PNA Diagnostic Use

Buchardt, Ole; Engholm, Michael; Berg, Rolf Henrik; Nielsen, Peter E.

Publication date: 2012

PNA DIAGNOSTIC USE

Inventors: Ole Buchardt, Hornbaek (DK); Michael Egholm, Madison, CT (US); Peter E. Nielsen, Frederiksberg (DK); Rolf H. Berg, Rungsted Kyst (DK)

Assignee: PNA Patent Owners (a/k/a CIG), Carlsbad, CA (US)

Appl. No.: 13/190,164

Filed: Jul. 25, 2011

Related U.S. Application Data

Continuation of application No. 11/029,005, filed on Jan. 5, 2005, now abandoned, which is a continuation of application No. 09/983,210, filed on Oct. 23, 2001, now abandoned, which is a continuation of application No. 08/150,156, filed on May 4, 1994, now Pat. No. 6,357,163, filed as application No. PCT/EP92/01220 on May 22, 1992.

Publication Classification

Int. Cl.
C12Q 1/68 (2006.01)
C07K 17/02 (2006.01)
C12N 9/96 (2006.01)
C07K 2/00 (2006.01)

U.S. Cl. .................... 435/6.11; 530/322; 530/391.1; 435/188

ABSTRACT

The present invention pertains to certain nucleic acid analogs and related kits that are useful for the capture, recognition, detection, identification, or quantification of certain chemical or biological entities.
FIG. 1(B)
FIG. 3 (A) 5'-GATCCATTTTTTTTTAGATC 3'-CTAGTTTTTTTTTTCCCTAG

FIG. 3 (B) 5'-GATCCATTTTTTTTTAGATC 3'-CTAGTTTTTTTTTTCCCTAG

FIG. 3 (C) 5'-GATCCATTTTTTTTTAGATC 3'-CTAGTTTTTTTTTTCCCTAG

FIG. 3 (D) 5'-GATCCATTTTTTTTTAGATC 3'-CTAGTTTTTTTTTTCCCTAG
FIG. 4
FIG. 7

1) SOCl₂/DMF (cat.) REFLUX
2) +H₂N(CH₂)₅CO₂CH₃/EI₃N/CH₂Cl₂ AT 0°C
3) PhOH/4-NO₂Ph-CONH(CH₂)₆NH₂ AT 120°C
4) DMF/aq. NaOH
5) DMF/CH₂Cl₂/PipOH/DCC
FIG. 8

ANCHORING

DEPROTECTION

COUPLING

REPEITIVE CYCLE

FINAL DEPROTECTION & CLEAVAGE

H₂NCO-□-NH{CO-□-NH}₁ CO-□-NH₂
FIG. 10
FIG. 11a

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>1</th>
<th>1</th>
<th>1</th>
<th>1</th>
<th>1</th>
<th>2</th>
<th>2</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{32}$P-oligo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oligo 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PNA-1</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PNA/DNA-complex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ssDNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dsDNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ssDNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIG. 11b

\begin{align*}
32P-\text{oligo} & : 1 \quad 1 \quad 1 \quad 1 \quad 1 \quad 1 \quad 2 \quad 2 \quad 2 \\
\text{oligo 2} & : - \quad - \quad - \quad + \quad + \quad + \quad - \quad - \quad - \\
\text{PNA-1} & : - \quad + \quad ++ \quad - \quad + \quad ++ \quad - \quad + \quad ++ \\
\end{align*}

\begin{tabular}{cccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9
\end{tabular}

PNA/DNA-complex

ssDNA
FIG. 12a

PNA-1
- + + - - +

<table>
<thead>
<tr>
<th>300nm</th>
<th>DHA</th>
<th>A/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$A_{10}$

[Image with gel electrophoresis results]
### FIG. 12c

<table>
<thead>
<tr>
<th>S₁-nuclease</th>
<th>5</th>
<th>50</th>
<th>500</th>
<th>5</th>
<th>50</th>
<th>500</th>
<th>A/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

![Image of gel electrophoresis](image-url)
FIG. 13
FIG. 14

\[
\begin{align*}
\text{aq. NaOH/MeOH} & \\
\text{+} & \\
\text{NH}_2 & \\
\end{align*}
\]
FIG. 15
FIG. 16
\[
\text{H}_2\text{N}-\text{NH}_2 + \begin{array}{c}
\text{O} \\
\text{\text{Boc}}
\end{array}\text{O} - \text{O} - \text{O} \\
\text{Boc-\text{NH-\text{NH}_2}} \\
\overset{\text{CH}_3\text{CN, REFLUX}}{\text{O}} \\
\text{Boc-\text{NH-\text{NCH}_3\text{CO}_2}} \\
\overset{\text{DMF/E}_3\text{N}}{\text{O}} \\
\text{Boc-\text{NH-\text{NCH}_3\text{CO}_2}} \\
\overset{\text{NaOH}}{\text{O}} \\
\text{Boc-\text{NH-\text{NCH}_3\text{CO}_2}}
\]

\text{FIG. 19}
FIG. 20

<table>
<thead>
<tr>
<th>Target PNA</th>
<th>$A_{10}$</th>
<th>$A_5GA_4$</th>
<th>$A_5GA_2GA_4$</th>
<th>all 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

PNA 1: $T_{10}$  PNA 2: $T_5CT_4$  PNA 3: $T_2CT_2CT_4$
FIG. 23

Inhibition of Restriction Enzyme Cleavage by PNA

PNA/DNA  0  0.006  0.02  0.06  0.2  0.6

PNA Target

5'---------GGATCCAAAAA---------AGGATCC---------
3'---------CCTAGGT------------TCTAGG---------

BamH1           BamH1
FIG. 24

Binding of $^{125}$I-Tyr-PNA-T$_{10}$ to dA$_{10}$

CT-DNA/oligo  0  0.3  1  3  10  30  100  300

Origin

Hybrid
**FIG. 25a**

PNA-T10 Sepharose

% Binding

Temperature

**FIG. 25b**

PNA-T9C Sepharose

% Binding

Temperature

**FIG. 25c**

PNA-T8C2 Sepharose

% Binding

Temperature
FIG. 26

PNA
- - - + + - - - + +
P X B P X P X B P X
1 2 3 4 5 6 7 8 9 10

F3-promoter

T10

Bam H1  Xba I  Pvu II
PNA DIAGNOSTIC USE

[0001] The present invention relates to the use of certain nucleic acid analogues in the field of diagnostics, for instance in the capture, recognition, detection, identification or quantification of one or more chemical or microbiological entities.

[0002] Oligo-deoxyribonucleotides (oligo-DNA) are finding increasing use in diagnostics procedures. They are for instance finding use in testing for the presence of specific micro-organisms or for testing for the presence of generic predispositions, for instance to disease, in forensic science and in microbiology generally. The uses of oligo-DNA's in this field are of course dependent upon the ability of such oligo-DNA's to hybridise to complementary nucleic acid sequences. By way of example, labelled oligo-DNA probes are used in hybridisation assays to probe immobilised target DNA's for the presence of specific sequences. In amplification procedures, the hybridisation property of oligo-DNA's is utilised to hybridise oligo-DNA primers to template molecules to be amplified.

[0003] Oligo-DNA's as long as 100 base pairs in length are now routinely synthesised using a solid support method and fully automatic synthesis machines are commercially available. Attention has nonetheless been given to the possibility of constructing synthetic DNA-analogues capable of hybridising to natural DNA in a sequence specific manner and yet having chemical properties advantageously distinct from DNA itself. This work has been largely motivated by the possible use of such compounds in "anti-sense" therapeutics where the use of conventional oligo-DNA's encounters difficulties because such unmodified oligonucleotides have a short half life in vivo due to the natural presence of nucleases, are difficult and costly to prepare in any quantity and are poor at penetrating cell membranes.

[0004] For instance, International (PCT) Patent Application WO86/05518 discloses DNA analogues having a backbone bearing a sequence of ligands, typically nucleotide bases, supposedly capable of sequence specific hybridisation to naturally occurring nucleic acids. A number of different backbone structures are disclosed. No specific exemplification of the provision of such compounds is given and there are no data showing the affinity of the claimed analogues for DNA.

[0005] International (PCT) Patent Application WO86/05519 claims diagnostic reagents and systems comprising DNA analogues of the same kind but once again, there is no exemplification.

[0006] International (PCT) Patent Application WO89/12060 describes oligonucleotide analogues based on various building blocks from which they are synthesised. Whilst there is exemplification of the building blocks, there is no example of actually preparing an oligonucleotide analogue from them and hence no indication of the performance of the analogues.

[0007] Furthermore, it is known to modify the DNA backbone with the aim of increasing resistance to nuclease and generally improving the suitability of the DNA for use in anti-sense therapeutic methods. Other attempts to design DNA analogues are discussed in the introductory portion of WO86/05518 mentioned above.

[0008] The universal experience has been that modifications of the backbone of natural DNA lead to a decrease in the stability of the hybrid formed between the modified oligonucleotide and its complementary normal oligonucleotide, assayed by measuring the $T_m$ value. Consequently, the conventional wisdom in this area is that modifications of the backbone always destabilise the hybrid, i.e. result in lower $T_m$ values, and therefore the modification should be as minor as possible in order to obtain hybrids with only a slight decrease in $T_m$ value as the best obtainable result.

[0009] The present invention relates to the use in diagnostics or in analysis of nucleic acid analogues of novel structure, preferably having the previously unknown property of forming hybrids with complementary sequence conventional nucleic acids which are more stable in terms of $T_m$ value than would be a similar hybrid formed by a conventional nucleic acid of corresponding sequence and/or exhibiting greater selectively for the complementary sequence compared to sequences involving a degrees of mis-match than would be exhibited by said corresponding conventional nucleic acid of corresponding sequence.

[0010] The invention provides a nucleic acid analogue for use in the capture, recognition, detection, identification or quantitation of one or more chemical or microbiological entities, which analogue is

(a) a peptide nucleic acid (PNA) comprising a polyamidyl backbone bearing a plurality of ligands at respective spaced locations along said backbone, said ligands being each independently naturally occurring nucleobases, non-naturally occurring nucleobases or nucleobase-binding groups, each said ligand being bound directly or indirectly to a nitrogen atom in said backbone, and said ligand bearing nitrogen atoms mainly being separated from one another in said backbone by from 4 to 8 intervening atoms.

(b) a nucleic acid analogue capable of hybridising to a nucleic acid of complementary sequence to form a hybrid which is more stable against denaturation by heat than a hybrid between the conventional deoxyribonucleotide corresponding to said analogue and said nucleic acid; or

(c) a nucleic acid analogue capable of hybridising to a double stranded nucleic acid in which one strand has a sequence complementary to said analogue, so as to displace the other strand from said one strand.

[0011] The separation of the nitrogen bearing atoms in the backbone of nucleic acid analogues defined in paragraph (a) above (PNA's) is preferably by five atoms. In nucleic acid analogues having the Formula I (below) this has been found to provide the strongest affinity to the modified DNA.

[0012] Preferably not more than 25% of interligand spacings will be 6 atoms or more. More preferably not more than 10 to 15% of interligand spacings will be 6 atoms or more. The azr nitrogen atoms which carry the ligands (directly or via linker groups are not themselves counted in the spacings referred to above.

[0013] An alternative or additional method for reducing the strength of DNA binding is to omit certain of the ligands, putting in their place a moiety which contributes little or nothing to the binding of DNA, e.g. hydrogen. Preferably, not more than 25% of the ligand positions will be occupied by non-binding moieties, e.g. not more than 10 to 15%.

[0014] Representative ligands include either the four main naturally occurring DNA bases (i.e., thymine, cytosine, adenine or guanine) or other naturally occurring nucleobases (e.g., inosine, uracil, 5-methylcytosine or thiomuracil) or arti-
ficial bases (e.g., bromouracil, azaadenines or azaguanines, etc.) attached to a peptide backbone through a suitable linker.

[0015] In preferred embodiments, the nucleic acid analogues used in the invention have the general formula (I):

\[
\begin{align*}
\text{Q} & \text{C}^{1} \text{C}^{1} \text{C}^{1} \text{C}^{1} \text{C}^{1} \text{C}^{1} \text{C}^{1} \\
\text{A}^{1} & \text{A}^{1} \text{A}^{1} \text{A}^{1} \text{A}^{1} \text{A}^{1} \text{A}^{1} \text{A}^{1} \\
\text{B}^{1} & \text{B}^{1} \text{B}^{1} \text{B}^{1} \text{B}^{1} \text{B}^{1} \text{B}^{1} \text{B}^{1} \\
\text{R}^{1} & \text{R}^{1} \text{R}^{1} \text{R}^{1} \text{R}^{1} \text{R}^{1} \text{R}^{1} \text{R}^{1} \\
\text{G}^{1} & \text{G}^{1} \text{G}^{1} \text{G}^{1} \text{G}^{1} \text{G}^{1} \text{G}^{1} \text{G}^{1} \\
\text{I} & \text{I} \text{I} \text{I} \text{I} \text{I} \text{I} \text{I} \\
\end{align*}
\]

wherein:

[0016] \( n \) is at least 2,

[0017] each of \( \text{L}^{1} \text{L}^{*} \) is independently selected from the group consisting of hydrogen, hydroxy, \((\text{C}_{1}-\text{C}_{3})\text{alkenyl, naturally occurring nucleobases, non-naturally occurring nucleobases,}

\text{aromatic moieties, DNA intercalators, nucleobase binding groups and reporter ligands, at least one of \( \text{L}^{1} \text{L}^{*} \) being a naturally occurring nucleobase, a non-naturally occurring nucleobase, a DNA intercalator, or a nucleobase binding group;}

[0018] each of \( \text{A}^{1} \text{A}^{*} \) is a single bond, a methylene group or a group of formula (IIa) or (IIb):

\[
\begin{align*}
\text{R}^{1} & \text{R}^{1} \\
\text{C} & \text{C} \\
\text{Y} & \text{Y} \\
\text{R}^{2} & \text{R}^{2} \\
\end{align*}
\]

or

\[
\begin{align*}
\text{R}^{1} & \text{R}^{1} \\
\text{C} & \text{C} \\
\text{X} & \text{X} \\
\text{R}^{2} & \text{R}^{2} \\
\end{align*}
\]

where:

[0019] \( X \) is O, S, Se, NR\(^3\), \( \text{CH}_2 \), or \( \text{C(CH}_3)_2 \);

[0020] \( Y \) is a single bond, O, S or NR\(^4\);

[0021] each of \( p \) and \( q \) is an integer from 1 to 5, the sum \( p+q \)

being not more than 10;

[0022] each of \( r \) and \( s \) is zero or an integer from 1 to 5, the

sum \( r+s \) being not more than 10;

[0023] each \( R^{2} \) and \( R^{2} \) is independently selected from the

group consisting of hydrogen, \((\text{C}_{1}-\text{C}_{3})\text{alkyl which may be hydroxy-}

\text{or alk oxy- or alkylthio-substituted, hydroxy, alk oxy, alkylthio, amino and halogen; and}

[0024] each \( R^{3} \) and \( R^{3} \) is independently selected from

the group consisting of hydrogen, \((\text{C}_{1}-\text{C}_{3})\text{alkyl, hydroxy-}

\text{or alk oxy- or alkylthio-substituted} \((\text{C}_{1}-\text{C}_{3})\text{alkyl, hydroxy, alk oxy, alkylthio and amino;}

[0025] each of \( B^{1} \text{B}^{*} \) is \( \text{N} \text{R}^{2} \text{N}^{*} \), where \( R^{2} \) is as defined above;

[0026] each of \( \text{C}^{1} \text{C}^{*} \) is \( \text{CR}^{2}\text{R}^{2}, \text{CHR}^{2}\text{CHR}^{2} \text{CHR}^{2}, \text{CH}_{2}\text{CHR}^{2} \text{CHR}^{2} \text{CHR}^{2}, \text{where \( R^{2} \) is hydrogen and \( R^{3} \) is selected from

the group consisting of the side chains of naturally occurring alpha amino acids, or \( R^{2} \) and \( R^{3} \) are independently selected from

the group consisting of hydrogen, \((\text{C}_{1}-\text{C}_{3})\text{alkyl, aryl, anil yl, heteroaryl, hydroxy,} \((\text{C}_{1}-\text{C}_{3})\text{alkylthio, NR}^{2}\text{R}^{2}\text{ and SRS, where \( R^{2} \) and \( R^{3} \) are as defined above, and \( R^{3} \) is hydrogen,}\((\text{C}_{1}-\text{C}_{3})\text{alkyl, hydroxy-}

\text{alkoxy- or alkylthio-substituted} \((\text{C}_{1}-\text{C}_{3})\text{alkyl, or \( R^{3} \) and \( R^{3} \) taken together complete an}

alicyclic or heterocyclic system;}

[0027] each of \( \text{D}^{1} \text{D}^{*} \) is \( \text{CR}^{2}\text{R}^{2}, \text{CHR}^{2}\text{CHR}^{2}, \text{CHR}^{2}\text{CHR}^{2}, \text{CH}_{2}\text{CHR}^{2} \text{CHR}^{2} \text{CHR}^{2}, \text{where \( R^{2} \) and \( R^{3} \) are as defined above;}

[0028] each of \( \text{G}^{1} \text{G}^{*} \) is

\[
\begin{align*}
\text{N} & \text{C} & \text{C} & \text{N} & \text{O} \\
\text{R}^{2} & & & \text{R}^{3} & or \\
\end{align*}
\]

in either orientation, where \( R^{3} \) is as defined above;

[0029] \( Q \) is \( \text{CO}_{2}\text{H, CONR}^{2}\text{R}^{2}, \text{SO}_{4}\text{H} \text{ or}

\text{SO}_{2}\text{NR}^{2}\text{R}^{2}\text{ or an activated derivative of} \text{CO}_{2}\text{H} \text{ or}

\text{SO}_{4}\text{H; H; and}

[0030] \( I \) is \( \text{NH}^{+}\text{R}^{3}\text{ or} \text{NR}^{2}\text{C(O)R}^{3}\text{, where \( R^{2}, R^{3} \) and \( R^{3} \) are independently selected from

the group consisting of hydrogen, alkyl, amino protecting groups, reporter ligands, intercalators, chelators, peptides, proteins, carbohydrates, lipids, steroids, oligonucleotides and soluble and non-soluble polymers. Alternatively, \( C \) and \( D \)

may be \( \text{CHR}^{2}\text{CHR}^{2}\text{CHR}^{2} \text{CHR}^{2} \text{CHR}^{2} \text{where \( S \) may be from 0 to 2.}

[0031] Preferred peptide nucleic acids have general formula (III):

\[
\begin{align*}
\text{R}^{4} & \text{O} & \text{O} & \text{O} \\
\text{L} & \text{R}^{2} & \text{R}^{2} & \text{R}^{2} \\
\text{O} & \text{O} & \text{O} \\
\text{R}^{4} & \text{R}^{4} & \text{R}^{4} & \text{R}^{4} \\
\text{NH} & \text{R}^{1} \\
\end{align*}
\]
wherein:

[0032] each L is independently selected from the group consisting of hydrogen, phenyl, heterocycles, e.g. of one, two or three rings, naturally occurring nucleobases, and non-naturally occurring nucleobases;

[0033] each R⁺ is independently selected from the group consisting of hydrogen and the side chains of naturally occurring alpha amino acids;

[0034] n is an integer from 1 to 60;

[0035] each of k, l and m is independently zero or an integer from 1 to 5; preferably the sum of k and m is 1 or 2, most preferably 1;

[0036] R² is OH, NH₂ or —NH-Lys-NH₂; and

[0037] R¹ is H or COCH₃.

Particularly preferred are compounds having formula (III) wherein each L is independently selected from the group consisting of the nucleobases thymine (T), adenine (A), cytosine (C), guanine (G) and uracil (U), in particular thymine, and n is an integer from 1 to 30, in particular from 4 to 20. An example of such a compound is provided in FIG. 1, which shows the structural similarity between such compounds and single-stranded DNA.

[0038] The peptide nucleic acids of the invention may be synthesized by adaptation of standard peptide synthesis procedures, either in solution or on a solid phase. The synths used may be specially designed monomer amino acids or their activated derivatives, protected by standard protecting groups. The oligonucleotide analogues also can be synthesized by using the corresponding diacids and diamines.

[0039] Thus, the monomer synths used to produce compounds for use on the invention may be selected from the group consisting of amino acids, diacids and diamines having general formulae:

[0040] Preferred monomer synths are amino acids having formula (VII):

![Diagram](image)

or amino-protected and/or acid terminal activated derivatives thereof, wherein L is selected from the group consisting of hydrogen, phenyl, heterocycles, naturally occurring nucleobases, non-naturally occurring nucleobases, and protected derivatives thereof; and R² is independently selected from the group consisting of hydrogen and the side chains of naturally occurring alpha amino acids. Especially preferred are such synths having formula (IV) wherein R² is hydrogen and L is selected from the group consisting of the nucleobases thymine (T), adenine (A), cytosine (C), guanine (G) and uracil (U) and protected derivatives thereof.

[0041] In accordance with the invention there is included the use of nucleic acid analogues as hereinbefore defined in the capture, recognition, detection, identification or quantitation of one or more chemical or micro-biological entities. Usually it is envisaged the entity which is detected in the first instance will be a nucleic acid and said entity will be detected via its characteristic sequences of nucleic acid bases by hybridisation.

[0042] Nucleic acid analogues as hereinbefore defined may be used in a method of capturing a nucleic acid comprising contacting under hybridising conditions said nucleic acid with a nucleic acid analogue for use in the invention immobilised to a solid support, which immobilised nucleic acid analogue has a sequence of ligands suitable to hybridise to said nucleic acid or nucleic acid analogue to be captured.

[0043] The solid support may take a wide variety of forms as is known in connection with the immobilisation of conventional oligo-nucleotides for use in affinity capture. A solid support may for instance be a plate, a filter, a multi-well plate or a dip stick. It may take the form of individual particles such as beads and such particles may be held in a column through which nucleic acid containing solutions may be run to allow the capture of desired species therefrom.

[0044] The captured nucleic acid may be recognised, detected, identified or quantitated by a wide variety of methods. Since after washing the captured nucleic acid may be the only nucleic acid remaining in the system, it may be detected by any reagent system suitable for demonstrating the presence of nucleic acid, whether or not specific for the captured sequence. Thus by way of example if the captured nucleic acid is DNA and is captured in single stranded form by a relatively short PNA, overhanging single stranded DNA may be digested by nuclease and the digestion products may be detected by conventional means. If the DNA is double stranded and the PNA is once again relatively short, that part of the DNA which remains in its original double stranded form (i.e., which is not displaced by the PNA) can be detected by conventional DNA intercalators that do not bind to the PNA-DNA duplex. Antibodies which recognise nucleic acids may be used to detect nucleic acids (RNA, dsDNA or ssDNA) bound to the immobilised nucleic acid analogue.
In the affinity capture of nucleic acid species using conventional oligonucleotides immobilised to a solid support, it is necessary normally to purify the target nucleic acid. Nucleases which may be present in the sample are liable to attack the immobilised nucleic acid. Little specific binding is obtained in practice with much non-specific binding. Furthermore, it is necessary to denature the DNA to single stranded form before the capture can take place.

The nucleic acid analogues of Formula 1 above are not susceptible to attack by nucleases and typically provide higher levels of specific binding by virtue of their higher affinity for nucleic acid of complementary sequence than is obtained using conventional oligonucleotides as the immobilised species. Furthermore, the nucleic acid analogues used in accordance with the invention are typically capable of hybridising to nucleic acids of complementary sequence without those nucleic acids first being denatured into single stranded form. Once the target nucleic acid has been captured, it may be released from the immobilised nucleic acid analogue by subjecting the immobilised nucleic acid analogue and captured nucleic acid to dehybridising conditions such as heat and dimethyl formamide.

By way of example, the immobilised nucleic acid analogue may comprise sequential ligands such as thymine, hybridisable to poly A tails of mRNA to capture the mRNA.

The invention includes an affinity capture column comprising immobilised nucleic acid analogues as described above.

Thus it can be seen that the present invention also pertains to the advantageous use of PNA molecules in solid-phase biochemistry (see, e.g., “Solid-Phase Biochemistry—Analytical and Synthetic Aspects”, W. I. Scuoton, ed., John Wiley & Sons, New York, 1983), notably solid-phase biosystems, especially bioassays or solid-phase techniques which concern diagnostic detection/quantitation or affinity purification of complementary nucleic acids (see, e.g., “Affinity Chromatography—A Practical Approach”, P. D. G. Dean, W. S. Johnson and F. A. Middle, eds., IRL Press Ltd., Oxford 1986; “Nucleic Acid Hybridization—A Practical Approach”, B. D. Harries and S. J. Higgins, IRL Press Ltd., Oxford, 1987). Present day methods for performing such bioassays or purification techniques almost exclusively utilize “normal” or slightly modified oligonucleotides either physically adsorbed or bound through a substantially permanent covalent anchoring linkage to beaded solid supports such as cellulose, glass beads, including those with controlled porosity (Mizutani, et al., J. Chromatogr. 1986, 356, 202), “Sephadex”, “Sephadex”, agarose, polyacrylamide, porous particulate alumina, hydroxylalkyl methacrylate gels, diol-bonded silica, porous ceramics, or contigous materials such as filter discs of nylon and nitrocellulose. One example employed the chemical synthesis of oligo-DT on cellulose beads for the affinity isolation of poly A tail containing mRNA (Gilham in “Methods in Enzymology”; L. Grossmann and K. Moldave, eds., vol. 21, part D, page 191, Academic Press, New York and London, 1971). All the above-mentioned methods are applicable within the context of the present invention. However, when possible, covalent linkage is preferred over the physical adsorption of the molecules in question, since the latter approach has the disadvantage that some of the immobilized molecules can be washed out (desorbed) during the hybridization or affinity process. There is, thus, little control of the extent to which a species adsorbed on the surface of the support material is lost during the various treatments to which the support is subjected in the course of the bioassay/purification procedure. The severity of this problem will, of course, depend to a large extent on the rate at which equilibrium between adsorbed and “free” species is established. In certain cases it may be virtually impossible to perform a quantitative assay with acceptable accuracy and/or reproducibility. Loss of adsorbed species during treatment of the support with body fluids, aqueous reagents or washing media will, in general, be expected to be most pronounced for species of relatively low molecular weight. In contrast with oligonucleotides, PNA molecules are easier to attach onto solid supports because they contain strong nucleophilic and/or electrophilic centers. In addition, the direct assembly of oligonucleotides onto solid supports suffers from an extremely low loading of the immobilized molecule, mainly due to the low surface capacity of the materials that allow the successful use of the state-of-the-art phosphoramidite chemistry for the construction of oligonucleotides. (Beaucage and Caruthers, Tetrahedron Lett., 1981, 22, 1859; Caruthers, Science, 1985, 232, 281). It also suffers from the fact that by using the alternative phosphite triester method (Letinger and Mahadevan, J. Am. Chem. Soc. 1976, 98, 356), which is suited for solid supports with a high surface/loading capacity, only relatively short oligonucleotides can be obtained. As for conventional solid-phase peptidyl synthesis, however, the latter supports are excellent materials for building up immobilized PNA molecules (the side-chain protecting groups may be removed from the synthesized PNA chain without cleaving the anchoring linkage holding the chain to the solid support). Thus, PNA species benefit from the above-described solid-phase techniques with respect to the much higher (and still sequence-specific) binding affinity for complementary nucleic acids and from the additional unique sequence-specific recognition of (and strong binding to) nucleic acids present in double-stranded structures. They also can be loaded onto solid supports in large amounts, thus further increasing the sensitivity/capacity of the solid-phase technique. Further, certain types of studies concerning the use of PNA in solid-phase biochemistry can be approached, facilitated, or greatly accelerated by use of the recently-reported “light-directed, spatially addressable, parallel chemical synthesis” technology (Fodor, et al., Science, 1991, 251, 767), a technique that combines solid-phase chemistry and photolithography to produce thousands of highly diverse, but identifiable, permanently immobilized compounds (such as peptides) in a substantially simultaneous way.

It has been found that PNA's according to Formula 1 exhibit a property never before observed which is that such a nucleic acid analogue is capable of hybridising to a conventional nucleic acid presented in double-stranded form and is capable under such conditions of hybridising to the strand which has a sequence complementary to the analogue and of displacing the other strand from the initial nucleic acid duplex. Such recognition can take place to dsDNA sequences 5-60 base pairs long. Sequences between 10 and 20 bases are of interest since this is the range within which unique DNA sequences of prokaryotes and eukaryotes are found. Reagents which recognize 17-18 bases are particular interest since this is the length of unique sequences in the human genome.

It has also been observed that when such a hybridisation reaction is conducted in solution, a second strand of the nucleic acid analogue having the same sequence as the first also hybridises to the nucleic acid strand of complementary sequence so as form a triple helix structure in which two
similar strands of PNA are hybridised to a single strand of conventional nucleic acid. It is believed that the first PNA strand hybridises by inter-base hydrogen bonding of the usual kind whilst the second strand of PNA is received in the major groove of the initial duplex by Hoogsteen pairing. Where the PNA is immobilised to a solid support, hybridisation to double stranded nucleic acid with displacement of one strand therefrom is observed but the formation of triple helix structures may be prevented by the immobilisation of the PNA.

[0052] The invention includes a nucleic acid analogue as defined above, incorporating or conjugated to a detectable label. Generally, all of these methods for labelling peptides, DNA and/or RNA which are presently known may in general terms be applied to PNA's also. Thus, methods of labelling will include the use of radio-isotope labels, enzyme labels, biotin, spin labels, fluorophores, chemiluminescence labels, antigen labels or antibody labels.

[0053] Labelled PNA's as described above may be used in methods of recognition, detection or quantitation of target nucleic acids comprising hybridising said target to a labelled nucleic acid analogue as defined above of sufficiently complementary sequence to hybridise therewith under hybridising conditions and detecting or quantitating said label of the nucleic acid analogue so hybridised to said target.

[0054] Optionally, the target may be immobilised on a substrate prior to the hybridisation.

[0055] In such a method, the target may be immobilised to the substrate by the hybridisation of the first region of the target to a capture nucleic acid or nucleic acid analogue having a sequence sufficiently complementary to said first region to hybridise therewith and which is itself immobilised to said substrate and the labelled nucleic acid analogue may be hybridised to a second region of the target.

[0056] The ability of at least preferred nucleic acid analogues according to the invention to hybridise to a double-stranded target nucleic acid and to displace one strand therefrom has been described above. The invention includes a method for displacing one strand from a nucleic acid duplex comprising hybridising to said duplex a nucleic acid analogue as defined above having an affinity for the other strand of said duplex sufficient to be able to displace said one strand therefrom.

[0057] The invention includes a method of detecting, identifying or quantitating a double-stranded target nucleic acid comprising hybridising thereto a displacing nucleic acid analogue as defined above capable of displacing one strand from a double-stranded target in which the other strand is of complementary sequence to said displacing nucleic acid analogue, wherein said displacing nucleic acid analogue is of sufficiently complementary sequence to said other strand of said double-stranded target to hybridise thereto so as to displace said one strand of said target in single stranded form, and detecting or quantitating the presence of said one strand after displacement from said double-stranded target.

[0058] The displaced strand may be broken down into fragments and the presence of said fragments may be detected. The displaced strand may preferably be broken down by attack by a nuclease. Thus, one may detect the present of a specific double-stranded target nucleic acid sequence by hybridising thereto a complementary PNA to produce strand displacement so as to produce single-stranded DNA in the reaction mixture and digest the single-stranded DNA by the use of a nuclease to produce nucleotides whose presence can be detected as an indicator that specifically the target double-stranded DNA was present initially.

[0059] The invention further includes kits for use in diagnostics incorporating at least one nucleic acid analogue as defined above and preferably comprising at least one such nucleic acid analogue which is labelled, e.g. a labelled PNA, and at least one detection reagent for use in detecting said label.

[0060] Generally, the nucleic acid analogues will be provided in solution in a hybridisation buffer. Such a kit will generally also include at least one wash buffer solution.

[0061] Where the nucleic acid analogue is indirectly labelled, e.g. by biotin, the kit may include a conjugate between an enzyme label and a material such as avidin able to bind to the label of the nucleic acid analogue.

[0062] Where the nucleic acid analogue is either directly or indirectly enzyme labelled, the kit may comprise a substrate for the enzyme which is suitable to undergo a monitorable reaction mediated by the enzyme.

[0063] The invention will be described in further detail and will be illustrated by reference to the accompanying figures, in which:

[0064] FIGS. 1(A) and 1(B) show a naturally occurring deoxyribooligonucleotide and a peptidic nucleic acid (PNA) of the invention, respectively.

[0065] FIGS. 2(A) and 2(B) provide examples of naturally occurring and non-naturally occurring nucleobases for DNA recognition and reporter groups.

[0066] FIGS. 3(A), 3(B), 3(C), and 3(D) provide a schematic illustration of photo cleavage by Acr1(-Taeg)10-Lys-NH2 (AcT10 Lys-NH2); photofragmentation of the diazo-linked acridine of Acr1(-Taeg)10-Lys-NH2 and preferred KMnO4 cleavage; and S1-nuclease enhanced cleavage and micrococcal nuclease cleavage of Acr1(-Taeg)10-Lys-NH2 binding site, respectively.

[0067] FIG. 4 provides examples of PNA monomer synths.

[0068] FIG. 5 shows the Acr1 ligand and a PNA, Acr1(-Taeg)10-Lys-NH2.

[0069] FIG. 6 provides a general scheme for the preparation of monomer synths.

[0070] FIG. 7 provides a general scheme for the preparation of the Acr1 ligand.

[0071] FIG. 8 provides a general scheme for solid-phase PNA synthesis illustrating the preparation of linear unprotected PNA amides.

[0072] FIG. 9 shows analytical HPLC chromatograms of: (A) crude H{-Taeg}15-NH2 after HF-cleavage (before lyophilization); (B) crude Acr1{-Taeg}15-NH2 after HF-cleavage (before lyophilization); and (C) purified Acr1{-Taeg}15-NH2. Buffer A, 5% CH3CN/95% H2O, 0.0445% TFA; buffer B, 60% CH3CN/40% H2O, 0.0390% TFA; linear gradient, 0-100% of B in 30 min; flow rate, 1.2 ml/min; column, Vydac C18 (5μm, 0.46×25 cm).

[0073] FIG. 10 shows analytical HPLC chromatograms of: (A) purified H{-Taeg}15-Lys-NH2 and (B) purified H{-Taeg}5-Caeg{-Taeg}15-Lys-NH2 employing the same conditions as in FIG. 9.

[0074] FIGS. 11a and 11b show binding of AcrT10-Lys to dA10, 5′-32P-labelled oligonucleotide (1) (5′-GATCCATG) was incubated in the absence or presence of AcrT10-Lys and in the absence or presence of oligonucleotide (2) (5′-GATCCATG) and the samples were analyzed by polyacry-
lumide gel electrophoresis (PAGE) and autoradiography under “native conditions” (FIG. 11a) or under “denaturating conditions” (FIG. 11b).

[0075] FIGS. 12a-c show chemical, photochemical and enzymatic probing of dsDNA-AcrT10-Lys-NH2 complex. Complexes between AcrT10-Lys-NH2 and a 32P-end labelled DNA fragment containing a dA10/dT10 target sequence were probed by affinity photo-cleavage (FIG. 12a, lanes 1-3; FIG. 12b, lanes 1-3), photofootprinting (FIG. 12a, lanes 5-6), potassium permanganate probing (FIG. 12b, lanes 4-6) or probing by *staphylococcus* nuclease (FIG. 12c, lanes 8-10) or by nuclease S1 (FIG. 12d). Either the A-strand (FIG. 12a) or the T-strand (FIGS. 12b,c) was probed.

[0076] FIG. 13 provides a procedure for the synthesis of protected PNA synthons.

[0077] FIG. 14 provides a procedure for the synthesis of a protected adenine monomer synthon.

[0078] FIG. 15 provides a procedure for the synthesis of a protected guanine monomer synthon.

[0079] FIG. 16 provides examples of PNA backbone alterations.

[0080] FIG. 17 provides a procedure for synthesis of thymine monomer synthons with side chains corresponding to the normal amino acids.

[0081] FIGS. 18a and 18b provide procedures for synthesis of an aminopropyl analogue and a propionyl analogue, respectively, of a thymine monomer synthon.

[0082] FIG. 19 provides a procedure for synthesis of an aminoethyl-β-alanine analogue of thymine monomer synthon.

[0083] FIG. 20 shows a PAGE autoradiograph demonstrating that PNA-T10, T-C and T-C bond to double stranded DNA with high sequence specificity.

[0084] FIG. 21 shows a graph based on densitometric scanning of PAGE autoradiographs demonstrating the kinetics of the binding of PNA-T10 to a double stranded target.

[0085] FIG. 22 shows a graph based on densitometric scanning of PAGE autoradiographs demonstrating the thermal stabilities of PNAs of varying lengths bound to an A10/T10 double stranded DNA target.

[0086] FIG. 23 show an electrophoretic gel staining demonstrating that restriction enzyme activity towards DNA is inhibited when PNA is bound proximal to the restriction enzyme recognition site.

[0087] FIG. 24 shows a PAGE autoradiograph demonstrating that 32P-labeled PNA-T10 binds to a complementary dA10 oligonucleotide.

[0088] FIGS. 25a to c show the percentage binding achieved at varying temperatures between an immobilised PNA and matching or one base mismatched oligo-DNAs.

[0089] FIG. 26 is an autoradiograph showing inhibition of transcription by RNA polymerase by T10 PNA on the transcribed strand.

[0090] FIG. 27 is an autoradiograph of a mixture of labelled plasmid dsDNA's captured on an immobilised complementary to one of them and washed at varying temperatures.

[0091] FIG. 28 combines an ethidium bromide gel and an autoradiograph thereof illustrating the quantitative determination of plasmid dsDNA by strand displacement by PNA.

[0092] In the PNAs of Formula I and monomer synthons used in their production, ligand L is primarily a naturally occurring nucleobase attached at the position found in nature, i.e., position 9 for adenine or guanine, and position 1 for thymine or cytosine. Alternatively, each of some of the ligands L may be a non-naturally occurring nucleobase (nucleobase analog), another base-binding moiety, an aromatic moiety, (C1-C6)alkanoyl, hydroxy or even hydrogen. Some typical nucleobase ligands and illustrative synthetic ligands are shown in FIG. 1. Furthermore, L can be a DNA intercalator, a reporter ligand such as, for example, a fluorophor, radio label, spin label, hapten, or a protein-recognizing ligand such as biotin.

[0093] In monomer synthons, L may be equipped with protecting groups. This is illustrated in FIG. 4, where P' is an acid, a base or a hydroxynicotinly or photochemically cleavable protecting group such as, for example, t-butoxy-carbonyl (Boc), fluorenylmethoxy carbonyl (Fmoc) or 2-nitrobenzyl (2NB).

[0094] Linker A can be a wide variety of groups such as

- CR1R2CO—,
- CR3R4CS—,
- CR2CNHR3—,
- CR3CNHR—,
- CR2C=CH2—,
- CR2C=C(CH3)2—,

where R1, R2 and R3 are as defined above. Preferably, A is a methylene carbonyl (—CH2CO—). Also, A can be a longer chain moiety such as propanoyl, butanoyl or pentanoyl, or a corresponding derivative, wherein O is replaced by another value of X or the chain is substituted with R2 or is heterogenous, containing Y. Further, A can be a (C1-C6)alkylene chain, a (C1-C6)alkylene chain substituted with R2 or can be heterogenous, containing Y. In certain cases, A can just be a single bond.

[0095] In the preferred form of the invention, B is a nitrogen atom, thereby presenting the possibility of an achiral backbone. B can also be R3N2 where R3 is as defined above.

[0096] In the preferred form of the invention, C is

- CR3R—,

but can also be a carbon unit, i.e.,

- CR1'CR1'CH2— or CR2RCH2—, where R2 and R3 are as defined above. R2 and R3 also can be a heteroaryl group such as, for example, pyrrol, furyl, thiophen, imidazolyl, pyridyl, pyrimidinyl, indolyl, or can be taken together to complete an alyclic system such as, for example, 1,2-cyclobutanediyl, 1,2-cyclopentanediyl, 1,2-cyclohexanediyl发电机。

[0097] In the preferred form of the invention, E in the monomer synthon is COOH or an activated derivative thereof, and G in the oligomer is —CONH2—. (Preferably in the orientation —R3NOC in Formula I). As defined above, G may also be CSOH, SOOH, SO3OH or an activated derivative thereof, whereby G in the oligomer becomes —CSNHR3—, —SOHR3— and —SONR3—, respectively. The activation may, for example, be achieved using an acid anhydride or an active ester derivative, wherein hydrogen in the groups represented by E is replaced by a leaving group suited for generating the growing backbone.

[0098] The amino acids which form the backbone may be identical or different. We have found that those based on 2-aminoethyl-glycine are especially well suited to the purpose of the invention.

[0099] In some cases it may be of interest to attach ligands at either terminus (Q, L) to modulate the binding characteristics of the PNAs. Representative ligands include DNA intercalators which will improve dsDNA binding or basic groups, such as lysine or polylysine, which will strengthen the binding of PNA due to electrostatic interaction. To decrease negatively charged groups such as carboxy and sulfo groups could be used. The design of the synthons further allows such other moieties to be located on non-terminal positions.

[0100] The PNA oligomers may be conjugated to low molecular weight effector ligands such as ligands having nuclease activity or alkylating activity or reporter ligands (fluorescent,
spin labels, radioactive, protein recognition ligands, for example, biotin or haptons). In a further aspect of the invention, the PNAAs are conjugated to peptides or proteins, where the peptides have signalling activity and the proteins are, for example, enzymes, transcription factors or antibodies. Also, the PNAAs can be attached to water-soluble or water-insoluble polymers. In another aspect of the invention, the PNAAs are conjugated to oligonucleotides or carbohydrates. When warranted, a PNA oligomer can be synthesized onto some moiety (e.g., a peptide chain, reporter, intercalator or other type of ligand-containing group) attached to a solid support.

The synthesis of the PNAAs for use in to the invention is discussed in detail in the following, where Fig. I illustrates one of the preferred PNA examples and compares its structure to that of a complementary DNA.

Synthesis of PNA Oligomers and Polymers

The principle of anchoring molecules onto a solid matrix, which helps in accounting for intermediate products during chemical transformations, is known as Solid-Phase Synthesis or Merrifield Synthesis (see, e.g., Merrifield, J. Am. Chem. Soc., 1963, 85, 2149 and Science, 1986, 232, 341). Established methods for the stepwise or fragmentwise solid-phase assembly of amino acids into peptides normally employ a bead matrix of slightly cross-linked styrene-divinylbenzene copolymer, the cross-linked copolymer having been formed by the pearl polymerization of styrene monomer to which has been added a mixture of divinylbenzenes. A level of 1-2% cross-linking is usually employed. Such a matrix also can be used in solid-phase PNA synthesis in accordance with the present invention (FIG. 8).

Concerning the initial functionalization of the solid phase, more than fifty methods have been described in connection with traditional solid-phase peptide synthesis (see, e.g., Barany and Merrifield in “The Peptides” Vol. 2, Academic Press, New York, 1979, pp. 1-284, and Stewart and Young, “Solid Phase Peptide Synthesis”, 2nd Ed., Pierce Chemical Company, Illinois, 1984). Reactions for the introduction of chloromethyl functionality (Merrifield resin; via a chloromethyl methyl ether/SnCl4 reaction), aminomethyl functionality (via an N-hydroxysuccinimide aldehyde reaction; see, Mitchell, et al., Tetrahedron Lett., 1976, 3795), and benzhydrylamino functionality (Pietta, et al., J. Chem. Soc., 1970, 650) are the most widely applied. Regardless of its nature, the purpose of the functionality is normally to form an anchoring linkage between the copolymer solid support and the C-terminus of the first amino acid to be coupled to the solid support. It is generally convenient to express the “concentration” of a functional group in terms of millimoles per gram (mmol/g). Other reactive functionalities which have been initially introduced include 4-methylbenzhydrylamo and 4-methoxybenzhydrylamino. All of these established methods are in principle useful within the context of the present invention. Preferred methods for PNA synthesis employ aminomethyl as the initial functionality, in that aminomethyl is particularly advantageous with respect to the incorporation of “spacer” or “handle” groups, owing to the reactivity of the amino group of the aminomethyl functionality with respect to the essentially quantitative formation of amide bonds to a carboxylic acid group at one end of the spacer-forming reagent. A vast number of relevant spacer- or handle-forming bifunctional reagents have been described (see, Barnay, et al., Int. J. Peptide Proteol. Res., 1987, 30, 705), especially reagents which are reactive towards amino groups such as found in the aminomethyl function. Representative bifunctional reagents include 4-(haloalkyl)aryl lower alkanoic acids such as 4-(bromomethyl)phenylacetic acid, Boc-aminocarbonyl-4-(oxymethyl)aryl-lower alkanoic acids such as Boc-aminooxyacetyl-4-(oxyethyl)phenylacetic acid, N-Boc-p-acylbenzhydrylamines such as N-Boc-p-glutarylbenzhydrylamine, N-Boc-4’-lower alkyl-p-acylbenzhydrylamines such as N-Boc-4’-methyl-p-glutaroylbenzhydrylamine, N-Boc-4’-lower alkoxy-p-acylbenzhydrylamines such as N-Boc-4’-methoxy-p-glutaroylbenzhydrylamine, and 4-hydroxyethylpiperazineacetic acid. One type of spacer group particularly relevant within the context of the present invention is the phenylacetamidomethyl (Pam) handle (Mitchell and Merrifield, J. Org. Chem., 1976, 41, 1957) which, deriving from the electron withdrawing effect of the 4-phenylacetamidomethyl group, is about 100 times more stable than the classical benzyl ester linkage towards the Boc-amino deprotection reagent trifluoroacetic acid (TFA).

Certain functionalities (e.g., benzhydrylamino, 4-methylbenzhydrylamino and 4-methoxybenzhydrylamino) which may be incorporated for the purpose of cleavage of a synthesized PNA chain from the solid support such that the C-terminal of the PNA chain is in amide form, require no introduction of a spacer group. Any such functionality may advantageously be employed in the context of the present invention.

An alternative strategy concerning the introduction of spacer or handle groups is the so-called “preformed handle” strategy (see, Tam, et al., Synthesis, 1979, 955-957), which offers complete control over coupling of the first amino acid, and excludes the possibility of complications arising from the presence of undesired functional groups not related to the peptide or PNA synthesis. In this strategy, spacer or handle groups, of the same type as described above, are reacted with the first amino acid desired to be bound to the solid support, the amino acid being N-protected and optionally protected at the other side-chains which are not relevant with respect to the growth of the desired PNA chain. Thus, in those cases in which a spacer or handle group is desirable, the first amino acid to be coupled to the solid support can either be coupled to the free reactive end of a spacer group which has been bound to the initially introduced functionality (for example, an aminomethyl group) or can be reacted with the spacer-forming reagent. The space-forming reagent is then reacted with the initially introduced functionality. Other useful anchoring schemes include the “multidetachable” resins (Tam, et al., Tetrahedron Lett., 1979, 4535 and J. Am. Chem. Soc., 1980, 102, 611; Tam, J. Org. Chem., 1985, 50, 5291), which provide more than one mode of release and thereby allow more flexibility in synthetic design.

(Brady, et al., *J. Org. Chem.*, 1977, 42, 143), Bic (Kemp, et al., *Tetrahedron*, 1975, 4624), the o-nitrophenylsulfenyl (Nps) (Zervas, et al., *J. Am. Chem. Soc.*, 1963, 85, 3660), and the dithiussuccinyl (Dts) (Barany, et al., *J. Am. Chem. Soc.*, 1977, 99, 7363). These amino protecting groups, particularly those based on the widely-used urethane functionality, successfully prohibit racemization (mediated by tautomerization of the readily formed oxazolone (azlactone) intermediates (Goodman, et al., *J. Am. Chem. Soc.*, 1964, 86, 2918)) during the coupling of most ε-amino acids. In addition to such amino protecting groups, a whole range of otherwise “worthless” nonurethane-type of amino protecting groups are applicable when assembling PNA molecules, especially those built from achiral units. Thus, not only the above-mentioned amino protecting groups (or those derived from any of these groups) are useful within the context of the present invention, but virtually any amino protecting group which largely fulfills the following requirements: (1) stability to mild acids (not significantly attacked by carboxyl groups); (2) stability to mild bases or nucleophiles (not significantly attacked by the amino group in question); (3) resistance to acylation (not significantly attacked by activated amino acids). Additionally: (4) the protecting group must be close to quantitatively removable, without serious side reactions, and (5) the optical integrity, if any, of the incoming amino acid should preferably be highly preserved upon coupling. Finally, the choice of side-chain protecting groups, in general, depends on the choice of the amino protecting group, since the protection of side-chain functionalities must withstand the conditions of the repeated amino deprotection cycles. This is true whether the overall strategy for chemically assembling PNA molecules relies on, for example, differential acid stability of amino and sidechain protecting groups (such as is the case for the above-mentioned “Boc- benzyl” approach) or employs an orthogonal, that is, chemoselective, protection scheme (such as is the case for the above-mentioned “Fmoc-tBu” approach).

[0107] Following coupling of the first amino acid, the next stage of solid-phase synthesis is the systematic elaboration of the desired PNA chain. This elaboration involves repeated deprotection/coupling cycles. The temporary protecting group, such as a Boc or Fmoc group, on the last-coupled amino acid is quantitatively removed by a suitable treatment, for example, by acidolysis, such as with trifluoroacetic acid, in the case of Boc, or by base treatment, such as with piperidine, in the case of Fmoc, so as to liberate the N-terminal amino functional group (Wieland, et al., *Angew. Chem., Int. Ed. Engl.*, 1971, 10, 336). Alternatively, the carboxyl group of the incoming amino acid can be reacted directly with the N-terminal of the last-coupled amino acid with the assistance of a condensation reagent such as, for example, diethylhexylcarbodiimide (Sheehan, et al., *J. Am. Chem. Soc.*, 1955, 77, 1067) or derivatives thereof. Benzotriazolyl N-oxytris-dimethylaminophosphonium hexafluorophosphate (BOP), “Castro’s reagent” (see, e.g., Rivaille, et al., *Tetrahedron*, 1980, 36, 3413) is recommended when assembling PNA molecules containing secondary amino groups. Finally, activated PNA monomers analogous to the recently-reported amino acid fluorides (Carpino, *J. Am. Chem. Soc.*, 1990, 112, 9651) hold considerable promise to be used in PNA synthesis as well.

[0109] Following assembly of the desired PNA chain, including protecting groups, the next step will normally be deprotection of the amino acid moieties of the PNA chain and cleavage of the synthesized PNA from the solid support. These processes can take place substantially simultaneously, thereby providing the free PNA molecule in the desired form. Alternatively, in cases in which condensation of two separately synthesized PNA chains is to be carried out, it is possible by choosing a suitable spacer group at the start of the synthesis to cleave the desired PNA chains from their respective solid supports (both peptide chains still incorporating their side-chain protecting groups) and finally removing the side-chain protecting groups after, for example, coupling the two sidechain protected peptide chains to form a longer PNA chain.

[0110] In the above-mentioned “Boc-benzyl” protection scheme, the final deprotection of side-chains and release of the PNA molecule from the solid support is most often carried out by the use of strong acids such as anhydrous HF (Sakakibara, et al., *Bull. Chem. Soc. Jpn.*, 1965, 38, 4921), boron tris (trifluoroacetate) (Pless, et al., *Helv. Chim. Acta*, 1973, 46, 1609), and sulfonic acids such as trifluoromethanesulfonic acid and methanesulfonic acid (Yajima, et al., *J. Chem. Soc., Chem. Commun.*, 1974, 107). This conventional strong acid (e.g., anhydrous HF) deprotection method, produces very reactive carboxylations that may lead to alklylation and acylation of sensitive residues in the PNA chain. Such side-reactions are only partly avoided by the presence of scavengers such as anisole, phenol, dimethyl sulphide, and mercaptoethanol and, therefore, the sulphide-assisted acidoytic S₂ deprotection method (Tam, et al., *J. Am. Chem. Soc.*, 1983, 105, 6442 and *J. Am. Chem. Soc.*, 1986, 108, 5242), the so-called “low”, which removes the precursors of harmful carbocations to form inert sulfonium salts, is frequently employed in peptide and PNA synthesis, either solely or in combination with “high” methods. Less frequently, in special cases, other methods used for deprotection and/or final cleavage of the PNA-solid support bond are, for example, such methods as base-catalyzed alcoholysis (Barton, et al., *J. Am. Chem. Soc.*, 1973, 95, 4501), and ammonolysis as well as hydrazinolysis (Bodanszky, et al., *Chem. Ind.*, 1964 1423), hydrolysis (Jones, *Tetrahedron Lett.* 1977 2853 and Schlatter, et al., *Tetrahedron Lett.* 1977 2861)), and photolysis (Rich and Gurwara, *J. Am. Chem. Soc.*, 1975 97, 1575).

[0111] Finally, in contrast with the chemical synthesis of “normal” peptides, stepwise chain building of achiral PNA polypeptides such as those based on aminoethyglycyl backbone units can start either from the N-terminus or the C-terminus, because the coupling reactions are free of racemization.
[0112] Based on the recognition that most operations are identical in the synthetic cycles of solid-phase peptide synthesis (as is also the case for solid-phase PNA synthesis), a new matrix, PEPS, was recently introduced (Berg et al., *J. Am. Chem. Soc.*, 1989, 111, 8024 and International Patent Application WO 90/02749) to facilitate the preparation of large numbers of peptides. This matrix is comprised of a polyethylene (PE) film with pendant long-chain polystyrene (PS) grafts (molecular weight on the order of 105). The loading capacity of the film is as high as that of a beaded matrix, but PEPS has the additional flexibility to suit multiple syntheses simultaneously. Thus, in a new configuration for solid-phase peptide synthesis, the PEPS film is fashioned in the form of discrete, labeled sheets, each serving as an individual compartment. During all the identical steps of the synthetic cycles, the sheets are kept together in a single reaction vessel to permit concurrent preparation of a multitude of peptides at a rate close to that of a single peptide by conventional methods. It was reasoned that the PEPS film support, comprising linker or spacer groups adapted to the particular chemistry in question, should be particularly valuable in the synthesis of multiple PNA molecules, these being conceptually simple to synthesize since only four different reaction compartments are normally required, one for each of the four “pseudonucleoside” units. Thus, the PEPS film support has been successfully tested in a number of PNA syntheses carried out in parallel and substantially simultaneous fashion. The yield and quality of the products obtained from PEPS were comparable to those obtained by using the traditional polystyrene beaded support. Also, experiments with other geometries of the PEPS polymer such as, for example, non-woven felt, knitted net, sticks or microcelluloids have not indicated any limitations of the synthetic efficacy.


[0114] While the conventional cross-linked styrene-dimethylbenzene copolymer matrix and the PEPS support are presently preferred in the context of solid-phase PNA synthesis, a non-limiting list of examples of solid supports which may be of relevance are: (1) Particles based upon copolymers of dimethylacrylamide cross-linked with N,N′-bisacycloxyethylenediamine, including a known amount of N-tertbutoxy-carbonyl-beta-alanylamide-N′-acryloyl-hexamethylenediamine. Several spacer molecules are typically added via the beta alanyl group, followed thereafter by the amino acid residue subunits. Also, the beta alanyl-containing monomer can be replaced with an acryloyl sarcosine monomer during polymerization to form resin beads. The polymerization is followed by reaction of the beads with ethylenediamine to form resin particles that contain primary amines as the covalently linked functionality. The polyaclrylamide-based supports are relatively more hydrophilic than are the polystyrene-based supports and are usually used with polar aprotic solvents including dimethylformamide, dimethyl-acetamide, N,N-dimethylpyrroolidine and the like (see Atherton, et al., *J. Am. Chem. Soc.*, 1975, 97, 6584, *Biorg. Chem.*, 1979, 8, 351, and J. C. S. Perkin I, 1381 (1981)); (2) a second group of solid supports is based on silica-containing particles such as porous glass beads and silica gel. One example is the reaction product of trichloro-[3-(4-chloromethyl)phenyl]propylsilane and porous glass beads (see Parr and Gröhn, *Angew. Chem. Internat. Ed. Engl.*, 1972, 11, 314) sold under the trademark “PORASIL E” by Waters Associates, Framingham, Mass., USA. Similarly, a mono ester of 1,4-dihydroxy-methylbenzene and silica (sold under the trademark “BIOPAK” by Waters Associates) has been reported to be useful (see Bayer and Jung, *Tetrahedron Lett.*, 1970, 4503); (3) a third general type of useful solid supports can be termed composites in that they contain two major ingredients: a resin and another material that is also substantially inert to the organic synthesis reaction conditions employed. One exemplary composite (see Scott, et al., *J. Chrom. Sci.*, 1971, 9, 577) utilized glass particles coated with a hydrophobic, cross-linked styrene polymer containing reactive chloromethyl groups, and was supplied by Northgate Laboratories, Inc., of Stamford, Conn., USA Another exemplary composite contains a core of fluorinated ethylene polymer onto which has been grafted polystyrene (see Kent and Merrifield, *Israel J. Chem.*, 1978, 17, 243) and van Rietshoenen in “Peptides 1974”, Y. Wolman, Ed., Wiley and Sons, New York, 1975, pp. 113-116); and (4) contiguously solid supports other than PEPS, such as cotton sheets (Lebl and Eichler, *Peptide Res.*, 1989, 2, 232) and hydroxypropylacrylate-coated polypropylene membranes (Daniels, et al., *Tetrahedron Lett.*, 1989, 4354), are suited for PNA synthesis as well.


[0116] While the solid-phase technique is presently preferred in the context of PNA synthesis, other methodologies or combinations thereof, for example, in combination with the solid-phase technique, apply as well: (1) the classical solution-phase methods for peptide synthesis (e.g., Bodanszky, “Principles of Peptide Synthesis”, Springer-Verlag, Berlin-New York 1984), either by stepwise assembly or by segment/fragment condensation, are of particular relevance when considering especially large scale productions (gram,
kilogram, and even tons) of PNA compounds; (2) the so-called "liquid-phase" strategy, which utilizes soluble polymeric supports such as linear polystyrene (Shemyakin et al., Tetrahedron Lett., 1965, 2323) and polyethylene glycol (PEG) (Mutter and Bayar, Angew. Chem., Int. Ed. Engl., 1974, 13, 88), is useful; (3) random polymerization (see, e.g., Odiann, "Principles of Polymerization," McGraw-Hill, New York (1970)) yielding mixtures of many molecular weights ("polydisperse") peptide or PNA molecules are particularly relevant for purposes such as screening for antiviral effects; (4) a technique based on the use of polymer-supported amino acid active esters (Fridkin et al., J. Am. Chem. Soc., 1965, 87, 4646), sometimes referred to as "inverse Merrifield synthesis" or "polymeric reagent synthesis," offers the advantage of isolation and purification of intermediate products, and may thus provide a particularly suitable method for the synthesis of medium-sized, optionally protected, PNA molecules, that can subsequently be used for fragment condensation into larger PNA molecules; (5) it is envisaged that PNA molecules may be assembled enzymatically by enzymes such as proteases or derivatives thereof with novel specificities (obtained, for example, by artificial means such as protein engineering). Also, one can envision the development of "PNA ligases" for the condensation of a number of PNA fragments into very large PNA molecules; (6) since antibodies can be generated to virtually any molecule of interest, the recently developed catalytic antibodies (enzymes), discovered simultaneously by the groups of Lerner (Tramontano et al., Science, 1986, 234, 1566) and of Schultz (Pollack et al., Science, 1986, 234, 1570), should also be considered as potential candidates for assembling PNA molecules. Thus, there has been considerable success in producing antibodies catalyzing acyl-transfer reactions (see, for example Shokat et al., Nature, 1989, 338, 269) and references therein). Finally, completely artificial enzymes, very recently pioneered by Stewart's group (Fall, et al., Science, 1990, 248, 1544), may be developed to suit PNA synthesis. The design of generally applicable enzymes, ligases, and catalytic antibodies, capable of mediating specific coupling reactions, should be more readily achieved for PNA synthesis than for "normal" peptide synthesis since PNA molecules will often be comprised of only four different amino acids (one for each of the four native nucleobases) as compared to the twenty natural by occurring (proteinogenic) amino acids constituting peptides. In conclusion, no single strategy may be wholly suitable for the synthesis of a specific PNA molecule, and therefore, sometimes a combination of methods may work best.

(a) Experimental for the Synthesis of Monomeric Building Blocks

The monomers preferably are synthesized by the general scheme outlined in FIG. 8. This involves preparation of either the methyl or ethyl ester of (Bocaminomethyl)glycine, by a protection/deprotection procedure as described in Examples 21-23. The synthesis of thymine monomer is described in Examples 24-25, and that of the protected cytosine monomer is described in Example 26.

The synthesis of the protected adenine monomer (FIG. 14) involved alkylation with ethyl bromoacetate (Example 27) and verification of the position of substitution by X-ray crystallography, as being the wanted 9-position. The N6-aminogroup then was protected with the benzoxycarbonyl group by the use of the reagent N-ethyl-benzyloxy carbonylimidazole tetrafluoroborate (Example 28). Simple hydrolysis of the product ester (Example 29) gave N6-benzyloxycarbonyl-9-carboxymethyl adenine, which then was used in the standard procedure (Examples 10-11, FIG. 8). The adenine monomer has been built into two different PNA-oligomers (Examples 30 and 31).

The synthesis of the protected G-monomer is outlined in FIG. 15. The starting material, 2-amino-6-chloropurine, was alkylated with bromoacetic acid (Example 32) and the chlorine atom was then substituted with a benzyloxyl group (Example 36). The resulting acid was coupled to the (bocaminomethyl)glycine methyl ester (Example 33) with agent PyBrop™, and the resulting ester was hydrolyzed (Example 23). The O3-benzyl group was removed in the final HCl-cleavage step in the synthesis of the PNA-oligomer. Cleavage was verified by finding the expected mass of the final PNA-oligomer, upon incorporation into an PNA-oligomer using diisopropyl carbodiimide as the condensation agent (Example 52).

The following abbreviations are used in the experimental examples: DMF, N,N-dimethylformamide; DCC, N,N-dicyclohexyl carbodiimide; DCCU, N,N-dicyclohexyl urea; THF, tetrahydrofuran; aeg, N-acetyl (2'-aminoethyl) glyoxime; pfp, pentafluorophenyl; Boc, tert-butoxycarbonyl; Z, benzoxycarbonyl; NMR, nuclear magnetic resonance; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet; b, broad; 8, chemical shift;

NMR spectra were recorded on either a JEOL FX 90Q spectrometer, or a Bruker 250 MHz with tetramethylsilane as internal standard. Mass spectrometry was performed on a Masslab VG 12-250 quadrupole instrument fitted with a VG FAB source and probe. Melting points were recorded on a Buchi melting point apparatus and are uncorrected. N,N-Dimethylformamide was dried over 4Å molecular sieves, distilled and stored over 4Å molecular sieves. Pyridine (BPLC quality) was dried and stored over 4Å molecular sieves. Other solvents used were either the highest quality obtainable or were distilled before use. Dioxane was passed through basic alumina prior to use. Bocanhydride, 4-nitrophenol, methyl bromoacetate, benzoyloxycarbonyl chloride, pentafluorophenol were all obtained through Aldrich Chemical Company. Thymine, cytosine, adenine were all obtained through Sigma.

Thin layer chromatography (Tlc) was performed using the following solvent systems: (1) chloroform:methanol, 7:1; (2) methylene chloride:methanol, 9:1; (3) chloroform:methanol:acetic acid 85:10:5. Spots were visualized by UV (254 nm) or by spraying with a ninhydrin solution (3g ninhydrin in 1000 ml 1-butanol and 30 ml acetic acid), after heating at 120°C for 5 min and, after spraying, heating again.

Extended Backbones

Variations of the groups A, C and D (FIG. 16) are demonstrated by the synthesis of monomeric building blocks and incorporation into PNA-oligomers.

In one example, the C group was a CH(CH3)2 group. The synthesis of the corresponding monomer is outlined in FIG. 17. It involves preparation of Boc-protected 1-amino-2, 3-propanediol (Example 35), which is cleaved by periodate to give bocaminooctadecaldehyde, which is used directly in the next reaction. The bocaminooctadecaldehyde can be condensed with a variety of amines; in Example 36, alamine ethyl ester was used. In Examples 17-19, the corresponding thymine monomer...
mers were prepared. The monomer has been incorporated into an 8-mer by the DCC-coupling protocol (Examples 30 and 31).

[0126] In another example, the D-group is a (CH₂)₂ CO group. The synthesis of the corresponding monomer is outlined in FIG. 18A and described in Examples 40 and 46.

[0127] In yet another example, the A-group is a (CH₂)₂ CO group. The synthesis of the corresponding thymine monomer is outlined in FIG. 18B and Examples 42 through 45.

[0128] In yet another example, the C-group is a (CH₂)₂ group. The synthesis of the thymine and protected cytosine monomer is outlined in FIG. 19 and Examples 46 through 51. Hybridization experiments with a PNA-oligomer containing one unit are described in Example 61, which shows a significant lowering of affinity but a retention of specificity.

EXAMPLE 1

tert-Butyl 4-nitrophenylobutonate

[0129] Sodium carbonate (29.14 g; 0.275 mol) and 4-nitrophenol (12.75 g; 91.66 mmol) were mixed with dioxane (250 ml). Boc-anhydride (20.0 g; 91.66 mmol) was transferred to the mixture with dioxane (50 ml). The mixture was refluxed for 1 h, cooled to 0°C, filtered and concentrated to ½, and then poured into water (350 ml) at 0°C. After stirring for ½ h, the product was collected by filtration, washed with water, and then dried over silicagel, in vacuo. Yield 21.3 g (97%). M.p. 73-74.5°C (lit. 78.5-79.5°C). Anal. for C₁₄H₁₁NO₃: Found: C 55.2 (55.23) H, 5.61 (5.48) N, 5.82 (5.85).

EXAMPLE 2
(N²-Boc-2'-aminoethyl)glycine (2)

[0130] The title compound was prepared by a modification of the procedure by Heinem, et al. N-(2-Aminoethyl)glycine (1, 3.00 g; 25.4 mmol) was dissolved in water (50 ml), dioxane (50 ml) was added, and the pH was adjusted to 11.2 with 2 N sodium hydroxide. tert-Butyl-4-nitrophényl carbonate (7.29 g; 30.5 mmol) was dissolved in dioxane (40 ml) and added dropwise over a period of 2 h, during which time the pH was maintained at 11.2 with 2 N sodium hydroxide. The pH was adjusted periodically to 11.2 for three more hours and then the solution was left overnight. The solution was cooled to 0°C, and the pH was carefully adjusted to 3.5 with 0.5 M hydrochloric acid. The aqueous solution was washed with chloroform (3×200 ml), the pH adjusted to 9.5 with 2 N sodium hydroxide and the solution was evaporated to dryness, in vacuo (14 mmHg). The residue was extracted with DMF (25×2×10 ml) and the extracts filtered to remove excess salt. The salt is not present in the solution of the title compound in about 60% yield and greater than 95% purity by tlc (system 1 and visualised with ninhydrin, RF=0.3). The solution was used in the following preparations of Boc-aeg derivatives without further purification.

EXAMPLE 3
N-1-4-Aminobenzylthymine (4)

[0131] This procedure is different from the literature synthesis, but is easier, gives higher yields, and leaves no unreacted thymine in the product. To a suspension of thymine (3, 4.0 g; 0.317 mol) and potassium carbonate (87.7 g; 0.634 mmol) in DMF (900 ml) was added methyl bromoacetate (30.00 ml; 0.317 mmol). The mixture was stirred vigorously overnight under nitrogen. The mixture was filtered and evaporated to dryness, in vacuo. The solid residue was treated with water (300 ml) and 4 N hydrochloric acid (12 ml), stirred for 15 min at 0°C, filtered, and washed with water (2×75 ml). The precipitate was treated with water (120 ml) and 2N sodium hydroxide (60 ml), and was boiled for 10 minutes. The mixture was cooled to 0°C, filtered, and the pure title compound was precipitated by the addition of 4N hydrochloric acid (70 ml). Yield after drying, in vacuo over sievets (37.1 g (64%); 1H-NMR: (90 MHz; DMSO-d₆): 11.33 ppm (s, 1H, NH); 7.49 (d, J=0.92 HZ, 1H, ArH); 4.38 (s, 2H, CH₂); 1.76 (d, J=0.92 HZ, T-CH₃).

EXAMPLE 4
N-1-Carboxymethylthymine pentafluorophenyl ester (5)

[0133] N-1-Carboxymethylthymine (4, 10.0 g; 54.3 mmol) and pentafluorophenol (10.0 g; 54.3 mmol) were dissolved in DMF (100 ml) and cooled to 5°C in ice water. DCC (13.45 g; 65.2 mmol) then was added. When the temperature passed below 5°C, the ice bath was removed and the mixture was stirred for 1 h at ambient temperature. The precipitated DCU was removed by filtration and washed twice with DMF (2×10 ml). The combined filtrate was poured into ether (1400 ml) and cooled to 0°C. Petroleum ether (1400 ml) was added and the mixture was left overnight. The title compound was isolated by filtration and washed thoroughly with petroleum ether. Yield: 14.8 g (78%). The product was pure enough to carry out the next reaction, but an analytical sample was obtained by recrystallization from 2-propanol. M.p. 200.4-206°C. Anal. for C₁₃H₁₂F₅N₂O₅: Found (Calc.) C, 44.79 (44.59); H, 2.14 (2.01) N, 8.13 (8.00). FAB-MS: 443 (M+14 glycerol), 351 (M+1). 1H-NMR (90 MHz, DMSO-d₆): 11.52 ppm (s, 1H, NH); 7.64 (s, 1H, ArH); 4.99 (s, 2H, CH₂); 1.76 (s, 3H, CH₃).

EXAMPLE 5
1-(Boc-aeg)thymine (6)

[0134] To the DMF-solution from above was added triethylamine (7.08 ml; 50.8 mmol) followed by N-1-carboxymethylthymine pentafluorophenyl ester (5, 4.45 g; 12.7 mmol). The resultant solution was stirred for 1 h. The solution was cooled to 0°C and treated with cation exchange material (Dowex 50W X-8”; 40 g) for 20 min. The cation exchange material was removed by filtration, washed with dichloromethane (2×15 ml), and dichloromethane (150 ml) was added. The resulting solution was washed with saturated sodium chloride, dried over magnesium sulfate, and evaporated to dryness, in vacuo, first by a water aspirator and then by a oil pump. The residue was shaken with water (50 ml) and evaporated to dryness. This procedure was repeated once. The residue then was dissolved in methanol (75 ml) and poured into ether (600 ml) and petroleum ether (1.4 L). After stirring overnight, the white solid was isolated by filtration, washed with petroleum ether. Drying over silicagel, in vacuo, gave 3.50 g (71.7%). M.p. 142-147°C. Anal. for C₁₄H₁₂N₂O₅: Found (Calc.) C, 49.59 (50.00) H, 6.34 (6.29) N, 14.58 (14.58). 1H-NMR (250 MHz, DMSO-d₆); Due to the limited rotation around the secondary amide bond several of the signals were doubled in the ratio 2:1 (indicated in the list by mj, for major and mi. for minor). 12.73 ppm (b, 1H,
—CO₂H); 11.27 ppm (s, m.j., imide); 11.25 ppm (s, m.j., imide); 7.30 ppm (s, m.j., ArH); 7.26 ppm (s, m.j., ArH); 6.92 ppm (unres. t, m.j., BocNH); 6.73 ppm (unres. t, m.j., BocNH); 4.64 ppm (s, m.j., T-CH₂—CO—); 4.47 ppm (s, m.j., T-CH₂— CO—); 4.19 ppm (s, m.j., CONRCH₂CO₂H); 3.97 ppm (s, m.j., CONRCH₂CO₂H); 3.41-2.89 ppm (unres. m., —CH₂CH₂— and water); 1.75 ppm (s, 3H, T-CH₃); 1.38 ppm (s, 9H, t-Bu). ¹³C-NMR: 170.68 ppm (CO); 170.34 (CO); 167.47 (CO); 167.34 (CO); 164.32 (C=O); 150.9 (C₆H₅); 141.92 (C₆H₂); 108.04 (C₂); 77.95 and 77.68 (Thy-CH₂CO₂); 48.96, 47.45 and 46.70 (—CH₂CH₂— and NCH₂CO₂H); 37.98 (Thy-Cl₃C); 28.07 (t-Bu). FAB-MS: 407 (M+Na⁺), 385 (M+H⁺).

EXAMPLE 6
1-(Boc-aeg)thymine pentafluorophenyl ester (7, Boc-TaeG.OtFP)

[0135] 1-(Boc-aeg)thymine (6) (2.00 g; 5.20 mmol) was dissolved in DMF (5 ml) and methylene chloride (15 ml) was added. Pentafluorophenol (1.05 g; 5.72 mmol) was added and the solution was cooled to 0°C in an ice bath. DCC then was added (1.29 g; 6.24 mmol) and the ice bath was removed after 2 min. After 3 h with stirring at ambient temperature, the precipitated DCU was removed by filtration and washed with methylene chloride. The combined filtrate was washed twice with aqueous sodium hydrogen carbonate and once with saturated sodium chloride, dried over magnesium sulfate, and evaporated to dryness, in vacuo. The solid residue was dissolved in dioxane (150 ml) and poured into water (200 ml) at 0°C. The title compound was isolated by filtration, washed with water, and dried over sodium sulfate, in vacuo. Yield: 2.20 g (77%). An analytical sample was obtained by recrystallisation from 2-propanol. M.p. 174-175.5°C. Analysis for C₂₅H₂₅N₃O₅, Found (Calc.): C, 56.22 (56.11); H, 5.27 (5.26); N, 6.97 (6.95). ¹³C-NMR (250 MHz, CDCl₃): Due to the limited rotation around the secondary amide bond several of the signals were doubled in the ratio 6:1 (indicated in the list by mj. for major and mi. for minor). 7.01 ppm (s, mj., ArH); 6.99 ppm (s, mj., ArH); 5.27 ppm (unres. t, BocNj); 4.67 ppm (s, mj., T-CH₂—CO—); 4.60 ppm (s, mj., T-CH₂—CO—); 4.45 ppm (s, mj., CONRCH₂CO₂Pb); 4.42 ppm (s, mj., CONRCH₂CO₂Pb); 3.64 ppm (t, 2H, BocNHCO₂H₂—); 3.87 ppm (q, 2H, BocNHCH₂CH₂—); 1.44 ppm (9H, t-Bu). FAB-MS: 551 (10; M+1); 495 (10; M+1-tBu); 451 (80; -Boc).

EXAMPLE 7
N⁴-Benzylxocarbonyl cytosine (9)

[0136] Over a period of about 1 h, benzylxocarbonyl chloride (52 ml; 0.36 mol) was added dropwise to a suspension of cytosine (8, 20.0 g; 0.18 mol) in dry pyridine (1000 ml) at 0°C under nitrogen in oven-dried equipment. The solution then was stirred overnight, after which the pyridine suspension was evaporated to dryness, in vacuo. Water (200 ml) and 4 N hydrochloric acid were added to reach pH 1. The resulting white precipitate was filtered off, washed with water and partially dried by air suction. The still-wet precipitate was boiled with absolute ethanol (500 ml) for 10 min, cooled to 0°C, filtered, washed thoroughly with ether, and dried, in vacuo. Yield 24.7 g (54%). M.p.>250°C. Analysis for C₁₄H₁₁N₃O₅, Found (Calc.): C, 58.59 (58.77); H, 4.55 (4.52); N, 17.17 (17.13). No NMR spectra were recorded since it was not possible to get the product dissolved.

EXAMPLE 8
N⁴-Benzylxocarbonyl-N¹-carboxymethylycytosine (10)

[0137] In a three necked round bottomed flask equipped with mechanical stirring and nitrogen coverage was placed methyl bromoacetate (7.82 ml; 82.6 mmol) and a suspension of N⁴-benzylxocarbonyl-cytosine (9, 21.0 g; 82.6 mmol) and potassium carbonate (11.4 g; 82.6 mmol) in dry DMF (900 ml). The mixture was stirred vigorously overnight, filtered, and evaporated to dryness, in vacuo. Water (300 ml) and 4 N hydrochloric acid (10 ml) were added, the mixture was stirred for 15 minutes at 0°C, filtered, and washed with water (2x75 ml). The isolated precipitate was treated with water (120 ml), 2N sodium hydroxide (60 ml), stirred for 30 min, filtered, cooled to 0°C, and 4 N hydrochloric acid (35 ml) was added. The title compound was isolated by filtration, washed thoroughly with water, recrystallized from methanol (1000 ml) and washed thoroughly with ether. This afforded 7.70 g (31%) of pure compound. The mother liquor from the recrystallization was reduced to a volume of 200 ml and cooled to 0°C. This afforded an additional 2.30 g of a material that was pure by tlc but had a reddish colour. M.p. 266-274°C. Analysis for C₁₄H₁₃N₃O₅, Found (Calc.): C, 55.41 (55.45); H, 4.23 (4.32); N, 14.04 (13.86). ¹H-NMR (90 MHz, DMSO-D₆): 8.02 ppm (d, J=7.32 Hz, 1H, H-6); 7.39 (s, 5H, Ph); 7.01 (d, J=7.32 Hz, 1H, H-5); 5.19 (s, 2H, PhCH₂—); 4.52 (s, 2H).

EXAMPLE 9
N⁴-Benzylxocarbonyl-N¹-carboxymethylcytosine pentafluorophenyl ester (11)

[0138] N⁴-Benzylxocarbonyl-N¹-carboxymethylcytosine (10, 4.00 g; 13.2 mmol) and pentafluorophenol (2.67 g; 14.5 mmol) were mixed with DMF (70 ml), cooled to 0°C with ice-water, and DCC (5.27 g; 15.8 mmol) was added. The ice bath was removed after 3 min and the mixture was stirred for 3 h at room temperature. The precipitated DCU was removed by filtration, washed with DMF, and the filtrate was evaporated to dryness, in vacuo (0.2 mmHg). The solid residu was treated with methylene chloride (250 ml), stirred vigorously for 15 min, filtered, washed twice with diluted sodium hydroxide solution, and once with saturated sodium chloride, dried over magnesium sulfate, and evaporated to dryness, in vacuo. The solid residue was recrystallized from 2-propanol (150 ml) and the crystals were washed thoroughly with ether. Yield 3.40 g (55%). M.p. 241-245°C. Analysis for C₂₅H₂₅N₃F₁₀O₅, Found (Calc.): C, 51.56 (51.18); H, 2.77 (2.58); N, 9.24 (8.95). ¹H-NMR (90 MHz, CDCl₃): 7.66 ppm (d, J=7.63 Hz, 1H, H-6); 7.37 (s, 5H, Ph); 7.31 (d, J=7.63 Hz, 1H, H-5); 5.21 (s, 2H, PhCH₂—); 4.97 (s, 2H, NCH₂—). FAB-MS: 470 (M+1).

EXAMPLE 10
N⁴-Benzylxocarbonyl-1-Boc-aeg-cytosine (12)

[0139] To a solution of (N-Boc-2-aminoethyl)glycine (2) in DMF, prepared as described above, was added triethyl amine (7.00 ml; 50.8 mmol) and N⁴-benzylxocarbonyl-N¹-carboxymethylcytosine pentafluorophenyl ester (11, 2.70 g; 5.75 mmol). After stirring the solution for 1 h at room tem-
perature, methylene chloride (150 ml), saturated sodium chloride (250 ml), and 4 N hydrochloric acid to pH 1~2 were added. The organic layer was separated and washed twice with saturated sodium chloride, dried over magnesium sulfate, and evaporated to dryness, in vacuo, first with a water aspirator and then with an oil pump. The oily residue was treated with water (25 ml) and was again evaporated to dryness, in vacuo. This procedure then was repeated. The oily residue (2.80 g) was then dissolved in methylene chloride (100 ml), petroleum ether (250 ml) was added, and the mixture was stirred overnight. The title compound was isolated by filtration and washed with petroleum ether. TLC (system 1) indicated substantial quantities of pentafluorophenol, but no attempt was made to remove it. Yield: 1.72 g (59%). M.p. 156°C. (decomp.). 1H-NMR (250 MHz, CDCl3): Due to the limited rotation around the secondary amide bond several of the signals were doubled in the ratio 2:1, (indicated in the list by mj. for major and mi. for minor). 7.88 ppm (dd, 1H, H-6); 7.39 ppm (m, 5H, Ph); 7.00 ppm (dd, 1H, H-5); 6.92 ppm (b, 1H, BocN;); 6.74 ppm (b, 1H, ZnN); 5.19 ppm (s, mi., Cyt-C-H); 4.81 ppm (s, mj., Cyt-C-H); 4.23 ppm (s, mi.; CONRCO2H); 3.98 ppm (s, mj.; CONRCO2H); 3.42-3.02 (m, 6H, CH2); and water; 1.37 ppm (s, 9H, tBu). FAB-MS: 504 (M+1); 448 (M+1-tBu).

EXAMPLE 11

N²-Benzyloxy carbonyl-1-Boc-aeg-cytosine pentafluorophenyl ester (13) [0140] N²-Benzylloxy carbonyl-1-Boc-aeg-cytosine (12, 1.50 g; 2.98 mmol) and pentafluorophenol (548 mg; 2.98 mmol) was dissolved in DFM (10 ml). Methylen chloride (10 ml) was added, the reaction mixture was cooled to 0°C in an ice bath, and DCC (676 mg; 3.28 mmol) was added. The ice bath was removed after min and the mixture was stirred for 3 h at ambient temperature. The precipitate was isolated by filtration and washed once with methylen chloride. The precipitate was dissolved in boiling dioxane (150 ml) and the solution was cooled to 15°C, whereby DUC precipitated. The DCU was removed by filtration and the resulting filtrate was poured into water (250 ml) at 0°C. The title compound was isolated by filtration, was washed with water, and dried over siecapent, in vacuo. Yield 1.30 g (65%). Analysis for C16H16N2O5F2. Found: Calc.: C, 52.63 (52.02); H, 4.41 (4.22); N, 10.55 (10.46). 1H-NMR (250 MHz; DMSO-d6): showed essentially the spectrum of the above acid, most probably due to hydrolysis of the ester. FAB-MS: 670 (M+1); 614 (M+1-tBu).

EXAMPLE 12

4-Chlorocarboxy-9-chloroacridine [0141] 4-Carboxyacridine (6.25 g; 26.1 mmol), thionyl chloride (25 ml), and 4 drops of DME were heated gently under a flow of nitrogen until all solid material had dissolved. The solution then was refluxed for 40 min. The solution was cooled and excess thionyl chloride was removed in vacuo. The last traces of thionyl chloride were removed by coevaporation with dry benzene (dried over Na-Pb) twice. The remaining yellow powder was used directly in the next reaction.

EXAMPLE 13

4-(5-Methoxycarbonyl)pentylamidocarboxy-9-chloroacridine [0142] Methion 6-aminohexanoate hydrochloride (4.70 g; 25.9 mmol) was dissolved in methylene chloride (90 ml), cooled to 0°C, triethyl amine (15 mL) was added, and the resulting solution was immediately added to the acid chloride from above. The round bottomed flask containing the acid chloride was cooled to 0°C in an ice bath. The mixture was stirred vigorously for 30 min at 0°C and 3 h at room temperature. The resulting mixture was filtered to remove the remaining solids, which were washed with methylene chloride (20 ml). The red-brown methylene chloride filtrate was subsequently washed twice with saturated sodium hydrogen carbonate, once with saturated sodium chloride, dried over magnesium sulfate, and evaporated to dryness, in vacuo. To the resulting oily substance was added dry benzene (35 ml) and ligroin (60-80°C, dried over Na-Pb). The mixture was heated to reflux. Activated carbon and celtite were added and mixture was refluxed for 3 min. After filtration, the title compound crystallised upon cooling with magnetic stirring. It was isolated by filtration and washed with petroleum ether. The product was stored over solid potassium hydroxide. Yield 5.0 g (50%).

EXAMPLE 14

4-(5-Methoxy carbonyl)pentylamidocarboxy-9-(6'- (4'-nitro-benzamido)hexylaminol)-aminoacridine [0143] 4-(5-Methoxy carbonyl)pentylamidocarboxy-9-chloroacridine (1.30 g; 3.38 mmol) and phenol (5 g) were heated to 80°C for min under a flow of nitrogen, after which 6'(4'-nitro-benzamido)-1-hexylamine (897 mg; 3.38 mmol) was added. The temperature raised to 120°C for 2 h. The reaction mixture was cooled and methylene chloride (80 ml) was added. The resulting solution was washed three times with 2N sodium hydroxide (60 ml portions) and once with water, dried over magnesium sulfate, and evaporated to dryness in vacuo. The resulting red oil (1.8 g) was dissolved in methylene chloride (40 ml), cooled to 0°C. Ether (120 ml) was added and the resultant solution was stirred overnight. This results in a mixture of solid material and an oil. The solid was isolated by filtration. The solid and the oil were redissolved in methylene chloride (80 ml) and added dropwise to cold ether (150 ml). After 20 minutes of stirring, the title compound was isolated by filtration in the form of orange crystals. The product was washed with ether and dried in vacuo over potassium hydroxide. Yield 1.60 g (77%). M.p. 145-147°C.

EXAMPLE 15

4-(5-Carboxy pentylamidocarboxy-9-[6'-4'-nitro-benzamido)-hexylaminol]-aminoacridine [0144] 4-(5-Methoxy carbonyl)pentylamidocarboxy-9-[6'-4'-nitro-benzamido)-hexylaminol]-aminoacridine (503 mg; 0.82 mmol) was dissolved in DME (30 ml), and 2 N sodium hydroxide (30 ml) was added. After stirring for 15 min, 2 N hydrochloric acid (35 ml) and water (50 ml) were added at 0°C. After stirring for 30 min, the solution was decanted, leaving an oily substance which was dissolved in boiling methanol (150 ml), filtered and concentrated to ½ volume. To the methanol solution were added ether (125 ml) and 5-6 drops of HCl in ethanol. The solution was decanted after 1 h of stirring at 0°C. The oily substance was redissolved in methanol (25 ml) and precipitated with ether (150 ml). The title compound
was isolated as yellow crystals after stirring overnight. Yield 417 mg (801). M.p. 173°C. (decomp.).

EXAMPLE 16

(a) 4-(5-pentfluorophenyl)oxyphényl-(pentyl)-aminocarboxyl-9-(6′,4′-nitrobenzamido)-hexylaminonoacrylimide (Acr-OOpf)

[0145] The acid from above (300 mg; 0.480 mmol) was dissolved in DMF (2 ml) and methylene chloride (8 ml) was added. Pentfluorophenol (97 mg; 0.53 mmol), transferred with 2×2 ml of the methylene chloride, was added. The resulting solution was cooled to 0°C, after which DCC (124 mg; 0.60 mmol) was subsequently added. The ice bath was removed after 5 minutes and the mixture was left with stirring overnight. The precipitated DCC was removed by centrifugation and the centrifugate was evaporated to dryness, in vacuo, first by a water aspirator and then by an oil pump. The residue was dissolved in methylene chloride (20 ml), filtered, and evaporated to dryness, in vacuo. The residue was again dissolved in methylene chloride and petroleum ether (150 ml). A 1 ml portion of 5 M HCl in ether was added. The solvent was removed by decanting after 30 min of stirring at 0°C. The residual oily substance was dissolved in methylene chloride (100 ml). Petroleum ether (150 ml) was added and the mixture was left with stirring overnight. The next day the yellow precipitated crystalline material was isolated by filtration and was washed with copious amounts of petroleum ether. Yield, after drying, 300 mg (78%). M.p. 97.5°C. (decomp.) All samples showed satisfactory elemental analysis, 1H- and 13C-NMR and mass spectra.

(b) Experimental for the Synthesis of PNA Compounds, cf. FIG. 8

[0146] Materials: Boc-Lys (Clz), benzhydrylamine-copoly(styrere-1%-divinylbenzene) resin (BHA resin), and p-methylbenzhydrylamine-copoly(styrere-1%-divinylbenzene) resin (MBHA resin) were purchased from Peninsula Laboratories. Other reagents and solvents were: Biograde trifluoroacetic acid from Halocarbon Products; disopropylethylamine (99%) was not further distilled) and N-acetylimidazole (98%) from Aldrich; H2O was distilled twice; anhydrous HF from Union Carbide; synthesis grade N,N-dimethylformamide and analytical grade methylene chloride (was not further distilled) from Merck; HPLC grade acetonitrile from Lab-San; purum grade anisole, N,N-dicyclohexylcarbodiimide, and puriss. grade 2,2,2-trifluoroethanol from Fluka.

(b) General Methods and Remarks

[0147] Except where otherwise stated, the following applies. The PNA compounds were synthesized by the stepwise solid-phase approach (Merrifield, J. Am. Chem. Soc., 1963, 85, 2149) employing conventional peptide chemistry utilizing the TFA-labile tert-butyloxycarbonyl (Boc) group for “temporary” N-protection (Merrifield, J. Am. Chem. Soc., 1964, 86, 304) and the more acid-stable benzoxylcarbonyl (Z) and 2-chlorobenzoxycarbonyl (Clz) groups for “permanent” side chain protection. To obtain C-terminal amides, the PNA were assembled onto the HF-labile BHA or MBHA resins (the MBHA resin has increased susceptibility to the final HF cleavage relative to the unsubstituted BHA resin (Matsue, et al., Peptides, 1981, 2, 45). All reactions (except HF reactions) were carried out in manually operated standard solid-phase reaction vessels fitted with a coarse glass frit (Merrifield, et al., Biochemistry, 1982, 21, 5020). The quantitative ninhydrin reaction (Kaiser test), originally developed by Merrifield and co-workers (Sarin, et al., Anal. Biochem., 1981, 117, 147) for peptides containing “normal” amino acids, was successfully applied (see Table I-III) using the “normally” employed effective extinction coefficient ε-15000 M⁻¹ cm⁻¹ for all residues to determine the completeness of the individual couplings as well as to measure the number of growing peptide chains. The theoretical substitution S0.1 upon coupling of residue number n (assuming both complete deprotection and coupling as well as neither chain termination nor loss of PNA chains during the synthetic cycle) is calculated from the equation:

\[ S_0.1 = \frac{1}{1 + \frac{1}{S_{0,1}} \times (1 + S_{0,1}) \times (\Delta MW \times 10^{-3}) \times (\text{mmol/mol})} \]

where ΔMW is the molecular weight (ΔMW = g/mol) and S0.1 is the theoretical substitution upon coupling of the preceding residue n-1 (S0 = mmol/g). The estimated value (%) on the extent of an individual coupling is calculated relative to the measured substitution (unless S was not determined) and include correction for the number of remaining free amino groups following the previous cycle. HF reactions were carried out in a Diaflon HF apparatus from Toho Kasei (Osaka, Japan). Vydac C18 (5 µm, 0.46x25 cm and 5 µm, 1.0x25 cm) reverse-phase columns, respectively were used for analytical and semi-preparative HPLC on an SP8000 instrument. Buffer A was 5% acetonitrile in water containing 445 µl trifluoroacetic acid per litre, and buffer B was 60% acetonitrile in water containing 300 µl trifluoroacetic acid per litre. The linear gradient was 0-100% of buffer B in 30 min, flow rate 1.2 ml/min (analytical) and 5 ml/min (semi-preparative). The eluents were monitored at 215 nm (analytical) and 230 nm (semi-preparative). Molecular weights of the PNA were determined by 25Cl plasma desorption time-of-flight mass spectrometry from the mean of the most abundant isotopes.

EXAMPLE 17

Solid-Phase Synthesis of Acr-[Taeg]15—NH2 and Shorter Derivatives

(a) Stepwise Assembly of Boc-(Taeg)15-BHA Resin

[0149] The synthesis was initiated on 100 mg of preswollen and neutralized BHA resin (determined by the quantitative ninhydrin reaction to contain 0.57 mmol NH2/g) employing single couplings (“Synthetic Protocol 1”) using 3.2 equivalents of BocTaeg-OPfp in about 33% DMF/CH2Cl2. The individual coupling reactions were carried out by shaking for at least 2 h in a manually operated 6 ml standard solid-phase reaction vessel and reacted amino groups were blocked by acetylation at selected stages of the synthesis. The progress of chain elongation was monitored at several stages by the quantitative ninhydrin reaction (see Table I). Portions of protected Boc-[Taeg]15-BHA, Boc-[Taeg]10-BHA, and Boc-[Taeg]5-BHA resins were taken out after assembling 5, 10, and 15 residues, respectively.
<table>
<thead>
<tr>
<th>Synthetic Residue</th>
<th>Substitution After Deprotection (nmol/g)</th>
<th>Remaining Free Amino Groups After (nmol/g)</th>
<th>Estimated Extent of Coupling (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mead</td>
<td>Theoreol</td>
<td>Single Coupling</td>
</tr>
<tr>
<td>0°</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.50</td>
</tr>
<tr>
<td>2 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.64</td>
</tr>
<tr>
<td>3 Boc</td>
<td>Taeg</td>
<td>0.29</td>
<td>0.39</td>
</tr>
<tr>
<td>4 Boc</td>
<td>Taeg</td>
<td>0.27</td>
<td>0.35</td>
</tr>
<tr>
<td>5 Boc</td>
<td>Taeg</td>
<td>0.26</td>
<td>0.32</td>
</tr>
<tr>
<td>6 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.30</td>
</tr>
<tr>
<td>7 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.28</td>
</tr>
<tr>
<td>8 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.26</td>
</tr>
<tr>
<td>9 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.24</td>
</tr>
<tr>
<td>10 Boc</td>
<td>Taeg</td>
<td>0.16</td>
<td>0.23</td>
</tr>
<tr>
<td>11 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.21</td>
</tr>
<tr>
<td>12 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.20</td>
</tr>
<tr>
<td>13 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.19</td>
</tr>
<tr>
<td>14 Boc</td>
<td>Taeg</td>
<td>ND</td>
<td>0.18</td>
</tr>
<tr>
<td>15 Boc</td>
<td>Taeg</td>
<td>0.07</td>
<td>0.17</td>
</tr>
</tbody>
</table>

(b) Synthesis of Acr¹-[Taeg]₁₅—BHA Resin

Following deprotection of the residual Boc-[Taeg]₁₅-BHA resin (estimated dry weight is about 30 mg; 7.002 mmol growing chains), the H-[Taeg]₁₅-BHA resin was reacted with about 50 equivalents (80 mg; 0.11 mmol) of Acr¹-OPfp in 1 ml of about 66% DMF/CH₂Cl₂ (i.e., a 0.11 M solution of the pentafluorophenyl ester) in a 3 ml solid-phase reaction vessel. As judged by a qualitative ninhydrin reaction, coupling of the acridine moiety was close to quantitative.

(c) Cleavage, Purification, and Identification of H-[Taeg]₁₅—NH₂

A portion of protected Boc-[Taeg]₁₅-BHA resin was treated with 50% trifluoroacetic acid in methylene chloride to remove the N-terminal Boc group (which is a precursor of the potentially harmful tert-butyl cation) prior to the HF cleavage. Following neutralization and washing (performed in a way similar to those of steps 2-4, in “Synthetic Protocol 1”), and drying for 2 h in vacuum, the resulting 67.1 mg (dry weight) of H-[Taeg]₁₅-BHA resin was cleaved with 5 ml of HF:anisole (9:1, v/v) stirring at 0° C. for 60 min. After removal of HF, the residue was stirred with dry diethyl ether (4×15 ml, 15 min each) to remove anisole, filtered under gravity through a fritted glass funnel, and dried. The PNA was then extracted into a 60 ml (4×15 ml, stirring 15 min each) 10% aqueous acetic acid solution. Aliquots of this solution were analyzed by analytical reverse-phase HPLC to establish the purity of the crude PNA. The main peak at 13.0 min accounted for about 93% of the total absorbance. The remaining solution was frozen and lyophilized to afford about 22.9 mg of crude material. Finally, 19.0 mg of the crude product was purified by five batches, each containing 3.8 mg in 1 ml of H₂O. The main peak was collected by use of a semi-preparative reverse-phase column. Acetonitrile was removed on a speed vac and the residual solution was frozen (dry ice) and subsequently lyophilized to give 13.1 mg of >99% pure H-[Taeg]₁₅—NH₂. The PNA molecule readily dissolved in water and had the correct molecular weight based on mass spectral determination. For (M+H)⁺ the calculated m/z value was 1349.3 and the measured m/z value was 1347.8.

(d) Cleavage, Purification, and Identification of H-[Taeg]₁₀—NR₂

A portion of protected Boc-[Taeg]₁₀-BHA resin was treated as described in section (c) to yield 11.0 mg of crude material upon HF cleavage of 18.9 mg dry H-[Taeg]₁₀-BHA resin. The main peak at 15.5 min accounted for about 53% of the total absorbance. About 1 mg of the crude product was purified repeatedly (for reasons described below) to give approximately 0.1 mg of at least 80% but presumably >99% pure H-[Taeg]₁₀—NH₂. A rather broad tail eluting after the target peak and accounting for about 20% of the total absorbance could not be removed (only slightly reduced) upon the repeated purification. Judged by the mass spectrum, which only confirms the presence of the correct molecular weight H-[Taeg]₁₀—NH₂, the tail phenomena is ascribed to more or less well-defined aggregational/conformational states of the target molecule. Therefore, the crude product is likely to contain more than the above-mentioned 53% of the target molecule. H-[Taeg]₁₀—NH₂ is readily dissolved in water. For (M+H)⁺ the calculated m/z value was 2679.6 and the measured m/z value was 2681.5.

(e) Cleavage, Purification, and Identification of H-[Taeg]₁₅—NH₂

A portion of protected Boc-[Taeg]₁₅-BHA resin was treated as described in section (c) to yield 3.2 mg of crude material upon HF cleavage of 13.9 mg dry H-[Taeg]₁₅-BHA resin. The main peak at 22.6 min was located in a broad bulge accounting for about 60% of the total absorbance (FIG. 12a). Again (see the preceding section), this bulge is ascribed to aggregational/conformational states of the target molecule H-[Taeg]₁₅—NH₂ since mass spectral analysis of the collected “bulge” did not significantly reveal the presence of other molecules. All of the crude product was purified collecting the “bulge” to give approximately 2.8 mg material. For (M+Na)⁺ the calculated m/z value was 4033.9 and the measured m/z value was 4032.9.

(f) Cleavage, Purification, and Identification of Acr¹-[Taeg]₁₅—NH₂

A portion of protected Acr¹-[Taeg]₁₅—BHA resin was treated as described in section (b) to yield 14.3 mg of crude material upon HF cleavage of 29.7 mg dry Acr¹-[Taeg]₁₅—BHA resin. Taken together, the main peak at 23.7 min and a “dimer” (see below) at 29.2 min accounted for about 40% of...
the total absorbance (Fig. 12C). The crude product was purified repeatedly to give approximately 1 mg of presumably >99% pure Acr-[Taeg]₅-NH₂ “contaminated” with self-aggregated molecules eluting at 27.4 min, 29.2 min, and finally as a huge broad bulge eluting with 100% buffer B (Fig. 12C). This interpretation is in agreement with the observation that those peaks grow upon standing (for hours) in aqueous acetic acid solution, and finally precipitate quantitatively. For [M+H]⁺ the calculated m/z value was 4593.6 and the measured m/z value was 4588.7.

(g) Synthetic Protocol

(0155) (1) Boc-deprotection with TFA/CH₂Cl₂ (1:1, v/v), 3 ml, 3x1 min and 1x30 min; (2) washing with CH₂Cl₂, 3 ml, 6x1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 3 ml, 3x2 min; (4) washing with CH₂Cl₂, 3 ml, 6x1 min, and drain for 1 min; (5) 2-5 mg sample of PNA-resin may be taken out and dried thoroughly for a quantitative ninhydrin analysis to determine the substitution; (6) addition of 3.2 equiv. (0.18 mmol; 100 mg) Boc-Taeg-OPfp dissolved in 1 ml CH₂Cl₂, followed by addition of 0.5 ml DMF (final concentration of pentfluorophenylester “0.12 M), the coupling reaction was allowed to proceed for a total of 12-24 h shaking at room temperature; (7) washing with DMF: 3 ml, 1x2 min; (8) washing with CH₂Cl₂, 3 ml, 4x1 min; (9) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 3 ml, 2x2 min; (10) washing with CH₂Cl₂, 3 ml, 6x1 min; (11) 2-5 mg sample of protected PNA-resin is taken out for a rapid qualitative ninhydrin test and further 2-5 mg is dried thoroughly for a quantitative ninhydrin analysis to determine the extent of coupling (after cycles 7, 10, and 15 unreacted amino groups were blocked by acetylation with N-acetylimidazole in methylene chloride).

EXAMPLE 18

Solid-Phase Synthesis of Acr-[Taeg]₅-Lys(NH₂) and Shorter Derivatives

(a) Stepwise Assembly of Boc-[Taeg]₅-Lys(NH₂)-BHA Resin

[0156] The synthesis was initiated by a quantitative loading (standard DCC in situ coupling in neat CH₂Cl₂) of Boc-Lys (CIZ) onto 100 mg of preswollen and neutralized BHA resin (0.57 mmol NH₂/g). Further extension of the protected PNA chain employed single couplings (“Synthetic Protocol 2”) for cycles 1 to 5 and cycles 10 to 15 using 3.2 equivalents of Boc-Taeg-OPfp in about 33% DMF/CH₂Cl₂. Cycles 5 to 10 employed an extra straight DCC (i.e., in situ) coupling of the free acid Boc-Taeg-OH in about 33% DMF/CH₂Cl₂. All coupling reactions were carried out by shaking for at least 12 h in a manually operated 6 ml standard solid-phase reaction vessel. Unreacted amino groups were blocked by acetylation at the same stages of the synthesis, as was done in Example 17. Portions of protected Boc-[Taeg]₅-Lys(CIZ)-BHA and Boc-[Taeg]₅-Lys(CIZ)-BHA resins were taken out after assembling 5 and 10 PNA residues, respectively. As judged by the analytical HPLC chromatogram of the crude cleavage product from the Boc-[Taeg]₅-Lys(CIZ)-BHA resin (see section (e)), an additional “free acid” coupling of PNA residues 5 to 10 gave no significant improvement of the synthetic yield as compared to the throughout single-coupled residues in Example 17.

(b) Synthesis of Acr-[Taeg]₅-Lys(CIZ)-BHA Resin

[0157] Following deprotection of a portion of Boc-[Taeg]₅-Lys(CIZ)-BHA resin (estimated dry weight is about 90 mg; 0.01 mmol growing chains), the H-[Taeg]₅-Lys(BHA resin was reacted with about 20 equivalents (141 mg; 0.19 mmol) of Acr-OPfp in 1 ml of about 661 DMF/CH₂Cl₂ in a 3 ml solid-phase reaction vessel. As judged by a qualitative ninhydrin reaction, coupling of the acridine moiety was close to quantitative.

(c) Synthesis of Acr-[Taeg]₅-Lys(CIZ)-BHA Resin

[0158] Following deprotection of the residual Boc-[Taeg]₅-Lys(CIZ)-BHA resin (estimated dry weight about 70 mg; 0.005 mmol growing chains), the H-[Taeg]₅-Lys(CIZ)-BHA resin was reacted with about 25 equivalents (91 mg; 0.12 mmol) of Acr-OPfp in 1 ml of about 661 DMF/CH₂Cl₂ in a 3 ml solid-phase reaction vessel. As judged by a qualitative ninhydrin reaction, coupling of the acridine moiety was close to quantitative.

(d) Cleavage, Purification, and Identification of H-[Taeg]₅-Lys(NH₂)

[0159] A portion of protected Boc-[Taeg]₅-Lys(CIZ)-BHA resin was treated as described in Example 17c to yield 8.9 mg of crude material upon HF cleavage of 19.0 mg dry H-[Taeg]₅-Lys(CIZ)-BHA resin. The main peak at 12.2 min (eluted at 14.2 min if injected from an aqueous solution instead of the 10% aqueous acetic acid solution) accounted for about 90% of the total absorbance. About 2.2 mg of the crude product was purified to give approximately 1.5 mg of 99% pure H-[Taeg]₅-Lys(NH₂).

(e) Cleavage, Purification, and Identification of H-[Taeg]₅-Lys(NH₂)

[0160] A portion of protected Boc-[Taeg]₅-Lys(CIZ)-BHA resin was treated as described in Example 17c to yield 1.7 mg of crude material upon HF cleavage of 7.0 mg dry H-[Taeg]₅-Lys(CIZ)-BHA resin. The main peak at 15.1 min (eluted at 17.0 min if injected from an aqueous solution instead of the 10% aqueous acetic acid solution) accounted for about 50% of the total absorbance. About 1.2 mg of the crude product was purified to give approximately 0.2 mg of >95% pure H-[Taeg]₅-Lys(NH₂). FIG. 13a, For [M+H]⁺ the calculated m/z value was 2807.8 and the measured m/z value was 2808.2.

(f) Cleavage, Purification, and Identification of Acr-[Taeg]₅-Lys(NH₂)

[0161] 99.1 mg protected Acr-[Taeg]₅-Lys(CIZ)-BHA resin (dry weight) was cleaved as described in Example 17c to yield 42.2 mg of crude material. The main peak at 25.3 min (eluted at 23.5 min if injected from an aqueous solution instead of the 10% aqueous acetic acid solution) accounted for about 45% of the total absorbance. An 8.87 mg portion of the crude product was purified to give approximately 5.3 mg of >95% pure H-[Taeg]₅-Lys(NH₂). For [M+H]⁺ the calculated m/z value was 2850.8 and the measured m/z value was 2849.8.

(g) Cleavage and Purification of Acr-[Taeg]₅-Lys(NH₂)

[0162] A 78.7 mg portion of protected Acr-[Taeg]₅-Lys(CIZ)-BHA resin (dry weight) was cleaved as described in Example 1 section (c) to yield 34.8 mg of crude material. The main peak at 23.5 min (about the same elution time if injected...
from an aqueous solution instead of the 10% aqueous acetic acid solution) and a “dimer” at 28.2 min accounted for about 35% of the total absorbance. About 4.5 mg of the crude product was purified to give approximately 1.6 mg of presumably >95% pure [L-Taeq]_{16}Lys—NH₂. This compound could not be free of the “dimer” peak, which grew upon standing in aqueous acetic acid solution.

(h) Synthetic Protocol 2

(0163) (1) Boc-deprotection with TFA/CH₂Cl₂ (1:1, v/v), 3 ml, 3x1 min and 1x30 min; (2) washing with CH₂Cl₂, 3 ml, 6x1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 3 ml, 3x2 min; (4) washing with CH₂Cl₂, 3 ml, 6x1 min, and drain for 1 min; (5) 2-5 mg sample of PNA-resin can be taken out and dried thoroughly for a qualitative ninhydrin analysis; (6) for cycles 1 to 5 and cycles 10 to 15 the coupling reaction was carried out by addition of 1.5 equiv. (0.18 mmol; 100 mg) BocTaqe-OPip dissolved in 1 ml CH₂Cl₂ followed by addition of 0.5 ml DMF (final concentration of pentfluorophenylester -0.12 M); the coupling reaction was allowed to proceed for a total of 12-24 h with shaking; cycles 5 to 10 employed an additional 0.12 M DCC coupling of 0.12 M BocTaqe-OH in 1.5 ml DMF/CH₂Cl₂ (1:2, v/v); (7) washing with DMF, 3 ml, 1x2 min; (8) washing with CH₂Cl₂, 3 ml, 4x1 min; (9) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 3 ml, 2x2 min; (10) washing with CH₂Cl₂, 3 ml, 6x1 min; (11) 2-5 mg of protected PNA-resin is taken out for a qualitative ninhydrin test (after cycles 7, 10, and 15 unreacted amino groups were blocked by acetylation with N-acetylimidazole in methylene chloride).

EXAMPLE 19

Improved Solid-Phase Synthesis of H-[Taeq]_{16}Lys—NH₂

(0164) The protected PNA was assembled onto an MBHA resin, using approximately half the loading of the BHA resin used in the previous examples. Furthermore, all cycles except one was followed by acetylation of uncoupled amino groups. The following describes the synthesis in full detail:

(a) Preparation of Boc-Lys(CIZ)-NH—CH₃—C₆H₄—C₆H₄ Resin (MBHA Resin) with an Initial Substitution of 0.3 mmol/g

(0165) The desired substitution of Boc-Lys(CIZ)-MBHA resin was 0.25-0.30 mmol/g. In order to get this value, 1.5 mmol of Boc-Lys(CIZ) was coupled to 5.0 g of neutralized and preswollen MBHA resin (determined by the quantitative ninhydrin reaction to contain 0.64 mmol NH₂/g) using a single “in situ” coupling (1.5 mmol of DCC) in 60 ml of CH₂Cl₂. The reaction was carried out by shaking for 3 h in a manually operated, 225 ml, standard, solid-phase reaction vessel. Unreacted amino groups were then blocked by acetylation with a mixture of acetic anhydride/pyridine/CH₂Cl₂ (1:1:2, v/v/v) for 18 h. A quantitative ninhydrin reaction on the neutralized resin showed that only 0.00093 mmol/g free amine remained (see Table I), i.e. 0.1% of the original amino groups. The degree of substitution was estimated by deprotection and ninhydrin analysis, and was found to be 0.32 mmol/g for the neutralized H-Lys(CIZ)-MBHA resin. This compares well with the maximum value of 0.28 mmol/g for a quantitative coupling of 0.30 mmol Boc-Lys(CIZ)/g resin (see Table II).

(b) Stepwise Assembly of Boc-[Taeq]_{16}Lys(CIZ)-MBHA Resin

(0166) The entire batch of H-Lys(CIZ)-MBHA resin prepared in section (a) was used directly (in the same reaction vessel) to assemble Boc-[Taeq]_{16}Lys(CIZ)-MBHA resin by single couplings (“Synthetic Protocol 3”) utilizing 2.5 equivalents of BocTaqe-OPip in neat CH₂Cl₂. The quantitative ninhydrin reaction was applied throughout the synthesis (see Table II).

(c) Stepwise Assembly of Boc-[Taeq]_{16}Lys(CIZ)-MBHA Resin

(0167) About 4.5 g of wet Boc-[Taeq]_{16}Lys(CIZ)-MBHA resin (~0.36 nmol growing chains; taken out of totally ~19 g wet resin prepared in section (b)) was placed in a 55 ml SPPS reaction vessel. Boc-[Taeq]_{16}Lys(CIZ)-MBHA resin was assembled by single couplings (“Synthetic Protocol 4”) utilizing 2.5 equivalents of BocTaqe-OPip in about 30% DMF/CH₂Cl₂. The progress of the synthesis was monitored at all stages by the quantitative ninhydrin reaction (see Table II).

(d) Stepwise Assembly of Boc-[Taeq]_{16}Lys(CIZ)-MBHA Resin

(0168) About 1 g of wet Boc-[Taeq]_{16}Lys(CIZ)-MBHA resin (~0.09 nmol growing chains; taken out of totally ~4 g wet resin prepared in section (c)) was placed in a 20 ml SPPS reaction vessel. Boc-[Taeq]_{16}Lys(CIZ)-MBHA resin was assembled by the single-coupling protocol employed in the preceding section utilizing 2.5 equivalents of BocTaqe-OPip in about 30% DMF/CH₂Cl₂. The reaction volume was 3 ml (vigorous shaking). The synthesis was monitored by the quantitative ninhydrin reaction (see Table II).

<table>
<thead>
<tr>
<th>Synthetic Residue</th>
<th>Substitution After Deprotection (mmol/g)</th>
<th>Remaining Free Amino Groups After (mmol/g)</th>
<th>Estimated Extent of Coupling (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Coupled</td>
<td>Meas</td>
<td>Theor</td>
</tr>
<tr>
<td>&quot;0&quot;</td>
<td>Boc[Lys(CIZ)]</td>
<td>0.32</td>
<td>0.28</td>
</tr>
<tr>
<td>1</td>
<td>BocTaqe</td>
<td>0.23</td>
<td>0.26</td>
</tr>
<tr>
<td>2</td>
<td>BocTaqe</td>
<td>0.21</td>
<td>0.24</td>
</tr>
<tr>
<td>3</td>
<td>BocTaqe</td>
<td>0.19</td>
<td>0.23</td>
</tr>
<tr>
<td>4</td>
<td>BocTaqe</td>
<td>0.18</td>
<td>0.21</td>
</tr>
<tr>
<td>5</td>
<td>BocTaqe</td>
<td>0.17</td>
<td>0.20</td>
</tr>
<tr>
<td>6</td>
<td>BocTaqe</td>
<td>0.15</td>
<td>0.19</td>
</tr>
<tr>
<td>7</td>
<td>BocTaqe</td>
<td>0.11</td>
<td>0.18</td>
</tr>
<tr>
<td>3</td>
<td>BocTaqe</td>
<td>0.12</td>
<td>0.17</td>
</tr>
</tbody>
</table>
(i) Synthetic Protocol 4

[0173] (1) Boc-deprotection with TFA/CH₂Cl₂ (1:1, v/v), 25 ml, 3x1 min and 1x30 min; (2) washing with CH₂Cl₂, 25 ml, 6x1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 25 ml, 3x2 min; (4) washing with CH₂Cl₂, 25 ml, 6x1 min; (5) 2-5 mg sample of PNA-resin is taken out and dried thoroughly for a quantitative ninhydrin analysis to determine the substitution; (6) addition of 2.5 equiv. (0.92 mmol; 9.506 g) Boc-Taeg-OPip dissolved in 6 ml CH₂Cl₂; followed by addition of 3 ml DMF (final concentration of pentfluoroanilolester -0.1 M); the coupling reaction was allowed to proceed for a total of 20-24 hrs with shaking; (7) washing with DMF, 25 ml, 1x2 min; (8) washing with CH₂Cl₂, 25 ml, 4x1 min; (9) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 25 ml, 2x2 min; (10) washing with CH₂Cl₂, 25 ml, 6x1 min; (11) 2-5 mg sample of protected PNA-resin is taken out for a rapid qualitative ninhydrin test and a further 2-5 mg is dried thoroughly for a quantitative ninhydrin analysis to determine the extent of coupling; (12) blocking of unreacted amino groups by acetylation with a 25 ml mixture of acetic anhydride/pyridine/CH₂Cl₂ (1:1:2, v/v/v) for 2 h; (13) washing with CH₂Cl₂, 100 ml, 6x1 min; (14) 2x2-5 mg samples of protected PNA-resin are taken out, neutralized with DIEA/CH₂Cl₂ (1:19, v/v) and washed with CH₂Cl₂ for qualitative and quantitative ninhydrin analyses.

(ii) Synthetic Protocol 3

[0172] (1) Boc-deprotection with TFA/CH₂Cl₂ (1:1, v/v), 100 ml, 3x1 min and 1x30 min; (2) washing with CH₂Cl₂, 100 ml, 6x1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 100 ml, 3x2 min; (4) washing with CH₂Cl₂, 100 ml, 6x1 min and drain for 1 min; (5) 2-5 mg sample of PNA-resin is taken out and dried thoroughly for a quantitative ninhydrin analysis to determine the substitution; (6) addition of 2.5 equiv. (3.75 mmol; 2.064 g) Boc-Taeg-OPip dissolved in 35 ml CH₂Cl₂ (final concentration of pentfluoroanilolester -0.1 M); the coupling reaction was allowed to proceed for a total of 20-24 h with shaking; (7) washing with DMF, 100 ml, 1x2 min (to remove precipitate of Boc-Taeg-OPi); (8) washing with CH₂Cl₂, 100 ml, 4x1 min; (9) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 100 ml, 2x2 min; (10) washing with CH₂Cl₂, 100 ml, 6x1 min; (11) 2-5 mg sample of protected PNA-resin is taken out for a rapid qualitative ninhydrin test and a further 2-5 mg is dried thoroughly for a quantitative ninhydrin analysis to determine the extent of coupling; (12) blocking of unreacted amino groups by acetylation with a 25 ml mixture of acetic anhydride/pyridine/CH₂Cl₂ (1:1:2, v/v/v) for 2 h; (13) washing with CH₂Cl₂, 100 ml, 6x1 min; (14) 2x2-5 mg samples of protected PNA-resin are taken out, neutralized with DIEA/CH₂Cl₂ (1:19, v/v) and washed with CH₂Cl₂ for qualitative and quantitative ninhydrin analyses.

(g) Cleavage, Purification, and Identification of Ac-[Taeg]₁₄₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉₋₋₉すこと
due was single-coupled. Incorporation of the C(Z)aeq-residue was accomplished by coupling with 2.0 equivalents of BenZ(Z)aeq-OPhp in TFE/CH$_2$Cl$_2$ (1:2, v/v). The progress of the synthesis was monitored at all stages by the quantitative ninhydrin reaction (see Table III).

<table>
<thead>
<tr>
<th>Synthetic Residue</th>
<th>Substitution After Deprotection (mol/l)</th>
<th>Remaining Free Amino Groups After (mol/l)</th>
<th>Estimated Extent of Coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Coupled, Meas.</td>
<td>Theoret.</td>
<td>1st Coupl</td>
</tr>
<tr>
<td>3</td>
<td>0.19, 0.23</td>
<td>1.00</td>
<td>0.57</td>
</tr>
<tr>
<td>4</td>
<td>0.17, 0.21</td>
<td>4.88</td>
<td>97.3</td>
</tr>
<tr>
<td>5</td>
<td>0.11, 0.20</td>
<td>70.20</td>
<td>27.98</td>
</tr>
<tr>
<td>6</td>
<td>0.10, 0.19</td>
<td>24.79</td>
<td>4.58</td>
</tr>
<tr>
<td>7</td>
<td>0.09, 0.18</td>
<td>8.55</td>
<td>1.61</td>
</tr>
<tr>
<td>8</td>
<td>0.08, 0.17</td>
<td>6.53</td>
<td>0.80</td>
</tr>
<tr>
<td>9</td>
<td>0.07, 0.16</td>
<td>9.26</td>
<td>3.66</td>
</tr>
<tr>
<td>10</td>
<td>0.07, 0.15</td>
<td>5.32</td>
<td>1.48</td>
</tr>
</tbody>
</table>

(b) Cleavage, Purification, and Identification of H$_2$-[Taeg]$_4$-Caeg-[Taeg]$_4$-Lys-NH$_2$

[0175] A portion of protected Boc-(Taeg)$_4$-Caeg-[Taeg]$_4$-Lys(C12)-BHA resin was treated as described in Example 1 section (c) to yield about 14.4 mg of crude material upon HCl cleavage of 66.9 mg dry H$_2$-[Taeg]$_4$-Caeg-(Taeg-4-Lys(C12)-BHA resin. The main peak at 14.5 min accounted for >50% of the total absorbance. A 100.0 mg portion of the crude product was purified (8 batches; each dissolved in 1 ml H$_2$O) to give approximately 9.1 mg of 95% pure H$_2$-[Taeg]$_4$-Caeg-[Taeg]$_4$-Lys-NH$_2$ (Fig. 136). For (M+H)$^+$ the calculated m/z value=2793.8 and the measured m/z value=2790.6.

EXAMPLE 21
N-Benzylxoxycarbonyl-N-Chinaaminoethylglycine

[0176] Aminoethyl glycine (52.86 g; 0.447 mol) was dissolved in water (500 ml) and dioxane (palladium ether) (1800 ml) was added. The pH was adjusted to 11.2 with 2N NaOH. While the pH was kept at 11.2, tert-butyl-p-nitrophenyl carbonate (128.4 g; 0.537 mol) was dissolved in dioxane (720 ml) and added dropwise over the course of 2 hours. The pH was kept at 11.2 for at least three more hours and then left with stirring overnight. The yellow solution was cooled to 0°C and the pH was adjusted to 3.5 with 2 N HCl. The mixture was washed with chloroform (4x100 ml) and the pH of the aqueous phase was readjusted to 9.5 with 2 N NaOH. The yellow xoxycarbonyl chloride (73.5 ml; 0.515 mol) was added over half an hour, while the pH was kept at 9.5 with 2 N NaOH. The pH was adjusted frequently over the next 4 hours, and the solution was left with stirring overnight. On the following day the solution was washed with ether (3x600 ml) and the pH of the solution was afterwards adjusted to 1.5 with 2 N HCl at 0°C. The title compound was isolated by extraction with ethyl acetate (5x1000 ml). The ethyl acetate solution was dried over magnesium sulfate and evaporated to dryness, in vacuo. This afforded 138 g, which was dissolved in ether (300 ml) and precipitated by the addition of ether (1800 ml). Yield 124.7 g (79%). M.p. 65.4-85°C. Anal. For C$_7$H$_{14}$N$_2$O$_2$: Found (Calc'd): C 58.40 (57.94); H 7.02 (6.86); N 7.94 (7.95). $^1$H-NMR (250 MHz, CDCl$_3$): 7.35 & 7.52 (m, Ph); 5.15 & 5.12 (2H, PhCH$_2$); 4.03 & 4.01 (2H, CH$_2$CO$_2$H); 3.46 (b, 2H, BocNHCH$_2$CH$_3$); 3.28 (b, 2H, BocNHCH$_2$CH$_3$); 1.43 & 1.40 (9H, "Bu). HPLC (260 nm) 20.71 min. (80.2%) and 21.57 min. (19.8%). The UV-spectra (200 nm-300 nm) are identical, indicating that the minor peak consists of Bis-Z-AEG.

EXAMPLE 22
N-Boc-aminooethyl glycine ethyl ester

[0177] N-Benzylxoxycarbonyl-N-Chinaaminoethyl)glycine (60.0 g; 0.170 mol) and N,N-dimethyl-4-aminopyridine (6.00 g) were dissolved in absolute ethanol (500 ml), and cooled to 0°C before the addition of DCC (42.2 g; 0.204 mol). The ice bath was removed after 5 minutes and stirring was continued for 2 more hours. The precipitated DCC (32.5 g) was removed by filtration and washed with ether (3x100 ml). The combined filtrate was washed successively with dilute potassium hydrogen sulfate (2x400 ml), diluted sodium hydrogencarbonate (2x400 ml) and saturated sodium chloride (1x400 ml). The organic phase was filtered, then dried over magnesium sulfate, and evaporated to dryness, in vacuo, which yielded 66.1 g of an oily substance which contained some DCC.

[0178] The oil was dissolved in absolute ethanol (600 ml) and was added 10% palladium on carbon (6.6 g) was added. The solution was hydrogenated at atmospheric pressure, where the reservoir was filled with 2 N sodium hydroxide. After 4 hours, 3.3 L was consumed out of the theoretical 4.2 L. The reaction mixture was filtered through celite and evaporated to dryness, in vacuo, affording 39.5 g (94%) of an oily substance. A 13 g portion of the oily substance was purified by silica gel (600 g SiO$_2$) chromatography. After elution with 300 ml 20% petroleum ether in methylene chloride, the title compound was eluted with 1700 ml of 5% methanol in methylene chloride. The solvent was removed from the fractions with satisfactory purity, in vacuo and the yield was 8.49 g. Alternatively 10 g of the crude material was purified by Kugel Rohr distillation. $^1$H-NMR (250 MHz, CD$_2$OD): 4.77 (b, s, NH); 4.18 (qu, 2H, MeCH$_2$-); 3.38 (6, 2H, NCH$_2$CO$_2$Et); 3.16 (2H, BocNHCH$_2$CH$_3$); 2.68 (2H, BocNHCH$_2$CH$_3$); 1.43 (s, 9H, "Bu) and 1.26 (3H, CH$_3$) $^1$C-NMR 171.4 (COEI); 156.6 (CO); 78.3 (CH$_3$CO$_2$H); 59.9 (CH$_3$); 49.0 (CH$_2$); 48.1 (CH$_2$); 39.0 (CH$_2$); 26.9 (CH$_2$) and 12.6 (CH$_3$).

EXAMPLE 23
N'-Boc-aminooethyl glycine methyl ester

[0179] The above procedure was used, with methanol being substituted for ethanol. The final product was purified by column purification.
EXAMPLE 24
1-(Boc-aeg)thymine ethyl ester

N'-Boc-aminomethyl glycine ethyl ester (13.5 g; 54.8 mmol), DihtOH (9.84 g; 60.3 mmol) and 1-carboxymethyl thymine (11.1 g; 60.3 mmol) were dissolved in DMF (210 ml). Methyline chloride (210 ml) then was added. The solution was cooled to 0° C. in an ethanol/ice bath and DCC (13.6 g; 65.8 mmol) was added. The ice bath was removed after 1 hour and stirring was continued for another 2 hours at ambient temperature. The precipitated DCC was removed by filtration and washed twice with methylene chloride (2 x 75 ml). To the combined filtrate was added more methylene chloride (650 ml). The solution was washed successively with diluted sodium hydrogen carbonate (3 x 500 ml), diluted potassium hydrogen sulfate (2 x 500 ml), and saturated sodium chloride (1 x 500 ml). Some precipitate was removed from the organic phase by filtration. The organic phase was dried over magnesium sulfate and evaporated to dryness, in vacuo. The oily residue was dissolved in methylene chloride (150 ml), filtered, and the title compound was precipitated by the addition of petroleum ether (350 ml) at 0°C. The methylene chloride/petroleum ether procedure was repeated once. This afforded 16.9 g (71%) of a material which was more than 99% pure by HPLC.

EXAMPLE 25
1-(Boc-aeg)thymine

N'-Boc-aminomethyl glycine ethyl ester (5.00 g; 20.3 mmol), DihtOH (3.64 g; 22.3 mmol) and N'-benzoyloxycarbonyl-1-carboxymethyl cytosine (6.77 g; 22.3 mmol) were suspended in DMF (100 ml). Methyline chloride (100 ml) then was added. The solution was cooled to 0° C. and DCC (5.03 g; 24.4 mmol) was added. The ice bath was removed after 2 h and stirring was continued for another hour at ambient temperature. The reaction mixture then was evaporated to dryness, in vacuo. The residue was suspended in ether (100 ml) and stirred vigorously for 30 min. The solid material was isolated by filtration and the ether wash procedure was repeated twice. The material was then washed with diethyl ether (1 x 15 min with dilute sodium hydroxide carbonate (approx. 4% solution, 100 ml), filtered and washed with water. This procedure was then repeated once, which after drying left 17.0 g of yellowish solid material. The solid was then boiled with dioxane (200 ml) and filtered while hot. After cooling, water (200 ml) was added. The precipitated material was isolated by filtration, washed with water, and dried. According to HPLC (observing at 260 nm) this material has a purity higher than 99%, besides the DCC. The ester was then suspended in THF (100 ml), cooled to 0° C., and 1 N LiOH (61 ml) was added. After stirring for 15 minutes, the mixture was filtered and the filtrate was washed with methylene chloride (2 x 150 ml). The alkaline solution then was cooled to 0° C. and the pH was adjusted to 2.0 with 1 N HCl. The title compound was isolated by filtration and was washed once with water, leaving 11.3 g of a white powder after drying. The material was suspended in methylene chloride (300 ml) and petroleum ether (300 ml) was added. Filtration and wash afforded 7.1 g (69%) after drying. HPLC showed a purity of 99% Rf=19.5 min., and a minor impurity at 12.6 min (approx. 1%) most likely the Z-de protected monomer. Anal. for C_{14}H_{18}N_{4}O_{2}. Found (Calc.): C, 54.16 (54.87); H, 5.76 (5.81); N, 13.65 (13.91). 1H-NMR (250 MHz, DMSO-d6): 10.78 (s, 1H, CO2H); 7.88 (2 overlapping doublets, 1H, CH=C=O); 7.41-7.52 (m, 5H, Ph); 7.01 (2 overlapping doublets, 1H, C-H); 6.94 & 6.78 (unres. triplets, 1H, BocN=H); 5.19 (s, 2H, PhCH2); 4.81 & 4.62 (s, 2H, CH2CO2); 4.17 & 3.98 (s, 2H, CH2CO2H); 3.42-3.05 (m, includes water, CH2CH2); and 1.38 & 1.37 (s, 9H, Boc).

EXAMPLE 26
N'-Benzoyloxycarbonyl-1-(Boc-aeg)cytosine

N'-Boc-aminomethyl glycine ethyl ester (5.00 g; 20.3 mmol), DihtOH (3.64 g; 22.3 mmol) and N'-benzoyloxycarbonyl-1-carboxymethyl cytosine (6.77 g; 22.3 mmol) were suspended in DMF (100 ml). Methyline chloride (100 ml) then was added. The solution was cooled to 0° C. and DCC (5.03 g; 24.4 mmol) was added. The ice bath was removed after 2 h and stirring was continued for another hour at ambient temperature. The reaction mixture then was evaporated to dryness, in vacuo. The residue was suspended in ether (100 ml) and stirred vigorously for 30 min. The solid material was isolated by filtration and the ether wash procedure was repeated twice. The material was then washed with diethyl ether (1 x 15 min with dilute sodium hydroxide carbonate (approx. 4% solution, 100 ml), filtered and washed with water. This procedure was then repeated once, which after drying left 17.0 g of yellowish solid material. The solid was then boiled with dioxane (200 ml) and filtered while hot. After cooling, water (200 ml) was added. The precipitated material was isolated by filtration, washed with water, and dried. According to HPLC (observing at 260 nm) this material has a purity higher than 99%, besides the DCC. The ester was then suspended in THF (100 ml), cooled to 0° C., and 1 N LiOH (61 ml) was added. After stirring for 15 minutes, the mixture was filtered and the filtrate was washed with methylene chloride (2 x 150 ml). The alkaline solution then was cooled to 0° C. and the pH was adjusted to 2.0 with 1 N HCl. The title compound was isolated by filtration and was washed once with water, leaving 11.3 g of a white powder after drying. The material was suspended in methylene chloride (300 ml) and petroleum ether (300 ml) was added. Filtration and wash afforded 7.1 g (69%) after drying. HPLC showed a purity of 99% Rf=19.5 min., and a minor impurity at 12.6 min (approx. 1%) most likely the Z-de protected monomer. Anal. for C_{14}H_{18}N_{4}O_{2}. Found (Calc.): C, 54.16 (54.87); H, 5.76 (5.81); N, 13.65 (13.91). 1H-NMR (250 MHz, DMSO-d6): 10.78 (s, 1H, CO2H); 7.88 (2 overlapping doublets, 1H, CH=C=O); 7.41-7.52 (m, 5H, Ph); 7.01 (2 overlapping doublets, 1H, C-H); 6.94 & 6.78 (unres. triplets, 1H, BocN=H); 5.19 (s, 2H, PhCH2); 4.81 & 4.62 (s, 2H, CH2CO2); 4.17 & 3.98 (s, 2H, CH2CO2H); 3.42-3.05 (m, includes water, CH2CH2); and 1.38 & 1.37 (s, 9H, Boc).

EXAMPLE 27
9'-Carboxymethyl adenine ethyl ester

Adenine (10.0 g, 74 mmol) and potassium carbonate (10.29 g, 74.0 mmol) were suspended in DMF and ethyl bromoacetate (8.24 ml, 74 mmol) was added. The suspension was stirred for 2.5 h under nitrogen at room temperature and then filtered. The solid residue was washed three times with DMF (10 ml). The combined filtrate was evaporated to dryness, in vacuo. The yellow-orange solid material was poured into water (200 ml) and 4 N HCl was added to pH=6. After stirring at 0°C for 10 min, the solid was filtered off, washed with water, and recrystallized from 96% ethanol (150 ml). The title compound was isolated by filtration and washed thoroughly with ether. Yield 3.4 g (20%), M.p. 215.5-220°C. Anal. for C_{18}H_{30}N_{4}O_{2}. Found (Calc.): C, 48.86 (48.65); H, 5.01 (4.91); N, 31.66 (31.42). 1H-NMR (250 MHz, DMSO-d6): 7.25 (b, 2H, H-N); 5.06 (s, 2H, NCH2); 4.17 (q, 2H, J=7.11 Hz, OCH2); and 1.21 (t, 3H, J=7.13 Hz, NCH). 13C-NMR: 152.70, 141.30, 61.41, 43.0, and 14.07. FAB-MS: 222 (M+). IR: Frequency in cm^{-1}.
EXAMPLE 28

N°-Benzoyloxycarbonyl-9-carboxymethyl adenine ethyl ester

[0185] 9-Carboxymethyladenine ethyl ester (3.40 g, 15.4 mmol) was dissolved in dry DMF (50 mL) by gentle heating, cooled to 20° C, and added to a solution of N-ethyl-benzoyloxycarbonylmidoazole tetrafluoroborate (62 mmol) in methylene chloride (50 mL) over a period of 15 min with ice-cooling. Some precipitation was observed. The ice bath was removed and the solution was stirred overnight. The reaction mixture was treated with saturated sodium hydrogen carbonate (100 mL). After stirring for 10 min, the phases were separated and the organic phase was washed successively with one volume of water, dilute potassium hydrogen sulfate (twice), and with saturated sodium chloride. The solution was dried over magnesium sulfate and evaporated to dryness, in vacuo, which afforded 11 g of an oily material. The material was dissolved in methylene chloride (25 mL), cooled to 0° C, and precipitated with petroleum ether (50 mL). This procedure was repeated once to give 3.45 g (63%) of the title compound. M.p. 132-133°C. Analysis for C₃₂H₂₅N₅O₄. Found: C, 56.95 (57.46); H, 4.71 (4.82); N, 19.35 (19.71).

1H-NMR (250 MHz, CDCl₃): 8.77 (s, 1H, H-2 or H-8); 7.99 (s, 1H, H-2 or H-8); 7.45-7.26 (m, 5H, Ph); 5.31 (s, 2H, N—C H₂); 4.96 (s, 2H, Ph—CH₂); 4.27 (q, 2H, J=7.15 Hz, CH₃—CH₂) and 1.30 (t, 3H, J=7.15 Hz, CH₃—CH₂). IR: frequency in cm⁻¹ (intensity). 3423 (52.1); 3182 (52.8); 3115 (52.1); 3031 (47.9); 2981 (38.6); 1747 (1.1); 1617 (4.8); 1587 (8.4); 1552 (25.2); 1511 (45.2); 1492 (37.9); 1465 (14.0) and 1413 (37.3).

EXAMPLE 29

N°-Benzoyloxycarbonyl-9-carboxymethyl adenine

[0186] N°-Benzoyloxycarbonyl-9-carboxymethyladenine ethyl ester (3.20 g; 9.01 mmol) was mixed with methanol (50 mL) cooled to 0° C. Sodium Hydroxide Solution (50 mL; 2N) was added, whereby the material quickly dissolved. After 30 min at 0° C, the alkaline solution was washed with methylene chloride (2x50 mL). The aqueous solution was brought to pH 1.0 with 4 N HCl at 0° C, whereby the title compound precipitated. The yield after filtration, washing with water, and drying was 3.08 g (104%). The product contained salt and elemental analysis reflected that. Anal. for C₃₂H₂₅N₅O₄. Found (Calc.): C, 56.32 (55.05); H, 4.24 (4.00); N, 18.10 (21.40) and C/N, 2.57 (2.56). 1H-NMR (250 MHz, DMSO-d₆): 8.70 (s, 2H, H-2 and H-8); 7.50-7.35 (m, 5H, Ph); 5.27 (s, 2H, N—C H₂); and 5.15 (s, 2H, Ph—CH₂). 13C-NMR: 168.77, 152.54, 151.36, 148.75, 145.13, 128.51, 128.17, 127.98, 66.76 and 44.67. IR (KBr): 3484 (18.3); 3109 (15.9); 3087 (15.0); 2966 (17.1); 2927 (19.9); 2383 (53.8); 1960 (62.7); 1739 (2.5); 1688 (5.2); 1655 (0.9); 1594 (1.17); 1560 (12.3); 1530 (26.3); 1499 (30.5); 1475 (10.4); 1455 (14.0); 1429 (24.5) and 1411 (23.6). FAB-MS: 328 (MH+) and 284 (MH+—CO₂). HPLC (215 nm, 260 nm) in system 1: 15.18 min, minor impurities all less than 2%.

EXAMPLE 30

N°-Benzoyloxycarbonyl-1-(Boc-aeg)adenine ethyl ester

[0187] N°-Boc-aminoethyl glycine ethyl ester (2.00 g; 8.12 mmol), DibohOB (1.46 g; 8.93 mmol) and N°-benzoyloxycarbonyl-9-carboxymethyl adenine (2.92 g; 8.93 mmol) were dissolved in DMF (15 mL). Methylene chloride (15 mL) then was added. The solution was cooled to 0° C, in an ethanol/ice bath. DCC (2.01 g; 9.74 mmol) was added. The ice bath was removed after 2.5 h and stirring was continued for another 1.5 hour at ambient temperature. The precipitated DCC was removed by filtration and washed once with DME (15 mL), and twice with methylene chloride (2x15 mL). To the combined filtrate was added more methylene chloride (100 mL). The solution was washed successively with dilute sodium hydrogen carbonate (2x100 mL), dilute potassium hydrogen sulfate (2x100 mL), and saturated sodium chloride (1x100 mL). The organic phase was evaporated to dryness, in vacuo, which afforded 3.28 g (73%) of a yellowish oily substance. HPLC of the raw product showed a purity of only 66% with several impurities, both more and less polar than the main peak. The oil was dissolved in absolute ethanol (50 mL) and activated carbon was added. After stirring for 5 minutes, the solution was filtered. The filtrate was mixed with water (30 mL) and was left with stirring overnight. The next day, the white precipitate was removed by filtration, washed with water, and dried, affording 1.16 g (26%) of a material with a purity higher than 98% by HPLC. Addition of water to the mother liquor afforded another 0.53 g with a purity of approx. 95%. Anal. for C₃₂H₃₅N₅O₃·H₂O. Found (Calc.): C, 55.01 (54.44); H, 6.85 (6.15) and N, 16.47 (17.09). 1H-NMR (250 MHz, CDCl₃): 8.74 (s, 1H, Ade-H-2); 8.18 (s, 1H, ZNH; 8.10 & 8.04 (s, 1H, H-8); 7.46-7.34 (m, 5H, Ph); 5.63 (unres. t, 1H, Boc-CH); 5.30 (s, 2H, Ph—CH₂); 5.16 & 5.00 (s, 2H, C H₂CON); 4.29 & 4.06 (s, 2H, CH₂—CO₂H); 2.92 (q, 2H, OC H₂CH₂); 3.67-3.29 (m, 4H, CH₂—CH₂); 1.42 (s, 9H, Boc) and 1.27 (t, 3H, OCH₂CH₂). The spectrum shows traces of ethanol and DCC.

EXAMPLE 31

N°-Benzoyloxycarbonyl-1-(Boc-aeg)adenine

[0188] N°-Benzoyloxycarbonyl-1-(Boc-aeg)adenine ethyl ester (1.48 g; 2.66 mmol) was suspended in THF (13 mL) and the mixture was cooled to 0° C. Lithium hydroxide (8 mL; 1 N) was added. After 15 min of stirring, the reaction mixture was filtered, extra water (25 mL) was added, and the solution was washed with methylene chloride (2x25 mL). The pH of the aqueous solution was adjusted to pH 2.0 with 1 N HCl. The precipitate was isolated by filtration, washed with water, and dried, and dried affording 0.82 g (58%). The product reprecipitated twice with methylene chloride/petroleum ether, 0.77 g (55%) after drying. M.p. 119° C. (decomp.) Anal. for C₃₂H₃₄N₅O₃·H₂O. Found (Calc.): C, 53.32 (52.84); H, 5.71 (5.73); N, 17.68 (17.97). FAB-MS: 528.5 (MH+). 1H-NMR (250 MHz, DMSO-d₆): 12.75 (very b, 1H, CO₂H); 10.65 (b, 1H, ZNH); 8.59 (d, 1H, J=2.14 Hz, Ade-H-2); 8.31 (s, 1H, Ade-H-8); 7.49-7.31 (m, 5H, Ph); 7.03 & 6.75 (unresol. t, 1H, Boc-NH); 5.33 & 5.16 (s, 2H, CH₂CON); 5.22 (s, 2H, PhC
H₃); 4.34-3.99 (s, 2H, CH₃CO₂H); 3.54-3.03 (m, s, includes water, CH₂CH₂); and 1.39 & 1.37 (s, 9H, βBu). ¹³C-NMR: 170.4; 166.6; 152.3; 151.5; 149.5; 145.2; 128.5; 128.0; 127.9; 66.32; 47.63; 47.03; 43.87 and 28.24.

**EXAMPLE 32**

2-Amino-6-chloro-9-carboxymethylpurine

[0189] To a suspension of 2-amino-6-chloropurine (5.02 g; 29.6 mmol) and potassium carbonate (12.91 g; 93.5 mmol) in DMF (50 ml) was added bromoacetic acid (4.70 g; 22.8 mmol). The mixture was stirred vigorously for 20 h under nitrogen. Water (150 ml) was added and the solution was filtered through Celite to give a clear yellow solution. The solution was acidified to a pH of 3 with 4 N hydrochloric acid. The precipitate was filtered and dried, over vacuo, on silica. Yield (3.02 g; 44.8%). ¹H-NMR (DMSO-d₆): d=4.88 ppm (s, 2H); 6.95 (s, 2H); 8.10 (s, 1H).

**EXAMPLE 33**

2-Amino-6-benzyl oxy-9-carboxymethylpurine

[0190] Sodium (2.0 g; 87.0 mmol) was dissolved in benzyl alcohol (20 ml) and heated to 130°C for 2 h. After cooling to 0°C, a solution of 2-amino-6-chloro-9-carboxymethylpurine (4.05 g; 18.0 mmol) in DMF (85 ml) was slowly added, and the resulting suspension stirred overnight at 20°C. Sodium hydroxide solution (1N, 100 ml) was added and the clear solution was washed with ethyl acetate (3×100 ml). The water phase then was acidified to a pH of 3 with 4 N hydrochloric acid. The precipitate was taken up in ethyl acetate (200 ml), and the water phase was extracted with ethyl acetate (2×100 ml). The combined organic phases were washed with saturated sodium chloride solution (2×75 ml), dried with anhydrous sodium sulfate, and taken to dryness by evaporation, in vacuo. The residue was recrystallized from ethanol (300 ml). Yield after drying, in vacuo, over silica: 2.76 g (52%). M.p. 159-165°C. Anal. (Calc. Found) C(56.18; 55.97), H (4.38; 4.32), N (23.4; 23.10). ¹H-NMR (DMSO-d₆): 4.82 ppm. (s, 2H); 5.51 (s, 2H); 6.45 (s, 2H); 7.45 (m, 5H); 7.82 (s, 1H).

**EXAMPLE 34**


[0191] 2-Amino-6-benzyl oxy-9-carboxymethyl-purine (0.50 g; 1.67 mmol), methyl-N-[2-[tert-butoxycarboxy]-l-phenyl]-l-alanine (0.65 g; 2.80 mmol), diisopropyl-ethyl amine (0.54 g; 4.19 mmol), and bromo-tris-pyridilino-phosphonium-hexahloro-phosphate (PyBroP+) (0.798 g; 1.71 mmol) were stirred in DMF (2 ml) for 4 h. The clear solution was poured into an ice-cooled solution of sodium hydrogen carbonate (1 N; 40 ml) and extracted with ethyl acetate (5×40 ml). The organic layer was washed with potassium hydrogen sulfate solution (1 N; 2x40 ml), sodium hydrogen carbonate (1 N; 1x40 ml) and saturated sodium chloride solution (60 ml). After drying with anhydrous sodium sulfate and evaporation, in vacuo, the solid residue was recrystallized from ethyl acetate/hexane (20 ml:21) to give the methyl ester in 63% yield (MS-FAB 514 (M+1)). Hydrolysis was accomplished by dissolving the ester in ethanol/water (30 ml:1:2) containing conc. sodium hydroxide (1 ml). After stirring for 2 h, the solution was filtered and acidified to a pH of 3, by the addition of 4 N hydrochloric acid. The title compound was obtained by filtration. Yield: 370 mg (72% for the hydrolysis). Purity by HPLC was more than 99%. Due to the limited rotation around the secondary amide several of the signals were doubled in the ratio 2:1 (indicated in the list by mj, for major and mi, for minor). ¹H-NMR (250 MHz, DMSO-d₆): δ=1.4 ppm. (s, 9H); 3.2 (m, 2H); 3.6 (m, 2H); 4.1 (s, mj, CONHCH₂COOH); 4.4 (s, mi, CONRCO); 5.0 (s, mi, Gua-CH₂COOH); 5.2 (s, mi, Gua-CO); 5.6 (s, 2H); 6.5 (s, 2H); 6.9 (m, mi, BocNH); 7.1 (m, mj, BocNH); 7.5 (m, 3H); 7.8 (s, 1H); 12.8 (s, 1H). ¹³C-NMR: 170.95; 170.52; 167.29; 166.85; 150.78; 155.84; 154.87; 140.63; 136.76; 128.49; 128.10; 113.04; 78.19; 77.86; 66.95; 49.22; 47.70; 46.94; 45.96; 43.62; 43.31 and 28.25.

**EXAMPLE 35**

3-Boc-amino-1,2-propanediol

[0192] 3-Amino-1,2-propanediol (40.00 g, 0.440 mol, 1.0 eq.) was dissolved in water (1000 ml) and cooled to 0°C. Di-tert-butyl dicarbonate (115.0 g, 0.526 mol, 1.2 eq.) was added in one portion. The reaction mixture was heated to room temperature on a water bath while stirring. The pH was maintained at 10-15 with a solution of sodium hydroxide (17.5 g, 0.440 mol, 1.0 eq.) in water (120 ml). When the addition of aqueous sodium hydroxide was completed, the reaction mixture was stirred overnight at room temperature. Subsequently, ethyl acetate (750 ml) was added to the reaction mixture, followed by cooling to 0°C. The pH was adjusted to 2.5 with 4 N sulphuric acid with vigorous stirring. The phases were separated and the water phase was washed with additional ethyl acetate (6×50 ml). The volume of the organic phase was reduced to 900 ml by evaporation under reduced pressure. The organic phase was then washed with a saturated aqueous solution of potassium hydrogen sulfate diluted to twice its volume (1×1000 ml) and with saturated aqueous sodium chloride (1×500 ml). The organic phase was dried (MgSO₄) and evaporated under reduced pressure to yield 50.12 g (60%) of the title compound. The product could be solidified by evaporation from methylene chloride and sublimation under vacuum. ¹H-NMR (CDCl₃/TMS): δ=1.43 (s, 9H, Me₂C); 3.25 (m, 2H, CH₂); 3.57 (m, 2H, CH₂); 3.73 (m, 1H, CH); ¹³C-NMR (CDCl₃/TMS): δ=28.2 (Me₂C), 42.6 (CH₂), 63.5, 71.1 (CH₂; CHO₂), 79.5 (Me₂C), 157.0 (C=O).

**EXAMPLE 36**

2-(Boc-amino)ethyl-L-alanine methyl ester

[0193] 3-Boc-amino-1,2-propanediol (20.76 g, 0.109 mol; 1 eq.) was suspended in water (150 ml). Potassium m-periodate (24.97 g, 0.109 mol, 1 eq.) was added and the reaction mixture was stirred for 2 h at room temperature under nitrogen. The reaction mixture was filtered and the water phase extracted with chloroform (6×250 ml) The organic phase was dried (MgSO₄) and evaporated to afford an almost quantitative yield of Boc-aminoacetaldehyde as a colourless oil, which was used without further purification in the following procedure.

[0194] Palladium-on-carbon (10%, 0.8 g) was added to MeOH (250 ml) under nitrogen with cooling to 0°C and vigorous stirring. Anhydrous sodium acetate (4.49 g, 5.07 mmol, 2 eq) and L-alanine methyl ester, hydrochloride (3.82
g. 27.4 mmol, 1 eqv) were added. Boc-aminoacetalddehyde (4.79 g, 30.1 mmol, 1 eqv) was dissolved in MeOH (150 mL) and added to the reaction mixture. The reaction mixture was hydrogenated at atmospheric pressure and room temperature until hydrogen uptake had ceased. The reaction mixture was filtered through celite, which was washed with additional MeOH. The MeOH was removed under reduced pressure. The residue was suspended in water (150 mL) and pH adjusted to 8.0 by dropwise addition of 0.5 N NaOH with vigorous stirring. The water phase was extracted with methylene chloride (4×250 mL). The organic phase was dried (MgSO4), filtered through celite, and evaporated under reduced pressure to yield 6.36 g (94%) of the title compound as a clear, slightly yellow oil. MS (FAB-MS): m/z (%)=247 (100, M+1, 191 (90), 147 (18). 1H-NMR (250 MHz, CDCl3): 1.18 (d, J=7.0 Hz, 3H, Me), 1.36 (s, 9H, MeC), 1.89 (br, 1H, NH), 2.51 (m, 1H, CH2), 2.66 (m, 1H, CH2), 3.10 (m, 2H, CH2), 3.27 (q, J=7.0 Hz, 1H, CH), 3.64 (s, 3H, OMe), 5.06 (br, 1H, carboxylate NH). 13C-NMR: d=18.8 (Me), 28.2 (MeC), 40.1, 47.0 (CH2), 51.6 (OMe), 56.0 (CH), 155.8 (carboxylate C=O), 175.8 (ester C=O).

EXAMPLE 37
N-(Boc-aminoethyl)-N-(1-thymylacetyl)-L-alanine methyl ester

To a solution of Boc-aminoethyl-L-alanine methyl ester (1.23 g, 5.0 mmol) in DMF (10 mL) was added Dib-HOCl (0.90 g, 5.52 mmol) and 1-thymylacetic acid (1.01 g, 5.48 mmol). When the 1-thymylacetic acid was dissolved, dichloromethane (10 mL) was added and the solution was cooled on an ice bath. After the reaction mixture had reached 0°C, DCC (1.24 g, 6.01 mmol) was added. Within 5 min after the addition, a precipitate of DCC was seen. After a further 5 min, the ice bath was removed. Two hours later, TLC analysis showed the reaction to be finished. The mixture was filtered and the precipitate washed with dichloromethane (100 mL). The resulting solution was extracted twice with 5% sodium hydrogen carbonate (150 mL) and twice with saturated potassium hydrogen sulfate (25 mL) in water (100 mL). After a final extraction with saturated sodium chloride (150 mL), the solution was dried with magnesium sulfate and evaporated to give a white foam. The foam was purified by column chromatography on silica gel using dichloromethane with a methanol gradient as eluent. This yielded a pure compound (>99% by HPLC) (1.08 g, 52.4%). FAB-MS: 413 (M+1) and 431 (M+1+water). 1H-NMR (CDCl3): 4.52 (s, 2H, CH2), 3.73 (s, 3H, OMe); 3.2-3.6 (m, 1H, ethyl CH2); 1.90 (s, 3H, Me in T); 1.49 (d, 3H, Me in Ala, J=7.3 Hz); 1.44 (s, 9H, Boc).

EXAMPLE 38
N-(Boc-aminoethyl)-N-(1-thymylacetyl)-L-alanine

The methyl ester of the title compound (2.07 g, 5.02 mmol) was dissolved in methanol (100 mL) and cooled on an ice bath. 2 M sodium hydroxide (100 mL) was added. After stirring for 10 min, the pH of the mixture was adjusted to 3 with 4 M hydrochloric acid. The solution was subsequently extracted with ethyl acetate (3×100 mL). The combined organic extracts were dried over magnesium sulfate. After evaporation, the resulting foam was dissolved in ethyl acetate (400 mL) and a few ml of methanol to dissolve the solid material. Petroleum ether then was added until precipitation started. After standing overnight at -20°C, the precipitate was removed by filtration. This afforded 1.02 g (67.3%) of the title compound, which was 99% pure according to HPLC. FAB-MS: 399 (M+1). 1H-NMR (CDCl3): 11.34 (s, 1H, COOH); 7.42 (s, 1H, CH); 4.69 (s, 2H, CH2); 4.40 (q, 1H, CH in Ala, J=7.20 Hz); 1.83 (s, 3H, Me in T); 1.52-1.40 (m, 12H, Boc+Me in Ala).

EXAMPLE 39
(a) N-(Boc-aminoethyl)-N-(1-thymylacetyl)-D-alanine methyl ester

To a solution of Boc-aminoethyl alanine methyl ester (2.48 g, 10.1 mmol) in DMF (20 mL) was added Dibt-BOC (1.80 g, 11.0 mmol) and thyminalic acid (2.14 g, 11.6 mmol). After dissolution of the 1-thyminalic acid, methylene chloride (20 mL) was added and the solution cooled on an ice bath. When the reaction mixture had reached 0°C, DCC (2.88 g, 14.0 mmol) was added. Within 5 min after the addition a precipitate of DCC was seen. After 5 min the ice bath was removed. The reaction mixture was filtered 3.5 h later and the precipitate washed with methylene chloride (200 mL). The resulting solution was extracted twice with 5% sodium hydrogen carbonate (200 mL) and twice with saturated potassium hydrogen sulfate in water (100 mL). After a final extraction with saturated sodium chloride (250 mL), the solution was dried with magnesium sulfate and evaporated to give an oil. The oil was purified by short column silica gel chromatography using methylene chloride with a methanol gradient as eluent. This yielded a compound which was 96% pure according to HPLC (1.05 g, 25.3%) after precipitation with petroleum ether. FAB-MS: 413 (M+1). 1H-NMR (CDCl3): 5.64 (t, 1H, Boc-NH-J=5.89 Hz); 4.56 (d, 2H, CH2); 4.35 (q, 1H, CH in Ala, J=7.25 Hz); 3.74 (s, 3H, OMe); 3.64-3.27 (m, 4H, ethyl H2); 1.90 (s, 3H, Me in T); 1.52-1.44 (t, 12H, Boc+Me in Ala).

(b) N-(Boc-aminoethyl)-N-(1-thymylacetyl)-D-alanine

The methyl ester of the title compound (1.57 g, 3.81 mmol) was dissolved in methanol (100 mL) and cooled on an ice bath. Sodium hydroxide (100 mL, 2 M) was added. After stirring for 10 min the pH of the mixture was adjusted to 3 with 4 M hydrochloric acid. The solution then was extracted with ethyl acetate (3×100 mL). The combined organic extracts were dried over magnesium sulfate. After evaporation, the oil was dissolved in ethyl acetate (200 mL). Petroleum ether was added (to a total volume of 600 mL) until precipitation started. After standing overnight at -20°C, the precipitate was removed by filtration. This afforded 1.02 g (67.3%) of the title compound, which was 94% pure according to HPLC. FAB-MS: 399 (M+1). 1H-NMR: 11.34 (s, 1H, COOH); 7.42 (s, 1H, CH); 4.69 (s, 2H, CH2); 4.40 (q, 1H, CH in Ala, J=7.20 Hz); 1.83 (s, 3H, Me in T); 1.52-1.40 (m, 12H, Boc+Me in Ala).

EXAMPLE 40
N-(N'-Boc-3'-aminopropyl)-N-(1-thymylacetyl) glycine methyl ester

N-(N'-Boc-3'-aminopropyl)glycine methyl ester (2.84 g, 0.0115 mol) was dissolved in DMF (35 mL), followed by addition of Dibt-BOC (2.07 g, 0.0127 mol) and 1-thyminalic acid (2.34 g, 0.0127 mol). Methylen chloride (35 mL) was added and the mixture cooled to 0°C on an ice bath.
After addition of DCC (2.85 g, 0.0138 mol), the mixture was stirred at 0°C for 2 h, followed by 1 h at room temperature. The precipitated DCU was removed by filtration, washed with methylene chloride (25 ml), and a further amount of methylene chloride (150 ml) was added to the filtrate. The organic phase was extracted with sodium hydrogen carbonate (1 volume saturated diluted with 1 volume water, 6x250 ml), potassium sulfate (1 volume saturated diluted with 4 volumes water, 3x250 ml), and saturated aqueous sodium chloride (1x250 ml), dried over magnesium sulfate, and evaporated to dryness in vacuo. The solid residue was suspended in methylene chloride (35 ml) and stirred for 1 h. The precipitated DCU was removed by filtration and washed with methylene chloride (25 ml). The filtrate was evaporated to dryness, in vacuo, and the residue purified by column chromatography on silica gel, eluting with a mixture of methanol and methylene chloride (gradient from 3:7% methanol in methylene chloride). This afforded the title compound as a white solid (3.05 g, 64%). M.p. 76-79°C. (decomp.). Anal. for C14H20O3S: Found (Calc.) C, 52.03 (52.42) H, 6.90 (6.84) N, 13.21 (13.58). The compound showed satisfactory 1H and 13C-NMR spectra.

**EXAMPLE 44**

N-[(N-Boc-3-aminopropyl)-N-[(1-thymyl)acetyl] glycine


[0204] N-[(N-Boc-aminooethyl)glycine ethyl ester (1.0 g, 0.0041 mol) was dissolved in DMF (12 ml). Dibutylamine (0.73 g, 0.0045 mol) and 3-(1-thymyl)propanoic acid (0.89 g, 0.0045 mol) were added. Methylene chloride (12 ml) was then added and the mixture was cooled to 0°C on an ice bath. After addition of DCC (1.01 g, 0.0049 mol), the mixture was stirred at 0°C for 2 h, followed by 1 h at room temperature. The precipitated DCU was removed by filtration, washed with methylene chloride (25 ml), and a further amount of methylene chloride (50 ml) was added to the filtrate. The organic phase was extracted with sodium hydrogen carbonate (1 volume saturated diluted with 1 volume water, 6x100 ml), potassium sulfate (1 volume saturated diluted with 4 volumes water, 3x100 ml), and saturated aqueous sodium chloride (1x100 ml), dried over magnesium sulfate, and evaporated to dryness in vacuo. The solid residue was suspended in methylene chloride (15 ml), and stirred for 1 h. The precipitated DCU was removed by filtration and washed with methylene chloride. The filtrate was evaporated to dryness, in vacuo, and the residue purified by column chromatography on silica gel, eluting with a mixture of methanol and methylene chloride (gradient from 1 to 6% methanol in methylene chloride). This afforded the title compound as a white solid (1.02 g, 59%). Anal. for C15H28N2O2. Found (Calc.) C, 53.15 (53.51) H, 6.90 (7.09) N, 12.76 (13.13). The compound showed satisfactory 1H and 13C-NMR spectra.

**EXAMPLE 45**

N-[(N-Boc-aminooethyl)-N-[(1-thymyl)propanoyl] glycine

[0205] N-[(N-Boc-aminooethyl)-N-[(1-thymyl)propanoyl]glycine ethyl ester (0.83 g, 0.00195 mol) was dissolved in methanol (25 ml). Sodium hydroxide (25 ml; 2 M) was added. The solution was stirred for 1 h. The methanol was removed by evaporation, in vacuo, and the pH adjusted to 2 with 4 M hydrochloric acid at 0°C. The product was isolated as white crystals by filtration, washed with water (3x10 ml), and dried over scopant, in vacuo. Yield 0.769 g (99%). M.p. 213°C. (decomp.).

**EXAMPLE 46**

Mono-Boc-ethylenediamine (2)

[0206] tert-Butyl-4-nitrophenyl carbonate (1) (10.0 g, 0.0418 mol) dissolved in DMF (50 ml) was added dropwise over a period of 30 min to a solution of ethylenediamine (27.9 ml; 0.418 mol) and DMF (50 ml) and stirred overnight. The mixture was evaporated to dryness, in vacuo, and the resulting oil dissolved in water (250 ml). After cooling to 0°C, pH was adjusted to 3.5 with 4 M hydrochloric acid. The solution then was filtered and extracted with chloroform (3x250 ml). The pH was adjusted to 12 at 0°C with 2 M sodium hydroxide, and the aqueous solution extracted with methylene chloride (3x50 ml). After treatment with sat. aqueous sodium chlo-
ride (250 ml), the methylene chloride solution was dried over magnesium sulfate. After filtration, the solution was evaporated to dryness, in vacuo, resulting in 4.22 g (63%) of the product (oil). $^1$H-NMR (90 MHz; CDCl$_3$): δ 1.14 (s, 9H); 2.87 (t, 2H); 3.1 (q, 2H); 3.62 (s, broad).

EXAMPLE 47
(N-Boc-aminoethyl)-β-alanine methyl ester, HCl

[0207] Mono-Boc-ethylenediamine (2) (16.28 g; 0.102 mol) was dissolved in acetonitrile (400 ml) and methyl acrylate (91.50 ml; 1.02 mol) was transferred to the mixture with acetonitrile (200 ml). The solution was refluxed overnight under nitrogen in the dark to avoid polymerization of methyl acrylate. After evaporation to dryness, in vacuo, a mixture of water and ethanol (200+200 ml) was added, and the solution was filtered and vigorously stirred. The aqueous phase was extracted one more time with ether and then freeze dried to yield solid acetate. Recrystallization from ethyl acetate yielded 13.09 g (46%) of the title compound. M.p. 138-140°C. Anal. for C$_4$H$_8$N$_2$O$_2$: C 58.98; H 8.65; N 12.13. Found: C 58.87; H 8.60; N 12.11.

EXAMPLE 48
N-[1-Thyminylacetyl]-N'-Boc-aminoethylyl-β-alanine methyl ester

[0208] (N-Boc-aminoethyl)-β-alanine methyl ester, HCl (3) (2.0 g; 0.0071 mol) and 1-thyminalcetic acid pentfluorophenyl ester (5) (2.82 g; 0.00812 mol) were dissolved in DMF (50 ml). Triethyl amine (1.12 ml; 0.00812 mol) was added and the mixture stirred overnight. After addition of methylene chloride (200 ml) the organic phase was extracted with aqueous sodium hydrogen carbonate (3x250 ml), half-sat. aqueous potassium hydrogen sulfate (3x250 ml), and sat. aqueous sodium chloride (250 ml) and dried over magnesium sulfate. Filtration and evaporation to dryness, in vacuo, resulted in 2.9 g (99%) yield (oil). $^1$H-NMR (250 MHz; CDCl$_3$); due to limited rotation around the secondary amide several of the signals were doubled: δ 1.35 (s, 9H); 2.9 (m, 8H); 3.64 (s, 3H).

EXAMPLE 49
N-[1-Thyminylacetyl]-N'-Boc-aminoethylyl-β-alanine

[0209] N-[1-Thyminylacetyl]-N'-Boc-aminoethylyl-β-alanine methyl ester (3.0 g; 0.0073 mol) was dissolved in 2 M sodium hydroxide (30 ml), the pH adjusted to 2 at 0°C, with 4 M hydrochloric acid, and the solution stirred for 2 h. The precipitate was isolated by filtration, washed three times with cold water, and dried over saccharine, in vacuo. Yield 2.23 g (77%). M.p. 170-176°C. Anal. for C$_{16}$H$_{12}$O$_3$N, H$_2$O: Found (Calc.) C 49.49 (49.03) H 6.31 (6.78) N 13.84 (13.45). $^1$H-NMR (90 MHz; DMSO-d$_6$): δ 1.48 (s, 9H); 1.76 (s, 3H); 2.44 and 3.29 (m, 8H); 4.55 (s, 2H); 7.3 (s, 1H); 11.23 (s, 1H). FAB-MS: 399 (M+1).

EXAMPLE 50
N-[1-(N$_3$-Z)-cytosyl]acetyl]-N'-Boc-aminoethyl-β-alanine methyl ester

[0210] (N-Boc-aminoethyl)-β-alanine methyl ester, HCl (3) (2.0 g; 0.0071 mol) and 1-(N$_3$-Z)-cytosylacetic acid pentfluorophenyl ester (5) (3.319 g; 0.0071 mol) were dissolved in DMF (50 ml). Triethylamine (0.99 ml; 0.0071 mol) was added and the mixture stirred overnight. After addition of methylene chloride (200 ml), the organic phase was extracted with aqueous sodium hydrogen carbonate (3x250 ml), half-sat. aqueous potassium hydrogen sulfate (3x250 ml), and sat. aqueous sodium chloride (250 ml), and dried over magnesium sulfate. Filtration and evaporation to dryness, in vacuo, resulted in 3.36 g of solid compound which was recrystallized from methanol. Yield 2.42 g (64%). M.p. 158-161°C. Anal. for C$_{25}$H$_{33}$N$_5$O$_3$: Found (Calc.) C 55.19 (56.49) H 6.19 (6.26) N 12.86 (13.18). $^1$H-NMR (250 MHz; CDCl$_3$); due to limited rotation around the secondary amide several of the signals were doubled: δ 1.43 (s, 9H); 2.57 (t, 1H); 3.60-3.23 (m, 6H); 3.60 (s, 1.5H); 3.66 (s, 1.5H); 4.80 (s, 1H); 4.88 (s, 1H); 5.20 (s, 2H); 7.80-7.25 (m, 7H). FAB-MS: 532 (M+1).

EXAMPLE 51
N-[1-(N$_3$-Z)-cytosyl]acetyl]-N'-Boc-aminoethyl-β-alanine

[0211] N-[1-(N$_3$-Z)-cytosyl]acetyl]-N'-Boc-aminoethyl-β-alanine methyl ester (0.621 g; 0.0012 mol) was dissolved in M sodium hydroxide (8.5 ml) and stirred for 2 h. Subsequently, pH was adjusted to 2 at 0°C, with 4 M hydrochloric acid and the solution stirred for 2 h. The precipitate was isolated by filtration, washed three times with cold water, and dried over saccharine, in vacuo. Yield 0.326 g (54%). The white solid was recrystallized from 2-propanol and washed with petroleum ether. M.p. 165°C. (decomp.). Anal. for C$_{26}$H$_{33}$N$_5$O$_3$: Found (Calc.) C 54.09 (54.03) H 6.19 (6.78) N 13.84 (13.45). $^1$H-NMR (250 MHz; CDCl$_3$); due to limited rotation around the secondary amide several of the signals were doubled: δ 1.40 (s, 9H); 2.57 (t, 1H); 2.65 (t, 1H); 3.60-3.32 (m, 6H); 4.85 (s, 1H); 4.98 (s, 1H); 5.21 (s, 2H); 5.71 (s, 1H, broad); 7.99-7.25 (m, 7H). FAB-MS: 518 (M+1).

EXAMPLE 52
Example of a PNA-Oligomer with a Guanine Residue

(a) Solid-Phase Synthesis of H-[Taeg]$_4$-[Gaeg]-[Taeg]$_3$-Lys-NH$_2$

[0212] The protected PNA was assembled onto a Boc-Lys (CIZ) modified MBHA resin with a substitution of approximately 0.15 mmol/g (determined by quantitative Ninhydrin reaction). Capping of uncoupled amino groups was only carried out before the incorporation of the BocGaeg-OH monomer.

(b) Stepwise Assembly of H-[Taeg]$_4$-[Gaeg]-[Taeg]$_3$-Lys-NH$_2$ (synthetic protocol)

[0213] Synthesis was initiated on 102 mg (dry weight) of pressurized polyurethane (overnight in DCM) and neutralized Boc-Lys (CIZ)-MBHA resin. The steps performed were as follows: (1) Boc-deprotection with TFA/DCM (1:1, v/v), 1x2 min and 1x1/2h, 3 ml; (2) washing with DCM, 4x20 sec, 3 ml; washing with DMF, 2x20 sec, 3 ml; washing with DCM, 2x30 sec, 3 ml, and drain for 30 sec; (3) neutralization with DIEA/DCM (1:19 v/v), 2x3 min, 3 ml; (4) washing with DCM, 4x20 sec, 3 ml, and drain for 1 min; (5) addition of 4 equiv. disopropylcarbodiimide (0.06 mmol; 9.7 μl) and equiv. (0.06 mmol; 24
mg) Boc-Taeg-OH or (0.06 mmol; 30 mg) Boc-Aeeg-OH dissolved in 0.6 ml DCM/DMF (1:1, v/v) (final concentration of monomer 0.1 M), the coupling reaction was allowed to proceed for 1/2 h shaking at room temperature; (6) suction was applied for 20 seconds (7) washing with DMF, 2x20 sec and 1x2 min; 3 ml; washing with DCM 4x20 sec, 3 ml; (8) neutralization with DIEA/DMC (1:19 v/v), 2x3 min, 3 ml; (9) washing with DCM 4x20 sec, 3 ml, and drain for 1 min; (10) qualitative Kaiser test; (11) blocking of unreacted amino groups by acetylation with Ac₂O/pyridine/DCM (1:1:2, v/v, 1x1/2 h, 3 ml; and (12) washing with DCM, 4x20 sec, 2x2 min and 2x20 sec, 3 ml. Steps 1-12 were repeated until the desired sequence was obtained. All qualitative Kaiser tests were negative (straw-yellow colour with no coloration of the beads) indicating near 100% coupling yield. The PNA-oligonucleotide was cleaved and purified by the normal procedure. FAB-MS: 2832.11 [M*+1] (Calc: 2832.15)

**EXAMPLE 54**

Solid-Phase Synthesis of H-Taeg-Aeeg-[Taeg]₂-Lys-NH₂

(a) Stepwise Assembly of Boc-[Taeg]₂-A(Z)aeeg-[Taeg]₂-Lys(CIZ)-MBHA Resin

[0214] About 0.3 g of wet Boc-[Taeg]₂-Lys(CIZ)-MBHA resin was placed in a 3 ml SPPS reaction vessel. Boc-Taeg-A(Z)aeeg-[Taeg]₂-Lys(CIZ)-MBHA resin was assembled by in situ DCC coupling (single) of the A(Z)aeeg residue utilizing 0.19 M of Boc-A(Z)aeeg-OH together with 0.15 M DCC in 2.5 ml 50% DMF/CH₂Cl₂ and a single coupling with 0.15 M Boc-OPbP in neat CH₂Cl₂ (“Synthetic Protocol 5”). The synthesis was monitored by the quantitative ninhydrin reaction, which showed about 50% incorporation of A(Z)aeeg and about 96% incorporation of Taeg.

(b) Cleavage, Purification, and Identification of H-Taeg-Aeeg-[Taeg]₂-Lys-NH₂

[0215] The protected Boc-Taeg-A(Z)aeeg-[Taeg]₂-Lys(CIZ)-BAH resin was treated as described in Example 40c to yield about 15.6 mg of crude material upon HF cleavage of 53.1 mg dry H-Taeg-A(Z)aeeg-[Taeg]₂-Lys(CIZ)-BAH resin. The main peak at 14.4 min accounted for less than 50% of the total absorbance. A 0.5 mg portion of the crude product was purified to give approximately 0.1 mg of H-Taeg-Aeeg-[Taeg]₂-Lys-NH₂. For (MH⁺) the calculated m/z value was 2816.16 and the measured m/z value was 2816.28.

(c) Synthetic Protocol 5

[0216] (1) Boc-deprotection with TFA/CH₂Cl₂ (1:1, v/v), 2.5 ml, 3x1 min and 1x30 min; (2) washing with CH₂Cl₂, 2.5 ml, 6x1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 2.5 ml, 3x2 min; (4) washing with CH₂Cl₂, 2.5 ml, 6x1 min, and drain for 1 min; (5) 2-5 mg sample of PNA-resin is taken out and dried thoroughly for a quantitative ninhydrin analysis to determine the substitution; (6) addition of 0.47 mmol (0.25 g) Boc-A(Z)aeeg-OH dissolved in 1.25 ml DCM followed by addition of 0.47 mmol (0.1 g) DCC in 1.25 ml CH₂Cl₂ or 0.36 mmol (0.20 g) Boc-Taeg-OPbP in 2.5 ml CH₂Cl₂; the coupling reaction was allowed to proceed for a total of 20-24 hrs shaking; (7) washing with DMF, 2.5 ml, 1x2 min; (8) washing with CH₂Cl₂, 2.5 ml, 4x1 min; (9) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 2.5 ml, 2x2 min; (10) washing with CH₂Cl₂, 2.5 ml, 6x1 min; (11) 2-5 mg sample of protected PNA-resin is taken out and dried thoroughly for a quantitative ninhydrin analysis to determine the extent of coupling; (12) blocking of unreacted amino groups by acetylation with a 25 ml mixture of acetic anhydride/pyridine/CH₂Cl₂ (1:1:2, v/v/v) for 2 h (except after the last cycle); and (13) washing with CH₂Cl₂, 2.5 ml, 6x1 min; (14) 2-5 mg samples of protected PNA-resin are taken out, neutralized with DIEA/CH₂Cl₂ (1:19, v/v) and washed with CH₂Cl₂ for ninhydrin analyses.

**EXAMPLE 54**

Solid-Phase Synthesis of R—[Taeg]₂-Aeeg-[Taeg]₂-Lys-NH₂

(a) Stepwise Assembly of Boc-[Taeg]₂-A(Z)aeeg-[Taeg]₂-Lys(CIZ)-MBHA Resin

[0217] About 0.5 g of wet Boc-[Taeg]₂-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]₂-A(Z)aeeg-[Taeg]₂-Lys(CIZ)-MBHA resin was assembled by in situ DCC coupling of both the A(Z)aeeg and the Taeg residues utilising 0.15 M to 0.2 M of protected PNA monomer (free acid) together with an equivalent amount of DCC in 2 ml neat CH₂Cl₂ (“Synthetic Protocol 6”). The synthesis was monitored by the quantitative ninhydrin reaction which showed a total of about 82% incorporation of A(Z)aeeg after coupling three times (the first coupling gave about 50% incorporation; a fourth HOBT-mediated coupling in 50% DMF/CH₂Cl₂ did not increase the total coupling yield significantly) and quantitative incorporation (single couplings) of the Taeg residues.

(b) Cleavage, Purification, and Identification of H-[Taeg]₂-Aeeg-[Taeg]₂-Lys-NH₂

[0218] The protected Boc-[Taeg]₂-A(Z)aeeg-[Taeg]₂-Lys(CIZ)-BAH resin was treated as described in Example 40c to yield about 16.2 mg of crude material upon HF cleavage of 102.5 mg dry H-[Taeg]₂-A(Z)aeeg-[Taeg]₂-Lys(CIZ)-BAH resin. A small portion of the crude product was purified. For (MH⁺)* the calculated m/z value was 2050.85 and the measured m/z value was 2050.90.

(c) Synthetic Protocol 6

[0219] (1) Boc-deprotection with TFA/CH₂Cl₂ (1:1, v/v), 2 ml, 3x1 min and 1x30 min; (2) washing with CH₂Cl₂, 2 ml, 6x1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 2 ml, 3x2 min; (4) washing with CH₂Cl₂, 2 ml, 6x1 min, and drain for 1 min; (5) 2-5 mg sample of PNA resin was taken out and dried thoroughly for a quantitative ninhydrin analysis to determine the substitution; (6) addition of 0.44 mmol (0.23 g) Boc-A(Z)aeeg-OH dissolved in 1.5 ml CH₂Cl₂, followed by addition of 0.44 mmol (0.09 g) DCC in 0.5 ml CH₂Cl₂ or 0.33 mmol (0.13 g) Boc-Taeg-OH in 1.5 ml CH₂Cl₂, followed by addition of 0.33 mmol (0.07 g) DCC in 0.5 ml CH₂Cl₂; the coupling reaction was allowed to proceed for a total of 20-24 hrs with shaking; (7) washing with DMF, 2 ml, 1x2 min; (8) washing with CH₂Cl₂, 2 ml, 4x1 min; (9) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 2 ml, 2x2 min; (10) washing with CH₂Cl₂, 2 ml, 6x1 min; (11) 2-5 mg sample of protected PNA resin is taken out and dried thoroughly for a quantitative ninhydrin analysis to determine the extent of coupling; (12) blocking of unreacted amino groups by acetylation with a 25 ml mixture of acetic anhydride/pyridine/CH₂Cl₂ (1:1:2, v/v/v) for 2 h (except after the last cycle); (13) washing with
EXAMPLE 55

[0220] An example with a “no base” substitution.

![Image of chemical structure]

<table>
<thead>
<tr>
<th>DNA</th>
<th>PNA</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>d(A)_{10}</td>
<td>H-T_{10}-LysNH_{2}</td>
<td>73°C</td>
</tr>
<tr>
<td>d(A)_{10}</td>
<td>H-T_{10}(Ac)T_{5}-LysNH_{2}</td>
<td>49°C</td>
</tr>
<tr>
<td>d(A)_{4}(G)</td>
<td>H-T_{4}(Ac)T_{5}-LysNH_{2}</td>
<td>37°C</td>
</tr>
<tr>
<td>d(A)_{4}(D)</td>
<td>H-T_{4}(Ac)T_{5}-LysNH_{2}</td>
<td>41°C</td>
</tr>
<tr>
<td>d(A)_{4}(D)</td>
<td>H-T_{4}(Ac)T_{5}-LysNH_{2}</td>
<td>41°C</td>
</tr>
<tr>
<td>d(A)_{5}(G)</td>
<td>H-T_{5}(Ac)T_{5}-LysNH_{2}</td>
<td>36°C</td>
</tr>
<tr>
<td>d(A)_{5}(D)</td>
<td>H-T_{5}(Ac)T_{5}-LysNH_{2}</td>
<td>40°C</td>
</tr>
</tbody>
</table>

[0221] Thus it can be seen that compared with H-T_{10}-LysNH_{2}, replacement of one thymine ligand by H results in a drop of Tm to 48°C from 73°C. The effect of also introducing a single base mismatch is also shown.

[0222] Certain biochemical/biological properties of PNA oligomers are illustrated by the following experiments.

1. Sequence Discrimination at the dsDNA Level (Example 63, FIG. 20).

[0223] Using the S_nuclease probing technique, the discrimination of binding of the T_{10}T_{5}CT_{4}(T_{5}C) & T_{5}CT_{4}T_{5}(T_{5}C) PNA to the recognition sequences A_{10}, A_{9}GA_{4}(A_{9}G) & A_{9}GA_{4}GA_{4}(A_{9}G) cloned into the BamHI site of plasmid pUC19 was analyzed. The results (FIG. 20) show that the three PNAS bind to their respective recognition sequences with the following relative efficiencies: PNA-T_{10} \rightarrow A_{10}, A_{9}G \rightarrow A_{9}G, PNA-T_{5}C: A_{9}G \rightarrow A_{9}G \rightarrow A_{9}G, PNA-T_{5}C: A_{9}G \rightarrow A_{9}G \rightarrow A_{9}G. Thus at 37°C one mismatch out of ten gives reduced efficiency (5-10 times estimated) whereas mismatches are not accepted.

2. Displacement of a Single Strand DNA from a ds-DNA by Hybridisation of PNAS T_{10}/T_{5}C/T_{5}C (FIG. 20)—Example 63.

3. Kinetics of PNA-T_{10}→dsDNA Strand Displacement Complex Formation (Example 64, FIG. 21).

[0224] Complex formation was probed by S_nuclease at various times following mixing of PNA and 32P-end-labelled dsDNA (FIG. 21).

4. Stability of PNA-dsDNA Complex (Example 65, FIG. 22).

[0225] Complexes between PNA-T_{10} and 32P-dsDNA (A_{10}/T_{10}) target were formed (60 min, 37°C). The complexes were then incubated at the desired temperature in the presence of excess oligo-dA_{10} for 10 min, cooled to RT and probed with KMnO_{4}. The results (FIG. 22) show that the thermal stability of the PNA-dsDNA complexes mirror that of the PNA oligonucleotide complexes in terms of “Tm”.

5. Inhibition of Restriction Enzyme Cleavage by PNA (Example 64, FIG. 23).

[0226] The plasmid construct, pT10, contains a d(A)_{9}dT_{10} tract cloned into the BamHI site in pUC19. Thus, cleavage of pT10 with BamHI and PvuII results in two small DNA fragments of 211 and 111 bp, respectively. In the presence of PNA-T_{10}, a 336 bp fragment is obtained corresponding to cleavage only by PvuII (FIG. 23). This cleavage by BamHI is inhibited by PNA bound proximal to the restriction enzyme site. The results also show that the PNA-dsDNA complex can be formed in 100% yield. Similar results were obtained using the pT8C2 plasmid and PNA-T8C2.

6. Binding of 125I-Labeled PNA to Oligonucleotides (Example 63, FIG. 24).

[0227] A Tyr-PNA-T_{10}-Lys-NH_{2} was labeled with 125I using Na125I and chloramine-T and purified by HPLC. The 125I-PNA-T_{10} was shown to bind to oligo-dA_{10} by PAGE and autoradiography (FIG. 24). The binding could be competed by excess denatured calf thymus DNA.

[0228] Reverting to point (1) above, the sequence-specific recognition of dsDNA is illustrated by the binding of a PNA, consisting of 10 thymine substituted 2-aminoethylglycyl units, which C-terminals in a lysine amide and N-terminals in a complex 9-aminoacridine ligand (9-Acr-1(Taq)_{7}Lys-NH_{2}, FIGS. 1I and b) to a d(A)_{9}dT_{10} target sequence. The target is contained in a 248 bp 32P-end-labelled DNA fragment.

[0229] Strand displacement was ascertained by the following type of experiments:

[0230] 1) The 9-Acr-1 ligand (FIG. 5), which is equipped with a 4-nitrobenzazido group to ensure cleavage of DNA upon irradiation, is expected only to cleave DNA in close proximity to its binding site. Upon irradiation of the DNA with the above 248 bp DNA fragment, selective cleavage at the d(A)_{9}dT_{10} sequence is observed (FIG. 3a).

[0231] 2) In a so-called photofootprinting assay, where a synthetic diazo-linked acridine under irradiation cleaves DNA upon interaction with DNA (except where the DNA is protected by said binding substance).

[0232] Such an experiment was performed with the above 248 bp dsDNA fragment, which showed clear protection against pho-toeleving of the PNA binding site (FIG. 3b).

[0233] 3) In a similar type of experiment, the DNA-cleaving enzyme micrococcal nuclease, which is also hindered in its action by most DNA-binding reagents, showed increased cleavage at the T_{10} target (FIG. 3c).
4) In yet another type of experiment, the well-known high susceptibility of single strand thymine ligands (as opposed to double strand thymine ligands) towards potassium permanganate oxidation was employed. Oxidation of the 248 bp in the presence of the reagent showed only oxidation of the T_{10} strand of the target (FIG. 36).

5) In a similar type of demonstration, the single strand specificity of 5′ nucleases clearly showed that only the T_{10} strand of the target was attacked (FIG. 36).

The very efficient binding of (Taeq)_{10} (Taeq)_{10} Lys-NH_{2} and Acr^{-}(Taeq)_{10}Lys-NH_{2} (FIG. 5) to the corresponding dA_{10} was furthermore illustrated in two ways:

1. As shown in Example 56 below PNA-oligonucleotide complexes will migrate slower than the single stranded oligonucleotide upon electrophoresis in polyacrylamide gels. Consequently, such experiments were performed with Acr^{-}(Taeq)_{10}Lys-NH_{2} and 32P-end-labelled dA_{10}. This showed retarded migration under conditions where a normal dA_{10}dT_{10} duplex is stable, as well as under conditions where such a duplex is unstable (denaturing gel). A control experiment was performed with a mixture of Acr^{-}(Taeq)_{10}Lys-NH_{2} and 32P-end-labelled dT_{10} which showed no retardation under the above conditions.

2. Upon formation of DNA duplexes (dsDNA) from single strand DNA, the extinction coefficient decreases (hypochromicity). Thus, the denaturation of DNA can be followed by measuring changes in the absorbance, for example, as a function of T_m, the temperature where 50% of a duplex has disappeared to give single strands.

Duplexes were formed from the single-stranded oligodeoxyribonucleotides and the PNA's listed below. Typically 0.3OD_{260} of the T-rich strand was hybridized with 1 equivalent of the other strand by heating to 90 C for 5 min, cooling to room temperature and kept for 30 min and finally stored in a refrigerator at 5 C for at least 30 min. The buffers used were all 10 mM in phosphate and 1 mM in EDTA. The low salt buffer contained no sodium chloride, whereas the medium salt buffer contained 140 mM NaCl and the high salt buffer 500 mM NaCl. The pH of all the buffers was 7.2. The melting temperature of the hybrids were determined on a Gilford Response apparatus. The following extinction coefficients were used: A: 15.4 ml/mmol/cm; T: 8.8; G: 11.7 and C: 7.3 for both normal oligonucleotides and PNA. The melting curves were recorded in steps of 0.5 C/min. The T_m were determined from the maximum of the 1st derivative of the plot of A_{260} vs temperature.

<table>
<thead>
<tr>
<th>Oligo/PNA</th>
<th>Low Salt</th>
<th>Medium Salt</th>
<th>High Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 + b</td>
<td>84.0</td>
<td>51.5</td>
<td>60.0</td>
</tr>
<tr>
<td>2 + a</td>
<td>73.0</td>
<td>72.5</td>
<td>73.0</td>
</tr>
<tr>
<td>2 + c</td>
<td>41.5</td>
<td>52.0*</td>
<td></td>
</tr>
<tr>
<td>2 + e</td>
<td>84.5</td>
<td>86.0</td>
<td>~90</td>
</tr>
<tr>
<td>2 + f</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 + a</td>
<td>60.0</td>
<td>59.0</td>
<td>61.5</td>
</tr>
<tr>
<td>4 + c</td>
<td>74.5</td>
<td>72.0</td>
<td>72.5</td>
</tr>
<tr>
<td>4 + f</td>
<td>62.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 + a</td>
<td>47.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 + c</td>
<td>57.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 + f</td>
<td>46.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 + a</td>
<td>46.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 + c</td>
<td>58.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 + f</td>
<td>43.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 + 12</td>
<td>23.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 + 14</td>
<td>39.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Two distinct melting temperatures are seen, indicating local melting before complete denaturation

The hybrid formed between RNA-A (poly rA) and PNA-T_{10}Lys-NH_{2} melts at such high temperature that it cannot be measured (>90 C). But specific hybridization is demonstrated by the large drop in A_{260} by mixing with RNA-A but not G, C and U. The experiment is done by mixing 1 ml of a solution of the PNA and 1 ml of a solution the RNA, each with A_{260}=0.6, and then measuring the absorbance at 260 nm. Thereafter the sample is heated to 90 C for 5 min, cooled to room temperature and kept at this temperature for 30 minutes and finally stored at 5 C for 30 min.
From the above measurements the following conclusions can be made. There is base stacking, since a melting curve is observed. The PNA-DNA hybrid is more stable than a normal DNA-DNA hybrid, and the PNA-RNA is even more stable. Mismatches cause significant drops in the $T_m$-value, whether the mispaired base is in the DNA or in the PNA-strand. The $T_m$-value is only slightly dependent on ionic strength, as opposed to normal oligonucleotides.

**EXAMPLE 56**

**Binding of Acr$^1$-(Taqe)$_{10}$-Lys-NH$_2$ to dA$_{10}$ (FIG. 11a)**

[0241] Acr$^1$-(Taqe)$_{10}$-Lys (100 ng) was incubated for 15 min at room temperature with 50 cpm 5'-32P-end-labelled oligonucleotide [d(GATCCAG-G)] in 20 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The sample was cooled in ice (15 min) and analyzed by gel electrophoresis in polyacrylamide (PAGE). To 10 μl of the sample was added 2 μl 50% glycerol, 5 μl TBE (TBE=90 mM Tris-borate, 1 mM EDTA, pH 8.3), and the sample was analysed by PAGE (15% acrylamide, 0.5% bisacrylamide) in TBE buffer at 4°C. A 10 μl portion of the sample was lyophilized and redissolved in 10 μl 80% formamide, 1 TBE, heated to 90°C (5 min), and analyzed by urea/PAGE (15% acrylamide, 0.5% bisacrylamide, 7 M urea) in TBE. [32P]-containing DNA bands were visualized by autoradiography using intensifying screens and Agfa Carp RfX-ray films exposed at -80°C for 2 h.

[0242] Oligonucleotides were synthesized on a Biosearch 7500 DNA synthesizer, labelled with γ-[32P]-ATP (Amerham, 5000 Ci/mmol) and polynucleotide kinase, and purified by PAGE using standard techniques (Maniatis et al. 1986): A laboratory manual, Cold Spring Harbor Laboratories.

[0243] In FIG. 11a and FIG. 11b, the 5'-32P-labelled oligonucleotide 1 is 5'-GATCCAG-G. This was incubated in the absence (lanes 1 and 4) or presence of Acr-T$_{10}$-Lys-NH$_2$ (in lanes 2 and 5, 25 pmol; lanes 3 and 6, 75 pmol) and also in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of “oligo-2” which was 5'-GATCCTT$_{10}$G. 5'-32P-labelled oligo 2 was incubated in the absence (lane 7) or presence of the same DNA (lane 8, 25 pmol; lane 9, 75 pmol) and analysed by PAGE as described above in detail.

[0244] The results in FIG. 11a show retardation of the ssDNA as it is hybridised by PNA (lanes 1 to 3) and the ability of PNA to compete with a DNA oligonucleotide, for labelled complementary oligonucleotide (lanes 4 to 6). The intensity of the band attributable to dsDNA grows faster as the PNA concentration is raised and is replaced by a band representing the slower moving PNA-DNA hybrid. Lanes 7 to 9 show that the PNA has no effect on the T$_{10}$ oligo DNA with which it is non-complementary.

[0245] In FIG. 11b, which was run under DNA denaturing conditions the PNA-DNA duplexes remain undenatured.

**EXAMPLE 57**

**Formation of Strand Displacement Complex**

[0246] A dA$_{10}$-dT$_{10}$ target sequence contained within a plasmid DNA sequence was constructed by cloning of two oligonucleotides (d(GATCCAG-G)+d(GATCCTT$_{10}$G)) into the BamHI restriction enzyme site of pUC19 using the Eschericia coli JM101 strain by standard techniques (Maniatis et al., 1986). The desired plasmid (designated p110) was isolated from one of the resulting clones and purified by the alkaline extraction procedure and CsCl centrifugation (Maniatis et al., 1986). A 34[32P]-end-labelled DNA fragment of 248 bp containing the dA$_{10}$/dT$_{10}$ target sequence was obtained by cleaving the pT10 DNA with restriction enzymes EcoRI and PvuII, labelling of the cleaved DNA with [32P]-dATP (4000 Ci/mmol, Amersham) using the Klenow fragment of E. coli DNA polymerase (Boehringer Mannheim), and purifying the 248 bp DNA fragment by PAGE (5% acrylamide, 0.66% bisacrylamide, TBE buffer). This DNA fragment was obtained with [32P]-end labelling at the 5'-end by treating the EcoRI-cleaved pT10 plasmid with bacterial alkaline phosphatase (Boehringer Mannheim), purifying the plasmid DNA by gel electrophoresis in low melting temperature agarose, and labelling with γ[32P] ATP and polynucleotide kinase. Following treatment with PvuII, the 248 bp DNA fragment was purified as above.

[0247] The complex between Acr$^1$-(Taqe)$_{10}$-Lys-NH$_2$ and the 248 bp DNA fragment was formed by incubating 50 ng of Acr$^1$-(Taqe)$_{10}$-Lys-NH$_2$ with 500 cpm 32P-labelled 248 bp fragment and 0.5 μg calf thymus DNA in 100 μl 25 mM Tris-HCl, 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, pH 7.4 for 60 min at 37°C. As described further below.

**EXAMPLE 58**

**Probing of Strand Displacement Complex with**

(a) *Staphylococcus* Nuclease (FIG. 12a Lanes 8 to 10)

[0248] The strand displacement complex was formed as described above. The complex was treated with *Staphylococcus* nuclease (Boehringer Mannheim) at 750 U/ml for 5 min at 20°C. The reaction was stopped by addition of EDTA to 25 mM. The DNA was precipitated with 2 vols. of ethanol, 2% potassium acetate redissolved in 80% formamide, TBE, heated to 90°C (5 min), and analyzed by high resolution PAGE (10% acrylamide, 0.3% bisacrylamide, 7 M urea) and autoradiography. Lane 8 contains zero DNA, lane 9 40 pmol and lane 10 120 pmol. As the PNA is included, we see the emergence of footprint indicating increasing susceptibility to digestion by *Staphylococcus* nuclease and hence increasing displacement of ssDNA from dsDNA.

(b) Affinity Photoligation (FIG. 12c Lanes 1 to 3 in Each Case)

[0249] The complex was formed in TE buffer. A sample contained in an Eppendorf tube was irradiated from above at 300 nm (Philips TL 20 W/12 fluorescent light tube, 24 Jm$^{-2}$-
1) for 30 min. The DNA was precipitated as above, taken up in 1 M piperidine, and heated to 90°C for 20 min. Following denaturing, the DNA was analysed by PAGE as above. Once again, in each lane 1 contains no PNA, and lanes 2 and 3 contain 40 pmol and 120 pmol of PNA respectively. The DNA strand bound to PNA (the A₁₀ strand) cleaves at the location of the acridine ester (lanes 1 to 3 of FIG. 12a) producing a new band (arrowed) whilst the strand displaced by PNA (the T₁₀ strand) cleaves randomly producing a footprint.

(c) Potassium Permanganate (FIG. 12b Lanes 4 to 6)

[0250] The complex was formed in 100 μl TE and 5 μl 20 mM KMnO₄ was added. After 15 s at 20°C, the reaction was stopped by addition of 50 μl 1.5 M sodium acetate, pH 7.0, 1 M 2-mercaptoethanol. The DNA was precipitated, treated with pipperidine and analyzed, as above. The same PNA concentrations are used in lanes 4 to 6 as in lanes 1 to 3. Once again, one can see the emergence of a footprint showing cleavage of displaced ssDNA by permanganate.

(d) Photofootprinting (FIG. 12c Lanes 5 to 6)

[0251] The complex was formed in 100 μl TE and diazo-linked acridine (0.1 μg/μl) (DHA, Nielsen et al. (1988) Nucl. Acids Res. 16, 3877-88) was added. The sample was irradiated at 365 nm (Philips TL 20 W/09N, 22 Jm⁻²s⁻¹) for 30 min and treated as described for “affinity photo cleavage”. In the presence of PNA, (lane 6) the DNA is protected and bands corresponding to cleavage in the protected region disappear.

(e) S₁-Nuclease (FIG. 12c Lanes 1 to 3)

[0252] The complex was formed in 50 mM sodium acetate, 200 mM NaCl, 0.5% glycerol, 1 mM MnCl₂, pH 4.5 and treated with nuclease S₁ (Boehringer Mannheim) at 0.5 U/ml for 5 min at 20°C. The reaction was stopped and treated further as described under “Staphylococcus nuclease”. The quantity of PNA used was zero (lanes 1 to 3) or 120 pmol (lanes 4 to 6) lane 7 shows size standards. Once again, cleavage of the T₁₀ DNA strand displaced by PNA is seen.

EXAMPLE 59

Sensitivity of Hybridisation to (1) Orientation (2) pH and (3) Sequence Mismatch

[0253] The PNA-oligomer H-T4C2TCTC-LysNH₂ was prepared by Synthetic Protocol 6, purified by reverse phase HPLC, and identified by FAB-mass spectrometry. Found (Calc.) 2746.8 (2447.15). Hybridization experiments with this sequence resolve the issue of orientation, since it is truly asymmetrical. Such experiments also resolve the issues of pH-dependency of the Tm, and the stoichiometry of complexes formed.

[0254] Hybridization experiments with the PNA-oligomer H-T₄C₂TCTC-LysNH₂ were performed as follows:

<table>
<thead>
<tr>
<th>Row Hybridized with</th>
<th>pH</th>
<th>Tm</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 5'-&lt; (DA)₂ (DG)₂ (DA) (DG) (DA) (DG)</td>
<td>7.2</td>
<td>55.5</td>
<td>2:1</td>
</tr>
<tr>
<td>2 5'-&lt; (DA)₂ (DG)₂ (DA) (DG) (DA) (DG)</td>
<td>9.0</td>
<td>98.5</td>
<td>2:1</td>
</tr>
<tr>
<td>3 5'-&lt; (DA)₂ (DG)₂ (DA) (DG) (DA) (DG)</td>
<td>5.0</td>
<td>92.6</td>
<td>2:1</td>
</tr>
</tbody>
</table>

[0255] These results show that a truly mixed sequence gave rise to well defined melting curves. The PNA-oligomers can actually bind in both orientations (compare row 1 and 4), although there is preference for the N-terminal/5'-orientation. Introducing a single mismatch opposite either T or C caused a lowering of Tm by more than 16°C at pH 7.2; at pH 5.0 the Tm-value was lowered more than 27°C. This shows that there is a very high degree a sequence-selectivity.

[0256] As indicated above, there is a very strong pH-dependency for the Tm-value, indicating that Hoogsteen basepairing is important for the formation of hybrids. Therefore, it is not surprising that the stoichiometry was found to be 2:1.

[0257] The lack of symmetry in the sequence and the very large lowering of Tm when mismatches are present show that the Watson-Crick strand and the Hoogsteen strand are parallel when bound to complementary DNA. This is true for both of the orientations, i.e., 5'/N-terminal and 3'/N-terminal.

EXAMPLE 60

Sequence Discrimination in Hybridisation

[0258] The results of hybridization experiments with H-T₄C₂TCTC-LysNH₂ to the deoxyoligomenucleotides shown below were as follows:
As shown by comparing rows 1, 3, and 6 with rows 2, 4, 5, and 7, G can in this mode discriminate between C/A and G/T in the DNA-strand, i.e., sequence discrimination is observed. The complex in row 3 was furthermore determined to be 2 PNA: 1 DNA complex by UV-mixing curves.

EXAMPLE 61
Sequence Specificity in Hybridisation Using a Modified Backbone

[0260] Hybridization data for a PNA-oligomer with a single unit with an extended backbone (the β-alanine modification) is as follows:

<table>
<thead>
<tr>
<th>PNA</th>
<th>DNA</th>
<th>T_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-T4(βT)T3-LysNH2</td>
<td>(dA)_{10}</td>
<td>73° C.</td>
</tr>
<tr>
<td>H-T4(βT)T3-LysNH2</td>
<td>(dA)_{10}</td>
<td>73° C.</td>
</tr>
<tr>
<td>H-T4(βT)T3-LysNH2</td>
<td>(dA)_{10}</td>
<td>73° C.</td>
</tr>
</tbody>
</table>

Although the melting temperature decreases, the data demonstrates that base specific recognition is retained.

EXAMPLE 62
Iodination Procedure—Radiolabelling

[0261] A 5 µg portion of Tyr-PNA-T15-Lys-NH2 is dissolved in 40 µl 100 mM Na-phosphate, pH 7.0, and 1 mM NaI and 2 µl chloramine-T (50 mM in CH3CN) are added. The solution is left at 20° C. for 10 min and then passed through a 0.5×5 cm Sephadex G10 column. The first 2 fractions (100 µl each) containing radioactivity are collected and purified by HPLC: reversed phase C-18 using a 0-60% CH3CN gradient in 0.1% CF3COOH in H2O. The 125I-PNA elutes right after the PNA peak. The solvent is removed under reduced pressure.

EXAMPLE 63
Binding of PNAa-T10/T4-C/T4-C to double stranded DNA targets A4/AgG/A4G3 (FIG. 20).

[0262] A mixture of 200 cps double stranded 32P-labeled EcoRI-PvuII fragment (the large fragment labeled at the 3'-end of the EcoRI site) of the indicated plasmid, 0.5 µg carrier calf thymus DNA, and 300 ng the indicated PNA in 100 µl buffer (200 mM NaCl, 50 mM Na-acetate, pH 4.5, 1 mM ZnSO4) was incubated at 37° C. for 120 min. 50 units of nuclease S1 were added and incubated at 20° C. for 5 min. The reaction was stopped by the addition of 3 µl 0.5 M EDTA and the DNA was precipitated by addition of 250 µl 2% potassium acetate in ethanol. The DNA was analyzed by electrophoresis in 10% polyacrylamide sequencing gels and the radiolabeled DNA bands visualized by autoradiography. The complete band patterns produced show the production by strand displacement of single stranded DNA which is attacked by the nuclease to produce a mixture of shorter oligonucleotides. Comparison of the results for the three PNA's used in each case shows the selectivity for each target which is obtained.

[0263] The target plasmid were prepared by cloning of the appropriate oligonucleotides into pUC19. Target A10: oligonucleotides GATCA_{10}G & GATCCT_{10}G cloned into the BamHI site (plasmid designated pT10). Target A10G14: oligonucleotides TCGACT_{10}C_{10}G & TCGACAG_{10}G cloned into the Sall site (plasmid pT19C). Target A10G14G14: oligonucleotides GA3GA3GA3TGCA & GT_{10}CT_{10}CT_{10}GCA into the PstI site (plasmid pT18C). The positions of the targets in the gel are indicated by bars to the left. A/G is an A4G sequence ladder of target P10.

EXAMPLE 64
Inhibition of restriction enzyme cleavage by PNA (FIG. 23)

[0264] A 2 µg portion of plasmid pT10 was mixed with the indicated amounts of PNA-T4 in 20 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and incubated at 37° C. for 120 min. 2 µl 10x concentrated buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 50 mM NaCl, 1 mM EDTA) and PvuII (2 units) and BamHI (2 units) were added and the incubation was continued for 60 min. The DNA was analyzed by gel electrophoresis in 5% polyacrylamide and the DNA was visualized by ethidium bromide staining.

[0265] In the presence of a significant proportion of PNA (0.2, 0.6), the cleavage pattern of enzyme Bam HI was changed indicating that the enzyme was inhibited by the presence of PNA alongside the cleavage site.

EXAMPLE 65
Kinetics of PNA-T10—dsDNA Strand Displacement Complex Formation (FIG. 21).

[0266] A mixture of 200 cps double stranded 32P-labeled EcoRI-PvuII fragment of pT10 (the large fragment labeled at the 3'-end of the EcoRI site), 0.5 µg carrier calf thymus DNA, and 300 ng of PNA-T10-Lys-NH2 in 100 µl buffer (200 mM NaCl, 50 mM Na-acetate, pH 4.5, 1 mM ZnSO4) were incubated at 37° C. At the times indicated, 50 U of S1 nuclease was added to each of 7 samples and incubation was continued for 5 min at 20° C. Any single stranded DNA produced by strand displacement is digested by the nuclease. The DNA was then precipitated by addition of 250 µl 2% potassium acetate in ethanol and analyzed by electrophoresis in a 10% polyacrylamide sequencing gel. The amount of strand displacement complex was calculated in arbitrary units from the intensity of the S1-cleavage at the target sequence, as measured by den-
sitometric scanning of autoradiographs. The formation of the complex over time can be seen.

EXAMPLE 66
Stability of PNA-dsDNA Complexes (FIG. 22)

[0267] A mixture of 200 cps ³²P-pT10 fragment (as in Example 65), 0.5 µg calf thymus DNA and 300 ng of the desired PNA (either T₁₀-LysNH₂, T₈-LysNH₂ or T₈-LysNH₂) was incubated in 100 µl 200 mM NaCl, 50 mM Na acetate, pH 4.5, 1 mM ZnSO₄ for 60 min at 37°C. A 2 µg portion of oligonucleotide CATCCAG was added to compete with the PNA for the labelled oligonucleotide and each sample was heated for 10 min at the temperature indicated, cooled in ice for 10 min and warmed to 20°C. 50 U of S₁ nuclease was added and the quantity of radio-activity liberated as single nucleotides was measured.

[0268] The expected Tₘ values for a T₁₀ DNA duplex would be 20°C, for T₈-16°C, and for T₈-12°C. The corresponding PNA/DNA values are shown to be T₁₀=70°C, T₈-60°C, and for T₈=37°C.

EXAMPLE 67
Imobilisation of DNA

[0269] For the preparation of PNA-Sepharose 10 mg of cyanogen bromide activated Sepharose (Sigma) was incubated with 10 µg PNA in 100 µl 50 mM Na-phosphate, pH 7.5 for 60 min at 37°C. The Sepharose was isolated by centrifugation and washed three times with 250 µl 50 mM Na-phosphate, pH 7.5.

EXAMPLE 68
Binding of ⁵⁻³²P-Endlabelled Oligonucleotide to PNA-Sepharose

[0270] 1 mg PNA-Sepharose (Example 67) in 100 µl TE was incubated with 50 cps (~100 ng) ³²P-labelled oligonucleotide for 16 hr at 20°C. The Sepharose was isolated by centrifugation and washed twice with 500 µl TE. Bound oligonucleotide was determined by liquid scintillation counting using the “Cerenkov” method. Results are shown in the Table below for three different PNA-Sepharose and four oligo-DNA’s. The specificity of the capture by the immobilised PNA’s is clearly seen, only one base pair mismatch being tolerated.

<table>
<thead>
<tr>
<th>% Binding of ³²P-oligo to PNA-Sepharose</th>
<th>PNA on solid support (CrBr-Sepharose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>T₁₀</td>
</tr>
<tr>
<td>----------------</td>
<td>-----</td>
</tr>
<tr>
<td>A₁₀</td>
<td>51</td>
</tr>
<tr>
<td>A₉G</td>
<td>11</td>
</tr>
<tr>
<td>A₈G₂</td>
<td>11</td>
</tr>
<tr>
<td>mix</td>
<td>11</td>
</tr>
</tbody>
</table>

[0271] In the table A₁₀ is ⁵-GATCCAAAAAAAG, A₉G is ⁵-TGCACAAAAAAG, A₈G₂ is ⁵-GAAGAAGAAGGTAG, & mix is ⁵-GATCACGCAG-

EXAMPLE 69
Temperature Dependence of Stability of ³²P-Oligonucleotide-Binding to PNA-Sepharose

[0272] Oligonucleotides were bound to PNA-Sepharose as described in Examples 67 and 68. In each case, the ³²P-oligonucleotide-PNA-Sepharose was subsequently washed at increasing temperatures. The Sepharose was isolated by centrifugation, the radioactivity determined by “Cerenkov” counting, and the Sepharose was again taken up in 500 µl TE, incubated at the next desired temperature, centrifuged etc. The figure shows the results normalised to initial binding. The oligonucleotide PNA-Sepharose were as described in the Table of Example 68. The shifts between the curves show the lowered stability of the oligonucleotide binding when one mismatch is present.

EXAMPLE 70
Inhibition of Transcription by PNA (FIG. 26)

[0273] A mixture of 100 ng plasmid DNA (cleaved with restriction enzyme PvuII (see below) and 100 ng of PNA in 15 µl 10 mM Tris-HCL, 1 mM EDTA, pH 7.4 was incubated at 37°C for 60 min. Subsequently, 4 µl 5x concentrated buffer (0.2 M Tris-HCL (pH8.0), 40 mM MgCl₂, 10 mM spermidine, 125 mM NaCl) were mixed with 1 µl NTP-mix (10 mM ATP, 10 mM CTP, 10 mM UTP, 10 mM GTP, 0.1 µg/ml ³²P-UTP, 5 mM DTT, 2 µg/ml RNA, 1 µg/ml heparin) and 3 units RNA polymerase. Incubation was continued for 10 min at 37°C. The RNA was then precipitated by addition of 60 µl 2% potassium acetate in 96% ethanol at -20°C, and analyzed by electrophoresis in 8% polyacrylamide sequencing gels. RNA transcripts were visualized by autoradiography. The plasmid used was: Lane 1 to 5; pA10KS (the A₁₀ target is positioned on the transcribed strand). Lane 6 to 10: pT10KS (the A₁₀ target is positioned on the non-transcribed strand). The plasmid was treated with the following restriction enzymes prior to the experiment: Lanes 1, 4, 6 and 9: PvuII; lanes 2, 5, 7 and 10: XbaI; lanes 3 and 8: SamHI. The samples of lanes 4, 5, 9 and 10 contained PNA, while the rest did not. It can be seen from the gel that when PNA T₁₀ is bound to the transcribed strand of the plasmid, transcription of RNA is arrested at the PNA binding site. If the PNA is bound to the non-transcribed strand of the plasmid, transcription is not arrested.

EXAMPLE 71
Affinity PNA—Sepharose (FIG. 27)

[0274] 1 mg of PNA-T₁₀ Sepharose (see Example 67) in 100 µl TE was incubated with ~50 cps of the following end-labelled oligonucleotides:

1: ⁵'-GAT CGG GCA AAT CGG CAA TAC GGC ATA ACG

GCT AAA CGG CTT TAC GGC TTA TCG GCC ATT CGG

CAT TTC GGC AAT TCG,
for 16 hrs at 20°C. The Sepharose was isolated by centrifugation and washed 3 times with TE. Oligonucleotides were subsequently washed off with 500 μl TE at increasing temperatures, precipitated with 1 ml ethanol, 2% potassium acetate and analysed by electrophoresis in 20% polyacrylamide, 7 M urea gel run in TBE buffer and detected by autoradiography (−70°C, 16 hrs. intensifying screen). The results are shown in FIG. 26. The lanes are as follows: — [0275] Lane 1: oligonucleotides not bound to the Sepharose; [0276] Lane 2: oligonucleotides washed off at 40°C C; [0277] Lane 3: oligonucleotides washed off at 60°C C; [0278] Lane 4: oligonucleotides washed off at 80°C C. [0279] Only plasmid 3 is complementary to the PNA. It can be seen that by washing at up to 80°C, essentially only the complementary plasmid is retained on the PNA-Sepharose. This example illustrates the extraction of an oligonucleotide from a mixture thereof by affinity capture using PNA.

EXAMPLE 72
Quantitative Assay of a DNA Sequence in a ds Plasmid by PNA-Strand Displacement
[0280] 3 μg plasmid DNA digested by the respective restriction enzymes indicated below in 10 μl 50 mM Tris-HCl, 1 mM EDTA, pH 7.4 was incubated with 5000 cpm (−10 ng) I-PNA-T167 Tyr and the quantity of cold PNA T107 Tyr indicated by the total PNA amounts given below for 60 mins at 37°C. The DNA was precipitated with 25 μl ethanol, 2% potassium acetate, and subsequently analysed by electrophoresis in 6% polyacrylamide in TBE buffer. The gel was stained with ethidium bromide and subsequently subject to autoradiography (−70°C., 16 hrs. intensifying screen). The results are seen in FIG. 28. “A” is the ethidium stained gel and “B” is the corresponding autoradiogram. The plasmids used were:
[0281] Lanes 1 to 3: pT167*HaeIII; [0282] Lanes 4 to 6: pT167*Hinfl; [0283] Lanes 7 to 9: pT167*PstII.

The total amount of PNA-T167 Tyr in each sample was:
[0284] Lanes 1, 4 and 7: −10 ng; [0285] Lanes 2, 5 and 8: 25 ng; [0286] Lanes 3, 6 and 9: 250 ng.

[0287] The ethidium bromide gel (a) shows the size of the DNA fragments produced (including DNA-PNA hybrids). The autoradiograph (b) shows which band in gel (a) contains PNA. The presence of a strand displacement complex can be seen in lane 1 as can the effect on the intensity of this band of increasing the proportion of cold PNA in lanes 2 and 3. Similar results are seen for each of the other two plasmids in lanes 4 to 6 and 7 to 9. The location of the PNA-DNA band in each case can be used to identify the plasmid and the based intensity can be used to quantitate the amount of plasmid present.

[0288] Those skilled in the art will appreciate that numerous changes and modifications may be made to the preferred embodiments of the invention and that such changes and modifications may be made without departing from the spirit of the invention. It is therefore intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.
(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AAAAGAAAA

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AAAACAAAA

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AAAATAAAAA

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) MOLECULE TYPE: DNA (genomic)
(iv) HYPOTHETICAL: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AAAAAGAAAA

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AAAAACAAAA

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAAAATAAAA

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GATCCAAAA  AAGGAATC

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 10 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AAGAGAAGAAAA

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 10 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: double
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AAAAAAAAAAA

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 10 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
(2) INFORMATION FOR SEQ ID NO: 12:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 8 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

   (x) PUBLICATION INFORMATION:
       (H) DOCUMENT NUMBER: WO PCT/EP92/01220
       (I) FILING DATE: 22-MAY-1992

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

   AAAAAAAAA

(2) INFORMATION FOR SEQ ID NO: 13:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 10 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

   (x) PUBLICATION INFORMATION:
       (H) DOCUMENT NUMBER: WO PCT/EP92/01220
       (I) FILING DATE: 22-MAY-1992

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

   AAAAGAAGAA

(2) INFORMATION FOR SEQ ID NO: 14:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 10 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

   (x) PUBLICATION INFORMATION:
       (H) DOCUMENT NUMBER: WO PCT/EP92/01220
       (I) FILING DATE: 22-MAY-1992

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

   TTTTCTTTTT

(2) INFORMATION FOR SEQ ID NO: 15:

   (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TTTCTCTTT

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTTCTCTTT

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTTCTCTCT

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TTCTTTTTTT

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 15 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TTTTTTTTTTT

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 15 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AAAAAAAAAAAAAA

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 16 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GATCCAAAA AAAAA

16

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GATCCCTTTTTTTTGG

16

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

AAAAAGAGAAG

10

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GAGAGGAAAA

10

(2) INFORMATION FOR SEQ ID NO: 25:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:
AAAAGTAGAG

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:
AAAAGGTUAG

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:
GAGATGAAA

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GAGTOGAAAA

10

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 16 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

TCGACTTTTC TTTTTG

16

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 16 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
   (H) DOCUMENT NUMBER: WO PCT/EP92/01220
   (I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

TCGACAAAAA GAAAAG

16

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 15 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GTTTTCTCTT TCTGCA 16

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GAAGAAGAAA CTGAC 15

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GATCACGCT ATACGCT 18
(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 77 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GATCCCGCCT ACCGCAATA CGGCGATCCG GCTAAACGCT GTACGCTTA

TCGCTATTC GCGATTTCG CAATTGG

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

GATCCGGTTC ACCGCAATT CGGCTATCG GCGATCCGG TTACTGCGAT

TAATCGGG

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO PCT/EP92/01220
(I) FILING DATE: 22-MAY-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

GACAAACATA CAATTTCGAGAACCAAAA AAAAAAAAA

(2) INFORMATION FOR SEQ ID NO: 38:
1. A nucleic acid analogue for use in the capture, recognition, detection, identification or quantification of one or more chemical or microbiological entities, which analogue is

(a) a peptide nucleic acid (PNA) comprising a polyamide backbone bearing a plurality of ligands at respective spaced locations along said backbone, said ligands being each independently naturally occurring nucleobases, non-naturally occurring nucleobases or nucleobase-binding groups, each said ligand being bound directly or indirectly to a nitrogen atom in said backbone, and said ligand bearing nitrogen atoms mainly being separated from one another in said backbone by from 4 to 8 intervening atoms;

(b) a nucleic acid analogue capable of hybridising to a nucleic acid of complementary sequence to form a hybrid which is more stable against denaturation by heat than a hybrid between the conventional deoxyribonucleotide corresponding to said analogue and said nucleic acid; or

(c) a nucleic acid analogue capable of hybridising to a double stranded nucleic acid in which one strand has a
sequence complementary to said analogue, so as to displace the other strand from said one strand.

2. A nucleic acid analogue as claimed in claim 1, having the general formula:

\[
\begin{align*}
&\text{wherein:} \\
&\text{n is at least 2,} \\
&\text{each of L}^{1}\text{--L}^{n} \text{ is independently selected from the group} \\
&\text{consisting of hydrogen, hydroxy, (C}_{1}\text{--C}_{n}\text{)alkanoyl,} \\
&\text{naturally occurring nucleobases, non-naturally occurring nucleobases, aromatic moiety, DNA intercalators,} \\
&\text{nucleobase-binding groups and reporter ligands; at least one of L}^{1}\text{--L}^{n} \text{ being a naturally occurring nucleobase,} \\
&\text{a non-naturally occurring nucleobase, a DNA intercalator; or a nucleobase-binding group;}
\end{align*}
\]

\[
\begin{align*}
\text{each of A}^{1}\text{--A}^{n} \text{ is a single bond, a methylene group or a} \\
\text{group of formula:}
\end{align*}
\]

\[
\begin{align*}
&\text{where:} \\
&X \text{ is O, S, Se, NR}^{3}, \text{CH}_{2} \text{ or C(CH}_{3}\text{)}_{2}; \\
&Y \text{ is a single bond, O, S or NR}^{5}; \\
&\text{each of } p \text{ and } q \text{ is an integer from 1 to 5, the sum } p+q \text{ being not more than 10;} \\
&\text{each of } r \text{ and } s \text{ is zero or an integer from 1 to 5, the sum } r+s \text{ being not more than 10;} \\
&\text{each } R^{1} \text{ and } R^{2} \text{ is independently selected from the group} \\
&\text{consisting of hydrogen, (C}_{1}\text{--C}_{n}\text{)alkyl which may be} \\
&\text{hydroxy- or alkoxy- or alkylthio-substituted, hydroxy,} \\
&\text{alkoxy, alkylthio, amino and halogen; and} \\
&\text{each } R^{2} \text{ each } R^{1} \text{ and } R^{2} \text{ is independently selected from the group} \\
&\text{consisting of hydrogen, (C}_{1}\text{--C}_{n}\text{)alkyl, hydroxy- or} \\
&\text{alkoxy- or alkylthio-substituted (C}_{1}\text{--C}_{n}\text{)alkyl, hydroxy,} \\
&\text{alkoxy, alkylthio and amino;}
\end{align*}
\]

\[
\begin{align*}
\text{each of } R^{3} \text{ is N or R}^{3} \text{N}^{+}, \text{where } R^{3} \text{ is as defined above;} \\
\text{each of } C^{1}\text{--C}^{n} \text{ is CR}^{3}\text{R}^{2}, \text{CHR}^{3}\text{CHR}^{2} \text{ or CR}^{3}\text{R}^{2}\text{CH}_{2}, \\
\text{where } R^{6} \text{ is hydrogen and } R^{7} \text{ is selected from the group} \\
\text{consisting of the side chains of naturally occurring alpha amino acids, or } R^{6} \text{ and } R^{7} \text{ are independently selected} \\
\text{from the group consisting of hydrogen, (C}_{1}\text{--C}_{n}\text{)alkyl,} \\
\text{aryl, aralkyl, heteroaryl, hydroxy, (C}_{1}\text{--C}_{n}\text{)alkoxy, (C}_{1}\text{--C}_{n}\text{)alkylthio, NR}^{3}\text{R}^{2} \text{ and SR}^{2}, \text{where } R^{3} \text{ and } R^{2} \text{ are as} \\
\text{defined above, and } R^{3} \text{ is hydrogen, (C}_{1}\text{--C}_{n}\text{)alkyl,} \\
\text{hydroxy-, alkoxy-, or alkylthio-substituted (C}_{1}\text{--C}_{n}\text{)alkyl, or } R^{6} \text{ and } R^{7} \text{ taken together complete an} \\
\text{acyclic or heterocyclic system;} \\
\text{each of } D^{1}\text{--D}^{n} \text{ is CR}^{6}\text{R}^{7}, \text{CHR}^{6}\text{CHR}^{7} \text{ or } \text{CHR}^{6}\text{CHR}^{7}, \\
\text{where } R^{6} \text{ and } R^{7} \text{ are as defined above;}
\end{align*}
\]

3. A nucleic acid analogue as claimed in claim 2, having the general formula:

\[
\begin{align*}
\text{wherein:} \\
\text{each } L \text{ is independently selected from the group consisting} \\
\text{of hydrogen, phenyl, naturally occurring nucleobases,} \\
\text{and non-naturally occurring nucleobases;}
\end{align*}
\]

\[
\begin{align*}
\text{each } R^{\text{IV}} \text{ is independently selected from the group consisting of} \\
\text{hydrogen and the side chains of naturally occurring alpha amino acids;}
\end{align*}
\]

\[
\begin{align*}
\text{n is an integer from 1 to 60,} \\
\text{each } k \text{ and } m \text{ is, independently, zero or one; and each } L \text{ is} \\
\text{independently from zero to 5;}
\end{align*}
\]

\[
\begin{align*}
\text{R}^{6} \text{ is OH, NH}_{2} \text{ or } \text{NHLysNH}_{2}; \text{ and} \\
\text{R}^{7} \text{ is H or COCH}_{3}.
\end{align*}
\]

4. A nucleic acid analogue as claimed in claim 3, having the general formula:

\[
\begin{align*}
\text{wherein:}
\end{align*}
\]
wherein:
each L is independently selected from the group consisting
of the nucleobases thymine, adenine, cytosine, guanine,
and uracil;
each R' is hydrogen; and
n is an integer from 1 to 30.
5. A nucleic acid analogue as claimed in any one of claims
1 to 4, incorporating or conjugated to a detectable label.
6. A labelled nucleic acid analogue as claimed in claim 5,
wherein said label is a radio isotope label, an enzyme label,
biotin, a fluorophore, a chemiluminescence label, an antigen,
an antibody or a spin label.
7. The use of nucleic acid analogue as defined in any one of
claims 1 to 6 in the capture, recognition, detection, identifi-
cation or quantitation of one or more chemical or microbio-
logical entities.
8. A method of capturing a nucleic acid comprising/con-
tacting under hybridising conditions said nucleic acid with a
nucleic acid analogue as claimed in any one of claims 1 to 6
immobilised to a solid support, which nucleic acid analogue
has a sequence of ligands suitable to hybridise to said nucleic
acid.
9. A method as claimed in claim 8, wherein said captured
nucleic acid is detected, recognised, quantitated or identified
by treatment with a nucleic acid recognition agent whilst
bound to said immobilised nucleic acid analogue.
10. A method as claimed in claim 9, wherein the captured
nucleic acid has a first region hybridised to said immobilised
nucleic acid analogue and a second region which is not so
hybridised and is treated with a labelled nucleic acid or
nucleic acid analogue which is adapted to hybridise to at least
part of said second region and said label is detected.
11. A method as claimed in claim 8, for capturing a mRNA
wherein said immobilised nucleic acid analogue comprises
sequential ligands hybridisable to poly A tails of said mRNA
to capture said mRNA.
12. A method as claimed in claim 11, wherein said sequen-
tial ligands are thymine.
13. A method as claimed in any one of claims 8 or 11 or 12,
wherein said nucleic acid once captured is released from said
immobilised nucleic acid analogue by subjecting the immo-
bilised nucleic acid analogue and captured nucleic acid to
dehybridising conditions.
14. A nucleic acid analogue as claimed in any one of claims
1 to 6; immobilised to a solid support.
15. An immobilised nucleic acid analogue as claimed in
claim 14, incorporated in an affinity capture column.
16. A method of recognition, detection or quantitation of a
target nucleic acid comprising hybridising said target to a
labelled nucleic acid analogue as claimed in claim 5 or claim
6 of sufficiently complementary sequence to hybridise there-
with under hybridising conditions and detecting or quantitat-
ing said label of the nucleic acid analogue so hybridised to
said target.
17. A method as claimed in claim 16, wherein said target
nucleic acid is immobilised on a substrate prior to said
hybridisation.
18. A method as claimed in claim 17, wherein said target
nucleic acid is immobilised to said substrate by the hybridi-
sation of a first region thereof to a capture nucleic acid or
nucleic acid analogue having a sequence sufficiently comple-
mentary to said first region to hybridise therewith and which
is itself immobilised to said substrate and wherein said
labelled nucleic acid analogue hybridises to a second region
of said target.
19. A method for displacing one strand from a nucleic acid
duplex comprising hybridising to said duplex a nucleic acid
analogue having an affinity for the other strand of said duplex
sufficient to be able to displace said one strand therefrom.
20. A method of detecting, identifying or quantitating a
double stranded target nucleic acid comprising hybridising
thereto a displacing nucleic acid analogue capable of displac-
ing one strand from a double stranded target in which the
other strand is of complementary sequence to said displacing
nucleic acid analogue, wherein said displacing nucleic acid
analogue is of sufficiently complementary sequence to said
other strand of said double stranded target to hybridise thereto
so as to displace said one strand of said target in single
stranded form, and detecting or quantitating the presence of
said one displaced strand.
21. A method as claimed in claim 20, wherein the displaced
strand is broken down into fragments and the presence of said
fragments is detected.
22. A method as claimed in claim 21, wherein said dis-
placed strand is broken down by attack by a nuclease.
23. A kit for use in a diagnostics procedure and comprising
at least one labelled nucleic acid analogue as claimed in claim
5 or claim 6, and at least one detection reagent for detecting
said labelled nucleic acid analogue.
24. A kit as claimed in claim 23, further including a nucleic
acid analogue as claimed in any one of claims 1 to 4 immo-
obilised on a solid support.
25. A kit comprising an immobilised nucleic acid analogue
as claimed in any one of claims 1 to 4, in combination with at
least one nucleic acid recognition agent for detecting the
presence of nucleic acid captured in use by said nucleic acid
analogue.
26. A kit as claimed in claim 25, wherein said nucleic acid
recognition agent is a labelled nucleic acid or a labelled
nucleic acid analogue.

* * * * *