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► **To cite this version:**

T Guerlavais, Albert Meyer, Françoise Debart, J-L Imbach, François Morvan, et al.. Use of MALDI-TOF mass spectrometry to monitor solid-phase synthesis of oligonucleotides. *Analytical and Bioanalytical Chemistry*, 2002, 374 (1), pp.57-63. 10.1007/s00216-002-1395-7 . hal-04600638

**HAL Id: hal-04600638**

**<https://hal.science/hal-04600638v1>**

Submitted on 4 Jun 2024

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## Use of MALDI–TOF mass spectrometry to monitor solid-phase synthesis of oligonucleotides

**Abstract** MALDI–TOF mass spectrometry has been used to characterize solid-supported oligonucleotides containing natural and non-natural and non-nucleoside moieties and a variety of internucleosidic linkages including phosphate and phosphite triesters and H-phosphonate diesters. This technique was used to follow the reactions involved in oligonucleotide synthesis; this enabled direct control of the elongation and optimization of the coupling process.

**Keywords** MALDI–TOF MS · Oligonucleotides · Analogs · Solid-phase synthesis

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### Introduction

The increasing demand for oligonucleotides (ON) as tools in molecular biology, for diagnostic purposes, and as potential therapeutics has led to an interest in their large-scale synthesis on solid supports. Monitoring solid-phase synthesis of oligonucleotides (ON) by common spectroscopic techniques is critical for analysis of the compounds while still anchored to the solid supports, to evaluate the efficiency of chain elongation process and to optimize reaction conditions involved in ON assembly.

Gel-phase  $^{31}\text{P}$  NMR [1] and  $^{31}\text{P}$  CPMAS NMR [2] have been used to analyze ON bound to polystyrene resin and controlled-pore-glass (CPG), respectively. Specific information on the chemistry of the phosphorus atom of the ON backbone is obtained but nothing about the nucleotide sequence. These NMR techniques were, furthermore, limited to short ON sequences (dimers and trimers) and required high quantities of material (5–17  $\mu\text{mol}$  supported ON). As a result of such a consuming process it does not

seem to us that  $^{31}\text{P}$  NMR could be used to monitor each individual step during ON synthesis.

Mass spectrometry and, especially, MALDI–TOF MS is a less product-consuming analytical method than NMR. Peptides [3, 4], carbohydrates [5], and, more recently, ON [6] covalently linked to a solid support by means of a photo-labile linker have been directly analyzed by MALDI–TOF MS. In these experiments the UV laser light was used to induce photo-cleavage of the analyte from the solid support and to promote its gas phase ionization. For ON, however, we found that the longer the ON the harder was the release of the full-length ON upon laser irradiation [7], certainly because of absorption of the UV light by the nucleobases. Furthermore, the *o*-nitrophenyl-1,3-propanediol moiety [8] used as the photo-labile linker connecting the solid support and the ON is not as common as the standard succinyl linker [9].

We recently reported [10] that MALDI–TOF MS of phosphotriester or phosphorothionotriester ON linked to a CPG solid support by means of the standard succinyl linker enabled direct and easy read-out of the nucleotide sequence of the ON and access to the internucleosidic backbone chemistry. We would like to show herein that this method is general and can be used to characterize still anchored ON bearing non-nucleosidic residues and a variety of internucleosidic linkages, including  $\text{P}_{\text{III}}$  H-phosphonate diester and phosphite triester linkages. MALDI can, moreover, be used to monitor oligonucleotide elongation step-by-step, thus enabling more rapid optimization of reaction conditions [11]. (Part of this work was presented at the XIV International Round Table on Nucleosides, Nucleotides, and their Biological Applications, San Francisco, USA, September 10–14, 2000 and published in the proceedings [11]).

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### Experimental

Oligonucleotide synthesis

ON were synthesized by use of an Applied Biosystems Model 381A DNA synthesizer, and procedures and reagents for standard

phosphoramidite or H-phosphonate chemistries. A solution of 3*H*-1,2-benzodithiol-3-one 1,1-dioxide [12] (0.05 mol L<sup>-1</sup>, Beaucage reagent) in acetonitrile was used as oxidizer for formation of thionophosphotriester internucleosidic linkages. Standard iodine-water or anhydrous *tert*-butyl hydroperoxide (Fluka) in dichloromethane were used as oxidizers to obtain phosphotriester ON.

5-*O*-Dimethoxytrityl-1,2-dideoxyribose-3-[(2-cyanoethyl)-(*N,N*-diisopropyl)]-phosphoramidite (dSpacer phosphoramidite), 3-*O*-dimethoxytrityl propyl-1-[(2-cyanoethyl)-(*N,N*-diisopropyl)]-phosphoramidite (Spacer C3 phosphoramidite), and [(3',6'-dipivaloylfluoresceinyl)-6-carboxymidohexyl]-1-*O*-(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidite were obtained from Glen Research and incorporated in ON as standard nucleoside phosphoramidites. The CPG-supported ON containing LNA T was obtained from Exiqon A/S (Vedbæk, Denmark).

#### MALDI-TOF MS analysis

MALDI-TOF mass spectra were recorded on a Voyager (Perseptive Biosystems, Framingham, MA, USA) mass spectrometer equipped with an N<sub>2</sub> laser (337 nm). MALDI conditions were: accelerating potential, 24,000 V; guide wire, 0.05% of accelerating voltage; grid voltage, 94% of accelerating voltage; delay extraction time, 550 ns. The spectra were not smoothed.

The supported oligonucleotides (0.2–1 mg) were suspended in 8–10 μL of the matrix 2,4,6-trihydroxyacetophenone [13] (THAP, 45 mg, ammonium citrate, 4 mg) in 500 μL acetonitrile–water, 50:50 (v/v). The beads–matrix mixture (1 μL) was spotted on the stainless steel probe tip and left to dry in air. The samples were then subjected to MALDI analysis.

## Results and discussion

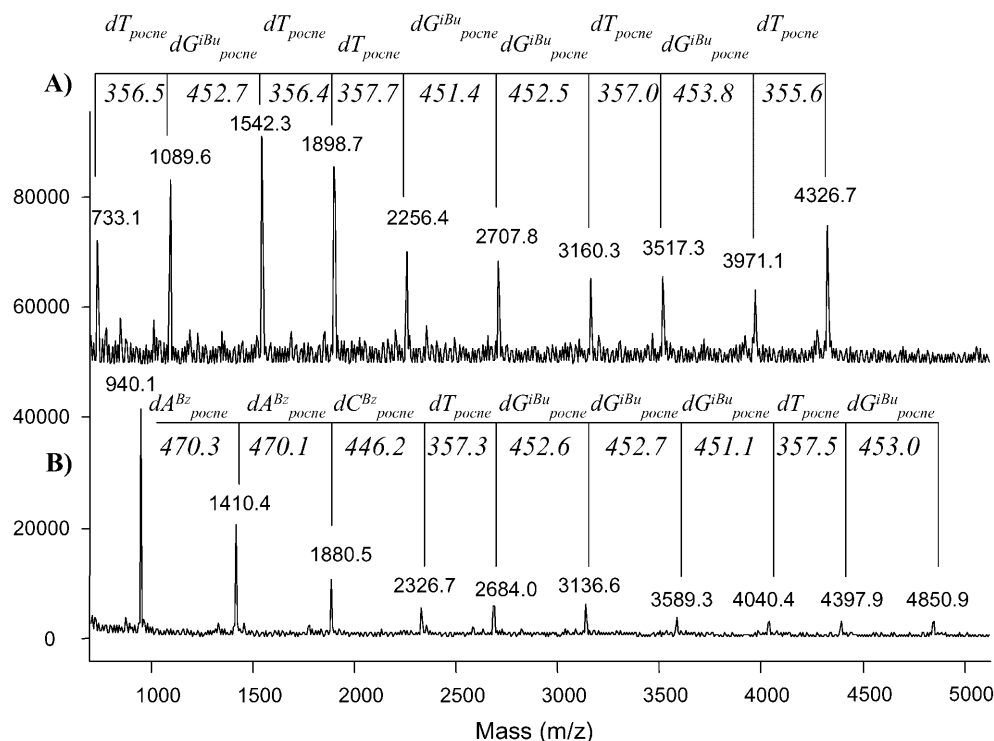
### Sequencing of ON

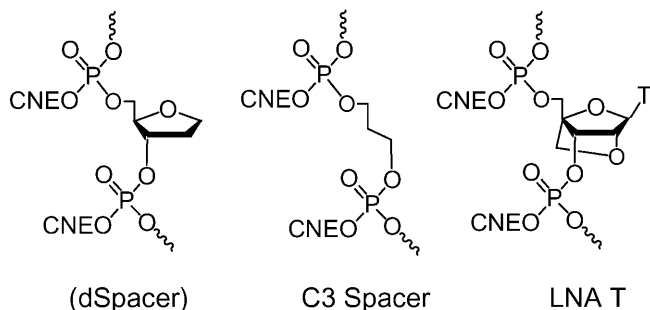
The solid-supported ON 5'-d(TTTGTTGGTGT)-3' and 5'-d(AGAAGTGGGTG)-3' were prepared on a DNA synthesizer by use of phosphoramidite methodology [14] with

the standard 5'-*O*-dimethoxytrityl (DMTr) 3'-*O*-cyanoethyl (CNE) *N,N*-diisopropyl-phosphoramidites of thymidine (dT), 6-*N*-benzoyl (Bz) 2'-deoxyadenosine (dA), 4-*N*-benzoyl 2'-deoxycytidine (dC) and 2-*N*-isobutyryl (iBu) 2'-deoxyguanosine (dG). Iodine–water was used to oxidize the phosphite triester intermediates to form cyanoethyl phosphotriester linkages (po-CNE). A capping step with acetic anhydride was usually used to prevent further elongation of unreacted material.

Analysis of ON anchored to a solid support by a succinyl linker bearing po-CNE and acyl nucleobase protections (Fig. 1), by MALDI-TOF MS (negative mode) with 2,4,6-trihydroxyacetophenone (THAP) as the matrix [13], results in the cleavage of the phosphotriester backbone between C-5' and O-5' of the nucleotide units. This fragmentation generates a set of d ions, i.e. ON fragments bearing a 3'-cyanoethyl phosphodiester moiety, as it is called in the standard nomenclature for ON fragment ions in mass spectrometry [15, 16]. The corresponding ladder of peaks that differ from each other by the mass of a nucleotide residue (consistent with calculated masses 357.26, 446.35, 452.38, and 470.38 Da for dT po-CNE, dC<sup>Bz</sup> po-CNE, dG<sup>iBu</sup> po-CNE, and dA<sup>Bz</sup> po-CNE, respectively), represents part of the sequence of the ON in the 5' to the 3' direction, except for the 3'-end nucleotide linked to the support and the 5'-end dinucleotide, for which only the nucleotide composition is directly accessible, because the abundance of the matrix ions in the lowest mass region (below *m/z* 600) does not enable observation of the 5'-end nucleotide d<sub>1</sub>. Thus, from the analysis of the solid-supported ON 5'-d(TTTGTTGGTGT)-3' and 5'-d(AGAAGTGGGTG)-3' (Fig. 1), part of their sequence 5'-(TT)TGT-TGGTGT and 5'-(AG or GA)AACTGGGT was easily

**Fig. 1** MALDI-TOF negative-ion mass spectra of the 3'-CPG-supported cyanoethyl phosphotriester (po-CNE) ON 5'-d(TTTGTTGGTGT)-3' (A) and 5'-d(AGAAGTGGGTG)-3' (B). Nucleobases A and C were protected with a benzoyl group (Bz) and G with an isobutyryl group (iBu)





**Fig. 2** Chemical structures of dSpacer, C3 Spacer, and LNA T in po-CNE ON

determined by reading the mass ladder of peaks. In these particular experiments molecular weights of d fragments were determined with accuracy in the range  $\pm(0.003-0.13)\%$ .

This technique could also be used for solid-supported ON bearing non- or modified nucleosidic residues. To illustrate this, three CPG-succinyl-supported ON 5'-d(ATCXGXAT)-3', where X were non-common residues dSpacer [17], Spacer C3 [18], and LNA T [19] (Fig. 2), were synthesized. dSpacer [17] and Spacer C3 [18] are non-nucleosidic residues that can be used to replace unstable 2'-deoxyribofuranose moieties of abasic sites in an ON or to substitute for an unknown base within the ON sequence in hybridization experiments (Fig. 2). The structure of dSpacer is a 1,2-dideoxy ribofuranose, and Spacer C3 is a 1,3-propanediol moiety. LNA (locked nucleic acids) [19] is a class of ON analogs with high affinity for DNA and RNA where the furanose ring of standard nucleosides is restricted by a methylene linker connecting the 2'-O position to the 4'-C position of the nucleoside (Fig. 2). These ON were synthesized by use of phosphoramidite methodology with the corresponding X CNE-phosphoramidite. As for regular solid-supported succinyl-linked ON, the sequences of the modified ON were determined by MALDI-TOF MS analysis of the supported ma-

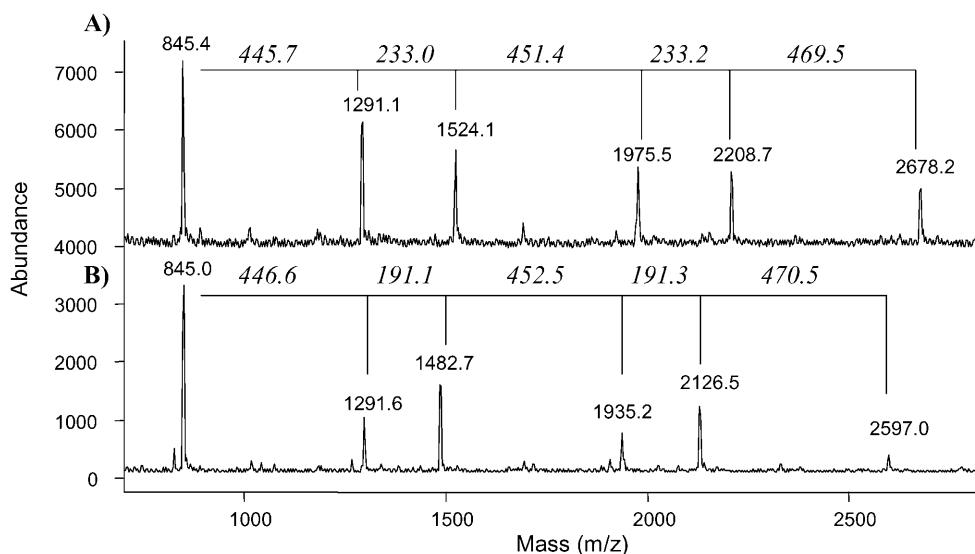
terials, resulting in a ladder of peaks from a fragmentation giving rise to d ions (Figs. 3 and 4).

For the ON bearing two dSpacer moieties (Fig. 3A), the observed mass differences of 233.0 Da between peaks at  $m/z$  1524.1 ( $d_5$  ion) and  $m/z$  1291.1 ( $d_4$  ion) and of 233.2 Da between peaks at  $m/z$  2208.7 ( $d_7$  ion) and  $m/z$  1975.5 ( $d_6$  ion) were characteristic of a dSpacer po-CNE mass loss (average mass 233.16 calculated for  $C_8H_{12}O_5NP$ ). Similarly, in the spectrum of the ON incorporating C3 Spacer (Fig. 3B), the observed mass differences of 191.1 and 191.3 Da were easily attributed to a C3 Spacer po-CNE (average mass 191.12 calculated for  $C_6H_{10}NO_4P$ ). Most of the proposed mechanisms for the fragmentation of ON induced by mass spectrometry, involved initial protonation or deprotonation of the nucleobases followed by loss of a base [20]. As the dSpacer and C3 Spacer do not have a nucleobase, the fragmentation pathway observed here for these ON is different.

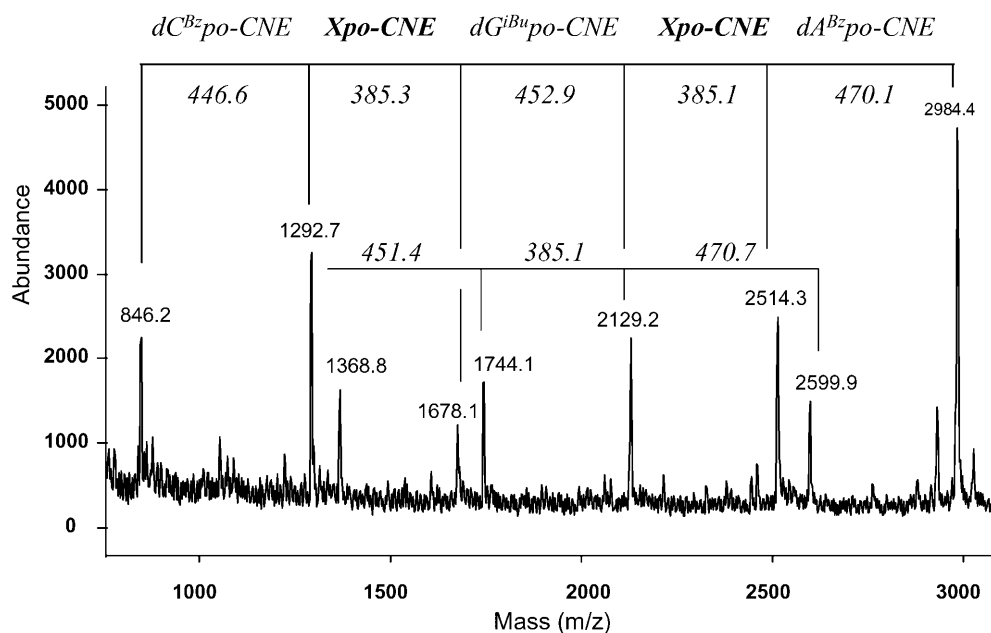
In MALDI-TOF MS analysis of the ON containing the LNA T nucleoside units (Fig. 4), a mass difference of 385.3 between  $d_3$  and  $d_4$  ion peaks and of 385.1 between  $d_6$  and  $d_7$  ion peaks was attributed to a LNA T po-CNE mass loss (average mass 385.27 calculated for  $C_{14}H_{16}N_3O_8P$ ). By analogy with MALDI fragmentation of oligonucleotide methylphosphonates giving rise to d ions [21], we proposed a mechanism involving H-4' proton abstraction of the ribose unit by the 5'-phosphotriester oxygen [10]. This hypothetical mechanism cannot, however, be used to explain the fragmentation of the current ON, because LNA do not have H-4' proton in the ribose moiety.

In addition to the set of peaks of the d ions corresponding to the 5' to the 3' sequence of the LNA-DNA ON 5'-d(ATCXGXAT)-3' ( $m/z$  846.2, 1292.7, 1678.1, 2129.2, 2514.3), the spectrum contained several other peaks corresponding to failure sequences (Fig. 4). The signal at  $m/z$  1368.8 was attributed to the truncated ON 5'-O-acetyl-d(GXAT)-3' resulting from incomplete coupling of the second LNA unit followed by acetylation under the capping step conditions ( $Ac_2O$ , *N*-methylimidazole) used

**Fig. 3** MALDI-TOF negative-ion mass spectra of the 3'-CPG-supported cyanoethyl phosphotriester ON 5'-d(ATCXGXAT)-3' with (A) X= dSpacer and (B) X= Spacer C3



**Fig. 4** MALDI-TOF negative-ion mass spectrum of 3'-CPG-supported po-CNE ON 5'-d(ATCXGXAT)-3' with X= LNA T



during oligonucleotide elongation. Two other peaks at  $m/z$  1678.1 and 2599.9 could belong to another series of ions corresponding to the d ions of 5'-d(ATCGXAT)-3' without the second LNA unit X. One possible explanation of this deleted sequence is that the acetylation of the failure sequence 5'-d(GXAT)-3', described above, was incomplete, and further elongation was possible. Thus MALDI-TOF MS analysis not only characterized the supported ON, but also provided information about chemical events on the solid support.

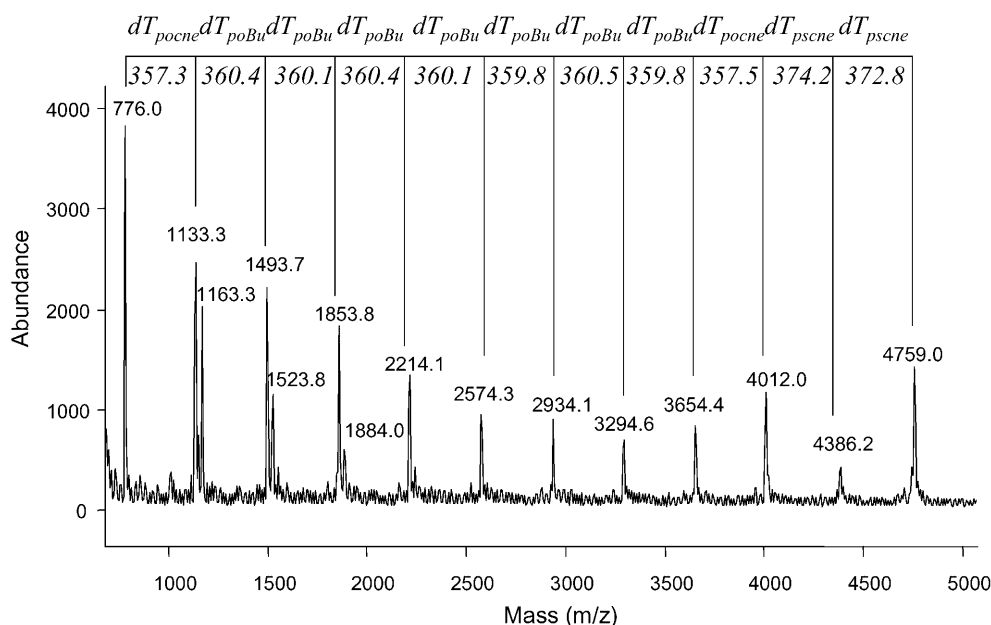
#### Internucleosidic linkage determination

A 3'-CPG-supported dodecathymidylate 5'-bis pivaloyl  $\text{Fluo}_{\text{poCNE}}\text{-d}[(\text{T}_{\text{poCNE}}(\text{T}_{\text{poBu}})_7\text{T}_{\text{poCNE}}(\text{T}_{\text{psCNE}})_2\text{T}]$  was pre-

pared. In this ON containing three different internucleosidic linkages the phenolic functions of the 5'- fluorescein (Fluo) are protected with pivaloyl groups. Commercial 5'-fluorescein cyanoethylphosphoramidite (6-FAM) was used to introduce the fluorescein moiety at 5'-end of the ON. Starting from the 3'-O-cyanoethyl and 3'-O-butyl phosphoramidites of thymidine, the corresponding cyanoethyl phosphotriester (po-CNE) and butyl phosphotriester (po-Bu) linkages were obtained by oxidation of the resulting phosphite triesters by oxidation with *tert*-butyl hydroperoxide [22] in anhydrous dichloromethane. For preparation of cyanoethyl phosphorothionotriester (ps-CNE) linkages the Beaucage reagent [12] was used as the oxidizer.

Here again, fragmentation laddering was observed during MALDI-TOF MS analysis (Fig. 5). Fragmentation of

**Fig. 5** MALDI-TOF negative-ion mass spectrum of 3'-CPG-supported ON 5'-bis pivaloyl  $\text{Fluo}_{\text{poCNE}}\text{-d}[(\text{T}_{\text{poCNE}}(\text{T}_{\text{poBu}})_7\text{T}_{\text{poCNE}}(\text{T}_{\text{psCNE}})_2\text{T}]$





this ON generated  $d_1$  to  $d_{12}$  ions. Information about the 5'-end monomer was accessible, because the mass of the  $d_1$  ion corresponding to the 5'-bis pivaloyl Fluo<sub>poCNE</sub> was >700 ( $m/z$  found 776.0,  $m/z$  calculated 775.8). For the phosphorothionotriester linkage the mass loss of the nucleotide group (average mass of dTps-CNE 373.33 calculated for  $C_{13}H_{16}N_3O_6PS$ ) differed from the phosphotriester (average mass of dTpo-CNE 357.26) by 16 Da corresponding to replacement of an oxygen atom by a sulfur atom in the internucleosidic linkage. The po-Bu linkage was easily differentiated from the two other linkages (dTpo-Bu mass loss, average mass 360.30 calculated for  $C_{14}H_{21}N_2O_7P$ ). This tells us that the MALDI MS analysis of solid-supported ON could be performed whatever the substituent of the phosphotriester backbone, and works also with phosphorothionotriester linkages. Compared with indirect methods for characterizing and sequencing ON [23], analysis of solid-supported ON does not require complex pre-MS sample treatment. One pretreatment, i.e. well-known exonuclease digestion generating a mixture of ON fragments, each progressively one nucleotide shorter in length [24, 25], could not be run with backbone-modified ON, because nucleases only recognize phosphodiester internucleosidic linkages. Even phosphorothioates need to be oxidized in phosphodiesters before enzymatic digestion [26]. Exonuclease digestion is, moreover, sensitive and sometimes inhibited by nucleosidic modifications [13].

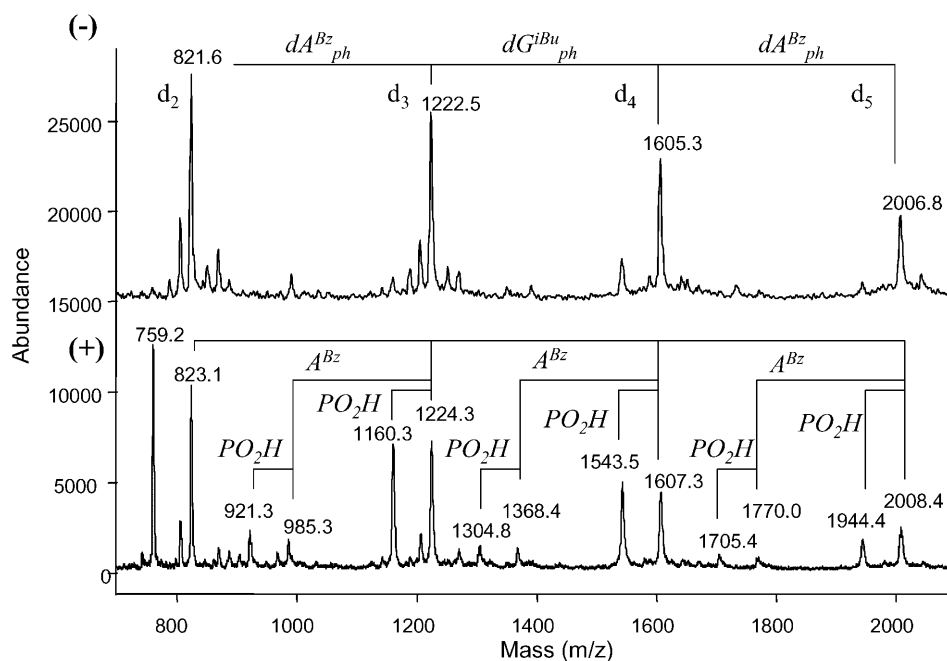
The spectrum of the solid-supported ON 5'-bis pivaloyl Fluo<sub>poCNE</sub>-d[T<sub>poCNE</sub>(T<sub>poBu</sub>)<sub>7</sub>T<sub>poCNE</sub>(T<sub>psCNE</sub>)<sub>2</sub>T] (Fig. 5) contained other peaks corresponding to the capped failure sequences 5'-O-acetyl d[T<sub>poCNE</sub>(T<sub>psCNE</sub>)<sub>2</sub>T] ( $d_3$  ion,  $m/z$  calculated 1163.0,  $m/z$  found 1163.3), 5'-O-acetyl d[T<sub>poBu</sub>T<sub>poCNE</sub>(T<sub>psCNE</sub>)<sub>2</sub>T] ( $d_4$  ion,  $m/z$  calculated 1523.0,  $m/z$  found 1523.8), 5'-O-acetyl d[(T<sub>poBu</sub>)<sub>2</sub>T<sub>poCNE</sub>(T<sub>psCNE</sub>)<sub>2</sub>T] ( $d_5$  ion,  $m/z$  calculated 1883.6,  $m/z$  found 1884.0). The

capped ON resulted from the lower coupling efficiency of dTpoBu phosphoramidite (<98%) compared with that of dTpoCNE phosphoramidite (>98%), determined after the removal of the 5'-O-DMTr group on acid treatment and subsequent dimethoxytrityl titration by spectrophotometry [27].

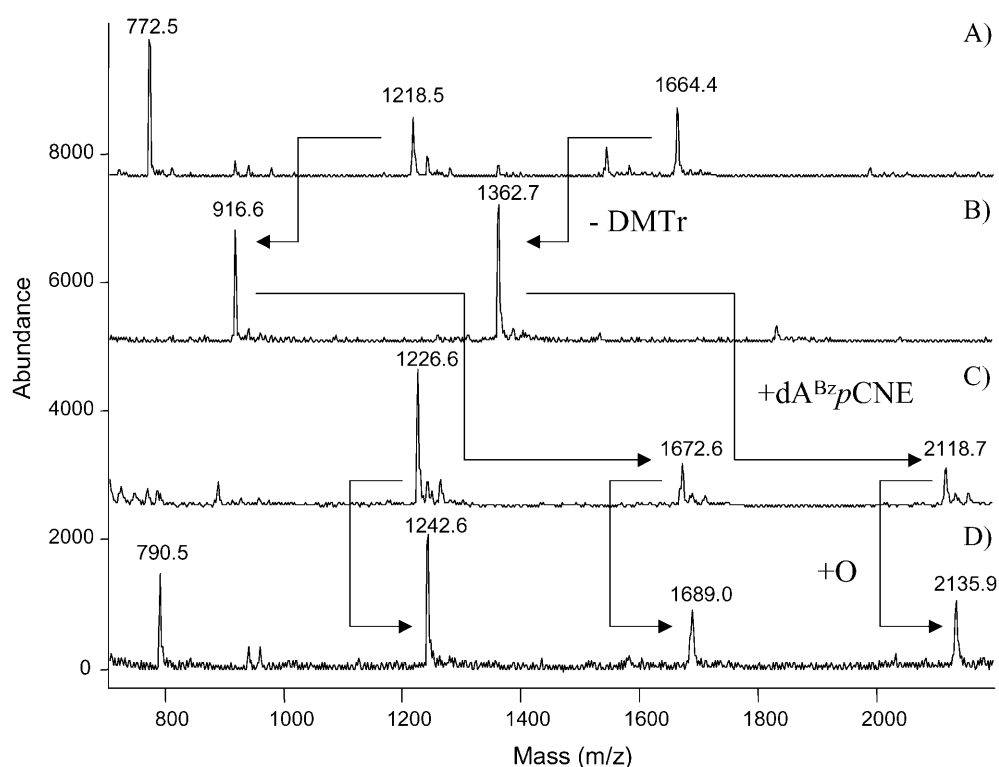
MALDI-TOF MS enabled direct read-out of backbone determination of solid-supported ON bearing P<sub>V</sub> internucleosidic linkages, but can be used to characterize still-anchored ON carrying P<sub>III</sub> internucleosidic linkages such as H-phosphonates (ph). For this purpose, the solid-supported H-phosphonate ON 5'-d[(Aph)<sub>3</sub>GphAphA]-3' was synthesized by use of H-phosphonate methodology [28] with the standard 5'-O-DMTr 3'-O-H-phosphonate monoesters of dA<sup>Bz</sup> and dG<sup>iBu</sup>.

The MALDI-TOF mass spectrum of the H-phosphonate diester ON (Fig. 6) revealed d ion fragmentation similar to P<sub>V</sub> oligos in negative mode. The dissociation pathway resulted in chain cleavage of the ON to produce one set of ions, the d series, representing its sequence in the 5' to the 3' direction. The mass ladder of peaks differed from one another by the mass of nucleotide residues dA<sup>Bz</sup>ph (average mass 401.32 calculated for  $C_{17}H_{16}N_5O_5P$ ) and dG<sup>iBu</sup>ph (average mass 383.30 calculated for  $C_{14}H_{18}N_5O_6P$ ). Interestingly, the positive mode spectrum contained other series of ions, one corresponding to the d ions without the 3'-H-phosphonate monoester moiety (average mass 63.98 calculated for PO<sub>2</sub>H) and another set of ions of smaller intensity resulting from the loss of the nucleobase A<sup>Bz</sup> (average mass 238.24 calculated for  $C_{12}H_8N_5O$ ) on the 5' side of the ON, as described elsewhere [10].

**Fig. 6** Comparison of negative and positive ion MALDI-TOF mass spectra of the H-phosphonate ON 5'-d((Aph)<sub>3</sub>GphAphA)-3'



**Fig. 7** MALDI–TOF negative-ion mass spectra of (A) 3'-CPG-supported ON 5'-*O*-DMTr d(G<sup>i</sup>Bu<sub>4</sub>poCNE (C<sup>Bz</sup>poCNE)<sub>2</sub>T), (B) obtained after detritylation of 5'-*O*-DMTr d(G<sup>i</sup>Bu<sub>4</sub>poCNE (C<sup>Bz</sup>poCNE)<sub>2</sub>T), (C) obtained after coupling of 5'-*O*-dimethoxytrityl (DMTr) 3'-*O*-cyanoethyl (CNE) phosphoramidite of 6-*N*-benzoyl 2'-deoxyadenosine, (D) after oxidation giving rise to 5'-*O*-DMTr d(A<sup>Bz</sup>poCNE G<sup>i</sup>Bu<sub>4</sub>poCNE(C<sup>Bz</sup>poCNE)<sub>2</sub>T)



### Monitoring DNA elongation

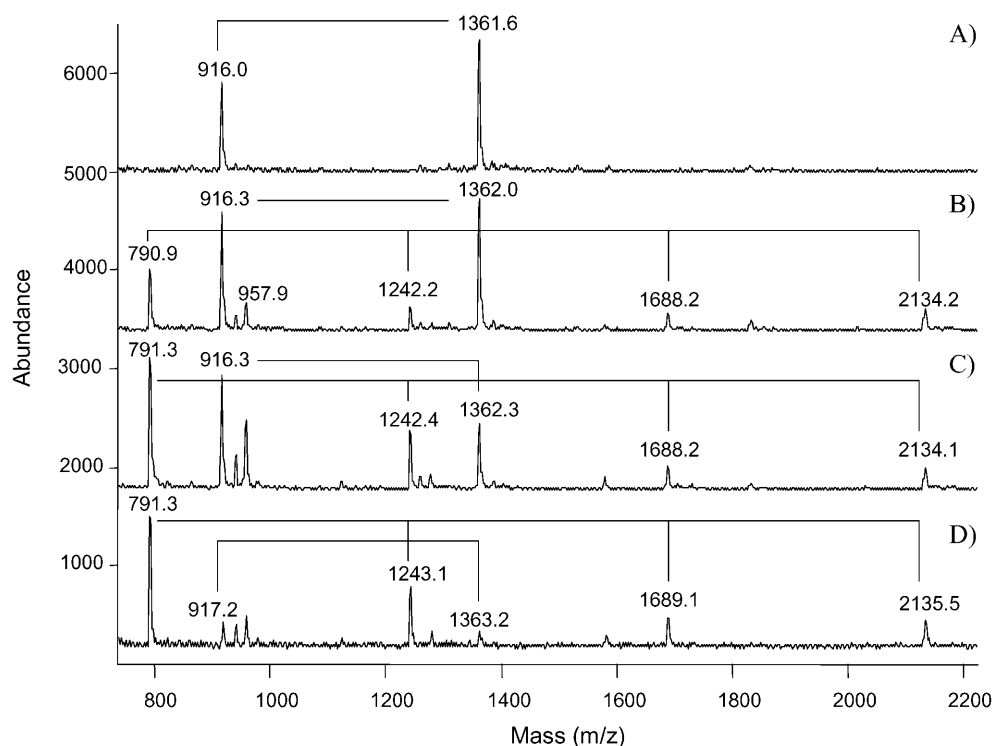
According to phosphoramidite methodology [14], typical DNA synthesis on a solid support involves acidic detritylation of the 5'-hydroxyl end of the supported ON, followed by coupling of an activated 5'-*O*-DMTr 3'-*O*-CNE *N*-diisopropyl phosphoramidite nucleoside with this free 5'-OH end. The coupling step, activated by tetrazole, generates an unstable CNE phosphite triester internucleosidic linkage. Finally, after capping of the uncoupled material, the phosphite triester is oxidized to a more stable phosphotriester. This cycle of reaction is repeated until complete elongation of the full length ON. Control over the elongation is usually performed by measurement of the dimethoxytrityl cation by spectrophotometry at each cycle entry [27]. This measurement does not, however, preclude modification of the structure of the growing ON as a result of side reactions or incomplete reactions during the DNA synthesis cycle. Because MALDI–TOF MS enabled characterization of a variety of P<sub>V</sub> and P<sub>III</sub> solid-supported ON, this technique would be a valuable tool for monitoring the different steps of solid-phase synthesis of an ON.

The step-by-step monitoring of incorporation of 5'-dA into a supported ON 5'-*O*-DMTr d[G<sup>i</sup>Bu<sub>4</sub>poCNE(C<sup>Bz</sup>poCNE)<sub>2</sub>T] to give 5'-*O*-DMTr d[A<sup>Bz</sup>poCNE G<sup>i</sup>Bu<sub>4</sub>poCNE (C<sup>Bz</sup>poCNE)<sub>2</sub>T] is illustrated in Fig. 7. In contrast with the set of d ions obtained by MALDI MS analysis of the starting dimethoxytritylated compound (Fig. 7A, d<sub>1</sub>, d<sub>2</sub>, d<sub>3</sub> ions at *m/z* calculated 771.8, 1218.1, 1664.5, respectively), the spectrum from the detritylated ON contained a series of d ions that differ by loss of the dimethoxytrityl moiety (average mass 302.4 calculated for C<sub>21</sub>H<sub>18</sub>O<sub>2</sub>) (Fig. 7B, d<sub>2</sub>, d<sub>3</sub> ions at

*m/z* calculated 915.7, 1362.1, respectively). Coupling of dA<sup>Bz</sup> CNE phosphoramidite, and after the capping step, resulted in a new spectrum (Fig. 7C) showing d ions containing a CNE phosphite triester linkage (d<sub>1</sub>, d<sub>2</sub>, d<sub>3</sub> ions at *m/z* calculated 1226.1, 1672.5, 2118.9, respectively). At this stage MALDI MS analysis needed to be performed rapidly after removal of a few beads of solid support, because extensive degradation was observed if the analysis was not performed less than 30 min after the coupling step. After oxidation with iodine–water (Fig. 7D) a new series of d ions that differ from the d series of the previous spectrum by 16 Da was observed, implying total conversion into the solid-supported CNE-phosphotriester ON 5'-*O*-DMTr d[A<sup>Bz</sup>poCNE G<sup>i</sup>Bu<sub>4</sub>poCNE(C<sup>Bz</sup>poCNE)<sub>2</sub>T] (d<sub>1</sub>, d<sub>2</sub>, d<sub>3</sub>, d<sub>4</sub> ions at *m/z* calculated 789.8, 1242.1, 1688.5, 2134.9, respectively).

Figure 8 shows that MALDI MS analysis could be used to monitor and optimize coupling efficiency. In that experiment the coupling efficiency of dA incorporation into the supported ON 5'-*O*-DMTr d[G<sup>i</sup>Bu<sub>4</sub>poCNE(C<sup>Bz</sup>poCNE)<sub>2</sub>T] to give 5'-*O*-DMTr d[A<sup>Bz</sup>poCNE G<sup>i</sup>Bu<sub>4</sub>poCNE (C<sup>Bz</sup>poCNE)<sub>2</sub>T] was monitored by MALDI–TOF MS analysis after the oxidation step without performing the capping step. Only four molar equivalents of 5'-*O*-DMTr dA<sup>Bz</sup> CNE-phosphoramidite were used instead of the twenty molar equivalents usually required. Because the coupling was inefficient, two series of ions were observed (Fig. 8B). One series corresponded to the starting material (Fig. 8A, d<sub>2</sub> and d<sub>3</sub> ions at *m/z* calculated 915.7 and 1362.1, respectively) and the other to the desired coupled compound (Fig. 8D, d<sub>1</sub>, d<sub>2</sub>, d<sub>3</sub> and d<sub>4</sub> ions at *m/z* calculated 789.8, 1242.1, 1688.5 and 2134.9 respectively). The coupling step was

**Fig. 8** MALDI-TOF negative-ion mass spectra of (A) 3'-CPG-supported ON 5'-d(G<sup>iBu</sup>poCNE(C<sup>Bz</sup>poCNE)<sub>2</sub>T), (B) obtained after one coupling run (coupling followed by oxidation) of 5'-O-dimethoxytrityl (DMTr) 3'-O-cyanoethyl (CNE) phosphoramidite of 6-N-benzoyl 2'-deoxyadenosine (4 molar equivalents) showing a mixture of the 3'-CPG-supported ON 5'-d(G<sup>iBu</sup>poCNE(C<sup>Bz</sup>poCNE)<sub>2</sub>T) and 5'-O-DMTr d(A<sup>Bz</sup>poCNE G<sup>iBu</sup>poCNE(C<sup>Bz</sup>poCNE)<sub>2</sub>T), (C) after two coupling runs, and (D) three coupling runs.



repeated three times until disappearance of the set of peaks of the starting material.

## Conclusion

MALDI-TOF mass spectrometry is a powerful technique for characterizing still-anchored oligonucleotides during their solid-phase elongation. Information is obtained about the sequence, including modified nucleosides, and about the character of the internucleosidic backbone, including P<sub>III</sub> linkages. This technique is very sensitive and does not require large quantities of material. We believe that understanding side reactions without release of the oligonucleotide from the solid support and monitoring step-by-step the reactions involved in the ON synthesis will be of particular interest in their large scale synthesis.

**Acknowledgments** We would like to thank the "Association pour la Recherche contre le Cancer" (ARC) for financial support.

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