Peptide Nucleic Acids

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PEPTIDE NUCLEIC ACIDS WITH POLYAMIDE-CONTAINING BACKBONES

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Related U.S. Application Data
Continuation of application No. 08/108,591, filed on Nov. 22, 1993, now Pat. No. 6,395,474.

References Cited
U.S. PATENT DOCUMENTS
5,340,716 A 8/1994 Ullman et al. ............. 435/6
5,539,082 A 7/1996 Nielsen et al. .......... 530/300

FOREIGN PATENT DOCUMENTS
WO WO 86/05518 9/1986

OTHER PUBLICATIONS

ABSTRACT
A novel class of compounds, known as peptide nucleic acids, bind complementary ssDNA and RNA strands more strongly than the corresponding DNA. The peptide nucleic acids generally comprise ligands such as naturally occurring DNA bases attached to a peptide backbone through a suitable linker.
OTHER PUBLICATIONS


Nielson, P.E. "Sequence-Specific transcription arrest by peptide nucleic acid bond to the DNA template strand", *Gene*, 1994, 149, 139-145.


* cited by examiner
FIGURE 2
300 nm radiation photocleavage

5'-GATCCAAAAAAAAAAAGGATC
3'-CTAGTTTTTTTTTTCCTAG

Diazoacridine photofootprint

5'-GATCCAAAAAAAAAAAGGATC
3'-CTAGTTTTTTTTTTCCTAG

KMnO₄ cleavage

5'-GATCCAAAAAAAAAAAGGATC
3'-CTAGTTTTTTTTTTCCTAG

S₁-nuclease cleavage

5'-GATCCAAAAAAAAAAAGGATC
3'-CTAGTTTTTTTTTTCCTAG

Micrococcus nuclease enhancement

FIGURE 3
FIGURE 4
FIGURE 5
Anchoring

Deprotection

Coupling

Final Deprotection & Cleavage

FIGURE 8
FIGURE 9
FIGURE 10
FIGURE 11 (a)
Figure 11 (b)

<table>
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<th></th>
<th>1</th>
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<th>1</th>
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<th>2</th>
<th>2</th>
<th>2</th>
</tr>
</thead>
<tbody>
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<td>$^{35}$P-oligo</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>
</tr>
<tr>
<td>AcrT10Lys</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

complex

ssDNA
Figure 12 (a)
FIGURE 12 (b)
$S_1$-nuclease 0.1 1 10 0.1 1 10

AcrT10Lys

FIGURE 12(c)
FIGURE 13
FIGURE 14
Cl
N
N
H₂N

Cl
N
N
H₂N

BrCH₂COOH
DMF, K₂CO₃

Cl
N
N
H₂N

CH₂COOH

NaOBzl / BzlOH
DMF, 0-20 °C, 16 h

OBzl
N
N
H₂N

CH₂COOH

1) (C₆H₅N)₃PBr⁺, PF₆⁻
BocAeg-OMe, DIPEA

OBzl
N
N
H₂N

2) NaOH, H₂O/EtOH

BocNH

FIGURE 15
Alterations of A, B, C and D

aminoethyl glycine

β-alanine

aminopropyl

propionyl

FIGURE 16
FIGURE 17
Synthesis of the aminopropyl analogue of the thymine monomer

\[
\begin{align*}
\text{H}_2\text{N}-\text{CH}_2\text{NH}_2 & \quad \rightarrow \quad \text{H}_2\text{N}-\text{CH}_2\text{NHCOOH} \\
\text{H}_2\text{N}-\text{CH}_2\text{NHCONH}_2 & \quad \rightarrow \quad \text{BocNH}-\text{CH}_2\text{NHCONH}_2 \\
\text{H}_2\text{N}-\text{CH}_2\text{CONH}_2 & \quad \rightarrow \quad \text{BocNH}-\text{CH}_2\text{NOCOOCO}_2 \\
\text{H}_2\text{N}-\text{CH}_2\text{CONH}_2 & \quad \rightarrow \quad \text{BocNH}-\text{CH}_2\text{NHCONH}_2 \\
\text{DHBzOH, DCC} & \quad \rightarrow \quad \text{NaOH} \\
\end{align*}
\]

FIGURE 18 (a)

Synthesis of the propionyl analogue of the thymine monomer

\[
\begin{align*}
\text{H}_2\text{N}\text{CONH}_2 + \text{CH}_2=\text{CHCN} & \quad \rightarrow \quad \text{NaOH/\text{H}_2\text{O}} \quad \text{reflux} \\
\text{H}_2\text{N}\text{CONH}_2 & \quad \rightarrow \quad \text{NaOH} \\
\text{DHBzOH, DCC} & \quad \rightarrow \quad \text{NaOH} \\
\end{align*}
\]

FIGURE 18 (b)
Synthesis of the aminoethyl-β-alanine analogue of the thymine monomer

\[
\text{H}_2\text{N} - \text{NH}_2 + \begin{array}{c}
\text{O} \\
\text{O} \\
\text{NO}_2
\end{array} \quad \text{Boc} \\
\text{Boc-NH} - \text{NH}_2 \quad \text{DMF} \\
\begin{array}{c}
\text{O} \\
\text{O} \\
\text{CH}_3\text{CN, reflux}
\end{array} \\
\text{Boc-NH} - \text{NH} - \text{CH}_2\text{COO} \\
\text{DMF/Et}_3\text{N} \\
\text{Boc-NH} - \text{NH} - \text{CH}_2\text{COO} \quad \text{NaOH} \\
\text{Boc-NH} - \text{NH} - \text{CH}_2\text{COO}
\]

FIGURE 19
FIGURE 20
Inhibition of Restriction Enzyme Cleavage by PNA

PNA/DNA  0  0.006  0.02  0.06  0.2  0.6

PNA Target

5'---------GGATCCAAAAAAAAAGGATCC---------
3'---------CCTAGGTTTTTTTTCTAGG---------

BamHI    BamHI

FIGURE 23
Binding of $^{125}$I-Tyr-PNA-T$_{10}$ to dA$_{10}$

<table>
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<tr>
<th>CT-DNA/oligo</th>
<th>0</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
<th>100</th>
<th>300</th>
</tr>
</thead>
</table>

Origin →

Hybrid →

FIGURE 24
Test of the Tosyl-group as N-protecting group in PNA-synthesis

Compound

compound 1 in
50 % TFA: 50 % Methylene chloride, 5 h, rt.

compound 1 in
100 % HF, 0 °C, 1 h

Quantitative de-benzylation

Quantitative de-benzylation and de-sulfonylation

FIGURE 27
PEPTIDE NUCLEIC ACIDS WITH POLYAMIDE-CONTAINING BACKBONES

RELATED APPLICATIONS


This invention was made subject to a joint research agreement between Isis Pharmaceuticals, University of Copenhagen, Ole Buchardt, Peter Nielsen, Michael Egholm and Rolf Berg.

1. Field of the Invention

This invention is directed to compounds that are not polynucleotides yet which bind to complementary DNA and RNA strands more strongly the corresponding DNA. In particular, the invention concerns compounds wherein naturally-occurring nucleobases or other nucleobase-binding moieties are covalently bound to a polyamide backbone.

2. Background of the Invention

Oligodeoxyribonucleotides as long as 100 base pairs (bp) are routinely synthesized by solid phase methods using commercially available, fully automatic synthesis machines. The chemical synthesis of oligoribonucleotides, however, is far less routine. Oligoribonucleotides also are much more stable than oligodeoxyribonucleotides, a fact which has contributed to the more prevalent use of oligodeoxyribonucleotides in medical and biological research directed to, for example, gene therapy or the regulation of transcription or translation.

The function of a gene starts by transcription of its information to a messenger RNA (mRNA) which, by interaction with the ribosomal complex, directs the synthesis of a protein coded for by its sequence. The synthetic process is known as translation. Translation requires the presence of various co-factors and building blocks, the amino acids, and their transfer RNAs (tRNA), all of which are present in normal cells.

Transcription initiation requires specific recognition of a promoter DNA sequence by the RNA-synthesizing enzyme, RNA polymerase. In many cases in prokaryotic cells, and probably in all cases in eukaryotic cells, this recognition is preceded by sequence-specific binding of a protein transcription factor to the promoter. Other proteins which bind to the promoter, but whose binding prohibits action of RNA polymerase, are known as repressors. Thus, gene activation typically is regulated positively by transcription factors and negatively by repressors.

Most conventional drugs function by interaction with and modulation of one or more targeted endogenous proteins, e.g., enzymes. Such drugs, however, typically are not specific for targeted proteins but interact with other proteins as well. Thus, a relatively large dose of drug must be used to effectively modulate a targeted protein. Typical daily doses of drugs are from $10^2$ to $10^5$ millimoles per kilogram of body weight or $10^3$ to 10 millimoles for a 100 kilogram person. If this modulation instead could be effected by interaction with and inactivation of mRNA, a dramatic reduction in the necessary amount of drug necessary could likely be achieved, along with a corresponding reduction in side effects. Further reductions could be effected if such interaction could be rendered site-specific. Given that a functioning gene continually produces mRNA, it would thus be even more advantageous if gene transcription could be arrested in its entirety.

Oligodeoxynucleotides offer such opportunities. For example, synthetic oligodeoxynucleotides could be used as antisense probes to block and eventually lead to the breakdown of mRNA. Thus, synthetic DNA could suppress translation in vivo. It also may be possible to modulate the genome of an animal by, for example, triple helix formation using oligonucleotides or other DNA recognizing agents. However, there are a number of drawbacks associated with triple helix formation. For example, it can only be used for homopurine sequences and it requires unphysiologically high ionic strength and low pH.

Furthermore, unmodified oligonucleotides are impractical both in the antisense approach and in the triple helix approach because they have short in vivo half-lives, they are difficult to prepare in more than milligram quantities and, thus, are prohibitively costly, and they are poor cell membrane penetrators.

These problems have resulted in an extensive search for improvements and alternatives. For example, the problems arising in connection with double-stranded DNA (dsDNA) recognition through triple helix formation have been diminished by a clever "switch back" chemical linking whereby a sequence of polypurine on one strand is recognized, and by "switching back", a homopurine sequence on the other strand can be recognized. See, e.g., McCurdy, Moulds, and Froehler, Nucleosides, in press. Also, good helix formation has been obtained by using artificial bases, thereby improving binding conditions with regard to ionic strength and pH.

In order to improve half life as well as membrane penetration, a large number of variations in polynucleotide backbones has been undertaken, although so far not with desired results. These variations include the use of methylphosphonates, monothiophosphates, dithiophosphates, phosphoramidates, phosphate esters, bridged phosphoramidates, bridged phosphorothioates, bridged methylene-phosphonates, diphosphor internucleotide analogs with siloxane bridges, carbonate bridges, carbamoylmethyl ester bridges, acetamide bridges, carbonate bridges, thioether, sulfoxyl, sulfono bridges, various "plastic" DNAs, $\alpha$-amino-meric bridges, and borane derivatives.

International patent application WO 86/05518 broadly claims a polymeric composition effective to bind to a single-stranded polynucleotide containing a target sequence of bases. The composition is said to comprise non-homopolymeric, substantially stereoregular polymer molecules of the form:

$$R_1 \text{--} R_2 \text{--} R_3 \text{--} R_4$$

B--B--B-- . . . B,

where:

(a) $R_1$--$R_4$ are recognition moieties selected from purine, purine-like, pyrimidine, and pyrimidine like heterocycles effective to bind by Watson/Crick pairing to corresponding, in-sequence bases in the target sequence;

(b) $n$ is such that the total number of Watson/Crick hydrogen bonds formed between a polymer molecule and target sequence is at least about 15;

(c) B--B are backbone moieties joined predominantly by chemically stable, substantially uncharged, predominantly achiral linkages.
(d) the backbone moiety length ranges from 5 to 7 atoms if the backbone moieties have a cyclic structure, and ranges from 4 to 6 atoms if the backbone moieties have an acyclic structure; and
(e) the backbone moieties support the recognition moieties at position which allow Watson-Crick base pairing between the recognition moieties and the corresponding, in-sequence bases of the target sequence.

According to WO 86/05518, the recognition moieties are various natural nucleobases and nucleobase-analogs and the backbone moieties are either cyclic backbone moieties comprising furan or morpholine rings or acyclic backbone moieties of the following forms:

![Chemical structure diagram]

where E is —CO— or —SO₂—. The specification of the application provides general descriptions for the synthesis of subunits, for backbone coupling reactions, and for polymer assembly strategies. However, the specification provides no example wherein a claimed compound or structure is actually prepared. Although WO 86/05518 indicates that the claimed polymer compositions can bind to target sequences and, as a result, have possible diagnostic and therapeutic applications, the application contains no data relating to the binding affinity of a claimed polymer.

International patent application WO 86/05519 claims diagnostic reagents and systems that comprise polymers described in WO 86/05518, but attached to a solid support. WO 86/05519 also provides no examples concerning actually preparation of a claimed diagnostic reagent, much less data showing the diagnostic efficiency of such a reagent.

International patent application WO 89/12060 claims various building blocks for synthesizing oligonucleotide analogs, as well as oligonucleotide analogs formed by joining such building blocks in a defined sequence. The building blocks may be either “rigid” (containing a ring) or “flexible” (lacking a ring). In both cases the building blocks contain a hydroxy group and a mercaptio group, through which the building blocks are said to join to form oligonucleotide analogs. The linking moiety in the oligonucleotide analogs is selected from the group consisting of sulfide (—S—), sulfone (—SO₂—), and sulfide (—SO₂—). WO 89/12060 provides a general description concerning synthesis of the building blocks and coupling reactions for the synthesis of oligonucleotide analogs, along with experimental examples describing the preparation of building blocks. However, the application provides no examples directed to the preparation of a claimed oligonucleotide analog and no data confirming the specific binding of an oligonucleotide analog to a target oligonucleotide.

Furthermore, oligonucleotides or their derivatives have been linked to intercalators in order to improve binding, to polyclin or other basic groups in order to improve binding both to double-stranded and single-strand DNA, and to peptides in order to improve membrane penetration. However, such linking has not resulted in satisfactory binding for either double-stranded or single-stranded DNA. Other problems which resulted from, for example, methylphosphonates and monothiophosphates were the occurrence of chirality, insufficient synthetic yield or difficulties in performing solid phase assisted syntheses.

In most cases only a few of these modifications could be used. Even then, only short sequences—often only diners—or monomers could be generated. Furthermore, the oligomers actually produced have rarely been shown to bind to DNA or RNA or have not been examined biologically.

The great majority of these backbone modifications led to decreased stability for hybrids formed between the modified oligonucleotide and its complementary native oligonucleotide, as assayed by measuring Tₘ values. Consequently, it is generally understood in the art that backbone modifications destabilize such hybrids, i.e., result in lower Tₘ values, and should be kept to a minimum.

OBJECTS OF THE INVENTION

It is one object of the present invention to provide compounds that bind ssDNA and RNA strands to form stable hybrids therewith.

It is a further object of the invention to provide compounds that bind ssDNA and RNA strands more strongly the corresponding DNA.

It is another object to provide compounds wherein naturally-occurring nucleobases or other nucleobase-binding moieties are covalently bound to a peptide backbone.

It is yet another object to provide compounds other than RNA that can bind one strand of a double-stranded polynucleotide, thereby displacing the other strand.

It is still another object to provide therapeutic and prophylactic methods that employ such compounds.

SUMMARY OF THE INVENTION

The present invention provides a novel class of compounds, known as peptide nucleic acids (PNAs), that bind complementary ssDNA and RNA strands more strongly than a corresponding DNA. The compounds of the invention generally comprise ligands linked to a peptide backbone via an aza nitrogen. 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 thieno-

In certain preferred embodiments, the peptide nucleic acids of the invention have the general formula (I):
wherein:

n is at least 2,

each of $L^1-L^n$ is independently selected from the group consisting of hydrogen, hydroxyl, (C$_1$-C$_4$)alkanoyl, naturally occurring nucleobases, non-naturally occurring nucleobases, aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, and reporter ligands, at least one of $L^1-L^n$ being a naturally occurring nucleobase, a non-naturally occurring nucleobase, a DNA intercalator, or a nucleobase-binding group;

each of $A^1-A^n$ is a single bond, a methylene group or a group of formula (Ia) or (Ib):

$$
\begin{align*}
\text{[R']_p} & \quad \text{C} \quad \text{Y} \quad \text{C} \quad \text{[R']_q} \\
\text{[R']_r} & \quad \text{c} \quad \text{C} \quad \text{[R']_s} \\
\end{align*}
$$

or

$$
\begin{align*}
\text{[R']_p} & \quad \text{C} \quad \text{Y} \quad \text{C} \quad \text{[R']_q} \\
\text{[R']_r} & \quad \text{c} \quad \text{C} \quad \text{[R']_s} \\
\end{align*}
$$

where:

X is O, S, Se, NR$, CH$_2$ or C(CH$_3$)$_2$;

Y is a single bond, O, S or NR$^2$; each of p and q is zero or an integer from 1 to 5, the sum p+q being not more than 10;

each of r and s is zero or an integer from 1 to 5, the sum r+s being not more than 10;

each R$^1$ and R$^2$ is independently selected from the group consisting of hydrogen, (C$_1$-C$_4$)alkyl which may be hydroxyl- or alkoxy- or alkylthio-substituted, hydroxy, alkoxy, alkylthio, amino and halogen; and

each R$^3$ and R$^4$ is independently selected from the group consisting of hydrogen, (C$_1$-C$_4$)alkyl, hydroxyl- or alkoxy- or alkylthio-substituted (C$_1$-C$_4$)alkyl, hydroxy, alkoxy, alkylthio and amino;

each of B$^1$-B$^n$ is N or R$^3$NR$, where R$^3$ is as defined above;

each of C$^1$-C$^n$ is CR$^2$R$^3$, CHR$^2$CHR$^4$ or CR$^2$CHR$^4$, where R$^2$ is hydrogen and R$^2$ is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or R$^3$ and R$^4$ are independently selected from the group consisting of hydrogen, (C$_2$-C$_6$)alkyl, aryl, aralkyl, heteroaryl, hydroxyl, (C$_1$-C$_4$)alkoxy, (C$_1$-C$_4$)alkylthio, NR$^3$R$^4$ and SR$^4$, where R$^3$ and R$^4$ are as defined above, and R$^2$ is hydrogen, (C$_1$-C$_4$)alkyl, hydroxyl-, alkoxy-, or alkylthio- substituted (C$_1$-C$_4$)alkyl, or R$^3$ and R$^4$ taken together complete an aliphatic or heterocyclic system;

each of D$^1$-D$^n$ is CR$^2$R$^3$, CHR$^2$CHR$^4$ or CHR$^2$CHR$^4$, where R$^3$ and R$^4$ are as defined above;

each of G$^1$-G$^{n+1}$ is —NR$^3$CO—, —NR$^3$CS—, —NR$^3$SO— or —NR$^3$SO$_2$—, Y in either orientation, where R$^3$ is as defined above;

Q is —CO$_2$H, —CONNR$^3$, —SO$_2$H or —SO$_2$NR$^3$R$^4$ or an activated derivative of —CO$_2$H or —SO$_2$H; and

I is —NHR$^3$R$^4$ or —NR$^3$C(O)R$^4$, where R$^3$, R$^4$, R$^5$ and R$^6$ 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.

The peptide nucleic acids of the invention differ from those disclosed in WO 86/05518 in that their recognition moieties are attached to anaza nitrogen atom in the backbone, rather than to an amide nitrogen atom, a hydrazine moiety or a carbon atom in the backbone.

Preferred peptide nucleic acids have general formula (III):

$$
\begin{align*}
\text{[CH}_2\text{H}_3\text{]} & \quad \text{N} \quad \text{[CH}_2\text{H}_3\text{]} \quad \text{O} \\
\text{[CH}_2\text{H}_3\text{]} & \quad \text{N} \quad \text{[CH}_2\text{H}_3\text{]} \\
\end{align*}
$$

wherein:

each L is independently selected from the group consisting of hydrogen, phenyl, heterocyclic moieties, naturally occurring nucleobases, and non-naturally occurring nucleobases;

each R$^5$ is independently selected from the group consisting of hydrogen and the side chains of naturally occurring alpha amino acids;

n is an integer from 1 to 60;

each of k, l and m is independently zero or an integer from 1 to 5;

R$^5$ is OH, NH$_2$ or —NHLysNH$_2$; and

R$^7$ is H or COCH$_3$.

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), and are zero
or 1, 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 com-
pounds and single-stranded DNA.

The peptide nucleic acids of the invention are synthesized
by adaptation of standard peptide synthesis procedures,
either in solution or on a solid phase. The synths used are
specifically designed monomer amino acids or their activated
derivatives, protected by standard protecting groups. The
oligonucleotide analogs also can be synthesized by using the
appropriate diacids and diamines.

Thus, the novel monomer synths according to the
invention are selected from the group consisting of amino
acids, diacids and diamines having general formulae:

\[
\begin{align*}
\text{(IV)} & \quad \text{E} & \quad \text{C} & \quad \text{B} & \quad \text{A} & \quad \text{D} & \quad \text{F} \\
\text{(V)} & \quad \text{E} & \quad \text{C} & \quad \text{B} & \quad \text{A} & \quad \text{D} & \quad \text{F} \\
\text{(VI)} & \quad \text{E} & \quad \text{C} & \quad \text{B} & \quad \text{A} & \quad \text{D} & \quad \text{F}
\end{align*}
\]

wherein \( L \), \( A \), \( B \), \( C \) and \( D \) are as defined above, except that
any amino groups therein may be protected by amino
protecting groups; \( E \) is \( \text{COOH}, \text{CSH}, \text{SOOH}, \text{SO}_2\text{OH} \) or an
activated derivative thereof; and \( F \) is \( \text{NH}_2R \) or \( \text{NPG}_2R \), where
\( R \) is as defined above and \( P \) is an amino protecting group.

Preferred monomer synths according to the invention are
amino acids having formula (VII):

\[
\begin{align*}
\text{(VII)} & \quad \text{L} & \quad \text{A} & \quad \text{B} & \quad \text{C} & \quad \text{D} & \quad \text{F} \\
\text{HOOC} & \quad \text{N} & \quad \text{R} & \quad \text{NH}_2
\end{align*}
\]

or amino-protected and/or acid terminal activated deriva-
tives thereof, wherein \( L \) is selected from the group consisting
of hydrogen, phenyl, heterocyclic moieties, naturally occur-
ing nucleobases, non-naturally occurring nucleobases, and
protected derivatives thereof; and \( R \) is independently
selected from the group consisting of hydrogen and the side
chains of naturally occurring amino acids. Especially
preferred are such synths having formula (VII) wherein
\( R \) 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.

Unexpectedly, these compounds also are able to recognize
duplex DNA by displacing one strand, thereby presumably
generating a double helix with the other one. Such recogni-
tion 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 of particular interest since this is
the length of unique sequences in the human genome. The
compounds of the invention also should be able to form
triplet helices with dsDNA.

Whereas the improved binding of the compounds of the
invention should render them efficient as antisense agents,
it is expected that an extended range of related reagents may
cause strand displacement, now that this surprising and
unexpected new behavior of dsDNA has been discovered.

Thus, in one aspect, the present invention provides meth-
ods for inhibiting the expression of specific genes in the
15 cells of an organism, comprising administering to said
organism a reagent as defined above which binds specifically
to sequences of said genes.

Further, the invention provides methods for inhibiting
transcription and/or replication of particular genes or for
inducing degradation of particular regions of double
stranded DNA in cells of an organism by administering to
said organism a reagent as defined above.

Still further, the invention provides methods for killing
cells or virus by contacting said cells or virus with a reagent
as defined above which binds specifically to sequences of
the genome of said cells or virus.

BRIEF DESCRIPTION OF THE DRAWINGS

The numerous objects and advantages of the present
invention may be better understood by those skilled in the art
reference to the accompanying figures, in which:

FIG. 1 shows a naturally occurring deoxyribonucleo-
tide (A) and a peptide nucleic acid (PNA) of the invention
(B).

FIG. 2 provides examples of naturally occurring and
non-naturally occurring nucleobases for DNA recognition
and reporter groups.

FIG. 3 provides a schematic illustration of (a) photocleavage
by Acr\(^1\)-(Taeg)\(_{10}\)-Lys-NH\(_2\) (Acr-Taeg\(_{10}\)-LysNH\(_2\)) of
3'-CTAGGTGTTTTTTTTTTCATG (SEQ ID NO: 40)/3'-
GATCCAAAAAAGGAATC (SEQ ID NO: 41); (b) photofootprint by the diazo-linked acridine of Acr-Taeg
10-Lys-NH\(_2\) and preferred KMnO\(_4\) cleavage; (c) S\(_1\) nuclease enhanced cleavage; and (d) micrococcal nuclease cleavage
of Acr\(^1\)-(Taeg)\(_{10}\)-Lys-NH\(_2\) binding site.

FIG. 4 provides examples of PNA monomer synths of
the invention.

FIG. 5 shows the Acr\(^1\) ligand and a PNA, Acr\(^1\)-(Taeg)\(_{10}\)-
Lys-NH\(_2\).

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

FIG. 7 provides a general scheme for the preparation of
the Acr\(^1\) ligand.

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

FIG. 9 shows analysis of HPLC chromatograms of:
(A) crude H-[Taeg]\(_{15}\)-NH\(_2\) after HF cleavage
(before lyophilization); (B) crude Acr\(^1\)-[Taeg]\(_{15}\)-NH\(_2\) after HF cleavage
(before lyophilization); and (C) purified Acr\(^1\)-[Taeg]\(_{15}\)-NH\(_2\).
Buffer A, 5% CH\(_3\)CN/95% H\(_2\)O/0.0445% TFA; buffer B,
60% CH\(_3\)CN/40% H\(_2\)O/0.0930% TFA; linear gradient,
0-100% of B in 30 min; flow rate, 1.2 ml/min; column,
Vydac C\(_{18}\) (5 μm, 0.46×25 cm).
FIG. 10 shows analytical HPLC chromatograms of: (A) purified H-[Taqel]_10-Lys-NH₂ and (B) purified H-[Taqel]_10-Cys-[Taqel]_4-Lys-NH₂ employing the same conditions as in FIG. 9.

FIGS. 11A and 11B show binding of AcT10-Lys to dA₁₀-\(5\,^{32}P\)-labeled oligonucleotide (1) (5\,^{32}P-GATCCCA\,G) (SEQ ID NO:1) in the absence or presence of AcT10-Lys-NH₂ and in the absence or presence of oligonucleotide (2) (5\,^{32}P-GATTCCTGT\,G) (SEQ ID NO:2) and the samples were analyzed by polyacrylamide gel electrophoresis (PAGE) and autoradiography under “native conditions” (FIG. 11A) or under “denaturing conditions” (FIG. 11B).

FIGS. 12A–C show chemical, photochemical and enzymatic probing of dsDNA-AcT10-Lys-NH₂ complex. Complexes between AcT10-Lys-NH₂ and a \(5\,^{32}P\)-end labeled DNA fragment containing a dA₁₀\/dT₁₀ target sequence were probed by affinity photocleavage (FIG. 12A, lanes 1–3; FIG. 12B, lanes 1–3), photolabeling (FIG. 12A, lanes 5–6), potassium permanganate probing (FIG. 12B, lanes 4–6) or probing by staphylococcus nuclease (FIG. 12B, lanes 8–10) or by nuclease S₁ (FIG. 12C). Either the A-strand (FIG. 12A) or the T-strand (FIG. 12B, C) was probed.

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

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

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

FIG. 16 provides examples of PNA backbone alterations.

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

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

FIG. 19 provides a procedure for synthesis of an aminoethyl-\(\beta\)-alanine analogue of thymine monomer synthon.

FIG. 20 shows a PAGE autoradiograph demonstrating that PNAs-T₁₀-T₁₀C and T₁₀C₁₀ bind to double stranded DNA with high sequence specificity.

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.

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.

FIG. 23 shows an electrophoretic gel staining demonstrating that restriction enzyme activity towards DNA (5\,^{32}P-GATCCCA\,GAGATCCCA\,G) (SEQ ID NO 42)\(5\,^{32}P\) CCTAGGTGTTTTTTTCTAGG (SEQ ID NO 43)) is inhibited when PNA is bound proximal to the restriction enzyme recognition site.

FIG. 24 shows a PAGE autoradiograph demonstrating that \(^{125}\)I-labeled PNA-T₁₀ binds to a complementary dA₁₀ oligonucleotide.

FIG. 25 shows a peptide nucleic acid according to the invention.

FIG. 26 shows the direction of synthesis for a peptide nucleic acid according to the invention.

FIG. 27 provides a test for the tosyl group as a nitrogen protecting group in the synthesis of peptide nucleic acids.
could be used. The design of the synths further allows such other moieties to be located on non-terminal positions.

In a further aspect of the invention, the PNA oligomers are conjugated to low molecular effector ligands such as ligands having nuclease activity or allylating activity or reporter ligands (fluorescent, spin labels, radioactive, protein recognition ligands, for example, biotin or hapten). In a further aspect of the invention, the PNA are conjugated to peptides or proteins, where the peptides have signaling activity and the proteins are, for example, enzymes, transcription factors or antibodies. Also, the PNA can be attached to water-soluble or water-insoluble polymers. In another aspect of the invention, the PNA 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.

Such conjugates can be used for gene modulation (e.g., gene targeted drugs), for diagnostics, for biotechnology, and for scientific purposes.

As a further aspect of the invention, PNA can be used to target RNA and ssDNA to produce both antisense-type gene regulating moieties and hybridization probes for the identification and purification of nucleic acids. Furthermore, the PNA can be modified in such a way that they can form triple helices with dsDNA. Reagents that bind sequence-specifically to dsDNA have applications as gene targeted drugs. These are foreseen as extremely useful drugs for treating diseases like cancer, AIDS and other virus infections, and may also prove effective for treatment of some genetic diseases. Furthermore, these reagents may be used for research and in diagnostics for detection and isolation of specific nucleic acids.

The triple helix principle is believed to be the only known principle in the art for sequence-specific recognition of dsDNA. However, triple helix formation is largely limited to recognition of homopurine-homopyrimidine sequences. Strand displacement is superior to triple helix recognition in that it allows for recognition of any sequence by use of the four natural bases. Also, in strand displacement recognition readily occurs at physiological conditions, that is, neutral pH, ambient (20–40 °C) temperature and medium (100–150 mM) ionic strength.

Gene targeted drugs are designed with a nucleobase sequence (containing 10–20 units) complementary to the regulatory region (the promoter) of the target gene. Therefore, upon administration of the drug, it binds to the promoter and block access thereto by RNA polymerase. Consequently, no mRNA, and thus no gene product (protein), is produced. If the target is within a vital gene for a virus, no viable virus particles will be produced. Alternatively, the target could be downstream from the promoter, causing the RNA polymerase to terminate at this position, thus forming a truncated mRNA/protein which is nonfunctional.

Sequence-specific recognition of ssDNA by base complementary hybridization can likewise be exploited to target specific genes and viruses. In this case, the target sequence is contained in the mRNA such that binding of the drug to the target hinders the action of ribosomes and, consequently, translation of the mRNA into protein. The peptide nucleic acids of the invention are superior to prior reagents in that they have significantly higher affinity for complementary ssDNA. Also, they possess no charge and water soluble, which should facilitate cellular uptake, and they contain amides of non-biological amino acids, which should make them biostable and resistant to enzymatic degradation by, for example, proteases.

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

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

Using the S1-nuclease probing technique, the discrimination of binding of the T10, T7, CTC(T7) and T7, CTC(T7, C7) PNA to the recognition sequences A10 G5 A8 G (SEQ ID NO: 4) and A5 G5 A5 G4 A8 G (SEQ ID NO: 5) cloned into the BamHI site and PstI of the plasmid pUC19 was analyzed. The results (FIG. 20) show that the three PNA bind to their respective recognition sequences with the following relative efficiencies: PNA-T10: A10 G5 A8 G A8 G; PNA-T7 C: A8 G A10 G5 A8 G; PNA-T7 C: A8 G A10 G5 A8 G. Thus at 37°C, one mismatch out of ten gives reduced efficiency (5–10 times estimated) whereas two mismatches are not accepted.

2. Kinetics of PNA-T0 dsDNA Strand Displacement Complex Formation (Example 66, FIG. 21).

Complex formation was probed by S1-nuclease at various times following mixing of PNA and 32P-end-labeled dsDNA fragment (FIG. 21).


Complexes between PNA-T10 and 32P-dsDNA (A10 G7 G) target were formed (60 min, 37°C). The complexes were then incubated at the desired temperature in the presence of excess oligo-dA10, for 10 min, cooled to RT and probed with KBrO4. 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".

4. Inhibition of Restriction Enzyme Cleavage by PNA (Example 65, FIG. 23).

The plasmid construct, pT10, contains a dA10/dT10 tract cloned into the BamHI site in pUC19. Thus, cleavage of pT10 with BamHI and PvuI results in two small DNA fragments of 211 and 111 bp, respectively. In the presence of PNA-T10, a 336 bp fragment is obtained corresponding to cleavage only by PvuII (FIG. 23). Thus 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.

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

A Tyr-PNA-T10, Lys–NH2 was labeled with 125I using Na125I and chloramine-T and purified by HPLC. The 5'-PNA-T10 was shown to bind to oligo-dA10 by PAGE and autoradiography (FIG. 24). The binding could be competed by excess denatured calf thymus DNA.

The sequence-specific recognition of dsDNA is illustrated by the binding of a PNA, consisting of 10 thymine substituted 2-aminoethylglycyl units, which C-terminates in a lysine amide and N-terminates in a complex 9-aminoacridine ligand (9-Acr1-(Taqe)1,Lys–NH2, FIGS. 11a, 11b) to the dA10/dT10 target sequence. The target is contained in a 248 bp 32P-end-labeled DNA fragment.

Strand displacement was ascertained by the following type of experiments:

1) The 9-Acr1 ligand (FIG. 5), which is equipped with a 4-nitrobenzamido 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 PNA with the above 248 bp DNA fragment, selective cleavage at the dA10/dT10 sequence is observed (FIG. 3a).
2) In a so-called photol footprinting assay, where a synthetic dino-linked acridine under irradiation cleaves DNA (except where the DNA is protected by said binding substance) upon interaction with DNA in the presence of a DNA-binding substance.

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

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 T10-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 T10-strand of the target (FIG. 3b).

5) In a similar type of demonstration, the single strand specificity of S1 nuclease clearly showed that only the T10-strand of the target was attacked (FIG. 3d).

The very efficient binding of (Taeg)10- (Taeg)10-Lys-NH2 and Acr(a-(Taeg)10-Lys-NH2 (FIGS. IIa, 1b) to the corresponding dA10, was furthermore illustrated in two ways:

1. Ligand-oligonucleotide complexes will migrate slower than the naked oligonucleotide upon electrophoresis in polyacrylamide gels. Consequently, such experiments were performed with Acr-(Taeg)10-Lys-NH2 and 32P-end-labelled dA10. This showed retarded migration under conditions where a normal dA10/dT10 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-(Taeg)10-Lys-NH2 and 32P-end-labelled dT10 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 denaturing of DNA can be followed by measuring changes in the absorbance, for example, as a function of Tm, 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 0.3 OD260 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 Gifford Response apparatus. The following extinction coefficients were used: A: 15.4 ml/mg/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 Tm were determined from the maximum of the 1st derivative of the plot of A260 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>56.0</td>
<td>51.5</td>
<td>50.0</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>84.5</td>
<td>80.0</td>
<td>90</td>
</tr>
<tr>
<td>2 + f</td>
<td>74</td>
<td>74</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>
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<tr>
<td>4 + f</td>
<td>62.0</td>
<td>62.0</td>
<td></td>
</tr>
<tr>
<td>5 + a</td>
<td>47.0</td>
<td>47.0</td>
<td></td>
</tr>
<tr>
<td>5 + c</td>
<td>57.5</td>
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<td></td>
</tr>
<tr>
<td>5 + f</td>
<td>46.5</td>
<td>46.5</td>
<td></td>
</tr>
<tr>
<td>7 + a</td>
<td>46.0</td>
<td>46.0</td>
<td></td>
</tr>
<tr>
<td>7 + c</td>
<td>58.0</td>
<td>58.0</td>
<td></td>
</tr>
<tr>
<td>7 + f</td>
<td>43.5</td>
<td>43.5</td>
<td></td>
</tr>
<tr>
<td>7 + 12</td>
<td>23.0</td>
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<td></td>
</tr>
<tr>
<td>13 + 14</td>
<td>39.0</td>
<td>39.0</td>
<td></td>
</tr>
</tbody>
</table>

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

The hybrid formed between RNA-A (poly rA) and PNA- T10-Lys-NH4 melts at such high temperature that it cannot be measured (>90 C). But specific hybridization is demonstrated by the large drop in A260 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 A260=0.6, and then measure 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 Tm value, whether the mispaired base is in the DNA or in the PNA-strand. The Tm value is only slightly dependent on ionic strength, as opposed to normal oligonucleotides.

The synthesis of the PNAAs according to the invention is discussed in detail in the following, where FIG. 1 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., Bannay 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 functionalization (Merrifield resin; via a chloromethyl methyl ether/SnCl4 reaction), aminomethyl functionality (via an N-hydroxysuccinimide reaction; see, Mitchell, et al., *Tetrahedron Lett.*, 1976, 3795), and benzyldiamino functionality (Pietta, et al., *J. Chem. Soc.*, 1970, 650) are the most widely applied. Regardless of its nature, the purpose of the functionalization 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. As will be recognized, anchoring linkages also can be formed between the solid support and the amino acid N-terminus. 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-methylbenzyldihydroamin and 4-methoxybenzyldihydroamin. 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, Bannay, et al., *Int. J. Peptide Protein 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 alkanolic acids such as 4-(bromomethyl)phenylacetic acid, Boc-aminoacetyl-4-(oxy methyl)aryl-lower alkanolic acids such as Boc-aminoacetyl-4-(oxy methyl)phenylacetic acid, N-Boc-p-acylbenzhydrylamines such as N-Boc-p-glutaroylbenzhydrylamine, N-Boc-4’-lower alkyl-p-acylbenz hydrylamines such as N-Boc-4’-methyl-p-glutaroylbenzhydrylamine, N-Boc-4’-lower alkoxy-p-acylbenz hydrylamines such as N-Boc-4’-methoxy-p-glutaroyl benzhydrylamine, and 4-hydroxymethylphenoxoacetic 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, 2015) 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-methyl benzhydrylamino and 4-methoxybenzyldihydroamin) 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, 4935 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.


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 side-chain protected peptide chains to form a longer PNA chain.

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 HCl (Sukakibara, et al., Bull. Chem. Soc. Jpn., 1965, 38, 4921), boron trifluoride (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 HCl) deprotection method, produces very reactive carbocations that may lead to alkylation and acylation of sensitive residues in the PNA chain. Side-reactions are only partly avoided by the presence of scavengers such as anisole, phenol, dimethyldulfide, and mercaptoethanol and, therefore, the sulfide-assisted acidolitic S2,2 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 diazotolysis (Bodanszky, et al., Chem. Ind., 1964 1423), hydrogenolysis (Jones, Tetrahedron
Finally, in contrast with the chemical synthesis of “normal” peptides, stepwise chain building of achiral PNA such as those based on aminomethylglycyl backbone units can start either from the N-terminus or the C-terminus, because the coupling reactions are free of racemization. Those skilled in the art will recognize that whereas synthesis commencing at the C-terminus typically employ protected amine groups and free or activated acid groups, syntheses commencing at the N-terminus typically employ protected acid groups and free or activated amine groups.

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) groups (molecular weight on the order of 10^6). 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 “pseudo-nucleotide” units. Thus, the PEPS film support has been successfully tested in a number of PNA syntheses carried out in a 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, experiences with other geometries of the PEPS polymer such as, for example, non-woven felt, knitted net, sticks or microwell-plates have not indicated any limitations of the synthetic efficacy.


While the conventional cross-linked styrene/divinylbenzene 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′-bisacyclolethylene-diamine, including a known amount of N-tertbutoxy-carbonyl-beta-alanyl-N′-acryloylhexamethylenediamine. 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 polyacrylamide-based supports are relatively more hydrophilic than are the polystyrene-based supports and are usually used with polar aprotic solvents including dimethylformamide, dimethylacetamide, N-methylpyrrolidone and the like (see Atherton, et al., J. Am. Chem. Soc., 1975, 97, 6584, Bioorg Chem. 1979, 8, 351), and J. C. S. Perkin 1538 (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 Grohmann, Angew. Chem. Internat. Ed. 1972, 11, 314) solid under the trademark “PORASIL E” by Waters Associates, Framingham, Mass., USA. Similarly, a mono ester of 1,4-dihydroxy-xymethylbenzene 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 Hamden, Conn., USA. Another exemplary composite contains a core of chlorinated ethylene polymer onto which has been grafted polystyrene (see Kent and Merrifield, Israel J. Chem. 1978, 17, 243) and van Rietschoen in “Peptides 1974”, Y. Wolman, Ed., Wiley and Sons, New York, 1975, pp. 113–116); and (4) contiguous solid supports other than PEPS, such as cotton sheets (Lebl and Eichler, Peptide Res. 1989, 2, 232) and hydroxypolycylate-coated polypolyene membranes (Daniels, et al., Tetrahedron Lett. 1989, 4345), are suited for PNA synthesis as well.

Whether manually or automatically operated, solid-phase PNA synthesis in the context of the present invention is normally performed batchwise. However, most of the syntheses may equally well be carried out in the continuous flow mode, where the support is packed into columns (Bayer, et al., Tetrahedron Lett., 1970, 4503 and Scott, et al., J. Chromatogr. Sci., 1971, 9, 577). With respect to continuous-flow solid-phase synthesis, the rigid poly(dimethylacrylamide)-Kieselgur support (Atherton, et al., J. Chem. Soc. Chem. Commun., 1981, 1151) appears to be particularly successful, but another valuable configuration concerns the
one worked out for the standard copoly (styrene-1%-divinylbenzene) support (Krchnak, et al., Tetrahedron Lett., 1987, 4469).

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 Bayer, Angew. Chem., Int. Ed. Engl., 1974, 13, 88), is useful; (3) random polymerization (see, e.g., Odian, “Principles of Polymerization” (McCraw-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 (Fredkin, 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 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 (abzymes), discovered simultaneously by the groups of Lerner (Tramantano, 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 enzymes 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 (Hahn, 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 achievable 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.

The present invention also is directed to therapeutic or prophylactic uses for peptide nucleic acids. Likely therapeutic and prophylactic targets include herpes simplex virus (HSV), human papillomavirus (HPV), human immunodeficiency virus (HIV), candida albicans, influenza virus, cytomegalovirus (CMV), intraacellular adhesion molecules (ICAM), 5-lipoxygenase (5-LO), phospholipase A2 (PLA2), protein kinase C (PKC), and RAS oncogene. Potential applications of such targeting include treatments for ocular, labial, genital, and systemic herpes simplex I and II infections; genital warts; cervical cancer; common warts; Kaposi’s sarcoma; AIDS; skin and systemic fungal infections; flu; pneumonia; retinitis and pneumonitis in immunosuppressed patients; mononucleosis; ocular, skin and systemic inflammation; cardiovascular disease; cancer; asthma; psoriasis; cardiovascular collapse; cardiac infarction; gastrointestinal disease; kidney disease; rheumatoid arthritis; osteoarthritis; acute pancreatitis; septic shock; and Crohn’s disease.

For therapeutic or prophylactic treatment, the peptide nucleic acids of the invention can be formulated in a pharmaceutical composition, which may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like in addition to peptide nucleic acid.

The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be done topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip or subcutaneous, intraperitoneal or intramuscular injection.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms may also be useful.

Compositions for oral administration may include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable.

Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

Dosing is dependent on severity and responsiveness of the condition to be treated, but will normally be one or more doses per day, with course of treatment lasting from several days to several months or until a cure is effected or a diminution of disease state is achieved. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates.

Treatments of this type can be practiced on a variety of organisms ranging from unicellular prokaryotic and eukaryotic organisms to multicellular eukaryotic organisms. Any organism that utilizes DNA-RNA transcription or RNA-protein translation as a fundamental part of its hereditary, metabolic or cellular control is susceptible to therapeutic and/or prophylactic treatment in accordance with the invention. Seemingly diverse organisms such as bacteria, yeast, protozoa, algae, all plants and all higher animal forms, including warm-blooded animals, can be treated. Further, since each cell of multicellular eukaryotes can be treated since they include both DNA-RNA transcription and RNA-protein translation as integral parts of their cellular activity. Furthermore, many of the organelles (e.g., mitochondria and chloroplasts) of eukaryotic cells also include transcription and translation mechanisms. Thus, single cells, cellular populations or organelles can also be included within the definition of organisms that can be treated with therapeutic or diagnostic phosphorothioate oligonucleotides. As used herein, therapeutics is meant to include the eradication of a
disease state, by killing an organism or by control of erratic or harmful cellular growth or expression.

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. H. Scoullar, ed., John Wiley & Sons, New York, 1983), notably solid-phase biocatalysts, especially bioassays or solid-phase techniques which concerns 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. Hames 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 (Minutani, et al., J. Chromatogr. 1986, 356, 202), "Sephadex", "Sephrose", agarose, polyacrylamide, porous particulate alumina, hydroxalkyl methacrylate gels, diol-bonded silica, porous ceramics, or contiguous materials such as filter discs of nylon and nitrocelulose. 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 more 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 (Letsinger and Mahadevan, J. Am. Chem. Soc. 1976, 98, 3655), which is suited for solid supports with a high surface/ loading capacity, only relatively short oligonucleotides can be obtained. As for conventional solid-phase peptide synthesis, however, the latter supports are excellent materials for building up immobilized PNA molecules (the side-chain protecting groups are 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.

Additional objects, advantages, and novel features of this invention will become apparent to those skilled in the art upon examination of the following examples thereof, which are not intended to be limiting.

**Synthesis of Monomeric Building Blocks**

The monomers preferably are synthesized by the general scheme outlined in FIG. 13. This involves preparation of either the methyl or ethyl ester of (Bocaminooethyl)glycine, by a protection/deprotection procedure as described in Examples 24-26. The synthesis of thymine monomer is described in Examples 27-28, and that of the protected cytosine monomer is described in Example 29.

The synthesis of the protected adenosine monomer (FIG. 14) involved alkylation with ethyl bromoacetate (Example 30) and verification of the position of substitution by X-ray crystallography, as being the wanted 9-position. The N9-amino group was protected by the benzoyloxyacar- bonyl group by the use of the reagent N9-ethylbenzoyloxy- carbonylimidazole tetrafluoroborate (Example 31). Simple hydrolysis of the product ester (Example 32) gave N9-ben- zoyloxyacarbonyl-n-carboxyethylmethyl adenine, which then was used in the standard procedure (Examples 33-34, FIG. 13). The adenosine monomer has been built into two different PNA-oligomers (Examples 56, 57, 71 and 73).

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 35) and the chlorine atom was then substituted with a benzoyloxy group (Example 36). The resulting acid was coupled to the (bocaminooethyl)glycine methyl ester (from Example 26) with agent PyBrop™, and the resulting ester was hydrolysed (Example 37). The O9-benzyl group was removed in the final HF-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 disopropyl carbodiimide as the condensation agent (Examples 55 and 71).

**Extended Backbones**

Alterations of the groups A, C and D (FIG. 16) is 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 38), which is cleaved by periodate to give bocaminooacetaldheyde, which is used directly in the next reaction. The bocaminooacetaldheyde can be condensed
with a variety of amines; in Example 39, alanine ethyl ester was used. In Examples 40–42, the corresponding thymine monomers were prepared. The monomer has been incorporated into an 8-mer (Example 60) by the DCC-coupling protocol (Examples 56 and 57).

In another example, the D group is a (CH$_2$)$_3$ group. The synthesis of the corresponding thymine monomer is outlined in FIG. 18A and described in Examples 43–44.

In another example, the A group is a (CH$_2$)$_3$CO group. The synthesis of the corresponding thymine monomer is outlined FIG. 18B and Examples 46 through 48.

In yet another example, the C group is a (CH$_2$)$_2$ group. The synthesis of the thymine and protected cytosine monomer is outlined in FIG. 19 and Examples 49 through 54. Hybridization experiments with a PNA-oligomer containing one unit is described in Examples 61 and 81, which shows a significant lowering of affinity but a retention of specificity.

General Remarks

The following abbreviations are used in the experimental examples: DMF, N,N-dimethylformamide; DCC, N,N-dicyclohexyl carbodiimide; DCE, N,N-dicyclohexyl urea; THF, tetrahydrofuran; aeg, N-acetyl (2”-aminoethyl)glycine; pfp, pentafluorophenyl; Boc, tert-butyloxycarbonyl; Z, benzoxycarbonyl; NMR, nuclear magnetic resonance; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet; b, broad; δ, 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 4 Å molecular sieves, distilled and stored over 4 Å molecular sieves. Pyridine (HPLC-quality) was dried and stored over 4 Å 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. Boc-anhydride, 4-nitrophthalaldehyde, benzyl chloride, and acetic acid were obtained through Aldrich Chemical Company. Thymine, cytosine, and adenine were all obtained through Sigma.

Thin layer chromatography (Tlc) was performed using the following solvent systems: (1) chloroform:trichloroethylene: methanol, 7:1:2; (2) ethylene dichloride: methanol, 9:1; (3) chloroform:methanol:acetic acid 85:10:5. Spots were visualized by UV (254 nm) or/and spraying with a ninhydrin solution (3 g ninhydrin in 1000 ml 1-butanol and 30 ml acetic acid), after heating at 100 °C. For 5 min and, after spraying, heating again.

EXAMPLE 1

tert-Butyl 4-nitrophthalaldehyde carbonate

Sodium carbonate (29.14 g; 0.275 mol) and 4-nitrophthalaldehyde (12.75 g; 91.6 mmol) were mixed with dioxane (250 ml). Boc-anhydride (20.0 g; 91.6 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 piperic acid, in vacuo. Yield 21.3 g (97%). M.p. 73.0–74.5 °C. (lit. 78.5–79.5 °C.). Anal. for C$_7$H$_6$N$_2$O$_2$: found (calc.) C, 55.20 (55.23); H, 5.61 (5.48); N, 5.82 (5.85).

EXAMPLE 2

(N’-Boc-2’-aminoethyglycine (2)

The title compound was prepared by a modification of the procedure by Heimer, et al. Int. J. Pept., 1984, 23, 203–211. 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-nitrophthalaldehyde (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 2N sodium hydroxide and the solution was evaporated to dryness, in vacuo (14 mm Hg). The residue was extracted with DMF (25×2×10 ml) and the extract filtered to remove excess salt. This resulted in a 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-Carboxymethylthymine (4)

This procedure is different from the literature synthesis, but is easier, gives higher yields, and leaves no unreacted thymine in the product. A suspension of thymine (3.40 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 4 N hydrochloric acid (70 ml). Yield after drying, in vacuo over silica: 37.1 g (64%). 1H-NMR: (90 MHz; DMSO-d$_6$): 11.33 ppm (s, 1H, NH); 7.49(d, J=0.9 Hz, 1H, ArH); 4.38 (s, 2H, CH$_2$); 1.76 (d, J=0.9 Hz, T-CH$_3$).

EXAMPLE 4

N-1-Carboxymethylthymine pentafluorophenyl ester (5)

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 3 h at ambient temperature. The precipitated DCC 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 was washed thoroughly with petroleum ether. Yield: 14.8 g (78%).
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product was pure enough to carry out the next reaction, but an analytical sample was obtained by recrystallization from 2-propanol. M.p. 200.5–206°C. Anal. for C₁₃H₁₂N₃O₄, Found(calc.): C, 44.79(44.59); H, 2.14(2.01); N, 8.15(8.00). FAB-MS: 443 (M+1+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)lthyline (6)

To the DME-solution from above was added triethylamine (7.08 ml; 50.8 mmol) followed by N₁-carboxybenzyl-methylamine pentaf luorophenyl ester (5; 4.45 g; 12.7 mmol). The resulting 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 (2x15 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 an 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 and washed with petroleum ether. Drying over silica gel, in vacuo, gave 3.50 g (71.7%). M.p. 142–147°C. Anal. for C₁₃H₁₂N₃O₄, 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). 12.75 ppm (5, 1H, —CO₂H); 11.27 ppm (s, mj. imide); 11.25 ppm (s, mi. imide); 7.30 ppm (s, mj. ArH); 7.26 ppm (s, mi. ArH); 6.92 ppm (unres. t, mj. BocNH₂); 6.73 ppm (unres. t, mi. BocNH₂); 4.64 ppm (s, mj. T-CH₃—CO—); 4.47 ppm (s, mi. T-CH₃—CO—); 4.19 ppm (s, mi. CONR₂CO₂H); 3.97 ppm (s, mj. CONR₂CO₂H); 3.41–2.89 ppm (unres. m, —CH₂CH₂— and water); 1.75 ppm (s, 3H, T-CH₃); 1.38 ppm (8, 9H, t-Bu).

C-NMR: 170.68 (CO); 170.34 (CO); 167.47 (CO); 167.08 (CO); 164.29 (CO); 150.9 (C⁵); 141.92 (C⁶); 108.04 (C²); 77.95 and 77.68 (Thy-CH₃CO₂); 48.96, 47.45 and 46.70 (—CH₂CH₂— and NCH₂CO₂H); 37.98 (Thy-CH₃); 28.07 (t-Bu). FAB-MS: 407 (M+Na⁺); 385 (M+H⁺).

EXAMPLE 6

1-(Boc-aeg)lthyline pentaf luorophenyl ester (7, Boc-Taeq.OPPp)

1-(Boc-aeg)lthyline (6) (2.00 g; 5.20 mmol) was dissolved in DME (5 ml) and methylene chloride (15 ml) was added. Pentfluorobenzophenol (1.05 g; 5.72 mmol) was added and the solution was cooled to 0°C in an ice bath. DDC 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 DDC was removed by filtration and washed with methylene chloride. The combined filtrate was washed twice with aqueous sodium hydroxide carbonate and once with saturated sodium chloride, dried over magnesium sulfate, and evacuated 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 scapent, 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 C₁₃H₁₂N₃O₄, Found(calc.): C, 48.22(48.01); H, 4.64(4.21); N, 9.67(10.18). ¹H-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, mi. ArH); 5.27 ppm (unres. t, BocNH₂); 4.67 ppm (s, mj. T-CH₂—CO—); 4.60 ppm (s, mj. T-CH₂—CO—); 4.45 ppm (s, mj. CONR₂CO₂H); 4.42 ppm (s, mi. CONR₂CO₂H); 3.64 ppm (s, 2H, BocNHCl₂CH₂—); 3.87 ppm (q”, 2H, BocNHCl₂CH₂—); 1.44 ppm (s, 9H, t-Bu). FAB-MS: 551 (10); M+1; 495 (10); M+1+0Bu; 451 (80); Boc.

EXAMPLE 7

N₄-Benzoyloxycarbonyl cytosome (9)

Over a period of about 1 h, benzoyloxycarbonyl chloride (52 ml; 0.36 mol) was added dropwise to a suspension of cytosome (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. Anal. for C₁₃H₁₂N₃O₄, 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-Benzoyloxycarbonyl-N₁-carboxymethyl cytosome (10)

In a three-necked round bottom flask equipped with mechanical stirring and nitrogen coverage was placed methyl bromacetate (7.82 ml; 82.6 mmol) and a suspension of N₂-benzoyloxycarbonyl-cytosome (9, 21.0 g; 82.6 mmol) and potassium carbonate (11.4 g; 82.6 mmol) in dry DME (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 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 color. M.p. 254–254°C. Anal. for C₁₃H₁₂N₃O₄, 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, PhH); 7.01 (d, J=7.32 Hz, 1H, H-5); 5.19 (s, 2H, PhCH₂); 4.52 (s, 2H).
EXAMPLE 9

N^4-Benzoylcarbonyl-N^1-carboxymethyl-cytosine pentfluorophenyl ester (11)

N^4-Benzoylcarbonyl-N^1-carboxymethyl-cytosine (10, 4.00 g; 13.2 mmol) and pentfluorophenol (2.67 g; 14.5 mmol) were mixed with DMF (70 ml), cooled to 0°C, with ice-water, and DCC (3.27 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 DCC was removed by filtration, washed with DMF, and the filtrate was evaporated to dryness, in vacuo (0.2 mmHg). The solid residue was treated with methylene chloride (250 ml), stirred vigorously for 15 min, filtered, washed twice with diluted sodium hydrogen carbonate 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 5.40 g (55%), M.p. 241-245°C. Anal. for C_{30}H_{27}N_{1}O_{4}F, Found: C, 51.56(51.18); H, 2.77(2.58); N, 9.24(8.95). 1H-NMR (90 MHz, CDC13): 7.66 ppm (d, J=7.63 Hz, 1H, H-6); 7.37 (s, 5H, Ph); 7.51 (d, J=7.63 Hz, 1H, H-5); 5.21 (s, 2H, PhCH=); 4.97 (s, 2H, NCH=). FAB-MS: 470 (M+1)

EXAMPLE 10

N^4-Benzoylcarbonyl-1-Boc-aeg-cytosine (12)

To a solution of (N-Boc-2-aminoethyl)glycine (2) in DMF, prepared as described above, was added triethylamine (7.00 ml; 50.8 mmol) and N^4-benzoylcarbonyl-N^1-carboxymethyl-cytosine pentfluorophenyl ester (11, 2.70 g; 5.75 mmol). After stirring the solution for 1 h at room temperature, methylene chloride (150 ml), saturated sodium chloride (250 ml), and 4 N hydrochloric acid to pH 1 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 pentfluorophenol, but no attempt was made to remove it. Yield: 1.72 g (59%). M.p. 156°C (dec.). 1H-NMR (250 MHz, CDCl3): Due to the limited rotation around the secondary amide bond several of the signals were in the ratio 2:1, identified in the list by mj. for major and mi. for minor). 7.88 ppm (dd, 1H, H-6); 7.39 (m, 5H, Ph); 7.00 (dd, 1H, H-5); 6.92 (b, 1H, BocNH); 6.74 (b, 1H, ZNH;); 5.19 (s, 2H, PhCH=); 4.81 ppm (s, mj., Cyt-CH2—CO—); 4.62 ppm (s, mi., CH2—CO—); 4.23 (s, mj., CONRCH2COH); 3.98 ppm (s, mi., CONRCH2COH); 3.42–3.02 (taures, m.—CH2—CH— and water); 1.37 (s, 9H, Boc). FAB-MS: 504 (M+1); 448 (M+1—Boc).

EXAMPLE 11

N^4-Benzoylcarbonyl-1-Boc-aeg-cytosine pentfluorophenyl ester (13)

N^4-Benzoylcarbonyl-1-Boc-aeg-cytosine (12, 1.50 g; 2.98 mmol) and pentfluorophenol (548 mg; 2.98 mmol) was dissolved in DMF (10 ml) Methylene 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 3 min and the mixture was stirred for 3 h at ambient temperature. The precipitate was isolated by filtration and washed once with methylene chloride. The precipitate was dissolved in boiling dioxane (150 ml) and the solution was cooled to 15°C, whereby DCU 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 sicapent, in vacuo. Yield 1.30 g (65%). Analysis for C_{30}H_{27}N_{1}O_{4}F, Found: 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—Boc).

EXAMPLE 12

4-Chlorocarboxylic acid (25 ml), and 4 drops of DMF were heated gently under a flow of nitrogen until all the solid material had dissolved. The solution then was refluxed for 40 min. The solution was cooled and excess thiouyl chloride were removed by coevaporation with dry benzene (dried over Na—Ph) twice. The remaining yellow powder was used directly in the next reaction.

EXAMPLE 13

4-(Methylxocarbonylpyrrolidin-9-chloroacridine

Methyl 6-aminoheptanate hydrochloride (4.70 g; 25.9 mmol) was dissolved in methylene chloride (90 ml), cooled to 0°C, triethylamine (15 ml) was added, and the resulting solution then was immediately, added to the acid chloride from above. The roundbottomed 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 methyll 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—Ph). The mixture was heated to reflux. Activated carbon and celite 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-(4-Methoxybenzoylpyrrolidin-9-[6'-

4-(3-Nitrobenzamido)hexylaminol]-aminoacridine

4-(6-Methoxybenzoylpyrrolidin-9-chloroacridine (1.30 g; 3.88 mmol) and phenol (5 g) were heated to 80°C for 30 min under a flow of nitrogen, after which
Materials: Boc-Lys(CI2), benzhydrylamino-copoly-(styr-
ene-1%-divinylbenzene) resin (BHA resin), and p-methyl-
benzhydrylamino-copolystyrene-1%-divinylbenzene resin
(MBHA resin) were purchased from Peninsula Laborato-
ries. Other reagents and solvents were: Biograde trifluoro-
acetic acid from Halocarbon Products; disopropylethyl-
amine (99%; not further distilled) and N-acetylaminidazole
(98%) from Aldrich; H2O was distilled twice; anhydrous HF
from Union Carbide; synthesis grade N,N-dimethylfor-
ma-mide and analytical grade methylene chloride (was
not further distilled) from Merck; HPLC grade acetonitrile
from Lab-San; purum grade anisole, N,N-dicyclohexylcarbodi-
imide, disopropylcarbodiimide, puriss. grade 2,2,2-triflu-
oroethanol from Fluka and trifluoromethanesulfonic acid
from Acr-oil.

(a) General Methods and Remarks

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 “tem-
porary” N-protection (Merrifield, J. Am. Chem. Soc., 1964,
86, 304) and the more acid-stable benzoxycarbonyl (Z)
and 2-chlorobenzoxycarbonyl (Clz) groups for “per-
manent” side chain protection. To obtain C-terminal amides,
the PNA’s 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
(Matsueda, et al., Peptides, 1981, 12, 45). All reactions (ex-
cept HF reactions) were carried out in manually operated
standard solid-phase reaction vessels fitted with a glass
coating (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”
primary amines, is successfully applied (see Table I-III)
using the “normally” employed effective extinction coeffi-
cient 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 theo-
retical substitution S0 is the coupling constant of residue n
(streaming both condensation and coupling as well as
either chain termination nor loss of PNA chains during the
synthetic cycle) is calculated from the equation:

\[ S_n = S_{0,n} \times (1 + (S_{0,n} \times \text{AMW} \times 10^{-3} \text{ mmol/mol})^{-1} \]

where AMW is the gain in molecular weight ([AMW]=g/mol)
and S0 is the theoretical substitution upon coupling of the
preceding residue n-1 ([S]-mmol/g). The estimated value (%)
was determined in the extent of an individual coupling is cal-
culated 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 Diaplan HF apparatus
from Toho Kasei (Osaka, Japan). Vydac C18 (5 μm, 0.46x25
cm and 5 μm, 1.0x25 cm) reverse-phase columns, respec-
tively were used for analytical and semi-preparative HPLC
on an S8000 instrument. Buffer A was 5 vol % aceto-
nitrile in water containing 445 μl trifluoroacetic acid per liter,
and buffer B was 60 vol % acetonitrile in water containing
390 μl trifluoroacetic acid per liter. The linear gradient was
0–100% of buffer B in 30 min, flow rates 1.2 ml/min (ana-
lytical) and 5 ml/min (semi-preparative). The eluents were
monitored at 215 nm (analytical) and 230 nm (semi-
preparative). Molecular weights of the PNA’s were deter-

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

EXAMPLE 15

4-(5-Carboxypentyl)-amidocarbonyl-9-(6'-4-ni-
 trobenzamido)-hexylaminio-aminoacridine (Acr-Polp)

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 2x2
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 centrifu-
gation 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 5M HCl in ether was added.
The solvent was removed by decanting after 30 min of stirring at
0°C. The residual oil substance was dissolved in methy-
lene 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 elemen-
tal analysis, H- and C-NMR and mass spectra.

EXAMPLE 16

4-(5-Phenanthrophenyl-oxycarbonyl-pentyl)-amidocar-
bonyl-9-(6'-4-nitrobenzamido)-hexylaminio-aminoacrid-
ine (Acr-Polp)

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 2x2
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 centrifu-
gation 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 5M HCl in ether was added.
The solvent was removed by decanting after 30 min of stirring at
0°C. The residual oil substance was dissolved in methyl-
ene 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 elemen-
tal analysis, H- and C-NMR and mass spectra.
mined by ²²⁴Cf plasma desorption time-of-flight mass spectrometry from the mean of the most abundant isotopes.

**EXAMPLE 17**

Solid-Phase Synthesis of Acr¹-[Taeg]₁₅-NH₂ and Shorter Derivatives

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

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 shimming for at least 12 h in a manually operated 6 ml standard solid-phase reaction vessel and unreacted 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 1). 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>Remaining Free Amino Groups After Deprotection</th>
<th>Estimated Extent of Coupling</th>
<th>(mmol/g)</th>
<th>Single Coupling (µmol/g)</th>
<th>Acetylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Coupled</td>
<td>Meas.</td>
<td>Theoret.</td>
<td>Coupling</td>
<td>Acetylation</td>
</tr>
<tr>
<td>&quot;u&quot;</td>
<td></td>
<td></td>
<td></td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>1 BocTaeg</td>
<td>ND</td>
<td>0.50</td>
<td>1.30</td>
<td>&lt;99.7</td>
<td></td>
</tr>
<tr>
<td>2 BocTaeg</td>
<td>ND</td>
<td>0.44</td>
<td>1.43</td>
<td>&lt;99.0</td>
<td></td>
</tr>
<tr>
<td>3 BocTaeg</td>
<td>0.29</td>
<td>0.39</td>
<td>3.33</td>
<td>99.3</td>
<td></td>
</tr>
<tr>
<td>4 BocTaeg</td>
<td>0.27</td>
<td>0.35</td>
<td>13.30</td>
<td>96.3</td>
<td></td>
</tr>
<tr>
<td>5 BocTaeg</td>
<td>0.26</td>
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<td>8.33</td>
<td>&gt;99.9</td>
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</tr>
<tr>
<td>6 BocTaeg</td>
<td>0.30</td>
<td>0.37</td>
<td>7.78</td>
<td>&gt;99.9</td>
<td></td>
</tr>
<tr>
<td>7 BocTaeg</td>
<td>ND</td>
<td>0.28</td>
<td>13.81</td>
<td>7.22</td>
<td>&gt;97.8</td>
</tr>
<tr>
<td>8 BocTaeg</td>
<td>ND</td>
<td>0.26</td>
<td>14.00</td>
<td>&lt;99.9</td>
<td></td>
</tr>
<tr>
<td>9 BocTaeg</td>
<td>ND</td>
<td>0.24</td>
<td>30.33</td>
<td>93.2</td>
<td></td>
</tr>
<tr>
<td>10 BocTaeg</td>
<td>0.16</td>
<td>0.23</td>
<td>11.67</td>
<td>2.67</td>
<td>&gt;99.9</td>
</tr>
<tr>
<td>11 BocTaeg</td>
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<td>4.58</td>
<td>&gt;99.9</td>
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<tr>
<td>12 BocTaeg</td>
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<td>5.87</td>
<td>&lt;99.0</td>
<td></td>
</tr>
<tr>
<td>13 BocTaeg</td>
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<td>1.67</td>
<td>&gt;99.9</td>
<td></td>
</tr>
<tr>
<td>14 BocTaeg</td>
<td>ND</td>
<td>0.18</td>
<td>14.02</td>
<td>&lt;93.0</td>
<td></td>
</tr>
<tr>
<td>15 BocTaeg</td>
<td>0.07</td>
<td>0.17</td>
<td>4.20</td>
<td>3.33</td>
<td>&gt;99.9</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 50 mg; 0.002 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., 0.11 M solution of the pentfluorophenylester) 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 HCl 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 HCl:anisole (9:1, v/v) stirring at 0°C for 60 min. After removal of HCl, the residue was stirred with dry diethyl ether (4x15 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 (4x15 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 from 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-NH₂

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 phenomenon 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}-NH\_2.

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 aggregation/conformational states of the target molecule H-[Taeg]_{15}-NH\_2 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 Acr-[Taeg]_{15}-NH\_2.

A portion of protected Acr-[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 Acr-[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. 12B). The crude product was purified repeatedly to give approximately 99% pure Acr-[Taeg]_{15}-NH\_2 "contaminated" with self-aggregated molecules eluting at 27.4 min, 29.2 min, and finally as a large 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 out quantitatively. For (M+H)^+ the calculated m/z value was 4593.6 and the measured m/z value was 4588.7.

(g) Synthetic Protocol 1

(1) Boc-deprotection with TFA/CH\_2Cl\_2 (1:1, v/v), 3 ml, 3x1 min and 1x30 min; (2) washing with CH\_2Cl\_2, 3 ml, 6x1 min; (3) neutralization with DIEA/CH\_2Cl\_2 (1:19, v/v), 3 ml, 3x2 min; (4) washing with CH\_2Cl\_2, 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-TaqeG-OPfp dissolved in 1 ml CH\_2Cl\_2 followed by addition of 0.5 mg DMF (final concentration of pentafluorophenylester-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 CH\_2Cl\_2, 3 ml, 4x1 min; (9) neutralization with DIEA/CH\_2Cl\_2 (1:19, v/v), 3 ml, 2x2 min; (10) washing with CH\_2Cl\_2, 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-acetylimidazol in methylene chloride).

**EXAMPLE 18**

**Solid-Phase Synthesis of Acr-[Taeg]_{15}-Lys-NH\_2 and Shorter Derivatives**

(a) Stepwise Assembly of Boc-[Taeg]_{15}-Lys(CIZ)-BRA Resin

The synthesis was initiated by a quantitative loading (standard DCC in situ coupling in neat CH\_2Cl\_2) of Boc-Lys (CIZ) onto 100 mg of press swollen and neutralized BHA resin (0.57 mmol NH\_2/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-TaqeG-OPfp in about 33% DMF/CH\_2Cl\_2. Cycles 5 to 10 employed an extra straight DCC (i.e., in situ) coupling of the free acid Boc-TaqeG-OF in about 33% DMF/CH\_2Cl\_2. 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]_{15}-Lys(CIZ)-BHA and Boc-[Taeg]_{15}-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]_{15}-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]_{15}-Lys(CIZ)-BRA Resin

Following deprotection of a portion of Boc-[Taeg]_{15}-Lys(CIZ)-BHA resin (estimated dry weight is about 90 mg; 0.01 mmol growing chains), the H-[Taeg]_{15}-BHA resin was reacted with about 20 equivalents (141 mg; 0.19 mmol) of Acr-OPfp in 1 ml of about 66% DMF/CH\_2Cl\_2 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]_{15}-Lys(CIZ)-BHA Resin

Following deprotection of the residual Boc-[Taeg]_{15}-Lys(CIZ)-BHA resin (estimated dry weight about 70 mg; 0.005 mmol growing chains), the H-[Taeg]_{15}-Lys(CIZ)-BHA resin was reacted with about 25 equivalents (91 mg; 0.12 mmol) of Acr-OPfp in 1 ml of about 66% DMF/CH\_2Cl\_2 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]_{15}-Lys-NH\_2

A portion of protected Boc-[Taeg]_{15}-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]_{15}-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]_{15}-Lys-NH\_2.

(e) Cleavage, Purification, and Identification of H-[Taeg]_{15}-Lys-NH\_2

A portion of protected Boc-[Taeg]_{15}-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]_{15}-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]_{15}-Lys-NH\_2, FIG. 13. 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]_{15}-Lys-NH\_2

99.1 mg protected Acr-[Taeg]_{15}-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 8.3 mg of crude product was purified to give approximately 5.3 mg of >97% pure H-Tae[l]_1-Lys-NH_2. 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 Acc]-[Tae[l]_1-Lys-NH_2 A 78.7 mg portion of protected Acc]-[Tae[l]_1-Lys(CIZ)]-BHA resin (dry weight) was cleaved as described in Example I section (c) to yield 34.8 mg of crude material. The main peak at 23.5 min (about the elution time if injected from an aqueous solution containing 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 H-[Tae[l]_1-Lys-NH_2. This compound could not be free of the “dimer” peak, which grew upon standing in aqueous acetic acid solution.

(h) Synthetic Protocol 2

(1) Boc-deprotection with TFA/CH_2Cl_2 (1:1 v/v), 3 ml, 3x1 min and 1x30 min; (2) washing with CH_2Cl_2, 3 ml, 6x1 min; (3) neutralization with DIEA/CH_2Cl_2 (1:1 v/v), 3 ml, 3x2 min; (4) washing with CH_2Cl_2, 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 3.2 equiv. (0.18 mmol; 100 mg) BocTae[3]-OPOEt dissolved in 1 ml CH_2Cl_2 followed by addition of 0.5 ml DMF (final concentration of pentfluorophenolester ~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 BocTae[2]-OH in 1.5 ml DMF/CH_2Cl_2 (1:2 v/v); (7) washing with DMF, 3 ml, 1x2 min; (8) washing with CH_2Cl_2, 3 ml, 4x1 min; (9) neutralization with DIEA/CH_2Cl_2 (1:1 v/v), 3 ml, 2x2 min; (10) washing with CH_2Cl_2, 3 ml, 6x1 min; (11) 2-5 mg sample of protected PNA-resin is taken out for a qualitative ninhydrin test (after cycles 7, 10, and 15 unretracted amino groups were blocked by acetylation with N-acetylomimidazole in methylene chloride).

EXAMPLE 19

Improved Solid-Phase Synthesis of H-[Tae[l]_1-Lys-NH_2

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 unreacted amino groups. The following describes the synthesis in full detail:

(a) Preparation of Boc-Lys(CIZ)-NH—CH (p-CH— C_6H_4)—C_6H_3 Resin (XBRA Resin) with an Initial Substitution of 0.3 mmol/g

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 NH_2/g) using a single “in situ” coupling (1.5 mmol of DCC) in 60 ml of CH_2Cl_2. 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/ CH_2Cl_2 (1:1:2, v/v/v) for 18 h. A quantitative ninhydrin reaction on the neutralized resin showed that only 0.00095 mmol/g free amine remained (see Table I), i.e. 0.15% 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-[Tae[l]_1-Lys(CIZ)]-MBHA Resin

The entire batch of H-Lys(CIZ)-MBHA resin prepared in section (a) was used directly (in the same reaction vessel) to assemble Boc-[Tae[l]_1-Lys(CIZ)]-MBHA resin by single couplings (“Synthetic Protocol 3”) utilizing 2.5 equivalents of BocTae[3]-OPOEt in neat CH_2Cl_2. The quantitative ninhydrin reaction was applied throughout the synthesis (see Table II).

(c) Stepwise Assembly of Boc-[Tae[l]_1-Lys(CIZ)]-MBHA Resin

About 4.5 g of wet Boc-[Tae[l]_1-Lys(CIZ)]-MBHA resin (~0.36 mmol growing chains; taken out of totally ~19 g wet resin prepared in section (b)) was placed in a 55 ml SPPS reaction vessel. Boc-[Tae[l]_1-Lys(CIZ)]-MBHA resin was assembled by single couplings (“Synthetic Protocol 4”) utilizing 2.5 equivalents of BocTae[3]-OPOEt in about 30% DMF/CH_2Cl_2. The progress of the synthesis was monitored at all stages by the quantitative ninhydrin reaction (see Table II).

(d) Stepwise Assembly of Boc-[Tae[l]_1-Lys(CIZ)]-MBHA Resin

About 1 g of wet Boc-[Tae[l]_1-Lys(CIZ)]-MBHA resin (~0.09 mmol growing chains; taken out of totally ~4 g wet resin prepared in section (c)) was placed in a 20 ml SPPS reaction vessel. Boc-[Tae[l]_1-Lys(CIZ)]-MBHA resin was assembled by the single-coupling protocol employed in the preceding section utilizing 2.5 equivalents of BocTae[3]-OPOEt in about 30% DMF/CH_2Cl_2. 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</th>
<th>Remaining Free Amino Groups After Coupling (mmol/g)</th>
<th>Estimated Extent of Coupling</th>
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<td>Meas</td>
<td>Theor</td>
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<td>Boc-[Taeg]_{10}-Lys</td>
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(e) Synthesis of Ac-[Taeg]_10-Lys(CIZ)-MBHA Resin

Following deprotection of a portion of Boc-[Taeg]_{10}-Lys(CIZ)-MBHA resin (estimated dry weight is about 45 mg), the resin was next acetylated quantitatively with a 2 ml mixture of acetic anhydride/pyridine/CHCl_3 (1:1:2, v/v/v) for 2 h in a 3 ml solid-phase reaction vessel.

(f) Cleavage, Purification, and Identification of H-[Taeg]_{10}-Lys-NH_2

A portion of protected Boc-[Taeg]_{10}-Lys(CIZ)-BHA resin was treated as described in Example 17c to yield about 24 mg of crude material upon HF cleavage of 76 mg dry H-[Taeg]_{10}-Lys(CIZ)-BHA resin. The main peak at 15.2 min (which includes impurities such as deletion peptides and various byproducts) accounted for about 78% of the total absorbance. The main peak also accounted for about 88% of the "main peak plus deletion peaks" absorbance, which is in good agreement with the overall estimated coupling yield of 90.1% obtained by summarizing the individual coupling yields in Table II. A 7.2 mg portion of the crude product was purified by two batches by use of a semi-preparative reverse-phase column, (collecting the main peak in a beaker cooled with dry ice/2-propanol). Each contained 3.6 mg in 1 ml of H_2O. The frozen solution was lyophilized directly (without prior removal of acetonitrile on a speed vac) to give 4.2 mg of 82% pure H-[Taeg]_{10}-Lys-NH_2.

(g) Cleavage, Purification, and Identification of Ac-[Taeg]_{10}-Lys-NH_2

A 400.0 mg portion of protected Ac-[Taeg]_{10}-Lys(CIZ)-BHA resin (dry weight) was cleaved as described in Example 17c, except for the TFA treatment to yield 11.9 mg of crude material. The main peak at 15.8 min accounted for about 75% of the total absorbance. A 4.8 mg portion of the crude product was purified to give approximately 3.5 mg of >95% pure Ac-[Taeg]_{10}-Lys-NH_2. For (M+H) the calculated m/z value = 2849.8 and the measured m/z value = 2848.8.

(h) Synthetic Protocol 3.

(1) Boc-deprotection with TFA/CH_2Cl_2 (1:1, v/v), 100 ml, 3x1 min and 1x30 min; (2) washing with CH_2Cl_2, 100 ml, 6x1 min; (3) neutralization with DIEA/CH_2Cl_2 (1:19, v/v), 100 ml, 3x2 min; (4) washing with CH_2Cl_2, 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. (0.92 mmol; 0.506 g) BocTaeg-OHp dissolved in 35 ml CH_2Cl_2 (final concentration of pentafluorophenyl ester -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 BocTaeg-OTf); (8) washing with CH_2Cl_2, 100 ml, 4x1 min; (9) neutralization with DIEA/CH_2Cl_2 (1:19, v/v), 100 ml, 2x2 min; (10) washing with CH_2Cl_2, 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 100 ml mixture of acetic anhydride/pyridine/CH_2Cl_2 (1:1:2, v/v/v) for 2 h; (13) washing with CH_2Cl_2, 100 ml, 6x1 min; (14) 2x2-5 mg samples of protected PNA-resin are taken out, neutralized with DIEA/CH_2Cl_2 (1:19, v/v) and washed with CH_2Cl_2 for qualitative and quantitative ninhydrin analyses.

(i) Synthetic Protocol 4.

(1) Boc-deprotection with TFA/CH_2Cl_2 (1:1, v/v), 25 ml, 3x1 min and 1x30 min; (2) washing with CH_2Cl_2, 25 ml, 6x1 min; (3) neutralization with DIEA/CH_2Cl_2 (1:19, v/v), 25 ml, 3x2 min; (4) washing with CH_2Cl_2, 25 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. (0.92 mmol; 0.506 g) BocTaeg-OHp dissolved in 6 ml CH_2Cl_2 followed by addition of 3 ml DMF (final concentration of pentafluorophenyl ester -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 CH_2Cl_2, 25 ml, 4x1 min; (9) neutralization with DIEA/CH_2Cl_2 (1:19, v/v), 25 ml, 2x2 min; (10) washing with CH_2Cl_2, 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/CH_2Cl_2 (1:1:2, v/v/v) for 2 h (except after the first cycle); (13) washing with CH_2Cl_2, 25 ml, 6x1 min; (14) 2-5 mg samples of protected PNA-resin are taken out, neutralized with DIEA/CH_2Cl_2 (1:19, v/v) and washed with CH_2Cl_2 for qualitative and quantitative ninhydrin analyses.

### EXAMPLE 20

Solid-Phase Synthesis of H-[Taeg]_4-Cae-[Taeg]_4-Lys-NR_3

(a) Stepwise Assembly of Boc-[Taeg]_4-C(z)ae-[Taeg]_4-Lys(CIZ)-MBHA Resin

About 2.5 g of wet Boc-[Taeg]_4-Lys(CIZ)-MBHA resin (~25% of the total remaining about 20 g wet resin ~0.75 g dry resin ~0.15 mmol growing chains) was placed in 6 ml
SSPS reaction vessel. Boc-[Taeg]₁₅-Caeg-[Taeg]₁₄-Lys(CIZ)-MBHA resin was assembled by double coupling of all Taeg-residues utilizing the usual 2.5 equivalents of Boc-Taeg-OPip in 2.5 mL about 30% DMF/CH₂Cl₂, except that the first residue was single-coupled. Incorporation of the (CIZ)taeg-residue was accomplished by coupling with 2.0 equivalents of BocC(Z)taeg-OPip in TFA/CH₂Cl₂ (1:2; v/v). The progress of the synthesis was monitored at all stages by the quantitative ninhydrin reaction (see Table III).

### TABLE III

<table>
<thead>
<tr>
<th>Synthetic Residue</th>
<th>Substitution After Deprotection (mmol/g)</th>
<th>Remaining Free Amino Groups After Coupling (mmol/g)</th>
<th>Estimated Extent of Coupling</th>
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</table>

(b) Cleavage, Purification, and Identification of H-[Taeg]₁₅-Caeg-[Taeg]₁₄-Lys-NH₂

A portion of protected Boc-[Taeg]₁₅-Caeg-[Taeg]₁₄-Lys(CIZ)-MBHA resin was treated as described in Example I section (c) to yield about 14.4 mg (crude material upon HPLC cleavage of 66.9 mg dry H-[Taeg]₁₅-Caeg-[Taeg]₁₄-Lys(CIZ)-MBHA 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 H₂O) to give approximately 9.1 mg of 96% pure H-[Taeg]₁₅-Caeg-[Taeg]₁₄-Lys-NH₂ (FIG. 13b). For (M+H) the calculated m/z value=2793.8 and the measured m/z value=2790.6.

### EXAMPLE 21

Binding of Ac⁻¹-[Taeg]₁₅-Lys-NH₂ to dA₁₀ (FIG. 11a)

Ac⁻¹-[Taeg]₁₅-Lys (100 ng) was incubated for 15 min at room temperature with 50 cpm ³²P-end-labelled oligonucleotide [d[GATCCA]₉dG] in 20 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The sample was loaded into ice (15 min) and analyzed by gel electrophoresis in polyacrylamide (PAGE). To 10 μL of the sample was added 2 μL 50% glycerol, 5 TBE (TBE=90 mM Tris-borate, 1 mM EDTA, pH 8.3), and the sample was analyzed by PAGE (15% acrylamide, 0.5% bisacrylamide) in TBE buffer at 45°C. A 10 μL portion of the sample was blotted, dried and resolved 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 Agfá Curix RPI X-ray films exposed at −80°C for 2 h.

Oligonucleotides were synthesized on a Biosearch 7500 DNA synthesizer, labelled with [³²P]-ATP (Amersham, 5000 Ci/mmol) and polyacrylamide kinase, and purified by PAGE using standard techniques (Maniatis et al., 1986). The desired plasmid (designated pT10) was isolated from one of the resulting clones and purified by the alkaline extraction procedure and CsCl centrifugation (Maniatis et al., 1986). A 3'-[³²P]-end-labelled DNA fragment of 248 bp containing the dA₁₀/dT₁₀ target sequence was obtained by cleaving the pT10 DNA with restriction enzymes EcoRI and PvuII, labelling the cleaved DNA with [³²P]-DATP (4000 Ci/mmole, Amersham) using the Klenow fragment of E. coli DNA polymerase (Boehringer Mannheim), and purifying the 248 bp DNA fragment by PAGE (5% acrylamide, 0.06% 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 agarose, and labelling with [³²P]-ATP and polynucleotide kinase. Following treatment with PvuII, the 248 bp DNA fragment was purified as above.

The complex between Ac⁻¹-[Taeg]₁₅-Lys-NH₂ and the 248 bp DNA fragment was formed by incubating 50 ng of Ac⁻¹-[Taeg]₁₅-Lys-NH₂ with 500 cpm ³²P-labelled 248 bp fragment and 0.5 μg calf thymus DNA in 100 μl buffer for 60 min at 37°C.

### EXAMPLE 23

Probing of Strand Displacement Complex with:

(a) *Staphylococcus* nuclease (FIG. 12B)

The strand displacement complex was formed in 25 mM Tris-HCl, 1 mM MgCl₂, 0.1 mM CaCl₂, pH 7.4 as described above. The complex was treated with *Staphylococcus* nuclease (Boehringer Mannheim) at 750 U/ml for 5 min at 20°C. and 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, 0.5% bisacrylamide, 7 M urea) and autoradiography.
(b) Affinity photocleavage (FIGS. 12A+12B)

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⁻²s⁻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 lyophilization, the DNA was analysed by PAGE as above.

(c) Potassium permanganate (FIG. 12B)

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 piperidine and analyzed, as above.

(d) Photofootprinting (FIG. 12B)

The complex was formed in 100 μl TE and diazo-linked acridine (0.1 μg/μl) (DHNAelsen 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 photocleavage".

(e) S1-nuclease (FIG. 12C)

The complex was formed in 50 mM sodium acetate, 200 mM NaCl, 0.5% glycerol, 1 mM ZnCl₂, 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".

EXAMPLE 24

N-Benzoyloxycarbonyl-N'-bocaminoethyl)glycine.

Aminoethyl glycine (52.86 g; 0.447 mol) was dissolved in water (900 ml) and dioxane (900 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 2N 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 at 0°C. Benzoyloxycarbonyl 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 petroleum ether (1800 ml). Yield 124.7 g (79%). M.p. 64.5-85°C. Anal. For C₁₁H₁₃N₂O₄: found (calc.) C 58.40(57.94); H 7.02(6.86); N 7.94(7.95); ¹H-NMR (250 MHz, CDCl₃) 7.33 & 7.52 (2H, PhH); 5.15 & 5.12 (2H, PhCH₂); 4.03 & 4.01 (2H, NCH₂CO₂H); 3.46 (2H, 2H, BocNHCH₂CH₃); 3.28 (2H, 2H, BocNHCH₂CH₃); 1.43 & 1.40 (9H, Bun). 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 25

N'-Boc-aminoethyl glycine ethyl ester.

N-Benzoyloxycarbonyl-N'-bocaminoethyl)glycine (60.0 g; 0.170 mol) and N,N-dimethyl-4-aminopyridine (0.00 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 dried) was removed by filtration and washed with ether (3x100 ml). The combined filtrate was washed successively with diluted potassium hydrogen sulfate (2x400 ml), diluted sodium hydrogen carbonate (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.

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 SiO₂) 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. ¹H-NMR (250 MHz, CD₃OD) 4.77 (b, s, NH); 4.18 (g, 2H, MeSCH₂—); 3.38 (s, 2H, NCH₂CO₂Et); 3.16 (t, 2H, BocNCH₂CH₂); 2.68 (t, 2H, BocNCH₂CH₂); 1.43 (s, 9H, Bun) and 1.26 (t, 3H, CH₃). ¹³C-NMR 171.4 (COEt); 156.6 (CO); 78.3 (CH₃CO₂Et); 59.9 (CH₃); 49.0 (CH₂); 48.1 (CH₂); 39.0 (CH₂); 26.9 (CH₂) and 12.6 (CH₃).

EXAMPLE 26

N'-Boc-aminoethyl glycine methyl ester.

The above procedure was used, with methanol being substituted for ethanol. The final product was purified by column purification.

EXAMPLE 27

1-(Boc-aeg)thymine ethyl ester.

N'-Boc-aminoethyl glycine ethyl ester (13.5 g; 54.8 mmol), DibOH (9.84 g; 60.3 mmol) and 1-carboxymethyl thymine (11.1 g; 60.3 mmol) were dissolved in DMF (210 ml). Methylene chloride (210 ml) then was added. The solution was cooled to 0°C in an 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 (2x75 ml). To the combined filtrate was added more methylene chloride (650 ml). The solution was washed successively with diluted sodium hydrogen carbonate (3x500 ml), diluted potassium hydrogen sulfate (2x500 ml) and saturated sodium chloride (1x500 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 meth-
ylene 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 16.0 g (71%) of a material which was more than 99% pure by HPLC.

**EXAMPLE 28**

1-(Boc-aeg)thymine.

The material from above was suspended in THF (194 ml, gives a 0.2 M solution), and 1 M aqueous lithium hydroxide (116 ml) was added. The mixture was stirred for 45 minutes at ambient temperature and then filtered to remove residual DCU. Water (40 ml) was added to the solution which was then washed with methylene chloride (300 ml). Additional water (30 ml) was added, and the alkaline solution was washed once more with methylene chloride (150 ml). The aqueous solution was cooled to 0 °C, and the pH was adjusted to 2 by the dropwise addition of 1 N HCl (approx. 110 ml). The title compound was extracted with ethyl acetate (9×200 ml), the combined extracts were dried over magnesium sulfate and were evaporated to dryness, in vacuo. The residue was evaporated once from methanol, which after drying overnight afforded a colorless glassy solid. Yield 9.57 g (64%), HPLC>98% Rr=14.8 min. Anal. for C14H16N2O2·H2O: Calc. (%) C, 49.29; H, 6.52; N, 14.11. 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).

**EXAMPLE 29**

Nα-Benzylxoycarbonyl-1-(Boc-aeg)cytosine.

Nα-Benzylxoycarbonyl-1-(Boc-aeg)cytosine 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 (2×150 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% Rr=19.5 min, and a minor impurity at 12.6 min (approx. 1%) most likely the Z-de protected monomer. Anal. for C23H26N2O4: Calc. (%) C, 54.16; H, 5.76; N, 13.65. IR: 3220 cm⁻¹ (NH), 1650 cm⁻¹ (C=O), 1120 cm⁻¹ (C-N).

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 C8H13N3O2: Calc. (%) C, 48.86; H, 5.10; N, 31.66. IR: 3220 cm⁻¹ (NH), 1650 cm⁻¹ (C=O).
residue which caused crystallisation of the title compound. The solid was recrystallised from 96% ethanol (600 ml). Yield after drying 53.7 (65.6%). HPLC (215 nm) purity > 99.5%.

EXAMPLE 31

N⁰-Benzoxycarbonyl-9-carboxymethyl adenine ethyl ester.

9-Carboxymethyl adenine ethyl ester (3.40 g, 15.4 mmol) was dissolved in dry DMSO (50 ml) by gentle heating, cooled to 20°C, and added to a solution of N-ethyl-benzoxycarbonyl bromide 85% (20 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 sulphate (twice), and with saturated sodium chloride. The solution was dried over magnesium sulfate and evaporated to dryness, in vacuo, which afforded 11 g of the 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. 132-135°C. Analysis for C₉₃H₇₇N₄O₂, found (calc.): C, 65.95(57.46); H, 4.71(4.82); N, 19.35(19.71). {H-NMR (250 MHz, CDCl₃) 8.77 (s, 11H, H-2 or H-8); 7.99 (s, 11H, H-2 or H-8); 7.45-7.26 (m, 5H, Ph); 5.31 (s, 2H, N-CH₃); 4.96 (s, 2H, Ph-CH₂); 4.27 (q, 2H, J=7.15 Hz, CH₂CH₂); and 1.30 (t, 3H, J=7.15 Hz, CH₂CH₂). 13C-NMR: 153.09; 143.11; 128.66; 67.84; 62.51; 44.24 and 14.09. FAB-MS: 356 (M+H) and 312 (M+H₂O). 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 32

N⁰-Benzoxycarbonyl-9-carboxymethyl adenine.

N⁰-Benzoxycarbonyl-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 C₁₀₂H₇₇N₄O₂, found (calc.): C, 46.32(55.05); H, 4.24(4.00); N, 18.10(21.40); and C/N, 2.57(2.56). {H-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-CH₃); 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 44.67JR (KBr) 4384(18.3); 3109 (15.9); 3087(15.0); 2968(17.1); 2927(19.9); 2383(53.8); 1960(62.7); 1739(2.5); 1688(5.2); 1655(9.9); 1594(11.7); 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 (M+H) and 284 (M+H₂O). HPLC (215 nm, 260 nm) in system 1:15:15 min, minor impurities all less than 2%.

EXAMPLE 34

N⁰-Benzyloxycarbonyl-1-(Boc-aeg) adenine ethyl ester.

N⁰-Benzyloxycarbonyl-1-(Boc-aeg) adenine ethyl ester (2.00 g; 8.12 mmol), DibutOH (1.46 g; 8.93 mmol) and N⁰-benzyloxycarbonyl-9-carboxymethyl adenine (2.92 g; 8.93 mmol) were dissolved in DMF (15 ml). Methylenne 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 DMF (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. A solution of water to the mother liquor afforded another 0.55 g with a purity of approx. 95%. Anal. for C₁₀₂H₁₁₄N₂O₁₂H₂O; found (calc.): C, 55.01(54.44); H, 6.85(6.15); and N, 16.47(17.09). {H-NMR (250 MHz; CDCl₃) 8.74 (s, 1H, Ade H-2); 8.18 (b, s, 1H, ZNH); 8.10 & 8.04 (1H, H-8); 7.46-7.34 (m, 5H, Ph); 5.63 (unres. t, 1H, BocNH); 5.30 (s, 2H, PhCH₂); 5.16 & 5.00 (s, 2H, CH₂CON); 4.29 & 4.06 (s, 2H, CH₂CO₂H); 4.20 (q, 2H, OCH₂CH₂); 3.67-3.29 (m, 4H, CH₂CH₂); 1.42 (s, 9H, 'B) and 1.27 (t, 3H, OCH₂CH₂). The spectrum shows traces of ethanol and DCC.
EXAMPLE 35

2-Amino-6-chloro-9-carboxyethylpurine.

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, in vacuo, over silica gel. Yield (3.02 g; 44.8%). 1H-NMR (DMSO-d6): δ=4.88 ppm (s, 2H); 6.95 (s, 2H); 8.10 (s, 1H).

EXAMPLE 36

2-Amino-6-benzoxyl-9-carboxyethylpurine.

Sodium (2.0 g; 87.0 mmol) was dissolved in benzyl alcohol (20 ml) and heated to 130°C. C. for 2 h. After cooling to 0°C, a solution of 2-amino-6-chloro-9-carboxyethylpurine (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 (3x100 ml). The water phase was then added in 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 (2x100 ml). The combined organic phases were washed with saturated sodium chloride solution (2x75 ml), dried with anhydrous sodium sulfate, and then dried by evaporation, in vacuo. The residue was recrystallized from ethanol (300 ml), Yield after drying, in vacuo, over silica gel: 2.76 g (52%). M.p. 159-160°C. Anal. calc.: found: C, 56.18; 55.97; H, 4.38; 4.32; N, 23.4; 23.10. 1H-NMR (DMSO-d6): δ=4.82 ppm (s, 2H); 5.51 (s, 2H); 6.45 (s, 2H); 7.45 (m, 5H); 7.82 (s, 1H).

EXAMPLE 37

N-[[2-Amino-6-benzoxyl-9-purine-yl]-acetyl]-N- 2-Boc-aminoethyl-glycine[BocGae-GOH monomer].

2-Amino-6-benzoxyl-9-carboxymethylpurine (0.50 g; 1.67 mmol), methyl N-[2-[tert-butoxycarbonyl]amino]-ethyl]-glycinate (0.65 g; 2.80 mmol), diisopropylethyl amine (0.54 g; 4.19 mmol) and bromo-tris-pyridyl-phosphonium-hexafluoro-phosphate (PyBOP) (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 (3x40 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:2:1) 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 (m, 1H, CONR2COOH); 4.4 (m, 1H, CONR2COOH); 5.0 (s, 1H, Guu-H2CO); 5.2 (s, mj., Guu-CH3CO); 5.6 (s, 2H); 6.5 (s, 2H); 6.9 (s, 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; 160.03; 159.78; 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 38

3-Boc-amino-1,2-propanediol.

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 during stirring. The pH was maintained at 10.5 with a solution of sodium hydroxide (17.56 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 (6x350 ml). The volume of the organic phase was reduced to 900 ml by evaporation under reduced pressure. The organic phase then was washed with a saturated aqueous solution of potassium hydrogen sulfate diluted to twice its volume (1x1000 ml) and with saturated aqueous sodium chloride (1x500 ml). The organic phase was dried (MgSO4) and evaporated under reduced pressure to yield 501.2 g (60%) of the title compound. The product could be solidified by evaporation from methylene chloride and subsequent freezing. 1H-NMR (CDCl3/TMS): δ=1.43 ppm (s, 9H, Me-C); 3.25 ppm (m, 2H, CH2); 3.57 ppm (m, 2H, CH2); 3.73 ppm (1H, CH). 13C-NMR (CDCl3/TMS): δ=28.2 ppm (Me-C); 42.6 ppm (CH2); 63.5 ppm, 71.1 ppm (CH2OH, CHOH); 79.5 ppm (Me-C); 157.0 ppm (C-O).

EXAMPLE 39

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

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 (6x250 ml). The organic phase was dried (MgSO4) and evaporated to afford an almost quantitative yield of Boc-aminooctocetdehyde as a colourless oil, which was used without further purification in the following procedure. Palladium-on-carbon (10%, 0.8 g) was added to MeOH (250 ml) under nitrogen with cooling (0°C) and vigorous stirring. Anhydrous sodium acetate (4.49 g, 54.7 mmol, 2 eqv) and L-alanine methyl ester, hydrochloride (3.82 g, 27.4 mmol, 1 eqv) were added. Boc-aminooctocetdehyde (4.79 g, 30.1 mmol, 1.1 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, Me;CH₃), 1.89 (b, 1H, NH), 2.51 (m, 1H, CH₂), 2.66 (m, 1H, CH), 3.10 (m, 2H, CH₂), 3.27 (q, J=7.0 Hz, 1H, CH), 3.64 (s, 3H, OMe), 5.06 (b, 1H, carbamate NH). ¹³C-NMR. d=18.8 (Me), 28.2 (Me(C)), 40.1, 47.0 (CH₂), 51.6 (OMe), 56.0 (CH), 155.8 (carbonate C=O), 175.8 (ester C=O).

EXAMPLE 40

N-(Boc-aminomethyl)-N-(1-thyminylacetyl)-L-alanine methyl ester.

To a solution of Boc-aminomethyl-(L)-alanine methyl ester (1.23 g, 5.0 mmol) in DMF (10 ml) was added Dib-H2O (0.90 g, 5.52 mmol) and 1-thyminylacetic acid (1.01 g, 5.48 mmol). When the 1-thyminylacetic 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 DCU 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 (250 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, 4H, 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 41

N-(Boc-aminomethyl)-N-(1-thyminylacetyl)-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 hydrogen chloride. 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 used until precipitation started. After standing overnight at -20°C, the precipitate was removed by filtration. This gave 1.01 g (50.5%) of pure compound (>99% by HPLC). The compound can be recrystallized from 2-propanol. FAB-MS: 399 (M+1). ¹H-NMR (DMSO-d₆). 11.35 (s, 1H, COOH); 7.42 (s, 1H, H₆); 4.69 (s, 2H, CH₂); 1.83 (s, 3H, Me in T); 1.50-1.40 (m, 12H, Me in Ala+Boc).

EXAMPLE 42

(a) N-(Boc-aminomethyl)-N-(1-thyminylacetyl)-Dalanine methyl ester.

To a solution of Boc-aminomethyl alanine methyl ester (2.48 g, 10.1 mmol) in DMF (20 ml) was added Dib-H2O (1.80 g, 11.0 mmol) and thyminylacetic acid (2.14 g, 11.6 mmol). After dissolution of the 1-thyminylacetic 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 DCU was seen. After 35 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, BocNH, J=5.89 Hz); 4.56 (d, 2H, CH₂); 4.35 (q, 2H, CH in Ala, J=7.25 Hz); 3.74 (s, 3H, OMe); 3.64-3.27 (m, 4H, ethyl H's); 1.90 (s, 3H, Me in T); 1.52-1.44 (t, 12H, Boc+Me in Ala).

(b) N-(Boc-aminomethyl)-N-(1-thyminylacetyl)-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 hydrogen chloride. 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, H₆); 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 43

N-(N'-Boc-3'-aminopropionyl)-N-[1-thyminylacetyl] glycine methyl ester.

N-(N'-Boc-3'-aminopropionyl)glycine methyl ester (2.84 g, 0.0115 mol) was dissolved in DMF (35 ml), followed by addition of Dib-H2O (2.07 g, 0.0127 mol) and 1-thyminylacetic acid (2.34 g, 0.0127 mol). Methylene 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
EXAMPLE 44


N-(N'-Boc-3'-aminopropyl)-N-[1-thyminyl]acetyl]glycine methyl ester (3.02 g, 0.0073 mol) was dissolved in methanol (25 ml) and stirred for 1.5 h with 2 M sodium hydroxide (25 ml). 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 (3 x 10 ml), and dried over isopentane, in vacuo. Yield 2.19 g (75%). Anal. for C₁₇H₁₉N₂O₄: C, 49.95 (50.03); H, 6.47 (6.29); N, 13.43 (13.45). The compound showed satisfactory ^1H and ^13C-NMR spectra.

EXAMPLE 45

3-(1-Thyminyl)-propanoic acid methyl ester.

Thymine (14.0 g, 0.11 mol) was suspended in methanol. Ethyl acetate (39.6 ml, 0.44 mol) was added, along with catalytic amounts of sodium hydroxide. The solution was refluxed in the dark for 45 h, evaporated to dryness, in vacuo, and the residue dissolved in methanol (8 ml) with heating. After cooling on an ice bath, the product was precipitated by addition of ether (20 ml), isolated by filtration, washed with ether (3 x 15 ml), and dried over isopentane, in vacuo. Yield 11.23 g (48%). M.p. 112-119°C. Anal. for C₁₇H₁₉N₂O₄: C, 51.14 (50.94); H, 5.78 (5.70); N, 11.52 (13.20). The compound showed satisfactory ^1H and ^13C-NMR spectra.

EXAMPLE 46

3-(1-Thyminyl)-propanoic acid.

3-(1-Thyminyl)-propanoic acid methyl ester (1.0 g, 0.0047 mol) was suspended in 2 M sodium hydroxide (15 ml), boiled for 10 min. The pH was adjusted to 0.3 with conc. hydrochloric acid. The solution was extracted with ethyl acetate (10 x 25 ml). The organic phase was extracted with saturated aqueous sodium chloride, dried over magnesium sulfate, and evaporated to dryness, in vacuo, to give the title compound as a white solid (0.66 g, 71%). M.p. 118-121°C. Anal. for C₁₇H₁₉N₂O₄: C, 48.38 (48.49); H, 5.09 (5.09); N, 13.93 (14.14). The compound showed satisfactory ^1H and ^13C-NMR spectra.

EXAMPLE 47


N-(N'-Boc-aminoethyl)glycine ethyl ester (1.0 g, 0.0041 mol) was dissolved in DMF (12 ml). DiisOBn (0.73 g, 0.0045 mol) and 3-(1-thyminyl)-propanoic acid (0.89 g, 0.0045 mol) were added. Methylene chloride (12 ml) then was added and the mixture was cooled to 0°C. After addition of isocyanate (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 with 1 volume water, 6 x 100 ml), potassium sulfate (1 volume saturated with 4 volumes water, 3 x 100 ml), and saturated aqueous sodium chloride (1 x 100 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 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 C₁₇H₁₉N₂O₄: C, 52.03 (52.42); H, 6.90 (6.84); N, 13.21 (13.38). The compound showed satisfactory ^1H and ^13C-NMR spectra.

EXAMPLE 48


N-(N'-Boc-aminoethyl)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 by filtration, washed with ether (3 x 15 ml), and dried over isopentane, in vacuo. Yield 0.769 g (99%). M.p. 213°C (decomp.).

EXAMPLE 49

Mono-Boc-ethylenediamine (2).

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, C₆H₆ was added and the solution was filtered and extracted with chloroform (3 x 250 ml). The pH was adjusted to 12 at 0°C with 2 M sodium hydroxide, and the aqueous solution was treated with methylene chloride (3 x 300 ml). After treatment with saturated aqueous sodium chloride (250 ml), the methylene chloride solution was dried over magnesium sulfate. After filtration, the solution was evaporated to dryness, in vacuo, resulting
EXAMPLE 50

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

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 ether (200+200 ml) was added, and the solution was filtered and vigorously stirred. The aqueous phase was extracted once more with ether and then freeze-dried to yield a yellow solid. Recrystallization from ethyl acetate yielded 13.09 g (46%) of the title compound. M.p. 138–140° C. Anal. for C₁₅H₂₁N₂O₃Cl found (calc.) C, 46.49 (46.72); H, 8.38 (8.20); N, 9.83 (9.91); Cl, 12.45 (12.54). 1H-NMR (90 MHz; DMSO-d₆): δ 1.39 (s, 9H); 2.9 (m, 8H); 3.64 (s, 3H).

EXAMPLE 51

N-[(1-Thyminyl)acetyl]-N'-Boc-aminoethyl-β-alanine methyl ester.

(N-Boc-aminoethyl)-β-alanine methyl ester, HCl (3) (2.0 g; 0.0071 mol) and 1-thyminylactic acid pentafluorophenyl 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). 1H-NMR (250 MHz; CDCl₃): due to limited rotation around the secondary amide several of the signals were doubled; δ 1.43 (s, 9H); 1.88 (s, 3H); 2.63 (t, 1H); 2.74 (t, 1H); 3.25–3.55 (4x, 4H); 3.65 (2x, 2H); 3.66 (s, 1.5); 3.72 (s, 1.5); 4.61 (s, 1H); 4.72 (s, 2H); 5.59 (s, 0.5H); 5.96 (s, 0.5H); 7.11 (s, 1H); 10.33 (s, 1H).

EXAMPLE 52

N-[(1-Thyminyl)acetyl]-N'-Boc-aminoethyl-β-alanine.

N-[(1-Thyminyl)acetyl]-N'-Boc-aminoethyl-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 silica gel, in vacuo. Yield 2.23 g (77%). M.p. 170–176° C. Anal. for C₁₄H₁₈N₂O₂·H₂O, found (calc.) C, 49.49 (49.03); H, 6.31 (6.78); N, 13.84 (13.45). 1H-NMR (90 MHz; DMSO-d₆): δ 1.38 (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 53

N-[(1-(N²-Z)-cytosyl)acetyl]-N'-Boc-aminoethyl-β-alanine methyl ester.

(N-Boc-aminoethyl)-β-alanine methyl ester, HCl (3) (2.0 g; 0.0071 mol) and 1-(N-4-Z)-cytosylactic acid pentafluorophenyl ester (5) (3.319 g; 0.0071 mol) were dissolved in DMF (50 ml). Triethyl amine (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 C₁₉H₂₁N₂O₅, found (calc.) C, 55.19 (56.49); H, 6.19 (6.26); N, 12.86 (13.18). 1H-NMR (250 MHz; CDCl₃): due to limited rotation around the secondary amide several of the signals were doubled; δ 1.43 (s, 9H); 2.57 (t, 1H); 3.60–3.32 (m’s, 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’s, 7H). FAB-MS: 532 (M+1).

EXAMPLE 54

N-[(1-(N⁴-Z)-cytosyl)acetyl]-N'-Boc-aminoethyl-β-alanine.

N-[(1-(N-4-Z)-cytosyl)acetyl]-N'-Boc-aminoethyl-β-alanine methyl ester (0.621 g; 0.0012 mol) was dissolved in 2 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 silica gel, 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 C₁₆H₂₃N₂O₅, found (calc.) C, 49.49 (49.03); H, 6.31 (6.78); N, 13.84 (13.45). 1H-NMR (250 MHz; CDCl₃): 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’s, 6H); 4.85 (s, 1H); 4.98 (s, 1H); 5.21 (s, 2H); 5.71 (s, 1H, broad); 7.99–7.25 (m’s, 7H). FAB-MS: 518 (M+1).

EXAMPLE 55

Example of a PNA-oligomer with a guanine residue


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 BocGaeOH monomer.

(b) Stepwise Assembly of H-[Taqε]₄-[Gaε]₄-[Taqε]₄-Lys-NH₂ (synthetic protocol)

Synthesis was initiated on 102 mg (dry weight) of pre-swollen (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), 1×2 min and 1×2½ h, 3 ml; (2) washing with DCM, 4×20 sec, 3 ml; washing with DCM, 2×20 sec, 3 ml; washing with DCM,
2×20 sec, 3 ml, and drain for 30 sec; (3) neutralization with DIEA/DCM (1:19 v/v), 2×3 min, 3 ml; (4) washing with DCM, 4×20 sec, 3 ml, and drain for 1 min.; (5) addition of 4 equiv. disopropyl carbodiimide (0.06 mmol; 9.7 μl) and 4 equiv. (0.06 mmol; 24 mg) BocTaeg-OH or (0.06 mmol; 30 mg) BocGae-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 ½ h shaking at room temperature; (6) drain for 20 sec; (7) washing with DMF, 2×20 sec and 1×2 min, 3 ml; washing with DCM 4×20 sec, 3 ml; (8) neutralization with DIEA/DCM (1:19 v/v), 2×3 min, 3 ml; (9) washing with DCM 4×20 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), 1×½ h, 3 ml; and (12) washing with DCM, 4×20 sec, 2×2 min and 2×20 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: 2852.11 [M⁺+1] (calc. 2852.15).

EXAMPLE 56

Solid-Phase Synthesis of H-[Taeg]-Aaeg-[Taeg]-Lys-NH₂.


About 0.3 g of wet Boc-[Taeg]₆-Lys(CIZ)-MBHA resin was placed in a 3 ml SPPS reaction vessel. Boc-Taeg-A(Z)aeq-[Taeg]₆-Lys(CIZ)-MBHA resin was assembled in situ DCC coupling (single) of the A(Z)aeq residue utilizing 0.19 M of BocA(Z)aeq-OH together with 0.15 M DCC in 2.5 ml 50% DME/CH₂Cl₂ and a single coupling with 0.15 M BocA(Z)aeq-OPic in neat CH₂Cl₂ (“Synthetic Protocol 5”).

The synthesis was monitored by the quantitative ninhydrin reaction, which showed about 50% incorporation of A(Z)aeq and about 96% incorporation of Taeg.

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

The protected Boc-Taeg-A(Z)aeq-[Taeg]₆-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)aeq-[Taeg]₆-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-Aaeg-[Taeg]₆-Lys-NH₂. For (M+H)⁺ the calculated m/z value was 2816.16 and the measured m/z value was 2816.28.

(c) Synthetic Protocol 5

(1) Boc-deprotection with TFA/CH₂Cl₂ (1:1, v/v), 2.5 ml, 3×1 min and 1×30 min; (2) washing with CH₂Cl₂, 2.5 ml, 6×1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 2.5 ml, 3×2 min; (4) washing with CH₂Cl₂, 2.5 ml, 6×1 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) BocA(Z)aeq-OH dissolved in 1.25 ml DME followed by addition of 0.47 mmol (0.13 g) DCC in 1.25 ml CH₂Cl₂ or 0.36 mmol (0.20 g) BocA(Z)aeq-OPic in 2.5 ml CH₂Cl₂; the coupling reaction was allowed to proceed for a total of 20–24 hrs with shaking; (7) washing with DME, 2.5 ml, 1×2 min; (8) washing with CH₂Cl₂, 2 ml, 4×1 min; (9) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 2.5 ml, 2×2 min; (10) washing with CH₂Cl₂, 2.5 ml, 6×1 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, 6×1 min; (14) 2×2–5 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 57


About 0.5 g of wet Boc-[Taeg]₆-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]₆-A(Z)aeq-[Taeg]₆-Lys(CIZ)-MBHA resin was assembled by in situ DCC coupling of both the A(Z)aeq 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)aeq after coupling three times (the first coupling gave about 50% incorporation; a fourth HOBt-mediated coupling in 50% DME/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]₆-Aaeg-[Taeg]₆-Lys-NK₂.

The protected Boc-[Taeg]₆-A(Z)aeq-[Taeg]₆-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]₆-A(Z)aeq-[Taeg]₆-Lys(CIZ)-BAH resin. A small portion of the crude product was purified. For (M+H)⁺, the calculated m/z value was 2802.85 and the measured m/z value was 2805.90.
The PNA-oligomer H-T4C2TCT-LysNH₂ was prepared as described in Example 93. Hybridization experiments with this sequence should resolve the issue of orientation, since it is truly asymmetrical. Such experiments should also resolve the issues of pH-dependency of the Tm, and the stoichiometry of complexes formed.

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

<table>
<thead>
<tr>
<th>Row</th>
<th>Hybridized With</th>
<th>pH</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-(-A₄)₂ (G₃) (A₄) (G₃) (A₄)</td>
<td>7.2</td>
<td>55.5</td>
</tr>
<tr>
<td>2</td>
<td>5'-(-A₄)₂ (G₃) (A₄) (G₃) (A₄)</td>
<td>9.0</td>
<td>26.0</td>
</tr>
<tr>
<td>3</td>
<td>5'-(-A₄)₂ (G₃) (A₄) (G₃) (A₄)</td>
<td>5.0</td>
<td>88.5</td>
</tr>
<tr>
<td>4</td>
<td>5'-(-G₄) (A₄) (G₃) (A₄) (G₃)</td>
<td>7.2</td>
<td>38.0</td>
</tr>
<tr>
<td>5</td>
<td>5'-(-G₄) (A₄) (G₃) (A₄) (G₃)</td>
<td>9.0</td>
<td>31.5</td>
</tr>
<tr>
<td>6</td>
<td>5'-(-G₄) (A₄) (G₃) (A₄) (G₃)</td>
<td>5.0</td>
<td>52.5</td>
</tr>
<tr>
<td>7</td>
<td>5'-(-A₄)₂ (G₃) (A₄) (G₃) (A₄)</td>
<td>7.2</td>
<td>39.0</td>
</tr>
<tr>
<td>8</td>
<td>5'-(-A₄)₂ (G₃) (A₄) (G₃) (A₄)</td>
<td>9.0</td>
<td>&lt;20</td>
</tr>
<tr>
<td>9</td>
<td>5'-(-A₄)₂ (G₃) (A₄) (G₃) (A₄)</td>
<td>5.0</td>
<td>51.5</td>
</tr>
<tr>
<td>10</td>
<td>5'-(-A₄)₂ (G₃) (A₄) (G₃) (A₄)</td>
<td>7.2</td>
<td>31.5</td>
</tr>
<tr>
<td>11</td>
<td>5'-(-A₄)₂ (G₃) (A₄) (G₃) (A₄)</td>
<td>5.0</td>
<td>50.5</td>
</tr>
<tr>
<td>12</td>
<td>5'-(-G₄) (A₄) (G₃) (A₄) (G₃)</td>
<td>7.2</td>
<td>24.5</td>
</tr>
<tr>
<td>13</td>
<td>5'-(-G₄) (A₄) (G₃) (A₄) (G₃)</td>
<td>9.0</td>
<td>&lt;20</td>
</tr>
<tr>
<td>14</td>
<td>5'-(-G₄) (A₄) (G₃) (A₄) (G₃)</td>
<td>5.0</td>
<td>57.0</td>
</tr>
<tr>
<td>15</td>
<td>5'-(-G₄) (A₄) (G₃) (A₄) (G₃)</td>
<td>7.2</td>
<td>25.0</td>
</tr>
<tr>
<td>16</td>
<td>5'-(-G₄) (A₄) (G₃) (A₄) (G₃)</td>
<td>5.0</td>
<td>59.5</td>
</tr>
</tbody>
</table>

$\dagger$: stoichiometry determined by UV-mixing curves
—: not determined

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 which should be a general feature for all PNA C/T sequences.

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.

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'/terminal.

EXAMPLE 59

The results of hybridization experiments with H-15G1₄-LysNH₂ to were performed as follows:

<table>
<thead>
<tr>
<th>Row</th>
<th>Deoxynucleoside</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-(-A₄) (5) (A₄) (A₄) (A₄)</td>
<td>55.0</td>
</tr>
<tr>
<td>2</td>
<td>5'-(-A₄) (5) (A₄) (A₄) (A₄)</td>
<td>47.0</td>
</tr>
<tr>
<td>3</td>
<td>5'-(-A₄) (5) (A₄) (A₄) (A₄)</td>
<td>35.6</td>
</tr>
<tr>
<td>4</td>
<td>5'-(-A₄) (5) (A₄) (A₄) (A₄)</td>
<td>46.5</td>
</tr>
<tr>
<td>5</td>
<td>5'-(-A₄) (5) (A₄) (A₄) (A₄)</td>
<td>48.5</td>
</tr>
<tr>
<td>6</td>
<td>5'-(-A₄) (5) (A₄) (A₄) (A₄)</td>
<td>55.5</td>
</tr>
<tr>
<td>7</td>
<td>5'-(-A₄) (5) (A₄) (A₄) (A₄)</td>
<td>47.0</td>
</tr>
</tbody>
</table>

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 60

The masses of some synthesized PNA-oligomers, as determined by FAB mass spectrometry, are as follows:

<table>
<thead>
<tr>
<th>SEQUENCE</th>
<th>CALC.</th>
<th>FOUND</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-T₄C₂TCTC-LysNH₂</td>
<td>2747.15</td>
<td>2746.78</td>
</tr>
<tr>
<td>H-T₄G₁₄-LysNH₂</td>
<td>2832.15</td>
<td>2822.11</td>
</tr>
<tr>
<td>H-T₄LysNH₂</td>
<td>2008.84</td>
<td>2040.84</td>
</tr>
<tr>
<td>H-T₄LysNH₂</td>
<td>2541.04</td>
<td>2540.84</td>
</tr>
<tr>
<td>H-T₄G₁₄-LysNH₂</td>
<td>2807.14</td>
<td>2806.69</td>
</tr>
<tr>
<td>H-T₄C₂TCTC-LysNH₂</td>
<td>2259.04</td>
<td>2250.18</td>
</tr>
<tr>
<td>H-T₄C₂TCTC-LysNH₂</td>
<td>2287.95</td>
<td>2288.60</td>
</tr>
<tr>
<td>H-T₄C₂TCTC-LysNH₂</td>
<td>2683.12</td>
<td>2683.09</td>
</tr>
</tbody>
</table>

EXAMPLE 61

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>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 H-T₄G₁₄-LysNH₂</td>
<td>(dA)₈₀</td>
<td>73°C</td>
</tr>
<tr>
<td>35 H-T₄G₁₄-LysNH₂</td>
<td>(dA)₈₀</td>
<td>25°C</td>
</tr>
<tr>
<td>35 H-T₄G₁₄-LysNH₂</td>
<td>(dA)₈₀</td>
<td>47°C</td>
</tr>
<tr>
<td>35 H-T₄G₁₄-LysNH₂</td>
<td>(dA)₈₀</td>
<td>49°C</td>
</tr>
</tbody>
</table>

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

EXAMPLE 62

An example with a "no base" substitution.
EXAMPLE 63

Iodination Procedure

A 5 µg portion of Tyr-PNA-Tc⁹⁰⁰⁰Lys-NH₂ is dissolved in 40 µl 100 mM Na-phosphate, pH 7.0, and 1mCi Na₂¹³¹I and 2 µl chloroamine-T (50 mM in CH₃CN) are added. The solution is left at 20°C for 10 min and then passed through a 0.5 x 5 cm Sephadex G10 column. The first 2 frctions (100 µl each) containing radioactive material are collected and purified by HPLC: reversed phase C-18 using a 0-60% CH₃CN gradient in 0.1% CF₃COOH in H₂O. The ¹³¹I-PNA elutes right after the PNA peak. The solvent is removed under reduced pressure.

EXAMPLE 64

Binding of PNA-T₁₀⁻¹₉-T₆₋₇₋₈₋₉₋₁₀ DNA targets A₆₁₋₉₋₉₋₈₋₉₋₁₀-G₅₂ (FIG. 10).

A mixture of 200 cps ³²P-labeled EcoRI-PvuII fragment (the large fragment labeled at the 5'-end of the EcoRI site) of the indicated plasmid, 0.5 µg carrier calf thymus DNA, and 300 ng PNA in 100 µl buffer (200 mM NaCl, 50 mM Na-acetate, pH 4.5, 1mM ZnSO₄) was incubated at 37°C for 120 min. A 50 µl portion of nuclease S₁ was added and incubated at 20°C for 5 min. The reaction was stopped by 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 target plasmids were prepared by cloning into the appropriate oligonucleotides into pUC19. Target A₁₀⁻¹₉ oligonucleotides GATCCAG₁₀⁻¹₉ & GATCCCT₁₀⁻¹₉ cloned into the BamHI site (plasmid designated pT10). Target A₆₁₋₉₋₉₋₈₋₉₋₁₀-G₅₂ (SEQ ID NO: 29) & TCGACT₁₀⁻¹₉₋₉₋₈₋₉₋₁₀ (SEQ ID NO: 30) cloned into the Sal site (plasmid pT10). Target A₆₁₋₉₋₉₋₈₋₉₋₁₀-G₅₂ (SEQ ID NO: 31) & GTG₁₀⁻¹₉₋₉₋₈₋₉₋₁₀-TGCA (SEQ ID NO: 32) into the Pst site (plasmid pT8C2). The positions of the targets in the gel are indicated by bars to the left. A/G is an A+G sequence ladder of target P10.

EXAMPLE 65

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

A 2 µg portion of plasmid pT10 was mixed with the indicated amount of PNA-T₁₀⁻¹₉-T₆₋₇₋₈₋₉₋₁₀ in 20 µl TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.4) and incubated at 37°C for 120 min. 2 µl buffer (10 mM Tris-HCl, pH 7.5, 10 mM, MgCl₂, 50 mM NaCl, 1mM DTT). 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.

EXAMPLE 66

Kinetics of PNA-T₁₀⁻¹₉+d dsDNA strand displacement complex formation (FIG. 21).

A mixture of 200 cps 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-T₁₀⁻¹₉-Lys-NH₂ in 100 µl buffer (200 mM NaCl, 50 mM Na-acetate, pH 4.5, 1mM ZnSO₄) were incubated at 37°C. At the times indicated, 50 µl of S₁ nucleases was added to each of 7 samples and incubation was continued for 5 min at 20°C. The DNA was then precipitated by addition of 250 µl 2% K-acetate in ethanol and analyzed by electrophoresis in a 5% polyacrylamide sequencing gel. The amount of strand displacement complex was calculated from the intensity of the S₁-cleavage at the target sequence, as measured by densitometric scanning of autoradiographs.

EXAMPLE 67

Stability of PNA-dsDNA complexes (FIG. 22).

A mixture of 200 cps ³²P-PvuII fragment, 0.5 µg calf thymus DNA and 300 ng of the desired PNA (either T₁₀⁻¹₉-Lys-NH₂, T6-Lys-NH₂ or T₄⁻¹₉-Lys-NH₂) was incubated in 100 µl 200 mM NaCl, 50 mM Na-acetate, pH 4.5, 1mM ZnSO₄ for 60 min at 37°C. A 2 µg portion of oligonucleotide GATCCAG₁₀⁻¹₉ was added and each sample was heated for 10 min at the temperature indicated, cooled in ice for 10 min and warmed to 20°C. A 50 µl portion of S₁ nucleases was added and the samples treated and analyzed and the results quantified.

EXAMPLE 68

Inhibition of Transcription by PNA

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, 1mM EDTA, pH 7.4) was incubated at 37°C for 60 min. Subsequently, 4 µl 5 × concentrated buffer (0.2 M Tris-HCl (pH 8.0), 40 mM MgCl₂, 10 mM spermidine, 125 mM NaCl) were mixed with 1 µl NTP-mix (10 mM ATP, 10 mM CTP, 10 mM GTP, 1mM UTP, 0.1 µg/ml ³²P-UTP, 5 mM DTT, 2 µg/ml tRNA, 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% postassium acetate in 96% ethanol at ~20°C and analyzed by electrophoresis in 8% polyacrylamide sequencing gels. RNA transcripts were visualized by autoradiography. The following plasmids were used: pT8C2-KS/pA8G2-KS: oligonucleotides GAG₁₀⁻¹₉₋₉₋₈₋₉₋₁₀-GTG₁₀⁻¹₉₋₉₋₈₋₉₋₁₀-GTC₁₀⁻¹₉₋₉₋₈₋₉₋₁₀-TGCA cloned into the Pst site of pBluescript-KS; pT10-KS/pA10-KS (both orientations of the insert were obtained), pT10UVS: oligonucleotides GATCCAG₁₀⁻¹₉ & GATCCCT₁₀⁻¹₉ cloned into the BamHI site of a pUC18 derivative in which the lac UV5 E.coli promoter had been cloned into the EcoRI site (Jeppeisen, et al., Nucleic Acids Res., 1988, 16, 9545).

Using T₃ RNA polymerase, transcription elongation arrest was obtained with PNA-T₁₀⁻¹₉-Lys-NH₂ and the pA8G2-KS plasmid having the PNA recognition sequence on the template strand, but not with pT8C2-KS having the PNA recognition sequence on the non-template strand. Similar results were obtained with PNA-T₁₀⁻¹₉-Lys-NH₂ and the plasmids pA10-KS and pT10-KS. (see FIG. 25) Using E.coli RNA polymerase and the pT10UVS Plasmid (A₁₀⁻¹₉ sequence on the template strand) transcription elongation arrest was obtained with PNA-T₁₀⁻¹₉-Lys-NH₂.
EXAMPLE 69

Biological stability of PNA

A mixture of PNA-Tε (10 μg) and a control, “normal” peptide (10 μg) in 40 μl 50 mM Tris-HCl, pH 7.4 was treated with varying amounts of peptidase from porcine intestinal mucosa or protease from Streptomyces caesius for 10 min at 37°C. The amount of PNA and peptide was determined by HPLC analysis (reversed phase C-18 column: 0–60% acetonitrile, 0.1% trifluoroacetic acid).

At peptidase/protease concentrations where complete degradation of the peptide was observed (no HPLC peak) the PNA was still intact.

EXAMPLE 70

Inhibition of Gene Expression

A preferred assay to test the ability of peptide nucleic acids to inhibit expression of the E2 mRNA of papillomavirus is based on the well-documented transactivation properties of E2. Spaldholz, et al., J. Virol., 1987, 61, 2128–2137. A reporter plasmid (E2RECAT) was constructed to contain the E2 responsive element, which functions as an E2 dependent enhancer. E2RECAT also contains the SV40 early promoter, an early polyadenylation signal, and the chloramphenicol acetyl transferase gene (CAT). Within the context of this plasmid, CAT expression is dependent upon expression of E2. The dependence of CAT expression on the presence of E2 has been tested by transfection of this plasmid into C127 cells transformed by BPV-1, uninfected C127 cells and C127 cells cotransfected with E2RECAT and an E2 expression vector.

A. Inhibition of BPV-1 E2 Expression

BPV-1 transformed C127 cells are plated in 12 well plates. Twenty four hours prior to transfection with E2RE1, cells are pretreated by addition of antisense PNA to the growth medium at final concentrations of 5, 15 and 30 mM. The next day cells are transfected with 10 μg of E2RE1CAT by calcium phosphate precipitation. Ten micrometers of E2RE1CAT and 10 μg of carrier DNA (PUC 19) are mixed with 62 μl of 2 M CaCl2 in a final volume of 250 μl of H2O, followed by addition of 250 μl of 2xBBS (1.5 mM Na2PO4, 10 mM KCl, 280 mM NaCl, 12 mM glucose and 50 mM HEPES, pH 7.0) and incubated at room temperature for 30 minutes. One hundred microliters of this solution is added to each test well and allowed to incubate for 4 hours at 37°C. After incubation, cells are glycerol shocked for 1 minute at room temperature with 15% glycerol in 0.75 mM Na2PO4, 5 mM KCl, 140 mM NaCl, 6 mM glucose and 25 mM HEPES, pH 7.0. After shocking, cells are washed 2 times with serum free DMEM and reseeded with DMEM containing 10% fetal bovine serum and antisense oligonucleotide at the original concentration. Forty eight hours after transfection cells are harvested and assayed for CAT activity.

For determination of CAT activity, cells are washed 2 times with phosphate buffered saline and collected by scraping. Cells are resuspended in 100 μl of 250 mM Tris-HCl, pH 8.0 and disrupted by freeze-thawing 3 times. Twenty four microliters of cell extract is used for each assay. For each assay the following are mixed together in an 1.5 ml Eppendorf tube and incubated at 37°C for one hour: 25 μl of cell extract, 5 μl of 4 mM acetyl coenzyme A, 18 μl H2O and 1 μl 14C-chloramphenicol, 40–60 mM/cm. After incubation, chloramphenicol (acetylated and nonacetylated forms) is extracted with ethyl acetate and evaporated to dryness. Samples are resuspended in 25 μl of ethyl acetate, spotted onto a TLC plate and chromatographed in chloroform: methanol (19:1). Chromatographs are analyzed by autoradiography. Spots corresponding to acetylated and nonacetylated 14C-chloramphenicol are eluted from the TLC plate and counted by liquid scintillation for quantitation of CAT activity. Peptide nucleic acids that depress CAT activity in a dose dependent fashion are considered positively.

B. Inhibition of HPV E2 Expression

The assay for inhibition of human papillomavirus (HPV) E2 by peptide nucleic acids is essentially the same as that for BPV-1 E2. For HPV assays appropriate HPV are co-transfected into either CV-1 or A431 cells with PSV2NEO using the calcium phosphate method described above. Cells which take up DNA are selected for by culturing in media containing the antibiotic G418. G418-resistant cells are then analyzed for HPV DNA and RNA. Cells expressing E2 are used as target cells for antisense studies. For each PNA, cells are pretreated as above, transfected with E2RE1CAT, and analyzed for CAT activity as above. Peptide nucleic acids are considered to have a positive effect if they can express CAT activity in a dose dependent fashion.

EXAMPLE 71


The protected PNA was assembled onto a Boc-Lys(CIZ) modified MBHA resin with a substitution of approximately 0.145 mmol/g. Capping of uncoupled amino groups was only carried out before the incorporation of the Boc-Gaeg-OH monomer.

Synthesis was initiated on 100 mg (dry weight) of neutralised Boc-Lys(CIZ)-MBHA resin that had been preswollen overnight in DCM. The incorporation of the monomers followed the protocol of Example 32, except at step 5 for the incorporation of the Boc-Aeg-OH monomer. Step 5 for the present synthesis involved addition of 4 equiv. disopropyl carbodiimide (0.06 ml; 9.7 μl) and 4 equiv. Boc-Aeg-OH (0.06 mmol; 32 mg) dissolved in 0.6 ml DCM/DMF (1:1, v/v) (final concentration of monomer 0.1M). The coupling reaction was allowed to proceed for 1x15 min and 1x60 min. (re coupling).

All qualitative Kaiser tests were negative (straw-yellow color with no coloration of the beads). The PNA-oligomer was cleaved and purified by the standard procedure. FAB-MS average mass found (calc.) (M+H) 4145.1 (4146.1).

EXAMPLE 72

Hybridization of H-TagTTATCTCTATCT-LysNH2

<table>
<thead>
<tr>
<th>DNA-target</th>
<th>pH</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-5′ 3′</td>
<td>5</td>
<td>60.5</td>
</tr>
<tr>
<td>5′-3′ 3′</td>
<td>7.2</td>
<td>43.0</td>
</tr>
<tr>
<td>5′-3′ 3′</td>
<td>9</td>
<td>38.5</td>
</tr>
<tr>
<td>3′-5′ 5′</td>
<td>5</td>
<td>64.5/49.0</td>
</tr>
</tbody>
</table>
The fact that there is almost no loss in Tm in going from pH 7.2 to 9.0 indicates that Hoogsteen basepairing is not involved. The increase in Tm in going from 7.2 to 5.0 is large for the parallel orientation and is probably due to the formation of a 2:1 complex. It is believed that the most favorable orientation in the Watson-Crick binding motif is the 3’/5’-orientation and that in the Hoogsteen motif the 5’/5’-orientation is the most stable. Thus, it may be the case that the most stable complex is with the two PNA’s strands anti parallel.

There is apparently a very strong preference for a parallel orientation of the Hoogsteen strand. This seems to explain why even at pH 9 a 2:1 complex is seen with the 5’/3’-orientation. Furthermore, it explains the small loss in going from pH 7.2 to 9 in the 3’/3’-orientation, as this is probably a 1:1 complex.

**EXAMPLE 73**


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

About 1 g of wet Boc-Lys(CIZ)-MBHA (0.28 mmol Lys/g) resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]2-A(Z)aeq-Taeg-C(Z)aeq-Taeg-C(Z)aeq-Taeg-Taeg-C(Z)aeq-Taeg-Lys(CIZ)-MBHA resin was assembled in situ DIC coupling of the five first residues utilizing 0.16 M of Boc(Z)-OH, BocTaeg-OH or BocA(Z)aeq-OH, together with 0.16 M DCC in 2 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol 9”) and by analogous in situ DIC coupling of the five last residues (“Synthetic Protocol 10”). Each coupling reaction was allowed to proceed for a total of 20-24 hrs with shaking. The reaction was monitored by the ninhydrin reaction, which showed nearly quantitative incorporation of all residues except of the first A(Z)aeq residue, which had to be coupled twice. The total coupling yield was about 96% (first coupling, about 89% efficiency).


The protected Boc-[Taeg]2-A(Z)aeq-Taeg-C(Z)aeq-A(Z) aeq-Taeg-C(Z)aeq-Taeg-C(Z)aeq-Taeg-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 53.4 mg of crude material upon HF cleavage of 166.1 mg dry Boc-[Taeg]2-A(Z)aeq-Taeg-C(Z)aeq-A(Z) aeq-Taeg-C(Z)aeq-Taeg-Lys(CIZ)-MBHA resin. The crude product (33.4 mg) was purified to give 18.3 mg of H-[Taeg] 2-Aaeg-Taeg-Caeg-Aaeg-Taeg-Caeg-Taeg-Taeg-Taeg-Taeg-Lys-NH₂. For (M+H)+, the calculated m/z value = 2780.17 and the measured m/z value = 2780.07.

**EXAMPLE 74**

Oligodeoxynucleotide  pH  Tm(°C)
5’-AAT AGT AGT G-3’ (SEQ ID NO: 35)  5  31.9°
5’-ATT AGT AGT G-3’ (SEQ ID NO: 36)  7.2  28.9°
5’-AAX AGT AGT G-3’  9  28.0°
5’-GAT ATG ATA A-3’  7.2  30.5°
5’-GAT ATG ATA A-3’  9  28.0°

†Low hypochromicity

**EXAMPLE 75**

Synthesis of a PNA With Two Parallel Strings Tied Together

![Chemical structure](image)

A 375 mg portion of MBHA resin (loading 0.6 mmol/g) was allowed to swell overnight in dichloromethane (DCM). After an hour in DME/DCM, the resin was neutralized by washing 2 times with 5% diisopropylethylamine in DCM (2 min.), followed by washing with DCM (2 ml; 6×1 min.) N,N'-di-Boc-aminomethyl glycine (41.9 mg; 0.132 mmol) dissolved in 2 ml DME was added to the resin, followed by DCC (64.9 mg; 0.315 mmol) dissolved in 1 ml of DCM. After 2.5 hours, the resin was washed with DME 3 times (1 min.) and once with DCM (1 min.). The unreacted amino groups were then capped by treatment with acetic anhydride/DCM/pyridine (1 ml 2:1 ml2 ml) for 72 hours. After washing with DCM (2 ml; 4×1 min.), a Kaiser test showed no amino groups were present. The resin was deprotected and washed as described above. This was followed by reaction with 6-(Bocamino)-hexanoic acid DHB ester (255.8 mg; 67 mmol) dissolved in DME/DCM 1:1 (4 ml) overnight. After washing and neutralization, a Kaiser test and an isatin test were performed. Both were negative. After capping, the elongation of the PNA-chains was performed according to standard procedures for DCC couplings. All Kaiser tests performed after the coupling reactions were negative (Yellow). Qualitative Kaiser tests were done after deprotection of PNA units number 1, 2, and 6. Each test was blue. The PNA oligomers were cleaved and purified by standard procedures. The amount of monomer and DCC used for each coupling was as follows (total volume 4.5 ml):

<table>
<thead>
<tr>
<th>Coupling</th>
<th>Monomer (μg)</th>
<th>DCC (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>173</td>
<td>95</td>
</tr>
<tr>
<td>2.</td>
<td>176</td>
<td>101</td>
</tr>
<tr>
<td>3.</td>
<td>174</td>
<td>97</td>
</tr>
<tr>
<td>4.</td>
<td>174</td>
<td>103</td>
</tr>
<tr>
<td>5.</td>
<td>178</td>
<td>97</td>
</tr>
<tr>
<td>6.</td>
<td>173</td>
<td>99</td>
</tr>
<tr>
<td>7.</td>
<td>174</td>
<td>95</td>
</tr>
<tr>
<td>8.</td>
<td>175</td>
<td>96</td>
</tr>
</tbody>
</table>
For the PNA having the Structure (70) where \( R_{11} = T_{6} \), there was 24.5 mg of crude product, which resulted in 6.9 mg after purification. For the PNA where \( R_{1} = T_{6} \) there was 28.8 mg of crude product, which resulted in 2.8 mg after purification. The products had a high tendency of aggregation, as indicated by a complex HPLC chromatogram after a few hours at room temperature in concentration above 1 mg/mL. The PNA-(T\(_6\)) and PNA-(T\(_6\))-5 were hybridised to (dA\(_5\)) and (dA\(_3\))\(_5\), respectively, with recorded Tm of 42°C and 59°C, respectively.

**EXAMPLE 76**

**Solid-Phase Synthesis of H-[Taeg\(_7\)]\(_{5}\)-Lys(CIZ)-MBHA Resin**

The PNA oligomer was assembled onto 500 mg (dry weight) of MBHA resin that had been preswollen overnight in DCM. The resin was initially substituted with approximately 0.15 mmol/g Boc-Lys(CIZ) as determined by quantitative ninhydrin reaction. The stepwise synthesis of the oligomer followed the synthetic protocol described in Example 32 employing 0.077 g (0.2 mmol) Boc-Taeg-OH and 3.13 \( \mu \)l (0.2 mmol) disopropyl carbodiimide in 2.0 ml 50% DMF/CH\(_2\)Cl\(_2\) in each coupling. Capping of uncoupled amino groups was carried out before deprotection in each step. All qualitative Kaiser tests were negative indicating near 100% coupling yield.

**EXAMPLE 77**

**Solid-Phase Synthesis of H-[Taeg\(_7\)]\(_{5}\)-[apgT]-[Taeg\(_7\)]\(_{5}\)-Lys-NH\(_2\)**

Synthesis was initiated on approximately 1/4 of the wet H-[Taeg\(_7\)]\(_{5}\)-Lys(CIZ)-MBHA resin from Example 76. In situ disopropyl carbodiimide (DIC) couplings of both Boc-(apgT)-OH and Boc-Taeg-OH were carried out in 1.2 ml 50% DMF/CH\(_2\)Cl\(_2\) using 0.048 g (0.12 mmol) and 0.046 g (0.12 mmol) monomer, respectively, and 18.7 \( \mu \)l (0.12 mmol) disopropyl carbodiimide in each coupling. All qualitative Kaiser tests were negative, indicating near 100% coupling yield. The PNA oligomer was cleaved and purified by standard procedures. For (M+H)*, the calculated m/z value was 2820.15 and the measured m/z value was 2820.92.

**EXAMPLE 78**

**Solid-Phase Synthesis of H-[Taeg\(_7\)]\(_{5}\)-[proT]-[Taeg\(_7\)]\(_{5}\)-Lys-NH\(_2\)**

Synthesis was initiated on approximately 1/4 of the wet H-[Taeg\(_7\)]\(_{5}\)-Lys(CIZ)-MBHA resin from Example 76. In situ disopropyl carbodiimide couplings of Boc-Taeg-OH were carried out in 1.2 ml 50% DMF/CH\(_2\)Cl\(_2\) using 0.046 g (0.12 mmol) monomer and 18.7 \( \mu \)l (0.12 mmol) disopropyl carbodiimide in each coupling. Due to solubility problems, Boc-(proT)-OH 0.048 g (0.12 mmol) was suspended in 2.5 ml 50% DMF/DMSO prior to coupling, the suspension filtered, and approximately 2 ml of the filtrate used in the overnight coupling. All qualitative Kaiser tests were negative, indicating near 100% coupling yields. The PNA oligomer was cleaved and purified by standard procedures.

**EXAMPLE 80**

**Solid-Phase Synthesis of H-[Taeg\(_7\)]\(_{5}\)_[bC]-[Taeg\(_7\)]\(_{5}\)-Lys-NH\(_2\)**

The PNA oligomer was assembled onto 100 mg (dry weight) MBHA resin that had been preswollen overnight in DCM. The resin was initially substituted with approximately 0.25 mmol/g Boc-Lys(CIZ) as determined by quantiative ninhydrin reaction. The stepwise synthesis of the oligomer followed synthetic Protocol 9 employing 0.023 g (0.06 mmol) Boc-Taeg-OH, 0.062 g (0.12 mmol) Boc(bCZ)-OH and 0.012 g (0.06 mmol) DCC in 1.2 ml 50% DMF/CH\(_2\)Cl\(_2\) in each coupling. Capping of uncoupled amino groups was carried out before deprotection in each step. All qualitative Kaiser tests were negative, indicating near 100% coupling yield. The PNA-oligomer was cleaved and purified by standard procedures.

**EXAMPLE 81**

**Hybridization properties of H-T\(_4\)bCT\(_4\)-Lys-NH\(_2\)**

<table>
<thead>
<tr>
<th>Oligodeoxynucleotide</th>
<th>Tm (° C.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-'AAA AAA AAA A</td>
<td>53.5</td>
</tr>
<tr>
<td>(SEQ ID NO: 3)</td>
<td></td>
</tr>
<tr>
<td>5'-'AAA AAG AAA A</td>
<td>44.0</td>
</tr>
<tr>
<td>(SEQ ID NO: 10)</td>
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<tr>
<td>5'-'AAA AAG AAA A</td>
<td>43.5</td>
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<tr>
<td>(SEQ ID NO: 8)</td>
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<tr>
<td>5'-'AAA ACA AAA A</td>
<td>46.5</td>
</tr>
<tr>
<td>(SEQ ID NO: 26)</td>
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<tr>
<td>5'-'AAA ATC AAA A</td>
<td>46.5</td>
</tr>
<tr>
<td>(SEQ ID NO: 27)</td>
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<tr>
<td>5'-'AAA AAT AAA A</td>
<td>46.0</td>
</tr>
<tr>
<td>(SEQ ID NO: 25)</td>
<td></td>
</tr>
</tbody>
</table>

**EXAMPLE 82**

**Stepwise Assembly of H-[Taeg\(_7\)]-[Taeg\(_7\)]-[Taeg\(_7\)]-[Aaeq]-[Taeg\(_7\)]-[Taeg\(_7\)]-[Taeg\(_7\)]-[Taeg\(_7\)]-Lys-NH\(_2\)**

Synthesis was initiated on a Boc-[Taeg\(_7\)]-Lys(CIZ)-MBHA resin (from example 76) that had been preswollen overnight in DCM. The resin resembled approximately 100 mg (dry weight) of Boc-Lys(CIZ)-MBHA resin (loading 0.15 mmol/g). The incorporation of the monomers followed the protocol of example 55, except for step 5 (incorporation
of the Boc(A)(Z)aeg-OH monomer). New step 5 (incorporation of A(Z)aeg) involved addition of 4 equiv. disopropyl carbodiimide (0.06 mmol; 9.7 μl) and 4 equiv. Boc(A)(Z)aeg-OH (0.06 mmol; 32 mg) dissolved in 0.6 ml DMF/DMF (1:1, v/v) (final concentration of monomer 0.1 M). The coupling reaction was allowed to proceed for 1×15 min. and 1×60 min. (recoupling).

Capping of uncoupled amino groups was only carried out before the incorporation of the Boc(A)(Z)aeg-OH monomer. The coupling reaction was monitored by qualitative ninhydrin reaction (Kaiser test). All qualitative Kaiser tests were negative (straw-yellow color with no coloration of the beads). The PNA oligomer was cleaved and purified by standard procedures.

EXAMPLE 84

Hybridization properties of H-T₄AT₅LysNH₂

<table>
<thead>
<tr>
<th>Oligodeoxynucleotide</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-AAA AAA AAA A</td>
<td>59.5</td>
</tr>
<tr>
<td>5'-AGA AGA AAA A</td>
<td>45.0</td>
</tr>
<tr>
<td>5'-AAA AAG AAA A</td>
<td>45.5</td>
</tr>
<tr>
<td>5'-AAA ACA AAA A</td>
<td>48.0</td>
</tr>
<tr>
<td>5'-AAA ATA AAA A</td>
<td>52.0</td>
</tr>
<tr>
<td>5'-AAA AAT AAA A</td>
<td>52.5</td>
</tr>
</tbody>
</table>

EXAMPLE 85

Stepwise Assembly of H-[Taeg]-[Taeg]-[Taeg]-[Gaeg]-[Gaeg]-[Gaeg]-[Gaeg]-[Gaeg]-Lys-NH₂

The protected PNA was assembled onto a Boc-Lys(CIZ) modified MBHA resin with a substitution of 0.15 mmol/g. The incorporation of the monomers followed the protocol of example 32, except that the capping step 11 and the washing step 12 were omitted. After the incorporation and deprotection of the first, second, and fourth G(BzI)aeg-monomer there were some difficulties getting the resin to swell properly. Three hours of shaking in neat DCM gave acceptable solubility. For the incorporation of residues Taeg-4, G(BzI)aeg-6, and Taeg-7 to Taeg-10, recoupling was necessary to obtain quantitative coupling yields. Taeg₄ (2x in 50% DMF/DCM), Gaeg (2x in 50% DMF/DCM), Taeg₃ (2x in 50% DMF/DCM), Taeg₂ (1x in 50% NMP/DCM and 1x in neat DCM), Taeg (1x in 50% DMF/DCM and 2x in neat DCM), Taeg₂ (2x in 50% DMF/DCM), Taeg₁ (2x in 50% DMF/DCM). All qualitative Kaiser tests were negative (straw-yellow color with no coloration of the beads). The PNA oligomer was cleaved and purified by standard procedures.

EXAMPLE 86

<table>
<thead>
<tr>
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<th>Tm</th>
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</tr>
<tr>
<td>5'-C4A2C4A2 (SEQ ID NO:39)</td>
<td>55</td>
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</tbody>
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(g) Synthetic Protocol 8 (General Protocol)
(1) Boc-deprotection with TFA/CH₂Cl₂, (1:1, v/v), 3×1 min and 1×30 min; (2) washing with CH₂Cl₂, 6×1 min; (3) neutralization with DIEA/CH₂Cl₂, (1:19, v/v), 3×2 min; (4) washing with CH₂Cl₂, 6×1 min, and drain for 1 min; (5) at some stages of the synthesis, 2–5 mg sample of PNA-resin is taken out and dried thoroughly for a ninhydrin analysis to determine the substitution; (6) addition of Boc-protected PNA monomer (Pip ester); the coupling reaction was allowed to proceed for a total of 4 hrs shaking; (7) washing with DMF, 1×2 min; (8) washing with CH₂Cl₂, 4×1 min; (9) neutralization with DIEA/CH₂Cl₂, (1:19, v/v), 2×2 min; (10) washing with CH₂Cl₂, 6×1 min; (11) occasionally, 2–5 mg sample of protected PNA-resin is taken out and dried thoroughly for a ninhydrin analysis to determine the extent of coupling; (12) at some stages of the synthesis, unreacted amino groups are blocked by acetylation with a mixture of acetic anhydride/pyridine/CH₂Cl₂, (1:1:2, v/v/v) for 2 h followed by washing with CH₂Cl₂, 6×1 min, and, occasionally, ninhydrin analysis.

EXEMPLARY 88

Solid-Phase Synthesis of H-[Taeg]4-[Caeg]-[Taeg]5-Lys(NH₂)₂

(a) Stepwise Assembly of Boc-[Taeg]4-[Caeg]-[Taeg]5-Lys(CIZ)-MBHA Resin.

About 1 g of wet Boc-[Taeg]5-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]4-[Caeg]-[Taeg]5-Lys(CIZ)-MBHA resin was assembled by in situ DCC coupling of all residues utilizing 0.16 M of BocC[Z]aeg-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ or 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol 9”). Each coupling reaction was allowed to proceed for a total of 20–24 hrs with shaking. The Boc-residue was coupled three times and the Boc residues were all coupled once. The synthesis was monitored by the ninhydrin reaction which showed >99% total incorporation of Boc (about 88% after the first coupling and about 93% after the second coupling) and close to quantitative incorporation of all the Taeg residues.

(b) Cleavage, Purification, and Identification of H-[Taeg]4-[Caeg]-[Taeg]5-Lys(NH₂)₂

The protected Boc-[Taeg]4-[Caeg]-[Taeg]5-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 22.5 mg of crude material upon HF cleavage of 108.9 mg dry H-[Taeg]4-[Caeg]-[Taeg]5-Lys(CIZ)-MBHA resin. Crude product (5.8 mg) was purified to give 3.1 mg of H-[Taeg]4-[Caeg]-[Taeg]5-Lys(NH₂)₂.

(c) Synthetic Protocol 9 (General Protocol)

(1) Boc-deprotection with TFA/CH₂Cl₂, (1:1, v/v), 3×1 min and 1×30 min; (2) washing with CH₂Cl₂, 6×1 min; (3) neutralization with DIEA/CH₂Cl₂, (1:19, v/v), 3×2 min; (4) washing with CH₂Cl₂, 6×1 min, and drain for 1 min; (5) at some stages of the synthesis, 2–5 mg sample of PNA-resin is taken out and dried thoroughly for a ninhydrin analysis to determine the substitution; (6) addition of Boc-protected PNA monomer (free acid) in X ml DMF followed by addition of DCC in X ml CH₂Cl₂; the coupling reaction was allowed to proceed for a total of Y hrs shaking; (7) washing with DMF, 1×2 min; (8) washing with CH₂Cl₂, 4×1 min; (9) neutralization with DIEA/CH₂Cl₂, (1:19, v/v), 2×2 min; (10) washing with CH₂Cl₂, 6×1 min; (11) occasionally, 2–5 mg sample of protected PNA-resin is taken out and dried thoroughly for a ninhydrin analysis to determine the extent of coupling; (12) at some stages of the synthesis, unreacted amino groups are blocked by acetylation with a mixture of acetic anhydride/pyridine/CH₂Cl₂, (1:1:2, v/v/v) for 2 h followed by washing with CH₂Cl₂, 6×1 min, and, occasionally, ninhydrin analysis.

EXEMPLARY 89


About 1 g of wet Boc-[Taeg]5-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]4-[Caeg]-[Taeg]5-Lys(CIZ)-MBHA resin was assembled by in situ DCC coupling utilizing 0.16 M of Boc(NBaeq)-OH together with 0.16 M DCC in 2.0 ml neat CH₂Cl₂ at 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ ("Synthetic Protocol 9"). Each coupling reaction was allowed to proceed for a total of 20–24 hrs with shaking. The NBaeq residue was coupled three times and the Taeg residues were all coupled once. The synthesis was monitored by the ninhydrin reaction which showed >99% total incorporation of NBaeq (about 88% after the first coupling and about 93% after the second coupling) and close to quantitative incorporation of all the Taeg residues.

(b) Cleavage, Purification, and Identification of H-[Taeg]4-[NBaeq]-[Taeg]5-Lys(NH₂)₂

The protected Boc-[Taeg]4-[NBaeq]-[Taeg]5-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 33.6 mg of crude material upon HF cleavage of 108.9 mg dry H-[Taeg]4-[NBaeq]-[Taeg]5-Lys(CIZ)-MBHA resin. Crude product (20.6 mg) was purified to give 1.6 mg of H-[Taeg]4-[NBaeq]-[Taeg]5-Lys(NH₂)₂. For (M+H)⁺, the calculated m/z value was 2683.12 and the measured m/z value was 2683.09.

EXEMPLARY 90

Solid-Phase Synthesis of H-[Taeg]4-[Caeg]-[Taeg]5-Lys(NH₂)₂

(a) Stepwise Assembly of Boc-[Taeg]4-[Caeg]-[Taeg]5-Lys(CIZ)-MBHA Resin.

About 1 g of wet Boc-[Taeg]5-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]4-[Caeg]-[Taeg]5-Lys(CIZ)-MBHA resin was assembled by in situ DCC single coupling of all residues utilizing: (1) 0.16 M of BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ or (2) 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ ("Synthetic Protocol 9"). Each coupling reaction was allowed to proceed for a total of 20–24 hrs with shaking. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all the residues.

(b) Cleavage, Purification, and Identification of H-[Taeg]4-[Caeg]-[Taeg]5-Lys(NH₂)₂

The protected Boc-[Taeg]4-[Caeg]-[Taeg]5-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 22.2 mg of crude material upon HF cleavage of 126.0 mg dry H-[Taeg]4-[Caeg]-[Taeg]5-Lys(CIZ)-MBHA resin. Crude product (22.2 mg) was purified to give 7.6 mg of H-[Taeg]4-[Caeg]-[Taeg]5-Lys(NH₂)₂. For (M+H)⁺, the calculated m/z value was 2641.11 and the measured m/z value was 2641.16.
EXAMPLE 91

Solid-Phase Synthesis of H-[Taeg]-4-Gly-[Taeg]-5-Lys-NH₂.

(a) Stepwise Assembly of Boc-[Taeg]-4-Gly-[Taeg]-5-Lys (CIZ)-MBHA Resin.

About 1 g of wet Boc-[Taeg]-5-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]-4-Gly-[Taeg]-5-Lys(CIZ)-MBHA resin was assembled by in situ DCC single coupling of all residues utilizing: (1) 0.16 M of BocGly-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ or (2) 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol 9”). Each coupling reaction was allowed to proceed for a total of 20–24 hrs with shaking. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all the residues.

(b) Cleavage, Purification, and Identification of H-[Taeg]-4-Gly-[Taeg]-5-Lys-NH₂.

The protected Boc-[Taeg]-4-Gly-[Taeg]-5-Lys(CIZ)-MBHA resin was treated as described in Example 18c to yield about 45.0 mg of crude material upon HF cleavage of 124.1 mg dry H-[Taeg]-4-Gly-[Taeg]-5-Lys(CIZ)+MBHA resin. Crude product (40.4 mg) was purified to give 8.2 mg of H-[Taeg]-4-Gly-[Taeg]-5-Lys-NH₂.

EXAMPLE 92

Solid-Phase Synthesis of H-[Taeg]-4-Gly-2-[Taeg]-5-Lys-NH₂.

(a) Stepwise Assembly of Boc-[Taeg]-4-Gly-2-[Taeg]-5-Lys (CIZ)-MBHA Resin.

About 1 g of wet Boc-[Taeg]-5-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]-4-Gly-[Taeg]-5-Lys(CIZ)-MBHA resin was assembled by in situ DCC single coupling of all residues utilizing: (1) 0.16 M of BocGly-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ or (2) 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol 9”). Each coupling reaction was allowed to proceed for a total of 20–24 hrs with shaking. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all the residues.

(b) Cleavage, Purification, and Identification of H-[Taeg]-4-Gly-2-[Taeg]-5-Lys-NH₂.

The protected Boc-[Taeg]-4-Gly-2-[Taeg]-5-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 32.6 mg of crude material upon HF cleavage of 156.6 mg dry H-[Taeg]-4-Gly-2-[Taeg]-5-Lys(CIZ)+MBHA resin. Crude product (30 mg) was purified to give 7.8 mg of H-[Taeg]-4-Gly-2-[Taeg]-5-Lys-NH₂. For (M+H)⁺ the calculated m/z value was 2655.09 and the measured m/z value was 2655.37.

EXAMPLE 93

Solid-Phase Synthesis of H-[Taeg]-4-[Caeg]-2-Taeg-Caeg-Taeg-Caeg-Lys-NH₂.

(a) Stepwise Assembly of Boc-[Taeg]-4-[Caeg]-2-Taeg-Caeg-[Z]-Caeg-Taeg-[Z]-Caeg-Lys(CIZ)-MBHA Resin.

About 1.5 g of wet Boc-Lys(CIZ)-MBHA (0.28 mmol Lys/g) resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]-4-[Caeg]-2-Taeg-Caeg-[Z]-Caeg-Taeg-[Z]-Caeg-Lys(CIZ)-MBHA resin was assembled by in situ DCC single coupling of all residues utilizing: (1) 0.16 M of BocC[2]-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂, or (2) 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol 9”). Each coupling reaction was allowed to proceed for a total of 20–24 hrs with shaking. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all the residues.

(b) Cleavage, Purification, and Identification of H-[Taeg]-4-[Caeg]-2-Taeg-Caeg-Taeg-Lys-NH₂.

The protected Boc-[Taeg]-4-[Caeg]-2-Taeg-Caeg-[Z]-Caeg-Taeg-[Z]-Caeg-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 56.1 mg of crude material upon HF cleavage of 255.0 mg dry H-[Taeg]-4-[Caeg]-2-Taeg-Caeg-[Z]-Caeg-Taeg-Lys(CIZ)-MBHA resin. Crude product (88.5 mg) was purified to give 46.2 mg of H-[Caeg]-4-[Caeg]-2-Taeg-Caeg-Lys-NH₂. For (M+H)⁺ the calculated m/z value was 2717.15 and the measured m/z value was 2716.93.

EXAMPLE 95


About 3 g of wet Boc-Lys(CIZ)-MBHA (0.28 mmol Lys/g) resin was placed in a 20 ml SPPS reaction vessel.
Boc-[Taeg]2-[C(Z)æg]3-[Taeg]2-[C(Z)æg]2-Lys(CIZ)-MBHA resin was prepared by reaction of all residues utilizing: (1) 0.16 M of Boc-[Z]-OH together with 0.16 M DCC in 3 ml 50% DMF/CH₂Cl₂ or (2) 0.16 M Boc-Taeg-OH together with 0.16 M DCC in 3 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol 9”). Each coupling reaction was allowed to proceed for a total of 20-24 hr with shaking. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all the residues. After deprotection of the N-terminal Boc group, half of the PNA-resin was coupled quantitatively onto Tyr(BrZ)-OH and a small portion was coupled quantitatively onto one more Caeg residue. Both couplings employed the above-mentioned synthetic protocol.

(b) Cleavage, Purification, and Identification of H-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(NH₃). The protected Boc-[Taeg]2-[C(Z)æg]3-[Taeg]2-[C(Z)æg]2-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 50.9 mg of crude material upon HF cleavage of 182.5 mg dry H-[Taeg]2-[C(Z)æg]3-[Taeg]2-C(Z)æg]2-Lys(CIZ)-MBHA resin. Crude product (50.9 mg) was purified to give 13.7 mg of H-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(NH₃). For (M+H)⁺ the calculated m/z value was 2466.04; the m/z value was not measured.

(c) Cleavage, Purification, and Identification of H-Tyr-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(NH₃). The protected Boc-Tyr(Brz)-[Z]æg]2-[C(Z)æg]3-[Taeg]2-[C(Z)æg]2-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 60.8 mg of crude material upon HF cleavage of 188.8 mg dry H-Tyr(Brz)-[Taeg]2-[C(Z)æg]3-[Taeg]2-[C(Z)æg]2-Lys(CIZ)-MBHA resin. Crude product (60.8 mg) was purified to give 20.7 mg of H-Tyr-[Taeg]2-[C(Z)æg]3-[Taeg]2-[C(Z)æg]2-Lys(NH₃). For (M+H)⁺ the calculated m/z value was 2629.11 and the measured m/z value was 2629.11.

(d) Cleavage, Purification, and Identification of H-Caeg-[Taeg]2-[C(Z)æg]3-[Taeg]2-[C(Z)æg]2-Lys(NH₃). The protected Boc-Caeg-[Taeg]2-[C(Z)æg]3-[Taeg]2-[C(Z)æg]2-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 11.7 mg of crude material upon HF cleavage of 42.0 mg dry H-Caeg-[Taeg]2-[C(Z)æg]3-[Taeg]2-[C(Z)æg]2-Lys(CIZ)-MBHA resin. Crude product (11.6 mg) was purified to give 3.1 mg of H-Caeg-[Taeg]2-[C(Z)æg]3-[Taeg]2-[C(Z)æg]2-Lys(NH₃). For (M+H)⁺ the calculated m/z value was 2717.15; the m/z value was not measured.

EXAMPLE 96


(a) Stepwise Assembly of Boc-[Z]-OH and Boc-Taeg-OH together with 0.16 M DCC in 3 ml 50% DMF/CH₂Cl₂ or (2) 0.16 M Boc-Taeg-OH together with 0.16 M DCC in 3 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol 9”). Each coupling reaction was allowed to proceed for a total of 20-24 hr with shaking. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all the residues. After deprotection of the N-terminal Boc group, half of the PNA-resin was coupled quantitatively onto Tyr(Brz)-OH and a small portion was coupled quantitatively onto one more Caeg residue. Both couplings employed the above-mentioned synthetic protocol.

(b) Cleavage, Purification, and Identification of H-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(NH₃). The protected Boc-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 57.6 mg of crude material upon HF cleavage of 172.7 mg dry H-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(CIZ)-MBHA resin. Crude product (57.6 mg) was purified to give 26.3 mg of H-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(NH₃). For (M+H)⁺ the calculated m/z value was 2466.04; the m/z value was not measured.

(c) Cleavage, Purification, and Identification of H-Tyr-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(NH₃). The protected Boc-Tyr(Brz)-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 57.6 mg of crude material upon HF cleavage of 172.7 mg dry H-Tyr(Brz)-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(CIZ)-MBHA resin. Crude product (47.1 mg) was purified to give 13.4 mg of H-Tyr-[C(Z)æg]2-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(NH₃). For (M+H)⁺ the calculated m/z value was 2629.11 and the measured m/z value was 2629.11.

(d) Cleavage, Purification, and Identification of H-Caeg-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(NH₃). The protected Boc-Caeg-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 53.4 mg of crude material upon HF cleavage of 42.4 mg dry H-Caeg-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(CIZ)-MBHA resin. Crude product (11.9 mg) was purified to give 4.3 mg of H-Caeg-[Taeg]2-[C(Z)æg]3-[Taeg]2-Lys(NH₃). For (M+H)⁺ the calculated m/z value was 2732.15; the m/z value was not measured.

(c) Synthetic Protocol 10 (General Protocol) Same protocol as “Synthetic Protocol 9”, except that DCC has been replaced with DIC.

EXAMPLE 97

Synthesis of the Backbone Moietry for Scale up by Reductive Amination

(a) Preparation of (bocamino)acetdehyde.

3-Amino-1,2-propanediol (80.0 g; 0.88 mol) was dissolved in water (1500 ml) and the solution was cooled to 4°C. Hereafter Boc anhydride (230 g; 1.05 mol) was added at once. The solution was gently heated to room temperature with a stirrer. The pH was kept at 10.5 by the dropwise addition of sodium hydroxide. Over the course of the reaction a total of 70.2 g NaOH, dissolved in 480 ml water, was added. After stirring overnight, ethyl acetate (1000 ml) was added and the mixture was cooled to 0°C and then was added to the solution with more ethyl acetate (8×500 ml). The combined ethyl acetate solution was reduced to a volume of 1500 ml using a rotary evaporator. The resulting solution was washed with half saturated potassium hydrogen sulphate (1500 ml) and then with saturated sodium chloride.
Solid-Phase Synthesis of Dansyl-[Taeg]_{10}-Lys-NH₂

(a) Stepwise Assembly of Dansyl-[Taeg]_{10}-Lys(CIZ)-MBHA Resin.
About 0.3 g of wet Boc-[Taeg]_{10}-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Dansyl-[Taeg]_{10}-Lys(CIZ)-MBHA resin was assembled by coupling of 0.5 M dansyl-Cl in 2.0 ml neat pyridine overnight. The ninhydrin reaction showed about 95% incorporation of dansyl.

(b) Cleavage, Purification, and Identification of Dansyl-[Taeg]_{10}-Lys-NH₂.
The protected dansyl-[Taeg]_{10}-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 12 mg of crude material upon HF cleavage of 71.3 mg dry dansyl-[Taeg]_{10}-Lys(CIZ)-MBHA resin. The crude product was purified to give 5.4 mg of dansyl-[Taeg]_{10}-Lys-NH₂.

EXAMPLE 100
Solid-Phase Synthesis of Gly-Gly-His-[Taeg]_{10}-Lys-NH₂

(a) Stepwise Assembly of Boc-Gly-Gly-His(Tos)-[Taeg]_{10}-Lys(CIZ)-MBHA Resin.
About 0.05 g of Boc-[Taeg]_{10}-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-Gly-Gly-His(Tos)-[Taeg]_{10}-Lys(CIZ)-MBHA resin was assembled by standard double in situ DCC coupling of Boc-protected amino acid (0.1 M) in 2.5 ml 25% DMF/CH₂Cl₂ except for the first coupling of BocHis(Tos), which was done by using a preformed symmetrical anhydride (0.1M) in 25% DMF/CH₂Cl₂. All couplings were performed overnight and ninhydrin reactions were not carried out.

(b) Cleavage, Purification, and Identification of Gly-Gly-His-[Taeg]_{10}-Lys-NH₂.
The protected Boc-Gly-Gly-His(Tos)-[Taeg]_{10}-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 10.3 mg of crude material (about 40% purity) upon HF cleavage of 34.5 mg dry Boc-Gly-Gly-His(Tos)-[Taeg]_{10}-Lys(CIZ)-MBHA resin. A small portion of the crude product (taken out before lyophilization) was purified to give 0.1 mg of Gly-Gly-His-[Taeg]_{10}-Lys-NH₂.

EXAMPLE 101
Solid-Phase Synthesis of H-[Taeg]_{1}-[Taeg]_{2}-NH₂

(a) Stepwise Assembly of Boc-[Taeg]_{1}-[C(Z)aeq]_{2}-MBHA Resin.
About 0.2 g of MBHA resin was placed in a 3 ml SPPS reaction vessel and neutralized. The loading was determined to be about 0.64 mmol/g. Boc(Z)aeq-OPip was coupled onto the resin using a concentration of 0.13 M in 2.5 ml 25% phenol/CH₂Cl₂. The ninhydrin analysis showed a coupling yield of about 40%. The remaining free amino groups were acetylated as usual. Boc-[Taeg]_{1}-[C(Z)aeq]_{2}-MBHA resin was assembled by single in situ DCC coupling of the next residue utilizing 0.11 M of Boc(CZ)aeq-OBzl together with 0.11 M DCC in 2.5 ml 50% DMF/CH₂Cl₂ and by coupling with 0.13 M BocTaeg-OPip in neat CH₂Cl₂ for the remaining residues (“Synthetic Protocol 8”). Each coupling reaction was allowed to proceed with shaking overnight. The
synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all the residues.

(b) Cleavage, Purification, and Identification of H-[Taeg]₃-[Caeg]-Lys-H₂O.

The protected Boc-[Taeg]₃-L-[Caeg]-Lys(CZ)-MBHA resin treated as described in Example 17c to yield about 21.7 mg of crude material (>80% purity) upon HF cleavage of 94.8 mg dry H-[Taeg]₃-L-[Caeg]-Lys(CZ)-MBHA resin. Crude product (7.4 mg) was purified to give 2.0 mg of H-[Taeg]₃-L-[Caeg]-Lys-H₂O (>99% purity).

EXAMPLE 102


(a) Stepwise Assembly of Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-MBHA Resin.

About 0.2 g of the above-mentioned MBHA resin was placed in a 5 ml SPPS reaction vessel and neutralized. Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-MBHA resin was assembled by single in situ DCC coupling of the C(Z)ae residue utilizing 0.13 M of BocC[Z]ae-g-0H together with 0.13 M DCC in 2.5 ml 50% DMF/CH₂Cl₂ and by coupling the Taeg residues with 0.13 M BocTaeg-OPfp in 2.5 ml neat CH₂Cl₂. Each coupling reaction was allowed to proceed with shaking overnight. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all the residues.

(b) Cleavage, Purification, and Identification of H-[Taeg]₃-Caeg-[Taeg]₃-Lys-H₂O.

The protected Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-MBHA resin was treated as described in Example 17c to yield about 44.4 mg of crude material upon HF cleavage of about 123 mg dry H-[Taeg]₃-C(Z)ae-[Taeg]₃-MBHA resin. Crude product (11.0 mg) was purified to give 3.6 mg of H-[Taeg]₃-Caeg-[Taeg]₃-Lys-H₂O.

EXAMPLE 103

Solid-Phase Synthesis of H-Taeg-Caeg-[Taeg]₃-Lys-H₂O.

(a) Stepwise Assembly of Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA Resin.

About 0.5 g of wet Boc-[Taeg]₃-Lys(CZ)-MBHA resin (substitution ~0.3 mmol Lys/g) was placed in a 3 ml SPPS reaction vessel. Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA resin was assembled by single in situ DCC coupling of the C(Z)ae residue (“Synthetic Protocol”) utilizing 0.2 M of BocC[Z]ae-g-0H together with 0.2 M DCC in 2.5 ml 50% DMF/CH₂Cl₂ (incorporation was about 80% as judged by ninhydrin analysis; remaining free amino groups were acetylated) and by overnight coupling the Taeg residue with 0.15 M BocTaeg-OPfp in 2.5 ml neat CH₂Cl₂ (nearly quantitatively).

(b) Cleavage, Purification, and Identification of H-Taeg-Caeg-[Taeg]₃-Lys-H₂O.

The protected Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA resin was treated as described in Example 17c to yield about 22.3 mg of crude material upon HF cleavage of about 76.5 mg dry H-Taeg-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA resin. Crude product (6.7 mg) was purified to give 2.6 mg of H-Taeg-Caeg-[Taeg]₃-Lys-H₂O. For M+H⁺ the calculated m/z value was 2792.15 and the measured m/z value was 2792.21.

EXAMPLE 104


(a) Stepwise Assembly of Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA Resin.

About 0.5 g of wet Boc-[Taeg]₃-Lys(CZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA resin was assembled by single in situ DCC coupling of all residues utilizing: (1) 0.12 M of BocC[Z]ae-g-0H together with 0.12 M DCC in 3.0 ml 50% DMF/CH₂Cl₂ or (2) 0.12 M BocTaeg-0H together with 0.12 M DCC in 3.0 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol”). Each coupling reaction was allowed to proceed overnight with shaking. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all the residues. During the synthesis, a small portion of H-[Taeg]₃-Lys(CZ)-MBHA resin was taken out for HF cleavage.

(b) Cleavage, Purification, and Identification of H-Caeg-[Taeg]₃-Lys-NH₂.

The protected Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA resin was treated as described in Example 17c to yield about 3.0 mg of crude material upon HF cleavage of 37.5 mg dry H-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA resin. About 0.7 mg of the crude product was purified to give about 0.5 mg of H-Caeg-[Taeg]₃-Lys-NH₂.


The protected Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA resin was treated as described in Example 17c to yield about 37.7 mg of crude material upon HF cleavage of 118.6 mg dry H-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA resin.

EXAMPLE 105

Solid-Phase Synthesis of H-Caeg₁₀-Lys(CZ)-MBHA Resin and Shorter Fragments.

About 5 g of wet Boc-[Taeg]₃-Lys(CZ)-MBHA resin (substitution ~0.3 mmol Lys/g) was placed in a 30 ml SPPS reaction vessel. Boc-[Taeg]₃-C(Z)ae-[Taeg]₃-Lys(CZ)-MBHA resin was assembled by single in situ DCC coupling of the first three residues with 0.1 M of BocC[Z]ae-g-0H together with 0.1 M DCC in 10 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol”) and by single in situ DCC coupling of the remaining seven residues with 0.1 M of BocC[Z]ae-g-0H together with 0.1 M DCC in 10 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol”). All the coupling reactions were allowed to proceed overnight. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of all residues. During the synthesis, portions of the shorter fragments H-[C(Z)ae]₉-Lys(CZ)-MBHA resin, H-[C(Z)ae]₈-Lys(CZ)-MBHA resin, H-[C(Z)ae]₇-Lys(CZ)-MBHA resin, H-[C(Z)ae]₆-Lys(CZ)-MBHA resin, and H-[C(Z)ae]₅-Lys(CZ)-MBHA resin were taken out for HF cleavage.

(b) Cleavage, Purification, and Identification of H-[Taeg]₃-Lys-NH₂.

The protected Boc-[Z]ae₁₀-Lys(CZ)-MBHA resin was treated as described in Example 17c to yield about 10.8
mg of crude material upon HF cleavage of 60.1 mg dry
H-[Zaeg]_{10}-Lys(CIZ)-MBHA resin.
(c) Cleavage, Purification, and Identification of
H-[Cae]_{10}-Lys-NH_{2}.

The protected Boc-[C(Zaeg)]_{10}-Lys(CIZ)-MBHA resin was
prepared as described in Example 17c to yield about 13.4
mg of crude material upon HF cleavage of 56.2 mg dry
H-[C(Zaeg)]_{10}-Lys(CIZ)-MBHA resin.
(d) Cleavage, Purification, and Identification of
H-[Cae]_{10}-Lys-NH_{2}.

The protected Boc-[C(Zaeg)]_{10}-Lys(CIZ)-MBHA resin was
prepared as described in Example 17c to yield about 65.6 mg dry
H-[IC(Zaeg)]_{10}-Lys(CIZ)-MBHA resin.
(e) Cleavage, Purification, and Identification of
H-[Cae]_{10}-Lys-NH_{2}.

The protected Boc-[C(Zaeg)]_{10}-Lys(CIZ)-MBHA resin was
prepared as described in Example 17c to yield about 142.4
mg of crude material upon HF cleavage of 441 mg dry
H-[C(Zaeg)]_{10}-Lys(CIZ)-MBHA resin.

EXAMPLE 106

Solid-Phase Synthesis of H-[Taeg]_{1}-Caeg-[Taeg]_{1}-
Caeg-[Taeg]_{2}-Lys-NH_{2}

(a) Stepwise Assembly of Boc-[Taeg]_{1}-C(Zaeg)-[Taeg]_{1}-
C(Zaeg)-[Taeg]_{2}-Lys(CIZ)-MBHA Resin.

About 0.3 g of wet H-[Taeg]_{1}-C(Zaeg)[Taeg]_{1}-Lys(CIZ)-
MBHA resin from the earlier synthesis of Boc-[Taeg]_{1}-C
(Zaeg)-[Taeg]_{2}-Lys(CIZ)-MBHA resin was placed in a 5 ml
SSPS reaction vessel. After coupling of the next residue five
times, a total incorporation of BocC(Zaeg) of 87% was
obtained. The five repeated couplings were carried out with
0.18 M BocC(Zaeg)-OPp in 2 ml of TFE/CH_{2}Cl_{2} (1:2, v/v),
2 ml of TFE/CH_{2}Cl_{2} (1:2, v/v), 2 ml of TFE/CH_{2}Cl_{2} (1:2,
v/v) with two drops of dioxane and two drops of DIEA (this
condition gave only a few per cent coupling yield), 2 ml of
TFE/CH_{2}Cl_{2} (1:2, v/v) plus 0.5 g phenol, and 1 ml of
CH_{2}Cl_{2} plus 0.4 g of phenol, respectively. The two final Taeg
residues were incorporated close to quantitatively by double
couplings with 0.25 M BocTaeg-OPp in 25% phenol/
CH_{2}Cl_{2}. All couplings were allowed to proceed overnight.
(b) Cleavage, Purification, and Identification of
H-[Taeg]_{1}-Caeg-[Taeg]_{1}-Caeg-[Taeg]_{2}-Lys-NH_{2}.

The protected Boc-[Taeg]_{1}-C(Zaeg)-[Taeg]_{1}-C(Zaeg-
[Caeg]_{2}-Lys(CIZ)-MBHA resin was prepared as described in
Example 17c to yield about 7 mg of crude material upon HF
cleavage of 80.7 mg dry H-[Taeg]_{1}-C(Zaeg)-[Taeg]_{1}-C(Zaeg-
[Caeg]_{2}-Lys(CIZ)-MBHA resin. The crude product was
purified to give 1.2 mg of H-[Taeg]_{1}-Caeg-[Taeg]_{1}-Caeg-
[Caeg]_{2}-Lys-NH_{2} (>99.9% purity).

EXAMPLE 107

Synthesis of a PNA with Two Anti Parallel Strands
Tied Together

Synthesis of H-[Taeg]_{1}-[Taeg]_{1}-[Taeg]_{1}-[Gaeg]_{1}-[Taeg]_{1}-[Taeg]_{1}[-AIA]_{1}[-Gaeg]_{1}-[AIA]_{1}[-Gaeg]_{1}-
[Taeg]_{1}[-Gaeg]_{1}-[Taeg]_{1}-Lys(NH_{2}, (6-AIA)-6-
aminohexanoic acid) (FIG. 26).

The protected PNA was assembled onto a Boc-Lys(CIZ)
modified MBHA resin with a substitution of approximately
0.30 mmol/g. Capping of uncoupled amino groups was only
carried out before the incorporation of the BocGaeg-OH
monomer. Synthesis was initiated on 1.00 g (dry weight) of
preswollen (overnight in DCM) and neutralized Boc-Lys
(CIZ)-MBHA resin. The incorporation of the monomers
followed the protocol of Example 32 and Example 71. The
coupling reaction was monitored by qualitative ninhydrin
reaction (kaiser test). In case of a positive Kaiser test, the
coupling reaction was repeated until the test showed no
coloration of the beads. Final deprotection, cleavage from
support, and purification were performed according to
standard procedures.

EXAMPLE 108

Alternative protecting group strategy for
PNA-synthesis (FIG. 27).

(a) Synthesis of test compounds.

2-amino-6-O-benzyl purine. To a solution of 2.5 g (0.109
mol) of sodium in 100 ml of benzyl alcohol was added 10.75
g (0.063 mol) of 2-amino-6-chloropurine. The mixture was
stirred for 12 h at 120° C. The solution was cooled to room
temperature and neutralized with acetic acid and extracted
with 10 portions of 50 ml of 0.2 N sodium hydroxide. The
collected sodium hydroxide phases were washed with 100
ml of diethyl ether and neutralized with acetic acid, whereby
precipitation starts. The solution was cooled to 0° C. The
yellow precipitate was collected by filtration. Recrystalliza-
tion from ethanol gave 14.2 g 92% of pure white crystals of
the target compound. 1H-NMR (250 MHz, DMSO-d6) d
ppm: 8-H: 7.92; benzylic aromatic, 7.60–7.40; 2NH_{2}, 6.36;
benzyl CH_{2}, 5.57.

(2-amino-6-O-benzyl purinyl)methyl ethanoate. A mixture
of 5 g (0.0207 mol) of 2-amino-6-O-benzyl purine, 30 ml of
DMF and 2.9 g (0.021 mol) of potassium carbonate
was stirred at room temperature. Methyl bromoacetate (3.2 g,
19 ml; 0.0209 mol) was added dropwise. The solution was
filtered after 4 h and the solvent was removed under reduced
pressure (4 mmHg, 40° C.). The residue was recrystallized
two times from ethyl acetate to give 3.7 g (57%) of the target
compound. 1H-NMR (250 MHz, DMSO-d6) d ppm: 8-H;
7.93; benzylic aromatic 7.4–7.6; 2NH_{2}, 6.61; benzylic CH_{2},
5.03; CH_{2}, 5.59; OCH_{3}, 3.78.

(2N-p-Toluenesulfonamido-6-O-benzyl purylin) methyl
ethanoate. To a solution of 0.5 g (1.6 mmol) of (2-amino-
6-O-benzyl purinyl) methyl ethanoate in 25 ml methane
chloride was added 0.53 g (1.62 mmol) of p-toluenesulfonyl
anhydride and 0.22 g (1.62 mmol) of potassium carbonate.
The mixture was stirred at room temperature. The mixture
was filtered and the solvent was removed at reduced pres-
sure (15 mmHg, 40°C). Diethyl ether was added to the oily
residue. The resulting solution was stirred overnight,
whereby the target compound (0.415 mg; 55%) precipitated
and was collected by filtration. 1H-NMR (250 MHz, DMSO-
d6) d ppm: 8-H: 8.97; aromatic 7.2–7.8; benzylic CH_{2}, 5.01;
CH_{2}, 4.24; OCH_{3}, 3.73; CH_{3}, 2.43.

(b) Stability of the tosyl protected base-residue in TFA
and HF.

The material was subjected to the standard deprotection
conditions (TFA-deprotection) and the final cleavage
conditions with HF. The products were then subjected to
HPLC-analysis using a 4 μm C2H 8x10 Nova pack column and
solvent A (0.1% TFA in water) and B (0.1% TFA in
acetonitrile) according to the following time gradient with a
flow of 2 ml/min.
The following retention times were found: (a) Compound 1: 30.77 min; (b) compound 2: 24.22 min; and (c) compound 3: 11.75 min. The analysis showed that the O6-Benzyl group was removed both by TFA and HF, whereas there was no cleavage of the tosyl group in TFA, but quantitative removal in HF under the standard cleavage conditions.

EXAMPLE 109

5-Bromouracil-N1-methyl acetate

5-Bromouracil (5.00 g; 26.2 mmol) and potassium carbonate (7.25 g; 52.3 mmol) were suspended in DMF (75 ml). Methyl bromoacetate (2.48 ml; 26.1 mmol) was added over a period of 5 min. The suspension was stirred for 2 h at room temperature, and then filtered. The solid residue was washed twice with DMF, and the combined filtrates were evaporated to dryness, in vacuo. The residue was an oil containing the title compound, DMF and some unidentified impurities. It is not necessary to purify the title compound before hydrolysis.

1H-NMR (DMSO-d6, 250 MHz): 8.55 (impurity); 8.27 (ClBr=CHN); 8.02 (impurity); 7.46 (impurity); 7.40 (impurity); 4.62 (NCH3-COCH3); 3.78 (COOCH3); 2.76 (DMF); 2.80 (DMF).13C-NMR (DMSO-d6, 250 MHz): 168.8 (COOCH3); 172.5 (C==Br=CON); 161.6 (DMF); 151.9 (NCON); 145.0 (CO—CBr=CHN); 95.6 (COOCH3=CHN); 52.6 (impurity); 52.5 (OCH3); 49.7 (impurity); 48.8 (NCH3COOOMe); 45.0 (impurity); 36.0 (DMF). UV (MeOH; nm): 228; 278; IR (KBr, cm−1): 3158s (NH2); 1743s (C=O, COOMe); 1701s (C=O, CONH); 1438s (OCH3, CH2O); 1223s (O—O, COOOMe); 864 m (OCH3, Br==C==H). FAB-MS m/z (assignment): 265/263 (M+H).

EXAMPLE 110

(5-Bromouracil)acetic acid

Water (30 ml) was added to the oil of the crude product from Example 109 and the mixture was dissolved by adding sodium hydroxide (2M, 60 ml). After stirring at 0°C, for 10 min, hydrochloric acid (4M, 45 ml) was added to pH2 and the title compound precipitated. After 50 min, the solid residue was isolated by filtration, washed once with cold water, and then dried in vacuum over siccant. Yield: 2.46 g (38%). Mp, 250°–251° C. Anal. for C9H5BrN2O4. Found (calc.): C, 52.1 (52.3); H, 2.00 (2.02); Br, 32.18 (32.09); N, 11.29 (11.25).1H-NMR (DMSO-d6, 250 MHz): 12.55 (1H, s, COOH); 11.97 (1H, s, NH); 8.30 (1H, s, C==C—H); 4.49 (2H, s, NCH3COOH).13C-NMR (DMSO-d6, 250 MHz): 169.4 (COOH); 159.8 (NHCOCBr==CH); 150.04 (NCON); 145.8 (COOCBr==CH); 94.6 (COOCBr==CH); 48.8 (NCH3COOCH3); UV (MeOH; nm): 228; 278. IR (KBr, cm−1): 3187s (NH2); 1708s (C==O, COOCH3); 1687s; 1654s (C==O, CONH); 1197s (C==O, COOCH3); 842 m (OCH3, Br==C==C==H). FAB-MS m/z (assignment, relative intensity): 479/477 (M+H, 5); 423/421 (M+2H—Br, 8); 379/377 (M+3H—Br, 103); 233/231 (M—backbone, 20).

EXAMPLE 112

N-(Boc-a-aminoethyl)-N-(5-bromouracil)-N1-methyl-ene-carboxyglycine ester

The product of Example 111 (1.96 g; 4.11 mmol) was dissolved in methanol (30 ml) by heating, and then cooled to 0°C. Sodium hydroxide (2M, 30 ml) was added, and the mixture stirred for 30 min. HCl (1M, 70 ml) was added to
pH=2.0. The water phase was extracted with ethyl acetate (3x65 ml + 7x40 ml). The combined ethyl acetate extracts were washed with saturated NaCl-solution (500 ml). The ethyl acetate phase was dried over magnesium sulphate, filtered and evaporated to dryness in vacuo. Yield: 1.77 g (96%). Mp. 92°-97° C. Anal. for C₉H₁₄Br₂N₂O₄. Found: (calc.) : C, 40.79 (40.10); H, 5.15 (4.71); Br, 14.64 (17.70); N, 11.35 (12.47). H-ν-MNR (DMSO-d₆, 250 MHz, J in Hz): 12.83 (1H, s, COOH); 11.93 & 11.91 (1H, s, C=ONHCH=O); 8.10 & 8.07 (1H, s, C===C==H); 7.00 & 6.81 (1H, t, BocNH); 4.79 & 4.65 (2H, s, NCH₂CON); 4.37 & 4.25 (2H, s, NCH₂COOH); 3.46-3.39 (2H, m's, BocNHCH₂CH₂N); 3.26-3.23 & 3.12-3.09 (2H, m's, BocNHCH₂CH₂N); 1.46 (9H, s, 'Bu). 13C-NMR (DMSO-d₆, 250 MHz): 13.04 (BuOCOOH); 166.9 (COOH); 159.7 (C==C==CON); 155.8 (NCH₂CON); 150.8 (NCH₂COOH); 140.4 (COBr=CH₂); 94.4 (COBr=CH₂); 78.1 (MeC=O); 49.1 & 48.0 (NCH₂COOH); 47.7 & 47.8 (NCH₂CON); 38.6 (BocNHCH₂CH₂N); 38.1 (Boc NHCH₂CH₂N); 28.2 (CH₂COO); UV (Methanol; mnm:); 226; 278; IR (KBr, cm⁻¹): 3316, 1685, 1593, 1542, 1372. The precipitate was washed once with the mother liquor and twice with cold water and dried in vacuo over conc. H₂SO₄. Yield: 6.66 g (82%). Mp. 288°-289° C. Anal. for C₉H₁₄Br₂N₂O₄. Found: (calc.) : C, 42.10 (42.36); H, 3.45 (3.55); N, 16.25 (16.47). H-ν-NMR (DMSO-d₆, 250 MHz, J in Hz): 13.19 (1H, s, COOH); 11.41 (1H, s, NH); 7.69 (1H, d, J₆₇=7.8 Hz); 7.36-7.5 (2H, CH₂); 4.49 (2H, s, NCH₂COOH). 13C-NMR (DMSO-d₆, 250 MHz): 169.9 (COOH); 163.9 (CH==CHCON); 151.1 (NCON); 146.1 (COCH==CHN); 100.9 (COCH==CHN); 48.7 (NCH₂COOH). UV (Methanol; mnm:); 246; 263, IR (KBr, cm⁻¹): 3122 (m, NH); 1703 (s, COOH); 1698 (s, C==O, CONH); 1205 (s, C==O, COOH). 676 (ΔCH, H==C==H). FAB-MS m/z (assignment): 171 (M+H+).

**EXAMPLE 115**

N-((Bocaminoethyl)-N-((uralc-N'-methylcarbonyl)glycine ethyl ester

(Bocaminoethyl)glycine ethyl ester (2.00 g, 8.12 mmol) was dissolved in DMF (10 ml). Dibut-OH (1.46 g, 8.93 mmol) was added and a precipitate was formed. DMF (2x10 ml) was added until all was dissolved. The product of Example 114 (1.52 g; 8.93 mmol) was added slowly to avoid precipitation. Methylene chloride (30 ml) was added, and the mixture was cooled to 0°C, whereafter DDC (2.01 g, 9.74 mmol) was added. The mixture was stirred for 1 h at 0°C, at 2 h at room temperature, and then filtered. The precipitated DUC was washed twice with methylene chloride. To combined filtrate was added methylene chloride (100 ml), and the solution washed with half-saturated NaHCO₃-solution (3x100 ml, H₂O:saturated NaHCO₃-solution 1:1 v/v), then with dilute KH₂SO₄-solution (2x100 ml, H₂O:saturated KH₂SO₄-solution 4:1 v/v) and finally with saturated NaCl-solution (1x100 ml). The organic phase was dried over magnesium sulphate, filtered and evaporated to dryness in vacuo (about 15 mmHg and then about 1 mmHg). The residue was suspended in methylene chloride (32 ml), and stirred for 35 min at room temperature, and 30 min at 0°C, and then filtered. The precipitate (DCU) was washed with methylene chloride. Petroleum ether (2 volumes) was added dropwise to the combined filtrate at 0°C, which caused separation of an oil. The mixture was decanted, the remaining oil was then dissolved in methylene chloride (20 ml), and then again precipitated by addition of petroleum ether (2 volumes). This procedure was repeated 5 times until an impurity was removed. The impurity can be seen by TLC with 10% MeOH/CH₂Cl₂ as the developing solvent. The resulting oil was dissolved in methylene chloride (20 ml) and evaporated to dryness in vacuo, which caused solidification of the title compound. Yield: 1.71 g (53%). Mp. 68.5°-75.7° C. Anal. for C₉H₁₄Br₂N₂O₄. Found: (calc.) : C, 50.61 (51.25); H, 6.48 (6.58); N, 13.33 (14.06). H-ν-NMR (DMSO-d₆, 250 MHz, J in Hz): 11.36 (1H, s, C==ONHCH==O); 7.51 & 7.47 (1H, d, J₆₇=7.8 Hz); 7.37 (1H, s, COOCH=CH₂); 7.00 & 6.80 (1H, t, BocNH); 5.83 & 5.66 (1H, d, J₆₇=7.8 Hz); 4.78 & 4.60 (2H, s, NCH₂CON); 4.37 & 4.12 (2H, s, NCH₂COOEt); 4.30-4.15 (2H, m's, COOCH₂CH₂); 3.49-3.46 (2H, m's, BocNHCH₂CH₂N); 3.27 3.23 & 3.11-3.09 (2H, m's, BocNHCH₂CH₂N); 1.46 (9H, s, 'Bu); 1.39-1.23 (3H, m's, U.S. 7,378,485 B2
N-(Bocaminoethyl)-N-(uracilmethylenecarboxonyl) glycine

The product of Example 115 (1.56 g; 3.91 mmol) was dissolved in methanol (20 ml) and then cooled to 0°C. Sodium hydroxide (2M, 20 ml) was added, and the mixture was stirred for 75 min at 0°C. Hydrochloric acid (1M, 46 ml) was added to pH 2. The water phase was extracted with ethyl acetate (3x50 ml+7x30 ml). The combined ethyl acetate extractions were washed with saturated NaCl solution (360 ml). The ethyl acetate phase was dried over magnesium sulphate, filtered, and evaporated to dryness, in vacuo. The residue was dissolved in methanol and evaporated to dryness, in vacuo. Yield: 0.55 g (38%). Mp 164°-170°C. Anal. for C12H2N2O5. Found (calc.): C, 46.68 (46.65); H, 6.03 (5.99); N, 14.61 (15.13). 1H-NMR (DMSO-d6, 250 MHz, J in Hz): 9.128 (1H, s, COOH); 11.36 (1H, s, C=ONHCH2CH3); 7.52-7.45 (1H, m, s, COOH); 5.17 & 6.82 (1H, t, J=6.5 Hz, BocNH); 5.67-5.62 (1H, m, s, COOH); 4.76 & 4.58 (2H, s, NCH2CON); 4.26 & 4.05 (2H, s, NCH2COOH); 3.46-3.39 (2H, m, m, BoschNCH2CH3); 3.25-3.23 & 3.15-3.09 (2H, m, m, BoschNCH2CH3); 1.96 (9H, s, tBu). 13C-NMR (DMSO-d6, 250 MHz): 167.9 (BuOC=O); 167.2 (COOH); 163.9 (C=CONH); 155.8 (NCH2CON); 151.1 (NCON); 146.4 (COOH); 140.8 (COOH); 100.8 (COOH); 71.1 (MeC3); 49.1 & 47.8 (NCH3, COOH); 47.6 & 46.1 (NCH2CON); 38.6 (BoscNH2CH2); 38.1 & 37.6 (BoscNHCH2CH3); 28.2 (CH3s). UV (BocNH); 226. 264. IR (KBr, cm-1): 3190 (NH); 2685, 1685s, bsroad (C=O, COOH, CONH); 1253s (C=O, COOH); 1171s (C=O, COOH); 682w (C=CH, C=O=CH=H). FAB-MS m/z assignment, relative intensity); 371 (M+H, 25); 271 (M+H-Boc, 100).

EXAMPLE 117
H-U10-LYS-NH2

Synthesis of the title compound was accomplished by using "Synthetic Protocol 10." The synthesis was initiated on approximately 100 mg Lys (CIZ)-MBHA-resin. The crude product (12 mg) was pure enough for hybridization studies. The hybrid between 5'-(dA)10 and H-U10 had Tm of 67.5°C.
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gagatgaaaa

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gagtggaaaa

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<400> SEQUENCE: 29

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16

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16

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<400> SEQUENCE: 37

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<210> SEQ ID NO 38
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<400> SEQUENCE: 39

cacaccaaaa

10

<210> SEQ ID NO 40
What is claimed is:

1. A compound having the formula:

```
\[ \begin{align*}
\text{Q} & \rightarrow \text{H} & \text{G}^1 & \text{C}^1 & \text{C}^2 & \text{C}^3 & \text{C}^4 & \text{D}^1 \\
\text{A}^1 & \rightarrow & \text{A}^2 & \rightarrow & \text{A}^3 & \rightarrow & \text{A}^4 & \\
\end{align*} \]
```

wherein:

- n is at least 2;
- each L<sup>1</sup>–L<sup>n</sup> is independently selected from the group consisting of hydrogen, hydroxy, (C<sub>1</sub>–C<sub>4</sub>) alkanoil, thymine, adenine, cytosine, guanine, and uracil;
- each pair of A<sup>1</sup>–A<sup>n</sup> and B<sup>1</sup>–B<sup>n</sup> is \( >\text{N}-(\text{C}(\text{O}))\text{-CH}_2 \) or \( >\text{N}^*(\text{R}^1)-(\text{C}(\text{O}))\text{-CH}_2 \);
- each R<sup>1</sup> is independently selected from the group consisting of hydrogen, (C<sub>1</sub>–C<sub>4</sub>) alkyl, hydroxy- or alkoxo- or alkylthio-substituted (C<sub>1</sub>–C<sub>4</sub>) alkyl, hydroxy, alkoxy, alkylthio and amino;
- each of \( T^1–T^n \) is CR<sup>5</sup>R<sup>7</sup>, CH<sub>2</sub>CR<sup>5</sup>R<sup>7</sup> or CR<sup>5</sup>R<sup>7</sup>CH<sub>2</sub>, where R<sup>5</sup> and R<sup>7</sup> are independently selected from the group consisting of hydrogen, (C<sub>2</sub>–C<sub>6</sub>) alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C<sub>1</sub>–C<sub>4</sub>) alkoxy, (C<sub>1</sub>–C<sub>4</sub>) alkylthio, NR<sup>3</sup>R<sup>4</sup> and SR<sup>5</sup>, where R<sup>3</sup> and R<sup>4</sup> are as defined above, and R<sup>5</sup> is hydrogen, (C<sub>1</sub>–C<sub>6</sub>) alkyl, hydroxy-, alkoxy-, or alkylthio- substituted (C<sub>1</sub>–C<sub>6</sub>) alkyl, or R<sup>5</sup> and R<sup>7</sup> taken together complete an alicyclic or heterocyclic system;
- each of \( D^1–D^n \) is CR<sup>5</sup>R<sup>7</sup>, CH<sub>2</sub>CR<sup>5</sup>R<sup>7</sup> or CH<sub>2</sub>CHR<sup>5</sup>R<sup>7</sup>, where R<sup>5</sup> and R<sup>7</sup> are as defined above;
- each of \( G^1–G^{n-1} \) is \(-\text{NR}^3\text{CO}–\), \(-\text{NR}^3\text{CS}–\), \(-\text{NR}^3\text{SO}–\) or \(-\text{NR}^3\text{SO}_2–\), in either orientation, where R<sup>3</sup> is as defined above;
- Q is \(-\text{CO}_2\text{H}, -\text{CONR}^3\text{R}^4, -\text{SO}_2\text{H} or -\text{SO}_2\text{NR}^3\text{R}^4\) or an activated derivative of \(-\text{CO}_2\text{H} or -\text{SO}_2\text{H} and l is \(-\text{NHR}^m\text{R}^m or -\text{NR}^m\text{CO} \text{R}^m\), where R<sup>m</sup>, R<sup>n</sup> and R<sup>m</sup> are independently selected from the group consisting of hydrogen, alkyl, amino protecting groups, reporter ligands, chelators, peptides, proteins, carbohydrates, lipids, steroids, and LysNH<sub>2</sub>.
2. A compound having the formula:

wherein:

each \( L \) is independently selected from the group consisting of hydrogen, thymine, adenine, cytosine, guanine, and uracil; each \( R^7 \) is hydrogen;

\( n \) is an integer from 1 to 60,

each \( k \) and \( m \) is, independently, zero or 1;

each \( l \) is 1;

\( R^b \) is OH, NH\(_2\) or -KHLysNH\(_2\); and

\( R^l \) is H or COCH\(_3\).

3. A compound having the formula:

wherein:

each \( L \) is independently selected from the group consisting of the nucleobases thymine, adenine, cytosine, guanine, and uracil;

\( n \) is an integer from 1 to 60;

\( R^b \) is OH, NH\(_2\) or -KHLysNH\(_2\); and

\( R^l \) is H or COCH\(_3\).
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In Column 101, lines 45 – 50, delete the formula in claim 1

and substitute with:

Signed and Sealed this

Eighth Day of June, 2010

David J. Kappos
Director of the United States Patent and Trademark Office