

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

G. N. Roviello · D. Musumeci · C. Pedone ·
E. M. Bucci

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Abstract In order to develop new oligodeoxyribo-nucleotide (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 -dabPNAs, 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 -dabPNA 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 -dabPNAs · 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, *amino-ethylglycyl*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 amino acid moieties at the *aeg*PNA ends (especially lysines and arginines) or directly into the PNA backbone (*ael*PNA, *amp*PNA, δ -ornPNA, γ -dabPNA, ϵ -lysPNA, etc., Fig. 1; Gangamani et al. 1999; Haimaa et al. 1996; Roviello et al. 2006, 2008; Sforza et al. 1999, 2002).

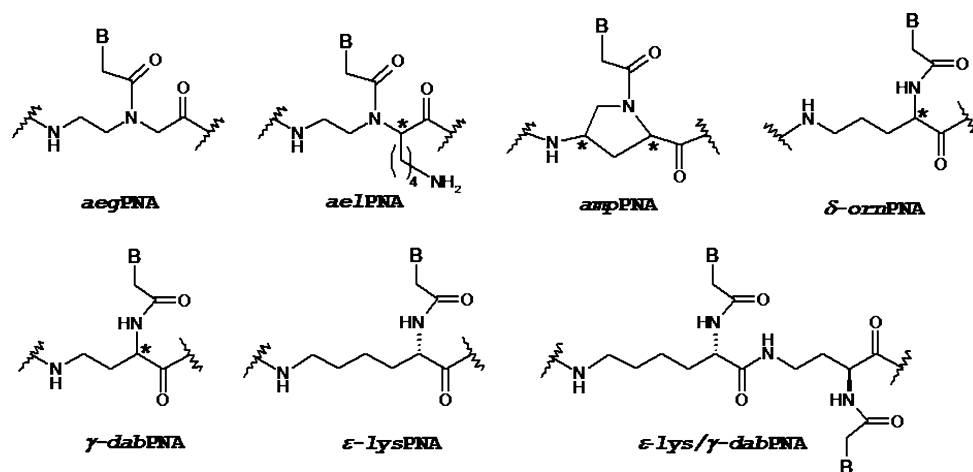
In continuing our research on nucleopeptides as ODN analogs, we recently reported the synthesis of nucleo- γ -peptides (γ -dabPNAs; Roviello et al. 2006, 2007) and

G. N. Roviello and D. Musumeci have contributed equally to this work

G. N. Roviello · D. Musumeci (✉) · C. Pedone
Istituto di Biostrutture e Bioimmagini—CNR,
Via Mezzocannone 16, 80134 Napoli, Italy
e-mail: domymusu@alice.it

E. M. Bucci
Bioindustry Park del Canavese SpA, Via Ribes 5,
10010 Colletterto Giacosa, Turin, Italy

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



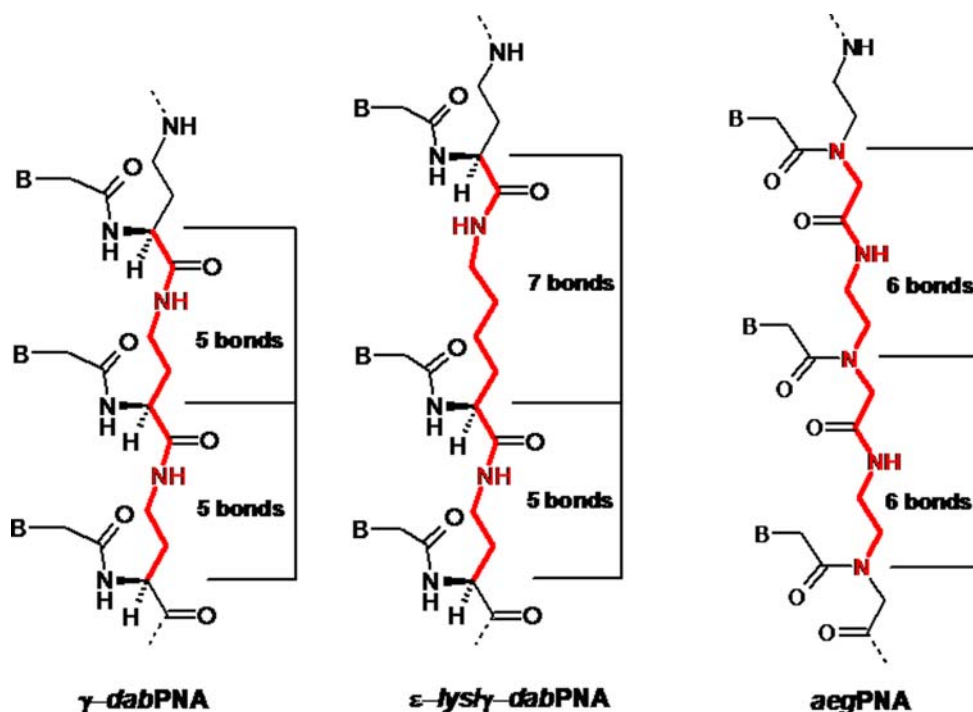
nucleo- ϵ -peptides (ϵ -lysPNAs; Roviello et al. 2008) (Fig. 1), based respectively on a L-diaminobutyric acid (DABA) and L-lysine (L-Lys) backbone, and their hybridization studies with natural nucleic acids. From these studies, it emerged that pure γ -dabPNAs, which have a backbone one-atom shorter than Nielsen *aeg*PNAs, were unable to bind DNA or RNA, while ϵ -lysPNAs, 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 -dabPNAs (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 -dabPNAs, 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 -dabPNA (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 -dabPNAs in antigene/antisense or diagnostic applications,

Fig. 2 Comparison between γ -dabPNA, ϵ -lys/ γ -dabPNA 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 -dabPNAs, 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. Anhydrosolan 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 μ m, 4.6 mm $\times$ 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 μ m, 10 mm $\times$ 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 $\times$ 0.4375 cm (Tandem cell).

Solid phase synthesis of oligomers 1 and 2

Thymine nucleaminoacids t_{L-dab} and t_{L-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 -dabPNA and ϵ -lysPNA monomers were checked by measuring the absorbance of the released Fmoc group ($\epsilon_{301} = 7,800$) 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 -dabPNA, average coupling yields were of about 75% for

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

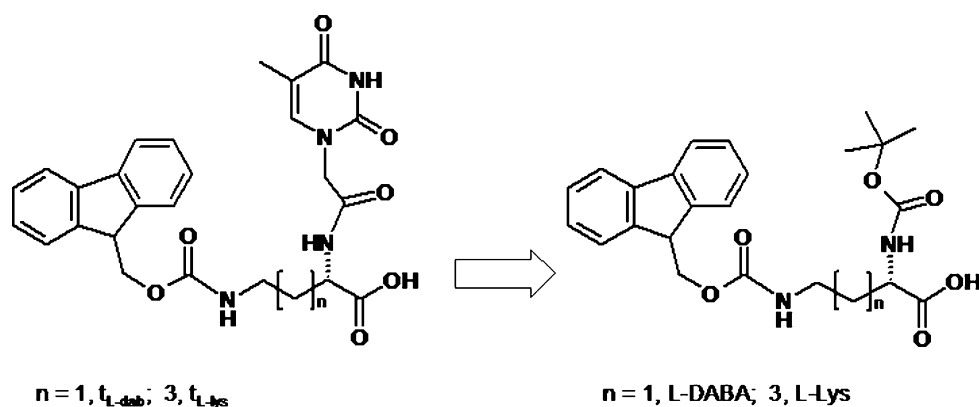
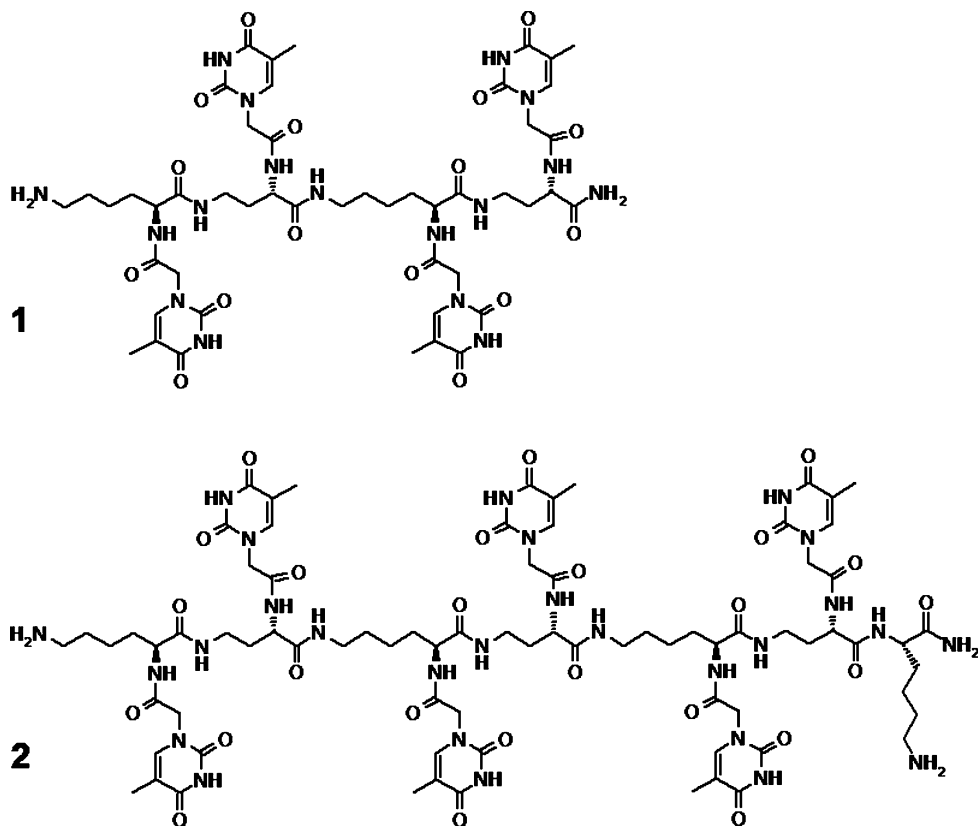


Fig. 4 Molecular structure for **1** and **2** nucleopeptides



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

$H-(t_{L-lys}-t_{L-dab})_2-NH_2$ (**1**, Fig. 4) was assembled on Rink-amide- NH_2 resin (0.5 mmol/g, 8 mg, 4 μ mol) using the following coupling protocol: a mixture of Fmoc-L- T_{lysPNA} -OH or Fmoc-L- T_{dabPNA} -OH (0.2 M in NMP, 60 μ l, 12 μ mol, 3 Eq), HATU (0.18 M in DMF, 60 μ l, 10.8 μ mol, 2.7 Eq) and 100 μ l of DMF was introduced into the reactor. Successively, 100 μ l (20 μ mol, 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 $[C_{48}H_{67}N_{17}O_{16} + H]^+$).

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

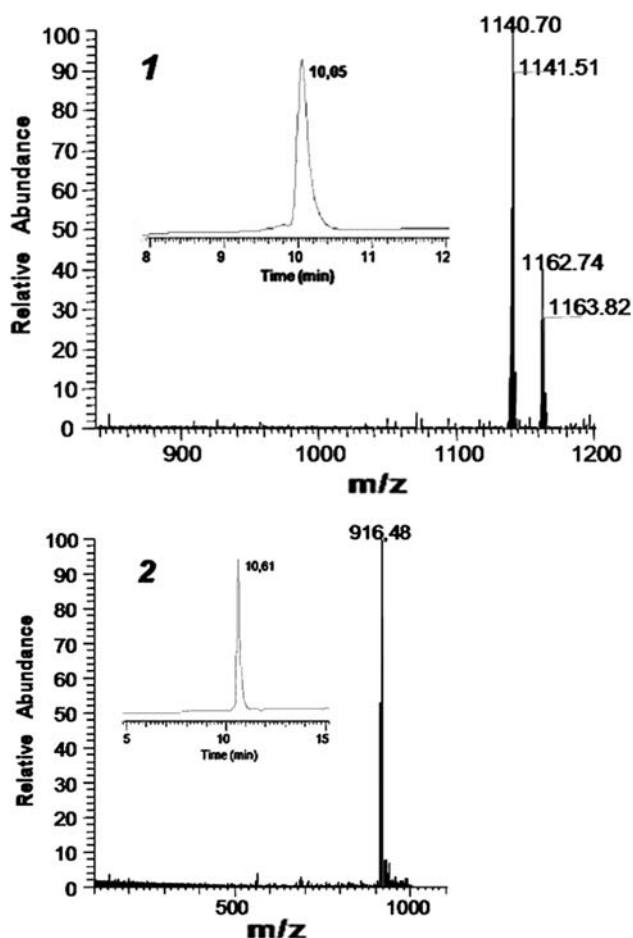


Fig. 5 LC-ESIMS of oligomers $H-(t_L\text{-lys-}t_L\text{-dab})_2\text{-NH}_2$ (**1**) and $H-(t_L\text{-lys-}t_L\text{-dab})_3\text{-Lys-NH}_2$ (**2**)

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 $[\text{C}_{78}\text{H}_{111}\text{N}_{27}\text{O}_{25} + 2\text{H}]^{2+}$).

UV and CD studies

Purified oligomers were dissolved in a known amount of MilliQ water and quantified by UV measurements ($T = 85^\circ\text{C}$, 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 *ae*gPNA 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 $0.5^\circ\text{C}/\text{min}$. 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 $\text{\AA}$ (5 μm , 4.6 mm $\times$ 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 nucleaminoacids 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 $\gamma\text{-dab}$ -PNA and $\epsilon\text{-lys}$ PNA monomers in the sequence, we synthesized on solid phase two $\epsilon\text{-lys}/\gamma\text{-dab}$ PNA nucleopeptides (**1** and **2**, Fig. 4) following a coupling protocol based on HATU/TMP which ensures retention of optical purity of the nucleaminoacid residues (Sforza et al. 2002).

During the synthesis of tetramer **1**, when $\gamma\text{-dab}$ PNA units were coupled, an average yield of about 75% was estimated, while yields for $\epsilon\text{-lys}$ 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 $\epsilon\text{-lys}$ - and $\gamma\text{-dab}$ PNA units, influenced by the distance of the NH_2 from the hindered C- α position, that is longer in the case of $\epsilon\text{-lys}$ PNA than in $\gamma\text{-dab}$ PNA monomer. Thus, probably due to steric 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 nucleoside 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 -*dab*PNA, (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 pre-organization 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 pre-organization 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 (dA₁₂) 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 dA₁₂ in the other one, and then the *mix*

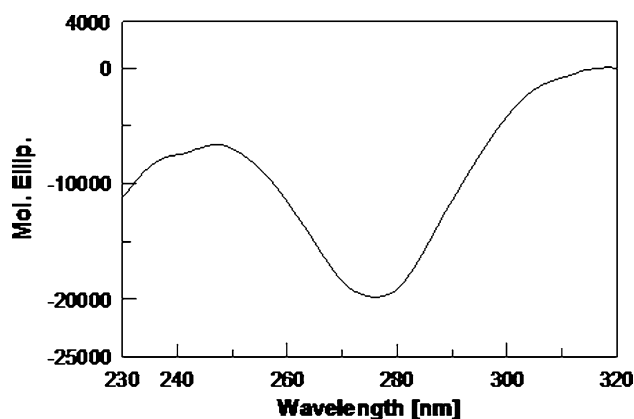


Fig. 6 CD spectrum of H-(*t*_L-lys-*t*_L-dab)₃-Lys-NH₂ (**2**) in 10 mM phosphate buffer (pH 7.5) at 5°C

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-lys-*t*_L-dab)₃/dA₁₂ complex having a structure which resembles that of the stacked dA₁₂ single strand.

UV melting experiments of the **2**/dA₁₂ 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-lys-*t*_L-dab)₃/polyA hybrid. Despite DNA, the structure of the complex formed between ϵ -*lys*/ γ -*dab*PNA and RNA was completely different

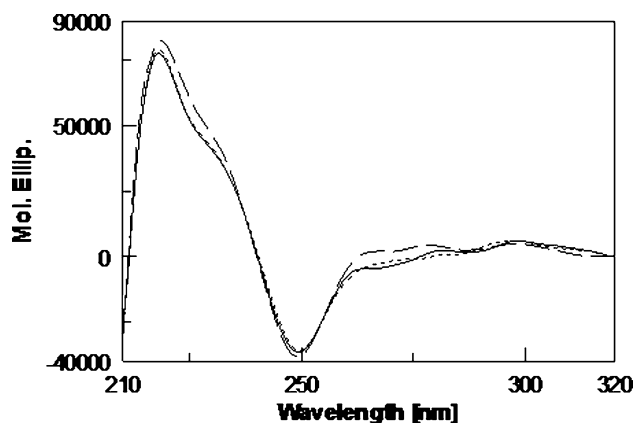


Fig. 7 CD spectra of **2**/dA₁₂ 8:1 ratio (4:1 in bases) in 10 mM phosphate buffer (pH 7.5) at 5°C (*sum*: dashed line; *mix* after 1 min: solid line; *mix* after 10 min: dotted line; tandem cell)

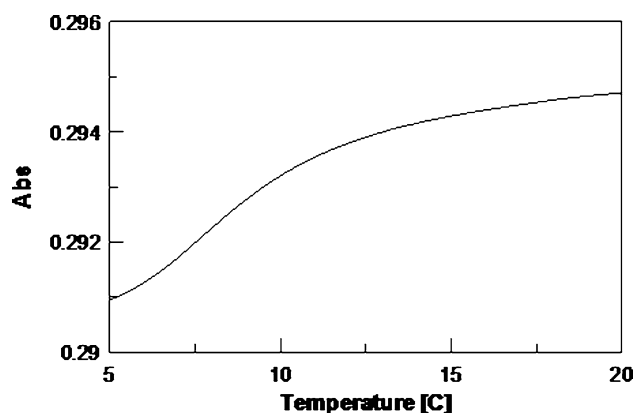


Fig. 8 UV melting (0.5°C/min) of the **2**/dA₁₂ 8:1 mixture (4:1 in bases) in 10 mM phosphate buffer (pH 7.5)

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 -dabPNA)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 -dabPNA 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 -dabPNA and polyA, the presence of only one transition

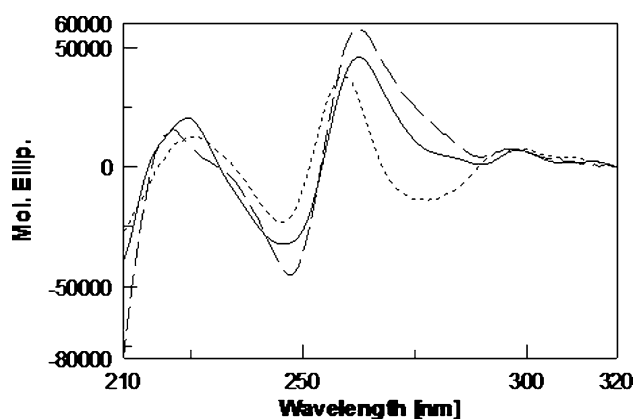


Fig. 9 CD spectra with tandem cell of **2**/polyA (10:1 in bases) in 10 mM phosphate buffer (pH 7.5) at 5 °C (*sum*: dashed line; *mix* after 1 min: solid line; *mix* after 10 min: dotted line)

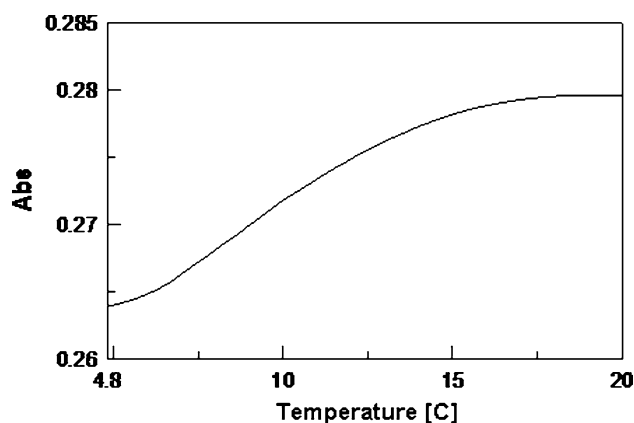


Fig. 10 UV melting (0.5°C/min) of the **2**/polyA complex (10:1 in bases) in 10 mM phosphate buffer (pH 7.5)

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 -dabPNA 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 -dabPNA.

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 -dabPNAs. 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 -dabPNA hexamer binds both DNA (dA₁₂) 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 -dabPNAs that did not bind natural nucleic acids (Roviello et al. 2006, 2007), and slightly different

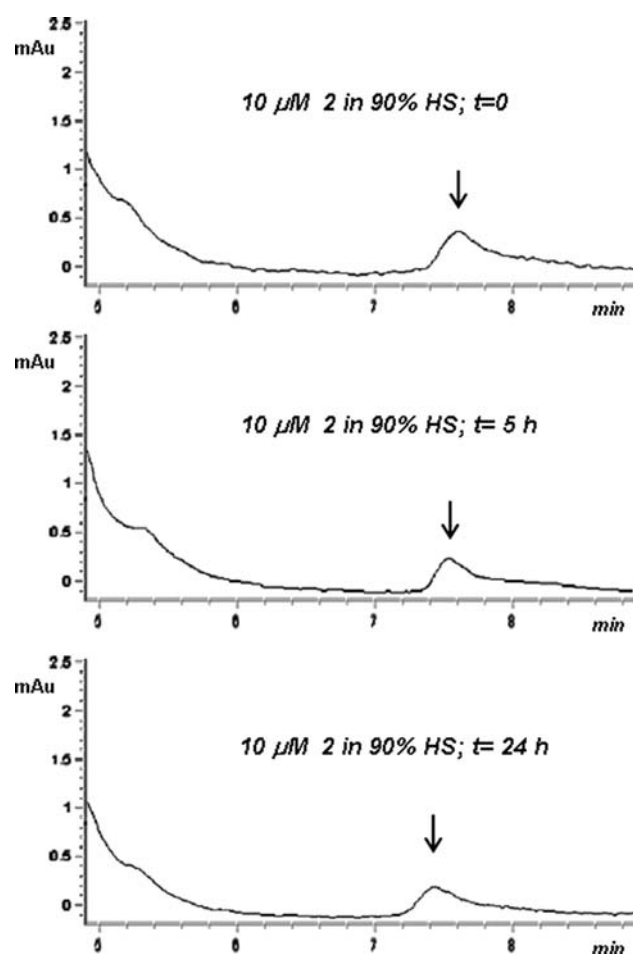


Fig. 11 Serum stability assay on ϵ -lys/ γ -dabPNA **2** performed by HPLC

from that presented by ϵ -lysPNAs 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 -dabPNA is not the result of the simple combination of the properties of γ -dabPNA and ϵ -lysPNA, 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 -dabPNAs, 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 -dabPNA oligomers of various lengths and with mixed nucleobase sequences: indeed, the realization of the ϵ -lysPNA and

γ -dabPNA 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 -dabPNAs, in which amino groups are capped by the nucleobases.

Finally, we propose that PNA molecules like our ϵ -lys/ γ -dabPNAs, 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; Vdoviykin 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 -dabPNAs 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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