The Prooligonucleotide Approach: Synthesis of Mixed Phosphodiester and SATE Phosphotriester Prooligonucleotides Using *H*-Phosphonate and Phosphoramidite Chemistries

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The synthesis of mixed SATE-phosphotriester and phosphodiester prooligonucleotides using both phosphoramidite and *H*-phosphonate chemistries is described. The key step is the selective oxidation of phosphite triester and *H*-phosphonate linkages to yield thiono- or oxo-triester SATE and thiono- or oxo-diester linkages. This approach allows the synthesis of any desired mixed prooligos bearing either *t*Bu- or Me-SATE enzymolabile protecting groups.

Introduction

Oligonucleotide-based therapy promises to be a highly specific tool for the treatment of numerous human diseases. However, the main hurdles to the use of oligonucleotides (oligos) as therapeutics are their rapid degradation by nucleases and their poor cellular uptake.[1] This latter limitation is mainly due to the polyanionic structures of oligos. The use of modified oligos (e.g. phosphorothioates) has partly resolved the problem of degradation. In the hope of overcoming their poor cellular uptake, we have been applying the prodrug concept[2] to oligos, thereby developing the prooligonucleotide (prooligo) approach.[3−5] The prooligo approach involves a temporary masking with enzymolabile *S*-Acyl-Thio-Ethyl (SATE) groups of the negative charges of each phosphodiester or phosphorothioate linkage forming a corresponding phosphotriester linkage. We have shown that such neutral prooligos are resistant to nucleases,[5] are less prone to non-specific interactions with proteins,[6] and, due to their increased lipophilicity, are taken up more efficiently by cells.[7] Once inside the cells, demasking proceeds by a carboxyesterase-mediated mechanism to release the polyanionic oligo, which can then interact with its target. Fully masked Me- or *t*Bu-SATE prooligos (12-mers) were found to be poorly soluble in water. Furthermore, the highly lipophilic *t*Bu-SATE prooligos, with either phospho- or thionophosphorotriester linkages, were found not to function as efficient substrates of carboxyesterases.[5] However, we have shown that two Me-SATE prooligos (12-mers) were fully demasked by carboxyesterases in cell extract with a half-life of about 21 h.[5] This prompted us to synthesize mixed phosphodiester/SATE phosphotriester prooligos so as to obtain compounds with an optimal balance between lipophilicity and water solubility. To this end, mixed prooligos were synthesized from *t*Bu-SATE[8] and commercial cyanoethyl phosphoramidites on a photolabile solid support.[9] The phosphodiester linkages were generated by a selective elimination of the cyanoethyl group from the phosphotriester by treatment with DBU in anhydrous tetrahydrofuran,[8] which induced the removal of cyanoethyl groups without affecting the *t*Bu-SATE enzymolabile groups. Such treatment proved convenient for prooligos with *t*Bu-SATE, but not for those with Me-SATE since this strong base was able to abstract a hydrogen from the methyl group, thereby cleaving the corresponding Me-SATE group.[10] To overcome this limitation, we have now developed a synthesis of mixed prooligos by combining hydrogen phosphonate and phosphoramidite chemistries. In this paper, we report the synthesis of mixed prooligos with either oxo- or thiono-phosphodiester (PO^- or PS^-) linkages and with either Me- or *t*Bu-SATE phosphotriester linkages. The introduction of Me-SATE groups in prooligos allows access to various oligomers exhibiting a wide range of lipophilicities. Hence, we hope to use these to gain more information about the relationship between lipophilicity of prooligos and their suitability as carboxyesterase substrates as well as their uptake capacities.

Results and Discussion

Synthesis of Mixed Phosphodiester SATE-Phosphotriester Prohexathymidines

To establish the optimal conditions for the synthesis of mixed prooligos using both phosphoramidite and *H*-phosphonate chemistries,[11] we prepared eight prohexathymidines containing either oxo- or thio-phosphodiester linkages and either oxo- or thiono- (Me- or *t*Bu-SATE) phosphotriester linkages (Figure 1). The SATE phosphotriester link-

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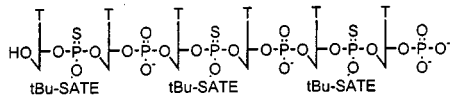
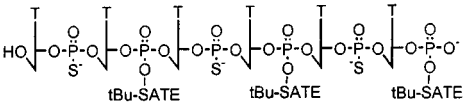
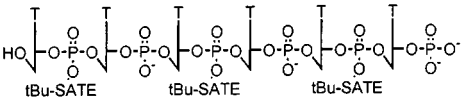
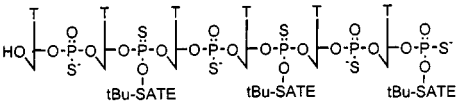
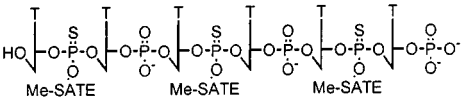
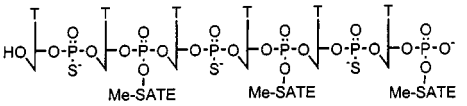
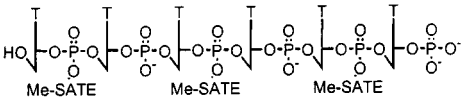
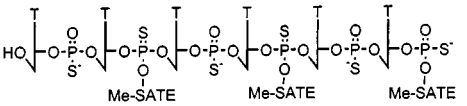
1		2323.4	2323.1
2		2323.2	2323.1
3		2275.0	2274.9
4		2371.6	2371.3
5		2197.4	2196.9
6		2198.4	2196.9
7		2148.8	2148.7
8		2244.1	2245.1

Figure 1. Schematic representation of the eight prohexathymidines and mass characterization

ages were introduced using a thymidine SATE phosphoramidite building block[5] by means of phosphoramidite chemistry, whereas the phosphodiester linkages were introduced using an *H*-phosphonate monoester thymidine building block[12] by means of *H*-phosphonate chemistry (Figure 2).

The eight prooligos were synthesized, starting from *H*-phosphonate thymidine[12] and Me- or *t*Bu-SATE thymid-

Table 1. Elongation cycle for the synthesis of mixed prooligos using both phosphoramidite and *H*-phosphonate chemistries

steps	SATE-phosphoramidite reagents and solvents	time [s]	*H*-phosphonate reagents and solvents	time [s]
wash	CH_3CN	10	CH_3CN	10
detritylation	3% TCA in CH_2Cl_2	60	3% TCA in CH_2Cl_2	60
wash	CH_3CN	15	CH_3CN/ Pyr (1:1, *v/v*)	30
coupling	SATE amidite (20 equiv., 0.1 M) and tetrazole (0.45 M) in CH_3CN	30 (180[a])	monomer (10 equiv., 0.05 M) and adamantoyl chloride (4%) in CH_3CN/Pyr (1:1, *v/v*)	30
wash			CH_3CN/Pyr	30
wash	CH_3CN	20	CH_3CN	20
oxidation[b]	*either* *t*BuOOH (1.1 M in CH_2Cl_2)	60	I_2/Pyr/H_2O (2%; 98:2, *v/v*)	120
oxidation[b]	*or* Beaucage reagent (0.05 M in CH_3CN)	30	S_8/CS_2/Pyr/Et_3N (1.25%, 5:5:0.2, *v/v/v*)	600

[a] Extended time for the first incorporation of phosphoramidite. – [b]Performed manually after synthesis of the *H*-phosphonate linkages.

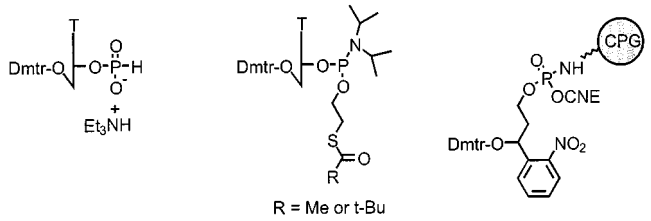


Figure 2. Building blocks and solid support used

ine phosphoramidites,[5] on a photolabile solid support[9] (Figure 2), from which the prooligo can be cleaved without any need for nucleophilic or basic treatment that would degrade the phosphotriester linkages.

Table 1 describes the standard elongation cycle performed on a DNA synthesizer (ABI 381A). Detritylation was accomplished by a standard treatment with trichloroacetic acid (TCA) (3% in dichloromethane) for 60 s to yield the free 5′-hydroxyl function. Then, either *H*-phosphonate thymidine monoester, activated with adamantoyl chloride, or SATE phosphoramidite thymidine (Me or *t*Bu), activated with tetrazole, was coupled, for 30 s in each case (an extended time of 180 s was employed for the first coupling to ensure its efficiency). In both cases, the coupling yields, calculated from the amount of dimethoxytrityl cation released, were between 98 and 99%.

The key step in the synthesis of such mixed prooligos is the selective oxidation of the SATE phosphite triesters and of the *H*-phosphonate diesters.[13] The SATE phosphotriester linkages were oxidized with *tert*-butyl hydroperoxide (*t*BuOOH)[14] (for 60 s) to generate oxo-phosphotriester linkages, or with the Beaucage reagent[15] (for 30 s) to generate the thiono-phosphotriester analogues. However, neither *tert*-butyl hydroperoxide[11] nor the Beaucage reagent[16] were able to oxidize the *H*-phosphonate linkages. Mass spectra of prooligos **2** and **6** did not reveal any side *O*-oxidation of *H*-phosphonate linkages during the oxidation of phosphite triesters with *t*BuOOH. Likewise, mass spectra of prooligos **1** and **5** did not show any sulfurization of the *H*-phosphonate linkages during the treatment with Beaucage reagent (Figure 1). A post-synthesis oxidation of the *H*-phosphodiester linkages (for 2−10 min) with iodine/water/pyridine[17] or with elemental sulfur in carbon disulfide/pyridine/triethylamine[18] gave the oxo- or thiono-phosphodiester linkages, respectively. We also tried to sulfurize the *H*-phosphonate linkages with a solution of the Beaucage reagent in triethylamine/water, as described by Stawinski et al.[16] However, this solution is very unstable and must be freshly prepared and used within 5 min. In our hands, this method also led to *O*-oxidation. Thus, we found sulfurization with elemental sulfur to be more convenient. No capping step was performed in the synthesis of alternating prooligos. Finally, they were released from the solid support by photolysis for 25 min,[5] thereby yielding the prooligos with a 3′-SATE-phosphodiester (**2**, **4**, **6**, or **8**) or a 3′-phosphomonoester linkage (**1**, **3**, **5**, or **7**) from the supported 3′-SATE phosphotriester and 3′-phosphodiester prooligos, respectively. Each prooligo was analyzed and purified by reversed-phase HPLC and characterized by mass spectrometry (electrospray ionization in negative mode) (Figure 1).

HPLC profiles of prooligos **1**, **3**, **5**, and **7** showed a small additional peak with a retention time greater than that of the corresponding prooligo (Figure 3). This peak corre-

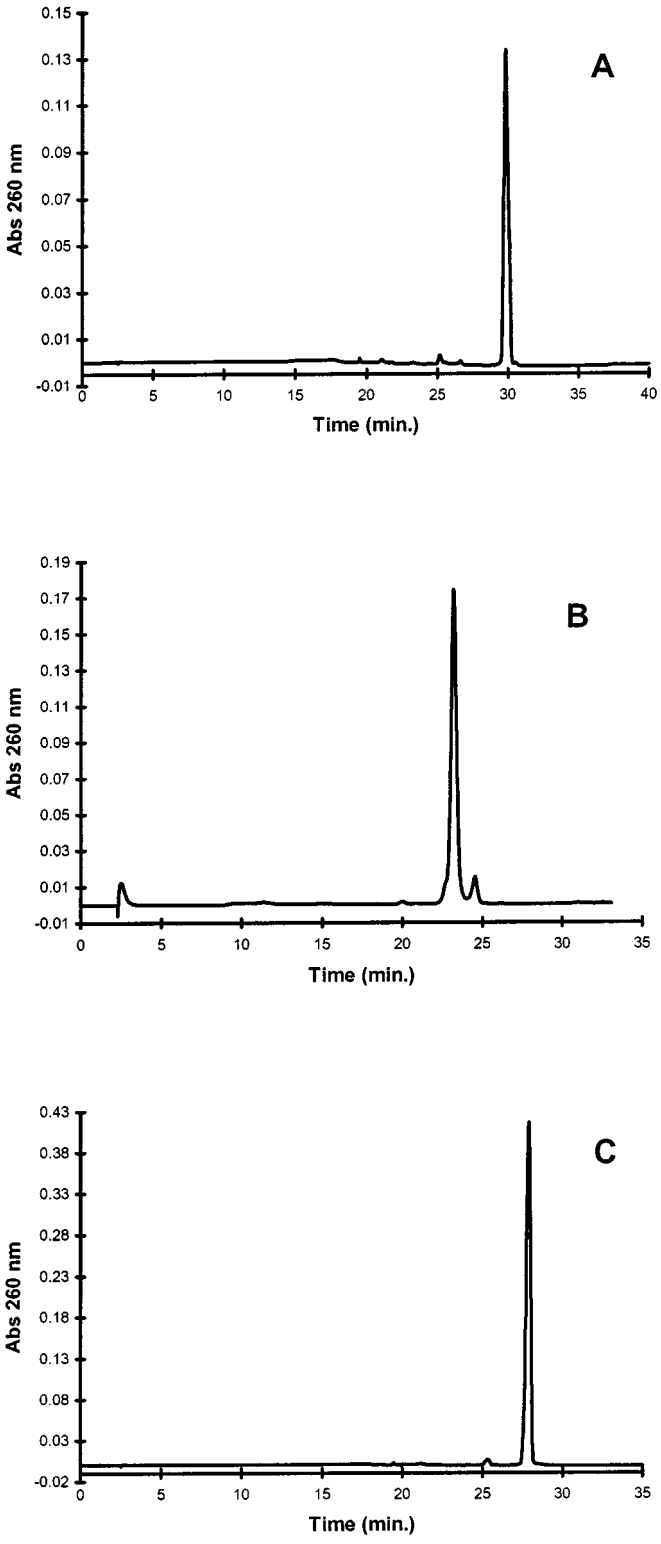


Figure 3. HPLC profiles after purification of A: **2**, B: **5**, and C: **8**

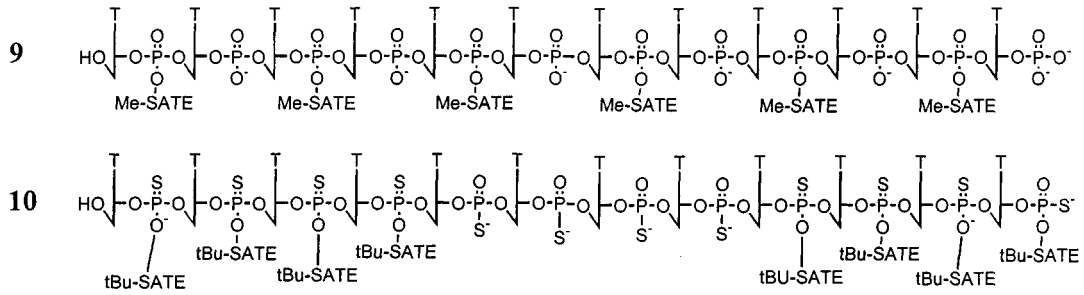


Figure 4. Structure of the prododecathymidines

sponds to a small amount of 3′-dephosphorylated prooligo, as was confirmed by the mass spectrum, which showed a signal at $[M - 80 - H]^-$. This was not seen for the other prooligos (**2**, **4**, **6**, and **8**) since these contain the enzymatically stable 3′-SATE phosphodiester linkage. The HPLC profiles did not show any peak at shorter retention times that would correspond to loss of a SATE group. Likewise, the mass spectra showed only multiply-charged ions corresponding to the expected prooligos without any loss of SATE groups or any desulfurization.

Since the synthesis of mixed prooligos using both phosphoramidite and *H*-phosphonate chemistry proved efficient for hexamers, we then prepared two dodecathymidine prooligos, one with Me-SATE (**9**) and the other with *t*Bu-SATE (**10**) (Figure 4), under the conditions established as above. Prooligo **9** represents an alternating phosphodiester Me-SATE phosphotriester and was synthesized in the same manner as the prooligos **1**−**8**. Prooligo **10** comprises a central section of four phosphorothioate diesters flanked by four SATE thionophosphotriesters at each wing (Figure 4). For **10**, we chose to perform a standard capping step with acetic anhydride (Ac_2O/NMI/lutidine in THF) after each coupling of phosphoramidite building block, then the four *H*-phosphonate monoesters were introduced without capping. At this stage, the *H*-phosphonate linkages were oxidized with elemental sulfur (as described above) and the synthesis was resumed with a capping by means of phosphoramidite chemistry.

The prooligos were finally released from the solid support by photolysis, purified by reversed-phase HPLC (C_{18}), and analyzed by mass spectrometry (ESI or MALDI-TOF).[19] As described above for **1**, **3**, **5**, and **7**, prooligo **9** evidently underwent a small amount of 3′-dephosphorylation (Figure 5, peak at t_R = 23.3 min; Figure 5A does not depict a nucleosidic compound). The HPLC profile of **10** (crude, data not shown) displayed only a broad peak at 72 min and, after ammonia treatment, a sharper peak corresponding to the phosphorothioate T_{12}. We did not notice any significant difference in the purities of the prooligos synthesized with or without the capping step.

Conclusion

We have shown that the synthesis of mixed prooligos can be achieved using both *H*-phosphonate and phosphoramidite chemistries. This approach allows the synthesis of any desired prooligos, i.e. with oxo or thiono SATE (Me or *t*Bu) triester linkages as well as with oxo or thiono phosphodiester linkages, since the SATE groups with either methyl or *tert*-butyl are stable throughout all the relevant processes. By applying this approach we will be able to synthesize various prooligos showing a wide range of lipophilicities. These should give information on the relationship between lipophilicity and the efficiency of demasking in total cell extracts, as well as on uptake and demasking in whole cells. Work along these lines is currently in progress.

Experimental Section

General Remarks: All commercial chemicals were reagent grade and were used without further purification, except where otherwise stated. DNA synthesis reagents, apart from the oxidants, were purchased from Perseptive Biosystems Ltd. (Voisins le Bretonneux, France). Anhydrous *tert*-butyl hydroperoxide (5.5 M in decane) was obtained from Fluka and was diluted with anhydrous dichloromethane (Aldrich). Elemental sulfur (99.998%) and carbon disulfide (99.9+%) were obtained from Aldrich. 3*H*-1,2-Benzodithiole-3-one-1,1-dioxide (Beaucage reagent) was a gift from Isis Pharmaceuticals (Carlsbad, CA).

Oligonucleotide Synthesis: Thymidine *H*-phosphonate monoester was prepared according to Stawinski et al.;[12] Me- and *t*Bu-SATE phosphoramidite thymidines were synthesized according to Tosquellas et al.[5] and the photolabile solid support used was prepared according to Dell′Aquila et al.[9]

All oligonucleotides (1 μmol scale) were synthesized on an ABI 381A DNA synthesizer, using a cycle involving both *H*-phosphonate and phosphoramidite chemistries (Table 1): a wash solution of acetonitrile/pyridine (1:1; *v/v*) was used in place of the iodine bottle, and oxidants for phosphite triester (*tert*-butyl hydroperoxide or Beaucage reagent), adamantoyl chloride, *H*-phosphonate, and SATE-phosphoramidite thymidine were placed in amidite bottles. The *H*-phosphonate linkages were oxidized externally from the synthesizer after the synthesis; solutions were removed by means of syringes and treated with a solution of iodine (2%) in pyridine/water (98:2, *v/v*) or with elemental sulfur (1.25%) dissolved in CS_2/pyridine/Et_3N (5:5:0.2, *v/v/v*) for 2−10 min to yield oxo- or thiono-phosphodiester linkages, respectively.

Photolysis: The CPG-supported prooligo was suspended in CH_3CN/H_2O (50:50 or 70:30, *v/v*, 1 mL) in a 1 cm pathlength quartz cell. The magnetically stirred suspensions were exposed to the Pyrex (thickness 2 mm) filtered output of a 125 W high-pressure Hg lamp (HPK 125, Philips) for 25 min at 20°C. The glass

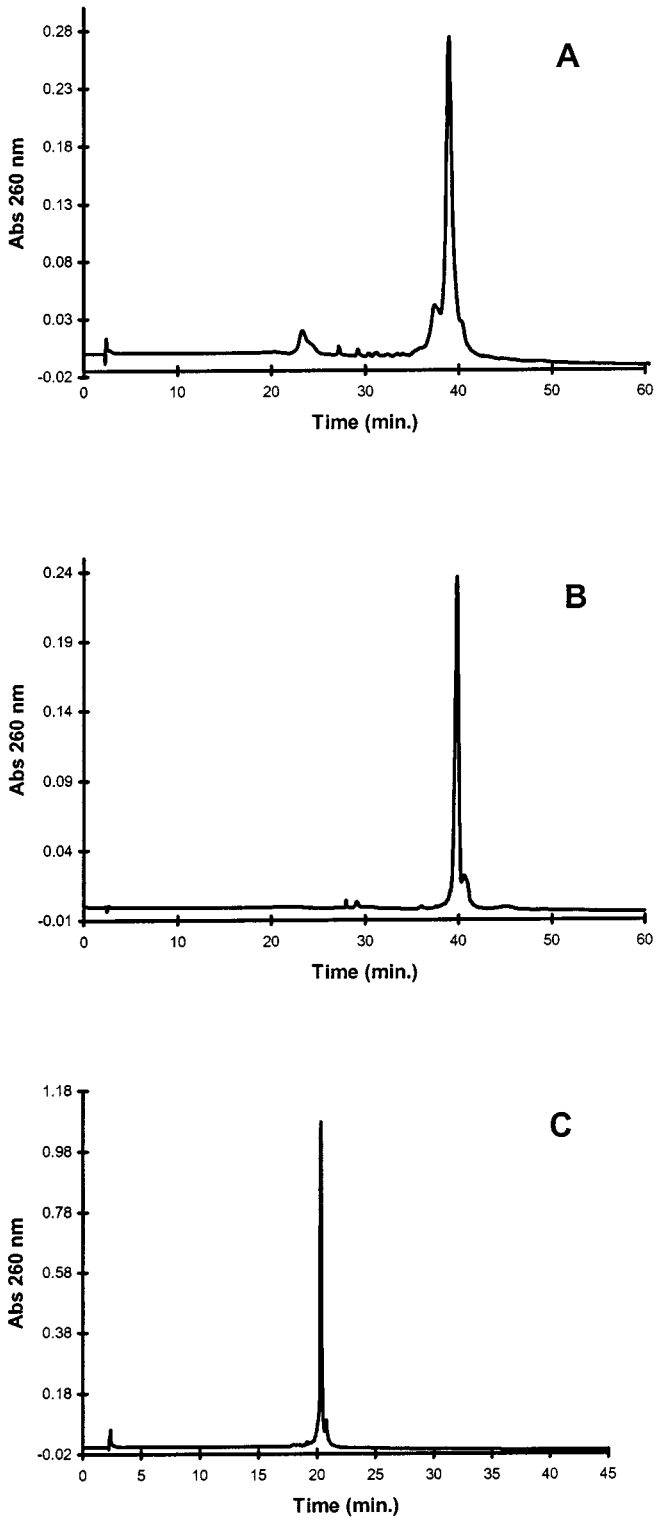


Figure 5. HPLC profiles of **9**; **A**: crude, **B**: purified, **C**: after ammonia treatment

beads were then filtered off and washed with the same solvent mixture (1 mL). The combined filtrate and washings were concentrated under reduced pressure, the residue was redissolved in water/dioxane (1:1, *v/v*, 500 μL), and lyophilized to afford a colorless powder.

HPLC Analysis and MS Characterization: High-performance liquid chromatography (HPLC) analyses were performed on a Waters-Millipore instrument equipped with a Model 600E solvent delivery system, a Model U6 K injector, and a Model 486 absorbance detector. A reversed-phase C_{18} (5 μm) Nucleosil column (150 (4.6 mm, Macherey−Nagel, Germany) was used at a flow rate of 1 mL·min^{-1}. Using a linear gradient of acetonitrile 0−80% in 0.05 M aqueous triethylammonium acetate (pH 7) for 80 min, oligos were purified by reversed-phase (C_{18}) HPLC on a DeltaPack (15 μm) column (300 × 7.8 mm, Waters).

For the ESI mass spectrometric analyses, the samples (1 OD^{260nm} in 1 mL water/acetonitrile, 50:50, *v/v*) were infused at 10−20 μL/min with a Harvard Apparatus model 22 pump into the ion-spray interface of a Finnigan MAT model SSQ 7000 mass spectrometer (San Jose, California, USA) equipped with an octapole ion guide. The mass spectrometer was operated in the negative-ion mode. Mass spectrometry conditions included a spray voltage of 4.5 kV, a capillary temperature of 250 °C, a nitrogen sheath gas pressure of 80 ψ, and an auxiliary gas pressure of 40 ψ. The spectra were acquired over the range m/z = 500−2500 at intervals of 1 s. Molecular masses of the prooligos were determined by analysis of the multiply-charged ion peaks.

MALDI-TOF mass spectra were recorded on a Voyager mass spectrometer (Perseptive Biosystems, Framingham, MA, USA) equipped with an N_2 laser. 1 μL of prooligo sample (0.1 OD^{260nm} in 100 μL water/acetonitrile, 50:50, *v/v*) was exchanged on ammonium DOWEX 50 W X8 resin prior to the addition of 10 μL of an acetonitrile/water (1:1, *v/v*) solution saturated with 2,4,6-trihydroxyacetophenone (THAP) and 1 μL of ammonium citrate (pH 9.4, 100 mM in water). This mixture was placed on a plate and dried at ambient temperature and pressure.

Acknowledgments

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[1] R. M. Crooke, "In vitro Cellular Uptake, Distribution, and Metabolism of Oligonucleotides", in: *Antisense Research and Application* (Ed.: S. T. Crooke), Springer-Verlag, Berlin, Heidelberg, New York, **1998**, Vol. 131, pp. 103−140.
[2] J. P. Krise, V. J. Stella, *Advanced Drug Delivery Reviews* **1996**, *19*, 287−310.
[3] I. Barber, B. Rayner, J.-L. Imbach, *Bioorg. Med. Chem. Lett.* **1995**, *5*, 563−568.
[4] N. Mignet, G. Tosquellas, I. Barber, F. Morvan, B. Rayner, J.-L. Imbach, *New J. Chem.* **1997**, *21*, 73−79.
[5] G. Tosquellas, K. Alvarez, C. Dell'Aquila, F. Morvan, J.-J. Vasseur, J.-L. Imbach, B. Rayner, *Nucleic Acids Res.* **1998**, *26*, 2069−2074.
[6] G. Tosquellas, A. Bryksin, K. Alvarez, F. Morvan, J.-J. Vasseur, B. Rayner, E. Rykova, P. Laktionov, V. V. Vlassov, J.-L. Imbach, *Antisense Nucleic Acid Drug Deliv.* **1999**, submitted.
[7] C. Dell'Aquila, E. Vivès, J.-L. Imbach, B. Rayner, *XIII International Round Table on Nucleosides, Nucleotides, and their Biological Applications. Poster 394*; Montpellier, **1998**.
[8] G. Tosquellas, J.-C. Bologna, F. Morvan, B. Rayner, J.-L. Imbach, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2913−2918.
[9] C. Dell'Aquila, J.-L. Imbach, B. Rayner, *Tetrahedron Lett.* **1997**, *38*, 5289−5292.
[10] L. H. Baptistella, J. F. Dos Santos, K. C. Ballabio, A. J. Marsaioli, *Synthesis* **1989**, 436−438.
[11] The use of both phosphoramidite and *H*-phosphonate chemistries has previously been described for the synthesis of alternating cationic/anionic oligos; P. M. Jung, G. Histand, R. L. Letsinger, *Nucleosides, Nucleotides* **1994**, *13*, 1597−1605.

[12] J. Stawinski, M. Thelin, *Nucleosides, Nucleotides* **1990**, *9*, 129−135.
[13] The different oxidation conditions were first evaluated on a trithymidine model with one *H*-phosphonate and one CNE-phosphotriester linkage on a standard CPG solid support with a succinyl spacer and subsequently applied to the hexamers.
[14] Y. Hayakawa, M. Uchiyama, R. Noyori, *Tetrahedron Lett.* **1986**, *27*, 4191−4194.
[15] R. P. Iyer, W. Egan, J. B. Regan, S. L. Beaucage, *J. Am. Chem. Soc.* **1990**, *112*, 1253−1254.
[16] J. Stawinski, M. Thelin, *J. Org. Chem.* **1991**, *56*, 5169−5175.
[17] J. Garegg, T. Regberg, J. Stawinski, R. Strömberg, *Nucleosides, Nucleotides* **1987**, *6*, 655−662.
[18] M. Matsukura, G. Zon, K. Shinozuka, C. A. Stein, H. Mitsuya, J. S. Cohen, S. Broder, *Gene* **1988**, *72*, 343−347.
[19] For **9**, $[M - H]^-$ calcd. 4280.3; found 4280.0 (ESI), 4284.4 (MALDI-TOF); for **10**, $[M - H]^-$ calcd. 5014.1; found 5012.3 (MALDI-TOF).

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