Synthetic Procedures for Peptide Nucleic Acids

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SYNTHETIC PROCEDURES FOR PEPTIDE NUCLEIC ACIDS

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C12Q 1/68; C07H 21/00

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Field of Search 435/5, 6, 810; 436/501; 530/300, 350; 536/23.1, 24.1, 24.3-24.33, 29.3; 935/77, 78

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

9 Claims, 31 Drawing Sheets
OTHER PUBLICATIONS


De Koning et al., “Unconventional Nucleotide Analogues V. Derivatives of 6-(1-pyrimidiny1) and 6-(9-purinyl)-2-aminoacrylic acid”, Recueil 1971, 90, 874–884.


* cited by examiner
Fig. 2(B)
**Fig. 3(a)**

5'-GATCCAAAAAAAAARAGGATC
3'-CTAGGTTTTTTTTTCTTAG

300 nm RADIATION
PHOTOCLEAVAGE

**Fig. 3(b)**

5'-GATCCAAAAAAAAARAGGATC
3'-CTAGGTTTTTTTTTCTTAG

DIAZOACRIDINE
PHOTOFOOTPRINT

**Fig. 3(c)**

5'-GATCCAAAAAAAAARAGGATC
3'-CTAGGTTTTTTTTTCTTAG

KMnO₄ CLEAVAGE

**Fig. 3(d)**

5'-GATCCAAAAAAAAARAGGATC
3'-CTAGGTTTTTTTTTCTTAG

S₁- NUCLEASE
CLEAVAGE

**Fig. 3(d)**

5'-GATCCAAAAAAAAARAGGATC
3'-CTAGGTTTTTTTTTCTTAG

MICROCOCCUS NUCLEASE
ENHANCEMENT
Fig. 4
Fig. 7

1) SOCl₂/DMF (cat.) reflux
2) H₂N(CH₂)₅CO₂H₂⁺, CH₂CL₂, CH₂Cl₂, Ethon, CH₂Cl₂, at 0°C
3) Pd(OH)₂, NaOH, NaOH, at 120°C
4) DMF/EtOH/PipOH/DCC
Fig. 8

ANCHORING

DEPROTECTION

COUPLING

REPETITIVE CYCLE

i CYCLES

FINAL DEPROTECTION & CLEAVAGE

$\text{H}_2\text{NCO-NH-CO-NH}_2 - \text{CO-NH}_2$
Fig. 10
\[ ^{32}\text{P-oligo} \quad 1 \quad 1 \quad 1 \quad 1 \quad 1 \quad 1 \quad 2 \quad 2 \quad 2 \]
\[ \text{oligo 2} \quad - \quad - \quad - \quad + \quad + \quad + \quad - \quad - \quad - \]
\[ \text{AcrT10Lys} \quad 0 \quad + \quad ++ \quad 0 \quad + \quad ++ \quad 0 \quad + \quad ++ \]

FIG. 11A
<table>
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<tr>
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<th>1</th>
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<th>1</th>
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<td>oligo 2</td>
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<td>-</td>
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<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>
<td>++</td>
</tr>
</tbody>
</table>

**FIG. 11B**
FIG. 12A
S₁-nuclease  0.1  1  10  0.1  1  10
AcrT₁₀Lys  - - - + + +

FIG. 12C
Fig. 13
Fig. 15

\[ \text{Reaction Scheme:} \]

1. Reaction with BrCH₂COOH in DMF, K₂CO₃
2. Reaction with NaOAc / BzIOH in DMF, 0-20°C, 16h
3. Subsequent reactions with (C₄H₈N)₃PBr⁺, PF₆⁻, BocAeg-OMe, DiEA
4. Final treatment with NaOH, H₂O/EtOH
Fig. 16
FIG. 20
PNA/DNA  0  0.006  0.02  0.06  0.2  0.6

PNA Target

5'----------GGATCCAAAAAAAAAAAGGATCC----------
3'----------CCTAGGTTTTTTTTTTTCTAGG----------

BamH1          BamH1

FIG. 23
Fig. 27
SYNTHETIC PROCEDURES FOR PEPTIDE NUCLEIC ACIDS

RELATED APPLICATIONS


FIELD OF THE INVENTION

This invention is directed to compounds that are not nucleosides yet which bind to complementary DNA and RNA strands more strongly than 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.

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 less 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 $10^{-3}$ millimoles per kilogram of body weight or $10^{-3}$ to $10^{-4}$ 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 in-vitro or in-vivo reducing 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.

Oligodeoxyribonucleotides offer such opportunities. For example, synthetic oligodeoxyribonucleotides 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 unpractical 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 polyurine 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 Frohlicher, 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 phosphororimidates, bridged phosphorothioates, bridged methylidylenephosphonates, dephospho intermucleotide analogs with siloxane bridges, carbonate bridges, carboxymethyl ester bridges, acetalide bridges, carbonate bridges, thioether, sulfoxyl, sulfone bridges, various "plastic" DNAs, α-anomeric 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:

$$R_1R_2R_3R_4$$

$$B-B-\ldots-$$

where:

(a) $R_1-R_4$ are recognition moieties selected from purine, purine-like, pyrimidine, and pyrimidine like heterocycles effective to bind to 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 moeity 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:

where E is $-\text{CO}$ or $-\text{SO}_2 \text{--}$. 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 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 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 mercapto 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 ($S\equiv S$), sulfoxide ($SO\equiv S\equiv S$), and sulfone ($SO_2\equiv S\equiv S$). 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 or a compound having 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 polyllysine or other basic groups in order to improve binding both to double-stranded and single-stranded DNA, and to polymers 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 dimers—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 $T_m$ values. Consequently, it is generally understood in the art that backbone modifications destabilize such hybrids, i.e., result in lower $T_m$ 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 azo 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 thiouracil) or artificial bases (e.g., bromothymine, azadiones or azaguanines, etc.) attached to a peptide backbone through a suitable linker.

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$_3$-C$_4$) alkynol,
naturally occurring nucleobases, non-naturally occurring nucleobases, aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, and reporter ligands, at least one of \( L' \) or \( L'' \) being a naturally occurring nucleobase, a non-naturally occurring nucleobase, a DNA intercalator, or a nucleobase-binding group;

each of \( A' \)–\( A'' \) is any single bond, a methylene group or a group of formula (IIa) or (IIb):

\[
\text{(IIa)} \quad \begin{array}{c}
\text{II} \\
\text{I}
\end{array}
\]

\[
\text{(IIb)} \quad \begin{array}{c}
\text{I} \\
\text{II}
\end{array}
\]

each of \( D' \)–\( D'' \) is \( CR^6R^7 \), \( CH_2CR^8R^7 \) or \( CHR^6CHR^7 \), where \( R^6 \) and \( R^7 \) are as defined above;

each of \( G^3G^4 \) is \( -NR^3CO- \) or \( -NR^3CS- \),\n
\[
\text{(III)} \quad \begin{array}{c}
\text{L} \\
\text{N}
\end{array}
\]

each of \( R^3 \) is 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 an azide 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):

where:

\( X \) is \( O, S, Se, NR^3, CH_2 \) or \( C(CH_3)_2 \);

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

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, \( (C_1-C_6) \)alkyl which may be hydroxy- or alkoxo- or alkylthio-substituted, hydroxy, alkoxy, alkylthio, amino and halogen; and

each \( R^3 \) is independently selected from the group consisting of hydrogen, \( (C_1-C_6) \)alkyl, hydroxy- or alkoxo- or alkylthio-substituted \( (C_1-C_6) \) alkyl, hydroxy, alkoxy, alkylthio and amino;

each of \( B' \)–\( B'' \) is \( N \) or \( R^6N^+ \), where \( R^6 \) is as defined above;

each of \( T' \)–\( T'' \) is \( CR^6R^7, CHR^6CHR^7 \) or \( CR^8R^7CH_2 \), where \( R^6 \) is hydrogen and \( R^7 \) is selected from the group consisting of the side chains of naturally occurring amino acids, or \( R^6 \) and \( R^7 \) are independently selected from the group consisting of hydrogen, \( (C_2-C_6) \)alkyl, aryl, aralkyl, heteroaryl, hydroxy, \( (C_1-C_6) \)alkoxy, \( (C_1-C_6) \)alkylthio, \( NR^3R^4 \), or \( SR^4 \), where \( R^3 \) and \( R^4 \) are as defined above, and \( R^3 \) is hydrogen, \( (C_1-C_6) \)alkyl, hydroxy-, alkoxy-, or alkylthio-substituted \( (C_1-C_6) \)alkyl, or \( R^4 \) and \( R^7 \) taken together complete an alicyclic or heterocyclic system;

wherein:

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

each \( R^7 \) 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;

\( R^6 \) is \( OH, NH_2 \) or \( -NH\text{lysNH}_2 \); 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), \( k \) and \( m \) 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 compounds 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 specially designed monomer amino acids or their activated derivatives, protected by standard protecting groups. The oligonucleotide analogs also can be synthesized by using the corresponding diacids and diamines.
Thus, the novel monomer synths according to the invention are selected from the group consisting of amino acids, dicarboxylic acids and diamines having general formulae:

\[ \text{(IV)} \]

wherein L, A, B, T and D are as defined above, except that any amino groups therein may be protected by amino protecting groups; E is COOH, CSOH, SOOH, SO$_2$OH or an activated derivative thereof; and G is NR$^3$ or NPGR$^3$, where R$^3$ as defined above and P$_G$ is an amino protecting group.

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

\[ \text{(VII)} \]

or amino-protected and/or acid terminal activated derivatives thereof, wherein L is selected from the group consisting of hydrogen, phenyl, heterocyclic moieties, naturally occurring nucleobases, non-naturally occurring nucleobases, and protected derivatives thereof; and R$^7$ is independently selected from the group consisting of hydrogen and the side chains of naturally occurring alpha amino acids. Especially preferred are such synths having formula (VII) wherein R$^7$ 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 recognition can take place to dsDNA sequences 5–60 base pairs long. Sequences between 10 and 20 bases are of interest since this is the range within which unique DNA sequences of prokaryotes and eukaryotes are found. Reagents which recognize 17–18 bases are 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 triple 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 methods for inhibiting the expression of particular genes in the 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 by reference to the accompanying figures, in which:

**FIGS.** 1A (I) and 1B (II) show a naturally occurring deoxyribonucleotide (A) and a peptide nucleic acid (PNA) of the invention (B).

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

**FIGS.** 3(a)–3(d) provide a schematic illustration of (a) photocleavage by Ac$_r$-[Tag$_g$]$_{10}$-Lys-NH$_2$ (Acr$_{10}$Lys-NH$_2$); (b) photosfootprint by the diazo-linked acridine of Ac$_r$-[Tag$_g$]$_{10}$-Lys-NH$_2$ and preferred Kmo$_r$-cleavage; and (c) S$_r$-nuclease enhanced cleavage and (d) micrococcus nuclease cleavage of Ac$_r$-[Tag$_g$]$_{10}$-Lys-NH$_2$ binding site.

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

**FIG.** 5 shows the Acr$_r$ ligand and a PNA, Ac$_r$-[Tag$_g$]$_{10}$-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$_r$ ligand.

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

**FIG.** 9 shows analytical HPLC chromatograms of: (A) crude H-[Tag$_g$]$_{12}$-Lys-NH$_2$ after HF-cleavage (before lyophilization); (B) crude Ac$_r$-[Tag$_g$]$_{12}$-Lys-NH$_2$ after HF-cleavage (before lyophilization); and (C) purified Ac$_r$-[Tag$_g$]$_{12}$-Lys-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; 0.0350% 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-[Tag$_g$]$_{10}$-Lys-NH$_2$ and (B) purified H-[Tag$_g$]$_{10}$-Cac$_g$-[Tag$_g$]$_{10}$-Lys-NH$_2$ employing the same conditions as in **FIG.** 9.

**FIGS.** 11A and 11B show binding of Acr$_{10}$-Lys to dA$_{10}$S-$^3$P-labeled oligonucleotide (1) ($^5$-GATCCAG$^5$G) was incubated in the absence or presence of Acr$_{10}$-Lys-NH$_2$ and in the absence or presence of oligonucleotide (2) ($^5$-GATCCTG$^5$G) 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, 12B, and 12C show chemical, photochemical and enzymatic probing of dsDNA-Acr$_{10}$-Lys-NH$_2$ complex. Complexes between Acr$_{10}$-Lys-NH$_2$ and a $^3$P-endlabeled DNA fragment containing a dA$_{10}$dT$_{10}$ target sequence were probed by affinity photocleavage (**FIG.** 12A,
lanes 1–3; FIG. 12B, lanes 1–3), photofootprinting (FIG. 12A, lanes 5–6), potassium permanganate probing (FIG. 12B, lanes 4–6) or probing by staphylococcus nuclease (FIG. 12B, lanes 8–10) or by nuclease S1 (FIG. 12C). Either the A-strand (FIG. 12A) or the T-strand (FIGS. 12B, C) was probed.

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

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

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

FIG. 16 provides examples of PNA backbone alterations.

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

FIGS. 18(a) and 18(b) provide procedures for synthesis of anaminopropyl analogue and a propionyl analogue, respectively, of a thymine monomer synth.

FIG. 19 provides a procedure for synthesis of an aminoethyl-β-alanine analogue of thymine monomer synth.

FIG. 20 shows a PAGE autoradiograph demonstrating that PNAS-T10-T2C and -T5C2 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 is inhibited when PNA is bound proximal to the restriction enzyme recognition site.

FIG. 24 shows a PAGE autoradiograph demonstrating that 125I-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.

DETAILED DESCRIPTION OF THE INVENTION

In the oligonucleotide analogs and monomer synths according to the invention, ligand L is primarily a naturally occurring nucleobase attached at the position found in nature, i.e., position 9 for adenine or guanine, and position 1 for thymine or cytosine. Alternatively, L may be a non-naturally occurring nucleobase (nucleobase analog), another base-binding moiety, aromatic moieties, C1-C6 alkylalkyl, hydroxy or even hydrogen. Some typical nucleobase ligands and illustrative synthetic ligands are shown in FIG. 2. Furthermore, L can be a DNA intercalator, a reporter ligand such as, for example, a fluorophor, radio label, spin label, hapten, or a protein-recognizing ligand such as biotin.

In one aspect of the invention naturally occurring and non-naturally occurring nucleobases include purines and pyrimidines. Purines and pyrimidines suitable for use include adenine, guanine, cytosine, uridine, and thymine, as well as other synthetic and natural nucleobases such as xanthine, hypoxanthine, 2-amino adenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halo uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudo uracil), 4-thiouracil, 8-halo, oxo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine. Further purines and pyrimidines include those disclosed in U.S. Pat. No. 5,687,808, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858–859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch, et al., Angewandte Chemie, International Edition 1991, 30, 613.

In monomer synths, L may be blocked with protecting groups. This is illustrated in FIG. 4, where Pr is an acid, a base or a hydrogogenetically or photochemically cleavable protecting group such as, for example, t-butoxycarbonyl (Boc), fluorenylmethyloxycarbonyl (Fmoc) or 2-nitrobenzyl (Nbt).

Linker A can be a wide variety of groups such as —CR₁R₂CO—, —CR₁R₂CS—, —CR₁R₂Se—, —CR₁R₂NHR₃—, —CR₁R₂C==CH₃—, and —CR₁R₂C≡C(CH₃)₂—, wherein R₁ and R₂ are as defined above. Preferably, A is methylene carbonyl (—CH₂CO—). Also, A can be a longer chain moiety such as propanoyl, butanoyl or pentanoyl, or corresponding derivative, wherein O is replaced by another value of X or the chain is substituted with R₃ or is heterogenous, containing Y. Further, A can be a (C₂-C₄)alkylene chain, a (C₃-C₅)alkene chain substituted with R₄R₅ or can be heterogenous, containing Y. In certain cases, A can just be a single bond.

In the preferred form of the invention, B is a nitrogen atom, thereby presenting the possibility of an achiral backbone. B can also be R₃N, wherein R₃ is as defined above.

In the preferred form of the invention, C is —CR₄R₅—, but can also be a two carbon unit, i.e. —CHR₄CHR₅— or —CR₄CH₂—, wherein R₄ and R₅ are as defined above. R₄ and R₅ also can be a heteroaryl group such as, for example, pyrrolyl, furyl, thiienyl, imidazolyl, pyridyl, pyrimidinyl, indolyl, or can be taken together to complete an alicyclic system such as, for example, 1,2-cyclobutanedicarly, 1,2-cyclopentanedicarly or 1,2-cyclohexanedicarly.

In the preferred form of the invention, E in the monomer synth is COOH or an activated derivative thereof, and G in the oligomer is —CONR₃—. As defined above, E may also be CSOH, SOOH, SO₂OH or an activated derivative thereof, whereby G in the oligomer becomes —CSNR₃—, —SONR₃— and —SO₃NR₃—, respectively. The activation may, for example, be achieved using an acid anhydride or an active ester derivative, wherein hydrogen in the groups represented by E is replaced by a leaving group suited for generating the growing backbone.

PNA oligomers of the invention can be synthesized with the G group in either orientation. The G group can be synthesized to have the carboxyl of —(O)NR₂— so oriented towards the N terminus or the C terminus of the oligomer.

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

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

In a further aspect of the invention, the PNA oligomers are conjugated to low molecular effector ligands such as ligands having nucleic activity or alkylating activity or reporter ligands (fluorescent, spin labels, radioactive, protein recognition ligands, for example, biotin or hapten). In a further aspect of the invention, the PNAs 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 PNAs can be attached to water-soluble or water-insoluble polymers. In another aspect of the invention, the PNAs 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, PNAs 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 PNAs 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. Sequence Discrimination at the dsDNA Level (Example 63, FIG. 20)

Using the S1-nuclease probing technique, the discrimination of binding of the T10 GACACCT(CT10) & T10 GACACCT(CT10) PNA to the recognition sequences A9G & A9G, respectively, cloned into the BamHI site of pUC19 was analyzed. The results (FIG. 20) show that the three PNAs bind to their respective recognition sequences with the following relative efficiencies: PNA-T10: A9G & A9G-> A9G, PNA-T10: C5G & A9G-> A9G, PNA-T10: C5G & A9G-> A9G. 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-T10–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).

3. Stability of PNA-dsDNA Complex (Example 67, FIG. 22)

Complexes between PNA-T10 and 32P-dsDNA (A9G/A9G) target were formed (60 min, 37°C). The complexes were then incubated at the desired temperature in the presence of excess oligo-dA9G for 10 min, cooled to RT and probed with K2Mo2O8. 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 da19dT19 tract cloned into the BamHI site in pUC19. Thus, cleavage of pT10 with BamHI and PvuII results in two small DNA fragments of 211 and 111 bp, respectively. In the presence of PNA-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 pT812 plasmid and PNA-T812.

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 125I-PNA-T10 was shown to bind to oligo-dA9G by PAGE and autoradiography (FIG. 24). The binding could be competed by excess denatured calf thymus DNA.

The sequence-specific recognition of dsDNAs is illustrated by the binding of a PNA, consisting of 10thymine substituted 2-aminoethylglycylc units which C-terminates in a lysine amide and N-terminates in a complex 9-aminoacridine ligand (9-Ac®(taeg))7-Lys-NH2, FIG. 11a, 1b) to a da19dT19 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-Ac® ligand (FIG. 5), which is equipped with a 4-nitrobenzamide group to ensure cleavage of DNA upon irradiation, is expected only to cleave DNA in close proximity of the binding site. Upon radiation of the PNA with the above 248 bp DNA fragment, selective cleavage at the da19dT19 sequence is observed (FIG. 3a).
2) In a so-called photofootprinting assay, where a synthetic diazo-linked adenine 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 photolysis 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. 3d).

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 (Taqc)10, (Taq5)10-Lys-NH2 and AcR′(Taqc)7-Lys-NH2 (FIGS. IIa, IIb) 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′(Taqc)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′(Taqc)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 denaturation 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 PNAs 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 G liford Response apparatus. The following extinction coefficients were used: A; 15.4 ml/mmol.cm; T; 8.8; G; 11.7 and C; 7.3 for both normal oligonucleotides and PNA. The melting curves were recorded in steps of 0.5 C.min. The Tm were determined from the maximum of the 1st derivative of the plot of A260 vs temperature.

List of Oligodeoxyribonucleotides

<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 + n</td>
<td>73.0</td>
<td>72.5</td>
<td>73.0</td>
</tr>
<tr>
<td>2 + c</td>
<td>41.5 and 52.0*</td>
<td>86.0</td>
<td>86.0</td>
</tr>
<tr>
<td>2 + e</td>
<td>48.5</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>4 + n</td>
<td>60.0</td>
<td>59.0</td>
<td>61.5</td>
</tr>
<tr>
<td>4 + c</td>
<td>74.5</td>
<td>72.0</td>
<td>72.5</td>
</tr>
<tr>
<td>4 + f</td>
<td>62.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 + n</td>
<td>47.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 + c</td>
<td>57.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 + f</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>7 + c</td>
<td>58.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 + f</td>
<td>43.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 + 12</td>
<td>23.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 + 14</td>
<td>39.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

The hybrid formed between RNA-A (poly rA) and PNA-T10-Lys-NH2 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 Gc 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 $T_m$ value, whether the mispaired base is in the DNA or in the PNA strand. The $T_m$ value is only slightly dependent on ionic strength, as opposed to normal oligonucleotides.

The synthesis of the PNAs 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 nucleotides 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 beaded 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 divinylbenzene-termine. 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-methylbenzhydrolamine and 4-methoxybenzhydrolamine. 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, Barany, 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-[(homoallyl)amino]-lower alkanolic acids such as 4-[(bromomethyl)phenylacetic acid]. Boc-aminomethyl-4-(oxymethyl)aryl-lower alkanolic acids such as Boc-aminomethyl-4-[(oxymethyl)phenylacetic acid, N-Boc-p-propylbenzhydrolamines such as N-Boc-4′-methyl-p-glutaroylbenzhydrolamine, N-Boc-4′-lower alkyl-p-acylbenzhydrolamines such as N-Boc-4′-methyl-p-glutaroylbenzhydrolamine, N-Boc-4′-lower alkoxyp-p-acylbenzhydrolamines such as N-Boc-4′-methoxy-p-glutaroyl-p-benzhydrolamine, and 4-hydroxymethylphenoxyacetic 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, 205) 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-aminodeprotection reagent trifluoroacetic acid (TFA).

Certain functionalities (e.g., benzhydrolamine, 4-methylbenzhydrolamine, and 4-methoxybenzhydrolamine) 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 “prefix-handle” strategy (see, e.g., Mitchell et al., *Synthesis*, 1979, 455–457), 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 spacing-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, 2591), which provide more than one mode of release and thereby allow more flexibility in synthetic design.

by tautomerization of the readily formed oxazolinone (azlactone) intermediates (Goodman, et al., J. Am. Chem. Soc., 1964, 86, 2918) during the coupling of most α-amino acids. In addition to such amino protecting groups, a whole range of otherwise “worthless” nonurethane-type of amino protecting groups are applicable when assembling PNA molecules, especially those built from achiral units. Thus, not only the above-mentioned amino protecting groups (or those derived from any of these groups) are useful within the context of the present invention, but virtually any amino protecting group which largely fulfills the following requirements: (1) stability to mild acids (not significantly attacked by carboxyl groups); (2) stability to mild bases or nucleophiles (not significantly attacked by the amino group in question); (3) resistance to acylation (not significantly attacked by activated amino acids). Additionally: (4) the protecting group must be close to quantitatively removable, without serious side reactions, and (5) the optical integrity, if any, of the incoming amino acid should preferably be highly preserved upon coupling. Finally, the choice of side-chain protecting groups, in general, depends on the choice of the amino protecting group, since the protection of side-chain functionalities must withstand the repeated amino deprotection cycles. This is true whether the overall strategy for chemically assembling PNA molecules relies on, for example, differential acid stability of amino and side-chain protecting groups (such as is the case for the above-mentioned “Boc-benzyl” approach) or employs an orthogonal, that is, chemoselective, protection scheme (such as is the case for the above-mentioned “Fmoc-bu” approach).

Following coupling of the first amino acid, the next stage of solid-phase synthesis is the systematic elaboration of the desired PNA chain. This elaboration involves repeated deprotection/coupling cycles. The temporary protecting group, such as a Boc or Fmoc group, on the last-coupled amino acid is quantitatively removed by a suitable treatment, for example, by acidolysis, such as with trifluoroacetic acid, in the case of Boc, or by base treatment, such as with piperidine, in the case of Fmoc, so as to liberate the N-terminal amino function.

The next desired N-protected amino acid is then coupled to the N-terminus of the last-coupled amino acid. This coupling of the C-terminus of an amino acid with the N-terminus of the last-coupled amino acid can be achieved in several ways. For example, it can be bound by providing the incoming amino acid in a form with the carboxyl group activated by any of several methods, including the initial formation of an active ester derivative such as a 2,4,5-trichlorophenyl ester (Pess, et al., Helv. Chim. Acta, 1963, 46, 1609, a phenylidamido ester (Nefkens, et al., J. Am. Chem. Soc., 1961, 83, 1263), a pentachlorophenyl ester (Kupiszewski, Rocz. Chem., 1961, 35, 395), a pentafluorophenyl ester (Kovacs, et al., J. Am. Chem. Soc., 1963, 85, 183), an α-nitrophenyl ester (Bodansky, Nature, 1955, 175, 685), an imidazole ester (Li, et al., J. Am. Chem. Soc., 1970, 92, 7608), and a 3-hydroxy-4-oxo-3,4-dihydroquinazoline (Dbht-OH) ester (Konig, et al., Chem. Ber., 1973, 103, 2024 and 2034), or the initial formation of an anhydride such as a symmetrical anhydride (Wieland, et al., Angew. Chem. Int. Ed. Engl., 1971, 10, 336). Alternatively, the carboxyl group of the incoming amino acid can be reacted directly with the N-terminal of the last-coupled amino acid with the assistance of a condensation reagent such as, for example, diacyloxyethylcarboximidate (Sheehan, et al., J. Am. Chem. Soc., 1955, 77, 607) or derivatives thereof. Benzotriazolyl N-oxytrisdimethylamino phosphonium hexafluorophosphate (BOP), “Castro’s reagent” (see, e.g., Rivaille, et al., Tetrahedron, 1980, 36, 3413) is recommended when assembling PNA molecules containing secondary amino groups. Finally, activated PNA monomers analogous to the recently reported amino acid fluorides (Carpino, J. Am. Chem. Soc., 1990, 112, 9651) hold considerable promise to be used in PNA synthesis as well.

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 either sequentially or 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.

The above-mentioned “Boc-benzyl” protection scheme, the final deprotection of side-chains and release of the PNA molecule from the solid support is most often carried out by the use of strong acids such as anhydrous HF (Sakakibara, et al., Bull. Chem. Soc. Jpn., 1965, 38, 4921), boron tris (trifluorooacetate) (Pless, et al., Helv. Chim. Acta, 1973, 46, 1609), and sulfonic acids such as trifluoromethanesulfonic acid and methanesulfonic acid (Yajima, et al., J. Chem. Soc. Chem. Commun., 1974, 107). This conventional strong acid (e.g., anhydrous HF) deprotection method, produces very reactive carbones that may lead to alkylation and acylation of sensitive residues in the PNA chain. Such side-reactions are only partly avoided by the presence of scavengers such as anisole, phenol, dimethyl sulfide, and mercaptoethanol and, therefore, the sulfide-assisted acidolytic Sn2-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 alcoholyisis (Barton, et al., J. Am. Chem. Soc., 1973, 95, 4501), and ammonolysis as well as hydrazinolysis (Bodansky, et al., Chem. Ind., 1964 1423), hydrogenolysis (Jones, Tetrahedron Lett. 1977 2853 and Schlatter, et al., Tetrahedron Lett. 1977 2861), and photolysis (Rich and Gurwara, J. Am. Chem. Soc., 1975 97, 1575).

Finally, in contrast with the chemical synthesis of “normal” peptides, stepwise chain building of achiral PNAS such as those based on aminoethyglycine 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 syntheses 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 Appli-
cation WO 90/02749) to facilitate the preparation of large numbers of peptides. This matrix is comprised of a poly-
ethylene (PE) film with pendant long-chain polystyrene (PS) 
grafts (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 com-
partment. 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 concep-
tually 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 
had 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, experiments 
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.

Two other methods proposed for the simultaneous syn-
thesis of large numbers of peptides also apply to the prep-
paration of multiple, different PNA molecules. The first of 
these methods (Geyser, et al., Proc. Natl. Acad. Sci. USA, 
1984, 81, 3998) utilizes acrylic acid-grafted polyethylene-
rods and 96-microliter wells to immobilize the growing 
peptide chains and to perform the compartmentalized syn-
thesis. While highly effective, the method is only applicable 
on a microgram scale. The second method (Houghten, Proc. 
Natl. Acad. Sci. USA, 1985, 82, 5131) utilizes a "tea bag" 
containing traditionally-used polymer beads. Other relevant 
proposals for multiple peptide or PNA synthesis in the 
context of the present invention include the simultaneous 
use of two different supports with different densities (Teague, 
175–178), combining of reaction vessels via a manifold 
(Gorman, Anal. Biochem., 1984, 136, 397), multicolunm 
solid-phase synthesis (e.g. Krichnak, et al., Int. J. Peptide 
Protein Res., 1989, 33, 209), and Holm and Meldal, in 
"Proceedings of the 20th European Peptide Symposium", G. 
Jung and E. Bayer, eds., Walter de Gruyter & Co., Berlin, 
1989 pp. 208–210), and the use of cellulose paper (Eichler, 

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 
copolymer of dimethylacrylamide cross-linked with N-N' 
-bisacryloylhexamethylenediamine, including a known amount of 
N-terbutoxycarbonyl-beta-allyl-N'-acryloylhexamethylen-
enediamine. 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 mono-
mer during polymerization to form resin beads. The poly-
merization is followed by reaction of the beads with ethyl-
ediamine to form resin particles that contain primary 
amines as the covalently linked functionality. The polyan
crylamide-based supports are relatively more hydro-
philic than are the polystyrene-based supports and are usu-
ally used with polar aprotic solvents including 
dimethylformamide, dimethylacetamide, N-methylpyrrolidone and the like (see Atherton, et al., J. 
Amer. Chem. Soc., 1975, 97, 6584, Bioorg. Chem. 1979, 8, 
351), and J. C. S. Perkin II 538 (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-chloromethylphenyl)] 
propylsilane and porous glass beads (see Parr and 
under the trademark "PORASIL E" by Waters Associates, 
Framingham, Mass., USA. Similarly, a mono ester of 1,4-
dihydroxyethylbenzene and silica (sold under the trade-
mark "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 
termed composites in that they contain two major ingre-
dients: a resin and another material that is also substantially 
inert to the organic synthesis reactions 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 exam-
ple composite contains a core of fluorinated ethylene 
polymer onto which has been grafted polystyrene (see Kent 
and Merrifield, Israel J. Chem. 1978, 17, 243) and van 
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 
hydroxypropylacrylate-coated polypropylene 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 syn-
theses may equally well be carried out in the continuous-
flow mode, where the support is packed into columns 
J. Chromatogr. Sci., 1971, 9, 577). With respect to 
continuous-flow solid-phase synthesis, the rigid poly-
(dimethylacrylamide)-Kieselguhr support (Atherton, et al., 
particularly successful, but another valuable configuration 
concerns the one worked out for the standard copoly-
(styrene-1%divinylbenzene) support (Krichnak, et al., 

While the solid-phase technique is presently preferred in 
the context of PNA synthesis, other methodologies or com-
binations 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 produc-
tions (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 Let., 1965, 2323) and 
polymethylene 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”, McGraw-Hill, New York (1970)) yielding mixtures of many molecular weights ("polydisperse") peptide or PNA molecules are particularly relevant for purposes such as screening for antiviral effects; (4) a technique based on the use of polymer-supported amino acid active esters (Fridkin, et al., J. Am. Chem. Soc., 1965, 87, 4646), sometimes referred to as "inverse Merrifield synthesis" or "polymeric reagent synthesis", offers the advantage of isolation and purification of intermediate products, and may thus provide a particularly suitable method for the synthesis of medium-sized, optionally protected, PNA molecules, that can subsequently be used for fragment condensation into larger PNA molecules; (5) it is envisaged that PNA molecules may be assembled enzymatically by enzymes such as proteases or derivatives thereof with novel specificities (obtained, for example, by artificial means such as protein engineering). Also, one can envision the development of "PNA ligases" for the condensation of a number of PNA fragments into very large PNA molecules; (6) since antibodies can be generated to virtually any molecule of interest, the recently developed catalytic antibodies (abzymes), discovered independently by the laboratories of Tramontano (Tramontano, et al., Science, 1986, 234, 1566) and of Schultz (Pollack, et al., Science, 1986, 234, 1570), should also be considered as potential candidates for assembling PNA molecules. Thus, there has been considerable success in producing abzymes 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 Stiewart'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 achieved for PNA synthesis than for "normal" peptide synthesis since PNA molecules will often be comprised of only four different amino acids (one for each of the four native nucleobases) as compared to the twenty natural by occurring (proteinogenic) amino acids constituting peptides. In conclusion, no single strategy may be wholly suitable for the synthesis of a specific PNA molecule, and therefore, sometimes a combination of methods may work best. 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), intracellular adhesion molecules (ICAM), 5-lipoxygenase (5-L0), 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; Kapo- si’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, e.g., 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 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 solvents. 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 organisms (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, therapeutic 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. Scoulen, ed., John Wiley & Sons, New York, 1983), notably solid-phase biosystems, especially bioassays or solid-phase techniques which concern diagnostic detection/quantitation or affinity purification of complementary nucleic acids (see, e.g., "Affinity Chromatography—A Practical Approach", P. D. G. Dean, W. S. Johnson and F. A. Middle, eds., IRL Press Ltd., Oxford 1986; "Nucleic Acid Hybridization—A Practical Approach", B. D. 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 porosi-
ity (Mizutani, et al., J. Chromatogr., 1986, 356, 202), “Sephadex”, “Sepharose”, agarose, polyaCRYLamide, porous particulate alumina, hydroxyalkyl methacrylate gels, diol-
bonded silica, porous ceramics, or contiguOUS materials such as filter discs of nylon and nitrocellulose. One example 
employed the chemical synthesis of oligo-OT on cellulose 
New York and London, 1971). All the abvo-
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 dis-
advantage 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. With the exception of the beads of the above-
discussed nature, the degree of the above- menioned 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 dis-
advantage 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 
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mentioned methods are applicable within the context of 
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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. With the exception of the beads of the above-
discussed nature, the degree of 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 dis-
advantage 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. With the exception of the beads of the above-
discussed nature, the degree of the above-

35 manently 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 
on 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 (Bocaminoethyl)glucine, 
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 adenine 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 
N°-amino group then was protected with the benzyloxyac-

conyl group by the use of the reagent N-ethyl-
benzyloxybenzylidazole tetrafluoroborate (Example 
31). Simple hydrolysis of the product (Examp 32) gave 
N°-carboxylic acid, which was then used in the standard 
procedure (Examples 
33-34, FIG. 13). The adenine 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 benzoxacyclic 
 moiety, and the product (Example 36). The resulting acid was coupled to the (bocaminoethyl) glyoxal alkyl ester (from Example 26) with agent PyP (Example 
37). The O°-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 condensa-
tion 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 32), which is cleaved by periodate 
to give bocaminoacetaldehyde, which is used directly in 
the next reaction. The bocaminoacetaldehyde 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 incorpo-
rated into an 8-mer (Example 60) by the DCC-coupling 
protocol (Examples 56 and 57). 

In another example, the D group is a (CH2)n group. The 
synthesis of the corresponding monomer is outlined in FIG. 
18A and described in Examples 43-44. 

In another example, the A group is a (CH2)nCO group. The 
synthesis of the corresponding thymine monomer is 
outlined FIG. 18.B and Examples 46 through 48. 

In yet another example, the C group is a (CH2)n group. The 
synthesis of the thymine and protected cytosine mono-
mer 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 specific-
ity.
General Remarks: Abbreviations are used in the experimental examples: DMF, N,N-dimethylformamide; DCC, N,N-dicyclohexyl carbodiimide; DCM, CHCl₃; tetrahydrofuran; aeg, N-acetyl (2′-aminomethyl)glycine; pfp, pentafluorophenyl; Boc, tert-butoxycarbonyl; Z, benzzyloxy carbonyl; 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-nitrophenol, methyl bromoacetate, benzzyloxy carbonyl chloride, pentafluorophenol were all obtained through Aldrich Chemical Company. Thymine, cytosine, adenine were all obtained through Sigma.

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

**Example 1**

**t-butyL 4-nitrophenyl Carbonate**

Sodium carbonate (29.14 g; 0.275 mol) and 4-nitrophenol (2.75 g; 0.016 mmol) were mixed with dioxane (250 ml). Boc-anhydride (20.0 g; 0.096 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 1/2, and then poured into wat.2 ml;8.26 mmol) and a suspension of N₂-Benzylxylcarbonyl-cytosine (9, 21.0 g;8.26 mmol) and potassium carbonate (11.4 g;8.26 mmol) in dry DME (900 ml). The mixture was stirred vigorously overnight, filtered, and evaporated to dryness, in vacuo. Water (500 ml) and 4 N hydrochloric acid (10 ml) were added; the mixture was stirred for 15 minutes at 0°C, filtered, and washed with water (2x5 ml). The isolated precipitate was treated with water (120 ml), 2N sodium hydroxide (60 ml), stirred for 30 min, filtered, cooled to 0°C, and 4 N hydrochloric acid (35 ml) was added. The title compound was isolated by filtration, washed thoroughly with water, recrystallized from methanol (1000 ml) and washed thoroughly with ether. This afforded 7.70 g (31%) of pure compound. The mother liquor from the recrystallization was reduced to a volume of 200 ml and cooled to 0°C. This afforded an additional 2.30 g of a material that was pure by tlc but had a reddish color. M.p. 266°-274°C. Anal. for C₁₅H₁₇N₂Os. Found (calcd.): C, 55.41 (55.45); H, 4.23 (4.32); N, 14.04 (13.88). ³H NMR (90 MHz; DMSO-d₆); 8.02 ppm (d,J=7.32 Hz,1H-H-6); 7.39 (8.5H, Ph); 7.01 (d,J=7.32 Hz,1H-H-5); 5.19 (s,2H,PhCH₂—); 4.52. (s,2H).

**Example 9**

N₂-Benzylxylcarbonyl-N₂-carboxymethyl-cytosine

**Pentafluorophenyl Ester (11)**

N₂-Benzylxylcarbonyl-N₂-carboxymethyl-cytosine (10, 4.00 g; 13.2 mmol) and pentafluorophenol (2.67 g; 14.5 mmol) were mixed with DMF (70 ml), cooled to 0°C, with ice-water, and DCC (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 mm Hg). The solid residue was treated with methyl 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 3.40 g (55%). M.p. 241°-245°C. Anal. for C₂₇H₂₁N₂O₂H₃. Found (calcd.); C, 51.56 (51.18); H, 2.72 (2.58); N, 9.24 (9.54). ³H NMR (90 MHz; CDCl₃); 7.66 ppm (d,J=7.63 Hz,1H-H-6); 7.37 (5.5H, Ph); 7.31 (d,J=7.63 Hz,1H-H-5); 5.21 (s,2H,PhCH₂—); 4.97 (s,2H,NCİ₂—). FAB-MS: 470 (M+1)

**Example 10**

N₂-Benzylxylcarbonyl-1-Boc-aeg-cytosine (12)

To a solution of (N-Benzyl-2-aminoethyl)glycine (2) in DME, prepared as described above, was added triethyl amine (7.00 ml; 50.8 mmol) and N₂-Benzylxylcarbonyl-N₂-carboxymethyl-cytosine pentafluorophenyl 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 pentafluorophenol, but no attempt was made to remove it. Yield: 1.72 g (59%). M.p. 156°C (decomp.). ³H NMR (250 MHz, CDCl₃); Due to the limited rotation around the secondary amine (500 ml) and 4 N hydrochloric acid (10 ml) were added; the mixture was stirred for 15 minutes at 0°C, filtered, and washed with water (2x5 ml). The isolated precipitate was treated with water (120 ml), 2N sodium hydroxide (60 ml), stirred for 30 min, filtered, cooled to 0°C, and 4 N hydrochloric acid (35 ml) was added. The title compound was isolated by filtration, washed thoroughly with water, recrystallized from methanol (1000 ml) and washed thoroughly with ether. This afforded 7.70 g (31%) of pure compound. The mother liquor from the recrystallization was reduced to a volume of 200 ml and cooled to 0°C. This afforded an additional 2.30 g of a material that was pure by tlc but had a reddish color. M.p. 266°-274°C. Anal. for C₁₅H₁₇N₂Os. Found (calcd.): C, 55.41 (55.45); H, 4.23 (4.32); N, 14.04 (13.88). ³H NMR (90 MHz; DMSO-d₆); 8.02 ppm (d,J=7.32 Hz,1H-H-6); 7.39 (8.5H, Ph); 7.01 (d,J=7.32 Hz,1H-H-5); 5.19 (s,2H,PhCH₂—); 4.52. (s,2H).

**Example 11**

N₂-Benzylxylcarbonyl-1-Boc-aeg-cytosine

**Pentafluorophenyl Ester (13)**

N₂-Benzylxylcarbonyl-1-Boc-aeg-cytosine (12, 1.50 g; 2.98 mmol) and pentafluorophenol (548 mg; 2.98 mmol) was dissolved in DMF (10 ml) Methylen chloride (10 ml) was added, the reaction mixture was cooled to 0°C. In an ice bath, and DCC (676 mg; 3.28 mmol) was added. The ice bath was removed after 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 precipi-
The solid was isolated by filtration. The solid and the oil were re-dissolved in methylene chloride (80 ml) and added dropwise to cold ether (150 ml). After 20 minutes of stirring, the title compound was isolated by filtration in the form of orange crystals. The product was washed with ether and dried in vacuo over potassium hydroxide. Yield 1.60 g (77%). M.p. 145–147° C.

**EXAMPLE 15**

4-(Carboxyphenyl)amidocarbonyl-9-[6-(4'-nitrobenzamido)hexylamino]-aminoacidine

4-(Methoxycarbonylmethyl)amidocarbonyl-9-[6-(4'-nitrobenzamido)hexylamino]aminoacidine (503 mg; 0.82 mmol) was dissolved in DMF (30 ml), and 2 N sodium hydroxide (30 ml) was added. After stirring for 15 min, 2 N hydrochloric acid (35 ml) and water (50 ml) were added at 0° C. After stirring for 30 min, the solution was decanted, leaving an oily substance which was dissolved in boiling methanol (150 ml), filtered and concentrated to ½ volume. To the methanol solution were added ether (125 ml) and 5–6 drops of HCl in ethanol. The solution was decanted after 1 h of stirring at 0° C. The oily substance was redissolved in methanol (25 ml) and precipitated with ether (150 ml). The title compound was isolated as yellow crystals after stirring overnight. Yield 417 mg (80%). M.p. 173° C. (decomp.)

**EXAMPLE 16**

(a) 4-(5-pentafluorophenylamidocarbonyl)-9-[6-(4'-nitrobenzamido)hexylamino]-aminoacidine (AcCl Oppl)

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

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

Materials: Boc-Lys(C12Z), benzhydroxime-copoly (styrone-1%-divinylbenzene) resin (BHA resin), and p-methylbenzhydroxime-copoly(styrone-1% divinylbenzene) resin (MBHA resin) were purchased from Peninsula Laboratories. Other reagents and solvents were: Biograde trichloroacetic acid from Halocarbon Products; dilsopropylethylamine (99%; was not further distilled) and N-acetylaminomethyl (98%) from Aldrich; H2O was distilled twice; anhydrous HF from Union Carbide; synthesis grade N,N-dimethylformamide and analytical grade methylene chloride (was not further distilled) from Merck; HPLC grade
acetonitrile from Lab-San; purum grade anisole, N,N-
dicyclohexylcarbodiimide, and puriss. grade 2,2,2-
trifluoroethanol from Fluka.

(b) General Methods and Remarks

Except where otherwise stated, the following applies. The PNA compounds were synthesized by the stepwise solid-phase approach (Merrifield, J. Am. Chem. Soc., 1963, 85, 2149) employing conventional peptide chemistry utilizing the TFA-labile tert-butyloxycarbonyl (Boc) group for "temporary" N-protection (Merrifield, J. Am. Chem. Soc., 1964, 86, 304) and the more acid-stable benzoyloxycarbonyl (Z) and 2-chlorobenzoyloxycarbonyl (Clz) groups for "permanent" side chain protection. To obtain C-terminal amides, the PNAs were assembled onto the H-frame 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, 2, 45). All reactions (except HF reactions) were carried out in manually operated standard solid-phase reaction vessels fitted with a coarse glass frit (Merrifield, et al., Biochemistry, 1982, 21, 5020).

The quantitative ninhydrin reaction (Kaiser test), originally developed by Merrifield and co-workers (Sarin, et al., Anal. Biochem., 1981, 117, 147) for peptides containing "normal" amino acids, was successfully applied (see Table I–III) using the "normally" employed effective extinction coefficient ε=15000 M⁻¹cm⁻¹ for all residues to determine the completeness of the individual couplings as well as to measure the number of growing peptide chains. The theoretical substitution S_n₋₁ upon coupling of residue number n (assuming both complete deprotection and coupling as well as neither chain termination nor loss of PNA chains during the synthetic cycle) is calculated from the equation:

\[ S_n = S_n × (1 + \frac{ΔMW}{M}) \times 10^{-2} \text{ mmol/mol} \]

where ΔMW is the gain in molecular weight ([ΔMW]g/mol) and S_n₋₁ is the theoretical substitution upon coupling of the preceding residue n–1 ([S]mmol/g). The estimated value (%) on the extent of an individual coupling is calculated relative to the measured substitution (unless S was not determined) and include correction for the number of remaining free amino groups following the previous cycle. HF reactions were carried out in a Difflon HF apparatus from Toho Kasei (Osaka, Japan). Vydac C18 (5 μm, 0.46×25 cm and 5 μm, 1.0×25 cm) reverse-phase columns, respectively, were used for analytical and semi-preparative HPLC on an SP8000 instrument. Buffer A was 5 vol % acetonitrile in water containing 45 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 (analytical) and 5 ml/min (semi-preparative). The eluents were monitored at 215 nm (analytical) and 230 nm (semi-preparative). Molecular weights of the PNAs were determined by ²⁵²Ct plasma desorption time-of-flight mass spectrometry from the mean of the most abundant isotopes.

EXAMPLE 17

Solid-Phase Synthesis of Acr⁻¹[Tag₄]⁺⁺-NH₂ and Shorter Derivatives

(a) Stepwise Assembly of Boc-[Tag₄]⁺⁺-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 Boc-Targ-OPfp in about 33% DMF/CH₂Cl₂. The individual coupling reactions were carried out by shaking 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 I). Portions of protected Boc-[Tag₄]⁺⁺-BHA, Boc-

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(b) Synthesis of Acr⁻¹[Tag₄]⁺⁺-BHA Resin

Following deprotection of the residual Boc-[Tag₄]⁺⁺-BHA resin (estimated dry weight is about 30 mg; 0.002 mmol growing chains), the H⁻¹[Tag₄]⁺⁺-BHA resin was reacted with about 50 equivalents (80 mg; 0.11 mmol) of Acr⁻¹-OPfp in 1 ml of about 66% DMF/CH₂Cl₂ (i.e., a 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⁻¹[Tag₄]⁺⁺-NH₂

A portion of protected Boc-[Tag₄]⁺⁺-BHA resin was treated with 50% trifluoroacetic acid in methane chloride to remove the N-terminal Boc group (which is a precursor of the potentially harmful tert-butylation cation) prior to the HF cleavage. Following neutralization and washing (performed in a way similar to those of steps 2–4 in "Synthetic Protocol 1"), and drying for 2 h in vacuum, the resulting 67.1 mg (dry weight) of H⁻¹[Tag₄]⁺⁺-BHA resin was cleaved with 5 ml of HF:anisole (9:1, v/v) stirring at 0°C. for 60 min. After removal of HF, the residue was stirred with dry diethyl ether (4×15 ml, 15 min each) to remove anisole, filtered under gravity through a flint glass funnel, and dried. The PNA was then extracted into a 60 ml (4×15 ml, stirring 15 min each) 10% aqueous acetic acid solution. Aliquots of this solution were analyzed by analytical reverse-phase HPLC to establish the purity of the crude PNA. The main peak at 13.0 min accounted for about 95% of the total absorbance. The remaining solution was frozen and lyophilized to afford about 22.9 mg of crude material. Finally, 19.0 mg of the crude product was purified by five batches, each containing 3.8 mg in 1 ml of H₂O. The main peak was collected by use of a semi-preparative reverse-phase column. Acetonitrile was removed on a speed vac and the residual solution was frozen (dry ice) and subsequently lyophilized to give 13.1
mg of >99% pure H-[Teag]$_3$-NH$_2$. 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-[Teag]$_3$-NH$_2$

A portion of protected Boc-[Teag]$_{13}$-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-[Teag]$_{13}$-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-[Teag]$_{13}$-NH$_2$. 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-[Teag]$_{13}$-NH$_2$, 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-[Teag]$_{13}$-NH$_2$ 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-[Teag]$_{13}$-NH$_2$

A portion of protected Boc-[Teag]$_{13}$-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-[Teag]$_{13}$-BHA resin. The main peak at 22.6 min was located in a broad bulk eluting for about 35% of the total absorbance (FIG. 12a). Again (see the preceding section), this bulge is ascribed to aggregational/conformational states of the target molecule H-[Teag]$_{13}$-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$_2$-[Teag]$_{13}$-NH$_2$

A portion of protected Acr$_2$-[Teag]$_{13}$-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$_2$-[Teag]$_{13}$-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 1 mg of presumably >99% pure Acr$_2$-[Teag]$_{13}$-NH$_2$, “contaminated” with self-aggregated molecules eluting at 27.4 min, 29.2 min, and finally as a huge broad bulge eluting with 100% buffer B (FIG. 12c). This interpretation is in agreement with the observation that those peaks grow upon standing (for hours) in aqueous acetic acid solution, and finally precipitate 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$_2$Cl$_2$ (1:1, v/v), 3 ml, 3x1 min and 1x30 min; (2) washing with CH$_2$Cl$_2$, 3 ml, 6x1 min; (3) neutralization with DIEA/CH$_2$Cl$_2$ (1:19, v/v), 3 ml, 3x2 min; (4) washing with CH$_2$Cl$_2$, 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) BocTeag-OHpf dissolved in 1 ml CH$_2$Cl$_2$, followed by addition of 0.5 ml DMF (final concentration of pentfluorophenylester–0.12 M); the coupling reaction was allowed to proceed for a total of 12–24 h shaking at room temperature; (7) washing with DMF, 3 ml, 1x2 min; (8) washing with CH$_2$Cl$_2$, 3 ml, 4x1 min; (9) neutralization with DIEA/CH$_2$Cl$_2$ (1:19, v/v), 3 ml, 2x2 min; (10) washing with CH$_2$Cl$_2$, 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-acetylilmidazol in methylene chloride).

EXAMPLE 18

Solid-Phase Synthesis of Acr$_2$-[Teag]$_{13}$-Lys-NH$_2$ and Shorter Derivatives

(a) Stepwise Assembly of Boc-[Teag]$_{13}$-Lys(CIZ)-BHA Resin

The synthesis was initiated by a quantitative loading (standard DCC in situ coupling in neat CH$_2$Cl$_2$ of Boc-Lys (CIZ) onto 100 mg of presswollen and neutralized BHA resin (0.57 mmol NH$_2$-). Further extension of the protected resin chain employed single couplings (“Synthetic Protocol 2”) for cycles 1 to 5 and cycles 10 to 15 using 3.2 equivalents of BocTeag-OHpf in about 33% DMF/CH$_2$Cl$_2$. Cycles 5 to 10 employed an extra straight DCC (i.e., in situ) coupling of the free acid BocTeag-OH in about 33% DMF/CH$_2$Cl$_2$. 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-[Teag]$_{13}$-Lys(CIZ)-BHA and Boc-[Teag]$_{13}$-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-[Teag]$_{13}$-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 throughput single-coupled, residues in Example 17.

(b) Synthesis of Acr$_2$-[Teag]$_{13}$-Lys(CIZ)-BHA Resin

Following depletion of a portion of Boc-[Teag]$_{13}$-Lys (CIZ)-BHA resin (estimated dry weight is about 90 mg; ~0.01 mmol growing chains), the H-[Teag]$_{13}$-BHA resin was reacted with about 20 equivalents (141 mg; 0.19 mmol) of Acr$_2$-OHpf in 1 ml of about 66% DMF/CH$_2$Cl$_2$ in a 3 ml solid-phase reaction vessel. As judged by a qualitative ninhydrin reaction, coupling of the acridine moieties was close to quantitative.

(c) Synthesis of Acr$_2$-[Teag]$_{13}$-Lys(CIZ)-BHA Resin

Following depletion of the residual Boc-[Teag]$_{13}$-Lys (CIZ)-BHA resin (estimated dry weight about 70 mg; ~0.005 mmol growing chains), the H-[Teag]$_{13}$-Lys(CIZ)-BHA resin was reacted with about 25 equivalents (91 mg; 0.12 mmol) of Acr$_2$-OHpf in 1 ml of about 66% DMF/CH$_2$Cl$_2$ in a 3 ml solid-phase reaction vessel. As judged by a qualitative ninhydrin reaction, coupling of the acridine moieties was close to quantitative.

(d) Cleavage, Purification, and Identification of H-[Teag]$_{13}$-Lys-NH$_2$

A portion of protected Boc-[Teag]$_{13}$-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-[Teag]$_{13}$-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-[Tæg]_10-Lys-NH₂.

(c) Cleavage, Purification, and Identification of H-[Tæg]_10-Lys-NH₂.

A portion of protected Boc-[Tæg]_10-Lys(CIZ)-BHA resin was treated as described in Example 17c to yield 7.0 mg of crude material upon HCl cleavage. The main peak at 17.0 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-[Tæg]_10-Lys-NH₂. FIG. 13r. For (M+H)^+ the calculated m/z value was 2807.8 and the measured m/z value was 2808.2.

(i) Cleavage, Purification, and Identification of Acr-[Tæg]_10-Lys-NH₂.

99.1 mg protected Acr-[Tæg]_10-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 25.3 min if injected from an aqueous solution instead of the 10% aqueous acetic acid solution) accounted for about 45% of the total absorbance. An 8.78 mg portion of the crude product was purified to give approximately 5.3 mg of >97% pure H-[Tæg]_10-Lys-NH₂. For (M+H)^+ the calculated m/z value was 2850.8 and the measured m/z value was 2849.8.

(g) Cleavage and Purification of Acr-[Tæg]_10-Lys-NH₂.

A 78.7 mg portion of protected Acr-[Tæg]_10-Lys(CIZ)-BHA resin was cleaved as described in Example 1 section (g) to yield 34.8 mg of crude material. The main peak at 28.2 min (about the same elution time if injected from an aqueous solution instead of the 10% aqueous acetic acid solution) and a “dimer” at 28.2 min accounted for about 35% of the total absorbance. About 4.5 mg of the crude product was purified to give approximately 1.6 mg of presumably >95% pure H-[Tæg]_10-Lys-NH₂. This compound could not be freed from the “dimer” peak, which grew upon standing in aqueous acetic acid solution.

(h) Synthetic Protocol 2.

(1) Boc-deprotection with TFA/CH₂Cl₂ (1:1, v/v), 3 ml, 3x(1 min and 1×30 min); (2) washing with DIEA/CH₂Cl₂, 3 ml, 6x1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 3 ml, 3x2 min; (4) washing with CH₂Cl₂, 3 ml, 6x1 min, and drain for 1 min; (5) 2–5 mg sample of PNA-resin can be taken out and dried thoroughly for a qualitative ninhydrin reaction; (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) Boc-OPFP dissolved in 1 ml CH₂Cl₂ followed by addition of 0.5 ml DMF (final concentration of pentahydroxypropaneester ~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 Boc-TEG-CH₂Cl in 1.5 ml DMF/CH₂Cl₂ (1:2, v/v); (7) washing with DMF, 3 ml, 1×2 min; (8) washing with CH₂Cl₂, 3 ml, 4x1 min; (9) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 3 ml, 2x2 min; (10) washing with CH₂Cl₂, 3 ml, 6x1 min; (11) 2–5 mg sample of protected PNA-resin is taken out for a qualitative ninhydrin test (after cycles 7, 10, and 15 unreacted amino groups were blocked by acetylation with N-acetylaminodiazin-methylene chloride).

EXAMPLE 19

Improved Solid-Phase Synthesis of H-[Tæg]_10-Lys-NH₂

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

(a) Preparation of Boc-Lys(CIZ)-NH—CH₂—CH₂—

CH₃—C₆H₅—C₆H₅ Resin (MBHA 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 presswollen MBHA resin (determined by the quantitative ninhydrin reaction to contain 0.64 mmol N₃/g) using a single “in situ” coupling (1.5 mmol of DCC) in 50 ml of CH₂Cl₂. The reaction was carried out by shaking for 3 h in a manually operated, 225 ml standard, solid-phase reaction vessel. Unreacted amino groups were then blocked by acetylation with a mixture of acetic anhydride/pyridine/CH₂Cl₂ (1:1:2, v/v/v) for 18 h. A quantitative ninhydrin reaction on the neutralized resin showed that only 0.00093 mmol/g free amine remained (see Table I), i.e. 0.156 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-[Tæg]_10-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-[Tæg]_10-Lys(CIZ)-MBHA resin by single couplings (“Synthetic Protocol 3”) utilizing 2.5 equivalents of Boc-OPFP in neat CH₂Cl₂. The quantitative ninhydrin reaction was applied throughout the synthesis (see Table II).

(c) Stepwise Assembly of Boc-[Tæg]_10-Lys(CIZ)-MBHA Resin

About 4.5 g of wet Boc-[Tæg]_10-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-[Tæg]_10-Lys(CIZ)-MBHA resin was assembled by single couplings (“Synthetic Protocol 4”) utilizing 2.5 equivalents of Boc-OPFP in about 30% DMF/CH₂Cl₂. The progress of the synthesis was monitored at all stages by the quantitative ninhydrin reaction (see Table II).

(d) Stepwise Assembly of Boc-[Tæg]_10-Lys(CIZ)-MBHA Resin

About 1 g of wet Boc-[Tæg]_10-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-[Tæg]_10-Lys(CIZ)-MBHA resin was assembled by the single-coupling protocol employed in the preceding section utilizing 2.5 equivalents of Boc-TEG-OPFP in about 30% DMF/CH₂Cl₂. The reaction volume was 3 ml (vigorous shaking). The synthesis was monitored by the quantitative ninhydrin reaction (see Table II).

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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 M mixture of acetic anhydride/pyridine/CH_{3}Cl_{2} (1:1.2:2, v/v/v) for 2 h in a 3 ml solid-phase reaction vessel.

(i) 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 from two batches by use of a semi-preparative reserve-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.

(b) Synthetic Protocol 3

(1) Boc-deprotection with TFA/CH\_3Cl\_2 (1:1, v/v), 100 ml, 3x1 min and 1x30 min; (2) washing with CH\_3Cl\_2, 100 ml, 6x1 min; (3) neutralization with DIEA/CH\_3Cl\_2 (1:19, v/v), 100 ml, 3x2 min; (4) washing with CH\_3Cl\_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. (3.75 mmol; 2.064 g) Boc-Taeg-OPfp dissolved in 35 ml CH\_3Cl\_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 Boc-Taeg-OH); (8) washing with CH\_3Cl\_2, 100 ml, 4x1 min; (9) neutralization with DIEA/CH\_3Cl\_2 (1:19, v/v), 100 ml, 2x2 min; (10) washing with CH\_3Cl\_2, 100 ml, 6x1 min; (11) 2-5 mg sample of protected PNA-resin is taken but 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\_3Cl\_2 (1:1.2, v/v/v) for 2 h; (13) washing with CH\_3Cl\_2, 100 ml, 6x1 min; (14) 2x2-5 mg samples of protected PNA-resin are taken out, neutralized with DIEA/CH\_3Cl\_2 (1:19, v/v) and washed with CH\_3Cl\_2 for qualitative and quantitative ninhydrin analyses.

(i) Synthetic Protocol 4

(1) Boc-deprotection with TFA/CH\_3Cl\_2 (1:1, v/v), 25 ml, 3x1 min and 1x30 min; (2) washing with CH\_3Cl\_2, 25 ml, 6x1 min; (3) neutralization with DIEA/CH\_3Cl\_2 (1:19, v/v), 25 ml, 3x2 min; (4) washing with CH\_3Cl\_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) Boc-Taeg-OPfp dissolved in 6 ml CH\_3Cl\_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\_3Cl\_2, 25 ml, 4x1 min; (9) neutralization with DIEA/CH\_3Cl\_2 (1:19, v/v), 25 ml, 2x2 min; (10) washing with CH\_3Cl\_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\_3Cl\_2 (1:1.2, v/v/v) for 2 h (except after the first cycle); (13) washing with CH\_3Cl\_2, 25 ml, 6x1 min; (14) 2x2-5 mg samples of protected PNA-resin are taken out, neutralized with DIEA/CH\_3Cl\_2 (1:19, v/v) and washed with CH\_3Cl\_2 for qualitative and quantitative ninhydrin analyses.

**EXAMPLE 20**

**Solid-Phase Synthesis of H-[Taeg]_{10}-Caeg-[Taeg]_{10}-Lys-NH\_2**

(a) Stepwise Assembly of Boc-[Taeg]_{10}-[CIZ]-Caeg-[Taeg]_{10}-Lys(CIZ)-MBHA Resin

About 2.5 g of wet Boc-[Taeg]_{10}-Lys(CIZ)-MBHA resin (~1/6 of the total remaining about 16 g wet resin; ~0.75 g dry resin ~0.15 mmol growing chains) was placed in a 6 ml SPPS reaction vessel. Boc-Taeg-[CIZ]-Caeg-[Taeg]_{10}-Lys(CIZ)-MBHA resin was assembled by double coupling of all Taeg-residues utilizing the usual 2.5 equivalents of Boc-Taeg-OPfp in 2.5 ml about 30% DMF/CH\_3Cl\_2 except that the first residue was single-coupled. Incorporation of the CIZ-raescue-residue was accomplished by coupling with 2.0 equivalents of BocC(Z)aeq-OPfp in TFE/CH\_3Cl\_2 (1:2, v/v). The progress of the synthesis was monitored at all stages by the quantitative ninhydrin reaction (see Table III).
(b) Cleavage, Purification, and Identification of H-[Taqg]-Caeg-[Taqg]-Lys-NH₂

A portion of protected Boc-[Taqg]-Caeg-[Taqg]-Lys (CIZ)-BHA resin was treated as described in Example I section (c) to yield about 14.4 mg of crude material upon HF cleavage of 66.9 mg dry H-[Taqg]-Caeg-[Taqg]-Lys(CIZ)-BHA resin. The main peak at 14.5 min accounted for more than 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-[Taqg]-Caeg-[Taqg]-Lys-NH₂ (FIG. 13b). For (M+H)+ the calculated m/z value = 2279.38 and the measured m/z value = 2279.6.

EXAMPLE 21

Binding of Acr⁺(Taqg)₁₀-Lys-NH₂ to dA₁₀ (FIG. IIa)

Acr⁺(Taqg)₁₀-Lys (100 ng) was incubated for 15 min at room temperature with 50 cpsi 5⁻³²P-end-labelled oligonucleotide [d(GATCCAG₉)] in 20 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The sample was cooled in ice (15 min) and analyzed by gel electrophoresis in polyacrylamide (PAGE). To 10 µl of the sample was added 2 µl of 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 4°C. A 10 µl portion of the sample was lysophilized and redissolved in 10 µl 80% formamide, 1 TBE, heated to 90°C (5 min), and analyzed by urca/PAGE (15% acrylamide, 0.5% bisacrylamide, 7 M urea) in TBE. [³²P]-containing DNA bands were visualized by autoradiography using intensifying screens and Agfa Curix 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; a laboratory manual, Cold Spring Harbor Laboratories).

EXAMPLE 22

Formation of Strand Displacement Complex

A dA₁₀(dG₉) target sequence contained within a plasmid DNA sequence was constructed by cloning of two oligonucleotides (d(GATCCAG₉)d(GATCCGG₉)) into the BamHI restriction enzyme site of pUC19 using the Escherichia coli JM101 strain by standard techniques (Maniatis et al., 1986). The desired plasmid (designated p110) was isolated from one of the resulting clones and purified by the alkaline extraction procedure and CsCl centrifugation (Maniatis et al., 1986). A 3⁻³²P-end-labelled DNA fragment of 248 bp containing the dA₁₀(dG₉) target sequence was obtained by cleaving the pT10 DNA with restriction enzymes EcoRI and PvuII, labelling of the cleaved DNA with [³²P]-dATP (4000 Ci/mmol, Amersham) using the Klenow fragment of E. coli DNA polymerase (Boehringer Mannheim), and purifying the 248 bp DNA fragment by PAGE (5% acrylamide, 0.06% 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]-dATP and polydeoxyribonucleotide kinase. Following treatment with PvuII, the 248 bp DNA fragment was purified as above.

The complex between Acr⁺(Taqg)₁₀-Lys-NH₂ and the 248 bp DNA fragment was formed by incubating 50 ng of Acr⁺(Taqg)₁₀-Lys-NH₂ with 500 cpsi 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, 0.36 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 fluorescent 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 30 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) (DHA, Nielsen et al., 1988) Nucl. Acids
Res. 16, 3877–88) was added. The sample was irradiated at 365 nm (Philips TL 20 W/09N, 22 Jm⁻²s⁻¹) for 30 min and treated as described for “affinity photocleavage”.

c) S₂-nucleoside (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 nucleoside S₂ (Boehringer Mannheim) at 0.5 U/ml for 5 min at 20°C. The reaction was stopped and treated further as described under “Staphylococcus nucleoside”.

EXAMPLE 24

N-Benzoxycarbonyl-N-(bocaminomethyl)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 2 N HCl. The mixture was washed with chloroform (4x100 ml) and the pH of the aqueous phase was readjusted to 9.5 with 2 N NaOH at 0°C. Benzyloxycarbonyl 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 C₂₁H₂₃N₂O₂. Found: C 58.4, 58.9; H, 7.02 (6.86); N, 7.94 (7.95). **H-NMR (250 MHz, CDCL₃) 7.33 & 7.32 (SH, Ph), 15.5 & 15.2 (2H, PhCH₃); 4.03 & 4.01 (2H, NCH₂CO₂H); 3.46 (b, 2H, BocNHC₁H₂); 3.28 (b, 2H, BocNHCH₂CH₂); 1.43 & 1.40 (9H, Bu).** HPLC (260 nm) 20.71 min. (80.2%) and 21.57 min. (19.8%). The UV-spectra (200 nm–300 nm) are identical, indicating that the minor peak consists of Bis-Z-AEG.

EXAMPLE 25

N-Benzoxycarbonyl glycine ethyl ester

N-Benzoxycarbonyl-N-(bocaminomethyl)glycine (60.0 g; 0.170 mol) and N,N-dimethyl-4-aminopyridine (6.00 g) were dissolved in absolute ethanol (500 ml) and cooled to 0°C before the addition of DCC (42.2 g; 0.204 mol). The ice bath was removed after 5 minutes and stirring was continued for 2 more hours. The precipitated DCC (32.5 g 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 65.1 g of an oily substance which contained some DCC.

The oil was dissolved in absolute ethanol (600 ml) and 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. **H-NMR (250 MHz, CD₂OD); 4.77 (b, s, NH); 4.18 (q, 2H, MeCH₂); 3.38 (s, 2H, NCH₂CO₂Et); 3.16 (t, 2H, BocNHCH₂CH₂); 2.68 (t, 2H, BocNHCH₂CH₂); 1.43 (s, 9H, Bu) and 1.26 (t, 3H, CH₃).** 13C-NMR 171.4 (COE); 156.6 (CO); 78.3 (CH₂); 59.9 (CH); 49.0 (CH₂); 48.1 (CH₂); 39.0 (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), Dibutylamine (9.84 g; 60.3 mmol) and 1-carboxymethyl thymine (11.1 g; 60.3 mmol) were dissolved in DME (210 ml). Methylene chloride (210 ml) then was added. The solution was cooled to 0°C in an ethanol/ice bath and DCC (13.6 g; 65.8 mmol) was added. The ice bath was removed after 1 hour and stirring was continued for another 2 hours at ambient temperature. The precipitated DCC was removed by filtration and washed twice with methylene chloride (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 methylene chloride (150 ml), filtered, and the title compound was precipitated by the addition of petroleum ether (350 ml) at 0°C. The methylene chloride/petroleum ether procedure was repeated once. This afforded 16.0 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 DCC. 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 (9x200 ml), the combined extracts were dried over magnesia...
sium 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 C=98% R=+4.8 min. Anal. for \( \text{C}_8\text{H}_4\text{N}_2\text{O}_6\); C 52.5 H 6.5 4.11 (14.11.41). Due to the limited rotation around the secondary amide, several of the signals were doublet in the ratio 2:1 (indicated in the list by mj. for major and mmr for minor). -H NMR (250 MHz, DMSO-d6): 12.75 (b.s., 1H, CO2H); 11.28 (s, “1H”, mj. imide NH); 11.26 (s, “1H”, mj. imide NH); 7.30 (s, “1H”, mj. H-6); 7.26 (s, “1H”, mj. H-6); 6.73 (s, “1H”, mj. BOCNH); 4.64 (s, “2H”, mj. CH2CON); 4.46 (s, “2H”, mj. CH2CON); 3.97 (s, “2H”, mj. CH2CO2H); 3.63-3.01 (unresolved m, includes water, CH3CH2); 1.75 (s, 3H, CH3) and 1.38 (s, 9H, Bu).

EXAMPLE 29

N4-Benzoylcarbonyl-1-(Boc-aeg)cytosine

N4-Boc-aminomethyl glycine ethyl ester (5.00 g; 20.3 mmol), DibutOH (3.64 g; 22.3 mmol) and N4-benzyloxycarbonyl-1-carboxyethyl cytosine (6.77 g; 22.3 mmol) were suspended in DMF (100 ml). Methylen chloride (100 ml) then was added. The solution was cooled to 0°C and DCC (5.03 g; 24.4 mmol) was added. The ice bath was removed after 2 h and stirring was continued for another hour at ambient temperature. The reaction mixture then was evaporated to dryness, in vacuo. The residue was suspended in ether (100 ml) and stirred vigorously for 30 min. The solid material was isolated by filtration and the ether wash was repeated twice. The material was then stirred vigorously for 15 min with dilute sodium hydroxide (approx. 4% solution, 100 ml), filtered and washed with water. This procedure was then repeated once, which after drying left 17.4 g of yellowish solid material. The solid was then boiled with dioxane (200 ml) and filtered while hot. After cooling, water (200 ml) was added. The precipitated material was isolated by filtration, washed with water, and dried. According to HPLC (observing at 260 nm) this material has a purity higher than 99%, besides the DCU. The ester was then suspended in THF (100 ml), cooled to 0°C, and 1 N LJOH (61 ml) was added. After stirring for 15 minutes, the mixture was filtered and the filtrate was washed with methylene chloride (2x150 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% R=19.5 min, and a minor impurity at 12.6 min (approx. 1%) most likely the Z-de protected monomer. Anal. for \( \text{C}_{16}\text{H}_{14}\text{N}_{10}\text{O}_6 \) found (calc.) C 54.16 (54.87); H 5.76 (5.81) and N 13.35 (13.91). -H NMR (250 MHz, DMSO-d6): 10.78 (b.s., 1H, CO2H) 7.88 (2 overlapping doublets, 1H, Cyt H-5); 7.41–7.32 (m, 5H, Ph); 7.01 (2 overlapping doublets, 1H, Cyt H-6); 6.94 & 6.78 (unres. triplets, 1H, BocNH); 5.19 (s, 2H, PhCH2); 4.81 & 4.62 (s, 2H, CH2CON); 4.17 & 3.98 (s, 2H, CH2CO2H); 3.45–3.03 (m, includes water, CH2Cl2); 1.38 & 1.37 (s, 9H, Bu). -C NMR (150.88; 128.52; 128.18; 127.96; 93.90; 66.53; 49.58 and 28.22. IR: Frequency in cm⁻¹ (intensity). 3423 (26.4), 3035 (53.2), 2978 (41.4), 1736 (17.3), 1658 (3.8), 1563 (23.0), 1501 (6.8) and 1456 (26.4).

EXAMPLE 30

9-Carboxymethyl Adenine Ethyl Ester

Adenine (10.0 g, 74 mmol) and potassium carbonate (10.29 g, 74.00 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. 215.5–220.5° C. Anal. for \( \text{C}_6\text{H}_7\text{N}_2\text{O}_6 \) found (calc.): C 48.86 (48.65); H 5.01 (4.91); N, 31.66 (31.42). -H NMR (250 MHz; DMSO-d6) (4H, 2H-H-2 & H-8); 7.25 (b.s., 2H, NH2); 5.06 (s, 2H, NCH2); 4.17 (q, 2H, J=7.7 Hz, OCH2) and 1.21 (t, 3H, J=7.13 Hz, NCH3). -C NMR (150.72, 140.30, 61.41, 43.97 and 14.07. FAB-MS. 222 (MH+): IR: Frequency in cm⁻¹ (intensity). 3855 (54.3), 3274 (10.4), 3246 (14.0), 3117 (5.3), 2989 (22.3), 2940 (33.9), 2876 (43.4), 2753 (49.0), 2346 (56.1), 2106 (57.1), 1899 (55.7), 1762 (42.4), 1742 (14.2), 1742 (10.0), 1671 (1.6), 1644 (10.9), 1606 (0.6), 1582 (7.1), 1522 (43.8), 1477 (7.2), 1455 (35.8) and 1422 (8.6). The position of alkylation was verified by X-ray crystallography on crystals, which were obtained by recrystallization from 96% ethanol.
EXAMPLE 32

N'-Benzoylcarbonyl-9-carboxymethyl Adenine

N'-Benzoylcarbonyl-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 C18H17N3O5: C, 46.32; H, 4.24; N, 18.10 (21.40) and C/N, 2.57 (2.56). 1H-NMR (250 MHz, DMSO-d6): 8.70 (s, 2H, H-12 and H-8); 7.50–7.35 (m, 5H, Ph); 5.27 (s, 2H, N-H); 5.15 (s, 2H, Ph-CH2). 13C-NMR, 168.77, 152.54, 151.36, 148.75, 145.13, 128.51, 128.17, 127.98, 66.76 and 44.67 (IR, kbr) 3484 (18.33); 3109 (15.90); 2966 (17.11); 2927 (19.97); 2838 (23.9); 1960 (62.7); 1739 (2.5); 1688 (5.2); 1655 (0.9); 1594 (11.7); 1560 (12.3); 1530 (26.3); 1499 (30.5); 1475 (10.4); 1455 (4.0); 1429 (24.5) and 1411 (23.6). FAB-MS: 328 (MH+ and 284 (MH+–CO2). HPLC (215 nm, 260 nm) in system 1:15.18 min, minor impurities all less than 2%.

EXAMPLE 33

N'-Benzoylcarbonyl-1-(Boc-aeg)adenine Ethyl Ester

N'-Boc-aminoethyl glycine ethyl ester (2.00 g; 8.12 mmol), DihyO (1.46 g; 8.93 mmol) and N'-benzoylcarbonyl-9-carboxymethyl adenine (2.92 g; 8.93 mmol) were dissolved in DMF (15 ml). Methylene chloride (15 ml) then was added. The solution was cooled to 0°C in an ethanol/ice bath. DCC (2.01 g; 9.74 mmol) was added. The ice bath was removed after 2.5 h and stirring was continued for another 1.5 hour at ambient temperature. The precipitated DCC was removed by filtration and washed once with 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. Addition of water to the mother liquor afforded another 0.53 g with a purity of approx. 95%. Anal. For C18H17N3O5·H2O found (calcld.) C, 53.81; H, 6.58 (6.15) and N, 16.47 (17.09). 1H-NMR (250 MHz, CDCl3) 8.74 (s, 1H, Ade H-2); 8.18 (b, s, 1H, ZNH); 8.10 & 8.04 (s, 1H, H-5); 7.63–7.34 (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, CH2COOH); 4.20 (q, 2H, OCH2CH3); 3.67–3.29 (m, 4H, CH2CH2O); 1.42 (s, 9H, Bu) and 1.27 (t, 3H, OCH2CH3). The spectrum shows traces of ethanol and DCU.

EXAMPLE 34

N'-Benzoylcarbonyl-1-(Boc-aeg)adenine ethyl ester (1.48 g; 2.66 mmol) was suspended in THF (13 ml) and the mixture was cooled to 0°C. Lithium hydroxide (8 ml; 1 N) was added. After 15 min of stirring, the reaction mixture was filtered, extra water (25 ml) was added, and the solution was washed with methylene chloride (2x25 ml). The pH of the aqeous solution was adjusted to pH 2.0 with 1 N HCl. The precipitate was isolated by filtration, washed with water, and dried, and yield 0.19 g (59%). The product precipitated twice with methylene chloride/petroleum ether, 0.17 g (55%) after drying. M.p. 119°C (decomp.) Anal. For C18H17N3O5·H2O found (calcld.) C, 53.82; H, 5.71 (5.73); N, 17.68 (17.97). FAB-MS: 528.5 (MH+). 1H-NMR (250 MHz, DMSO-d6), 12.75 (very b, 1H, CO2H); 10.65 (b, 1H, ZNH); 8.59 (d, 1H, J=2.14 Hz, Ade H-2); 8.31 (s, 1H, Ade H-8); 7.49–7.31 (m, 5H, Ph); 7.05 & 6.75 (unsres. t, 1H, BocNH); 5.33 & 5.16 (s, 2H, CH2CON); 3.22 (s, 2H, PhCH2); 4.34–3.99 (s, 2H, CH2COOH); 3.54–3.03 (m, includes water, CH2CH3); 1.39 & 1.37 (s, 9H, Bu). 13C-NMR, 170.4; 166.6; 152.3; 145.1; 145.2; 128.5; 128.0; 127.9; 133.2; 47.63; 47.03; 44.38 and 28.24.

EXAMPLE 35

2-Amino-6-chloro-9-carboxymethylpurine

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 sacopent. Yield (3.02 g; 44.8%). 1H-NMR(DMSO-d6): d=4.88 ppm (6,2H); 6.95 (s,2H); 8.10 (s,1H).

EXAMPLE 36

2-Amino-6-benzoxyl-9-carboxymethylpurine

Sodium (2.0 g; 87.0 mmol) was dissolved in benzyl alcohol (20 ml) and heated to 130°C for 2 h. After cooling to 0°C, a solution of 2-amino-6-chloro-9-carboxymethylpurine (4.05 g; 18.0 mmol) in DMF (85 ml) was slowly added, and the resulting suspension stirred overnight at 20°C. Sodium hydroxide solution (1N, 100 ml) was added and the clear solution was washed with ethyl acetate (3x100 ml). The water phase was then acidified to a pH of 3 with 4 N hydrochloric acid. The precipitate was taken up in acetate (200 ml), and the water phase was
extracted with ethyl acetate (2×100 ml). The combined organic phases were washed with saturated sodium chloride solution (2×75 ml), dried with anhydrous sodium sulfate, and taken to dryness by evaporation, in vacuo. The residue was recrystallized from ethanol (300 ml). Yield after drying, in vacuo, over scavenger 2.76 g (52%). M.p. 159–61° C. Anal. (calc., found) C,56.18; 55.97; H,(4.38; 4.32); N,(23.4; 23.10). 1H-NMR (DMSO-d6): 4.82 ppm. (s,2H), 5.51 (s,2H); 6.45 (s,2H); 7.45 (m,5H); 7.82 (s,1H).

EXAMPLE 37
N-[2-Amino-6-benzoyloxypyr-9-yl]-acetyl-N-(2-Boc-aminoethyl)-glycine [BocGaeG-OH Monomer]

2-Amino-6-benzoyloxypyr-9-carboxyethyl-purine (0.50 g; 1.67 mmol), methyl-N-[2-(tet-butoxycarbonylamino)ethyl]-glycinate (0.65 g; 2.80 mmol), diisopropyl ethylamine (0.54 g; 4.19 mmol), and bromo-tris-pyridylphospholine-hexafluorophosphate (PyBroP) (0.798 g; 1.71 mmol) were stirred in DMF (2 ml) for 4 h. The clear solution was poured into an ice-cooled solution of sodium hydrogen carbonate (1 N; 40 ml) and extracted with ethyl acetate (3×40 ml). The organic layer was washed with potassium hydrogen sulfate solution (1 N; 2×40 ml), sodium hydrogen carbonate (1 N; 1×40 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+H). 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 pH 3 by the addition of 4 N hydrochloric acid. The title compound was obtained by filtration. Yield: 370 mg (72% for the purity). 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 m, for major and m, for minor). 1H-NMR(250 MHz, DMSO-d6): δ=1.4 ppm. (s,9H); 3.2 (m,2H); 3.6 (m,2H); 4.1 (s, m), CONR2CH2COOH; 4.4 (s, m), CONR2CH2COOH; 5.0 (s, m), Gua-CH2COO−; 5.2 (s, m), Gua-CH2COO− (s,2H); 6.5 (s,2H, 6.9 (s, m), BoeCH2N; 7.1 (m, m); BoeN; 7.5 (m,3H); 7.8 (s,1H); 8.5 (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; 76.19; 77.86; 66.95; 49.22; 47.70; 46.94; 45.95; 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-tet-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 and Vigorous stirring. The phases were separated and the water phase was washed with additional ethyl acetate (6×350 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 (1×1000 ml) and with saturated aqueous sodium chloride (1×500 ml). The organic phase was dried (MgSO4) and evaporated under reduced pressure to yield 50.12 g (600%) of the title compound. The product could be solidified by evaporation from methylene chloride and subsequent freezing. 1H-NMR (CDCl3/TMS): δ=1.43 (s, 9H, Me-C), 3.25 (m, 2H, CH2), 3.57 (m, 2H, CH2), 3.73 (m, 1H, CH) 13C-NMR (CDCl3/TMS): δ=28.2 (Me-C), 42.6 (CH2), 63.5; 71.1 (CH2OH, CHOH), 79.5 (Me-C), 157.0 (C=O).

EXAMPLE 39
2-(Boc-amino)ethyl-L-alanine Methyl Ester

3-Bocamino-1,2-propanediol (20.76 g, 0.109 mol, 1 eq.) was suspended in water (150 ml). Potassium m-periodate (24.97 g, 0.109 mol, 1 eq.) was added and the reaction mixture was stirred for 2 h at room temperature under nitrogen. The reaction mixture was filtered and the water phase extracted with chloroform (6×250 ml) The organic phase was dried (MgSO4) and evaporated to afford an almost quantitative yield of Boc-aminoacetalddehyde 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 eq) and L-alanine methyl ester, hydrochloride (3.82 g, 27.4 mmol, 1 eq) were added. Boc-aminoacetalddehyde (4.79 g, 30.1 mmol, 1.1 eq) 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-C), 1.49 (6H, 1H), 2.51 (m, 1H, CH2), 2.46 (m, 1H, CH2), 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: δ=18.8 (Me), 28.2 (Me-C), 40.1, 47.0 (CH2), 51.6 (OMe), 56.0 (CH), 155.8 (carbamate C=O), 175.8 (ester C=O).

EXAMPLE 40
N-(Boc-a-methoxy)-N-(1-thyminyacyl)-L-alanine Methyl Ester

To a solution of Boc-a-methoxy-(L)-alanine methyl ester (1.23 g, 5.0 mmol) in DMF (10 ml) was added DiBt-OH (0.90 g, 5.52 mmol) and 1-thyminyacyl acid (1.01 g, 5.48 mmol). When the 1-thyminyacyl acid was dissolved, dichloromethane (10 ml) was added and the solution was cooled on an ice bath. After the reaction mixture had reached 0° C, DCC (1.24 g, 6.01 mmol) was added. Within 5 min after the addition, a precipitate of DCC was seen. After a further 5 min, the ice bath was removed. Two hours later, TLC analysis showed the reaction to be finished. The
mixture was filtered and the precipitate washed with dichlo-romethane (100 ml). The resulting solution was extracted twice with 5% sodium hydrogen carbonate (150 ml) and twice with saturated potassium hydrogen sulfate (25 ml) in water (100 ml). After a final extraction with saturated sodium chloride (150 ml), the solution was dried with magnesium sulfate and evaporated to give a white foam. The foam was purified by column chromatography on silica gel using dichloromethane with a methanol gradient as eluent. This yielded a pure compound (>99% by HPLC) (1.08 g, 52.4%). FAB-MS: 413 (M+1) and 43 (M+1+water).

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 hydrochloric acid. The solution then was extracted with ethyl acetate (3×100 ml). The combined organic extracts were dried over magnesium sulfate. After evaporation, the residue was dissolved in ethyl acetate (200 ml) and petroleum ether (20 ml) was added. The mixture was filtered through a 1 cm column of silica gel. The filtrate was concentrated to dryness in vacuo. The resulting compound was recrystallized from 2-propanol. FAB-MS: 399 (M+1). 1H-NMR (DMSP-d6, δ): 11.55 (s, 1 H, COOH); 7.42 (s, 1 H, H2); 4.69 (s, 2 H, CH2); 1.83 (s, 3 H, Me in T); 1.50–1.40 (m, 12 H, Me in Ala+Boc).

EXAMPLE 42

(a) N-(Boc-aminomethyl)-N-(1-thyminylacetyl)-d-alanine Methyl Ester

To a solution of Boc-aminomethyl alanine methyl ester (2.48 g, 10.1 mmol) in DMF (20 ml) was added dibutyl ether (1.80 g, 11.0 mmol) and thyminylacetic acid (2.14 g, 11.6 mmol). After dissolution of the thyminylacetic acid, methyl 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 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, δ): 5.64 (s, 1 H, BocNH, J=5.89 Hz); 4.56 (d, 2 H, CH2); 4.35 (q, 1 H, CH in Ala, J=7.25 Hz); 3.74 (s, 3 H, OMe); 3.64–3.27 (m, 4, 2 H, ethyl HS); 1.90 (s, 3 H, Me in T); 1.52–1.44 (t, 12 H, 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 hydrochloric acid. The solution then was extracted with ethyl acetate (3×100 ml). The combined organic extracts were dried over magnesium sulfate. After evaporation, the oil was dissolved in ethyl acetate (200 ml). Petroleum ether was added (to a total volume of 600 ml) until precipitation started. After standing overnight at 20°C, the precipitate was removed by filtration. This afforded 1.02 g (67.3%) of the title compound, which was 94% pure according to HPLC. FAB-MS: 399 (M+1). 1H-NMR: 11.34 (s, 1 H, COOH); 7.42 (s, 1 H, H2); 4.69 (s, 2 H, CH2); 4.40 (q, 1 H, CH in Ala, J=7.20 Hz); 1.83 (s, 3 H, Me in T); 1.52–1.40 (m, 12 H, Boc+Me in Ala).

EXAMPLE 43

N-(N'-Boc-3-aminopropyl)-N'-(1-thyminylacetyl) glycine methylester

N-(N'-Boc-3-aminopropyl)-N'-[(1-thyminylacetyl)] glycine methylester (2.84 g, 0.0115 mol) was dissolved in DMF (35 ml), followed by addition of Diisobutyl ether (2.07 g, 0.0127 mol) and 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 water, 6×250 ml), potassium sulfate (1 volume saturated diluted with 4 volumes water, 3×250 ml), and saturated aqueous sodium chloride (1×250 ml), dried over magnesium sulfate, and evaporated to dryness, in vacuo. The solid residue was suspended in methylene chloride (35 ml) and stirred for 1 h. The precipitated DCU was removed by filtration and washed with methylene chloride (25 ml). The filtrate was evaporated to dryness, in vacuo, and the residue purified by column chromatography on silica gel, eluting with a mixture of methanol and methylene chloride (gradient from 3–7% methanol in methylene chloride). This afforded the title compound as a white solid (3.05 g, 64%). M.p. 76–79°C (decompy.). Anal. for C11H16N2O4, found (calc.) C, 52.03; (52.42); H, 6.90; (6.84); N, 13.21 (13.58). The compound showed satisfactory 1H and 13C-NMR spectra.

EXAMPLE 44

N-(N'-Boc-3-aminopropyl)-N'-(1-thyminylacetyl) glycine

N-(N'-Boc-3-aminopropyl)-N'-(1-thyminylacetyl) glycine methylester (3.02 g, 0.00732 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 pH adjusted to 2 with 4 M hydrochloric acid at 0°C. The product was isolated as white crystals by filtration, washed with water (3×10 ml), and dried over sixcapentane, in vacuo. Yield 2.19 g (75%). Anal. for C12H17N2O4, H2O, found (calc.) C, 49.95; (49.03); 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. Methyl acrylate (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 (3×15 ml), and dried over silicic acid, in vacuo. Yield 11.23 g (48%). M.p. 112–119°C. Anal. for C₈H₂₈NO₂ found (calc.) 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 (10×25 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 C₈H₂₈NO₂ found (calc.) 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)-N-[(1-thyminyl)propanoyl] glycine Ethyl Ester

N-(N'-Boc-aminoethyl)glycine ethyl ester (1.0 g, 0.0041 mol) was dissolved in DMF (12 ml). DibutOH (0.73 g, 0.0045 mol) and 3-(1-thyminyl)-propanoic acid (0.49 g, 0.0045 mol) were added. Methylene chloride (12 ml) was then added and the mixture was cooled to 0°C on an ice bath. After addition of DCC (1.01 g, 0.0049 mol), the mixture was stirred at 0°C for 2 h, followed by 1 h at room temperature. The precipitated DCC was removed by filtration, washed with methylene chloride (25 ml), and a further addition of methylene chloride (50 ml) was added to the filtrate. The organic phase was extracted with sodium hydrogen carbonate (1 volume saturated diluted with 1 volume water, 6×100 ml), potassium sulfate (1 volume saturated diluted with 4 volumes water, 3×100 ml), and saturated aqueous sodium chloride (1×100 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 DCC was removed by filtration and washed with methylene chloride. The filtrate was evaporated to dryness, in vacuo, and the residue purified by column chromatography on silica gel, eluting with a mixture of methanol and methylene chloride (gradient from 1 to 6% methanol in methylene chloride). This afforded the title compound as a white solid (1.02 g, 59%). Anal. for C₈H₂₈NO₂ found (calc.) C, 53.15; (53.51); H, 6.90; (7.09); N, 12.76; (13.13). The compound showed satisfactory ¹H and ¹³C-NMR spectra.

**EXAMPLE 48**

N-(N'-Boc-aminoethyl)-N-[(1-thyminyl)propanoyl] glycine

N-(N'-Boc-aminoethyl)-N-[(1-thyminyl)propanoyl] glycine ethyl ester (0.83 g, 0.00195 mol) was dissolved in methanol (25 ml). Sodium hydroxide (25 ml; 2 M) was added. The solution was stirred for 1 h. The methanol was removed by evaporation, in vacuo, and the pH adjusted to 2 with 4 M hydrochloric acid at 0°C. The product was isolated by filtration, washed with ether (3×15 ml), and dried over silicic acid, in vacuo. Yield 0.769 g, 99%. M.p. 213°C (decomp.).

**EXAMPLE 49**

Mono-Boc-ethylendiamine (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, pH was adjusted to 3.5 with 4 M hydrochloric acid. The solution then was filtered and extracted with chloroform (3×250 ml). The pH was adjusted to 12 at 0°C with 2 M sodium hydroxide, and the aqueous solution extracted with methylene chloride (3×300 ml). After treatment with sat. aqueous sodium chloride (250 ml), the methylene chloride solution was dried over magnesium sulfate. After filtration, the solution was evaporated to dryness, in vacuo, and the residue resulting in 4.22 g (63%) of the product (oil). ¹H-NMR (90 MHz; CDCl₃): δ1.44 (s, 9H); 2.87 (t, 2H); 3.1 (q, 2H); 5.62 (s, broad).

**EXAMPLE 50**

(N-Boc-aminoethyl)-β-alanine Methyl Ester, HCl

Mono-Boc-ethylendiamine (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 one more time 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 C₈H₂₈NO₂Cl 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): δ1.39 (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-amino-ethyl)-β-alanine methyl ester, HCl (3) (2.0 g; 0.0071 mol) and 1-thyminylacetic acid pentafluorophenyl ester (5) (2.82 g; 0.00812 mol) were dissolved in DMF (50 ml). Trichloroacetic amine (1.12 ml; 0.000812 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 (3×250 ml), half-sat. aqueous potassium hydrogen sulfate (3×250 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; δ1.43 (s, 9H); 1.88 (s, 3H); 2.63 (t, 1H); 2.74 (t, 1H); 3.25–3.55 (in 4x, 8H); resulting (2xt, 2H); 3.66 (s, 1.5x); 3.72 (s, 1.5x); 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-aminomethyl-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 sievacip, in vacuo. Yield 2.23 g (77%). M.p. 170–176°C. Anal. for C₉H₁₂N₂O₅.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-(N⁵-Z)-cytosyl]acetyl]-N'-Boc-aminomethyl-β-alanine Methyl Ester

(N-Boc-aminomethyl-β-alanine methyl ester, HCI (3) (2.0 g; 0.0071 mol) and 1-(N⁵-Z)-cytosylacetic acid pentfluoro-phenyl 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 hydroxide (3×250 ml), half-sat. aqueous potassium hydrogen carbonate (3×250 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 C₂₁H₂₄N₄O₅ found (calc.) C, 55.19; (56.49); H, 6.19; (6.26); N, 12.86; (13.81). 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); 3.60–3.23 (m, 6H); 3.60 (s, 1.5H); 3.66 (s, 1.5H); 4.80 (s, 1H); 4.88 (s, 1H); 5.20 (s, 2H); 7.80–7.25 (m, 7H). FAB-MS: 532 (M+1).

EXAMPLE 54

N-[1-(N⁵-Z)-cytosyl]acetyl]-N'-Boc-aminomethyl-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 sievacip, in vacuo. Yield 0.326 g (54%). The white solid was recrystallized from 2-propanol and washed with petroleum ether. M.p. 163°C. (decomp.). Anal. for C₂₁H₂₄N₄O₅.0.5H₂O. 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, 6H); 4.85 (s, 1H); 4.98 (s, 1H); 5.21 (s, 2H); 5.71 (s, 1H, broad); 7.79–7.25 (m, 7H). FAB-MS: 518 (M+1).

EXAMPLE 55

Example of a PNA-oligomer with a Guanine Residue

(a) Solid-Phase Synthesis of H-[Taeg]₁-[Gaeg]₁-[Taeg]₁-Lys-NH₂

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

(b) Stepwise Assembly of H-[Taeg]₁-[Gaeg]₁-[Taeg]₁-Lys-NH₂ (Synthetic Protocol)

Synthesis was initiated on 102 mg (dry weight) of pre-swollen (overnight in DCM) and neutralized Boc-Lys(CIZ)-MBHIA resin. The steps performed were as follows: (1) Boc-deprotection with TFA/DCM (1:1, v/v), 1×2 min and 1×½ h, 3 ml; (2) washing with DCM, 4×20 sec, 3 ml; washing with DMC, 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 µg) BocTaeg-OH or (0.06 mmol; 30 mg) BocGaeg-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 DMC, 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-oligomer was eluted and purified by the normal procedure. FAB-MS: 2832.11 [M+1] (calc. 2832.15).

EXAMPLE 56

Solid-Phase Synthesis of H-Taeg-Aaeg-[Taeg]₁-[Lys]-NHN₃ (CIZ)-MBHIA Resin

About 0.3 g of wet Boc-[Taeg]-Lys(CIZ)-MBHIA resin was placed in a 3 ml SPSS reaction vessel. Boc-Taeg-Aaeg-[Taeg]₁-[Lys]-NHN₃ (CIZ)-MBHIA resin was absorbed by a 0.19 M of BocAaeg-OH together with 0.15 M DCC in 2.5 ml 50% DCM/CH₂Cl₂ and a single coupling with 0.15 M BocTaeg-OH in neat CH₂Cl₂ (“Synthetic Protocol 5”). The synthesis was monitored by the quantitative ninhydrin reaction, which showed about 50% incorporation of A(Z)aeg and about 90% incorporation of Taeg.

(b) Cleavage, Purification, and Identification of H-Taeg-Aaeg-[Taeg]₁-[Lys]-NHN₃

The protected Boc-Taeg-[Z]aeg-[Taeg]₁-[Lys]-NHN₃(CIZ)-BAH resin was treated as described in Example 40c to yield about 15.6 g of crude material upon HF cleavage of 53.1 mg, dry H-Taeg-Aaeg-[Taeg]₁-[Lys]-NHN₃(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]₁-[Lys]-NHN₃(CIZ)-BAH. For (MH⁺) 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, 3x1 min and 1×30 min; (2) washing with CH₂Cl₂, 2.5 ml, 6x1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 2.5 ml, 3x2 min; (4) washing with CH₂Cl₂, 2.5 ml, 6x1 min, and drain for 1 min; (5) 2–5 mg sample of PNA-resin is
taken out and dried thoroughly for a quantitative ninhydrin analysis to determine the substitution; (6) addition of 0.47 mmol (0.25 g) Boc(AZ)(aeg-OH) dissolved in 1.25 ml DMF followed by addition of 0.47 mmol (0.1 g) DCC in 1.25 ml CH₂Cl₂ or 0.36 mmol (0.20 g) BocAeg-OPfp in 2.5 ml CH₂Cl₂; the coupling reaction was allowed to proceed for a total of 20–24 hrs shaking; (7) washing with DMF, 2.5 ml, 1×2 min; (8) washing with CH₂Cl₂, 2.5 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**

**Solid-Phase Synthesis of H-[T(aeg)-A(aeg)-[T(aeg)]₂-**

**Lys(CIZ)-MBHA Resin**

A stepwise assembly of Boc-[T(aeg)-A(aeg)-[T(aeg)]₂- Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[T(aeg)-A(aeg)-[T(aeg)]₂- Lys(CIZ)-MBHA resin was assembled in situ DCC coupling of both the Z[Aeg] and the Target 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] 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 Target residues.

(b) Cleavage, Purification, and Identification of H-[T(aeg)-A(aeg)-[T(aeg)]₂- Lys(NH₂).

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

(c) Synthetic Protocol 6

(1) Boc-deprotection with TFA/CH₂Cl₂ (1:1, v/v), 2 ml, 3×1 min and 1×30 min; (2) washing with CH₂Cl₂, 2 ml, 6×1 min; (3) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 2 ml, 3×2 min; (4) washing with CH₂Cl₂, 2 ml, 6×1 min, and drain for 1 min; (5) 2–5 mg sample of PNA-resin was taken out and dried thoroughly for a quantitative ninhydrin analysis to determine the substitution; (6) addition of 0.44 mmol (0.23 g) Boc(AZ)(aeg-OH) dissolved in 1.5 ml CH₂Cl₂, followed by addition of 0.44 mmol (0.09 g) DCC in 0.5 ml CH₂Cl₂ or 0.33 mmol (0.13 g) BocAeg-OH in 1.5 ml CH₂Cl₂, followed by addition of 0.33 mmol (0.07 g) DCC in 0.5 ml CH₂Cl₂; the coupling reaction was allowed to proceed for a total of 20–24 hrs with shaking; (7) washing with DMF, 2 ml, 1×2 min; (8) washing with CH₂Cl₂, 2 ml, 4×1 min; (9) neutralization with DIEA/CH₂Cl₂ (1:19, v/v), 2 ml, 2×2 min; (10) washing with CH₂Cl₂, 2 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); (13) washing with CH₂Cl₂, 2 ml, 6×1 min; and (14) 2×2–5 mg samples of protected PNA-resin were taken out, neutralized with DIEA/CH₂Cl₂ (1:19, v/v) and washed with CH₂Cl₂ for ninhydrin analyses.

**EXAMPLE 58**

The PNA-oligomer H-T₄C₂TCTC-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:

| Row Hybridized With | pH | Tm | $|$ |
|---------------------|----|----|----|
| 1 5′-(DA)(G)₃( DA)( D) (DA) (DG) | 7.2 | 55.5 | 2:1 |
| 2 5′-(DA)(G)₃( DA)( D) (DA) (DG) | 9.0 | 26.0 | 2:1 |
| 3 5′-(DA)(G)₃( DA)( D) (DA) (DG) | 5.0 | 88.5 | 2:1 |
| 4 5′-(DA)(G)₃( DA)( D) (DA) (DG) | 7.2 | 38.0 | 2:1 |
| 5 5′-(DG) (DA) (DG) (DG) (DA) (DG) | 9.0 | 31.5 | 1:1 |
| 6 5′- (DA) (DG) (DA) (DG) (DA) (DG) | 5.0 | 52.5 | 1:1 |
| 7 5′-(DA)(G)₃( DT)( DA)( D) (DA) (DG) | 7.2 | 39.0 | 1:1 |
| 8 5′-(DA)(G)₃( DT)( DA)( D) (DA) (DG) | 9.0 | <20 | 1:1 |
| 9 5′-(DA)(G)₃( DT)( DA)( D) (DA) (DG) | 5.0 | 50.5 | 1:1 |
| 10 5′-(DA)(G)₃( DT)( DA)( D) (DA) (DG) | 5.0 | 52.5 | 1:1 |
| 11 5′-(DA)(G)₃( DT)( DA)( D) (DA) (DG) | 7.2 | 24.5 | 1:1 |
| 12 5′-(DA)(G)₃( DT)( DA)( D) (DA) (DG) | 9.0 | <20 | 1:1 |
| 13 5′-(DA)(G)₃( DT)( DA)( D) (DA) (DG) | 5.0 | 57.0 | 1:1 |
| 14 5′-(DA)(G)₃( DT)( DA)( D) (DA) (DG) | 7.2 | 25.0 | 1:1 |
| 15 5′-(DA)(G)₃( DT)( DA)( D) (DA) (DG) | 5.0 | 39.5 | 1:1 |

$|$ = 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 the 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 the Tm when mismatches are present show that the Watson-Crick strand and the Hoogsteen strand are parallel when bound to complementary DNA. This is true for both of the orientations, i.e., 5′/N-terminal and 3′/N-terminal.

**EXAMPLE 59**

The results of hybridization experiments with H-T₄GT₄₄-LysNH₂ to were performed as follows:

| Row Hybridized With | Tm | $|$ |
|---------------------|----|----|
| 1 5′-(DA) 5′-(DA)(G)₃( DA)( D) (DA) (DG) | 55.0 | 55.0 |
| 2 5′-(DA) 5′-(DA)(G)₃( DA)( D) (DA) (DG) | 47.0 | 47.0 |
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-TcTC-TCTC-LysNH₂</td>
<td>2747.15</td>
<td>2746.78</td>
</tr>
<tr>
<td>H-TcCTcTC-LysNH₂</td>
<td>2832.15</td>
<td>2832.11</td>
</tr>
<tr>
<td>H-TcTcTC-LysNH₂</td>
<td>2508.84</td>
<td>2509.84</td>
</tr>
<tr>
<td>H-TcTcLcTc-LysNH₂</td>
<td>2541.04</td>
<td>2540.84</td>
</tr>
<tr>
<td>H-TcTcLcTc-LysNH₂</td>
<td>2507.14</td>
<td>2506.89</td>
</tr>
<tr>
<td>H-TcTcLcTc-LysNH₂</td>
<td>2259.94</td>
<td>2259.18</td>
</tr>
<tr>
<td>H-TcTcTcTC-LysNH₂</td>
<td>2287.95</td>
<td>2288.60</td>
</tr>
<tr>
<td>H-TcTcTcTC-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>H-TcTcC-LysNH₂</td>
<td>(DA)₀</td>
<td>73° C</td>
</tr>
<tr>
<td>H-TcTcC-LysNH₂</td>
<td>(DA)₀</td>
<td>73° C</td>
</tr>
<tr>
<td>H-TcC-LysNH₂</td>
<td>(DA)₀</td>
<td>47° C</td>
</tr>
<tr>
<td>H-TcC-LysNH₂</td>
<td>(DA)₀</td>
<td>47° 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-T₁₀-Lys-NH₂ is dissolved in 40 μl 100 mM Na-phosphate, pH 7.0, and 1 mCi Na¹²⁵I and 2 μl chloramine-T (50 mM in CH₂CN) are added. The solution is left at 20° C. for 10 min and then passed through a 0.5-5 cm Sephadex G10 column. The first 2 fractions (100 μl each) containing radioactivity are collected and purified by HPLC: reversed phase C-18 using a 0-60% CH₂CN gradient in 0.1% CF₃COOH in H₂O. The¹²⁵I-PNA elutes right after the PNA peak. The solvent is removed under reduced pressure.

**EXAMPLE 64**

Binding of PNAAs-T₁₀/T₂₀/C₁₀/C₂₀ to Double Stranded DNA Targets A₂₀/A₂₀/A₂₀/G₂₀ (FIG. 20)

A mixture of 200 cpm ³²P-labeled EcoRI-PvuII fragment (the large fragment labeled at the 3'-end of the EcoRI site) of the indicated plasmid, 0.5 μg carrier calf thymus DNA, and 300 ng PNA in 100 μl buffer (200 mM NaCl, 50 mM Na-acetate, pH 4.5, 1 mM ZnSO₄) was incubated at 37° C. for 120 min; A 50 unit portion of nuclease S₁ was added and incubated at 40° 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 of the appropriate oligonucleotides into pUC19. Target A₂₀-oligonucleotides GATCCA₉₅G & GATCCT₁₀₀G cloned into the BamHI site (plasmid designated pT₁₀). Target A₂₀GA₉₅ oligonucleotides TCGACT₁₀₀G & TCGACA₁₀₀GA₉₅ cloned into the SalI site (plasmid pTCTL). Target A₂₀GA₉₅ oligonucleotides GA₉₅GA₉₅GA₉₅G & GT₁₀₀CT₁₀₀CT₁₀₀GCA into the PsiI 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 in 20 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and incubated at 37° C. for 120 min. 2 μl 10xbuffer (10 mM Tris-HCl, pH 7.5, 10 mM, 50 mM NaCl, 10 mM EDTA, pH 7.5) was added and incubated at 37° C.
57 MgCl₂, 50 mM NaCl, 1 mM 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-T₁₀₀−dsDNA Strand Displacement Complex Formation (Fig. 21)**

A mixture of 200 cpm 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 30.0 ng of PNA-T₁₀₀-LysNH₂ in 100 µl buffer (200 mM NaCl, 50 mM Na-acetate, pH 4.5, 1 mM ZnSO₄) were incubated at 37°C. At the times indicated, 50 U of S₁ nuclease was added to each of 7 samples and incubation was continued for 5 min at 20°C. The DNA was then precipitated by addition of 250 µl 2% K-acetate in ethanol and analyzed by electrophoresis in a 10% 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 cpm ³²P-pT10 fragment, 0.5 µg calf thymus DNA and 300 ng of the desired PNA (either T₁₀₀-LysNH₂, T₁₀₀-LysNH₂ or T₁₀₀-LysNH₂) was incubated in 100 µl 200 mM NaCl, 50 mM Na-acetate, pH 4.5, 1 mM ZnSO₄ for 60 min at 37°C. A 2 µg portion of oligonucleotide GATCCA₁₀₀₋₀G 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 U portion of S₁ nuclease 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, 1 mM EDTA, pH 7.4 was incubated at 37°C for 60 min. Subsequently, 4 µl 5x concentrated buffer (0.2 M Tris-HCl (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, 1 mM UTP, 0.1 µCi/µl ³²P-UTP, 5 mM DTT, 2 µg/ml RNA, 1 µg/ml heparin) and 3 units RNA polymerase. Incubation was continued for 10 min at 37°C. The DNA 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: pT10C-2KS/pA82KS: oligonucleotides GA₂₆-GA₂₆ GTGAC & GTTCCTCTCTGCA cloned into the PsI site of pBluescript-KS², pT10C-pA100-KS (both orientations of the insert were obtained): pT10UVS: oligonucleotides GATCCA₁₀₀₋₀G & GATCTTCCTG cloned into the BamHI site of a pUC18 derivative in which the lac UVS E.coli promoter had been cloned into the EcoRI site (Jeppesen, et al., *Nucleic Acids Res.*, 1988, 16, 9545).

Using T₁₀₀RNA polymerase, transcription elongation arrest was obtained with PNA-T₁₀₀-C₅₀-LysNH₂ and the pA82KS 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-T₁₀₀-LysNH₂ and the plasmids pA10-KS and pT10-KS. (see, Fig 25) Using E.coli RNA polymerase and the pT10UV5 plasmid (A₁₀₀₋₀ sequence on the template strand) transcription elongation arrest was obtained with PNA-T₁₀₀-LysNH₂.

**EXAMPLE 69**

**Biological Stability of PNA**

A mixture of PNA-T₁₀₀ (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 caespitosus 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. Spahlholz, 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 micrograms 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 2×HBSS (1.5 mM Na₂PO₄, 10 mM KCI, 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 KCI, 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 refed 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-shattering 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 mCi/mM. 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 excised 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 positives.

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 quantitation of 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 depress CAT activity in a dose dependent fashion.

EXAMPLE 71

Synthesis of PNA 15-mer Containing Four Naturally Occurring Nucleobases; H-[Taqg]-[Aaqg]-
[Gaqg][Taqg][Taqg][Aaqg][Taqg][Caqg][Taqg][Aaqg][Caqg][Taqg][Aaqg][Caqg][Taqg][Aaqg][Caqg]-LYS—
NH2

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-Aaqg
OH monomer.

Synthesis was initiated on 100 mg (dry weight) of neutralised
Boc-Lys(CIA)-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-Aaqg-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-Aaqg-OH
(0.06 mmol; 32 mg) dissolved in 0.6 ml DCM/DMF (1:1, by
v/v) (final concentration of monomer 0.1M). The coupling
reaction was allowed to proceed for 1×15 min and 1×60 min.
(recoupling).

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'--3'</td>
<td>5</td>
<td>60.5</td>
</tr>
<tr>
<td>5'--3'</td>
<td>7.2</td>
<td>43.0</td>
</tr>
<tr>
<td>5'--3'</td>
<td>9</td>
<td>36.5</td>
</tr>
<tr>
<td>5'--3'</td>
<td>5</td>
<td>64,549.0</td>
</tr>
<tr>
<td>3'--3'</td>
<td>7.2</td>
<td>53.5</td>
</tr>
<tr>
<td>3'--3'</td>
<td>9</td>
<td>51.5</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 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'/N-orientation and that in the Hoogsteen motif the
5'/N-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'/N-
orientation. Furthermore, it explains the small loss in going
from pH 7.2 to 9 in the 3'/N, as this is probably a 1:1
complex.

EXAMPLE 73

Solid-Phase Synthesis of H-[Taqg]-Aaqg-Taqg-Caqg-Aaqg-Taqg-Caqg-Lys-NH2

(a) Stepwise Assembly of Boc-[Taqg]-[Aaqg]-Taqg-Caqg-Aaqg-Caqg-Taqg-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-[Taqg]-[Aaqg]-Taqg-Caqg-Aaqg-Caqg-Taqg-Lys(CIZ)-MBHA resin was assembled in situ DCC coupling of the five first residues utilizing
0.16 M of Boc-[Taqg]-OH, Boc-Aaqg-OBH or Boc-Aaqg-OH,
together with 0.16 M DCC in 2.0 ml 50% DMF/CH3Cl
(“Synthetic Protocol 9”) and by analogous in situ DCC coupling of the last five residues (“Synthetic Protocol 10”).
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 nearly quantitative incor-
poration of all residues except of the first A[Z] residue,
which had to be coupled twice. The total coupling yield was
about 96% (first coupling, about 89% efficiency).

(b) Cleavage, Purification, and Identification of H-[Taqg]-
Aaqg-Taqg-Caqg-Aaqg-Taqg-Caqg-Lys-NH2

The protected Boc-[Taqg]-[Aaqg]-Taqg-Caqg-Aaqg-Caqg-Taqg-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about
53.4 mg of crude material upon HCl cleavage of 166.1 mg dry.
Boc-[Taqg]-[Aaqg]-Taqg-Caqg-Aaqg-Caqg-Taqg-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about
53.4 mg of crude material upon HCl cleavage of 166.1 mg dry.
For (M+H)+, the calculated m/z value = 2780.17 and the measured m/z value=2780.07.
EXAMPLE 74

Hybridization Properties of H-TTA TCA TCT C-Lys-NH₂

The title compound hybridized with the following oligonucleotides:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>pH</th>
<th>Tm(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-AAT AGT AGT G-3'</td>
<td>5</td>
<td>31.51</td>
</tr>
<tr>
<td>5'-ATT AGT AGT G-3'</td>
<td>7.2</td>
<td>28.51</td>
</tr>
<tr>
<td>5'-AAT AGT AGT G-3'</td>
<td>9</td>
<td>28.01</td>
</tr>
<tr>
<td>5'-GTO AGT AFA A-3'</td>
<td>7.2</td>
<td>30.5</td>
</tr>
<tr>
<td>5'-GTO AGT AFA A-3'</td>
<td>9</td>
<td>28.0</td>
</tr>
</tbody>
</table>

†Low hypochromicity

EXAMPLE 75

Synthesis of a PNA with Two Parallel Strings Tied Together

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% disopropylethylamine in DCM (2 min.), followed by washing with DCM (2 ml; 6x1 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 DDC (64.9 mg; 0.315 mmol) dissolved in 1 ml of DCM. After 2.5 hours, the resin was washed with DCM 3 times (1 min.) and once with DCM (1 min.). The uncleaved amino groups were then capped by treatment with acetic anhydride/DCM/pyridine (1 ml:2 ml:2 ml) for 72 hours. After washing with DCM (2 ml; 4x1 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 DHBT 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 coupling, 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, 4, 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(T)</th>
<th>DCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>173 mg</td>
<td>95 mg</td>
</tr>
<tr>
<td>2.</td>
<td>176 mg</td>
<td>101 mg</td>
</tr>
<tr>
<td>4.</td>
<td>174 mg</td>
<td>97 mg</td>
</tr>
</tbody>
</table>

For the PNA having the Structure (70) where R₂₆₆=₆₆, there was 24.5 mg of crude product which resulted in 6.9 mg. after purification. For the PNA where R₂₆₆=₂₆, 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-(T₂₆₆) and PNA-(T₂₆) were hybridized to (d6A)₆ and (d6A)₆, respectively, with recorded Tm of 42° C. and 59° C., respectively.

EXAMPLE 76

Solid-Phase Synthesis of H-[Taqg]₄-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 sulfosuccinimidyl 6-(Bocamino)-hexanoic acid DHBT 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 coupling, 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, 4, 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(T)</th>
<th>DCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>173 mg</td>
<td>95 mg</td>
</tr>
<tr>
<td>2.</td>
<td>176 mg</td>
<td>101 mg</td>
</tr>
<tr>
<td>4.</td>
<td>174 mg</td>
<td>97 mg</td>
</tr>
</tbody>
</table>

EXAMPLE 77

Solid-Phase Synthesis of H-[Taqg]₄-[apgT]-[Taqg]₄-Lys-NH₂

Synthesis was initiated on approximately ¼ of the wet H-[Taqg]₄-Lys(CIZ)-MBHA resin from Example 76. In situ diisopropyl carbodiimide (DIC) couplings of both Boc-(apgT)-OH and Boc-Taqg-OH were carried out in 1.2 ml 50% DME/CH₂Cl₂ using 119.04 mg (0.12 mmol) and 0.046 g (0.12 mmol) monomer, respectively, and 18.7 μl (0.12 mmol) diisopropyl 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 2830.15 and the measured m/z value was 2830.92.

EXAMPLE 78

Solid-Phase Synthesis of H-[Taqg]₄-[proT]-[Taqg]₄-Lys-NH₂

Synthesis was initiated on approximately ¼ of the wet H-[Taqg]₄-Lys(CIZ)-MBHA resin from Example 76. In situ diisopropyl carbodiimide couplings of Boc-Taqg-OH were carried out in 1.2 ml 50% DME/CH₂Cl₂ using 119.04 mg (0.12 mmol) monomer and 18.7 μl (0.12 mmol) diisopropyl carbodiimide in each coupling. Due to solubility problems, Boc-(proT)-OH 0.048 g (0.12 mmol) was suspended in 2.5 ml 50% DME/DMF 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 yield. The PNA oligomer was cleaved and purified by standard procedures.

**EXAMPLE 79**

Hybridization Properties of H-[Taeg]-[proT]-[Taeg]_5-Lys-NH₂

<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>5'-AAA AAG AAA A</td>
<td>44.0</td>
</tr>
<tr>
<td>5'-AAA ACA AAA A</td>
<td>43.5</td>
</tr>
<tr>
<td>5'-AAA AAC AAA A</td>
<td>46.5</td>
</tr>
<tr>
<td>5'-AAA ADA AAA A</td>
<td>46.5</td>
</tr>
<tr>
<td>5'-AAA AAT AAA A</td>
<td>46.0</td>
</tr>
</tbody>
</table>

**EXAMPLE 80**

Solid-Phase Synthesis of H-[Taeg]_5-[bC]-[Taeg]_5-Lys-NH₂

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 quantitative ninhydrin reaction. The stepwise synthesis of the oligomer followed synthetic Protocol 9 employing 0.023 g (0.06 mmol) BocTaeg-OH, 0.062 g (0.12 mmol) Boc(bC)Z-OH and 0.012 g (0.06 mmol) DCC in 1.2 ml 50% DME/CH₂Cl₂ 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-T₄₆bC[T₄₆bC]₅-Lys-NH₂

<table>
<thead>
<tr>
<th>Oligodeoxynucleotide</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-AAA AAA AAA A</td>
<td>43.5</td>
</tr>
<tr>
<td>5'-AAA AAG AAA A</td>
<td>55.0</td>
</tr>
<tr>
<td>5'-AAA ACA AAA A</td>
<td>60.0</td>
</tr>
<tr>
<td>5'-AAA AAC AAA A</td>
<td>34.5</td>
</tr>
<tr>
<td>5'-AAA ACA AAA A</td>
<td>34.5</td>
</tr>
<tr>
<td>5'-AAA ADA AAA A</td>
<td>34.0</td>
</tr>
<tr>
<td>5'-AAA AAT AAA A</td>
<td>36.0</td>
</tr>
</tbody>
</table>

**EXAMPLE 82**

Stepwise Assembly of H-[Taeg]_5-[Taeg]-[Taeg]-[Aaeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-Lys-NH₂

Synthesis was initiated on a Boc-[Taeg]₂₅-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)₅-Taeg-OH-monomer). New step 5 (incorporation of A(Z)₅-Taeg) involved addition of 4 equiv. diisopropyl carbodiimide (0.06 mmol; 9.7 μl) and 4 equiv. BocA(Z)₅-Taeg-OH (0.06 mmol; 32 mg) 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 1x15 min. and 1x60 min. (recoupling).

Capping of uncoupled amino groups was only carried out before the incorporation of the Boc(A(Z)₅-Taeg-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-T₄₆AT₅-LysNH₂

<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'-AAA AOA AAA A</td>
<td>44.0</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 AAC AAA A</td>
<td>48.0</td>
</tr>
<tr>
<td>5'-AAA ADA 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]-[Aaeg]-[Gaeg]-[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 53, 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(Bz)Taeg-monomer there were some difficulties getting the resin to swell properly. Three hours of shaking in neat DCM gave acceptable swelling. For the incorporation of residues Taeg-4, G(Bz) aeg-6, and Taeg-7 to Taeg-10, recoupling was necessary to obtain near quantitative coupling yields. Taeg₅ (2x in 50% DME/DMF),Gaeg₅ (2x in 50% DME/DMF), Taeg₇ (2x in 50% DME/DMC, 1x in 50% NMP/DCM and 1x in neat DCM), Taeg₉ (1x in 50% DME/DMC and 2x in neat DCM), Taeg₁₀ (2x in 50% DME/DMC). 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**

Hybridization Properties of Crude (approx. 50%) H-T₄₆G₅₂TG₅-G-LysNH₂

<table>
<thead>
<tr>
<th>Oligodeoxynucleotide</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-AAC2ACAC</td>
<td>38</td>
</tr>
<tr>
<td>5'-CAC2AC2A4</td>
<td>55</td>
</tr>
</tbody>
</table>

**EXAMPLE 87**


(a) Stepwise Assembly of Boc-[Taeg]₅₁₅₁-Lys(CIZ)-MBHA Resin and Shorter Fragments

About 9 g of wet Boc-[Taeg]₅₁₅₁-Lys(CIZ)-MBHA (see, Example 19b) resin was placed in a 60 ml SPPS reaction
vessel. Boc-[Taeg]-Lys(CIZ)-MBHA resin was assembled by single coupling of both residues with 0.15 M of Boc-Taeg-OPfp in 10 ml neat CH_2Cl_2 ("Synthetic Protocol 8"). Both coupling reactions were allowed to proceed overnight. The synthesis was monitored by the ninhydrin reaction, which showed close to quantitative incorporation of both residues. After deprotection of the N-terminal Boc group, about 4.5 g of H-[Taeg]-Lys(CIZ)-MBHA was placed in a 20 ml SPPS reaction vessel and elongated to Boc-[Taeg]-Lys(CIZ)-MBHA by single in situ DCC coupling of all residues (close to quantitative, except for residue number eight) overnight with 0.2 M of Boc-Taeg-OH together with 0.2 M DCC in 7.5 ml neat CH_2Cl_2 ("Synthetic Protocol 9"). Before coupling of Taeg residues number seven and eight, respectively, small portions of H-[Taeg]-Lys(CIZ)-MBHA and H-[Taeg]-Lys(CIZ)-MBHA, respectively, were taken out for HF cleavage. Taeg residue number eight was coupled twice (overnight) to give close to quantitative incorporation. After deprotection of the N-terminal Boc group, a large portion of H-[Taeg]-Lys(CIZ)-MBHA was taken out for HF cleavage. Boc-[Taeg]-Lys(CIZ)-MBHA resin was assembled by double in situ DCC coupling of 0.16 M Boc-Taeg-OH, together with 0.16 M DCC in 20 ml 50% DMF/CH_2Cl_2 ("Synthetic Protocol 9"). Before coupling of the final residue, a small portion of H-[Taeg]-Lys(CIZ)-MBHA was taken out for HF cleavage.

(b) Cleaveage, Purification, and Identification of H-[Taeg]-Lys-NH_2.

The protected Boc-[Taeg]-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 14.0 mg of crude material upon HF cleavage of 52.4 mg dry H-[Taeg]-Lys(CIZ)-MBHA resin. The crude product was not purified (about 99% purity).

(c) Cleaveage, Purification, and Identification of H-[Taeg]-Lys-NH_2.

The protected Boc-[Taeg]-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 5.2 mg of crude material upon HF cleavage of 58.4 mg dry H-[Taeg]-Lys(CIZ)-MBHA resin.

(d) Cleaveage, Purification, and Identification of H-[Taeg]-Lys-NH_2.

The protected Boc-[Taeg]-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 114 mg of crude material upon HF cleavage of about 604 mg dry H-[Taeg]-Lys(CIZ)-MBHA resin.

(e) Cleaveage, Purification, and Identification of H-[Taeg]-Lys-NH_2.

The protected Boc-[Taeg]-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 19.3 mg of crude material upon HF cleavage of 81.0 mg dry H-[Taeg]-Lys(CIZ)-MBHA resin.

(f) Cleaveage, Purification, and Identification of H-[Taeg]-Lys-NH_2.

The protected Boc-[Taeg]-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 141 mg of crude material upon HF cleavage of about 417 mg dry H-[Taeg]-Lys(CIZ)-MBHA resin.

(g) Synthetic Protocol 8 (General Protocol)

1. Boc-deprotection with TFA/CH_2Cl_2 (1:1, v/v), 3x1 min and 1x30 min; (2) washing with CH_2Cl_2, 6x1 min; (3) neutralization with DIEA/CH_2Cl_2 (1:19, v/v), 3x2 min; (4) washing with CH_2Cl_2, 6x1 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 (Pfp ester); the coupling reaction was allowed to proceed for a number of hours with shaking; (7) washing with DMF, 1x2 min; (8) washing with CH_2Cl_2, 4x1 min; (9) neutralization with DIEA/CH_2Cl_2 (1:19, v/v), 2x2 min; (10) washing with CH_2Cl_2, 6x1 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/CH_2Cl_2 (1:1:2, v/v/v) for 2 h followed by washing with CH_2Cl_2, 6x1 min, and, occasionally, ninhydrin analysis.

EXAMPLE 88

Solid-Phase Synthesis of H-[Taeg]-Lys(CIZ)-MBHA Resin

(a) Stepwise Assembly of Boc-[Taeg]-Lys(CIZ)-MBHA Resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]-Lys(CIZ)-MBHA resin was assembled by in situ DCC coupling of all residues utilizing 0.16 M of Boc-[Taeg]-OH together with 0.16 M DCC in 20 ml 50% DMF/CH_2Cl_2 or 0.16 M Boc-Taeg-OH together with 0.16 M DCC in 20 ml 50% DMF/CH_2Cl_2 ("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 about 98% incorporation of [CZ]taeg and close to quantitative incorporation of all the Taeg residues.

(b) Cleaveage, Purification, and Identification of H-[Taeg]-Lys(CIZ)-MBHA Resin was treated as described in Example 17c to yield about 22.5 mg of crude material upon HF cleavage of 128.2 mg dry H-[Taeg]-Lys(CIZ)-MBHA resin. Crude product (5.8 mg) was purified to give 3.1 mg of H-[Taeg]-Lys(CIZ)-MBHA resin.

(c) Synthetic Protocol 9 (General Protocol)

1. Boc-deprotection with TFA/CH_2Cl_2 (1:1, v/v), 3x1 min and 1x30 min; (2) washing with CH_2Cl_2, 6x1 min; (3) neutralization with DIEA/CH_2Cl_2 (1:19, v/v), 3x2 min; (4) washing with CH_2Cl_2, 6x1 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 CH_2Cl_2; the coupling reaction was allowed to proceed for a total of Y hrs shaking; (7) washing with DMF, 1x2 min; (8) washing with CH_2Cl_2, 4x1 min; (9) neutralization with DIEA/CH_2Cl_2 (1:19, v/v), 2x2 min; (10) washing with CH_2Cl_2, 6x1 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/CH_2Cl_2 (1:1:2, v/v/v) for 2 h followed by washing with CH_2Cl_2, 6x1 min, and, occasionally, ninhydrin analysis.

EXAMPLE 89

Solid-Phase Synthesis of H-[Taeg]-[N-Baeg]-[Taeg]-Lys(NH_2) (NB=COCH_3)

(a) Stepwise Assembly of Boc-[Taeg]-[N-Baeg]-[Taeg]-Lys(CIZ)-MBHA Resin was assembled by

About 1 g of wet Boc-[Taeg]-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]-[N-Baeg]-[Taeg]-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 or 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DME/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 95% after the second coupling) to close quantitative incorporation of all the Taeg residues.  

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

The protected Boc-[Taeg]-[NBaeq]-[Taeg]-[5-Lys]-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]-[NBaeq]-[Taeg]-[5-Lys]-MBHA resin. Crude product (2.06 mg) was purified to give 4.6 mg of H-[Taeg]-[NBaeq]-[Taeg]-[5-Lys]-NH₂. For (M+H)+, the calculated m/z value was 2683.12 and the measured m/z value was 2683.09.

EXAMPLE 90

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

(a) Stepwise Assembly of Boc-[Taeg]-4-aeg-[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-aeg]-[Taeg]-[5-Lys](CIZ)-MBHA resin was assembled by in situ DCC single coupling of all residues utilizing: (1) 0.16 M of Bocaeq-OH together with 0.16 M DCC in 2.0 ml 50% DME/CH₂Cl₂ or (2) 0.16 M BocTaeg-OH together with (2) 0.16 M DCC in 2.0 ml 50% DME/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-aeg-[Taeg]-[5-Lys]-NH₂

The protected Boc-[Taeg]-4-aeg-[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-aeg-[Taeg]-[5-Lys](CIZ)-MBHA resin. Crude product (22.2 mg) was purified to give 7.6 mg of H-[Taeg]-4-aeg-[Taeg]-[5-Lys]-NH₂. 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% DME/CH₂Cl₂ or (2) 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DME/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. For (M+H)+, the calculated m/z value was 2747.15 and the measured m/z value was 2746.78.

EXAMPLE 92

Solid-Phase Synthesis of H-[Taeg]-4-Gly2-[Taeg]-[5-Lys]-NH₂

(a) Stepwise Assembly of Boc-[Taeg]-4-Gly2-[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% DME/CH₂Cl₂ or (2) 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DME/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-Gly2-[Taeg]-[5-Lys]-NH₂

The protected Boc-[Taeg]-4-Gly2-[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-Gly2-[Taeg]-[5-Lys](CIZ)-MBHA resin. Crude product (30 mg) was purified to give 7.8 mg of H-[Taeg]-4-Gly2-[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-[Caeg2]-2-Taeg-Caeg-Taeg-Caeg-Lys-NH₂

(a) Stepwise Assembly of Boc-[Taeg]-[Caeg2]-2-Taeg-[CZaeg]-Taeg-[CZaeg]-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]-[Caeg2]-2-Taeg-[CZaeg]-Taeg-[CZaeg]-Lys(CIZ)-MBHA resin was assembled by in situ DCC single coupling of all residues utilizing: (1) 0.16 M of Boc[Zh]-OH together with 0.16 M DCC in 2.0 ml 50% DME/CH₂Cl₂ or (2) 0.16 M BocTaeg-OH together with 0.16 M DCC in 2.0 ml 50% DME/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]-[Caeg2]-2-Taeg-Caeg-Taeg-Lys-NH₂

The protected Boc-[Taeg]-[Caeg2]-2-Taeg-CZaeg-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 52.1 mg of crude material upon HF cleavage of 216.7 mg dry H-[Taeg]-[Caeg2]-2-Taeg-CZaeg-Lys(CIZ)-MBHA resin. Crude product (50.6 mg) was purified to give 6.2 mg of H-[Taeg]-[Caeg2]-2-Taeg-Caeg-Taeg-Lys-NH₂. For (M+H)+, the calculated m/z value was 2747.15 and the measured m/z value was 2746.78.
EXAMPLE 94

Solid-Phase Synthesis of H-Caeg-Taeg-Caeg-Taeg-[Caeg]3-Taeg-Caeg-Taeg-Lys-NH2

(a) Stepwise Assembly of Boc-(CZ)Zaeg-Taeg-(CZ)Zaeg-Taeg-[C Zaeg3]-Taeg-(CZ)Zaeg-Taeg-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-(CZ)Zaeg-Taeg-(CZ)Zaeg-Taeg-(CZ)Zaeg-Taeg-[C Zaeg3]-Taeg-(CZ)Zaeg-Taeg-Lys(CIZ)-MBHA resin was assembled by situ DCC single coupling of all residues utilizing: (1) 0.16 M of Boc-(CZ)OH together with 0.16 M DCC in 2.0 ml 50% DME/CH2Cl2 or (2) 0.16 M Boc-Taeg-OH together with 0.16 M DCC in 2.0 ml 50% DME/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-Caeg-Taeg-Caeg-Taeg-[Caeg]3-Taeg-Caeg-Taeg-Lys-NH2

The protected Boc-(CZ)Zaeg-Taeg-[C Zaeg3]-Taeg-(CZ)Zaeg-Taeg-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-(CZ)Zaeg-Taeg-[C Zaeg3]-Taeg-(CZ)Zaeg-Taeg-Lys(CIZ)-MBHA resin. Crude product (85.8 mg) was purified to give 46.2 mg of H-Caeg-Taeg-Caeg-Taeg-[Caeg]3-Taeg-Caeg-Taeg-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-(CZ)Zaeg-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA resin was assembled by situ DCC single coupling of all residues utilizing: (1) 0.16 M of Boc-(CZ)OH together with 0.16 M DCC in 3.0 ml 50% DME/CH2Cl2 or (2) 0.16 M Boc-Taeg-OH together with 0.16 M DCC in 3.0 ml 50% DME/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. 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-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys-NH2

The protected Boc-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-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-(CZ)Zaeg-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA resin. Crude product (50.9 mg) was purified to give 13.7 mg of H-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys-NH2. 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-Tyr2-[C Zaeg3]-Taeg-[C Zaeg2]-Lys-NH2

The protected Boc-(BrZ)-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-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-(BrZ)-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA resin. Crude product (60.8 mg) was purified to give 20.7 mg of H-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys-NH2. 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-[C Zaeg3]-Taeg-[C Zaeg2]-Lys-NH2

The protected Boc-(BrZ)-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-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-(CZ)Zaeg-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA resin. Crude product (11.6 mg) was purified to give 3.1 mg of H-Caeg-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys-NH2. 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-(CZ)Zaeg-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA Resin, Boc-Taeg-(BrZ)-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA Resin, and Boc-(BrZ)-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA Resin

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-(CZ)Zaeg-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA resin was assembled by situ DCC single coupling of all residues utilizing: (1) 0.16 M of Boc-(CZ)OH together with 0.16 M DCC in 3.0 ml 50% DME/CH2Cl2 or (2) 0.16 M Boc-Taeg-OH together with 0.16 M DCC in 3.0 ml 50% DME/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. 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 Taeg residue. Both couplings employed the above-mentioned synthetic protocol.

(b) Cleavage, Purification, and Identification of H-(Taeg2)-Caeg-[Taeg3]-3-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys-NH2

The protected Boc-(CZ)Zaeg-Taeg-[C Zaeg3]-3-Taeg-[C Zaeg2]-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-(CZ)Zaeg-Taeg-[C Zaeg3]-3-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA resin. Crude product (57.6 mg) was purified to give 26.3 mg of H-(Taeg2)-Caeg-[Taeg3]-3-Taeg-[C Zaeg2]-Lys-NH2. 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-Taeg2-[C Zaeg3]-Taeg-[C Zaeg2]-Lys-NH2

The protected Boc-(BrZ)-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-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-(BrZ)-Taeg-[C Zaeg3]-Taeg-[C Zaeg2]-Lys(CIZ)-MBHA resin. Crude product (47.1 mg) was purified to give 13.4 mg...
of H-Tyr-[Caeg]-[Taaeg]-Lys-NH₂. 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-Tae-[C(Z)aaeg]-[Taaeg]-[C(Z)aaeg]-[Taaeg]-Lys-NH₂.

The protected Boc-Taeg-[C(Z)aaeg]-[Taaeg]-[C(Z)aaeg]-[Taaeg]-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-Taeg-[C(Z)aaeg]-[Taaeg]-Lys(CIZ)-MBHA resin. Crude product (11.9 mg) was purified to give 4.3 mg of H-Taeg-[C(Z)aaeg]-[Taaeg]-[C(Z)aaeg]-[Taaeg]-Lys-NH₂. 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 Moiety for Scale Up by Reductive Amination

(a) Preparation of (bocamino)acetalddehyde

3-Amino-1,2-propanediol (80.0 g; 0.88 mmol) was dissolved in water (1500 ml) and the solution was cooled to 4 °C, whereafter Boc anhydride (230 g; 1.05 mol) was added at once. The solution was gently heated to room temperature with a water bath. 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 the pH was adjusted to 2.5 by the addition of 4 M hydrochloric acid. The ethyl acetate layer was removed and the acidic aqueous solution was extracted with more ethyl acetate (6x500 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.

It then was dried over magnesium sulphate and evaporated to dryness, in vacuo. Yield. 145.3 g (86%).

(b) Preparation of (N-bocaminoethyl)glycine Methyl Ester Palladium on carbon (10%; 2.40 g) was added to a solution of (bocamino)acetalddehyde (10.0 g; 68.9 mmol) in methanol (150 ml) at 0 °C. Sodium acetate (113.3 g; 138 mmol) in methanol (150 ml), and glycine methyl ester hydrochloride (8.65 g; 68.9 mmol) in methanol (75 ml) then were added. The mixture was hydrogenated at atmospheric pressure for 2.5 h, then filtered through celite and evaporated to dryness, in vacuo. The material was redissolved in water (150 ml) and the pH was adjusted to 8.0 with 0.5 N NaOH. The aqueous solution was extracted with methylene chloride (5x130 ml). The combined extracts were dried over sodium sulphate and evaporated to dryness, in vacuo. This resulted in 14.1 g (88%) of (N-bocaminoethyl)glycine methyl ester.

The crude material was purified with kuglerrohr distillation at 84 °C and 0.3 mmHg in two portions. The yield 79 g (77%) of a colorless oil. The product had a purity that was higher than the material produced in example 26 according to tlc-analysis (10% methanol in methylene chloride).

Alternatively, sodium cyanoborohydride can be used as reducing agent instead of hydrogen (with Pd(C) as catalyst), although the yield (42%) was lower.

(c) Preparation of (N-bocaminoethyl)glycine Ethyl Ester

The title compound was prepared by the above procedure with glycine ethyl ester hydrochloride substituted for glycine methyl ester hydrochloride. Also, the solvent used was ethanol. The yield was 78%.

EXAMPLE 98

Solid-Phase Synthesis of H-Tyr-[Taeeg]-Lys-NH₂

(a) Stepwise Assembly of Boc-Tyr(Bz)-[Taaeg]-Lys(CIZ)-MBHA Resin

About 0.2 g of wet Boc-[Taeeg]-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-Tyr(Bz)-[Taaeg]-Lys(CIZ)-MBHA resin was assembled by standard in situ DCC coupling utilizing 0.32 M of BocTyr(Bz)-OH together with 0.32 M DCC in 3.0 ml neat CH₂Cl₂ overnight. The ninhydrin reaction showed about 95% incorporation of BocTyr(Bz).

(b) Cleavage, Purification, and Identification of H-Tyr-[Taaeg]-Lys-NH₂

The protected Boc-Tyr(Bz)-[Taaeg]-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 5.5 mg of crude material upon HF cleavage of 20.7 mg dry H-Tyr(Bz)-[Taaeg]-Lys(CIZ)-MBHA resin. The crude product was purified to give 2.5 mg of H-Tyr-[Taaeg]-Lys-NH₂.

EXAMPLE 99

Solid-Phase Synthesis of Dansyl-[Taeeg]-Lys-NH₂

(a) Stepwise Assembly of Dansyl-[Taaeg]-Lys(CIZ)-MBHA Resin

About 0.3 g of wet Boc-[Taeeg]-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Dansyl-[Taaeg]-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-[Taaeg]-Lys-NH₂

The protected dansyl-[Taaeg]-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-[Taaeg]-Lys(CIZ)-MBHA resin. The crude product was purified to give 5.4 mg of dansyl-[Taaeg]-Lys-NH₂.

EXAMPLE 100

Solid-Phase Synthesis of Gly-Gly-His-[Taeeg]-Lys-NH₂

(a) Stepwise Assembly of Boc-Gly-Gly-His(Tos)-[Taaeg]-Lys(CIZ)-MBHA Resin

About 0.05 g of Boc-[Taaeg]-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-Gly-Gly-His(Tos)-[Taaeg]-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 (0.1 M) 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]₉-Lys-NH₂

The protected Boc-Gly-Gly-His(Tos)-[Taeg]₉-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]₉-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]₉-Lys-NH₂.

EXAMPLE 101

Solid-Phase Synthesis of H-[Taeg]₉-[Caeg]₉-NH₂

(a) Stepwise Assembly of Boc-[Taeg]₉-[C(Z)aeq]₉-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. BocC(Z)aeq-OH 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]₉-[C(Z)aeq]₉-MBHA resin was assembled by single in situ DCC coupling of the next residue utilizing 0.11 M of BocC(Z)aeq-OH together with 0.11 M DCC in 2.5 ml 50% DMF/CH₂Cl₂ and by coupling with 0.13 M BocTaeg-OH 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]₉-[Caeg]₉-NH₂

The protected Boc-[Taeg]₉-[C(Z)aeq]₉-MBHA resin was 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 Boc-[Taeg]₉-[C(Z)aeq]₉-MBHA resin. Crude product (7.4 mg) was purified to give 2.0 mg of H-[Taeg]₉-[Caeg]₉-NH₂ (>99% purity).

EXAMPLE 102

Solid-Phase Synthesis of H-[Taeg]₉-[Caeg]-[Taeg]₉-NH₂

(a) Stepwise Assembly of Boc-[Taeg]₉-[C(Z)aeq]-[Taeg]₉-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]₉-[C(Z)aeq]-[Taeg]₉-MBHA resin was assembled by single in situ DCC coupling of the C(Z)aeq residue utilizing 0.13 M of BocC(Z)aeq-OH together with 0.13 M DCC in 2.5 ml 50% DMF/CH₂Cl₂ and by coupling the Taeg residues with 0.13 M BocTaeg-OH 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]₉-[Caeg]-[Taeg]₉-NH₂

The protected Boc-[Taeg]₉-[C(Z)aeq]-[Taeg]₉-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]₉-[C(Z)aeq]-[Taeg]₉-MBHA resin. Crude product (11.0 mg) was purified to give 3.6 mg of H-[Taeg]₉-[Caeg]-[Taeg]₉-NH₂.

EXAMPLE 103

Solid-Phase Synthesis of H-Taeg-Caeg-[Taeg]₉-Lys(NH₂)

(a) Stepwise Assembly of Boc-[Taeg]-C(Z)aeq-[Taeg]₉-Lys(CIZ)-MBHA Resin

About 0.3 g of wet Boc-[Taeg]₉-Lys(CIZ)-MBHA resin was placed in a 3 ml SPPS reaction vessel. Boc-Taeg-C(Z)aeq-[Taeg]₉-Lys(CIZ)-MBHA resin was assembled by single in situ DCC coupling overnight of the C(Z)aeq residue (“Synthetic Protocol 9”) utilizing 0.2 M of BocC(Z)aeq-OH 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-OH in 2.5 ml neat CH₂Cl₂ (nearly quantitatively).

(b) Cleavage, Purification, and Identification of H-Taeg-Caeg-[Taeg]₉-Lys(NH₂)

The protected Boc-Taeg-C(Z)aeq-[Taeg]₉-Lys(CIZ)-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)aeq-[Taeg]₉-Lys(CIZ)-MBHA resin. Crude product (6.7 mg) was purified to give 2.6 mg of H-Taeg-Caeg-[Taeg]₉-Lys(NH₂). For (M+H)+ the calculated m/z value was 2792.15 and the measured m/z value was 2792.21.

EXAMPLE 104

Solid-Phase Synthesis of H-Caeg-[Taeg]-Lys-NH₂ and H-[Taeg]₉-Caeg-[Taeg]-[Taeg]-Lys(NH₂)

(a) Stepwise Assembly of Boc-[Taeg]₉-[C(Z)aeq]-[Taeg]-Lys(CIZ)-MBHA Resin

About 0.5 g of wet Boc-[Taeg]₉-Lys(CIZ)-MBHA resin was placed in a 5 ml SPPS reaction vessel. Boc-[Taeg]-C(Z)aeq-[Taeg]-Lys(CIZ)-MBHA resin was assembled by single in situ DCC coupling of all residues utilizing: (1) 0.12 M of BocC(Z)aeq-OH together with 0.12 M DCC in 3.0 ml 50% DMF/CH₂Cl₂ or (2) 0.12 M BocTaeg-OH together with 0.12 M DCC in 3.0 ml 50% DMF/CH₂Cl₂ (“Synthetic Protocol 9”). 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-[C(Z)aeq]-[Taeg]-Lys(CIZ)-MBHA resin was taken out for HF cleavage.

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

The protected Boc-[Taeg]-C(Z)aeq-[Taeg]-Lys(CIZ)-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-[C(Z)aeq]-[Taeg]-Lys(CIZ)-MBHA resin. About 0.7 mg of the crude product was purified to give about 0.5 mg of H-Caeg-[Taeg]-Lys(NH₂).

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

The protected Boc-[Taeg]-C(Z)aeq-[Taeg]-Lys(CIZ)-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]-C(Z)aeq-[Taeg]-Lys(CIZ)-MBHA resin.
EXAMPLE 105

Solid-Phase Synthesis of H-[Caeg]-Lys-NH₂, H-[Caeg]-Lys-NH₂, H-[Caeg]-Lys-NH₂, and H-[Caeg]-Lys-NH₂

(a) Stepwise Assembly of Boc-[C(Z)age]₃₀ '-Lys(CIZ)-MBHA Resin and Shorter Fragments

About 5 g of wet Boc-Lys(CIZ)-MBHA resin (substitution=0.5 mmol Lys/g) was mixed in a 50 ml SPS reaction vessel. Boc-[C(Z)age]₃₀ '-Lys(CIZ)-MBHA resin was assembled by single in situ DCC coupling of the first three residues with 0.1 M of Boc(C)Zage-OH together with 0.1 M DCC in 10 ml 50% DMF/CH₂Cl₂ ("Synthetic Protocol 9") and by single in situ DCC coupling of the remaining seven residues with 0.1 M of Boc(C)Zage-OH together with 0.1 M DCC in 1.0 ml 50% DMF/CH₂Cl₂ ("Synthetic Protocol 10"). 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 the residues. During the synthesis, portions of the shorter fragments H-[C(Z)age]₀'-Lys(CIZ)-MBHA resin, H-[C(Z)age]₀'-Lys(CIZ)-MBHA resin, H-[C(Z)age]₀'-Lys(CIZ)-MBHA resin, and H-[C(Z)age]₀'-Lys(CIZ)-MBHA resin were taken out for HF cleavage.

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

The protected Boc-[C(Z)age]₃₀ '-Lys(CIZ)-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-[C(Z)age]₀'-Lys(CIZ)-MBHA resin.

(c) Cleavage, Purification, and Identification of H-[Caeg]-Lys-NH₂

The protected Boc-[C(Z)age]₃₀ '-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 13.4 mg of crude material upon HF cleavage of 56.2 mg dry H-[C(Z)age]₀'-Lys(CIZ)-MBHA resin.

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

The protected Boc-[C(Z)age]₃₀ '-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 16.8 mg of crude material upon HF cleavage of 65.6 mg dry H-[C(Z)age]₀'-Lys(CIZ)-MBHA resin.

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

The protected Boc-[C(Z)age]₃₀ '-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 142.4 mg of crude material upon HF cleavage of 441 mg dry H-[C(Z)age]₀'-Lys(CIZ)-MBHA resin.

EXAMPLE 106

Solid-Phase Synthesis of H-[Taeg]-Caeag-[Taeg]-Caeag-[Taeg]-Lys-NH₂

(a) Stepwise Assembly of Boc-[C(Z)age]₃₀ 'Lys(CIZ)-MBHA Resin

About 0.3 g of wet H-[Taeg]-C(Z)age-[Taeg]-Lys(CIZ)-MBHA resin from the earlier synthesis of Boc(C)Zage-[Taeg]-Lys(CIZ)-MBHA resin was placed in a 5 ml SPS reaction vessel. After coupling of the next residue five times, a total incorporation of Boc(C)Zage of 87% was obtained. The five repeated couplings were carried out with 0.18 M Boc(C)Zage-OPip in 2 ml of TFE/CH₂Cl₂ (1:2, v/v), 2 ml of TFE/CH₂Cl₂ (1:2, v/v), 2 ml of TFE/CH₂Cl₂ (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/CH₂Cl₂ (1:2, v/v) plus 0.5 g phenol, and 1 ml of CH₂Cl₂ 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-OPip in 25% phenol/CH₂Cl₂. All couplings were allowed to proceed overnight.

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

The protected Boc-[Taeg]-C(Z)age-[Taeg]-C(Z)age-[Taeg]-Lys(CIZ)-MBHA resin was treated as described in Example 17c to yield about 7 mg of crude material upon HF cleavage of 80.7 mg dry H-[Taeg]-C(Z)age-[Taeg]-C(Z)age-[Taeg]-Lys(CIZ)-MBHA resin. The crude product was purified to give 1.2 mg of H-[Taeg]-Caeag-[Taeg]-Caeag-[Taeg]-Lys-NH₂ (>99.9% purity).

EXAMPLE 107

Synthesis of a PNA with Two Anti Parallel Strands Tied Together Synthesis of H-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-[Taeg]-Lys(NH₂)₆-6-AHA-6-amino-hexanoic 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 uncleaved amino groups was only carried out before the incorporation of the Boc(C)Zage-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 and the yellow precipitate was collected by filtration. Recrystallization from ethanol gave 14.2 g 92% of white pure crystals of the target compound. 1H-NMR (250 MHz, DMSO-d₆) d ppm: 8-H, 7.92; benzyl aromatic, 7.60–7.40; 2NH₂, 6.36; benzyl CH₂, 5.57.

(2-amino-6-O-benzyl purinyl)methylthioanato. 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; 1.9 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 twice 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; benzyl aromatic 7.4–7.6; 2NH₂, 6.61; benzyl CH₂, 5.03; CH₂, 5.59; OCH₃, 3.78.
(2-N-Toluene sulfonamido-6-O-benzyl purine)methyl ethanoate. To a solution of 0.5 g (1.6 mmol) of (2-amino-6-O-benzyl purine)methyl ethanoate in 25 ml methylene chloride was added 0.53 g (1.62 mmol) of p-toluene-sulfonyl 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 pressure (15 mmHg, 40°C). Dicyethyl 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) δ ppm: 8-H: 8.47, aromatic 7.2-7.8; benzyl CH2: 5.01; CH2: 4.24; OCH3: 3.73; CH3: 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 RCM 8×10 Nova pack column and solvents 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.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A</th>
<th>% B</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
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<td>35</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>37</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>39</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

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 (7.23 g; 52.3 mmol) were suspended in DMF (75 ml). Methyl bromacetate (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 (C5H=CH); 8.02 (impurity); 4.76 (impurity); 4.70 (impurity); 4.62 (CNC=OAcO); 3.78 (COClCH3); 2.96 (DMF); 2.80 (DMF). 13C-NMR (DMSO-d6, 250 MHz): 168.8 (COOCH3); 172.5 (CH=CBrCON); 161.6 (DMF); 151.9 (NCON); 145.0 (CO=C=BrCH3); 95.6 (COBr=CH); 52.6 (impurity); 52.5 (OCH3); 49.7 (impurity); 48.8 (NCH2COOMe); 43.0 (impurity); 36.0 (DMF). UV(MeTHLol, max nm): 226; 278. IR (KBr/cm-1): 3158s (C=C, O=COOME); 1701s (C=C, CONH); 1438s(δ CH, CH2O); 1223s (C=O, COOMe); 864 m (δ CH, Br=C=H). FAB-MS m/z (assignment): 265/263 (M+H).
EXAMPLE 112

N-(Boc-aminoethyl)-N-(5-bromomaurycyl-N'-methylcarbonyl)glycine

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. The water phase was extracted with ethyl acetate (3x65 ml+7x40 ml). The combined acetate extractions were washed with saturated NaCl-solution (500 ml). The ethyl acetate phase was dried over magnesium sulfate, filtered, and evaporated to dryness in vacuo. Yield: 1.77 g (96%).

C: 40.79; (40.10); H: 15.24; (15.17); N: 11.35; (12.47).

H-NMR (DMso-d6, 125 MHz, J in Hz):
- 12.85 (1H,H,COOH); 11.93 & 11.91 (2H,CH=C=ONH=CO); 8.10 & 8.07 (2H,CH=C=CH=CH=CO); 7.00 & 6.81 (2H,H,BOC-NH); 4.79 & 4.61 (2H,CH=N,CONH); 4.37 & 4.25 (2H,CH=N,CONH); 3.46 - 3.39 (2H,CH=NH,CH2,N); 3.26 - 3.23 & 3.12 - 3.09 (2H,CH=NH,CH2,N); 1.46 (9H,H,BOC).

EXAMPLE 113

Uracil-N4-methyl Acetate

Uracil (10.0 g; 82.92 mmol) and potassium carbonate (24.7 g; 178 mmol) were suspended in DMF (250 ml). Methyl bromoacetate (8.45 ml; 89.22 mmol) was added over a period of 5 min. The suspension was stirred overnight under nitrogen at room temperature, and then filtered. TLC (10% methanol in ethyl chloride) indicated incomplete conversion of uracil. The solid residium was washed twice with DMF, and the combined filtrates were evaporated to dryness in vacuo. The precipitate was suspended in water (60 ml) and HCl (2.5 ml, 4M) was added to pH = 2. The suspension was stirred for 30 min at 0°C, and then filtered. The precipitated title compound was washed with water and dried, in vacuo, over sicapent. Yield: 9.91 g (60%).

Mp: 182° - 183° C. Anal. for C11H12N4O4. Found (calc.): C, 45.38; (45.66); H, 4.29; (4.38); N, 15.00; (15.21). 1H-NMR (DMso-d6, 250 MHz, J in Hz):
- 7.17 (1H,H,CH=NH); 6.78 (1Hd, Jpc = 10.6, 7.9); 5.69 (1Hd, Jpc = 10.6, Jpe = 1.9); 5.17 (CH=CH=CH=CH=CO); 5.10 (1H,H,CH=CH=CH=CH=CO); 4.57 (1Hd, Jpc = 10.6, Jpe = 1.9); 3.76 (3Hs, COOCOMe); 3.18 (NH, CONH); 2.68 (COOMe); 164.0 (C=O); 149.0 (COON) 146.1 (COCH=CH); 101.3 (COCH=CH); 52.5 (COCH=CH); 48.7 (NCH=COOMe).

UV (Methanol; nm): 266, 261. IR (KBr; cm⁻¹): 3164s (NH); 1738s (CH=O, CONH); 1450vs (CH=CH, HO); 1243vs (C=O, COOMe); 701m (CH, H—C=C—H). FAB-MS m/z (assignment, relative intensity): 185 (M+1).

EXAMPLE 114

Uracilacetamide

Water (90 ml) was added to the product of Example 113 (8.76 g; 47.5 mmol), followed by sodium hydroxide (2M, 40 ml). The mixture was heated for 40 min, until all the methyl ester has reacted. After stirring at 0°C for 15 min, hydrochloric acid (4M, 25 ml) was added to pH = 2. The title compound precipitated and the mixture was filtered after 2-3 h. The precipitate was washed twice with the mother liquor and twice with cold water and dried in vacuo over sacipent. Yield: 6.66 g (82%).

Mp: 288° - 289° C. Anal. for C16H13N3O4. Found (calc.): C, 42.10; (42.36); H, 3.43; (3.55); N, 16.25; (16.47). 1H-NMR (DMso-d6, 250 MHz, J in Hz):
- 13.19 (1Hs,COOH); 11.41 (1Hs,CONH); 7.69 (1Hd, Jpc = 10.6, Jpe = 7.9); 5.17 (1Hd, Jpc = 10.6, Jpe = 7.9); 4.49 (2H,CH=N,CONH); 3.18 (3Hs,COOMe); 2.68 (COOMe); 169.9 (COOH); 163.9 (CH=CHCON); 151.1 (CON); 146.4 (COCH=CH); 140.9 (COOH); 48.7 (NCH=COOH). UV (Methanol; nm): 264; 263. IR (KBr; cm⁻¹): 3122s (NH); 1703vs (C=O, COOH); 1698vs (C=O, CONH); 1205s (C=O, COOMe); 676 (CH, H—C=C—H). FAB-MS m/z (assignment): 171 (M+1).

EXAMPLE 115

N-(Boc-aminoethyl)-N-ureidyl-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 DCC was washed twice with methylene chloride. To combined filtrate was added methylene chloride (100 ml), and the solution was 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 1:1 v/v) and finally with saturated NaCl-solution (1x100 ml). The organic phase was dried over magnesium sulfate, filtered and evaporated to dryness in vacuo (about 15 mmHg and then about 1 mmHg). The residue was dissolved in methylene chloride (32 ml), and stirred for 35 min at room temperature, and 30 min at 0°C, and then filtered. The precipitated (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/CHCl3 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° - 75.5° C. Anal. for C16H13N3O4. Found (calc.): C, 50.61; (51.25); H, 6.48; (6.58); N, 13.33; (14.06). 1H-NMR


**EXAMPLE 116**

N-(Bocaminoethyl)-N-(uracilmenthoxycarbonyl) 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=2.0. The water phase was extracted by ethyl acetate (3x50 ml+7<30 ml). The combined ethyl acetate was washed with saturated NaCl solution (360 ml). The ethyl acetate phase was then evaporated to dryness, 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 C₉H₈N₂O₄: Found (calc.), C, 46.68; (48.65); H, 6.03; (5.90); N, 41.46; (15.13). ¹H-NMR (DMSO-d₆, 250 MHz, J in Hz): 12.83 (1H, s, COOD); 11.36 (1H, s, C=OHNJCOC=O); 7.52-7.45 (1H, m, COOH=C=OH); 7.00 & 6.82 (1H, t, Boc=N); 5.07 & 5.62 (1H, M's, COCH=CH); 4.76 & 4.58 (2H, s, N=COHN); 4.26 & 4.05 (2H, s, N=COH); 3.46-3.39 (2H, m's, Boc=NHCH=CHNH); 3.25-3.23 & 3.15-3.09 (2H, t, Boc=N=CHN=CH=CHN); 1.46 (9H, s, 'Bu). ¹³C-NMR (DMSO-d₆, 250 MHz): 170.5 (BaCO₃; 0); 167.2 (COOH); 163.9 (C=O); 155.8 (N=COH); 151.1 (N=COH); 146.4 (COHN); 130.8 (COOH=C=OH); 78.1 (MeC=O); 49.1 & 47.8 (N=CH₂COOH); 47.6 & 46.9 (N=COH); 38.6 & 38.1 (O=COH); 38.1 & 37.6 (O=COH); 28.2 (C=CH₂). UV (Methanol; maxnm): 226; 264, IR (KBr, cm⁻¹): 3053m (N=H); 1685vs, vbroad (∼C=O), 1125s (C=O), 1372s (COH); 1178 (COOH); 1581 (N=COH); 147.1 (N=COH). FAB-MS m/z (assignment, relative intensity): 399 (M+H⁺; 35); 343 (M+2H⁺-H₂O, 200); 299 (M+2H⁺-Boc, 100); 153 (M-backbone, 30).

**EXAMPLE 117**

H-U10-LysNH₂

Synthesis of the title compound was accomplished by using “Synthetic Protocol 10”. The synthesis was initiated on approximately 100 mg Lys-(C12)-MBHA-resin. The crude product (12 mg) was pure enough for hybridization studies. The hybrid between 5'-dA10 and H-U10 had Tm of 67.5°C.

**EXAMPLE 118**

N-(Carbamoylmethyl)-β-alanine Ethyl ester

A suspension of 2-chloroacetamide (2.34 g, 25.0 mmol), β-alanine ethyl ester hydrochloride (15.36 g, 100 mmol) and K₂CO₃ (42.0 g) in dry DMF (200 ml) was heated at 90°C for 24 hours. After cooling to room temperature the reaction mixture was filtered to remove any unreacted β-alanine ethyl ester and the solvent was removed by distillation under reduced pressure (50°C, 1 Torr). The resulting yellow oil (6.0 g) was purified by silica gel flash column chromatography using ethyl acetate, ethyl acetate/ethanol (2:1, v/v), and finally ethanol as the eluent, to give 2.2 g (56%) of the title compound as a colorless oil. TLC (methanol, det. ninyhydrin, Rf=0.65).

¹H NMR (DMSO-d₆) δ 8.18 (t, δ=7.1 Hz, 3H, C₅H₃); 2.42, 2.70 (2J=6.7 Hz, 4H, CH₂Cl₂); 3.03 (s, 2H, CH₂); 4.06 (q, J=7.1 Hz, 2H, OCH₂); 7.02, 7.24 (2J=2.4 Hz, 2H, NH₂) ppm. ¹³C NMR (DMSO-d₆) δ 14.1 (CH₃), 34.5 (CH₂CO), 44.7 (N=CH₂), 51.8 (N=COH); 59.8 (OCH₃), 172.1 (C=O) ppm. FAB-MS: m/z 175.1 (M+1)⁺.

A solution of N-carbamoylmethyl)-β-alanine ethyl ester (530 mg) in diethyl ether was treated with HCl (4.7 M in diethyl ether). The precipitated HCl of N-(carbamoylmethyl)-β-alanine ethyl ester was filtered, rinsed with cold diethyl ether and dried in vacuo, to a white powder.

¹H NMR (DMSO-d₆) δ 8.12 (t, J=7.1 Hz, 3H, CH₃); 2.90, 3.16 (2J=6.7 Hz, 4H, CH₂Cl₂); 3.69 (s, 2H, CH₂); 4.10 (q, J=7.1 Hz, 2H, OCH₂); 7.53, 7.96 (2J=2.4 Hz, 2H, NH₂); 9.22 (s, 1H, NH₂). ¹³C NMR (DMSO-d₆) δ 84.0 (CH₂), 30.2 (CH₂CO), 44.2 (N=CH₂), 47.6 (N=COH), 60.5 (OCH₃), 166.8 (amide C=O), 170.0 (ester C=O). Anal. Calcd for C₉H₈ClN₂O₃; 210.66; C, 39.91; H, 7.18; N, 13.30. Found, C, 39.45; H, 7.10; N, 13.22.

**EXAMPLE 119**

N-(Carbamoylmethyl)-N-(thymyl-1-yacetetyl)-β-alanine Ethyl Ester

DibHOH (9.37 g, 57.4 mmol), N₂-thiazinyl acetic acid (10.57 g, 57.4 mmol) and CH₂Cl₂ (100 ml) were added to a solution of N-(carbamoylmethyl)-β-alanine ethyl ester (10.0 g, 57.4 mmol) in dry DMF (100 ml). The solution was cooled to 0°C in an ice bath and a solution of DCC (1.90 g, 9.18 mmol) in DMF/CH₂Cl₂ (100 ml, 1:1 v/v) was added dropwise within 20 minutes. The ice bath was removed and after stirring for 1 hour at room temperature. The reaction mixture was filtered and the precipitate (DCC) was rinsed with CH₂Cl₂ (50 ml). The organic solution and the CH₂Cl₂ rinses were combined. A colorless precipitate formed overnight which was filtered and rinsed with CH₂Cl₂. The precipitate was dried in vacuo to give 12.0 g (61%) (mp 207-208°C) of the title compound.

Due to restricted conformations at the tertiary amide group two sets of signals (ratio 1:1) are observed for most protons and carbons in the NMR spectra, the coalescence temperature is 85°C. ¹H NMR (DMSO-d₆) δ 81.16 to 1.18 (2J=7.1 Hz, 3H, CH₂Cl₂); 1.73 (s, 3H, T-CH₃); 2.49, 2.70, 3.45, 3.58 (4J=6.7 Hz, 4H, CH₂Cl₂); 3.84, 4.04 (2J=2.4 Hz, CH₂); 4.03, 4.07 (2J=6.7 Hz, 2H, OCH₂); 4.42, 4.65 (2J=2.4 Hz, T(N)=CH₂); 7.01, 7.22, 7.26, 7.54 (4J=2.4 Hz, 2H, NH₂); 7.28, 7.32 (2J=2.4 Hz, 1H, CH₂); 11.22, 11.24 (2J=2.4 Hz, T(NH) ppm. ¹³C NMR (DMSO-d₆) δ 81.19 to 1.15 (2J=2.4 Hz, 1H, CH₂); 32.1, 32.7 (CH₃CO); 43.4, 43.9 (N=CH₂); 47.85, 47.91 (N=COH); 48.5, 50.2 (T(N)=CH₂); 60.1, 60.2 (OCH₂); 108.0, 108.1 (CH₂), 142.2, 142.3 (CH); 151.0, 151.1 (T,
NC(O)N, 164.4 (T, C=O), 167.1, 167.7 (tert. amide C=O), 169.8, 170.0 (prim. amide C=O), 171.2 (ester C=O) ppm. FAB-MS: m/z=341.1 (M+1)*. Anal. Calcd for C_{32}H_{23}N_{2}O_{4}: 49.41; H, 5.92; N, 16.46. Found C, 48.76; H, 5.76; N, 16.34.

**EXAMPLE 120**

N-(Carbamoylimethyl)-N-(N6-benzoylcarbonyl) adenin-9-ylacetyl)-β-alanine Ethyl Ester

As per the above procedure for the preparation of N-(carbamoylimethyl)-N-(thymin-1-ylacetyl)-β-alanine ethyl ester, N-(carbamoylimethyl)-β-alanine ethylester (2.00 g, 11.5 mmol) was reacted with N9-(N6-benzoylcarbonyl) adeninyl acetic acid (3.76 g, 11.5 mmol), DibutO (1.88 g, 11.5 mmol) and DCC (2.27 g, 11.5 mmol). The crude product was purified by silica gel flash chromatography using ethylacetate/methanol 3:1 as the eluent, to give 4.0 g (75% of theoretical yield).

**EXAMPLE 122**

N-(N-tert-Butyloxycarbonyl-a-monomethyl)-N-(N6-benzoylcarbonyl-adenin-9-ylacetyl)-β-alanine Ethylester

A solution of N-(carbamoylimethyl)-N-(N6-benzoylcarbonyl) adenin-9-ylacetyl)-β-alanine ethyl ester (6.00 g, 5.38 mmol) and (bis[trifluoroacetyl]iodo) benzene (2.77 g, 6.45 mmol) in CH_{3}CN/H_{2}O 1:1, v/v (50 mL) was stirred for 1 hour at room temperature. The mixture was filtered and the solvent was evaporated to a residue under reduced pressure. 1,4-Dioxane (50 mL) and Na_{2}CO_{3} (5.0 g) were added, followed by addition of a solution of di-tert-butyl dicarbonate (1.65 g, 7.56 mmol) in 1,4-dioxane (10 mL). The resulting mixture was stirred at room temperature for 24 hours and then filtered. The filtrate was concentrated in vacuo to a residue. The residue was purified by silica gel column chromatography using ethyl acetate/methanol 9:1 to give 2.24 g (75%) of the title compound. TLC (MeOH/ethyl acetate 1:1, det. UV, ninhydrin): R_{f}(N-(carbamoylimethyl)-N-(N6-benzoylcarbonyl)adenin-9-ylacetyl)-β-alanine ethylester)=0.65. H NMR (DMSO-d6) δ1.23, 1.30 (2H, CH_{2}Ph), 7.12, 7.67 (2H, 1H, NH), 7.40-7.55 (5H, arom), 8.41 (1H, A-H), 8.67, 8.68 (2H, 1H, A-H), 8.71 (1H, HNCCH) ppm. C_{33}H_{33}N_{2}O_{4}F_{2} (587.57) requires C, 54.50; H, 5.59; N, 19.54.

**EXAMPLE 123**

N-(N-tert-Butyloxycarbonyl-a-monomethyl)-N-(N1-thyminylacetyl)-β-alanine Ethylester

A solution of [bis[trifluoroacetyl]iodo] benzene (16.71 g, 38.8 mmol) in CH_{3}CN (55 mL) was added dropwise within 10 minutes to a stirred solution of N-(carbamoylimethyl)-N-(thymin-1-ylacetyl)-β-alanine ethyl ester (11.5 g, 33.8 mmol) in CH_{3}CN/H_{2}O (275 mL, 2.3 v/v). After 20 hours at room temperature the volume was reduced to about 50 mL under reduced pressure (1 Torr, 30°C) and the solution was extracted with diethyl ether (150 mL). The aqueous phase was evaporated to dryness in vacuo (1 Torr, 30°C). The remaining solid was rinsed with diethyl ether (20 mL) and dried in vacuo. 1,4-Dioxane (150 mL), K_{2}CO_{3} (12.2 g) and a solution of di-tert-butyldicarbonate (8.4 g, 38.5 mmol) in 1,4-dioxane (50 mL) were added. After stirring for 1 hour at room temperature the reaction mixture was filtered and the solvent was removed under reduced pressure. The resulting residue was purified by silica gel flash column chromatography using hexane/acetonitrile 1:1, v/v and subsequent precipitation from MeOH (60 mL) by addition of hexane (300 mL) gave 8.6 g (64%) of the title compound, mp 174-175°C. TLC (MeOH, det. UV, ninhydrin): R_{f}(N-(carbamoylimethyl)-N-(thymin-1-ylacetyl)-β-alanine ethyl ester)=0.73. 1H NMR (DMSO-d6) δ1.17 (T, J=7 Hz, 2H, CH_{2}Ph), 1.38, 1.42* (2H, 9H, t-Bu), 1.75 (s, 3H, T-CH_{3}), 2.50, 2.72 (2H, 7J=7.9 Hz, 2H, NCH_{2}Ph), 3.31* + 3.50 (2H, NCH_{2}Ph), 4.05*, 4.08 (2H, OCH), 4.58, 4.61* (2H, CH_{2}Ph), 4.61 (overlap), 4.74* (2H, CH_{2}Ph), 7.26*, 7.35 (2H, 1H, CH), 7.4, 7.8 (2H, 1H, NH), 11.25 (s, 1H, NH) ppm. 13^C NMR (major rotamer) (DMSO-d4) δ91.9 (T, CH_{3}), 14.1 (CH_{2}Ph), 28.0 (Bu-CH_{2}), 32.2 (CH_{2}CO), 41.4 (NCH_{2}CH), 48.1 (T(N=CH)), 53.1 (NCH_{2}N), 60.0 (OCH_{2}), 78.8 (C(=N)C), 108.2 (T, CC=C), 142.0 (CH), 151.1 (T, NC(O)), 155.7 (carbonyl C=O), 164.4 (T, C=O). Anal. Calcd for C_{33}H_{33}N_{2}O_{4}: 54.32; H, 6.68; N, 13.58. Found C, 52.37; H, 6.68; N, 13.57.
with ethyl acetate. The aqueous layer was acidified to pH 3.5 by dropwise addition of hydrochloric acid (4 M) and kept at 4°C for 3 hours. The colorless precipitate was filtered, rinsed with water and dried in vacuo to give 4.40 g of the title compound, mp 196°C (dec.). The filtrate was extracted with ethyl acetate, the organic phase dried over Na₂SO₄ and the solvent was removed under reduced pressure. The residue was crystallized from MeOH/ethyl acetate/hexane to give 280 mg additional title compound for an overall yield of 92%. TLC (CHCl₃/MeOH/NH₄OH, 7:2:1, UV-detection): Rf (N-(N-tert-butyloxy carbonyl-aminomethyl)-N(N1-thymineacyl)-β-alanine ethylester)=0.94, Rf (the title compound)=0.52. 1H NMR (DMSO-d₆) (two rotamers, ratio 3:1) 81.46, 1.50* (2s, 9H, t-Bu), 1.83 (s, 3H, T-CH₃), 2.52*, 2.73 (2t, J=7.5 Hz, 2H, NCH₂CH₃), 3.54*, 3.64 (2t, J=7.5 Hz, 2H, NCH₂CH₃), 4.64, 4.69 (2d, J=6.4 Hz, J=7.0 Hz). 13C NMR (major rotamer) (DMSO-d₆) 81.19 (T, CH₃), 28.1 (t-CH₂), 32.2 (CH₂CO), 41.6 (NCH₂CH₃), 48.1 (T(N1)CH₃), 53.2 (NCH₂N), 60.0 (OCH₃), 78.9 (C(CH₃)₂), 108.2 (T, C₃H₂), 142.0 (CH), 151.1 (T, NC(O)N), 155.7 (carbamate C=O), 164.4 (T, C=O), 166.9 (tert. amide C=O), 172.7 (carboxylic acid C=O) ppm. FAB-MS: m/z = 385.15 (M+H)*. Anal. Calcd. for C₃₇H₃₆N₅O₉ (384.39): C, 50.00; H, 6.29; N, 14.58. Found C, 49.95; H, 6.40; N, 14.23.

**EXAMPLE 124**

N-(N-tert-Butyloxy carbonyl-aminomethyl)-N-(N6-benzoyl carbamoyl adenin-9-yl)alanine (4b)

A mixture of N-(N-tert-butyloxy carbonyl-aminomethyl)-N-(N9-benzoyl carbamoyl adenin-9-yl)alanine ethylester (2.34 g, 4.21 mmol) in THF (25 mL) and aqueous LiOH (0.5 M, 25 mL) was stirred at room temperature. At 1.5 hour TLC indicated that hydrolysis was complete. The reaction mixture was extracted with CH₂Cl₂, acidified with HCl (2N) and extracted with CH₂Cl₂ to give 2.1 g (95%) of the title compound. 1H NMR (DMSO-d₆) 81.47, 1.53* (2s, 9H, t-Bu), 2.51*, 2.78 (2t, J=7.5 Hz, 2H, NCH₂CH₃), 3.57*, 3.75 (2t, J=7.2 Hz, 2H, NCH₂CH₃), 4.65, 4.83* (2d, NCH₂N), 5.29 (s, 2H, CH₂Ph), 5.44, 5.54* (2s, 2H, CH₂), 7.4-7.6 (5H, aromat.), 8.02 (NH), 8.39, 8.67 (2s, aromat.), 10.7 ppm. FAB-MS: m/z=528.1 (M+1)*. Anal. Calcd. for C₃₇H₃₆N₅O₉ (527.53): C, 54.64; H, 5.54; N, 18.59. Found C, 54.86; H, 5.50; N, 18.81.

**EXAMPLE 125**

General coupling procedures for amino alkyl-β-alanine PNA’s having letters attached through acetyl linkage

A. Preparation of the C Terminus Fragment

In general the PNA monomer, dimer or larger fragment such as N-(N-tert-butyloxy carbonyl-aminomethyl)-N-(N1-thymineacyl)-β-alanine ethylester, or N-(N-tert-butyloxy carbonyl-aminomethyl)-N-(N9-benzoyl carbamoyl adenin-9-yl)alanine ethylester prepared above, to be attached at the C terminus of another PNA monomer or larger fragment is dissolved in trifluoroacetic acid/CH₂Cl₂ (0.2-2 mL, 1:1, v/v). After stirring for 20 min at room temperature the clear solution is evaporated to dryness in vacuo (0.5 Torr) Diethyl ether (2 mL) is added and the mixture is evaporated to dryness. This process removes the t-Boc protecting group from the N terminus.

B. Preparation of the N Terminus Fragment

In general a PNA monomer, dimer or larger fragment having a free carboxyl group at the C terminus such as N-(N-tert-butyloxy carbonyl-aminomethyl)-N-(N1-thymineacyl)-β-alanine, or N-(N-tert-butyloxy carbonyl-aminomethyl)-N-(N6-benzoyl carbamoyl adenin-9-yl)acetamide-β-alanine prepared above, is mixed with an equimolar amount of HRTU (0.1-1 mmol) in a solution of DMF/pyridine (0.5-5 mL, 1:1, v/v) and diisopropylethylamine (0.2-2 mL).

C. Coupling of the Two Fragments

The solution prepared in part B is mixed with the solid residue obtained in part A and the mixture is stirred for 2 hours at room temperature. The reaction mixture is purified by silica gel flash chromatography using a gradient of methanol and ethyl acetate as the eluent to give the corresponding oligomer.

Using the procedures illustrated a number of PNA’s having the amino alkyl-β-alanine backbone were prepared. These PNA’s are listed in Table IV, as is MS characterization data and yields.

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*Purity established by HPLC

[1+1]-

**EXAMPLE 126**

General Procedure for Hydrolysis Amino Alkyl-β-alanine PNA’s Having Letters Attached Through Acetyl Linkage

In general the PNA monomer, dimer or larger fragment having its carbonyl group as an ester is hydrolyzed by adding (0.1-1 mmol) to a mixture of THF (0.8-8 mL) and LiOH (0.5 M, 0.8-8 mL). The mixture is stirred at room temperature until TLC indicated that hydrolysis is complete (ca. 2 hours). After extraction with n-butanol, the aqueous phase is acidified with HCl (2N). A precipitate is isolated by filtration and/or the solution is extracted with n-butanol a second time. The organic layer is separated and dried over Na₂SO₄, evaporated and the crude products are combined and purified by silica gel flash column chromatography using a methanol/ethyl acetate gradient as eluent.

**EXAMPLE 127**

PNA Uptake and Protein Inhibition by PNA 20-mer

The PNA 20-mer H-Gly-TTT AGG ATT GCT GCT CAT GG-Lys-CONH₂ was labeled with fluorescein isothiocyan-
ate (FITC) and used in an uptake study. Transformed human hepatocytes (H8Ad17) expressing HCV sequences were seeded onto 4-chamber slides at approximately 4x10^5 cells per well in 10% FBS media. FITC labeled PNA was prehybridized with 4 different lengths of oligodeoxynucleotides with a first region having from 6 to 12 deoxyribonucleotides that are complementary to the PNA and a second region having 10 deoxyribonucleotides that are not hybridized e.g. a tail. The sequences of the oligodeoxymucleotides used were 5'-ACG AAT CAC AAA CAA AGA A-3' (SEQ ID NO: 40) 5'-GAA TCC TAA ACC TCA AAG AAA A-3' (SEQ ID NO: 41) 5'-ATG CTA AAC CTC AAG AAA GAA A-3' (SEQ ID NO: 42) and 5'-CCT AAAA CCT CAAAG A A-3' (SEQ ID NO: 43) with the underlined portion denoting the portion of the oligodeoxynucleotide that is complementary to the PNA 20-mer. Each deoxyribonucleotide (22, 20, 18, and 16-mer) was independently hybridized with 5 equivalents of the above PNA 20-mer in 100nm NaCl and 100mm tris-HCl buffer. The mixture was preheated to 65°C for 15 minutes and cooled to 37°C for 30 minutes. The cells were washed twice with 1 ml of optimem. Then 1 ml of optimem with lipofectin was placed on the cells. Cells were treated with DNA prehybridized with oligodeoxyribonucleotides for 4 hours. The cells were rinsed with complete media and wet mounts were prepared immediately in order to study the cells under a fluorescent scope.

The cells treated with FITC labeled PNA 20-mer that were prehybridized with 20-mer 5'-GAA TCC TAA ACC TCA AAG AAA A-3' (SEQ ID NO: 41) where the underlined portion is hybridized to the PNA deoxyribonucleotide, showed staining consistent with cellular uptake. The cells treated with FITC labeled PNA 20-mer that were not prehybridized with deoxyribonucleotides did not show staining.

In a similar study the effects of the above PNA on HCV core protein were examined. Cells were seeded in 6-well plates at approximately 3x10^5 cells per well, and the harvesting was done in 100 ml per well of laemmli buffer. Other than that the process was exactly the same as the uptake study above.

The results show that unhybridized PNA had an inhibitory affect on the HCV core protein. The PNA hybridized to the oligodeoxynucleotides also showed inhibition.

EXAMPLE 128
Preparation of N-(trifluoroacetyl)-3-[O-(dimethoxytrityl)-1-amino propan-3-ol]
To a solution of 3-amino-1-propanol (1 g, 13.31 mmol) in dichloromethane (50 ml) was added ethyl trifluoromethacacetate (1.58 ml, 13.31 mmol). The reaction was complete as indicated by TLC (10% MeOH/CH2Cl2) in 2 hours. The solvent was removed by evaporation in vacuo. The residue was coevaporated four times with dry pyridine. The residue was then dissolved in a mixture of dry pyridine (50 ml) and triethylamine (1.87 ml, 13.31 mmol). While the reaction mixture was stirred, dimethoxytrityl chloride (4.51 g, 13.31 mmol) was added in four portions. The reaction was complete in 20 hours as indicated by TLC (25% EtOAc/hexanes). The solvent was reduced in vacuo and the residue was partitioned between dichloromethane and water. The dichloromethane was collected and washed with water (2x25 ml) and once with brine (25 ml). The dichloromethane was dried (MgSO4) and concentrated in vacuo. The residue was neutralized with a solution of 5% EtOH in hexanes with 1% pyridine and was purified by silica gel chromatography eluted with 5% EtOH in hexanes. The appropriate fractions were collected and concentrated in vacuo to yield title compound in 84% yield (5.22 g). 1H NMR (DMSO) δ: 1.79 (m, 2H, CH2), 3.01 (t, 2H, CH2), 3.75 (s, 6H 2xCH3), 6.88-7.36 (m, 13H, aromatics), 9.36 (bs, 1H, NH).

EXAMPLE 129
Preparation of Methyl N-(3-O-dimethoxytritylprop-1-yl)-N-(trifluorooacetyl)glycinate
To a solution of N-(trifluoroacetyl)-3-[O-dimethoxytrityl]-1-amino propan-3-ol (40 g, 84.50 mmol) in DMF (550 ml) at 0°C, was added NaH (4 g, 101.40 mmol) in four 1 g portions. The reaction mixture was stirred until the evolution of gas stopped. Then methylv bromoacetate (10.31 ml, 92.95 mmol) was added to the reaction mixture dropwise via addition funnel. The reaction was complete as indicated by TLC (20% EtOAc in hexanes) in 20 hours. The solvent volume was reduced in vacuo to approximately 200 ml and then partitioned between water (100 ml) and dichloromethane (200 ml). The aqueous layer was extracted with dichloromethane (4x75 ml). The organic fractions were collected and dried over MgSO4 and concentrated in vacuo. The residue was purified by silica gel flash chromatography eluted with 20% EtOAc in Hexanes. The appropriate fractions were collected and concentrated in vacuo to yield title compound 35.90 g (83%). 13N NMR (CDCl3) δ: 1.90 (m, 2H, CH2), 3.18 (m, 2H, CH2), 3.65 (m, 2H, CH2), 3.78 (d, 3H, CH3), 3.83 (s, 6H, 2xCH3), 4.10 (s, 2H, CH2), 6.81-7.48 (m, 13H, aromatics).

EXAMPLE 130
Preparation of Sodium Salt of N-[3-O-(dimethoxytrityl)prop-1-yl]-N-(trifluoroacetyl)glycinate
Methyl N-(3-O-dimethoxytritylprop-1-yl)-N-(trifluoroacetyl)glycinate (1.57 g, 3.08 mmol) was suspended in 20% aq. NaOH (50 ml) and the reaction mixture was refluxed. The reaction was complete as indicated by TLC (20% EtOAc in hexanes) in 4 hours. The reaction was allowed to cool to room temperature and was extracted with EtOAc (4x25 ml). The organic fractions were collected and washed once with brine (25 ml) and dried over MgSO4, then concentrated in vacuo to yield 1.19 g (85%) of the title compound. ES/MS Positive mode m/z=434.30. 31N NMR (DMSO) δ: 1.65 (m, 2H, CH2), 2.79 (bt, 2H, CH2), 2.47 (m, 6H, CH2+DMSO), 3.02 (bt, 2H, CH2), 3.78 (s, 6H, 2xCH3), 6.88-7.41 (m, 13H, aromatics).

EXAMPLE 131
Preparation of Sodium Salt of N-[3-O-(dimethoxytrityl)prop-1-yl]-N-[thymidyldimethyleneacarbonyl]glycine
N-acetyl acid thymidine (722 mg, 1.58 mmol), hydroxybenzyltriazole (74 mg, 0.482 mmol), EDC HCl salt (92 mg, 0.482 mmol) were dissolved in pyridine (25 ml) and DMF (25 ml) and stirred for 1 hour. The sodium salt of N-[3-O-(dimethoxytrityl)prop-1-yl]glycine was then added and reaction mixture was stirred for 20 hours at which time TLC (10% MeOH in CH2Cl2) indicated that the reaction was complete. Dichloromethane (50 ml) and water (50 ml) were added to the reaction mixture and separated. The water was extracted with dichloromethane (3x50 ml). The organic layers were pooled and washed with brine (1x50 ml), dried over MgSO4, and concentrated in vacuo. The product was
puriﬁed by silica gel ﬂash chromatography eluting with 10% MeOH in CH₂Cl₂ then 15% MeOH in CH₂Cl₂. The appropriate fractions were collected and concentrated in vacuo to yield title compound 164 mg (60%). ¹H NMR (DMSO) δ: 1.65–1.71 (bs, 5H, CH₅+CH₉), 2.95 (m, 2H, CH₂), 3.33 (m, 2H, CH₂), 3.71 (bs, 8H, CH₂+2xCH₃), 4.50 (d, 2H, CH₂), 6.84–7.5 (m, 14H, aromatics), 11.3 (bs, 1H, NH).

EXAMPLE 132
Preparation of N-(trifluoroacetyl)-3-[O-(dimethoxytrityl)]-1-aminobutan-4-ol

The procedure of Example 128 was repeated using 4-amino-1-propanol as the starting material. ¹H NMR (DMSO) δ: 1.53 (m, 4H, 2xCH₂), 2.98 (t, 2H, CH₂), 3.27 (m, 2H, CH₂), 3.73 (s, 6H, 2xCH₃), 6.85–7.40 (m, 13H, aromatics), 9.40 (bt, 1H, NH).

EXAMPLE 133
Preparation of Methyl N-(3-O-dimethoxytrityl)prop-1-yl-N-(trifluoroacetyl)glycinate

The procedure of Example 129 was repeated using N-(trifluoroacetyl)-3-[O-(dimethoxytrityl)]-1-aminobutan-4-ol as the starting material. ¹H NMR (DMSO) δ: 1.60 (m, 4H, 2xCH₂), 2.99 (t, 2H, CH₂), 3.45 (m, 2H, CH₂), 3.71 (d, 3H, CH₃), 3.78 (s, 6H, 2xCH₃), 4.30 (d, 2H, CH₂), 6.89–7.41 (m, 13H, aromatics)

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What is claimed is:

1. A process for preparing a compound comprising a polyamide backbone bearing a plurality of ligands that are individually bound to aza nitrogen atoms located within said backbone, comprising the steps of:

A) providing a polymer substrate, said polymer being functionalized with a chemical group capable of forming an anchoring linkage with an amino acid;
B) coupling said polymer with a first amino acid through said anchoring linkage, said first amino acid having formula (IV):

![Diagram of formula IV]

wherein:
L is selected from the group consisting of naturally occurring nucleobases, non-naturally occurring nucleobases, provided that at least one L is a naturally occurring nucleobase; aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, and reporter ligands, wherein amino groups are, optionally, protected by amino protecting groups; provided that at least one L is a naturally occurring nucleobase;
A is a single bond or a group of the formula:

![Diagram of formula X]

where:
X is O, S, Se, NR², CH₂ or C(CH₃)₂;
Y₁ is a single bond, O, S or NR⁴;
Y₂ is single bond, O or S;
p and q are zero or integers from 1 to 5;
r and s are zero or integers from 1 to 5;
R¹ and R² are independently selected from the group consisting of hydrogen, (C₃–C₆)alkyl, hydroxy- or alkoxy- or alkylthio-substituted (C₃–C₆)alkyl, hydroxy, alkoxy, alkylthio, amino and halogen; and
R³ and R⁴ are independently selected from the group consisting of hydrogen, (C₃–C₆)alkyl, hydroxy- or
alkoxy- or alkylthio-substituted (C₅-C₆)alkyl, hydroxy, alkoxy, alkylthio and amino;
B is N or R²N⁺, where R² is as defined above;
T is CR³R⁷, CHR³CHR⁷ or CR³R⁷CH₂, where R⁶ is hydrogen and R⁷ are independently selected from the group consisting of hydrocarbon, (C₂-C₆)alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C₁-C₆)alkoxy, (C₁-C₆)alkylthio, NR³R⁴ and SR³,
where R⁶ and R⁷ are as defined above, and R⁵ is hydrogen or (C₁-C₆)alkyl, hydroxy-, alkoxy-, or alkylthio-substituted (C₁-C₆)alkyl, or R⁶ and R⁷ taken together complete an alicyclic or heterocyclic system;
D is CR³R⁷, CH₂CR³R⁷ or CHR³CHR⁷, where R⁶ and R⁷ are as defined above;
E is COOH or an activated or protected derivative thereof; and
G is NPgR³ where R³ is as defined above and Pg is an amino protecting group;
C) removing said amino protecting group from said coupled first amino acid to generate a free amino group; and
D) reacting said free amino group with a second amino acid having formula (IV) to form said compound.

2. The process of claim 1 further comprising the steps of:
E) removing said amino protecting group from said second amino acid to generate a terminal free amino group on said peptide chain; and
F) reacting said free amino group on said peptide chain with a further amino acid having formula (IV) to lengthen said peptide chain.

3. The process of claim 2 wherein steps E and F are performed a plurality of times.

4. The process of claim 2 further comprising removing at least one protecting group remaining on the amino acid moieties of the peptide chain.

5. The process of claim 1 further comprising cleaving said anchoring linkage without substantially degrading said peptide chain.

6. The process of claim 1 wherein the polymer substrate contains polystyrene, polyacrylamide, silica, a composite material, or cotton.

7. The process of claim 1 wherein the chemical group capable of forming said anchoring linkage is chloro-, bromo- and iodo-substituted alkyl, amino-substituted alkyl, amino and aryl-substituted alkyl, amino- and alkylaryl-substituted alkyl, hydroxy-substituted alkyl, or a derivative thereof having a spacer group that can be cleaved substantially without degradation of said polypeptide.

8. The process of claim 7 wherein chloro-substituted alkyl is chloromethyl, amino-substituted alkyl is aminomethyl, amino- and alkyl-substituted aryl is α-aminobenzyl, amino- and alkylaryl-substituted alkyl is selected from the group consisting of α-aminomethyl and α-aminomethylbenzyl, and hydroxy-substituted alkyl is hydroxymethyl.

9. The process of claim 7 wherein:
the chemical group is derived from an amino-containing moiety selected from amino-substituted alkyl, amino- and aryl substituted alkyl, and amino- and alkylaryl-substituted alkyl; and
the chemical group includes a spacer group derived from the group consisting of 4-(haloalkyl)aryl-lower alkanolic acids, Boc-aminoacetyl-4-(oxygenmethyl)aryl-lower alkanolic acids, N-Boc-p-acylbenzydrylamines, N-Boc-4′-(lower alky)aryl-p-acylbenzydrylamines, and 4-hydroxyethylbenzydrylamines.

* * * * *
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

**Column 103,**
Line 65, please delete “provided that at least one L is a naturally occurring nucleobase;”;

**Column 106,**
Line 8, please delete “polystrene” and insert therefor -- polystyrene --.

Signed and Sealed this

Fifth Day of April, 2005

[Signature]

JON W. DUDAS

Director of the United States Patent and Trademark Office