Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 14 (2004) 4435–4438
Bioorganic & Medicinal Chemistry Letters

Synthesis and cellular uptake of a fluorescently labeled cyclic PNA-based compound

Sergio Caldarelli,[a] Geoffrey Depecker,[a] Nadia Patino,[a] Audrey Di Giorgio,[a] Thibault Barouillet,[b] Alain Doglio[b] and Roger Condom[a,*]

[a]*Laboratoire de Chimie Bioorganique UMR-CNRS 6001, Université de Nice-Sophia Antipolis, Parc Valrose, 06108 Nice Cédex 2, France*
[b]*Laboratoire de Virologie, INSERM U526, Faculté de Médecine, Avenue de Valombrose, 06107 Nice Cédex, France*

Received 30 April 2004; revised 1 June 2004; accepted 17 June 2004

Abstract—A cyclic hexameric PNA-based compound labeled with fluorescein has been prepared following the liquid phase FPB strategy. Its cellular uptake, without and with electroporation, has been investigated by fluorescence microscopy.

1. Introduction

Single-stranded RNAs adopt frequently folded hairpin structures, which contain apical loops often involved in biological processes through specific interactions with proteins or complementary RNA sequences. Several examples of interactions between two natural or synthetic RNA loops have been reported in literature.[1–6] These interactions forming very stable 'kissing-loop' complexes resulted from complementary base pairing of the residues of the two loops.

In previous papers, some of us reported the elaboration of cyclic PNA (polyamide nucleic acids)-based compounds, which target HIV RNA hairpins involved in crucial steps of its replication.[7–10] The objectives were to establish whether HIV replication can be efficiently inhibited through kissing-loop interactions. Although one of these compounds was shown in vitro to interfere with the dimerization process of the HIV-1 genome, it displayed no anti-HIV activity in infected cells,[8] probably as a result of a poor cellular uptake. To confirm or infirm this hypothesis, it was necessary to investigate the cellular uptake of such cyclic PNA-based compounds. Aiming at this goal, we synthesized the fluorescent-labeled loop **2** (Fig. 1), a close analog of the previously reported cyclic hexameric 5′-UCUCCU-3′ PNA sequence **1**,[7] which can be taken as being representative of such types of molecules. The labeled loop **2** contains a lysine residue into the linker onto which a fluorescein moiety has been connected.

In this paper, we report on the liquid-phase synthesis of the fluorescein-labeled PNA **2** following the fully protected backbone (FPB) approach,[11] as well as on its cellular uptake, which was investigated using fluorescence microscopy.

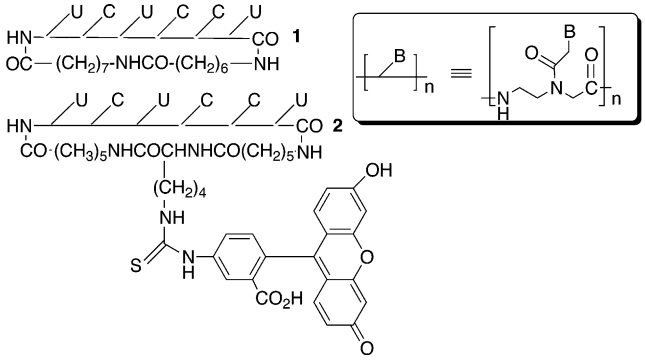


Figure 1. Synthetic loops structures.

Keywords: Cyclic PNA; Cellular uptake; Fluorescence microscopy.
* Corresponding author. Tel.: +33-(0)4-9207-6152; fax: +33-(0)4-9207-6151; e-mail: condom@unice.fr

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.06.055

2. Chemistry

The FPB methodology developed in our laboratory is a liquid-phase procedure that allows to prepare PNA from fully amino-protected (aminoethylglycinamide) polymers such as **3**–**6** (Fig. 2).[7–11] The retrosynthetic pathway to compounds **1** and **2**, which contain two different kinds of nucleic bases (U and C), is illustrated in Figure 2. These compounds result from their respective cyclic precursors **3** and **4** in which the six secondary amine groups of the backbone are protected by two kinds of orthogonal P_A and P_B groups. These cyclic precursors stem, respectively, from linear backbones **5** and **6** whose N and C-extremities are protected by P_1 and P_2 groups, and from the corresponding linkers **7** and **8**.

The synthetic procedure to compound **2** was adapted from the procedure applied to the synthesis of compound **1**. This derivative was previously constructed by using the set of protecting groups: Boc (P_1), methyl (P_2), Troc (P_A) and Alloc (P_B) (Fig. 2).[7,13] However, the cadmium-promoted Troc cleavage required a tedious purification step to remove the cadmium salts. These drawbacks led us to develop an alternative strategy for the synthesis of compound **2**, in which the Boc group replaces the Troc one as P_A. Consequently, we selected the Mmt group as P_1, because its cleavage can be achieved without affecting the other protecting groups (Boc, Alloc, Me) present in the protected hexameric key synthon **6**. The synthesis of **6** was performed via a divergent approach using the monomer protected 2-aminoethylglycine building blocks **9**–**12** as starting materials[8] (Scheme 1). Coupling the acid monomers **9** and **11** with the amine components **10** and **12**, respectively, afforded, after N- and C-deprotections, acid **13** and amine **14** dimers, respectively. Condensation of **13** with **14** led, after N-deprotection, to tetramer **15**, which was conjugated to acid dimer **13** to afford the desired hexamer **6**. Mmt deprotection (step c) was performed in mild conditions by means of 2% TFA in CH_2Cl_2. Saponification (step b) was carried out using 1 N LiOH. The condensation steps

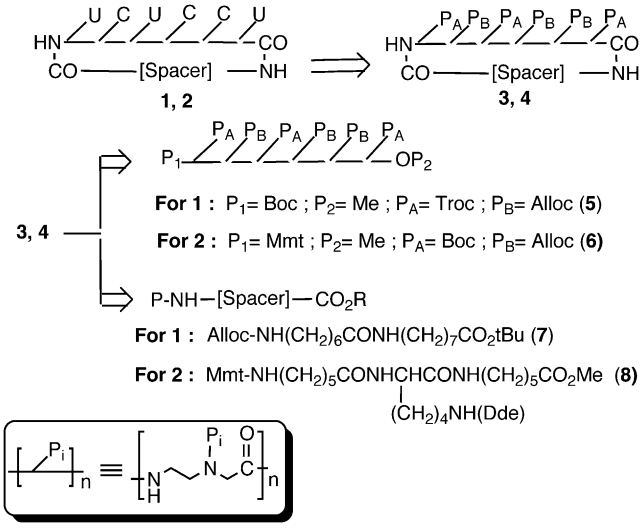
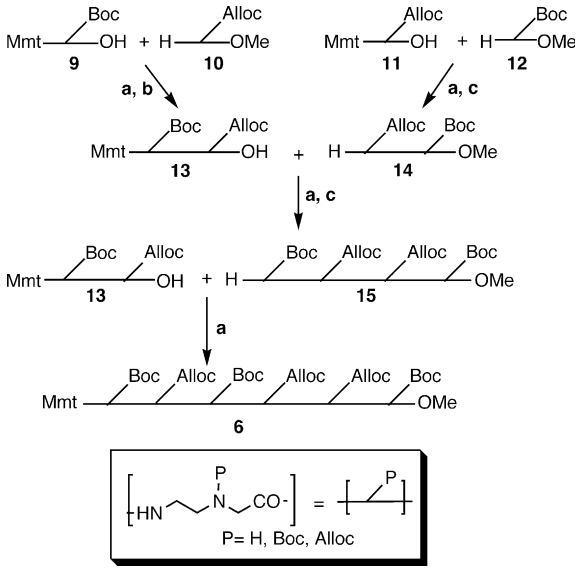


Figure 2. Retrosynthetic route to compounds **1** and **2**.

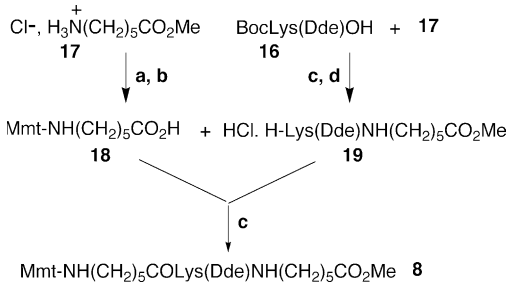


Scheme 1. Reagents and conditions: (a) iBuOCOCl, TEA, CH_2Cl_2 (80%); (b) LiOH (1N), THF (80%); (c) 2% TFA/CH_2Cl_2 (85–90%).

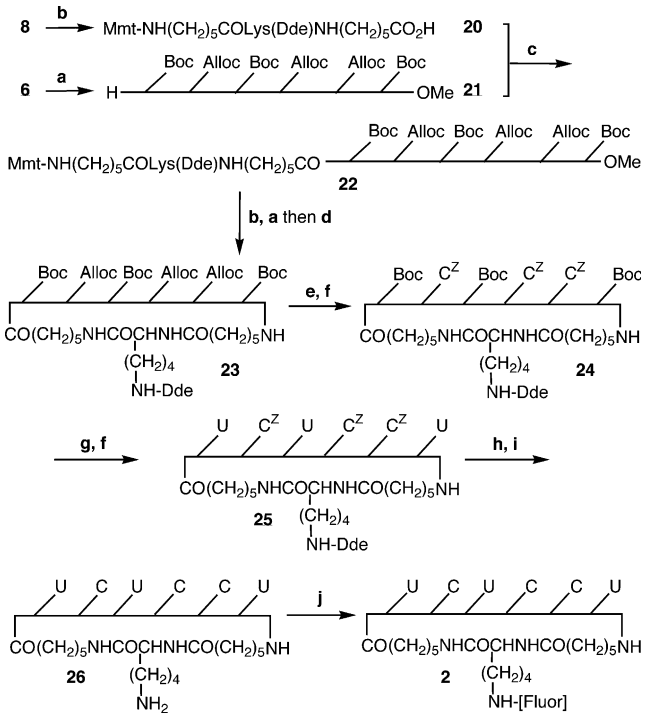
(step a) between the monomers (**9**+**10** and **11**+**12**) or dimers (**13**+**14**), or dimer and tetramer moieties (**13**+**15**) were all performed via chloroformate activation (iBuOCOCl). All the reactions described in Scheme 1 occurred in high yields (from 80% to almost quantitative).

Concerning the protected lysine-based linker **8**, the ε-amine function of lysine was masked with the Dde protecting group (Fig. 2), which was selected for its stability towards Mmt, Boc and Alloc cleavage conditions. Compound **8** was constructed starting from Boc-Lys(Dde)OH **16** and from aminocaproic methyl ester **17** (Scheme 2). A series of classical protection, condensation and deprotection steps gave acid **18** and amine **19**, which were subsequently condensed to afford the desired protected linker **8** in good overall yield (60%).

The last stages of the synthesis of **2**, starting from hexa-PNA **6** and linker **8** are described in Scheme 3. These two reagents were first deprotected into the acid linker **20** and amine PNA moiety **21** in 75% yield. It should be mentioned that saponification of **8** was cleanly achieved with LiOH in presence of Ca^{2+} ions, which circumvented the OH^- catalyzed Dde cleavage. Conden-



Scheme 2. Reagents and conditions: (a) Mmt-Cl, TEA, CH_2Cl_2 (100%); (b) 1N LiOH, THF (75%); (c) Bop, DIPEA, DMF (80–85%); (d) AcCl, MeOH (93%).



Scheme 3. Reagents and conditions: (a) 2% TFA/CH_2Cl_2 (75–87%); (b) 1 N LiOH, $CaCl_2$ (0.8 M)/iPrOH/H_2O (7/3, v/v) (75%); (c) Bop, DIPEA, CH_2Cl_2, (82%); (d) HATU, HOAt, DIPEA, DMF (62%); (e) $Pd[PPh_3]_4$, DEA, TFA/CH_2Cl_2 (99%); (f) $C^ZCH_2CO_2H$ or U-CH_2CO_2H, HATU, HOAt, DIPEA, DMF (63% and 86%); (g) TFA/$CHCl_3$ (99%); (h) 1% N_2H_4/DMF (98%); (i) 1/3 TFMSA/TFA (90%); (j) FITC, DIPEA, DMF (30% after HPLC purification).

sation of the acid linker **20** with **21** by means of Bop reagent afforded the linear precursor **22** in 82% yield. Saponification of **22** with a LiOH/Ca^{2+} mixture, followed by Mmt-deprotection with a 2% TFA/CH_2Cl_2 solution, then head-to-tail cyclization via a HATU/HOAt activation and semi-high dilution conditions (10 mM) afforded the cyclic protected precursor **23** (respectively in 75%, 87% and 62% yields).

Selective cleavage of the three Alloc protecting groups by means of $Pd(PPh_3)_4$/DEA, then condensation of N-Z-cytosine acetic acid units onto the amine function thus generated, via a HATU/HOAt activation, gave derivative **24** in 63% overall yield. Boc-deprotection (quantitative), then HATU-mediated condensation of three uracil acetic acid units afforded compound **25** (86% yield). The Dde-deprotection of the lysine side chain using a freshly prepared 1% hydrazine/DMF solution, and Z-deprotection of the cytosine residues by means of a 1/3 TFMSA/TFA solution led to **26** (88% for the two steps). The target fluorescein-labeled PNA **2** was then obtained by condensing fluorescein isothiocyanate on the ε-amine of the lysine residue of **26**. Compound **2** was isolated in 30% yield after semi preparative HPLC. Its purity was demonstrated by HPLC analyses and its structure confirmed by MS experiments.[12]

3. Cellular uptake study

The cellular uptake of the fluorescein-labeled PNA compound **2** was examined by fluorescence microscopy after incubating this derivative (1 mM) with adherent HeLa cells and with suspension Jurkat cells at 37 °C during 48 h.[14] Under these conditions, no fluorescently labeled cells were seen, indicating likely that no cellular uptake has occurred. To further confirm that the cyclic PNA **2** cannot cross cell membranes, cell penetration of **2** into suspension Jurkat cells was examined after electroporation, which is known to permeabilize cell and nuclear membranes. Cells (10^5) were suspended in a PNA-containing RPMI solution (40 μM) and placed into a gap cuvette. Electroporation was performed with a single electric shock and the system was further incubated for 36 h. Fluorescence microscopy (Fig. 3) shows a homogeneous distribution of PNA **2** within the cell. Apparently, there is no preferred nuclear versus cytosolic localization. Furthermore, it is noteworthy that once inside the cells after electroporation, **2** was not toxic at the tested concentration.

4. Conclusion

The synthesis of a fluorescein-labeled cyclic PNA has been successfully performed following a liquid-phase FPB methodology. This labeling has been performed onto the lysine residue that is present in the linker moiety of the cyclic PNA. It allowed us to demonstrate that such cyclic PNAs do not enter cells. Conjugation of the cyclic PNA lysine residue to cell-permeant peptides[15] or to cationic lipids[16] in view of improving its cell delivery is currently under investigation.

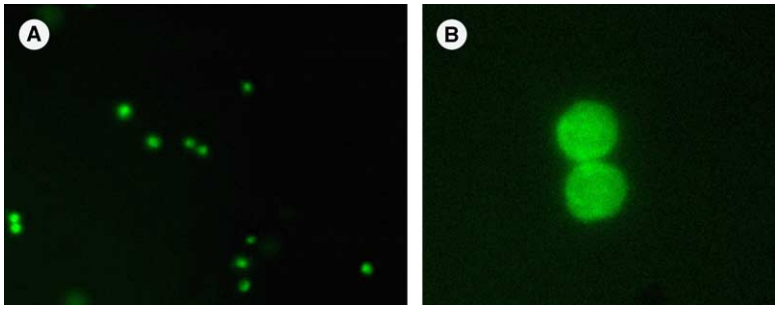


Figure 3. Fluorescence of **2** in Jurkat cells in vitro (**A**: ×40; **B**: ×100).

References and notes

1. Gatignol, A. *Gene Expr.* **1996**, *5*, 217–228.
2. Tomizawa, J. I. *J. Mol. Biol.* **1990**, *212*, 695–708.
3. Mujeed, A.; Clever, J. L.; Billeci, T. M.; James, T. L.; Parslow, T. G. *Nat. Struct. Biol.* **1998**, *5*, 432–436.
4. Eisinger, J. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 854–861.
5. Comolli, L. R.; Pelton, J. G.; Tinoco, I. *Nucleic Acids Res.* **1998**, *26*, 4688–4695.
6. Brunel, C.; Marquet, R.; Romby, P.; Ehresmann, C. *Biochimie* **2002**, *84*, 925–944.
7. Depecker, G.; Schwergold, C.; Di Giorgio, C.; Patino, N.; Condom, R. *Tetrahedron Lett.* **2001**, *42*, 8303–8306.
8. Schwergold, C.; Depecker, G.; Di Giorgio, C.; Patino, N.; Jossinet, F.; Ehresmann, B.; Terreux, R.; Cabrol-Bass, D.; Condom, R. *Tetrahedron* **2002**, *58*, 5675–5687.
9. Di Giorgio, C.; Depecker, G.; Schwergold, C.; Leroux, M. L.; Caldarelli, S.; Farese Di Giorgio, A.; Patino, N.; Condom, R. *Recent Res. Dev. Nucleosides Nucleotides* **2003**, *1*, 81–95.
10. Depecker, G.; Patino, N.; Di Giorgio, C.; Terreux, R.; Cabrol-Bass, D.; Bailly, C.; Aubertin, A. M.; Condom, R. *Org. Biomol. Chem.* **2004**, *2*, 74–79.
11. Di Giorgio, C.; Pairot, S.; Schwergold, C.; Patino, N.; Condom, R.; Farese Di Giorgio, A.; Guedj, R. *Tetrahedron* **1999**, *55*, 1937–1958.
12. Compound **2** analyses: MS: $C_{99}H_{120}N_{32}O_{29}S$; ESI(+): 1127.32 (M + 2H/2); HPLC: column (250*4 mm) packed with Lichrospher 100-RP-18 (5 μm), (H_2O 0.1%TFA/CH_3CN 0.1%TFA) from 80/20 to 0/100 in 30 min, with a 1 mL/min flow, $t_r = 10.6$ min, $\lambda_{max1} = 212.8$ nm, $\lambda_{max2} = 271.0$ nm.
13. *Abbreviations*: Alloc: allyloxycarbonyl; Boc: tertiobutyloxycarbonyl; DEA: diethylamine; DIPEA: diisopropylethylamine; DMF: dimethylformamide; FITC: fluorescein isothiocyanate; HATU: *O*-(7-aza-1-benzotriazolyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexa-fluorophosphate; HOAt: 1-hydroxy-7-azabenzotriazole; Mmt: monomethoxytrityl; TFA: trifluoroacetic acid; TFMSA: trifluoromethane sulfonic acid; Troc: trichloroethyloxycarbonyl.
14. Fluorescence microscopy studies were performed with a Zeiss Axiovert 200 microscope with shutter-controlled illumination and a cooled, digital CCD camera (Roper Scientific) using a 100× lens.
15. Koppelhus, U.; Nielsen, P. E. *Adv Drug Deliv. Rev.* **2003**, *55*, 267–280.
16. Filipovska, A.; Eccles, M. R.; Smith, R. A.; Murphy, M. P. *FEBS Lett.* **2004**, *556*, 180–186.