JOC*Note*

New Method To Prepare Peptide−Oligonucleotide Conjugates through Glyoxylic Oxime Formation

Yashveer Singh, Eric Defrancq,* and Pascal Dumy

LEDSS, UMR CNRS 5616, ICMG FR2607, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France

Eric.Defrancq@ujf-grenoble.fr

Received August 24, 2004

Abstract: A new method to prepare peptide−oligonucleotide conjugates through chemoselective glyoxylic oxime linkage is reported. A novel phosphoramidite reagent, readily accessible from serine, was prepared and used in automated DNA synthesis to prepare oligonucleotides carrying a glyoxylic aldehyde functionality at the 5′ terminus. This was efficiently coupled to a peptide functionalized with an aminooxy group. The method could be of general use to prepare a broad range of oligonucleotide conjugates.

Oligonucleotides and their analogues constitute an important class of therapeutic agents that are being investigated for their use as specific inhibitors of gene expression.[1] The efficacy of oligonucleotides in cell culture is, however, often limited by poor cellular uptake.[2] Certain peptide carriers have been reported to enhance the cell delivery of the oligonucleotides.[3] These peptides have been either used as additives or covalently conjugated to the oligonucleotide to achieve an improved cell delivery.[4] In this context, the synthesis of peptide−oligonucleotide conjugates (POCs) has attracted considerable interest, and various synthetic approaches have been developed for POC synthesis.[4c] The total stepwise solid-phase synthesis that involves complete synthesis of the two fragments on the same or different support[5] as well as fragment coupling on solid support has been proposed.[6] These methods, however, do not find much favor on account of poor compatibility between the peptide and oligonucleotide chemistries. A postassembly conjugation strategy (the fragment-coupling approach) that involves separate synthesis and deprotection of appropriately functionalized peptide and oligonucleotide fragments followed by a coupling reaction in the solution phase is therefore preferred. To date, however, only a limited number of such reactions are known. For instance, formation of a disulfide bond,[7] reaction of cysteine peptide with maleimido oligonucleotide,[8] reaction of bromoacetyl peptide with thiol-functionalized oligonucleotide,[9] and formation of amide bonds[10] have been reported. These established routes still suffer from certain disadvantages.

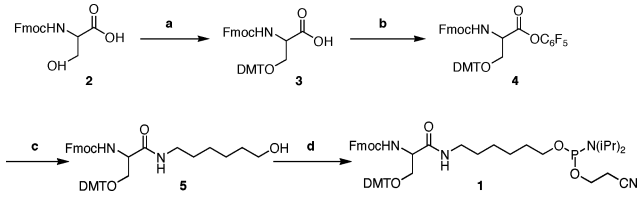
Earlier work from our laboratory[11] and others' laboratories[12] has focused on developing the use of chemoselective oxime, thiazolidine, or hydrazone linkages for efficient preparation of oligonucleotide conjugates. Herein, the oligonucleotides functionalized with an aldehyde group are reacted with the peptides containing nucleophilic groups such as aminooxy, aminothiol, or hydrazine. The conjugation reactions are efficient but suffer from the fact that oligonucleotide aldehyde has poor stability and a known propensity to react with the purine bases.

In a search for an alternative strategy to prepare POCs, we decided to explore the glyoxylic aldehyde functionality. The advantage of using the glyoxylic aldehyde is that it is highly stable to air oxidation and does not react with lysine side chains or α-amino groups during ligation or storage, unlike the oligodeoxynucleotide (ODN) aldehydes. These are extensively used in protein engineering.[13] However, there is only one report on the use of oligonucleotide functionalized with glyoxylic aldehyde for conjugation through a hydrazone linkage.[12c] Unfortunately, no data on the hydrolytic stability has been reported. Herein, we describe a new, convenient, and straightforward method to prepare oligonucleotides functionalized with glyoxylic aldehyde at the 5′ end. The efficiency of the method is illustrated by coupling the ODN-glyoxylic aldehyde to a nuclear localizing signal (NLS) peptide sequence functionalized with an aminooxy function. Also, the hydrolytic stability of the glyoxylic

(1) Crooke, S. T. *Annu. Rev. Med*. **2004**, *55*, 61−95.
(2) Stein, C. A.; Narayan, R. *Perspect. Drug Discovery Des.* **1996**, *4*, 41−50.
(3) Valkna, A.; Soomets, U.; Langel, Ü. In *Cell Penetrating Peptides: Process and Applications*; Langel, Ü., Ed.; CRC Press: Boca Raton, FL, 2002; pp 347−363.
(4) (a) Midoux, P.; Kichler, A.; Boutin, V.; Maurizot, J.-C.; Monsigny, M. *Bioconjugate Chem*. **1998**, *9*, 260−267. (b) Morris, M. C.; Vidal, P.; Chaloin, L.; Heitz, F.; Divita, G. *Nucleic Acids Res*. **1997**, *25*, 2730−2736. (c) Tung, C.-H.; Stein, S. *Bioconjugate Chem*. **2000**, *11*, 605−618.
(5) (a) Stetsenko, D. A.; Gait, M. J. *Org. Lett.* **2002**, *4*, 3259−3262. (b) Antopolsky, M.; Azhayeva, E.; Tengvall, U.; Azhayev, A. *Tetrahedron Lett.* **2002**, *43*, 527−530. (c) Antopolsky, M.; Azhayev, A. *Tetrahedron Lett.* **2000**, *41*, 9113−9117. (d) Soukchareun, S.; Tregear, G. W.; Haralambidis, J. *Bioconjugate Chem.* **1995**, *6*, 43−53.
(6) (a) Kubo, T.; Morikawa, M.; Obha, H.; Fujii, M. *Org. Lett*. **2003**, *5*, 2623−2626. (b) Peyrottes, S.; Mestre, B.; Burlina, F.; Gait, M. J. *Tetrahedron* **1998**, *54*, 12513−12522. (c) Tetzlaff, C. N.; Schwope, I.; Bleczinski, C. F.; Steinberg, J. A.; Richert, C. *Tetrahedron Lett*. **1998**, *39*, 4215−4218.
(7) (a) Vives, E.; Lebleu, B. *Tetrahedron Lett*. **1997**, *38*, 1183−1186. (b) Antopolsky, M.; Azhayeva, E.; Tengvall, U.; Auriola, S.; Jaaskelainen, I.; Ronkko, S.; Honkakoski, P.; Urtti, A.; Lonnberg, H.; Azhayev, A. *Bioconjugate Chem*. **1999**, *10*, 598−606.
(8) (a) Mier, W.; Eritja, R.; Mohammed, A.; Haberkorn, U.; Eisenhut, M. *Bioconjugate Chem*. **2000**, *11*, 855−860. (b) Soukcharen, S.; Haralambidis, J.; Tregear, G. *Bioconjugate Chem.* **1998**, *9*, 466−475. (c) Harrison, J. G.; Balasubramanian, S. *Nucleic Acids Res*. **1998**, *26*, 3136−3145.
(9) Arar, K.; Aubertin, A.-M.; Roche, A.-C.; Monsigny, M.; Mayer, M. *Bioconjugate Chem*. **1995**, *6*, 573−577.
(10) (a) Stetsenko, D. A.; Gait, M. J. *J. Org. Chem*. **2000**, *65*, 4900−4908. (b) McMinn, D. L.; Matray, T. J.; Greenberg, M. M. *J. Org. Chem*. **1997**, *62*, 7074−7075.
(11) (a) Edupuganti, O. P.; Singh, Y.; Defrancq, E.; Dumy, P. *Chem. Eur. J.* **2004**, in press. (b) Edupuganti, O. P.; Renaudet, O.; Defrancq, E.; Dumy, P. *Biorg. Med. Chem. Lett.* **2004**, *14*, 2839−2842. (c) Forget, D.; Boturyn, D.; Defrancq, E.; Lhomme, J.; Dumy, P. *Chem. Eur. J.* **2001**, *7*, 3976−3984.
(12) (a) Zatsepin, T. S.; Stetsenko, D. A.; Arzumanov, A. A.; Romanova, E. A.; Gait, M. J.; Oretskaya, T. S. *Bioconjugate Chem*. **2002**, *13*, 822−830. (b) Cebon, B.; Lambert, J. N.; Leung, D.; Mackie, K.; McCluskey, K. L.; Nguyen, H.; Tassone, C. *Aust. J. Chem*. **2000**, *53*, 333−340. (c) Ollivier, N.; Olivier, C.; Gouyette, C.; Huynh-Dinh, T.; Gras-Masse, H.; Melnyk, O. *Tetrahedron Lett.* **2002**, *43*, 997−999.
(13) (a) Melnyk, O.; Fehrentz, J.-A.; Martinez, J.; Gras-Masse, H. *Biopolymers* **2000**, *55*, 165−186. (b) Shao, J.; Tam, J. P. *J. Am. Chem. Soc*. **1995**, *117*, 3893−3899. (c) Rose, K. *J. Am. Chem. Soc*. **1994**, *116*, 30−33.

10.1021/jo0485177 CCC: $27.50
Published on Web 11/02/2004

SCHEME 1[a]



[a] Reagents and conditions: (a) 4,4′-dimethoxytrityl chloride/pyridine, rt, overnight (**3**, 78%); (b) pentafluorophenol/DCC/CH_2Cl_2, rt, 4 h (**4**, 95%); (c) 6-aminohexanol/NEt(^{i}Pr)$_2$/CH_2Cl_2, rt, 3 h (**5**, 57%); (d) 2-cyanoethyl-*N*,*N*-diisopropylaminochlorophosphoramidite/NEt(^{i}Pr)$_2$/CH_2Cl_2, 0 °C, 1 h, then rt for 2 h (**1**, 65%).

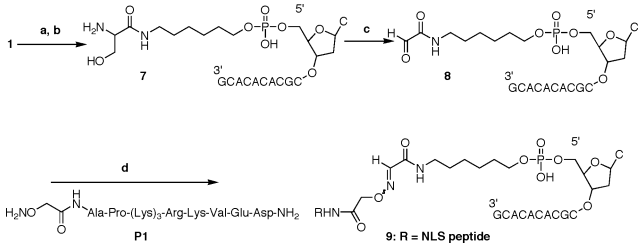
oxime linkage in the conjugate has been investigated in buffer solutions at different pH levels.

To obtain oligonucleotides containing the glyoxylic aldehyde functionality at the 5′ end, a new phosphoramidite reagent **1**, bearing a protected serine residue, was prepared. The use of phosphoramidite **1** was considered because oxidative cleavage of an N-terminal serine residue has been widely applied to produce glyoxylic aldehyde at the N terminus of the peptides. Furthermore, the (fluorenylmethoxy)carbonyl (Fmoc)-protected amino group was preferred because it could be easily removed by applying the conventional conditions used for the deprotection of the nucleobases. The phosphoramidite **1** was prepared from commercially available *N*-α-Fmoc-L-serine **2** in a few simple steps by employing routine chemical procedures (Scheme 1). The free hydroxyl group on the serine was DMT-protected using the described procedure.[14] The appropriately protected serine **3** was converted to pentafluorophenyl ester **4** using pentafluorophenol/DCC. N acylation of **4** with 6-aminohexanol-1 gave the linker-attached serine derivative **5**. Standard phosphitylation of **5** gave phosphoramidite **1** in a 65% yield. Compounds **1**−**5** were characterized by satisfactory NMR and mass spectroscopic data (see the Supporting Information).

The advantage of the phosphoramidite reagent **1** reported herein is that it can be directly incorporated into the growing oligonucleotide chain using the standard DNA synthesis protocol. The usual workup procedures involved in standard synthesis give oligonucleotide sequences bearing a free 1,2-amino alcohol group, a precursor for the aldehyde group. This is contrary to the earlier approach,[12c] where the oligonucleotides were first functionalized with a protected amino group and then further reacted with diacetyl-L-tartaric anhydride after deprotection.

To evaluate the efficiency of the method, undecamer d-5′XCGCACACACGC-3′ (X = **1**) was prepared. The phosphoramidite **1** was coupled in the final step of the automated DNA synthesis following the standard oligonucleotide assembly (Scheme 2). The oligonucleotide chains were cleaved from the support and released into the solution by treatment with ammonia. The deprotection of nucleobases and removal of the Fmoc group were achieved by keeping the ammonia solution at 55 °C for 16 h. Oligonucleotide **6**, incorporating the phosphoramidite **1** with a free amino group, was obtained after

SCHEME 2[a]



[a] Reagents and conditions: (a) automated DNA synthesis incorporating modified phosphoramidite **1** during the last coupling step, then 28% ammonia solution, 55 °C, 16 h; (b) 80% AcOH, rt, 1 h (**7**, 92%); (c) 20 equiv of $NaIO_4$/H_2O, rt, 1 h (**8**, 84%); (d) 2.5 equiv of NLS-ONH_2 **P1**/0.1 M ammonium acetate buffer (pH 4.6), rt, 6−8 h (**9**, 55%).

HPLC purification. Removal of the DMT group was effected by treatment with acetic acid to obtain the oligonucleotide **7** bearing the free 1,2-amino alcohol group. Oxidative cleavage with sodium *m*-periodate in aqueous solution gave the oligonucleotide **8** bearing the glyoxylic aldehyde at the 5′ end. The oxidative cleavage reaction was found to be clean and led to the exclusive formation of the ODN-glyoxylic aldehyde. These aldehydes were found to be stable in storage (−20 °C) for months, consistent with the earlier observation.[12c]

The suitability of ODN-glyoxylic aldehyde for oligonucleotide conjugation was assessed by preparing the peptide conjugate **9**. The peptide **P1** used in the present work incorporates a simian virus 40 antigen-NLS sequence with the basic peptide sequence of APKKKRKV. The ability of this peptide sequence for gene delivery has been investigated.[15] The synthesis of **P1** with a nucleophilic aminooxy group was described earlier.[11c] The conjugation reaction with the NLS-peptide **P1** was carried out in a 0.1 M ammonium acetate buffer at slightly acidic pH (4.6). The acidic pH helps to keep the free amino group in the peptide chain protonated, and this aids in solubilizing the peptide in an aqueous medium. The reaction was monitored by reverse-phase HPLC and shows exclusive formation of the product (see the Supporting Information). The POC **9** with a glyoxylic oxime linkage was purified by HPLC and obtained in good yield. The oligonucleotide derivatives and the conjugates **6**−**9** prepared herein were characterized by ESIMS analysis, and an excellent agreement between the observed molecular weights and calculated values is observed (Table 1).

The hydrolytic stability of the glyoxylic oxime linkage was studied as a function of pH. Purified conjugate **9** was incubated at 37 °C in a suitable phosphate buffer solution with pH adjusted to 4.0−9.0. The incubated samples were analyzed by HPLC on an analytical column after periods of 24, 48, and 72 h. The results obtained from HPLC analysis were further authenticated by ESIMS analysis. The percentage of hydrolysis of the conjugate as a function of pH is given in Table 2.

The data show that glyoxylic oxime is stable over the pH range of 4−7 because less than 4% hydrolysis is

(14) Nielsen, J.; Brenner, S.; Janda, K. D. *J. Am. Chem. Soc*. **1993**, *115*, 9812−9813.

(15) (a) Zanta, M. A.; Belguise-Valladier, P.; Behr, J.-P. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 91−96. (b) Bremmer, K. H.; Seymour, L. W.; Logan, A.; Read, M. L. *Bioconjugate Chem*. **2004**, *15*, 152−161.

TABLE 1. ESIMS Characterization of the Oligonucleotides[a]

compound	calcd mass	found mass
6	3839.77	3837.85
7	3537.40	3535.68
8	3506.34	3505.85
9	4758.82	4758.77

[a] Analysis was carried out in negative mode. $CH_3CN/H_2O/NEt_3$ [50/50/2 (v/v/v)] was used as the eluent at a flow rate of 8 μL min^{-1}.

TABLE 2. Percentage of Hydrolysis of Glyoxylic Oxime Conjugate 9 at Different pH

	% hydrolysis[a]		
pH	24 h	48 h	72 h
4.0	<1.0	<1.0	<1.0
5.0	<1.0	<2.0	<4.0
6.0	<1.0	<2.0	<3.0
7.0	<4.0	<6.0	<10.0
8.0	<10.0	<13.0	<23.0
9.0	<15.0	<24.0	<32.0

[a] Percentage hydrolysis was estimated from the area under the peak in the HPLC chromatogram.

observed after 24 h of incubation. However, some hydrolysis is noticed at alkaline pH levels (8.0−9.0), where 10−15% of the conjugate is hydrolyzed in 24 h. The results could be compared to the stability data reported earlier for the aldo-oxime linkage where 5−30% hydrolysis of the conjugate has been reported at pH 4−9 in 24 h.[11a]

In conclusion, a new and convenient method to prepare oligonucleotides with glyoxylic aldehyde at the 5′ end has been described. The method involves preparation of key phosphoramidite reagent **1**, which is readily accessible from serine in a few chemical steps. The use of this phosphoramidite reagent is compatible with the solid-phase oligonucleotide synthesis and directly incorporates the masked precursor of the aldehyde group into the oligonucleotide sequence. The ODN-glyoxylic aldehyde could be efficiently coupled to a NLS-peptide sequence. The hydrolytic stability studies show that the glyoxylic oxime bonds are stable in acidic to neutral pH and show improved stability when compared to the aldo-oximes. The NLS sequences are known to carry the cellular and viral proteins efficiently into the nucleus through intracellular transport receptors such as importins and facilitate transfer through nuclear pores. The NLS-oligonucleotide conjugate prepared in the present work can be potentially useful in investigations aimed at developing nonviral gene delivery methods to improve the nuclear delivery of DNA.

Acknowledgment. This work was supported by the Region Rhone-Alpes and Centre National de la Recherche Scientifique (CNRS). The "Institut Universitaire de France" is gratefully acknowledged for their financial support. A postdoctoral fellowship from EGIDE (to Y.S.) is gratefully acknowledged.

Supporting Information Available: Experimental procedures, spectroscopic data, and HPLC profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0485177