Electrospray–mass spectrometry characterization and measurement of far-UV-induced thymine photoproducts 1

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Abstract

Far-UV-induced formation of dimeric pyrimidine photoproducts within DNA is a major cause of the carcinogenic effects of solar light. The chemical structure of this class of lesion has been mostly determined by studies on model compounds. The present work is aimed at providing mass spectrometry data on the thymine–thymine photoproducts, including the diastereoisomers of the cyclobutane dimer, the (6–4) adduct, the related Valenwic valence isomer and the spore photoprodout. Fragmentation mass spectra of the modified bases, nucleosides, dinucleoside monophosphates and dinucleotides were recorded following electrospray ionization with either triple-quadrupolar or ion-trap detection. The results showed differences in fragmentation pattern between the different types of photoproducts. In addition, a drastic effect of the diastereoisometry was observed for the cyclobutane dimers. A sensitive detection technique has been developed for the analysis of dinucleoside monophosphates and dinucleotides recorded following electrospray ionization with either triple-quadrupolar or ion-trap apparatus. The latter MS system allows the determination of fragment ions that may be further fragmented. The process was applied to the characterization of thymine–thymine photoproducts at the base, nucleoside, dinucleoside monophosphate and dinucleotide level. Analyses were carried out on both a triple-quadrupolar spectrometer and an ion-trap apparatus. The latter MS system allows the determination of fragment ions that may be further fragmented. The process can be repeated up to ten times (MSn analysis, n < 10). In addition, the coupling of high-performance liquid chroma-

1. Introduction

The mutagenic and carcinogenic effects of solar light are mainly mediated by the UV portion of its spectrum [1]. In particular, UV-B radiation is highly lethal and mutagenic to cells. At these wavelengths, the mutagenicity and lethality action spectra are identical to the DNA absorption spectrum. This strongly supports the involvement of photochemical reactions induced by the direct absorption of UV-B light by DNA components. The main class of UV-B-induced lesions consists of dimeric pyrimidine photoproducts (Fig. 1). Efforts have been devoted in the last four decades to elucidating the chemical nature of these photolesions [2,3]. These include cyclobutane-type dimers arising from the cycloadition of the C5–C6 double bond of two adjacent pyrimidines, and the pyrimidine (6–4) pyrimidone adducts, which result from the addition of the C5–C6 double bond of the 5′-end pyrimidine to the C4 carbonyl or imine group of the 3′-end pyrimidine. Subsequently, the (6–4) photoproducts can photoisomerize into their Dewar valence isomers upon UV-B irradiation. It may be added that, in dry materials, an additional photoproduct, known as the spore photoprodout [4], is generated through the addition of the methyl group of a thymine to the C5 position of an adjacent thymine. These photoproducts have been studied using various model compounds including bases, nucleosides and dinucleoside monophosphates [5–17]. Extensive characterization has been carried out by UV absorption, fluorescence, NMR and mass spectrometry measurements. The last technique has mostly been used to determine the molecular weight of isolated photoproducts, but only a few extensive studies of the fragmentation patterns have been reported [18–21]. In the present work, electrospray tandem mass spectrometry (ES/MS–MS) was applied to the characterization of thymine–thymine photoproducts at the base, nucleoside, dinucleoside monophosphate and dinucleotide level. Analyses were carried out on both a triple-quadrupolar spectrometer and an ion-trap apparatus. The latter MS system allows the determination of fragment ions that may be further fragmented. The process can be repeated up to ten times (MSn analysis, n < 10). In addition, the coupling of high-performance liquid chroma-

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2. Materials and methods

2.1. Chemicals

Thymidine was purchased from Sigma (St Louis, MO). Thymidine was obtained from Pharma-Waldhof. Ammonium formate, acetonitrile and formic acid were purchased from Aldrich (Milwaukee, WI). TpT and pTpT (5'-monophosphate thymidylyl(3'-5')thymidine) were prepared by using a phosphotriester method. Water was purified on a Milli Q system (Millipore, Molsheim, France).

2.2. Synthesis of the cyclobutane dimers

Thymidine (20 mg) was irradiated in a quartz vial in 30 ml of a 10 mM aqueous solution of acetonophenone. UV-B light (300 nm) was provided by 16 lamps of a Rayonet photoreactor (The Southern New England Ultraviolet Company, Handem, MA). Then, the volume was reduced to 2 ml under vacuum. The six diastereoisomers of the thymidine cyclobutane dimers were separated by successive HPLC purifications [22] with a UV spectrometer set at 230 nm as the detector. A first separation was performed on an Inertsil ODS 2 octadecylsilyl silica gel column (250×4 mm ID, 5 μm particle size, Interchim, Montluçon, France) with a [90:10] mixture of a 25 mM aqueous solution of ammonium formate and methanol used as the isocratic eluent. The cis-syn and trans-anti diastereoisomers were isolated as pure material (retention times 9.6 and 25.6 min, respectively). Two fractions that contained a mixture of the (−)trans-syn and the (−)cis-anti on one hand, and the (+)trans-syn and the (+)cis-anti diastereoisomers on the other hand were collected at retention times of 11.2 and 12 min, respectively. The two latter fractions were concentrated under vacuum and injected on a Hypersil NH₂ amino column (250×4.6 mm ID, 5 μm particle size, Interchim, Montluçon, France) with a [7:93] mixture of a 25 mM aqueous solution of ammonium formate and acetonitrile used as the isocratic eluent. The retention times of the thymidine cyclobutane dimers were the following: (−)trans-syn, 8; (−)cis-anti, 11.2; (+)trans-syn, 8.8 and (+)cis-anti, 12 min. Diastereoisomers of thymine cyclobutane dimers were prepared by 88% formic acid hydrolysis (140°C, 2 h) of the corresponding purified thymidine photoproducts. Formic acid was evaporated under vacuum. The sample was then solubilized in water. TpT and pTpT cyclobutane dimers were prepared by photosensitization of 5 mg of the starting material in 20 ml of a 10 mM aqueous solution of acetonophenone using the procedure used for the synthesis of thymidine dimers. The photoproducts were isolated by reverse-phase HPLC as reported elsewhere [23]. The photoproducts were identified by comparison of their retention times and UV spectra with those of authentic standards.

2.3. Synthesis of the (6-4) photoproducts and their related Dewar valence isomers

The (6-4) photoproduct of thymine was obtained by irradiation of the base in frozen aqueous solution (20 mg in 20 ml solution). The sample was placed in a 20 cm diameter Petri dish and subsequently frozen on dry ice. It was then exposed to the 254 nm light emitted by a germicidal lamp for 2 h. Then, the solution was concentrated to 5 ml, centrifuged and the liquid phase injected on an Inertsil ODS2 octadecylsilyl silica gel column (250×4.6 mm ID, 5 μm particle size, Interchim, Montluçon, France). The gradient of elution consisted of a mixture of a 25 mM aqueous solution and acetonitrile. The proportion of the latter solvent linearly increased from 0 to 5% within 15 min. The elution was monitored at 315 nm. The retention time of the thymine (6-4) photoprodut was 11.1 min. The two diastereoisomers of the thymidine (6-4) adducts were prepared by irradiation at room temperature of a saturated aqueous solution of thymidine with the 254 nm light emitted by a germicidal lamp. Then, the solution was freeze-dried overnight and the resulting residue was dissolved in a [90:10] mixture of acetonitrile and water. The
two diastereoisomers were then isolated on a semi-preparative Hypersil NH₂ amino column (250×10 mm ID, 5 μm particle size) with the UV detector set at 320 nm. The retention times of the two diastereoisomers were 24 and 32 min, respectively, when a [90:10] mixture of acetonitrile and 25 mM aqueous solution of ammonium formate was used as the isocratic eluent. The (6–4) photoproducts of TpT and pTpT were obtained by exposure to 254 nm light for 2 h of 5 mg of starting material in aqueous solution (20 ml) placed in a 20 cm diameter Petri dish. The solution was then concentrated under vacuum and the photoproducts isolated by reverse-phase HPLC as previously reported [13,16]. The thyminde valence isomers of thymine, thymidine, TpT and pTpT were all prepared using the same protocol. A 1 ml solution of the corresponding (6–4) photoproduct was placed in a UV spectrophotometer cell and the UV absorbance at 320 nm was measured. Then, the quartz cell was placed in a Rayonet photoreactor equipped with 16 300 nm lamps. After increasing irradiation times, the 320 nm absorbance of the solution was measured. The absorption was stopped when no residual 320 nm radiation remained.

2.4. Synthesis of the spore photoproducts

Three methods were used for the preparation of the thymidine spore photoproducts. (i) Thymidine (100 mg) was solubilized in 50 ml of ethanol. The resulting solution was placed in a 4 cm×30 cm vial and evaporated overnight. Then, the dry residue was irradiated for 24 h with a 2×15 W germicidal lamp. A hair dryer was blown on the dry film throughout the irradiation to prevent moisture absorption. In a subsequent step, 5 ml of water was added to the solid film. The resulting suspension was centrifuged and the liquid phase collected. (ii) Thymidine (100 mg) was solubilized in 50 ml of ethanol together with 50 mg of benzophenone. The resulting solution was placed in a 4 cm×30 cm vial and evaporated overnight. The dry residue was then irradiated for 17 h in a Rayonet photoreactor equipped with ten 350 nm lamps. Then, 5 ml of water was added to the solid film. The resulting suspension was centrifuged and the liquid phase collected. (iii) Thymidine (300 mg) was solubilized in 50 ml of water. The solution was frozen on dry ice in a 12 cm diameter Petri dish and then irradiated for 2 h with a 254 nm germicidal lamp. The solution was thawed and irradiated for 30 min in a Rayonet photoreactor equipped with ten 300 nm lamps in a 150 ml quartz vial. The latter step was aimed at converting (6–4) photoproducts into their Dewar valence isomers in order to facilitate the subsequent HPLC purification. The solution was then concentrated to 5 ml under vacuum. Following any of the three procedures described above, the thymidine spore photoproducts were purified by reverse-phase HPLC on a Uptisphere ODB octadecysilil silica gel column (250×4.6 mm ID, 5 μm particle size, Interchim, Montluçon, France). The chromatogram was a [85:15] mixture of a 25 mM aqueous solution of ammonium formate and methanol used at a flow rate of 1 ml/min. The retention times of the two diastereoisomers of the thymidine spore photoproduct were 18 and 23 min. The two first syntheses provided only the slowly eluting isomer. The two diastereoisomers were characterized by comparison of their UV absorption and 1H NMR features with published data [2,3]. The thymine spore photoproduct was obtained by hot formic acid hydrolysis of the related thymidine derivatives.

2.5. ES/MS–MS analyses

All photoproducts were analysed by electrospray mass spectrometry in the positive mode. For this purpose, the products were solubilized in a [1:1] mixture of acetonitrile and water that contained 0.1% formic acid. The photoproducts of TpT and pTpT were also analysed in the negative mode in a [1:1] mixture of acetonitrile and water that contained 25 mM ammonium formate. All spectra were recorded with unity resolution. The same ionization and fragmentation conditions were used within a homologous series of photoproducts. Fragmentation mass spectra on the triple-quadrupole system were recorded on a Quattro II spectrometer (Micromass, UK). The argon pressure in the collision cell was set between 5×10⁻⁶ and 10⁻³ mTorr. Simultaneous acquisitions of spectra with different settings of the collision energy were performed. Mass spectra were also recorded on an ion-trap LCQ spectrometer (Finnigan MAT, San Jose, CA). MS² and MS³ spectra were recorded for all compounds on the latter apparatus. When the intensity of the signal was high enough, MSⁿ (n≤5) analyses were also carried out. TpT photoproducts were detected by HPLC coupled to tandem mass spectrometry performed on an API3000 spectrometer (Perkin–Elmer/Sciex, Toronto, Canada). The HPLC system consisted of a 7100 Hitachi–Merck pump (Merck, Darmstadt, Germany) associated with an SIL-9 automatic injector (Shimadzu, Tokyo, Japan). The column was an Uptisphere ODB (150×2 mm ID, 5 μm particle size) octadecysilil silica gel column (Interchim, Montluçon, France). The mobile phase was a gradient of 0.5 mM ammonium formate and methanol with a flow rate of 200 μl/min. The proportion of methanol rose from 0 to 2% within 8 min and reached 28% after 20 min. Methanol was also added at the outlet of the column and prior to the inlet of the mass spectrometer at a flow rate of 0.2 ml/min. The spectrometer was operated in the negative mode and the analyses were performed in the MRM mode. Two transitions were monitored: 545→447 and 545→532. The dwell time was set at 1 s for both signals.

3. Results and discussion

3.1. Preparation of the photoproducts

The synthesis and purification of the cyclobutane dimers and the (6–4) adducts of thymine, thymidine, TpT and pTpT were achieved using published procedures [7,13,23–25]. The preparation of the Dewar valence isomers by photoiso-
merization of solutions of (6−4) photoproducts allowed a comparison of the influence of the structure of the adduct (base, nucleoside or dinucleoside monophosphate) on the efficiency of the photoisomerization reaction. The main effect was observed for the thymine (6−4) photoproduct, which was found to be converted significantly more slowly than the related thymidine, TpT and pTpT adducts. This reflects a significant change in the photochemical properties of this class of photoproduct when a free rotation along the C6−C4 bond is possible. A much higher fluorescence quantum yield has already been reported for the free base (6−4) adduct with respect to the related modified dinucleoside monophosphate [26].

Emphasis was also placed on the mechanism of formation of the spore photoprodut. The latter compound has been reported to be obtained from thymidine upon UV-C irradiation either in the dry state or in frozen solution [9]. Interestingly, only one of the two possible diastereoisomers was obtained when thymidine was irradiated as a dry film, whereas the two photoproducts were obtained in frozen solution. This clearly shows the importance of the stacking of the nucleoside in the stereochemistry of the photoreaction. Two mechanisms have been proposed for the formation of the spore photoprodut [2]. The first one involves the recombination of the photogenerated 5-(uracil) methyl and the 5,6-dihydrothymin-5-yl radicals. A concerted mechanism has also been proposed and has been shown actually to take place in the frozen state on the basis of experiments involving irradiation of methyl deuterated thymidine [2]. The present photosensitization experiment of thymidine by benzophenone in the dry state strongly suggests that a concerted mechanism is also involved in the formation of the spore photoprodut in dry films of thymidine. Indeed, the energy of the triplet state of benzophenone is close to that of thymine [27]. Therefore, triplet energy transfer from excited benzophenone to thymine is an efficient process. This has been widely used for the preparation of cyclobutane dimers in aqueous solution of bases, nucleosides, dinucleoside monophosphates and DNA [16,24,27]. The observation of the formation of the spore photoprodut upon irradiation of thymidine in the dry state shows the involvement of the triplet state of the nucleoside, and rules out the radical mechanism. It is worth mentioning that irradiation of TpT in the dry state did not allow the isolation of the related spore photoprodut. A likely explanation for this might be the presence of water bound to the phosphodiester group. Indeed, humidity has been shown to be a key parameter in the formation of the spore photoprodut within DNA films [28].

3.2. Mass spectra of the photoproducts of bases and nucleosides

The syntheses described above provided 7 thymine–thymine and 12 thymidine–thymidine photoproducts. It should be remembered that, for a given series, all types of photoproducts exhibit the same molecular weights, which are 252 and 484, respectively. It is therefore of interest to determine whether the fragmentation pattern of each individual compound allows the different photoproducts to be differentiated. The interpretation of the data presented below is based on fragmentation mass spectra obtained on a quadrupolar spectrometer. The data provided by the analyses carried out on the ion-trap system will be discussed when they provide additional insight into the understanding of the fragmentation pattern of the various photoproducts. However, it must be emphasized that both types of mass spectra exhibited the same ions for a given product.

All thymine photoproducts were analysed by ES/MS–MS (Fig. 2). The main fragment was observed at m/z = 235 for the (6−4) photoproduct of thymine, which corresponds to the dehydration of the C5−C6 bond of the 5′-end thymine. The same fragmentation was obtained for the Dewar valence isomer. In contrast, the spore photoprodut underwent the loss of 17 mass units. No explanation could be found for this loss from the spore photoproducts. To rule out a poor calibration of the spectrometer, a mixture of the (6−4) adduct and the spore photoprodut was analysed and two distinct ions were observed at m/z = 235 and 236. The fragmentation pattern of the cyclobutane dimers was found to depend on the respective orientation of the two C5−C6 bonds (Table 1).

![Fig. 2. Mass spectra of thymine–thymine photoproducts. All spectra were recorded under the same conditions on a triple-quadrupole Quattro II spectrometer. The argon pressure in the collision cell was set to 3 × 10⁻⁴ mTorr and the collision energy was 25 V.](image)

**Table 1**

<table>
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<tr>
<th>Ion</th>
<th>cis–syn</th>
<th>trans–syn</th>
<th>cis–anti</th>
<th>trans–anti</th>
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<tr>
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<td>4</td>
<td>5</td>
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<td>100</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>127</td>
<td>n.d.</td>
<td>n.d.</td>
<td>100</td>
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</tr>
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</table>

Relative abundance of the main ions observed on the mass spectra of thymine cyclobutane dimers. The analyses were performed on a triple-quadrupolar Quattro II spectrometer. The argon pressure in the collision cell was 9 × 10⁻⁴ mTorr and the collision energy was 25 V. n.d. = not detected.
Indeed, the spectra of the cis–syn and the trans–syn isomers were very similar and exhibited a main ion at m/z = 210. This corresponds to the loss of 43 mass units, which can be rationalized in terms of the loss of a HCNO fragment from the pyrimidine ring. This result is in agreement with those obtained by using laser-desorption Fourier-transform mass spectrometry. Indeed, the loss of a HCNO fragment from the [M−H]⁺ pseudo-molecular ion of the cis–syn cyclobutane dimer was observed [18–21]. In contrast to the observation made on the syn diastereoisomers, the main ion observed in the ES/MS–MS fragmentation spectrum of the cis–anti and trans–anti cyclobutane dimers is at m/z = 127. The latter ion corresponds to protonated thymine. This is indicative of a lower stability of the cyclobutane ring of the anti cyclobutadithymines with respect to that of the syn diastereoisomers.

Fragmentation spectra of the diastereoisomeric spore photoproducts of thymidine exhibited two main ions (m/z = 369 and 253) corresponding to the successive loss of the 2-deoxyribose units ([MH−116]⁺ and [MH−232]⁺). The interpretation of the loss of 232 mass units as the fragmentation of the two N-glycosidic bonds was confirmed by ion-trap analysis. Indeed, fragmentation of the [MH−116]⁺ ion yielded the [MH−232]⁺ ion. No significant differences were observed between the spectra of the two diastereoisomers of the thymidine spore photoproducts. The ions at m/z = 369 and m/z = 253 were also observed on the MS–MS spectra of the two (6–4) photoproducts, together with their dehydration products. Analysis with the ion-trap spectrometer showed that the loss of a 2-deoxyribose was favoured, since it is the only fragmentation observed on the MS² spectrum. The loss of one molecule of water is observed on the MS³ spectrum of the [MH−116]⁺ ion, together with the loss of the second 2-deoxyribose unit and a second molecule of water. A similar fragmentation pattern was obtained for the related Dewar valence isomers.

The loss of the 2-deoxyribose unit was also the major fragmentation pathway for the syn thymidine cyclobutane dimers (Table 2). With a low collision energy, only loss of one 2-deoxyribose was observed. However, the abundance of the ions corresponding to the loss of the second sugar ring was found to increase with the collision energy. It should be mentioned that the ion of the protonated 2-deoxyribose (m/z = 117) was observed for collision energies higher than 25 V. Interestingly, the MS³ spectrum of the cis–syn cyclobutane dimer showed that the loss of 232 mass units was more facile than the loss of 116 mass units.

Table 2
Relative abundance of main ions observed on the fragmentation spectrum of the diastereoisomers of the thymidine cyclobutane dimers. The experimental conditions were identical for all dimers. In particular, the argon pressure in the collision cell and the collision energy were set at 8×10⁻⁴ mTorr and 25 V, respectively. n.d. = not detected

<table>
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<tr>
<th>Ion</th>
<th>(−) cis–anti</th>
<th>(−) trans–syn</th>
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<td>485</td>
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<td>10</td>
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<tr>
<td>369</td>
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<tr>
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</tr>
<tr>
<td>117</td>
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</tbody>
</table>

Fig. 3. Ion-trap mass spectra. Left panel: MS² mass spectrum of the thymine cis–syn cyclobutane dimer (parent ion: 253.1). Right panel: MS³ mass spectrum of thymidine cis–syn cyclobutane dimer (parent ion: 485.1 → 369.1 → 253.1). The difference in the relative abundance of the ions is due to a different setting of the collision energy (13 and 11%, respectively).
butadithymidine acquired on the ion-trap spectrometer was identical to the MS’ spectrum of the corresponding thymine photoproduct (Fig. 3). This was expected, since MS’ spectrometry yielded the pseudo-molecular ion of the thymine photoproduct from the related thymidine derivative by successive loss of the two 2-deoxyribose units. This example shows the interest of the ion-trap technology in the characterization of complex molecules. As already observed with thymine photoproducts, the anti thymidine cyclobutane dimers exhibited different spectra from the syn diastereoisomers. Indeed, a significant signal corresponding to protonated thymidine (m/z = 243) was observed together with the ion arising from the loss of the 2-deoxyribose units. Even at low collision energy, two other ions were observed, corresponding to the protonated 2-deoxyribose (m/z = 117) and to protonated thymine (m/z = 127), respectively. The latter ions are likely to arise from the fragmentation of thymidine. Indeed, a complete loss of the 2-deoxyribose ring was observed on the MS–MS spectrum of pure thymidin under the conditions used for the analysis of the photoproducts. These observations confirm that the cyclobutane ring is more stable in the syn cyclobutadithymidine than in the anti diastereoisomers. It should be pointed out that the six thymidine cyclobutane dimers have already been extensively studied by FAB mass spectrometry in both the positive and the negative modes [19]. In contrast to the present results, no major difference was observed between the mass spectra of the different diastereoisomers that exhibited protonated thymine as the main fragmentation product. This can be explained by the higher energy provided to the molecule by the FAB ionization technique, leading to a less selective fragmentation. This comparison illustrates another advantage of the soft electrospray ionization technique.

3.3. Fragmentation of modified dinucleoside monophosphates and dinucleotides

The photoproducts of TpT and pTpT were also analysed by ES/MS–MS in the positive mode. In addition, the presence of the easily ionized phosphate groups allowed their characterization as negatively charged ions. It should be mentioned that not all possible diastereoisomers could be isolated. Only one (6→4) photoproduct isomer is obtained upon exposure of TpT and pTpT to far-UV light. No anti cyclobutadipyrimidine diastereoisomers can be obtained because of the fixed parallel orientation of the C5–C6 double bond of the thymine rings. The cis–syn cyclobutane dimer is the major cyclobutane dimer obtained with both TpT and pTpT, while one trans–syn isomer is produced in lower yield. The minor trans–syn II diastereoisomer of TpT described by Kao et al. [29] could not be isolated, probably because of a too low amount of starting material.

A general trend is that the fragmentation spectra of TpT and pTpT photoproducts are richer in the positive mode than in the negative mode. This was clearly illustrated by the observation that, within the range of collision energy applied to the other photoproducts, no fragmentation was observed for the cis–syn cyclobutane dimer of pTpT in the negative mode. Use of higher collision energy led to a decrease in the signal of the pseudo-molecular ion with no appearance of fragment signals. In contrast, peaks at m/z = 431 and m/z = 449 were observed in a relative abundance of 30% in the mass spectrum of the latter photoprodut recorded in the positive mode (Fig. 4). Similarly, the pseudo-molecular ion was the only signal observed on the MS–MS spectrum of the (6→4) photoprodut of pTpT recorded in the negative mode with a collision energy of 15 V. This energy had to be raised to 30 V to induce significant fragmentation. In contrast, dehydration was found to take place for a collision energy of 15 V in the positive mode. These observations can be explained by the fact that positively charged pseudo-molecular ions are obtained by protonation of either the base or the sugar moiety. This is likely to destabilize the molecule and favour its fragmentation. In contrast, the negative ions arise from the ionization of the phosphate group while the nucleosidic part remains unchanged. It can also be added that TpT photoproducts have been analysed by thermospray mass spectrometry in both the positive and the negative modes [21]. The thermospray spectra exhibit a higher number of fragments with lower masses when compared with the ES/MS–MS data. This is in agreement with the observation made with thymidine photoproducts that ES/MS–MS is a soft ionization technique, which allows a better control of the fragmentation of the analysed compounds.

The mass spectra of the (6→4) photoproduts of TpT and pTpT recorded in the positive mode exhibit mainly fragments arising from the dehydration of the 5′-end thymine, as observed for the related base lesion. This indicates that the presence of a phosphodiester bond in modified TpT and pTpT reinforces the N-glycosidic bond. Indeed, loss of the 2-deoxyribose unit was the main fragmentation observed with thymidine photoproducts (vide supra). The fragmentation...
spectrum of the Dewar valence isomer also exhibits the dehydrated ion as the main product, together with several unidentified peaks at \( m/z < 250 \). The interpretation of the fragmentation of the cyclobutane dimers of TpT and pTpT is less obvious. Indeed, the main ions observed upon fragmentation of the [M + H]^+ pseudo-molecular ion of TpT cis-syn and trans-syn cyclobutane dimers correspond to the loss of two 98 and two 116 mass units. This leads to the formation of ions at \( m/z = 449 \), 431, 351 and 333. A first explanation is the loss of fragments of the 2-deoxyribose units. Indeed, 98 mass units corresponds to the mass of a 2-deoxyribose ring that has lost a hydroxyl group involved in the phosphodiester bond. An alternative fragmentation pathway may involve the loss of CO–NH–CO–C–CH₃ fragments from the pyrimidine ring on both the 5' and 3' ends. Loss of a molecule of water from the [MH–98]^+ ion could account for the [MH–116]^+ ion. Support for the latter reaction was provided by the MS³ analysis of the cis-syn and the trans-syn cyclobutane dimers on the ion-trap spectrometer. Indeed, fragmentation of the ion at \( m/z = 449 \) gave rise to the ion at \( m/z = 431 \). The ions at \( m/z = 449 \) and 431 were also the main peaks observed on the spectrum of the pTpT cis-syn cyclobutane dimer. Again, this could be explained by either the loss of a phosphorylated 2-deoxyribose ring or of a phosphate group and a CO–NH–CO–C–CH₃ fragment. Definitive conclusions about the fragmentation pattern could only be achieved by the analysis of isotopically labelled photoproducts.

The interpretation of the spectra recorded in the negative mode was facilitated by the reduced number of product ions (Fig. 5). The main signal in the spectrum of the (6–4) photoproducts of TpT and pTpT and their Dewar valence isomers was at [M–H–113]^−. This can be accounted for by a fragmentation of the 5'-saturated pyrimidine ring. Interestingly, no dehydration reaction was observed, in contrast to the result obtained in the positive mode. As already mentioned, no significant fragmentation could be obtained for the cis-syn cyclobutane dimer of pTpT. In contrast, the corresponding TpT photoproduc yielded a predominant ion at \( m/z = 447 \), corresponding to the loss of a 98 mass units fragment as observed in the positive mode. The same rearrangement was also observed for the trans-syn isomer. It may be added that a significant ion at \( m/z = 195 \) was observed for all TpT derivatives, which corresponds to a 2-deoxyribose monophosphate moiety.

3.4. Detection of TpT photoproducts by HPLC coupled to tandem mass spectrometry

Even though the cis-syn cyclobutadithymine was identified almost 40 years ago, one still lacks a method for the simultaneous detection of the main base photolesions within DNA. The formation of cyclobutadithymine has been monitored by using formic acid hydrolysis of [³H]-thymine-labelled DNA, followed by a chromatographic separation [30–32]. Cis-syn cyclobutane dimers have also been measured within acid-hydrolysed DNA by applying a GC–MS isotopic dilution technique [33]. The direct detection of (6–4) photoproducts is hampered by their low stability under usual acidic hydrolysis conditions. However, the rate of formation of the latter lesions was determined within DNA using a mild HF–pyridine hydrolysis followed by a HPLC–fluorescence detection [34]. None of these techniques allows the simultaneous measurement of the cyclobutane dimers, the (6–4) adducts and the Dewar valence isomer. The bulk of the results obtained on the formation of far-UV-

![Fig. 5. ES/MS-MS spectra of the cis-syn cyclobutane dimer (upper panel) and the (6–4) photoproduct (lower panel) of TpT. Spectra were recorded in the negative mode on a triple-quadrupolar API 3000 spectrometer. Detection conditions were identical for both analyses.](image-url)
induced dimeric photoproducts within isolated and cellular DNA have been obtained by using indirect techniques including immunological approaches [35–37] and the use of repair enzyme as a way to convert the lesions into easily detectable DNA strand breaks [38]. Even though they are highly sensitive, the latter methods do not allow the individual quantification of each bipyrimidine lesion for a given class of photoproducts. Therefore, a technique allowing the simultaneous detection of the three types of photoproducts arising from the different bipyrimidine sequences is still needed.

The results presented above show that the use of an electrospray ionization technique is particularly suitable for the mass-spectrometric analysis of the various thymine photoproducts. Therefore, the latter technique combined with liquid chromatography might provide a new and efficient analytical tool for the detection of far-UV-induced thymine photoproducts in hydrolysed DNA. Interestingly, TpT photoproducts are well separated by reverse-phase HPLC. In addition, the latter compounds can be analysed in the negative mode, which is more sensitive than the detection of positive ions because of a reduced background. Therefore, a method was optimized for the analysis of TpT photoproducts by HPLC coupled to a triple quadrupolar spectrometer used in the negative mode with a MRM detection. A 2 mm column was used to avoid splitting of the HPLC eluent and a gradient was applied to provide an optimal separation. As already mentioned, the major signal of the fragmentation spectrum of the TpT cyclobutane dimers was observed at m/z = 447 in the negative mode. In contrast, the pseudo-molecular ion of the (6–4) photoproduct and of its Dewar valence isomer yielded a daughter ion at m/z = 432. The spectrometer was thus set to monitor the transitions 545 → 447 and 545 → 432.

This analytical technique allowed the simultaneous detection of a mixture of the four photoproducts (Fig. 6). Indeed, the (6–4) adduct and the trans–syn cyclobutane dimer were separated as single peaks with a high proportion of methanol in the mobile phase. In contrast, the Dewar valence isomer and the cis–syn cyclobutane dimer were eluted close to each other. However, their difference in favoured fragmentation pathway still allowed them to be resolved. The sensitivity of the detection is high, since a signal-to-noise ratio of 10 is obtained for 200 fmol of product injected on the column. The detection of TpT photoproducts thus appears to be an interesting approach for the measurement of far-UV-induced DNA lesions. The key point remains the optimization of the release of the photoproducts from DNA as dinucleoside monophosphates. This might be achieved by the use of both 3’- and 5’-phosphodiesterases. Indeed, cyclobutane dimers and (6–4) photoproducts were found to be quantitatively released as dinucleoside monophosphate from far-UV-irradiated short oligonucleotides [23,39].

4. Conclusions

The present study allows two interesting comparisons to be made. First, it clearly shows the advantages of the ES/MS–MS approach for the acquisition of fragmentation spectra of thymine photoproducts. Indeed, when compared with previous data based on the use of FAB or thermospray ionization, the ES/MS–MS spectra show a more controlled fragmentation. No differences in fragmentation pattern were observed between the (6–4) adducts and their Dewar valence isomers. However, a significant influence of the diastereoisomeric
mery of the cyclobutane dimer was observed for both the thymine and the thymidine derivatives. In addition, a comparison could be done between the triple-quadrupole and the ion-trap technologies. The latter approach was unambiguously more efficient for the determination of the fragmentation pathways. It also appeared to be slightly more sensitive for the recording of full spectra than the triple-quadrupolar spectrometer. However, the latter system provided a very sensitive detection when used in the MRM mode, which, in contrast, only slightly improved the sensitivity of the ion-trap spectrometer.

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