Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 17 (2007) 6576–6578
Bioorganic & Medicinal Chemistry Letters

Synthesis of phosphorothioate oligonucleotide–peptide conjugates by solid phase fragment condensation

Irmina Diala,[a] Akira Osada,[a] Shinji Maruoka,[a] Takashi Imanisi,[a] Satoshi Murao,[a] Taishi Ato,[b] Hideki Ohba[b,c] and Masayuki Fujii[a,*]

[a]*School of Humanity Oriented Science and Technology, Kinki University, 11-6 Kayanomori, Iizuka, Fukuoka 820-8555, Japan*
[b]*Carefor Inc. 807-1 Shuku-machi, Tosu, Saga 841-0052, Japan*
[c]*National Institute for Advanced Industrial Science and Technology (AIST) Kyushu, 807-1 Shuku-machi, Tosu, Saga 841-0052, Japan*

Received 25 June 2007; revised 11 September 2007; accepted 21 September 2007
Available online 5 October 2007

Abstract—Phosphorothioate oligonucleotide–peptide conjugates were synthesized by solid phase fragment condensation (SPFC). Arginine rich peptides could be successfully conjugated in 2.8–13.4% isolated yields. All the products were fully characterized by reversed phase HPLC and MALDI–TOF–MS to give satisfactory results.

Phosphorothioate oligonucleotides are useful tools for genetic analyses and antisense genetic medicines.[1–5] Advantages of phosphorothioate oligonucleotides as antisense drugs include an increased resistance against intracellular nucleases and a restored ability of RNase H activation. On the other hand, disadvantages of them include a decreased binding affinity with a target sequence of mRNA because of an increased electrostatic repulsion between phosphorothioate backbone and phosphate linkage of mRNA. It has been also pointed out that phosphorothioate oligonucleotides might interact with intracellular proteins and other organs in a non-selective manner, which often causes a reduction of silencing efficiency and unexpected side effects.

Therefore, further chemical modifications and/or conjugations with other functional molecules on phosphorothioate oligonucleotides are required for the application to genetic medicines. From a synthetic point of view, phosphorothioate oligonucleotides have some difficulties when they are conjugated with cationic peptides or cellular membrane penetrating peptides. Using a conventional synthetic method of conjugates of oligonucleotides and peptides, thiol groups on peptides are often reacted with a maleimide group or an activated disulfide group linked to oligonucleotides.[6,7] Sulfur atom in phosphorothioate group, however, is nucleophilic enough to react with a maleimide or an activated disulfide group to give complicated products. Therefore, this common synthetic method of conjugation using thiol groups on peptides should be avoided in the case of phosphorothioate oligonucleotides, and only other few methods can be used for this purpose.[8–12] Moreover, conjugation of phosphorothioate oligonucleotides with highly cationic peptides is often interfered by an electrostatic interaction of anionic phosphorothioate backbone and cationic peptide side chains. These problems inhibit further innovation of antisense technology using phosphorothioate oligonucleotides in spite of their highly attractive properties as antisense reagents. In this paper, a facile and conventional synthesis of conjugates of phosphorothioate oligonucleotides and a variety of peptides is achieved.

Solid phase synthesis of peptide fragments: Peptides were prepared by a standard fmoc-chemistry using 100 mg of Wang resin (novabiochem, 100–200 mesh, 0.50–1.30 mmol/g). Each peptide is modified with lysine, which is initially protected by *tert*-butyloxycarbonyl (boc) group and finally deprotected after the cleavage, at the second terminal position of carboxyl terminus to put a free reactive amino group on peptides. The obtained peptide fragments were fully characterized by RP-HPLC and MALDI–TOF–MS to give satisfactory

Keywords: Phosphorothioate oligonucleotide–peptide conjugates; Solid phase fragment condensation; Synthesis.
* Corresponding author. Tel.: +81 948 22 5655; fax: +81 948 23 0536; e-mail: mfujii@fuk.kindai.ac.jp

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.09.101

Table 1. Synthesized peptides

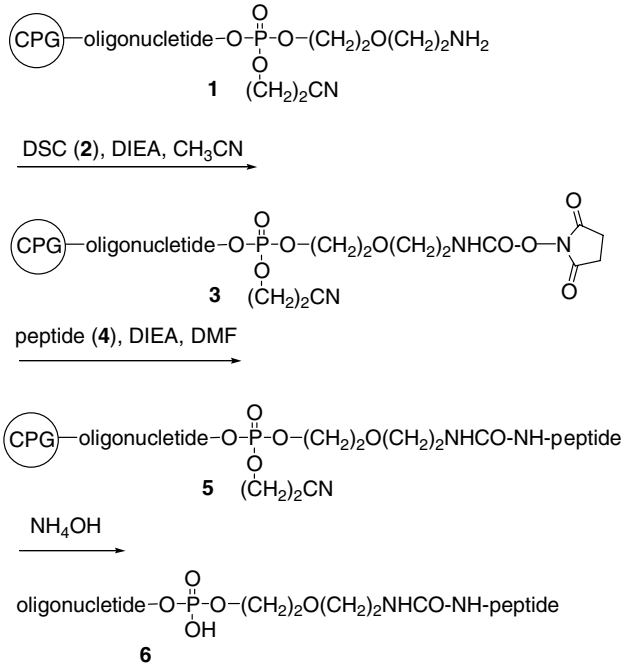
Peptide	Sequence (origin)	Yield[a] (%)	MALDI–TOF–MS (found/calcd)
4a	Ac-GPK̲K̲K̲R̲K̲VKG-OH (SV40LT-ant NLS)	5.6	1552.69/1551.56
4b	Ac-GRK̲K̲RRQRRRPPGKG-OH (HIV-1 tat NLS)	4.3	2196.11/2194.36
4c	Ac-LPPLERLTLKG-OH (HIV-1 rev NES)	11.8	1278.45/1278.59
4d	Ac-LRALLRALLRALLRALKG-OH (designed)	4.1	2063.18/2058.64

K̲; ε-amino group is protected by trifluoroacetyl (tfa) group.
[a] Isolated yields.

results (Table 1). The peptide **4a** is a nuclear localization signal (NLS) sequence derived from SV40 large T antigen,[13] the peptide **4b** is an NLS from HIV-1 tat protein,[14] peptide **4c** is a nuclear export signal (NES) sequence from HIV-1 rev protein[14], and the peptide **4d** is a designed peptide with a repeated LRAL sequence which is supposed to form a cationic α-helix in the presence of dsDNA.[15,16]

Synthesis of s-DNA–peptide conjugates by SPFC: The syntheses of the conjugates involve a solid phase fragment condensation (SPFC)[17] as shown in Scheme 1.

A phosphorothioate oligonucleotide assembled in 1 mmol scale on CPG (PROLIGO, 500 Å, 30–40 μmol/g) support was modified at 5′-end of the oligonucleotides by an amino group using the commercially available phosphoramidite (Glen Research 5′-Amino Modifier 5) (**1**). Next a bifunctional linker molecule, di(*N*-succinimidyl)carbonate (DSC) (**2**), was reacted with the terminal amino group on solid phase, and then a partially protected peptide fragment (**4**) bearing a single free reactive amino group, independently synthesized and purified, was reacted with CPG-linked phosphorothioate oligonucleotides (**3**) to give phosphorothioate oligonucleotide–peptide conjugates still attached to CPG (**5**). In the peptide fragments, ε-amino groups of lysine (K) except



Scheme 1. Synthesis of oligonucleotide–peptide conjugates by SPFC.

Table 2. Phosphorothioate oligonucleotide[a]–peptide conjugates

Conjugate	Peptide	Yield[b] (%)	MALDI–TOF–MS (found/calcd)
6a	**4a**	8.5	5582.24/5581.96
6b	**4b**	2.8	6420.67/6418.85
6c	**4c**	13.4	5695.16/5694.06
6d	**4d**	8.7	6475.67/6475.12

[a] Oligonucleotide sequence 5′-s(CAGTTAGGGTTAG)-3′.
[b] Isolated yields determined on A_{260}.

for a reactive site are protected by trifluoroacetyl (tfa) group, thiols of cysteine (C) and hydroxyls of serine (S) and threonine (T) are protected by acetyl (ac) group, and guanidyl and carboxyl groups are already deprotected before the condensation. Finally, CPG-linked products are treated with concentrated aqueous ammonia at 55 °C for 4 h to give fully deprotected product. Reversed phase HPLC[18] purification gives a single peak pure products in 3–13% overall yields in >95% purities (determined on RP-HPLC analyses) and all the products are fully characterized by MALDI–TOF–MS[19] to give satisfactory results (Table 2). The phosphorothioate oligonucleotide used in this study has an antisense sequence against the RNA template in human telomerase (hTR). All the peptides include lysine and/or arginine. Especially, although peptides **4a** and **4b** are highly cationic, coupling reactions were not inhibited at all. Therefore, this study proved that SPFC can be widely used for the conjugation of phosphorothioate oligonucleotides and cationic peptides.

Study on intracellular delivery and antisense inhibition of human telomerase by these phosphorothioate oligonucleotide–peptide conjugates is now in progress in our laboratory and will be reported somewhere shortly.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.09.101.

References and notes

1. Crooke, S. T. *Annu. Rev. Med.* **2004**, *55*, 61.
2. Crooke, S. T. *Antisense & Nucl. Acid Drug Develop.* **1998**, *8*, 115.
3. Kurreck, J. *Eur. J. Biochem.* **2003**, *270*, 1628.
4. Wilson, C.; Keefe, A. D. *Curr. Opin. Chem. Biol.* **2006**, *10*, 607.
5. Katesan, N.; Kim, B. H. *Chem. Rev.* **2006**, *106*, 3712.
6. Eritja, R.; Pons, A.; Escarceller, M.; Giralt, E.; Albericio, F. *Tetrahedron* **1991**, *47*, 4113.

7. Chen, C.-P.; Zhang, L.; Peng, Y.-F.; Wang, X.-B.; Wang, S.-Q.; Zhang, L.-H. *Bioconjug. Chem.* **2003**, *14*, 532.
8. Antopolsky, M.; Azhayeva, E.; Tengvall, U.; Azhayev, A. *Tetrahedron Lett.* **2002**, *43*, 527.
9. Antopolsky, M.; Azhayev, A. *Tetrahedron Lett.* **2000**, *41*, 9113.
10. Antopolsky, M.; Azhayev, A. *Helv. Chim. Acta* **1999**, *82*, 2130.
11. Basu, S.; Wickstrom, E. *Tetrahedron Lett.* **1995**, *36*, 4943.
12. Aubert, Y.; Bourgerie, S.; Meunier, L.; Mayer, R.; Roche, A. C.; Monsigny, M.; Thoung, N. T.; Asseline, U. *Nucleic Acids Res.* **2000**, *28*, 818.
13. Goldfarb, D. S.; Gariepy, J.; Schoolnik, G.; Komberg, R. D. *Nature* **1986**, *322*, 641.
14. Dingwell, C.; Laskey, R. A. *Annu. Rev. Cell Biol.* **1986**, *2*, 367.
15. Fujii, M.; Yokoyama, K.; Kubo, K.; Ueki, R.; Abe, S.; Goto, K.; Niidome, T.; Aoyagi, H.; Iwakuma, K.; Ando, S.; Ono, S. *Peptide Sci. 2000* **2001**, 109.
16. Kubo, T.; Fujii, M. *Nucleosides, Nucleotides Nucleic Acids* **2001**, *20*, 1313.
17. Kubo, T.; Morikawa, M.; Ohba, H.; Fujii, M. *Org. Lett.* **2003**, *5*, 2623.
18. RPHPLC conditions; ODS 4×125 mm, 1 ml/min, A: 0.1% TFA in H_2O, pH 2.5, B: CH_3CN, a linear gradient of B from 10% to 100% in 60 min.
19. MALDI–TOF–MS conditions; Voyager DE, PE Biosystems, matrix: saturated α-cyano-4-hydroxycinnamic acid in 50% CH_3CN/H_2O containing 1% TFA.