



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EXAMPLE VII

Repeating the nucleoside precursor synthesis and 5'-hydroxyl masking procedures of Examples I-VI, but replacing 5-chloromercuri-2'-deoxyuridine with 5-chloro-mercuriuridine is productive of the corresponding 5'-dimethoxytrityl-5-substituted uridines.

Examples VIII to XI illustrate the synthesis of modified cytosine nucleosides. Since cytosine nucleosides, as well as adenosine nucleosides, have reactive groups on their bases unlike the uracil nucleosides, such reactive groups are masked to prevent unwanted reactions therewith. These examples illustrate masking of reactive groups on the cytosine base moiety as in Reaction 1, as well as masking of the 5'-hydroxyl as in Reaction 2.

EXAMPLE VIII

5-(3-trifluoroacetylaminopropenyl)-
N⁴-benzoyl-2'-deoxycytidine

Repeating the nucleoside precursor synthesis procedure of Example I, but replacing 5-chloromercuri-2'-deoxyuridine with 5-chloromercuri-2'-deoxycytidine is productive of 5-(3-trifluoroacetylaminopropenyl)-2'-deoxycytidine (UV λ_{max} 287 nm). Purified 5-(3-trifluoroacetylaminopropenyl)-2'-deoxycytidine (1.3 g, 4.6 mmol) is stirred in 80 ml anhydrous ethanol, benzoyl anhydride (1.5 g, 7 mmol) is added, and the reaction



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refluxed. Five additional 1.5 g portions of benzoyl anhydride are added hourly. After the reaction is judged complete by thin layer chromatography [silica plates eluting n-butanol/methanol/conc $\text{NH}_4\text{OH}/\text{H}_2\text{O}$ (60: 5 20:1:20)] in 6-10 hours, the reaction is cooled and concentrated under reduced pressure to a semisolid. The solid is triturated with ether three times, decanted and dried. The crude product is crystallized from water to give chromatographically pure N^4 -benzoyl-5- 10 (3-trifluoroacetylaminopropenyl)-2'-deoxycytidine as a white solid. The product decomposes above 120°C ; UV max 311 nm.

EXAMPLE IX

15 5'-Dimethoxytrityl-5-(3-trifluoroacetylaminopropenyl)- N^4 -benzoyl-2'-deoxycytidine

Repeating the 5'-hydroxyl masking procedure of Example III, but replacing 5-(3-trifluoroacetylaminopropenyl)-2'-deoxyuridine with 5-(3-trifluoroacetylaminopropenyl)- N^4 -benzoyl-2'-deoxycytidine is productive 20 of 5'-dimethoxytrityl-5-(3-trifluoroacetylaminopropenyl)- N^4 -benzoyl-2'-deoxycytidine.

EXAMPLE X

Repeating the nucleoside precursor synthesis and 5' hydroxyl masking procedures of Examples VIII and 25 IX, but replacing N-allyltrifluoroacetamide with the N-alkyltrifluoroacetamides of Example V is productive of the corresponding 5'-dimethoxytrityl-5-(trifluoroacetyl aminoalkyl)- N^4 -benzoyl-2'-deoxycytidines, viz:

30 5'-dimethoxytrityl-5-(4-trifluoroacetylaminobuten-1-yl)- N^4 -benzoyl-2'-deoxycytidine

5'-dimethoxytrityl-5-(6-trifluoroacetylaminohexen-1-yl)- N^4 -benzoyl-2'-deoxycytidine

5'-dimethoxytrityl-5-(3-trifluoroacetyl-amino-2-methylpropen-1-yl)- N^4 -benzoyl-2'-deoxycytidine



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- 5'-dimethoxytrityl-5-[2-(4-trifluoroacetylaminomethyl-phenyl)ethen-1-yl]-N⁴-benzoyl-2'-deoxycytidine
- 5'-dimethoxytrityl-5-(4-trifluoroacetyl-amino-4-methyl-buten-1-yl)-N⁴-benzoyl-2'-deoxycytidine
- 5 5'-dimethoxytrityl-5-[N-(12-trifluoroacetylaminodecyl)-1-acrylamido]-N⁴-benzoyl-2'-deoxycytidine
- 5'-dimethoxytrityl-5-[N-(pertrifluoroacetylpolylsyl)-1-acrylamido]-N⁴-benzoyl-2'-deoxycytidine

EXAMPLE XI

- 10 Synthesis of 5'-dimethoxytrityl-N⁴-benzoyl-5-(2-carbmethoxyethenyl)-2'-deoxycytidine

- 5-(2-Carbmethoxyethenyl)-2'-deoxycytidine (0.82 g, 2.6 mmol) is stirred in 50 ml anhydrous ethanol. Benzoic anhydride (500 mg, 2.2 mmol) is added, and the
- 15 reaction heated to reflux. Five additional 500 mg portions of benzoic anhydride are added hourly. After the reaction is judged complete by thin layer chromatography (usually 6-8 hours) the reaction is cooled, and evaporated under reduced pressure to a yellow semi-
- 20 solid. Chromatography on silica gel eluting a linear 1:19 to 1:3 methanol/chloroform mixture followed by thorough evaporation of appropriately combined fractions gives N⁴-benzoyl-5-(2-carbmethoxyethenyl)-2'-deoxycytidine as an amorphous white solid. UV $\lambda_{\max}$ 296 nm,
- 25 $\lambda_{\min}$ 270 nm. The solid is dried thoroughly, and dissolved in 20 ml pyridine. Dimethoxytrityl chloride (1.1 eq) is added, and the reaction stirred at ambient temperature for six hours. Concentration to a solid followed by column chromatography on silica gel eluting
- 30 10% methanol in chloroform yields 5'-dimethoxytrityl-N⁴-benzoyl-5-(2-carbmethoxyethenyl)-2'-deoxycytidine as a fluffy off-white solid.



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EXAMPLE XII

Repeating the nucleoside precursor synthesis procedure of Example XI, but replacing 5-(2-carbmethoxyethenyl)-2'-deoxycytidine with the following compounds

5 numbered 19 through 27 below is productive of the corresponding compounds numbered 19' through 27' below, respectively, i.e., substituting:

- 19 5-(2-carbmethoxyethyl)-2'-deoxycytidine
20 5-(3-carbmethoxyprop-1-yl)-2'-deoxycytidine
10 21 5-(4-carbmethoxy-2-methylbuten-1-yl)-2'-deoxycytidine
22 5-(3-cyanopropen-1-yl)-2'-deoxycytidine
23 5-(4-cyano-2-methylbuten-1-yl)-2'-deoxycytidine
24 5-[2-(4-carbmethoxyphenyl)ethen-1-yl]-2'-deoxycytidine
15 25 5-(4-acetoxybuten-1-yl)-2'-deoxycytidine
26 5-(4-acetoxybut-1-yl)-2'-deoxycytidine
27 5-[4-(2,4-dinitrophenyl)butyl]-2'-deoxycytidine

is productive of the following 5'-dimethoxytrityl-N⁴-benzoyl-5-alkyl-2'-deoxycytidines:

- 20 19' 5'-DMT-N⁴-benzoyl-5-(2-carbmethoxyethen-1-yl)-2'-deoxycytidine
20' 5'-DMT-N⁴-benzoyl-5-(3-carbmethoxyprop-1-yl)-2'-deoxycytidine
25 21' 5'-DMT-N⁴-benzoyl-5-(4-carbmethoxy-2-methylbuten-1-yl)-2'-deoxycytidine
22' 5'-DMT-N⁴-benzoyl-5-(3-cyanopropen-1-yl)-2'-deoxycytidine
23' 5'-DMT-N⁴-benzoyl-5-(4-cyano-2-methylbuten-1-yl)-2'-deoxycytidine
30 24' 5'-DMT-N⁴-benzoyl-5-[2-(4-carbmethoxyphenyl)ethen-1-yl]-2'-deoxycytidine
25' 5'-DMT-N⁴-benzoyl-5-(4-acetoxybuten-1-yl)-2'-deoxycytidine



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- 26' 5'-DMT-N⁴-benzoyl-5-(4-acetoxybut-1-yl)-2'-deoxycytidine
27' 5'-DMT-N⁴-benzoyl-5-[4-(2,4-dinitrophenyl)butyl]-2'-deoxycytidine

5 Similarly, the use of the other acid anhydrides, e.g., acetic anhydride, anisoyl anhydride, or tolyl anhydride, is productive of the corresponding N⁴-acyl or N⁴-acetyl 5-alkyl-2'-deoxycytidines of Examples X and XI wherein benzoyl is replaced by acetyl or acyl.

10

EXAMPLE XIII

Repeating the nucleoside precursor synthesis and 5'-hydroxyl masking procedures of Examples VIII to X, but replacing 5-chloromercuri-2'-deoxycytidine with 5-chloromercuricytidine is productive of the corresponding 5'-dimethoxytrityl-N⁴-benzoyl-5-substituted cytidines.

EXAMPLE XIV

This example typifies the masking of reactive base moieties and the masking of 5'hydroxyl of adenine nucleosides.

N⁶-benzoyl-8-(6-aminohexyl)amino-2'-deoxyadenosine (4 mmol) is stirred in 60 ml anhydrous ethanol. Tri-fluoroacetic anhydride (6 mmol) is added, and the reaction stirred at room temperature. Two additional portions of trifluoroacetic anhydride are added hourly.



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After four hours, the reaction is concentrated to a solid residue, and lyophilized overnight. The crude N⁶-benzoyl-8-(6-trifluoroacetylaminohexyl)amino-2'-deoxyadenosine is dried thoroughly and concentrated to a solid residue twice from pyridine. The solid is stirred in 40 ml of pyridine, and dimethoxytrityl chloride (6.5 mmol) is added. After four hours, the reaction is concentrated to leave a solid residue. Purification by column chromatography on silica gel eluting a multi-step gradient of 0 to 15% methanol in chloroform gives 5'-dimethoxytrityl-N⁶-benzoyl-8-(6-trifluoroacetylaminohexyl)amino-2'-deoxyadenosine as an off-white solid.

15 Examples XV to XVII typify the activation of 5'-masked 5-substituted, and naturally occurring nucleosides, to their respective phosphomonochloridites, as illustrated in Reaction 3 of the diagram.

EXAMPLE XV

20 Preparation of 3'-phosphomonochloridite of 5'-DMT-5-(3-trifluoroacetylaminoprop-1-yl)-2'-deoxyuridine

25 Dry 5'-DMT-5-(3-trifluoroacetylaminoprop-1-yl)-2'-deoxyuridine (1.54 g, 2.2 mmol) is lyophilized from 20 ml benzene three times for more than twelve hours each to remove residual water and solvents. The resulting very fluffy white power is transferred to a nitrogen



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atmosphere while still under vacuum and dissolved in anhydrous acetonitrile containing 5 vol % 2,6-lutidine to a final nucleoside concentration of 30 mM. While swirling vigorously under nitrogen, one rapid bolus of methylphosphodichloridite (1.0 eq) is added by syringe. The reaction is swirled for about one minute under nitrogen. The resulting crude 5'-DMT-5-(3-trifluoroacetylaminoprop-1-yl)-2'-deoxyuridine 3'-methylphosphomonochloridite reaction solution is then used directly for deoxyoligonucleotide synthesis (Example XVIII) with no further purification, [^{31}P -NMR($\text{CH}_3\text{CN}/\text{CDCl}_3$) generally indicates 40-70 mol % desired product (167.5 ppm); remainder is composed of bis-3',3'-[5'-DMT-5-(3-trifluoroacetylaminoprop-1-yl)-2'-deoxyuridylyl] methylphosphite (140 ppm) and 5'-DMT-5-(3-trifluoroacetylaminoprop-1-yl)-2'-deoxyuridine 3'-methylphosphonate (9.5 ppm), the latter product being formed in amounts reflecting the presence of water in the reaction.]

EXAMPLE XVI

Preparation of 3'-phosphomonochloridites of the naturally-occurring 2'-deoxynucleosides

Repeating the procedure of Example XV, but replacing 5'-DMT-5-(3-trifluoroacetylaminoprop-1-yl)-2'-deoxyuridine with:

- 5'-DMT-2'-deoxythymidine
- 5'-DMT- N^4 -benzoyl-2'-deoxycytidine
- 5'-DMT- N^6 -benzoyl-2'-deoxyadenosine
- 5'-DMT- N^2 -isobutyryl-2'-deoxyguanosine

is productive of the corresponding phosphomonochloridites, viz.:

5'-DMT-2'-deoxythymidine 3'-methylphosphomonochloridite
5'-DMT- N^4 -benzoyl-2'-deoxycytidine 3'-methylphosphomonochloridite



5'-DMT-N⁶-benzoyl-2'-deoxyadenosine 3'-methylphospho-
monochloridite

5'-DMT-N²-isobutyryl-2'-deoxyguanosine 3'-methylphosphomonochloridite

EXAMPLE XVII

10 3'-phosphomonochloridites, viz.:

5'-DMT-5-(3-trifluoroacetylaminopropyl)-2'-deoxyuridine
3'-o-chlorophenylphosphomonochloridite

5'-DMT-2'-deoxythymidine 3'-o-chlorophenylphosphomono-
chloridite

15 5'-DMT-N⁴-benzoyl-2'-deoxycytidine 3'-o-chlorophenyl-
phosphomonochloridite

5'-DMT-N⁶-benzoyl-2'-deoxyadenosine 3'-o-chlorophenyl-
phosphomonochloridite

5'-DMT-N²-isobutyryl-2'-deoxyguanosine 3'-o-chloro-
20 phenylphosphomono-chloridite

Similarly, the use of p-chlorophenylphosphodichloridite is productive of the analogous 3'-p-chlorophenylphosphomonochloridite adducts. [^{32}P]NMR ($\text{CH}_3\text{CN CDCl}_3$) of o-chlorophenylphosphomonochloridite products 160.7,

25 160.5 ppm (diastereomers).

Examples XVIII - XXIV typify the chemical synthesis of oligonucleotides which incorporate modified bases, as illustrated by Reactions 4 and 5 in the diagram.



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EXAMPLE XVIII

Synthesis of deoxyoligonucleotides containing
5-(3-aminopropyl)-uracil and naturally-
occurring nucleotide units

5 The phosphomonochloridite synthesis procedures of
Examples XV and XVI are accomplished immediately
before deoxyoligonucleotide synthesis, and the result-
ing products are used directly as 30 mM crude 3'-methyl-
phosphomonochloridites in anhydrous acetonitrile/5
10 vol % 2,6-lutidine.

Solid support (5-DMT-N⁶-benzoyl-2'-deoxyadenosine
3'-succinamidepropyl silica, 250 mg, 20 μ eq) is put
into a suitable reaction flow vessel (glass or Teflon[®]
column or funnel). The solid support is preconditioned
15 by successive treatments with acetonitrile/5 vol % luti-
dine, 2 w/v % iodine in tetrahydrofuran/water/lutidine
for 2 minutes, acetonitrile/5% lutidine, chloroform,
4 vol % dichloroacetic acid in chloroform for 2.5 min-
utes, and acetonitrile/5% lutidine, where treatments are
20 total volumes of 5-15 ml in either 2 or 3 portions or
by constant flow, as desired.

The deoxyoligonucleotide is synthesized in accord-
ance with Reaction 4 by sequential addition of the
desired activated 5'-DMT-nucleoside 3'-methylphospho-
25 monochloridite monomer and coupling thereof to the free
5'-hydroxyl of the terminal unit of the growing nucleo-
tide chain, which unit is initially the only unit of
the chain, i.e., the deoxyadenosine based unit compris-
ing the solid support. Additions are by reaction of
30 10 ml of the crude 30 mM monochloridites chosen from
Examples XV and XVI with the now-unprotected 5' hydroxyl
of the chain in either 2 or 3 portions or by constant
flow, for 2 to 6 minutes. The first phosphomonochlori-
dite addition followed by one complete reagent cycle
35 consists of sequential treatments with:



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- 5'-DMT-5-(3-trifluoroacetylaminopropyl)-2'-deoxyuridine
3'-methylphosphomonochloridite
-acetonitrile/lutidine wash
-capping for 5 minutes with 0.3 M 4-dimethylaminopyridine
5 in acetic anhydride/lutidine/tetrahydrofuran (1:3:2)
-acetonitrile/5% lutidine wash
-oxidation with 2% iodine in tetrahydrofuran/water/
lutidine (6:2:1) for 2 minutes
-acetonitrile/5% lutidine wash
10 -chloroform wash
-removal of DMT by 2.5 minute treatment with 4 vol %
dichloroacetic acid in chloroform
-chloroform wash
-acetonitrile/lutidine wash
15 The above cycle is repeated thirteen times, each time
replacing 5'-DMT-5-(3-trifluoroacetylaminopropyl)-2'-
deoxyuridine 3'-methylphosphomonochloridite with a
different one of the following 3'-methylphosphomono-
chloridites:
20 5'-DMT-2'-deoxythymidine 3'-methylphosphomonochloridite
5'-DMT-5-(3-trifluoroacetylaminopropyl)-2'-deoxyuridine
3'-methylphosphomonochloridite
5'-DMT-N⁶-benzoyl-2'-deoxyadenosine 3'-methylphosphomono-
chloridite
25 5'-DMT-N⁴-benzoyl-2'-deoxycytidine 3'-methylphosphomono-
chloridite
5'-DMT-N²-isobutyryl-2'-deoxyguanosine 3'-methylphospho-
monochloridite
5'-DMT-5-(3-trifluoroacetylaminopropyl)-2'-deoxyuridine
30 3'-methylphosphomonochloridite
5'-DMT-2'-deoxythymidine 3'-methylphosphomonochloridite
5'-DMT-5-(3-trifluoroacetylaminopropyl)-2'-deoxyuridine
3'-methylphosphomonochloridite



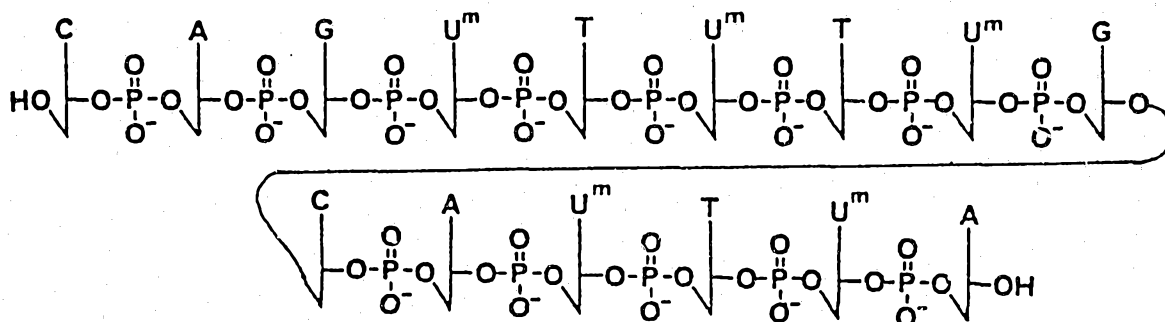
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- 5'-DMT-deoxythmidine 3'-methylphosphomonochloridite
5'-DMT-5-(3-trifluoroacetylaminopropyl)-2'-deoxyuridine
3'-methylphosphomonochloridite
5'-DMT-N²-isobutyryl-2'-deoxyguanosine 3'-methylphosphomonochloridite
5'-DMT-N⁶-benzoyl-2'-deoxyadenosine 3'-methylphosphomonochloridite
5'-DMT-N⁴-benzoyl-2'-deoxycytidine 3'-methylphosphomonochloridite
- 10 in respective order, deleting dichloroacetic acid treatment during the last reagent cycle. The support is transferred and treated with 2 ml concentrated ammonium hydroxide for 4 hours at ambient temperature to release the product from the support. The supernatant is
15 removed, the solid washed three times with 0.5 ml concentrated ammonium hydroxide, and the combined supernatants are sealed and heated at 50°C overnight. The clear yellow supernatant is lyophilized thoroughly. Initial purification is accomplished by reverse phase
20 high pressure liquid chromatography (HPLC) on an RP-8 (C-8) column eluting a 60 minute linear gradient of 0 to 30% vol % acetonitrile in 25 mM ammonium acetate, pH 6.8. The 5'-DMT-terminated product, eluting as a sharp peak at about 40 minutes, is collected; all shorter chains,
25 both capped and uncapped, elute before 25 minutes. The collected product is evaporated to a solid residue, treated with 80% acetic acid at ambient temperature for 20 minutes (to remove DMT), lyophilized to a solid residue, and dissolved in a small amount of aqueous
30 buffer. The product, generally greater than 90% homogeneous after HPLC, is further purified by conventional electrophoresis on 20% polyacrylamide gels (1 to 6 mm thick) by excision and extraction of the appropriate product band (product generally migrates slower than



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unmodified deoxyoligonucleotides of similar length). The purified 5-aminopropyl-uracil-containing pentadeca-deoxyoligonucleotide product illustrated diagrammatically below, wherein U^m = 5-(3 aminopropyl)uracil, is thereby produced.



Note the conventional deprotection of the oligonucleotide with ammonia has also removed the trifluoroacetyl masking group on the substituent.

The length and sequence of this oligonucleotide may then be determined by ^{32}P -kinasing and sequencing using suitable protocols, for example the protocols heretofore used to determine the length and sequence of the prior art oligonucleotides in which the bases of the nucleotide units therein are unmodified.

Similarly, intentional variation of the order and number of methylphosphomono-chloridite additions employed here is productive of other 5-(modified)uracil-containing deoxyoligonucleotides which vary in selected length and base sequence. In addition, replacement of the nucleoside 3'-methylphosphomono-chloridite adducts of Examples XV and XVI with the corresponding 3'-o- or p-chlorophenylphosphomono-chloridite adducts of Example XVII and inclusion of pyridinium oximate treatment to remove chlorophenyl blocking groups (at the end of the deoxyoligonucleotide synthesis and before concentrated



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ammonium hydroxide treatment) is productive of the same deoxyoligonucleotide products.

EXAMPLE XIX

Repeating the phosphomonochloridite and deoxyoligo-
 5 nucleotide synthesis procedures of Examples XV to XVIII,
 but replacing 5'-DMT-5-(3-trifluoroacetylaminopropyl)-2'-
 deoxyuridine with the 5'-DMT-5-alkyl-2'-deoxyuridines
 numbered 28 through 36 below is productive of the
 corresponding oligonucleotides having uracil bases U^m
 10 numbered 28' through 36' below, respectively, i.e.,
 substituting:

- 28 5'-dimethoxytrityl-5-(3-trifluoroacetylaminopropen-
1-yl)-deoxyuridine
- 29 5'-dimethoxytrityl-5-(4-trifluoroacetylaminobut-
 15 1-yl)-2'-deoxyuridine
- 30 5'-dimethoxytrityl-5-(4-trifluoroacetylaminobuten-
1-yl)-2'-deoxyuridine
- 31 5'-dimethoxytrityl-5-(6-trifluoroacetylaminohex-1-
yl)-2'-deoxyuridine
- 20 32 5'-dimethoxytrityl-5-(6-trifluoroacetylaminohexen-1-
yl)-2'-deoxyuridine
- 33 5'-dimethoxytrityl-5-(2-trifluoroacetylaminoprop-2-
yl)-2'-deoxyuridine
- 34 5'-dimethoxytrityl-5-(3-trifluoroacetyl-amino-2-
 25 methyl-propen-1-yl)-2'-deoxyuridine
- 35 5'-dimethoxytrityl-5-(3-trifluoroacetyl-amino-2-
methyl-prop-1-yl)-2'-deoxyuridine
- 36 5'-dimethoxytrityl-5-[2-(4-trichluoroacetyl-amino-
methylphenyl)ethen-1-yl]-2'-deoxyuridine
- 30 37 5'-dimethoxytrityl-5-[N-(pertrifluoroacetylpolyl-
lysyl)-1-acrylamido]-2'-deoxyuridine
- 38 5'-DMT-5-[N-(7-trifluoroacetylaminohexyl)-1-
acrylamido]-2'-deoxyuridine



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is productive of the deoxynucleotides corresponding to the product of Example XVIII, wherein U^m is:

- 28' 5-(3-aminopropen-1-yl) uracil
- 29' 5-(4-aminobut-1-yl) uracil
- 5 30' 5-(4-aminobuten-1-yl) uracil
- 31' 5-(6-aminohe~~x~~-1-yl) uracil
- 32' 5-(6-aminohexen-1-yl) uracil
- 33' 5-(3-aminoprop-2-yl) uracil
- 34' 5-(3-amino-2-methylpropen-1-yl) uracil
- 10 35' 5-(3-amino-2-methylprop-1-yl) uracil
- 36' 5-[2-(4-aminoethylphenyl)ethen-1-yl] uracil
- 37' 5-[N-(polylysyl)-1-acrylamido] uracil
- 38' 5-[N-(7-aminoheptyl)-1-acrylamido] uracil
- 15 Similarly, by employing other 5'-DMT-5-(acylaminoalkyl)-2'-deoxyuridines, the analogous deoxyoligonucleotides are produced.

EXAMPLE XX

- Repeating the phosphomonochloridite and deoxyoligo-
- 20 nucleotide synthesis procedures of Examples XV to XVIII, but replacing 5'-DMT-5-(3-acetylaminopropyl)-2'-deoxyuridine with 5-substituted-2'-deoxyuridines numbered 37 through 46 below is productive of the corresponding oligonucleotides having the U^m uracil bases numbered
- 25 37' through 46', below respectively; i.e., substituting:
- 37 5'-DMT-5-(propen-1-yl)-2'-deoxyuridine
 - 38 5'-DMT-5-(2-carbmethoxyethyl)-2'-deoxyuridine
 - 39 5'-DMT-5-(3-carbmethoxyprop-1-yl)-2'-deoxyuridine
 - 40 5'-DMT-5-(4-carbmethoxy-2-methylbuten-1-yl)-2'-
 - 30 deoxyuridine
 - 41 5'-DMT-5-(3-cyanopropen-1-yl)-2'-deoxyuridine
 - 42 5'-DMT-5-(4-cyano-2-methylbuten-1-yl)-2'-deoxyuridine

- 43 5'-DMT-5-[2-(4-carbomethoxyphenyl)ethen-1-yl]-2'-
deoxyuridine.
44 5'-DMT-5-(4-acetoxybuten-1-yl)-2'-deoxyuridine
45 5'-DMT-5-(4-acetoxybut-1-yl)-2'-deoxyuridine
5 46 5'-DMT-5-[4-(2,4-dinitrophenyl)butyl]-2'-deoxy-
uridine

is productive of the products wherein, U^m is:

- 37' 5-(propen-1-yl)uracil
38' 5-(2-carboxyethyl)uracil
10 39' 5-(3-carboxyprop-1-yl)uracil
40' 5-(4-carboxy-2-methylbuten-1-yl)uracil
41' 5-(3-cyanopropen-1-yl)uracil
42' 5-(4-cyano-2-methylbuten-1-yl)uracil
43' 5-[2-(4-carboxyphenyl)ethen-1-yl]uracil
15 44' 5-(4-hydroxybuten-1-yl)uracil
45' 5-(4-hydroxybut-1-yl)uracil
46' 5-[4-(2,4-dinitrophenyl)butyl]uracil

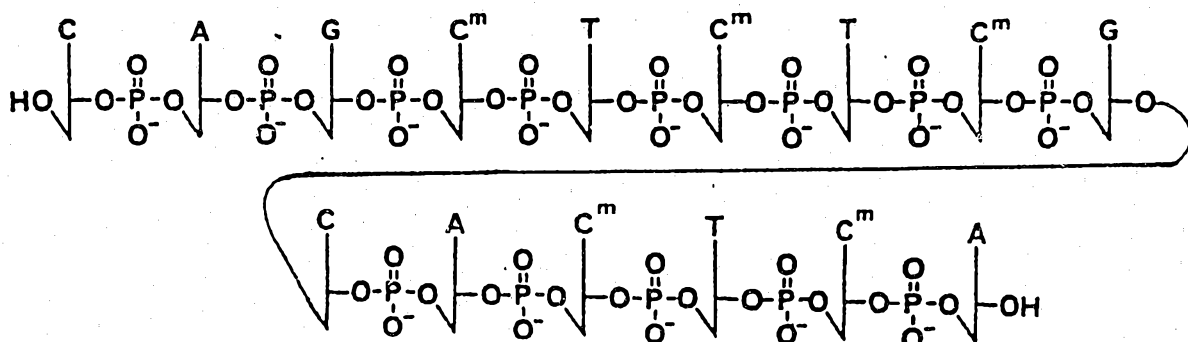
Note: In 46' the dinitrophenyl is a ligand recognition
type reporter group, i.e. use of antidinitrophenyl
20 antibody as the ligand. Similarly, by employing other
appropriate 5'-DMT-5-alkyl-2'-deoxyuridines, the analo-
gous deoxyoligonucleotides are produced.

EXAMPLE XXI

Repeating the procedures of Examples XV to XVIII,
25 but replacing 5'-DMT-5-(3-trifluoroacetylaminopropyl)-
2'-deoxyuridine with 5'-DMT- N^4 -benzoyl-5-(3-trichloro-
acetylaminopropyl)-2'-deoxycytidine is productive of
deoxyoligonucleotides as in Example XVIII wherein U^m
[5-(3-aminopropyl)-uracil] is replaced by 5-(3-amino-
30 propyl)cytosines. For example,



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where C^m = 5-(3-aminopropyl)cytosine.

EXAMPLE XXII

Repeating the deoxyoligonucleotide synthesis procedure of Example XXI but replacing 5'-DMT-N⁴-benzoyl-5-(3-trichloroacetylaminopropyl)-2'-deoxycytidine with compounds numbered 47 through 57 below is productive of the corresponding oligonucleotides having the C^m cytosine bases numbered 47' through 57' below, respectively, i.e., substituting:

- 5 47 5'-DMT-N⁴-benzoyl-5-(3-trifluoroacetylaminopropen-1-yl)-2'-deoxycytidine
- 10 48 5'-DMT-N⁴-benzoyl-5-(4-trifluoroacetylaminobut-1-yl)-2'-deoxycytidine
- 49 5'-DMT-N⁴-benzoyl-5-(4-trifluoroacetylaminobuten-1-yl)-2'-deoxycytidine
- 15 50 5'-DMT-N⁴-benzoyl-5-(6-trifluoroacetylaminohex-1-yl)-2'-deoxycytidine
- 51 5'-DMT-N⁴-benzoyl-5-(6-trifluoroacetylaminohexen-1-yl)-2'-deoxycytidine
- 52 5'-DMT-N⁴-benzoyl-5-(3-trifluoroacetylaminoprop-2-yl)-2'-deoxycytidine
- 20 53 5'-DMT-N⁴-benzoyl-5-(3-trifluoroacetyl-amino-2-methylpropen-1-yl)-2'-deoxycytidine
- 54 5'-DMT-N⁴-benzoyl-5-(3-trifluoroacetyl-amino-2-methylprop-1-yl)-2'-deoxycytidine
- 25 55 5'-DMT-N⁴-benzoyl-5[2-(4-trifluoroacetylaminomethyl-phenyl)ethen-1-yl]-2'-deoxycytidine

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- 56 5'-DMT-N⁴-benzoyl-5-[N-(pertrifluoroacetylpolyl-lysyl)-1-acrylamido]-2'-deoxycytidine
- 57 5'-DMT-N⁴-benzoyl-5-[N-(trifluoroacetylaminoheptyl)-acrylamido]-2'-deoxycytidine

5 is productive of the products wherein C^m is:

- 47' 5-(3-aminopropen-1-yl)cytosine
- 48' 5-(4-aminobut-1-yl)cytosine
- 49' 5-(4-aminobuten-1-yl)cytosine
- 50' 5-(6-aminohept-1-yl)cytosine
- 10 51' 5-(6-aminohepten-1-yl)cytosine
- 52' 5-(3-aminoprop-2-yl)cytosine
- 53' 5-(3-amino-2-methylpropen-1-yl)cytosine
- 54' 5-(3-amino-2-methylprop-1-yl)cytosine
- 55' 5-[2-(4-aminomethylphenyl)ethen-1-yl]cytosine
- 15 56' 5-[N-(polylysyl)-1-acrylamido]cytosine
- 57' 5-[N-(7-aminoheptyl)-1-acrylamido]cytosine

Similarly, by employing other N⁴-acyl-5-(acylaminoalkyl)-2'-deoxycytidines the analogous deoxyoligonucleotides are produced.

20

EXAMPLE XXIII

Repeating the deoxyoligonucleotide synthesis procedure of Example XXI, but replacing 5'-DMT-N⁴-benzoyl-5-(3-trifluoroacetylaminopropyl)-2'-deoxycytidine with the compounds numbered 58 through 68 below is productive of the corresponding oligonucleotides having the C^m cytosine bases numbrerred 58' through 68' below, respectively, i.e., substituting:



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- 58 5'-DMT-N⁴-benzoyl-5-(propen-1-yl)-2'-deoxycytidine
 59 5'-DMT-N⁴-benzoyl-5-(2-carbmethoxyethyl)-2' deoxy-
 cytidine
 60 5'-DMT-N⁴-benzoyl-5-(2-carbmethoxyethen-1-yl)-2'-
 5 deoxycytidine
 61 5'-DMT-N⁴-benzoyl-5-(3-carbmethoxyprop-1-yl)-2'-
 deoxycytidine
 62 5'-DMT-N⁴-benzoyl-5-(4-carbmethoxy-2-methylbuten-
 1-yl)-2'-deoxycytidine
 10 63 5'-DMT-N⁴-benzoyl-5-(3-cyanopropen-1-yl)-2'-deoxy-
 cytidine
 64 5'-DMT-N⁴-benzoyl-5-(4-cyano-2-methylbuten-1-yl)-
 2'-deoxycytidine
 65 5'-DMT-N⁴-benzoyl-5-[2-(4-carbmethoxyphenyl)ethen-
 1-yl]-2'-deoxycytidine
 15 66 5'-DMT-N⁴-benzoyl-5-(4-acetoxybuten-1-yl)-2'-
 deoxycytidine
 67 5'-DMT-N⁴-benzoyl-5-(4-acetoxybut-1-yl)-2'-
 deoxycytidine
 20 68 5'-DMT-N⁴-benzoyl-5-[4-(2,4-dinitrophenyl)butyl]-
 2-deoxycytidine

is productive of the products wherein C^m is;

- 58' 5-(propen-1-yl)cytosine
 59' 5-(2-carboxyethyl)cytosine
 25 60' 5-(2-carboxyethen-1-yl)cytosine
 61' 5-(3-carboxyprop-1-yl)cytosine
 62' 5-(4-carboxy-2-methylbuten-1-yl)cytosine
 63' 5-(3-cyanopropen-1-yl)cytosine
 64' 5-(4-cyano-2-methylbuten-1-yl)cytosine



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65' 5-[2-(4-carboxyphenyl)ethen-1-yl]cytosine

66' 5-(4-hydroxybuten-1-yl)cytosine

67' 5-(4-hydroxybut-1-yl)cytosine

68' 5-[4-(2,4-dinitrophenyl)butyl]cytosine

- 5 Similarly, by employing other appropriate 5'-DMT-N⁴-acyl-5-alkyl-2'-deoxycytidines the analogous deoxy-oligonucleotides are produced.

EXAMPLE XXIV

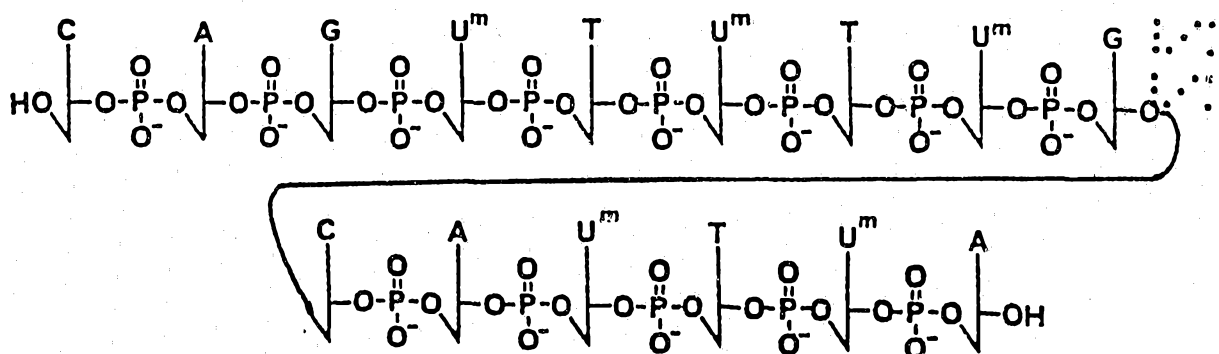
- 10 Repeating the phosphomonochloridite and deoxyoligo-nucleotide synthesis procedures of Examples XV-XXIII, but replacing 5'-DMT-5-(3-trifluoroacetylaminopropyl)-2'-deoxyuridine with 5'-DMT-N⁶-benzoyl-8-(6-trifluoroacetylaminohexyl)amino-2'-deoxyadenosine is productive
15 of deoxyoligonucleotides as in Example XVIII, except that the U^m is replaced by A^m, and A^m = 8-(6-aminohexyl)amino-2'-deoxyadenosine.

- Examples XXV to XXVIII typify the binding of reporter groups to oligonucleotides containing appropriately
20 modified bases, as illustrated in Reaction 6.

EXAMPLE XXV

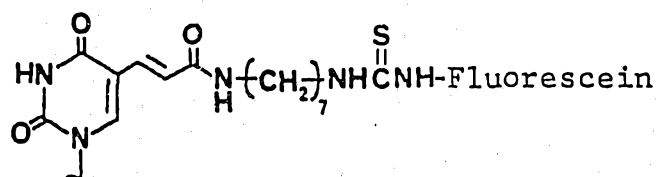
Fluoresceinated deoxyoligonucleotides

A purified pentadeca-nucleotide (from Example XVIII) of the structure



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[where U^m is 5-[N-(7-aminoheptyl)-1-acrylamido]uracil is dissolved at 25 A_{260} units per ml in aqueous 300mM sodium borate or sodium carbonate buffer, pH 9.5, containing 30 mM sodium chloride. Solid fluorescein isothiocyanate (0.5 mg per ml) is added, and the mixture sealed and shaken gently at 4 C to 25 C overnight. The reaction is chromatographed directly on a column of G-50 Sephadex[®] to separate unbound fluorescein adducts which are retained; the fluoresceinated deoxyoligo-nucleotide adducts elute near the void volume. Early fractions containing significant A_{260} units are combined and lyophilized to solid product of structure similar to the starting pentadecadeoxynucleotide where U^m is now either



or unreacted 5-[N-(7-aminoheptyl)-1-acrylamido]uracil. λ_{max} (H_2O) 262 nm, 498 nm.

Repeating the procedure on compounds recited in Examples XIX, XXI, XXII, and XXIV is productive of the corresponding fluoresceinated or polyfluoresceinated deoxyoligonucleotides in like manner.

EXAMPLE XXVI

Attachment of reporter groups other than fluorescein can be accomplished by repeating the procedure of Example



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~~XXV, but replacing fluorescein isothiocyanate with,~~

For example:

2,4-dinitrophenyl isothiocyanate

1-fluoro-2,4-dinitrobenzene

5 aminoethyl isoluminol isothiocyanate

aminoethylaminonaphthalene-1,2-carboxylic hydrazide
isothiocyanate

N,N'-bis (alkylsulfonyl)-N-aryl-N'-isothiocyanatoaryl-
dioxamide

10 m-sulfonyl aniline isothiocyanate

9-(N-hydroxysuccinimidyl) biotin

9-(N-hydroxysuccinimidyl carboxy)-N-methylacridine,
or cyanogenbromide-activated Sepharose[®]

is productive of the corresponding adducts wherein the

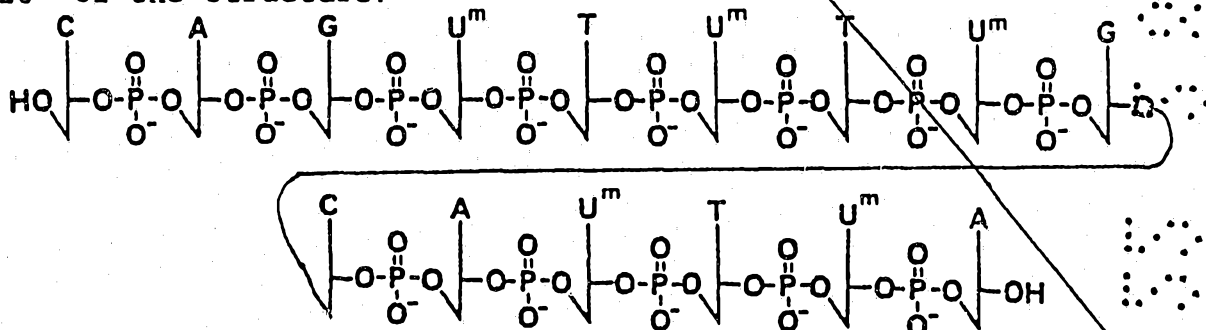
15 attached group is other than fluorescein.

EXAMPLE XXVII

Attachment of isoluminol and free primary
amine-containing reporter groups

A purified pentadecanucleotide from Example XVIII

20 of the structure:



[where U^m is 5-(2-carboxyethyl)uracil] is dissolved
in water at 30 A_{260} units per ml., and diluted with one
~~volume pyridine. Aminobutyl ethyl isoluminol is added~~



XXV, but replacing fluorescein isothiocyanate with,
for example:

2,4-dinitrophenyl isothiocyanate

1-fluoro-2,4-dinitrobenzene

5 aminoethyl isoluminol isothiocyanate

aminoethylaminonaphthalene-1,2-carboxylic hydrazide
isothiocyanate

N,N'-bis (alkylsulfonyl)-N-aryl-N'-isothiocyanatoaryl-
dioxamide

10 m-sulfonyl aniline isothiocyanate

N-hydroxysuccinimidyl biotin

9-(N-hydroxysuccinimidyl carboxy)-N-methylacridine,
or cyanogenbromide-activated Sepharose[®]

is productive of the corresponding adducts wherein the

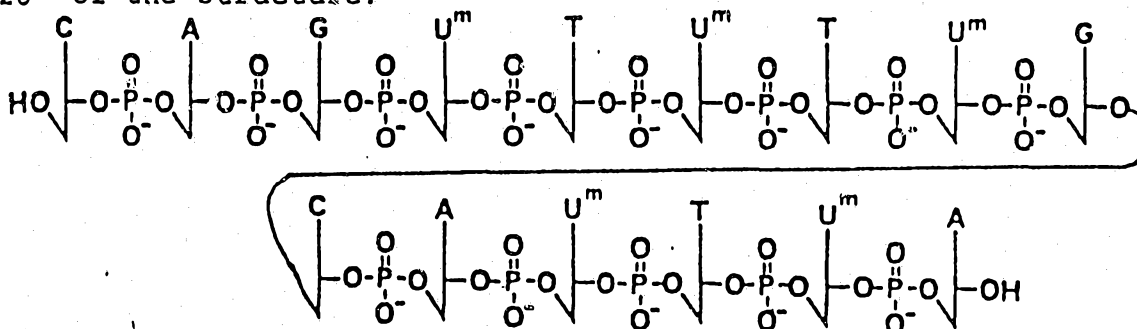
15 attached group is other than fluorescein.

EXAMPLE XXVII

Attachment of isoluminol and free primary
amine-containing reporter groups

A purified pentadecanucleotide from Example XVIII

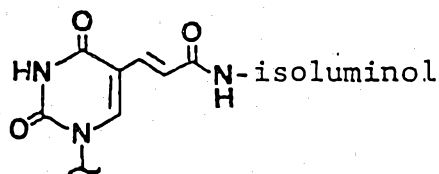
20 of the structure:



[where U^m is 5-(2-carboxyethenyl)uracil] is dissolved
in water at 30 A_{260} units per ml., and diluted with one
volume pyridine. Aminobutyl ethyl isoluminol is added

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to a final concentration of 1 mg/ml, followed by addition of a five-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The reaction is sealed and shaken gently in the dark for 12 to 48 hours. The reaction mixture is concentrated under reduced pressure to a solid residue, and chromatographed directly on a column of G-50 Sephadex[®]; the isoluminol-deoxyoligonucleotide conjugates elute near the void volume. Early fractions containing significant A₂₆₀ units are combined and lyophilized to solid product of structure similar to the starting deoxyoligonucleotide where U^m is now either



or unreacted 5-(2-carboxyethenyl)uracil.

Repeating the procedure on compounds from Examples XV and XXIII wherein R₂ contains carboxy is productive of the corresponding deoxyoligonucleotide-isoluminol adducts in like manner.

Repeating the procedure, but replacing aminobutyl isoluminol with other reporter groups containing a free primary amine is productive of the corresponding deoxyoligonucleotide-reporter adducts in like manner.

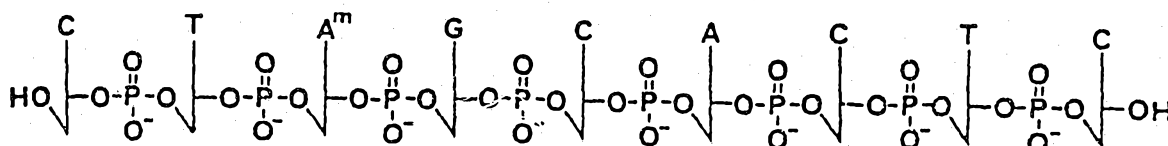


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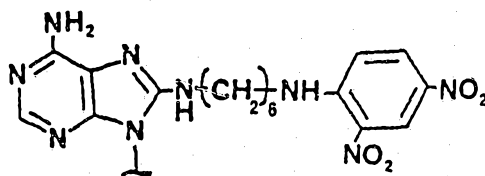
EXAMPLE XXVIII

Attachment of dinitrophenyl reporter groups

A purified nonanucleotide of the structure:



where A^m = 8-(6aminohexyl)aminoadenine is dissolved at
20 A_{260} units per ml in 250 mM sodium carbonate buffer,
pH 9, and 1-fluoro-2,4-dinitrobenzene is added. The
reaction solution is shaken at ambient temperature
overnight, then chromatographed directly on a column
of Sephadex[®] G-50. Early fractions containing signifi-
cant A_{260} units are combined and concentrated to give
an oligonucleotide product similar to the starting
decanucleotide wherein A^m is now either



or unreacted 8-(6-aminohexyl)aminoadenine.

Repeating the procedure, but replacing 8-(6-amino-
15 hexyl)aminoadenine with other modified bases containing
a free primary amine is similarly productive of the
corresponding dinitrophenylated oligonucleotide adducts.



The claims defining the invention are as follows:

1. A substantially pure single-stranded oligonucleotide comprising a preselected sequence of not more than approximately 200 nucleotides wherein at least one of said nucleotides is at a pre-selected position in said sequence and includes a base having a covalently attached linker arm attached to the base at a sterically tolerant site and having two or more carbon atoms if attached to a pyrimidine and one or more carbon atoms if attached to a purine, said linker arm containing or capable of binding at least one reporter group or a solid support.

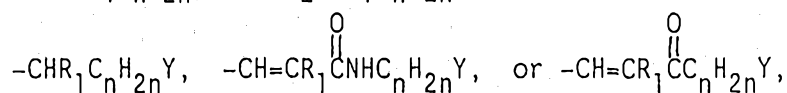
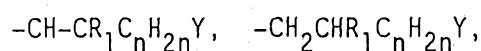
2. The substantially pure single-stranded oligonucleotide of Claim 1 wherein said base is a pyrimidine and said linker arm is attached to the C-5 position thereof.

3. The substantially pure single-stranded oligonucleotide of Claim 1 wherein said base is a purine and said linker arm is attached to the C-8 position thereof.

4. The substantially pure single-stranded oligonucleotide of any one of Claims 1 to 3 wherein said reporter group is capable of detection by colorimetric, fluorescent, luminescent, radioactive or ligand-binding/mediated means.

5. The substantially pure single-stranded oligonucleotide of Claim 1 wherein said preselected sequence is between 10 to 40 nucleotides in length.

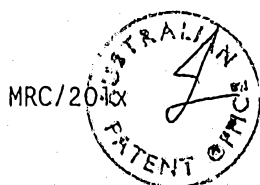
6. The substantially pure single-stranded oligonucleotide of Claim 1 wherein the linker arm is



wherein R_1 is hydrogen or C_{1-6} lower alkyl,
 n is 1 to 20 and

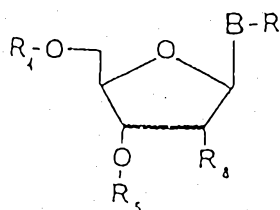
Y is at least one amine or masked amine, carboxy or masked carboxy, hydroxy or masked hydroxy, carboxyphenyl or masked carboxyphenyl, aminoalkylphenyl or masked aminoalkylphenyl, or an adduct thereof.

7. A process for the chemical synthesis of a substantially pure single-stranded oligonucleotide comprising a preselected sequence of not more than approximately 200 nucleotides wherein at least one of said nucleotides is at a preselected position in said sequence and contains a base having a covalently attached linker arm attached to the base a



sterically tolerant site and having two or more carbon atoms if attached to a pyrimidine and one or more carbon atoms if attached to a purine, said linker arm containing or capable of binding at least one reporter group or a solid support or a moiety capable of binding at least one reporter group or a solid support, the process comprising the stepwise addition of selected reactive nucleotides to a substantially pure single-stranded oligonucleotide having less than approximately 200 nucleotides in a preselected sequence, wherein said nucleotide or said oligonucleotide contains a reactive phosphorus-containing group attached to a 3' or a 5' hydroxyl group and wherein at least one of said reactive nucleotides includes a base having a covalently attached linker arm containing or capable of binding at least one reporter group or a solid support.

8. A reactive nucleotide monomer useful as an intermediate in the chemical synthesis of a substantially pure single-stranded oligonucleotide comprising the structure:



wherein B is a pyrimidine or purine base;

R is a linker arm attached to B at a sterically tolerant site and containing or capable of binding at least one reporter group or a solid support wherein said linker arm has two or more carbon atoms if attached to a pyrimidine and one or more carbon atoms if attached to a purine;

and provided that

when R_4 is a masking group then

R_5 is a reactive phosphorus-containing group or H; and

when R_5 is a masking group then

R_4 is a reactive phosphorus-containing group or H; and

R_8 is a H or masked hydroxyl group.

9. The reactive nucleotide monomer of Claim 8

wherein R is $-\text{CH}_2\text{CHR}_1\text{C}_n\text{H}_{2n}\text{Y}$; $-\text{CH}=\text{CR}_1\text{C}_n\text{H}_{2n}\text{Y}$; $-\text{CHR}_1\text{C}_n\text{H}_{2n}\text{Y}$;

$-\text{CH}=\text{CR}_1\overset{\text{O}}{\parallel}\text{C}_n\text{H}_{2n}\text{Y}$; or $-\text{CH}=\text{CR}_1\overset{\text{O}}{\parallel}\text{CC}_n\text{H}_{2n}\text{Y}$;

wherein R_1 is H or alkyl,

n is 0 to 20 and

Y contains at least one blocked amino or blocked carboxyl or



blocked hydroxy or blocked thio group or at least one reporter group or solid support.

10. The activated nucleotide monomer of Claim 6 wherein R is attached at the C-5 position when B is a pyrimidine or the C-8 position when B is a purine.

11. A substantially pure single stranded oligonucleotide as defined in Claim 1 and substantially as hereinbefore described with reference to any one of the Examples.

12. A process for the chemical synthesis of a substantially pure single-stranded oligonucleotide, which process is defined in Claim 7 and substantially as hereinbefore described with reference to any one of the Examples.

13. A reactive nucleotide monomer as defined in Claim 8 and substantially as hereinbefore described with reference to any one of the Examples.

DATED this NINETEENTH day of JANUARY 1990

Molecular Biosystems Inc

Patent Attorneys for the Applicant
SPRUSON & FERGUSON

