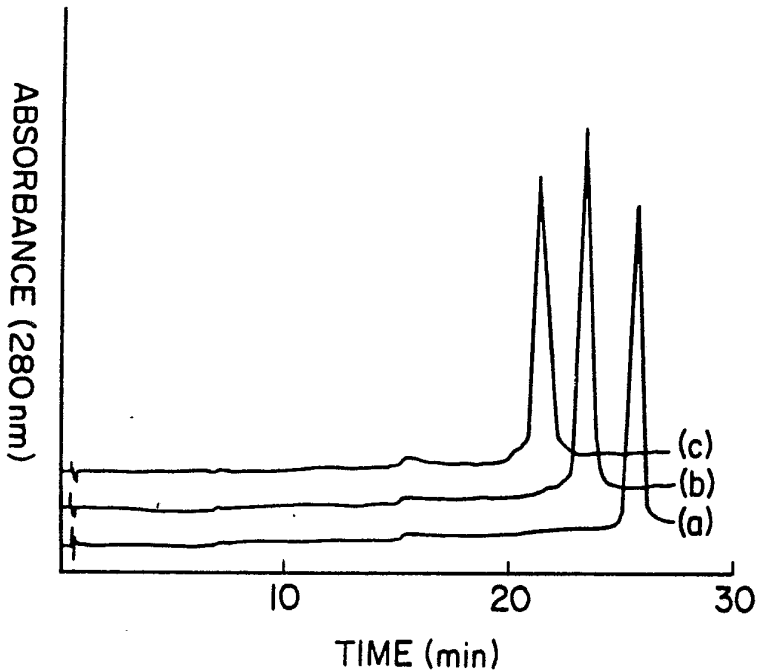




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<b>(54) Title:</b> SITE-SPECIFIC FUNCTIONALIZATION OF OLIGODEOXYNUCLEOTIDES FOR NON-RADIOACTIVE LABELLING    <b>(57) Abstract</b>  A method of producing a synthetic oligodeoxynucleotide of selected nucleotide sequence which is internally labelled with a nonradioactive material at at least one selected location, as well as oligodeoxynucleotides produced by the method.		

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SITE-SPECIFIC FUNCTIONALIZATION OF  
OLIGODEOXYNUCLEOTIDES FOR NON-RADIOACTIVE LABELLING

Description

Background of the Invention

- 05        There is at present growing interest in non-radioactively labelled modified oligodeoxy-nucleotides. Biotin (Agrawal, S. et al., Nucleic Acid Research, 14:6227-6245 (1986); Agrawal, S. Tet. Lett., 30:7025-7028 (1989)), fluorophores (Cardullo, 10 R.A. et al., Proc. Natl. Acad. Sci. USA, 85:8790-8794 (1988); Agrawal, S. et al., J. Cell Biology, 107:468 (1988); Haralambidis, J. et al., Nucleic Acids Res., 18(3):501-505 (1989)), intercalating (Helene, C. and J.J. Toulme, 15 Oligodeoxynucleotides-Antisense Inhibitors of Gene Expression, Ed. J.S. Cohen, Macmillan Press, 137-166 (1989)) and chelating (Oser, A. et al., G. Nucleic Acid Research, 16:1181-1196 (1988)) reagents attached to synthetic oligonucleotides are becoming 20 important tools of molecular biology. A variety of enzymatic and chemical procedures have been developed for their synthesis (Matthews, J.S. and L.J. Kricka, Anal. Biochem., 169:1-25 (1988)). Central to some of these procedures are (a) the

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introduction of a reactive group at either the 3'- or 5'-terminus of the oligonucleotide (Agrawal, S. et al., Nucleic Acid Research, 14:6227-6245 (1986); Agrawal, S. Tet. Lett., 30:7025-7028 (1989);

05 Fidanza, J.A. and L.W. McLaughlin, J. Am. Chem. Soc., 111:9117-9119 (1989); Nelson, P.S. et al., Nucleic Acid Research, 17:7187-7194 (1989)) or (b) the synthesis of modified nucleosides which contain the masked reactive group and are incorporated into

10 the nucleic acid (Fidanza, J.A. and L.W. McLaughlin, J. Am. Chem. Soc., 111:9117-9119 (1989)). The presently-available methods are useful, but are limited in their usefulness for site specific internal non-radioactive labelling of synthetic

15 oligonucleotides possible.

#### Summary of the Invention

The present invention relates to a method of site specific functionalization of oligodeoxy-nucleotides for non-radioactive labelling, as well

20 as to functionalized oligodeoxynucleotides and non-radioactively labelled oligodeoxynucleotides produced by the method. This method makes it possible to modify one or more selected internucleo-side phosphate(s) in a synthetic oligodeoxynucleo-

25 tide in such a manner that it (they) can be used to incorporate a non-radioactive material into the synthetic oligodeoxynucleotide. In particular, the method of the present invention is used to modify one or more selected internucleoside phosphates in a

30 synthetic oligodeoxynucleotide, to give aminoalkyl-phosphoramidate residues or aminoalkylphosphotri-

-3-

ester residues. The amino group(s) of the resulting modified residue(s) is then further reacted with a non-radioactive label, such as biotin, fluorescein or rhodamine (e.g., N-hydroxysuccinimide ester of  
05 biotin, N-caproyl amidobiotin, and a variety of fluorophore isothiocyanates), to produce a non-radioactively labelled oligodeoxynucleotide in which the label is present at a predetermined location or locations.

10 In the present method, an H-phosphonate internucleoside linkage is oxidized with an appropriately protected diamine, such as

N-1-trifluoroacetylhexanediamine, ( $\text{CF}_3\text{CO NH}(\text{CH}_2)_6\text{NH}_2$ ), in the presence of an appropriate  
15 solvent, such as carbon tetrachloride, to give a phosphoramidate internucleoside linkage (Zwierzak, A., Synthesis, 507-508 (1975); Froehler, B. et al., Nucleic Acid Research, 16:4831-4839 (1989); Letsinger, R.L. et al., J. Am Chem. Soc.,  
20 110:4470-4471 (1988); Agrawal, S. et al., Proc. Natl. Acad. Sci. USA, 85:7079-7083 (1988); Jager, A. et al., Biochemistry, 27:7237-7246 (1988)). The resulting phosphoramidate internucleoside linkage is stable under oligonucleotide assembly conditions  
25 using phosphoramidite chemistry (Caruthers, M.H. et al., Methods in Enzymology, 154:287-313 (1987)) and to subsequent deprotection steps. Alternatively, the H-phosphonate internucleoside can be oxidized, to give a phosphotriester internucleoside linkage,  
30 with an appropriately protected aminoalkyl alcohol in N-methylimidazole-triethylamine-carbon tetrachloride (e.g., 5:5:90).

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The present method can be used to produce non-radioactively labelled oligodeoxynucleotides which include a non-radioactive material at one or more sites and are useful in research and in the diagnosis and treatment of diseases and conditions of interest.

#### Brief Description of the Drawings

Figure 1 is a graphic representation of results of HPLC of oligomers 1, 2 and 5. Figure 1A shows results of analytical ion exchange chromatogram of oligomers 1 (a); 2 (b); and 5 (c). Figure 1B shows results of reversed phase HPLC of ion exchange purified oligomers 1 (chromatogram (d)); 2 (chromatogram (e)); and 5 (chromatogram (f)).

Figure 2 shows reversed phase HPLC traces of oligomer 2 (a); reaction mixture of oligomer 2 with biotin N-hydroxysuccinimide (b); oligomer 5 (c); and reaction mixture of oligomer 5 with biotin N-hydroxysuccinimide (d).

#### Detailed Description of the Invention

The present invention relates to a method of producing oligodeoxynucleotides which have a desired (selected) nucleotide sequence and which are labelled internally with a non-radioactive material or reporter group at one or more internucleoside linkages. In the method of the present invention, one or more selected internucleoside phosphate residues are modified to produce aminoalkylphosphoramidate residues or aminoalkylphosphotriester residues which are present in an

-5-

oligodeoxynucleotide at selected positions. The amino group(s) in such modified (functionalized) residues is further reacted with a label or reporter group, resulting in production of a

05 non-radioactively labelled oligodeoxynucleotide labelled internally at selected location(s).

Briefly, the present method is carried out by oxidizing an H-phosphonate internucleoside linkage using an appropriately protected diamine,

10 represented by the formula  $XCONH(CH_2)_nNH_2$ , in which X is a base labile protecting group and n can be 2 or more. For example, an H-phosphate

internucleoside linkage is oxidized using N-1-trifluoroacetylhexanediamine ( $CF_3CONH(CH_2)_6NH_2$ )

15 in the presence of an appropriate solvent, such as anhydrous carbon tetrachloride. As a result, a primary aliphatic amine is incorporated at the internucleoside phosphate as phosphoramidate. In

the case of phosphotriester linkages, oxidation is  
20 carried out using a suitably protected amino alcohol, represented by the formula  $XNH(CH_2)_nOH$ , in which X is a base labile protecting group and n can be 2 or more. For example, an H-phosphonate inter-

25 N-1-fluoroenylmethoxycarbonylaminohexanol

F-MOC-NH(CH<sub>2</sub>)<sub>6</sub>OH in the presence of

N-methylimidazole-triethylamine-carbon

tetrachloride. The remaining nucleotides needed to produce the desired nucleotide sequence are added

30 using art-recognized techniques, such as

phosphoramidate, H-phosphonate chemistry or methyl

phosphoramidate. (Caruthers, M.H. et al., Methods

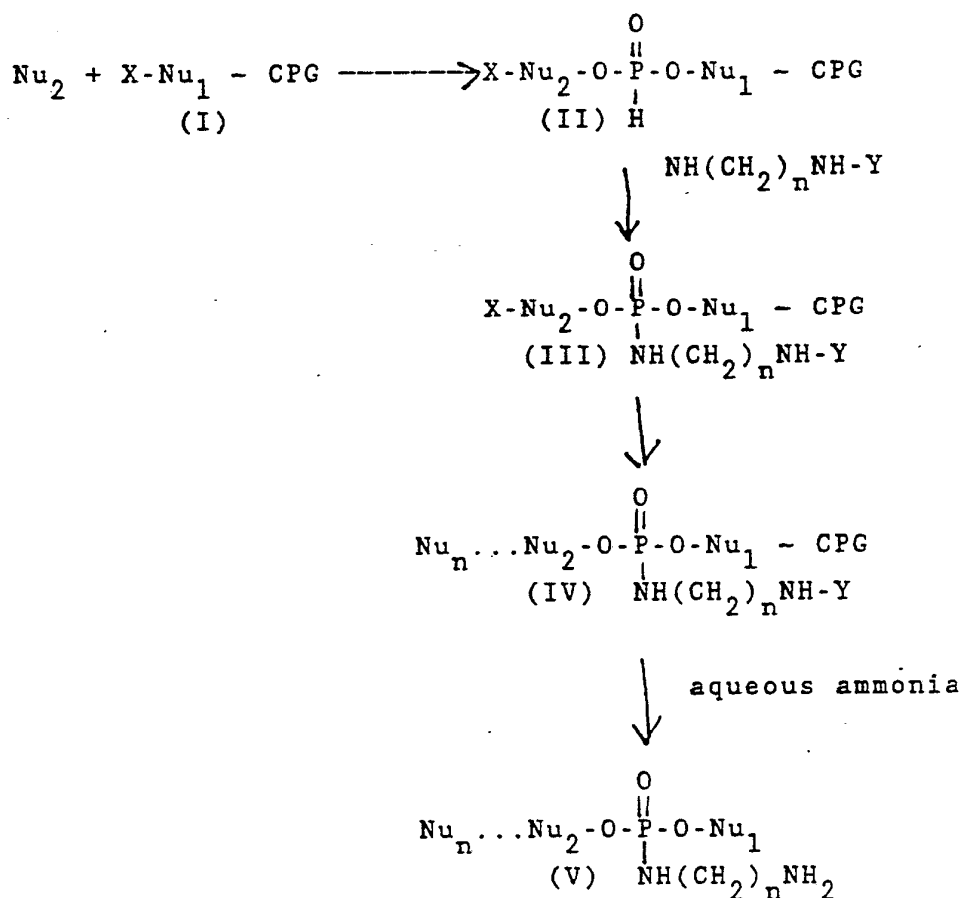
-6-

in Enzymology, 154:287-313 (1987); co-pending U.S. patent application Serial No. 07/334,679 (Method of Synthesizing Oligonucleotides and Their Analogs Adaptable to Large Scale Synthesis, by S. Agrawal and P. Zamecnik, filed April 6, 1989, the teachings of which are incorporated herein by reference; Agrawal, S. and J. Goodchild, Tet. Lett., 28(31):3539-3542 (1987)). After the desired oligodeoxynucleotide is produced, the protecting group present on the primary aliphatic amine is removed. The unmasked amino group can now react with one or more selected labels or reporter groups. As a result, the oligodeoxynucleotide is labelled, non-radioactively, at one or more selected internal locations. One or both amino groups present in the diamine react with the selected label.

The method of the present invention is represented in a series of steps below. The following is an explanation of those steps, with reference to the respective reactants and steps represented below.



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- 05 1. Initial coupling of two nucleotides (designated  
Nu<sub>1</sub> and Nu<sub>2</sub>) is carried out, using  
H-phosphonate chemistry. Generally, Nu<sub>1</sub> is  
bound to a solid support, such as CPG and  
terminates in a dimethoxytrityl residue  
10 (designated X). As a result, a support-bound  
dinucleoside H-phosphonate is produced (desig-  
nated (II)).
2. The support-bound dinucleoside H-phosphonate  
(II) is subsequently oxidized by being combined  
15 with an appropriately protected diamine in the

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- presence of a suitable solvent, resulting in formation of a phosphoramidate internucleoside linkage or a phosphotriester internucleoside linkage (and linking of the protected diamine to the dinucleoside through the unprotected amino group of the diamine). The resulting product is designated (III).
- 05
3. The dimethoxytrityl residue present on the unbound end of (I) is removed and the remaining deoxynucleotides of the desired oligodeoxy-
- 10 nucleotide to be produced are added at the now free end, using phosphoramidate chemistry or H-phosphonate chemistry, producing a support-bound oligodeoxynucleotide (IV) which includes the phosphoramidate linkage produced in step
- 15 (2).
4. The protecting group Y present on the diamine is removed and the compound is removed from the solid support. This results in production of
- 20 an unbound functionalized oligodeoxynucleotide (i.e., an aminoaliphatic oligomer or an oligodeoxynucleotide having a desired nucleotide sequence and an alkyl amino group present at the selected internucleoside phosphate(s) as a phosphoramidate or phosphotriester).
- 25
5. The unbound functionalized oligodeoxynucleotide is reacted with an appropriate form of a non-radioactive material, which becomes bound to the amino group and serves as a label or
- 30 reporter group on the oligodeoxynucleotide. This results in production of an oligodeoxynucleotide labelled site specifically

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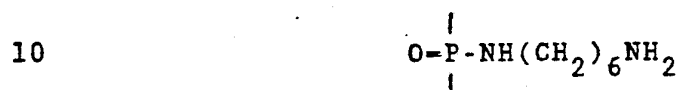
with a non-radioactive material. The non-radioactive material can be a fluorophore, a spin label, an enzyme, a chelator, a heterocyclic molecule, a protein, a lipid, a drug derivative, an antigen, an intercalator or other organic or inorganic moiety.

In a specific embodiment of the present invention, which has been used to produce non-radioactively labelled oligodeoxynucleotides, the following steps were carried out to produce a non-radioactively labelled oligodeoxynucleotide:

1. Initially, (I) and  $\text{Nu}_2$  were coupled, using art-recognized H-phosphonate chemistry, resulting in production of a support-bound dinucleoside H-phosphonate.
2. The support-bound dinucleoside H-phosphonate was oxidized, using N-1-trifluoroacetyldiaminohexane ( $\text{NH}(\text{CH}_2)_6\text{NH}-\text{CO}-\text{CF}_3$ ) in carbon tetrachloride-dioxane, resulting in formation of a phosphoramidate internucleoside linkage between  $\text{Nu}_2$  and  $\text{Nu}_1$ .
3. The remainder of the nucleotide sequence of the oligodeoxynucleotide was produced in a two-step procedure in which the dimethoxytrityl residue [DMTrO] was removed from the nucleotide now bound to the solid support ( $\text{Nu}_2$  in the reaction scheme above) and the desired nucleotides were added stepwise (i.e., to the now free end of the dinucleoside which, for convenience, can be referred to as the 5' end).

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4. The protecting group ( $-\text{CO}-\text{CF}_3$ ) present on  $\text{NH}(\text{CH}_2)_6\text{NH}-\text{CO}-\text{CF}_3$  was removed during deprotection of oligonucleotides in aqueous ammonia, resulting in production of a functionalized oligodeoxynucleotide (one in which the previously protected amino group is unprotected) of the desired sequence, in which there is an aminoalkylphosphoramidate residue of the formula



present at the desired internucleoside phosphate linkage(s).

5. The unbound modified oligodeoxynucleotide with the aminoalkylphosphoramidate residue was reacted with a non-radioactive material, such as biotin, fluorescein or rhodamine in appropriate form (e.g., N-hydroxysuccinimide ester of biotin, N-caproyl amidobiotin, fluorophore isothiocyanates), which becomes bound to the amino group of the aminoalkylphosphoramidate internucleoside linkage.

Alternatively, a functionalized oligodeoxynucleotide of the desired sequence, in which there is an aminoalkylphosphotriester residue present at the desired internucleoside phosphate linkage(s) can be produced. In this case, the non-radioactive material becomes bound to the amino

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group of the aminoalkylphosphotriester internucleoside linkage.

05 The production of oligodeoxynucleotides labelled at a selected site or sites by the present method is described in greater detail in the Exemplification.

10 It is possible, using the present method, to produce oligodeoxynucleotides of desired sequence which are labelled internally at one or more nucleosides. The oligodeoxynucleotide backbone can be unmodified (e.g., as it occurs in nature) or modified (e.g., amidate, methylphosphate, phosphothioate, phosphotriester backbones). The label present at two or more sites can be the same  
15 (e.g., biotin) or different and can be present at as many sites as desired. As described in the Exemplification, a 17-mer of the sequence shown has been produced, functionalized at a selected site or sites and labelled at the site(s) with a  
20 non-radioactive material.

In the above description of the present method, two single nucleotides (designated  $Nu_1$  and  $Nu_2$ ) are initially joined using H-phosphonate chemistry and the resulting oligodeoxynucleotide is functionalized  
25 at the internucleoside phosphate linkage formed between  $Nu_1$  and  $Nu_2$ . However, any number of nucleotides can be joined, using art-recognized techniques such as H-phosphonate chemistry, before modification of a selected internucleoside phosphate  
30 linkage is carried out. This has been carried out, as described in the Exemplification, to produce a 17-mer functionalized at a central internal site (oligomer 3) and a 17-mer functionalized at two

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internal phosphate linkages (oligomer 5). Using H-phosphonate chemistry, for example, an internal nucleotide (e.g., a support-bound nucleotide such as Nu<sub>1</sub>) can be added to, resulting in production of a longer sequence (e.g., Nu<sub>10</sub>Nu<sub>9</sub>...Nu<sub>1</sub>). The longer sequence can then be functionalized, by the method described above, resulting in production of a functionalized oligodeoxynucleotide (e.g., Nu<sub>10</sub><sup>+</sup>Nu<sub>9</sub>...Nu<sub>1</sub>, in which the internucleoside phosphate linkage between Nu<sub>9</sub> and Nu<sub>10</sub> is modified). The modified oligodeoxynucleotide can then be further elongated by addition of selected nucleotides to produce a modified oligodeoxynucleotide of desired sequence. The protecting group present can be removed, as described above. Alternatively, the functionalized oligodeoxynucleotide initially produced can be extended (by addition of selected nucleotides), one or more additional internucleoside phosphate linkages can be modified and a functionalized oligodeoxynucleotide which has two or more sites at which non-radioactive material can be added is, thus, produced.

Protecting groups other than trifluoroacetyl (-CO-CF<sub>3</sub>), as described above, can also be used to protect the diamine. Other base labile protecting groups, such as F-moc and t-boc, may also be used. The linker present between the two amino groups in the diamine used can be of any suitable length (e.g., -(CH<sub>2</sub>)<sub>2</sub>- to -(CH<sub>2</sub>)<sub>n</sub>-); the length used in a particular case can be determined empirically. The diame can be branched or unbranched and bi-functional or multifunctional.

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EXEMPLIFICATIONPreparation of N-1-trifluoroacetylhexanediamine and  
Its Use for Site-Specific Introduction of Amino  
Groups into Oligodeoxynucleotides

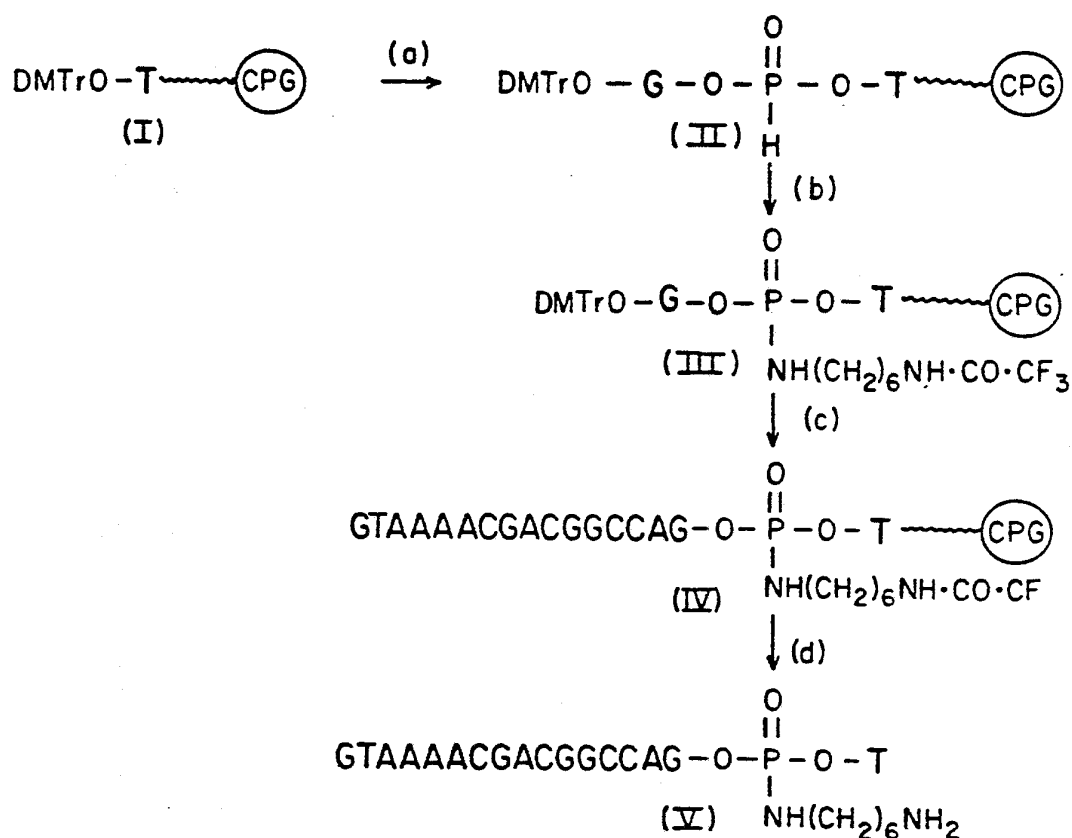
05        N-1-trifluoroacetylhexanediamine,  
          $\text{CF}_3\text{CONH}(\text{CH}_2)_6\text{NH}_2$ , was prepared by adding ethyltri-  
         fluoroacetate (1.2 ml, 10 mmol) dropwise over one  
         hour to a stirred mixture of hexanediamine (1.16g;  
         10 mmol) and triethylamine (1 ml; 7 mmol) in 20 ml  
10    methanol. The solution was stirred overnight.  
         After removal of solvents, the reaction mixture was  
         flash chromatographed on silica using 0-25% methanol  
         in dichloromethane. The fractions containing the  
         desired product were pooled and concentrated to give  
15    a colorless powder (1.1 gm, yield-42.6%), M.pt. 52°.  
          $^1\text{H}$  NMR ( $\text{CDCl}_3$ , d, TMS=0.00) 7.1-7.2 (m, 3H,  $\text{NH}_2$ , NH)  
         3.2-3.3 (m, 2H, CO-NH- $\text{CH}_2$ ), 2.8-2.9 (m 2H,  $\text{CH}_2$ - $\text{NH}_2$ )  
         1.2-1.6 (m, 8H, - $\text{CH}_2$ -( $\text{CH}_2$ )<sub>2</sub>- $\text{CH}_2$ -).

         To test the efficacy of ( $\text{CF}_3\text{CONH}(\text{CH}_2)_6\text{NH}_2$ ) for  
20    amino group introduction at specific sites of  
         oligonucleotides, a 17-mer sequence, GTA AAA CGA CGG  
         CCA GT, (oligomer 1) was made. Oligomers  
         (designated 2-5 below) carrying aminohexyl residues  
         at different sites, as shown by (<sup>†</sup>), were also made.

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<u>Oligomer #</u>	<u>Sequence</u>
1	GTA AAA CGA CGG CCA GT
2	GTA AAA CGA CGG CCA G <sup>↓</sup> T
3	GTA AAA CG <sup>↓</sup> A CGG CCA GT
05 4	G <sup>↓</sup> TA AAA CGA CGG CCA GT
5	G <sup>↓</sup> TA AAA CGA CGG CCA G <sup>↓</sup> T

The steps involved for labelling, for sequence 2, are shown below:





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The first coupling (represented by (a) in the above reaction) was carried out using H-phosphonate chemistry. This resulted in a production of a support-bound dinucleoside H-phosphonate (II), which  
05 was then oxidized with 4%  
N-1-trifluoroacetyldiaminohexane (I) in carbon tetrachloride-dioxane (8:2, v/v) for 30 minutes, resulting in production of (III). After oxidation with  $\text{CF}_3\text{CONH}(\text{CH}_2)_6\text{NH}_2$  (step (b) of the above  
10 reaction), the assembly of the rest of the oligodeoxynucleotide sequence was carried out (step (c)) using phosphoramidite or H-phosphonate chemistry. This resulted in production of the oligonucleotide (IV), which was deprotected in  
15 aqueous ammonia for 6 hours at 55°C (step (d)), resulting in formation of the aminohexyl oligomer (V).

Assessment of the oligomers 1-5 was carried out. Analytical ion exchange HPLC of oligomer 2  
20 showed the major peak eluting earlier than that of oligomer 1 with the same gradient (Figure 1a and 1b), confirming that in oligomer 2, one of the internucleoside linkages is a phosphoramidate linkage, which is non-ionic at phosphores.

25 Sequences 3 and 4, which are functionalized at different sites, also showed a HPLC profile similar to that of oligomer 2. Oligomer 5, which is functionalized at two sites, was eluted even earlier (Figure 1c).

30 When ion exchange HPLC purified oligomer 2 was checked on reversed phase HPLC, it gave a doublet peak in ratio of 1:2 (Figure 1e) compared to 1

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(Figure 1d). This results from the diastereoisomeric nature of phosphoramidate internucleoside linkage. Similarly, oligomer 5 eluted as a broad peak because of two such diastereoisomeric linkages (Figure 1f). Both oligomers 2 and 5 had retention times longer than that of oligomer 1 because of the hydrophobic nature of the alkyl chain present in oligomers 2 and 5.

Reaction of oligomer 2 was carried out with biotin N-hydroxysuccinimide using reported conditions (Agrawal, S. et al., Nucleic Acid Research, 14:6227-6245 (1986)). The reaction mixture after gel filtration (Sephadex G-25) showed two new peaks of the diastereomeric biotin adducts (Figure 2b). Similarly, reaction of oligomer 5 gave a broad peak as a doublet eluting later than the unreacted material (Figure 2c and Figure 2d).

The method described herein provides a way for functionalizing oligonucleotides at one or more selected or specified sites. As described above, the subject method of introducing reporter groups has been carried out by reaction of the functionalized oligodeoxynucleotide with biotin active ester, resulting in production of an oligodeoxynucleotide labelled at the selected site(s) with biotin. In addition, the aminohexyl residue present was reacted in high yield with fluorescein and rhodamine isothiocyanate, to produce a fluorescent hybridization probe. Multiple labelling may increase the sensitivity of detection in diagnostic assays.

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CLAIMS

1. A method of producing a synthetic oligodeoxynucleotide of selected nucleotide sequence which is internally labelled at at least one selected internucleoside linkage, comprising the steps of:
- 05
- a) modifying at least one internucleoside linkage of the synthetic oligodeoxynucleotide to produce an aminoalkyl residue selected from the group consisting of aminoalkylphosphoramidate residues and aminoalkylphosphotriester residues, in which a protected primary alkyl amine is present;
- 10
- b) deprotecting the protected primary alkyl amine present in the aminoalkyl residue, thereby producing a functionalized synthetic oligodeoxynucleotide with a deprotected amino group; and
- 15
- c) reacting the functionalized synthetic oligodeoxynucleotide with a deprotected amino group with a selected non-radioactive label, under conditions appropriate for joining of the selected non-radioactive label and the deprotected amino group, thereby producing a synthetic oligodeoxynucleotide in which the amino group is joined with the non-radioactive label.
- 20
- 25

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- 05 2. A synthetic oligodeoxynucleotide of selected nucleotide sequence which is internally labelled at at least one selected internucleoside linkage, produced by the method of Claim 1.
- 10 3. A method of producing a synthetic oligodeoxy-nucleotide, having a selected nucleotide sequence, which is internally labelled at at least one selected H-phosphonate internucleo-  
side linkage, comprising the steps of:
- 15 a) producing a support-bound oligodeoxynucleotide having an H-phosphonate internucleoside linkage present between the two nucleotides present at the unbound end of the support-bound oligodeoxynucleotide;
- 20 b) combining the support-bound oligodeoxy-nucleotide with an appropriately protected diamine and an appropriate solvent, under conditions appropriate for oxidation of the H-phosphonate internucleoside linkage to produce an aminoalkylphosphoramidate internucleoside linkage in which there is an aminoalkyl having a protected terminal  
25 amine group, thereby producing a modified oligodeoxynucleotide;
- 30 c) extending the nucleotide sequence of the modified oligodeoxynucleotide produced in step (b) as needed to produce the selected nucleotide sequence;

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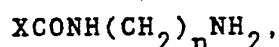
- d) deprotecting the protected terminal amino group present on the aminoalkylphosphoramidate internucleoside linkage, thereby producing a functionalized oligodeoxynucleotide; and
- 05 e) combining the functionalized oligodeoxynucleotide produced in step (d) with a selected non-radioactive label, under conditions appropriate for joining of the functionalized oligodeoxynucleotide and the non-radioactive label.
- 10
4. The method of Claim 3 wherein step (a), (b) and (c) are repeated as needed to produce a modified oligodeoxynucleotide having more than one aminoalkylphosphoramidate linkage in which there is a primary aminoalkyl having a protected terminal amino group.
- 15
5. A synthetic oligodeoxynucleotide of selected nucleotide sequence which is internally labelled at at least one selected internucleoside linkage, produced by the method of Claim 4.
- 20
6. The method of Claim 3 wherein the appropriately protected diamine has a base labile protecting group; the appropriate solvent is carbon tetrachloride; and the selected non-radioactive label is selected from the group consisting of: fluorophores, spin labels, drug derivatives, antigens and intercalators.
- 25

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7. The method of Claim 2 wherein the appropriately  
protected diamine is N-1-trifluoroacetylhexane-  
diamine; the appropriate solvent is carbon  
tetrachloride; and the selected non-radioactive  
05 label is a fluorophore selected from the group  
consisting of: biotin, fluorescein and  
rhodamine.

8. A synthetic oligodeoxynucleotide of selected  
nucleotide sequence which is internally  
10 labelled at at least one selected  
internucleoside linkage, produced by the method  
of Claim 7.

9. A protected diamine of the formula:



15 wherein X is a base labile protecting group and  
n is at least two.

10. The protected diamine of Claim 9 wherein X is  
 $\text{CF}_3$  and n is 6.

11. A protected amino alcohol of the formula:



wherein X is a base labile protecting group and  
n is at least two.

12. The protected amino alcohol of Claim 11 wherein  
X is F-MOC and n is 6.

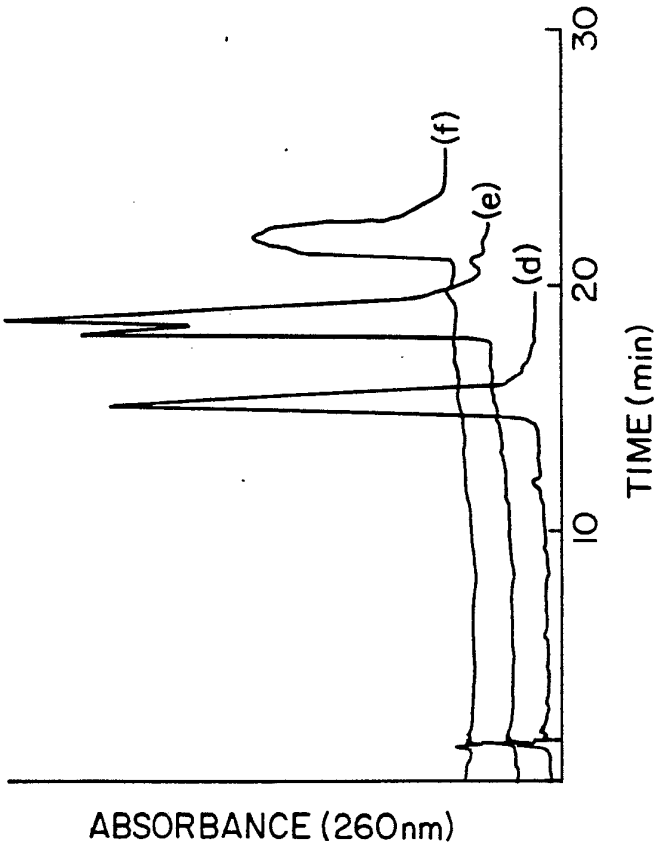


Fig. 1b

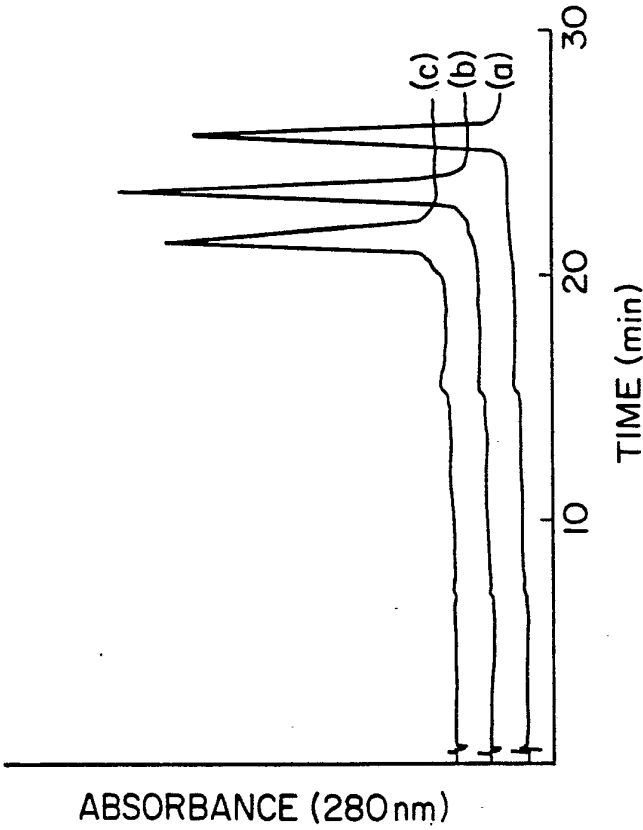


Fig. 1a

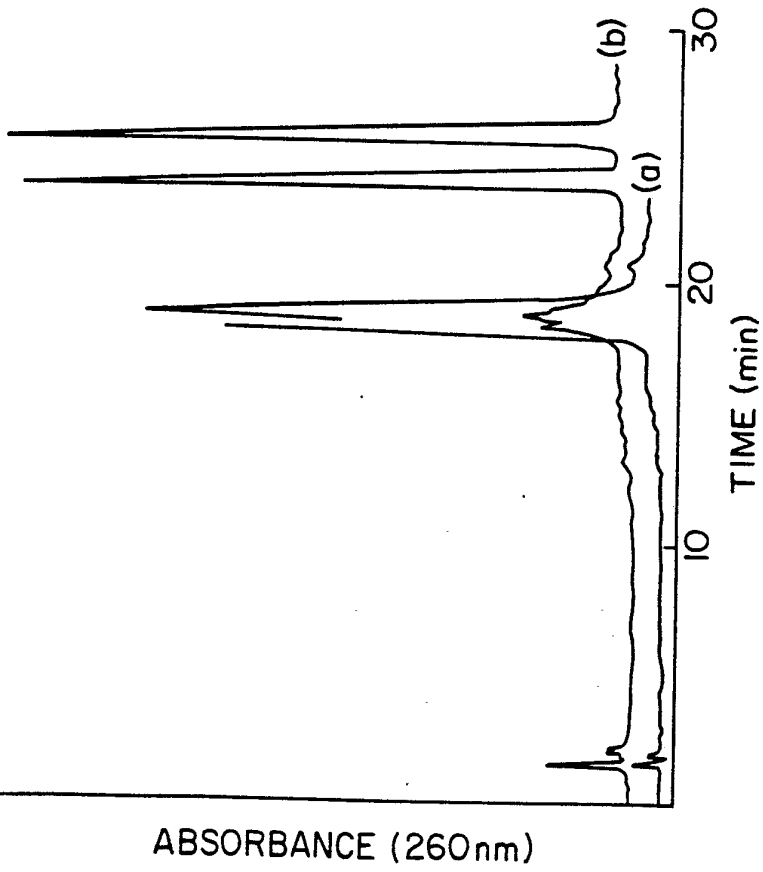


Fig. 2a

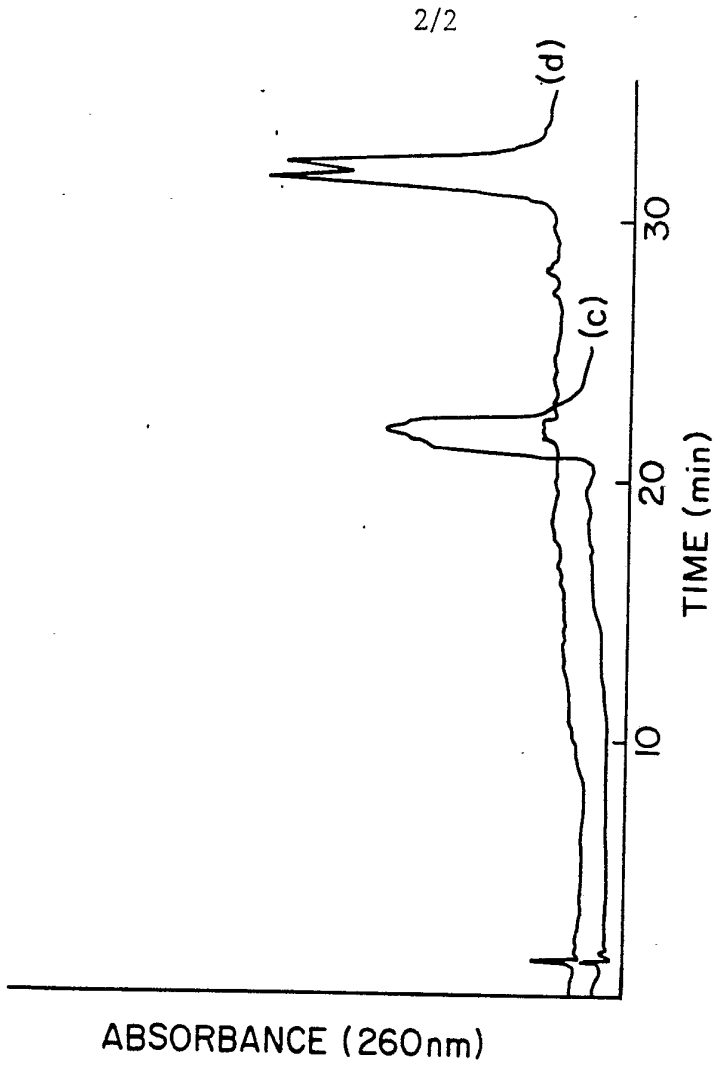


Fig. 2b