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Short communication

HPLC electrospray mass spectrometric characterization of trimeric building blocks for oligonucleotide synthesis

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Abstract

Trimeric nucleotide building blocks are valuable in synthesis of randomized oligonucleotides. In this study, we have developed HPLC-MS and HPLC-MS/MS methods for quality control of protected trinucleotides. C18 reversed-phase HPLC was used for purity evaluation, and base sequences were verified using negative ion electrospray ionization mass spectrometry (ESI-MS) and collision induced dissociation (CID) MS/MS. The principal dissociation pathway was formation of w-ions, which represent the 3′–5′ direction. The other major fragments were d-ions, which are formed by cleavage of 5′ C–O bonds of the sugars. The developed method was suitable for verification of purity and structure of the 29 trinucleotides studied.

Keywords: Oligonucleotides; Electrospray; Mass spectrometry; Fragmentation

1. Introduction

Large peptide libraries for drug discovery programs can be created using phage display methods. Oligonucleotide-directed mutagenesis is the favoured method for preparing these libraries. For this purpose, randomized oligonucleotides are usually prepared using mixtures of nucleotides at each step of synthesis. However, this strategy often results in incorporation of unwanted amino acids, or stop codons into the sequence. A significant solution to this problem has been introduction of trimeric nucleotide building blocks (Fig. 1), which correspond to desired codons [1].

Liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) is a widely accepted tool in analysis of thermally unstable and polar biopolymers, such as proteins and oligonucleotides. However, successful application of ESI-MS for analysis of oligonucleotides requires careful control of sample and instrument parameters to obtain high quality results. The main problems in oligonucleotide analysis include low sensitivity especially for long oligomers, and formation of metal cation adduct ions [2]. Oligonucleotides are usually analyzed using negative electrospray ionization, because the phosphate groups are readily deprotonated in solution. The

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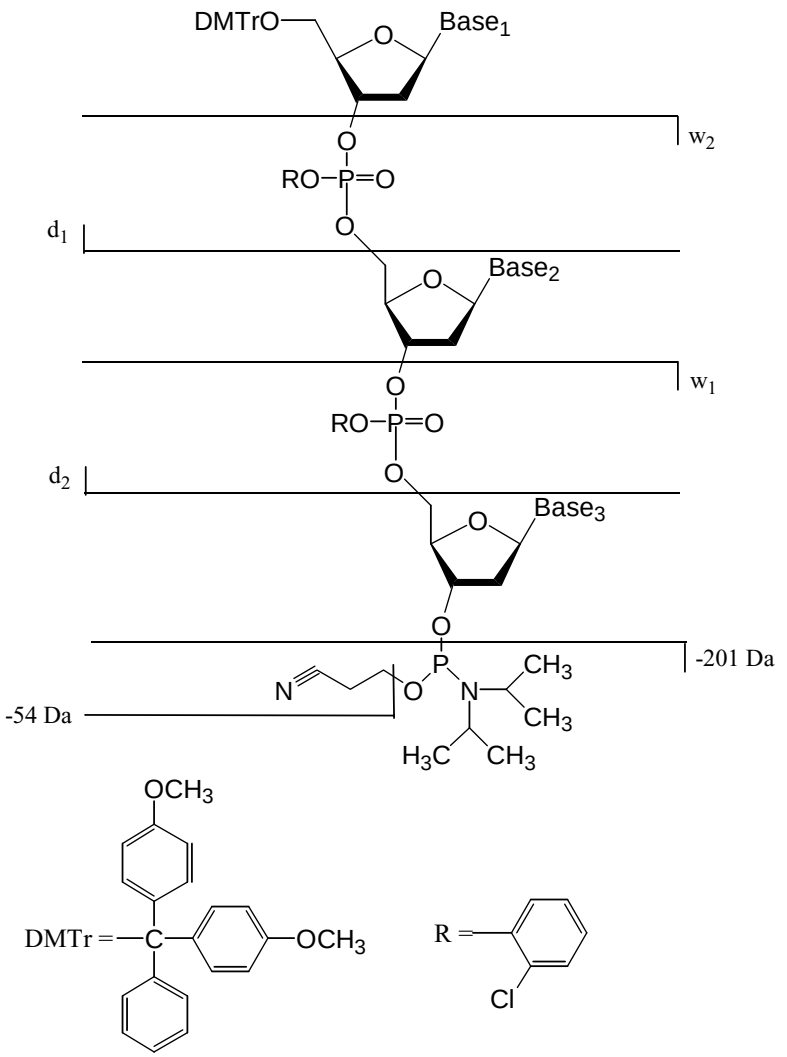

Fig. 1. Structure of trimeric oligonucleotide building blocks. The ions are labelled as described in Fig. 2.

effect of instrument tuning on oligonucleotide ionization has been recently studied by Oberacher et al. [3]. In general, high concentration of organic solvent, such as isopropanol or acetonitrile, in the eluent, is needed to obtain good sensitivity. The formation of adduct ions can be suppressed either by using on line chromatography before ESI, or by using solvent additives to displace sodium ions. The widely used 0.1 M ammonium acetate buffer cannot be used in combination of HPLC-MS, as the high ion concentration will result in severe signal supression. The most commonly used LC methods are based on use of reversed-phase columns, and water–acetonitrile gradients with addition of some short chain trialkylamine at low millimolar concentration [4,5]. The chromatographic resolution can be further enhanced by using hexafluoro-2-propanol as mobile phase additive [6,7].

The MS/MS decomposition of oligonucleotide anions has been extensively studied by McLuckey and Habibi-Goudarzi [8], and their nomenclature for fragment ions widely used in various publications (Fig. 2). The major ions observed are normally a-base ions, which are formed by loss of a nucleobase followed by cleavage at the 3′ C–O bond of the sugar from which

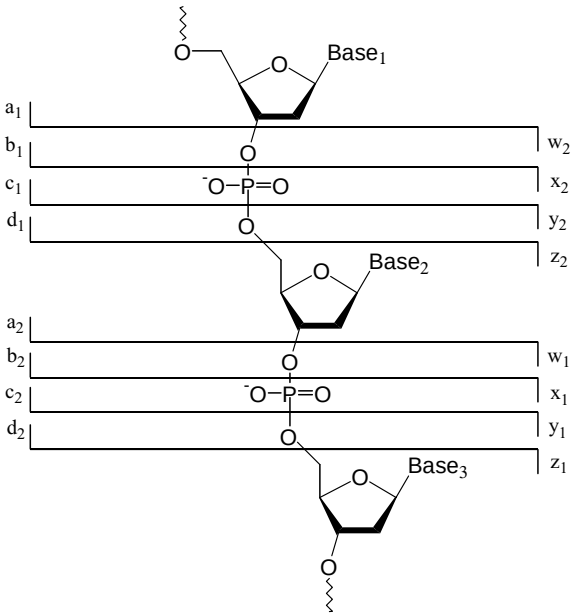

Fig. 2. Structure of DNA oligonucleotide fragment. The fragment ions are labelled according to McLuckey and Habibi-Goudarzi [8].

the base is lost. The complementary w-ion contains the remaining nucleotide sequence with a phosphate group on the 5′-end [8,9]. The interpretation strategy of oligonucleotide mass spectra has been extensively described by Ni et al. [10].

In this study, we have developed a rapid HPLC-MS method for quality control of trimeric nucleotide building blocks. The method is based on separation of the compounds by reversed-phase chromatography using ammonium acetate buffer and acetonitrile gradient. The purity of the compounds was based on full scan MS monitoring of the chromatographic peaks. The structures of the compounds were verified by using negative ion ESI and measurement of collision induced MS/MS spectra with a LCQ ion trap instrument.

2. Experimental

2.1. Sample preparation

The samples were prepared by dissolving trimetric blocks in acetonitrile–water (1:1) solution. The final sample concentration was 100 pg/μl.

2.2. Chromatographic system for HPLC-ESI-MS

In HPLC-MS analyses a Phenomenex C18 column (50 mm × 2.0 mm, particle size 5 μm) was used. Solvent A was 10 mM ammonium acetate (pH 7.2) and solvent B was acetonitrile. The HPLC gradient was 7–100% of B in 5 min and then column was washed with B for 3 min. The flow was set to 160 μl/min and the injection volume was 20 μl.

2.3. Mass spectrometry

The system used for HPLC-MS analysis was a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) using electrospray ion source equipped with Ultimate HPLC pump (LC Packings, Amsterdam, The Netherlands). In negative ionization mode electrospray was stabilized using nitrogen sheath gas (flow 100 arbitary units) and the spray needle voltage was −4 kV. The inlet capillary was heated to 225 °C and the tube lens offset was set to 15 V. In positive ion mode, the spray needle voltage was set to +4 kV, and the tube lens offset was set to 30 V. The full scan mass spectra from *m*/*z* 400 to 2000 were measured using 500 ms for collection of the ions in the trap, and five microscans were summed. The MS/MS data was collected from the most abundant ion. The collision energy was set to 35% in negative ion mode. In case of positive ion MS/MS, also source-induced collision (energy 40%) was used to fragment the protonated molecule.

3. Results and discussion

Reversed-phase HPLC using simple acetonitrile gradient was suitable for purity control of protected nucleotides. Fig. 3 shows a typical base peak ion chromatogram of synthetic oligonucleotides. No wrong sequences were detected though some minor impurities were observed just before the main peak. The impurities are mainly formed by the cleavage of phosporamidite functions from the 2′-deoxyguanosine and 2′-deoxythymidin residues at 3′-termini.

Negative electrospray mass spectrometry can be used for molecular weight determination of the

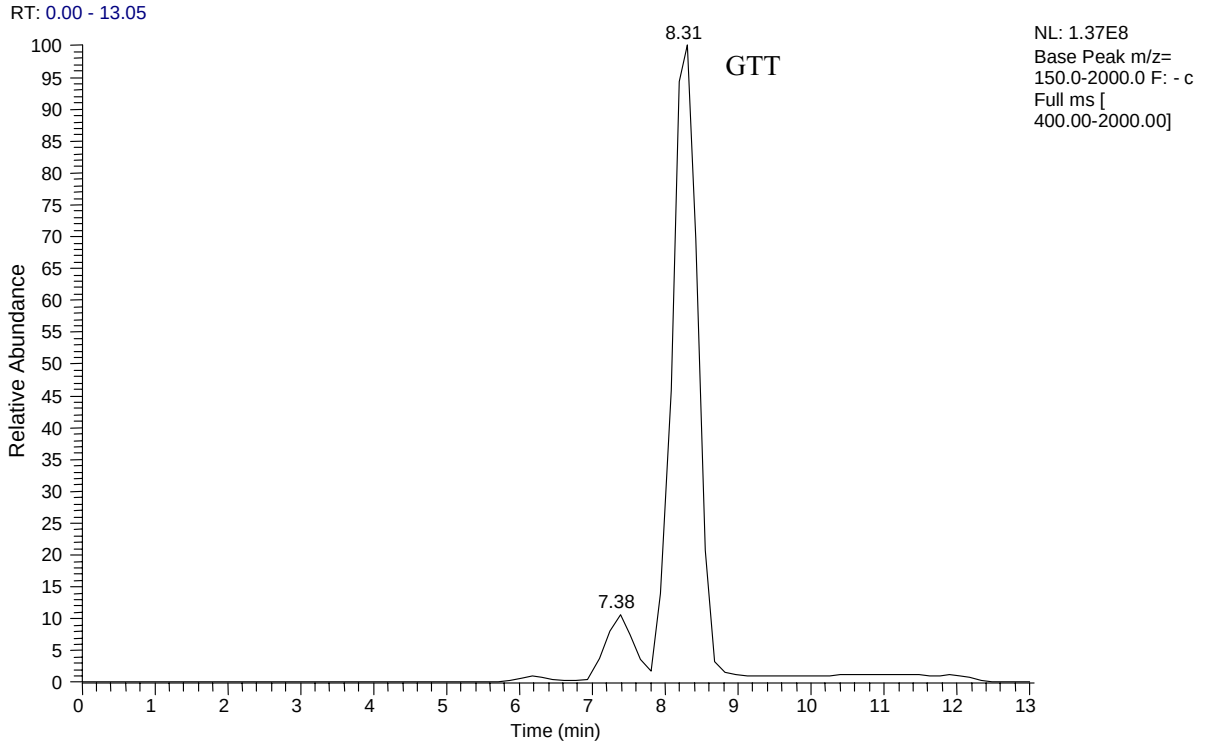

Fig. 3. HPLC-ESI-MS full scan chromatogram of a protected GTT trinucleotide building block. Conditions: Phenomenex C18 column (50 mm × 2.0 mm, particle size 5 μm). Solvent A was 10 mM ammonium acetate (pH 7.2) and solvent B was acetonitrile. The HPLC gradient was 7–100% of B in 5 min. The flow was set to 160 μl/min.

compounds (Fig. 4A, Table 1), because the negative ion spectrum shows singly deprotonated molecule as the base peak with high sensitivity (signal-to-noise ratio >100). The monoisotopic molecular weights were measured using zoom scan mode (Fig. 4B, Table 1). The zoom scan spectra show isotopic pattern typical to chlorine containing compounds. Negative ion mode electrospray gave also good results in structural analysis by MS/MS. In negative ion mode MS/MS analysis of the deprotonated molecule gives two types of complementary fragment ions, which are indicative to the base sequence (Fig. 4C). The formation of 3′-terminal w-ions is the main fragmentation pathway as in case of MS/MS of unprotected nucleotides [8]. According to the generally adopted fragmentation mechanism the negatively charged phosphodiester is initially engaged in the reaction at the cleavage site. However, Bartlett et al. have shown, that formation of w-ions is possible also at neutral alkylated phosphate groups by transfer of charge from a remote site [11]. In the present study, formation of a-fragments or a-base-fragments was not favored (Fig. 4C). The main 5′-terminal fragments were d-ions, which are formed by cleavage of 5′ C–O bonds of the sugars. Table 1 shows that for almost all protected trinucleotides full coverage complementary w and d sequence ions were obtained. For six trimers, one of the four possible fragment ions was not found. However, also in these cases the whole base sequence could be verified.

In positive ion mode, the compounds show protonated molecules as major peaks (Fig. 5A), but the MS/MS spectra were not very informative. When CID in the ion trap was used for MS/MS analysis, no significant fragment ions were obtained in the scan range *m*/*z* 475–2000. Due to instrumental limitations, fragment ions with *m*/*z* value lower than 30% of the parent ion cannot be stored in the ion trap. However, by using source-induced collision, the full range

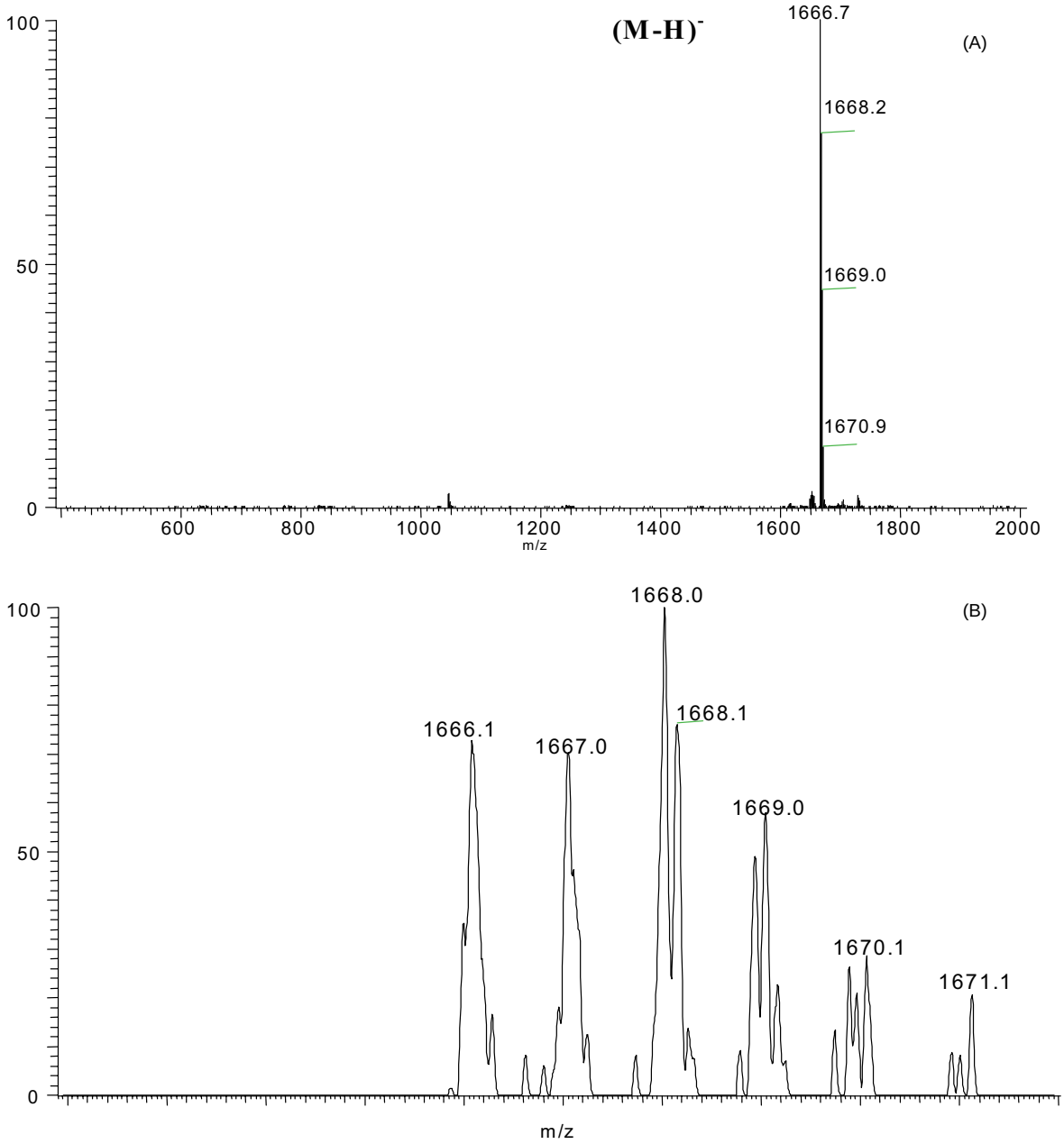

Fig. 4. (A) Negative ion full scan ESI mass spectrum of a protected GTT trinucleotide building block. (B) Negative ion zoom scan ESI spectrum of GTT showing the isotopic pattern of the molecule. Ion at *m/z* 1666.1 corresponds to the singly deprotonated monoisotopic molecule. (C) Collision induced negative ion MS/MS spectrum of the GTT trinucleotide building block. The ions are labelled as described in Fig. 2.

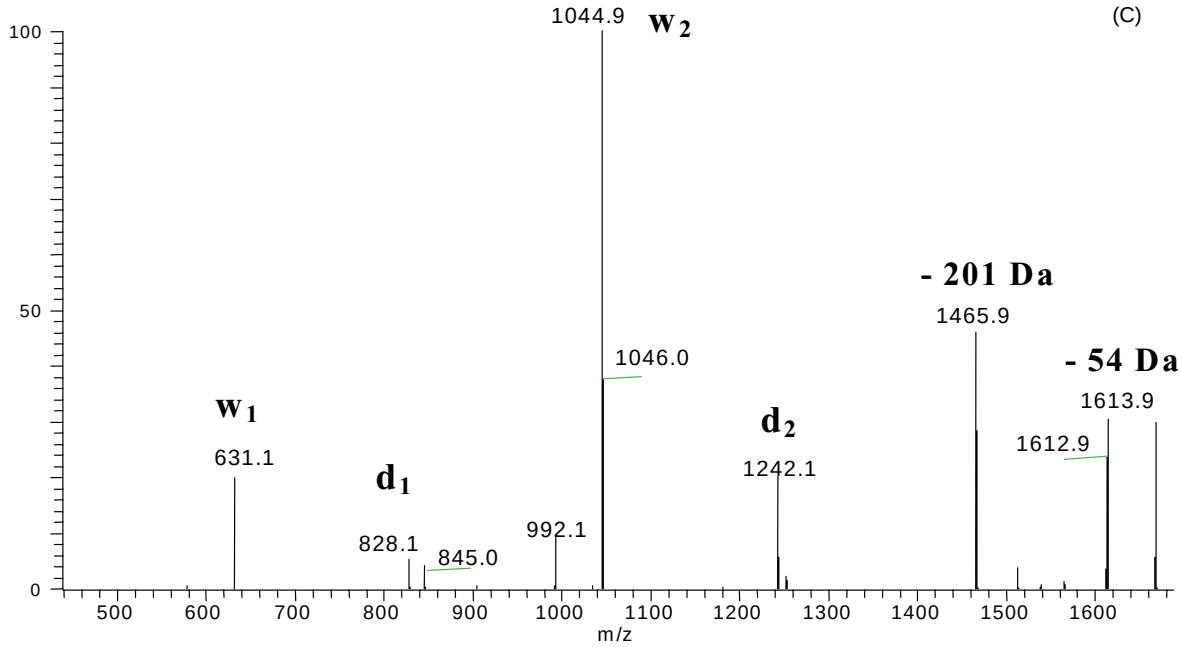

Fig. 4. (*Continued*).

Table 1
Calculated and measured monoisotopic molecular weights of protected trinucleotides and most abundant fragment ions

	M (calculated)	*M* (measured)	w_1	w_2	d_1	d_2
TAC	1774.5	1774.2	720.3	1247.0	733.1	[a]
ACT	1774.5	1774.2	631.2	1133.7	846.0	1348.9
AAA	1911.5	1911.2	744.3	1271.0	846.1	1373.1
ATC	1774.5	1774.2	720.2	1134.0	846.2	1134.0
TTC	1661.4	1661.1	720.1	1134.0	733.1	1147.0
GCT	1756.5	1756.2	631.2	1134.0	828.1	1331.1
GGC	1851.5	1851.1	720.1	1228.9	828.1	1337.0
GAA	1893.5	1893.2	744.2	1271.0	828.0	1355.0
GGT	1762.5	1762.3	630.8	1140.1	828.2	1337.2
AAG	1893.5	1893.2	726.5	1253.1	846.0	1373.1
AAC	1887.5	1887.2	720.0	1247.0	846.0	1373.1
AGA	1893.5	1893.2	744.2	1253.0	846.0	1355.2
TGG	1762.5	1762.2	726.3	1235.3	735.3	1242.1
CTG	1756.5	1756.1	726.3	1140.1	822.2	1236.1
CGT	1756.5	1756.2	631.2	1140.0	822.0	1330.9
GAC	1869.5	1869.2	720.2	1247.0	[a]	1355.1
ATG	1780.5	1780.1	726.4	1140.2	[a]	1259.9
ACC	1863.5	1863.1	720.1	1222.9	846.2	1348.9
CCG	1845.5	1845.0	726.3	1228.9	822.2	1324.9
CCC	1839.5	1839.2	720.1	1223.0	821.6	[a]
CAG	1869.5	1869.2	[a]	1253.0	822.0	1349.0
GCC	1845.5	1845.0	720.1	1222.9	828.2	1331.0

Table 1 (*Continued*)

	M (calculated)	*M* (measured)	w_1	w_2	d_1	d_2
GTG	1762.5	1762.1	726.3	1140.2	828.2	1242.1
AGC	1869.5	1869.2	720.3	1229.1	846.2	1355.2
CAT	1774.5	1774.2	631.1	1158.0	[a]	1350.0
TGC	1756.5	1756.2	720.4	1229.0	733.1	1242.1
GTT	1667.5	1667.1	631.1	1044.9	828.1	1242.1
TCT	1661.4	1661.2	631.2	1134.0	733.1	1236.1
CGC	1845.5	1845.2	720.1	1228.9	822.1	1331.1

Ions are labelled as described in Fig. 2. Molecular weights were calculated with MolE—Mass Calculator v 2.0 (http://rna.rega.kuleuven.ac.be/masspec/mole.htm).

[a] Not detected.

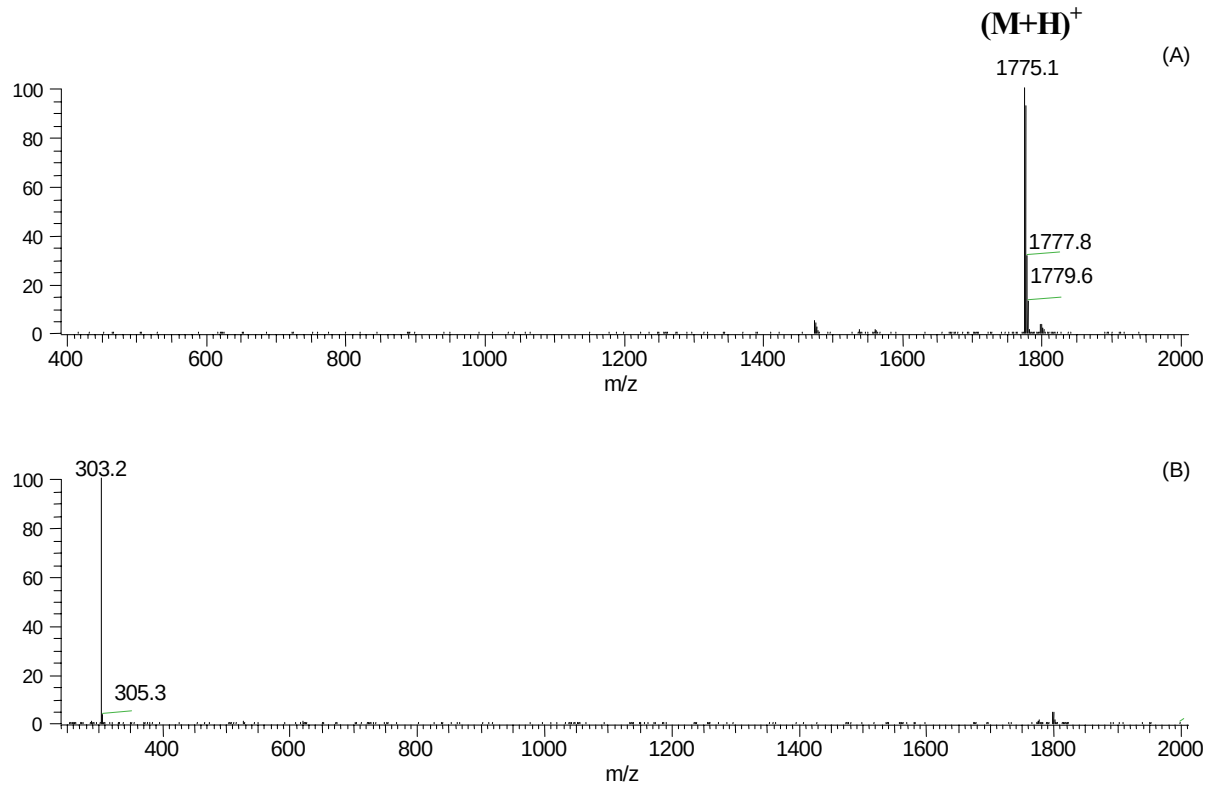

Fig. 5. (A) Positive ion full scan electrospray mass spectrum of protected TAC trinucleotide building block. (B) Positive ion MS/MS spectrum of TAC trinucleotide building block obtained by source-induced collision. The peak at *m*/*z* 303 corresponds to the dimethoxyltrityl fragment.

of product ions may be scanned [12]. Fragmentation of protonated molecular ion results in production of very abundant dimethoxytrityl ion at *m*/*z* 303, but no other ions were suitable for sequence verification (Fig 5B).

4. Conclusions

HPLC and negative ion electrospray mass spectrometry is a suitable tool for quality control and characterization of trimeric oligonucleotide building blocks. In

this study, the structures of 29 protected trinucleotides were successfully verified. The most prominent sequence ions were of w- and d-type, whereas a-ions were of low abundance.

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