International Journal of Pharmaceutics 415 (2011) 206–210

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Synthesis and characterization of a novel ester-based nucleoamino acid for the assembly of aromatic nucleopeptides for biomedical applications

Giovanni N. Roviello[a,*], Domenica Musumeci[a], Enrico M. Bucci[a], Carlo Pedone[b]

[a] Istituto di Biostrutture e Bioimmagini – CNR, Via Mezzocannone 16, 80134 Napoli, Italy
[b] Dipartimento delle Scienze Biologiche, Università di Napoli "Federico II", 80134 Napoli, Italy

ARTICLE INFO

Article history:
Received 16 February 2011
Received in revised form 1 June 2011
Accepted 3 June 2011
Available online 13 June 2011

Keywords:
Tyrosine
Peptide
Aromatic

ABSTRACT

In this work, we report a technological approach to a novel Fmoc-protected nucleoamino acid, based on L-tyrosine, carrying the DNA nucleobase on the hydroxyl group by means of an ester bond, suitable for the solid-phase synthesis of novel aromatic nucleopeptides of potential interest in biomedicine. After ESI-MS and NMR characterization this building block was used for the assembly of a thymine-functionalized tetrapeptide, composed of nucleobase-containing and underivatized L-tyrosine moieties alternated in the backbone.

1. Introduction

In the last decades, a great importance has been attributed to the realization of hydrogels as well as water-soluble macromolecular networks suitable for the incorporation and the release of genes and drugs (Shuai et al., 2005). These systems are non-covalent polymers based on one or more types of monomeric units governed by weak interactions occurring between the subunits. Among the many classes of molecules used to form supramolecular architectures, it is worth to mention polynucleobase molecules such as nucleic acids, of particular importance in nanomedicine (Chhabra et al., 2010) and peptide nucleic acids (Moccia et al., 2009; Roviello et al., 2011a,b). Nevertheless also systems characterized by monomeric units bringing a single nucleobase are known to form gels based on the cooperative effects of the complementary nucleobases (Snip et al., 2002).

Several investigations on oligonucleotide analogues have demonstrated the possibility of replacing the ribose phosphodiester linkage by various modifications (Bell and Micklefield, 2009). For example, it is worth to mention nucleobase-containing polyesters, stable in water solutions and fully resistant to enzymatic degradation (Efimov et al., 1999; Murata and Wada, 2006), and nucleobase-containing polyamides carrying positively charged residues, with remarkable cell permeability properties (Dragulescu-Andrasi et al., 2006; Katritzky and Narindoshvili, 2008), or aromatic moieties, which were found to bind DNA and RNA (Fader and Tsantrizos, 2002). Several attempts to use real peptides as alternative oligonucleotide linkages have also been reported and chiral alpha nucleopeptides with interesting properties were obtained in several cases (Diederichsen, 1996; Geotti-Bianchini et al., 2008; Roviello et al., 2009, 2010a,b,c). As a general rule, a potential nucleic acid-binding nucleopeptide is built of an alpha-aminoacid pair containing a nucleobase-substituted and a proteinogenic aminoacid or another nucleobase-containing monomer or combination of amino acids allowing for a distance between the nucleobase-bearing atoms of the nucleopeptide backbone, similar to that present in DNA, i.e. six bonds (Diederichsen, 1996). In particular, short dinucleobase tetrapeptides based on nucleobase-bearing and underivatized serines alternated in the backbone were shown to base-specifically interact with complementary nucleic acids (Yamazaki et al., 1997). Regarding the distance between the nucleobases and the backbone, DNA-binding ability was found in those cases in which such distance was similar to that found in natural nucleic acids. However, also peptide-like DNA analogues with long side chains were reported in literature to form stable complexes with natural oligonucleotides with high sequence specificities (Wada et al., 2000; Sawa et al., 2010). Taking into account all these considerations, together with the interesting properties of the aromatic nucleobase-bearing polyamides (Fader and Tsantrizos, 2002), as well as the possibility to form supramolecular systems of potential biomedical importance by using nucleobase-containing molecules (Snip et al., 2002; Roviello et al., 2011a,b), we realized and fully characterized a novel

* Corresponding author. Tel.: +39 0 81 2534585; fax: +39 0 81 2534574.
E-mail address: giroviel@unina.it (G.N. Roviello).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.06.007

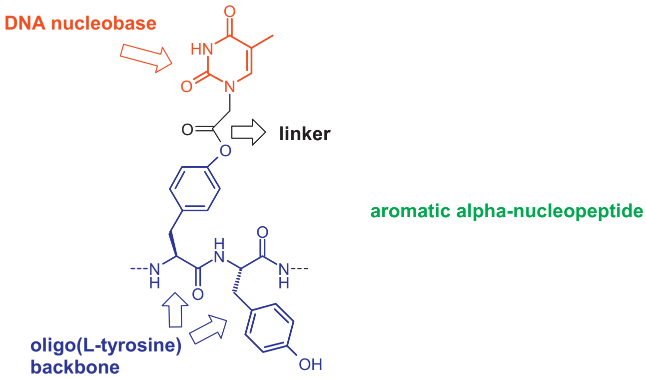


Fig. 1. Structural representation of the repeating unit of L-tyrosine-based nucleopeptide.

nucleoamino acid based on L-tyrosine. A short tetrapeptide was also synthesized, whose backbone comprised both nucleobase-containing and underivatized L-tyrosine moieties alternated in the sequence (Fig. 1).

In this oligomer the DNA bases were anchored to the phenolic hydroxyl groups by means of ester bonds, while underivatized L-tyrosines were introduced as spacers between the nucleobase-carrying aminoacids in order to achieve the same number of bonds between the atoms bringing the nucleobases (i.e. 6) found in DNA backbone. In analogy to the serine-based nucleopeptides already described by us (Roviello et al., 2011a,b) the novel tyrosine-based aromatic nucleopeptide could be employed for the realization of supramolecular networks, based on both aromatic interactions and hydrogen bonding, useful in biomedicine as drug delivery systems.

2. Materials and methods

2.1. Chemicals

Fmoc-L-Tyr(*t*Bu)-OH and PyBop, were purchased from Novabiochem. Anhydroscan DMF and NMP were from LabScan. Piperidine was from Biosolve. CH_3CN for HPLC chromatography and acetic anhydride were from Reidel-de Haën. Thyminyl acetic acid, TFA, TMP, Rink-MBHA-amide resin were from Fluka. DCM and TFA (for HPLC) were from Romil. Deuterated DMSO, DIPC, DIPEA, DMAP and TIS were from Sigma–Aldrich. Diethyl ether was from Carlo Erba.

2.2. Apparatus

^{1}H 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). Proton chemical shifts were referenced to residual CHD_2SOCD_3 (δ = 2.49, quin) signals. ^{13}C NMR chemical shifts were referenced to the solvent (CD_3SOCD_3: δ = 39.5, sept). Crude samples containing the nucleopeptide were centrifuged for 4 min at 4000 rpm (Z 200 A, Hermle). Products were analysed 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 mm × 150 mm) column. Gradient elution was performed (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/Agilent 1100 series, 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 25 °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 the nucleopeptide (crude or purified), were lyophilized in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h. Ultraviolet (UV) spectra were recorded on a UV–vis Jasco model V-550 spectrophotometer using a Hellma quartz cell with a light path of 1 cm.

2.3. Synthesis of the Fmoc-L-Tyr(T)-OH monomer (3)

The nucleobase-containing monomer was synthesized starting from the commercially available Fmoc-L-Tyr(tBu)-OH (**3**, Fig. 2). About 100 mg of **1** (0.217 mmol) were treated with TFA/DCM 1:1 (3 ml, RT, 2 h) to selectively remove the tert-butyl group. After removal of TFA and DCM under nitrogen stream and vacuum-evaporation, Fmoc-L-Tyr-OH (**2**) was recovered in almost quantitative yield (86 mg, 0.213 mmol,) and characterized by ^{1}H/^{13}C NMR and ESI-MS. δ_H (400 MHz, DMSO-d$_6$), 8.97 (2H, bs, OH, COOH), 7.92–7.32 (9H, m, aromatic CH Fmoc, Fmoc-NH), 7.11 (2H, d, $^3J_{H,H}$ = 8.0 Hz, aromatic CH_{tyr}CO), 6.72 (2H, d, $^3J_{H,H}$ = 8.4 Hz, aromatic CH_{tyr}), 4.27–4.13 (4H, m, FmocCH-CH_2, FmocCH-CH_2, CH_{alpha}), 3.00 (1H, bdd, CH_AHCH_{alpha}), 2.81 (1H, bdd, CH_BHCH_{alpha}), δ_C (100 MHz, DMSO-d$_6$) 177.4, 159.9, 159.8, 147.7, 144.6, 133.9, 131.5, 130.9, 129.2, 129.1, 123.9, 118.9, 68.8, 59.8, 50.5, 39.7. *LC–ESI-MS characterization of Fmoc-L-Tyr-OH*. Method: 15% (5 min) to 95% B in A over 15 min, t_R = 13.69 min. ESI-MS *m/z*: 427.46 (found), 426.43 (expected for $[C_{24}H_{21}NO_5 + Na]^+$); 405.58 (found), 404.45 (expected for $[C_{24}H_{21}NO_5 + H]^+$). Subsequently, compound **2** (86 mg, 0.213 mmol) was dissolved in 1 ml DMF, treated with TMP (39.4 μl, 0.298 mmol), and coupled with TCH_2COOH (1.8 equiv., 71 mg), which was previously preactivated with DIPC (1.8 equiv., 60 μl)/DMAP (cat., ca. 1 mg) in DMF (1 ml), at room temperature

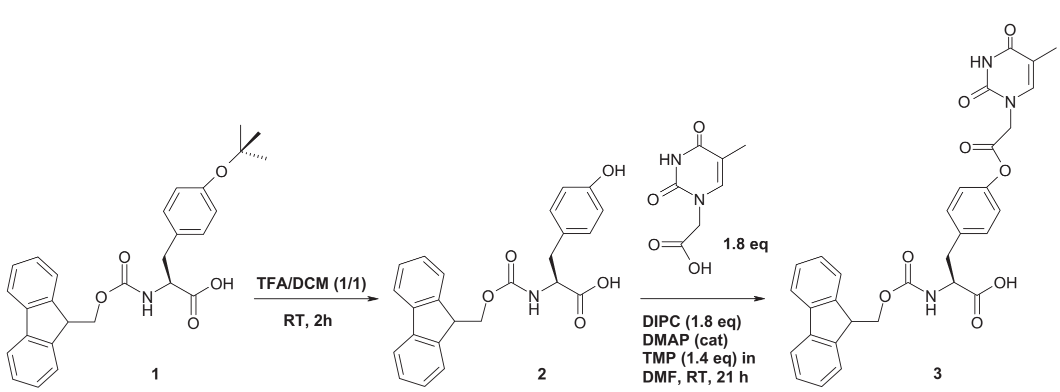


Fig. 2. Synthesis of monomer **3**.

(Fig. 2). After 21 h, the reaction was quenched by adding water, freezing and lyophilizing the mixture. The crude material was resuspended in 20% aqueous CH_3CN and purified by semipreparative HPLC on a C_{18} column using a linear gradient of 20% (5 min) to 80% B in A over 20 min; t_R = 19.9 min. The desired product **3** was obtained in 34% yield (42 mg, 0.073 mmol) and characterized by $^1H/^{13}C$ NMR and ESI-MS (Fig. 3). δ_H (400 MHz, DMSO-d$_6$) 11.53 (1H, s, NH thymine), 7.92 (2H, d, $^3J_{H,H}$ = 7.6 Hz, aromatic CH_{tyr}CO), 7.74–7.30 (10H, m, aromatic CH Fmoc, Fmoc-NH, CH thymine), 7.10 (2H, d, $^3J_{H,H}$ = 8.4 Hz, aromatic CH_{tyr}), 4.79 (2H, s, NCH_2CO), 4.27–4.15 (4H, m, FmocCH-CH_2, FmocCH-CH_2, CH_{alpha}), 3.14 (1H, bdd, CH_AHCH_{alpha}), 2.92 (1H, bdd, CH_BHCH_{alpha}), 1.82 (3H, s, CH_3 thymine), δ_C (100 MHz, DMSO-d$_6$) 177.2, 171. 2, 168.3, 159.9, 155.0, 152.5, 147.7, 145.4, 144.6, 140.0, 134.2, 131.6, 131.0, 129.2, 125.0, 124.0, 112.7, 69.6, 59.4, 52.6, 50.5, 39.7, 15.9. *LC–ESI-MS characterization of Fmoc-L-Tyr(T)-OH* (*3*). Method: 15% (5 min) to 95% B′ in A′ over 15 min; t_R = 13.33 min. ESI-MS (Fig. 3) *m*/*z*: 609.35 (found), 608.68 (expected for $[C_{31}H_{27}N_3O_8 + K]^+$); 593.61 (found), 592.57 (expected for $[C_{31}H_{27}N_3O_8 + Na]^+$); 571.30 (found), 570.59 (expected for $[C_{31}H_{27}N_3O_8 + H]^+$).

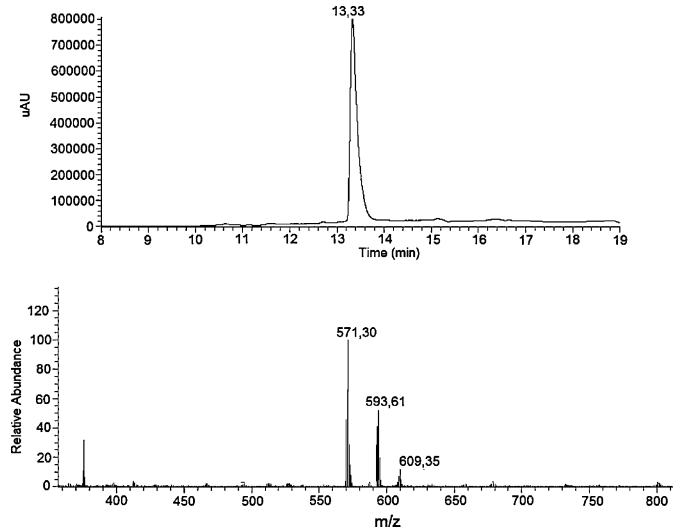
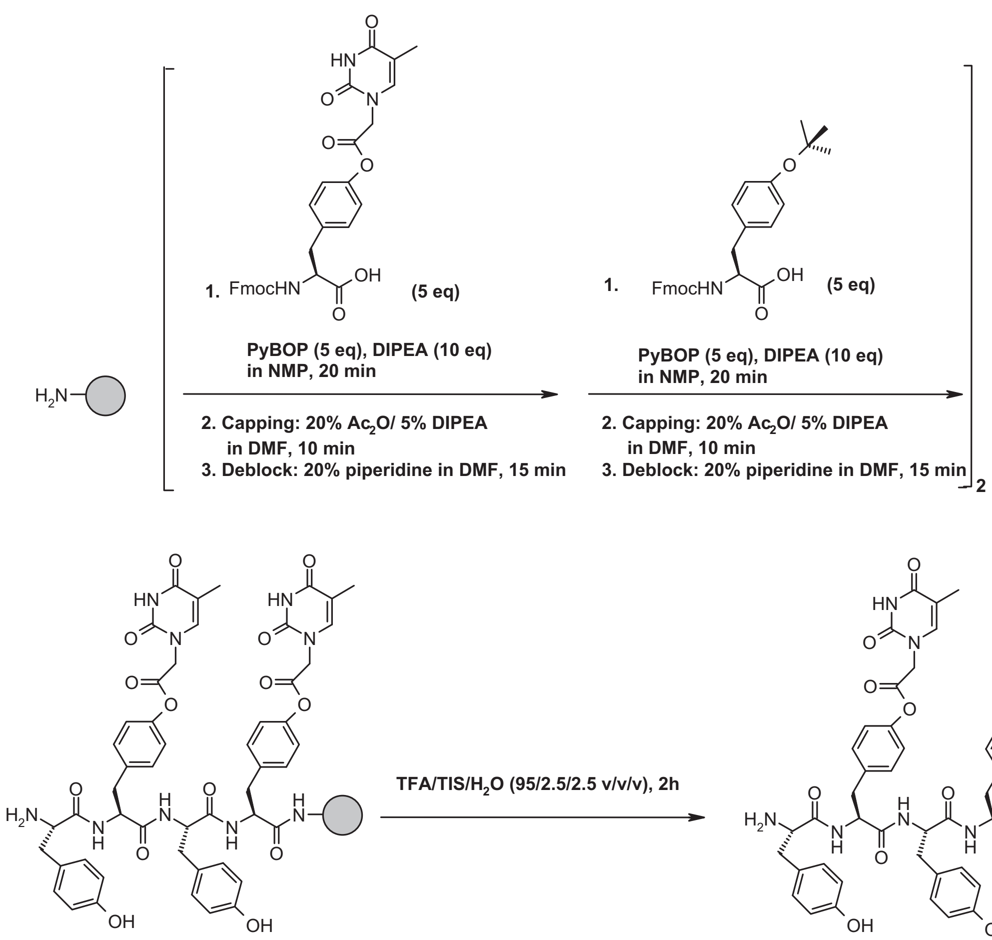


Fig. 3. LC–ESI-MS (positive ions) of Fmoc-L-Tyr(T)-OH (**3**); t_R = 13.33 min; method: 15% (5 min) to 95% B in A over 15 min (A = 0.05% TFA in H_2O, B = 0.05% TFA in CH_3CN).

2.4. Solid phase synthesis of nucleopeptide 4

The tetrapeptide H–[Tyr–Tyr(T)]$_2$–NH_2 (**4**, Fig. 4) was assembled on Rink-amide-NH_2 resin (0.5 mmol/g, 20 mg, 10 μmol) by alternatively coupling Fmoc-L-Tyr(T)-OH (0.2 M in NMP, 5 equiv., 250 μl) and the commercial Fmoc-L-Tyr(*t*Bu)-OH (5 equiv., 23 mg), and using PyBOP (5 equiv., 26 mg)/DIPEA (10 equiv., 17 μl) as activating system in NMP (about 400 μl for 20 min). Capping was performed with 20% $(Ac)_2O$/5% DIPEA for 10 min, while Fmoc deprotection of the amino groups was obtained with 20% piperidine in DMF for 15 min. Subsequently, oligomer **4** was detached from the resin by TFA/TIS/H_2O (95:2.5:2.5) treatment (2 h) and



4

Fig. 4. Solid phase synthesis of the nucleo-tetrapeptide **4**.

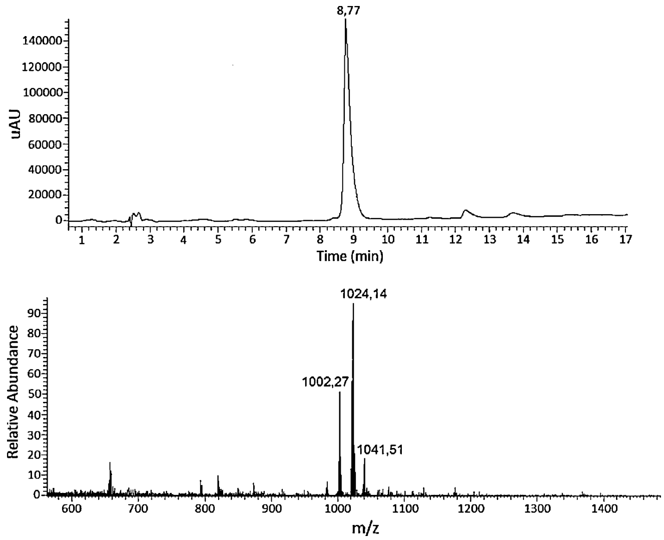


Fig. 5. LC–ESI-MS (positive ions) of nucleopeptide **4**; t_R = 8.77 min; method: 15% (5 min) to 95% B in A over 15 min (A = 0.05% TFA in H_2O, B = 0.05% TFA in CH_3CN).

recovered by precipitation with cold diethyl ether, centrifugation and lyophilization. The crude material was purified by semipreparative HPLC on a C_{18} column using a linear gradient of 25% (for 5 min) to 70% B′ in A′ over 25 min: t_R = 12.3 min; The purified oligomer **4** was dissolved in MilliQ water and quantified by UV measurements (T = 80 °C, absorbance value at λ = 260 nm). The epsilon value used for the quantification of the oligomer (17.2 m M^{-1}) was calculated using the molar extinction coefficient of thymine PNA monomer (8.6 m M^{-1}); UV quantification of the purified product gave 3.2 μmol of **1** (3.2 mg; 32% yield). *LC–ESI-MS characterization of nucleopeptide 4*. Method: 15% (5 min) to 95% B′ in A′ over 15 min; t_R = 8.77 min. ESI-MS (Fig. 5) *m*/*z*: 1041.51 (found), 1041.12 (expected for $[C_{50}H_{51}N_9O_{14} + K]^+$); 1024.14 (found), 1025.01 (expected for $[C_{50}H_{51}N_9O_{14} + Na]^+$); 1002.27 (found), 1003.03 (expected for $[C_{50}H_{51}N_9O_{14} + H]^+$).

3. Results and discussion

3.1. Synthesis of the Fmoc-protected monomer 3

We realized a convenient and fast synthetic route to a chiral Fmoc nucleo-L-tyrosine monomer, in which the (S)-2-amino-3-(4′-hydroxyphenyl)-propanoic moiety was connected to the DNA nucleobase by an ester bond, suitable for the solid phase synthesis of aromatic nucleopeptides. The nucleobase-containing monomer was synthesized starting from the commercially available Fmoc-L-Tyr(*t*Bu)-OH (**1**, Fig. 2). In the first synthetic step the *tert*-butyl group was selectively removed with trifluoroacetic acid to give the intermediate **2** with the free phenolic hydroxyl group (Fig. 2).

Subsequently, Fmoc-L-Tyr-OH **2** was coupled with the commercially available nucleobase acetic acid under different synthetic conditions with the best results coming from the use of DIPC/DMAP in DMF as solvent in the presence of TMP (Fig. 2). After RP-HPLC purification the desired product **3** was obtained in 34% yield and characterized by $^{13}C/^{1}H$ NMR and ESI-MS (Fig. 3).

3.2. Synthesis of the aromatic nucleopeptide 4

Subsequently, the nucleo-tetrapeptide **4** was assembled in solid phase using a synthetic strategy employing the coupling of both monomer **3** and the commercial Fmoc-L-Tyr(*t*Bu)-OH achieved by using PyBop/DIPEA as activating system (Fig. 4). Nucleopeptide was purified by RP HPLC, quantified by UV and characterized by ESI-MS (positive ions) which confirmed the identity of the oligomer. The nucleopeptide showed a good water solubility and a good chemical stability in the aqueous solutions.

4. Conclusion

In conclusion, a Fmoc-protected thymine nucleoamino acid (**3**, Fig. 2), useful for the solid phase assembly of aromatic nucleopeptides, was prepared for the first time by a convenient synthetic route and fully characterized by NMR and ESI-MS. Furthermore, we realized by a synthetic strategy employing the novel Fmoc-protected nucleoamino acid, a dithymine tetrapeptide (**4**, Fig. 4), made of both thymine-containing and unfunctionalized L-tyrosine units alternated in the sequence. The synthetic procedure designed and realized in this work is flexible and should allow for the introduction of the other three protected nucleobase acetic acids. Moreover, future efforts will be directed towards the study of the properties of both the nucleoamino acid and the nucleopeptide here described in analogy to the reports on single-nucleobase-bearing and nucleopeptide-based networks (Snip et al., 2002; Moccia et al., 2009; Roviello et al., 2011a,b) in view of their employment for the realization of supramolecular structures beneficial in the biomedical research as drug and gene delivery tools.

Acknowledgments

We thank Dr. Mariangela Castiglione, Dr. Annalisa Cesarani and Dr. Valentina Roviello for their precious suggestions, and Dr. Giuseppe Perretta and Mr. Leopoldo Zona for their invaluable technical assistance. We are also grateful to the institutions that supported our laboratory (*Consiglio Nazionale delle Ricerche and Università degli Studi di Napoli 'Federico II'*).

References

Bell, N.M., Micklefield, J., 2009. Chemical modification of oligonucleotides for therapeutic, bioanalytical and other applications. Chembiochem 17, 2691–2703.

Chhabra, R., Sharma, J., Liu, Y., Rinker, S., Yan, H., 2010. DNA self-assembly for nanomedicine. Adv. Drug Deliv. Rev. 62, 617–625.

Diederichsen, U., 1996. Pairing properties of alanyl peptide nucleic acids containing an amino acid backbone with alternating configuration. Angew. Chem. Int. Ed. 35, 445–448.

Dragulescu-Andrasi, A., Rapireddy, S., He, G., Bhattacharya, B., Hyldig-Nielsen, J.J., Zon, G., Ly, D.H., 2006. Cell-permeable peptide nucleic acid designed to bind to the 5′-untranslated region of E-cadherin transcript induces potent and sequence-specific antisense effects. J. Am. Chem. Soc. 128, 16104–16112.

Efimov, V.A., Buryakova, A.A., Choob, M.V., Chakhmakhcheva, O.G., 1999. Polyester and N-methyl analogues of peptide nucleic acids: synthesis and hybridization properties. Nucleosides Nucleotides 18, 2533–2549.

Fader, L.D., Tsantrizos, Y.S., 2002. Hybridization properties of aromatic peptide nucleic acids: a novel class of oligonucleotide analogues. Org. Lett. 10, 63–66.

Geotti-Bianchini, P., Beyrath, J., Chaloin, O., Formaggio, F., Bianco, A., 2008. Design and synthesis of intrinsically cell-penetrating nucleopeptides. Org. Biomol. Chem. 6, 3661–3663.

Katritzky, A.R., Narindoshvili, T., 2008. Chiral peptide nucleic acid monomers (PNAM) with modified backbones. Org. Biomol. Chem. 6, 3171–3176.

Moccia, M., Musumeci, D., Roviello, G.N., Fusco, S., Valente, M., Bucci, E.M., Sapio, R., Pedone, C., Netti, P.A., 2009. Preliminary studies on noncovalent hyperbranched polymers based on PNA and DNA building blocks. J. Pept. Sci. 15, 647–653.

Murata, A., Wada, T., 2006. Synthesis of a novel ester analog of nucleic acids bearing a serine backbone. Bioorg. Med. Chem. Lett. 16, 2933–2936.

Roviello, G.N., Gröschel, S., Pedone, C., Diederichsen, U., 2009. Synthesis of novel MMT/acyl-protected nucleo alanine monomers for the preparation of DNA/alanyl-PNA chimeras. Amino Acids 38, 1301–1309.

Roviello, G.N., Musumeci, D., De Cristofaro, A., Capasso, D., Di Gaetano, S., Bucci, E.M., Pedone, C., 2010a. Alternate dab-aegPNAs: synthesis, nucleic acid binding studies and biological activity. Mol. Biosyst. 6, 189–195.

Roviello, G.N., Crescenzo, C., Capasso, D., Di Gaetano, S., Franco, S., Bucci, E.M., Pedone, C., 2010b. Synthesis of a novel fmoc-protected nucleoaminoacid for the solid phase assembly of 4-piperidyl glycine/L-arginine-containing nucleopeptides and preliminary RNA interaction studies. Amino Acids 39, 795–800.

Roviello, G.N., Benedetti, E., Pedone, C., Bucci, E.M., 2010c. Nucleobase-containing peptides: an overview of their characteristic features and applications. Amino Acids 39, 45–57.
Roviello, G.N., Ricci, A., Bucci, E.M., Pedone, C., 2011a. Synthesis, biological evaluation and supramolecular assembly of novel analogues of peptidyl nucleosides. Mol. Biosyst. 7, 1773–1778.
Roviello, G.N., Musumeci, D., Bucci, E.M., Pedone, C., 2011b. Evidences for supramolecular organization of nucleopeptides: synthesis, spectroscopic and biological studies of a novel dithymine L-serine tetrapeptide. Mol. BioSyst. 7, 1073–1080.
Sawa, N., Wada, T., Inoue, Y., 2010. Synthesis and DNA-recognition behavior of a novel peptide ribonucleic acid with a serine backbone (oxa-PRNA). Tetrahedron 66, 344–349.
Shuai, X., Merdan, T., Unger, F., Kissel, T., 2005. Supramolecular gene delivery vectors showing enhanced transgene expression and good biocompatibility. Bioconjug. Chem. 16, 322–329.
Snip, E., Koumoto, K., Shinkai, S., 2002. Gel formation properties of a uracil-appended cholesterol gelator and cooperative effects of the complementary nucleobases. Tetrahedron 58, 8863–8873.
Wada, T., Minamimoto, N., Inaki, Y., Inoue Y, 2000. Peptide ribonucleic acids (PRNA) 2. A novel strategy for active control of DNA recognition through borate ester formation. J. Am. Chem. Soc. 122, 6900–6910.
Yamazaki, T., Komatsu, K., Umemiya, H., Hashimoto, Y., Shudo, K., Kagechika, H., 1997. Dinucleotide-analogous tetrapeptides. Specific triplex formation with complementary polynucleotides. Tetrahedron Lett. 38, 8363–8366.