ORGANIC LETTERS

2002
Vol. 4, No. 19
3259−3262

Total Stepwise Solid-Phase Synthesis of Oligonucleotide-(3′→*N*)-Peptide Conjugates

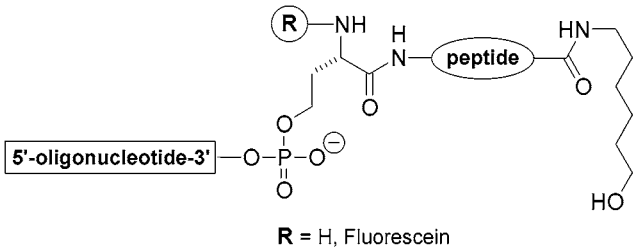
Dmitry A. Stetsenko, Andrey D. Malakhov, and Michael J. Gait*

Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

mgait@mrc-lmb.cam.ac.uk

Received July 11, 2002

ABSTRACT



R = H, Fluorescein

An efficient total stepwise solid-phase synthesis of oligonucleotide-(3′→*N*)-peptide conjugates is described that makes use of either a controlled pore glass support or macroporous polystyrene beads. Extending our previous homoserine linker approach, we prepared a range of conjugates containing one of four different cell or nuclear penetration peptides together with oligonucleotides containing 2′-deoxynucleoside or 2′-*O*-methylribonucleoside phosphodiesters, or gapmers containing 2′-deoxyphosphorothioates. The route also allows incorporation of a fluorescent label within the conjugate for cell uptake studies.

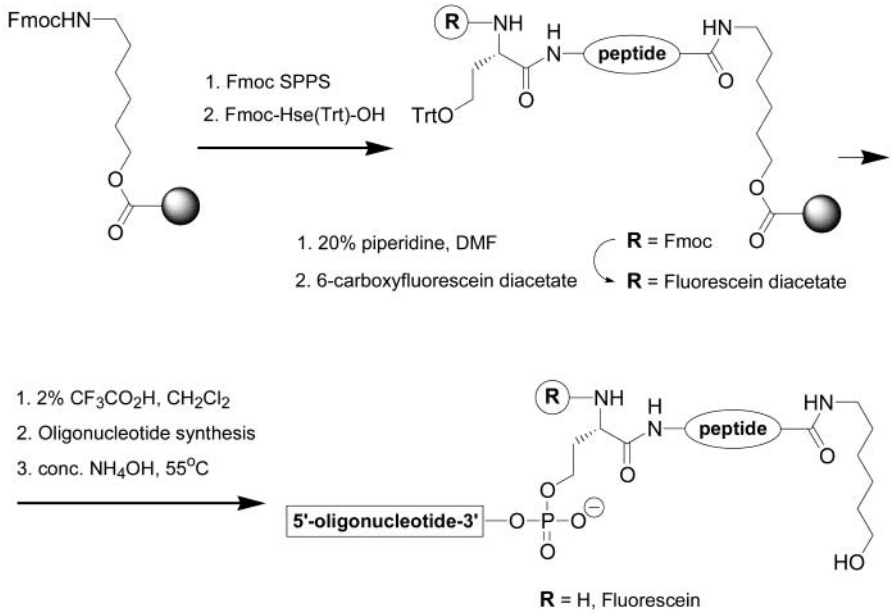
Oligonucleotides and their analogues have been studied as specific inhibitors of gene expression in cells for almost a quarter of a century.[1] However, their use as gene regulation agents within cultured cells has been much hampered by poor cellular uptake, which has led to investigations of many different transfection agents.[2] One promising approach is to conjugate the oligonucleotide or analogue to a peptide that possesses cell penetration properties to facilitate cellular delivery and/or to alter internal localization.[3] Among alternative methods for the preparation of peptide−oligonucleotide conjugates, total stepwise solid-phase synthesis seems to be the most direct and a number of routes have been suggested.[4] Of particular significance have been methods based on the use of branched linkers that allow successive peptide and oligonucleotide assembly from the branch point.[5] We described recently a novel and facile method of synthesis of oligonucleotide 3′-conjugates, including some short peptides, using an ω-aminoalkyl succinate/L-homoserine combination linker.[6] Now we report the modification and extension of this method toward the total stepwise solid-phase synthesis of longer cell penetration peptides 3′→*N* conjugated to antisense oligonucleotides and their 2′-*O*-methyl analogues (see Scheme 1).

As antisense oligonucleotide models (Table 1) we have used a 15-mer 2′-deoxyoligonucleotide and 12-mer and 16-mer 2′-*O*-methyloligoribonucleotide (OMe) sequences. We have shown recently that OMe oligonucleotides complementary to the apical stem-loop of the trans-activation

(1) Stein, C. A.; Krieg, A. M. *Applied Antisense Oligonucleotide Technology;* Wiley-Liss: New York, 1998.
(2) Reviewed in: Hughes, M. D.; Hussain, M.; Nawaz, Q.; Sayyed, P.; Akhtar, S. *Drug Discovery Today* **2001**, *6*, 303
(3) Reviewed in: Stetsenko, D. A.; Arzumanov, A. A.; Korshun, V. A.; Gait, M. J. *Mol. Biol (Russ.)* **2000**, *34*, 852. Tung, C.-H.; Stein, S. *Bioconj. Chem.* **2000**, *11*, 605.
(4) Reviewed in: Zubin, E. M.; Romanova, E. A.; Oretskaya, T. S. *Russ. Chem. Rev.* **2002**, *71*, 239.
(5) (a) Antopolsky, M.; Azhayev, A. *Helv. Chim. Acta* **1999**, *82*, 2130. (b) Antopolsky, M.; Azhayev, A. *Tetrahedron Lett.* **2000**, *41*, 9113. (c) Antopolsky, M.; Azhayev, A. *Tetrahedron Lett.* **2002**, *43*, 527.
(6) Stetsenko, D. A.; Gait, M. J. *Bioconj. Chem.* **2001**, *12*, 576.

10.1021/ol026502u CCC: $22.00
Published on Web 08/28/2002

Scheme 1



responsive element TAR RNA inhibit in vitro transcription of HIV-1 by steric competition with HIV Tat-mediated viral transcription to decrease the amount of full-length transcript.[7] 3′-Fluorescein-labeled oligonucleotide analogues delivered by a cationic lipid were also tested in a HeLa cell reporter assay, where cellular activity was found for chimeric oligonucleotides containing both OMe and locked nucleic acid (LNA) units.[8] As further models (Table 1), we chose

Table 1. Sequences of Antisense Oligonucleotides[a]

sequence	no.
cucccaggcuca	**I**
CTCCCAGGCTCAAAT	**II**
GCTCCCAGGCTCAAA	**III**
AGCTCCCAGGCTCAA	**IV**
ugugc**TATTCTGT**gaauu	**V**
uaagc**TGTTCTAT**guguu	**VI**
cucccaggcucagauc	**VII**
agcucccaggcucaga	**VIII**

[a] 2′-Deoxynucleotides are in capitals, 2′-deoxy phosphorothioates in bold capitals, and 2′-*O*-methyls in lower case.

antisense and mismatch gapmers directed against survivin mRNA[9] consisting of a 2′-deoxy phosphorothioate octamer sequence flanked 3′ and 5′ by OMe pentanucleotide wings.

For initial conjugate synthesis, we have chosen three peptides (Table 2) that were reported to have cellular membrane penetration activity: a 16-mer fragment of the HIV-1 gp41 fusion sequence (**1**),[10] 15-mer Kaposi fibroblast growth factor peptide (K-FGF) (**2**),[11] and a 21-mer short version of Transportan (a chimeric galanin/mastoparan peptide) (**3**).[12] An 8-mer peptide **4** that constitutes part of the nucleoplasmin nuclear localization signal (NLS)[13] was also included. The first two peptides do not require amino acid side-chain protection, By contrast, Transportan includes asparagine and tyrosine residues as well as lysines, the ϵ-amino groups of which are normally protected as trifluoroacetamides.[6] Since satisfactory base-labile protection for the tyrosine phenol group compatible with Fmoc/*tert*-butyl chemistry[14] is lacking, we opted for the mild acid-labile

Table 2. Sequences of Cell-Penetrating Peptides[a]

sequence	no.
AVGIGALFLGFLGAAG	**1**
AVALLPAVLLALLAP	**2**
AGYLLGK(Ac)INLKALAALAKKIL	**3**
AKKKKLDK	**4**

[a] All peptides are *C*-terminal 6-hydroxyhexylamides and contain L-homoserine residue at the *N*-terminus, optionally *N*-acylated with 6-carboxyfluorescein.

(7) Arzumanov, A.; Walsh, A. P.; Liu, X.; Rajwanshi, V. K.; Wengel, J.; Gait, M. J. *Nucleosides, Nucleotides Nucl. Acids* **2001**, *20*, 471.
(8) Arzumanov, A.; Walsh, A. P.; Rajwanshi, V. K.; Kumar, R.; Wengel, J.; Gait, M. J. *Biochemistry* **2001**, *40*, 14645.
(9) Chen, J.; Wu, W.; Tahir, S. K.; Kroeger, P. E.; Rosenberg, S. H.; Cowsert, L. M.; Bennett, F.; Krajewski, S.; Krajewski, M.; Welsh, K.; Reed, J. C.; Ng, S.-C. *Neoplasia* **2000**, *2*, 235.
(10) Soukchareun, S.; Tregear, G. W.; Haralambidis, J. *Bioconj. Chem.* **1995**, *6*, 43.
(11) Dokka, S.; Toledo-Velasquez, D.; Shi, X.; Wang, L.; Rojanasakul, Y. *Pharm. Res.* **1997**, *14*, 1759.
(12) Soomets, U.; Kilk, K.; Land, T.; Langel, U. In *Innovations and Perspectives in Solid-Phase Synthesis and Combinatorial Libraries 2000*; Epton, R., Ed.; Mayflower Press: Kingswinford, UK, 2001.
(13) de la Torre, B.; Albericio, F.; Saison-Behmoaras, E.; Bachi, A.; Eritja, R. *Bioconj. Chem.* **1999**, *10*, 1005.
(14) Chan, W. C.; White, P. D. *Fmoc Solid-Phase Peptide Synthesis: A Practical Approach*; Oxford University Press: Oxford, UK, 2000.

2-chlorotrityl protecting group instead.[15] The appropriate monomer, Fmoc-Tyr(2-ClTrt)-OH, is available from Novabiochem. The asparagine residue was incorporated as its amide-unprotected form via use of Fmoc-asparagine pentafluorophenyl ester to avoid side reactions resulting from carboxylic activation. The rest of the amino acids were coupled via the HATU/DIEA/DMF in situ activation protocol and Fmoc groups were removed throughout by use of 20% piperidine/DMF.[14] The NLS sequence contains aspartic acid of which the β-carboxy group requires protection. Here, we found the recently described base-labile Dmab ester[16] to be a good solution to this problem.

For initial trials, manual peptide assemblies were carried out on 500 Å long-chain alkylamine controlled pore glass (LCAA-CPG) support functionalized by sarcosine followed by an aminohexyl succinate linker as described previously.[6] Peptide **1** was assembled by fragment coupling with tripeptides Fmoc-AAG-OH and Fmoc-FLG-OH, and dipeptides Fmoc-IG-OH and Fmoc-VG-OH. For peptide **4**, all monomer couplings were used. For both peptides, coupling steps were followed by acetic anhydride/*N*-methylimidazole/2,4,6-collidine capping. Synthesis efficiency was monitored by measurement of *N*-(9-fluorenylmethyl)piperidine absorbance values[14] and double couplings were used when yields dropped below 95%. The average stepwise yield was >96%. Then, we explored two alternative polymer supports that are based on macroporous polystyrene beads: PS200 resin (Amersham Biosciences), 30 μm particle size, and ArgoPore low loading resin obtained from Aldrich, >100 μm bead size. To our knowledge, the latter has not been tested previously in oligonucleotide synthesis. Peptide sequence **2** was assembled first on ArgoPore resin manually to check its performance, using two dipeptide blocks Fmoc-AP-OH and Fmoc-LP-OH, the rest being monomer couplings. Again, Fmoc deprotection yields were carefully checked, and double couplings were used when appropriate. PS200 resin was used for Transportan (**3**) synthesis. The first two amino acids were introduced manually to test the support under peptide synthesis conditions. Then, the resin was subjected to automated peptide assembly on a Pioneer Peptide Synthesizer (Applied Biosystems), except for the asparagine residue which was coupled manually. Double couplings with Fmoc-amino acid monomers and manufacturer's standard protocols were used throughout the automated syntheses. Acetic anhydride capping steps were omitted, except before and after all manual coupling steps. In the case of peptide **3**, after assembly the 2-chlorotrityl group on the tyrosine residue was removed by treatment with 2% $CF_3CO_2H-CH_2Cl_2$ (v/v) for 2 min, and the resin was capped, but using isobutyric anhydride instead of acetic anhydride, to protect the phenolic group of tyrosine with a base-labile isobutyryl group.

After all peptide assemblies, Fmoc deprotection was followed by Fmoc-Hse(Trt)-OH manual coupling and an optional fluorescein label could then be introduced by further Fmoc deprotection and coupling with 6-carboxyfluorescein diacetate. Homoserine incorporation also could be accomplished in a machine-assisted way by using the conditions for normal Fmoc-amino acid coupling, as was demonstrated by the synthesis of peptide **2** on PS200 support. Finally, 2% $CF_3CO_2H-CH_2Cl_2$ (v/v) was used to remove the trityl group.

Then, peptide-loaded resins were subjected to standard oligonucleotide chain assembly on a ABI 394 DNA/RNA Synthesizer with either 2′-deoxyribonucleoside or 2′-*O*-methyl ribonucleoside phosphoramidites and manufacturer's protocols. Beaucage's reagent (Glen Research) was used for phosphorothioate synthesis. Conjugates were cleaved from the resin and deprotected by concentrated aqueous ammonia treatment at 55 °C overnight, conditions we have found to be safe for a range of peptides.[6,17] The resulting products were isolated in good to moderate yield and analyzed by reversed-phase HPLC, and their respective molecular masses checked by MALDI-TOF mass spectrometry (Table 3).

Table 3. Properties of Oligonucleotide-(3′→*N*)-peptide Conjugates

no.[a]	FAM[b]	MALDI-TOF, calcd/found	RP-HPLC retention time, min	purity, %[c]
I.1	no	5591.5/5591.3	25.2	69.4
I.2	yes	5961.8/5958.7	57.3	22.5
I.3	yes	6741.3/6739.3	56.3	26.9
I.4	no	5117.5/5118.6	16.5	79.0
II.1	yes	6548.8/6545.8	27.7	54.7
II.2	no	6201.9/6198.6	54.3	90.6
II.3	no	6981.3/6984.2	57.8	17.7
II.4	no	5715.6/5711.5	11.4	73.9
II.4	yes	6073.6/6073.4	18.2	70.4
III.2	no	6226.9/6225.3	30.7	54.7
III.3	no	7006.3/7002.5	58.0	11.6
IV.3	no	7006.3/7003.6	33.0	20.0
V.2	yes	7944.9/7943.7	30.7	50.5
V.3	yes	8724.4/8720.8	31.0	19.2
VI.2	yes	7944.9/7944.4	30.6	48.9
VI.3	yes	8724.4/8722.6	31.0	18.5
VII.2	yes	7303.0/7297.7	30.8	59.4
VII.3	yes	8082.5/8078.7	31.2	35.6
VIII.1	yes	6996.9/6992.9	27.5	45.8

[a] Oligonucleotides are designated by Roman numbers, peptides by Arabic. [b] 6-Carboxyfluorescein label. [c] Integrated from HPLC traces of crude conjugates.

Examples of typical RP-HPLC traces are shown in Figure 1.

Comparison of the results of conjugate synthesis on the different supports showed unequal performance in peptide and oligonucleotide assemblies. The PS200 support (Amersham Biosciences) gave the best overall purity/yield balance in both oligonucleotide and peptide synthesis. CPG-500 (conjugates **I.1**, **I.4**, **II.1**, **II.4**, and **VIII.1**) showed comparable or slightly better results in oligonucleotide synthesis,

(15) Athanassopoulos, P.; Barlos, K.; Gatos, D.; Hatzi, O.; Tsavara, C. *Tetrahedron Lett.* **1995**, *36*, 5645.

(16) Chan, W. C.; Bycroft, B. W.; Evans, D. J.; White, P. D. *J. Chem. Soc., Chem. Commun.* **1995**, 2209.

(17) Stetsenko, D. A.; Williams, D.; Gait, M. J. *Nucl. Acids Res. Suppl.* **2001**, 1, 153.

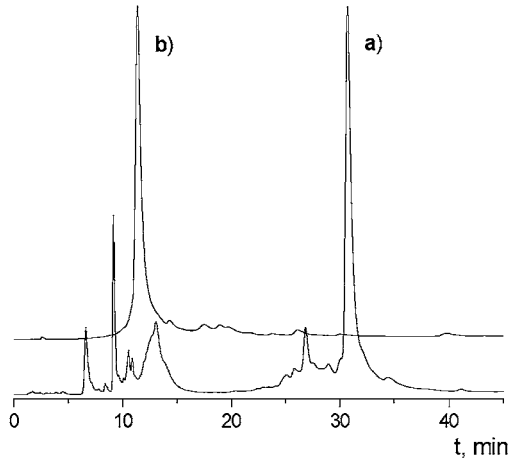


Figure 1. RP-HPLC traces of crude oligonucleotide-(3′→N)-peptide conjugates: **I.2** (a) and **II.4** (b).

but was noticeably inferior in peptide synthesis. ArgoPore resin (conjugate **II.2**) gave excellent product purity, but low isolated yield (10% calculated from initial peptide loading), which may indicate that it is more suitable for peptide synthesis than for oligonucleotide synthesis.

In conclusion, we have presented a new approach toward the total stepwise solid-phase synthesis of peptide−oligonucleotide conjugates based on our previously reported homoserine linker. This approach is an effective and expeditious way to obtain cell-penetrating peptide−oligonucleotide conjugates necessary for antisense inhibition and cell delivery studies, or for other applications such as improving hybridization properties of oligonucleotides in vitro.[18] Several amino acids that are common in such peptides and which require side-chain protection were successfully incorporated by using ammonia-labile protecting groups (lysine, aspartic acid) or transient protection (tyrosine). The incorporation of arginine remains problematic, but one possible solution through use of protected ornithine has been published.[5b,c] Cellular studies with these antisense peptide-2′-*O*-methyl-oligonucleotide and gapmer conjugates are in progress and will be reported elsewhere.

Acknowledgment. One of us (A.D.M.) thanks Astra-Zeneca Ltd for financial support. The authors thank Per Denker (Amersham Biosciences) for the generous gift of PS200 support, and Donna Williams for advice and for carrying out some oligonucleotide assemblies.

Supporting Information Available: Synthesis details, HPLC traces, and MALDI-TOF spectra for the conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

OL026502U

(18) Corey, D. *J. Am. Chem. Soc.* **1995**, *117*, 9373.