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Rapid Characterization of Cross-links, Mono-adducts, and Non-covalent Binding of Psoralens to Deoxyoligonucleotides by LC-UV/ESI-MS and IRMPD Mass Spectrometry

Suncerae I. Smith and Jennifer S. Brodbelt

Department of Chemistry and Biochemistry University of Texas at Austin Austin, TX 78712

Summary

Upon UV photoactivation, psoralen analogs form covalent mono-adducts and cross-links with DNA at thymine residues. Electrospray ionization mass spectrometric analysis allowed rapid and efficient determination of the reaction percentages of each psoralen analog with DNA duplexes containing different binding sites after exposure to UV irradiation. The distribution of cross-linked products and mono-adducts was monitored by both LC-UV and IRMPD-MS methods with the highest ratio of cross-linked products to mono-adducts obtained for 8-methoxypsoralen (8-MOP), psoralen (P), and 5-methoxypsoralen (5-MOP). Reactions at 5'-TA sites were favored over 5'-AT sites, and duplexes containing two and three binding sites showed extensive binding by the psoralens. 4'-aminomethyl-4,5',8-trimethylpsoralen (AMP) bound non-selectively via non-covalent interactions and was the only psoralen analog to show significant binding in the absence of UV irradiation. 8-MOP binding displayed the greatest sequence selectivity among the psoralen analogs. The sites of interstrand cross-linking were determined by fragmentation of the duplex/psoralen complexes by infrared multiphoton dissociation (IRMPD), which produced cross-linked product ions containing an intact single strand, the psoralen analog, and either a w_n or a_n-B portion of the complementary strand. IRMPD of DNA/AMP complexes after UV irradiation also produced high abundances of the intact single strands with the AMP ligand attached, products indicative of a significant population of mono-adducts.

Introduction

A wide array of DNA interactive compounds have been developed as chemotherapy agents,^{1,2} with some associating with DNA via non-covalent interactions and others covalently binding to DNA. As one example of the latter, bifunctional alkylating agents may react with two different nucleophilic centers in DNA.³ If the two sites are on opposite polynucleotide strands, interstrand DNA cross-links result. Interstrand cross-linking agents represent an important class of compounds that damage DNA by preventing strand separation, therefore prohibiting DNA replication and transcription. It is for this reason that a number of agents such as mitomycin,⁴ nitrogen and sulfur mustards,⁵ platinum derivatives,⁶ and photoactivated psoralens⁷ have been used in cancer chemotherapy.

Psoralens are naturally occurring linear furocoumarins synthesized in plants that have been used in the treatment of skin diseases for thousands of years.⁸ The most widely used psoralen,

Correspondence to: Jennifer S. Brodbelt, jbrodbelt@mail.utexas.edu.

Contents Image

The formation of non-covalent complexes and both mono-adducts and cross-links between DNA and a series of psoralen analogs was evaluated by infrared multiphoton dissociation (IRMPD) and LC-UV-MS.

8-methoxypsoralen, has been used more recently for the treatment of vitiligo, psoriasis, and cutaneous T cell lymphoma.⁹ Psoralens are actively used for the treatment of these diseases because of their ability to interact with DNA. In the absence of light, the planar tri-cyclic structure of psoralen first intercalates between base pairs in the DNA double helix to form a non-covalent complex. Cycloaddition products are formed with pyrimidine bases, typically thymine, upon absorption of a photon of long wavelength ultraviolet light (~320-410 nm). The cycloaddition occurs between the 5,6 double bond of a thymine base and the 3,4 (pyrone) or 4',5' (furan) double bond of the psoralen.^{10,11} Absorption of a second photon by the furan side mono-adduct can result in formation of interstrand cross-links via cycloaddition at both ends of the psoralen.^{12,13} The yields of mono-adducts and cross-links are dependent upon several conditions, including the structure of the psoralen, the irradiation time, and the wavelength of the irradiation source.¹⁴

Psoralens are most reactive with TA-rich duplex DNA sequences.^{15,16} Binding to single strands occurs to a much smaller extent,¹⁷ with no preference for the sequence surrounding the reactive thymine.¹⁸ Several studies agree that the 5'-TA site is more reactive than the 5'-AT site,^{11,15,16,19-24} and that repeated T-A sequences are hot spots for reaction due to the increased number of potential binding sites.^{15,24-26}

Determination of sequence selectivities and characterization of the resulting adducts and/or cross-linked products remains a significant analytical challenge, both for psoralens and for other classes of DNA interactive agents that engage in covalent binding to DNA.

Wang and coworkers have used P1 nuclease digestion in combination with mass spectrometry to digest DNA deoxyoligonucleotides modified with TMP to a four nucleotide product containing the TMP adduct.²⁷ Recently, they have extended this technique using P1 nuclease digestion LC-MS/MS with isotope dilution to quantify the formation of mono-adducts and interstrand cross-links in human cells upon treatment with 8-MOP or S-59 psoralen and UV light.^{28,29}

Electrospray ionization mass spectrometry (ESI-MS) has been increasingly utilized for the analysis of DNA adducts due to its high sensitivity and fast analysis time. During the ESI process, both non-covalent³⁰⁻⁵³ and covalent⁵⁴⁻⁵⁷ DNA ligand complexes can be transferred from the solution to the gas phase with conservation of many of the intermolecular interactions formed in the solution environment. The successful transfer of these complexes to the gas phase allows determination of binding stoichiometries, sequence selectivities, and relative binding affinities. In addition, tandem mass spectrometry techniques such as collisionally induced dissociation (CID)^{35,39,41,46-49,52} and infrared multiphoton dissociation (IRMPD)^{50, 52, 57} can be used to reveal information about the binding mode and often the binding site. The present study reports the development and application of ESI-MS and IRMPD methods for screening the DNA binding interactions of a series of psoralen analogs (Figure 1) well characterized by other analytical techniques.¹⁰⁻²⁶

Results and Discussion

To characterize the DNA binding properties of the psoralen analogs, a multi-faceted approach was followed. ESI-MS was used to evaluate the stoichiometries of the duplex/psoralen complexes and assess the sequence selectivities of the psoralen analogs. Two activation techniques, IRMPD and CID, were used to map the dissociation patterns of the complexes and confirm the binding sites of the covalently bound psoralen analogs. Liquid chromatography with UV detection was used to determine the distributions of DNA psoralen cross-links versus DNA psoralen mono-adducts, with comparison to rapid quantitative screening by IRMPD-MS. The sequences of the duplexes used in this study are shown in Table 1.

Binding Stoichiometries of DNA/Psoralen complexes

Psoralens are known to intercalate between the base pairs of DNA prior to UV irradiation, with association constants on the order of 10^4 M^{-1} .⁵⁸ The probability of psoralen cross-link formation is dependent on the solubility of the psoralen, the ability of the psoralen to intercalate into the base pairs of duplexes, as well as the probability of mono-adduction once intercalation has occurred. Therefore, the initial limiting factor for formation of DNA/psoralen products stems from the rather low solubilities and low association constants of psoralens with nucleic acids.

The ESI-mass spectrum of each duplex alone is dominated by ions of the 5-, 6-, and 7- charge states of the intact duplex, in addition to some unannealed single strand species of low abundance in the 3- and 4- charge states, as shown for D1 in Figure 2a. Incubation of each duplex with either P, 5-MOP, 8-MOP, or TMP without UV exposure led to no detectable products by ESI-MS. Previous equilibrium dialysis and spectrophotometric studies have demonstrated the relatively low association constants for the intercalation of furocoumarins into DNA.^{58,59} The non-covalent DNA-psoralen complexes may be too low in abundance for detection by ESI-MS or may be too fragile to survive the electrospray process. After UV irradiation of the incubates for 30 minutes at 365 nm, DNA/psoralen complexes are readily detected by ESI-MS, as illustrated in Figure 2b for 8-MOP. Each psoralen analog has the ability to intercalate, form mono-adducts, or cross-link DNA. Because the 4',5'-furan and 3,4-pyrone double bonds of psoralen undergo addition reactions with the 5,6 double bond of thymine to form the mono-adducts or cross-links,^{11,12} the resulting DNA/psoralen complexes all have identical masses whether formed via intercalation, mono-adduction, or cross-linking. However, these three distinctive types of products can be differentiated by MS/MS experiments, as discussed later. The duplex/8-MOP complexes are produced with solely 1:1 binding stoichiometries under these reaction conditions. Psoralen and two other analogs, P, 5-MOP, and TMP, formed less abundant complexes than 8-MOP, also with 1:1 binding stoichiometries. Due to the solubility of the saturated stock solutions of P, 5-MOP, and 8-MOP, the final DNA to psoralen molar ratio approximated 1:1; however, TMP is significantly less soluble than the other psoralen derivatives, affording a final molar ratio of 100:1 DNA to TMP for the solutions. The psoralen/duplex complexes generally maintain the same charge state pattern as observed for the free duplexes (mainly 5- and 6-). Binding to single strands is not observed, indicating a high degree of duplex selectivity.

Upon incubation of each duplex with AMP (without UV exposure), abundant duplex/AMP complexes with stoichiometries ranging from 1:1 to 1:5 were detected by ESI-MS. The positively charged amino methyl group of AMP, originally incorporated to increase the solubility of psoralen in aqueous solution,¹³ likely enhances binding to DNA via electrostatic interaction with the negatively charged phosphate backbone. After 30 minutes of UV irradiation of the incubate, a small increase in complexation is observed (Figure 2c) with stoichiometries still ranging from 1:1 to 1:5. Due to the high solubility of AMP, the final molar ratio of AMP to DNA is 100:1, and may account for the high binding stoichiometry observed. In addition, AMP may self-aggregate prior to or during DNA binding or engage in a high degree of non-specific non-covalent binding to DNA, prior to forming covalent complexes. Binding to single strands is not observed, again confirming a high degree of duplex selectivity.

Relative binding selectivities

A comparison of the relative binding selectivities of the psoralen analogs for four duplexes with varying AT base pair composition is summarized in bar graph form in Figure 3 based on the relative binding percentages determined by ESI-MS. The first duplex (D1) contains a preferred psoralen binding site, 5'-TA. The second and third duplexes (D2 and D3) have two and three potential binding sites, respectively. The fourth duplex (D4) has a reversed 5'-AT

binding site. Prior studies have shown that 5'-TA is the preferred binding site over 5'-AT.^{12, 15,16,19-24} Molecular modeling experiments have shown that 5'-TA is better oriented for T-psoralen-T cross-linking than 5'-AT.²² Several reports have indicated that a greater number of binding sites also increases psoralen binding^{15,24-26}. The relative binding percentages allow comparison of the extent of ligand binding for different ligands bound to selected duplexes; however, due to the different solubilities of psoralen derivatives, comparisons *between* psoralens are limited. Higher values of the relative binding percentages suggest greater DNA/psoralen binding affinities. Three replicates were undertaken for each solution.

The relative binding percentages in Figure 3 indicate that P, 5-MOP, 8-MOP, and to a lesser extent TMP are selective for specific DNA sequences. Binding for all of the psoralen analogs generally follows the pattern D3 > D2 > D1 > D4. Duplexes used in this study are 14 base pairs long, and although they are relatively short compared to nuclear DNA, the direct binding sites of the psoralens consist of only two base pairs, so that the local environment of the psoralen should be similar to in vivo samples. In addition, the sequences chosen are not representative of actual DNA sequences, rather the unique sequences were chosen to allow evaluation of site-specific binding effects. Boyer and coworkers found that the same sites in fragments of the *lacI* gene of *Escherichia coli* were targeted with relatively similar frequencies by 8-MOP, 5-MOP, and other psoralen analogs not used in the present study.²⁴ This past result suggests that certain DNA sequences are likely preferred by all furocoumarins, consistent with the present ESI-MS findings.

For P, the relative binding percentage increases with the number of 5'-TA binding sites (D1 versus D2 versus D3). The binding percentage is lower for D4 compared to D1, consistent with the reversal of the AT binding site. For complexes containing P and D1, D2, or D4, the duplex:psoralen product stoichiometries are 1:1. However, for the duplex containing three binding sites (D3), stoichiometries of both 1:1 and 1:2 are observed for the resulting D3/P complexes. The solubility of P corresponds to a molar ratio of 1:1 DNA to P in solution, which may not allow complete saturation of all binding sites. In addition, the close proximity of the binding sites in D2 and D3 may sterically inhibit two psoralens from binding to D2 and three psoralens from binding to D3. For 5-MOP and 8-MOP, the relative binding percentages similarly increase with the number of binding sites. Moreover, binding of 5-MOP and 8-MOP to D4 is significantly reduced, suggesting a high degree of selectivity for 5'-TA binding sites over 5'-AT sites. A 1:1 DNA:5-MOP binding stoichiometry is consistently observed for all duplexes. The solubility of 5-MOP and 8-MOP correspond to a molar ratio of 1:1 DNA to psoralen in solution, but does not fully account for the differences in stoichiometry observed for 5-MOP and 8-MOP. The proximity of the methoxy group to the reactive site on 5-MOP compared to 8-MOP may decrease overall relative binding and produce a strained DNA/5-MOP complex that sterically inhibits the binding of another psoralen at an adjacent site. In contrast, the DNA:psoralen binding stoichiometries are 1:1 for D1, D2 and D4 with 8-MOP, and 1:2 for duplex D3, as observed for the P complexes described above. The duplex/TMP products all maintain 1:1 binding stoichiometries. TMP is significantly less soluble, resulting in only 1 TMP ligand available for every 100 DNA duplexes. The relative binding percentages obtained for AMP were uniformly the highest among all the psoralens, yet with little selectivity observed among the four duplexes. Upon reaction with duplexes D1, D2, and D4, the stoichiometries of the resulting duplex/AMP complexes ranged from 1:1 up to 1:5, and the stoichiometries increased to up to 1:6 for complexes containing D3. These large stoichiometries are suggestive of non-specific binding or self-aggregation of AMP.

The relative binding percentages across the series of psoralens are significantly affected by their solubilities. In our study using saturated psoralen solutions, the psoralen:DNA molar ratios are approximately 1:1 for P, 5-MOP, and 8-MOP. The molar ratio of AMP to DNA is 100:1, but the molar ratio of TMP to DNA is only 1:100 which may account for its low binding

percentage in Figure 3. Previous studies based on equilibrium dialysis and gel electrophoresis have shown that the binding of 8-MOP to DNA is greater than that of 5-MOP^{24,26} which may be due to slightly different average orientations in the intercalative complexes,⁶⁰ presumably due to the specific location of the methoxy substituent. The binding of AMP is also greater than that of TMP.¹³ Hearst and coworkers found that the number of covalently bound psoralen molecules per 1000 base pairs of DNA followed the trend AMP > 8-MOP > TMP > P.⁶¹ Methoxy groups slowed the photochemistry, with 8-MOP (and presumably 5-MOP) reacting more slowly with DNA but also resisting photobreakdown about ten times better than P, AMP, and TMP.⁶¹ The photochemistry of methylated psoralens (like TMP) is relatively fast compared to that of unsubstituted psoralen,^{16,23,59,62} but the former are more susceptible to photobreakdown.^{58,62} AMP is the only psoralen analog to show binding by ESI-MS without UV irradiation in the present study, indicating substantial non-covalent association, presumably through intercalation and likely enhanced by the presence of the aminomethyl moiety.¹³ The formation and survival of both non-covalent and covalent complexes would account for the high binding percentages obtained for AMP compared to the other psoralen analogs in our ESI-MS study. As demonstrated in this study, the use of ESI-MS allows the binding selectivities of the psoralens and the stoichiometries of the products to be screened rapidly and with minimal sample consumption.

Impact of irradiation time on the formation of DNA/psoralen complexes

To evaluate the impact of the irradiation time on the abundances of DNA/psoralen complexes, ESI-MS spectra were obtained for solutions containing 10 μ M of duplex D1 and 1 μ L of saturated psoralen solution after incubation in the dark for 1 hour and after exposure to UV irradiation (on ice) for 0-90 minutes (Supplementary Figure 1a). The binding stoichiometries of the resulting DNA/psoralen complexes were 1:1 regardless of irradiation time for P, 5-MOP, 8-MOP, and TMP, suggesting all binding was specific to the one binding site. The binding stoichiometries of the DNA/AMP complexes ranged from 1:1 to 1:5 irrespective of irradiation time. For all of the psoralen analogs, the relative binding percentages increased with UV irradiation time. A leveling effect occurred at longer irradiation times depending on the psoralen analog. P, TMP, and AMP all yielded the near maximum relative binding percentages after only 5 minutes of UV irradiation, with only a slight increase in binding at longer irradiation times. The relative binding percentages obtained for 5-MOP and 8-MOP increased more slowly with irradiation time. These results are consistent with the relative rates of reaction determined by Hearst and coworkers which showed that the addition of 8-MOP to calf thymus DNA occurred about ten times slower compared to AMP, TMP and P.⁵⁹

For incubations with AMP, the relative binding percentage for D1 was greater than 70% prior to UV irradiation, supporting the significant formation of non-photoactivated non-covalent complexes. In fact, CID of the (D1 + AMP)⁶⁻ complex formed in the incubate without UV irradiation showed the loss of AMP in addition to strand separation. This pattern is characteristic of non-covalent complexes which simply disassemble into their constituents upon energization in the gas phase.

A second set of time-dependent experiments was undertaken for reactions of 8-MOP, selected as a representative psoralen analog, with duplexes D1, D2, D3, and D4. These experiments were aimed at assessing whether the specific DNA sequence had a significant impact on product formation. ESI-MS spectra were obtained for solutions containing 10 μ M of D1, D2, D3, or D4 and 1 μ L of saturated 8-MOP solution after incubation in the dark or after exposure to UV irradiation (on ice) for 0-90 minutes (Supplementary Figure 1b). The relative binding percentages for each of the duplexes with 8-MOP varied substantially, as expected based on the earlier findings described in Figure 3, with a greater binding percentage as the irradiation time increased. The binding stoichiometries of the DNA/8-MOP complexes remained

exclusively 1:1 for D1 and D4 regardless of irradiation time. After 45 minutes of irradiation, however, a small percentage of the products had 1:2 stoichiometries for D2 and both 1:2 and 1:3 stoichiometries for D3.

IRMPD of duplex DNA/psoralen complexes

In the present study, we exploit the benefits of IRMPD to characterize the DNA/psoralen complexes along with acquisition of CID mass spectra for comparative purposes. IRMPD has proven useful as an alternative approach to traditional CID for gaining sequence information of DNA as well as elucidation of sites of ligand adduction.^{46,50,52} IRMPD offers two main benefits over CID in ion traps. The non-resonant process of ion activation by IR absorption results in rapid conversion of the uninformative base loss ions, ones that often dominate CID mass spectra acquired in quadrupole ion traps, into *a-B* and *w* sequence ions without the need for sequential stages of ion activation. Secondly, during IRMPD, ion activation is independent of the *rf* trapping voltage, extending the trapping range to lower *m/z* values and thus allowing detection of single nucleobases and modified ones too. This important lower *m/z* range is lost due to constraints on the *rf* trapping voltage during conventional collision activation. CID of non-covalent duplex DNA/intercalator complexes has been reported previously.^{35,41,46,47,49,52} Weakly bound duplex/intercalator complexes dissociate by loss of the intercalating ligand, leaving the intact duplex.^{41,46,47,49,57} Ligands that bind covalently to DNA often show significantly different MS/MS patterns.⁵⁴⁻⁵⁷ Depending on charge state, covalent DNA-ligand complexes may dissociate via strand separation (if they are mono-adducts, not cross-linked species) and upon sequential CID steps, dissociation of the modified single strands results in formation of *a - B* and *w* ions, with the covalent modification being retained on specific diagnostic sequence ions. Another common fragmentation pathway that is observed when the ligand is covalently bound to a nucleobase is loss of the entire modified base. Cross-linked deoxyoligonucleotides often dissociate into products that contain an intact single strand, the cross-linker, and a smaller portion of the complementary single strand.⁵⁶

For this study, each DNA/psoralen complex was isolated and subjected to IRMPD or CID. Examples of the IRMPD mass spectra are shown in Figure 4 for complexes containing duplex D1 with two of the psoralen analogs, 8-MOP and AMP. Use of a very short IR irradiation period inhibits secondary dissociation, and the resulting spectra are similar to the conventional CID mass spectra. For example, DNA complexes containing P, 5-MOP, 8-MOP, or TMP dissociate primarily via loss of a nucleobase, a process that is not particularly informative and gives no insight into the specific structures of the cross-linked complexes (in terms of the cross-linking sites) (data not shown). Use of longer IR irradiation times results in secondary dissociation of the primary base loss ions, resulting in a far more diagnostic array of cross-linked product ions, most of which incorporate an intact single strand, the psoralen cross-linker, and a segment of the complementary second strand (Figure 4a). Significantly higher irradiation times results in extensive secondary dissociation of all products, leading to formation of smaller backbone sequence ions, some of which still retain the covalently bound psoralen moiety. The IRMPD pattern exemplified by the mass spectrum shown in Figure 4a is highly diagnostic for cross-linked complexes. In contrast, unmodified duplexes typically dissociate by base loss or strand separation (spectra not shown). Strand separation is clearly impeded by cross-linking, as evidenced by the fragmentation pattern shown in Figure 4a. Low abundances of the intact single strands resulting from strand separation are observed, indicating ions that likely originate from the presence of a small population of non-covalent DNA/8-MOP complexes.

In addition to the fragmentation pathways described above for the complexes containing P, 5-MOP, 8-MOP, or TMP, the complexes containing AMP also dissociate by one other noteworthy route (Figure 4b). This pathway entails strand separation of the duplex, with retention of AMP by either strand leading to the observed *ss* + AMP and *ss* + AMP - B ions.

This process is consistent with mono-adduction of AMP to the duplex, resulting in covalent attachment of AMP to either strand.

A comparison of dissociation trends for CID and IRMPD of the D1/8-MOP complexes (6-charge state) is shown in Supplementary Figure 2. Peak area abundances were summed for the precursor ions, the base loss ions, the sequence ions, the sequence ions containing an 8-MOP adduct, mono-adducted ions, and cross-linked ions, as illustrated for the energy-variable CID (Supplementary Figure 2a) and time-resolved IRMPD (Supplementary Figure 2b) distributions. For the energy-variable CID data, the portion of sequence ions, mono-adducted ions, and cross-linked ions increases slightly and the portion of base loss ions increases dramatically until the precursor ion population is exhausted at 13% normalized collision energy (41 mV CID voltage), after which no significant change in ion distribution is observed. In contrast, IRMPD results in a population of base loss ions from the precursor between 3.0 and 5.0 ms irradiation, cross-linked ions and mono-adducted ions between 4.0 and 6.0 ms irradiation, and then these ions undergo secondary dissociation upon further irradiation (beyond 6.0 ms). The striking difference in sequence ion abundance between CID and IRMPD methods illustrates the greater tunability of the IRMPD method.

To evaluate the potential of IRMPD to pinpoint the exact location of the psoralen cross-link sites, IRMPD mass spectra were collected for each duplex/psoralen complex. A summary of the product ions obtained by IRMPD for (D1 + 8-MOP) is shown in Scheme 1 in which the slash marks along the DNA sequences represent the detection of an intact single strand containing the 8-MOP adduct plus the *a* – *B* or *w* portions from the complementary single strand. The summary shown in Scheme 1 represents a compilation of all products observed upon dissociation of all the (duplex + 8-MOP) ions in all charge states. High sequence coverage of the duplexes is obtained by IRMPD. This series of cross-linked product ions can be used to identify conclusively the specific binding sites of the psoralens to thymines. Sites of psoralen binding are confirmed by detection of two complementary series of cross-linked product ions, e.g., (ss + 8-MOP + *w*) and (ss + 8-MOP + *a* – *B*), stemming from specific thymine binding sites. Thymines that are shown in bold font in Scheme 1 are the sites of 8-MOP binding. The two duplexes that contain more than one thymine (D2 and D3) exhibit binding at each of the potential sites, indicating a mixture of populations. All sites of cross-linking solely occur at thymines.

The IRMPD mass spectra for the other duplex/psoralen products containing P, 5-MOP, TMP or AMP resulted in a similar series of cross-linked product ions. High sequence coverage of the cross-linked product ions conclusively reveals that the central thymines are the sole binding sites for each psoralen with D1 and D4. Each psoralen analog exhibits a mixture of binding at several thymine sites for D2 and D3.

Quantitative analysis of product formation

In order to determine the relative distributions of mono-adducts and cross-linked products, each DNA/psoralen incubate was subjected to LC-UV, as well as IRMPD-MS analysis described earlier. During the HPLC separation, the column was heated at 55°C to disrupt all of the non-covalent interactions and allow ample resolution of the cross-linked products from unreacted duplexes or non-covalent products. (This means that both the unreacted DNA duplexes and any non-covalent DNA/psoralen complexes are converted similarly to single strands and are thus not distinguishable during LC-UV analysis.) Figure 5 shows the chromatograms obtained for the incubates containing duplex D1 with 8-MOP or AMP. Cross-linked DNA elutes first, followed by the unmodified single strands and various single strand mono-adducts. The identities of the various eluting products were confirmed by ESI-MS. The relative distribution of cross-linked products to mono-adducts was calculated by integration of the appropriate chromatographic peaks, then normalizing the relative areas based on the

extinction coefficients for the mono-adducts (assumed to be similar to single strands) versus the crosslinked products (in which the extinction coefficients for the constituent single strands were summed). The resulting product distributions are summarized in Figure 6. Both unmodified duplexes and non-covalent DNA/psoralen complexes are disrupted at the elevated temperature of the LC separation and thus both collectively contribute to the portion of products detected as unmodified single strands (i.e. labeled as ssG and ssC in Figure 5). It is estimated that the contribution of non-covalent DNA/psoralen complexes is lower for P, 5-MOP, 8-MOP, and TMP because these psoralens exhibited no evidence of non-covalent adduction in the experiments described earlier. From the LC-UV chromatographic profiles shown in Figure 5a for 8-MOP, the portion of unreacted D1 plus non-covalent D1/8-MOP complexes is 76%, the portion of mono-adducts is 5%, and the portion of cross-linked products is 19%. For P, 5-MOP, 8-MOP, and TMP, the resulting DNA products consist of ~4% mono-adducts with varying contributions from cross-linked products as summarized in Figure 6. The overall trend for cross-link formation is 8-MOP > P ~ 5-MOP > TMP. For AMP, the product distribution is 49% unreacted D1 and non-covalent D1/AMP complexes, 32% mono-adducts, and 19% crosslinked species.

We also compared the ability of IRMPD-MS to evaluate the distribution of DNA/psoralen products (i.e. non-covalent complexes and mono-adducts and cross-linked products) relative to LC-UV analysis. The mono-adducts and cross-linked products have distinctive IRMPD fragmentation patterns, and thus we envisioned the capability for rapidly monitoring the product formation based on IRMPD mass spectra rather than far more time-consuming HPLC separation and detection of products. Moreover, the mass-selective ion isolation step prior to IRMPD means that only the reacted DNA (i.e. both non-covalent DNA/psoralen complexes and covalent DNA/psoralen products but not unreacted duplexes) is activated and analyzed. This allows assessment of the three distinctive types of DNA/psoralen products, including non-covalent complexes, mono-adducts, and cross-links, all which have the same mass due to the attachment of the psoralen without any interference from the unreacted DNA (which has no attached psoralen). The ESI-IRMPD-MS method requires only a few minutes for data collection, whereas the LC-UV strategy entails several hours for analysis of the whole series of samples. Estimations of product distributions are based on the integration of specific peaks in the IRMPD mass spectra after 5.0-9.0 ms of IR irradiation following exposure of the DNA psoralen incubate to 30 minutes of UV radiation. The abundances of the $(ssG)^{3-}$, $(ssG - GH)^{3-}$, $(ssC)^{3-}$, $(ssC - CH)^{3-}$, and $(ds)^{6-}$ ions were summed to reflect the portion of non-covalent DNA/psoralen complexes. The abundances of the $(ssG + psoralen)^{3-}$, $(ssG + psoralen - GH)^{3-}$, $(ssC + psoralen)^{3-}$, and $(ssC + psoralen - CH)^{3-}$ products were summed to reflect the mono-adducts. The abundances of the $(ds + psoralen - GH)^{6-}$ ion and the crosslinked product ions were summed to represent the portion of the cross-linked products. The IRMPD analysis of the $(D1 + psoralen\ analog)^{6-}$ complexes at optimized IRMPD irradiation times indicate that the distribution of D1/8-MOP products consists of 60% cross-links, 14% mono-adducts, and 26% non-covalent complexes. For P, 5-MOP, and TMP, the resulting DNA products consist of 46-56% cross-links, 22-32% mono-adducts, and 20-25% non-covalent complexes. The overall trend for cross-link formation mirrors the LC-UV results, with 8-MOP > 5-MOP ~ P > TMP. The distribution of products observed for each psoralen analog with D1 by both IRMPD and LC-UV methods is summarized in Table 2. In addition, the final column tabulates the ratio of cross-links to mono-adducts produced for each psoralen analog upon interaction with D1, with impressive agreement between the IRMPD-MS and LC-UV methods.

With respect to the reactions of AMP with DNA, the ESI-MS and IRMPD-MS evidence presented earlier suggested that AMP interacted with DNA via significant formation of non-covalent complexes, as well as mono-adducts and cross-linked products. The unusual behavior of AMP relative to the other psoralens prompted a more detailed evaluation of its time-dependent product formation. Solutions containing 10 μ M of D1 and 1 μ L of AMP after

incubation in the dark (1 hour, room temperature) and subsequent exposure to UV irradiation (365 nm, 0-30 minutes, on ice) were analyzed in parallel by ESI-IRMPD-MS and capillary LC-UV (Figure 7). The IRMPD mass spectrum of the (D1 + AMP)⁶⁻ complex acquired for the incubate prior to UV irradiation shows exclusively the disassembly of the complex (loss of AMP, strand separation) (see Figure 7a). This spectrum is characteristic of non-covalent complexes. The corresponding UV chromatogram obtained for the same incubate shows the presence of only the single strands, confirming that the only species in solution are non-covalent complexes (including both unreacted DNA and non-covalent DNA/AMP complexes) (Figure 7e). After 5 minutes of UV irradiation, the resulting (D1 + AMP)⁶⁻ products were again subjected to IRMPD, and the fragmentation pattern is very different (Figure 7b). The newly emerging (ss + AMP) products [i.e. (ssG + AMP)³⁻, (ssG + AMP - GH)³⁻, (ssC + AMP)³⁻, and (ssC + AMP - CH)³⁻] are characteristic of mono-adducts. In addition, some low abundant cross-linked species are observed [i.e. (ds + AMP - GH)⁶⁻ and (ss + AMP + w_n)⁵⁻ and (ss + AMP + a_n-B)⁵⁻]. The corresponding UV chromatogram mirrors this product distribution (Figure 7f).

Figure 7d and 7h show the distribution of products (after irradiation of the incubate for 30 minutes) by IRMPD and LC-UV, respectively. The IRMPD-MS analysis of the (D1 + AMP)⁶⁻ complexes indicates that upon 30 minutes of exposure to UV irradiation, the distribution consists of 28% cross-links, 35% mono-adducts, and 37% non-covalent DNA/AMP complexes (Figure 7d). The distribution of products obtained from the complementary LC-UV data (Figure 7h) indicates 19% cross-links, 32% mono-adducts, and 49% non-covalent complexes plus unreacted DNA. The LC-UV chromatogram does not allow differentiation of unreacted DNA from non-covalent DNA/AMP complexes because both of these species are converted to single strands in the heated column. It should be noted that a large population of the precursor ion was not dissociated at the low irradiation time used for the IRMPD mass spectra, and therefore the distribution may not be representative of the entire population of DNA/AMP products. A longer irradiation time would have significantly increased the secondary dissociation, thereby complicating assignment of low m/z products. For example, at longer irradiation times, it is not apparent whether a w₃ ion is the primary product of the non-covalent duplex/AMP complex, or if the w₃ ion is the secondary product of either a mono-adduct or cross-link. In any case, the ratio of cross-linked products to mono-adducts shows remarkably good agreement between the fast IRMPD-MS analysis and the traditional LC-UV analysis, as shown in the last column of Table 2.

Experimental

Chemicals

Saturated stock solutions of psoralen (P), 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), 4,5',8-trimethylpsoralen (TMP), and 4'-aminomethyl-4,5',8-trimethylpsoralen (AMP) were purchased from Sigma Aldrich (St. Louis, MO) and prepared in ethanol and stored in the freezer in the dark. The following oligodeoxynucleotides (ODNs) were purchased from Integrated DNA Technologies (Coralville, IA) on the 1.0 μM scale and used without further purification: 5'-GCGGGGTAGGGGCG-3', 5'-CGCCCCTACCCCGC-3', 5'-GCGGGTATAGGGGCG-3', 5'-CGCCCTATACCCCGC-3', 5'-GCGGTATATAGGCG-3', 5'-CGCCTATATACCCG-3', 5'-GCGGGGATGGGGCG-3' and 5'-CGCCCCATCCCCGC-3'. DNA single strand concentrations were determined spectrophotometrically by Beer's Law using the extinction coefficients provided by the manufacturer. Annealing was performed by preparing stock solutions containing 1 mM of each complementary ODN in 200 mM ammonium acetate. The solutions were heated to 90°C for 10 min and slowly cooled to room temperature overnight. The following duplexes were created: duplex 1 = 5'-GCGGGGTAGGGGCG-3' / 5'-CGCCCCTACCCCGC-3', duplex 2 = 5'-GCGGGTATAGGGGCG-3' / 5'-CGCCCTATACCCCGC-3', duplex 3 = 5'-

GCGGTATATAGGCG-3' / 5'-CGCCTATATACCGC-3', and duplex 4 = 5'-GCGGGGATGGGGCG-3' / 5'-CGCCCCATCCCCGC-3'.

Duplex/psoralen complexes were formed by incubating 100 μ M DNA with 1-5 μ L (typically 1 μ L) saturated psoralen analog in 200 mM ammonium acetate. The total volume of each incubate was maintained at 20 μ L regardless of the amount of saturated psoralen added. In water, the solubilities (mole/liter) for each psoralen derivative are as follows: P – 1.9×10^{-4} , 5-MOP/8-MOP – 1.7×10^{-4} , TMP – 2.6×10^{-6} , and AMP – 3.4×10^{-2} .⁶¹ Due to the different solubilities of each psoralen ligand, the final psoralen:DNA concentration ratio was 0.95:1.0 for P, for 5-MOP and 8-MOP, the psoralen:DNA concentration ratio was 0.85:1.0, for TMP, the psoralen:DNA concentration ratio was 0.013:1.0, corresponding to 1 TMP per 100 DNA duplexes; and for AMP, the psoralen:DNA concentration ratio was 170:1, affording complete saturation of the DNA. The incubate was allowed to sit in the dark for 1 hour at room temperature to allow for optimal intercalation. The incubate was then subjected to UV irradiation (UVP BlackRay XX-20 BLB UV Bench Lamp, Upland, CA) using two Sankyo Denki 20W bulbs centered around 365 nm for 5-60 minutes (typically 30 minutes) while on ice. For ESI-MS analysis, the incubate was diluted to a final concentration of 10 μ M of DNA in 20 mM ammonium acetate.

Liquid Chromatography

HPLC was performed using a Dionex UltiMate 3000 capillary HPLC System (pump module LPG-3400M, Chromeleon Data System 6.80 SP2 Build 2284, Sunnyvale, CA) with a UV detector (VWD-3100) set to monitor 260 nm. A 300 μ m $\times$ 15 cm Dionex Acclaim PepMap 100 C18 column (3 μ m in particle size and 100 Å in pore size, LC Packings, Dionex) was used. 10 mM ammonium acetate buffer (solution A), a mixture of 10 mM ammonium acetate and acetonitrile (50/50, v/v, solution B), and a solution of acetonitrile and water (80/20 v/v, solution C) were employed as mobile phases. Following a 100 nL sample injection, a gradient of 40 min 1.0-15.0% B followed by 10 min 99% C was employed, and the flow rate was 4 μ L/min. The column was heated to 55 °C by using a Dionex FLM-3300 column heater. The identities of the eluted LC oligomers were verified by LC-MS. Quantification of the relative distribution of cross-links, mono-adducts, and non-covalent complexes was performed using LC-UV instead of LC-MS due to a suppression of the cross-linked products in the MS.

Mass spectrometry

Electrospray ionization mass spectra were collected on a ThermoFinnigan LTQ XL mass spectrometer (San Jose, CA). A Harvard syringe pump (Holliston, MA) set at a flow rate of 3 μ L/min was used to introduce the sample solutions into the mass spectrometer when direct infusion was employed. The ESI source was operated in the negative ion mode with an electrospray voltage of 3.5 kV and a heated capillary temperature of 90 °C. To assist in desolvation, nitrogen sheath and auxiliary gas were applied at 40 and 20 arbitrary units, respectively. Spectra were acquired by summing 20 scans. The extent of DNA binding, expressed as a “relative binding percentage” that equates with the relative binding affinity of each psoralen analog, was determined based on the following equation:⁶³

$$\text{Relative Binding Percentage} = \frac{A_{(1:1)} + A_{(1:2)} + A_{(1:3)} + \dots}{A_{(\text{DNA})} + A_{(1:1)} + A_{(1:2)} + A_{(1:3)} + \dots} \times 100\%$$

where A_{DNA} is the peak area of the free duplex and $A_{(1:n)}$ are the peak areas of all DNA/psoralen complexes in the ESI-mass spectra. All calculated relative binding percentages of bound DNA assume the peak areas of the free and bound DNA ions are proportional to their relative concentrations in solution.

The ion trap mass spectrometer was adapted for IRMPD as described previously.⁶⁴ IRMPD was carried out with a model 48-5 Synrad 50 W CO₂ continuous wave laser (Mukilteo, WA). The laser was triggered during the activation portion of the scan function for photodissociation using a q_z value of 0.10 for IRMPD experiments. This low q_z value resulted in a broader m/z storage range (i.e. extension of lower m/z range) than for CID experiments. The typical irradiation times for IRMPD were 5-10 ms at 10 W for DNA/psoralen complexes unless otherwise stated.

In the CID experiments, activation voltages were applied at a level required to reduce the isolated precursor ion to ~10-20% of its original abundance. The default activation time of 30 ms was used in all CID experiments with a q_z value of 0.25.

Conclusions

Electrospray ionization mass spectrometry and infrared multiphoton dissociation were used to characterize the products obtained upon reaction of selected DNA duplexes with five psoralen analogs. The fragmentation patterns of the products confirmed the formation of cross-linked products upon UV irradiation of the DNA/psoralen incubates. The fragmentation patterns of the duplex/psoralen products also confirmed that AMP was the least selective psoralen with respect to the types of products formed, yielding a large portion of mono-adducts, non-covalent complexes, and cross-links. In contrast, 8-MOP produced a similar percentage of cross-links compared to AMP, in addition to exhibiting higher sequence selectivity. With respect to the interactions of the psoralen analogs with duplexes containing different binding motifs, the relative binding selectivities of the psoralen analogs followed the pattern 5'-TATATA > 5'-TATA > 5'-TA > 5'-AT with AMP showing the greatest but least selective interaction with DNA. IRMPD-MS allowed rapid assessment of the portion of cross-links, monoadducts, and non-covalent complexes based on the abundances of the unique fragment ions attributed to each of these types of products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Waring MJ. *Drugs under Experimental and Clinical Research* 1986;12:441. [PubMed: 2427296]
2. Avendano, C. 1st ed. Elsevier; Amsterdam ; Boston: 2008.
3. Lawley PD. *Progress in nucleic acid research and molecular biology* 1966;5:89. [PubMed: 5337700]
4. Borowy-Borowski H, Lipman R, Tomasz M. *Biochemistry* 1990;29:2999. [PubMed: 2110821]
5. Chun, Edward H. L.; Gonzales, Leon; Lewis, Florence S.; Jones, Judith; Rutman, Robert J. *Cancer Research* 1969;29:1184. [PubMed: 5797106]
6. Chu G. *Journal of Biological Chemistry* 1994;269:787. [PubMed: 8288625]
7. Cole RS. *Biochimica et Biophysica Acta, Nucleic Acids and Protein Synthesis* 1971;254:30.
8. Perone VB. *Microbial Toxins* 1972;8:67.
9. Parrish JA, Fitzpatrick TB, Tanenbaum L, Pathak MA. *The New England journal of medicine* 1974;291:1207. [PubMed: 4422691]
10. Straub K, Kanne D, Hearst JE, Rapoport H. *Journal of the American Chemical Society* 1981;103:2347.
11. Kanne D, Straub K, Rapoport H, Hearst JE. *Biochemistry* 1982a;21:861. [PubMed: 7074056]

12. Kanne D, Straub K, Hearst JE, Rapoport H. *Journal of the American Chemical Society* 1982b; 104:6754.
13. Isaacs ST, Shen CJ, Hearst JE, Rapoport H. *Biochemistry* 1977;16:1058. [PubMed: 849407]
14. Cimino GD, Gamper HB, Isaacs ST, Hearst JE. *Annual review of biochemistry* 1985;54:1150.
15. Sage E, Moustacchi E. *Biochemistry* 1987;26:3307. [PubMed: 3651386]
16. Esposito F, Brankamp RG, Sinden RR. *Journal of Biological Chemistry* 1988;263:11466. [PubMed: 2841329]
17. Thompson JF, Bachellerie JP, Hall K, Hearst JE. *Biochemistry* 1982;21:1363. [PubMed: 6176270]
18. Piette J, Hearst J. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine* 1985;48:381.
19. Gamper H, Piette J, Hearst JE. *Photochemistry and Photobiology* 1984;40:29. [PubMed: 6091158]
20. Sinden RR, Hagerman PJ. *Biochemistry* 1984;23:6299. [PubMed: 6529552]
21. Tessman JW, Isaacs ST, Hearst JE. *Biochemistry* 1985;24:1669. [PubMed: 4005221]
22. Zhen WP, Buchardt O, Nielsen H, Nielsen PE. *Biochemistry* 1986;25:6598. [PubMed: 3024713]
23. Yeung AT, Jones BK, Capraro M, Chu T. *Nucleic Acids Research* 1987;15:4957. [PubMed: 3299260]
24. Boyer V, Moustacchi E, Sage E. *Biochemistry* 1988;27:3011. [PubMed: 3401463]
25. Miolo G, Dall'Acqua F, Moustacchi E, Sage E. *Photochemistry and Photobiology* 1989;50:75. [PubMed: 2762383]
26. Gia O, Marciari MS, Garbesi A, Colonna FP, Palumbo M. *Biochemistry* 1992;31:11818. [PubMed: 1445915]
27. Wang Y, Wang Y. *Analytical Chemistry* 2003;75:6306–6313. [PubMed: 14616015]
28. Cao H, Hearst JE, Corash L, Wang Y. *Analytical Chemistry* 2008;80:2932–2938. [PubMed: 18324836]
29. Lai C, Cao H, Hearst JE, Corash L, Luo H, Wang Y. *Analytical Chemistry* 2008;80:8790–8798. [PubMed: 18947205]
30. Gale DC, Goodlett DR, Light-Wahl KJ, Smith RD. *Journal of the American Chemical Society* 1994;116:6027.
31. Gale DC, Smith RD. *Journal of the American Society for Mass Spectrometry* 1995;6:1154.
32. Triolo A, Arcamone FM, Raffaelli A, Alvaradori P. *Journal of Mass Spectrometry* 1997;32:1186. [PubMed: 9373959]
33. Gabelica V, De Pauw E, Rosu F. *Journal of Mass Spectrometry* 1999;34:1328. [PubMed: 10587629]
34. Kapur A, Beck JL, Sheil MM. *Rapid Communications in Mass Spectrometry* 1999;13:2489. [PubMed: 10589098]
35. Wan KX, Gross ML, Shibue T. *Journal of the American Society for Mass Spectrometry* 2000;11:450. [PubMed: 10790849]
36. Gabelica V, Rosu F, Houssier C, De Pauw E. *Rapid Communications in Mass Spectrometry* 2000;14:464. [PubMed: 10717657]
37. Iannitti-Tito P, Weimann A, Wickham G, Sheil MM. *Analyst* 2000;125:627. [PubMed: 10892019]
38. Beck JL, Colgrave ML, Kapur A, Iannitti-Tito P, Ralph SF, Sheil MM, Weimann A, Wickham G. *Advances in Mass Spectrometry* 2001;15:175.
39. Reyzer ML, Brodbelt JS, Kerwin SM, Kumar D. *Nucleic Acids Research* 2001;29:e103/1. [PubMed: 11691940]
40. Rosu F, Gabelica V, Houssier C, De Pauw E. *Nucleic Acids Research* 2002;30:e82/1. [PubMed: 12177310]
41. Gabelica V, De Pauw E. *Journal of the American Society for Mass Spectrometry* 2002;13:91. [PubMed: 11777205]
42. Colgrave ML, Beck JL, Sheil MM, Searle MS. *Chemical Communications* 2002;6:556. [PubMed: 12120121]
43. Gabelica V, Galic N, Rosu F, Houssier C, De Pauw E. *Journal of Mass Spectrometry* 2003;38:491. [PubMed: 12794869]
44. Beck JL, Gupta R, Urathamakul T, Williamson NL, Sheil MM, Aldrich-Wright JR, Ralph SF. *Chemical Communications* 2003;9:1106.

45. Oehlers L, Mazzitelli CL, Brodbelt JS, Rodriguez M, Kerwin S. *Journal of the American Society for Mass Spectrometry* 2004;15:1593. [PubMed: 15519226]
46. Keller KM, Zhang J, Oehlers L, Brodbelt JS. *Journal of Mass Spectrometry* 2005;40:1362. [PubMed: 16220501]
47. Rosu F, Pirotte S, Pauw E, Gabelica V. *International Journal of Mass Spectrometry* 2006;253:156.
48. Sherman CL, Pierce SE, Brodbelt JS, Tuesuwan B, Kerwin SM. *Journal of the American Society for Mass Spectrometry* 2006;17:1342. [PubMed: 16872840]
49. Smith SI, Guziec LJ, Guziec FS Jr, Hasinoff BB, Brodbelt JS. *Journal of Mass Spectrometry* 2007;42:681. [PubMed: 17405184]
50. Wilson JJ, Brodbelt JS. *Analytical Chemistry* 2007;79:2067. [PubMed: 17249688]
51. Mazzitelli CL, Chu Y, Reczek JJ, Iverson BL, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* 2007;18:311. [PubMed: 17098442]
52. Mazzitelli CL, Brodbelt JS. *Analytical Chemistry* 2007;79:4636. [PubMed: 17508717]
53. Mazzitelli CL, Rodriguez M, Kerwin SM, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* 2008;19:209. [PubMed: 17583529]
54. Yu E, Fabris D. *Journal of Molecular Biology* 2003;330:211. [PubMed: 12823962]
55. Colgrave ML, Iannitti-Tito P, Wickham G, Sheil MM. *Australian Journal of Chemistry* 2003;56:401.
56. Wang Y, Zhang Q, Wang Y. *Journal of the American Society for Mass Spectrometry* 2004;15:1565. [PubMed: 15519223]
57. Smith SI, Guziec FS Jr, Guziec L, Brodbelt JS. *Analyst* 2009;134:2058. [PubMed: 19768213]
58. Beaumont PC, Land EJ, Navaratnam S, Parsons BJ, Phillips GO. *Biochimica et Biophysica Acta, Nucleic Acids and Protein Synthesis* 1980;608:182.
59. Dall'Acqua F, Terbojevich M, Marciani S, Vedaldi D, Recher M. *Chemico-Biological Interactions* 1978;21:103. [PubMed: 566637]
60. Palumbo M, Capasso L, Palu G, Magno S, Marciani. *Nucleic Acids Research* 1984;12:8567. [PubMed: 6504703]
61. Hearst JE. *Annual Review of Biophysics and Bioengineering* 1981;10:69.
62. Isaacs ST, Chun C, Hyde JE, Rapoport H, Hearst JE. *Trends Photobiol* (8th) 1982:279.
63. Mazzitelli CL, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* 2006;17:676. [PubMed: 16516486]
64. Gardner MW, Vasicek LA, Shabbir S, Anslyn EV, Brodbelt JS. *Analytical Chemistry* 2008;80:4807. [PubMed: 18517224]

Figure 1.
Structures, abbreviations, and molecular weights of the psoralen analogs.

Figure 2.

ESI-mass spectra of incubates containing: (a) D1, (b) D1 + 8-MOP, and (c) D1 + AMP. The incubates were prepared by adding 1 μ L of saturated psoralen to a solution of 10 μ M D1 in 20 mM ammonium acetate (20 μ L total), and subjecting the incubate to UV irradiation (365 nm, 30 minutes, on ice) after an equilibrium period in the dark (1 hour, room temperature).

Figure 3. Relative binding percentage of the psoralens to each duplex, based on ESI-MS peak areas. The relative binding percentages include all possible DNA/psoralen products: mono-adducts, cross-links, and non-covalent complexes.

Figure 4.

IRMPD mass spectra of (a) $(D1 + 8MOP)^{6-}$ and (b) $(D1 + AMP)^{6-}$. The star denotes the precursor ion. The asterisk denotes an internal product ion. The product ions, w_n and a_n -B, that are determined to be products of the guanine-rich single strand (ssG) or the cytosine-rich single strand (ssC) are labeled w_n ssG or w_n ssC. Product ions that may be a product of either strand are labeled with both strands separated by a slash mark, as " w_n ssG/ssC". Fragment ions from cross-links incorporate a w_n or a_n -B portion plus the psoralen and the corresponding single strand, ssG or ssC. The IRMPD irradiation time was 9 ms and 5 ms for (a) and (b), respectively.

Figure 5. LC chromatograms for incubates containing D1 with (a) 8-MOP and (b) AMP after UV irradiation. XL = cross-linked DNA; M = mono-adducted DNA, ssG and ssC = single strands arising from unreacted DNA plus non-covalent DNA/psoralen complexes disrupted into single strands. After 30 minutes of irradiation, for 8-MOP, ssG + ssC = 76%, M = 5%, XL = 19%; for AMP, ssG + ssC = 49%, M = 32%, XL = 19% (with normalization for the different extinction coefficients of the products).

Figure 6.

Relative distribution of single strand DNA (arising from noncovalent DNA/psoralen complexes and unreacted DNA that are disrupted into single strands due to the temperature of the HPLC column) to cross-linked DNA to mono-adducted DNA for each psoralen with D1 based on summed LC peak areas. The temperature of the LC column was maintained at 55°C in order to disrupt any non-covalent interactions, including DNA base-pairing and non-covalent DNA psoralen complexes. Cross-linked duplexes do not melt at this temperature. Mono-adducted duplexes melt, but the adducted psoralen remains bound to either strand which allows them to be resolved from non-adducted single strands. Three replicates were performed and the relative standard deviation was less than 3% in each case.

Figure 7. Conversion of non-covalent AMP/D1 complexes to covalent AMP/D1 complexes as a function of UV irradiation time monitored by (a-d) IRMPD MS (3.0 ms irradiation time) and (e-h) LC-UV detection. At zero minutes of UV irradiation (a,e), interaction between AMP and the duplex is purely non-covalent.. XL = cross-link; M = mono-adduct. The precursor ion is denoted with a star.

Summary of the cross-linked products observed upon IRMPD of all four duplexes and 8-MOP. The fragments observed are a summation of IRMPD ions observed for the duplex/8-MOP precursors in the 7-, 6-, and 5- charge states. Although two or three 8-MOP binding sites are shown for D2 and D3, respectively, only precursor ions containing a single 8-MOP moiety was subjected to IRMPD. The cross-linked products observed for D2 + 8-MOP and D3 + 8-MOP indicate a mixed population of cross-linked products.

Table 1

DNA sequences used in this study.

Name	DNA Sequence	No. Binding Sites
D1	5'-GCGGGGTAGGGGCG (ssG) 5'-CGCCCCTACCCCGC (ssC)	1
D2	5'-GCGGGTATAGGGGCG (ssG) 5'-CGCCCTATACCCCGC (ssC)	2
D3	5'-GCGGTATATAGGGGCG (ssG) 5'-CGCCTATATACCCCGC (ssC)	3
D4	5'-GCGGGGATGGGGGCG (ssG) 5'-CGCCCCATCCCCCGC (ssC)	1

Table 2

Distribution of types of psoralen adducts determined by IRMPD-MS and LC-UV. AMP was monitored after several different UV exposure times. Standard deviations were less than 5% in each case.

IRMPD-MS distributions	% Cross-links	% Mono-adducts	% Non-covalent	Cross-links/Mono-adducts
P (30 min)	53	22	25	2.4
5-MOP (30 min)	56	24	20	2.3
8-MOP (30 min)	60	14	26	4.3
TMP (30 min)	46	32	22	1.4
AMP (30 min)	28	35	37	0.8
AMP (15 min)	24	37	39	0.6
AMP (5 min)	3	56	42	< 0.1
AMP (0 min)	0	1	99	< 0.1

LC-UV distributions	% Cross-links	% Mono-adducts	% Non-covalent, Unadducted	Cross-links/Mono-adducts
P (30 min)	7	3	90	2.3
5-MOP (30 min)	8	4	89	2.0
8-MOP (30 min)	19	5	76	3.8
TMP (30 min)	5	4	92	1.2
AMP (30 min)	19	32	49	0.6
AMP (15 min)	8	21	71	0.4
AMP (5 min)	1	14	85	< 0.1
AMP (0 min)	0	1	99	< 0.1