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Synthesis, characterization and hybridization studies of new nucleo-γ-peptides based on diaminobutyric acid

G. N. ROVIELLO,[a] M. MOCCIA,[b] R. SAPIO,[c] M. VALENTE,[c] E. M. BUCCI,[a,c] M. CASTIGLIONE,[b] C. PEDONE,[a,b] G. PERRETTA,[a] E. BENEDETTI[b] and D. MUSUMECI[a]*

[a] Istituto di Biostrutture e Bioimmagini-CNR, Via Mezzocannone 16, Napoli 80134, Italy
[b] Università degli Studi di Napoli 'Federico II', Dip. Scienze Biologiche sez. Biostrutture, via Mezzocannone 16, Napoli 80134, Italy
[c] Bionucleon Srl, via Ribes 5, Colleretto Giacosa (TO) 10010, Italy

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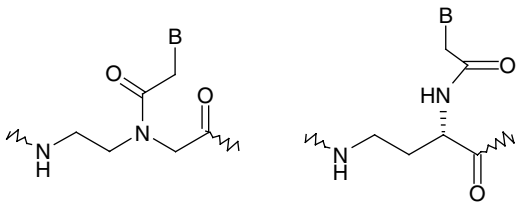
Abstract: In the present work, we report the synthesis and the characterization of a new chiral nucleoaminoacid, in which a diaminobutyric moiety is connected to the DNA nucleobase by an amidic bond, and its oligomerization to give the corresponding nucleo-γ-peptide. The ability of this synthetic polymer to bind complementary DNA was studied in order to explore its possible use in antigene/antisense or diagnostic applications. Our interest in the presented DNA analogue was also supported by the importance of γ-aminoacid-containing compounds in natural products of biological activity and by the known stability of γ-peptides to enzymatic degradation. Furthermore, our work could contribute to the study of the role of nucleopeptides as prebiotic material in a PNA world that could successively lead to the actual DNA/RNA/protein world, as recently assumed. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: γ-peptides; DABA; DNA analogue; PNA

INTRODUCTION

Aminoethylglycyl peptide nucleic acids (*aeg*PNAs **1**, Figure 1) emerged more than a decade ago as strong and specific DNA/RNA binding agents. Owing to their remarkable properties, they have triggered much research interest in the development of PNA-based antisense/antigene therapeutic or diagnostic agents [1]. Much effort has been directed mainly toward the refinement of *aeg*PNA properties such as binding affinity to DNA/RNA, water solubility, cellular uptake and discrimination between parallel and antiparallel binding modes. Different PNA modifications are widely described in the scientific literature [2–9], having met in some cases with success.



*aeg*PNA **1** *dab*PNA **2**

Figure 1 *dab*PNAs characterized by a chiral γ-peptide skeleton instead of the *aeg*PNA pseudopeptide backbone.

The most explored PNA modifications concern the aminoethylglycine backbone and the methylene carbonyl linker (first-generation modifications). In these cases, hybridization properties of PNA towards natural nucleic acids are negatively influenced by modifications to the aminoethylglycine backbone or changes in the distances between the nitrogen atoms of the backbone, as well as the distance from the tertiary amide nitrogen to the nucleobase or to the carbonyl of the glycine fragment [3,9]. In the first-generation modifications, the only tolerated substitution concerns the α-position of the glycine. For example, substitution of the glycine with an alanine (D or L) resulted in a slightly lower binding affinity towards DNA [3]. Introduction of D-lysine resulted in a slight increase of T_m in the binding to DNA, while the L-isomer had the opposite effect [3].

Many second-generation PNA-like molecules have been designed, synthesized and evaluated for interesting or improved properties. For example, much work has been devoted to investigating various cyclic backbone-based PNAs [2,4–7,9] with the goal of increasing the selectivity or strength of interaction with natural nucleic acids. These types of modifications, by connecting different parts of the PNA backbone, introduce conformational restrictions and stereocenters into the polymer, which can present remarkable hybridization properties. Among the most successful modifications, aminoprolyl PNA-containing oligomers are very interesting [4–7]. *aeg*PNAs with a single aminoprolyl PNA unit at the *N*-terminus show discrimination of antiparallel *versus* parallel binding to DNA. However,

*Correspondence to: D. Musumeci, Istituto di Biostrutture e Bioimmagini-CNR, Via Mezzocannone 16, Napoli 80134, Italy;
e-mail: domymusu@alice.it

a fully modified sequence does not bind to the nucleotidic target. Interestingly, an alternate *aeg*/aminoprolyl PNA oligomer shows a higher binding affinity than pure *aeg*PNA [7].

Another class of PNA analogues is based on a diamino acid backbone carrying the nucleobase by means of an amidic bond to one amino group. In particular, the oligothymine PNA analogue based on D- or L-ornithine was previously shown to bind to RNA by forming a triplex [8].

In the present work we report the synthesis and characterization of a new nucleoamino acid monomer, which is formed by a 2,4-diaminobutyric acid (DABA) moiety connected to the DNA nucleobase by an amidic bond, and its oligomerization to give a new nucleo-γ-peptide (*dab*PNA **2**, Figure 1).

DABA is a nonprotein amino acid found in the seeds and vegetative tissues of flatpea (*Lathyrus sylvestris L.*) distributed intracellularly in mature leaves with about 15% of the cellular DABA contained in chloroplasts and at least 75% in vacuoles [10]. Interestingly, poly(2,4-diaminobutyric acid), (PDBA), was recently used in a novel nonviral cytokine gene delivery system, which makes use of PDBA instead of polylysine resulting in high transfection efficiency. In particular, *in vivo* antitumor effects of dendritic cell immunotherapy with poly(D,L-2,4-diaminobutyric acid)-mediated intratumoral delivery of the interleukin-12 gene were evaluated with the conclusion that this system suppresses tumor growth significantly [11].

The ability of the presented synthetic polymer to bind to complementary DNA was studied in order to explore its possible use in antigene/antisense or diagnostic applications. The importance of this class of polymers for this type of applications arises from the known stability of γ-peptides to enzymatic degradation and from the biological activity of γ-amino acid containing compounds. Furthermore, our work could contribute to the study of the role of nucleopeptides as prebiotic material in a PNA world that could successively lead to the actual DNA/RNA/protein world, as recently hypothesized [12,13].

MATERIALS AND METHODS

Chemicals

Fmoc-Gly-OH, HATU, Fmoc-Lys(Boc)-OH, and PyBOP were purchased from Novabiochem. Anhydroscan DMF and NMP were from LabScan. Piperidine was from Biosolve. Solvents for HPLC and Ac_2O were from Reidel-de Haën. TFA, Rink-amide resin, and TCH_2COOH were from Fluka. Perspective Biosystem PNA kit (Fmoc/Bhoc monomers, HATU activator, Base solution, Wash B, DIEA, Cap Solution, Deblock solution) was purchased from PRIMM (Milan, Italy). Boc-DAB(Fmoc)-OH was from Bachem. DCM and TFA (for HPLC) were from Romil. Deuterated solvents (DMSO, methanol) were from Aldrich. Thin-layer chromatography (TLC) analyses were performed on silica gel Macherey–Nagel G-25 UV_{254} plates (0.25-mm thick) visualized by UV light and by a ninhydrin staining solution. The reactions were monitored by TLC until all starting material had been consumed. Solvents for TLC analyses, and diethyl ether were from Carlo Erba. dA_{12} was purchased from Biomers (Ulm, Germany).

Apparatus

^1H NMR and ^{13}C NMR spectra were recorded at 25 °C on Varian unity 300 MHz and Varian Inova 600 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; CD_3OD: $\delta = 49.0$, sept). *aeg*PNA oligomers were assembled on solid phase with an Applied Biosystems Expedite 8909 oligosynthesizer. Crude samples containing PNA oligomers 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) 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.

Semipreparative purifications were performed by RP-HPLC on a Shimadzu LC-8A equipped with an SPD-10A VP UV–vis detector, and on a Hewlett Packard/Agilent 1100 series equipped with a diode array detector, by using a Phenomenex Jupiter C18 300 Å (10 μm, 10 × 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 containing PNAs (crude or purified) were lyophilized in an FD4 freeze dryer (Heto Lab Equipment) for 16 h.

Circular dichroism (CD) spectra were obtained at 20 °C on a Jasco J-810 spectropolarimeter using a 1-cm quartz cuvette (Hellma). Ultraviolet (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 a 1-cm quartz cuvette (Hellma).

Synthesis of the (L) *dab* PNA Monomer

N$^{\gamma}$-Fmoc-L-2,4-diaminobutyric acid, 4 (Scheme 1). Commercial Fmoc/Boc-protected 2,4-diaminobutyric acid **3** (200 mg, 0.45 mmol) was treated with a solution of TFA/DCM/H_2O 4.5 : 4.5 : 1 (8 ml) at 50 °C. After stirring for 1 h, the pH of the solution was adjusted to 5 by dropwise addition of 1 M aqueous KOH. The obtained white precipitate was filtered, washed with DCM and coevaporated three times with dry CH_3CN (138 mg of **4**, 0.40 mmol, 90% yield). The crude precipitate contained mostly one product as evidenced by TLC, which showed only one spot at $R_f = 0.14$ (6 : 4 CH_2Cl_2/CH_3OH), and by LC-MS. ESI-MS m/z: 340.4 (found),

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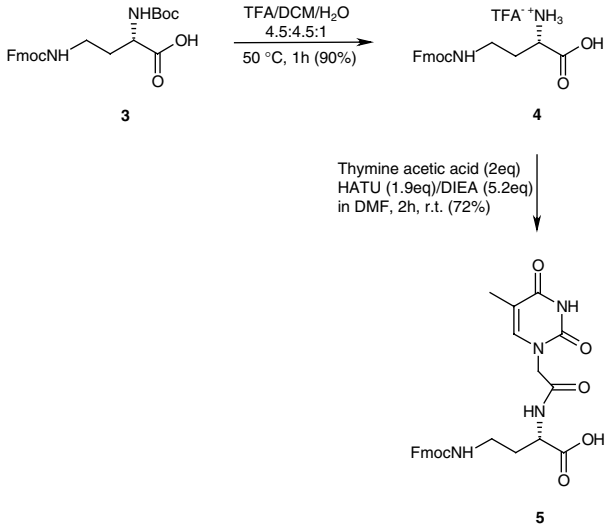

Scheme 1 Synthesis of the new *dab*PNA monomer **5**.

341.38 (expected for MH^+); δ_H (300 MHz, DMSO-d_6) 8.27 (3H, br s, NH_3^+), 7.29–7.89 (9H, aromatic CH Fmoc and Fmoc-NH), 4.32 (2H, m, FmocCH-CH_2), 4.22 (1H, m, FmocCH-CH_2), 3.90 (1H, m, CH_α), 3.14 (2H, m, CH_2NH), 1.92 (2 H, m, C$H_2$$CH_\alpha$).

N^γ-Fmoc-N^α-(thymin-1-ylacetyl)-L-2,4-diaminobutyric acid, 5 (Scheme 1). Product **4** (1 equiv., 138 mg, 0.40 mmol) was dissolved in dry DMF (6 ml) and DIEA (1.2 equiv., 82.5 µl, 0.49 mmol) and reacted with TCH_2COOH (2 equiv., 149 mg, 0.81 mmol), which was previously preactivated with HATU (1.9 equiv., 293 mg, 0.77 mmol) and DIEA (4 eq, 275 µl, 1.62 mmol) for 2 min. After 2 h, the reaction was quenched by adding 1 ml of water and the solvent was removed *in vacuo*. The crude material was purified by semipreparative HPLC on a Shimadzu instrument using increasing amounts (15–70% in 25 min) of solution B in A, giving the pure sample **5** (148 mg, 0.29 mmol, 72% yield) as a white powder; $R_f = 0.50$ (8 : 1:1 butanol : acetic acid : water); LC-ESI-MS (Figure 2) m/z: 507.32 (found), 507.52 (expected for MH^+); δ_H (600 MHz, DMSO-d_6) 11.35 (1H, br s, NH thymine), 8.54 (1H, br s, NH amide), 7.98 (2H, d, $J = 7.3$, aromatic CH Fmoc), 7.77 (2H, d, $J = 7.3$, aromatic CH Fmoc), 7.51 (2H, t, $J = 7.3$, aromatic CH Fmoc), 7.49–7.51 (1H, m, Fmoc-NH), 7.42 (2H, t, $J = 7.3$, aromatic CH Fmoc), 7.41 (1H, s, CH thymine), 4.43 (2H, s, CH_2 acetyl linker), 4.05–4.50 (4H, m, FmocCH-CH_2 and CH_α), 3.14 (2H, m, CH_2NH), 2.00 (1H, m, part of an AB system centred at 1.91, C$H_2$$CH_\alpha$), 1.83 (1H, m, part of an AB system centred at 1.91, C$H_2$$CH_\alpha$), 1.82 (3H, s, CH_3 thymine); δ_H (600 MHz, CD_3OD) 7.78 (2H, d, $J = 7.3$, aromatic CH Fmoc), 7.64 (2H, d, $J = 7.3$, aromatic CH Fmoc), 7.38 (2H, t, $J = 7.3$, aromatic CH Fmoc), 7.36 (1H, s, CH thymine), 7.30 (2H, t, $J = 7.3$, aromatic CH Fmoc), 4.47 (2H, s, CH_2 acetyl linker), 4.19–4.50 (4H, m, FmocCH-CH_2 and CH_α), 3.19 (1H, m, part of an AB system centred at 3.22, CH_2NH), 3.25 (1H, m, part of an AB system centred at 3.22, CH_2NH), 2.11 (1H, m, part of an AB system centred at 1.98, C$H_2$$CH_\alpha$), 1.85 (1H, m, part of an AB system centred at 1.98, C$H_2$$CH_\alpha$), 1.84 (3H, s, CH_3 thymine); δ_C (150 MHz, DMSO-d_6) 170.98 (COOH), 168.48 (CH_2CONH), 160.10 (thymine C-4), 155.00 (OCONH), 147.96 (aromatic Fmoc 2C), 147.89 (thymine C-2), 146.36 (aromatic Fmoc 2C), 144.73 (thymine C-6), 131.65 (aromatic Fmoc 2CH),

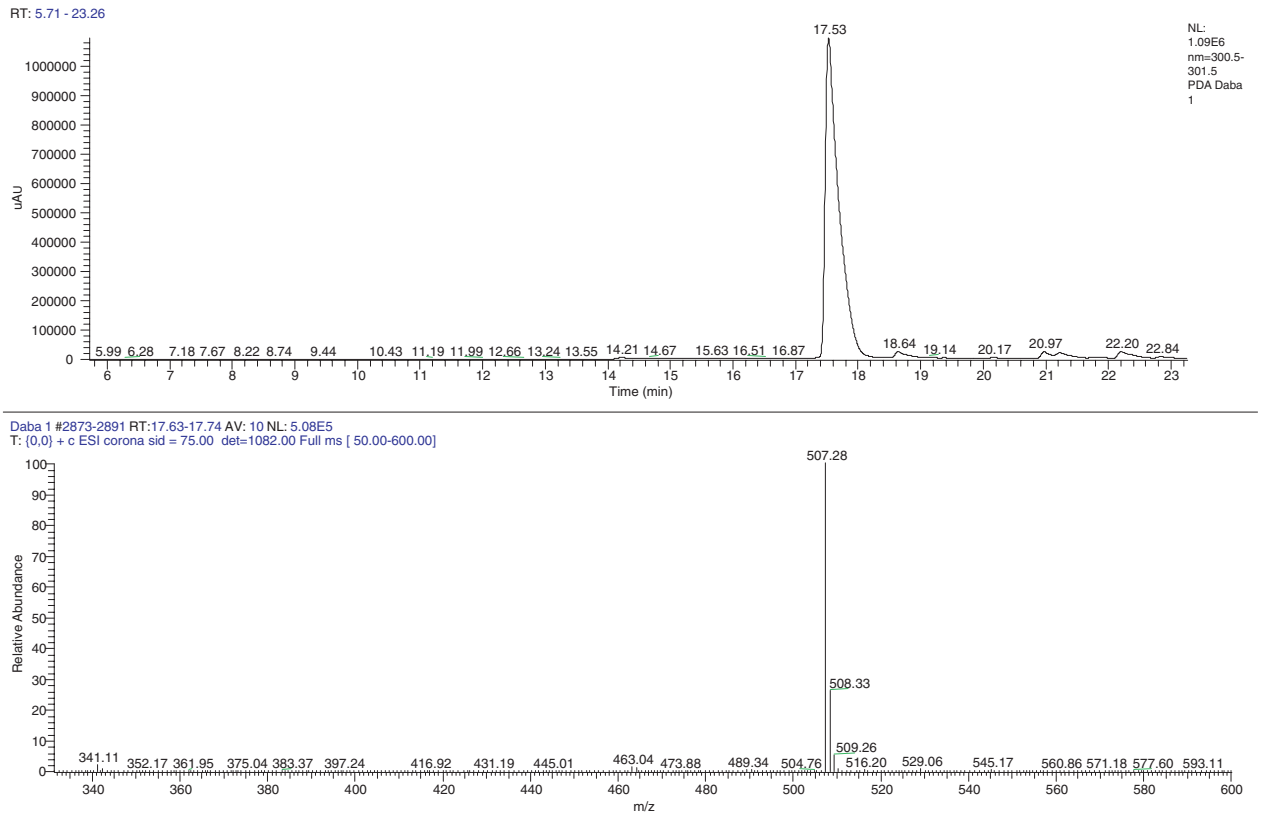

Figure 2 LC-ESI-MS of compound **5**.

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131.11 (aromatic Fmoc 2CH), 129.18 (aromatic Fmoc 2CH), 124.15 (aromatic Fmoc 2CH), 111.96 (thymine C-5), 69.40 (Fmoc CH_2), 53.02 (CH_2 acetyl linker), 50.73 (CH_α), 44.1 (Fmoc CH), 41.30 (CH_2NH), 22.56 (CH_2CH_α), 15.91 (thymine CH_3).

Solid-phase Synthesis of Oligomers 6–9 (Table 1)

Solid support functionalization and manual solid-phase oligomerizations were carried out in short PP columns (4 ml) equipped with a PTFE filter, a stopcock and a cap. Solid support functionalization: Rink-amide resin (0.50 mmol NH_2/g, 128 mg) was functionalized with a lysine (Fmoc-Lys(Boc)-OH, 0.5 equiv., 14.8 mg, 32 μmol) using PyBOP (0.5 equiv., 16.8 mg, 32 μmol) as activating agent and DIEA (1 equiv., 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 functionalization. Automatic solid-phase assembly of the *aeg*PNAs was performed on an Expedite 8909 nucleic acid synthesis system using a standard 2-μmol-scale protocol and Fmoc chemistry leaving the final Fmoc on, in order to evaluate SPS yields by the UV Fmoc test. Manual couplings of *dab*PNA monomers were monitored by the UV Fmoc test in order to evaluate the incorporation yields of the new monomer. A glycine residue (3 equiv.) was attached to the *N*-terminus of all the oligomers by using PyBOP (3 equiv.)/DIEA (6 equiv.) in DMF as the activating system. After removal and quantification of the Fmoc group, all oligomers were cleaved from the resin and deprotected under acidic conditions (TFA/*m*-cresol 4 : 1 v/v). The oligomers were isolated by precipitation with cold diethyl ether, centrifugation and lyophilization. Purified oligomers (semipreparative HPLC) were quantified and characterized by LC-ESI-MS.

***H-Gly-(t_{dab})$_{12}$-Lys-NH$_2$* 6.** $(t_{dab})_{12}$ was assembled manually on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) using the following protocol: 0.2 M Fmoc-t_{dab}-OH monomer in NMP (3 equiv., 120 μl, 24 μmol), 0.2 M PyBOP in DMF (3 equiv., 120 μl, 24 μmol) and 0.2 M NMM in DMF (6 equiv., 240 μl, 48 μmol) were added in sequence to the resin and the coupling was left for 30 min with shaking at room temperature. Successively, the unreacted amino groups were capped with PNA Cap solution for 5 min. Fmoc removal was accomplished with PNA Deblock solution (5 min) and was monitored at every step by UV measurements: average yield for the first six steps was 96%, while for the last six steps a lowering of the yields from 96 to 70% was observed probably because of the enhanced aggregation tendency of the increasing homothymine chain (overall yield: 25%). Yield of the last coupling with glycine was 97%. After cleavage and deprotection, the oligomer **6** was purified by semipreparative RP-HPLC using a linear gradient of 8% (for 5 min) to 25% B in A over 30 min: $t_R = 31.0$ min; UV quantification of the purified product gave 100 nmol of **6**; ESI-MS (Figure 3) m/z: 1134.8 (found), 1133.4 (expected for $[M + 3H]^{3+}$).

Table 1 Polyamidic sequences and T_m relative to hybridization with dA_{12}

Sequences	T_m (°C) ± 0.5
H-G-t_d t_d t_d t_d t_d t_d t_d t_d t_d t_d t_d t_d-K-NH_2 **(6)**	—
H-G-t t t t t t t t t t t t-K-NH_2 **(7)**	86.2
H-G-t_d t t t t t t t t t t t-K-NH_2 **(8)**	84.8
H-G-t t t t t t t_d t t t t t-K-NH_2 **(9)**	72.0

***H-Gly-t$_{12}$-Lys-NH$_2$* 7.** Standard t_{12} *aeg*PNA was assembled on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) on an automatic synthesizer. After the synthesis was complete, the final Fmoc was left on and removed manually to perform the Fmoc UV test. The overall yield of t_{12} was only 18% probably because the *flow through* synthesis of the automatic synthesizer enhances the aggregation problems of the homothymine sequence. Glycine was incorporated manually as the last residue with high efficiency (98% yield). The cleaved and deprotected oligomer was purified by semipreparative RP-HPLC using a linear gradient of 8% (for 5 min) to 20% B in A over 30 min: $t_R = 27.9$ min; UV quantification of the purified product gave 60 nmol of **7**; ESI-MS m/z: 1132.0 (found), 1697.1 (found), 1133.4 (expected for $[M + 3H]^{3+}$), 1699.7 (expected for $[M + 2H]^{2+}$).

***H-Gly-t$_{dab}$t$_{11}$-Lys-NH$_2$* 8.** The *aeg*PNA part of **8** was assembled on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) in an automatic synthesizer leaving the final Fmoc on (yield: 20%), while the *dab*PNA monomer was attached manually as the last residue following the protocol described for **6** (yield: 95%). After the last coupling with glycine, an overall yield of 18% was obtained. After cleavage and deprotection, the oligomer was purified by semipreparative RP-HPLC using a linear gradient of 8% (for 5 min) to 17% B in A over 35 min: $t_R = 29.8$ min; UV quantification of the purified product gave 70 nmol of **8**; ESI-MS m/z: 1133.0 (found), 1699.8 (found), 1133.4 (expected for $[M + 3H]^{3+}$), 1699.7 (expected for $[M + 2H]^{2+}$).

***H-Gly-t$_6$t$_{dab}$t$_5$-Lys-NH$_2$* 9.** The synthesis of the *aeg*PNA part of **9** was performed on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) by the automatic synthesizer except for the *dab*PNA monomer, which was attached manually in the middle of the *aeg*PNA sequence (overall yield: 20%). Glycine was incorporated manually as the last residue with 95% yield. The cleaved and deprotected oligomer was purified by semipreparative RP-HPLC using a linear gradient of 7% (for 5 min) to 19% B in A over 40 min: $t_R = 30.1$ min; UV quantification of the purified product gave 80 nmol of **9**; ESI-MS m/z: 1132.8 (found), 1698.5 (found), 1133.4 (expected for $[M + 3H]^{3+}$), 1699.7 (expected for $[M + 2H]^{2+}$).

UV and CD Studies

Purified oligomers were dissolved in a known quantity of milliQ water and quantified by UV measurements ($T = 80$ °C, absorbance value at $\lambda = 260$ nm). The epsilon used for the quantification of the oligomers (103.2 mM^{-1}) was calculated using the molar extinction coefficient of thymine *aeg*PNA monomer (8.6 mM^{-1}).

Annealing of all PNA oligomers with complementary dA_{12} was performed by mixing equimolar amounts of the two strands in 10 mM phosphate buffer at pH 7.5 to achieve a

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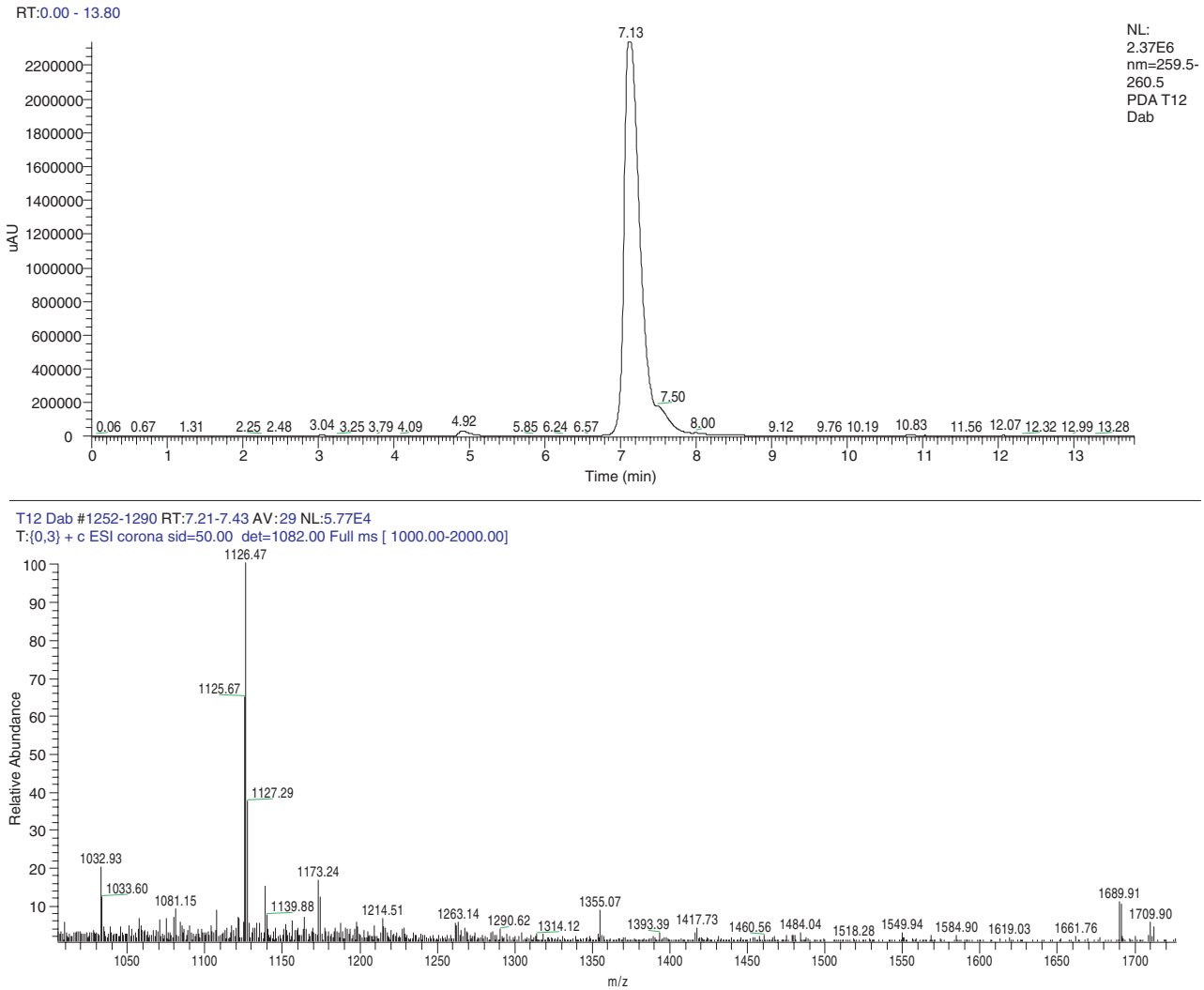

Figure 3 LC-ESI-MS of (L) *dab*PNA **6**.

duplex concentration of 4 μM. The solution was heated at 85 °C (5 min) and then allowed to cool slowly to room temperature.

Thermal melting curves (Figure 4) were obtained by recording the UV absorbance at 260 nm while the temperature was ramped from 20 to 97 °C at a rate of 0.5 °C/min. T_m values, calculated by the first derivative method, are reported in Table 1.

CD spectrum of **6** (Figure 5) was recorded from 320 to 200 nm at 20 °C under the following conditions: scan speed 50 nm/min; data pitch 2 nm; bandwidth 2 nm; response 4 s; accumulations 3.

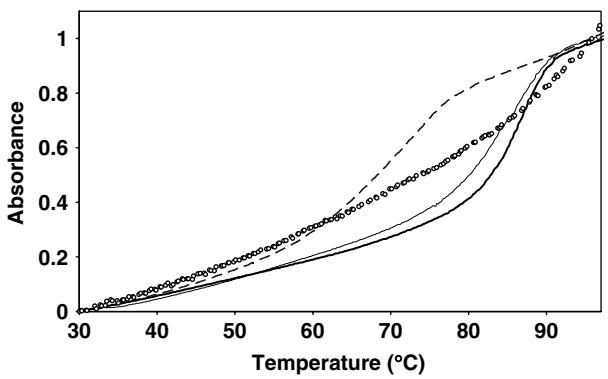

Figure 4 Melting curves, normalized between 0 and 1, for the duplexes (4 μM, in 10 mM phosphate buffer, pH 7.5) formed between dA_{12} and the PNA oligomers: $(t_{dab})_{12}$ **6** (open circle), t_{12} **7** (solid line), $t_{11}t_{dab}$ **8** (thin line) and $t_6t_{dab}t_5$ **9** (dashed line).

RESULTS AND DISCUSSION

In continuing our research in the field of new DNA analogues as therapeutic or diagnostic agents, we designed a new monomer constituted by a γ-amino acid carrying in the α-position an amino group that allows for the linkage to the carboxymethylated nucleobase. Different from *aeg*PNA monomers, the *dab*PNA monomer (Figure 1) presents a shorter backbone (3C between the nitrogen atom and the carbonyl) and an enhanced distance between the nucleobase and the backbone,

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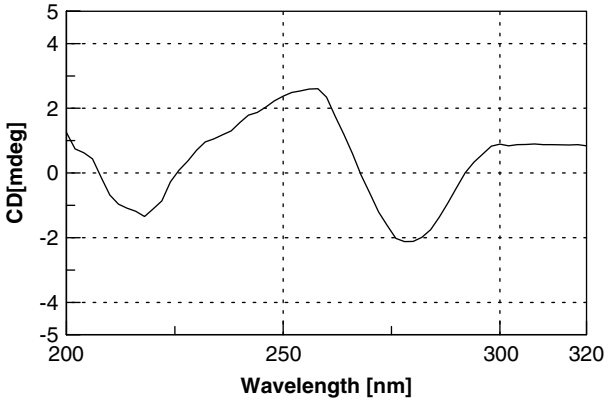

Figure 5 CD spectrum of (L) *dab*PNA **6** (4 μM) in 10 mM phosphate buffer, pH 7.5.

i.e. three atoms (1N + 2C) instead of 2C in *aeg*PNAs. Furthermore, in our case the amidic NH that carries the carboxymethylated nucleobase protrudes from the backbone. The polymerization of such a building block gives a nucleopolyamide with a γ-peptide skeleton, different from the pseudopeptide skeleton of *aeg*PNA.

We chose as starting material for the synthesis of the new monomer the L-enantiomer of the natural diaminobutyric acid, in analogy to the L-ornithine-based PNAs, proposed by Petersen *et al.* [14], which formed a triplex structure with a complementary RNA. The thymine-containing monomer was synthesized starting from the commercially available Boc-(*S*)-DAB(Fmoc)-OH diaminoacid (**3**, Scheme 1). In the first synthetic step, the Boc group was selectively removed with TFA to give the free amino group in the α-position. The obtained product **4**, characterized by ^{1}H NMR and LC-ESI-MS, was coupled with the thymine-1-acetic acid under different synthetic conditions. The best results were obtained using HATU/DIEA as the activating system in DMF as solvent, leading to the compound **5** in 72% yield.

The Fmoc-protected monomer **5,** characterized by ^{1}H/^{13}C NMR and LC-ESI-MS (Figure 2), was oligomerized manually on solid phase to the corresponding *dab*PNA dodecamer (**6**, Table 1) using PyBOP/NMM as the activating system in NMP/DMF (1 : 4, v/v). Since the base-mediated deprotonation of the carboxylic group and the time for its preactivation in basic conditions are critical points to avoid racemization during the amidation of carboxylic acids with a chiral carbon in the α-position [15,16], we chose as coupling reactants a mild base (NMM) and an activator (PyBOP) that does not require preactivation. The manual synthesis of the new oligomer allowed us to check, by UV Fmoc test, the coupling efficiency, which was high in the first six steps (about 96%) and decreased gradually from 96% to 70% in the last six steps, probably because of the enhanced aggregation tendency of the increasing homothymine chain. L-Lysine and glycine residues were incorporated in the strand at the *C*- and *N*-termini, respectively, to improve the solubility of the homothymine polymer (overall yield: 24%).

The t_{12} *aeg*PNA (**7**, Table 1) was also synthesized as the reference oligomer for hybridization studies. Automatic synthesis on 2-μmol scale using the standard protocol gave **7** in poor yield (18%) probably owing to aggregation problems concerning the homothymine sequence and enhanced by the *flow through* synthesis.

The dodecamers carrying a single t_{dab}PNA unit at the terminal and central position of *aeg*PNA chain (**8** and **9**, respectively, Table 1) were realized with the aim to explore the ability of *aeg*PNAs, modified by a single *dab*PNA insertion, to bind natural targets and to study the stability of their complexes with nucleic acids. PNAs **8** and **9** were assembled on the automatic synthesizer using a standard 2-μmol-scale protocol for the *aeg*PNA part, with the manual single insertion of *dab*PNA monomer using the protocol described for **6**. The overall yields of **8** and **9** were 18% and 19%, respectively.

After cleavage and deprotection, all oligomers were purified by semipreparative RP-HPLC, and successively characterized by LC-ESI-MS, which confirmed the identity of the products. The LC-ESI-MS profile of the new polymer **6** is reported in Figure 3.

Since single strands of PNAs containing chiral units show CD signals (even though the bands are very weak), as reported previously for ornithine-based PNAs [8], we performed a CD study on the (L) $(t_{dab})_{12}$ polymer. The profiles of CD spectra for single strands of L- and D-ornithine-based PNAs showed a perfect mirror–image relationship in the 260–290 nm region. In particular the H-$(t_{L-Orn})_{10}$K-NH_2 PNA exhibited a negative band at 260–290 nm, while H-$(t_{D-Orn})_{10}$K-NH_2 PNA showed a positive band at the same wavelength range. In perfect analogy with these results, the CD spectrum for our H-Gly$(t_{L-dab})_{12}$K-NH_2 (Figure 5) showed a negative band at 260–290 nm (4 μM in 10 mM phosphate buffer, pH 7.5). A positive CD band in 260–290 nm region should be expected for H-Gly$(t_{D-dab})_{12}$K-NH_2, as we hope to demonstrate after the synthesis of the (D) Fmoc-t_{dab}-OH enantiomer, in continuation of our research in this field.

In order to verify the ability of PNAs **6**–**9** to bind to complementary DNA, UV melting experiments involving an dA_{12} and oligomers **6**, **7**, **8** and **9** were performed on the annealed strands in 10 mM phosphate buffer at pH 7.5 (4 μM duplex concentration). The overlapped melting curves are shown in Figure 4 and the corresponding T_m values are summarized in Table 1. From our preliminary results, the *dab*PNA homopolymer **6** does not convincingly bind to the complementary dA_{12}. The incorporation of *dab*PNA monomer in the middle or at the *N*-terminus of the homothymine *aeg*PNA oligomer leads to a decreased binding efficiency for the oligomers **8** and **9** to the target DNA sequence in comparison to *aeg*PNA **7**. Since *dab*PNA did not convincingly form

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duplexes with complementary oligonucleotides as seen by UV, no further CD studies were performed.

CONCLUSIONS AND PERSPECTIVES

Even though no encouraging results, relative to the ability of (L) *dab*PNA oligomer to bind DNA, came from the current investigation, its binding to other natural targets and to complementary *dab*PNA sequences will be the subject of our future research, in order to develop new, interesting biomedical tools and novel drug delivery systems. Moreover, our future interest will be focused on realizing polymers based on the (D)-DABA enantiomer, which will be studied in analogy with what we have described in this work. A linker different from the methylene carbonyl moiety will also be examined for connecting the nucleobase to the peptidic backbone. Finally, new oligomers with alternate *aeg*PNA/*dab*PNA (and possibly even in other combinations) will be realized and studied with the aim of finding novel nucleopeptides with useful biotechnological characteristics.

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