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Oligonucleotide covalent modifications by estrogen quinones evidenced by use of liquid chromatography coupled to negative electrospray ionization tandem mass spectrometry

L. Debrauwer*, E. Rathahao, C. Couve, S. Poulain, C. Pouyet, I. Jouanin, A. Paris

Laboratoire des Xénobiotiques, INRA, B.P. 3, 31931 Toulouse Cedex 9, France

Abstract

Liquid chromatography coupled to tandem mass spectrometry has been used for the detection and the structural characterization of T-rich model oligonucleotides covalently modified by estradiol-2,3-quinone. After separation by gradient elution, adducts were analyzed by negative electrospray mass spectrometry, enabling to evidence and localize the modifications in the oligonucleotide sequence. Modifications by one molecule of estrogen were evidenced on purines (A, G) whereas no reaction was observed on pyrimidic bases (T). Isomeric adducts were differentiated using tandem mass spectrometry, and energy resolved mass spectrometry allowed to underline differences in the behavior of the adducts towards collisional excitation into an ion trap device.

Keywords: Mass spectrometry; Oligonucleotides; Estrogen quinones; Quinones; Steriods

1. Introduction

Hydroxylation via cytochrome P450 enzymes, occurring on the preferred positions C16α, C2 and C4 of the steroid skeleton, represents one of the major metabolic routes of estrogens [1,2]. When hydroxylation on the aromatic A ring (positions C2 or C4) is concerned, catechol estrogens are formed. In most cases, these catechol estrogens are inactivated via catechol O-methyl transferases, leading to O-methylated derivatives. If not, catechol estrogens can accumulate in the cell and undergo further enzymatic or chemical oxidation processes to be converted into quinoid forms. Owing to their high electrophilic properties, these quinoid species are extremely reactive towards major cellular constituents and thus can be responsible for cytotoxic and genotoxic effects [1,2]. Although the conjugation with glutathione is known to constitute another biological protective barrier towards estrogen quinones [3,4], these latter species are able to form DNA adducts by covalent binding with nucleophilic sites of DNA bases [1,2,5]. This metabolic activation process can induce mutagenicity phenomena and is reported as a critical initiating event of several hormono-dependent cancers [1,2,6]. Depending upon the involved reactive site of the DNA base, two different kinds of adducts can be formed whether or not the nucleophilic attack induces the destabilization of the deoxyribonucleotide glycosidic bond [5,7]. This finally gives rise to the formation of either an alkylated or an abasic site on the DNA chain. In this context, the development of analytical tools intended for providing information on the nature of the

*Corresponding author.
E-mail address: laurent.debrauwer@toulouse.inra.fr (L. Debrauwer).

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modifications are of particular importance. Indeed, isomeric adducts may possess different mutagenic activities since the biological response to the presence of those adducts (DNA repairing processes) may depend upon the nature and the conformation of the formed adduct.

Most of previous works on the modification of DNA by estrogen quinones has been devoted to the study of the reactivity of estrogen quinones towards individual model deoxyribonucleosides [5,8]. From these works, puric bases (dG, dA) have been shown [5,8] to be more reactive towards estrogen quinones than pyrimidic bases (dC, dT). They give several isomeric adducts which can be distinguished and structurally characterized using LC–electrospray ionization (ESI) MS coupling [8]. On the other hand, only one adduct could be evidenced with dC while no reaction was observed with dT [5,8]. A particular emphasis has been given to 4-hydroxylated estrogens because they have been shown to induce cancers in hamsters [9] or rats [6] whereas 2-hydroxylated estrogens did not. This difference was attributed to the ability of 4-hydroxylated estrogens to form depurinating adducts whereas 2-hydroxylated estrogens were reported to lead only to stable adducts [5,6]. However, other results made evident the formation of depurinating adducts by reaction of estradiol-2,3-quinone with nucleosides [8,10,11], showing that they also may induce formation of abasic sites in DNA.

The detection of DNA modifications occurring in vivo is usually achieved by DNA digestion and subsequent analysis of the resulting oligonucleotides. This approach implies the development of powerful analytical tools for the characterization of modified oligonucleotides. Carcinogen-DNA adducts can be analyzed with a great sensitivity using immunoassays and ^{32}P post-labeling [12,13], but these methods cannot provide any structural information. Because of its sensitivity and specificity, mass spectrometry appears as a powerful tool for exploring this field [14–16] since it is often the only source of structural information taking into account the very small amounts of available adducts. Moreover, the possibility of on-line coupling to separation techniques, such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), enhances the detection capabilities of this method [17–20]. Negative ESI and tandem mass spectrometry (MS–MS) are now widely used for the analysis of non-modified [21–23] as well as modified oligonucleotides [24–26].

In the continuation of our work on the reactivity of estradiol-2,3-quinone towards DNA constituents [8,10,11,27], we present hereafter the development of analytical tools using liquid chromatography (LC) coupled to an ion trap mass spectrometer for the characterization of isomeric adducts resulting from the reaction of estradiol-2,3-quinone with T-rich model oligonucleotides containing one reactive (TTTTTATTTTTT, TTTTTGTTTTTT) or two reactive (TTTTTATTTATT and TTTTATTTGTTTT) bases. The various adducts separated by liquid chromatography are then characterized by mass spectrometry. The use of tandem mass spectrometry and energy resolved mass spectrometry experiments presented herein clearly illustrates the usefulness of this methodology for discriminating isomeric adducts, which may possess different biological significance.

2. Experimental

2.1. Chemicals

2-Hydroxyestradiol (2OH-E_2) and activated MnO_2 used for syntheses were from Sigma (Sigma–Aldrich Chimie, L'Isle d'Abeau Chesnes, France). Model oligonucleotides ($^{5'}$TTTTTATTTTTT$^{3'}$, $^{5'}$TTTTTGTTTTTT$^{3'}$, $^{5'}$TTTTTATTTATT$^{3'}$, $^{5'}$TTTTATTTGTTTT$^{3'}$) were purchased from Sigma-Genosys (Pampisford, UK). Solvents used were of highest purity available from Merck (Merck Eurolab, Fontenay sous Bois, France) or Sharlau (Barcelona, Spain). Water was from a Milli-Q system (Millipore, Saint Quentin en Yvelines, France).

2.2. Syntheses

2-Hydroxyestradiol was oxidized into estradiol-2,3-quinone (E_2-2,3-Q) in acetonitrile at −40 °C using activated MnO_2 according to previously published procedures [5,28]. After 10 min, the resulting suspension was filtered through a Whatman GF/B

filter over a solution of the model oligonucleotide (60–70 μg in 0.5 ml of 5 m*M* triethylammonium acetate) and left to react for 6 h under stirring. The resulting mixture (15–20 nmol of oligonucleotide in 2 ml solution) was then stored at −18 °C until analysis.

2.3. LC-MS

All experiments were performed on a Finnigan LCQ ion trap mass spectrometer (Thermo Quest, Les Ulis, France) equipped with an ESI source operated in the negative mode using typical following conditions: needle voltage (−3 kV); heated capillary temperature (230 °C); capillary voltage (−29 V). All spectra were acquired using AGC (automatic gain control). Multiple MS (MS^n) experiments were carried out using He as collision gas. Liquid chromatography was achieved using a Thermo Separation P4000 pump (Thermo Quest) fitted with a Rheodyne injector. The LC column used was an Uptispher ODSB 5 μm C_{18} column (250×2 mm) from Interchim (Montluçon, France). The following gradient elution was used: 100 to 95% A from 0 to 15 min, then 95 to 92% A from 15 to 25 min, 92 to 88% A from 25 to 35 min, and 88% A to 100% B from 35 to 60 min, using mixtures of ammonium triethylacetate (5 m*M*)–CH_3CN (95:5) for A and (10:90) for B. Typical LC/MS analyses were carried out by injections of 20 μl of the final solutions, i.e., 150–200 pmol equiv. oligonucleotide injected.

3. Results and discussion

3.1. TTTTTGTTTTTT

The ion chromatograms obtained from the LC–MS analysis of the crude reaction mixture of TTTTTGTTTTTT with estradiol-2,3-quinone are reported in Fig. 1. Under our optimal conditions, the unmodified oligonucleotide was characterized by its *m/z* 1203 $[M\text{-}3H]^{3-}$ triply charged molecular ion detected as the major multiply charged species (Fig. 1a). The signal observed at *m/z* 1298 allowed us to evidence the occurrence of three isomeric adducts (G1, G2, G3) formed by addition of one molecule of 2OH-E_2 on the oligonucleotide, which were also represented by their $[M\text{-}3H]^{3-}$ ion, as indicated in Fig. 1b. No oligonucleotide bearing an abasic site was observed, suggesting that only ''stable'' adducts were formed in the reaction. This was rather unexpected since unstable adducts giving rise to abasic sites on the DNA chain have already been observed on model systems involving E_2-2,3-Q and deoxyguanosine [8,10,11]. From this observation, one should conclude that the poly-T environment of deoxyguanosine could influence the reactivity of this nucleoside in the model sequence chosen in this work.

In order to get structural information on the various formed adducts, MS–MS experiments were carried out on isomeric G1, G2 and G3 adducts by collisional excitation of their $[M\text{-}3H]^{3-}$ ions into the ion trap device. The different fragment ions observed

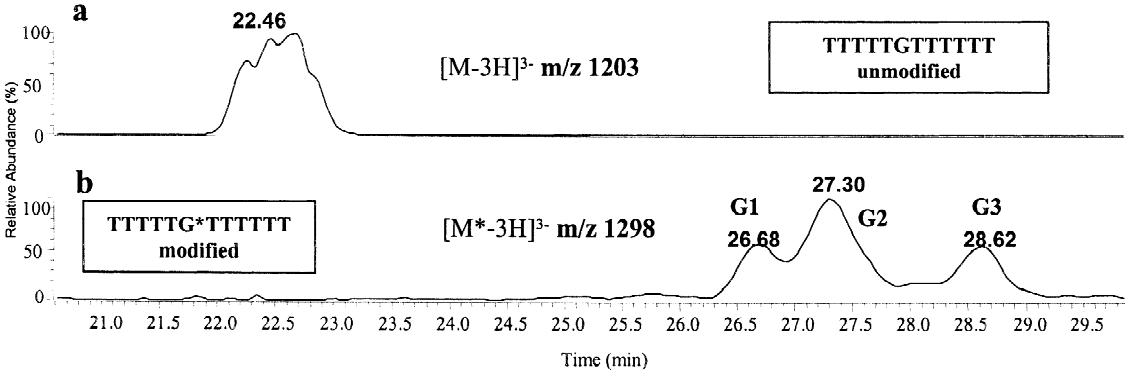

Fig. 1. LC–negative ion ESI-MS chromatograms obtained from the injection of a crude reaction mixture of E_2-2,3-Q with TTTTTGTTTTTT: reconstituted ion chromatograms for the $[M\text{-}3H]^{3-}$ ions of (a) the unmodified (*m/z* 1203) and (b) the modified oligonucleotide (*m/z* 1298).

Table 1
Fragment ions obtained from the MS–MS analysis of negative ESI produced $[M\text{-}3H]^{3-}$ ions from unmodified oligonucleotides TTTTTATTTTTT, TTTTTGTTTTTT, and their corresponding adducts

Compound	TTTTTATTTTTT	Adducts (A1/A2/A3)	TTTTTGTTTTTT	Adducts (G1/G2/G3)
Selected parent ion	$[M\text{-}3H]^{3-}$ *m/z* 1198	$[M^*\text{-}3H]^{3-}$ *m/z* 1293 $[M^*\text{-}3H\text{-}St]^{3-}$ (**1198**)	$[M\text{-}3H]^{3-}$ *m/z* 1203	$[M^*\text{-}3H]^{3-}$ *m/z* 1298 $[M^*\text{-}3H\text{-}St]^{3-}$ (**1203**)
Fragment ions (***m/z***)	$[M\text{-}3H\text{-}(A)]^{3-}$ (**1153**) $[a_6\text{-}B_6]^{-}$ (**1617**) w_6^{2-} (**920**)	$[M^*\text{-}3H\text{-}(A^*)]^{3-}$ (**1153**) $[a_6\text{-}B_6^*]^{-}$ (**1617**) w_6^{2-} (**920**)	$[M\text{-}3H\text{-}(G)]^{3-}$ (**1153**) $[a_6\text{-}B_6]^{-}$ (**1617**) w_6^{2-} (**920**)	$[M^*\text{-}3H\text{-}(G^*)]^{3-}$ (**1153**) $[a_6\text{-}B_6^*]^{-}$ (**1617**) w_6^{2-} (**920**)

from this MS–MS analysis are summarized in Table 1 for the unmodified oligonucleotide as well as the three isomeric adducts. As reported in Fig. 2, the two isomeric G1 and G2 adducts displayed similar collision-induced dissociation (CID) mass spectra. Characteristic decompositions were observed, such as the loss of the modified G* base leading to the *m/z* 1153 ion. Other diagnostic fragment ions were detected at *m/z* 920 and 1617, corresponding to the $[w_6]^{2-}$ and $[a_6\text{-}B_6^*]^{-}$ complementary ions, respectively, according to the nomenclature established by Mc Luckey et al. [21]. This represents a specific fragmentation pattern already reported for modified oligonucleotides [24], in which the oligonucleotide preferentially fragments at the modified site [25]. These features allowed us to locate the modification on the reactive G base. On the other hand, the fragmentation pattern of G3 was very different and mainly led to the loss of the steroid moiety (*m/z* 1203 fragment ion), whereas the $[w_6]^{2-}$ and $[a_6\text{-}B_6^*]^{-}$ ions were not observed for the collision energy used in this experiment (Fig. 2c). Although a weak signal was observed at *m/z* 1153, corresponding to the loss of the modified G* base, at this stage, the localization of the modified base remained uncertain from these fragment ions, since the *m/z* 1153 fragment may arise from the consecutive loss of G regardless of the elimination of the steroid (*m/z* 1203 fragment ion). Although important information was obtained by these MS–MS experiments, we decided to get more precise structural information by monitoring the abundances of the various fragment ions produced by decomposition of the three isomeric $[M\text{-}3H]^{3-}$ ions as a function of the energy of these precursor ions. The use of tandem mass spectrometry in such a way is referred to as energy resolved mass spectrometry (ERMS) experiments, and is known to be a powerful tool for the differentiation of constitutional as well as stereoisomers [29]. ERMS experiments can be carried out by using up-front collision-induced dissociation with ion sources operating at atmospheric pressure [30–32] or by using MS–MS in triple quadrupole [32] or ion trap [33] instruments. When ion trap mass analyzers are concerned, this is achieved by varying the voltage applied to the ion trap end-cap electrodes for the collisional activation of the isolated precursor ions. In this work, ERMS experiments were carried out on the various $[M\text{-}3H]^{3-}$ parent ions within the elution of the chromatographic peaks during the LC–MS–MS run, by increasing the ion trap end-cap electrodes voltage from 0.25 V_{p-p} to 1 V_{p-p} with 0.05 V increments. The resulting energy resolved breakdown graphs are reported in Fig. 3. Firstly, they indicate that the formation of the $[w_6]^{2-}$ and $[a_6\text{-}B_6^*]^{-}$ complementary fragment ions is also observed in the decomposition of the $[M\text{-}3H]^{3-}$ parent ions from G3, when submitted to higher collision energies (i.e., >0.5 V). As previously emphasized, the occurrence of these fragment ions can be considered as characteristic of the modification of the G base within the oligonucleotidic sequence, and thus, G3 can be characterized as a third isomeric modified TTTTTGTTTTTT in which G is the modified base. Fig. 3 also provides additional data for the discrimination of G1 and G2. By scrutinizing the breakdown graphs of G1 and G2 (Fig. 3a and b), one can observe that the (*m/z* 1617)/(*m/z* 1153) abundance ratio is much higher for G2 than for G1 in the 0.7–0.9 V region. In other words, the consecutive decomposition of the *m/z* 1153 fragment ion into the *m/z* 1617 (and 920) ions is favored for G2 compared to its G1 isomer. Thus,

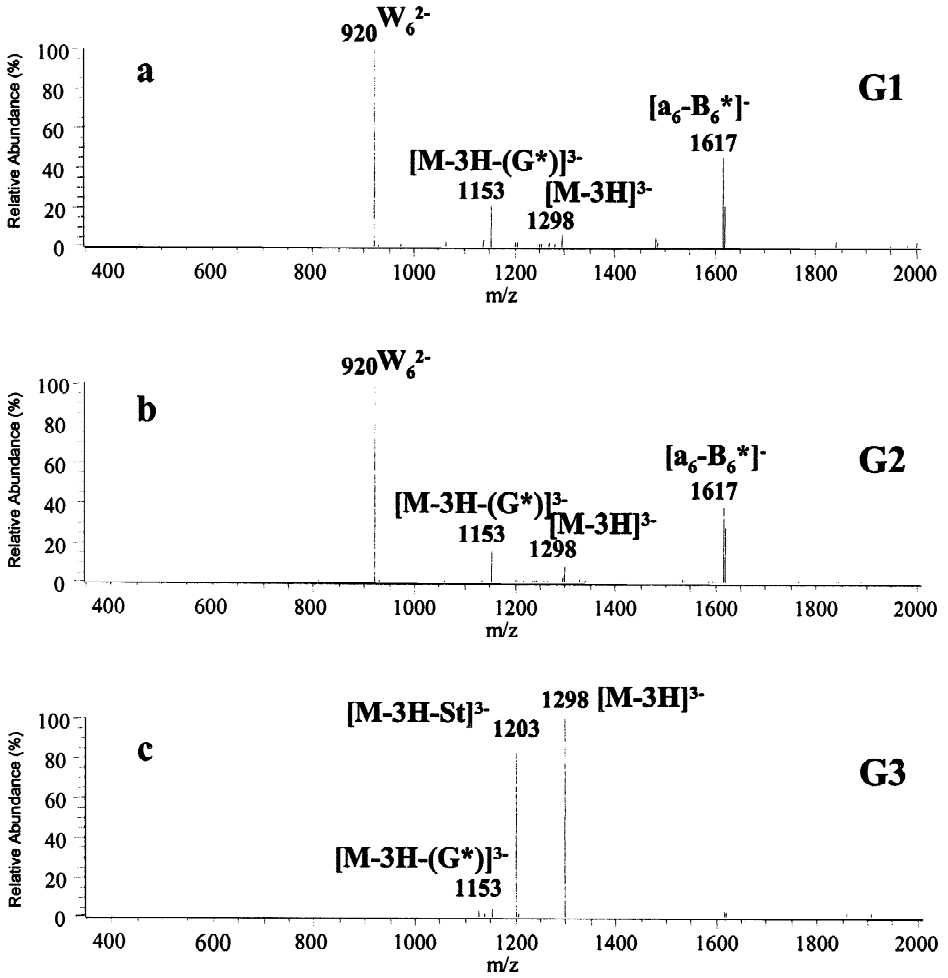

Fig. 2. CID mass spectra obtained from the isomeric m/z 1298 $[M\text{-}3H]^{3-}$ ions of (a) adduct G1, (b) adduct G2 and (c) adduct G3 from modified TTTTTGTTTTTT.

according to these results, G1 and G2 could be better distinguished by recording a CID spectrum at 0.8 V., corresponding to the voltage value for which the difference between the (m/z 1617)/(m/z 1153) abundance ratio for G1 and G2 is maximized.

From this information, it can be concluded that the reaction of TTTTTGTTTTTT with E_2-2,3-Q produced three stable adducts corresponding to the addition of one molecule of steroid to the oligonucleotide. The decomposition of these adducts in MS–MS experiments provided diagnostic fragment ions, which allowed us to localize the modified site as the G base. This result is in agreement with previous data obtained on individual model nucleosides, which showed that several isomeric adducts were formed with dG whereas no reaction occurred with dT [5,8]. In these previous works, dG was shown to give two major stable adducts by linkage of the base on the C6α and C6β faces of the steroid, via its exocyclic N^2 nitrogen atom, and a third minor adduct resulting from the nucleophilic attack of the N^2 atom of dG on the aromatic A ring of the steroid. Taking this information into account, one could deduce that G1 and G2 represent stereoisomeric adducts in which the steroid is attached to the base of the oligonucleotide via a C6α (C6β)/N^2 linkage whereas in G3, the attachment may involve the C9 or the aromatic A ring (C1 or C4) positions

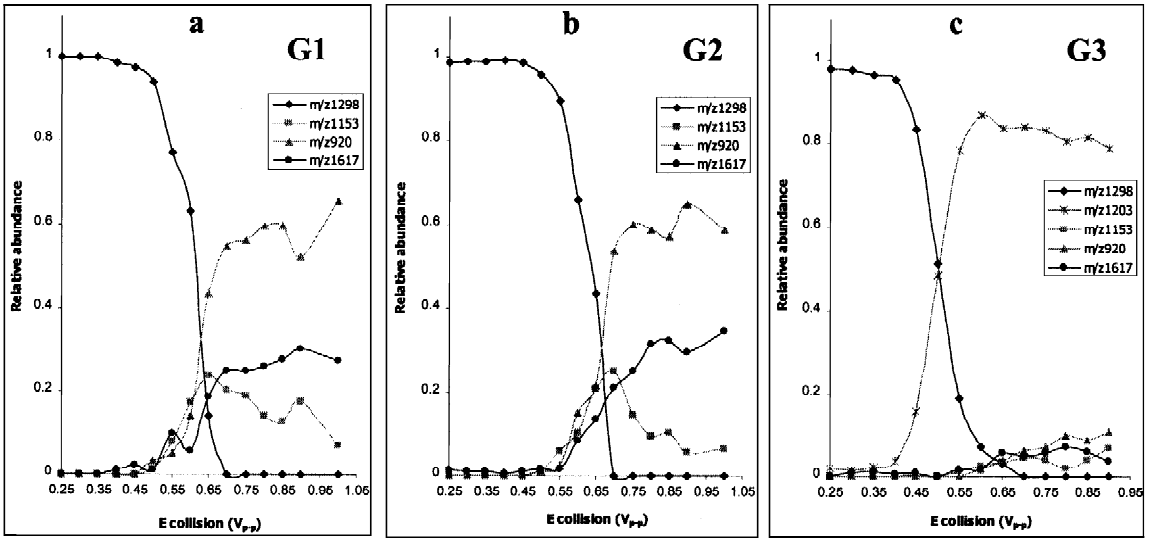

Fig. 3. Energy resolved breakdown graphs obtained from the isomeric m/z 1298 $[M\text{-}3H]^{3-}$ ions of (a) adduct G1, (b) adduct G2 and (c) adduct G3 from modified TTTTTGTTTTTT. .

[5,8,11] of the estrogen. However, no further information could be obtained on the nature of the steroid–base linkage of the adducts studied in this work, due to the absence of negatively charged steroid–base moiety in the MS–MS spectra. Indeed, the decomposition of the modified oligonucleotides always led to fragment ions in which the negative charges were located on the nucleotidic part of the adduct. Besides, it is noteworthy that all data previously obtained on adducts generated from individual nucleosides were obtained using positive ionization modes, either fast atom bombardment (FAB) [5,7] or ESI [8,11,27]. Therefore, they could not be used for the interpretation of the decomposition processes in the present work carried out on oligonucleotides using negative ionization mode, since the behavior of positive protonated species and negative ions towards collisional activation can be totally different.

3.2. TTTTTATTTTTT

The LC–ESI-MS analysis of the crude mixture obtained after reaction of E_2-2,3-Q with TTTTTATTTTTT resulted in the detection of $[M\text{-}3H]^{3-}$ ions at m/z 1198 and m/z 1293 (A1, A2, A3), corresponding, respectively, to the unmodified and three modified forms of the oligonucleotide. As observed for TTTTTGTTTTTT, the mass shift from m/z 1198 to m/z 1293 also corresponded to the addition of one molecule of 2OH-E_2 to the oligonucleotide. When selected and submitted to collisional excitation, the $[M\text{-}3H]^{3-}$ ions of the three adducts (A1, A2, A3) decomposed according to the same fragmentation pathway (Table 1). The loss of the steroid moiety leading to the m/z 1198 ion confirmed the presence of 2OH-E_2 on the modified oligonucleotide. The specific fragmentation leading to the complementary m/z 920 $[w_6]^{2-}$ and m/z 1617 $[a_6\text{-}B_6^*]^-$ ions, was also observed as for the adducts from TTTTTGTTTTTT. Thus, the fragmentation data provided by the MS–MS spectra showed that for the three adducts, A was the modified base on this oligonucleotide. Energy resolved mass spectrometry experiments were then conducted on the various $[M\text{-}3H]^{3-}$ parent ions. The three isomeric adducts were differentiated on the basis of the relative intensities of the respective $[M\text{-}3H\text{-}St]^{3-}$ (m/z 1198), $[M\text{-}3H\text{-}A^*]^{3-}$ (m/z 1153) and $[w_6]^{2-}$ (m/z 920) fragment ions for a collision energy of 0.65 V (data not shown). Under these conditions, the m/z 920 $[w_6]^{2-}$ was the most intense fragment ion for A1 and A2 adducts whereas A3 mainly decomposed into the m/z 1198 fragment ion (loss of the steroid moiety). A1 and A2 behaved differential-

ly with regard to the ratio of the m/z 1153 to m/z 1198 fragment ion relative abundances which was >1 for A1 and <1 for A2. Thus, the additional data provided by the ERMS experiments clearly evidenced different behavior of the three isomeric modified oligonucleotides.

3.3. TTTTTATTTATT and TTTTATTTGTTTT

The 12-mer TTTTTATTTATT and the 13-mer TTTTATTTGTTTT crude reaction mixtures were analyzed using LC–ESI-MS, as described above. Four isomeric adducts (AG1, AG2, AG3, AG4) were separated and detected as their $[M\text{-}3H]^{3-}$ ions at m/z 1403 for the modified 13-mer as indicated in Fig. 4 and Table 2, and two adducts (AA1, AA2) were evidenced at m/z 1296 from modified TTTTTATTTATT (Table 2). In both cases, this corresponded to adducts in which one molecule of 2OH-E_2 is linked to the oligonucleotide, since the unmodified oligonucleotides were represented by their $[M\text{-}3H]^{3-}$ ion at m/z 1201 for the 12-mer (Table 2) and m/z 1308 for the 13-mer (Fig. 4a). Despite the fact that both oligonuceotides bear two reactive bases, no signal corresponding to $[M\text{-}3H]^{3-}$ ions at m/z 1391 or m/z 1498 for doubly modified TTTTTA*TTTA*TT and TTTTA*TTTG*TTTT, respectively, was detected, meaning that no adduct containing two steroid moieties was formed in the reaction conditions we used in this study. It should be noted that in this case again, no abasic site was generated in the reaction of E_2-2,3-Q with these oligonucleotides. Although the formation of depurinating adducts has already been reported from model reactions between estradiol-2,3-quinone and puric bases (dA, dG) [8,11], the occurrence of such adducts was never evidenced in vivo with estrogen-2,3-quinones contrary to estrogen-3,4-quinones [6]. Indeed, considering that different reaction media and stoechiometries were used for the syntheses of adducts with nucleosides and oligonucleotides, the chemical model studies carried out on nucleosides [5,8] may not accurately predict the adducts formed with DNA [6] or model oligonucleotides.

MS–MS experiments were also carried out on the various isomeric adducts. As an example, the corresponding CAD spectra obtained for modified TTTTATTTGTTTT are reported in Fig. 5. Data obtained from the MS–MS analysis of the isomeric adducts are reported in Table 2. Adducts AG1 and AG2 displayed similar spectra concerning the m/z ratios of the main fragment ions arising from the decomposition of their $[M\text{-}3H]^{3-}$ ions. However, it should be noted that the $[M\text{-}3H]^{3-}$ ion from AG1 proved to be much more stable than the $[M\text{-}3H]^{3-}$ ion from AG2, as shown by the relative intensities of the m/z 1403 ions displayed in Fig. 5a and b. The same trend could be observed for AG3 and AG4, which displayed similar CID spectra, and a more stable $[M\text{-}3H]^{3-}$ ion for AG3 compared to AG4 (see Fig. 5c and 5d). As expected for an oligonucleotide bearing two reactive bases, modifications could be evidenced and located on both reactive A and G bases along the oligonucleotide chain. Indeed, the

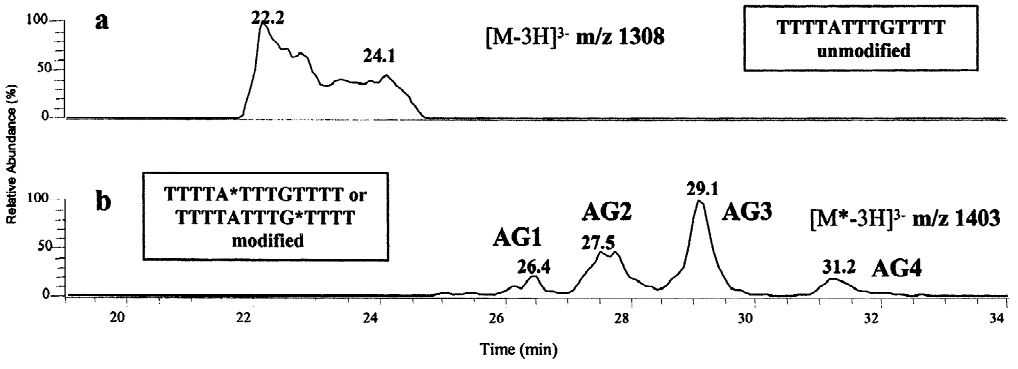

Fig. 4. LC–negative ion ESI-MS chromatograms obtained from the injection of a crude reaction mixture of E_2-2,3-Q with TTTTATTTGTTTT: reconstituted ion chromatograms for the $[M\text{-}3H]^{3-}$ ions of (a) the unmodified (m/z 1308) and (b) the modified oligonucleotide (m/z 1403).

Table 2
Fragment ions obtained from the MS–MS analysis of negative ESI produced $[M\text{-}3H]^{3-}$ ions from unmodified oligonucleotides TTTTATTTGTTTT, TTTTTATTTATT, and their corresponding adducts

	TTTTATTTGTTTT	Adducts (AG1/AG2)	Adducts (AG3/AG4)
Selected parent ion	$[M\text{-}3H]^{3-}$ m/z 1308	$[M^*\text{-}3H]^{3-}$ m/z 1403	
		$[M^*\text{-}3H\text{-}St]^{3-}$ (**1308**)	$[M^*\text{-}3H\text{-}St]^{3-}$ (**1308**)
Fragment ions (***m/z***)	$[M\text{-}3H\text{-}(A)]^{3-}$ (**1263**) $[M\text{-}3H\text{-}(G)]^{3-}$ (**1257**) $[a_5\text{-}B_5]^-$ (**1313**) $[a_9\text{-}B_9]^{2-}$ (**1269**) w_8^{2-} (**1237**) w_4^- (**1233**)	$[M^*\text{-}3H\text{-}(A)]^{3-}$ (**1358**) $[M^*\text{-}3H\text{-}(G^*)]^{3-}$ (**1257**) $[a_5\text{-}B_5]^-$ (**1313**) $[a_9\text{-}B_9^*]^{2-}$ (**1269**) $(w_8^*)^{2-}$ (**1379**) w_4^- (**1233**)	$[M^*\text{-}3H\text{-}(A^*)]^{3-}$ (**1263**) $[M^*\text{-}3H\text{-}(G)]^{3-}$ (**1353**) $[a_5\text{-}B_5^*]^-$ (**1313**) $[a_9^*\text{-}B_9]^{2-}$ (**1412**) w_8^{2-} (**1237**) w_4^- (**1233**)
	TTTTTATTTATT	Adducts (AA1)	Adducts (AA2)
Selected parent ion	$[M\text{-}3H]^{3-}$ m/z 1201	$[M^*\text{-}3H]^{3-}$ m/z 1296	
Fragment ions (***m/z***)	$[M\text{-}3H\text{-}(A)]^{3-}$ (**1156**) $[a_6\text{-}B_6]^-$ (**1617**) $[a_6\text{-}B_6]^{2-}$ (**808**) $[a_{10}\text{-}B_{10}]^{2-}$ (**1421**) w_6^{2-} (**925**) w_2^- (**625**)	$[M^*\text{-}3H\text{-}(A^*)]^{3-}$ (**1156**) $[a_6\text{-}B_6^*]^-$ (**1617**) $[a_6\text{-}B_6^*]^{2-}$ (**808**) $[a_{10}^*\text{-}B_{10}]^{2-}$ (**1564**) w_6^{2-} (**925**) w_2^- (**625**)	$[M^*\text{-}3H\text{-}(A^*)]^{3-}$ (**1156**) $[a_6\text{-}B_6]^-$ (**1617**) $[a_6\text{-}B_6]^{2-}$ (**808**) $[a_{10}\text{-}B_{10}^*]^{2-}$ (**1421**) $(w_6^*)^{2-}$ (**1068**) w_2^- (**625**)

decompositions of the $[M\text{-}3H]^{3-}$ ion led to diagnostic fragment ions, respectively at m/z 1379, 1313, 1269 and 1233 for AG1 and AG2 (Fig. 5a and b), and at m/z 1412, 1313, 1237 and 1233 for AG3 and AG4 (Fig. 5c and d). These fragment ions arose from the characteristic decompositions occurring on modified oligonucleotides [24,25]. As summarized in Fig. 6a for AG1 and AG2, the fragment ions displayed at m/z 1233 and 1379 could be attributed to $[w_4]^-$ and $[w_8^*]^{2-}$ ions, respectively, which indicated that the m/z 1379 ion arising from the cleavage of the 3′ C–O bond at the fifth base from the 5′ end (A_5) still contained the steroid moiety whereas the m/z 1233 ion, corresponding to the cleavage of the 3′ C–O bond at the ninth base from the 5′ end (G_9^*) did not. Furthermore, the corresponding complements to these ions were observed at m/z 1313 for $[a_5\text{-}B_5]^-$ and m/z 1269 for $[a_9\text{-}B_9^*]^{2-}$. This indicated that the m/z 1269 fragment ion contained the modified base whereas the m/z 1313 ion did not, which confirmed the previous information. In the same manner, non modified $[w_4]^-$ (m/z 1233) and $[w_8]^{2-}$ (m/z 1237), as well as modified $[a_5\text{-}B_5^*]^-$ (m/z 1313) and $[a_9^*\text{-}B_9]^{2-}$ (m/z 1412) fragment ions could be observed on the CAD spectra of AG3 and AG4 (Fig. 5c and d) which provided evidence for the modification of the A base. Moreover, the presence of the m/z 1257 and 1358 fragment ions (elimination of modified G_9^* and non-modified A_5 bases, respectively) on the CAD spectra of AG1 and AG2 (Fig. 5a and b) on the one hand, and the m/z 1263 and 1353 ions (elimination of modified A_5^* and non-modified G_9 bases, respectively) on the CAD spectra of AG3 and AG4 (Fig. 5c and d) on the other hand, constitute additional data supporting the above conclusions. Thus, AG1 and AG2 could be identified as isomeric adducts of TTTTATTTGTTTT modified on the G base whereas A was the modified base in AG3 and AG4.

In the same manner, MS–MS experiments were carried out on the m/z 1296 $[M\text{-}3H]^{3-}$ ions of AA1 and AA2 from modified TTTTTATTTATT (see Table 2). The CAD spectrum of AA1 was characterized by diagnostic fragment ions at m/z 625 $[w_2]^-$, m/z 925 $[w_6]^{2-}$, m/z 1564 $[a_{10}^*\text{-}B_{10}]^{2-}$ and m/z 1617 $[a_6\text{-}B_6^*]^-$ which revealed the modification of the sixth base (A_6) from the 5′ end as indicated on Fig. 7a. On the other hand, fragment ions present at m/z 625 $[w_2]^-$, m/z 1068 $[w_6^*]^{2-}$, m/z 808 $[a_6\text{-}$

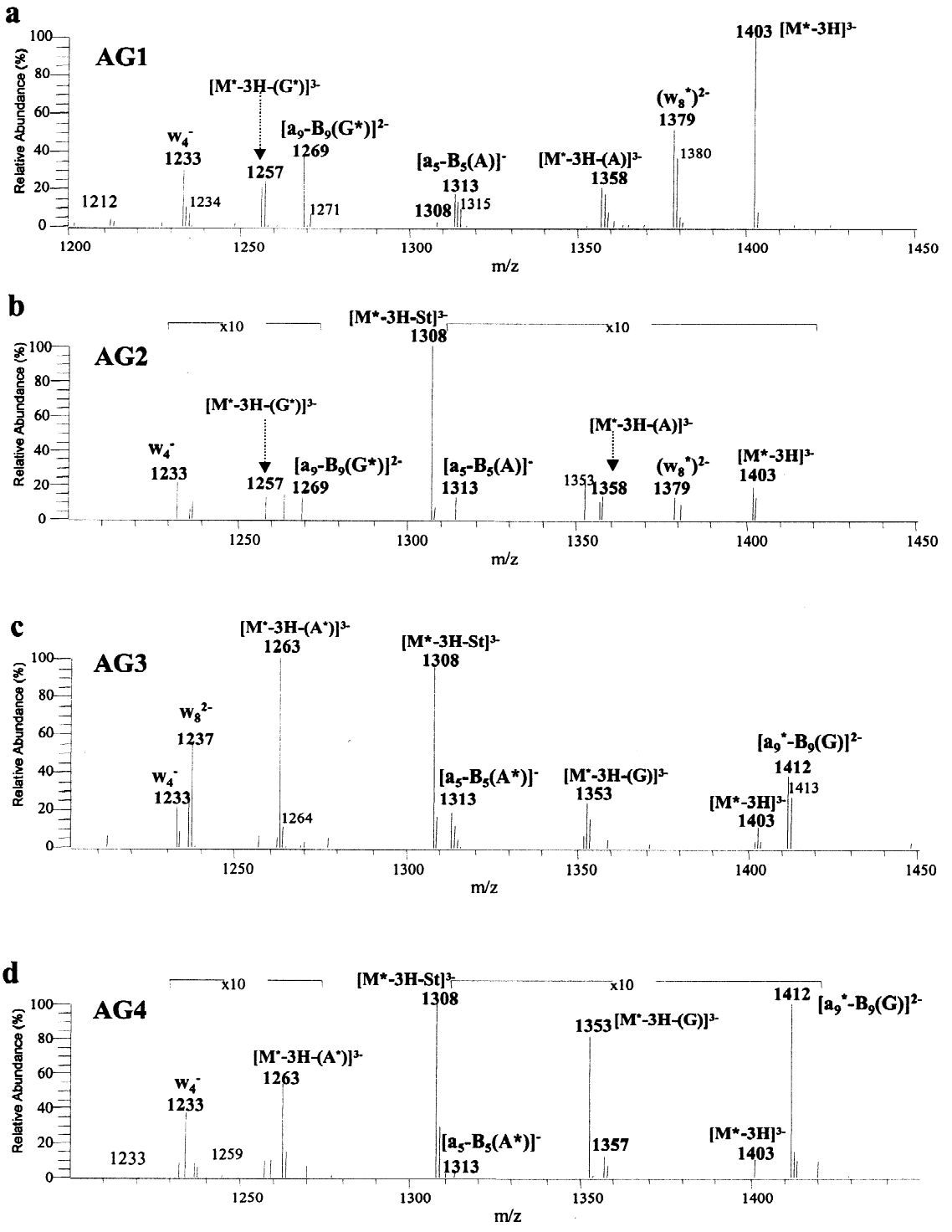

Fig. 5. CID mass spectra obtained from the isomeric m/z 1403 $[M\text{-}3H]^{3-}$ ions of (a) adduct AG1, (b) adduct AG2, (c) adduct AG3 and (d) adduct AG4 from modified TTTTATTTGTTTT.

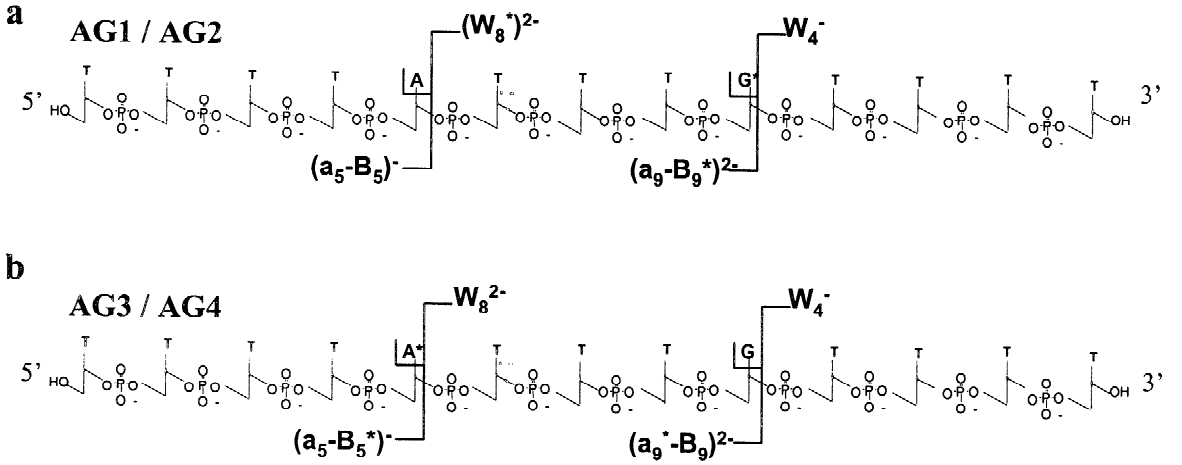

Fig. 6. Summary of fragmentation of (a) AG1 and AG2, and (b) AG3 and AG4 isomeric adducts.

$B_6]^{2-}$ and *m/z* 1421 $[a_{10}\text{-}B^{*}_{10}]^{2-}$ provided evidence for a modification of the A_{10} base on AA2 (see Fig. 7b). Thus, the fragmentation patterns observed for AA1 and AA2 showed that modification could occur on both reactive A bases along the oligonucleotide chain, but that only one A base was modified for each adduct formed. Besides, only one adduct was detected for each modified position in this case, suggesting that the reaction of E_2-2,3-Q with TTTTTATTTATT was regiospecific. As a matter of fact, previous studies carried out on nucleosides have shown that dG gave more isomeric adducts than dA [5,7,8], and thus, compared to the previous 13-mer described above, this 12-mer could be expected to give less isomeric forms of adducts. However, the co-elution of isomeric AA adducts in the chromatographic conditions used in this work cannot be totally ruled out.

4. Conclusion

As previously described for other modified

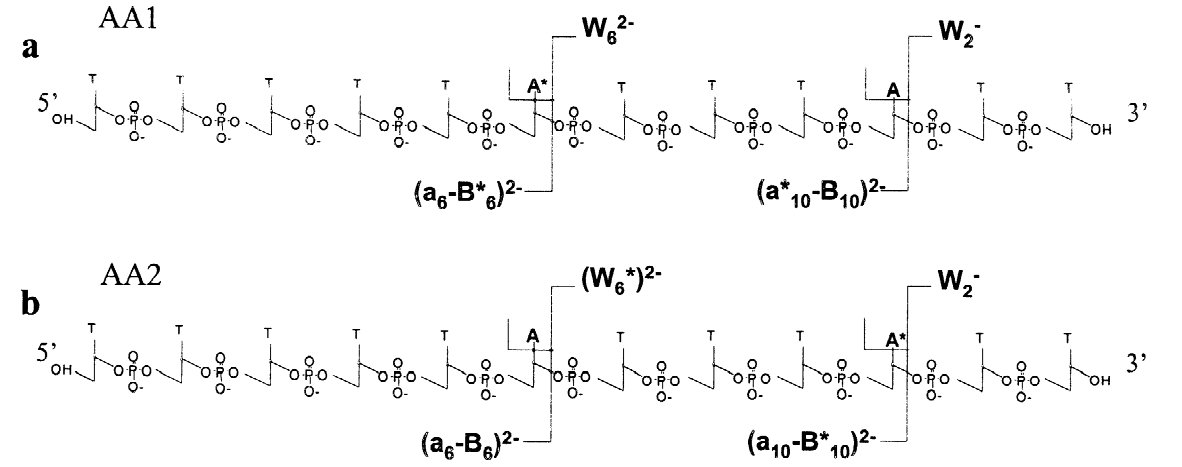

Fig. 7. Summary of fragmentation of (a) AA1 and (b) AA2 isomeric adducts.

oligonucleotiodes, liquid chromatography coupled to negative electrospray ion trap spectrometry provides a powerful tool for the detection and the characterization of isomeric covalent adducts corresponding to the addition of one molecule of 2OH-E_2 to the oligonucleotide. In this work, T-rich model oligonucleotides were studied in order to assess the feasibility of our methodology for the positional mapping of the covalent modifications. As expected from the reactivity of puric bases compared to pyrimidines, MS–MS experiments carried out on the triply charged molecular species demonstrated that the linkage always occurred on the reactive base (i.e., A or G) whereas no attachment of the steroid on T bases was observed. In the experimental conditions we used for the chemical preparation of the adducts, no more than one reactive base was modified on the oligonucleotides, and no abasic site was formed. Although the precise structure of each adduct could not be determined, isomeric differentiation could be achieved using ERMS experiments on each isomeric adduct. These studies clearly demonstrate that negative electrospray coupled to ion trap tandem mass spectrometry constitutes a very powerful method for the location of oligonucleotide modifications by estrogen quinones and the differentiation of isomeric adducts.

Studies are now in progress in order: (i) to improve analytical conditions using micro-HPLC [20,34,35] for the detection of modified oligonucleotides from in vitro or in vivo samples, and (ii) to develop mass spectrometric experiments allowing further investigation of the modified base moiety formed in the MS–MS experiments under negative ionization conditions, thus providing more structural information about the precise sites involved in the linkage between the catechol estrogen and the oligonucleotide.

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