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(54) Title: POLYAMIDE NUCLEIC ACID DERIVATIVES, AGENTS AND METHODS FOR PRODUCING SAME

(57) **Abrégé/Abstract:**

The invention relates to PNA derivatives which carry a phosphoryl radical on the N terminus of the PNA backbone, for example a phosphate or a substituted phosphoryl radical, substituted phosphoryl derivatives optionally carrying one or more marker groups or groups for cross-linking or groups which favor intracellular take-up or groups which increase the binding affinity of the PNA derivative to nucleic acids. The invention also relates to a method for producing the aforementioned PNA derivatives and to their use as medicaments and diagnostic agents.



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(54) TITLE: POLYAMIDE NUCLEIC ACID DERIVATIVES, AGENTS AND METHODS FOR PRODUCING SAME

(54) TITRE: DÉRIVÉS D'ACIDE POLYAMIDONUCLÉIQUE, MOYENS ET PROCÉDÉS POUR LEUR PRODUCTION

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(57) Abstract: The invention relates to PNA derivatives which carry a phosphoryl radical on the N terminus of the PNA backbone, for example a phosphate or a substituted phosphoryl radical, substituted phosphoryl derivatives optionally carrying one or more marker groups or groups for cross-linking or groups which favor intracellular take-up or groups which increase the binding affinity of the PNA derivative to nucleic acids. The invention also relates to a method for producing the aforementioned PNA derivatives and to their use as medicaments and diagnostic agents.

(57) Zusammenfassung: Gegenstand der vorliegenden Erfindung sind PNA-Derivate, die am N-Terminus des PNA-Rückgrates einen Phosphoryl-Rest tragen, beispielsweise einen Phosphat- oder einen substituierten Phosphorylrest, wobei substituierte Phosphorylderivate gegebenenfalls eine oder mehrere Markergruppen, oder Gruppen zur Quervernetzung, oder Gruppen, die die intrazelluläre Aufnahme begünstigen, oder Gruppen, die die Bindungsaffinität des PNA-Derivats an Nukleinsäuren steigern, tragen. Gegenstand der Erfindung ist ferner ein Verfahren zur Herstellung der oben genannten PNA-Derivate und ihre Verwendung als Arzneimittel und diagnostische Mittel.

WO 01/79249

PCT/EP01/04027

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Polyamide Nucleic Acid Derivatives, Agents and Methods for Producing Same

5 The present invention relates to N-terminally phosphorylated polyamide nucleic acid (PNA) derivatives having improved properties, to their use and to agents and processes for preparing them.

10 Polyamide nucleic acids, also termed peptide nucleic acids (PNA), bind to complementary target sequences (DNA or RNA) with a higher affinity than do natural oligonucleotides and, furthermore, have the advantage, as compared with natural DNA, that they are very stable in serum. PNA were originally described as unnatural nucleic acid analogs in which the entire sugar-phosphate backbone is replaced with N-(2-aminoethyl)glycine units (M. Egholm et al. (1991) Science 254, 1497-1500; WO 92/20702; M. Egholm et al. Nature (1993) 365, 566-568; P. Nielsen, (1994) Bioconjugate Chem. 5, 3-7; E. Uhlmann et al. (1998) Angewandte Chemie Int. Ed. Engl. 37, 2796-2823). The bases employed are the nucleobases which occur naturally and are customary in nucleotide chemistry or else nucleobases which do not occur naturally, or their prodrug forms, that is precursors which are only converted into the free base by biotransformation in the body. In addition to this, PNAs have been described in which not all the positions in the backbone carry base residues (Greiner et al. (1999) Helv. Chim Acta 82, 2151), and in which aminoethylglycine is replaced by more complex units (Uhlmann et al. (1998) Angewandte Chem. Int. Ed. 37, 2796; Falkiewicz (1999) Biochim. Pol., 46, 509-529).

25 The fact that the PNA backbone does not have any net charge is an important feature of this class of substances and has far-reaching consequences. The fact that PNA binds to complementary DNA and RNA even at low salt concentration (e.g. Peptide Nucleic Acids: Protocols and Applications; Peter E. Nielsen and Michael Egholm (Edit.) Horizon Scientific Press, 1999, 3), with the Watson-Crick base pairing rules being obeyed, is ascribed to the neutral character of the PNA and the decrease in charge repulsion which is associated

30

therewith. For this reason, PNA can, in principle, be used for numerous applications in which natural oligonucleotides or oligonucleotide derivatives would otherwise be employed. However, in addition to this, because of the unique binding properties, a large number of applications which are not possible with natural oligonucleotides also ensue (see, for example: Peptide Nucleic Acids: Protocols and Applications; Peter E. Nielsen and Michael Egholm (Edit.) Horizon Scientific Press, 1999). For example, a strand invasion of double-stranded DNA has been observed when using PNA.

Typical examples of applications for PNA include its use for inhibiting gene expression by binding, in a sequence-specific manner, to cellular DNA or RNA. "Antisense agents" are short, single-stranded nucleic acid derivatives which bind, by means of Watson-Crick base pairing, to a complementary mRNA whose translation into the corresponding protein is to be inhibited (Uhlmann and Peyman (1990) Chem. Rev. 90, 543; Larsen et al. (1999) Biochem. Biophys. Acta 1489, 159). "Anti-gene agents" bind, by way of Hoogsteen base pairing, in the major groove of the DNA double helix with the formation of a triple helix, resulting in transcription of the genes being inhibited in a sequence-specific manner (Praseuth et al. (1999) Biochem. Biophys. Acta 1489, 181). Gene expression can also be specifically inhibited by so-called decoy oligomers, which mimic the regions for binding transcription factors. By treating with decoy agents, particular transcription factors can be captured in a sequence-specific manner and activation of transcription thereby prevented (Mischiati et al. (1999) J. Biol. Chem. 274, 33114). Another group of oligonucleotide derivatives which act intracellularly, i.e. the chimeraplasts, are used for specific gene proof-reading (Cole-Strauss et al. (1996) Science 273, 1386-1389).

PNAs can, therefore, be used as pharmaceuticals and/or diagnostic agents or for producing pharmaceuticals and/or diagnostic agents.

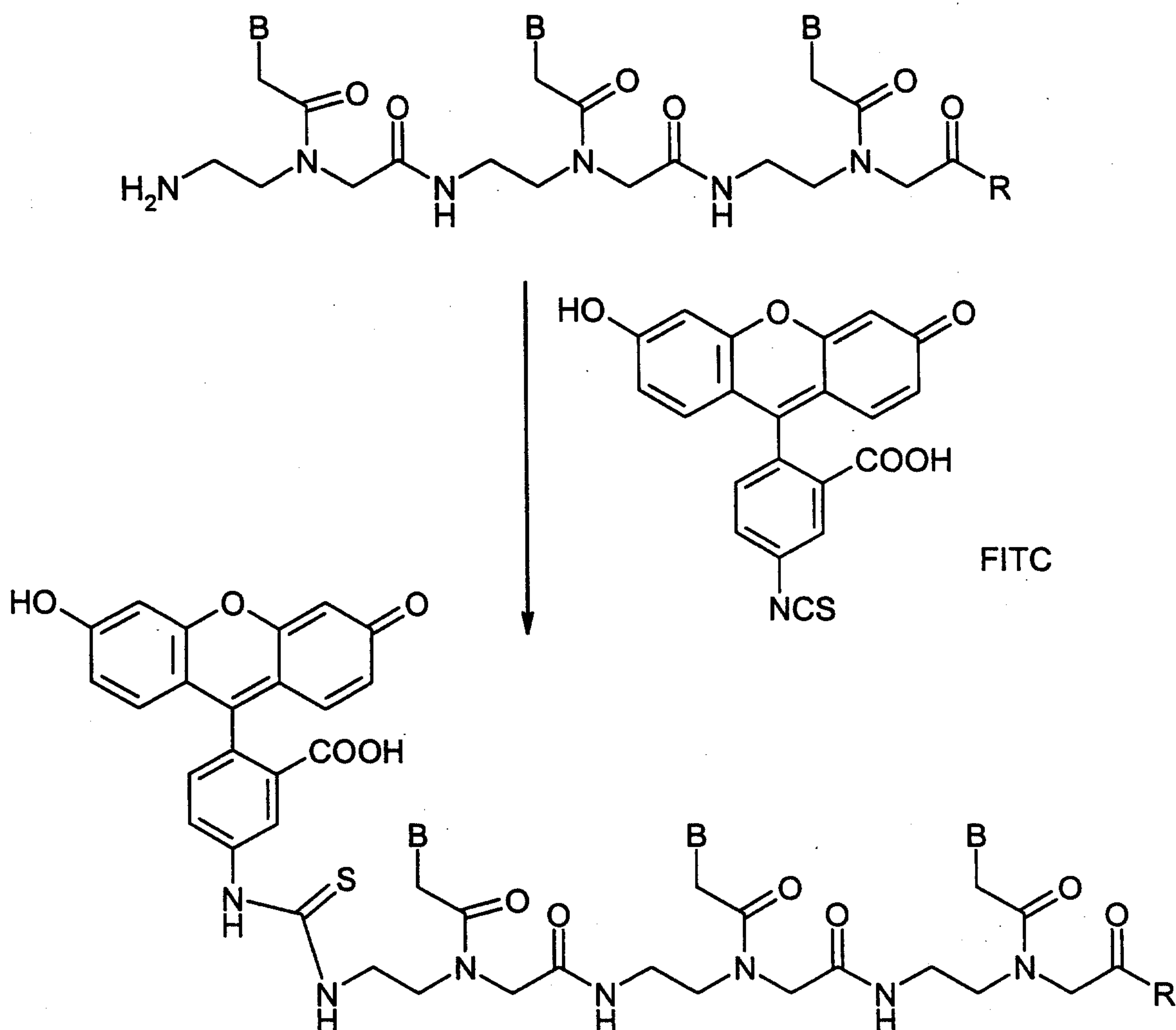
For example, after having been labeled with biotin, fluorescein or other labels, PNA can be used, for diagnostic purposes and in molecular biology, as a

specific hybridization probe. Four methods have so far been described in the literature for introducing the labeling groups (Oerum et al. (1999), in Peptide Nucleic Acids: Protocols and Applications, pages 81-86; Lohse et al. (1997) Bioconjugate Chem. 8, 503). The first method is based on labeling the free
5 (deprotected) PNA after it has been synthesized in solution. In this method, the aminotermminus of the PNA is reacted with an activated carboxylic acid or an isothiocyanate. However, additional lysine residues are frequently introduced into the PNA, with these residues then being reacted with fluorescein isothiocyanate (FITC).

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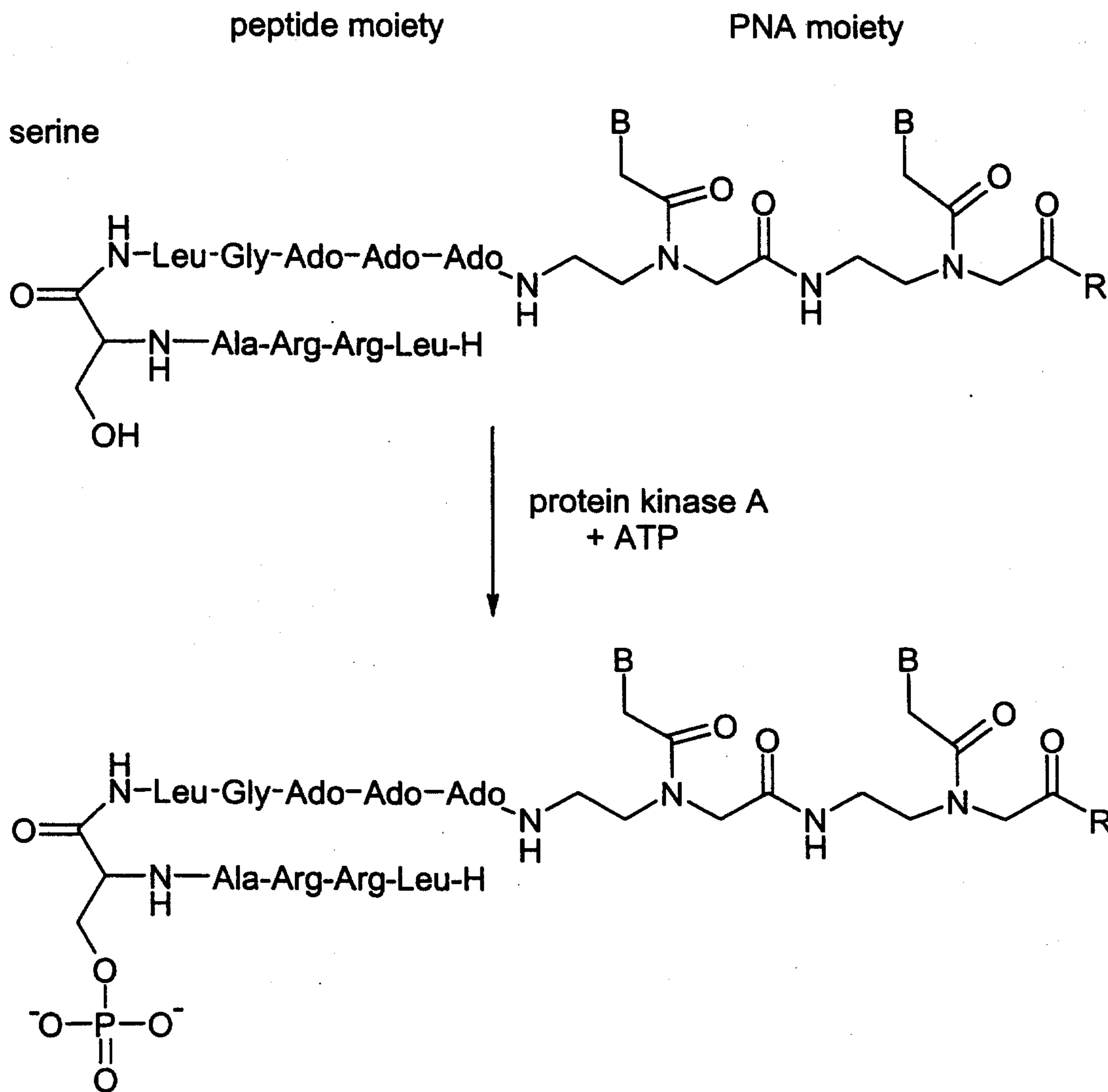
In the second method, the protected PNA is modified at its aminotermminus with activated carboxylic acid derivatives or isothiocyanates while it is still on the solid phase. This method is only suitable for labeling groups which are stable under the conditions which pertain during deprotection of the PNA and during its
15 cleavage from the support. The reactive reagents which are preferably used in both cases are isothiocyanates (P. Wittung et al., (1995) FEBS Lett. 375, 27) and activated carboxylic acids, such as N-hydroxysuccinimide esters (NHS) (Oerum et al., 1999). A disadvantage of the reaction using the NHS derivatives is that it is frequently only accomplished with poor yields. For this reason, 8-
20 amino-3,6-dioxaoctanoic acid is frequently condensed, as a linker or spacer, between the PNA and the labeling group (Oerum et al., 1999). Both linkages are effected by way of amide bonds or thiourea bonds, which, as such, are, however, more likely to lead to insolubility. Alternatively, the carboxylic acids are caused to react using activators which are customary in peptide chemistry, such
25 as HBTU, TBTU or HATU.

4



- In a third method, fluorescein-conjugated monomers are used during the synthesis of the PNA on the solid phase, with the fluorescence labeling being effected by way of an amide bond (Lohse et al. (1997) *Bioconjugate Chem.* 8, 503), which once again leads to conjugates which are relatively difficult to dissolve.
- 10 A fourth method uses PNA peptide conjugates in which the peptide moiety forms a substrate for a protein kinase (Koch et al. (1995) *Tetrahedron Lett.* 36, 6933). In this way, therefore, it is not the PNA moiety which is modified; rather, the serine residue in the peptide segment is phosphorylated enzymically. When this method is used, therefore, it is only possible to introduce radioactive

phosphate, and not, for example, any fluorescein or biotin, into the peptide segment of the PNA-peptide conjugate.



It is known that PNA tends to aggregate in aqueous solution, that is under physiological conditions as well. PNA is therefore poorly soluble in aqueous buffer and is then unavailable for hybridizing to complementary sequences. Furthermore, PNA has a high affinity for various materials such as Sephadex® (from Pharmacia), Bond Elut® (from Varian) or various HPLC chromatograph materials which are used in purifying oligomers, which means that PNA can frequently only be isolated in poor yields. It is therefore necessary to conjugate PNA with lysine or other positively charged amino acids (by way of the

C terminus) (Egholm et al (1992) J. Am. Chem. Soc. 114, 1895). Guanine-rich PNA sequences have a very particular tendency to aggregate, for which reason the advice is not to use such PNA (see "Guidelines for sequence design of PNA oligomers" in Peptide Nucleic Acids: Protocols and Applications (1999) pages 5 253-255). For example, relatively long fluorescein-labeled PNA oligomers are particularly difficult to dissolve, with the addition of an organic solvent and heating to 50°C being recommended.

- It is particularly difficult to purify the poorly soluble lipophilic PNA derivatives.
- 10 Several peaks which are due to PNA aggregates are frequently detected in the HPLC. The technique of polyacrylamide (PAA) gel electrophoresis, which is frequently employed for purifying and separating nucleic acids, cannot be used for these PNA derivatives.
- 15 In the methods of derivatizing PNA which are described above, the labeling group is always introduced by forming an amide bond or a thioamide bond, with PNA derivatives being formed which are relatively difficult to dissolve. Poorly soluble PNA derivatives are formed, in particular, when lipophilic residues, such as fluorescein, are introduced. Since, furthermore, the labeling reactions
- 20 frequently proceed with poor yields, an object to be achieved consists in making available novel PNA derivatives which it should be possible to prepare in high yields, which should exhibit advantageous properties, such as improved solubility, improved binding behavior and better cellular uptake, and which, in addition, make it possible to use efficient methods for purifying the PNA
- 25 oligomers.

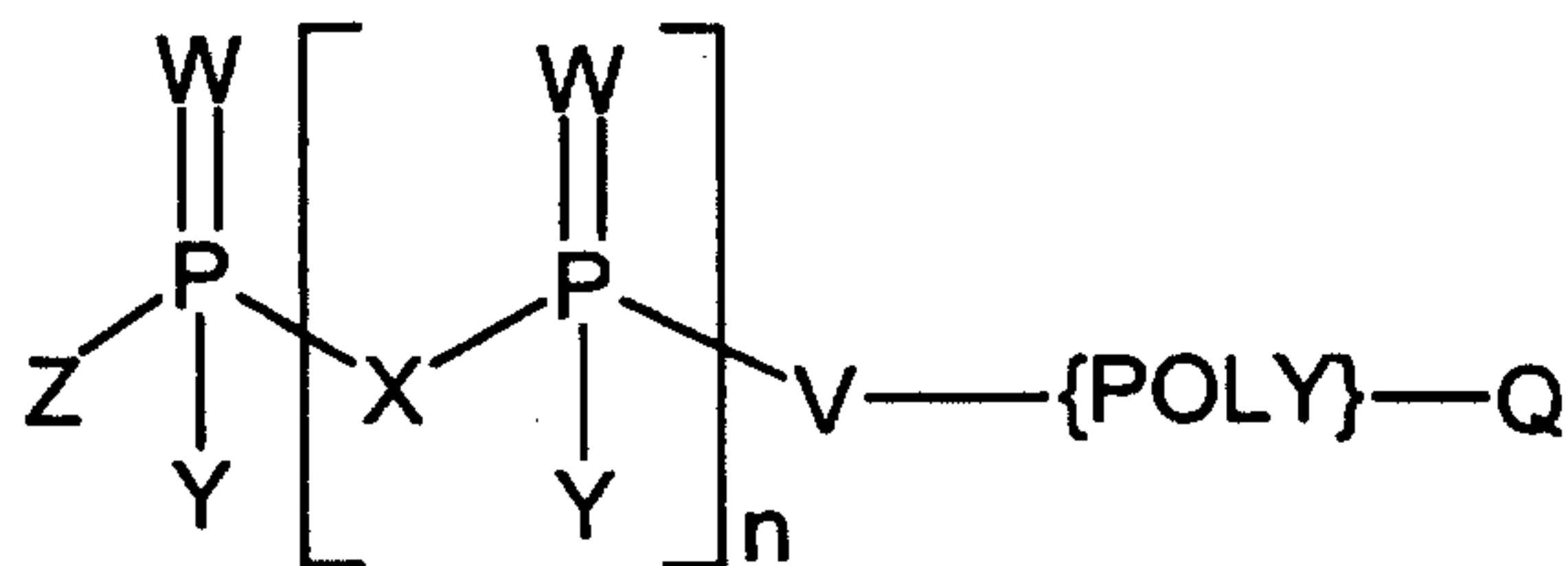
According to the invention, this object is achieved by making available PNA derivatives which carry one or more phosphoryl radicals at the N terminus of the PNA backbone, with thio derivatives and imino derivatives also being included

30 in addition to oxo derivatives, and with at least one of the phosphoryl radicals carrying one or more deprotonatable groups, preferably hydroxyl groups or mercapto groups. The phosphoryl radicals are linked to the PNA backbone by

way of an oxygen-phosphorus bond, sulfur-phosphorus bond or nitrogen-phosphorus bond, either directly or by way of a spacer, with it being possible for the spacer to be, for example, an alkanoylamide, a poly(alkoxy)carboxamide or an amino acid which, where appropriate, carry a side chain at the α -C or β -C atom, with this side chain not being allowed to carry any phosphoryl radical. Examples of phosphoryl radicals are phosphate, phosphonate, thiophosphate, phosphoamidate and substituted phosphoryl radicals, with substituted phosphoryl radicals carrying, where appropriate, one or more labeling groups, or groups for crosslinking, or groups which promote intracellular uptake, or groups which increase the binding affinity of the PNA derivative for nucleic acids.

In this connection, labeling groups (labels) are understood as being groups which enable the chemical or biological activity of the PNA derivatives to be assessed qualitatively or quantitatively, for example biotin or fluorescein. Crosslinking is understood as being the formation of intramolecular or intermolecular bonds between spatially adjacent functionalities. An example of a group for crosslinking is the psoralen group.

The invention preferably relates to PNA derivatives of the formula I



Formula I

where

V is oxygen, sulfur, NR_1 , a group $\text{U}-(\text{CR}_3\text{R}_4)_{\text{u}}'-\text{C}(\text{O})-\text{NH}$ or a group $\text{U}-(\text{CH}_2\text{CH}_2\text{O})_{\text{u}}'-\text{CH}_2-\text{C}(\text{O})-\text{NH}$,

U is, independently of each other, oxygen, sulfur or NH ,

5

u' is, independently of each other, from 1 to 10, preferably from 1 to 4, particularly preferably 1,

W is, independently of each other, oxygen, sulfur or NR_1 ,

10

Y is, independently of each other, hydroxyl, mercapto, oxyanion, thioate or NR_1R_2 ,

R_1 and R_2 are, independently of each other, a radical consisting of hydrogen or C_1 - C_6 -alkyl, preferably hydrogen,

15

R_3 and R_4 are, independently of each other, a radical consisting of hydrogen or C_1 - C_6 -alkyl, or the radical of an amino acid side chain, preferably hydrogen,

20

X is, independently of each other, a group $\text{U}-(\text{C}_2\text{-C}_{22}\text{-alkanediyl})-\text{U}$ or a group $\text{U}-(\text{CH}_2\text{CH}_2\text{-O})_{\text{u}}'$,

25

or is a labeling group or a group for crosslinking, or a group which promotes intracellular uptake, or a group which increases the binding affinity of the PNA derivative for nucleic acids, for example a bifunctional fluorescein, rhodamine, TAMRA, biotin, pyrene, dinitrophenyl, cholesteryl, acridine, adamantyl, vitamin E, cyanine dye, Dabcyl, Edans, lexitropsin, psoralen, BODIPY, ROX, R6G or digoxigenin radical,

Z is hydroxyl, mercapto, oxyanion, thioate or NR_1R_2 , $\text{C}_1\text{-C}_{22}\text{-alkyl}$, $\text{C}_1\text{-C}_8\text{-arylalkyl}$, $\text{C}_1\text{-C}_{22}\text{-alkyl-U}$, $\text{C}_1\text{-C}_8\text{-arylalkyl-U}$, hydroxy- $\text{C}_1\text{-C}_{18}\text{-U}$, aminoalkyl-U or mercaptoalkyl-U, or a group of the formula $\text{R}_9(\text{CH}_2\text{CH}_2\text{-O})_m$, where R_9 is hydroxyl, amino or $\text{C}_1\text{-C}_{22}\text{-alkoxy}$, and m is from 1 to 100, preferably from 2 to 10, or is a labeling group, or a group for crosslinking, or a group which promotes intracellular uptake, or a group which increases the binding affinity of the PNA derivative for nucleic acids, for example a monofunctional or bifunctional fluorescein, rhodamine, TAMRA, biotin, pyrene, dinitrophenyl, cholesteryl, acridine, adamantyl, vitamin E, cyanine dye, Dabcyl, Edans, Lexitropsin, Psoralen, BODIPY, ROX, R6G or digoxigenin radical,

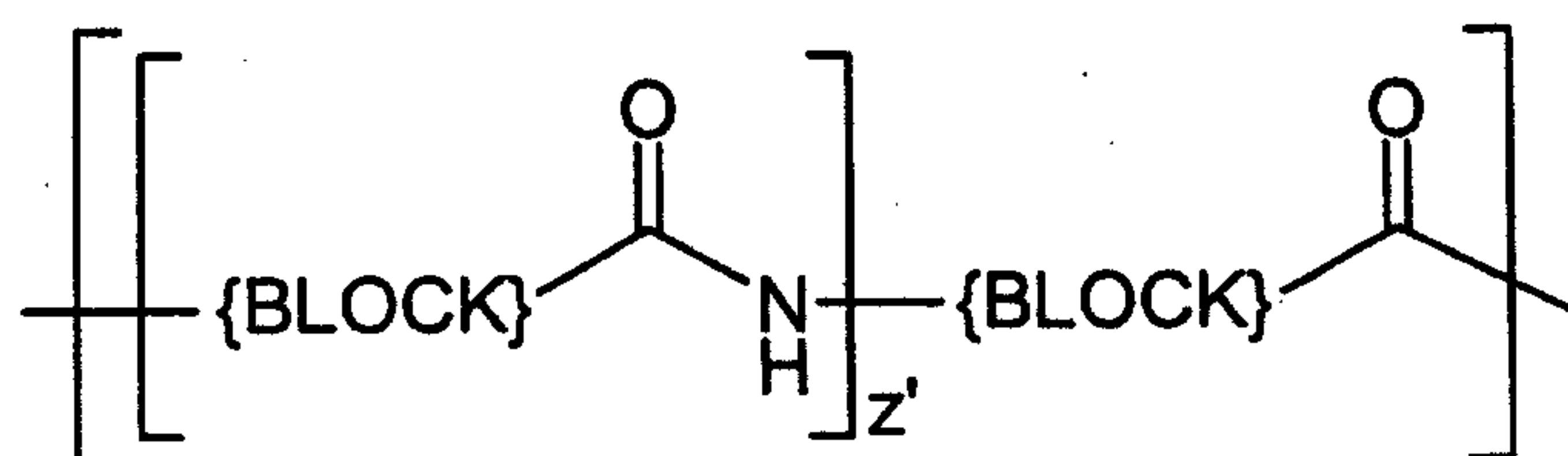
n is from 0 to 10, preferably from 0 to 3,

Q is hydroxyl, amino, NHR_7 , NR_7R_8 , an amino acid derivative or a peptide radical,

R_7 and R_8 are, independently of each other, $\text{C}_1\text{-C}_{18}\text{-alkyl}$ or hydroxy- $\text{C}_1\text{-C}_{18}\text{-alkyl}$,

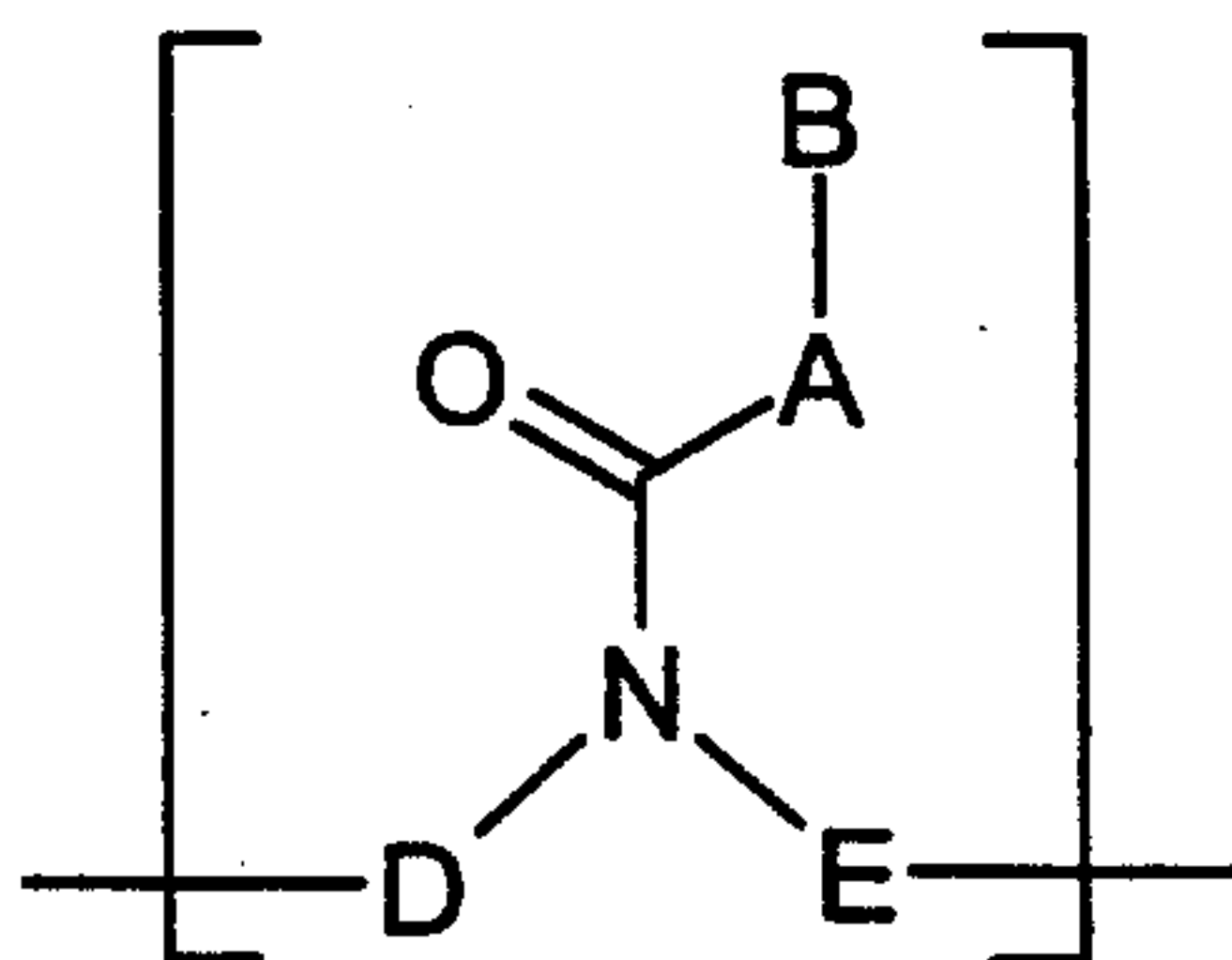
with the proviso that at least one Y or Z radical is hydroxyl, mercapto, oxyanion or thioate.

{POLY} is described by the formula II.



Formula II

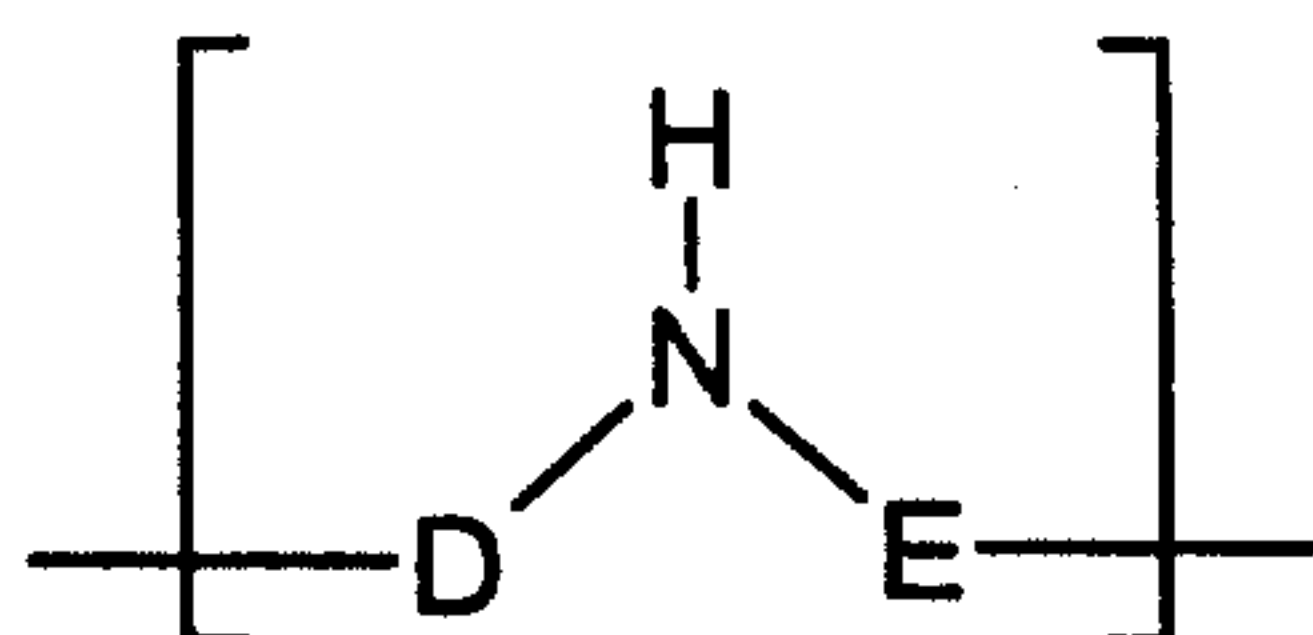
The PNA backbone is consequently composed of $z'+1$ monomers, where {BLOCK} is, independently of each other, a group selected from formula IIIA,



Formula IIIA

5

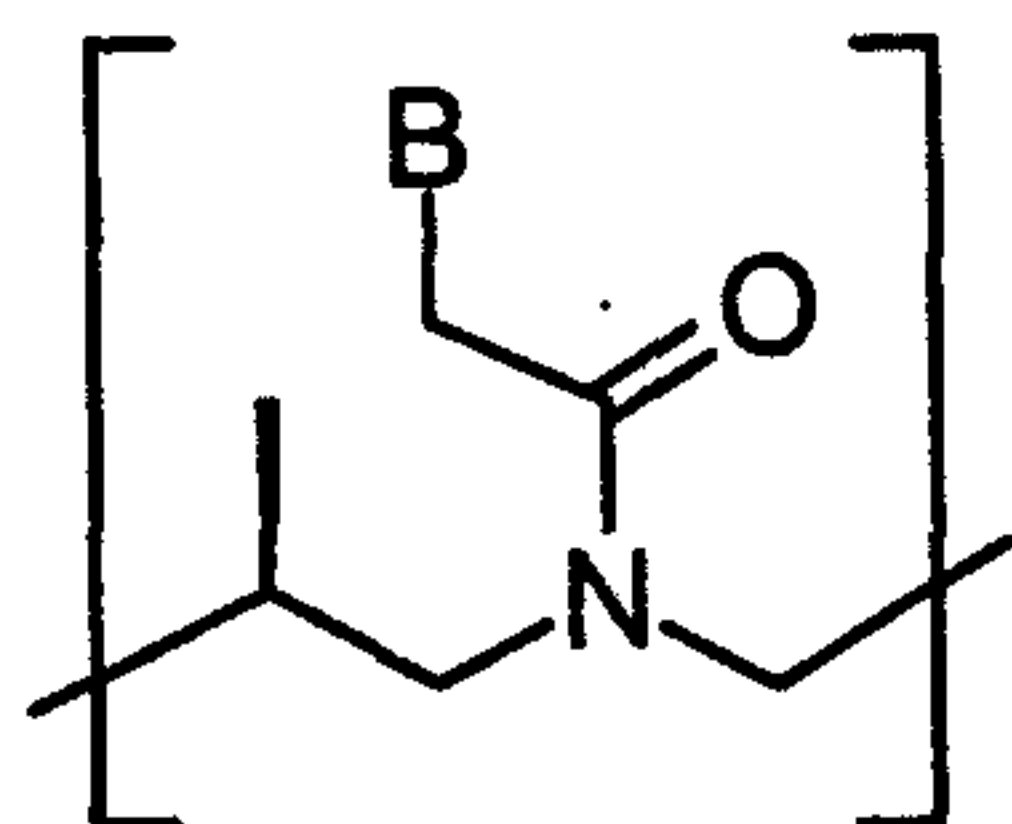
or from formula IIIB (Greiner et al. (1999) *Helv. Chim Acta* 82, 2151),



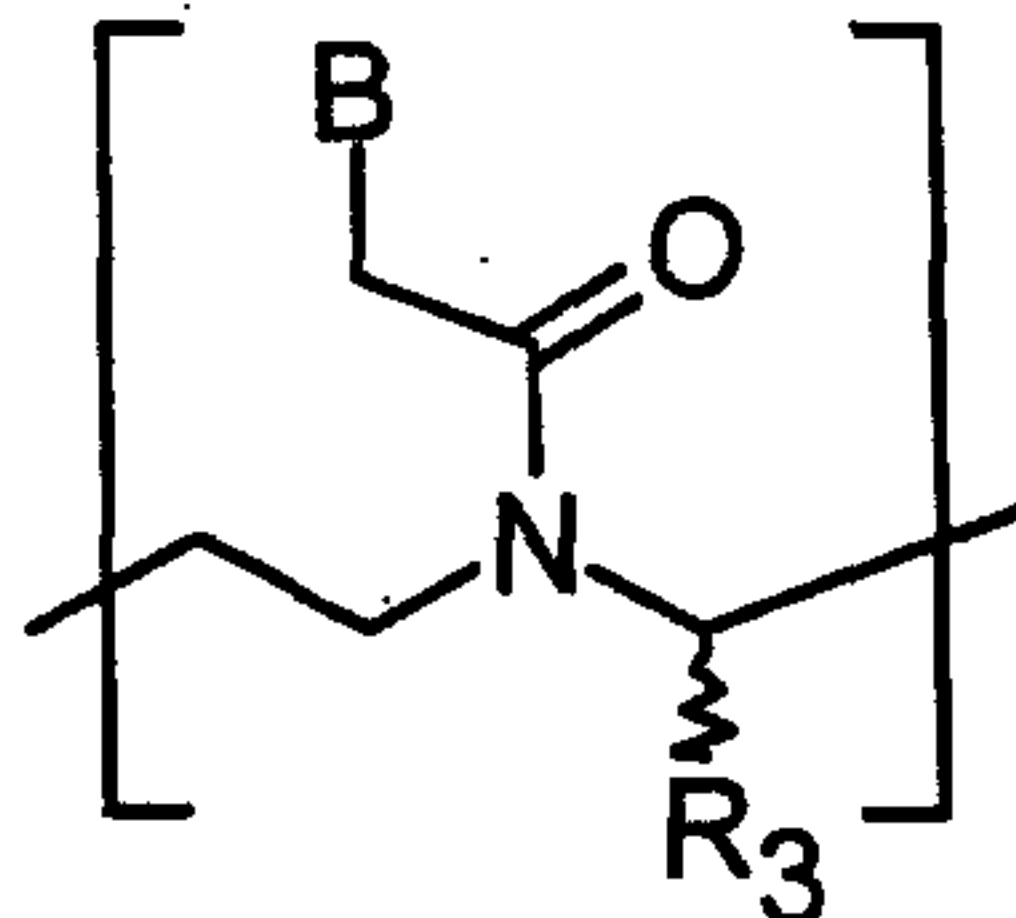
Formula IIIB

10

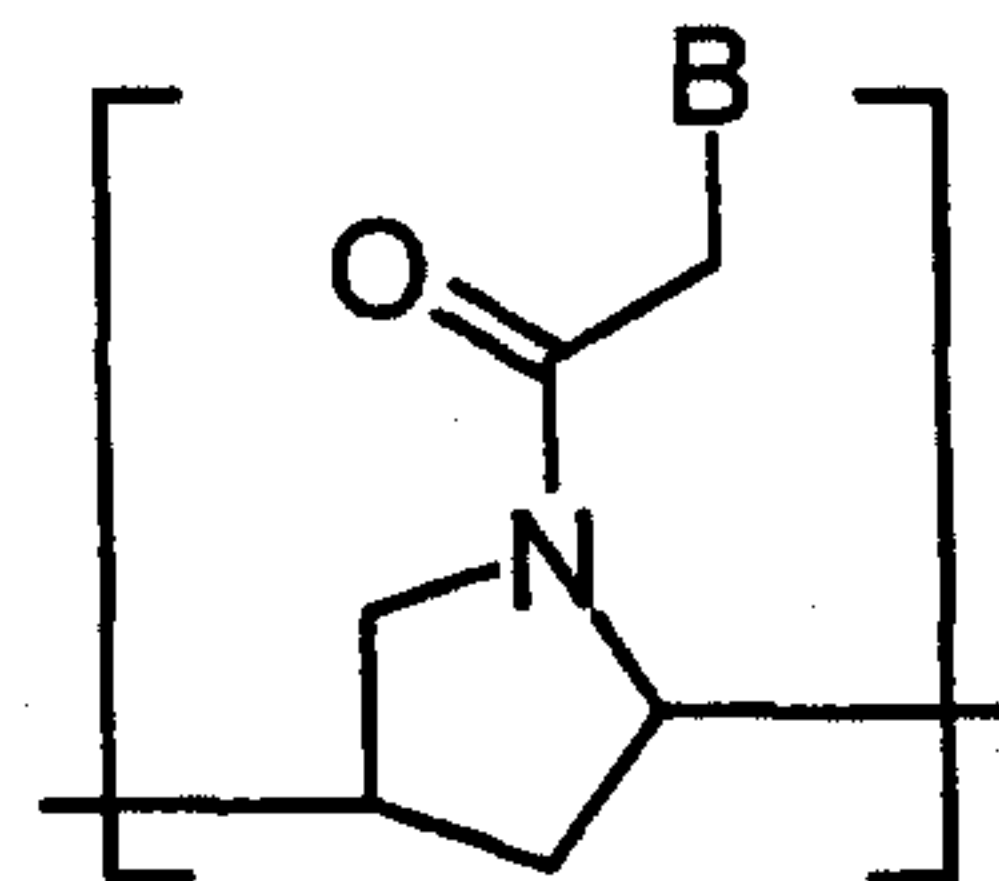
or from the formulae IV A to IV G (Uhlmann et al. (1998) *Angewandte Chem. Int. Ed.* 37, 2796; Falkiewicz (1999) *Biochim. Pol.*, 46, 509-529),



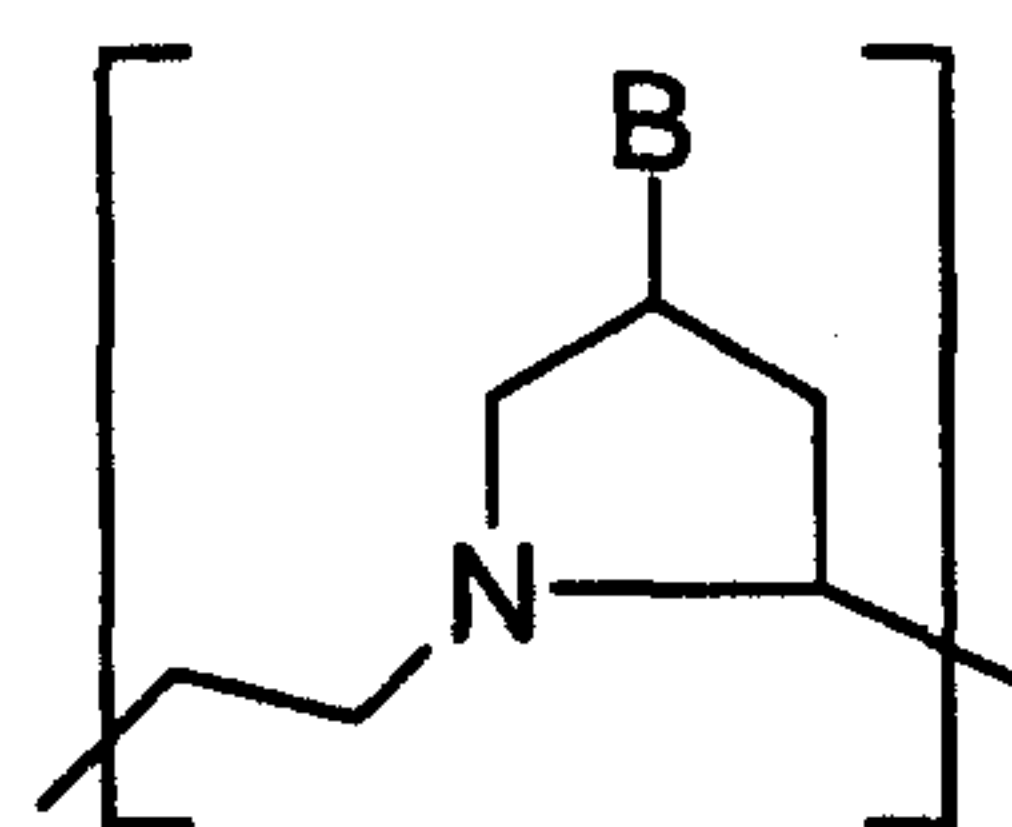
Formula IV A



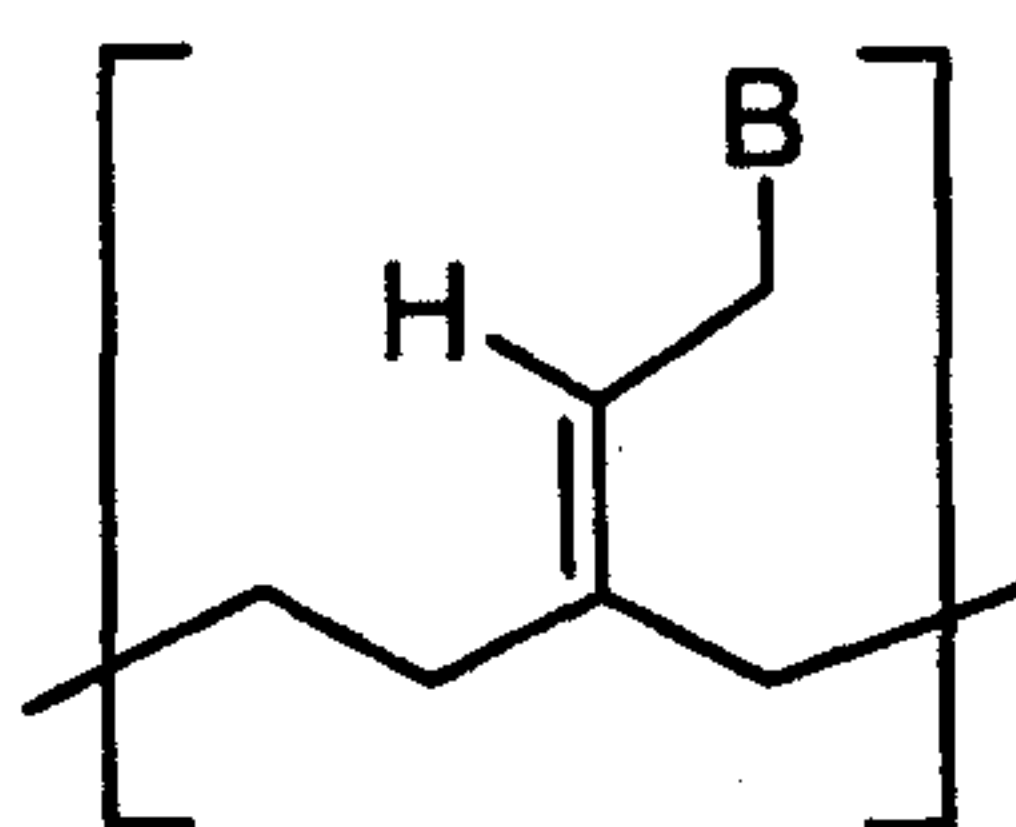
Formula IV B



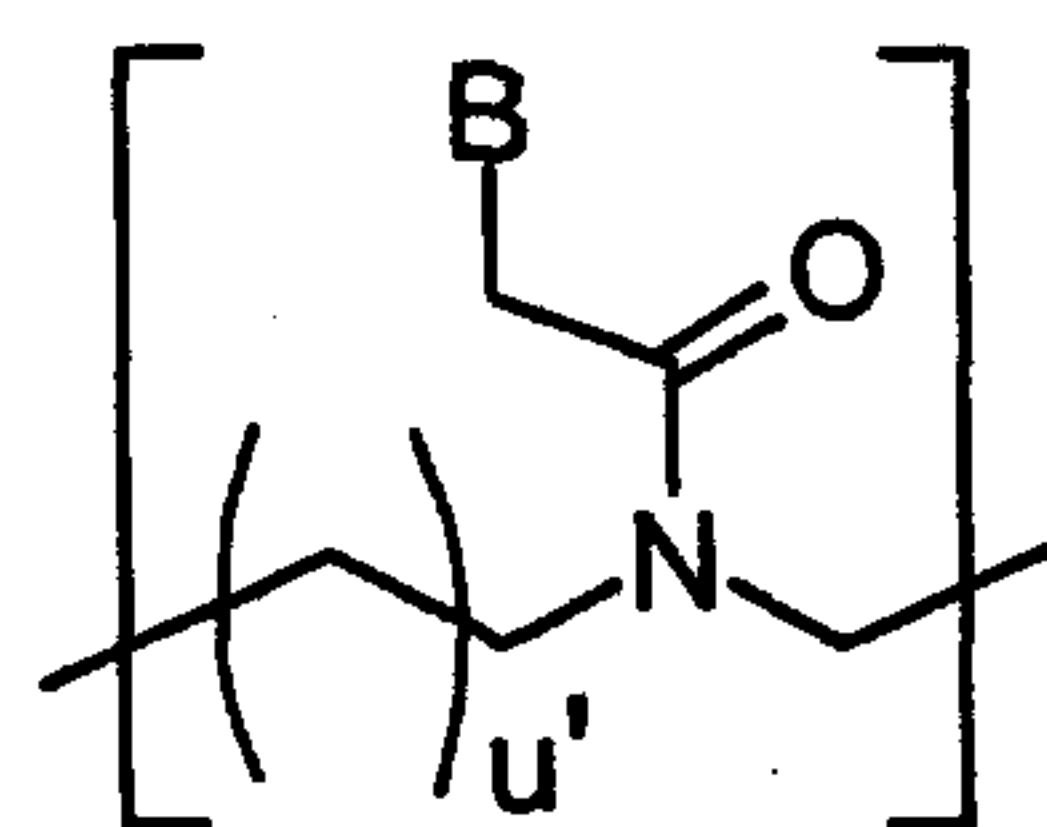
Formula IV C



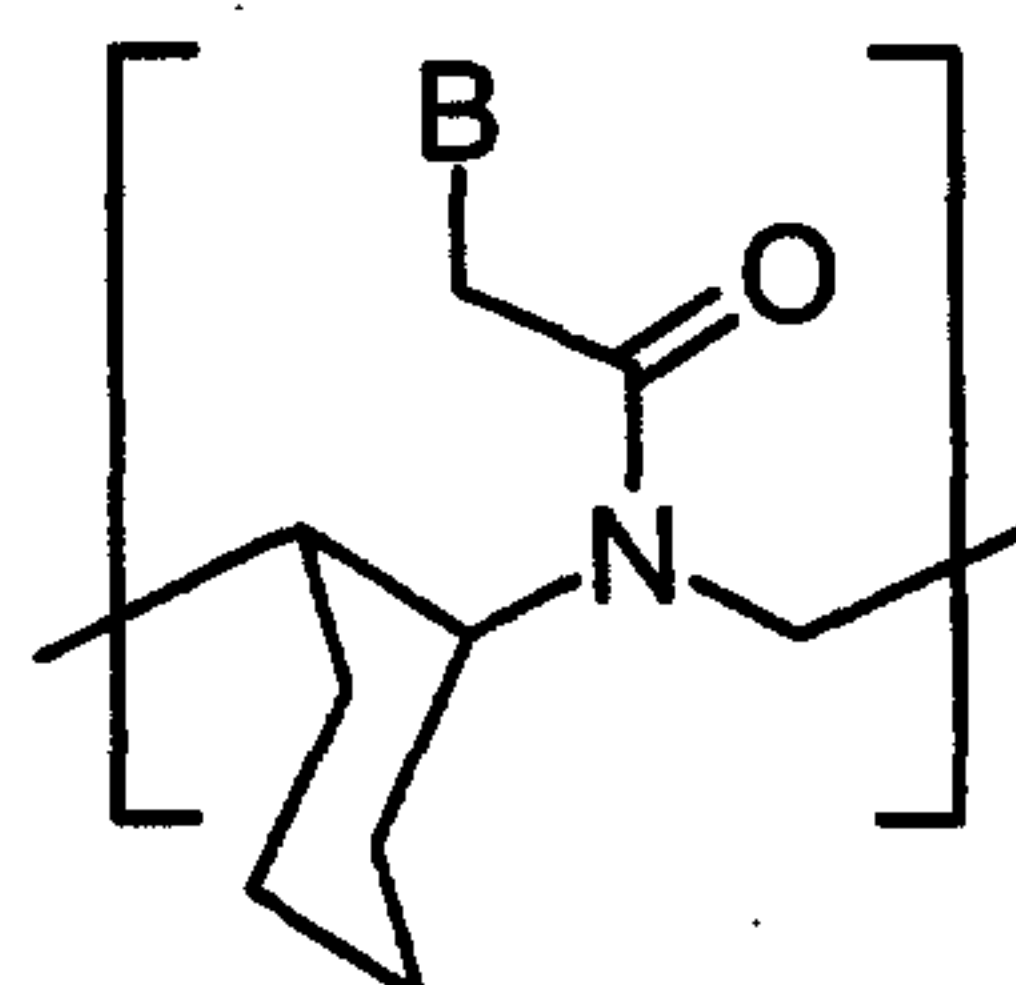
Formula IV D



Formula IV E



Formula IV F



Formula IV G

where each {BLOCK} building block can be different,

5 and where

A is, independently of each other, a group $(CR_1R_2)_s$, where s is from 1 to 3, preferably 1,

10 B is, independently of each other, either an aromatic radical, which can also possess heteroaromatic character, or hydrogen, or hydroxyl or C_1 - C_{18} -alkyl,
or a nucleobase which occurs naturally, and is customary in nucleotide chemistry, or which does not occur naturally, or its prodrug form,
15 where at least one B radical in formula II is a nucleobase,

D is, independently of each other, a group $(CR_3R_4)_t$, where t is from 2 to 10, preferably from 2 to 4, particularly preferably 2, where R_3 and R_4 have the abovementioned meaning, and two adjacent D radicals can also form a C₅-C₈-cycloalkyl ring,

5

E is, independently of each other, a group $(CR_5R_6)_u$, where two adjacent R_5 and R_6 radicals can also form a C₅- to C₈-cycloalkyl ring or a spiro compound,

10 R_5 and R_6 are, independently of each other, a radical consisting of hydrogen or C₁-C₆-alkyl, preferably hydrogen, or the radical of an amino acid side chain,

z' is from 0 to 100, preferably 1-20, particularly preferably 4-15.

15

In addition, the invention relates to physiologically tolerated salts of the PNA derivatives of the formula I. Physiologically tolerated salts are described, inter alia, in Remingtons Pharmaceutical Science (1985) Mack Publishing Company, Easton, PA, USA, page 1418. Preference is given to ammonium salts, 20 trialkylammonium salts, alkali metal salts (such as sodium salts and potassium salts) and alkaline earth metal salts (such as magnesium salts and calcium salts). Particular preference is given to sodium salts.

It has been found, surprisingly, that a negative partial charge on the phosphoryl radical is already sufficient to improve decisively the properties of the 25 compounds of the formula I. Whereas PNA itself does not migrate during PAA gel electrophoresis, the compounds of the formula I migrate to the anode. Since the phosphoryl radical is only introduced in the last cycle during the synthesis of the compounds of the formula I, it is only the compounds according to the 30 invention which migrate in the electrical field during the PAA gel electrophoresis,

while all the erroneous sequences and byproducts do not migrate and can consequently be separated off in an extremely simple manner.

The hydroxy or mercapto substituents of the phosphoryl radicals of the DNA derivatives according to the invention can be deprotonated in a pH range of from 4.5 to 14, preferably of from 6.5 to 12, particularly preferably of from 6.5 to 9. The property of the ionizability of the phosphoryl radicals can advantageously be exploited for purifying the compounds of the formula I. On the one hand, the compounds of the formula I can be purified by electrophoresis, for example polyacrylamide gel electrophoresis. On the other hand, it is also possible to purify them using anion exchangers. In this case, the desired products are preferably eluted by using a salt gradient, for example a sodium chloride gradient, or a pH gradient. The PNA derivatives of the formula I according to the invention can be particularly simply and efficiently purified using anion exchangers. It was found that the uncharged byproducts are not retarded on the anion exchanger whereas the charged product adhered to the column. After washing with water, it was possible to isolate the desired product in pure form using acetic acid or a sodium chloride solution. The anion exchangers employed are preferably strong anion exchangers or mixed-mode phases, such as ®Oasis MAX (Waters GmbH, Eschborn).

It was furthermore found that the compounds of the formula I according to the invention are in general more readily soluble in aqueous medium than are the corresponding PNA oligomers which do not possess the phosphoryl radical. This makes itself very particularly apparent, in the form of a greatly improved solubility in aqueous medium, in the case of the lipophilic derivatives, such as the fluorescein derivatives or the hexadecyl derivatives.

An additional surprising, positive effect which was found was that the introduction of a phosphoryl radical, for example as phosphate or else in the form of a lipophilic derivatization (e.g. as a hexadecyl phosphodiester) increases the affinity of the PNA for complementary DNA or RNA. This effect was

unexpected since the strong bonding of PNA to complementary DNA or RNA was attributed to the neutral character of the PNA and the reduced charge repulsion which was associated therewith (e.g. Peptide Nucleic Acids: Protocols and Applications; Peter E. Nielsen and Michael Egholm (Edit.) Horizon Scientific Press, 1999, 3).

The biotin was introduced particularly efficiently by way of a phosphoryl radical. When used as hybridization probes, the biotinylated PNA of the formula I (X and/or Z = biotin radical) displayed better binding properties and fewer spurious, nonspecific background effects than did corresponding biotinylated DNA probes.

In contrast to the uncharged PNA, the PNA derivatives of the formula I according to the invention can also migrate in an electric field, thereby making it possible to microlocate them and concentrate them on immobilized complementary nucleic acid derivatives. In the case of the polyanionic oligonucleotides, this method using the electrical field has already been described for rapidly determining base mismatches (Sosnowski et al. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 1119).

The invention relates, in particular, to PNA derivatives in which A and E are CH_2 . The invention furthermore relates, in particular, to PNA derivatives in which D substituents are $(\text{CH}_2)_2$. Preference is also given to PNA derivatives of the formula I in which V, W and Y are oxygen.

Examples of natural bases are adenine, cytosine, 5-methylcytosine, guanine, thymine and uracil. Examples of unnatural bases are purine, 2,6-diaminopurine, N^4N^4 -ethanocytosine, N^6N^6 -ethano-2,6-diaminopurine, 5-($\text{C}_3\text{-C}_6$)-alkynyluracil, 5-($\text{C}_3\text{-C}_6$)-alkynylcytosine, 5-(1-propargylamino)uracil, 5-(1-propargylamino)cytosine, phenoxazine, 9-aminoethoxyphenoxazine, 5-fluorouracil or pseudoisocytosine, 5-(hydroxymethyl)uracil, 5-aminouracil,

pseudouracil, dihydrouracil, 5-(C₁-C₆)-alkyluracil, 5-(C₁-C₆)-alkylcytosine, 5-(C₂-C₆)-alkenylcytosine, 5-fluorocytosine, 5-chlorouracil, 5-chlorocytosine, 5-bromouracil, 5-bromocytosine, 7-deazaadenine, 7-deazaguanine, 8-azapurine, and 7-deaza-7-substituted purines.

5

Q is preferably a hydroxyalkylamino radical, in particular a hydroxyhexylamino radical, or a derivative of a known natural or unnatural amino acid or of a peptide. Suitable peptide sequences are preferably those which optimize the organ distribution or the cellular location of the PNA, such as transportan,
 10 insulin-like growth factor, nuclear localization signals or other carrier sequences (Larsen et al. (1999) Biochim. Biophys. Acta 159-166). The peptide can also be used as an affinity tag, as, for example, a (His)₆ chain.

The present invention enables the X and Z radicals to be varied broadly
 15 (Examples are given in Figures 1a, 1b, 2a, 2b, 3a and 3b) and thereby makes it possible to introduce different specific functional features into the PNA.

A preferred embodiment of Z is a C₁- to C₂₂-alkyl radical. Preference is also given to C₁- to C₂₂-alkoxy radicals, in particular C₁₆-alkoxy radicals. Other
 20 preferred radicals are hydroxy (C₁-C₁₈-alkoxy) radicals, in particular HO(CH₂)₃₋₁₂O. Preference is also given to aminoalkoxy radicals, in particular 6-aminohexoxy and 5-aminopentoxo radicals. Preference is also given to radicals of the formula R₉(CH₂CH₂-O)_m, where R₉ is hydroxyl, amino or C₁-C₂₂-alkoxy, preferably, however, hydroxyl, and m is from 0 to 100,
 25 preferably from 2 to 10. Particular preference is given to HO(CH₂CH₂-O)₂, HO(CH₂CH₂-O)₆ and H₂N-(CH₂CH₂-O)₂. Other preferred examples of Z include mercaptoalkoxy radicals, in particular 6-mercaptohexyloxy.

In another preferred embodiment, Z comprises a fluorescent group, such as fluorescein, rhodamine, TAMRA or a cyanine dye. Preferred fluorescent groups can be found in Figures 1a to 3b. Very particular preference is also given to Z being biotin. Other preferred groups for Z include Dabcyl, psoralen, acridine, DNP and cholesterol (Figures 1b and 2b), BODIPY-, ROX- or R6G radicals (Su-Chun Hung et al. (1998) Analytical Biochemistry 255, 32-38) and digoxigenin (Tarrason et al., Methods in Enzymology (1999) Vol. 313, 257-268). In addition to this, Z can be a group consisting of a monofunctional or a bifunctional fluorescein, rhodamine, TAMRA, biotin, pyrene, dinitrophenyl, cholesteryl, acridine, adamantyl, vitamin E, cyanine dye, Dabcyl, Edans, lexitropsin, or psoralen radical. Monofunctional end groups are executed by way of example in Figures 1a, 1b, 2a and 3a, while bifunctional bridging groups are listed in Figures 2b and 3b. In another preferred embodiment, n is 0, i.e. the PNA moiety carries only one phosphate or phosphoryl radical.

15

A preferred embodiment of X is U-(C₂-C₂₂-alkanediyl)-U, in particular O-(C₂-C₂₂-alkanediyl)-O, particularly preferably O-(CH₂)₂₋₆-O. Another preferred embodiment of X is a group of the formula U-(CH₂CH₂-O)_u', where u' is from 1 to 10, preferably from 1 to 6, and where U is preferably oxy. In a further preferred embodiment, X comprises a fluorescent group such as fluorescein, rhodamine, TAMRA or a cyanine dye, for example @Cy3 (from Amersham Pharmacia Biotech). Preferred bifunctional groups can be found in Figures 2a and 3a. Very particular preference is also given to X being biotin. Other groups which are preferred for X include Dabcyl, psoralen, acridine, DNP, cholesterol, BODIPY, digoxigenin, ROX- and R6G radicals.

25

The different radicals for X and Z in formula I can fulfill different functions. The fluorescein radicals have far-reaching applications in DNA sequencing and signal amplification or as markers for determining the cellular uptake of PNA. The cyanine dye radicals (@Cy3 and @Cy5) give a substantially more intense and longer-lasting fluorescence signal than does fluorescein itself. The psoralen

30

radical is employed for crosslinking with complementary nucleic acids. The acridine radical is an effective intercalator and can thereby augment the binding affinity of the PNA. Biotin, acridine and psoralen derivatives can also be used for antisense experiments. In addition, hexadecyloxy and cholesterol derivatives
5 can be used for increasing the ability of the PNA to traverse membranes. DNP-labeled compounds of the formula I can be detected using anti-DNP antibodies. Aminoalkoxy radicals can be used for coupling on other groups, for example lexitropin (cf. Example 17; PNA-16). In a similar way, mercaptoalkoxy groups can also be used for further derivatization.

10

The invention furthermore relates to the use of the PNA derivatives of the formula I as pharmaceuticals. These pharmaceuticals can be used for preventing and/or treating diseases which are accompanied by the expression or overexpression of particular genes. The invention furthermore relates to the
15 use of PNA derivatives of the formula I as diagnostic agents. These diagnostic agents can be used for diagnosing the abovementioned diseases at an early stage.

20

When being employed as pharmaceuticals or diagnostic agents, the PNA derivatives of the formula I can be used as antisense agents, anti-gene agents, decoy agents and chimeraplast agents, depending on their sequence. The PNA derivatives according to the invention are used, in particular, for producing pharmaceuticals for treating diseases in which defined genes are the cause, or are involved, as a result of their overexpression.

25

These pharmaceuticals can, for example, be used for treating diseases which are provoked by viruses, for example by CMV, HIV, HSV-1, HSV-2, influenza, VSV, hepatitis B or papilloma viruses, with the corresponding virus sequence being the target.

30

Antisense PNA derivatives according to the invention which are active against these targets have, for example, the following base sequences.

a) against CMV, e.g.

SEQ ID NO. 1 5'-G C G T T T G C T C T T C T T C T T G C G -3'

5

b) against HIV, e.g.

SEQ ID NO. 2 5'-A C A C C C A A T T C T G A A A A T G G -3'

SEQ ID NO. 3 5'-A G G T C C C T G T T C G G G C G C C A -3'

10

c) against HSV-1, e.g.

SEQ ID NO. 4 5'-G C G G G G C T C C A T G G G G G T C G -3'

15 Such pharmaceuticals are also suitable, for example, for treating cancer. In this connection, it is preferably possible to use sequences which are directed against targets which are responsible for the carcinogenesis or the growth of a cancer, in particular by inhibiting the telomerase (E. Matthes et al. (1999) Nucleic Acids Res. 27, 1152). Additional targets of this nature are:

20 1) Nuclear oncoproteins, such as c-myc, N-myc, c-myb, c-fos, c-fos/jun, PCNA, p120,

2) Cytoplasmic/membrane-associated oncoproteins, such as EJ-ras, c-Ha-ras, N-ras, rrg, bcl-2, cdc-2, c-raf-1, c-mos, c-src, c-abl, c-ets,

25

3) Cell receptors, such as EGF receptor, Her-2, c-erbA, VEGF receptor (KDR-1), retinoid receptors, protein kinase regulatory subunit, c-fms, Tie-2, c-raf-1 kinase, PKC-alpha, and protein kinase A (R1 alpha),

30 4) Cytokines, growth factors and extracellular matrix, such as CSF-1, IL-6, IL-1a, IL-1b, IL-2, IL-4, IL-6, IL-8, bFGF, VEGF, myeloblastin and fibronectin.

Antisense PNA derivatives which are active against such targets have, for example, the following base sequences:

a) against c-Ha-ras, e.g.

5

SEQ ID NO. 5	5'-CAGCTGCAACCCAGC-3'
SEQ ID NO. 6	5'-TATTCCGTCAT-3'
SEQ ID NO. 7	5'-TTCCGTCATCGCTCCTCAGGGG-3'

10 b) bFGF, e.g.

SEQ ID NO. 8	5'-GGCTGCCATGGTCCC-3'
--------------	-----------------------

c) c-myc, e.g.

15

SEQ ID NO. 9	5'-GGCTGCTGGAGCGGGGCACAC-3'
SEQ ID NO. 10	5'-AACGTTGAGGGGGCAT-3'

d) c-myb, e.g.

20

SEQ ID NO. 11	5'-GTGCCGGGGTCTTCGGGC-3'
---------------	--------------------------

e) c-fos, e.g.

25

SEQ ID NO. 12	5'-CGAGAACATCATCGTGG-3'
SEQ ID NO. 13	5'-GGAGAACATCATGGTCGAAAG-3'
SEQ ID NO. 14	5'-CCCGAGAACATCATGGTCGAAAG-3'
SEQ ID NO. 15	5'-GGGGAAAGCCCGGCAAGGGG-3'

30 f) p120, e.g.

SEQ ID NO. 16	5'-CACCCGCCTTGGCCTCCAC-3'
---------------	---------------------------

g) EGF receptor, e.g.

SEQ ID NO. 17 5'-G G G A C T C C G G C G C A G C G C -3'
5 SEQ ID NO. 18 5'-G G C A A A C T T T C T T T T C C T C C -3'

h) p53 tumor suppressor, e.g.

SEQ ID NO. 19 5'-G G G A A G G A G G A G G A T G A G G -3'
10 SEQ ID NO. 20 5'-G G C A G T C A T C C A G C T T C G G A G -3'

i) bcl-2, e.g.

SEQ ID NO. 21 5'-T C T C C C A G C G T G C G C C A T -3'

15 k) VEGF, e.g.

SEQ ID NO. 22 5'-G C G C T G A T A G A C A T C C A T G -3'
SEQ ID NO. 23 5'-G G A G G C C C G A C C -3'
SEQ ID NO. 24 5'-G G T T T C G G A G G C -3'
SEQ ID NO. 25 5'-T G G T G G A G G T A G -3'
20 SEQ ID NO. 26 5'-G C A T G G T G G A G G -3'
SEQ ID NO. 27 5'-T T G G C A T G G T G G -3'
SEQ ID NO. 28 5'-G C C T G G G A C C A C -3'
SEQ ID NO. 29 5'-C A G C C T G G G A C C -3'
SEQ ID NO. 30 5'-T G C A G C C T G G G A -3'
25 SEQ ID NO. 31 5'-G T G C A G C C T G G G -3'
SEQ ID NO. 32 5'-G G T G C A G C C T G G -3'
SEQ ID NO. 33 5'-A T G G G T G C A G C C -3'
SEQ ID NO. 34 5'-G G C T T G A A G A T G -3'
SEQ ID NO. 35 5'-G C A G C C C C C G C A -3'
30 SEQ ID NO. 36 5'-G C A G C A G C C C C C -3'

l) c-raf kinase, e.g.

SEQ ID NO. 37 5'-T C C C G C C T G T G A C A T G C A T T -3'

m) PKC-alpha, e.g.

5

SEQ ID NO. 38 5'-G T T C T C G C T G G T G A G T T T C A -3'

n) protein kinase A, e.g.

SEQ ID NO. 39 5'-G C G T G C C T C C T C A C T G G C -3'

10

Pharmaceuticals which comprise PNA derivatives of formula I are furthermore suitable, for example, for treating diseases which are effected by integrins or cell-cell adhesion receptors, for example by VLA-4, VLA-2, ICAM, VCAM or ELAM.

15 Antisense PNA derivatives which are active against such targets have, for example, the following base sequences:

a) VLA-4, e.g.

20 SEQ ID NO. 40 5'-G C A G T A A G C A T C C A T A T C -3'

b) ICAM-1, e.g.

SEQ ID NO. 41 5'-G C C C A A G C T G G C A T C C G T C A -3'
25 SEQ ID NO. 42 5'-C C C C C A C C A C T T C C C C T C T C -3'
SEQ ID NO. 43 5'-C T C C C C C A C C A C T T C C C C T C -3'
SEQ ID NO. 44 5'-G C T G G G A G C C A T A G C G A G G -3'

c) ELAM-1, e.g.

30

SEQ ID NO. 45 5'-A C T G C T G C C T C T T G T C T C A G G -3'
SEQ ID NO. 46 5'-C A A T C A A T G A C T T C A A G A G T T C -3'

d) Integrin alpha(V), e.g.

SEQ ID NO. 47 5'-G C G G C G G A A A G C C A T C G -3'

5

Pharmaceuticals which comprise PNA derivatives of the formula I are also suitable, for example, for preventing restenosis. In this connection, it is possible to use PNA sequences which are directed against targets which are responsible for proliferation or migration. Examples of such targets are:

10

1) Nuclear transactivator proteins and cyclins, such as c-myc, c-myb, c-fos, c-fos/jun, cyclins and cdc2 kinase

15

2) Mitogens or growth factors, such as PDGF, bFGF, VEGF, EGF, HB-EGF and TGF- β .

3) Cell receptors, such as bFGF receptor, EGF receptor and PDGF receptor.

20

Antisense PNA derivatives which are active against such targets have, for example, the following base sequences:

a) c-myb, e.g.

SEQ ID NO. 48 5'-G T G T C G G G G T C T C C G G G C -3'

25

b) c-myc, e.g.

SEQ ID NO. 49 5'-C A C G T T G A G G G G C A T -3'

30

c) cdc2 kinase, e.g.

SEQ ID NO. 50 5'-G T C T T C C A T A G T T A C T C A -3'

d) PCNA (proliferating cell nuclear antigen of rat), e.g.

SEQ ID NO. 51 5'-G A T C A G G C G T G C C T C A A A-3'

5

PNA derivatives can likewise be used for treating vitiligo and other depigmentation diseases or depigmentation disturbances (e.g. of the skin, the hair and the eyes), such as albinism and psoriasis, or for treating asthma, with expression of the adenosine A1 receptor, the adenosine A3 receptor or the
10 bradykinin receptor, or of IL-13, being inhibited using suitable antisense agents. An example of such a base sequence is:

SEQ ID NO. 52 5'-G A T G G A G G G C G G C A T G G C G G G-3'

15 Pharmaceuticals which comprise a PNA derivative of the formula I can be used, for example, in the form of pharmaceutical preparations which can be administered orally, for example in the form of tablets, coated tablets, hard or soft gelatin capsules, solutions, emulsions or suspensions. They can also be administered rectally, e.g. in the form of suppositories, or parenterally, e.g. in
20 the form of solutions for injection. In order to produce pharmaceutical preparations, these compounds can be processed in therapeutically inert organic and inorganic excipients. Examples of such excipients for tablets, coated tablets and hard gelatin capsules are lactose, cornstarch or derivatives thereof, tallow and stearic acid or salts thereof. Suitable excipients for preparing
25 solutions are water, polyols, sucrose, invert sugar and glucose. Suitable excipients for injection solutions are water, alcohols, polyols, glycerol and vegetable oils. Suitable excipients for suppositories are vegetable oils and hydrogenated oils, waxes, fats and semiliquid polyols. The pharmaceutical preparations can also comprise preservatives, solvents, stabilizers, wetting
30 agents, emulsifiers, sweeteners, dyes, flavorants, salts for altering the osmotic pressure, buffers, coating agents, antioxidants and, where appropriate, other therapeutic active compounds.

Preferred administration forms are topical applications, local applications, for example using a catheter or by inhalation, injections or infusions and oral administration. For injection, the PNA derivatives of the formula I are formulated in a liquid solution, preferably in a physiologically acceptable buffer, such as
5 Hank's solution or Ringer's solution. However, the oligonucleotides can also be formulated in solid form and dissolved or suspended before use. The doses which are preferred for systematic administration are from about 0.01 mg/kg to about 50 mg/kg of bodyweight and per day.

10 The invention furthermore relates to pharmaceutical preparations which comprise PNA derivatives of the formula I and/or their physiologically tolerated salts in addition to pharmaceutically unobjectionable excipients and/or additives. The PNA derivatives of the formula I and/or their physiologically tolerated salts can be administered to animals, preferably to mammals, and in particular to
15 humans, as pharmaceuticals, on their own, in mixtures with each other, or in the form of pharmaceutical preparations which permit topical, percutaneous, parenteral or enteral use and which comprise, as the active constituent, an effective dose of at least one PNA derivative together with customary, pharmaceutically unobjectionable excipients and additives. The preparations
20 normally comprise from about 0.1 to 90% by weight of the therapeutically active compound. A topical application, for example in the form of ointments, lotions or tinctures, emulsions or suspensions, is preferred for treating skin diseases.

The pharmaceutical preparations are produced in a manner known per se (e. g.
25 Remingtons Pharmaceutical Sciences, Mack Publ. Co., Easton, PA.), with pharmaceutically inert inorganic and/or organic excipients being used. It is possible, for example, to use lactose, cornstarch and/or derivatives thereof, tallow, stearic acid and/or its salts, etc., for producing pills, tablets, coated tablets and hard gelatin capsules. Examples of excipients for soft gelatin
30 capsules and/or suppositories are fats, waxes, semisolid and liquid polyols, natural and/or hydrogenated oils, etc. Suitable excipients for producing solutions and/or syrups are, for example, water, sucrose, invert sugar, glucose,

polyols, etc. Suitable excipients for producing solutions for injection are water, alcohols, glycerol, polyols, vegetable oils, etc. Suitable excipients for microcapsules, implants and/or rods are copolymers consisting of glycolic acid and lactic acid. Liposome formulations which are known to the skilled person (N. Weiner, Drug Develop Ind Pharm 15 (1989) 1523; "Liposome Dermatics, Springer Verlag 1992), for example HVJ liposomes (Hayashi, Gene Therapy 3 (1996) 878) are also suitable. Dermal application can also be effected, for example, using ionophoretic or methods and/or using electroporation.

10 In addition to the active compounds and excipients, a pharmaceutical preparation can also contain additives, such as fillers, extenders, disintegrants, binders, glidants, wetting agents, stabilizers, emulsifiers, preservatives, sweeteners, dyes, flavorants or aromatizing agents, thickeners, diluents and buffering substances, and, furthermore, solvents and/or solubilizing agents and/or agents for achieving a sustained release effect, and also salts for altering the osmotic pressure, coating agents and/or antioxidants. They can also comprise two or more different PNA derivatives of the formula I and/or their physiologically tolerated salts and also, furthermore, in addition to at least one PNA derivative of the formula I, one or more different therapeutically active substances. The dose can vary within wide limits and is to be adjusted to the individual circumstances in each individual case.

The invention furthermore relates to the use of PNA derivatives of the formula I as diagnostic agents, in particular as aids in DNA diagnosis and in molecular biology (see, for example: Peptide Nucleic Acids: Protocols and Applications; Peter E. Nielsen and Michael Egholm (Edit.) Horizon Scientific Press, 1999). In DNA diagnosis, gene probes, also termed DNA probes or hybridization probes, play an important role in the sequence-specific detection of particular genes. In general, a gene probe consists of a recognition sequence and one or more suitable labeling groups (labels). The specificity with which a target sequence in an analytical sample is identified by means of hybridization with a complementary gene probe is determined by the recognition sequence and its

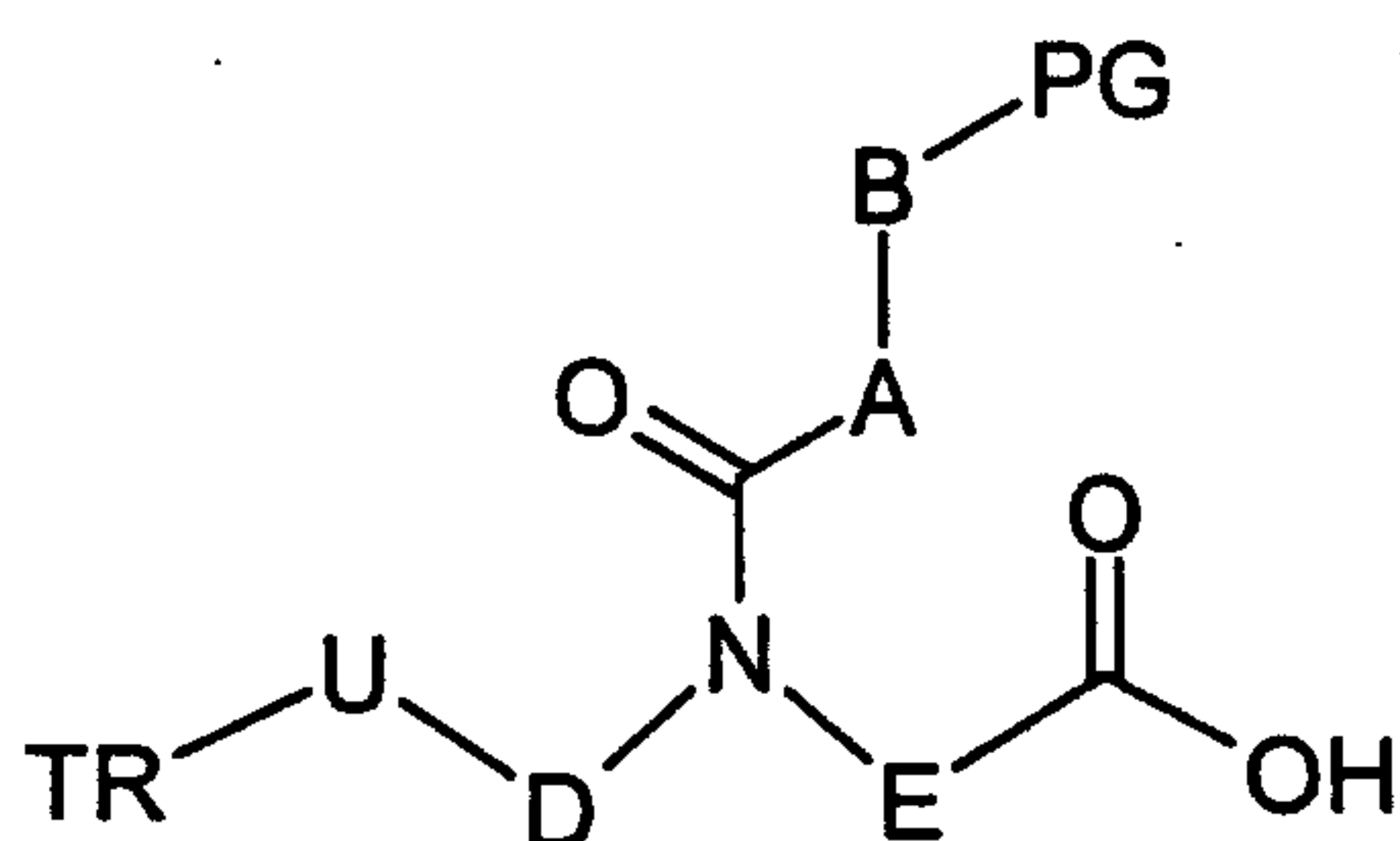
chemical structure. This technique can also be applied to PNA. As compared with oligonucleotides having a natural structure, PNA has the advantage that it has a higher affinity for the target sequence and a greater ability to discriminate between bases.

5

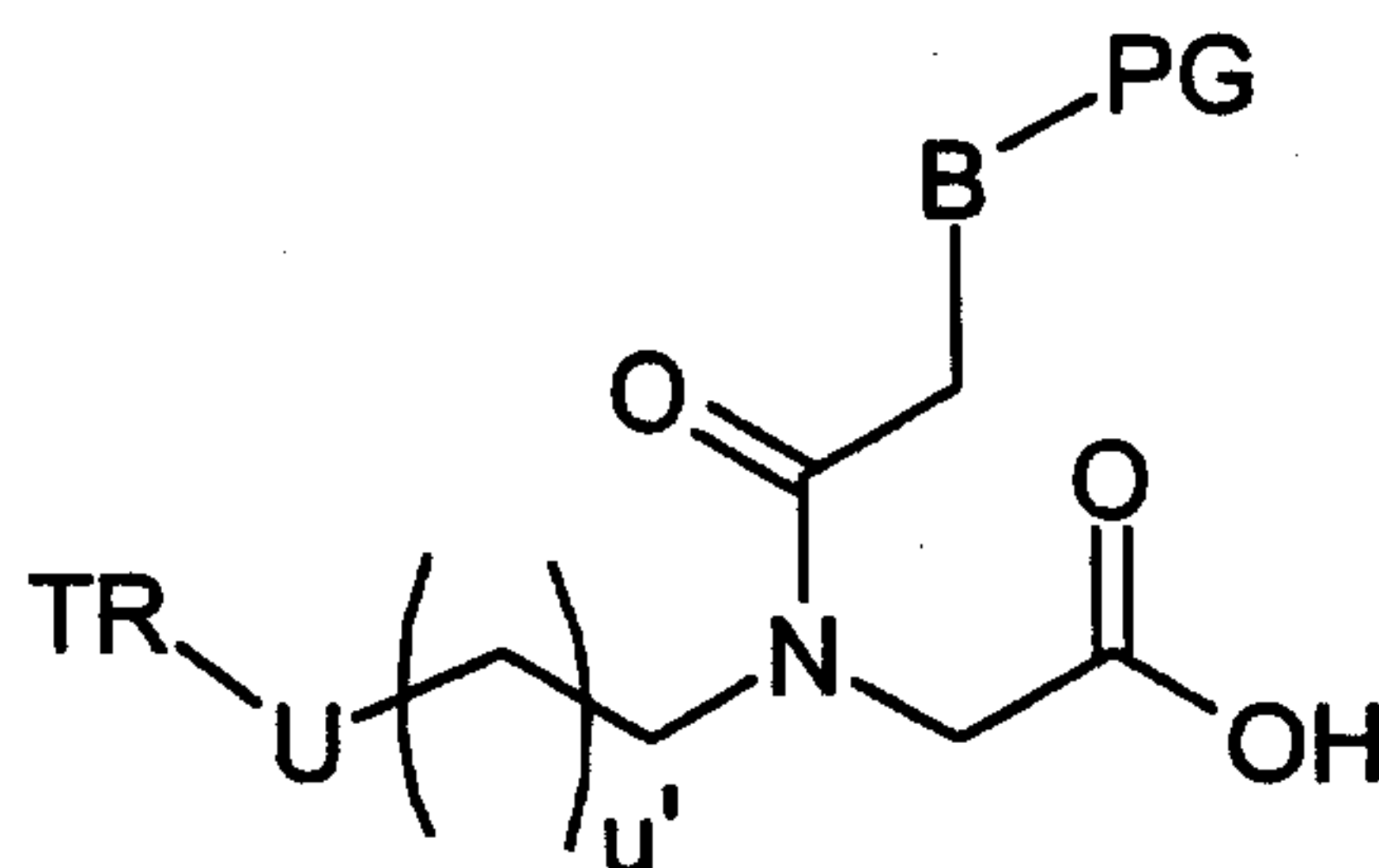
The use of the compounds of the formula I therefore also relates to in-situ hybridization and fluorescence in-situ hybridization (FISH). In-situ hybridization can also be used, for example, for detecting microorganisms and viruses (Just et al. (1998) J. Vir. Method. 73, 163-174). Another application of the compounds
10 of the invention relates to detecting and quantifying nucleic acids. For this, use is preferably also made of array technology (Strother et al. J. Am. Chem. Soc. (2000) 122, 1205-1209; Niemeyer et al., Angew. Chem. (1999) 111, 3039-3043; Pirrung (1997) Chem. Rev. 97, 473-488), which provides high sample throughput and a high degree of sensitivity. In this case, the PNA probes are
15 fixed on a suitable support or PNA chip. To achieve this, PNA can be synthesized as described in the examples and subsequently fixed onto the support or PNA chip. Alternatively, the PNA can be prepared directly on the support. Another application is the use of the compounds of the formula I as biosensors for detecting nucleic acids (Wang et al (1996) J. Am. Chem. Soc. 118, 7667). The use of PNA of the formula I possessing an affinity label, such
20 as histidyl-PNA, is another application for purifying nucleic acids (Oerum et al. (1999), in Peptide Nucleic Acids: Protocols and Applications).

The PNA backbone is synthesized using the methods described in the
25 literature, for example using the tert-butyloxycarbonyl(BOC), 9-fluorenylmethoxycarbonyl (Fmoc) or monomethoxytrityl (Mmt) protecting group strategy (Peptide Nucleic Acids: Protocols and Applications; Peter E. Nielsen and Michael Egholm (Edit.) Horizon Scientific Press, 1999). Preference is given to using the Mmt protecting group for temporarily protecting the amino function
30 of the aminoethylglycine and base-labile protecting groups on the heterocyclic nucleobases (D. Will et al. (1995) Tetrahedron 51, 12069; Breipohl et al. (1997) Tetrahedron 53, 14671-14686). Examples of monomeric building blocks are

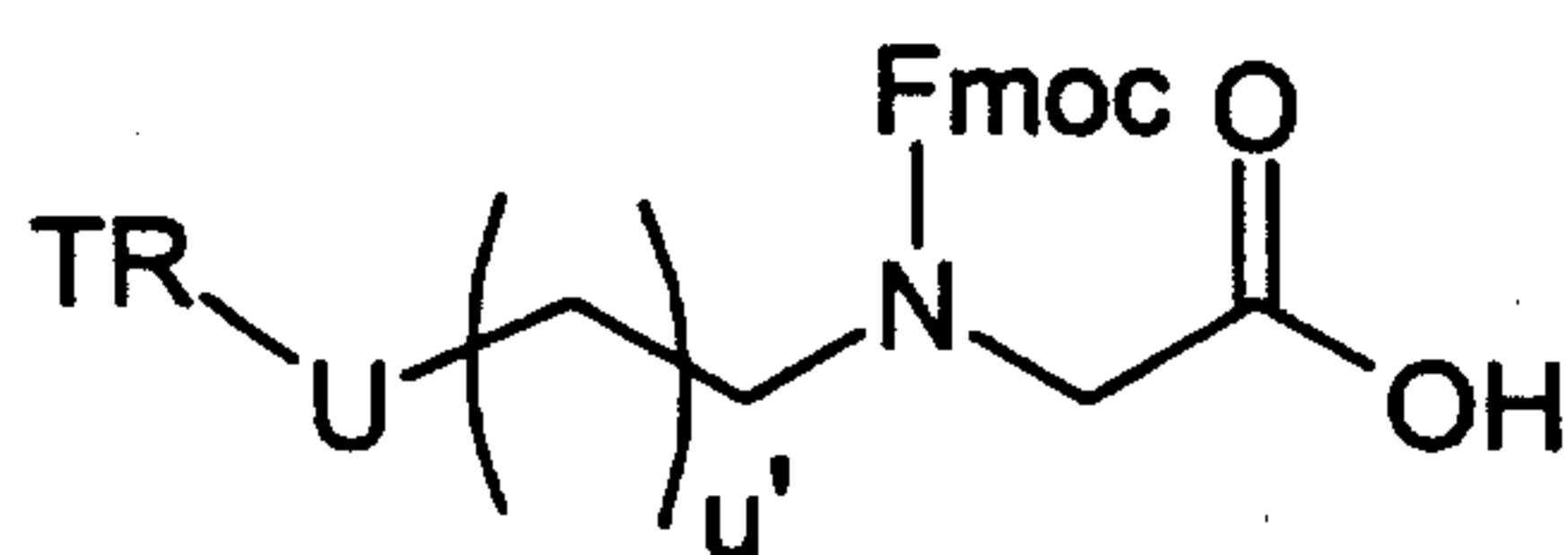
compounds of the formulae V to V B, with A, B, D, E, u' and U having the above meaning. PG is an amino-protecting group such as benzoyl, anisoyl-, isobutyroyl-, acetyl- or tert-butylbenzoyl (Breipohl et al. (1997) Tetrahedron 53, 14671-14686). TR is an acid-labile protecting group such as dimethoxytrityl (Dmt) (for U = O and S) or Mmt (for U = NH).



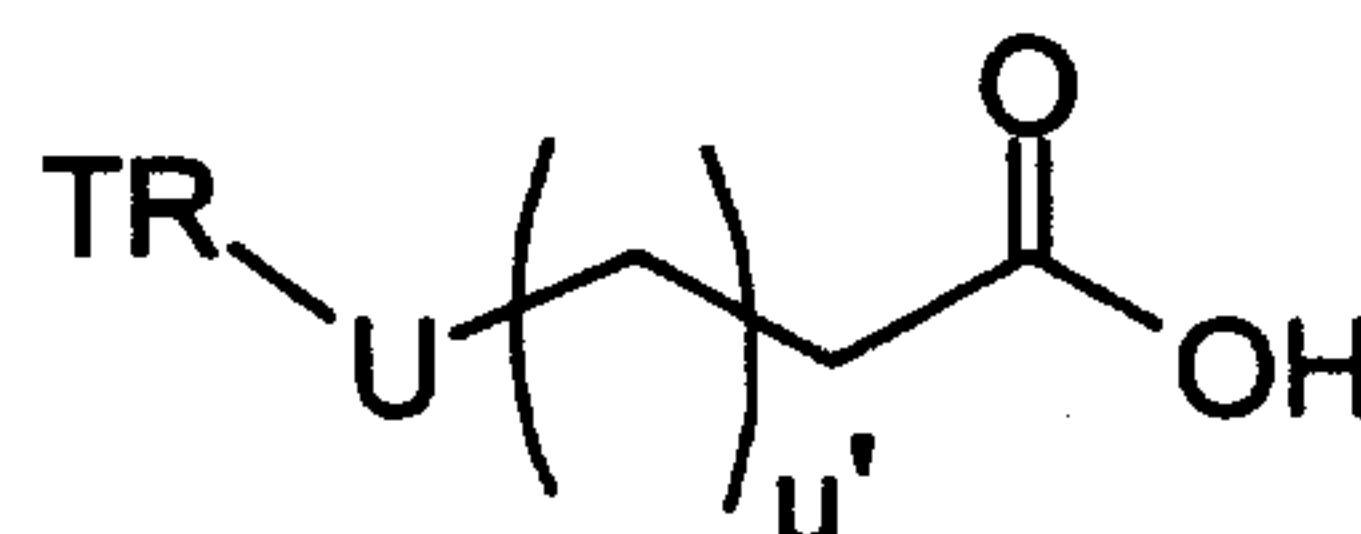
Formula V



Formula V A



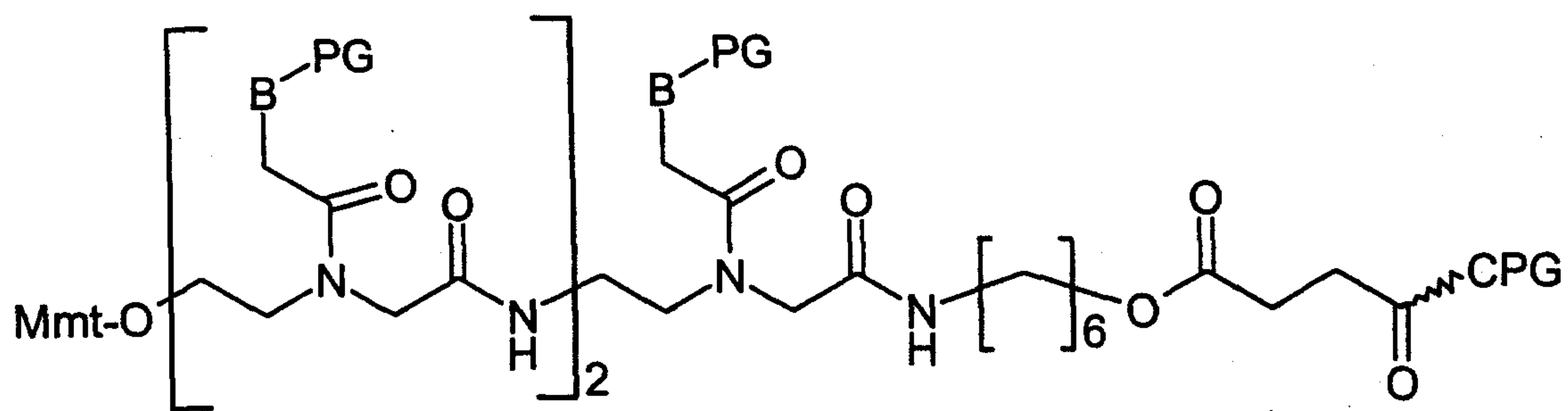
Formula V B



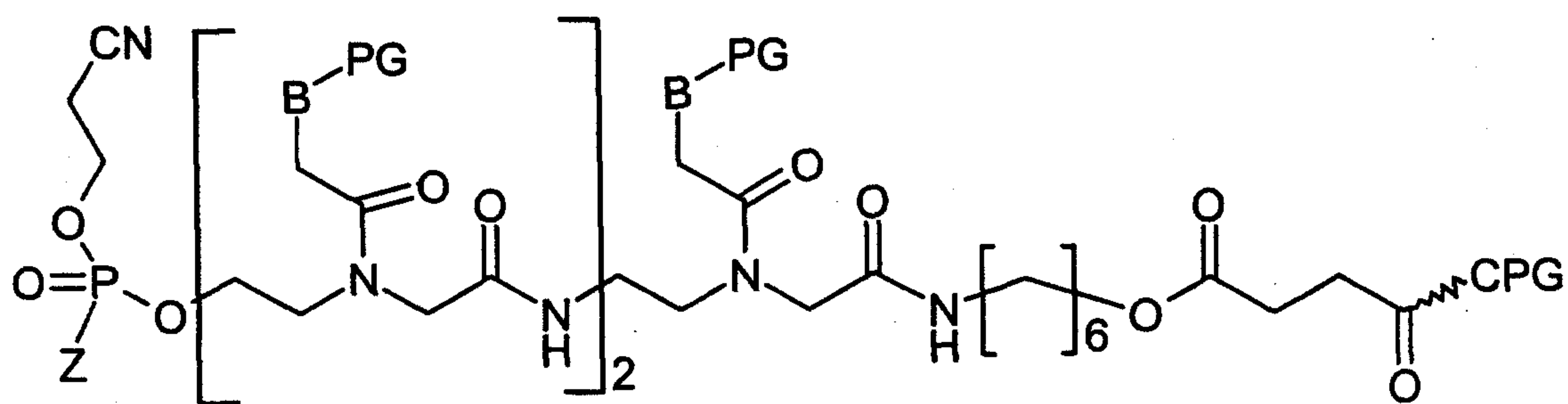
Formula V C

After the PNA backbone has been constructed, the free amino function of the N terminus can be reacted directly with an appropriate phosphorylating reagent, for example to give the corresponding phosphoramidate (U = NR₁ in formula I).

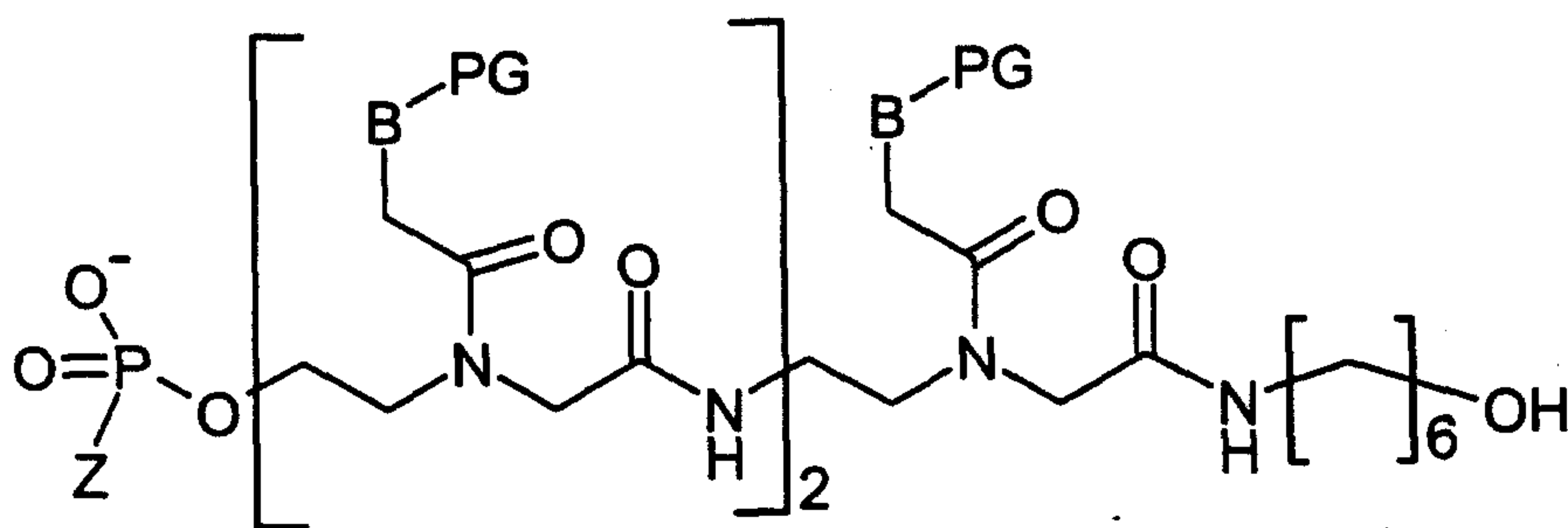
28



1. 3% TCA in DCM
2. Tetrazole + $\text{NC-CH}_2\text{CH}_2\text{O-P(=O)(Z)-N(CH}_2\text{CH(CH}_3\text{))}_2$ phosphoramidite
3. $\text{I}_2/\text{H}_2\text{O}$ oxidation



NH_4OH



Thus, PNA-7 in Example 8 ($U = NH$) was obtained, for example, by reacting the N terminus (at the building block which was coupled on last, where $B = \text{adenine}$) with biotin-phosphoramidite 5 (Figure 4b). Alternatively, a Dmt-protected hydroxyalkyl building block (for $U = O$) or a Dmt-protected mercaptoalkyl building block (for $U = S$) of the formula V A can be coupled on in the last cycle. After the Dmt group has been eliminated, the free hydroxy or mercapto function can be reacted, for example, with phosphorylation reagent 1 (Figure 4a). Following oxidation, cleavage from the support and removal of the protecting groups, the phosphate ($V = W = X = Y = O$) or thiophosphate ($V = S, W = X = Y = O$) of the formula I is obtained. If the amino terminal unit does not contain any nucleobase (B is hydrogen), the building block shown in formula V B is used in the condensation reaction of the last cycle, with the Fmoc protecting group being eliminated with ammonia at the end of the synthesis. The amino terminus of the PNA can be extended by condensing building blocks of the formula V C before the actual phosphorylation reaction is carried out.

The phosphoryl radicals can be introduced using the reagents which are customarily employed in nucleotide chemistry, for example by means of the phosphoramidite method, the H-phosphonate method or the phosphotriester method (E. Sonveaux (1986) *Bioorganic Chemistry* 14, 274; S.L. Beaucage and R.P. Iyer (1993) *Tetrahedron* 49, 1925; E. Uhlmann and A. Peyman (1990) *Chem. Rev.* 90, 543). There are a large number of phosphorylating reagents available (Glen Research Corporation, Sterling, VA 20164, USA) which can be used for preparing the compounds of the formula I. A selection of the reagents is shown in Figures 4a to 4d, with the invention not, however, being restricted to these special derivatives. In the above-described phosphorylation reaction, Z can also have the meaning of X , with reactive functions being protected in intermediate steps with suitable protecting groups.

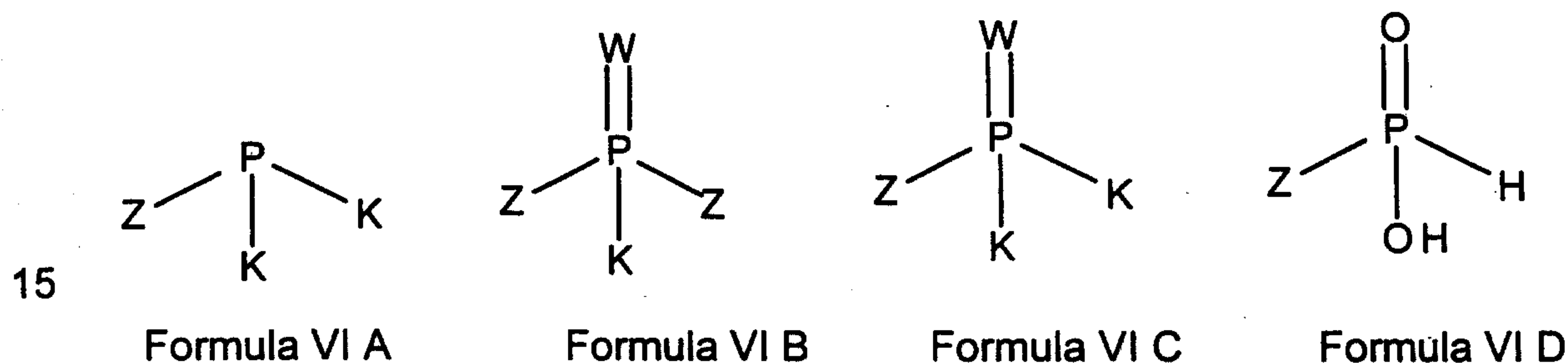
Just how varied the amino terminal modification is can be illustrated on the basis of the synthesis of PNA-6, PNA-12, PNA-13 and PNA-14. First of all, the PNA t(oeg)-at tcc gtc at-hex-CPG (PNA bases are abbreviated with small letters

for the corresponding nucleobases; oeg stands for hydroxyethylglycine) is synthesized, in fully protected form, on the CPG (controlled pore glass) support, which carries a hydroxyethylglycine-(oeg)-acetylthymine as the last building block. The hydroxyl group can now be reacted individually with

5 phosphoramidites 1, 5, 7 and 3, respectively. Following oxidation, elimination of the protecting groups and cleavage of the product from the CPG support, PNA-6, PNA-12, PNA-13 and PNA-14, respectively, are correspondingly obtained. ®TentaGel (from Rapp Polymers GmbH, Tübingen) and aminomethylpolystyrene are also used as solid supports.

10

While, in principle, all the reagents which are known in nucleotide chemistry are suitable for introducing the phosphoryl function, those which are particularly suitable are the following reagents of the formulae VI A, VI B, VI C and VI D

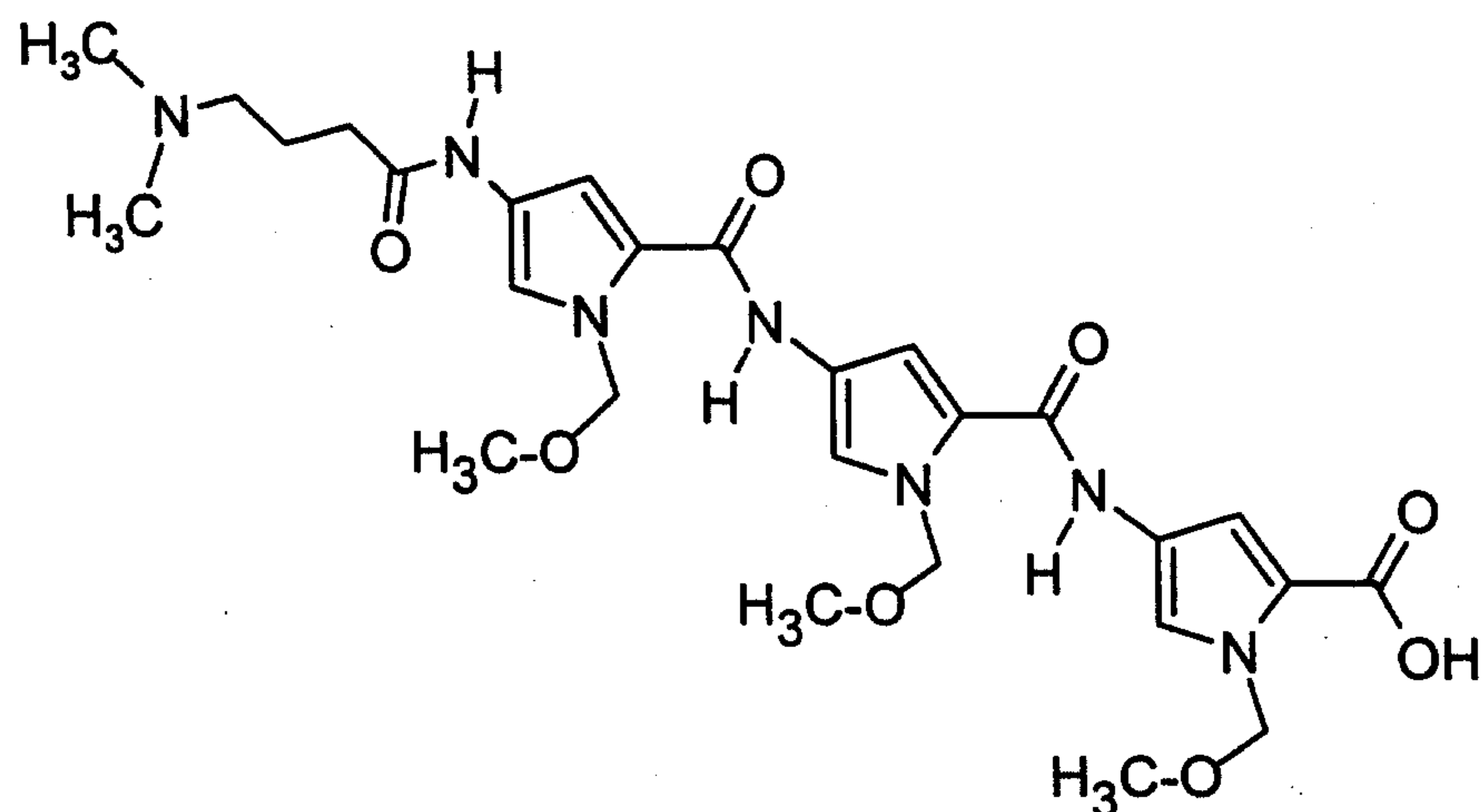


where K is halogen, preferably Cl, triazolyl, imidazolyl or dialkylamino, and Z has the abovementioned meaning or the meaning of X, with reactive groups
20 being appropriately protected. For example, the hydroxyl groups of the fluorescein-phosphoramidite 3 (Figure 4a) are protected by esterifying with pivalic acid.

The compounds of the formula VI are only to be regarded as being examples of
25 such reagents, which react, where appropriate, in the added presence of other auxiliary reagents such as bases, acids or condensing reagents. Particular preference is given to the reagents of the formula VI A, which react in accordance with the phosphoramidite method (Beaucage and Iyer, 1993). These reagents are reacted as the phosphorus (III) compound and

subsequently oxidized. If, for example, the oxidation is carried out using iodine/water/pyridine or tert-butyl hydroperoxide, the phosphoryl derivatives ($W = O$) are then obtained. If, on the other hand, the oxidation is carried out using elemental sulfur or Beaucage reagent, the corresponding thiophosphoryl compound ($W = S$) is then obtained.

Among the reagents (Figures 4a to 4d), are also to be found "bifunctional reagents" which, because of the possession of a second function, which is initially protected, can be caused to react several times. The phosphoramidites 4, 6 and 8 to 13 are examples of such bifunctional reagents. In this connection, it can be a matter of the multiple conjugation of a reagent or else of successive reaction with different reagents. Thus, for example, the fluorescein-phosphoramidite 3 can only be caused to react once. By comparison, the fluorescein-phosphoramidite 4 possesses a Dmt group-protected hydroxyl function which can be reacted once again with a phosphorylating reagent after the Dmt group has been eliminated. In this way, it is possible to introduce one and the same group or else different groups several times. PNA-16 is a typical example of a multiple conjugation. After the PNA chain had been synthesized, a hydroxyethyleneglycine-C building block was coupled on in the last cycle, with this building block being reacted successively twice with the spacer 18 phosphoramidite 9, the amino modifier 5 phosphoramidite 12 and finally with lexitropsin of the formula VII while activating with peptide coupling reagents such as HBTU. The coupling on of an amino modifier permits the subsequent introduction of a further residue, which can be introduced in the form of an activated carboxylic acid derivative. In this connection, it is also possible, for example, to use isothiocyanates and N-hydroxysuccinimide esters.



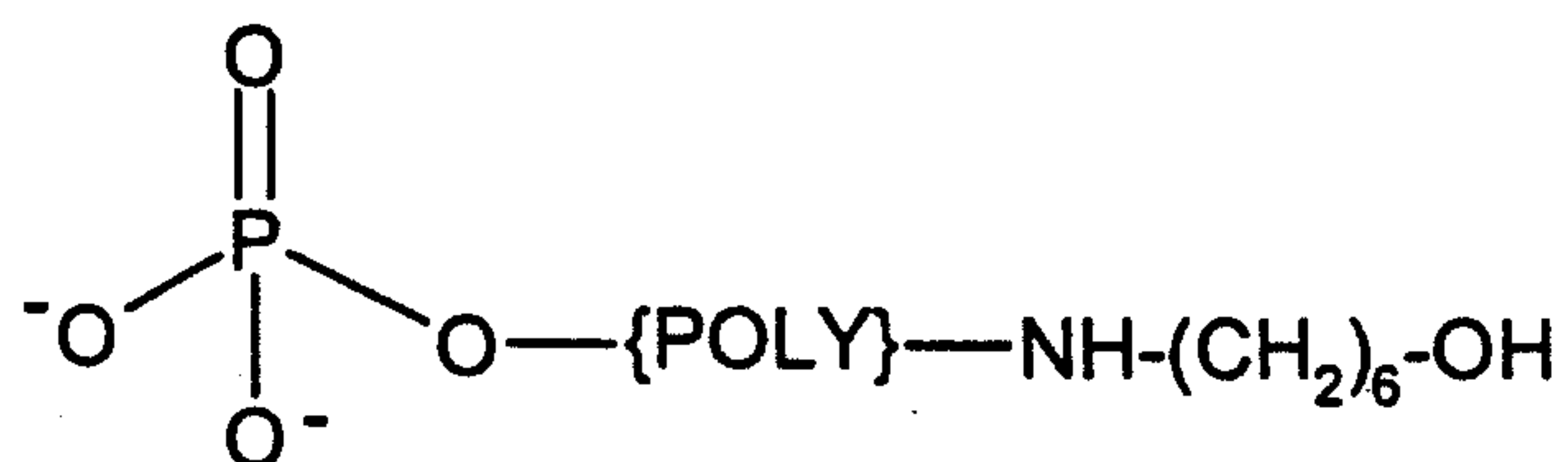
Formula VII

- Figures 5a and 5b show some examples of compound types for the N-terminal modification of the compounds of the formula I. Compound type A is obtained by reacting the terminal hydroxyl group of the PNA with the phosphorylation reagent 1. Compound type B is obtained by reacting the terminal amino group of the PNA with the biotin-phosphoramidite 5. Compound type C is obtained by successively reacting the PNA having a terminal hydroxyl group with the spacer-18 phosphoramidite 9, amino modifier-5 phosphoramidite 12 and lexitropsin.
- Compound type D is obtained by successively reacting the PNA having a terminal hydroxyl group with the spacer-9 phosphoramidite 8 and the cyanine-3 phosphoramidite 10. Compound type E is obtained by successively reacting the PNA having a terminal hydroxyl group with the bifunctional fluorescein-phosphoramidite 4, the spacer-9 phosphoramidite 8, and the C16-phosphorylating reagent 7. Compound type F is obtained by reacting the terminal amino group of the PNA with 4-hydroxybutyric acid, whose hydroxyl function is temporarily protected with a Dmt group, and subsequently reacting with the fluorescein-phosphoramidite 3. The steps which additionally have to be carried out, such as oxidation and protecting group elimination are described in the examples.

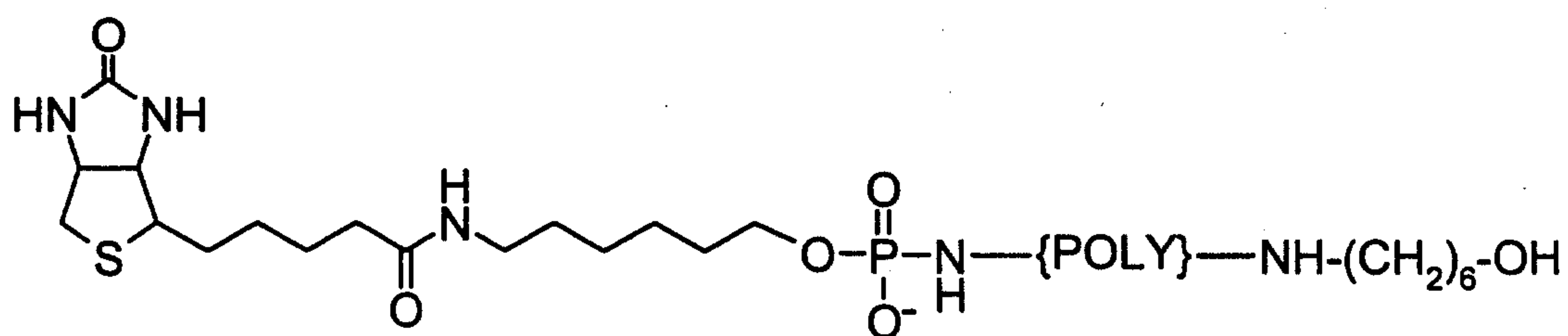
Examples:

The preparation of the following compounds is described by way of example:

5 PNA-1 to PNA-6:

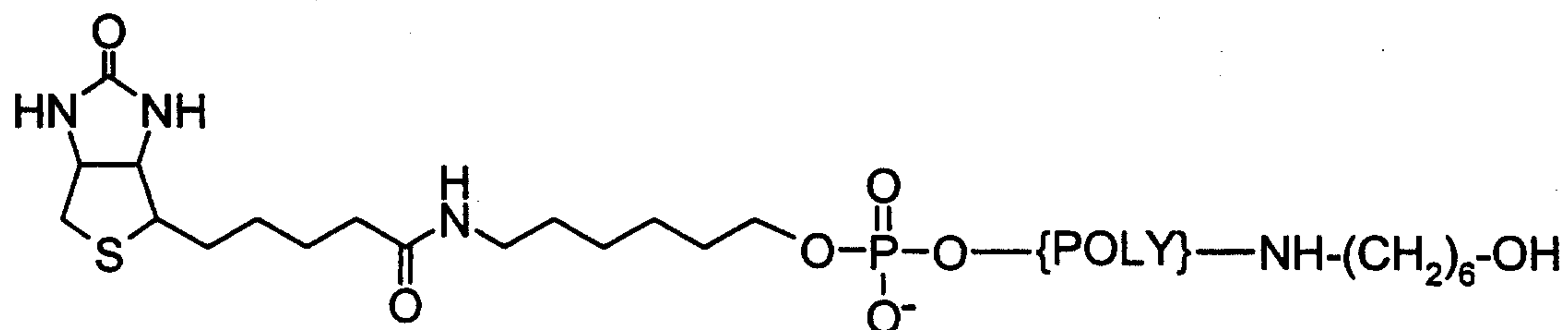


10 PNA-7:

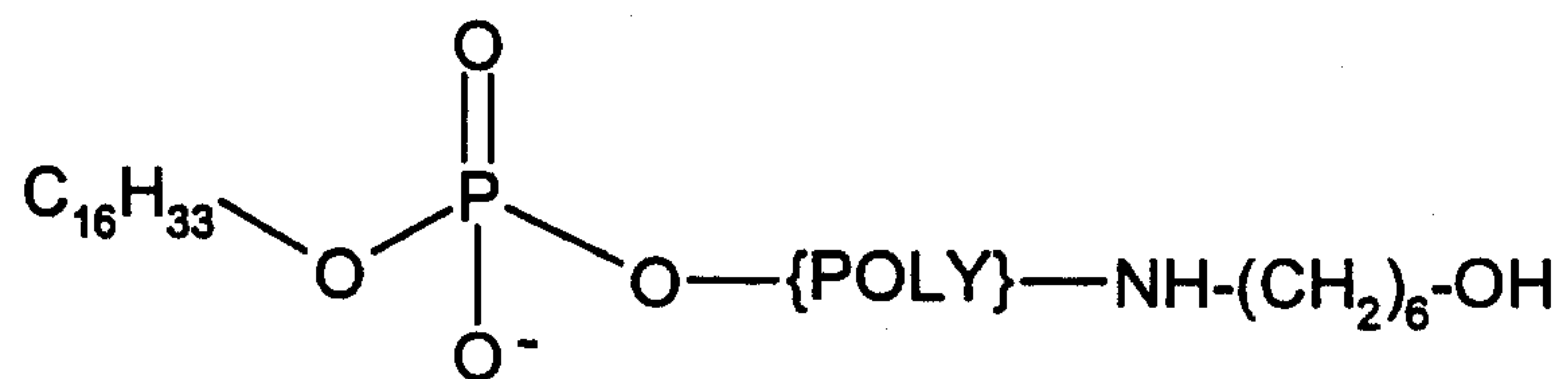


PNA-8 to PNA-12:

15



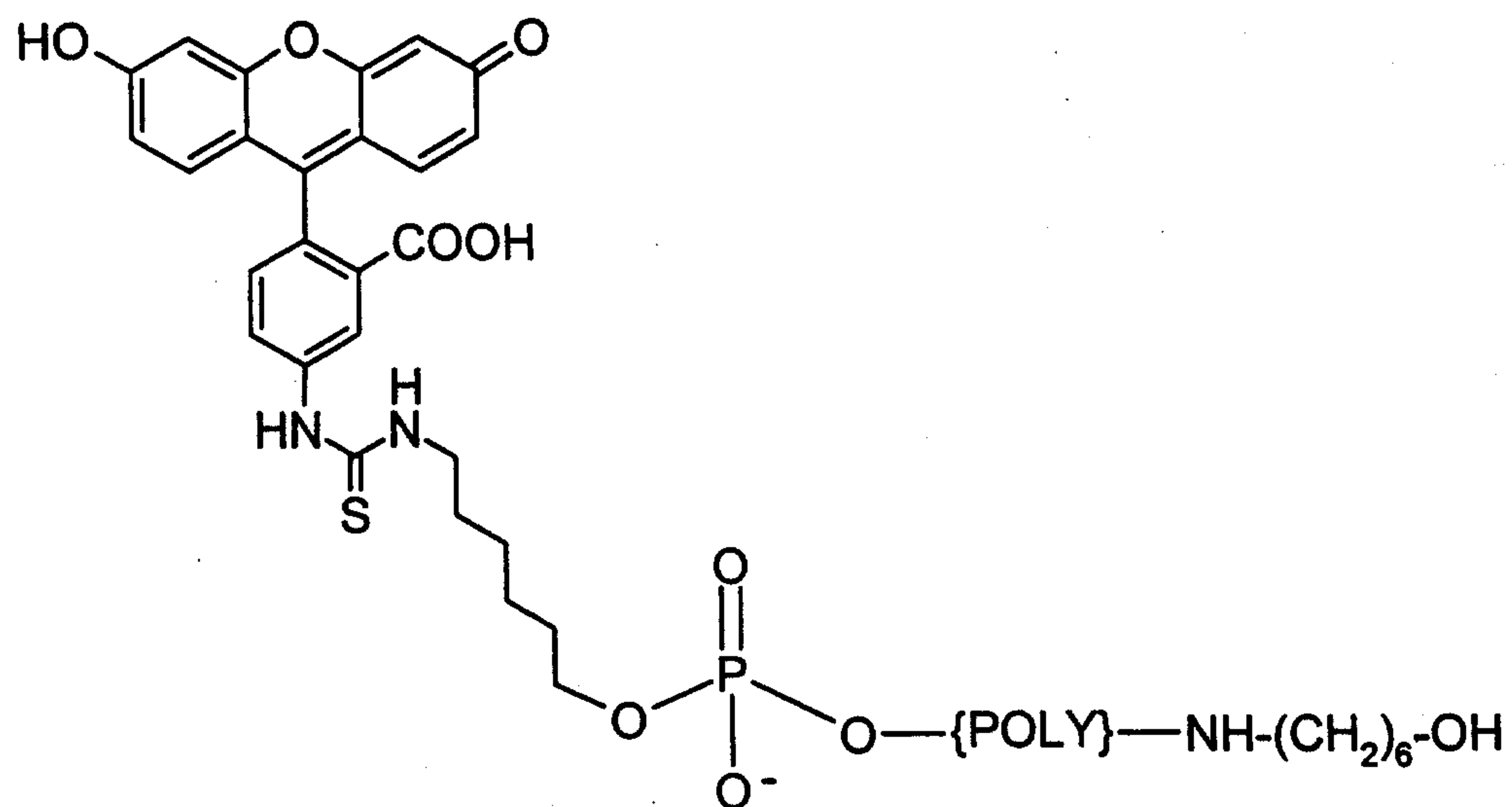
PNA-13:



20

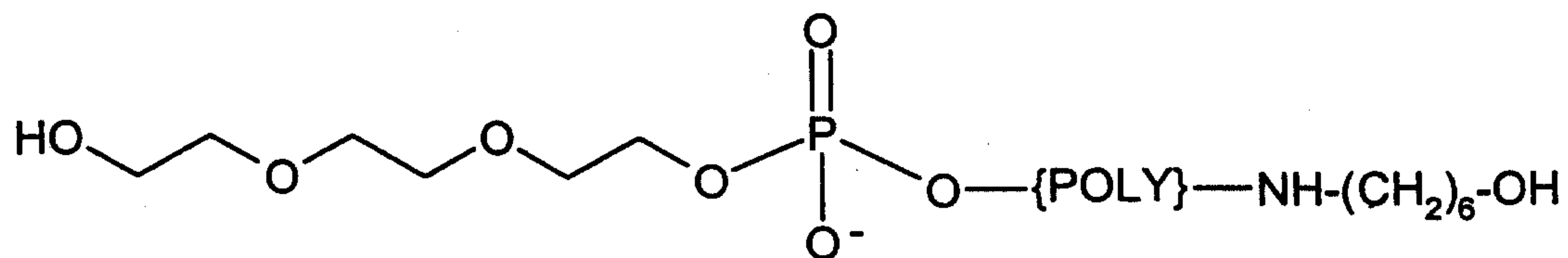
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PNA-14:

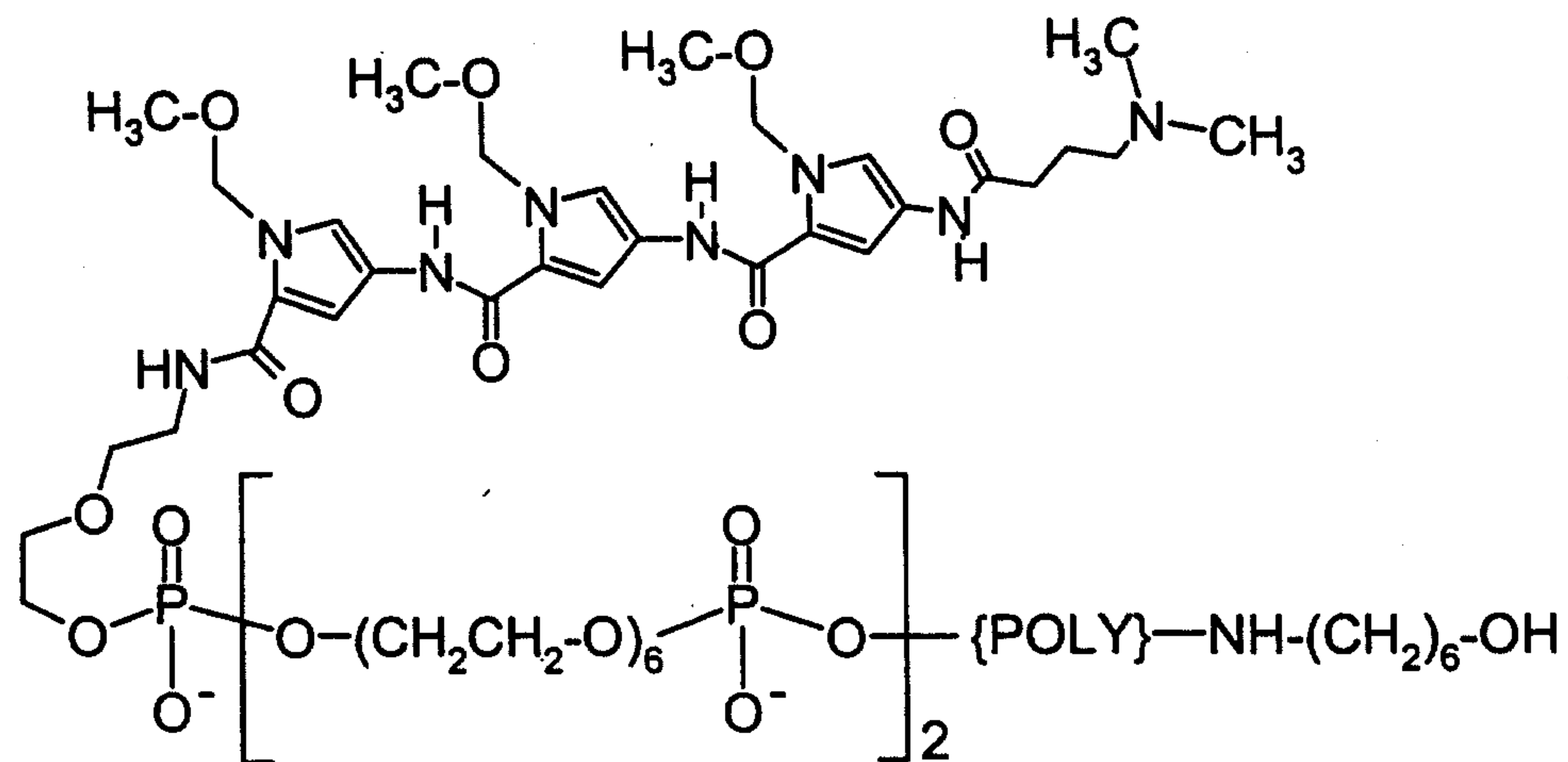


PNA-15:

5



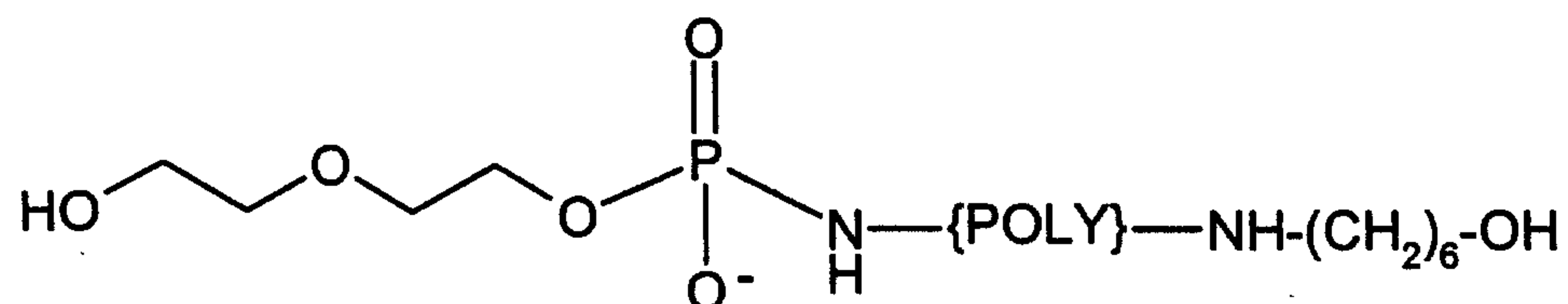
PNA-16:



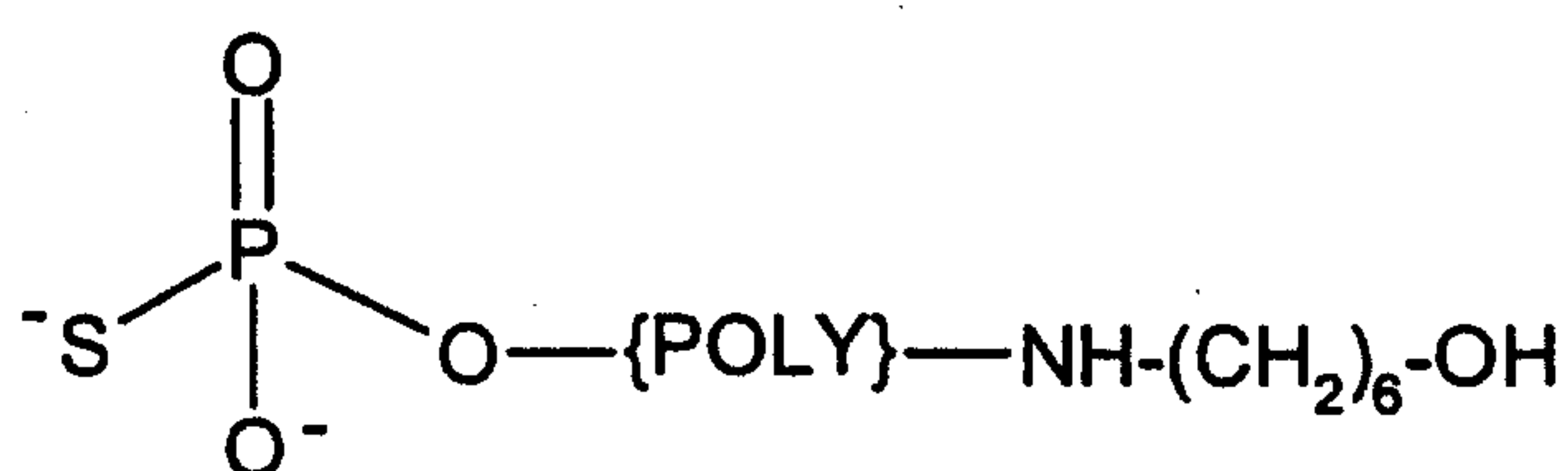
10

35

PNA-17:



5 PNA-18:



10 where the base sequences in each case are described by the following sequences,

SEQ ID NO. 53	5'-A A C T-3'	(PNA-1)
SEQ ID NO. 54	5'-A C A T C A T G G T C G-3'	(PNA-2)
SEQ ID NO. 55	5'-C C A C G A T G A T G T-3'	(PNA-3)
15 SEQ ID NO. 56	5'-G A G C C A T G T A T A G T G A C-3'	(PNA-4)
SEQ ID NO. 57	5'-T C G G T T T G A G A T C T G G-3'	(PNA-5)
SEQ ID NO. 58	5'-T A T T C C G T C A T-3'	(PNA-6, PNA-12, PNA-13, PNA-14, PNA-18)
SEQ ID NO. 59	5'-A C T G A T G T A G T C-3'	(PNA-7)
20 SEQ ID NO. 60	5'-G C T G A T G T A G T C-3'	(PNA-8)
SEQ ID NO. 61	5'-G G T A T G G G A T A T-3'	(PNA-9, PNA-11)
SEQ ID NO. 62	5'-T G A A G G A A G A G G-3'	(PNA-10)
SEQ ID NO. 63	5'-G T T A G G G T T A G-3'	(PNA-15, PNA-17)
SEQ ID NO. 64	5'-C C C C T T C C-3'	(PNA-16)

25

and where {POLY} is described by formula II and {BLOCK} is in each case described by formula IIIA, and where, in addition, A and E are CH₂ and D is

(CH₂)₂, and z' in each case ensues from the length of the sequence of the oligomer.

Example 1: Synthesizing the PNA chain

5

The following reagents were used for preparing the PNA moiety:

1. Phosphoramidite reagent (0.1 M in acetonitrile (ACN))
2. Mmt-PNA monomers and/or Dmt-oeg-PNA monomers (0.2 M in DMF:ACN (1:1; v:v))
- 10 3. Anhydrous ACN (≤ 30 ppm of water)
4. Trichloroacetic acid (3%) in dichloromethane (DCM)
5. Acetic anhydride, 2,6-lutidine in THF (1:1:8; v:v:v); (Cap A)
6. N-Methylimidazole (16%) in THF; (Cap B)
7. Iodine solution (0.05 M) in THF, water, pyridine (7:2:1; v:v:v)
- 15 8. Washing solution (THF, water, pyridine (7:2:1; v:v:v))
9. Tetrazole (0.3 M) in ACN
10. HBTU; 0.2 M in DMF:ACN (1:1; v:v)
11. DIPEA; 0.2 M in DMF:ACN (1:1; v:v)
12. DMF (> 99.5%)
- 20 13. Solid phase support: aminopropyl-CPG (550 Å) loaded with Mmt-amino-hex-1-yl hemisuccinate (for PNA-hexylamides).

The Mmt/acyl-protected or Dmt/acyl-protected oeg monomers were prepared as has already been described (Breipohl et al. (1997) Tetrahedron 53, 14671-14686). The loading of aminopropyl-CPG with the Mmt-amino-hex-1-yl hemisuccinate has likewise already been described (Will et al. (1995) Tetrahedron 51, 12069-12082). The PNA syntheses were in general carried out on a scale of from 2 to 5 μ mol.

30 The following cycle was used for the PNA synthesis:

1. Step of washing with ACN

2. Deprotecting the Mmt group or the Dmt group by treating with 3% TCA in DCM; 110 sec.
3. Step of washing with DMF/ACN (1:1)
4. Neutralizing with DIPEA in DMF/ACN (1:1)
- 5 5. Coupling on the monomeric building block by preactivating (15 min) with HBTU/DIPEA/PNA monomer (ratio 1:1:1; total volume 450 μ l) charging the solid phase and coupling (45 min)
6. Step of washing with ACN
7. Capping with acetic anhydride/N-methylimidazole
- 10 8. Step of washing with ACN
9. New cycle

Example 2: Synthesizing phosphate-{a(oeg)act}-hex (PNA-1)

- 15 The PNA moiety is prepared by solid phase synthesis as described in Example 1 (2 μ mol synthesis). In the last cycle, a hydroxyethylglycine (oeg)-based building block having an adenine as the nucleobase is coupled on (Formula V A; where TR is Dmt, U is oxygen, u' is 2, PG is anisoyl and B is adenine). After the terminal Dmt group has been eliminated with 3% TCA, the free
- 20 hydroxyl function is reacted with phosphorylating reagent 1 (Figure 4a) using tetrazole as catalyst. This reaction employs an excess of the phosphorylating reagent 1 (approx. 25-fold), as a 0.3 M solution in acetonitrile/tetrahydrofuran (1:1; v:v) and the tetrazole (approx. 50-fold; 0.5 M in acetonitrile). After the condensation has taken place, oxidation is effected using an iodine solution
- 25 (0.05 M in tetrahydrofuran/water, pyridine (7:2:1; v:v:v)). The PNA is then cleaved from the support, and the protecting groups removed at the same time, by treating with conc. ammonia at 50°C overnight. 83 OD (260 nm) are obtained. Of these, 40 OD are purified by preparative polyacrylamide (PAA) gel electrophoresis. The band of desired product is eluted with 0.2 M
- 30 triethylammonium bicarbonate buffer and desalted through a Bond-Elut C18 column (1 g). 13.5 OD are obtained. The product was analyzed by negative ion

mass spectrometry, which confirmed the calculated mass (calc. 1266.2; found 1265.9).

Example 3: Synthesizing phosphate-{a(oeg) ca tca tgg tcg}-hex (PNA-2)

5

The preparation is effected, in a 2 μ mol synthesis, in an analogous manner to that described in Example 2. Following cleavage with ammonia, 122 OD of crude product are obtained, with this crude product being purified by electrophoresis through a 15% PAA gel. 27 OD are obtained. The product was
10 analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3450.3; found 3450.4).

Example 4: Synthesizing phosphate-{c(oeg) ca cga tga tgt}-hex (PNA-3)

15 The preparation is effected, in a 2 μ mol synthesis, in an analogous manner to that described in Example 2, with a hydroxyethylglycine-based building block having cytosine as the nucleobase being coupled on in the last cycle. Following cleavage with ammonia, 124 OD of crude product are obtained, with this crude product being purified by electrophoresis through a 15% PAA gel. 19 OD are
20 obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3450.3; found 3450.1).

Example 5: Synthesizing phosphate-{g(oeg)-ag cca tgt ata gtg ac}-hex (PNA-4)

25 The preparation is effected, in a 2 μ mol synthesis, in an analogous manner to that described in Example 2, with a hydroxyethylglycine-based building block having guanine as the nucleobase being coupled on in the last cycle. Following cleavage with ammonia, 120 OD of crude product are obtained. 60 OD of the crude product were purified by electrophoresis through a 15% PAA gel. 10 OD
30 are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 4848.6; found 4849.9).

Example 6: Synthesizing phosphate-{t(oeg)cg gtt tga gat ctg g}-hex (PNA-5)

The preparation is effected, in a 2 μ mol synthesis, in an analogous manner to that described in Example 2, with a hydroxyethylglycine-based building block
5 having thymine as the nucleobase being coupled on in the last cycle. Following cleavage with ammonia, 250 OD of crude product are obtained. 60 OD of the crude product were purified by eletrophoresis through a 15% PAA gel. 22.9 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 4595.4; found 4596.3).

10

Example 7: Synthesizing phosphate-{t(oeg)-at tcc gtc at}-hex (PNA-6)

The preparation is effected in a 0.5 μ mol synthesis, in an analogous manner to that described in Example 2, with a hydroxyethylglycine-based building block
15 having thymine as the nucleobase being coupled on in the last cycle. Following cleavage with ammonia, 63 OD of crude product are obtained. 30 OD of the crude product were purified by electrophoresis through a 15% PAA gel. 4.2 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3124.5; found 3124.8).

20

Example 8: Synthesizing biotin-p-{act gat gta gtc}-hex (PNA-7)

The PNA moiety is prepared by solid phase synthesis as described in Example 1 (2 μ mol synthesis). In the last cycle, a normal PNA building block, having
25 adenine as the nucleobase, is coupled on (formula V A; where TR is Mmt, U is NH, u' is 2, PG is anisoyl and B is adenine). After eliminating the terminal Mmt group with 3% TCA, the free amino function is reacted with biotin-phosphoramidite 5 (Figure 4b) using tetrazole as catalyst. This reaction employs an excess of the phosphorylating reagent (approx. 10-fold) as an 0.12 M
30 solution in acetonitrile and the tetrazole (approx. 5-fold; 0.5 M in acetonitrile). After the condensation has taken place, oxidation is effected using an iodine solution (0.05 M in tetrahydrofuran/water, pyridine (7:2:1; v:v:v)). After cleaving

with ammonia, 288 OD of crude product are obtained. The crude product was purified by electrophoresis through a 15% PAA gel. 17.5 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3789.6; found 3789.8).

5

Example 9: Synthesizing biotin-p-{g(oeg)-ct gat gta gtc}-hex (PNA-8)

The PNA moiety is prepared by solid phase synthesis as described in Example 1 (1 μ mol synthesis). A hydroxyethyleneglycine-based building block having
10 guanine as the nucleobase (formula V A; where TR is Dmt, U is oxygen, u' is 2, PG is anisoyl and B is guanine) is coupled on in the last cycle. After eliminating the terminal Dmt group with 3% TCA, the free hydroxyl function is reacted with biotin-phosphoramidite 5 (Figure 4b) using tetrazole as catalyst. This reaction employs an excess of the phosphorylating reagent (approx. 10-fold) as a 0.12 M
15 solution in acetonitrile and the tetrazole (approx. 50-fold; 0.5 M in acetonitrile). After the condensation has taken place, oxidation is effected using an iodine solution (0.05 M in tetrahydrofuran/water, pyridine (7:2:1; v:v:v)). After cleaving with ammonia, 63 OD of crude product are obtained. The crude product was purified by electrophoresis through a 15% PAA gel. 11.2 OD are obtained. The
20 product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3806.8; found 3807.2).

Example 10: Synthesizing biotin-p-{g(oeg) gt atg gga tat}-hex (PNA-9)

25 The preparation was effected, in a 2 μ mol synthesis, in an analogous manner to that described in Example 9, with a hydroxyethylglycine-based building block having guanine as the nucleobase being coupled on in the last cycle. The biotin residue was introduced with biotin-phosphoramidite 5 (Figure 4b). After cleaving with ammonia, 274 OD of crude product are obtained. The crude product was
30 purified by electrophoresis through a 15% PAA gel. 25.8 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3870.8; found 3869.7).

Example 11: Synthesizing biotin-p-{t(oeg) ga agg aag agg}-hex (PNA-10)

5 The preparation was effected, in a 2 μ mol synthesis, in an analogous manner to that described in Example 9, with a hydroxyethylglycine-based building block having thymine as the nucleobase being coupled on in the last cycle. The biotin residue was introduced with biotin-phosphoramidite 5 (Figure 4b). After cleaving with ammonia, 190 OD of crude product are obtained. The crude product was purified by electrophoresis through a 12% PAA gel. 29 OD are obtained with the
10 expected molecular weight (calc. 3913.9; found 3913.7).

Example 12: Synthesizing biotin-p-{g(oeg)-gt atg gga tat}-hex (PNA-11)

15 The preparation was effected, in a 2 μ mol synthesis, in an analogous manner to that described in Example 9, with a hydroxyethylglycine-based building block having guanine as the nucleobase being coupled on in the last cycle. The biotin residue was introduced with biotin-phosphoramidite 5 (Figure 4b). After cleaving with ammonia, 162 OD of crude product are obtained. The crude product was purified by electrophoresis through a 12% PAA gel. 21 OD are obtained. The
20 product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3870.8; found 3870.8).

Example 13: Synthesizing biotin-p-{t(oeg) at tcc gtc at}-hex (PNA-12)

25 The preparation was effected, in a 0.5 μ mol synthesis, in an analogous manner to that described in Example 9, with a hydroxyethylglycine-based building block having thymine as the nucleobase being coupled on in the last cycle. The biotin residue was introduced with biotin-phosphoramidite 5 (Figure 4b). After cleaving with ammonia, 67 OD of crude product are obtained. The crude product was
30 purified by electrophoresis through a 12% PAA gel. 8.5 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3449.5; found 3449.9).

Example 14: Synthesizing hexadecyl-O-p-{t(oeg) at tcc gtc at}-hex (PNA-13)

5 The preparation was effected, in a 0.5 μ mol synthesis, in an analogous manner to that described in Example 9, with a hydroxyethylglycine-based building block having thymine as the nucleobase being coupled on in the last cycle. The hexadecyl residue was introduced with C16-phosphorylating reagent 7 (Figure 4c). After cleaving with ammonia, 58 OD of crude product are obtained. The crude product (30 OD) was purified by electrophoresis through a 12% PAA
10 gel. 2 OD are obtained with the expected molecular weight (calc. 3349.4; found 3349.7).

Example 15: Synthesizing fluorescein-p-{t(oeg) at tcc gtc at}-hex (PNA-14)

15 The preparation was effected, in a 0.5 μ mol synthesis, in an analogous manner to that described in Example 9, with a hydroxyethylglycine-based building block having thymine as the nucleobase being coupled on in the last cycle. The fluorescein residue was introduced with fluorescein-phosphoramidite 3 (Figure 4a). After cleaving with ammonia, 62 OD of crude product are obtained.
20 The crude product (30 OD) was purified by electrophoresis through a 12% PAA gel. 4.2 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3581.5; found 3582.4).

25 Example 16: Synthesizing HO-spacer9-p-{g(oeg)-tt agg gtt ag}-hex (PNA-15)

The preparation was effected, in a 1 μ mol synthesis, in an analogous manner to that described in Example 9, with a hydroxyethylglycine-based building block having thymine as the nucleobase being coupled on in the last cycle. Spacer-9
30 was introduced with the corresponding phosphoramidite 8 (Figure 4c). After cleaving with ammonia, 52 OD of crude product are obtained. The crude product (30 OD) was purified by electrophoresis through a 15% PAA gel.

1.8 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3402.3; found 3401.8).

5 Example 17: Synthesizing lexitropsin-aminolinkC5-p-spacerC18-p-spacerC18-p-{c(oeg)-c cct tcc}-hex (PNA-16; where c is a pseudo-iso-cytosine PNA building block)

10 The preparation was effected, in a 1 μ mol synthesis, in an analogous manner to that described in Example 9, with a hydroxyethylglycine-based building block having cytosine as the nucleobase being coupled on in the last cycle. After that, spacer-18 phosphoramidite 9 is condensed on twice in succession and amino modifier-5 phosphoramidite 12 (Figure 4d) is then condensed on once. After the coupling reactions, the Dmt and/or Mmt group was in each case eliminated by
15 treating with 3% trichloroacetic acid. In order to couple on the lexitropsin, 300 μ l of the corresponding active ester (prepared from 0.1 M lexitropsincarboxylic acid, 0.1 M TBTU and 0.4 M diisopropylethylamine; 1:1:1 = v:v:v; 30 min preactivation) are added and the mixture is left to react for 3 hours. Washing is then effected using DMF. After cleaving with ammonia, 43 OD of crude product
20 are obtained. The crude product (35 OD) was purified by electrophoresis through a 15% PAA gel. 5.3 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3583.5; found 3583.1).

25 Example 18: Determining the melting temperatures

The melting temperatures were determined using an HP 8452A diode-array spectrophotometer, an HP 89090A Peltier element and HP Temperature Control Software Rev. B5.1 (from Hewlett Packard). Measurement takes place
30 in 0.5°C/min steps in 140 mM KCl, 10 mM sodium dihydrogen phosphate, 0.1 mM EDTA (pH 7.4) as the buffer. The oligomer concentration is from 0.5 to 1 OD₂₆₀ per ml.

Surprisingly, the phosphoryl-modified PNA-6 and PNA-12 to PNA-14 derivatives exhibited a higher degree of binding towards complementary DNA and RNA than did the uncharged PNA (reference substance).

5

PNA derivative		T _m (DNA)	T _m (RNA)
Reference	Ac-HN-tat tcc gtc at-hex	41.9°C	56.6°C
PNA-6	p-{t(oeg)-at tcc gtc at}-hex	44.0°C	57.5°C
PNA-13	Hexadecyl-O-p-{t(oeg) at tcc gtc at}-hex	46.7°C	58.9°C
PNA-14	Fluorescein-p-{t(oeg) at tcc gtc at}-hex	42.5°C	56.7°C
PNA-12	Biotin-p-{t(oeg) at tcc gtc at}-hex	43.6°C	57.9°C

Example 19: Determining cell uptake after fluorescence labelling

COS cells are allowed to grow to confluence in Dulbecco's MEM (DMEM), which has been supplemented with 10% FCS, in 5 cm Petri dishes. The cells are washed twice with serum-free DMEM. An area of approx. 1 cm² is scratched out in the middle of the Petri dish using a sterile needle. The PNA solution (10 µM) under investigation is applied in this area. The dish is incubated at 37°C under a CO₂ atmosphere. After 2, 4 and 16 hours, the cells are examined by fluorescence microscopy. For this, the cells are washed four times with serum-free DMEM, covered with a cover slip and evaluated under the fluorescence microscope or by phase contrast.

In order to investigate the cell uptake, PNA-6 and PNA-13 were labeled with fluorescein at the C terminus and then examined microscopically.

p-{t(oeg)-at tcc gtc at}-fluorescein
hexadecyl-O-p-{t(oeg) at tcc gtc at}-fluorescein

In this connection, it was found that the hexadecyl-PNA derivative (PNA-13) was taken up more efficiently into the cells.

Example 20: Inhibiting cell proliferation with PNA-13

The sequence of PNA-13 is directed against the translation start of the Ha-ras mRNA. REH cells (human pre-B leukemia cells, DSM ACC 22) or A549 tumor cells were cultured, at 37°C and under 5% CO₂, in OptiMEM (Gibco BRL) containing 10% fetal calf serum (FCS, GIBCO-BRL). The cell density for the assay was approx. 1×10^6 /ml. The PNA-13 (10 µM) was incubated with the cells in 24-well plates. After incubating at 37°C and under 5% CO₂ for 96 hours, the cell density was determined. Mean values for the cell density were determined from 3 individual wells at a given PNA concentration. It was found that PNA-13 inhibits proliferation of the REH cells. After > 4 days of incubation, the inhibition brought about by PNA-13 is greater than that brought about by a corresponding phosphorothioate oligonucleotide.

15

Example 21: Synthesizing HO-spacer9-p-{gtt agg gtt ag}-hex (PNA-17)

The preparation was effected, in a 0.67 µmol synthesis, in an analogous manner to that described in Examples 9 and 16, with a normal 2-aminoethylglycine building block, having thymine as the nucleobase, being coupled on in the last cycle of the PNA synthesis. Spacer-9 was subsequently introduced using the corresponding phosphoramidite 8 (Figure 4c) (3 × 5 min reaction time). After cleaving with ammonia, 108 OD of crude product are obtained. The crude product was purified by electrophoresis through a 15% PAA gel. 5.7 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the calculated mass (calc. 3401.3; found 3399.8).

25

Example 22: Synthesizing thiophosphate-{t(oeg)-at tcc gtc at}-hex (PNA-18)

30

The preparation is effected, in a 1 µmol synthesis, in an analogous manner to that described in Examples 2 and 7, with a hydroxyethylglycine-based building

block having thymine as the nucleobase being coupled on in the last cycle. After eliminating the terminal Dmt group with 3% TCA, the free hydroxyl function is reacted with phosphorylating reagent 1 (Figure 4a) using tetrazole as catalyst. After the condensation has been completed, oxidation is effected using the

5 Beaucage reagent (0.075 M 3H-1,2-benzodithiol-3-one 1,1-dioxide in acetonitrile) in order to introduce the thio function. After cleaving with ammonia, 66.5 OD of crude product are obtained. 61 OD of the crude product were purified by electrophoresis through a 15% PAA gel. 12.4 OD are obtained. The product was analyzed by negative ion mass spectrometry, which confirmed the

10 calculated mass (calc. 3141; found 3140).

List of abbreviations:

ACN	acetonitrile
BOC	tert-butyloxycarbonyl
<u>C</u> , <u>c</u>	pseudo-iso-cytosine
COS	CV1 origin SV 40
CPG	controlled pore glass
DCM	dichloromethane
DIPEA	diisopropylethylamine
DMEM	Dulbecco's MEM
DMF	dimethylformamide
Dmt	dimethoxytrityl
DNA	deoxyribonucleic acid
DNP	dinitroaryl
FITC	fluorescein isothiocyanate
Fmoc	fluorenylmethoxycarbonyl
HATU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

hex	-NH-(CH ₂) ₆ -OH
MEM	modified Eagle's minimal essential medium
Mmt	monomethoxytrityl
OD	optical density
oeg	N-(2-hydroxyethyl)glycine
PAA	polyacrylamide
PG	protecting group
PNA	polyamide nucleic acid
RNA	ribonucleic acid
TBTU	O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TCA	trichloroacetic acid
THF	tetrahydrofuran
TR	acid-labile protecting group

Figures 1a, 1b, 2b and 3b show examples of terminal Z radicals.

Figures 2a and 3a show examples of bridging X radicals.

5

Figures 4a, 4b, 4c and 4d show examples of phosphorylating reagents.

Figures 5a and 5b show examples of single (A, B) and multiple (C to F) N-terminal derivatization of PNA.

SEQUENCE LISTING

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<120> Peptide nucleic acid derivatives with a negative charge, agents and methods for producing them

<130> 9982-729

<140> CA 2,406,168

<141> 2000-04-18

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15

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49

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18

<210> 4

<211> 17

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<400> 4

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17

<210> 5

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52

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<400> 13
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21

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<400> 16
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12

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12

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57

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gcagcccccg ca

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58

gttctcgctg gtgagtttca

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59

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Oligonucleotide

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<222> (1)..(19)

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62

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<400> 51
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12

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64

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17

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11

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12

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11

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66

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tgaaggaaga gg

12

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11

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8

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67

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ttccgtcatc gctcctcagg gg

22

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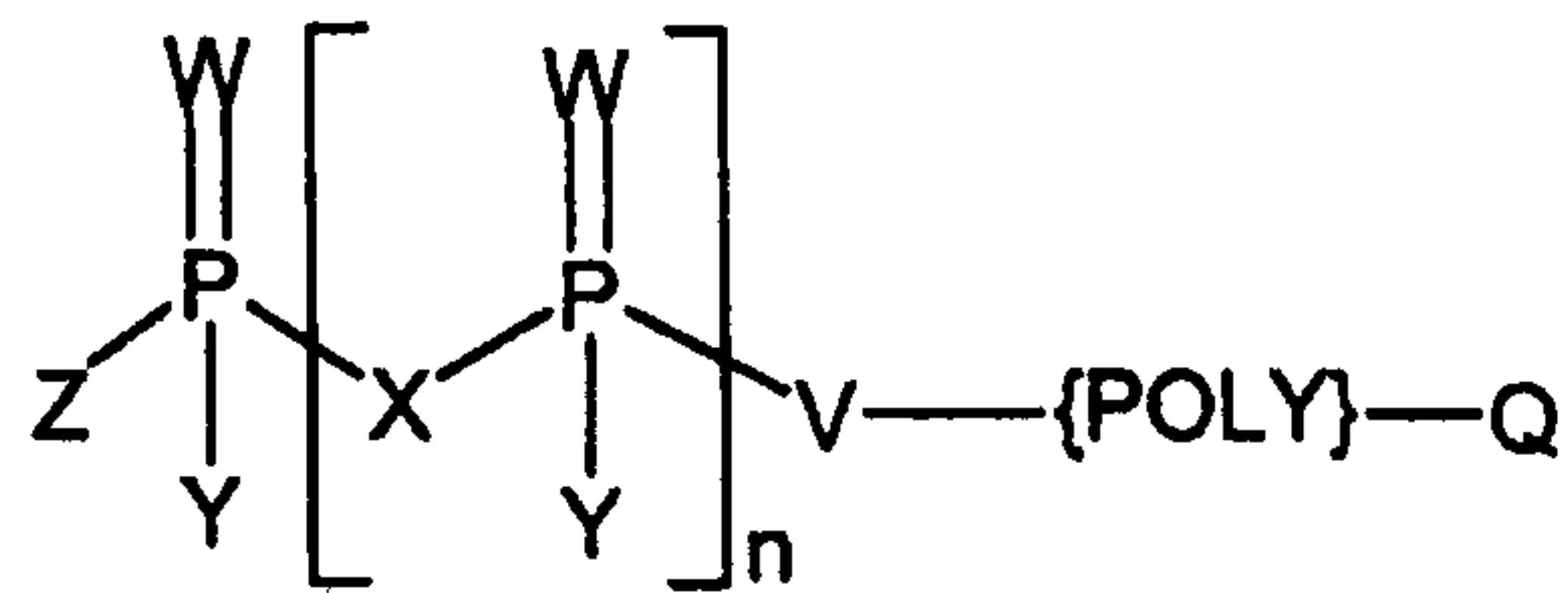
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21

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A PNA derivative of the formula I

5



Formula I

where

- 10 V is oxygen, sulfur, NR_1 , a group $\text{U}-(\text{CR}_3\text{R}_4)_{u'}-\text{CH}_2-\text{C}(\text{O})-\text{NH}$ or a group $\text{U}-(\text{CH}_2\text{CH}_2\text{O})_{u'}-\text{C}(\text{O})-\text{NH}$,

U is, independently of each other, oxygen, sulfur or NH ,

- 15 u' is, independently of each other, from 1 to 10,

W is, independently of each other, oxygen, sulfur or NR_1 ,

- 20 Y is, independently of each other, hydroxyl, mercapto, oxyanion, thioate or NR_1R_2 ,

R_1 and R_2 are, independently of each other, a radical consisting of hydrogen or C_1 - C_6 -alkyl,

- 25 R_3 and R_4 are, independently of each other, a radical consisting of hydrogen or C_1 - C_6 -alkyl, or the radical of an amino acid side chain,

- 30 X is, independently of each other, a group $\text{U}-(\text{C}_2\text{-C}_{22}\text{-alkanediyl})-\text{U}$ or a group $\text{U}-(\text{CH}_2\text{CH}_2\text{-O})_{u'}$,

or X is a bifunctional labeling group,

or X is a bifunctional group for crosslinking with complementary nucleic acids,

or X is a bifunctional group which promotes intracellular uptake,

or X is a bifunctional group which increases the binding affinity of the PNA derivative for a target nucleic acid,

Z is hydroxyl, mercapto, oxyanion, thioate, NR_1R_2 , $\text{C}_1\text{-C}_{22}\text{-alkyl}$, $\text{C}_1\text{-C}_8\text{-arylalkyl}$, $\text{C}_1\text{-C}_{22}\text{-alkyl-U}$, $\text{C}_1\text{-C}_8\text{-arylalkyl-U}$, hydroxy- $\text{C}_1\text{-C}_{18}\text{-U}$, aminoalkyl-U, arylalkyl-U or mercaptoalkyl-U,

or a group of the formula $\text{R}_9(\text{CH}_2\text{CH}_2\text{-O})_m$, where R_9 is hydroxyl, amino or $\text{C}_1\text{-C}_{22}\text{-alkoxy}$, and m is from 1 to 100,

or Z is a monofunctional or bifunctional labeling group,

or Z is a monofunctional or bifunctional crosslinking group,

or Z is a monofunctional or bifunctional group which promotes intracellular uptake,

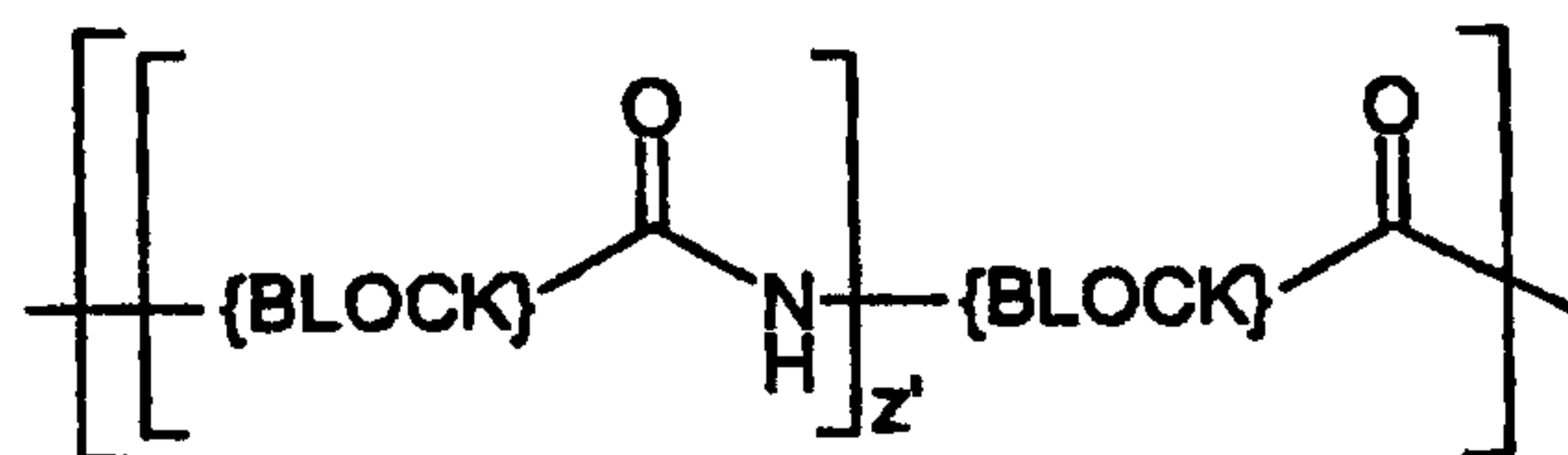
or Z is a monofunctional or bifunctional group which promotes the binding affinity of the PNA derivative for a target nucleic acid,

n is from 0 to 10,

Q is hydroxyl, amino, NHR_7 , NR_7R_8 , an amino acid derivative or a peptide radical,

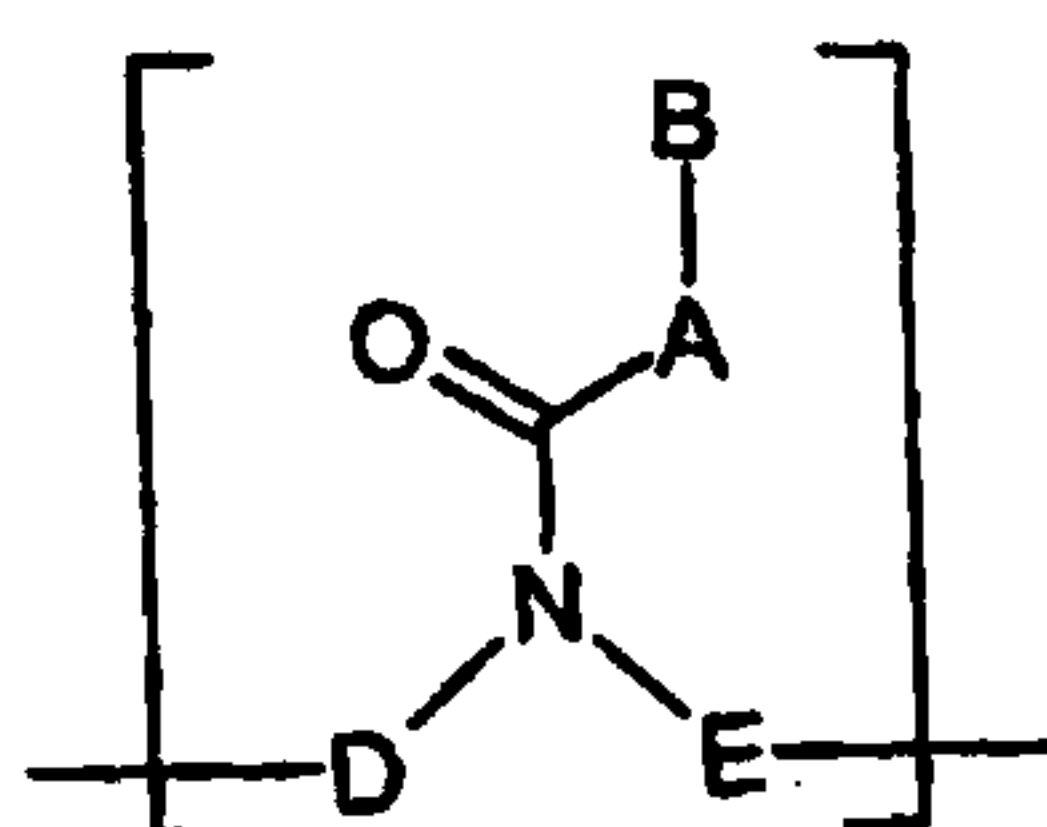
R_7 and R_8 are, independently of each other, $\text{C}_1\text{-C}_{18}\text{-alkyl}$ or hydroxy- $\text{C}_1\text{-C}_{18}\text{-alkyl}$,

and where {POLY} is described by the formula II



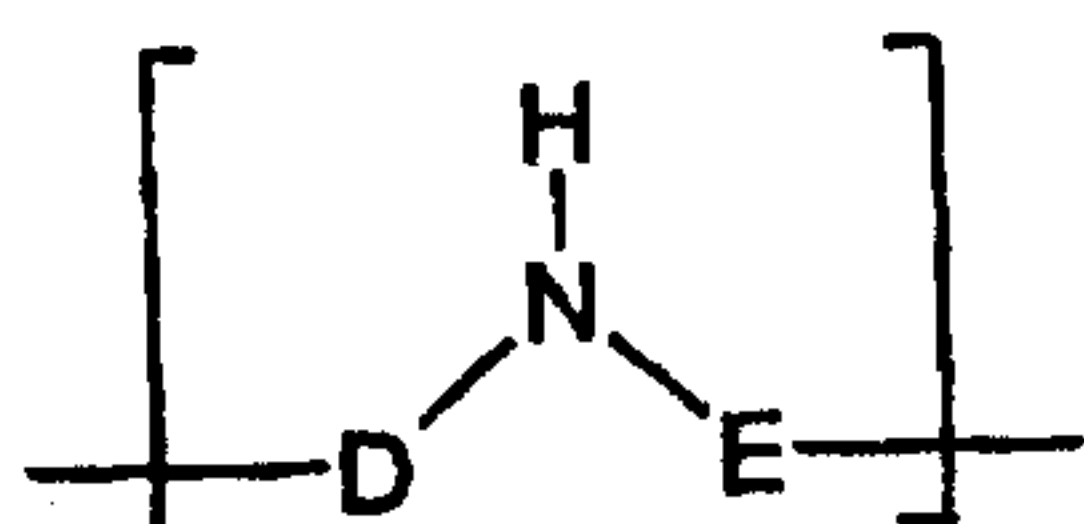
Formula II

where {BLOCK} is, independently of each other, either described by formula
5 IIIA,



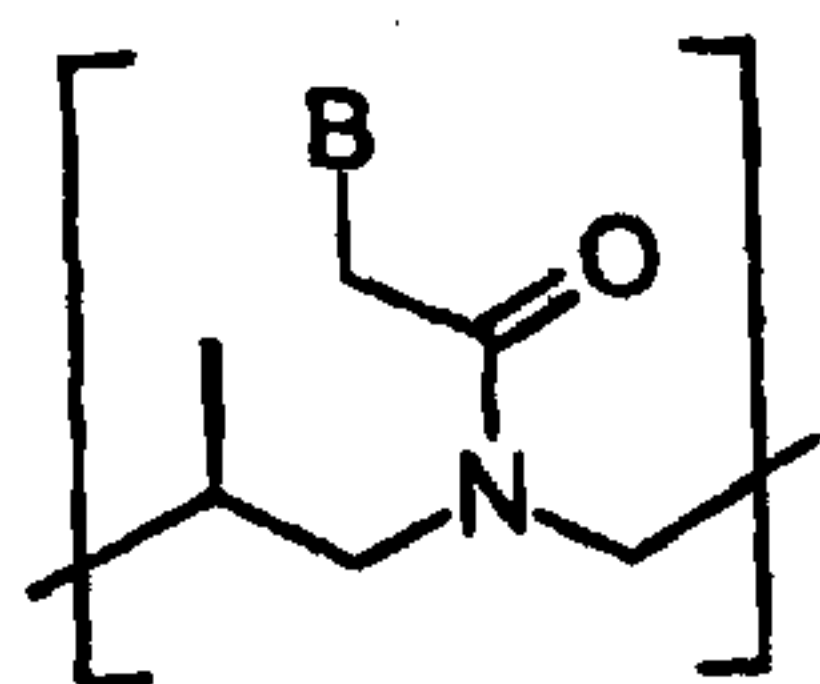
Formula IIIA

or formula IIIB,

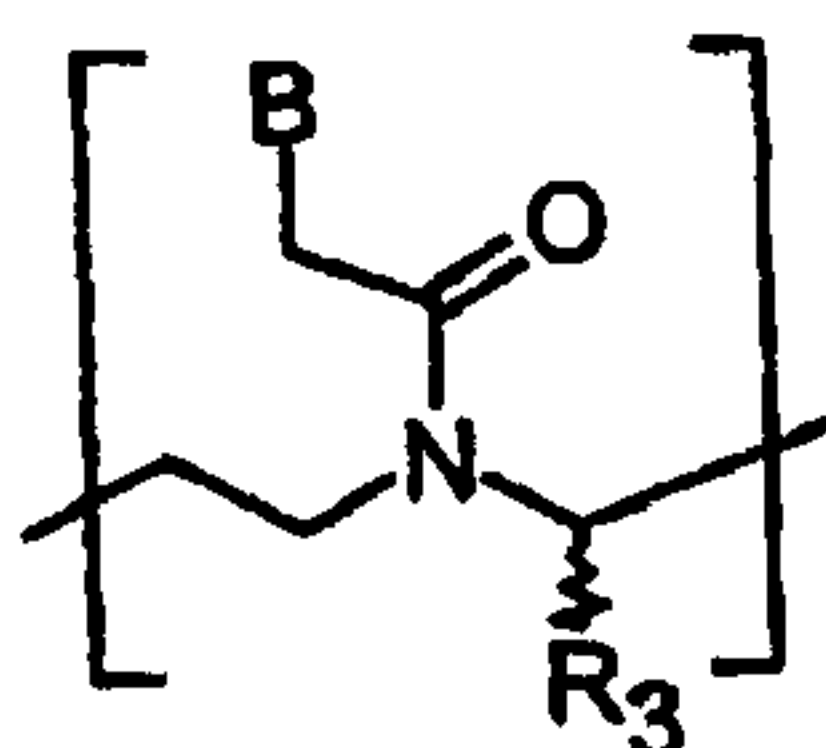


Formula IIIB

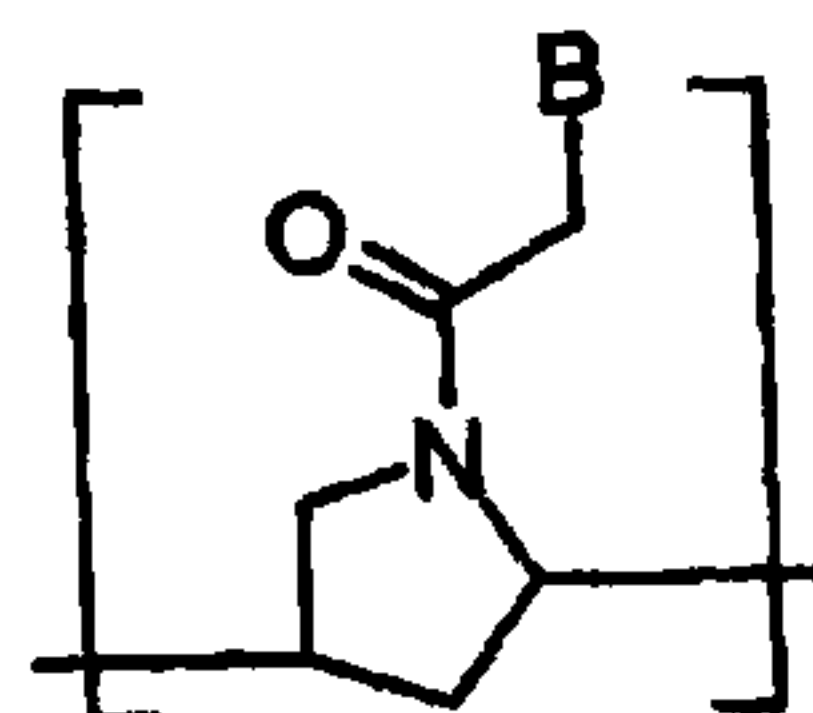
or formulae IV A to IV G,



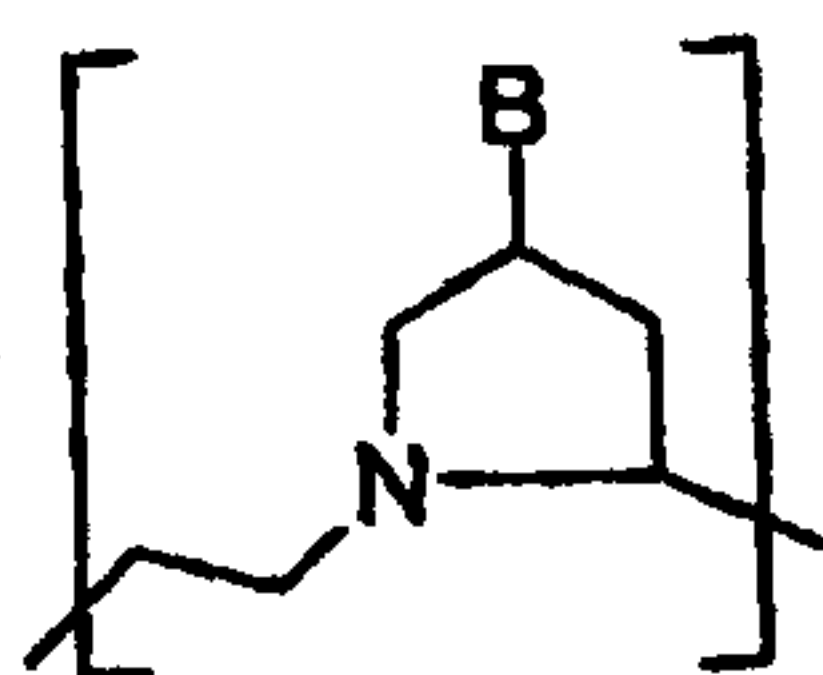
Formula IV A



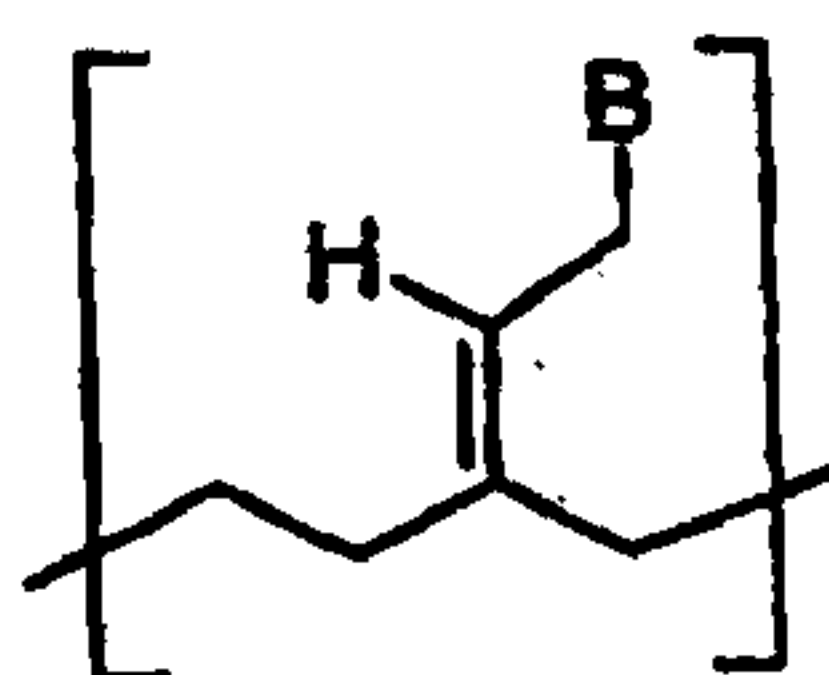
Formula IV B



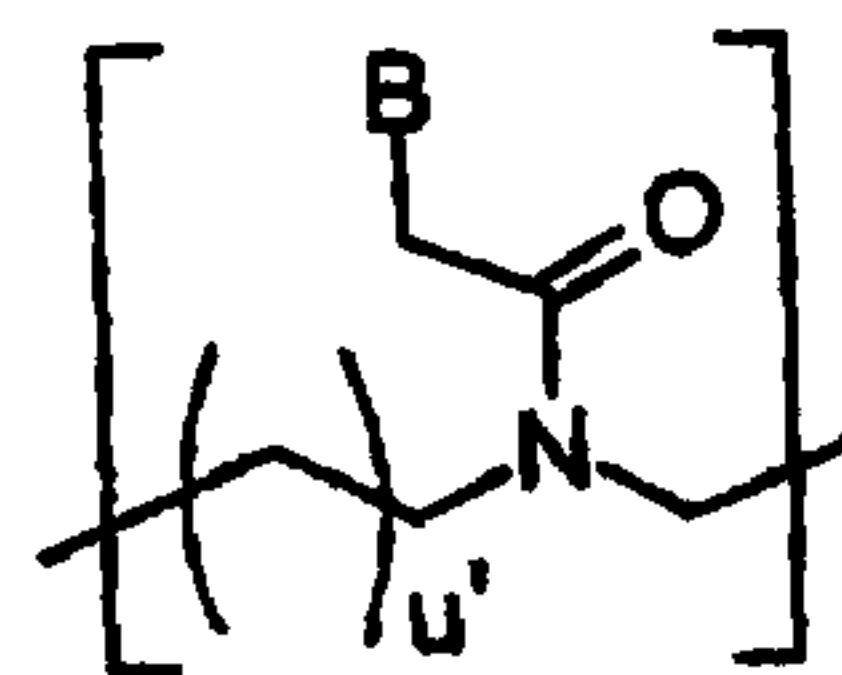
Formula IV C



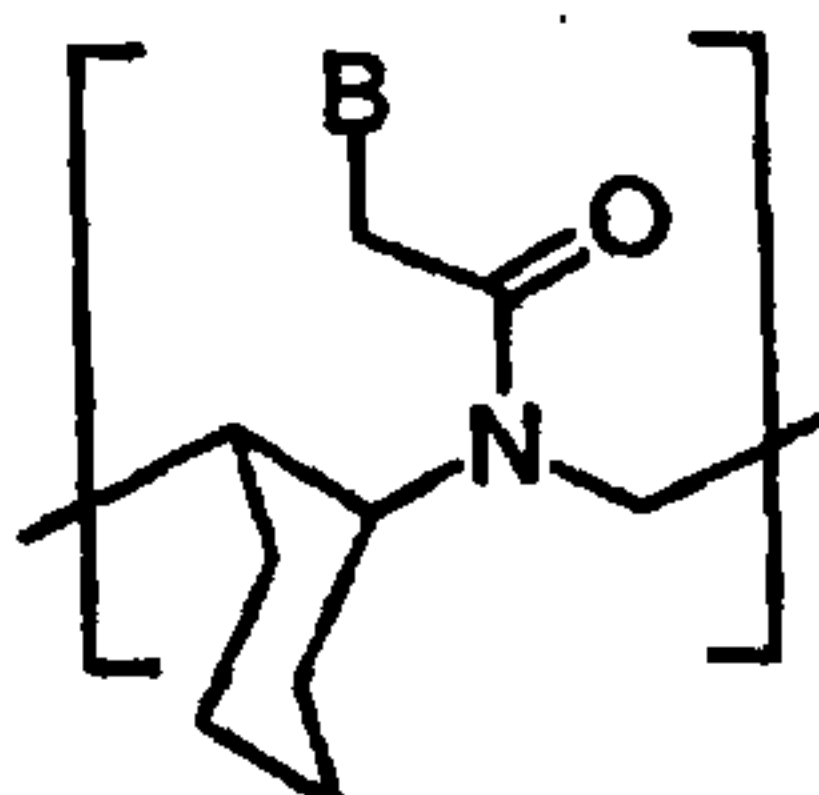
Formula IV D



Formula IV E



Formula IV F



Formula IV G

where each building block {BLOCK} is optionally different,

and where it furthermore applies that

z' is from 0 to 100,

5

A is, independently of each other, a group $(CR_1R_2)_s$, where s is from 1 to 3,

10

B is, independently of each other, either an aromatic radical, which optionally possesses heteroaromatic character, hydrogen, hydroxyl or C_1 - C_{18} -alkyl, or a nucleobase which occurs naturally, and is customary in nucleotide chemistry, or which does not occur naturally, or its prodrug form, where at least one B radical is a nucleobase,

15

D is, independently of each other, a group $(CR_3R_4)_t$, where t is from 2 to 10, where two adjacent R_3 and R_4 radicals optionally form a C_5 - C_8 -cycloalkyl ring,

20

E is, independently of each other, a group $(CR_5R_6)_{u'}$, where two adjacent R_5 and R_6 radicals optionally form a C_5 - C_8 -cycloalkyl ring or a spiro compound,

25

R_5 and R_6 are, independently of each other, a radical consisting of hydrogen, C_1 - C_6 -alkyl, or an amino acid side chain,

and also physiologically tolerated salts of the PNA derivative of the formula I, with the proviso that at least one Y or Z radical is hydroxyl, mercapto, oxyanion or thioate.

30

2. The PNA derivative as claimed in claim 1, wherein u' is from 1 to 4.

3. The PNA derivative as claimed in claim 2, wherein u' is 1.

4. The PNA derivative as claimed in any one of claims 1 to 3, wherein R_1 and R_2 are hydrogen.
5. The PNA derivative as claimed in any one of claims 1 to 4, wherein R_3 and R_4 are hydrogen.
6. The PNA derivative as claimed in any one of claims 1 to 5, wherein the bifunctional labeling group, X, is selected from the group consisting of fluorescein, rhodamine, TAMRA, biotin, pyrene, dinitrophenyl, acridine, cyanine dye, Dabcyl, digoxigenin and Edans derivative.
7. The PNA derivative as claimed in claim 6, wherein the bifunctional labeling group is a biotin derivative.
8. The PNA derivative as claimed in any one of claims 1 to 5, wherein the bifunctional group, X, for crosslinking with complementary nucleic acids is a psoralen derivative.
9. The PNA derivative as claimed in any one of claims 1 to 5, wherein the bifunctional group, X, which promotes intracellular uptake is selected from the group consisting of cholesteryl, adamantyl and vitamin E derivatives.
10. The PNA derivative as claimed in any one of claims 1 to 5, wherein the bifunctional group, X, which increases the binding affinity of the PNA derivative for a target nucleic acid is acridine or lexitropsin derivatives.
11. The PNA derivative as claimed in any one of claims 1 to 10, wherein m is from 2 to 10.
12. The PNA derivative as claimed in any one of claims 1 to 11, wherein the monofunctional or bifunctional labeling group Z is selected from the group consisting of fluorescein, rhodamine, TAMRA, biotin, pyrene, dinitrophenyl, acridine, cyanine dye, Dabcyl, digoxigenin and Edans derivative.

13. The PNA derivative as claimed in claim 12, wherein the bifunctional labeling group is a biotin derivative.

14. The PNA derivative as claimed in any one of claims 1 to 11, wherein
5 the monofunctional or bifunctional crosslinking group Z is a psoralen derivative.

15. The PNA derivative as claimed in any one of claims 1 to 11, wherein
10 the monofunctional or bifunctional group, Z, which promotes intracellular uptake is selected from the group consisting of cholesteryl, adamantyl and vitamin E derivatives.

16. The PNA derivative as claimed in any one of claims 1 to 11, wherein
15 the monofunctional or bifunctional group, Z, which promotes the binding affinity of the PNA derivative for a target nucleic acid is a lexitropsin derivative.

17. The PNA derivative as claimed in any one of claims 1 to 16, wherein n
is from 0 to 3.

20

18. The PNA derivative as claimed in any one of claims 1 to 17, wherein z'
is from 1 to 20.

19. The PNA derivative as claimed in claim 18, wherein z' is from 4 to 15.

25

20. The PNA derivative as claimed in any one of claims 1 to 19, wherein t
is from 2 to 4.

21. The PNA derivative as claimed in claim 20, wherein t is 2.

30

22. The PNA derivative as claimed in any one of claims 1 to 21, wherein R₅
and R₆ are hydrogen.

23. A PNA derivative as claimed in claim 1, wherein at least one Y or Z radical in formula I is oxyanion or thioate in a pH range from 4.5 to 14.

24. The PNA derivative as claimed in claim 23, wherein the pH range is
5 from 6.5 to 12.

25. The PNA derivative as claimed in claim 23, wherein the pH range is from 6.5 to 9.

10 26. A PNA derivative as claimed in any one of claims 1 to 25, wherein D is $(\text{CH}_2)_2$.

27. A PNA derivative as claimed in one of claims 1 to 26, wherein A and E are CH_2 .

15

28. A PNA derivative as claimed in any one of claims 1 to 27, wherein Q is a hydroxyaminoalkyl radical, a carrier sequence, insulin-like growth factor, nuclear localization signals or an affinity tag.

20 29. The PNA derivative as claimed in claim 28, wherein the hydroxyaminoalkyl radical is a hydroxyaminohexyl radical.

30. The PNA derivative as claimed in claim 28, wherein the carrier sequence is a transportan.

25

31. The PNA derivative as claimed in claim 28, wherein the affinity tag is a $(\text{His})_6$ chain.

30 32. A PNA derivative as claimed in one of claims 1 to 31, wherein B is adenine, cytosine, 5-methylcytosine, guanine, thymine, uracil, purine, 2,6-diaminopurine, N^4N^4 -ethanocytosine, N^6N^6 -ethano-2,6-diaminopurine, 5-(C_3 - C_6)-alkynyluracil, 5-(C_3 - C_6)-alkynylcytosine, 5-(1-propargylamino)uracil, 5-(1-propargylamino)cytosine, phenoxazine, 9-aminoethoxyphenoxazine, 5-fluorouracil, pseudoisocytosine, 5-(hydroxymethyl)uracil, 5-aminouracil,

pseudouracil, dihydrouracil, 5-(C₁-C₆)-alkyluracil, 5-(C₁-C₆)-alkylcytosine, 5-(C₂-C₆)-alkenylcytosine, 5-fluorocytosine, 5-chlorouracil, 5-chlorocytosine, 5-bromouracil, 5-bromocytosine, 7-deazaadenine, 7-deazaguanine, 8-azapurine or a 7-deaza-7-substituted purine.

5

33. A PNA derivative as claimed in one of claims 1 to 32, wherein W is oxygen and Y is hydroxyl or oxyanion.

34. A PNA derivative as claimed in any one of claims 1 to 33, wherein X is
10 a group U-(C₂-C₂₂-alkanediyl)-U, or is a group U-(CH₂CH₂-O)_u', where u' is from 1 to 6.

35. The PNA derivative as claimed in claim 34, wherein the group U-(C₂-C₂₂-alkanediyl)-U is O-(C₂-C₂₂-alkanediyl)-O.

15

36. The PNA derivative as claimed in claim 35, wherein the group O-(C₂-C₂₂-alkanediyl)-O is O-(CH₂)₂₋₆O.

37. The PNA derivative as claimed in claim 34, wherein the group U-
20 (CH₂CH₂-O)_u' is O(CH₂CH₂-O)_u'.

38. A PNA derivative as claimed in any one of claims 1 to 37, wherein Z is
a phosphate, a C₁- to C₂₂-radical, a C₁-C₂₂-U radical, hydroxy-C₁-C₁₈-U, an
aminoalkyl-U radical, a group of the formula R₉-(CH₂CH₂-O)_m, where R₉ is OH
25 or NH₂ and m is from 1 to 6, or a mercaptoalkyl-U radical.

39. The PNA derivative as claimed in claim 38, wherein the C₁-C₂₂-U
radical is C₁-C₂₂-alkoxy radical.

30 40. The PNA derivative as claimed in claim 39, wherein the C₁-C₂₂-alkoxy
radical is a C₁₆-alkoxy radical.

41. The PNA derivative as claimed in claim 38, wherein the hydroxy-C₁-
C₁₈-U radical is a hydroxy-C₁-C₁₈-O radical.

42. The PNA derivative as claimed in claim 41, wherein the hydroxy-C₁-C₁₈-O radical is a HO-(CH₂)₃₋₁₂-O radical.

5 43. The PNA derivative as claimed in claim 38, wherein the aminoalkyl-U radical is an aminoalkoxy radical.

44. The PNA derivative as claimed in claim 43, wherein the aminoalkoxy radical is a 6-aminohexoxy or 5-aminopentoxy radical.

10

45. The PNA derivative as claimed in claim 38, wherein R₉-(CH₂CH₂-O)_m is HO(CH₂CH₂-O)₂, HO(CH₂CH₂-O)₆ or H₂N-(CH₂CH₂-O)₂.

15 46. The PNA derivative as claimed in claim 38, wherein the mercaptoalkyl-U radical is a mercaptoalkoxy radical.

47. The PNA derivative as claimed in claim 46, wherein the mercaptoalkoxy radical is a 6-mercaptohexyloxy radical.

20 48. A PNA derivative as claimed in any one of claims 1 to 47, wherein X and Z are, independently of each other, selected from the group consisting of biotin, fluorescein and lexitropsin derivatives.

25 49. A PNA derivative as claimed in any one of claims 1 to 48, wherein X and Z are selected, independently of each other, from the group consisting of rhodamine, TAMRA and cyanine dye.

30 50. A PNA derivative as claimed in one of claims 1 to 49, wherein X and Z are selected, independently of each other, from the group consisting of Dabcyl, psoralen, acridine, DNP and cholesterol.

51. A PNA derivative as claimed in any one of claims 1 to 50, wherein {POLY} comprises a nucleotide base sequence which is directed against parts

of tumor suppressor genes, oncogenes or telomerases, or their transcription products.

52. A PNA derivative as claimed in claim 51, wherein the base sequence of
5 the PNA moiety is directed against the translation start of HA-ras mRNA.

53. The use of a PNA derivative as claimed in one of claims 1 to 52 for producing a pharmaceutical for tumor therapy.

10 54. A PNA derivative as claimed in any one of claims 1 to 52 for use as a diagnostic agent.

55. The use of a PNA derivative as claimed in any one of claims 1 to 52 for detecting microorganisms and/or viruses.

15

56. The use of a PNA derivative as claimed in any one of claims 1 to 52 for detecting and/or quantifying nucleic acids.

57. The use of a PNA derivative as claimed in any one of claims 1 to 52 as
20 a detection reagent for in-situ hybridization or fluorescence in-situ hybridization.

58. The use of a PNA derivative as claimed in any one of claims 1 to 52 as an antisense agent, anti-gene agent, decoy agent or chimeraplast agent.

25

59. A detection reagent, comprising a PNA derivative as claimed in any one of claims 1 to 52.

60. A PNA chip, comprising a PNA derivative as claimed in any one of
30 claims 1 to 52.

61. A biosensor, comprising a PNA derivative as claimed in any one of claims 1 to 52.

62. A pharmaceutical composition, comprising a PNA derivative as claimed in any one of claims 1 to 52 and other pharmacologically tolerated additives and/or excipients.

5 63. An antisense agent, comprising a PNA derivative as claimed in any one of claims 1 to 52.

64. A process for preparing a PNA derivative as claimed in any one of claims 1 to 52, wherein

- 10 a) the PNA backbone is synthesized, starting from the C terminus, using activated amidonucleic acids, and
- b) the N terminus is reacted with a phosphorylating reagent.

15 65. The process as claimed in claim 64, wherein the PNA is prepared using the t-butyloxycarbonyl (BOC), 9-fluorenylmethoxycarbonyl (Fmoc) or monomethoxytrityl (Mmt) protecting groups.

66. The process as claimed in claim 65, wherein the PNA is prepared using solid supports.

20

67. The process as claimed in claim 66, wherein CPG, tentagel or aminomethylpolystyrene is used as the solid support.

68. A process for producing a pharmaceutical, wherein

- 25 a) a PNA derivative as prepared in any one of claims 64 to 67 is prepared, and
- b) further pharmacologically tolerated additives and/or excipients are optionally added to it.

30 69. A process for producing a PNA chip, wherein, as claimed in any one of claims 64 to 67, a PNA derivative is firstly prepared and then fixed onto a solid support, or the PNA derivative is prepared directly on the support.

70. A process for preparing a PNA derivative of the formula I as claimed in any one of claims 64 to 67, wherein, in addition, the PNA is purified by means of chromatography or electrophoresis while exploiting the acid character of the phosphonss radical.

5

71. The process as claimed in claim 70, wherein a PNA derivative is purified by means of chromatography using a basic stationary phase and an acid or salt-containing eluent.

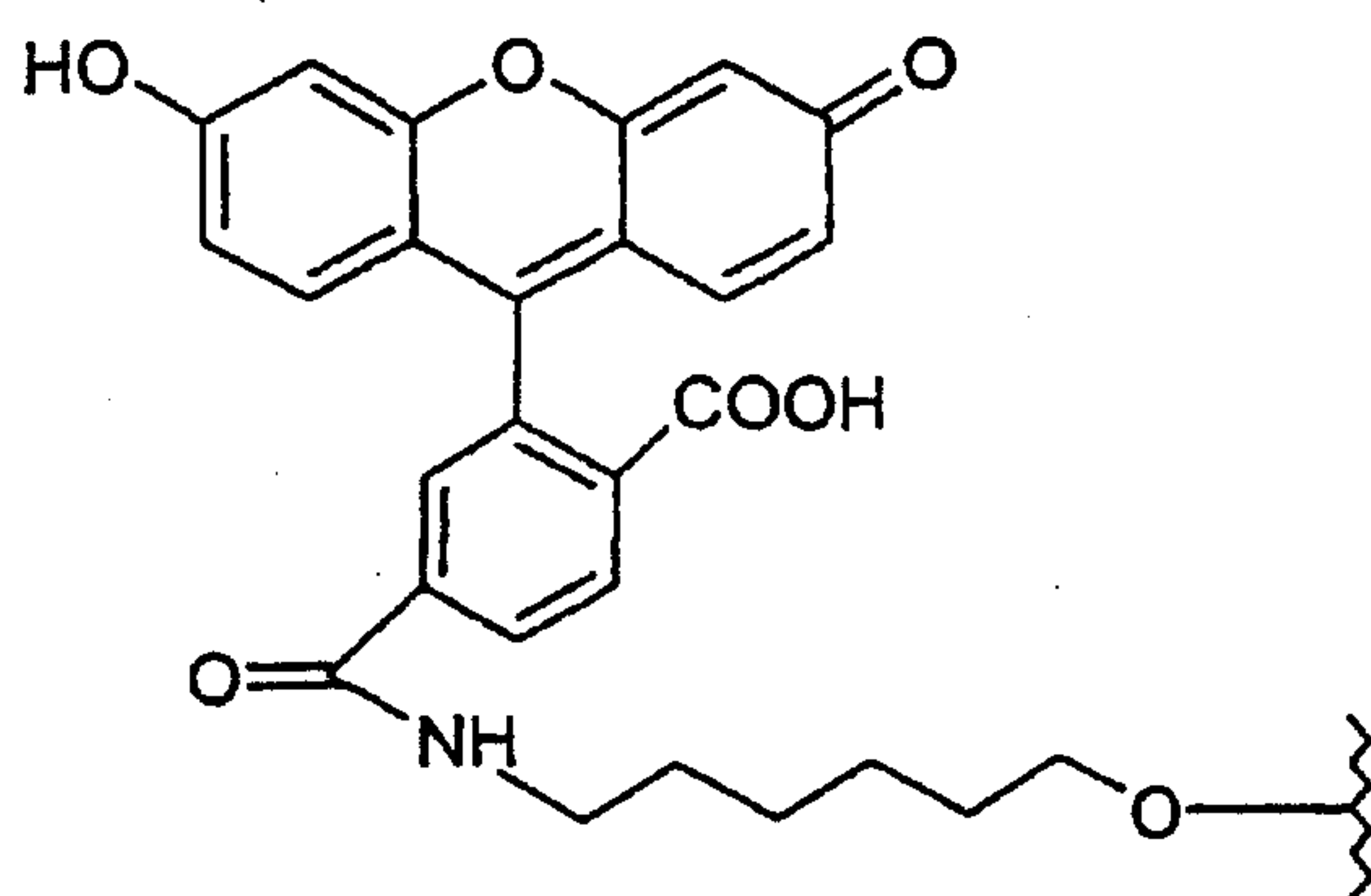
10 72. The process as claimed in claim 71, wherein the stationary phase is an anion exchanger or a mixed-mode phase.

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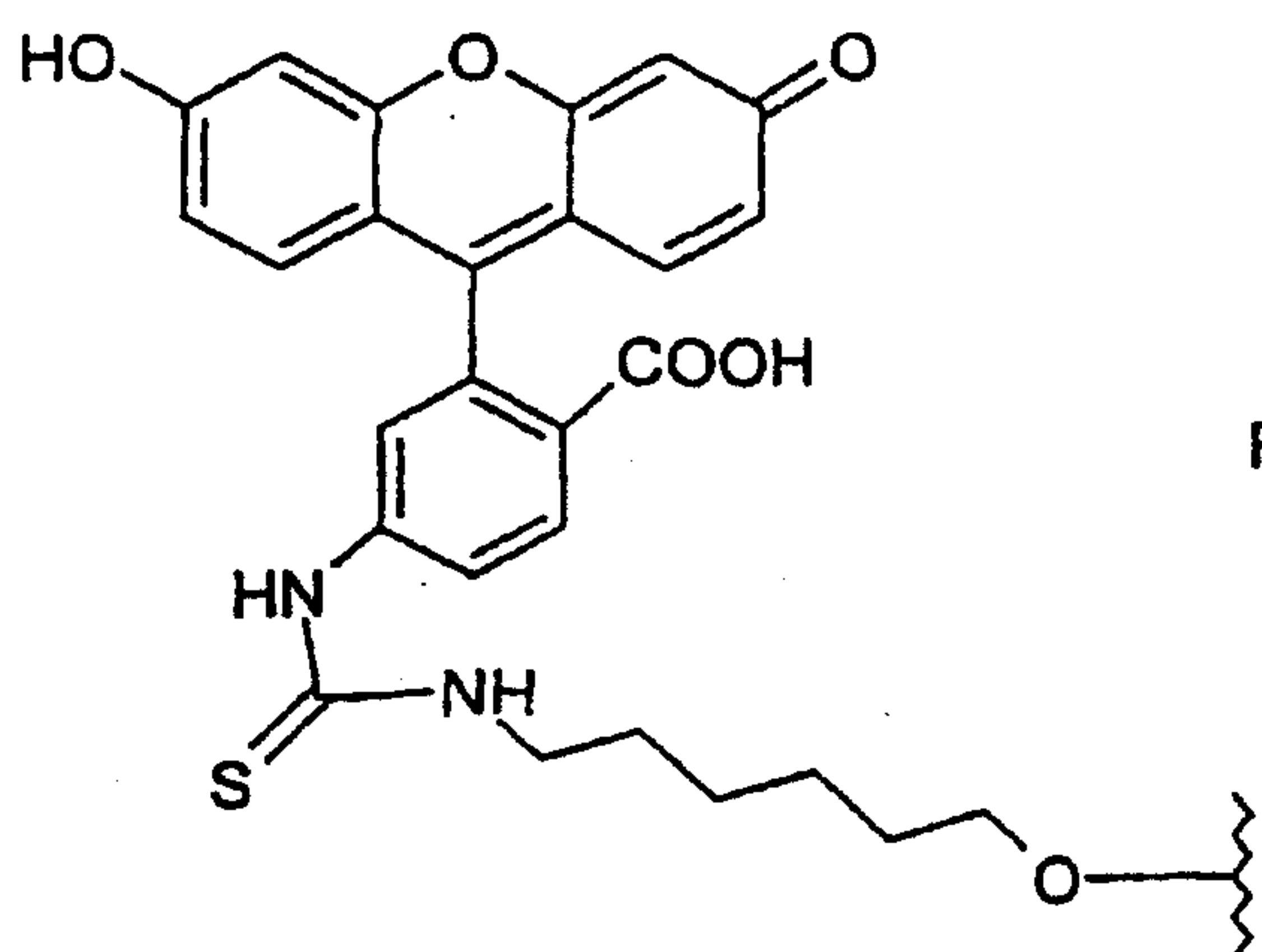
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1/14

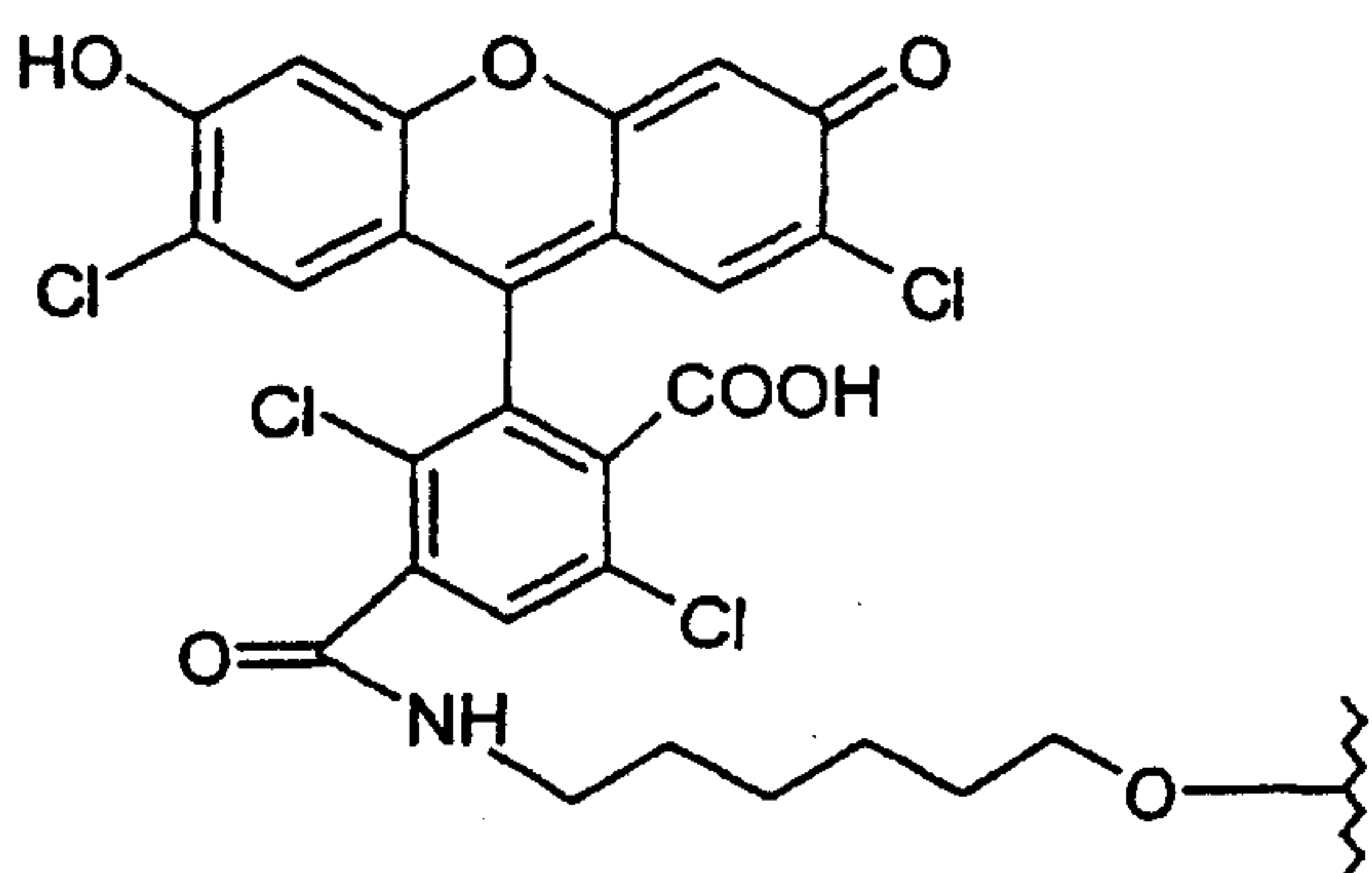
Fig. 1a:



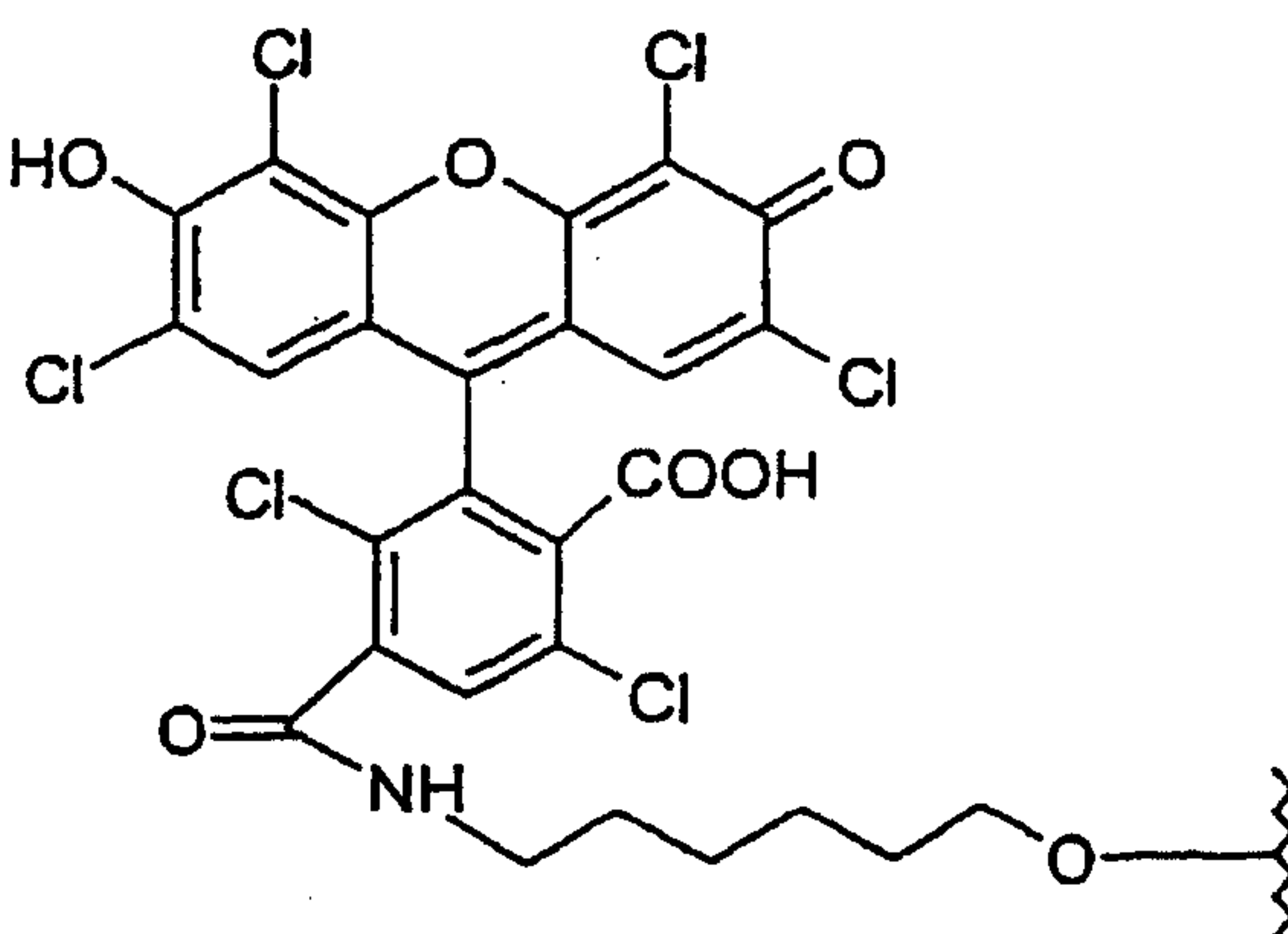
Fluorescein (amide)



Fluorescein (thiourea)



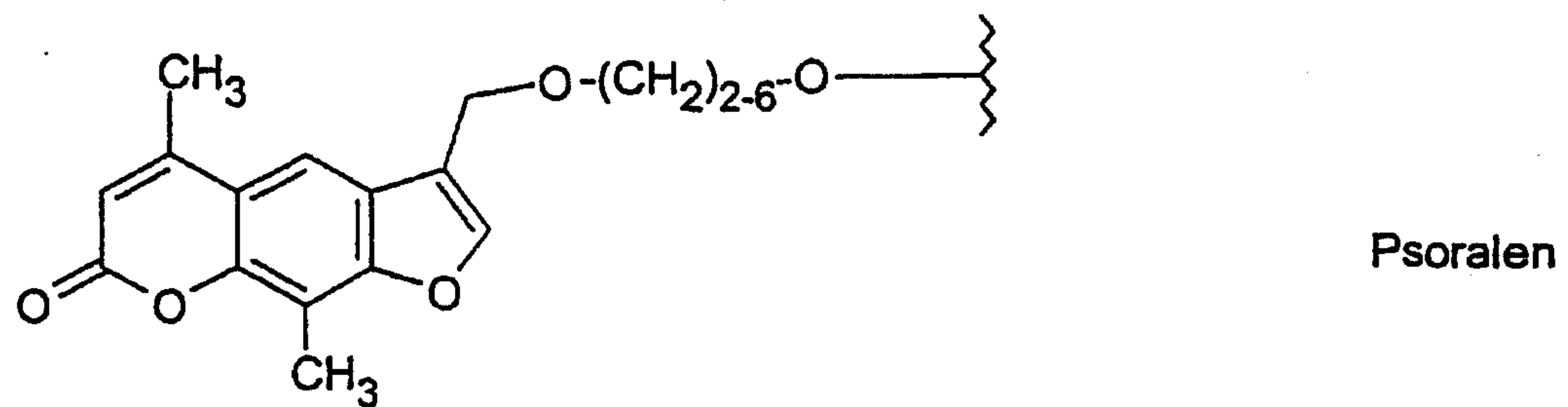
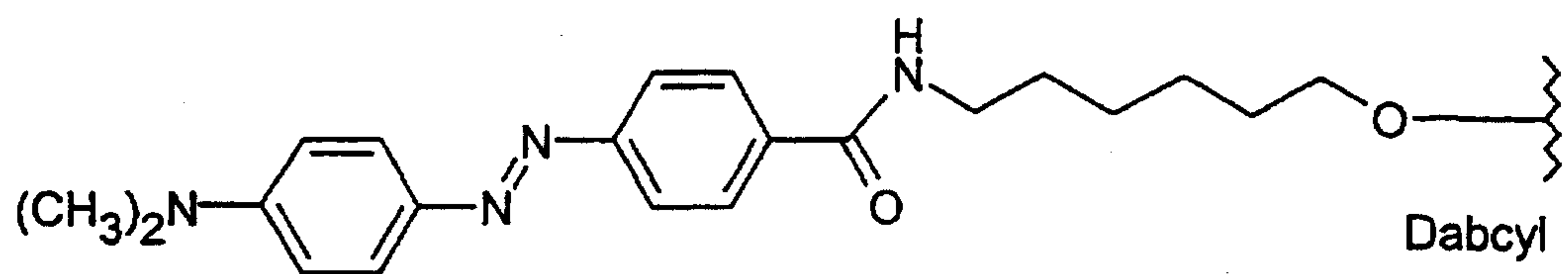
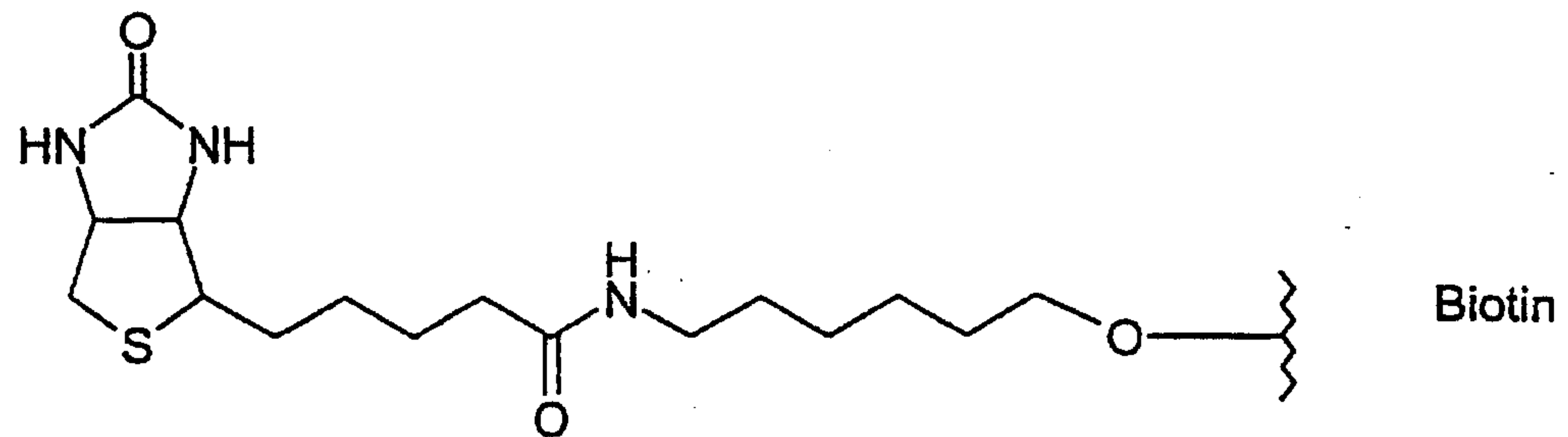
Tetrachlorofluorescein



Hexachlorofluorescein

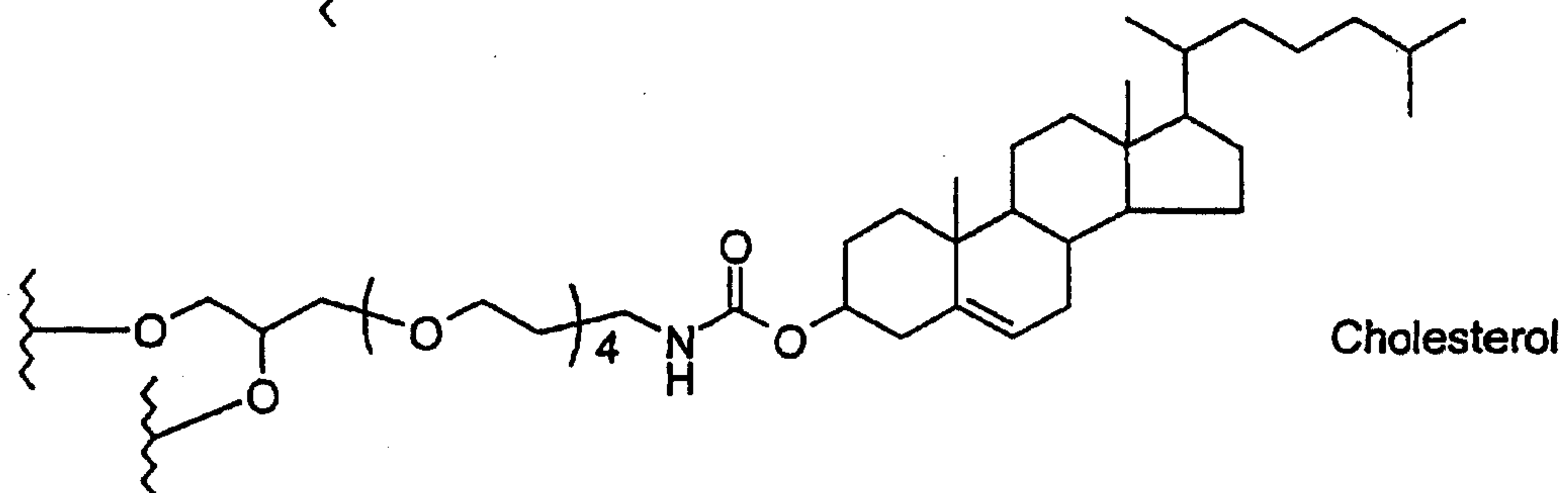
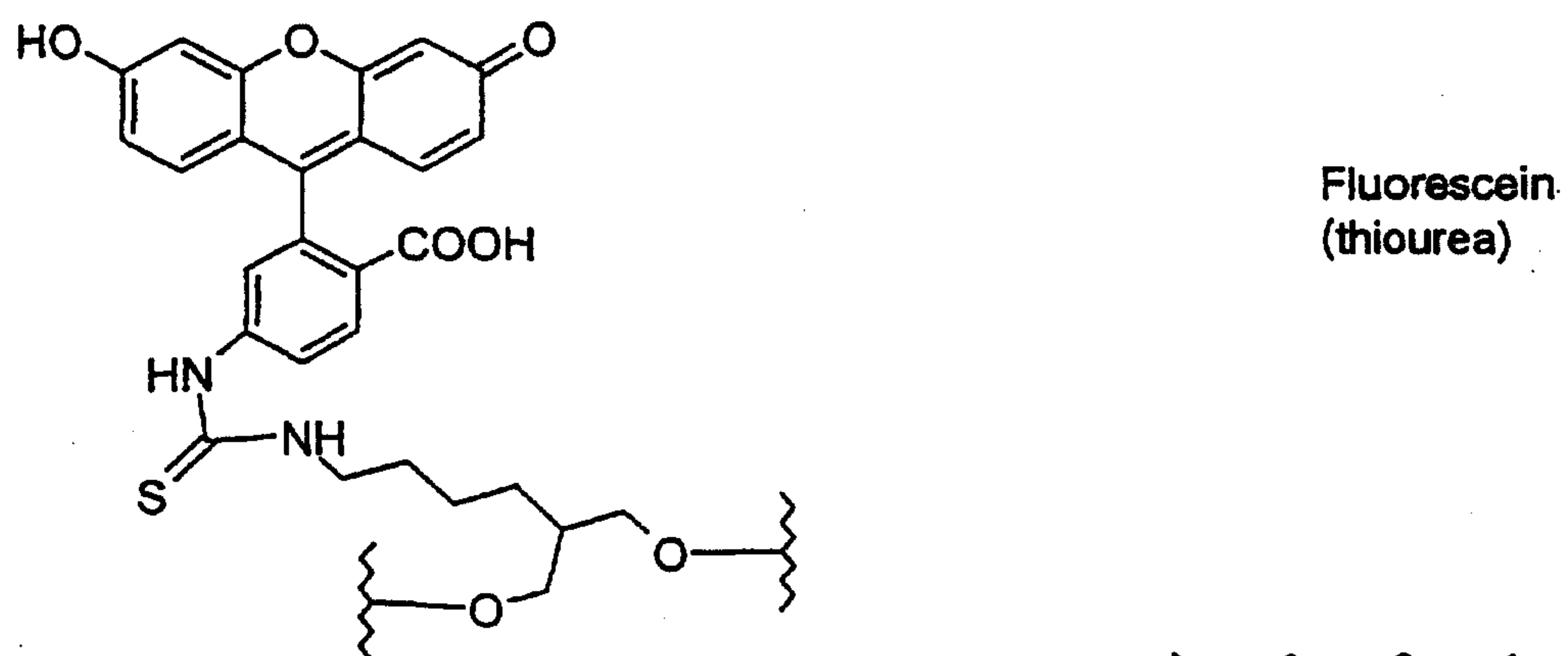
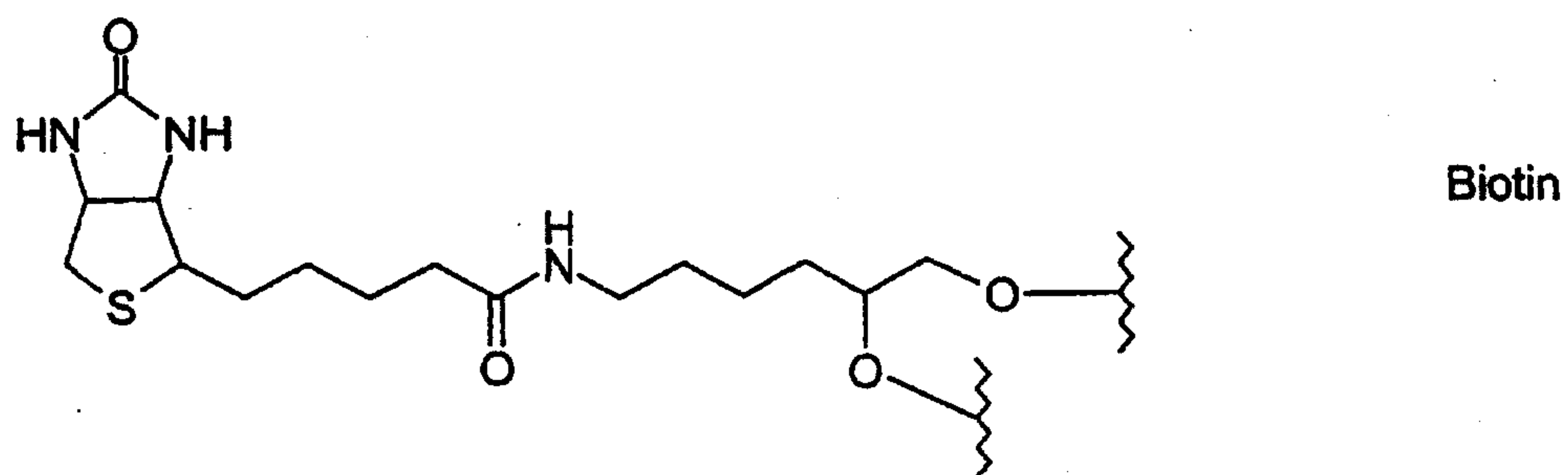
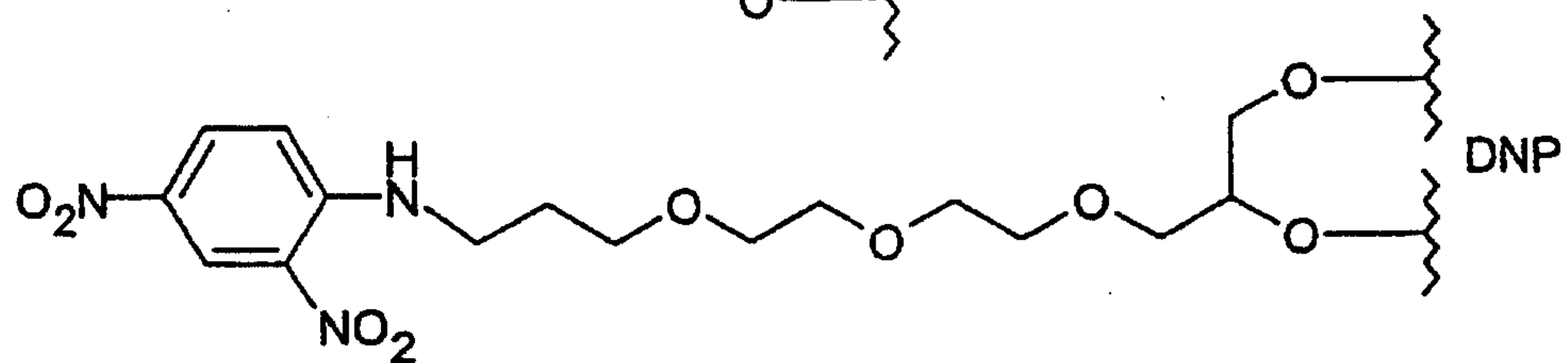
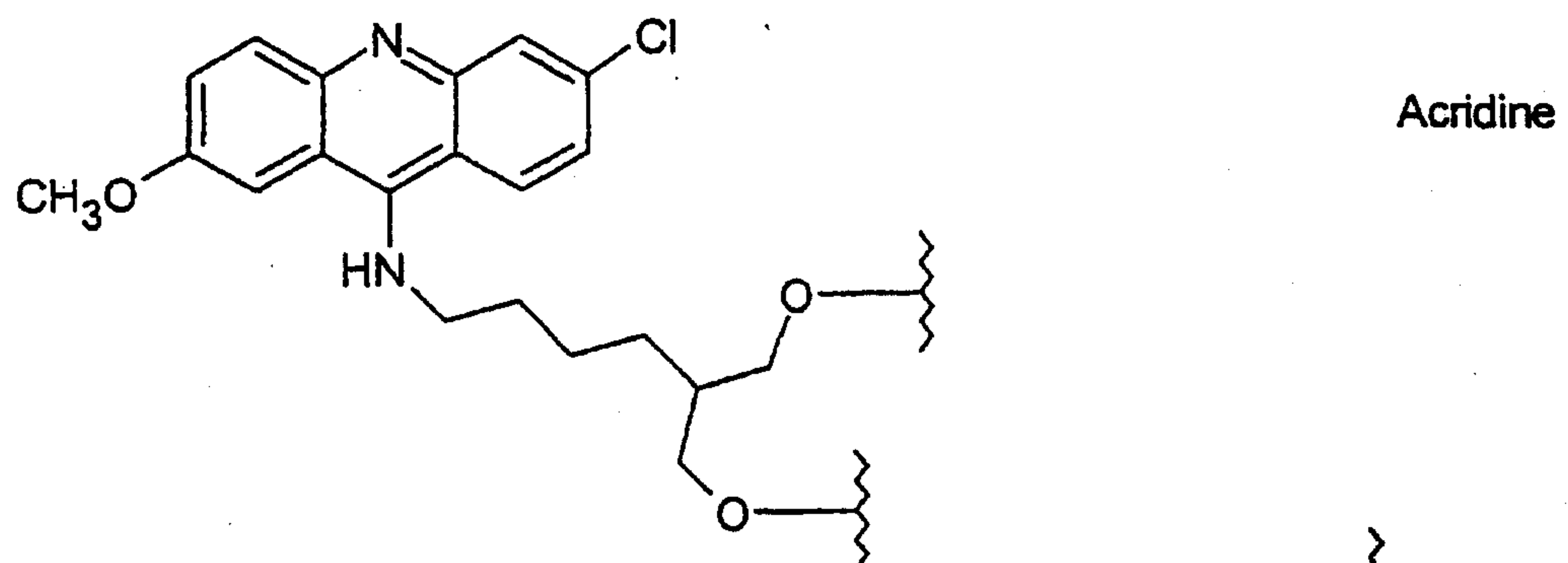
2/14

Fig. 1b:



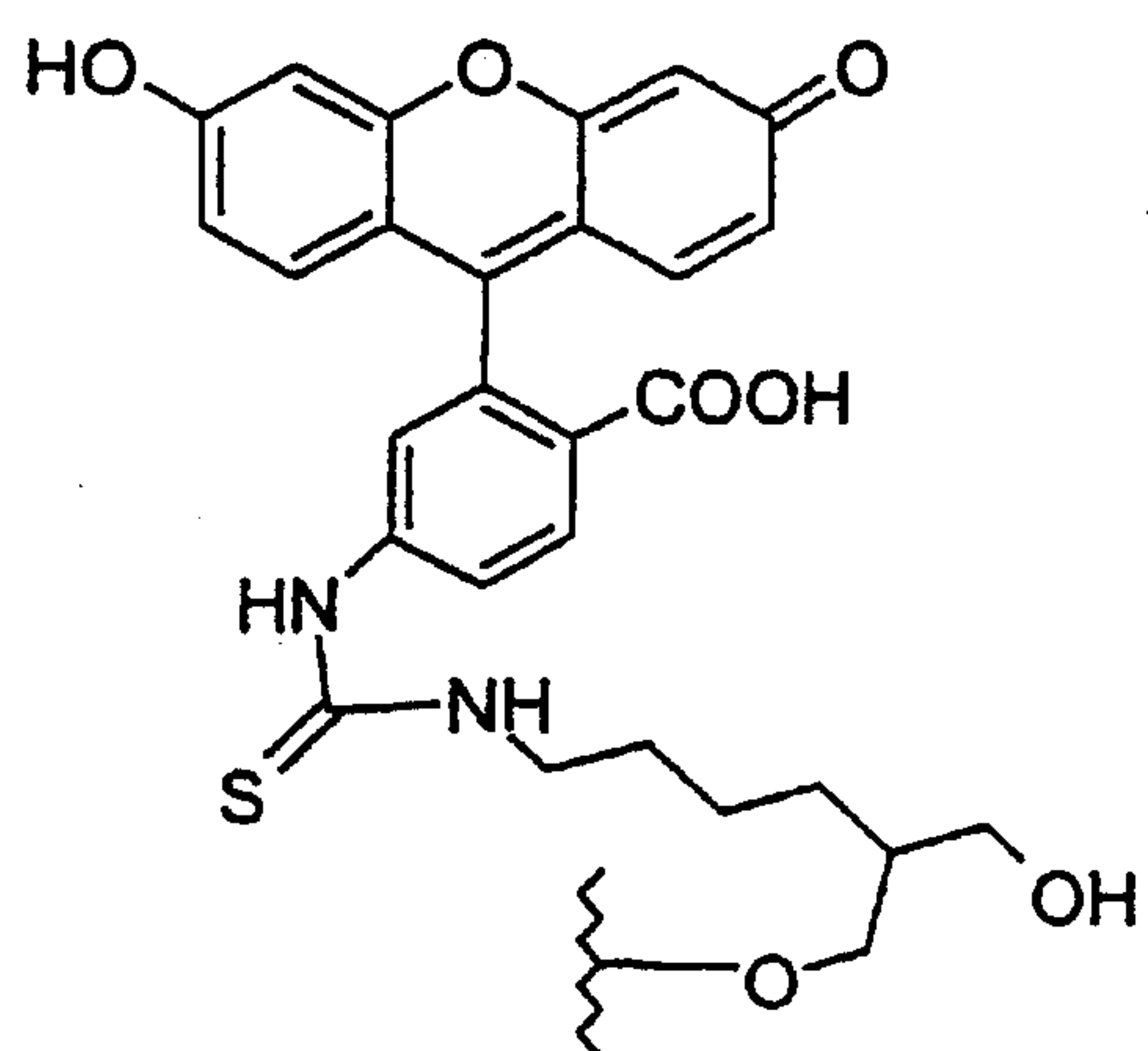
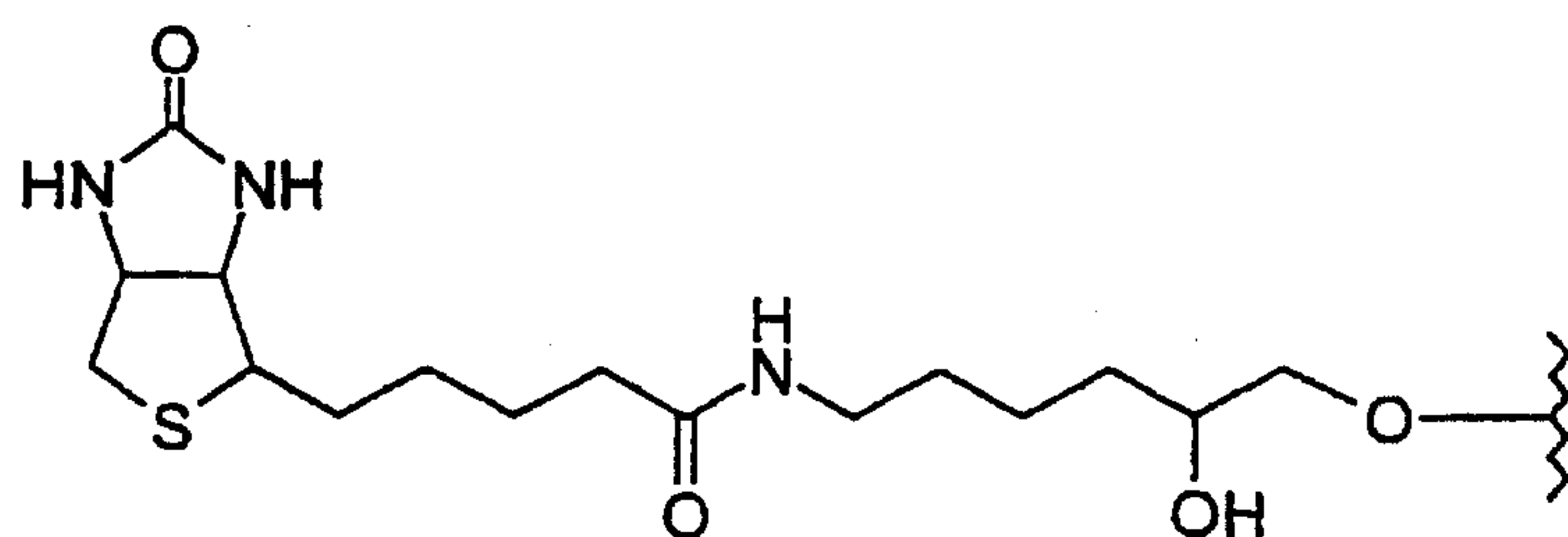
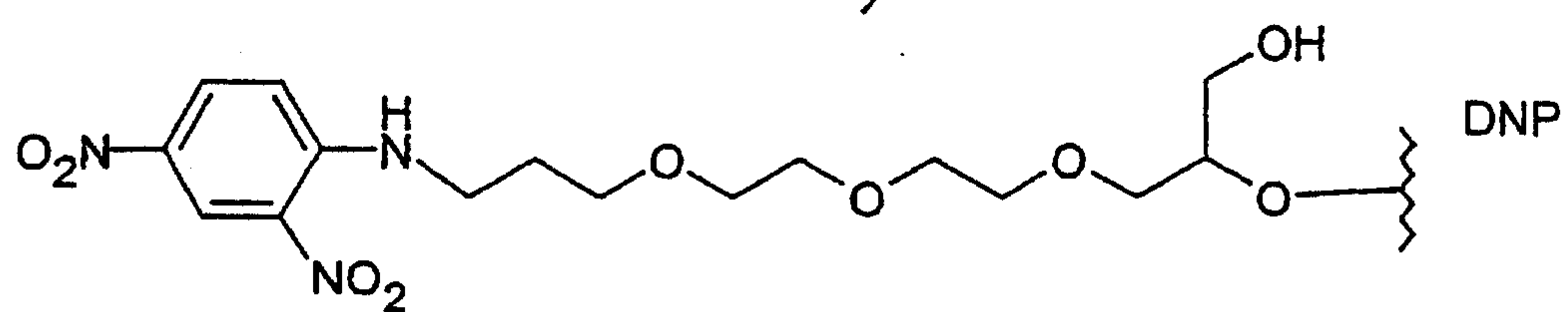
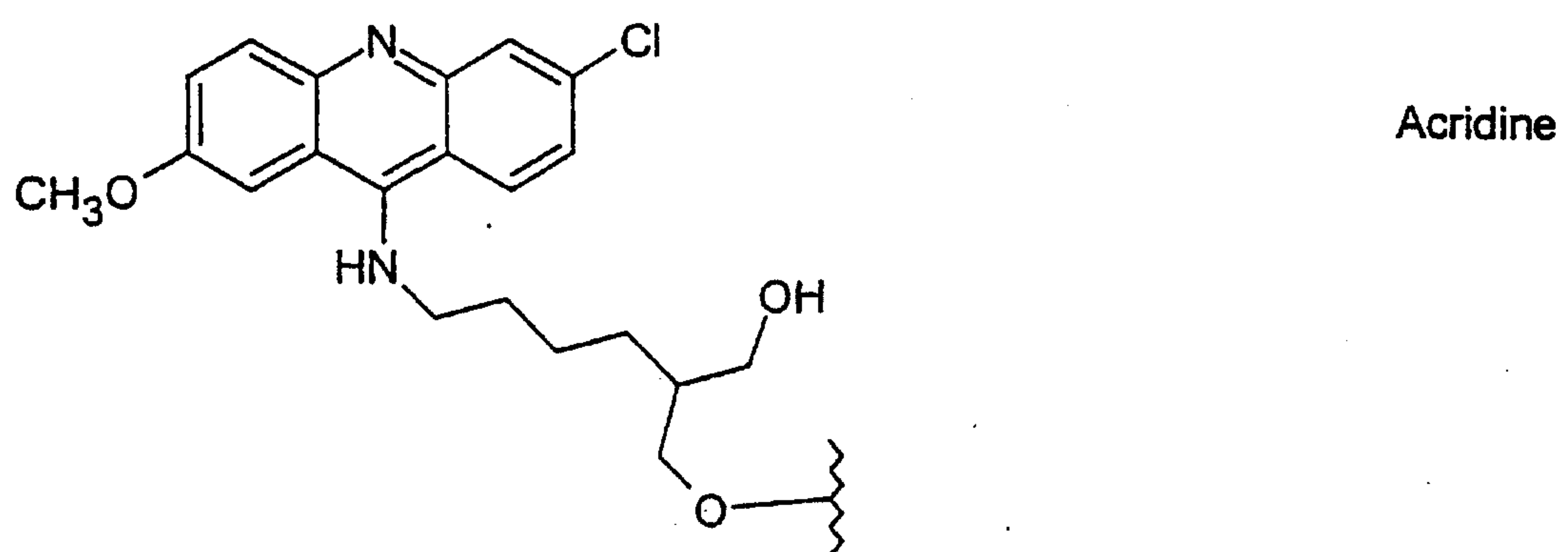
3/14

Fig. 2a:

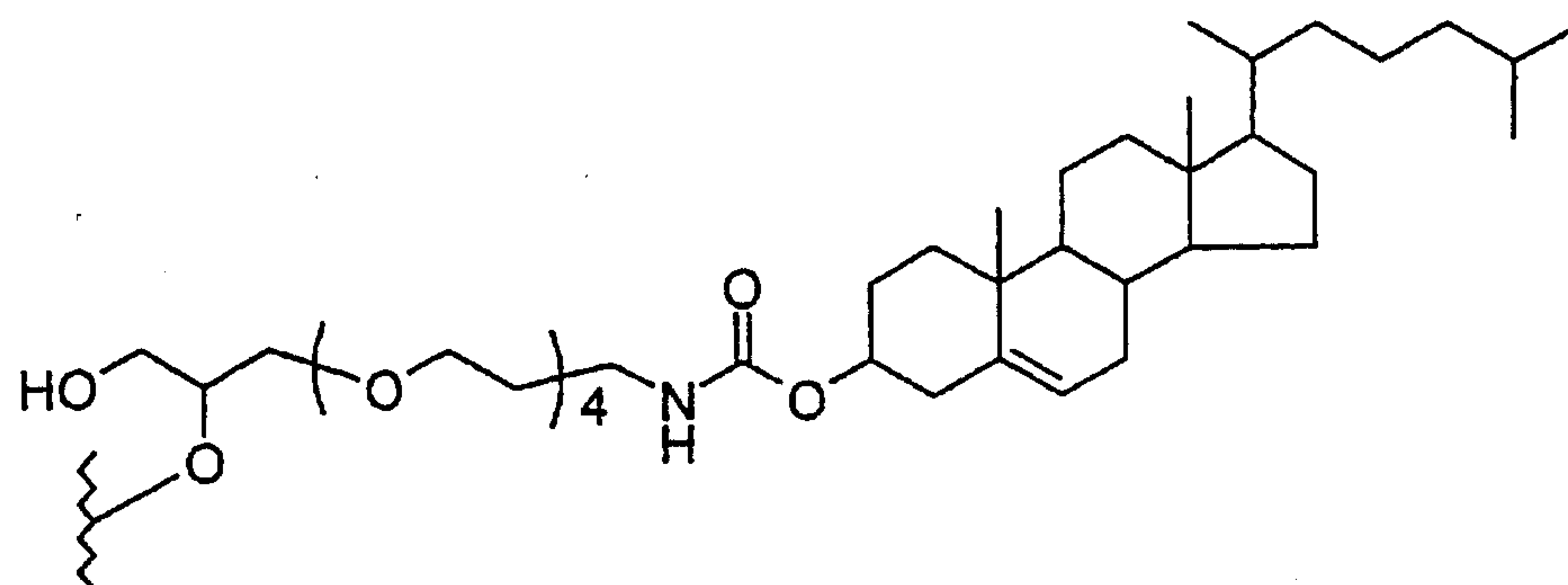


4/14

Fig. 2b:



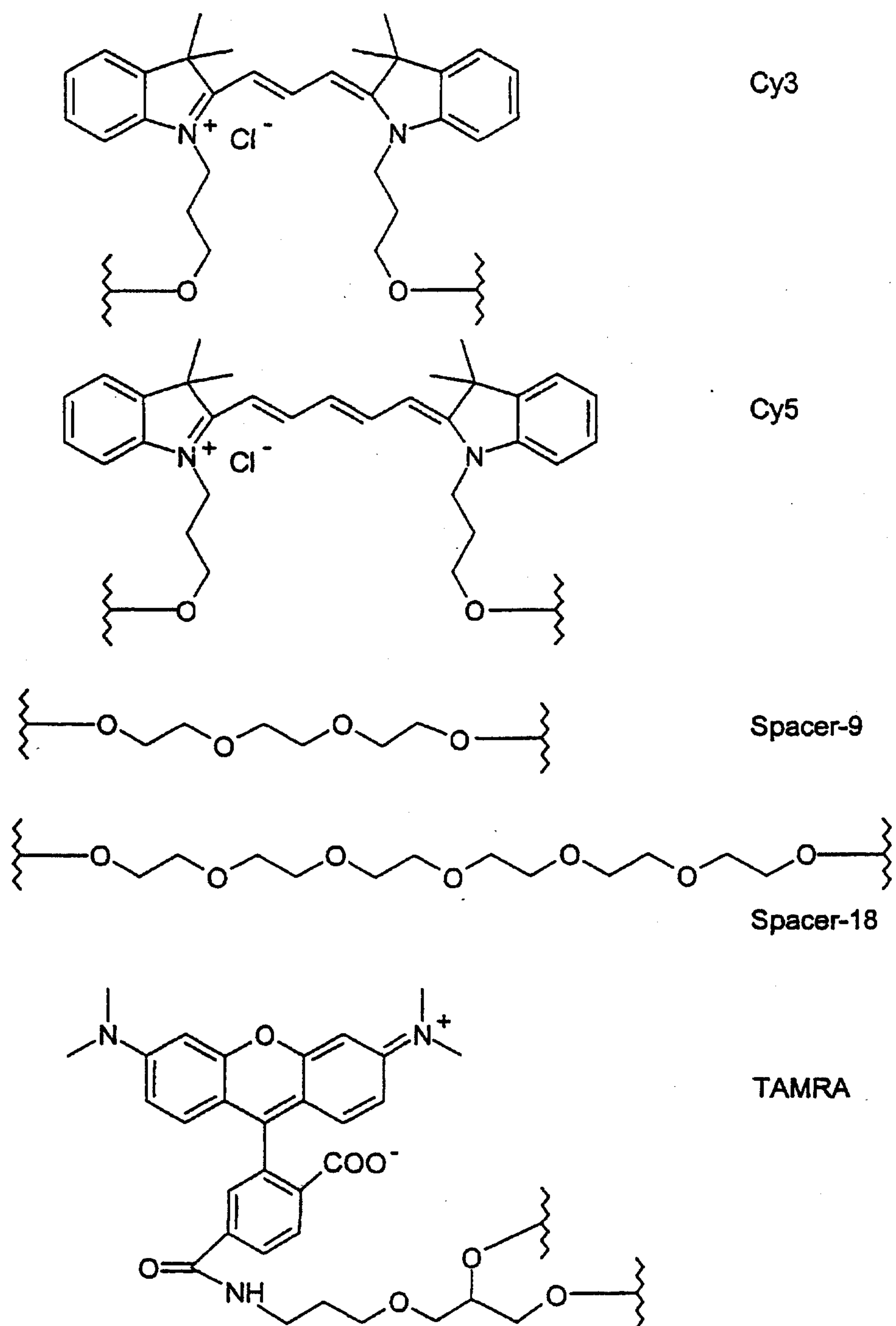
5/14



6/14

Fig. 3a:

Cholesterol



7/14

Fig. 3b:

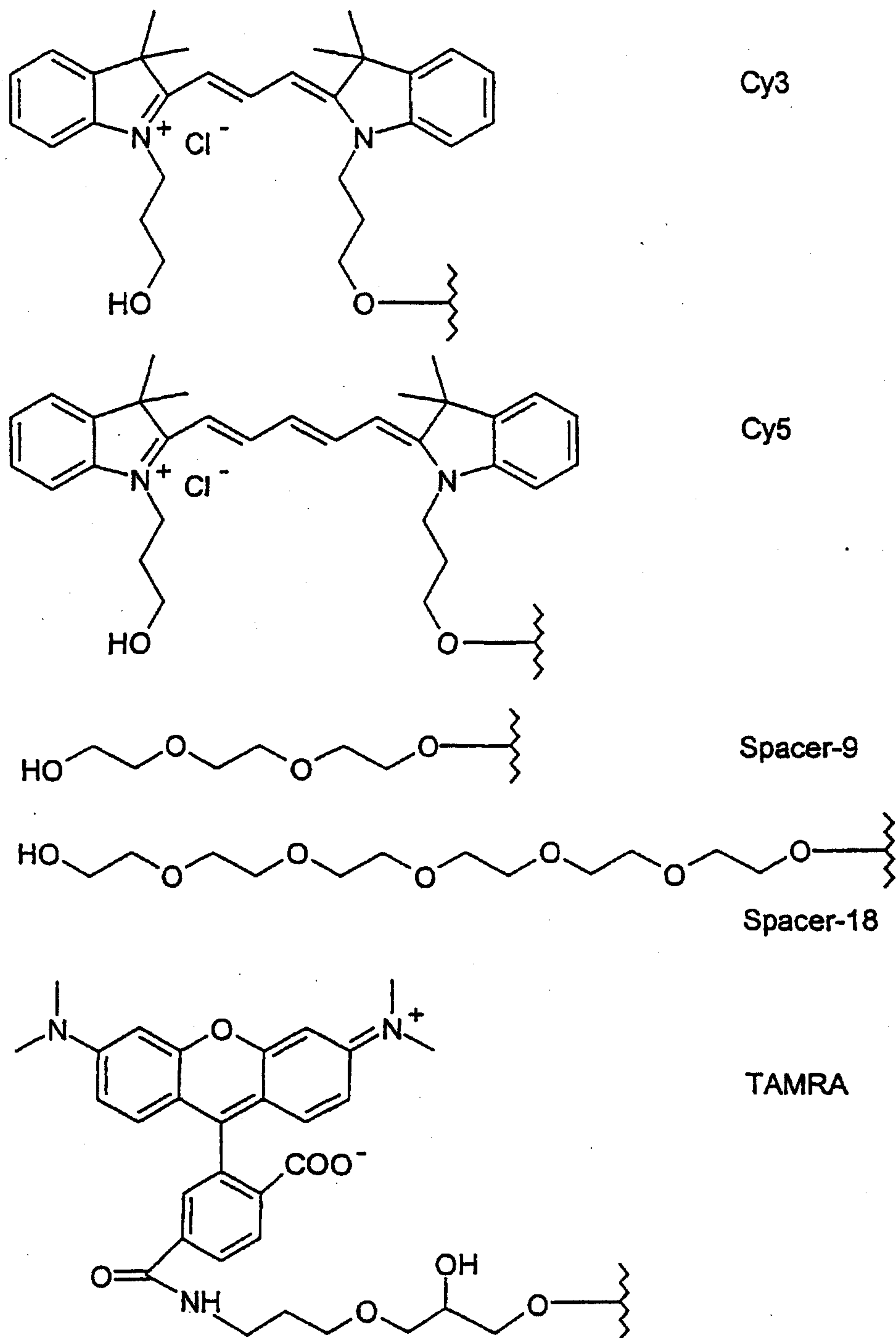
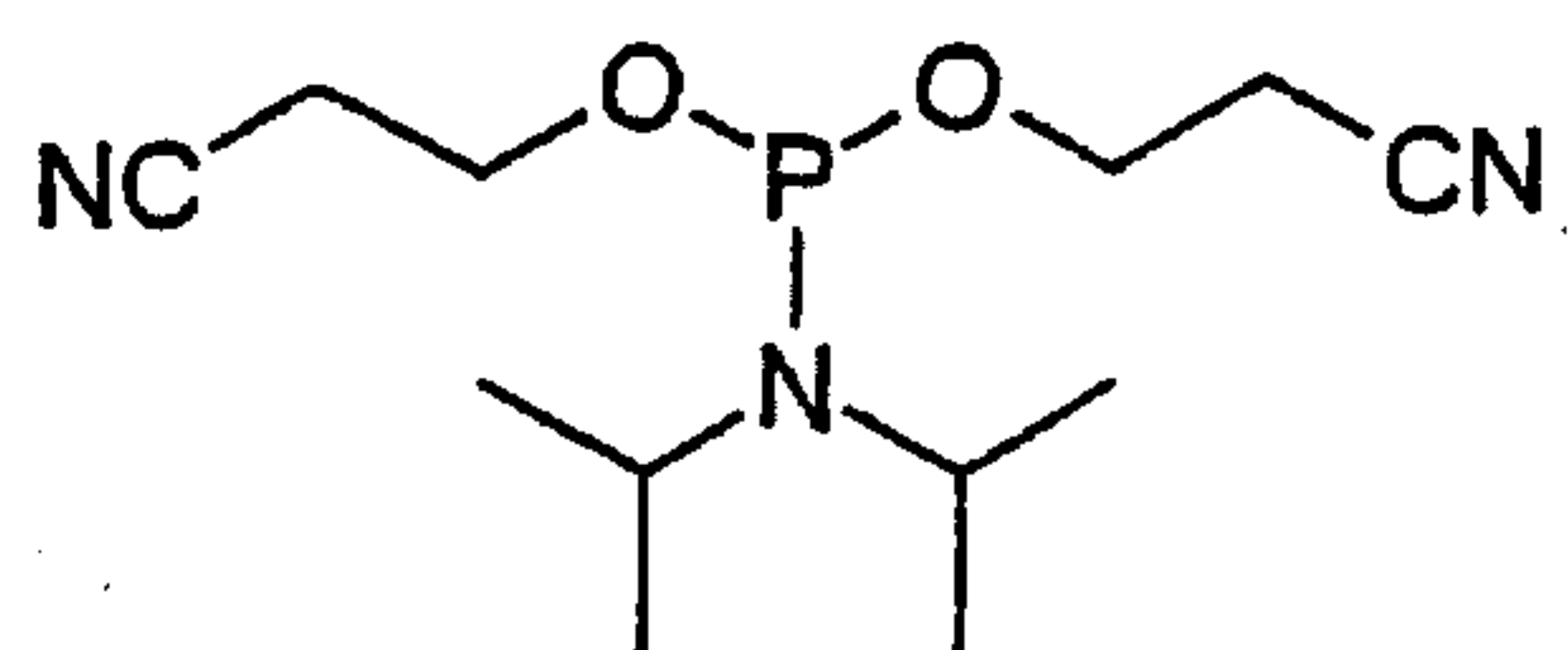
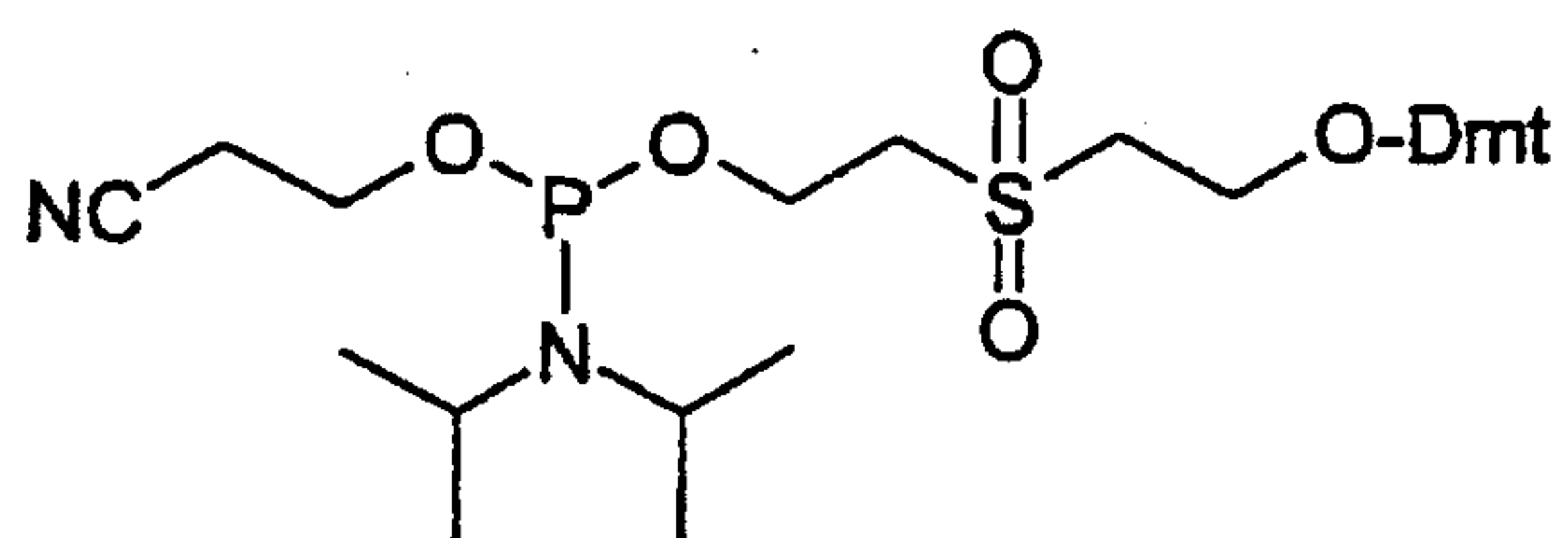


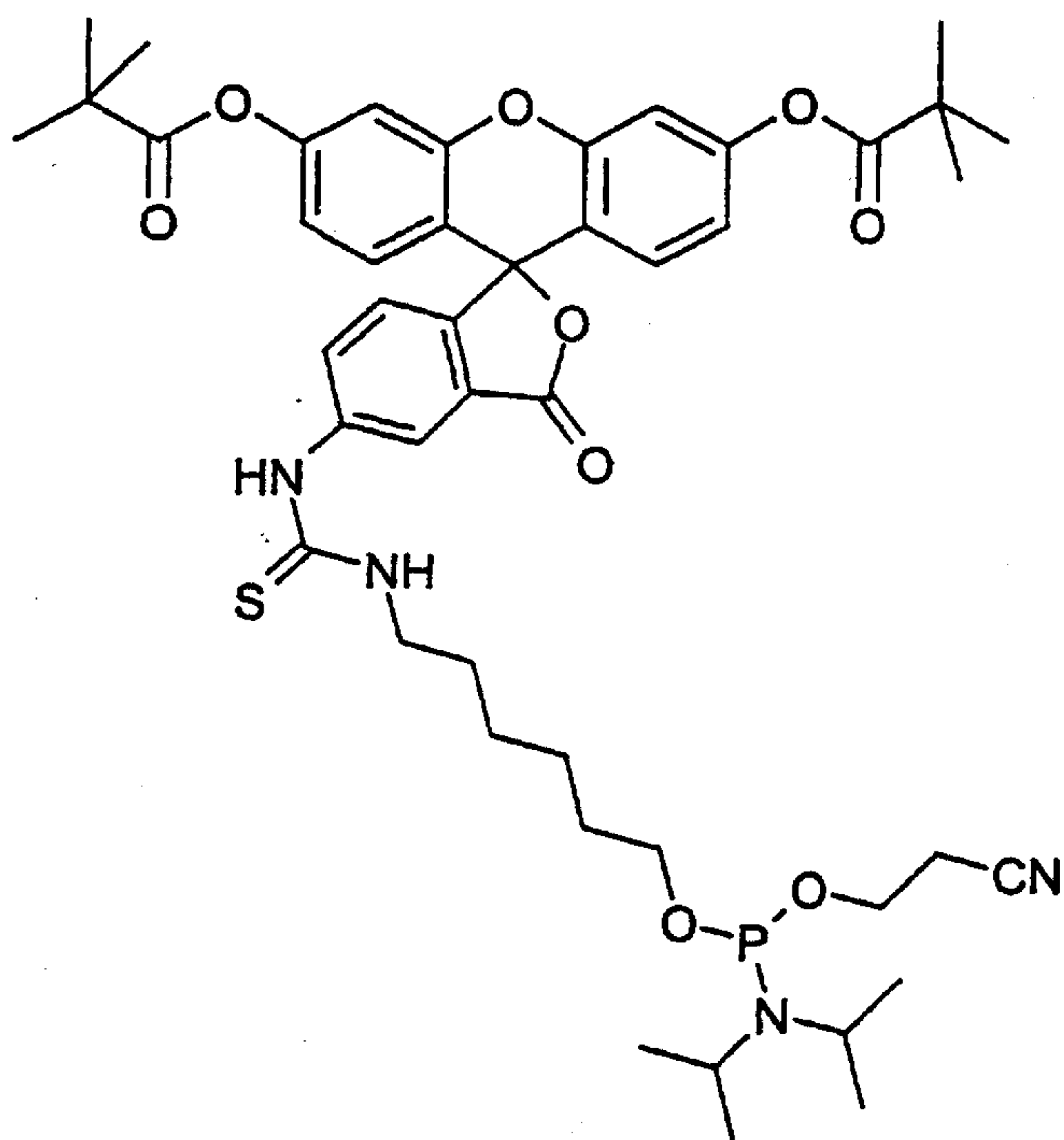
Fig. 4a:



Phosphorylating reagent 1



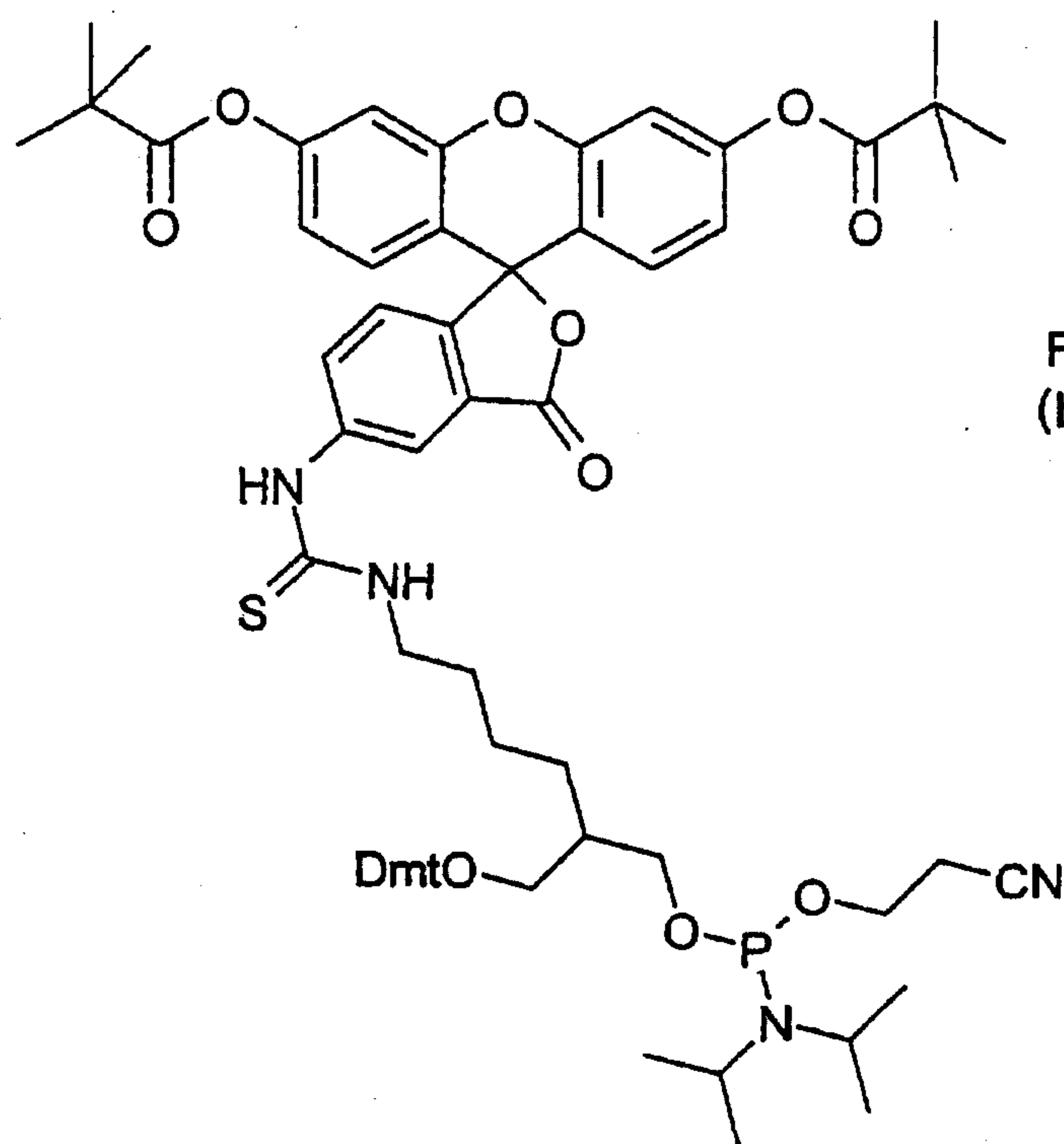
Phosphorylating reagent 2



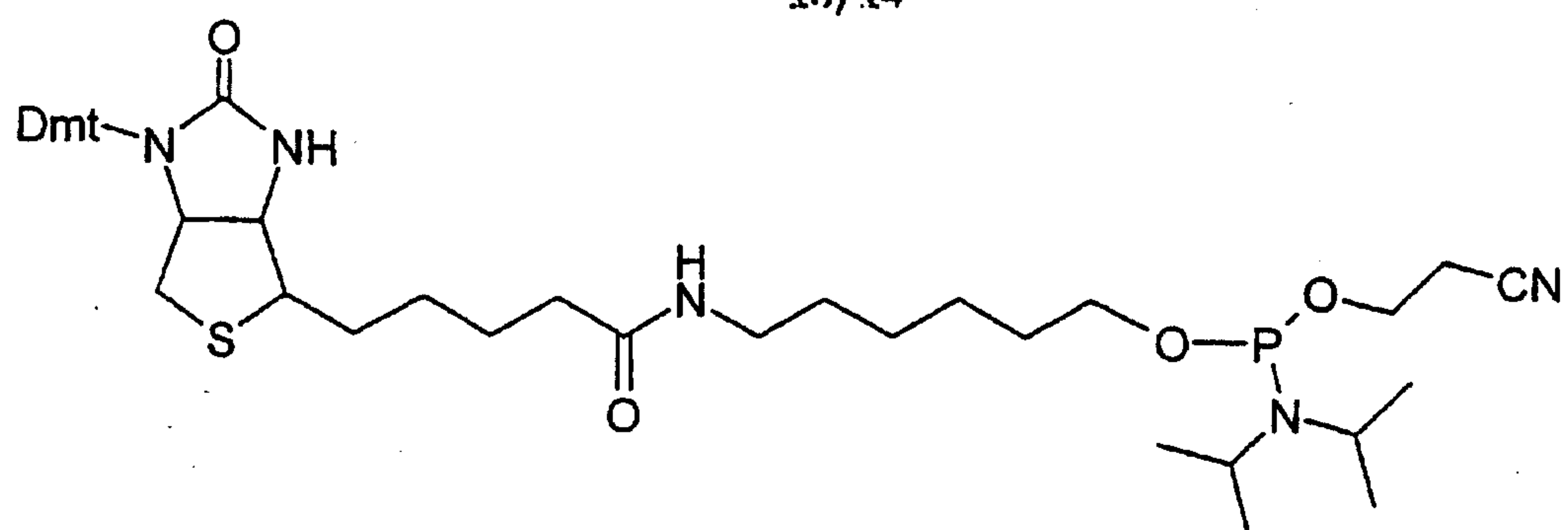
Fluorescein phosphoramidite 3 (monofunctional)

9/14

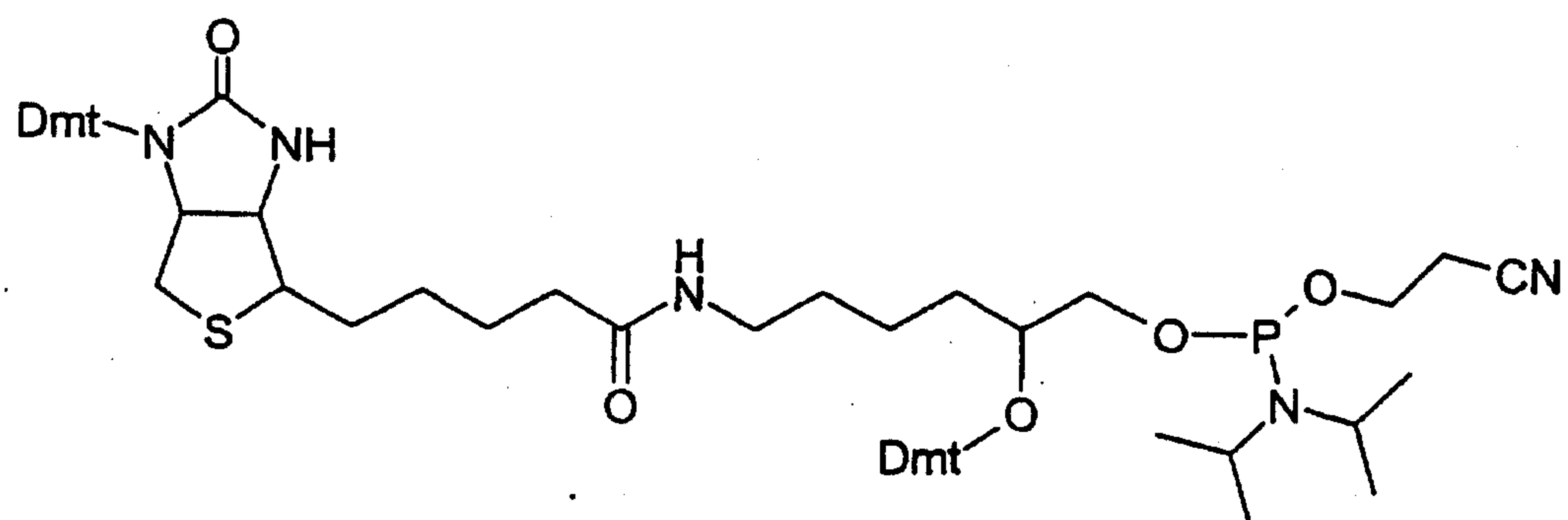
Fig. 4b:

Fluorescein phosphoramidite 4
(bifunctional)

10/14

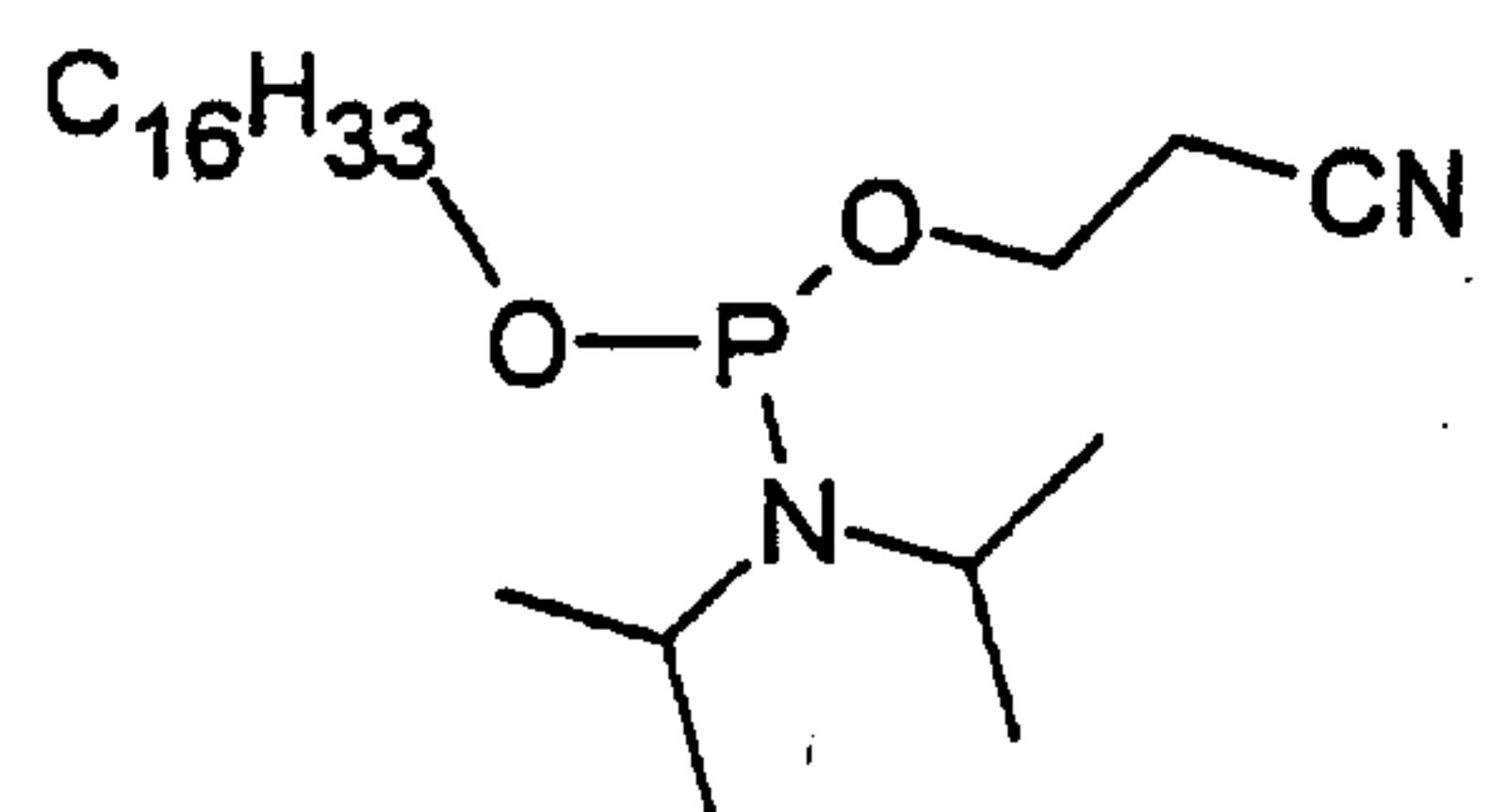


Biotin phosphoramidite 5 (monofunctional)

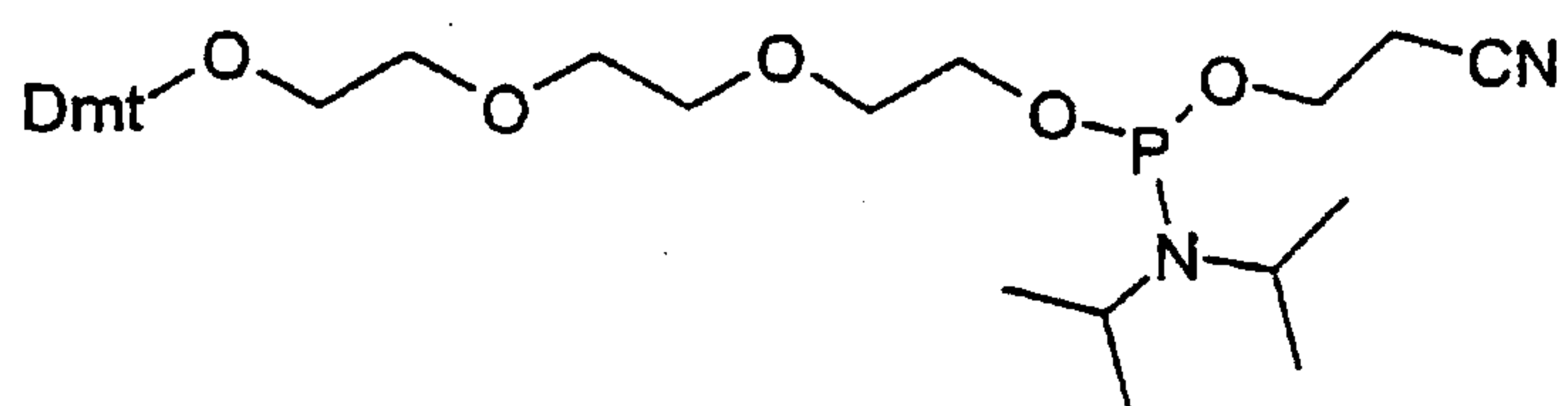


Biotin phosphoramidite 6 (bifunctional)

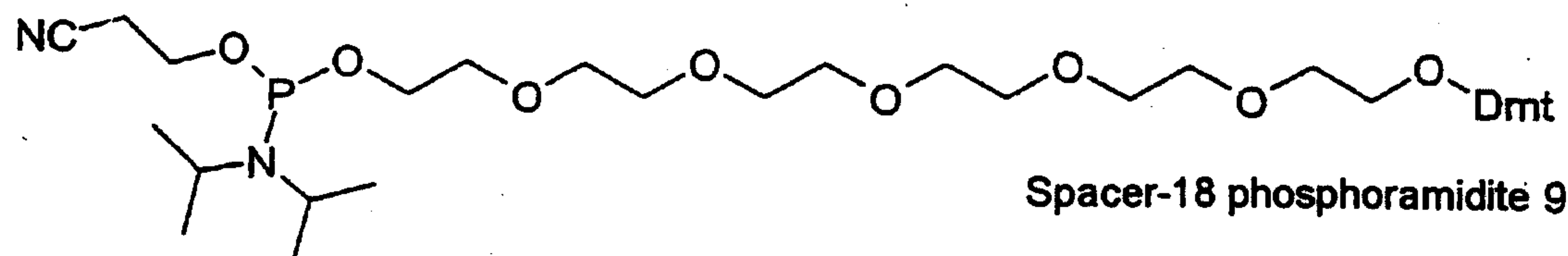
Figure 4c:



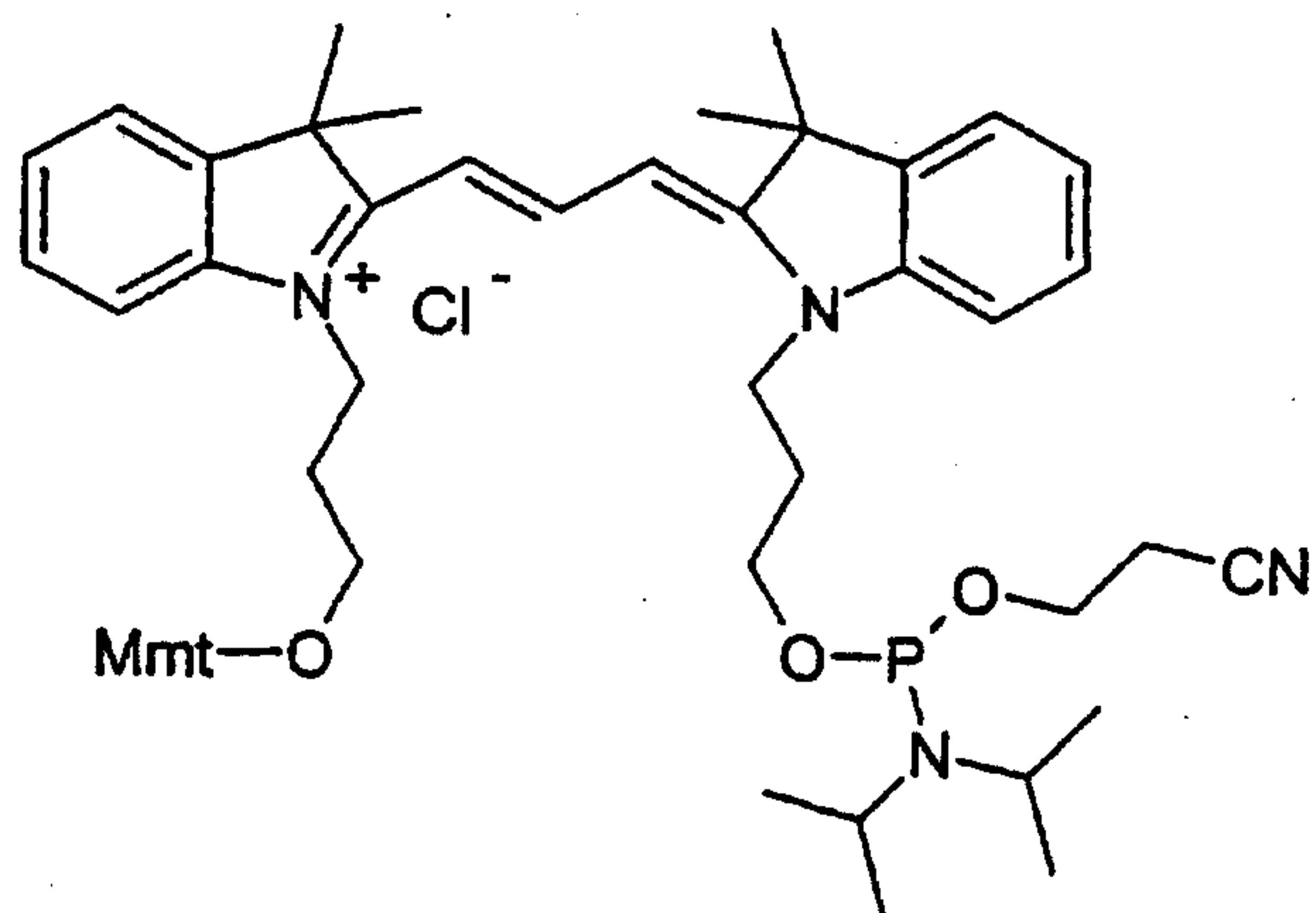
C16-phosphorylating reagent 7



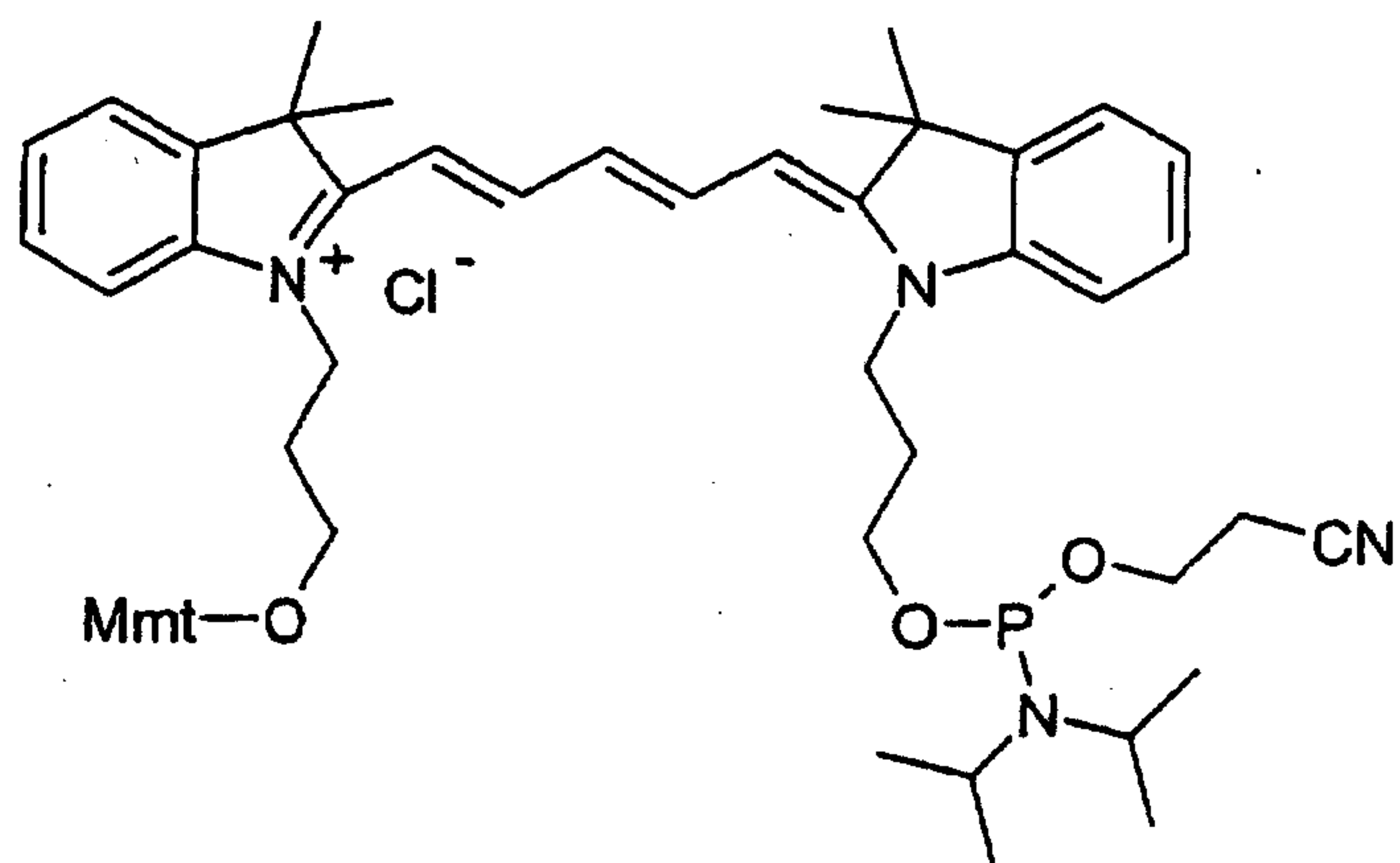
Spacer-9 phosphoramidite 8



Spacer-18 phosphoramidite 9



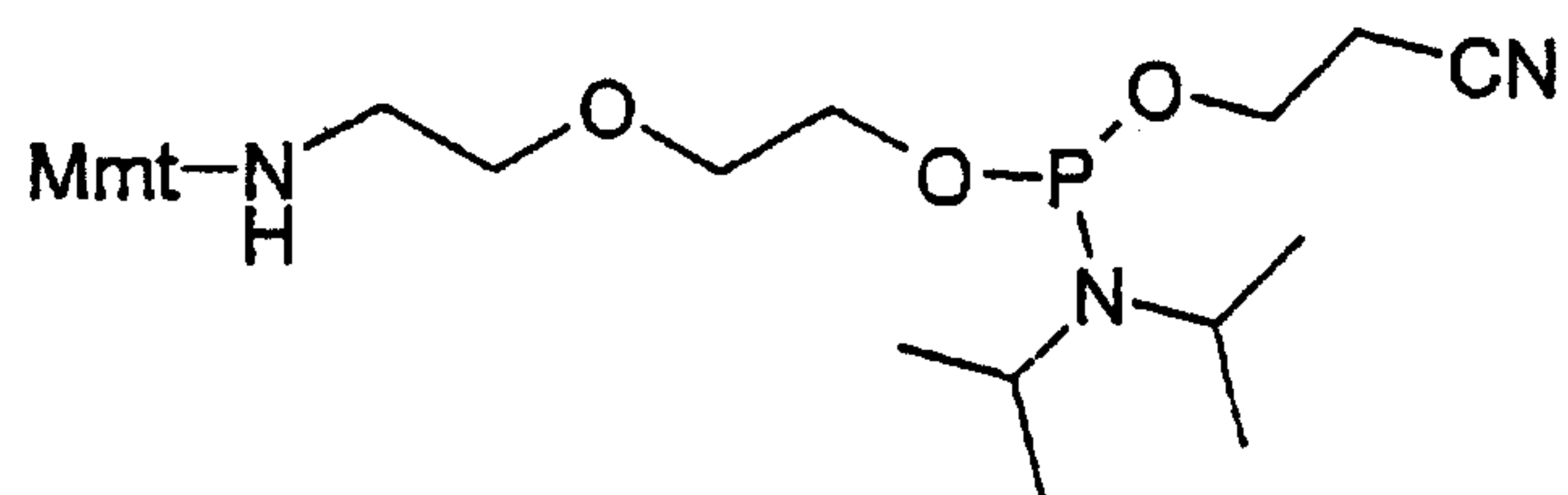
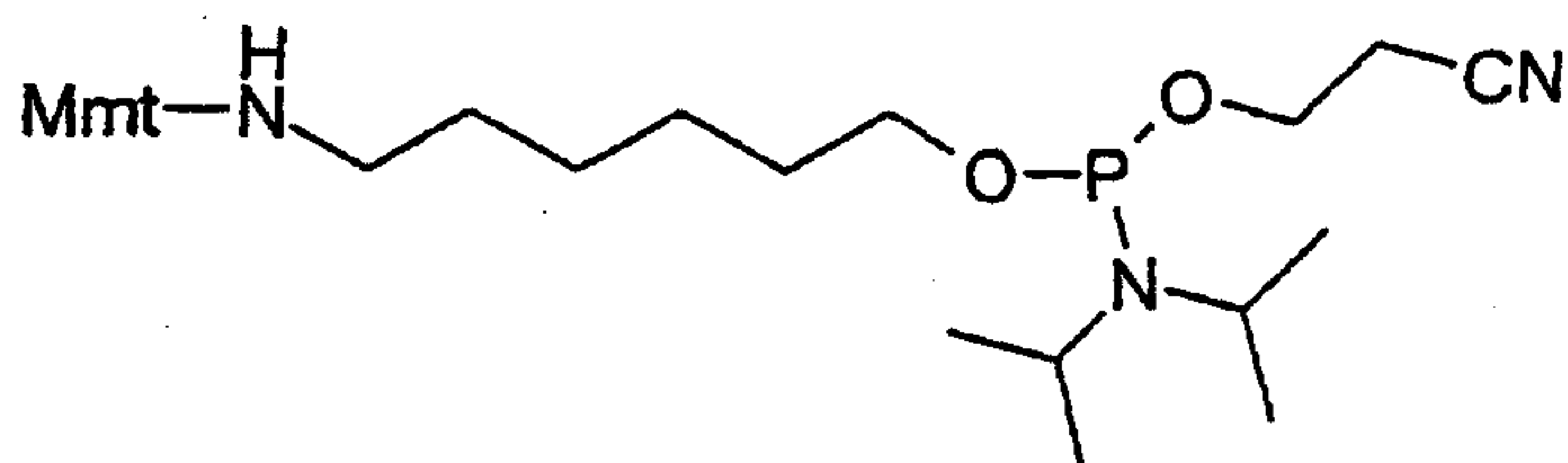
Cyanine-3 phosphoramidite 10



Cyanine-5 phosphoramidite 11

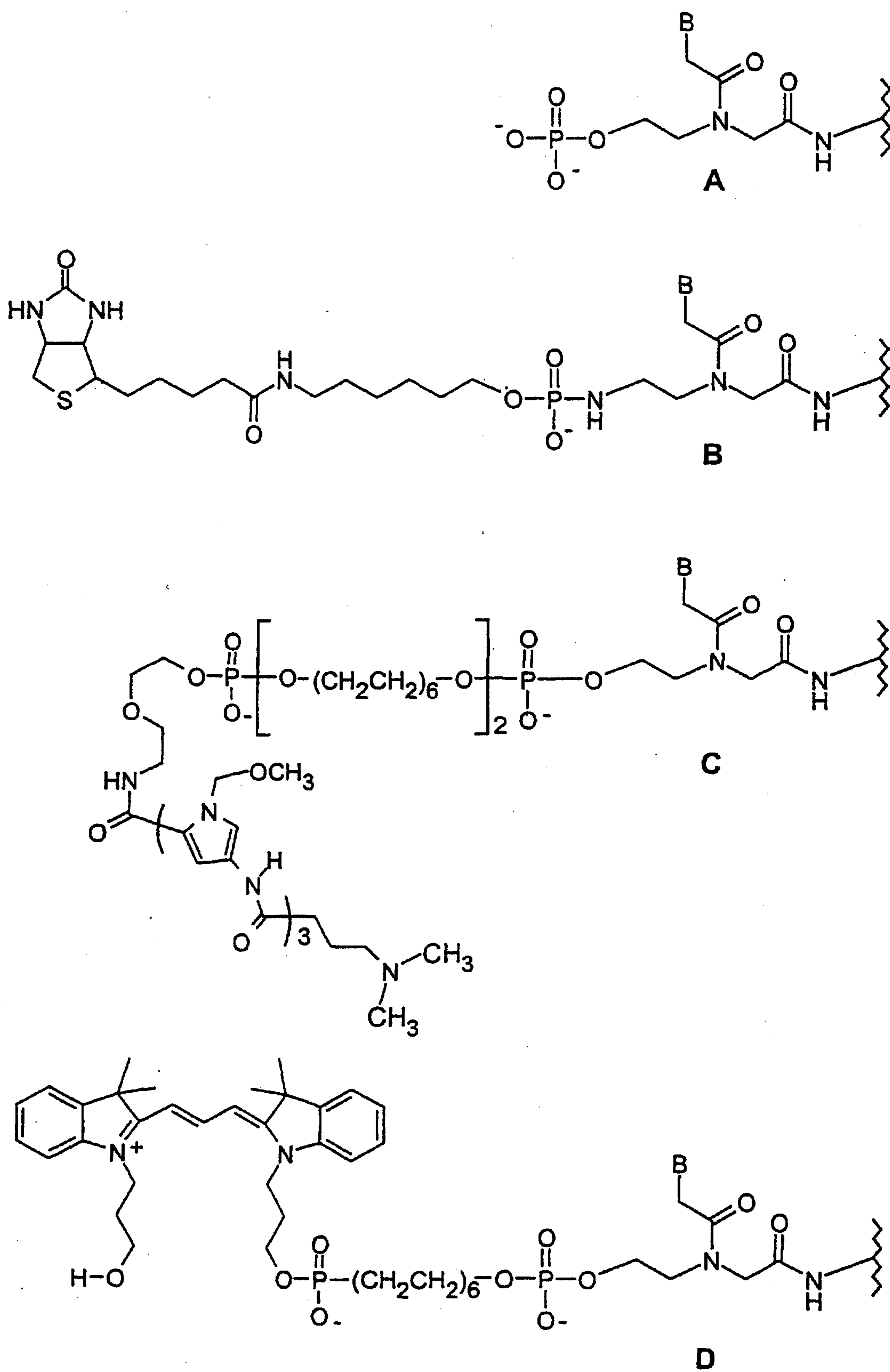
12/14

Fig. 4d:

Aminomodifier-5
phosphoramidite 12Aminomodifier-C6
phosphoramidite 13

13/14

Fig. 5a:



14/14

Fig. 5b:

