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MINIREVIEW ARTICLE

Nucleobase-containing peptides: an overview of their characteristic features and applications

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Abstract Reports on nucleobase-containing chiral peptides (both natural and artificial) and achiral pseudopeptides are reviewed. Their synthesis, structural features, DNA and RNA-binding ability, as well as some other interesting applications which make them promising diagnostic/therapeutic agents of great importance in many areas of biology and therapy are taken into critical consideration.

Keywords Nucleobase · Peptide · Nucleopeptide · Nucleic acid

Introduction

Nucleobase-containing peptides, also referred to as nucleopeptides, represent a promising class of molecules of important biomedical significance presenting a peptide-like backbone conjugated to nucleobases through different linker moieties. Interestingly, some of them are natural, such as willardiine-containing nucleopeptides (**1a**, Fig. 1) and peptidyl nucleosides (also indicated as nucleoside peptides, **1b**), which are well-known for their antimicrobial activity. There are, however, a number of artificial nucleobase-containing peptides (**1c**) or pseudopeptides (**1d**) which were prepared and studied for their binding properties, as described below in this review.

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Natural nucleobase-containing peptides

Naturally-occurring nucleopeptides include peptidyl nucleosides, hybrid molecules comprising short peptide (mainly dipeptide) moieties conjugated to purine or pyrimidine nucleobases, which were isolated from different *Streptomyces* bacteria and willardiine-based peptides, which were isolated from vegetal sources.

Pyrimidine-containing compounds

Nikkomycin Z (**2a**, Fig. 2), an uracyl-containing peptidyl nucleoside produced by *Streptomyces tendae*, showed a significant antifungal activity due to its ability to inhibit chitin synthase, the fungal enzyme responsible for the formation of chitin, an essential component of the fungal cell wall (Gooday 1990; Nix et al. 2009). Nikkomycin Z, which can be synthesized using a direct three-component Mannich reaction (Toure and Hall 2009) or four-component Ugi reaction (Plant et al. 2009), was able to eradicate Valley Fever infections in mice (Hector et al. 1990) and showed antifungal activity against *Candida albicans* in combination with caspofungin, voriconazole or amphotericin (Sandovsky-Losica et al. 2008).

Blasticidin S (**2b**) is another natural peptidyl nucleoside antibiotic, which was isolated from *Streptomyces griseochromogenes*. This cytosine-containing molecule is able to interfere with the peptide bond formation in the ribosomal machinery and therefore to specifically inhibit the protein synthesis in both prokaryotes and eukaryotes (Yamaguchi and Tanaka 1966). This antibiotic finds application also in genetic engineering, being used to select transformed cells (Itaya et al. 1990). Interestingly, the first total synthesis of this peptidyl nucleoside antibiotic was achieved by

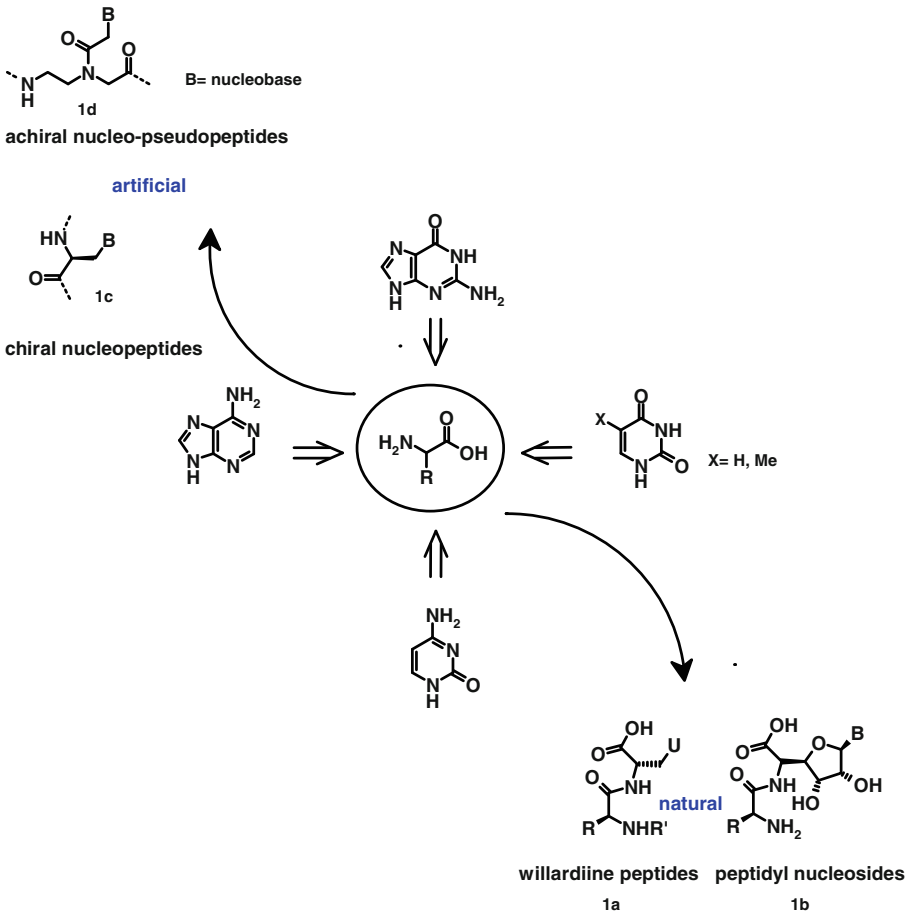


Fig. 1 Overview of nucleobase-containing peptides

Ichikawa et al. by coupling cytosinine and blastidic acid (Ichikawa et al. 2004).

Another class of natural pyrimidine-containing peptides is based on 3-(1-uracil)-L-alanine (L-willardiine, **1a**). More particularly, gamma-L-glutamyl-L-willardiine and gamma-L-glutamyl-L-phenylalanyl-L-willardiine peptides were isolated from seeds of *Fagus silvatica* (Kristensen and Larsen 1974). The structures for the above pyrimidine-containing peptides have been proposed on the basis of a study of their chemical and spectroscopic properties.

Purine-containing compounds

Besides the above-mentioned nucleopeptides based on pyrimidines, some purine-containing aminonucleosides are also known, such as puromycin (**2c,** Fig. 2), an antibiotic isolated from *Streptomyces alboniger*, which inhibits peptidyl transfer in both prokaryotic and eukaryotic ribosomes during translation, causing the premature chain release termination (Azzam and Algranati 1973). Even if the exact mechanism of action of this analog of the 3′ end of aminoacyl-tRNA (which is a nucleobase-containing aminoacyl ester) is not clear, a key role is probably played by the amide linkage of the 3′ position instead of the normal ester linkage present in a normal tRNA, which makes the molecule much more resistant to hydrolysis, thus causing the ribosome machinery to stop. Furthermore, recent studies (Oguma et al. 2009) indicated an induction of cell death in thymic lymphoma cells, by low-dose puromycin treatment, due to endoplasmic reticulum stress. Cystocin (**2d**) is another recently discovered aminonucleoside which shares similar properties with puromycin. However, cystocin exhibits about two folds higher antibiotic activity than puromycin and possesses also a significant antitumor activity (Lee et al. 2003). The antifungal antibiotic chryscandin (**2e**), produced by *Chrysosporium pannorum*, is another purine-containing peptidyl nucleoside which resembles puromycin from the point of chemical structure. However, a notable difference from puromycin is found in the 3-aminoribofuranuronic acid moiety which is characteristic of chryscandin. Some other differences are observed in the biological properties of the two antibiotics. Chryscandin is active against *Candida albicans* and has low toxicity in mice, whereas puromycin is only active against bacteria and fairly toxic in mice (Yamashita et al. 1984; Liu and Arora 2008).

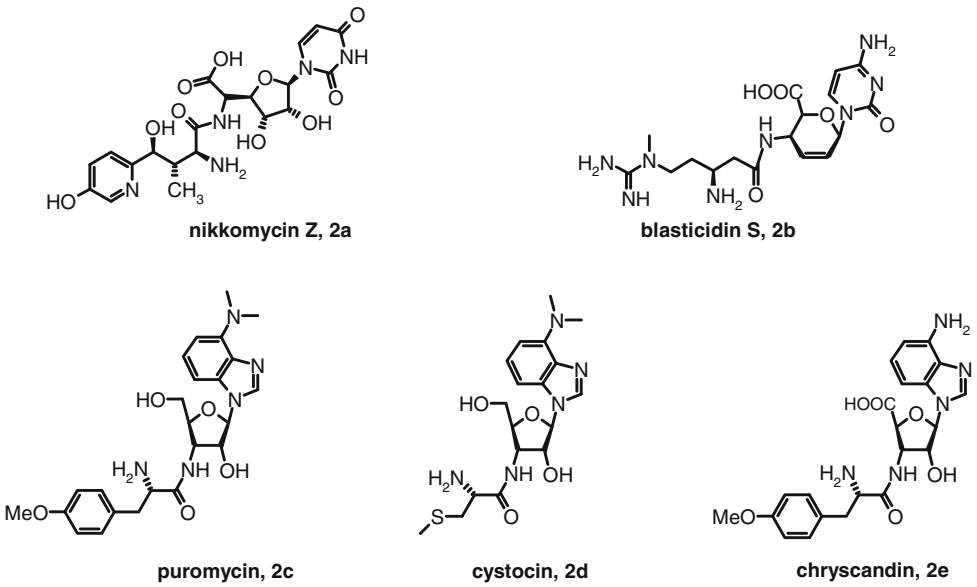


Fig. 2 Molecular structures of some peptidyl and amino nucleosides

Artificial nucleobase-containing peptides

In the last decades the synthesis and the study of artificial nucleic acids, based on a peptide skeleton, has gained a considerable attention in the scientific community for the possibility of achieving novel powerful molecules able to (1) interact with natural nucleic acids or to (2) form biomaterials, which are useful features in various biomedical applications.

Synthesis of α-nucleopeptides

A general strategy to synthesize nucleopeptides makes use of nucleobase-containing monomers, i.e. nucleoamino acids, to be employed in peptide synthesis in solution, solid phase synthesis or polycondensation reactions of carboxyl-activated derivatives. On the other hand, nucleoamino acids are synthesizable by strategies in which the purine or pyrimidine ring is formed during the synthesis of the compound (Shvachkin et al. 1982) or by methods in which complete purines or pyrimidines are variously functionalized as reported for example in the scientific work of Nollet et al. (1969). More particularly, these authors described the synthesis of "unconventional nucleotide analogues", i.e. α-amino acids (homoalanines) containing adenine, hypoxanthine, guanine and xanthine bases (**3a**, Fig. 3).

In the same year, Doel et al. used the term "nucleopeptide" to indicate peptide analogs of oligonucleotides synthesized in his laboratory by using nucleobase-bearing alanines (**3b**, Fig. 3; Doel et al. 1969) and 5 years later reported the synthesis of several other nucleopeptides made of the same "base-bearing amino acids" (**3b**) functionalized with uracil, thymine, cytosine and adenine (Doel et al. 1974). Furthermore, Buttrey et al. (1975) described the synthesis of homothymine polymers based on D, L-mixture as well as optically active D and L nucleoamino acids **3b** (Buttrey et al. 1975). Three years later, Draminski and Pitha reported the preparation of several polypeptides containing adenine and uracil residues based on nucleobase-containing alanines (**3b**) polymerized by different methods (Draminski and Pitha 1978). Raukas et al. (1982) described some short peptides containing basic amino acids as well as residues carrying uracil and adenine nucleobases anchored to alanine units (**3b**), which they designated by the term "nucleopeptides". Interestingly, other α-nucleopeptides realized by alternating α-nucleoamino acids **3b** with glycines were also synthesized by Korshunova et al. (1997). Furthermore, poly-L, D- and DL-lysine derivatives carrying nucleobases as pendants (**3c**), were obtained by a polymer modification reaction by Takemoto (1985). Returning to the issue of the nucleobase-containing oligopeptides, Lenzi et al. (1995) reported the synthesis of a "real chiral peptide nucleic acid", as defined by the same authors, which was prepared with a self-complementary nucleobase sequence by alternating α-nucleoamino acids (**3a**) and proteogenic α-amino acids. The same α-nucleoamino acids (**3a**), were alternated with several proteogenic α-amino acids to give short chiral nucleopeptides also by Yamazaki et al. (1997). Similar

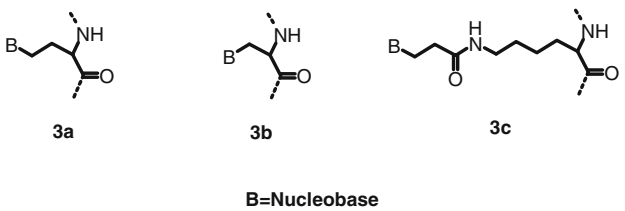


Fig. 3 Nucleoamino acid moieties present in various literature examples

chiral nucleopeptides, containing nucleobase-functionalized homoalanines (**3a**), were obtained by Brasun et al. (2001) who alternated α-nucleoamino acids and glycines, and by Matsumura et al. (2003). Another important contribution to the research on nucleopeptides is the study reported by Diederichsen (1996) on the alanyl nucleoamino acids (**3b**) and the so-called "alanyl PNAs" synthesized with an alternating configuration of the nucleoamino acid residues. Furthermore, a nucleopeptide based on adeninyl D-homoalanine (**3a**) alternated with thyminyl L-alanine residues (Diederichsen and Schmitt 1996), as well as other interesting peptide analogs of oligonucleotides were realized and studied by the group of Diederichsen. In particular, they described a series of nucleopeptides based on nucleobase-containing alanyl (**3b**), homoalanyl (**3a**) and norvalyl monomers (**4a,** Fig. 4) in which the nucleobase was separated from the peptide backbone by 1, 2 and 3 methylene moieties, respectively (Diederichsen et al. 2005), and also reported on nucleopeptides composed of both *β*-amino acids and *β*-nucleoamino acids (**4b**, Weiss and Diederichsen 2007).

Further studies on nucleobase-containing alanines (**3b**) were performed recently from the same research team (Roviello et al. 2009c) who realized protected monomers suitable for the solid phase synthesis of DNA–nucleopeptide chimeras. The interest in nucleopeptides has increased rapidly in recent years as attested by the growing literature on these analogs. For example, in a recent work Huang reported on a novel synthetic approach to α-nucleoamino acid-containing monomers for the synthesis of α-nucleopeptides (Huang 2008) and, not less important, new α-nucleopeptides, made of α-nucleoamino acids and different α-amino acids (Fig. 5), were presented very recently by Geotti-Bianchini et al. (2008). In both cases the nucleopeptides of interest were based on the repeating unit **3b**.

Diamino acid-based nucleopeptides

In 1996, two different research teams reported on nucleopeptides based on a chiral natural diamino acid, i.e.

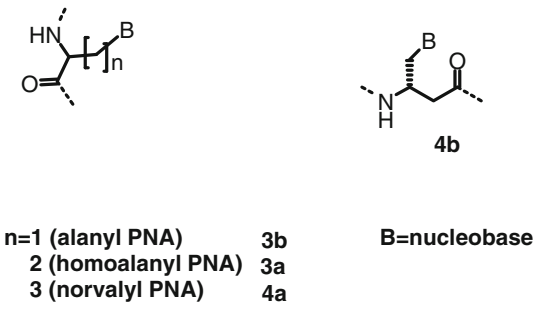


Fig. 4 Nucleoamino acids present in the α-nucleopeptides (**3a, 3b, 4a**) and helix-forming *β*-peptides (**4b**) presented by the Diederichsen group

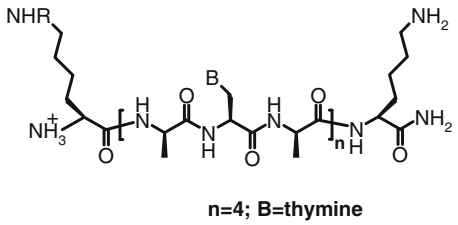


Fig. 5 Molecular representation of an "intrinsically cell-penetrating" α-nucleopeptide

ornithine (**6a,** Fig. 6), which was functionalized with thymine nucleobase through a methyl carbonyl linker by using thymine acetic acid. More in detail, Lioy and Kessler prepared three chiral nucleopeptides by making use of both L- and D-ornithine nucleoamino acids by a Fmoc solid phase synthetic strategy (Lioy and Kessler 1996), whereas Petersen et al. reported the synthesis and the evaluation of the RNA-binding properties of a decamer based solely on the L-enantiomer (Petersen et al. 1996).

Subsequently, Korshunova et al. (1997) reported the design and the synthesis of several "oligonucleopeptides", i.e. *δ*-nucleopeptides based on ornithine with an ethyl linker to the nucleobase (**6b,** Korshunova et al. 1997). Ornithine-containing *δ*-nucleopeptides based on repeating unit **6a** were also the object of researches performed by Inaki et al. (1998), which demonstrated by CD and NMR the presence of hydrogen-bonding between thymine and ornithine moieties (Inaki et al. 1998) in such nucleopeptides, and by van der Laan et al., who in the same year prepared several chirally-pure ornithine nucleopeptides (van der Laan et al. 1998). Four years later Mandrugin et al. reported the synthesis of "new hetero organic nucleopeptides", whose base sequence was complementary to specific viral mRNA regions (Mandrugin et al. 2002). Such nucleopeptides were characterized by a *δ*-ornithine-backbone, with some residues carrying also free amino groups in the linker to nucleobase (**6c**). In the same year a study by Sforza et al. demonstrated the need of modified coupling conditions to obtain by solid phase synthesis optically pure D- or L-ornithyl *δ*-peptides (based on **6a** moieties) which presented novel binding characteristics

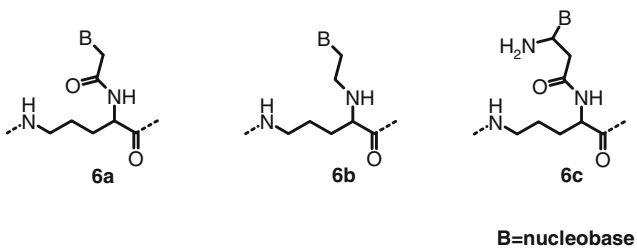


Fig. 6 Molecular representation of ornithine-based repeating units in some literature *δ*-nucleopeptides

towards nucleic acids, as they demonstrated by CD spectroscopy (Sforza et al. 2002). More in detail, the use of HATU as a coupling agent, and collidine (TMP) as a base, to be added in portions and without preactivation of the nucleoamino acid monomer, led to the lowest degree of epimerization, as reported by Corradini et al. (2001) for the synthesis of ornithine-containing nucleopeptides. Subsequently, Roviello et al. reported the synthesis of diaminobutyric acid-based and lysine-based γ- and ε-nucleopeptides that were studied for their binding properties (Roviello et al. 2006, 2007, 2008a, b, 2009a). The same research group studied also diaminobutyric-based nucleopeptides with alternate configuration of the nucleoamino acid residues (Roviello et al. 2009b) as well as alternated L-lysine/L-diaminobutyric acid nucleopeptides (Roviello et al. 2010b). Finally, the synthesis and biological properties of alternate 2-aminoethylglycine/L-diaminobutyric acid as well as 4-piperidyl glycine/L-arginine and alpha/epsilon L-lysine-based nucleopeptides were also described by Roviello et al. (2009d, 2010a, c).

Nucleic acid-binding properties of nucleopeptides

Besides the synthesis of nucleopeptides, particular attention was also paid to the study of their nucleic acid-binding properties. For example, RNA-binding studies were performed on polymers based on D,L-mixture as well as optically active D and L nucleobase-containing alanines (**3b**). However, these studies showed that the nucleoamino acid-containing polymers were not able to bind polyadenylic RNA (Buttrey et al. 1975). On other hand, RNA-binding ability was demonstrated by UV and CD spectroscopies for other polymers containing adenine and uracil residues, based on nucleobase-containing alanines (**3b**), synthesized by Draminski and Pitha (1978). Subsequently, the study of Raukas et al. (1982) demonstrated that short peptides containing both basic amino acids and nucleobase-bearing alanines (**3b**) were able to interact with complementary DNA, but not with RNA. Moreover, chiral nucleopeptides obtained by alternating the α-nucleoamino acids **3a** with different proteogenic α-amino acids bound to complementary DNA and RNA, as evidenced in studies performed on diadenylic tetrapeptides in complex with poly dT and poly U (Yamazaki et al. 1997).

Furthermore, δ-nucleopeptides based on L-ornithine were shown to form complexes with RNA (Petersen et al. 1996; van der Laan et al. 1998) and DNA (Sforza et al. 2002). No DNA or RNA-binding ability was found in case of L- and D-diaminobutyric acid-based γ-nucleopeptides (Roviello et al. 2006, 2007, 2008b), whereas alternate L-diaminobutyric acid/2-aminoethylglycine and L-diaminobutyric acid/L-lysine-containing nucleopeptides bound complementary strands of both natural nucleic acids (Roviello et al. 2010a, b). Furthermore, homothymine 4-piperidyl glycine/L-arginine-containing nucleopeptides as well as ε-nucleopeptides, but not alternate α/ε-nucleopeptides, based on L-lysine were shown to interact with complementary RNA (Roviello et al. 2009a, d, 2010c). On the basis of the above findings and also considering the previous studies of Diederichsen (1996) it can be concluded that, as a general rule, in a nucleopeptide backbone isomorphous to DNA and RNA, six bonds of the sugar–phosphate backbone are correlated, in terms of their length, to two α-amino acids or equivalently to one δ-amino acid residue (but also to one 2-aminoethylglycine residue in case of PNA). Therefore, the repeating unit of a potential nucleic acid-binding nucleopeptide can be built by (1) an α-amino acid pair containing a nucleobase substitution and an unsubstituted amino acid, or (2) by another nucleobase-containing monomer or combination of amino acids allowing for a distance between the atoms of the nucleopeptide backbone which bear the nucleobases, similar to that present in DNA (i.e. six bonds). Furthermore, the presence of basic residues in the nucleopeptide (such as underivatized lysines or arginines), which are known to interact strongly with the phosphate groups of DNA or RNA, may lead to more effective nucleopeptide/nucleic acid interactions.

Nucleopeptide-based molecular recognition

Apart from their possible interaction with DNA and RNA, nucleobase-containing peptides are also interesting as materials for the possibility to form molecular networks based on hydrogen-bonding schemes between complementary (or self-complementary) nucleopeptide strands.

This property was studied in 1985 by Takemoto who reported not only a study on the conformation of poly-L, -D- and DL-lysine derivatives carrying nucleobases as pendants (**3c**), but also an investigation on the polymer–polymer interactions revealing the formation of complexes by specific base pairing between complementary poly-α-lysine polymers (Takemoto 1985). Subsequently, an oligopeptide with a self-complementary nucleobase sequence obtained by alternating α-nucleoamino acids (**3a**) and proteogenic α-amino acids was studied by Lenzi et al. (1995) who demonstrated the formation of a complex with a Tm of 19°C due to the self recognition of this molecule and suggested a DNA-like self-aggregation in solution. Similar chiral nucleopeptides, obtained by alternating α-nucleoamino acids (**3a**) and glycines, were shown to be ligands for Cu^{2+} and Ni^{2+} ions much more effective than simple peptides. The same authors also observed the induction of highly ordered structures based on such chiral nucleopeptides, formed as a consequence of the metal ion binding (Brasun et al. 2001). Furthermore, studies on nucleopeptides

based on alanyl nucleoamino acids (**3b**) revealed that oligomers with an alternating configuration of the residues were able to form complexes by a base recognition system different from that of DNA, being based on unnatural base pairs (Diederichsen 1996). Also studies on self-pairing nucleopeptides based on adeninyl D-homoalanine (**3a**) alternated with thyminyl L-alanine (**3b**) residues suggested the formation of a linear, antiparallel A–T pairing complex (Diederichsen and Schmitt 1996). The complementary nucleobase recognition was at the basis of the research of Matsumura et al. (2003), who described several peptides, containing nucleobase-functionalized homoalanines (**3a**), able to form interesting peptide architectures reinforced by complementary nucleobase recognition. The interaction between the nucleobases also led to the acceleration of the self-replication reactions of these nucleopeptides. Further nucleopeptides based on nucleobase-containing alanyl (**3b**), homoalanyl (**3a**), norvalyl monomers (**4a,** Fig. 4) were also examined by Diederichsen et al. (2005) and, in particular, the stabilities of the various double strands obtained by using these nucleopeptides were compared. The authors concluded that side chain homology affected the pairing selectivity because of the altered orientation of Hoogsteen sites and also the donor/acceptor positions at the Watson–Crick positions. Two years later Weiss and Diederichsen reported on β-peptide helices formed by nucleopeptides composed both of β-amino acids and β-nucleoamino acids (**4b**) which showed specific base pair recognition or non-specific hydrogen bonding/aromatic interaction for the cases in which Watson–Crick pairing was not permitted (Weiss and Diederichsen 2007). PNA-nucleopeptide interactions were investigated also by Roviello et al. (2008a), who also demonstrated the formation of molecular networks in solutions of diaminobutyric acid-based γ-nucleopeptides with alternate configuration of residues having (1) a self-complementary nucleobase (Roviello et al. 2009b) or (2) a homothymine sequence in the presence of free adenine in solution (Roviello et al. 2008b). In conclusion nucleopeptides can form molecular complexes of different nature, thanks to the complementary nucleobase-recognition, which can be beneficial for example in promoting peptide–peptide (or protein–protein) interaction or molecular networking in biocompatible gels to be employed in drug delivery strategies.

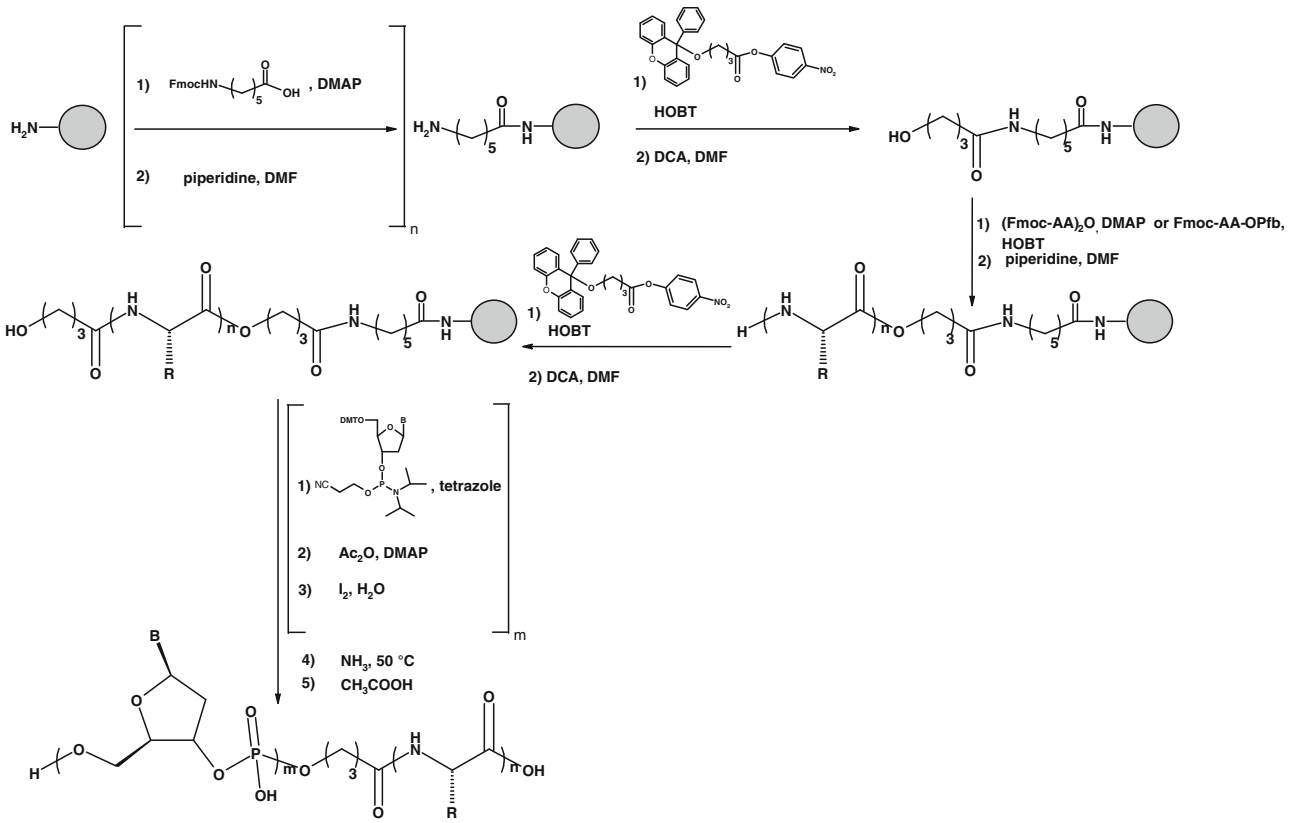
Biological properties and hyphothesized prebiotic role of nucleopeptides

Apart from the nucleic acid-binding ability presented by some nucleopeptides, other biological properties (such as cell permeability, nucleopeptide/biomolecule interaction ability and serum stability) of this class of oligonucleotide analogues are also interesting in view of their use in biomedicine. For example it is possible to realize "intrinsically cell-penetrating nucleopeptides", such as those presented recently by Geotti-Bianchini et al. (2008), made of α-nucleoamino acids and different α-amino acids (Fig. 5), which are water soluble, able to penetrate into cells and reach the nucleus without giving any cytotoxic effect, which are fundamental characteristics for increasing the efficiency of nucleopeptide-based diagnostic and therapeutic strategies. Nucleopeptides can also interact with proteins as suggested by the recent studies of Roviello et al. (2009d) and are much more stable to enzymatic degradation than oligonucleotides (Roviello et al. 2009a, b). Finally it is worthy to mention that, after the detection of several nucleobases and diamino acids (but not ribose) in meteorite soil, diamino acid-containing nucleopeptides were proposed as primordial genetic material (Meierhenrich et al. 2004; Nielsen 1993; Strasdeit 2005), perhaps delivered to earth via meteorites. In this hypothesis nucleopeptides would have acted as self-replicating genetic molecules, with also a key role in the transition to the later RNA world.

Oligonucleotide-peptide conjugates

It is important to underline that the term "nucleopeptide" was also used in literature to indicate a class of oligonucleotide conjugates which are composed of an oligonucleotide part (mainly DNA) connected to a peptide component which can facilitate the membrane transport of the antisense or antigene moiety or can function as a cell signalling entity or also serve as nuclease (De la Torre et al. 1994; McMinn and Greenberg 1999; Astriab-Fisher et al. 2000). A significant contribution in this field came from Haralmbidis's group who developed methods for the synthesis of oligonucleotide–peptide chimeras containing fusion peptides derived from the gp41 glycoprotein of HIV. This strategy allows the preparation of conjugates comprising peptide moieties which facilitate the intracellular delivery of the antisense oligonucleotides and also provide protection against 3′-exonuclease digestion (Soukchareun et al. 1995).

In the convenient synthesis (Scheme 1) of oligonucleotide–peptide conjugates reported by Soukchareun et al. (1995), controlled pore glass (CPG) solid support, aminated with (3-aminopropyl)triethoxysilane, was functionalized with Fmoc-protected aminohexanoic acid. After Fmoc removal, the amino terminus was reacted with the pentafluorophenyl (Pfp) active ester of O-pixyl-4-hydroxybutyric acid. After removal of the pixyl protecting group, the first amino acid, was introduced as the symmetrical anhydride using DMAP as the catalyst. Subsequently, standard Fmoc peptide chemistry employing the p-nitrophenyl esters of amino acids was used for the remainder of the synthesis.



Scheme 1 Oligonucleotide–peptide conjugate synthesis based on the method of Soukchareun et al. (1995)

After the completion of peptide assembly, the terminus was converted to a protected aliphatic hydroxy group by reaction with the already mentioned 4-hydroxybutyric acid derivative. Automated DNA synthesis was then performed directly on the derivatized peptidyl resin utilizing the standard p-cyanoethyl phosphoramidite chemistry. Finally, the cleavage of the peptide ester bond to liberate the oligonucleotide-peptide conjugate and the removal of the protecting groups from nucleobases were afforded by treating the resin with aqueous concentrated ammonia, while, after HPLC purification, the detritylation of the conjugate was achieved by acetic acid.

Finally, it is worthy to mention the interesting examples of peptide conjugates of oligonucleotides reported by Turner et al. (2007) who described RNA targeting oligonucleotides covalently conjugated to Penetratin or Tat cell-penetrating peptides.

Artificial nucleobase-containing pseudopeptides

Among the artificial nucleobase-bearing pseudopeptides described in literature, PNAs (peptide nucleic acids), presented by Nielsen et al. (1991), are the most successful DNA analogs based on a polyamide backbone prepared to date. These achiral oligonucleotide analogs are characterized by a nucleobase-containing 2-aminoethyl glycine repeating unit (Fig. 7) in which the DNA base is anchored to the glycine nitrogen by means of a methylene carbonyl linker (Corey 1997; Nielsen and Egholm 1999; Egholm et al. 1992) and, as specified also by Nielsen, can not be considered real peptides. Nevertheless, the name "Peptide Nucleic Acids" was chosen for these innovative nucleotide molecules in order to underline their polyamide nature.

General properties of PNA

All intramolecular distances, as well as the configuration of bases in PNA, resemble those present in natural DNA, while the uncharged nature of PNA prevents any electrostatic repulsion during the complexation of the target. Thus, PNAs are able to form complexes with both DNA and RNA molecules, based on hydrogen bond formation with high melting temperatures (as a general rule Tm of PNA/DNA duplex in 100 mM NaCl is 1°C higher per base pair than Tm of a DNA/DNA duplex with the same base composition). These complexes are stable also under low salt conditions, because no cations are needed to counteract the interstrand repulsion typical of the duplex formation between the two negatively charged strands of natural

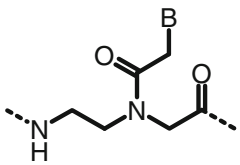
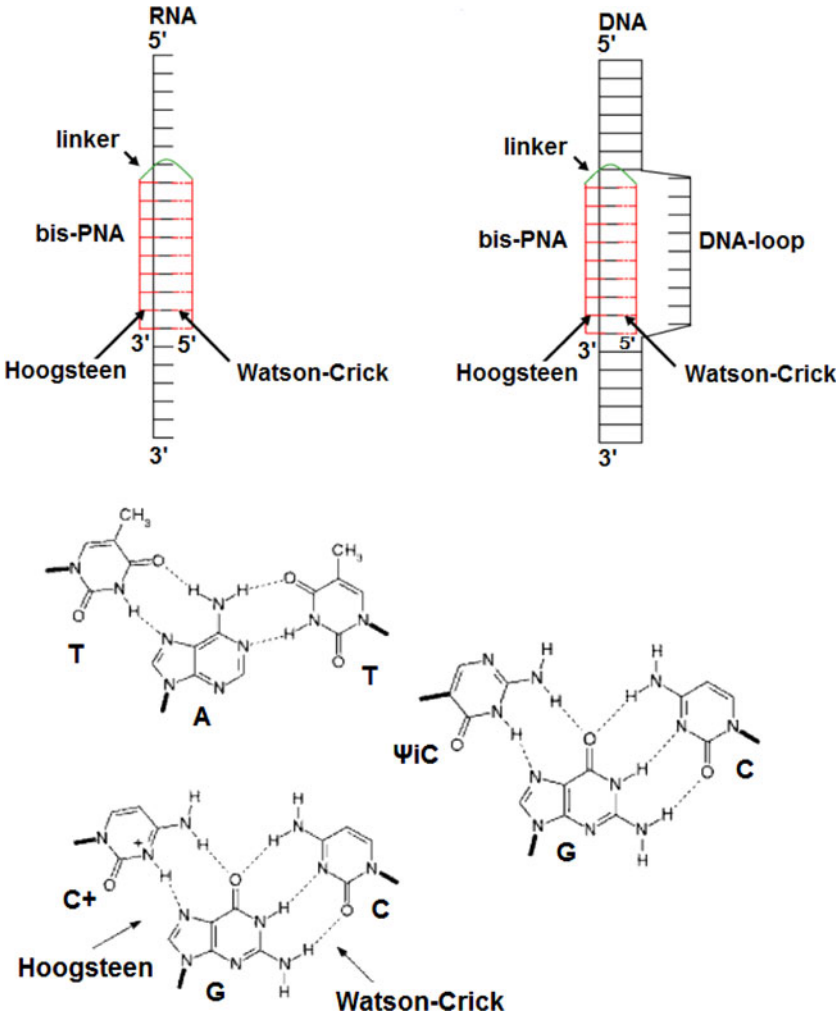


Fig. 7 *N*-(2-aminoethyl) glycine-based repeating unit of peptide nucleic acids

oligonucleotides. It is also important to underline that PNAs are stable across a wide range of pH, unlike a natural oligonucleotide which depurinates under acidic conditions, and is also thermoresistant (Uhlmann et al. 1998). PNAs show also a remarkable specificity in binding to complementary natural oligonucleotides; indeed a single mismatch in a PNA/DNA duplex is more destabilizing than a DNA/DNA mismatch. Furthermore, pyrimidine-rich PNAs form with complementary strands of DNA, (but also RNA or PNA) complexes characterized by a 2:1 PNA/DNA (but also PNA/RNA or PNA/PNA) stoichiometry corresponding to triplex structures (Betts et al. 1995, Egholm et al. 1993). The possibility to form stable triplexes with DNA allows PNA to "strand invade" DNA/DNA double helices (Scheme 2). Another favourable aspect of PNAs resides in the high stability toward nucleases and proteases (Demidov et al. 1994), which is an important feature for their applicability in in vivo strategies.

Synthesis of PNA

The synthesis of PNA can be performed in analogy to peptides (Merrifield 1986), with solid phase strategies making use of a solid support, e.g. a (methylbenzhydryl)amine polystyrene resin (Christensen et al. 1995; Pothukanuri et al. 2008; Lee et al. 2007), and of Fmoc(9-fluorenylmethoxycarbonyl group)-protected PNA monomers in which the exocyclic amino groups of A, G and C bear Bhoc (benzyhydryloxycarbonyl) protecting groups which are removed at the end of the synthesis by treatment with trifluoroacetic acid. The primary amino group in the monomer backbone can be deprotected from the Fmoc-group by treatment with 20% piperidine in DMF (dimethylformamide) at the end of each coupling step. After the synthesis is accomplished, the PNA can be



Scheme 2 Triplexes are formed between one homopurine DNA or RNA strand and two sequence-complementary PNAs. One PNA strand binds by Watson–Crick base pairing and the other via Hoogsteen base pairing. The two PNA strands can be covalently connected to each other by a flexible linker to create bis-PNAs. Furthermore, the binding can be rendered pH independent by replacing cytosines in the Hoogsteen strand of modified PNAs with pseudoisocytosines (ΨiC)

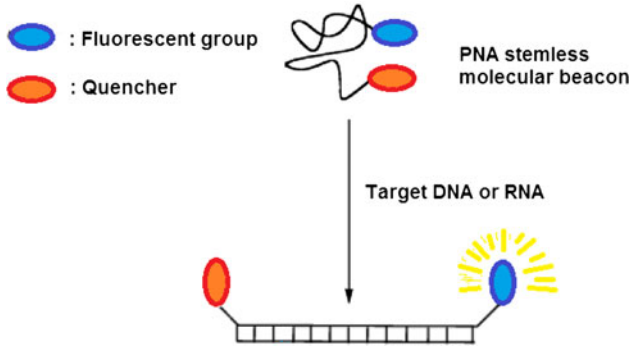
cleaved from the resin by treatment with trifluoroacetic acid and m-cresol (4/1 v/v) and precipitated by cold diethyl ether. Subsequently, HPLC purification can be performed and pure PNA oligomers are characterized by mass spectrometry (Christensen et al. 1995). Taking into account the importance of the orthogonality of PNA synthesis with other chemistries, Pothukanuri et al. performed a thorough investigation of six types of protecting groups for the terminal nitrogen atom and five protecting groups for the nucleobases of PNA monomers (Pothukanuri et al. 2008). Furthermore, the synthesis of self-activated PNA monomers as well as an efficient route to PNAs using a benzothiazole-2-sulfonyl group as an amine-protecting group and an acid-activating group were also reported by Lee et al. (2007). Since the synthesis of homothymine PNA segments, required for the construction of PNA-based triplex structures, is plagued by the occurrence of a significant amount of truncation products, Altenbrunn and Seitz investigated the use of novel allyl-protected thymine PNA monomers which provided significant improvements to the yields with the standard protocols used in the automated PNA synthesis (Altenbrunn and Seitz 2008). Interestingly, the synthesis of a Fmoc/Boc pseudoisocytosine monomer for peptide nucleic acid assembly was recently described by Hudson and Wojciechowski (2008).

PNA modifications

Among the many possible modifications, PNA can be derivatized by insertion of lysine or cysteine amino acids in the pseudopeptide skeleton (Dueholm et al. 1994, Thomson et al. 1995). Furthermore, bis-PNA can be prepared by connecting two PNA strands by a flexible linker such as the 6-aminohexanoic acid (Egholm et al. 1995). Further remarkable characteristics come from the modification of the PNA skeleton by DNA tracts as reported in the case of the so-called PNA–DNA chimeras that can be assembled in solution by ligation of pre-assembled PNA and DNA molecules, or alternatively by solid phase synthesis making use of suitable linkers (Uhlmann et al. 1998, Musumeci et al. 2004).

Applications of PNA in molecular biology and medicine

PNAs can be used as therapeutic drugs in antigene and antisense strategies (Hanvey et al. 1992; Nielsen et al. 1993; Nielsen 1999a). In an antigene approach, PNAs are designed to hybridize to complementary DNA sequences in a particular gene. The structural hindrance, due to the strand invasion of DNA, blocks the activity of the prokaryotic/eukaryotic RNA polymerase interfering with the transcription of the gene of interest (Nielsen et al. 1994). Of course, since PNA_2DNA triplexes are formed only with polypyrimidine PNA, a polypurine tract must be present in the gene to be targeted. In an antisense strategy, PNA are used to inhibit the translation of complementary mRNA. Both duplex and triplex-forming PNA molecules can inhibit the translation at initiation codon targets but only triplex-forming PNAs can be used to cause the translation elongation arrest (Knudsen and Nielsen 1996). PNA–peptide conjugates, able to penetrate into cells, were used in anticancer applications in which they inhibited telomerase activity in human melanoma cells and tumour specimens (Villa et al. 2000). PNAs find application also as tools for molecular biology and functional genomics (Nielsen 1999b). For example, they can be used in combination with a nuclease as an "artificial restriction enzyme". Indeed, PNAs can be designed and prepared to hybridize a complementary target on a DNA duplex via a strand invasion mechanism. The single-stranded DNA fragments looped out by the PNA are thus easily degraded by the nuclease employed in combination with the PNA in this strategy (Demidov 2001). Futhermore, short PNA sequences, preferably bis-PNAs, in combination with methylation and restriction enzymes can be used as rare genome cutters. The PNA molecule will shield the host site on DNA from enzymatic methylation, whereas the other regions of DNA will be methylated. The methylation will protect from enzymatic digestion all DNA sites except those regions previously bound to PNA (Veselkov et al. 1996). PNAs can be used also for PCR amplification. Indeed, PNAs do not interact with DNA polymerase but are able to terminate the elongation of oligonucleotide primers by competing with them or binding to the template (Demers et al. 1995). Interestingly, PNA–DNA chimeras show interaction with DNA polymerase and find application as primers for PCR reactions (Fiandaca et al. 2001). Due to their high binding affinity, PNA can also be used to detect single base pair mutation by PCR (Orum et al. 1993). PNA can also be employed in an improved version of the southern hybridization technique, which is daily used in molecular biology to predict sequence and size characteristics of DNA. With respect to the conventional technique, the use of PNA in southern hybridization allows a much faster analysis (Perry-O'Keefe et al. 1996). PNAs also find application in the purification of target nucleic acids, as in the case of PNA carrying six histidines which are used in combination with nickel affinity columns (taking advantage of the affinity of nickel for the histidine tag) or biotinylated PNA with streptavidin-coated magnetic beads (Orum et al. 1995; Kerman et al. 2004). Besides all the innovative PNA applications heretofore cited, it is also worth mentioning the PNA molecular beacons and more particularly the stemless PNA beacons (Scheme 3), which are less sensitive to ionic strength than DNA molecular



Scheme 3 Generic representation of stemless PNA molecular beacons. On their own, PNA molecular beacons are non-fluorescent, because they present the fluorophore close to the quencher. When the probe sequence hybridizes to its DNA/RNA target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, restoring fluorescence

beacons and do not present important drawbacks, such as the influence of DNA-binding proteins on the quenched fluorescence (Ortiz et al. 1998; Socher et al. 2008).

Moreover, PNA-based chips are also known to present a number of advantages with respect to oligo-DNA chips, being fast, reusable and presenting high accuracy and reproducibility as well as a longer storage than traditional DNA chips (Brandt and Hoheisel 2004). Another interesting application of PNAs was given in the recent study of Corriveau et al. (2009), which describes the detection of *Staphylococcus aureus* within nasal tissue collected in patients with chronic rhinosinusitis using the peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH) technique (Corriveau et al. 2009).

Drawbacks of PNA and development of analogues with improved properties

Besides the many positive characteristics of PNA, some drawbacks of these powerful biomimetics should also be considered. Indeed, PNAs are poorly soluble in water and have a certain tendency to aggregate. Furthermore, PNAs penetrate with difficulty into the cell membrane. However, the introduction of charged amino acids can give some improvements in the solubility of PNAs (Zhou et al. 2003; Katritzky and Narindoshvili 2008) and, as in the case of PNAs modified with arginines, can facilitate the cell uptake (Dragulescu-Andrasi et al. 2006; Calabretta et al. 2009). Among the many PNA analogues with improved properties (Corradini et al. 2007; Wojciechowski and Hudson 2007), Oxy-peptide nucleic acids (OPNAs), introduced by Kuwahara et al. (1999), are oligonucleotide analogs containing ether linkages in the main chain. These nucleobase-containing pseudopeptides, characterized by a better water solubility than PNAs, are able to bind complementary DNA molecules (Kuwahara et al. 1999, Kitamatsu et al. 2002). Not less importantly, PNA conjugated with specific peptides, which can be obtained by automated orthogonal microwave synthesis (Svensen et al. 2008), but also with antibodies and steroids, have also been used to improve the PNA delivery efficiency (Simmons et al. 1997; Boado et al. 1998; Rebuffat et al. 2002).

Conclusions

The present work gives an overview of nucleobase-containing peptides. This interesting class of chimeric molecules includes some natural molecules with antimicrobial activity as well as many synthetic biomimetics prepared with the aim of conjugating the nucleic acid-binding ability of oligonucleotides with other characteristics, such as the chemical and thermal stabilities, typical of peptides. Not all of the nucleopeptides obtained to date showed the favourable hybridization properties found in the case of PNA, a potent nucleo-pseudopeptide which currently finds important applications in the field of medicine. In any case, some PNA limitations, such as insolubility and poor cell permeability, need to be overcome, for example by the chemical modification of the aminoethylglycine backbone or the design and the preparation of novel nucleopeptides which will increase the potential of nucleobase-containing peptides in many areas of biology and therapy.

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