



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(51) International Patent Classification<sup>4</sup> :</b><br><b>C12Q 1/68, C12P 19/34</b><br><b>C07H 15/12, 19/06</b>  | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 89/ 02933</b><br><b>(43) International Publication Date:</b> 6 April 1989 (06.04.89)   |
| <b>(21) International Application Number:</b> PCT/US88/03275<br><b>(22) International Filing Date:</b> 26 September 1988 (26.09.88)<br><br><b>(31) Priority Application Number:</b> 104,330<br><b>(32) Priority Date:</b> 2 October 1987 (02.10.87)<br><b>(33) Priority Country:</b> US<br><br><b>(71) Applicant:</b> ML TECHNOLOGY VENTURES, L.P.<br>[US/US]; Merrill Lynch R&D Management Inc.,<br>North Tower, World Financial Center, New York,<br>NY 10281-1201 (US).<br><br><b>(72) Inventors:</b> ARNOLD, Lyle, John ; 5439 Noah Way, San<br>Diego, CA 92117 (US). BHATT, Ram, Saroop ; 3578<br>San Toro Way, San Diego, CA 92130 (US).   |           | <b>(74) Agents:</b> STEWART, Gordon, M. et al.; Lyon & Lyon,<br>611 West Sixth Street, 34th Floor, Los Angeles, CA<br>90017 (US).<br><br><b>(81) Designated States:</b> AU, DK, FI, JP, KR, NO.<br><br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the</i><br><i>claims and to be republished in the event of the receipt</i><br><i>of amendments.</i> |
| <b>(54) Title:</b> NON-NUCLEOTIDE REAGENTS FOR SUBSTITUTING TERMINI OF OLIGONUCLEOTIDES<br><br><b>(57) Abstract</b><br><br>A reagent which can couple with the 5' hydroxy position of a nucleotide, typically the terminal nucleotide in a nucleotide multimer, under the same conditions used in known standard solid-phase nucleotide multimer synthesis. When so coupled, the reagent produces a substituted nucleotide multimer with a protected linking moiety which is deprotected under the same alkaline conditions typically used to deprotect nucleotide exocyclic amines of a completed synthetic nucleotide multimer. A single one of the class of reagents can be so coupled to any nucleotide, and since the reagents have a non-nucleotide skeleton, the resulting substituted nucleotide multimer does not have an additional nucleotide which could potentially interfere with hybridization with a target nucleotide sequence. |           |  |

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DESCRIPTIONNon-Nucleotide Reagents For Substituting  
Termini of OligonucleotidesField of the Invention

This invention relates to reagents of a type which are suitable for preparing a 5' hydroxy substituted nucleotide, the substituent of which can then be conveniently deprotected under the same alkaline conditions used to deprotect nucleotide exocyclic amines during standard solid-phase nucleotide multimer synthesis, to leave a linking group to which can then be attached useful moieties, for example a label or an intercalator.

10 Technology Review

In clinical research and diagnosis, a known technique for determining the presence of a particular nucleotide sequence (the "target nucleotide sequence" or simply the "target sequence") in either RNA or DNA polynucleotides, is to perform a nucleic acid hybridization assay. In such an assay, a nucleotide multimer probe (typically an oligonucleotide probe) is chosen which has a nucleotide sequence complimentary to at least a portion of the target sequence. Typically, the probe is labelled, that is, it is provided with an atom or a group linked thereto, the presence of which can be readily detected. When the labelled probe is exposed to a test sample suspected of containing the target nucleotide sequence, under hybridizing conditions, the two will hybridize. The presence of the target sequence in the sample can be determined qualitatively or quantitatively usually by separating hybridized and non-hybridized probe, then determining the amount of labelled probe which hybridized, either by determining the presence of label in probe hybrids, or by determining the quantity of label in non-hybridized probes.

A known labelling technique involves incorporating a radioactive label, such as  $^{125}\text{I}$  or  $^{32}\text{P}$  into the probe. Use of radiolabelled probes is associated with certain drawbacks, however, including probe instability and the usual hazards associated with handling radioisotopes. In an effort to overcome these drawbacks, probes have more recently been labeled with non-radioactive tags, such as biotin, haptens, or fluorophores. The utility of such non-radioactive tags has already seen widespread application in the area of immunodiagnostics. Use of non-radioactive tags in detection of DNA probes has also already been reported in the literature. Examples of detection protocols include use of enzyme-antibody conjugates, which recognize a hapten tag, or enzyme-avidin conjugates, which recognize biotin tags.

Early methods developed for attachment of non-radioactive labels to synthetic nucleotide multimer probes involve chemical modifications of the probe after it has been synthesized and/or purified ("post-modification"). For example, R.P. Viscidi et al. (J. Clin. Microbiol., 1986, 23, pp. 311-317) used a bisulfite-catalyzed transamination reaction to attach 1,2-diaminoethane at the C-6 position of cytosine residues in lambda phage DNA. Reaction of the transaminated DNA with biotinyl-epsilon-aminocaproic acid N-hydroxysuccinimide ester, produced biotinylated DNA which could be hybridized to complementary DNA on nitrocellulose and detected using a streptavidin-alkaline phosphatase complex. We have attempted labeling a synthetic DNA oligonucleotide by this method, and have observed a dramatic lowering of the melting temperature associated with its RNA hybrid. Thus, attachment of labels on cytosine appears to interfere with normal base pairing associated with hybridization and may not be a suitable method in the case of short synthetic oligonucleotides.

Another post-modification method involves use of a photo-activatable analog of biotin which randomly labels

DNA (A.C. Forster et al., Nucl. Acids Res., 1985, 13, pp. 745-761). Again, this method is not particularly suited to labelling synthetic oligonucleotides due to interference with base pairing and resulting destabilization of the hybrid formed with the target oligonucleotide.

In a further post-modification method, various nucleotide and deoxynucleotide triphosphates have been prepared with amine-terminated linkers which can be incorporated into short DNA probes by enzymatic means. Amine linkers have been substituted at the N6-position of adenine (L. Klevon, PCT application number WO 86/02929, published May 22, 1986, the CS position of cytosine (D. Ward et al., European Patent publication number 0063879, published March 11, 1982). In one method, a biotinylated form of C5-modified dUTP has been attached to the 3'-end of an oligonucleotide primer with a complementary oligonucleotide as template using the enzyme DNA polymerase (A. Murasugi and R.B. Wallace, DNA, 1984, 3, pp. 269-277; P.R. Langer, A.A. Waldrop and D.C. Ward, Proc. Natl. Acad. Sci. USA, 1981, 78, pp. 6633-6637). Another method employs the use of the enzyme terminal deoxynucleotidyl transferase to add these linker-modified triphosphates to the 3'-end of oligonucleotides (see, for example, L. Riley et al. DNA, Vol. 5, p. 333, 1986). Drawbacks to these methods include the number of purification steps required, limitations to scale-up inherent in working with pre-purified oligonucleotides, and the potential for mis- or non-pairings associated with the additional or modified nucleotide residues under hybridization conditions.

In another post-modification method, amine linkers have been attached to the 5'-end of the 5'-phosphorylated oligonucleotides using carbonyldiimidazole (B. Chu, G. Wahl and L.E. Orgel, Nucl. Acids Res., 1983, 11, pp. 6513-6529). This method has been used to attach biotin for use in non-radioactive detection formats (B. Chu and L.E. Orgel, DNA, 1985, 4, pp. 327-331; A. Chollet and E.H. Kawashima, Nucl. Acids Res., 1985, 13, pp. 1529-1541).

Attachment of a label at the 5'-end has the advantage of generally reducing interference of the probe in hybridization reactions. However, the method still suffers from the number of steps required to attach the linker, namely, enzymatic phosphorylation, reaction with imidazole, reaction with 1,2-diaminoethane or 1,6-diaminohexane, and column purification.

A more direct labelling method is incorporation of the attachment of an amine linker, as a step in automated solid-phase nucleotide multimer synthesis. Of the several methods which have been described in the literature, none is without limitations. Wachter et al. (Nucl. Acids Res., 1986, 14, pp. 7985-7995) used carbonyldiimidazole to achieve aminoalkylation of the 5'-hydroxyl of a synthetic oligonucleotide at the end of a step-wise automated synthesis; the oligonucleotide was still attached to the polymer support. Although this method has the advantage of simplifying purification, the reaction conditions required that the last steps be done manually. Kempe, et al. (Nucl. Acids Res., 1985, 13, pp. 45-57) attached 2-(biotinylamide) ethanol to a 5'-phosphorylated synthetic oligonucleotide attached to a solid support in the presence of a condensing agent. In addition to being somewhat laborious, this method requires that the attached label survive the conditions of deprotection and removal from the solid support. Thus, it may not be suited for attachment of chemically sensitive labels.

The procedure of Smith et al. (Nucl. Acids. Res., 1985, 13, pp. 2399-2412) is more directly applicable to automated oligonucleotide synthesis in that a 5'-protected-5'-amino-5'-deoxythymidine-3'-O-phosphoramidite is attached in a final standard coupling reaction. A free amino group is generated during deprotection and removal from the solid support, which is then available for attachment of a variety of labels. Since a nucleotide skeleton (i.e. thymidine) is used, in order to preserve a complimentary relationship between a probe and a target

sequence, any of 8 different linkers of the type described by Smith et al. would have to be prepared. Otherwise, an additional thymidine residue could, in some cases, cause a loss in specificity for the target sequence due to mis-hybridization.

Compounds have been suggested which can be used to insert a primary amine-modified digonucleotide during standard automated synthesis procedures. Such compounds include analogs of deoxythymidine, deoxyadenine, and deoxyguanine (G.B. Dreyer, et al., Proc. Natl. Acad. Sci. USA, Vol. 82, p. 968, 1985; J.L. Ruth, PCT Application No. WO84/03285 published August 30, 1985). However, these methods suffer from the same limitations as the Smith et al method.

Agrawal et al. (Nucl. Acids Res., 1986, 14, pp. 6227-6245) recently reported two methods of attaching labels which are applicable to automated solid-phase DNA synthesis. In the first method, a uridine-2',3'-protected-5'-phosphoramidite or 5'-phosphite is added to the 5'-end of the oligonucleotide as a last coupling step. The 2'- and 3'-hydroxyls are subsequently deprotected and oxidized to a dialdehyde using periodate. Biotin hydrazide is then condensed to form a cyclic linkage after treatment with borohydride. This method suffers from the disadvantage of requiring several post-modification steps. In the second method, an N-protected-O-phosphoramidite derivative of ethanolamine is added in the last coupling cycle during automated synthesis. N-protection is accomplished by means of a O-fluorenylmethoxycarbonyl groups.

#### Definitions

As used in this disclosure and claims, the following terms are defined as:

nucleotide: a subunit of a nucleic acid consisting of a phosphate group, a 5 carbon sugar and a nitrogen containing base. In RNA the 5 carbon sugar is ribose. In

DNA, it is a 2- deoxyribose. The term also includes analogs of such subunits.

nucleotide multimer: a chain of nucleotides linked by phosphodiester bonds or analogs thereof.

5     oligonucleotide: a nucleotide multimer generally about 10 to about 100 nucleotides in length, but which may be greater than 100 nucleotides in length. They are usually considered to be synthesized from nucleotide monomers, but may also be obtained by enzymatic means.

10     polynucleotide: a nucleotide multimer generally about 100 nucleotides or more in length. These are usually of biological origin or are obtained by enzymatic means.

15     nucleotide multimer probe: a nucleotide multimer having a nucleotide sequence complementary with a target nucleotide sequence contained within a second nucleotide multimer, usually a polynucleotide. Usually the probe is selected to be perfectly complementary to the corresponding base in the target sequence. However, in some  
20 cases it may be adequate or even desirable that one or more nucleotides in the probe not be complementary to the corresponding base in the target sequence, or that various moieties of synthetic origin either replace a nucleotide within the probe or be inserted between bases of the  
25 probe. Typically, the probe is labeled.

oligonucleotide probe: a probe of synthetic or enzymatic origin having less than 100 nucleotides, but which may contain in excess of 200 nucleotides.

hybridization: the formation of a "hybrid", which is  
30 the complex formed between two nucleotide multimers by Watson-Crick base pairings between the complementary bases.

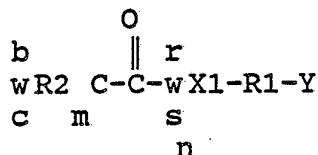
#### Summary of the Invention

35     The present invention then, provides a reagent which is suitable for preparing a 5' hydroxy substituted nucleotide. The foregoing nucleotide is usually the last



nucleotide in a biological or synthetic nucleotide multimer, typically the latter. After the desired sequence has been synthesized employing a standard nucleotide multimer solid phase synthesis, the 5' hydroxy of the last nucleotide is deprotected, and it may then react with a suitable coupling group on the present reagent to provide a substituted nucleotide multimer. A linking moiety on the reagent is protected by an acyl functional group, which inhibits reagent polymerization during the foregoing coupling step. The linking moiety can be subsequently deprotected under the same non-adverse alkaline conditions typically used to deprotect exocyclic amines, following solid-phase synthesis of a nucleotide multimer. Thus deprotection of the acyl protected linking moiety and exocyclic amines can be accomplished simultaneously, without need of any steps additional to those used in standard nucleotide solid-phase synthesis. The resulting substituted nucleotide multimer then, has a 5' "linking arm" which can be linked to useful moieties, such as non-isotopic labels.

Accordingly, the present invention provides, in its broad aspect, a reagent suitable for preparing a 5' hydroxy substituted nucleotide (again, typically a terminal nucleotide on a nucleotide multimer), which reagent has the formula:



if X1 = sulfur, n = 1

if X1 = amino group, n = 1 or 2

m = either of 1, 2 and 3

In the foregoing formula, Y is a non-nucleotide based coupling group, R1 is a non-nucleotide skeleton, X1 is a protected linking moiety selected from sulfur or an amino group (which includes substituted amino groups in which the amino N is bonded to R1 and the carbonyl carbon of the

protecting group). The acyl group  $R_2^m \overset{\text{O}}{\parallel} \text{C}-\text{C}-$ , is a protecting group for X1, which can be cleaved (under  
5 conditions described below), to leave a linking group H-X1-. By any constituent being "non-nucleotide" is meant that the structure of that constituent is such that it "does not significantly participate in hybridization, said structure must not, for example, participate in any  
10 significant hydrogen bonding with a nucleotide, and would exclude structures having as a component one of the 5 nucleotide bases or analogs thereof. Y is selected such that it is capable of coupling R1 to the 5' hydroxy of a nucleotide, under coupling conditions which are non-  
15 adverse. By "non-adverse" conditions, is meant that the conditions are such that they do not substantially adversely affect the polymer backbone and its sugar, base, linker arm or other components, nor the monomeric reagents.

20 Further, in the above formula, m represents an appropriate number of R2 to satisfy the normal tetravalence of carbon. Each R2 may be part of a cyclic group. Each R2 may also be the same or they may be different, the important consideration being that they are  
25 selected such that the alpha carbon of the acyl group, has an electronegativity such that X1 is not substantially deprotected under the coupling conditions (and thus reagent polymerization is thereby inhibited), but is deprotected under non-adverse alkaline conditions to allow  
30 X1 to link to some useful moiety under non-adverse conditions. In this regard, one measure of electronegativity of  $R_2^m$  is given by the Hammett Equation (for a review on the use of this equation and a table of substituent parameters, see J. Connolly Martin, "Quantitative Drug  
35 Design, A Critical Introduction," Marcell Dekker, Inc., NY, 1978):

$$\log (k/k_o) = \rho \sigma$$

5             $k$         =        equilibrium constant of ionization of  
                 parasubstituted benzoic acid.

$k_o$       =        equilibrium constant of ionization of  
                 benzoic acid.

$P$         =        a constant, defined as 1.00 for the  
                 ionization of benzoic acids in water at  
10            25°C.

$\sigma_p$       =        an empirically determined value for a  
                 particular para-substituent.

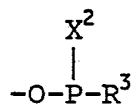
15 In the preferred case of the present invention where  
each R2 is F (i.e., the protecting group is trifluoro-  
acetyl),  $\sigma_p$  (F-) is 0.15, and  $\sigma_p$  (CF<sub>3</sub>-) = 0.54. Thus it  
would be predicted that other suitable R<sub>2m</sub>C- groups might  
include Cl<sub>3</sub>C- ( $\sigma_p$  = 0.33), N C ( $\sigma_p$  = 0.66), CF<sub>3</sub>CF<sub>2</sub>- ( $\sigma_p$  =  
20 0.52), CH<sub>3</sub>C- ( $\sigma_p$  = 0.50), CH<sub>3</sub>O-C- ( $\sigma_p$  = 0.45), or where X1  
= N and n = 2, might also include groups which form cyclic  
N groups.

Several of the amino protecting groups commonly used in the art for protein modifications might also be predicted to serve as suitable amino protecting groups in the present invention. (For a review of protein amino protecting groups, see "The Peptides, Vol. 3, Protection of Functional Groups in Peptide Synthesis", ed. E. Gross and J. Meinhofer, Academic Press, 1981). These may include trifluoracetyl, pthaloyl and maleoyl.

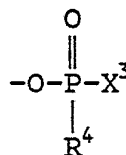
Preferably then,  $X1=N$ , and at least one, and preferably each  $R2$  is selected from a halogen (most preferably each  $R2$  is fluorine). Typically,  $R1$  will be alkyl, desirably being an acyclic hydrocarbon chain of 35 from 1 to 10 carbon atoms.

Preferably, -Y is selected from groups having the formula:

10



and



5

In the foregoing formula:

- $\text{X}^2$  = halogen (preferably Cl) or substituted amino (preferably a dialkyl amino or heterocyclic N-amine);
- 10  $\text{X}^3$  = halogen (preferably Cl); amino (preferably a heterocyclic N-amino); or  $\text{O}^-$ ;
- $\text{R}^3$  = alkyl; preferably an alkoxy selected from methoxy, ethoxy, chlorophenoxy, or betacyanoethoxy); or aryloxy
- 15  $\text{R}^4$  = alkyl; preferably alkoxy selected from methoxy, ethoxy, or beta-cyanoethoxy); aryloxy, such as chlorophenoxy; or H only if  $\text{X}^3 = -\text{O}^-$ .

A method of preparing a nucleotide multimer, which

20 has a linking moiety at the 5' hydroxy position, is also disclosed, which method uses reagents of the type described above and is performed under non-adverse conditions. Broadly, the method comprises first coupling  $\text{R}^1$  through Y (that is Y serves as the coupling group and

25 all or a portion of Y may or may not remain after actual coupling), to the 5' hydroxy of a terminal nucleotide in a nucleotide multimer. Then, the resulting product is exposed to non-adverse alkaline conditions, so as to simultaneously deprotect any of the linking moiety and any

30 protected exocyclic amines and phosphates.

### Drawings

Embodiments of the present invention will now be described with reference to the drawings in which:

Fig. 1 illustrates the synthesis of a reagent of the

35 present invention;

Fig. 2 illustrates the synthesis of a substituted nucleotide multimer of the present invention; and

Fig. 3 illustrates a method of labeling the substituted nucleotide multimer produced by the method shown in Fig. 2, by linking a biotin label to the amine linking moiety on the former.

5 Detailed Description Of The Embodiments Of The Invention

Examples 1-5 below illustrate various aspects of the present invention. In particular, Example 1 describes in detail the synthesis of a reagent of the present invention. Example 2 describes the synthesis of a substituted  
10 nucleotide multimer of the present invention, by a coupling reaction between the reagent produced in Example 1 and a synthetic nucleotide multimer. Example 3 further illustrates the synthesis of other substituted nucleotide multimers of the present invention, while Examples 4 and  
15 5 illustrates linking of a label to such a substituted nucleotide multimer.

Example 1

Figure 1 illustrates the scheme of the following synthesis.

20 Materials: 6-amino-1-hexanol, S-ethyltrifluorothioacetate, N,N-diisopropylethylamine and N,N-diisopropylamino-chloromethoxyphosphine were products of Aldrich Fine Chemicals, Milwaukee, WI.

(a) 6-Trifluoroacetamido-1-hexanol (IV)

25 To a stirring slurry of 6-amino-hexanol (II) (1.17g, 10 mmol) in anhydrous ethylacetate (15 ml) was added dropwise S-ethyltrifluorothioacetate (2 ml, 15 mmol). A clear solution was obtained as soon as the addition of S-ethyltrifluorothioacetate was completed. After stirring  
30 the reaction mixture at room temperature for 5 hours, 0.5 ml more of S-ethyltrifluorothioacetate was added. After stirring the reaction mixture for another 1/2 hour, silica gel thin-layer chromatography in acetone-dichloromethane, 5:7 ratio, indicated that the starting amino-hexanol (II).  
35 had disappeared (Ninhydrin spray). Visualization of the

TLC plate after spraying with 0.1 M piperidine followed, after 15 minutes, by ninhydrin spray and heating the plate at 110°C for 2-3 minutes displayed a faster moving broad spot near the solvent front ( $R_f$  0.85). Petroleum ether (60 ml) was added to the reaction mixture and the turbid solution was stored at -20°C for 16 hours to give colorless powder which was washed with cold (-20°C) petroleum ether (3x5 ml). The product was then dried briefly under vacuum and then treated with 5 ml of 10:1 pyridine-water mixture for 10 minutes to hydrolyze off any O-trifluoroacetyl group which may have been formed resulting in III. The reaction mixture was concentrated to dryness, the residue left was co-evaporated with pyridine (4x10 ml) to give 1.1 g (47%) of compound IV in the form of a gum.

(b) 6-Trifluoroacetamidohexane-1-N, N-diisopropylamino-methoxyphosphine I

To a stirring solution of compound IV (0.426 g, 2 mmol) in dry THF (10 ml) was added, under argon, N, N-diisopropylethylamine (1.4 ml, 8 mmol) followed by the addition of N, N-diisopropylamino-chloro-methoxyphosphine (0.79 g, 4 mmol). The reaction was stirred at room temperature for 1/2 hour after which the white precipitate was filtered off. The filtrate was diluted with ethylacetate (60 ml) and washed with 5% sodium carbonate solution (3x15 ml). The organic layer was then dried over  $MgSO_4$  and evaporated to give a mobile syrup which was dried under vacuum for 16 hours to give 0.7 g (94%) of desired compound I which was used without any purification. The presence of the desired phosphoramidite (I) was confirmed by  $^{31}P$  NMR (in  $CD_3CN$ , trimethylphosphate, external standard): (ppm) 117.4.

Example 2: Automatic Coupling of Amino-Linker I With the Synthetic DNA Fragments

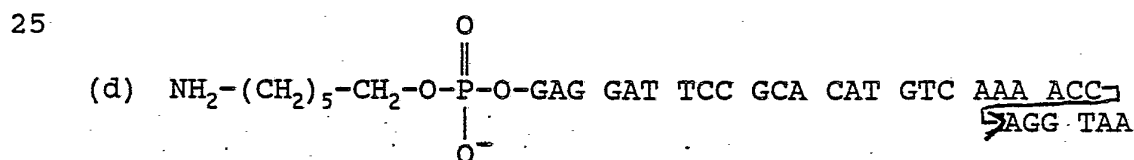
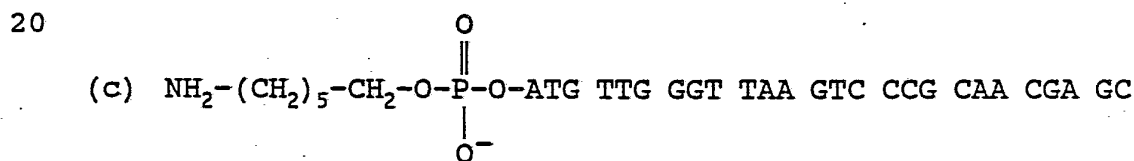
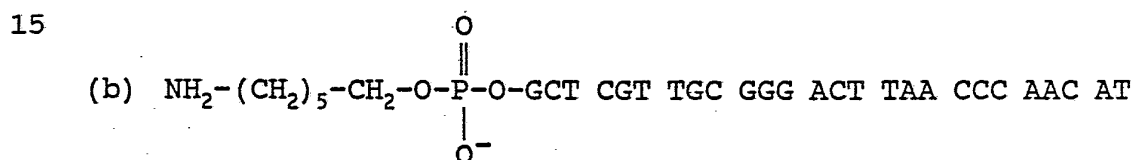
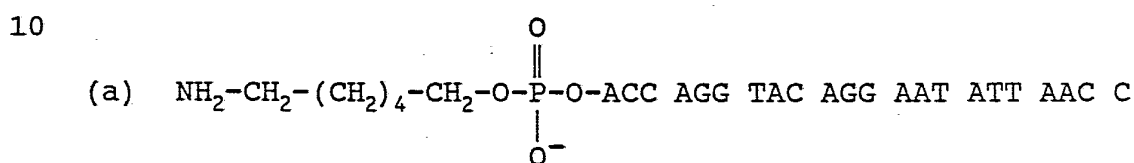
The scheme of Figure 2 outlines the steps involved in the automated synthesis of a nucleotide multimer followed by the automated addition of the amine-linker reagent I. Polymer-support synthesis of a nucleotide multimer with

defined sequence was first accomplished using either an Applied Biosystems ("ABI") Model 380A or a Biosearch Model 8600 DNA synthesizer, using a modified version of the manufacturer's synthesis program. This modification makes use of a basic aqueous wash (10% water in 2% pyridine - THF) step prior to the oxidation step (see Figure 2). This modification was included to minimize side reactions in the overall synthesis.

The automated coupling of the amine-linker I was accomplished by loading it as a 0.2 molar solution in acetonitrile into position #6 on the ABI 380A DNA synthesizer. Using the method, the amino-linker was added to the 5'-hydroxyl group of the resin-bound N-protected oligomer in the same way as 5'-dimethoxytrityl-N-protected-nucleoside beta-cyanoethylphosphoramidites of general structure V are added. Following a final basic aqueous wash and oxidation step, the resin-bound oligomer was cleaved from the resin by treatment with concentrated aqueous  $\text{NH}_4\text{OH}$  at room temperature for 1 hour. The oligomer was then deprotected by heating the  $\text{NH}_4\text{OH}$  solution at  $55^\circ\text{C}$  for 16 hours. This step simultaneously deprotects exocyclic amines of the nucleotides, and the linking amine moiety (by cleaving the trifluoroacetyl to leave a primary amine) After evaporating the ammonium hydroxide solution, the crude product was then purified by electrophoresis on a 12% polyacrylamide - 7M urea gel. Bands were visualized by a UV shadowing technique, whereby a fluorescent thin-layer chromatography plate was placed underneath the gel, and a UV lamp was held over the gel. The linker modified oligomer gave a band which migrated slower on the gel than the unmodified oligomer. The linker modified band was then excised from the gel and extracted with 0.1M  $\text{NH}_4\text{OAc}$  (pH 7.5) and then desalted through a column of Sephadex G-25 to obtain the pure 5' hydroxy substituted nucleotide X. Based on relative band intensities from the polyacrylamide gel (UV shadowing method), the coupling of the amine linker was estimated at greater than 90%.

Example 3

Using the above procedure, the following substituted synthetic nucleotide multimers with the amino-linker at the 5'- end, were synthesized and purified. It will be appreciated that substituted nucleotide X, or any of those substituted nucleotides below, could be used as probes to detect a complementary target nucleotide sequence. Of course in such cases, a suitable label would typically be linked to the amine linking moiety.



30 In each case, the substituted synthetic oligomer moved slower than the parent oligomer as shown by the UV shadowing of 12% acrylamide-7 M urea gel.

Example 4

35 To illustrate the suitability of the amine moiety of the foregoing substituted nucleotides, for linking to a label, it was linked to biotin. The scheme for such linking, and subsequent conjugation of the biotin labeled substituted nucleotide, is illustrated in Figure 3.



(a) Biotinylation:

To facilitate monitoring, the 5'-amino-linked synthetic deoxyoligonucleotide detailed in Example 3(b) was first labelled at its 3'-OH terminal using alpha-<sup>32</sup>P(TTP) and terminal deoxynucleotidyl transferase by following a standard protocol of Tu and Cohen (Gene, 10, 177) The result is compound XI.

For biotinylation, 5 pmoles lyophilized 3' -<sup>32</sup>P labelled probe (XI) was incubated with 0.2M sodium borate (pH 9.0) (5 ml), dimethyl sulfoxide (4 ml) and 30 mmoles biotin-x-NHS (calbiochem-Belving Corp., San Diego, CA, USA) (1 ml) at room temperature for 1 hour. The reaction mixture was then twice ethanol precipitated by adding 250 ml of 0.3M sodium acetate, 750 ml of ethanol and, 20 ug of glycogen. The pellet of the product XIII was dissolved in 20 ml H<sub>2</sub>O.

(b) Streptavidin - Conjugate:

The verification of the presence of biotin moiety on the foregoing labelled probe XIII was accomplished by conjugation of the biotinylated probe with streptavidin-agarose according to the following procedure:

In two 1.5 ml Eppendorfh tubes was taken 100 ul each of streptavidine-agarose slurry (Bethesda Research Labs, Inc., Gaithersburg, MD, USA) To one tube was added 1 mg of biotin (Calbiochem-Behring Corp., San Diego, CA, USA) in 500 ul of wash buffer (wash buffer consisted of 500 mM phosphate buffer, 2 mM EDTA, 0.5 M sodium chloride pH 7.4). After agitating at room temperature for 15 minutes, 2 ul of the solution by labeled probe XII was added and the reaction mixture was agitated at room temperature for 30 minutes.

To the second tube was added 500 ul of wash buffer and 2 ul of the solution of biotinylated probe and stirred at room temperature for 45 minutes.

Both the tubes were then centrifuged and the supernant removed and labelled as S1 and S2. The pelleted

agarose gels were washed again with 500 ul of wash buffer and the supernant combined with S1 and S2 respectively. The residues left were suspended in 500 ul each of wash buffer and the amounts of  $^{32}\text{p}$  were measured by Cerenkov  
 5 scintillation counting.

|             | <u>CPM</u> | <u>% Bound.</u> |
|-------------|------------|-----------------|
| Tube I      | 1095]      |                 |
|             | ]          | 6.02            |
| Supernate 1 | 17588]     |                 |
| 10 Tube 2   | 9901]      |                 |
|             | ]          | 54.4            |
| Supernate 2 | 9231]      |                 |

The results clearly demonstrated the formation of biotin-streptavidin adduct which in turn further  
 15 illustrated the presence of amino-linkage on the probe.

#### Example 5

#### B. Labelling of Amine Linker-Arm Probe with Acridinium Ester and Subsequent Purification

A 25mM stock solution of acridinium ester (for composition refer to I. Weeks et al., Clin. Chem., Vol. 29, p. 1474, 1983) was prepared in distilled DMSO. The amine linker probe described above in Example 3(b) was evaporated to dryness in a 1.5 ml conical polypropylene tube. The following cocktail was constructed by adding the  
 25 following ingredients in the order listed:

3ul H<sub>2</sub>O

1ul 1M HEPES (8.0)

4ul DMSO (distilled)

2ul 25mM acridinium ester in DMSO (distilled)

30 The mixture was vortexed, spun in a microcentrifuge for 2 seconds (to bring the contents to the bottom of the tube), and incubated at 37°C for 20 minutes. At that point, the following components were added to the reaction cocktail in the order listed:

35 3.0 ul 25 mM acridinium ester in DMSO (distilled)

1.5 ul H<sub>2</sub>O

0.5 ul 1M HEPES (8.0)

The cocktail again was vortexed, spun, and incubated an additional 20 minutes at 37°C. The unreacted label was quenched using a 5-fold excess of lysine by adding 5 ul of  
5 0.125M lysine in 0.1M HEPES (8.0), 50% DMSO, and incubated 5 minutes at room temperature.

At this point the acridinium ester-labelled oligomer was purified using the following method. To the 20ul quenched reaction mixture 30ul 3M NaOAc (5.0), 245ul H<sub>2</sub>O  
10 and 5ul glycogen was added as a carrier (the glycogen was pre-treated to remove any nuclease activity). The sample was vortexed briefly and 64 0ul of absolute EtOH was added. The sample was vortexed briefly and incubated on ice 5-10 minutes, then centrifuged 5 minutes at 15,000 rpm  
15 in a microcentrifuge. The supernatant was carefully removed and the pellet was redissolved in 20ul of 0.1M NaOAc (5.0), 0.1% SDS. The sample was further purified by ion-exchanged high performance liquid chromatography (HPLC) as follows: the 20ul redissolved pellet was  
20 injected onto a Nucleogen-DEAE 60-7 ion-exchange HPLC column mounted in an IBM, 9533 HPLC system. All buffers used in the process were made with HPLC grade water, acetonitrile (CH<sub>3</sub>CN) and sodium acetate (NaOAc), and reagent grade glacial acetic acid (HOAc) and LiCl.  
25 Additionally, all buffers were filtered through 0.45um pore size Nylon-66 filters before use. Buffer A was 20mM NaOAc, pH 5.5, 20% CH<sub>3</sub>CN, and 1 M LiCl. Elution was achieved with a linear gradient from 55% Buffer A, 45% Buffer B to 30% Buffer A, 70% Buffer B in 25 minutes at a  
30 flow rate of 1 ml/min. Absorbance at 260 nm was monitored during the run; fractions of 0.5 ml were collected in 1.5 ml conical polypropylene tubes. Immediately after the run, 5ul of 10% SDS was added to each tube followed by vortexing of each tube (this was done to ensure that the  
35 acridinium ester-labelled probe did not stick to the walls of the tube). A 0.5ul aliquot was removed from fractions 21-42 and added to 200ul water in a 12x75mm tube (a

separate pipet tip was used for each aliquot to avoid a carryover problem). The chemiluminescence of each aliquot was then determined in a Berthold Clinilumat by automatic injection of 200ul of 0.25N HNO<sub>3</sub>, 0.1% H<sub>2</sub>O<sub>2</sub>, followed after  
5 a 1 second delay by 200ul of 1N NaOH and reading of chemiluminescence for 10 seconds.

Fractions 29-33 were EtOH precipitated by adding to each 5ul glycogen, vortexing, adding 1 ml EtOH to each, vortexing, incubating 5-10 minutes on ice, and  
10 centrifuging 5 minutes at 15,000 rpm in a microcentrifuge. Each supernatant was carefully removed, each pellet was redissolved in 20ul 0.1M NaOAc, pH5, 0.1% SDS and these separate fractions were then pooled.

Based upon a comparison of UV absorbance and  
15 chemiluminescence output, the chemiluminescence specific activity of the labelled probe and the starting label reagent were comparable on a molar basis.

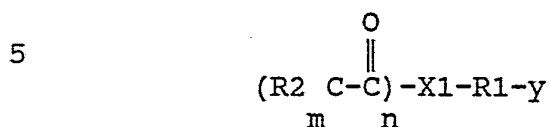
It will be seen then, that reagents have been described which can readily couple to the 5' hydroxy  
20 position of a nucleotide, typically the terminal nucleotide of a nucleotide multimer, to produce a substituted nucleotide multimer with a linking moiety which is readily deprotected under the same conditions as deprotection of exocyclic amines in standard solid-phase synthesis. As  
25 illustrated, such reagents can be coupled to a nucleotide multimer as a final step in well known solid-phase synthesis procedures using standard chemistry of such procedures. Furthermore, no special deprotecting step is required of the linking moiety in the resulting substituted  
30 nucleotide multimer, since the acyl protecting group, being cleavable under the same alkaline conditions used to deprotect exocyclic nucleotide amines in the multimer, is deprotected simultaneously therewith.

Although specific embodiments of the present  
35 invention have been described, variations and modifications to such embodiments will be conceivable to those skilled in the art. Accordingly, the scope of the present

invention is not limited by the embodiments described in detail above.

Claims:

1. A reagent suitable for preparing a 5' hydroxy substituted nucleotide, which reagent has the formula:

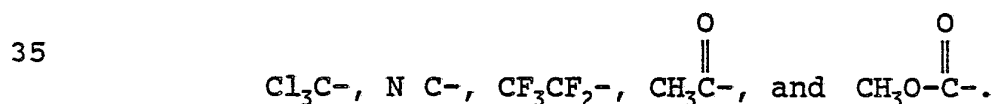


wherein:

- 10 (a) y is a non-nucleotide based coupling group which is capable of coupling R1 to the 5' hydroxy of a nucleotide, under coupling conditions which are non-adverse;
- (b) R1 is a non-nucleotide skeleton;
- 15 (c) X1 is a protected linking moiety selected from S or an amino group;
- (d)  $\begin{array}{c} \text{O} \\ \parallel \\ \text{R}_2 \text{ C}-\text{C}- \\ \text{m} \end{array}$  is an X1 protecting group, with m being
- 20 an appropriate number of R2, each of which may be different, and is comprised of one or more atoms of carbon, oxygen, nitrogen or halogen, or a combination thereof, such that R2 C- has an
- 25 electronegativity so that X1 is not deprotected under the coupling conditions but is deprotected under non-adverse alkaline conditions.
- (e) n=1 when X1=S; n=1 or 2 when X1 is an amino group.

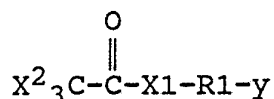
30 2. A reagent as defined in claim 1 wherein X1=N and at least one of R2 is selected from a halogen.

3. A reagent as defined in claim 1 wherein R2<sub>m</sub>C is selected from:



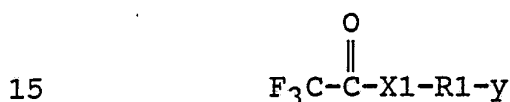
4. A reagent as defined in claim 1 wherein  $R_2C-C(=O)-$  is selected from trifluoroacetyl, phthaloyl, and maleoyl.

5. A reagent as defined in claim 1 wherein  $X^1=N$  and the reagent formula is:



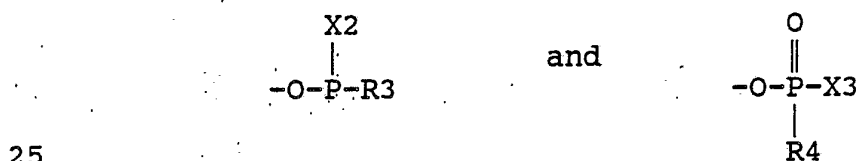
10 in which each  $X^2$  is selected from a halogen and  $R^1=alkyl$ .

6. A reagent as defined in claim 1 wherein  $X^1=N$  and  $R^1=alkyl$ , and the reagent formula is selected from:



7. A reagent as defined in claim 1 wherein  $R^1$  is an acyclic hydrocarbon chain of from 1 to 10 carbon atoms.

8. A reagent as defined in any of claims 1 to 7 wherein  $X$  is an amino group, and wherein  $-y$  is selected from:

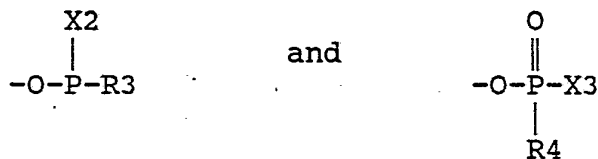


in which:

- (a)  $X^2$  = halogen or substituted amino
- (b)  $X^3$  = halogen; amino; or  $O^-$
- (c)  $R^3$  = alkyl; alkoxy; aryloxy;
- 30 (d)  $R^4$  = alkyl; alkoxy; aryloxy; or H only if  $X^3=O-$

9. A reagent as defined in any of claims 1 to 7 wherein  $R^1 = alkyl$ ,  $X^1$  is an amino group, and  $-Y$  is selected from:

22

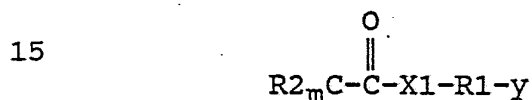


5

in which:

- (a) X2 = Cl or a secondary amino;
- (b) X3 = Cl; a secondary amino; or O<sup>-</sup>;
- (c) R3 = alkoxy; aryloxy
- 10 (d) R4 = alkoxy; or H only if X<sup>3</sup> = O<sup>-</sup>.

10. A method of preparing a nucleotide multimer with a linking moiety at a 5' hydroxy, using a reagent of the formula:



15

in which:

- (i) Y is a non-nucleotide based coupling group;
- (ii) R1 is a non-nucleotide skeleton;
- 20 (iii) X1 is a protected linking moiety selected from sulfur or an amino;

(iv)

$$\begin{array}{c} \text{O} \\ || \\ \text{R2}_m\text{C}-\text{C}- \end{array}$$
 is an X1 protecting group, in which m is an appropriate number of R2 each of which may be different but which is selected such that R2<sub>m</sub>C- is more electronegative than an alkyl;

25

the method comprising:

- (a) first coupling R1 through Y, to the 5' hydroxy of a terminal nucleotide of the multimer under non-adverse conditions;
- 30 (b) then exposing the resulting product to non-dverse alkaline conditions so as to simultaneously deprotect X1 and any protected exocyclic amines.

35

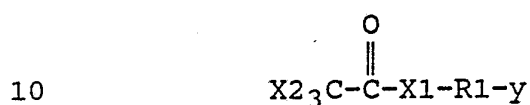
11. A method as defined in claim 10 wherein X1=N and a least one of R2 is selected from a halogen.



12. A method as defined in claim 10 wherein  $X1=N$  and

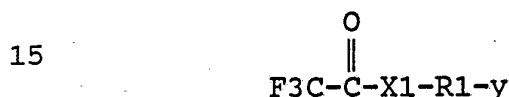
$R2_m \overset{\overset{O}{\parallel}}{C}-$  is selected from trifluoracetyl, phthaloyl, and  
5 maleoyl.

13. A method as defined in claim 10 wherein  $X1=N$  and  
 $R1=alkyl$ , and wherein the reagent formula is:

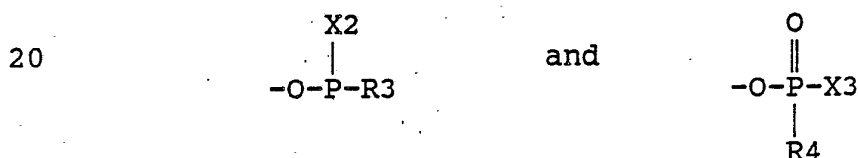


in which each  $X2$  is selected from a halogen.

14. A method as defined in claim 10 wherein  $X1=N$  and  
 $R1=alkyl$ , and the reagent formula is:



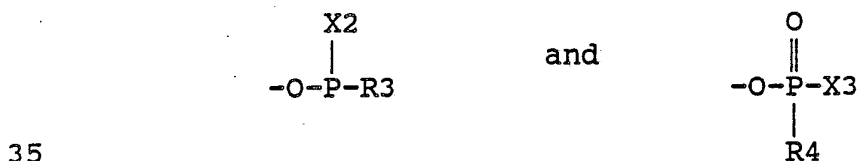
15. A method as defined in any of claims 10 to 14  
wherein  $X$  is an amino, and wherein  $-Y$  is selected from:



in which:

- 25 (a)  $X2 =$  halogen or substituted amino  
(b)  $X3 =$  halogen; amino; or  $O-$   
(c)  $R3 =$  alkyl; alkoxy; aryloxy  $-O-$ ;  
(d)  $R4 =$  alkyl; alkoxy; aryloxy; or  $H$  only if  $X^3=O-$

16. A method as defined in any of claims 10 to 14  
30 wherein  $-Y$  is selected from:



in which:

- 5 (a) X2 = Cl or a secondary -amino;  
(b) X3 = Cl; a secondary amino; or O<sup>-</sup>;  
(c) R3 = alkoxy;  
(d) R4 = alkoxy or H only if X3 = O-  
(e) R1 = alkyl

17. A method of preparing a labelled nucleotide multimer comprising:

- 10 (a) preparing a nucleotide multimer with a linking moiety at a 5' hydroxy, by the method of any of claims 9 to 15;  
(b) linking a chemiluminescent acridinium ester to the linking moiety.

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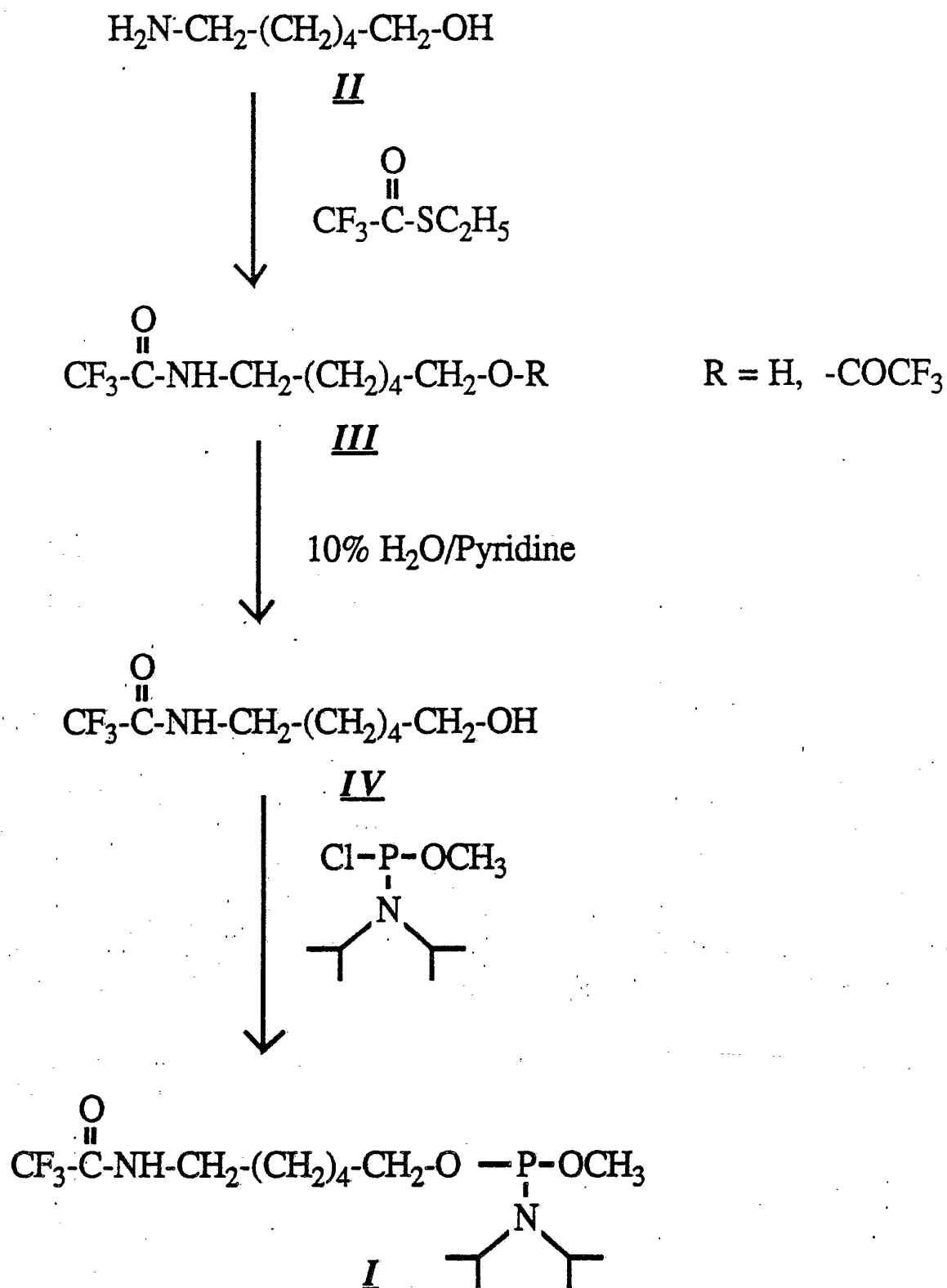
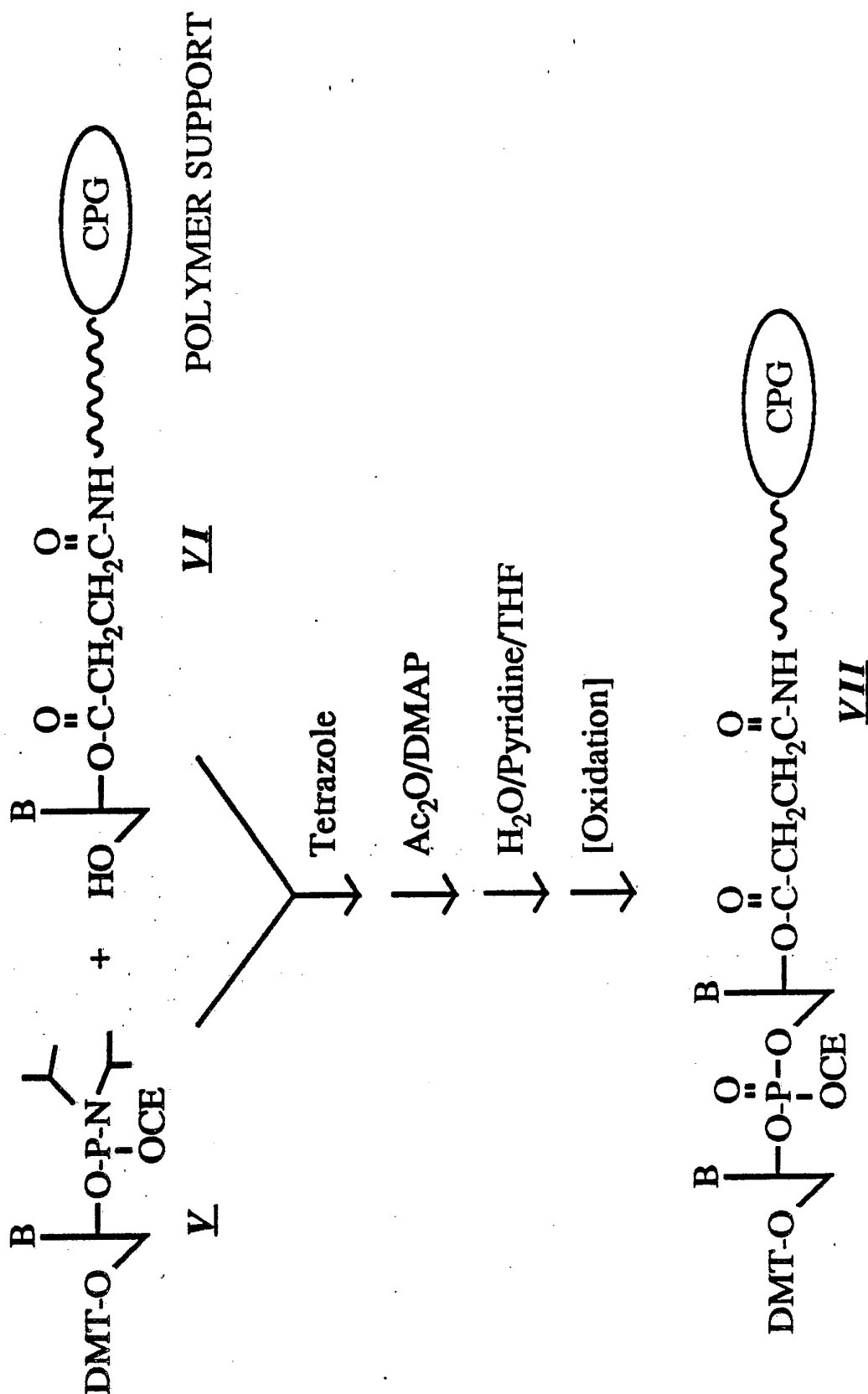


FIG. 1.

FIG. 2a.





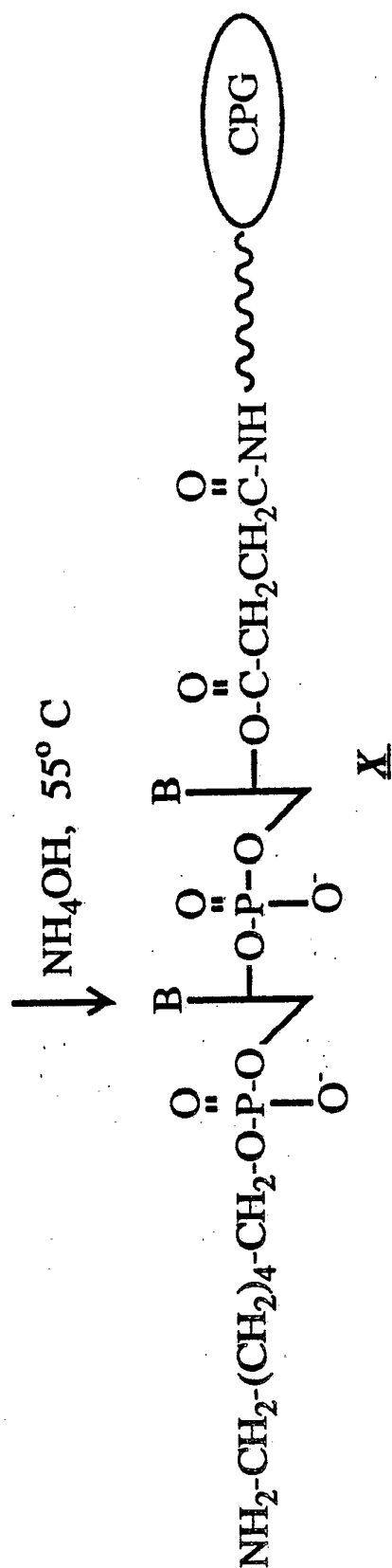


FIG. 2c.

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FIG. 3a.

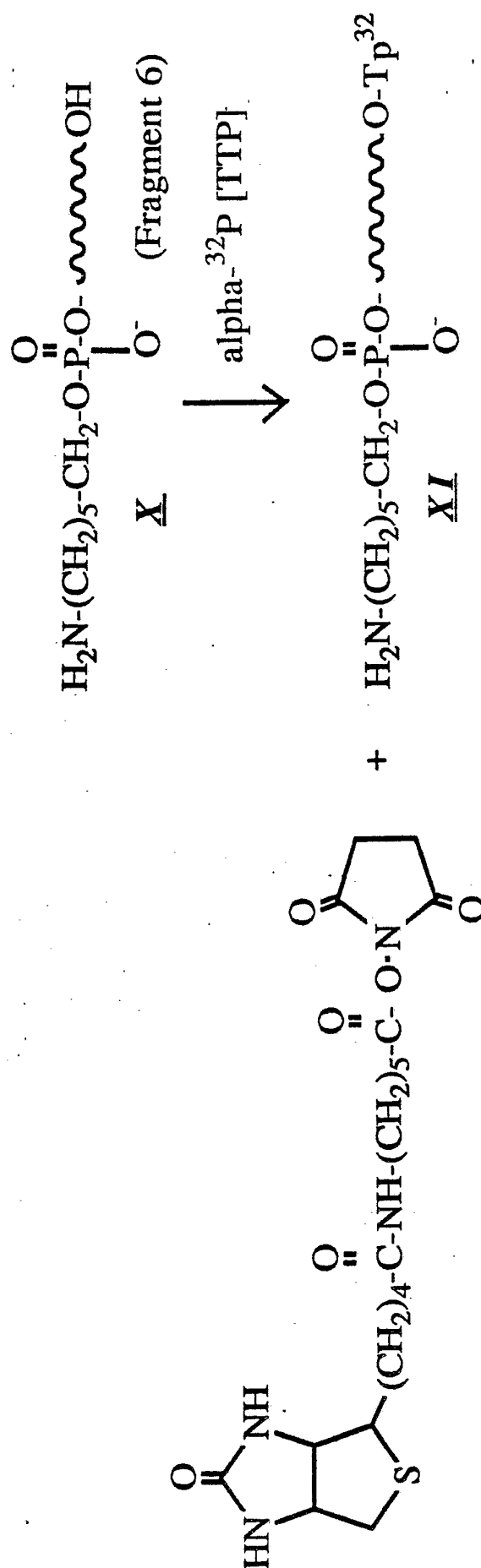
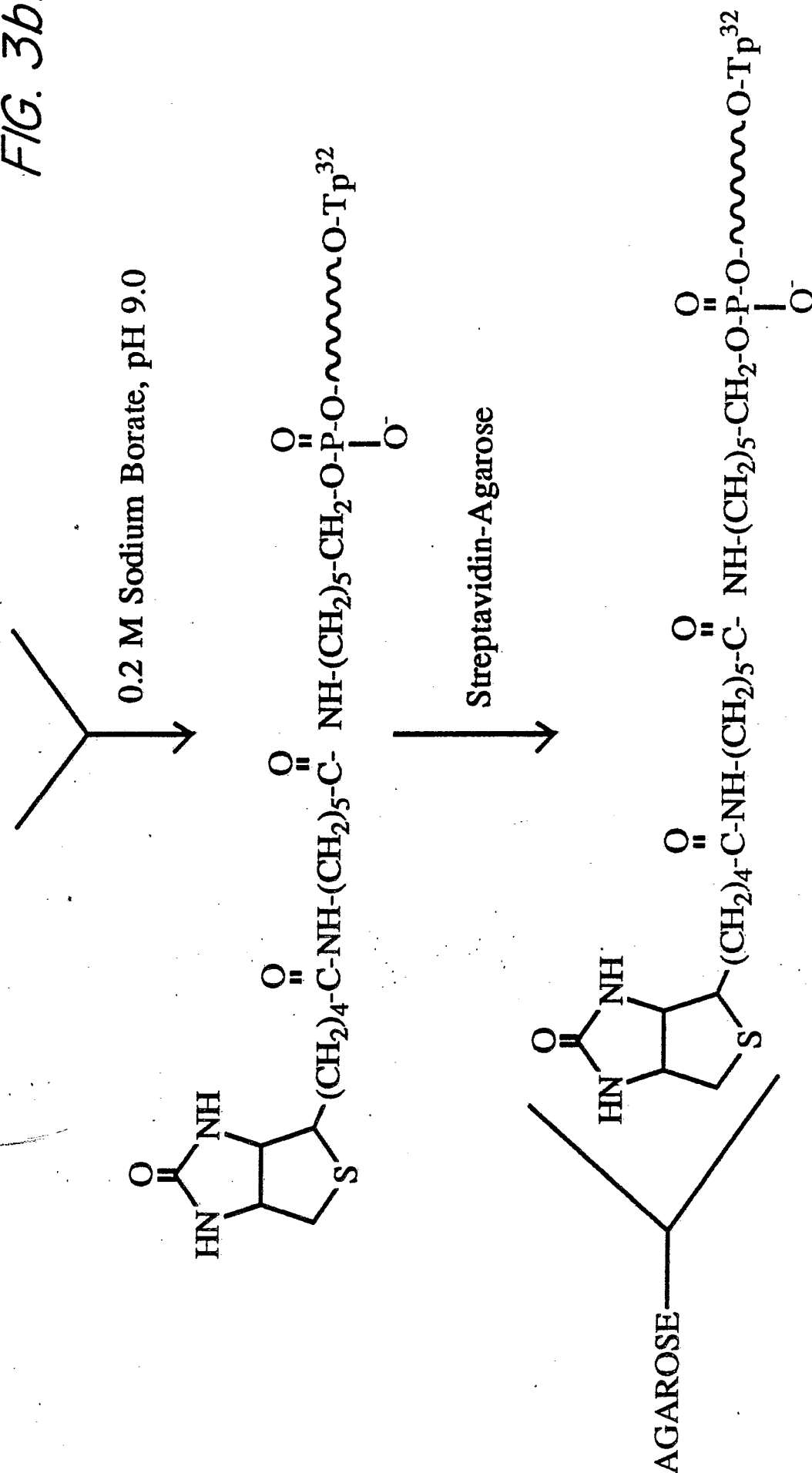


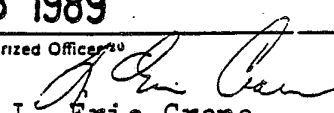
FIG. 3b.

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SUBSTITUTE SHEET



|  |  |                                     |
|--|--|-------------------------------------|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>3</sup>  |  |                                     |
| According to International Patent Classification (IPC) or to both National Classification and IPC<br>Int. Cl. (4th Ed.): C12Q 1/68; C12P 19/34; C07H 15/12; C07H 19/06<br>U.S. Cl.: 558/170; 435/6; 435/91; 536/27; 536/28; 536/29.  |  |                                     |
| <b>II. FIELDS SEARCHED</b>   |  |                                     |
| Minimum Documentation Searched <sup>4</sup>  |  |                                     |
| Classification System <sup>1</sup>   | Classification Symbols   |                                     |
| U.S.   | 558/170; 536/27; 536/28; 536/29; 435/6.  |                                     |
| Documentation Searched other than Minimum Documentation<br>to the extent that such Documents are included in the Fields Searched <sup>4</sup>  |  |                                     |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>  |  |                                     |
| Category <sup>1</sup>  | Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>   | Relevant to Claim No. <sup>18</sup> |
| P,Y  | US A 4,762,779 (David L. Snitman)<br>09 August 1988; see column 9 lines 1-60.  | 1-17                                |
| Y  | Nucleic Acids Research, Volume 13, Issued<br>1985, (Oxford, England), Smith et al.,<br>The synthesis of oligonucleotides<br>containing an aliphatic group at the 5'<br>terminus: synthesis of fluorescent DNA<br>primers for use in DNA sequence analysis,<br>see pp. 2399-2412. | , 1-17                              |
| Y  | Nucleic Acids Research, Volume 13, Issued<br>1985, (Oxford, England), Kempe et al.,<br>Chemical and enzymatic biotin-labeling<br>of oligonucleotides, see pp. 45-57.   | 1-17                                |
| Y  | Nucleic Acids Research, Volume 13, Issued<br>1985, (Oxford, England); Chollet et al,<br>Biotin-labeled synthetic oligodeoxyribo-<br>nucleotides: chemical synthesis and uses<br>as hybridization probes, see pp. 1529 -<br>1541  | 1-17                                |
| <p>* Special categories of cited documents: <sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> |  |                                     |
| <b>IV. CERTIFICATION</b>   |  |                                     |
| Date of the Actual Completion of the International Search <sup>3</sup>   | Date of Mailing of this International Search Report <sup>3</sup>   |                                     |
| 09 January 1989  | 16 FEB 1989  |                                     |
| International Searching Authority <sup>1</sup>   | Signature of Authorized Officer <sup>20</sup>  |                                     |
| ISA/US   | <br>L. Eric Crane   |                                     |

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

|      |  |      |
|------|--|------|
| Y    | Nucleic Acids Research, Volume 11,<br>Issued 1983, (Oxford, England),<br>Chu et al., Derivatization of<br>unprotected polynucleotides, see pp.<br>6513-6529.         | 1-17 |
| Y    | DNA, Volume 4, Issued 1985, (U.S.A.),<br>Chu et al., LABORATORY METHODS: Detection<br>of Specific DNA Sequences with Short<br>Biotin-Labeled Probes, see pp. 327-331 | 1-17 |
| P, Y | US A 4,500,707 (Marvin H. Caruthers et al)<br>19 February 1985, see columns 5-6.   | 1-17 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>10</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>11</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.