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Studies on the chemical synthesis of oligodeoxynucleotides containing the s^{5}T(6-4)T photoproduct: side reactions derived from the methylsulfenyl thiol protection elucidated by MALDI mass spectrometry †

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Attempts to incorporate the phosphoramidite of the thymine-thymine (6-4) photoproduct C5 thiol analogue (s^{5}T(6-4)T PP), whose sulfur atom was protected with the methylsulfenyl group, into oligodeoxynucleotides (ODNs), are reported. Using matrix-assisted laser desorption-ionisation mass spectrometry (MALDI-MS) coupled to enzymatic digestion, accurate mass measurements and tandem mass spectrometry experiments, we demonstrated that ODNs containing the (2-cyanoethylthio)^{5}T(6-4)T PP were obtained. Supported by model reactions, these results were explained 1) by the incorporation, during oligonucleotide synthesis, of the sulfur deprotected phosphoramidite that arose from a Michaelis–Arbusov-type rearrangement, and 2) the Michael addition to the thiol of acrylonitrile released upon the cyanoethyl phosphotriester deprotection. To avoid the formation of the cyanoethyl adduct, the phosphotriester deprotection was carried out in the presence of a thiol in excess. This afforded the ODN containing the h^{5}T(6-4)T PP.

Introduction

Sulfur analogues at position C5 of (6-4) photoproducts (s^5(6-4) PPs) constitute attractive surrogates of (6-4) photoproducts ((6-4) PPs),[1] one of the major mutagenic class of DNA damage formed at dipyrimidine sites upon UV exposure (Fig. 1).[2]

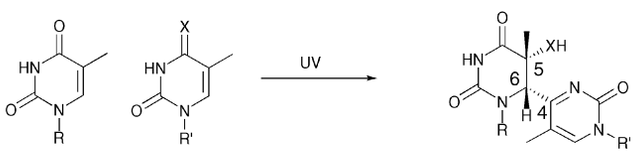
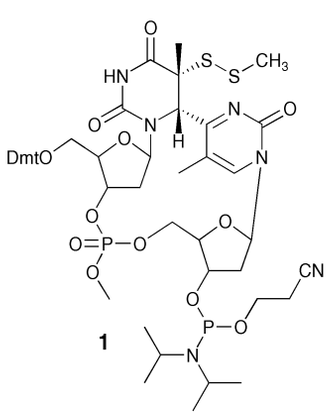


Fig. 1 Formation of (6-4) PP at adjacent thymine site (X = O; T(6-4)T PP) and of its C5 sulfur analogue (X = S; s^{5}T(6-4)T PP). R and R′ represent the polynucleotide chain.

s^5(6-4) PPs have been proved to be valuable tools to investigate some of the properties of the photobiologically relevant adducts such as their formation or repair mechanism[3,4] and their ability to bend the DNA double helix.[5] However, the potential of these unique tools to probe other essential biophysical or biological processes linked to (6-4) PPs is currently limited by the lack of convenient methods allowing their site specific incorporation into ODNs. Indeed, s^5(6-4) PP-containing ODNs have been obtained so far by direct photolysis of ODNs containing the thymine 4-thiothymine (Ts^{4}T) sequence.[4a,c,5] Obviously, site specific incorporation of a phosphoramidite building block should obviate the tedious purification step required to eliminate the photoproducts formed unavoidably within the Ts^{4}T sequence and between s^{4}T and the adjacent 3′ nucleobase.[7] Therefore, we recently designed and synthesized the phosphoramidite building block of the s^{5}T(6-4)T PP **1** whose C5 thiol function was protected as a mixed disulfide,[7] as commonly performed to incorporate thiol-containing derivatives into ODNs by phosphoramidite-based solid-phase synthesis.[8] The stability of the chosen methylsulfenyl protection had been carefully examined with regard to the conditions used for the phosphoramidite-based ODN synthesis.[7] Herein, we describe our attempts to incorporate **1** into oligonucleotides and the problems associated with the thiol protection.



Results and discussion

Oligonucleotide synthesis

We decided to evaluate the incorporation of **1** into ODNs by preparing a 10-mer, then a 30-mer to ascertain that our method had no sequence limitation. We chose to synthesize decamer 5′-d(CGCA[s^{5}T(6-4)T]ACGC)-3′ (**ODN 1**) and 30-mer 5′-d(GCACGACCAA*CGCA[s^{5}T(6-4)T]ACGC*AACCAGCACG)-3′ containing the 10-mer sequence at the central position (**ODN 2**) since the phosphoramidite-based synthesis of the corresponding sequences containing the biologically relevant T(6-4)T adduct had been previously reported[6,9] and due to the availability of biophysical and biological data concerning

† Electronic supplementary information (ESI) available: HPLC chromatograms of the oligonucleotides; time-dependent MALDI mass spectra of exonucleases digestion products. See http://www.rsc.org/suppdata/ob/b3/b314831a/

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these ODNs.[9–11] Each synthesis was performed on a 1 μmol scale according to the following strategy. Although the (4-*tert*-butylphenoxy)acetyl group, due to its removal with ammonia at room temperature, is recommended for the protection of the exocyclic amino function of dA, dG and dC when incorporating base sensitive modified nucleotides such as (6-4) PPs,[6,12] we decided to use standard 2-cyanoethyl phosphoramidites since conventional protecting groups (benzoyl for dA and dC and isobutyryl for dG) are removed with concentrated aqueous ammonia at room temperature,[13] conditions compatible with the s^5T(6-4)T PP stability.[7] Previous stability studies on the methylsulfenyl group of **1** had indicated that modifications of the synthesis protocol had to be done.[7] Hence, the oxidation step was performed using a 0.02 M iodine solution instead of the 0.1 M conventional solution.[7,14] The reaction time for the coupling step of **1** was extended to 20 min. Upon these conditions, the coupling efficiency of **1** was approximately 59% and 90% for the 10- and 30-mer, respectively, compared to approximately 98% for the standard phosphoramidites (25 s coupling). After chain assembly, in each series, the 5′-*O*-dimethoxytrityl (Dmt) ODN was cleaved from the support and deprotected upon treatment with concentrated aqueous ammonia at room temperature for 24 h. The 5′-Dmt-oligomers were purified by reverse-phase HPLC then the Dmt group was removed by treatment with 40% aqueous acetic acid for 1 hour.[7] Final purification was performed by reverse-phase HPLC. Deprotection of the thiol function was planned to be done at a later stage.

Oligonucleotide characterisation

In each 10- and 30-mer series, examination of the HPLC chromatogram of the detritylation reaction products revealed the formation of two oligonucleotides **ODN 3**, **ODN 4** and **ODN 5**, **ODN 6**, respectively. MALDI-MS measurements showed that oligonucleotide **ODN 3** ($[M - H]^-$ ion at *m*/*z* 2379.8) corresponded to the 8-mer 5′-d(CGCAACGC)-3′ (calculated *m*/*z* 2379.6) and **ODN 5** ($[M - H]^-$ at *m*/*z* 8514.4) corresponded to the 28-mer 5′-d(GCACGACCAACGCAACGCAACCAGCACG)-3′ (calculated *m*/*z* 8515.6). The lack of the s^5T(6-4)T PP moiety in these oligomers was likely due to an inefficient capping step of the remaining unphosphitylated nascent ODN after the coupling step of **1**. Both oligonucleotides **ODN 4** and **ODN 6** exhibited a UV absorption in the 325 nm region and were fluorescent, attesting the successful incorporation of the pyrimidone moiety.[15] MALDI mass spectrum of **ODN 4** exhibited an ion peak at *m*/*z* 3055.3 ($[M - H]^-$) corresponding to an excess of 52 amu compared to the *m*/*z* value expected for **ODN 1** (3003.1 amu). This mass excess was consistent with the addition of a cyanoethyl residue on the expected ODN. In the 30-mer series, the molecular mass of the compound required the acquisition of the mass spectrum in the linear mode, leading to an uncertainty on the mass measurement accuracy (±5 amu in this mass range). Under such conditions, **ODN 6** exhibited a signal at *m*/*z* 9195.1 ($[M - H]^-$) (calculated *m*/*z* 9139.1 for **ODN 2**). This difference was also in accordance with the presence of a cyanoethyl moiety within the expected ODN. Thus, it appeared that both **ODN 4** and **ODN 6** contained the s^5T(6-4)T PP together with an additional cyanoethyl residue.

To further confirm the molecular weight of **ODN 4**, we decided to use accurate mass measurement and high resolution mass spectrometry (HRMS), a combination of techniques now frequently applied to characterize and identify biological molecules.[16–18] Under these conditions, the monoisotopic $[M - H]^-$ ion peak of **ODN 4** was found at *m*/*z* 3054.5386, in excellent agreement with a cyanoethyl adduct structure (calc. 3054,5378).

The sequence and location of the modification within **ODN 4** were determined by exonuclease digestions coupled to accurate mass measurement by MALDI mass spectrometry.[18,19] In a first step, **ODN 4** was submitted to digestion by bovine spleen phosphodiesterase (BSP) that hydrolyses the ODN chain from the 5′ side. The mass difference between the successive ion peaks of the digestion mixture recorded at different reaction times attested the ODN sequence (data not shown). The final product of the BSP digestion of **ODN 4** gave an ion peak at *m*/*z* 1834.47, corresponding, as expected,[20,21] to the sequence 5′-d([X4]ACGC)-3′ (where X4 represents the modified dinucleotide incorporated into **ODN 4**). Snake venom phosphodiesterase (SVP) was used in a second step, directly on the 5′-d([X4]ACGC)-3′ BSP digest, to generate the 3′-to-5′ sequence information. This confirmed the oligonucleotide sequence from its 3′ side, the final product of the digestion reaction being identified as 5′-d([X4]A)-3′ (*m*/*z* 927.48) (Fig. 2). It appeared that the 3′-exonuclease was unable to hydrolyse the 3′-phosphodiester bond adjacent to [X4]. Indeed, the ability of exonucleases to cleave the phosphodiester bond adjacent to the modification depends on the type of modification. Such behaviour has already been observed in the 5′,6-cyclo-5,6-dihydropyrimidine series.[22]

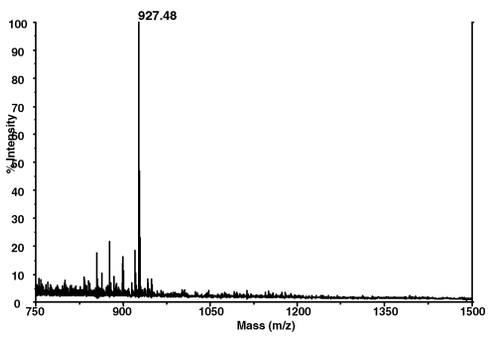


Fig. 2 MALDI mass spectrum of the tandem digestion final product of **ODN 4**.

Structural assignment of modifications within ODNs is usually performed in a two-step procedure based on electrospray ionisation.[23,24] After complete hydrolysis of ODNs into their nucleoside constituents, the mixture is identified by combined liquid-chromatography and MS/MS. We decided to use MALDI tandem mass spectrometry (MS/MS) experiments to ascertain the structure of the modification present in **ODN 4** since this method has the advantage to primarily generate singly charged ions which can be analysed directly and individually by MS/MS, reducing the need for pre-analytical separations. To our knowledge, such an approach has never been used to characterise modifications within ODNs. The digestion product ion of 5′-d([X4]A)-3′ (*m*/*z* 927.18) was thus submitted to collisional activation. The collision-induced dissociation (CID) spectrum of the $[M - H]^-$ ion at *m*/*z* 927.18 (Fig. 3) displayed a series of fragment ion peaks which were easily identified on the basis of the proposed structure (Scheme 1). Ions containing the 3′-terminal deoxyadenosyl moiety appeared at *m*/*z* 330, 314, 195, 177 whereas ions corresponding to the 5′-end of 5′-d([X4]A)-3′ appeared at *m*/*z* 792, 694, 676, 614, 596, 498. This unambiguously confirmed the presence of the cyanoethyl residue within [X4].

At this stage, the cyanoethyl group within [X4] could be possibly located on the C5-thiol or on the N^3 atom of the 5,6-dihydropyrimidine moiety although we suspected an *S*-substitution due to the stronger nucleophile character of the sulfur atom (*vide infra*).

Hypothesis for the cyanoethyl adduct formation supported by a model reaction

The origin of the cyanoethyl moiety had to be ascertained in order to be able to avoid its formation. Two hypotheses could be proposed, each beginning by the deprotection of the thiol function resulting from an intermolecular Michaelis–Arbusov-type reaction[25,26] between the disulfide bond of **1** and the P III atom of another molecule of phosphoramidite **1** (Scheme 2).

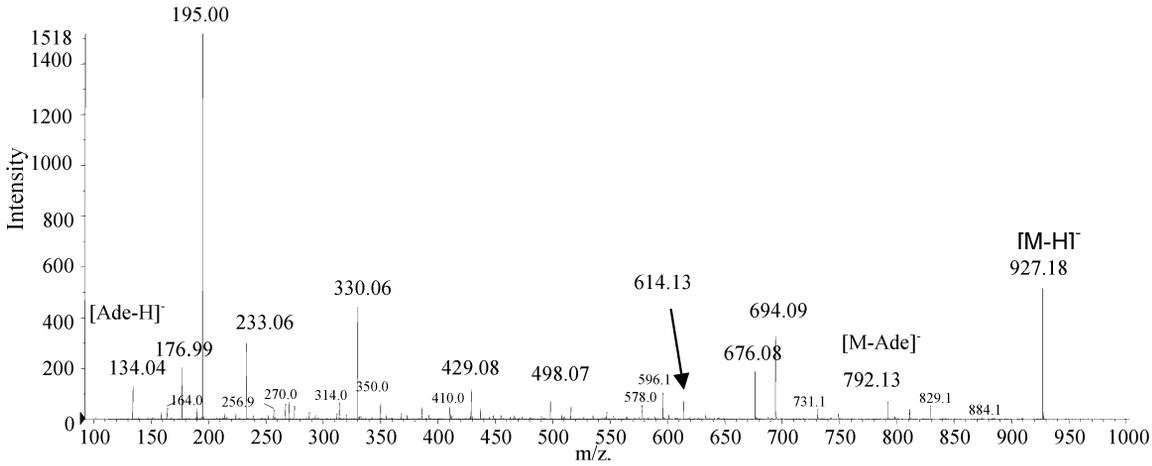
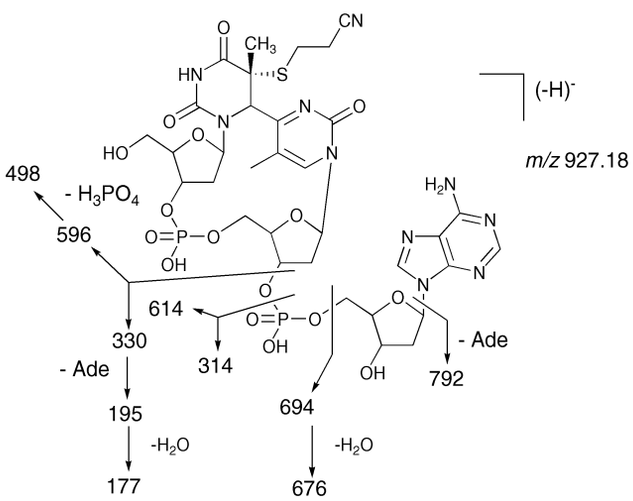


Fig. 3 MALDI-MS/MS spectrum of the ion at *m/z* 927.18 corresponding to the $[M-H]^-$ ion of the end product of the tandem digestion of **ODN 4** (5′-d([X4]A)-3′).

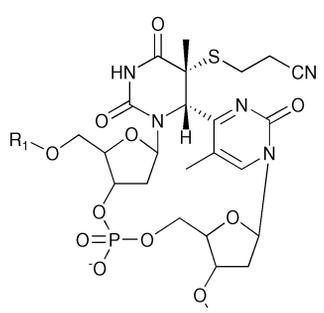


Scheme 1

This rearrangement would lead to the free thiol-containing phosphoramidite (**3**), the unreactive phosphorus-containing S-methyl phosphoramidothiolate (**4a**), and acrylonitrile (**2**). Michael addition between **3** and acrylonitrile could lead to **5**. Concomitantly, compound **4a** could also undergo a Michaelis–Arbusov reaction leading to **4b** whose free thiol could trap the acrylonitrile released during Michaelis–Arbusov reactions leading to **4c** (Scheme 2). Therefore, either **3** or **5** could be coupled to the nascent ODN. After coupling of **3**, the thiol function could be subsequently capped (Ac_2O). At the end of ODN synthesis, deacetylation would afford a free thiol that could undergo a Michael addition with acrylonitrile released during the cyanoethyl phosphate deprotection, leading to **ODN 4**. Such side reaction leading to an *S*-cyanoethyl adduct has already been reported to occur when deprotecting cyanoethyl phosphate in the presence of a free thiol.[27] Coupling of **5** would directly lead to **ODN 4**.

To demonstrate that **1** could undergo a Michaelis–Arbusov-type reaction, we first designed and studied a model reaction in which the disulfur bond and the P III belonged to two distinct molecules for the ease of analysis. For this, we used the disilyl derivative of 5-mercapto-5,6-dihydrothymidine **6** that was available in our laboratory.[28] Treatment of **6** with methyl methanethiosulfonate in the presence of K_2CO_3 afforded the mixed disulfide **7** in 80% yield (Scheme 3).

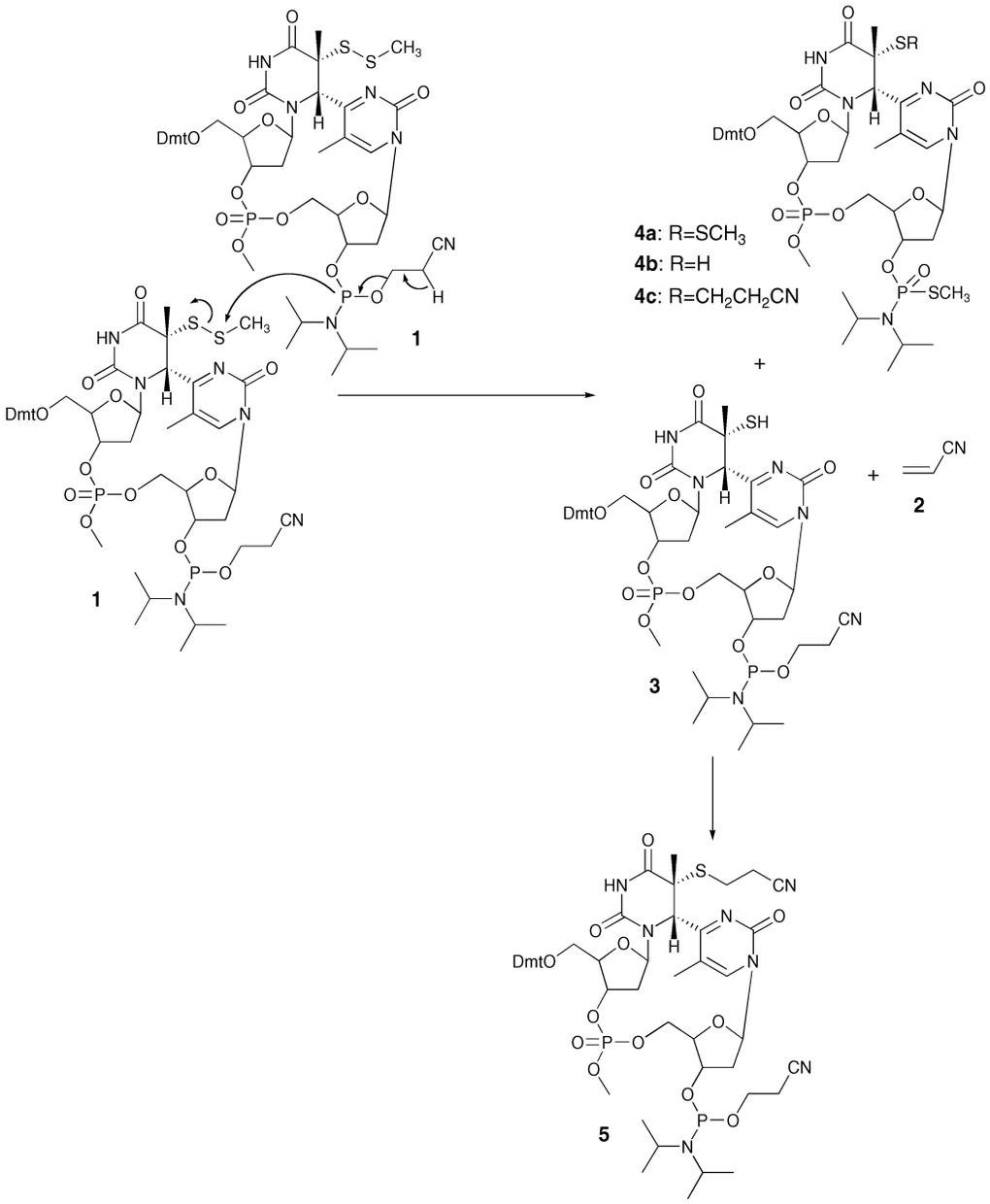
Treatment of 5′-*O*-dimethoxytritylthymidine 3′-cyanoethyl-*N*,*N*-diisopropylphosphoramidite **8** with compound **7** (0.5 eq) gave **6** in trace amount together with **9** and **10a**,**b** in 40% and 58% yield respectively. Unreacted **7** was also isolated (40%). The electrospray ionisation (ESI) mass spectrum of **9** exhibited an ion at *m/z* 580 ($[M + Na]^+$), confirming the addition of acrylonitrile. Compared to the ^{1}H NMR spectrum of **7**, the ^{1}H NMR spectrum of **9** lacked the SMe proton signal but displayed two additional vicinal groups of two protons at δ 2.63 and 2.95. These chemical shifts revealed that the cyanoethyl group was located on the sulfur atom since the protons of a CH_2 substituting the N^3 atom of thymine would resonate near δ 4.[26] This observation confirmed our initial assumption that the SH function was more likely to be alkylated than the N^3 position and unambiguously settled the structure of **ODN 4** and **ODN 6**.



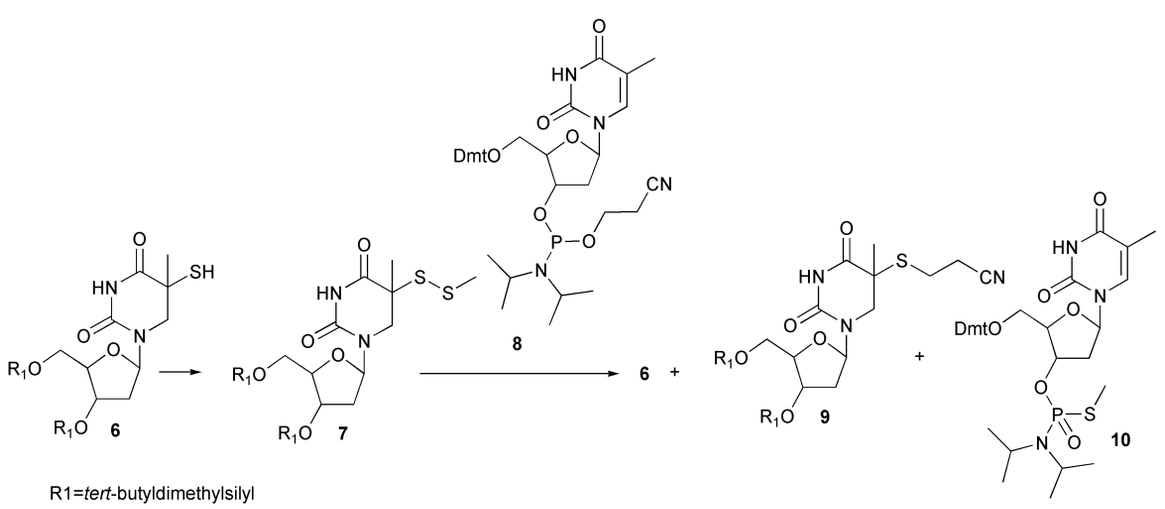
ODN 4: R_1=5′-d(CGCAp); R_2= (pACGC)-3
ODN 6: R_1=5′-d(GCACGACCAA*CGCAp*);
R_2= (*pACGC*AACCAGCACG)-3′

Compounds **10a**,**b** turned out to be diastereomers. One of the diastereomers could be isolated in pure form and its ^{1}H NMR spectrum, compared to the one of **8**, evidenced the absence of the 2-cyanoethyl proton signals. Conversely, one doublet at δ 2.22 (3H, J = 15 Hz) was present. Analysis of the ^{31}P NMR spectrum revealed the presence of one phosphorus resonance at δ 34.16, a chemical shift consistent with a *S*-methyl phosphoramidothiolate structure[30] and therefore, the methyl signal at δ 2.22 was attributed to the P-SMe group. The ESI (+) mass spectrum of this diastereomer exhibited a major peak at *m/z* 760 ($[M + Na]^+$) consistent with the structure assigned to **10** (Scheme 3). Therefore, this model reaction demonstrated that the *S*-methylsulfenyl group, in this structure context, is prone to undergo a Michaelis–Arbusov rearrangement with a phosphoramidite, leading to a *S*-methyl phosphoramidothiolate.

These results triggered the careful examination of the reactivity of **1**. Upon storage in acetonitrile, phosphoramidite **1** slowly decomposed leading to a complex mixture of products as revealed by ^{1}H NMR analysis. Among these latter, the major compound turned out to be an equimolecular diastereomeric



Scheme 2



Scheme 3

mixture. On its ^{1}H NMR spectrum, the most important feature was the absence of the SMe and cyanoethyl proton signals. Conversely, two doublets near δ 2.3 (3H, J = 15 Hz) that collapsed to a broad singlet upon broad band phosphorus decoupling were present. Analysis of the ^{31}P NMR spectrum revealed the presence of two phosphorus resonances at δ 35.6

and 35.9. Finally, the ESI (+) mass spectrum of the mixture of diastereomers exhibited a major peak at *m*/*z* 1094 ([M + Na]$^+$). Taken together, these data confirmed that the two compounds corresponded to **4b**, therefore evidencing that the disulfide bond of phosphoramidite **1** can undergo a Michaelis–Arbusov rearrangement.

Attempts to avoid the Michael addition to the C5 thiol of acrylonitrile: characterisation of the new ODNs

Although we did not obtain the expected ODNs, **ODN 1** and **ODN 2**, our study indicated that it was possible to incorporate the pyrimidine (6-4) pyrimidone motif. Indeed, if **ODN 4** and **ODN 6** had been produced by incorporation of **3** and trapping of the acrylonitrile released during the phosphate deprotection step, it should be possible to obtain the desired ODNs by quenching the acrylonitrile with an excess of thiol during the final deprotection of the oligonucleotide.[27a,31] Thus, we modified our ODN synthesis protocol and deprotection conditions. The 10-mer of sequence **ODN 1** was synthesised. To avoid the elongation of the nascent ODN having misincorporated the building block resulting in the formation of **ODN 3**, we performed a double capping step after the coupling of **1** with 81% efficiency. The cleavage from the support and the deprotection of the ODN was carried out using concentrated aqueous ammonia containing 50 mM dithiothreitol (DTT) or 2% β-mercaptoethanol (BME). The 5′-*O*-Dmt oligomer was purified by reverse-phase HPLC then the Dmt group was removed by treatment with 40% aqueous acetic acid for 1 h and the resulting ODN purified by RP HPLC. Two ODNs (**ODN 7** and **ODN 8**) were obtained.

Oligodeoxynucleotide **ODN 7** was fluorescent, attesting again the incorporation of the pyrimidone motif. Its HR MALDI mass spectrum showed an [M − H]$^-$ ion peak at *m*/*z* 2969.5364, a difference of 31.9749 compared to the calculated mass for **ODN 1** (M 3002.5191). This difference evidenced the loss of the sulfur atom at C5. Thus, **ODN 7** could correspond to the 10-mer containing the h^{5}T(6-4)T PP.

The HR MALDI mass spectrum of the second oligonucleotide (**ODN 8**) showed an [M − H]$^-$ ion peak at *m*/*z* 2986.5629, 17.0265 amu higher than that of **ODN 7**. As the pyrimidone motif was not present (no fluorescence), this suggested that this oligomer could correspond to the addition, in **ODN 7**, of ammonia at C6 of the pyrimidone, leading to an amino-substituted dihydropyrimidone moiety.

As for **ODN 4**, the sequence and location of the modification within **ODN 7** and **ODN 8** were gathered from time dependent MALDI mass spectra of the BSP digestion followed by the SVP digestion. The MALDI mass spectra of the tandem digestion final products of **ODN 7** and **ODN 8** afforded 5′-d([X7]A)-3′ (*m*/*z* 842.98) and 5′-d([X8]A)-3′ (*m*/*z* 859.43), respectively (Fig. 4 and 5, respectively). As in the case of **ODN 4**, SVP was unable to cleave the 3′-phosphodiester bond adjacent to the modification. The excess of 17 Da between the end-products of

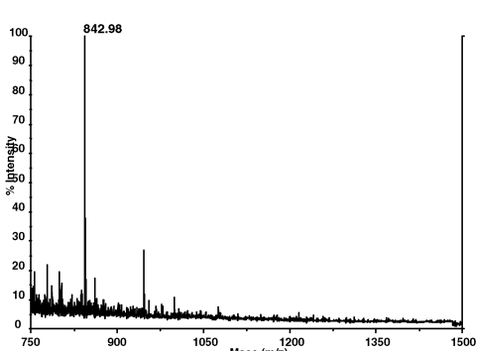
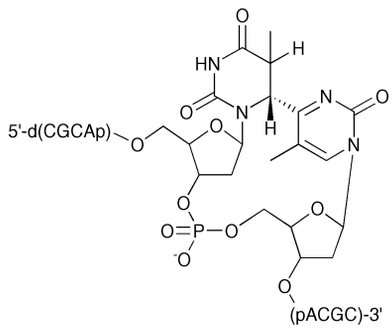
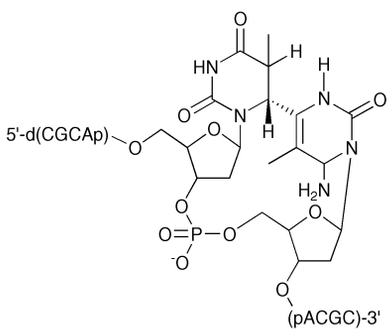


Fig. 4 MALDI mass spectrum of the tandem digestion final product of **ODN 7**.



ODN 7



ODN 8

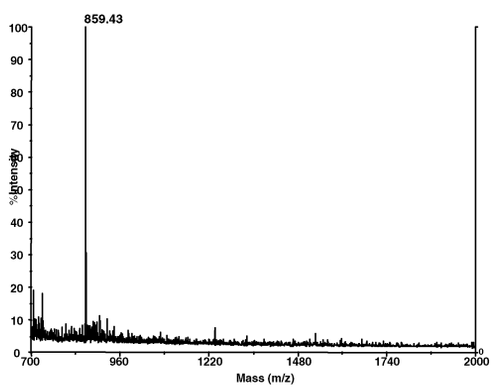


Fig. 5 MALDI mass spectrum of the tandem digestion final product of **ODN 8**.

ODN 7 and **ODN 8** confirmed the location of the ammonia molecule on the 5′-d([X8]A)-3′ sequence.

As in the case of **ODN 4**, the structural assignment of the photomodified bases within oligodeoxynucleotides **ODN 7** and **ODN 8** was supported by MALDI-MS/MS experiments performed on the tandem digestion product ions at *m*/*z* 842.19 (5′-d([X7]A)-3′) and 859.21 (5′-d([X8]A)-3′), respectively. The CID spectrum of the [M − H]$^-$ ion at *m*/*z* 842.19 (Fig. 6), displayed a series of fragment ion peaks which were easily identified on the basis of the proposed structure of this ODN.

The assignment of the fragment ions was in agreement with those observed on the MS/MS spectrum of the [5′-d([X4]A)-3′-H]$^-$ ion (Fig. 3). All ions containing the 3′-terminal 2′-deoxyadenosyl moiety appeared at the same *m*/*z* values (330, 314, 195, 177) whereas ions corresponding to the 5′-end of 5′-d([X7]A)-3′ were shifted at lower *m*/*z* ratios by a 85 amu difference with regard to those arising from collision-induced dissociation of the deprotonated 5′-d([X4]A)-3′ molecule (*m*/*z* 707, 609, 591, 529, 511, 413).

The 5′-d([X8]A)-3′ digest exhibited a significantly different behaviour under collisional activation conditions (Fig. 7). Whereas ions containing the 3′-terminal deoxyadenosyl moiety appeared at the same *m*/*z* values as observed for 5′-d([X4]A)-3′ and 5′-d([X7]A)-3′ (*m*/*z* 330, 314, 195, 177), the high mass range of the MS/MS spectrum displayed specific fragmentation pathways. The loss of 43 mass units from the deprotonated

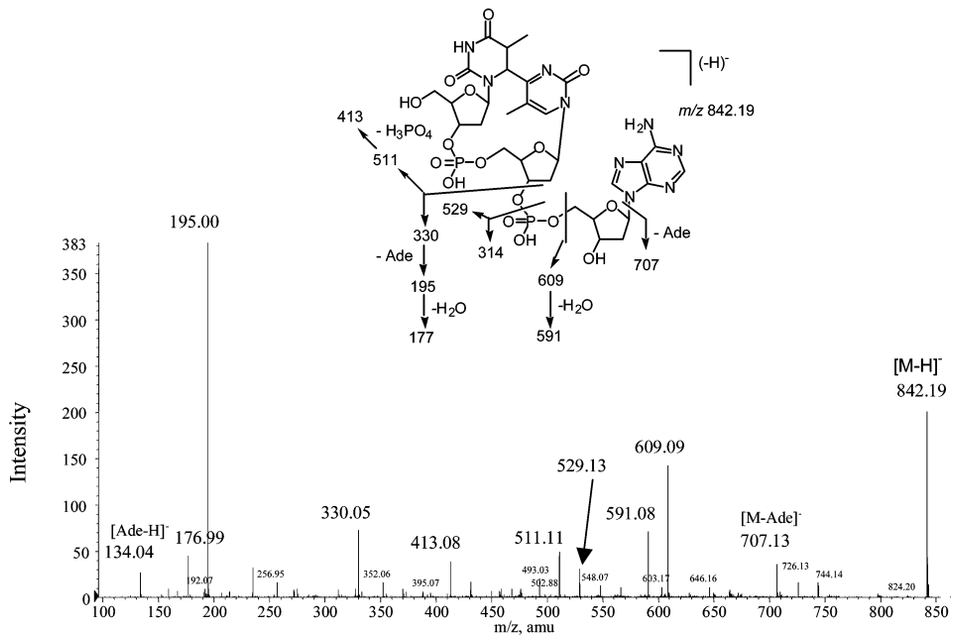


Fig. 6 MALDI-MS/MS spectrum of the ion at *m*/*z* 842.19 corresponding to the $[M - H]^-$ ion of the end product of the tandem digestion of **ODN 7** (5′-d([X7]A)-3′).

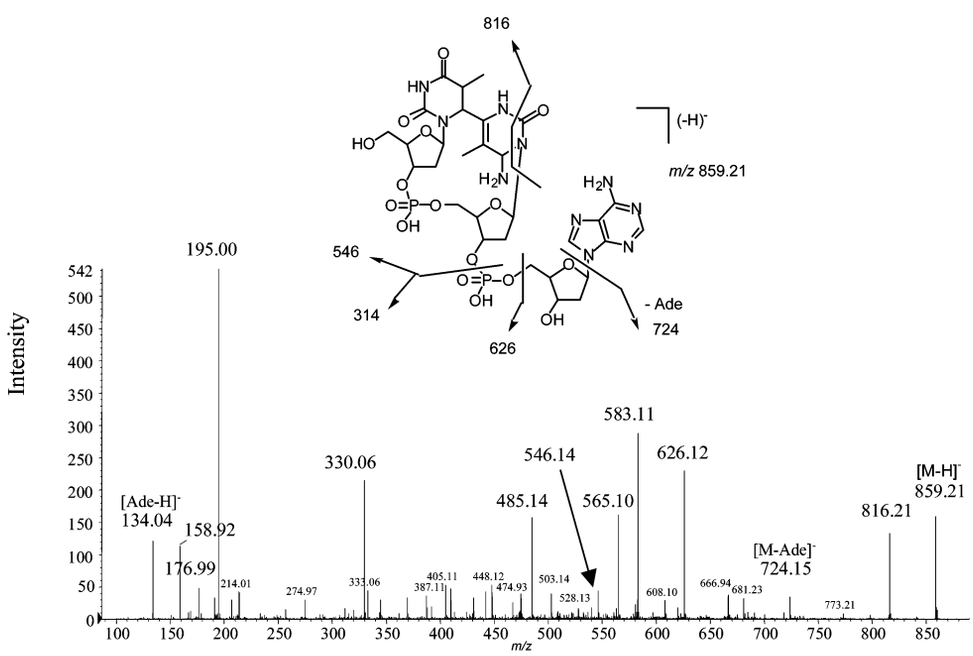


Fig. 7 MALDI-MS/MS spectrum of the ion at *m*/*z* 842.19 corresponding to the $[M - H]^-$ ion of the end product of the tandem digestion of **ODN 8** (5′-d([X8]A)-3′).

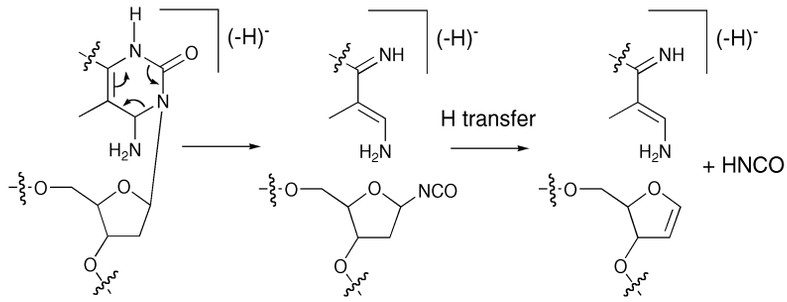
molecule (*m*/*z* 816) was also observed for the fragment ion at *m*/*z* 626 (corresponding to the *m*/*z* 609 ion for 5′-d([X7]A)-3′ (Fig. 6) and *m*/*z* 694 ion for 5′-d([X4]A)-3′ (Fig. 3)), giving rise to a second generation fragment ion at *m*/*z* 583. This latter underwent two further decompositions attributed to H_2O and H_3PO_4 neutral losses (*m*/*z* 565 and 485, respectively).

The initial loss of 43 mass units from both the $[M - H]^-$ and the *m*/*z* 626 ions could be rationalized by the presence, consecutively to the addition of an ammonia molecule, of a dihydropyrimidone moiety in 5′-d([X7]A)-3′. Rearrangement of the dihydropyrimidone followed by an intramolecular proton transfer thus allowed the loss of HNCO (43 amu) as shown in Scheme 4.

Discussion

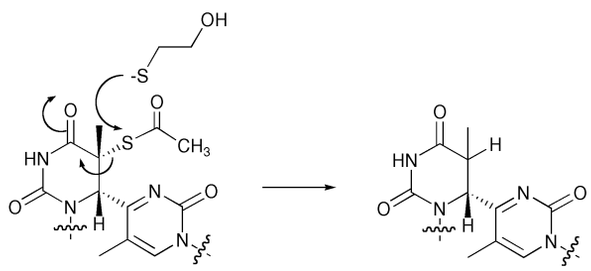
Formation of **ODN 7** and **ODN 8** indicates that the presence of DTT or BME, during the ammonia deprotection, is efficient to prevent the formation of **ODN 4** (removal of the cyanoethyl adduct by BME or DTT and subsequent reactions is unlikely[27a]) but does not allow the formation of **ODN 1**. Thus, since no *S*-cyanoethyl adduct-containing oligonucleotide was observed upon ammonia deprotection in the presence of BME or DTT, it is likely that **ODN 4** derived from acrylonitrile generated in the course of the phosphate deprotection and not in the course of the Michaelis-Arbusov rearrangement. Hence, this indicates that **1** has been incorporated under its thiol deprotected form (**3**) and hence that the methylsulfenyl thiol protection is not incompatible with the phosphoramidite-based oligonucleotide synthesis but requires that, either the phosphate protecting groups have to be changed, or another system has to be used to quench the acrylonitrile released during the cyanoethyl phosphotriester deprotection.

Although unexpected, formation of **ODN 7** is interesting since this ODN could be a valuable tool to probe the structure–mutagenicity relationships of (6-4) PP.[32] This synthetic pathway



Scheme 4

can be considered as an alternative to the approach consisting to site specifically incorporate the known h[5](6-4) derivative formed from the s[5](6-4) PP by Raney nickel treatment[33] into ODNs. The formation mechanism of the h[5](6-4) derivative, during ODN synthesis, could be possibly due to the nucleophilic attack of the thiolate ion of BME on the sulfur atom of the C5 acetylthio derivative of the (6-4) adduct (Scheme 5).[34]



Scheme 5

Formation of **ODN 8** is also somewhat puzzling although it has been previously suggested that ammonia could add across the double bond of the pyrimidone.[35] Since addition of ammonia was not observed during the first syntheses, it can be proposed that the strongly nucleophilic sulfur atom of BME adds first to position C6 of the pyrimidone of **ODN 7** and that, through iminium formation, it is replaced by an ammonia molecule to lead to **ODN 8** (Scheme 6).

It is worth mentioning that our study demonstrates that the ability of the *S*-methylsulfenyl group to undergo a Michaelis–Arbusov reaction with phosphoramidites is quite different from that of the *tert*-butylsulfenyl group[8] or other alkylsulfenyl groups.[14] Interestingly, the methylsulfenyl group has been used to protect the thiocarbonyl function of 4-thiothymine for its incorporation into oligonucleotides by the phosphoramidite route.[35] The authors observed that a significant amount of 5-methylcytosine- and thymine-containing oligonucleotides was obtained consecutively to the cleavage of the disulfide bond. From our results, it is possible that their *S*-methyl disulfide had undergone a Michaelis–Arbusov reaction leading to its cleavage.

Finally, the total regioselectivity observed during the Michaelis–Arbusov reaction leading to the preferential scission of the unsymmetrical disulfide bond can be rationalized by the polarization of this bond.[26] The electron withdrawing character of the C4 carbonyl at position α with respect to the C5 thiol in **1** likely polarizes the disulfide bond in order to favor the nucleophilic attack of the P III on the methylsulfenyl group leading to the deprotection of the dihydropyrimidine C5 thiol. The ease of highly polarized disulfide bonds to undergo Michaelis–Arbusov reactions has been used to synthesize intra and inter nucleoside phosphorothiolate linkages[36] and more recently to prepare 2′,3′-cyclic phosphate terminated oligonucleotides.[37]

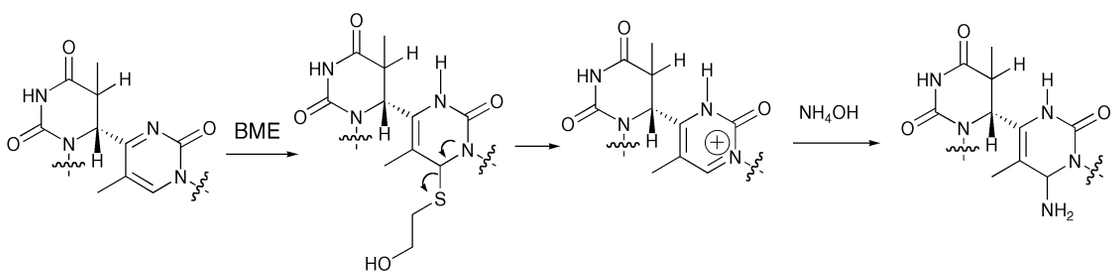
Conclusion

This study has clearly evidenced that **1** can be introduced in ODNs. However, the stability of the *S*-methylsulfenyl protecting group is not sufficient to give rise to the expected ODN using standard final deprotection procedures and the use of ordinary thiol scavengers does not represent a suitable solution. Analysing the synthetic ODNs by HR MALDI mass spectrometry combined with exonuclease digestions and tandem mass spectrometry has allowed to understand the reactions that have occurred. Cleavage of the disulfide bond of **1** arises from a Michaelis–Arbusov rearrangement that probably occurs during its incorporation although a partial and iterative cleavage of the disulfide bond during each coupling step by the incoming phosphoramidites cannot be totally ruled out. In both cases, the generated free thiol is acetylated during the subsequent capping step leading finally to an *S*-acetyl derivative. This signifies that the synthetic remaining difficulty is the *S*-deacetylation with respect of the s[5]T(6-4)T integrity. For this purpose, the use of 2,2′-dithiodipyridine[27a] or 2,2′-dithiodipyridine and phenol[27b] with ammonia has already been proposed. Precise conditions allowing efficiently such a deprotection are currently investigated in our laboratory.

Experimental

General

CH_3CN was dried by heating under reflux with P_2O_5. Methanol was distilled over magnesium methoxide. *N*,*N*-Diisopropylethylamine and CH_2Cl_2 were dried by distillation from calcium hydride. Thin-layer and column chromatography were carried out on silica gel 60 F_{254} 60–15 μm and silica gel 6–35 μm or 35–70 μm, respectively, from SDS (Peypin, France). ^1H and ^{13}C NMR spectra were recorded on a Bruker AM300 instrument. ^1H Chemical shifts (δ) are reported in ppm relative to TMS



Scheme 6

(δ 0.00) in $CDCl_3$. ^{13}C Chemical shifts are reported in ppm relative to solvent peak ($CDCl_3$ δ 77.7). ^{31}P NMR spectra were recorded on a Bruker Avance 600 instrument. Chemical shifts are reported relative to an external capillary standard of 85% phosphoric acid (δ 0.00). HR FAB mass spectra (glycerol matrix) were carried out using a ZabSpec/T spectrometer (Micromass, Manchester, UK). HR MALDI mass spectra (2,4,6-trihydroxyacetophenone matrix) were carried out using a Perseptive Voyager DE STR MALDI time-of-flight mass spectrometer (Perseptive Biosystems, Framingham, Ma).

3′5′-Bis-*O*-(*tert*-butyldimethylsilyl)-5-mercapto-5,6-dihydrothymidin-5-yl methyl disulfide 7

To an anhydrous methanolic solution of **6** (153 mg, 0.30 mmol; 4 ml) was added K_2CO_3 (91 mg, 2.2 eq) then $CH_3SO_2SCH_3$ (75.7 µL; 2 eq) in CH_3OH (100 µL). The reaction was stirred at room temperature for 5 min. The mixture was concentrated and the crude product was purified by flash chromatography on silica gel using a gradient of AcOEt in heptane (0–20%) to give compound **7** (133 mg, 80%). ^1H NMR (300 MHz, $CDCl_3$): δ 0.08 and 0.09 (12H, 2 s, *t*-BDMS), 0.90 and 0.92 (18H, 2 s, *t*-BDMS), 1.58 (3H, s, CH_3), 2.04 (2H, m, H2′, H2″), 2.42 (3H, s, $SSCH_3$), 3.50 (2H, m, H6a, H6b), 3.71 (2H, m, H5′, H5″), 3.78 (1H, m, H4′), 4.37 (1H, m, H3′), 6.34 (1H, dd, J = 6, 8 Hz, H1′), 7.70 (1H, s, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ −4.7, −4.1, −3.9 (*t*-BDMS), 18.7 and 19.1 (*t*-BDMS), 20.7 (CH_3), 24.6 ($SSCH_3$), 26.4 and 26.7 (*t*-BDMS), 38.6 (C2′), 47.6 (C6), 50.8 (C5), 63.6 (C5′), 72.6 (C3′), 84.3 (C1′), 87.2 (C4′), 152.5 (C2), 170.9 (C4). HRMS (FAB) $(M + H)^+$ Calcd for $C_{23}H_{47}O_5N_2Si_2S_2$ 551.2465, found 551.2458.

Reaction of 7 with phosphoramidite 8

To compound **7** (42 mg, 0.076 mmol) was added 53 µL of *N*,*N*-diisopropylethylamine then 1.1 mL of anhydrous CH_2Cl_2 followed by phosphoramidite **8** (115.5 mg, 0.152 mmol). After 48 h of stirring, the reaction was diluted with AcOEt, washed with a saturated aqueous solution of $NaHCO_3$ then dried over sodium sulfate. The crude reaction was purified by silica gel chromatography using a gradient of AcOEt in heptane (30 to 60%).

Compound **7**: 16 mg, yield 40%.

Compound **9**: 17 mg, yield 40% (based on **7**); ^1H NMR (300 MHz, $CDCl_3$): δ 0.08 and 0.09 (12H, 2s, *t*-BDMS), 0.90 and 0.92 (18H, 2 s, *t*-BDMS), 1.57 (3H, s, CH_3), 2.05 (2H, m, H2′, H2″), 2.63 (2H, m, SCH_2CH_2CN), 2.95 (2H, m, SCH_2CH_2CN), 3.44 (2H, m, H6a, H6b), 3.71 (2H, m, H5′, H5″), 3.78 (1H, m, H-4′), 4.35 (1H, m, H-3′), 6.34 (1H, dd, J = 6; 8 Hz, H-1′), 7.73 (1H, s, NH). HRMS (MALDI) $(M + Na)^+$ Calcd for $C_{25}H_{47}O_5N_3Si_2SNa$ 580.2656, found 580.2672.

Compound **10**: 33 mg, yield 58% (based on **7**). Data of one isomer: ^1H NMR (300 MHz, $CDCl_3$): δ 1.25 (12H, d, J = 7 Hz, isopropyl), 1.37 (3H, s, CH_3), 2.22 (3H, d, J = 15 Hz, $PSCH_3$), 2.43 (1H, m, H2′a), 2.60 (1H, m, H2″a), 3.46 (2H, m, H5′, H5″), 3.54 (2H, m, isopropyl), 3.79 (6H, s, DMT), 4.27 (1H, br s, H4′), 5.23 (1H, m, H3′), 6.45 (1H, dd, J = 9; 5 Hz, H1′), 6.83, (4H, J = 8 Hz, DMT), 7.41–7.22 (m, 9H, DMT), 7.61 (1H, s, H6), 8.09 (1H, br s, NH). ^{31}P NMR (243 MHz, CD_3CN): δ 34.16. HRMS (MALDI) $(M + Na)^+$ Calcd for $C_{38}H_{48}O_8N_3SPNa$ 760.2791, found 760.2797.

Synthesis and purification of ODNs

ODNs were synthesised on an Applied Biosystems, 392 DNA/RNA synthesizer in 1 µmol-scale using standard phosphoramidites purchased from Applied Biosystems (Courtaboeuf, France) and **1**. Phosphoramidite **1** was dissolved in CH_3CN at a concentration of 0.1 M. The coupling time used for incorporation of **1** was extended to 20 min and was followed, in the second series of synthesis, by a double capping. After chain elongation (DMT ON), ODNs were cleaved from the CPG support and were deprotected simultaneously by treatment with a concentrated aqueous ammonia solution or a concentrated aqueous ammonia solution containing 50 mM DTT or 2% BME at room temperature for 24 h in the dark. The ammonia was removed by evaporation and tritylated ODNs were purified by reverse-phase C-18 chromatography using a Waters Prepak Delta-Pak C4 25 × 100 mm (15 µm, 300 Å) column, at a flow rate of 6 mL min^{-1}. Tritylated ODNs of the first series of synthesis were purified using 5% of acetonitrile in 0.1 M triethylammonium acetate, (pH 7) for 15 min followed by a linear gradient (5 to 10% for 20 min), a plateau for 10 min, a gradient (10 to 40% for 15 min) and a plateau for 10 min for the 10-mer and 5% of acetonitrile in 0.1 M triethylammonium acetate, (pH 7) for 15 min followed by a linear gradient (5 to 40% during 50 min) for the 30-mer. The tritylated 10-mer of the second series of synthesis was purified using a linear gradient of acetonitrile (5 to 40% for 40 min) in 0.1 M triethylammonium acetate, (pH 7). After concentration, the 5′-Dmt ODNs were treated with 40% aqueous acetic acid for 1 h. After concentration to dryness, deprotected ODNs of the first synthesis series were purified by reverse-phase HPLC on a Waters Delta-Pak C18 3.9 × 150 mm (5 µm, 300 Å) column, at a flow rate of 1 mL min^{-1} using a linear gradient of acetonitrile (6 to 16% during 35 min) in 0.1 M triethylammonium acetate, (pH 7) (**ODN 3** retention time: 14 min; **ODN 4** retention time: 19 min) or a linear gradient of acetonitrile (8 to 16% during 40 min) in 0.1 M triethylammonium acetate, (pH 7) (**ODN 5** retention time: 31 min, **ODN 6** retention time: 33 min). Deprotected ODNs of the second synthesis series were purified on Waters Prepak Delta-Pak C18 25 × 100 mm (15 µm, 300 Å) column using a linear gradient of acetonitrile (5 to 20% for 55 min) in 0.1 M triethylammonium acetate (pH 7) at a flow rate of 6 mL min^{-1} (**ODN 7** retention time 23 min; **ODN 8** retention time 21 min). Oligonucleotides were lyophilised and stored at −20 °C.

Enzymatic digestions

Snake Venom Phosphodiesterase (SVP), and Bovine Spleen Phosphodiesterase (BSP) were obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). In a first step, 10 µL of BSP solution (1.85 mU μL^{-1}) were added to 50 µL of the ODN solution (10 pmol μL^{-1}). The digestion reaction mixture was kept at room temperature. A 2 µL aliquot of the digest solution was removed every 10 min during the first hour, and every half an hour thereafter over a total digestion period of 2 h. The sample was held on dry ice for 5 s to quench the reaction, mixed with 8 µL of the MALDI matrix solution, immediately spotted on the MALDI plate, and air-dried for analysis. In a second step, 10 µL of SVP solution (3 mU μL^{-1}), 10 µL of 100 mM $MgCl_2$, and finally 10 µL of 100 mM ammonium citrate (pH adjusted to 9.4 with NH_4OH) were added to the BSP digestion end product. The conditions of the time-dependent studies were the same as those of the BSP digestion.

MALDI mass spectrometry measurements

Materials and reagents. The matrix component 2,4,6-trihydroxyacetophenone (2,4,6-THAP) was purchased from Aldrich (Saint Quentin Fallavier, France). This matrix was of the highest grade available and used without purification. Magnesium chloride hexahydrate and ammonium citrate were obtained from Aldrich. Water was purified by a Millipore water purification system and had a resistivity ≥ 18 MΩ cm^{-1}. All synthetic oligonucleotides (dT5, dT10) were purchased from MWG Biotech (Courtaboeuf, France) except for 5′-d(CGCATTACGC)-3′ which was synthesized in our laboratory.

Mass spectrometry measurements. The matrix mixture was prepared by dissolving 2,4,6-THAP (10 mg, 0.05 mmol) in 100 µL of methanol and 50 µL water, and further adding 50 µL

of a 100 mM ammonium citrate solution. The analyte (1 μL, 10 pmol μL^{-1}) and 9 μL of the matrix solution were mixed together and 1 μL of the resulting solution was spotted on the MALDI plate and air dried. 1 μL of dT10 and 5′-d(CGCATTACGC)-3′ (10 μM aqueous solutions) were used as internal standards for accurate mass measurements.

Low resolution MALDI mass spectra were obtained with a Voyager Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, Ma, USA) using trihydroxyacetophenone as matrix.

A Perseptive Voyager DE STR MALDI time-of-flight mass spectrometer (Perseptive Biosystems, Framingham, Ma), equipped with a Tektronix TDS 540C digital oscilloscope (500 MHz, digitization rate 2 Gigasamples s^{-1}) was used for accurate mass measurements and to follow the ODN digestions by BSP and SVP exonucleases. The instrument was used in the reflector mode (RDE) with a delayed extraction.

A hybrid tandem mass spectrometer API QSTAR® Pulsar i (MDS SCIEX, Toronto, Canada), equipped with the oMALDI™ ion source was used for MS/MS experiments on the resulting digestion products. Laser pulses were generated by a nitrogen laser source (337 nm) and transferred to the target by an optic fibre. MALDI-generated ions were extracted with 50 V into a quadrupole ion guide (q0) which provided collisional cooling. For all experiments, a mass window of 1 *m*/*z* value was set for the precursor ion selection by the analysing quadrupole (q1). Nitrogen was used as collision gas in the quadrupolar collision cell (q2) at a pressure of 0.382 mTorr. The collision energy ranged from 40 to 80 eV. An acceleration potential of 4 kV with a repetition rate of 6 kHz was used to introduce the ions into the orthogonal reflectron TOF mass analyser.

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28 A. Loukaci, J.-L. Fourrey, P. Clivio, to be published. Spectral data of compound **6**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.08 and 0.09 (12H, 2s, *t*-BDMS), 0.90 and 0.92 (18H, 2 s, *t*-BDMS), 1.61 (3H, s, CH_3), 2.05 (2H, m, H2′, H2″), 2.51 (1H, s, SH), 3.43 (2H, m, H6a, H6b), 3.71 (2H, m, H5′, H5″), 3.80 (1H, m, H-4′), 4.37 (1H, m, H-3′), 6.37 (1H, dd, J = 6; 8 Hz, H-1′), 8.20 (1H, s, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ −4.7; −4.2; −4.0 (*t*-BDMS), 18.6 and 19.0 (*t*-BDMS), 24.0 (CH_3), 26.4 and 26.6 (*t*-BDMS), 38.7 (C2′), 43.9 (C5), 49.5 (C6), 63.6 (C5′), 72.6 (C3′), 84.2 (C1′), 87.2 (C4′), 152.9 (C2), 172.2 (C4). HRMS (FAB) $(M + H)^+$ Calcd for $C_{22}H_{45}O_5N_2Si_2S$ 505.2588, found 505.2582.
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