Research Article

Journal of PeptideScience

Received: 13 June 2008 Revised: 23 July 2008 Accepted: 31 July 2008 Published online in Wiley Interscience: 4 November 2008

(www.interscience.com) DOI 10.1002/psc.1072

Solid phase synthesis and RNA-binding studies of a serum-resistant nucleo-ε-peptide‡

G. N. Roviello,[a]* D. Musumeci,[b] M. Castiglione,[c] E. M. Bucci,[b] C. Pedone[a,c] and E. Benedetti[c]

In the present work we report the synthesis of a new Fmoc-protected L-lysine-based nucleo-amino acid suitable for the solid phase assembly and its oligomerisation to the corresponding nucleo-ε-peptide that we called *ε-lys*PNA. The ability to bind complementary RNA and the stability in serum of this synthetic nucleo-ε-peptide were studied to explore its possible use in antisense or diagnostic applications. Our interest to the presented oligonucleotide analogue was also supported by the importance of ε-peptides and other ε-amino acid-containing compounds in natural products with biological activity such as the poly-ε-lysines produced by *Streptomyces albulus* that possess a highly selective antimicrobial activity. Another aspect we intended to evaluate by this work is the possible prebiotic implication of these nucleopeptides, since ε-peptides, and not α-peptides, were mainly obtained among the other thermal prebiotic polypeptides in pyrocondensation of lysine, a diamino acid also detected in Mighei meteorite. Besides this intriguing question, all the remarkable properties emerged from the present investigation on *ε-lys*PNAs encourage, without doubts, interest in the therapeutic and diagnostic potential of these bioinspired nucleopeptides.

Keywords: nucleo-ε-peptides; RNA analogue; *ε-lys*PNA; CD

Introduction

The development of new oligonucleotide analogues that are able to bind DNA and RNA sequences with good sequence specificity and thermal stability has gained a great scientific interest in recent years towards a number of artificial nucleotidic molecules, with various modifications both in backbone and in nucleobases. Among these analogues, *aminoethylglycyl* peptide nucleic acids (*aeg*PNAs), proposed by Nielsen in 1991, with an artificial pseudopeptide backbone in place of the sugar-phosphate one, emerged as the most promising candidates for their ability to form sequence-specific complexes (double and triple helices) of high thermal stability with DNA and RNA [1,2].

Besides the remarkable binding properties of *aeg*PNAs, however, some drawbacks, such as low water solubility, poor cell permeability, costly precursors and also ambiguity in nucleic acids recognition (parallel/antiparallel) [2], are expected to be overcome.

In particular, the possibility to discriminate between parallel/antiparallel binding modes is an important feature for a nucleotidic analogue to achieve the best specificity in the detection of complementary strands of natural targets. To introduce this favourable characteristic into PNAs, different approaches were reported in which aminoacidic moieties, especially lysines or arginines, were added to the *aeg*PNA ends [2,3]. In this way the presence of the stereogenic centres allowed for improved binding-mode discrimination, while the positive charge of the aminoacidic side chain favoured the solubility of the PNA oligomers. Furthermore, lysine units were also inserted in the *aeg*PNA backbone in place of the glycine unit creating a chiral box inside the PNA oligomer [4].

In order to develop new oligonucleotide analogues overcoming some of the *aeg*PNA drawbacks, we realised a chiral nucleopeptide, indicated as *ε-lys*PNA, in which the backbone is entirely constituted by L-lysine moieties with the α-amino groups carrying the nucleobases through a methylenecarbonyl bridge (Figure 1).

Despite *aeg*PNAs that have a pseudopeptide backbone (Figure 1), the analogue we propose is bioinspired because it can be considered as an ε-peptide (Figure 1), associable with the natural ε-poly-L-lysines, with nucleobases as pendant groups.

Interestingly, dendritic α,ε-poly-lysines were reported to act as delivery agents for antisense oligonucleotides as demonstrated very recently in experiments of antisense oligonucleotide delivery to He–La cells [5].

Furthermore, the ε-poly-L-lysines that find application in food conservation for their antimicrobial activity towards some food pathogens are naturally secreted by *Streptomycetaceae* bacteria [6] and have attracted increasing attention in recent years due to their various functions, as well as their non-toxicity and biodegradability.

* *Correspondence to: G. N. Roviello, Istituto di Biostrutture e Bioimmagini-CNR, Via Mezzocannone 16, Napoli 80134, Italy. E-mail: giroviel@unina.it*

a *Istituto di Biostrutture e Bioimmagini-CNR, Via Mezzocannone 16, Napoli, Italy*

b *Bionucleon srl, via Montesano 49, Napoli, Italy*

c *Università degli Studi di Napoli 'Federico II,' Dip. Scienze Biologiche sez. Biostrutture, via Mezzocannone 16, Napoli, Italy*

Abbreviations used: *Ac_2O, acetic anhydride; Boc, tert-butoxycarbonyl; DCM, dichloromethane; DIEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate; NMP, 4-methylpyrrolidone; PyBOP, (1H-benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate; TCH_2COOH, thymin-1-yl acetic acid; TFA, trifluoroacetic acid; TMP, 2,4,6-trimethylpyridine.*

‡ *11th Naples Workshop on Bioactive Peptides.*

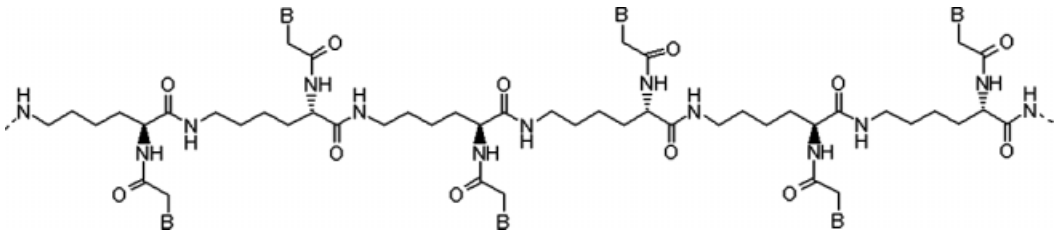


Figure 1. Molecular structure of ε-*lys*PNA.

Indeed, materials based on ε-poly-L-lysine are biodegradable from the PL-tolerant *Chryseobacterium* sp. [7].

Here we present the synthesis, purification and characterisation of a novel Fmoc-protected nucleo-amino acid based on L-lysine, suitable for the solid phase synthesis, and its oligomerisation to the corresponding nucleopeptide. Our preliminary results based on CD and UV experiments, regarding the ability of these oligomers to bind complementary RNA are encouraging in view of a possible future employment of ε-*lys*PNAs in antisense applications and open the door also to further studies on binding-mode discrimination and cell permeability of these oligonucleotide analogues.

Materials and Methods

Chemicals

Boc-L-Lys(Fmoc)-OH, Fmoc-Gly-OH, Fmoc-L-Lys(Boc)-OH, HATU and PyBOP were purchased from Novabiochem. Anhydroscan DMF and NMP were from LabScan. Piperidine was from Biosolve. Solvents for HPLC chromatography and acetic anhydride were from Reidel-de Haën. PolyA, TCH_2COOH, TFA, TMP and Rink-amide resin were from Fluka. DCM and TFA (for HPLC) were from Romil. Deuterated solvents (DMSO and methanol) were from Aldrich.

Apparatus

^1H NMR and ^{13}C NMR spectra were recorded at 25 °C on Varian unity 400 MHz spectrometers. Chemical shifts (δ) are given in parts per million (ppm) and all coupling constants (J) in Hz. Proton chemical shifts were referenced to residual CHD_2SOCD_3 ($\delta = 2.49$, quin) and CHD_2OD ($\delta = 3.30$, quin) signals. ^{13}C NMR chemical shifts were referenced to the solvent (CD_3SOCD_3: $\delta = 39.5$, sept). Crude samples containing nucleopeptides were centrifuged for 4 min at 4000 rpm (Z 200 A, Hermle). Products were analyzed by LC-MS, performed 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 × 150 mm^2) column. Gradient elution was performed at 40 °C (monitoring at 260 nm) by building up a gradient starting with buffer A (0.05% TFA in water) and applying buffer B (0.05% TFA in acetonitrile) with a flow rate of 0.8 ml/min.

Semi-preparative purifications were performed by RP-HPLC on a Hewlett Packard (HP)/Agilent 1100 series, equipped with a diode array detector, by using a Phenomenex Juppiter C18 300 Å (10 µm, 10 × 250 mm^2) 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. Stability in serum was analysed by RP-HPLC by using a Phenomenex Juppiter C18 300 Å (5 µm, 4.6 × 250 mm^2) column with a linear gradient of 5% (for 5 min) to 20% B in A over 20 min. Samples containing nucleopeptides (crude or purified) were lyophilised in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h. CD spectra were obtained at 20 °C on a Jasco J-810 spectropolarimeter using 1-cm quartz cuvette (Hellma). 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 using 1-cm quartz cuvette (Hellma).

Synthesis of the Fmoc-L-t_ε-*lys*PNA-OH Monomer

The L-lysine-based thymine monomer (**3**), suitably protected for peptide solid phase synthesis (Fmoc chemistry), was obtained in analogy with the synthesis of our L-DABA monomer [8], but starting from Boc-L-Lys(Fmoc)-OH as a precursor.

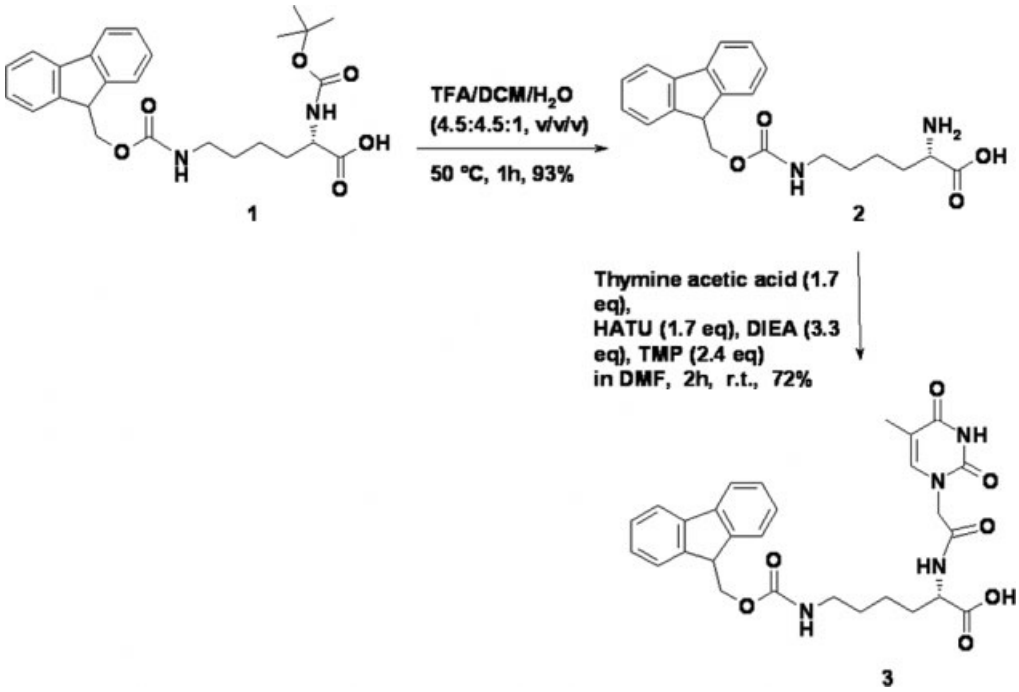
N^ε-Fmoc-L-2,6-diaminohexanoic acid, **2**

Commercial Fmoc/Boc-protected 2,4-diaminohexanoic acid **1** (Boc-L-Lys(Fmoc)-OH: 200 mg, 0.43 mmol) was treated with a solution of TFA/DCM/H_2O 4.5 : 4.5 : 1 (8 ml) at 50 °C (Scheme 1). After 1 h stirring, the solvent was removed under vacuum. The obtained crude was treated with cold diethyl ether and after centrifugation a white precipitate was obtained. Subsequently, this material was filtered, washed with diethyl ether and DCM and coevaporated three times with dry CH_3CN (147 mg of **2**, 0.40 mmol, 93% yield). This precipitate contained prevalently one product, as evidenced by LC-MS. ESI-MS *m/z*: 368.29 (found), 369.45 (expected for $[C_{21}H_{24}N_2O_4 + H]^+$); *m/z* 390.30 (found), 391.44 (expected for $[C_{21}H_{24}N_2O_4 + Na]^+$); *m/z* 737.25 (found), 737.89 (expected for $[2(C_{21}H_{24}N_2O_4) + H]^+$).

N^ε-Fmoc-N^α-(thymin-1-ylacetyl)-L-2,6-hexanoic acid, **3**

Product **2** (1 eq, 147 mg, 0.40 mmol) was dissolved in dry DMF (2 ml), treated with DIEA (1.6 eq, 108.6 µl, 0.64 mmol) and TMP (0.7 eq, 35.8 µl, 0.27 mmol) and reacted with TCH_2COOH (1.7 eq, 125 mg, 0.68 mmol) which was previously pre-activated with HATU (1.7 eq, 252 mg, 0.66 mmol) and DIEA (1.7 eq, 115 µl, 0.68 mmol)/TMP (1.7 eq, 90 µl, 0.68 mmol) in NMP (1 ml) for 2 min. After 2 h the solvent was removed under vacuum (Scheme 1). The recovered crude was treated with water (50 ml) and after sonication the aqueous solution was removed from the white material that was purified by semi-preparative HPLC on Agilent instrument by using increasing amounts (25–70% in 25 min) of solution B in A, giving pure sample **3** (155 mg, 0.29 mmol, 72% yield) as a white powder; LC-ESI-MS (Figure 2) *m/z* 534.56 (found), 535.58 (expected for $[C_{28}H_{30}N_4O_7 + H]^+$); *m/z* 556.60 (found), 557.57 (expected for $[C_{28}H_{30}N_4O_7 + Na]^+$); δ_H (400 MHz,

Journal of PeptideScience



Scheme 1. Synthesis of the Fmoc-protected thymine monomer **3**.

DMSO-d_6) 11.23 (1H, s, NH thymine), 8.40 (1H, d, J = 8.0, NH amide), 7.86 (2H, d, J = 7.2, aromatic CH Fmoc), 7.66 (2H, d, J = 7.2, aromatic CH Fmoc), 7.41–7.22 (6H, m, aromatic CH Fmoc, Fmoc-NH, CH thymine), 4.31 (2H, s, CH_2 acetyl linker), 4.12–4.27 (4H, m, FmocCH-CH_2 and CH_α), 2.94 (2H, m, CH_2NH), 1.72 (3H, s, CH_3 thymine), 1.67 (1H, m, CH_2CH_α), 1.56 (1H, m, CH_2CH_α) 1.37 (2H, m, $CH_2CH_2CH_\alpha$), 1.26 (2H, m, CH_2CH_2NH); δ_H (400 MHz, CD_3OD) 7.81 (2H, d, J = 7.6, aromatic CH Fmoc), 7.67 (2H, br d, aromatic CH Fmoc), 7.40 (2H, t, J = 7.2, aromatic CH Fmoc), 7.36 (1H, s, CH thymine), 7.32 (2H, t, J = 7.2, aromatic CH Fmoc), 4.19–4.55 (6H, m, CH_2 acetyl linker, FmocCH–CH_2 and CH_α), 3.10–3.16 (2H, m, CH_2NH), 1.87 (3H, s, CH_3 thymine), 1.94–1.68 (2H, m, CH_2CH_α), 1.58–1.38 (4H, m, $CH_2CH_2CH_\alpha$ and CH_2CH_2NH); δ_C (100 MHz, DMSO-d_6) 177.34 (COOH), 170.95 (thymine C-4), 168.47 (CH_2CONH), 160.11 (OCONH), 154.98 (aromatic Fmoc 2C), 147.94 (thymine C-2), 146.43 (aromatic Fmoc 2C), 144.75 (thymine

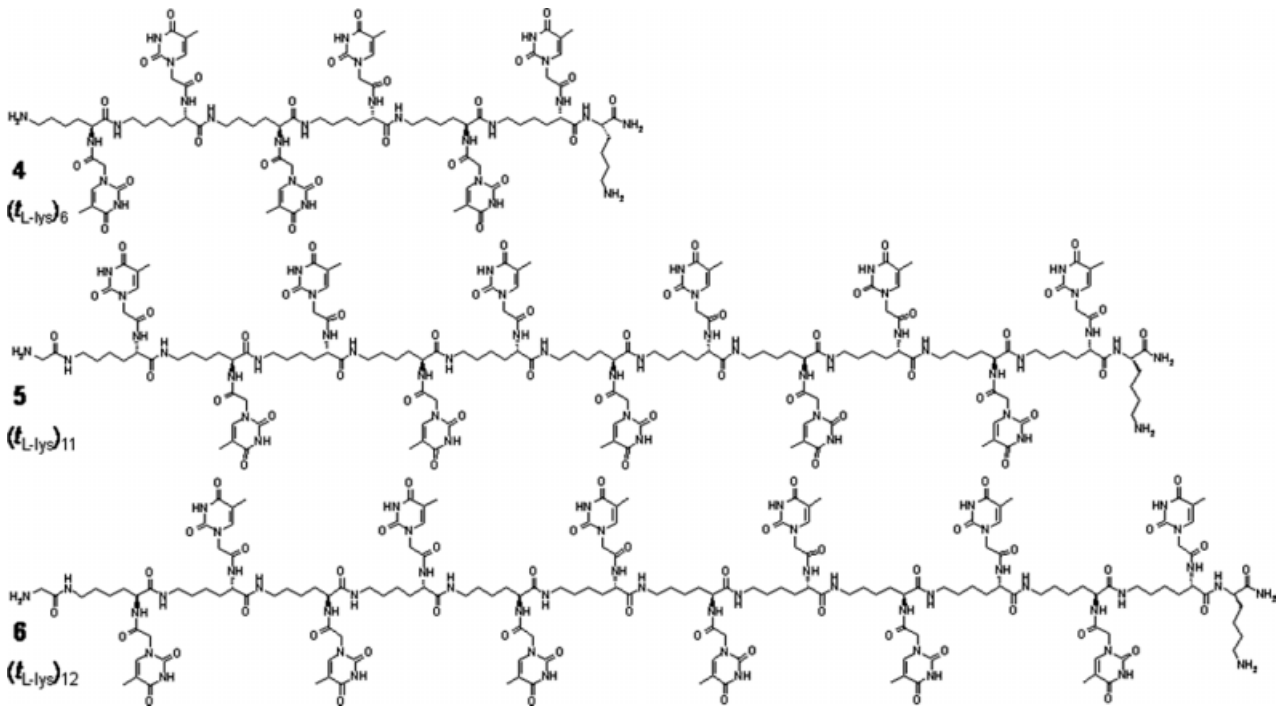


Figure 2. Molecular structures for **4**, **5** and **6** nucleopeptides.

C-6), 131.62 (aromatic Fmoc 2CH), 131.07 (aromatic Fmoc 2CH), 129.16 (aromatic Fmoc 2CH), 124.12 (aromatic Fmoc 2CH), 111.87 (thymine C-5), 69.21 (Fmoc CH_2), 56.04 (CH_2 acetyl linker), 53.01 (CH_2NH), 50.78 (CH_α), 44.04 (Fmoc CH), 34.98 (CH_2CH_2NH), 32.99 (CH_2CH_α), 26.59 ($CH_2CH_2CH_\alpha$), 15.90 (thymine CH_3).

Solid Phase Synthesis of Oligomers 4–6

Solid support functionalisation and manual solid phase oligomerisations were carried out in short PP columns (4 ml) equipped with a polytetrafluoroethylene (PTFE) filter, a stopcock and a cap. Solid support functionalisation: Rink-amide resin (0.50 mmol NH_2/g, 128 mg) was functionalised with a L-lysine (Fmoc-L-Lys(Boc)-OH, 0.5 eq, 14.8 mg, 32 µmol) using PyBOP (0.5 eq, 16.8 mg, 32 µmol) as activating agent and DIEA (1 eq, 12 µl, 64 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. Loading of the resin was checked by measuring the absorbance of the released Fmoc group ($\varepsilon_{301} = 7800$, quantitative yield) after treatment with a solution of piperidine (30%) in DMF (UV Fmoc test) and the resultant reduced to 0.25 mmol/g with respect to the initial functionalisation.

This value is generally appropriate for the synthesis of PNA to avoid aggregation effects during chain elongation. Then, we performed the coupling steps with ε-*lys*PNA monomer **3** using a procedure reported in the literature [9] that minimises racemisation during the coupling reaction (HATU/TMP). Couplings of ε-*lys*PNA monomers were monitored by UV Fmoc test to evaluate the incorporation yields of each monomer. As an improvement with respect to the synthetic route followed for oligomer **4**, we introduced a glycine unit at *N*-terminus of oligomers **5** and **6** for avoiding the N-migration of the carboxylated thymine. The glycine residue (3 eq) was attached by using PyBOP (3 eq)/DIEA (6 eq) as activating system in DMF. All oligomers were cleaved from the resin and deprotected under acidic conditions (TFA/*m*-cresol 4 : 1 v/v) and recovered by precipitation with cold diethyl ether, centrifugation and lyophilisation. Purified oligomers (semi-preparative HPLC) were quantified and characterized by LC-ESIMS.

*H-(t_ε-lys*PNA)$_6$-*Lys-*NH_2 (**4**)

H-(t_ε-*lys*PNA)$_6$-Lys-NH_2 (**4**) was assembled on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) using the following protocol: a mixture of Fmoc-L-$t_{\varepsilon-lysPNA}$-OH **3** (120 µl of a 0.2 M solution in DMF, 24 µmol, 3 eq), HATU (120 µl of a 0.2 M solution in DMF, 24 µmol, 3 eq) and 100 µl of DMF was introduced into the reactor. Subsequently, 120 µl (24 µmol, 3 eq) of a TMP solution (0.2 M in NMP) was added to the stirred reaction in four portions over 1 h. After the coupling steps, unreacted amino groups were capped with PNA Cap solution for 5 min. Fmoc removal was accomplished with PNA Deblock solution (8 min) and was monitored for every step by UV measurement. After cleavage and deprotection, oligomer **4** was purified by semi-preparative RP-HPLC using a linear gradient of 5% (for 5 min) to 20% B in A over 20 min: $t_R = 16.6$ min; UV quantification of the purified product gave 1.4 µmol of **4** (18% yield); ESI-MS (Figure 3) *m/z*: 1934.10 (found), 1934.07 (expected for $[C_{84}H_{123}N_{27}O_{25} + Na]^+$); 956.75 (found), 956.55 (expected for $[C_{84}H_{123}N_{27}O_{25} + 2H]^{++}$).

*H-Gly-(t_ε-lys*PNA)$_{11}$-*Lys-*NH_2 **5**

Undecamer **5** was assembled on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) following the procedure above described for hexamer **4**. However, as an improvement to this synthetic route, here we introduced a glycine residue at *N*-terminus for avoiding the N-migration of the carboxylated thymine. The cleaved and deprotected oligomer was purified by semi-preparative HP HPLC using a linear gradient of 10% (for 5 min) to 30% B in A over 25 min: $t_R = 22.6$ min; UV quantification of the purified product gave 1.0 µmol of **5** (13% yield); ESI-MS *m/z*: 1721.62 (found), 1721.86 (expected for $[C_{151}H_{216}N_{48}O_{46} + 2H]^{++}$); 1148.15 (found), 1147.57 (expected for $[C_{151}H_{216}N_{48}O_{46} + 3H]^{+++}$).

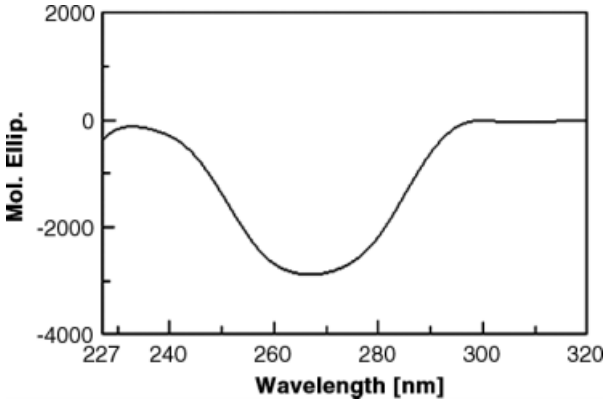


Figure 3. CD spectrum of the L-lysine nucleopeptide **5** (10 °C, 5 µM, 10 mM phosphate buffer, pH 7.5).

*H-Gly-(t_ε-lys*PNA)$_{12}$-*Lys-*NH_2 **6**

Dodecamer **6** was obtained on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) following the same procedure above reported for undecamer **5**. After cleaving and deprotection, the nucleopeptide was purified by semi-preparative HP HPLC using a linear gradient of 10% (for 5 min) to 30% B in A over 25 min: $t_R = 23.6$ min; UV quantification of the purified product gave 880 nmol of **6** (11% yield); ESI-MS *m/z*: 1869.64 (found), 1868.02 (expected for $[C_{164}H_{234}N_{52}O_{50} + 2H]^{++}$); 1245.93 (found), 1245.68 (expected for $[C_{164}H_{234}N_{52}O_{50} + 3H]^{+++}$).

UV and CD Studies

Purified oligomers were dissolved in a known amount of milliQ water and quantified by UV measurements ($T = 80$ °C, absorbance value at $\lambda = 260$ nm). The epsilon values used for the quantification of the oligomers **4**, **5** and **6** (51.6, 94.6 and 103.2 mM^{-1}) were calculated using the molar extinction coefficient of thymine *aeg*PNA monomer (8.6 mM^{-1}). Thermal melting curve (Figure 4) was obtained by recording the UV absorbance at 260 nm by increasing the temperature at a rate of 0.5 °C/min. T_m value was calculated by the first derivative method. 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

Nucleopeptide solution (oligomer **4**) was adjusted to 36 µM in phosphate buffer (pH = 7.5) and 20 µl added to 80 µl of fresh human serum. A 10-µl sample was removed immediately and the remainder incubated at 37 °C. Aliquots (10 µl) were taken at 1, 2, 24, 72 and 168 h, and quenched with 10 µl of 7 M urea solution at a temperature of 95 °C for 2 min. Stability was analysed by RP-HPLC by using a Phenomenex Juppiter C18 300 Å (5 µm, 4.6 × 250 mm^2)

Journal of PeptideScience

column with a linear gradient of 5% (for 5 min) to 20% B in A over 20 min.

Results and Discussion

The synthesis of the new L-lysine-based thymine monomer (**3**), suitably protected for peptide solid phase synthesis (Fmoc chemistry), is reported in Scheme 1 and is analogous to that recently described in our previous work for the synthesis of the L-DABA monomer [8], but starting from the commercial Boc-(L)-Lys(Fmoc)-OH (**1**). LC-ESIMS characterisation confirmed the identity of both compound **2** and monomer **3**. The novel Fmoc-protected nucleo-amino acid **3** (Fmoc-L-$t_{\varepsilon-lys}$PNA-OH) was also examined by $^{1}H/^{13}C$-NMR spectroscopy.

Three homothymine ε-*lys*PNA oligomers, **4**, **5** and **6**, with 6, 11 and 12 thymine residues, respectively (Figure 2), were synthesised in solid phase by using the monomer **3** with a protocol which minimises racemisation during the coupling steps (HATU/TMP) [8,9]. All oligomers were cleaved from the solid support by acidic treatment (TFA/m-cresol, 4 : 1, v/v) and purified by RP-HPLC on a C-18 column with a linear gradient of CH_3CN (0.1% TFA) in H_2O (0.1% TFA).

LC-ESIMS characterisation confirmed the identity of the new molecules obtained. Subsequently, the structural characteristics of the L-lysine-based nucleopeptides as well as their ability to form complexes with complementary RNA were investigated by CD spectroscopy. First, the CD profile of the nucleopeptidic single strand in 10 mM phosphate buffer (pH 7.5) was analyzed to evaluate an helical pre-organisation of the ε-*lys*PNA molecules. By examining the CD behaviour of the molecules **4**, **5** and **6** in the range 190–240 nm at different temperatures (5, 10 and 25 °C), no significant α-helical contribution was detected for the single strands (data not shown). Anyway, a weak CD signal was detected for the chiral oligomers and, more exactly, a negative band centred at 272 nm was observed (Figure 3).

This finding is in agreement with the CD behaviour already reported for other nucleopeptides, like *orn*PNA and *dab*PNA, with L-stereocentres, and would suggest a certain structural pre-organisation for the nucleopeptides **4**–**6** in aqueous medium.

CD-binding experiments on the homothymine ε-*lys*PNAs with the complementary RNA (polyA) were then performed to evaluate their potential use as antisense tools. The results obtained for all oligomers with polyA by CD spectroscopy were in perfect analogy with each other. Thus, here we report, as an example, the CD behaviour of oligomer **5**. As expected, the CD of ε-*lys*PNA single strands (red line, Figure 4) resulted very weak when compared with the strong bands of the RNA (blue line, Figure 4).

When an equimolar amount in nucleobase of thymine ε-*lys*PNA was added to a solution of polyadenylic RNA in 10 mM phosphate (pH 7.5) at 10 °C, a certain change was detected in the shape and intensity of the CD profile of the polyA (green line, Figure 4). Anyway, a more evident variation in CD signal was observed only when a further equivalent in thymine of ε-*lys*PNA was added to form a 2 : 1 = T : A complex (magenta line, Figure 4). These findings clearly show the formation of a complex between the ε-*lys*PNA and RNA, confirmed also by UV melting studies that revealed also a T_m of 2.2 °C per A/T base pair (Figure 5).

We intended also to investigate the stability of our nucleopeptide in human serum and, more particularly, analysed the oligomer **4** for degradation products by RP-HPLC (Figure 6). A slight decrease in the amount of nucleopeptide was observed after 60 min, but no significant degradation products appeared even after 24 h. After 72 h of incubation in serum, the nucleopeptide was still intact and only after 1 week a certain degradation was noticed even if oligomer **4** was still present.

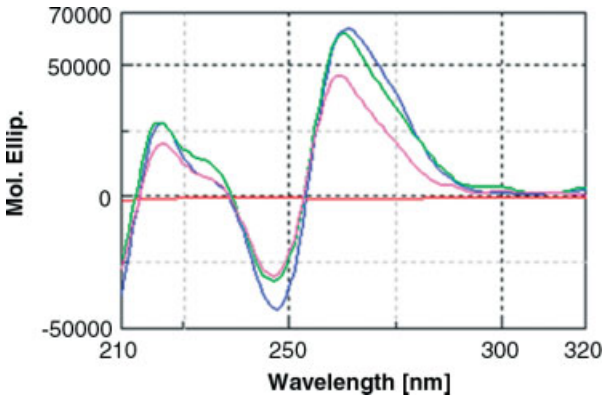


Figure 4. CD-binding experiments between ε-*lys*PNA and RNA in 10 mM phosphate buffer (pH 7.5) at 10 °C: 8 μM in thymine of **5** (red); 4 μM in adenine of PolyA RNA (blue); 4 μM in thymine of **5** + 4 μM in adenine of PolyA RNA (green); 8 μM in thymine of **5** + 4 μM in adenine of PolyA RNA (magenta).

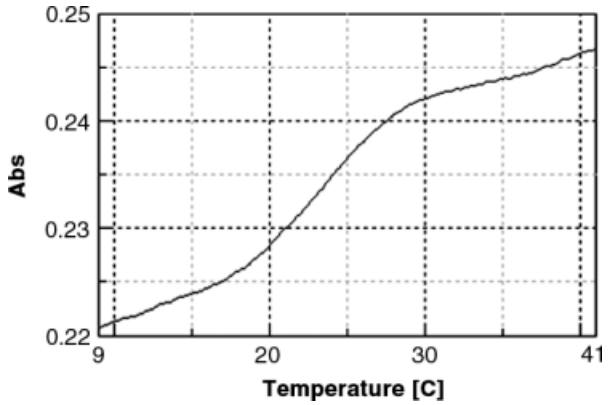


Figure 5. UV melting of the ε-*lys*PNA/RNA complex (14 μM in thymine of **5** + 4 μM in adenine of PolyA RNA) in 10 mM phosphate buffer (pH 7.5).

Conclusions and Perspectives

In conclusion, we reported the synthesis, purification and characterisation of a novel Fmoc-protected nucleo-amino acid based on L-lysine, suitable for solid phase synthesis, and its oligomerisation to nucleopeptides **4**, **5** and **6** with, respectively, 6, 11 and 12 thymine monomers making use of a synthetic route that avoids racemisation during every coupling step. Binding experiments, performed by CD and UV spectroscopies, showed that all the chiral nucleopeptides were able to interact with complementary RNA molecules. These findings support the use of this class of nucleopeptides for the realisation of novel antisense tools based on chiral diaminocarboxylic acids. The present work gives also a contribution to the investigation on the role of nucleopeptides as a hypothetical primordial genetic material that preceded the actual nucleic acids in the prebiotic 'PNA world' [10]. In fact, it is important to underline that 2,6-diaminohexanoic acid was found not only on the earth, but also in the soil of the Mighei meteorite [11] that fell in Ukraine in 1889. Not less importantly,

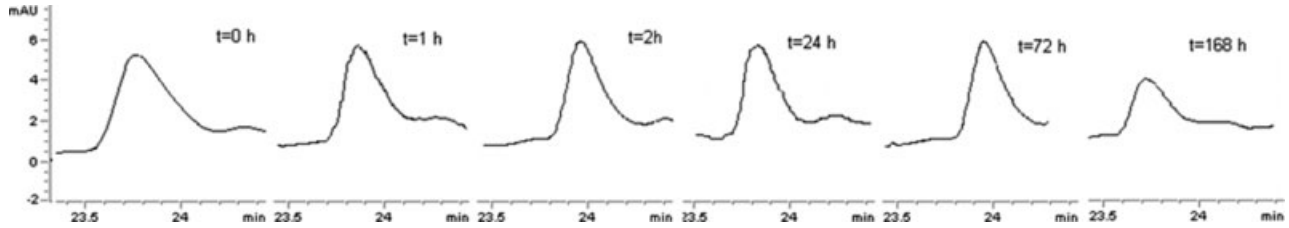


Figure 6. Serum stability profiles (experiments performed on nucleopeptide **4** in 80% fresh human serum).

the ε-peptide bonds largely predominated over α-peptide bonds in thermal syntheses of polypeptides that clearly showed the formation of peptide links involving mainly (70%) the ε-group of lysine in simulated prebiotic conditions [12]. In other words, on the basis of our data, we can suggest that lysine-containing ε-nucleopeptides that are able to interact with RNA could have been involved in the transition from the already hypothesised 'PNA world' to the actual based on the DNA/RNA/proteins system. Anyway, this point needs to be deeply investigated to obtain more detailed information on the role that ε-peptides could have played in the evolution of life.

On the basis of all these positive considerations, we are encouraged to further study ε-*lys*PNAs that we plan to synthesise not only with homothymine sequences, but also with mixed nucleobase compositions to test them as antisense or diagnostic tools. Nevertheless, the possibility to discriminate between parallel and antiparallel binding mode, because of the chirality of the backbone, and several other desirable characteristics of these nucleopeptides such as cell permeability and non-toxicity will be evaluated in view of their usage in *in vivo* approaches.

Acknowledgements

We thank Prof. Antonio Roviello for his precious suggestions, Dr Giuseppe Perretta and Leopoldo Zona for their invaluable technical assistance.

References

1. Nielsen PE, Egholm M, Berg RH, Buchardt O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 1991; **254**: 1497–1500.
2. Ray A, Norden B. Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. *FASEB J.* 2000; **14**: 1041–1060.
3. Silvester NC, Bushell GR, Searles DJ, Brown CL. Effect of terminal amino acids on the stability and specificity of PNA–DNA hybridisation. *Org. Biomol. Chem.* 2007; **5**: 917–923.
4. Sforza S, Corradini R, Ghirardi S, Dossena A, Marchelli R. DNA binding of a D-Lysine-Based chiral PNA: direction control and mismatch recognition. *Eur. J. Org. Chem.* 2000; **16**: 2905–2913.
5. Eom KD, Park SM, Tran HD, Kim MS, Yu RN, Yoo H. Dendritic α,ε-poly(L-lysine)s as delivery agents for antisense oligonucleotides. *Pharm. Res.* 2007; **24**(8): 1581–1589.
6. Saimura M, Takehara M, Mizukami S, Kataoka K, Hirohara H. Biosynthesis of nearly monodispersed poly(epsilon-L-lysine) in Streptomyces species. *Biotechnol. Lett.* 2008; **30**(3): 377–385.
7. Kito M, Takimoto R, Onji Y, Yoshida T, Nagasawa T. Purification and characterization of an ε-poly-L-lysine-degrading enzyme from the ε-poly-L-lysine-tolerant *Chryseobacterium sp.* OJ7. *J. Biosci. Bioeng.* 2003; **96**(1): 92–94.
8. Roviello GN, Moccia M, Sapio R, Valente M, Bucci EM, Castiglione M, Pedone C, Perretta G, Benedetti E, Musumeci D. Synthesis, characterization and hybridization studies of new nucleo-gamma-peptides based on diaminobutyric acid. *J. Pept. Sci.* 2006; **12**: 829–835.
9. Sforza S, Galaverna G, Dossena A, Corradini R, Marchelli R. Role of chirality and optical purity in nucleic acid recognition by PNA and PNA analogs. *Chirality* 2002; **14**: 591–598.
10. Nelson KE, Levy M, Miller SL. Peptide nucleic acids rather than RNA may have been the first genetic molecule. *Proc. Natl. Acad. Sci. U.S.A.* 2000; **97**: 3868–3871.
11. Vdovykin GP. The Mighei meteorite. *Space Sci. Rev.* 1973; **14**: 832–879.
12. Temussi PA, Paolillo L, Ferrara L, Benedetti L, Andini S. Structural characterization of thermal prebiotic polypeptides. *J. Mol. Evol.* 1976; **7**: 105–110.

