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ORIGINAL ARTICLE

Synthesis, characterization and hybridization studies of an alternate nucleo-ε/γ-peptide: complexes formation with natural nucleic acids

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Abstract In order to develop new oligodeoxyribonucleotide (ODN) analogs to be used in biotechnological applications, we report here the synthesis, characterization and nucleic acid binding studies of novel nucleopeptides, that we called *ε-lys/γ-dab*PNAs, containing a backbone of alternated L-diaminobutyric acid and L-lysine moieties. Exploring the hybridization properties of the new ODN analog, we found, by circular dichroism and UV spectroscopies, that a homothymine *ε-lys/γ-dab*PNA hexamer binds both DNA and RNA of complementary sequence. Furthermore, human serum stability assays on the alternate nucleopeptide evidenced a noteworthy degradation resistance. These results encourage us to deepen the knowledge of this analog, in order to evaluate its possible use in antigene/antisense or diagnostic applications.

Keywords Nucleopeptides · ODN analogs · *ε-lys/γ-dab*PNAs · Binding studies · Serum stability

Introduction

The development of new synthetic oligodeoxyribonucleotide (ODN) analogs able to bind complementary natural nucleic acids has received considerable scientific interest for decades in view of their biomedical and bioengineering applications and has lead to a number of artificial molecules containing modifications in the backbone and/or in nucleobases respect to natural oligonucleotides (Briones and Martin-Gago 2006; Helene and Le Doan 1991; Kurreck 2003). Among the many ODN mimics, *aminoethylglycyl*peptide nucleic acids (*aeg*PNAs), introduced for the first time in 1991 (Nielsen et al. 1991) and presenting an artificial pseudo-peptide chain in place of the sugar–phosphate backbone (Fig. 1), emerged as the most promising candidates, forming with DNA and RNA sequence-specific complexes characterized by high thermal stabilities (Uhlmann et al. 1998). However, besides these important features, *aeg*PNAs present some drawbacks, like poor water solubility, low cell permeability and also ambiguity in nucleic acids recognition (parallel/antiparallel mode), that are expected to be overcome (Uhlmann et al. 1998). In fact, an important characteristic for obtaining high specificity in the recognition of complementary nucleotide targets resides in the ability of an ODN analog to discriminate between parallel and antiparallel orientations during the binding. Interestingly, the presence of stereogenic centers in PNA molecules allows for an improved binding mode discrimination, as reported in several literature examples describing PNA analogs with polyamide backbones entirely or partially chiral. In many cases chiral centers are provided by the insertion of aminoacid moieties at the *aeg*PNA ends (especially lysines and arginines) or directly into the PNA backbone (*ael*PNA, *amp*PNA, *δ-orn*PNA, *γ-dab*PNA, *ε-lys*PNA, etc., Fig. 1; Gangamani et al. 1999; Haimaa et al. 1996; Roviello et al. 2006, 2008; Sforza et al. 1999, 2002).

In continuing our research on nucleopeptides as ODN analogs, we recently reported the synthesis of nucleo-γ-peptides (*γ-dab*PNAs; Roviello at al. 2006, 2007) and

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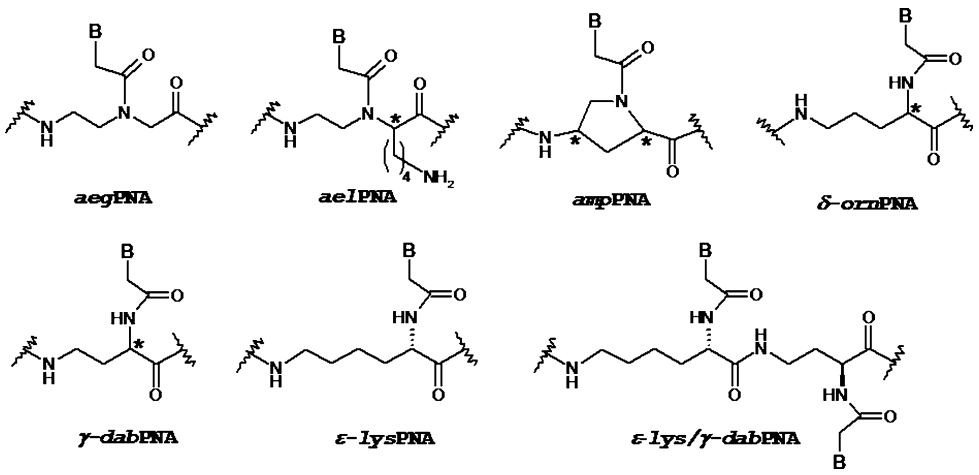

Fig. 1 Repeating units of synthetic DNA mimics based on polyamide backbones

nucleo-ε-peptides (ε-*lys*PNAs; Roviello at al. 2008) (Fig. 1), based respectively on a L-diaminobutyric acid (DABA) and L-lysine (L-Lys) backbone, and their hybridization studies with natural nucleic acids. From these studies, it emerged that pure γ-*dab*PNAs, which have a backbone one-atom shorter than Nielsen *aeg*PNAs, were unable to bind DNA or RNA, while ε-*lys*PNAs, with a backbone one-atom longer than *aeg*PNAs, bound complementary RNA but did not show significant interaction with DNA.

In the present work, we report the synthesis and characterization of novel chiral nucleopeptides, indicated as ε-*lys*/γ-*dab*PNAs (Fig. 1), realized alternating L-DABA- and L-Lys-based nucleoaminoacids. These monomers are linked together by peptide bonds involving the γ- and ε-side-chain amino groups, respectively of the DABA and Lys moieties. The NH_2 in α position of both L-diaminoacids carries the nucleobase through a methylene carbonyl linker. Since the backbone of ε-*lys* and γ-*dab* nucleoaminoacids contains respectively one atom more and one atom less than the aminoethylglycine unit of *aeg*PNAs, by alternating these two monomers in ε-*lys*/γ-*dab*PNAs, the average number of bonds between the atoms bearing the nucleobases was the same found in *aeg*PNAs (i.e., 6), while for the previously reported γ-*dab*PNA (Roviello et al. 2006, 2007), which did not bind DNA/RNA, it was lower (i.e., 5; Fig. 2).

Subsequently, in order to explore the possible use of ε-*lys*/γ-*dab*PNAs in antigene/antisense or diagnostic applications,

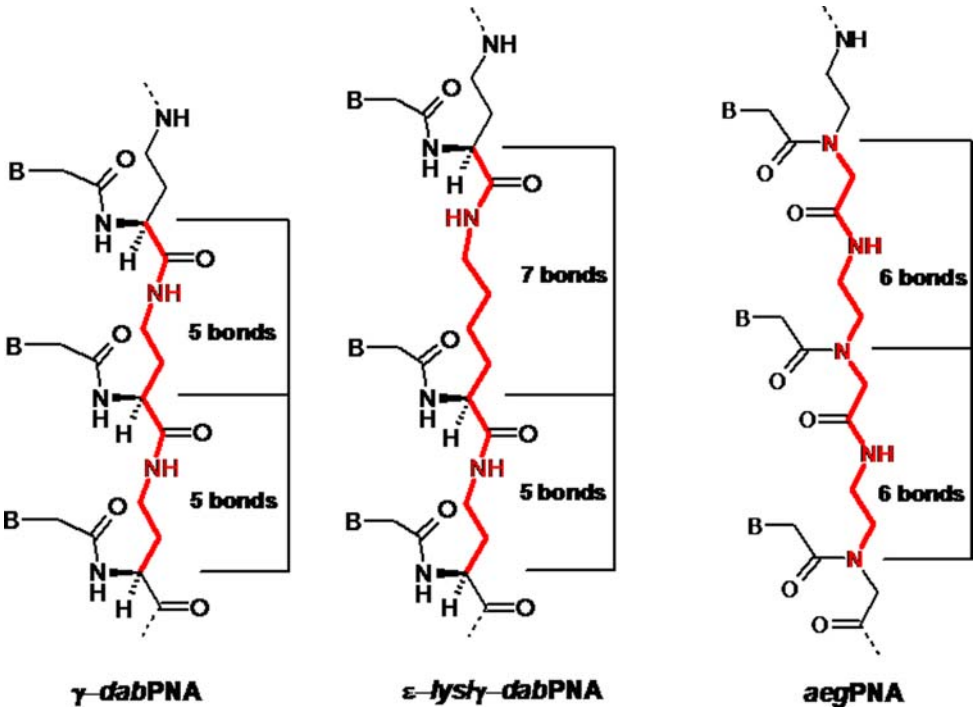

Fig. 2 Comparison between γ-*dab*PNA, ε-*lys*/γ-*dab*PNA and *aeg*PNA structures: the bonds between the atoms bearing nucleobases are evidenced in *red*

we investigated the ability of the new analogs to bind DNA and RNA by UV and CD spectroscopies, and evaluated their enzymatic resistance by serum stability assays.

It is noteworthy to underline that, despite *aeg*PNAs which are artificial pseudopeptides, the analog proposed here is a real peptide containing ε/γ-peptide bonds, and is bio-inspired because based on the proteogenic L-lysine and on L-DABA, a natural diaminoacid occurring, for example, in seeds and vegetative tissues of flatpea (*Lathyrus sylvestris L.*; Foster et al. 1987).

Furthermore, recent important findings on the antimicrobial activities and non-toxicity of γ-poly-L-DABA (Iwashita et al. 2005; Takehara et al. 2008) and ε-poly-L-lysines (Saimura et al. 2008; Hiraki et al. 2003), which resulted also biodegradable (Kito et al. 2003; Yoshida and Nagasawa 2003), encourage us to investigate in future experiments other possible biotechnological applications of our nucleo-ε/γ-peptides, such as the inhibitory action against microorganisms.

Finally, the present work could contribute to the study of the prebiotic implication of nucleopeptide hetero-oligomers, like our alternated *ε-lys/γ-dab*PNAs, in a scenario preceding the RNA world in which PNAs could have acted as self-replicating molecules with also a key role in the transition from the PNA world to the later genetic system based on nucleic acids.

Materials and methods

Abbreviations

*Aminoethylglycyl*PNA (*aeg*PNA), *tert*-butoxycarbonyl (Boc), circular dichroism (CD), diaminobutyric acid (DABA), *diaminobutyryl*PNA (*dab*PNA), *N,N*-diisopropylethylamine (DIEA), *N,N*-dimethylformamide (DMF), 9-fluorenylmethoxycarbonyl (Fmoc), *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU), 4-methylpyrrolidone (NMP), polytetrafluoroethylene (PTFE), (1H-benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyBOP), trifluoroacetic acid (TFA), 2,4,6-trimethylpyridine (TMP).

Chemicals

Fmoc-L-Lys(Boc)-OH, HATU, PyBOP were purchased from Novabiochem. Boc-L-DABA(Fmoc)-OH was from Bachem. Anhydroscan DMF and NMP were from Lab-Scan. Piperidine was from Biosolve. Solvents for HPLC chromatography and acetic anhydride were from Reidel-de Haën. PolyA, TFA, TMP, Rink-amide resin were Fluka. TFA (for HPLC) was from Romil. Diethyl ether was from Carlo Erba.

Apparatus

Centrifugations were performed on a Z 200 A Hermle centrifuge. Products were analysed and characterized by LC–MS on an MSQ mass spectrometer (ThermoElectron, Milan, Italy) equipped with an ESI source operating at 3-kV needle voltage and 320°C, and with a complete Surveyor HPLC system, comprising an MS pump, an autosampler and a PDA detector, by using a Phenomenex Jupiter C18 300 Å (5 μm, 4.6 mm × 150 mm) column. Gradient elution was performed by using increasing amounts of acetonitrile (0.05% TFA) in water (0.05% TFA), monitoring at 260 nm, with a flow rate of 0.8 ml/min. Semi-preparative purifications were performed on a Hewlett Packard/Agilent 1100 series HPLC, equipped with a diode array detector, by using a Phenomenex Jupiter C18 300 Å (10 μm, 10 mm × 250 mm) column. Gradient elution was performed at 45°C (monitoring at 260 nm) by building up a gradient starting with buffer A (0.1% TFA in water) and applying buffer B (0.1% TFA in acetonitrile) with a flow rate of 4 ml/min.

Samples were lyophilized in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h. CD spectra were obtained on a Jasco J-810 spectropolarimeter. UV spectra and UV melting experiments were recorded on a UV–Vis Jasco model V-550 spectrophotometer equipped with a Peltier ETC-505T temperature controller. UV and CD measurements were performed in Hellma quartz Suprasil cells, with a light path of 1 cm and 2 cm× 0.4375 cm (Tandem cell).

Solid phase synthesis of oligomers **1** and **2**

Thymine nucleoaminoacids $t_{L\text{-}dab}$ and $t_{L\text{-}lys}$ were realized starting from the commercial Boc-L-DABA(Fmoc)-OH and Boc-L-Lys(Fmoc)-OH, respectively (Fig. 3), following the synthetic procedure that we previously reported (Roviello et al. 2006, 2007, 2008).

Solid phase synthesis was carried out in short PP columns (4 ml) equipped with a PTFE filter, a stopcock and a cap on a Rink-amide resin using the peptide-like Fmoc chemistry. Every synthetic cycle was composed by (1) a coupling step of the monomers to the NH_2 on the resin by using the protocol described below; (2) a capping of the unreacted NH_2 with 5% $(Ac)_2O$/6% Lutidine for 5 min; (3) a deblocking step to remove the Fmoc group from the NH_2 of the coupled monomer for 8 min. Couplings of *γ-dab*PNA and *ε-lys*PNA monomers were checked by measuring the absorbance of the released Fmoc group ($\varepsilon_{301} = 7{,}800$) after treatment with a solution of piperidine (20%) in DMF (UV Fmoc test) in order to evaluate the incorporation yields of each monomer. In the case of *γ-dab*PNA, average coupling yields were of about 75% for

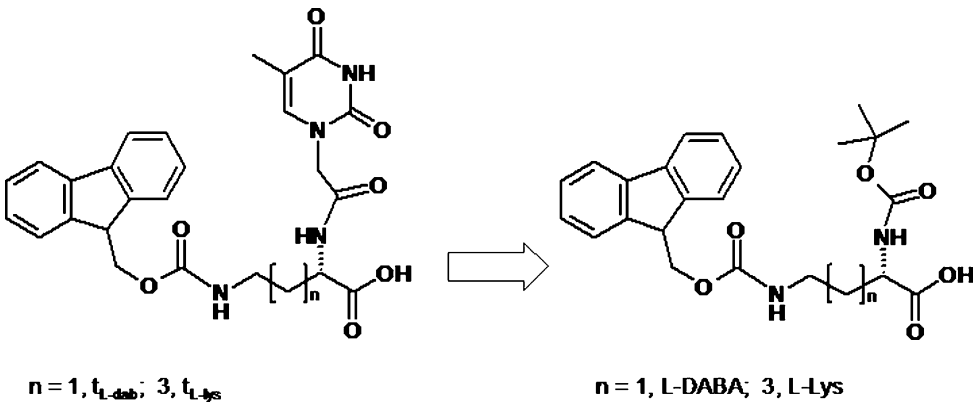

Fig. 3 L-DABA/L-Lys thymine nucleoaminoacids and their starting precursors

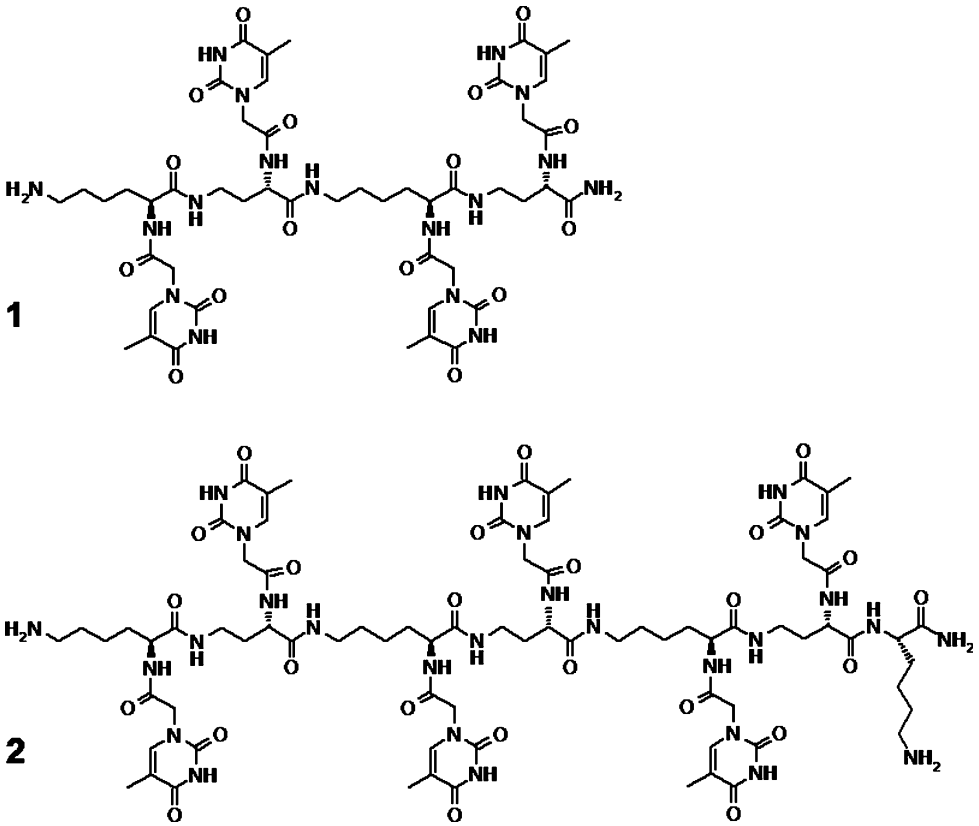

Fig. 4 Molecular structure for **1** and **2** nucleopeptides

1 and 90% for **2**; while, in the case of ε-*lys*PNA, yields were of about 40% for **1** and 60% for **2**.

H-($t_{L\text{-}lys}$–$t_{L\text{-}dab}$)$_2$-NH_2 (**1**, Fig. 4) was assembled on Rink-amide-NH_2 resin (0.5 mmol/g, 8 mg, 4 μmol) using the following coupling protocol: a mixture of Fmoc-L-T_{lys}PNA-OH or Fmoc-L-T_{dab}PNA-OH (0.2 M in NMP, 60 μl, 12 μmol, 3 Eq), HATU (0.18 M in DMF, 60 μl, 10.8 μmol, 2.7 Eq) and 100 μl of DMF was introduced into the reactor. Successively, 100 μl (20 μmol, 5 Eq) of a TMP solution (0.2 M in NMP) was added to the stirred reaction in four portions over 1 h. The overall yield of **1** calculated on the basis of the UV Fmoc test was 10%. Oligomer **1** was cleaved from the resin and deprotected under acidic conditions (TFA/m-cresol/H_2O 80:18:2 v/v, 1 ml) and recovered by precipitation with cold diethyl ether, centrifugation and lyophilization. The tetramer **1** was purified by semipreparative HPLC using a linear gradient of 6% (for 5 min) to 23% B in A over 25 min: t_R = 25.0 min; UV quantification of the purified product gave 320 nmol of **1**; ESI–MS (Fig. 5) *m/z*: 1162.74 (found), 1161.16 (expected for $[C_{48}H_{67}N_{17}O_{16} + Na]^+$); 1140.70 (found), 1139.18 (expected for $[C_{48}H_{67}N_{17}O_{16} + H]^+$).

H-($t_{L\text{-}lys}$–$t_{L\text{-}dab}$)$_3$-Lys-NH_2 (**2**, Fig. 4). Rink-amide resin (0.5 mmol/g, 16 mg, 8 μmol) was functionalized with a L-lysine (Fmoc-L-Lys(Boc)-OH, 0.5 Eq, 1.9 mg, 4 μmol) using PyBOP (0.5 Eq, 2.1 mg, 4 μmol) as activating agent

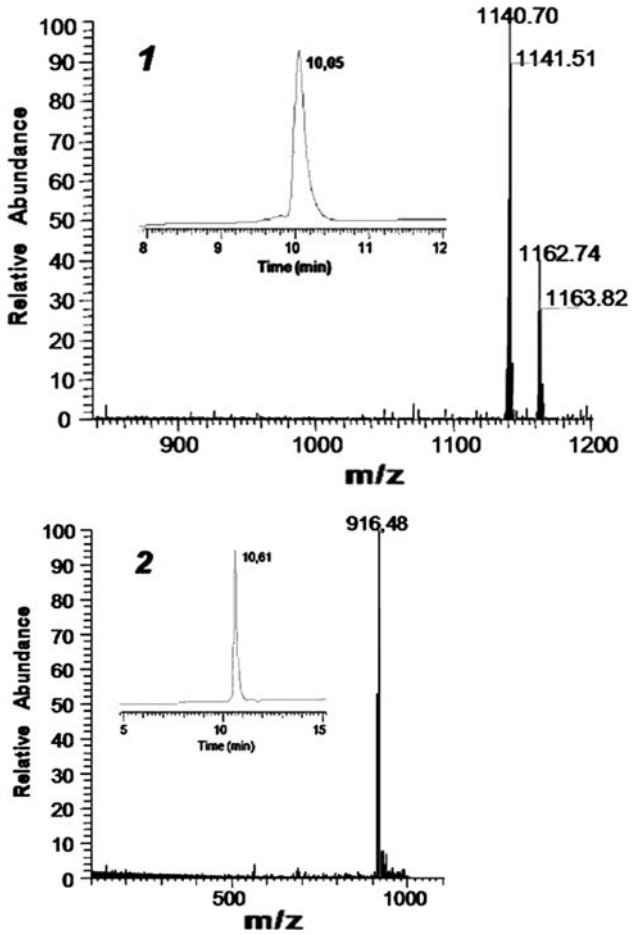

Fig. 5 LC-ESIMS of oligomers H-($t_{L\text{-}lys}$–$t_{L\text{-}dab}$)$_2$-NH_2 (**1**) and H-($t_{L\text{-}lys}$–$t_{L\text{-}dab}$)$_3$-Lys-NH_2 (**2**)

and DIEA (1 Eq, 1.4 µl, 8 µmol) as base for 30 min at room temperature. Capping of the unreacted amino groups was performed with Ac_2O (20%)/DIEA (5%) in DMF. The UV Fmoc test (deprotection with 30% piperidine in DMF) revealed a quantitative yield respect to the added lysine (0.25 mmol/g). Hexamer **2** was assembled following the procedure described above for tetramer **1**. The overall yield of **2** calculated on the basis of the UV Fmoc test was 17%. The cleaved and deprotected oligomer was purified by semipreparative HPLC using a linear gradient of 6% (for 5 min) to 23% B in A over 25 min: $t_R = 28.7$ min; UV quantification of the purified product gave 500 nmol of **2**; ESI-MS (Fig. 5) *m/z*: 916.48 (found), 914.47 (expected for $[C_{78}H_{111}N_{27}O_{25} + 2H]^{2+}$).

UV and CD studies

Purified oligomers were dissolved in a known amount of MilliQ water and quantified by UV measurements ($T = 85$°C, absorbance value at $\lambda = 260$ nm). The epsilon values used for the quantification of the oligomers **1** and **2** (34.4 and 51.6/mM) were calculated using the molar extinction coefficient of thymine *aeg*PNA monomer (8.6/mM).

Thermal melting curves were obtained by recording the UV absorbance at 260 nm by increasing the temperature at a rate of 0.5°C/min. T_m values were calculated by the first derivative plot. CD spectra were recorded from 320 to 200 nm: scan speed 50 nm/min, data pitch 2 nm, band width 2 nm, response 4 s, five accumulations.

Serum stability assays

Ten microliters of nucleopeptide **2** (100 µM in phosphate buffer, pH = 7.5) was added to 90 µl of 100% fresh human serum (**2** was 10 µM in 90% serum) in a micro-vial and the mixture was incubated at 37°C. Aliquots (10 µl) were taken at 0, 1, 2, 3, 4, 5 and 24 h, quenched by adding 10 µl of 7 M urea solution, kept at 95°C for 2 min and then stored at −20°C until subsequent analysis. The withdrawn samples were analyzed by HPLC on a Phenomenex Jupiter C18 300 Å (5 µm, 4.6 mm × 250 mm) column using a linear gradient of 5% (for 5 min) to 40% B in A over 10 min.

Results and discussion

The synthesis of the thymine nucleoaminoacids based on L-DABA and L-lysine ($t_{L\text{-}dab}$ and $t_{L\text{-}lys}$, Fig. 3), suitably protected for Fmoc solid-phase peptide-like synthesis, was accomplished, as reported in our previous works (Roviello et al. 2006, 2007, 2008) starting from the commercial Boc-L-DABA(Fmoc)-OH and Boc-L-Lys(Fmoc)-OH (Fig. 3).

NMR and ESIMS spectra were in agreement with our previous published data (Roviello et al. 2006, 2007, 2008) confirming the identity of both monomers.

By alternating the above mentioned thymine γ-*dab*-PNA and ε-*lys*PNA monomers in the sequence, we synthesized on solid phase two ε-*lys*/γ-*dab*PNA nucleopeptides (**1** and **2**, Fig. 4) following a coupling protocol based on HATU/TMP which ensures retention of optical purity of the nucleoaminoacid residues (Sforza et al. 2002).

During the synthesis of tetramer **1**, when γ-*dab*PNA units were coupled, an average yield of about 75% was estimated, while yields for ε-*lys*PNA insertions were <40%. A possible explanation for this difference resides in the different reactivity of the ε- and γ-amino groups, respectively of the ε-*lys*- and γ-*dab*PNA units, influenced by the distance of the NH_2 from the hindered C-α position, that is longer in the case of ε-*lys*PNA than in γ-*dab*PNA monomer. Thus, probably due to sterical hindrance, couplings of the Fmoc-protected *lys*PNA monomers to the free amino groups of *dab*PNAs on the resin are less favored

than couplings of the Fmoc-protected *dab*PNA monomers on the *lys*PNA amino groups on the resin.

Furthermore, in order to confer better water solubility to the detached *ε-lys*/*γ-dab*PNA oligomer, we introduced a L-lysine unit at the C-terminus of oligomer **2**. The functionalization of the resin was twofold reduced with the lysine to 0.25 mmol/g, to ensure a longer distance between the growing chains and consequently to avoid the typical aggregation problems occurring during nucleoaminoacid couplings (Altenbrunn and Seitz 2008). In this way, we revealed an increase in the average coupling yields of thymine *γ-dab*PNA and thymine *ε-lys*PNA monomers to about 90 and 60%, respectively. We proceeded with the synthetic cycles until the sixth monomer to obtain hexamer **2**.

Both oligomers **1** and **2** were cleaved from the solid support and deprotected by TFA treatment, followed by precipitation with cold diethyl ether. HPLC purification, performed on a C-18 column, revealed for alternate *ε-lys*/*γ-dab*PNA oligomer **2** a retention time intermediate between that of full *γ-dab*PNA and full *ε-lys*PNA thymine hexamers, as expected. In particular, the order of increasing apolarity was: (1) full *γ-dab*PNA, (2) alternate *ε-lys*/*γ-dabPNA*, (3) full *ε-lys*PNA.

LC-ESIMS characterization confirmed the identity of the new molecules **1** and **2** (Fig. 5). Successively, the structural characteristics of the *ε-lys*/*γ-dab*PNAs as well as their ability to form complexes with complementary natural nucleic acids were investigated by CD and UV spectroscopies. Firstly, the CD spectrum of the nucleopeptide single strands in 10 mM phosphate buffer (pH 7.5) was recorded in order to evaluate a possible helical preorganization of the *ε-lys*/*γ-dab*PNA alternate molecule. By examining the CD profile of hexamer **2** in the range 190–240 nm at different temperatures (5, 10 and 25°C) no significant α-helical contribution was detected for the oligomer. Anyway, a weak CD signal for the chiral nucleopeptide and, more exactly, a negative band centered at 277 nm was observed (Fig. 6) in analogy to the CD behavior already reported for other nucleopeptides, like *δ-orn*PNA (Sforza et al. 2002), *γ-dab*PNA (Roviello et al. 2006, 2007) or *ε-lys*PNA (Roviello et al. 2008), containing L-stereocenters. Thus, a certain degree of structural preorganization for the *ε-lys*/*γ-dab*PNA **2** in aqueous medium could be suggested. The shorter nucleopeptide **1** furnished an analogous CD spectrum, but with a lower intensity, respect to hexamer **2** (data not shown).

Subsequently, CD binding experiments on the homothymine oligomer **2** with complementary DNA (dA_{12}) and RNA (polyA) were performed in a tandem cell.

We firstly recorded the *sum* CD spectrum of the separated oligomers, *ε-lys*/*γ-dab*PNA **2** in one compartment of the tandem cell and dA_{12} in the other one, and then the *mix*

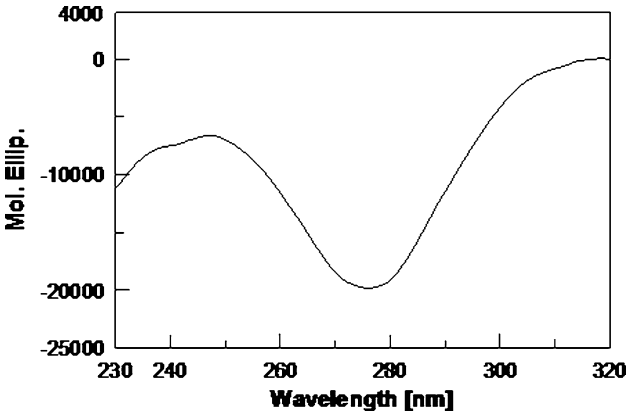

Fig. 6 CD spectrum of H-$(t_{L\text{-}lys}$–$t_{L\text{-}dab})_3$-Lys-NH_2 (**2**) in 10 mM phosphate buffer (pH 7.5) at 5°C

spectrum of the solution obtained after mixing the two samples. Some differences between *sum* and *mix* CD spectra (Fig. 7) suggested the formation of a $(t_{L\text{-}lys}$–$t_{L\text{-}dab})_3$/dA_{12} complex having a structure which resembles that of the stacked dA_{12} single strand.

UV melting experiments of the **2**/dA_{12} 8:1 mixture (4:1 in bases) evidenced a sigmoidal profile of the denaturation curve with a T_m of 8.7°C (Fig. 8), suggesting the formation of a weak complex based on hydrogen bonds and base stacking. The process is reversible and the pairing is completed in about 12°C (24 min at 0.5°C/min).

Circular dichroism binding experiments on the *ε-lys*/*γ-dab*PNA **2** with RNA (polyA) in the tandem cell also revealed the formation of a complex, as evidenced by the comparison of the *sum* and *mix* CD spectra (Fig. 9). CD signal of the *mix* spectrum stabilized after approximately 10 min, at 5°C (dotted line, Fig. 9), suggesting a kinetic in this time range for the formation of the $(t_{L\text{-}lys}$–$t_{L\text{-}dab})_3$/polyA hybrid. Despite DNA, the structure of the complex formed between *ε-lys*/*γ-dab*PNA and RNA was completely different

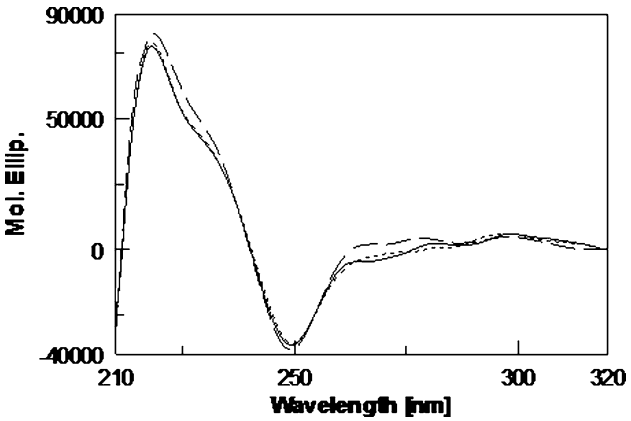

Fig. 7 CD spectra of **2**/dA_{12} 8:1 ratio (4:1 in bases) in 10 mM phosphate buffer (pH 7.5) at 5°C (sum: *dashed line*; mix after 1 min: *solid line*; mix after 10 min: *dotted line*; tandem cell)

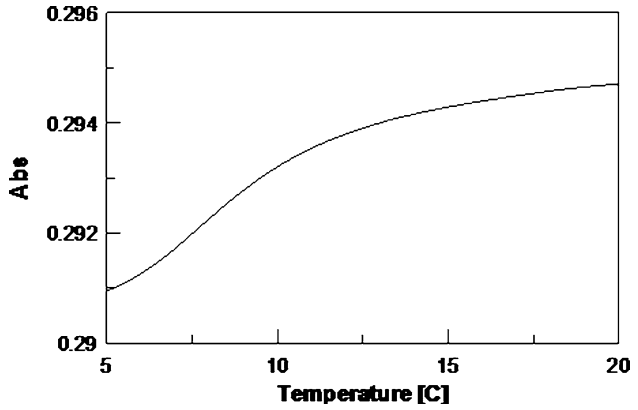

Fig. 8 UV melting (0.5°C/min) of the **2**/dA_{12} 8:1 mixture (4:1 in bases) in 10 mM phosphate buffer (pH 7.5)

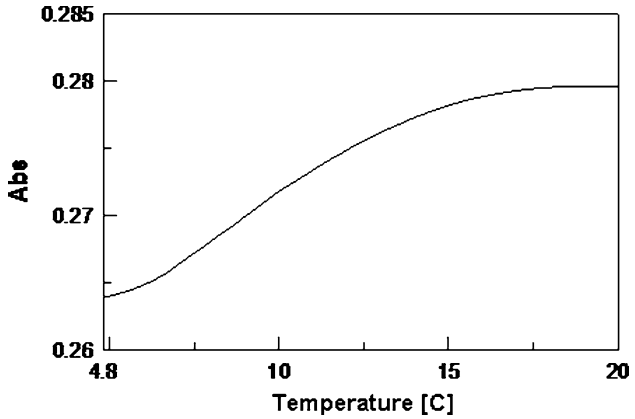

Fig. 10 UV melting (0.5°C/min) of the **2**/polyA complex (10:1 in bases) in 10 mM phosphate buffer (pH 7.5)

from that of the stacked polyA single strand, as revealed by the differences between *sum* and *mix* CD spectra. More particularly, the *mix* CD spectrum presented positive maxima at about 220, 260 and 290 nm. Since homopyrimidine PNA generally form $(PNA)_2$/RNA triplexes with complementary RNA sequences (Knudsen and Nielsen 1996; Uhlmann et al. 1998), we suggest for our case the formation of a (ε-*lys*/γ-*dab*PNA)$_2$/RNA triple helix (T-A-T triplets) with probably a left-handed structure in accord to previous studies (Sforza et al. 1999; Lagriffoule et al. 1997) that demonstrated as complexes involving PNA containing S-stereocenters, or equivalently L-aminoacids, were characterized by left-handed helices, predominant in solution.

The formation of a complex between ε-*lys*/γ-*dab*PNA and RNA was also confirmed by UV denaturation experiments which evidenced a sigmoidal profile of the melting curve relative to the **2**/polyA complex (10:1 in bases) with a T_m of 12°C (Fig. 10).

In the hypothesis of a triplex formation between ε-*lys*/γ-*dab*PNA and polyA, the presence of only one transition in the UV melting curve (Fig. 10) would suggest that no duplex intermediate was effectively formed, in agreement with the reported behavior of other PNA triplexes.

In the case of nucleopeptide **1**, no melting sigmoidal curve was obtained by UV denaturation, because of the low stability of the complexes of the tetrathymine nucleopeptide with nucleic acids.

The demonstration of the interesting binding properties of the ε-*lys*/γ-*dab*PNA nucleopeptide towards DNA and RNA pushed us to investigate also the stability of this ODN analog to enzymatic degradation, in view of its possible biomedical applications in vitro or in vivo. Thus, we incubated oligomer **2** in fresh human serum at 37°C and analyzed by HPLC several samples withdrawn from the reaction mixture at various times (Fig. 11). No significant degradation products appeared even after 24 h supporting the nuclease resistance of ε-*lys*/γ-*dab*PNA.

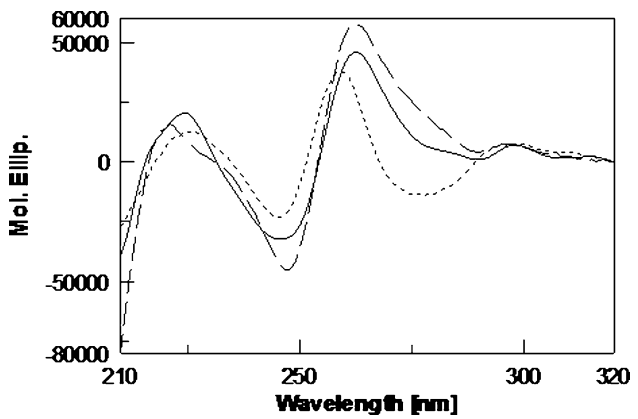

Fig. 9 CD spectra with tandem cell of **2**/polyA (10:1 in bases) in 10 mM phosphate buffer (pH 7.5) at 5 °C (sum: *dashed line*; mix after 1 min: *solid line*; mix after 10 min: *dotted line*)

Conclusions and perspectives

In order to develop new synthetic ODN analogs to be used in biotechnological applications, we designed and realized novel chiral nucleopeptides that we called ε-*lys*/γ-*dab*PNAs. To the best of our knowledge, this is the first report of an alternate ε/γ-peptide containing L-lysine and L-DABA diaminoacids linked together through their ε- and γ-side-chain amino groups and functionalized with DNA nucleobases in their α-positions.

Exploring the hybridization properties of this new ODN analog, we found, by using CD and UV spectroscopies, that a homothymine ε-*lys*/γ-*dab*PNA hexamer binds both DNA (dA_{12}) and RNA (polyA) of complementary sequence forming complexes based on hydrogen bonds and base stacking. Interestingly, this behavior is deeply far from that shown by γ-*dab*PNAs that did not bind natural nucleic acids (Roviello et al. 2006, 2007), and slightly different

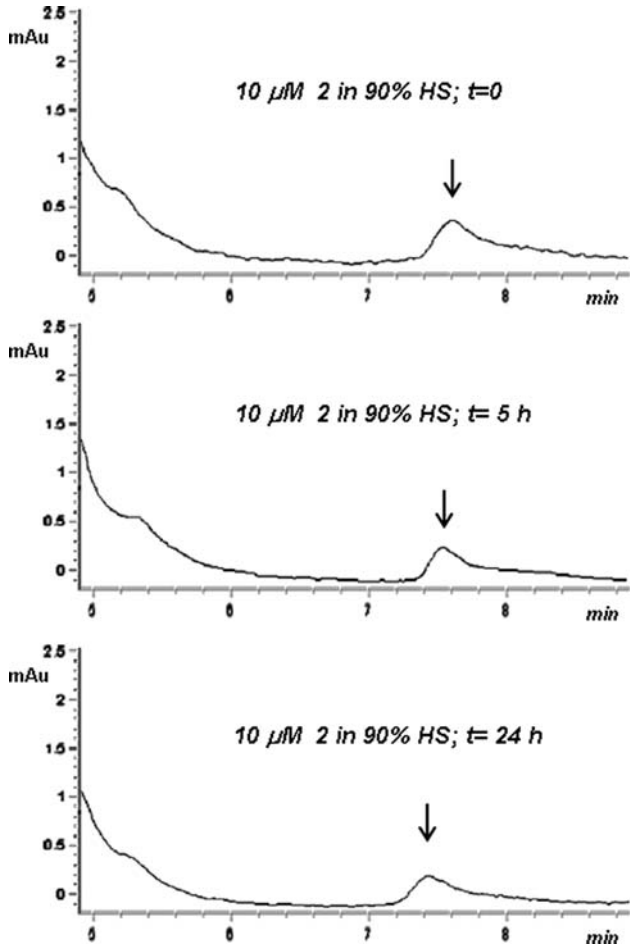

Fig. 11 Serum stability assay on *ε-lys*/*γ-dab*PNA **2** performed by HPLC

from that presented by *ε-lys*PNAs that bound complementary RNA but did not seem to bind DNA molecules (Roviello et al. 2008). In other words, the ODN-binding ability of the *ε-lys*/*γ-dab*PNA is not the result of the simple combination of the properties of *γ-dab*PNA and *ε-lys*PNA, but is more probably associable exclusively to the alternate molecular structure of the *ε*/*γ*-nucleopeptide.

Furthermore, human serum stability assays on the alternate nucleopeptide evidenced that the new ODN analog is more resistant than natural oligonucleotides, remaining substantially stable after 1 day.

All the remarkable characteristics of *ε-lys*/*γ-dab*PNAs, emerged from the present work, strongly encourage further scientific research in the study of the therapeutic and diagnostic potentiality of these alternate bio-inspired nucleopeptides. In particular, in order to verify the ability of our chiral nucleopeptides to discriminate between parallel and antiparallel binding to nucleic acids, in our future investigation we intend to synthesize *ε-lys*/*γ-dab*PNA oligomers of various lengths and with mixed nucleobase sequences: indeed, the realization of the *ε-lys*PNA and *γ-dab*PNA monomers containing all four bases is in progress. Nevertheless, several other desirable features of these nucleopeptides like cell permeability and non-toxicity will be evaluated in view of their usage in in vivo applications.

Interestingly, since the well-known antimicrobial activity of the natural poly(*ε*-L-lysine) and poly(*γ*-L-diaminobutyric acid) (Saimura et al. 2008; Takehara et al. 2008) was shown to be not necessarily due to their polycationic nature, determined by free amino groups (Takehara et al. 2008), this property should be explored also in the case of our mixed *ε-lys*/*γ-dab*PNAs, in which amino groups are capped by the nucleobases.

Finally, we propose that PNA molecules like our *ε-lys*/*γ-dab*PNAs, based on a mixed composition of diaminoacids, perhaps delivered to Earth via meteorites, could have been involved in the already hypothesized PNA world (Meierhenrich et al. 2004; Nelson et al. 2000; Nielsen 1993), in which they could have acted as self-replicating molecules, with also a key role in the transition to the later RNA world. The following observations are in favor of this hypothesis: (1) the recovery in meteoritic soil of DABA (Meierhenrich et al. 2004), lysine (Meierhenrich et al. 2004; Vdovykin 1973) and nucleobases (Glavin and Bada 2004); (2) the obtainment of DABA under simulated prebiotic conditions (Wolman et al. 1972); (3) the proposal of DABA-containing nucleopeptides as primordial genetic material (Meierhenrich et al. 2004; Nielsen 1993; Strasdeit 2005); (4) the predominant formation of *ε*-peptide bonds over *α*-peptide bonds in thermal synthesis of polypeptides under simulated prebiotic conditions (Temussi et al. 1976); (5) the ability, demonstrated in the present work, of *ε-lys*/*γ-dab*PNAs oligomers to bind RNA, which is an important requirement for a candidate as an RNA precursor (Strasdeit 2005).

However, it should be also investigated the catalytic potential of diaminoacids-containing nucleopeptides, for example in acting, similarly to *aeg*PNAs (Schmidt et al. 1997), as template for the synthesis of RNA molecules, an expected feature for an RNA precursor in the already proposed transition from the PNA world to the genetic system based on nucleic acids.

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References

Altenbrunn F, Seitz O (2008) *O*-Allyl protection in the Fmoc-based synthesis of difficult PNA. Org Biomol Chem 6:2493–2498. doi:10.1039/b805165h

Briones C, Martin-Gago JA (2006) Nucleic acids and their analogs as nanomaterials for biosensor development. Curr Nanosci 2:257–273

Foster JG, Cress WD, Wright SF, Hess JL (1987) Intracellular localization of the neurotoxin 2, 4-diaminobutyric acid in Lathyrus sylvestris L. leaf tissue. Plant Physiol 83(4):900–904

Gangamani BP, Kumar VA, Ganesh KN (1999) Chiral analogues of peptide nucleic acids: synthesis of 4-aminoprolyl nucleic acids and DNA complementation studies using UV/CD spectroscopy. Tetrahedron 55:177–192. doi:10.1016/S0040-4020(98)01024-2

Glavin DP, Bada JL (2004) Isolation of purines and pyrimidines from the Murchison meteorite. 35th Lunar and planetary science conference, Houston, Abstract 1022

Haimaa G, Lohse A, Buchardt O, Nielsen PE (1996) Peptide nucleic acids (PNAs) containing thymine monomers derived from chiral amino acids: hybridization and solubility properties of D-lysine PNA. Angew Chem Int Ed Engl 35:1939–1942. doi:10.1002/anie.199619391

Helene C, Le Doan T (1991) Oligonucleotides: problems and frontiers of practical applications. Trends Biotechnol 9:339–340. doi:10.1016/0167-7799(91)90115-X

Hiraki J, Ichikawa T, Ninomiya S, Seki H, Uohama K, Seki H, Kimura S, Yanagimoto Y, Barnett JW Jr (2003) Use of ADME studies to confirm the safety of ε-polylysine as a preservative in food. Regul Toxicol Pharmacol 37:328–340. doi:10.1016/S0273-2300(03)00029-1

Iwashita Y, Goto S, Tominaga M, Sasaki A, Ohmori N, Goto T, Sato S, Ohta M, Kitano S (2005) Dendritic cell immunotherapy with poly(d, l-2, 4-diaminobutyric acid)-mediated intratumoral delivery of the interleukin-12 gene suppresses tumor growth significantly. Cancer Sci 96:303–307. doi:10.1111/j.1349-7006.2005.00049.x

Kito M, Takimoto R, Onji Y, Yoshida T, Nagasawa T (2003) Purification and characterization of an ε-poly-L-lysine-degrading enzyme from the ε-poly-L-lysine-tolerant *Chryseobacterium* sp. OJ7. J Biosci Bioeng 96(1):92–94

Knudsen H, Nielsen PE (1996) Antisense properties of duplex- and triplex-forming PNAs. Nucleic Acids Res 24:494–500. doi:10.1093/nar/24.3.494

Kurreck J (2003) Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem 270:1628–1644. doi:10.1046/j.1432-1033.2003.03555.x

Lagriffoule P, Wittung P, Jensen K, Eriksson M, Norden B, Buchardt O, Nielsen PE (1997) Peptide nucleic acids with a conformationally constrained chiral cyclohexyl-derived backbone. Chem Eur J 3:912–919. doi:10.1002/chem.19970030613

Meierhenrich UJ, Muñoz Caro GM, Bredehöft JH, Jessberger EK, Thiemann WHP (2004) Identification of diamino acids in the Murchison meteorite. Proc Natl Acad Sci USA 101:9182–9186. doi:10.1073/pnas.0403043101

Nelson KE, Levy M, Miller SL (2000) Peptide nucleic acids rather than RNA may have been the first genetic molecule. Proc Natl Acad Sci U S A 97:3868–3871. doi:10.1073/pnas.97.8.3868

Nielsen PE (1993) Peptide nucleic acid (PNA): a model structure for the primordial genetic material? Orig Life Evol Biosph 23:323–327. doi:10.1007/BF01582083

Nielsen PE, Egholm M, Berg RM, Buchardt O (1991) Sequence-selective recognition of DNA by strand displacement with thymine-substituted polyamide. Science 254:1497–1500. doi:10.1126/science.1962210

Roviello GN, Moccia M, Sapio R, Valente M, Bucci EM, Castiglione M, Pedone C, Perretta G, Benedetti E, Musumeci D (2006) Synthesis, characterization and hybridization studies of new nucleo-gamma-peptides based on diaminobutyric acid. J Pept Sci 12:829–835. doi:10.1002/psc.819

Roviello GN, Musumeci D, Moccia M, Castiglione M, Sapio R, Valente M, Bucci EM, Perretta G, Pedone C (2007) *dab*PNA: design, synthesis and DNA binding studies. Nucleosides Nucleotides Nucleic Acids 26:1307–1310. doi:10.1080/15257770701530640

Roviello GN, Musumeci D, Castiglione M, Bucci EM, Pedone C, Benedetti E (2008) Solid phase synthesis and RNA-binding studies of a serum-resistant nucleo-ε-peptide. J Pept Sci. doi:10.1002/psc.1072 (published online on 4 Nov 2008)

Saimura M, Takehara M, Mizukami S, Kataoka K, Hirohara H (2008) Biosynthesis of nearly monodispersed poly(epsilon-L-lysine) in *Streptomyces* species. Biotechnol Lett 30(3):377–385. doi:10.1007/s10529-007-9563-7

Schmidt JG, Nielsen PE, Orgel LE (1997) Information transfer from peptide nucleic acids to RNA by template-directed syntheses. Nucleic Acids Res 25:4797–4802. doi:10.1093/nar/25.23.4797

Sforza S, Haaima G, Marchelli R, Nielsen PE (1999) Chiral peptide nucleic acids (PNAs): helix handedness and DNA recognition. Eur J Org Chem 1999:197–204. doi:10.1002/(SICI)1099-0690(199901)1999:1<197::AID-EJOC197>3.0.CO;2-N

Sforza S, Galaverna G, Dossena A, Corradini R, Marchelli R (2002) Role of chirality and optical purity in nucleic acid recognition by PNA and PNA analogs. Chirality 14:591–598. doi:10.1002/chir.10087

Strasdeit H (2005) New studies on the Murchison meteorite shed light on the pre-RNA world. Chembiochem 6:801–803. doi:10.1002/cbic.200400435

Takehara M, Saimura M, Inaba H, Hirohara H (2008) Poly(γ-L-diaminobutanoic acid), a novel poly(amino acid), coproduced with poly(ε-L-lysine) by two strains of *Streptomyces cellulofla-vus*. FEMS Microbiol Lett 286:110–117. doi:10.1111/j.1574-6968.2008.01261.x

Temussi PA, Paolillo L, Ferrara L, Benedetti L, Andini S (1976) Structural characterization of thermal prebiotic polypeptides. J Mol Evol 7:105–110. doi:10.1007/BF01732469

Uhlmann E, Peyman A, Breipohl G, Will DW (1998) PNA: synthetic polyamide nucleic acids with unusual binding properties. Angew Chem Int Ed Engl 37:2796–2823. doi:10.1002/(SICI)1521-3773(19981102)37:20<2796::AID-ANIE2796>3.0.CO;2-K

Vdovykin GP (1973) The Mighei meteorite. Space Sci Rev 14:832–879

Wolman Y, Haverland WJ, Miller SL (1972) Nonprotein amino acids from spark discharges and their comparison with the Murchison meteorite amino acids. Proc Natl Acad Sci USA 69:809–811. doi:10.1073/pnas.69.4.809

Yoshida T, Nagasawa T (2003) ε-Poly-L-lysine: microbial production, biodegradation and application potential. Appl Microbiol Biotechnol 62:21–26. doi:10.1007/s00253-003-1312-9