The Analysis of Photochemical Nitrile Imine Cross-Linking for Ribonucleosides in Gas-Phase Ions

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Abstract

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This research presents the exploration of nitrile imine cross-linking related to ribonucleoside ions in the gas phase. Riboguanosine was tagged with 4-(2-phenyl-2H-tetrazol-5-yl)benzoic acid at the 5'O-ribose position. The tagged nucleosides were analyzed by liquid chromatography mass spectrometry (LC-MS) and tandem mass spectrometry including ultraviolet photodissociation tandem mass spectrometry (UVPD-MS) and collision-induced dissociation tandem mass spectrometry (CID-MS). Crosslinking was found to occur in tetrazole-guanosine ions upon N_2 loss under CID-MS^2 and UVPD-MS^2. The spectra from CID-MS^3 and UVPD-CID-MS^3 also suggested that the cross-linking involved interactions between the guanine carbonyl group and the nitrile imine.

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or in graduate school, which makes me no longer feel lonely in this foreign country. When you guys are here, we can always have a solution on any issue.

DEDICATION

To my beloved MOM and DAD, Grandpa and Grandma, and anyone who has supported me.

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Chapter 1. INTRODUCTION

1.1 Mass Spectrometry Analysis

Mass Spectrometry (MS) is a sensitive technology in analytical chemistry for the analysis of biomolecules including RNA, and provides characterization of the samples.[1] This technology can also be used to analyze interactions between proteins, peptides, and RNA in order to study the translation, stability, and structural features of peptides and ribonucleosides.[2] In the pharmaceutical industry, a variety of quantitative mass spectrometry methods are widely used in toxicological studies and detection of metabolites to facilitate the process of drug discovery.[3]

The advantage of MS technology is that it can also provide information on both non-covalent and covalent interactions between biomolecules, especially proteins and nucleotides.[4,5] With the progress of MS technology, scientists have developed new MS methods to test samples in solution as well as in the gas phase. For example, time-of-flight mass spectrometry (ToF-MS) and electrospray technology have been used to determine mass changes in biomolecules.[6,7] The technology of mass spectrometry has become a bedrock for today's biomolecule structure and cross-linking analysis.

For the analysis of oligonucleotide crosslinks, mass spectrometry offers several advantages.[8] The most significant one is that mass spectrometry can analyze the oligonucleotide complexes at a very low mass and concentration, which can provide important information on the fragmentation of the compounds.[8,9] In addition, mass spectrometry can provide sensitive and precise data for molecules over a wide mass range up to 20 kDa, which can give a more accurate view to estimate structure changes in oligonucleotides.[10] Also, tandem MS can be used to selectively dissociate stable ions by collisions with neutral gas.[11] This method can fragment ionized compounds at the same time, and it can provide information about compounds through ionization cross-section based on

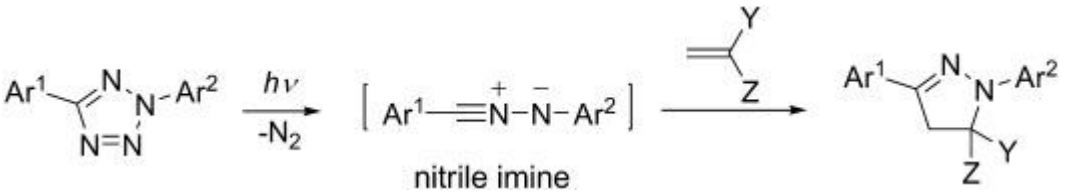
these fragments.[11]

In this research, collision-induced dissociation mass spectrometry (CID-MS) and ultraviolet photodissociation mass spectrometry (UVPD-MS), carried out in both the ion trap and Orbitrap, were used to analyze tetrazole labeled ribonucleoside conjugates which were synthesized with the goal of studying the mechanism of nitrile imine related crosslinking.

1.2 Photo-Crosslinking of Diaryl Tetrazole

In 1959, Huisgen et al. discovered that nitrile imines can be the intermediates in the thermolysis of 2,5-disubstituted tetrazoles.[12] About fifty years later, in 2008, Song et al. proposed the mechanism that makes diaryl tetrazoles release N_2 upon photoirradiation reaction with ultraviolet (UV) light at 290 nm.[13] The nitrile imine reacts as a dipolarophile, which was utilized for labeling with 2,5-diaryl tetrazole of the polypeptide chain in lysozyme.[13]

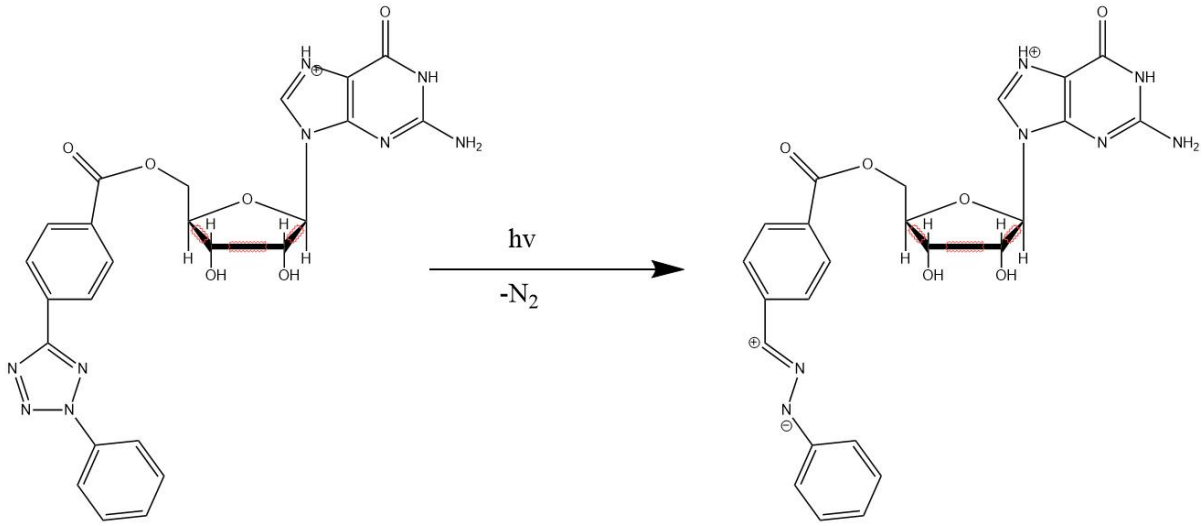
These properties of nitrile imine intermediates make diaryl tetrazoles a useful label group for achieving cross-links between different biomolecules like peptides and nucleotides. Testing their photochemical dissociations and interactions after nitrogen loss has been an active area of research. With the use of UVPD-MS, the Turecek group have generated a method in which 2,5-diaryltetrazole-labeled peptide complexes were converted to nitrile imine crosslinkers by UV irradiation, and the ions that lost N_2 were analyzed for intramolecular cross-linking.[14]



Scheme 1. The mechanism for the photolysis of 2,5-diaryltetrazole to nitrile imine and addition to a dipolarophile.[13]

However, gas-phase nitrile-imine based crosslinking has been focused on peptide conjugates and only rarely has been applied to nucleotide complexes to determine their reactivity. In this research project, 4-(2-phenyl-2H-tetrazol-5-yl) benzoic acid was used as a stable label that was attached to the 5'-O ribose position in riboguanosine and cross-linking reactions of the guanosine ribonucleosides conjugate with the nitrile imine were analyzed

with the use of electrospray ionization mass spectrometry (ESI-MS), CID-MS and UVPD-MS.

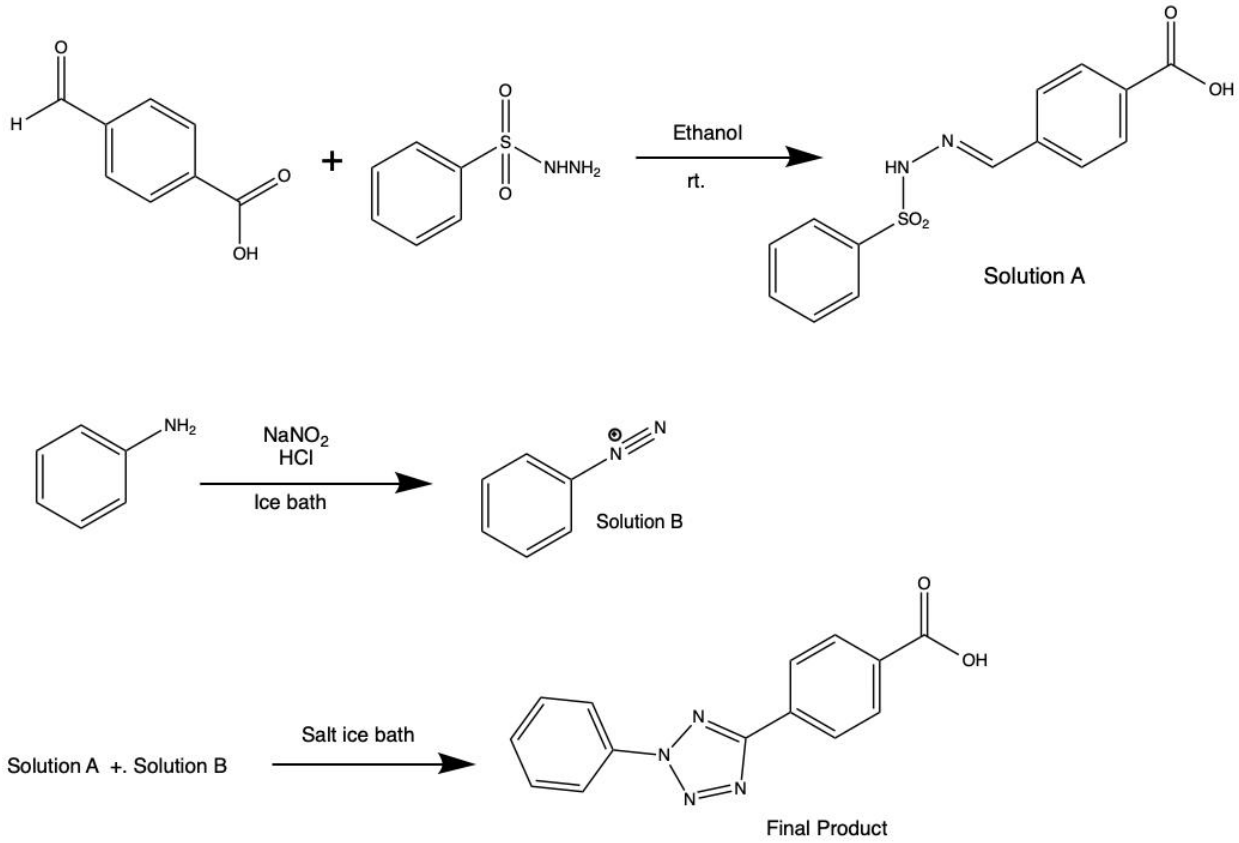


Scheme 2. The proposed mechanism for the loss of N_2 in the photochemical dissociation of a tetrazole-guanosine conjugate under UV light.[15]

Chapter 2. Background

2.1 Synthesis of Diaryltetrazole

Diaryltetrazole acid is an important label for photochemical cross-linking of ribonucleosides as it can form a nitrile imine under the UV light and thus allow for crosslinking.[13] In 2008, Song et al. established a procedure for synthesizing the diaryltetrazole acid,[13] but some modification was made in this project.



Scheme 3. Synthesis of 4-(2-phenyl-2H-tetrazol-5-yl) benzoic acid.[13]

The synthesis was performed with two parts, solution A and solution B, and the solution A was made by dissolving 0.75 g 4-formylbenzoic acid and 0.86 g benzenesulfonylhydrazide in 50 mL of ethanol and continuously stirring the solution for 30 minutes at room temperature, then adding 100 mL of water to precipitate the intermediate.[13] After filtration, the intermediate was dissolved in 30 mL of pyridine.[13]

For solution B, 0.345 g of sodium nitrite was dissolved in 2 mL of deionized water, then dissolving 0.456 mL of aniline into 8mL ethanol solvent (ethanol:water = 1:1), and slowly adding 1.3 mL of 37.7% hydrochloric acid in an ice bath, and pipetting the sodium nitrite dropwise into the aniline solution.[13]

For the final synthesis, solution A was added into solution B with a syringe at five drops every 10 seconds while the temperature was maintained by a salt-ice bath.[13] The solution was extracted with ethyl acetate three times, and upon adding 250 mL of 3N hydrochloric acid solution into the collected organic layer, the final product precipitated and was filtered out and dried.[13] The final product was characterized by ^{1}H-NMR on a Bruker AV301 spectrometer, and the spectrum of the compound was found to match that reported by Song et al.[13] This product was used for the guanosine-tetrazole conjugate synthesis.[15]

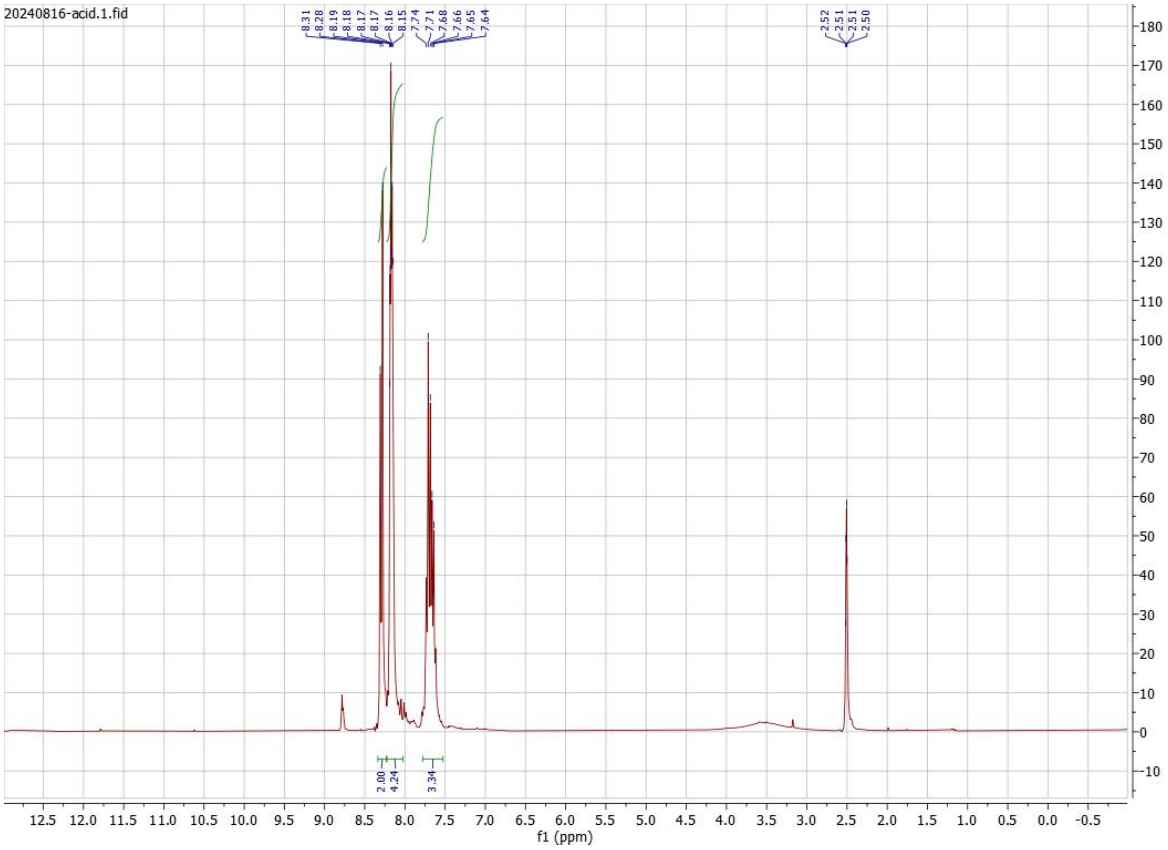
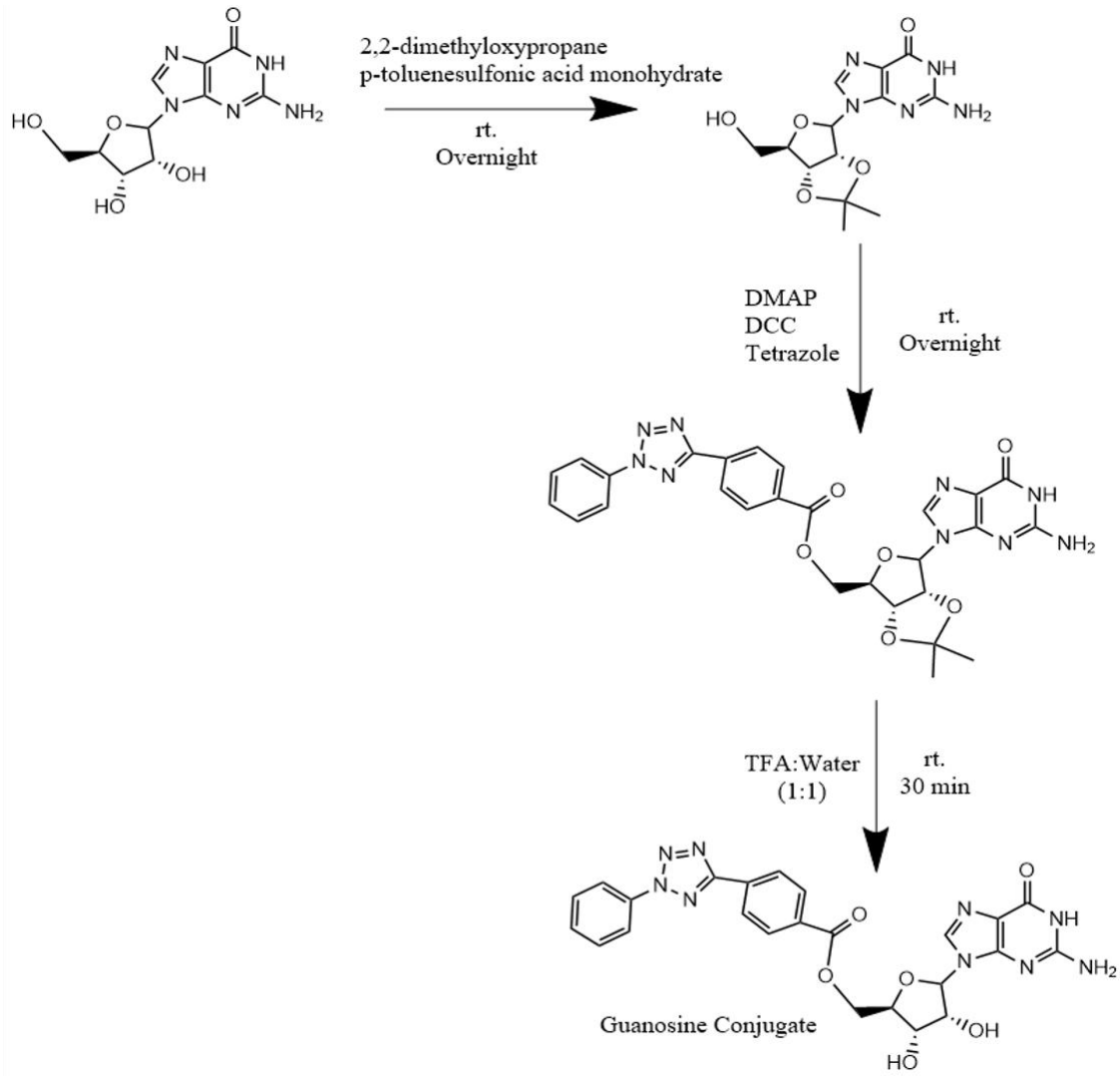


Figure 1. The H-NMR spectra for 4-(2-Phenyl-2H-tetrazol-5-yl) benzoic acid (300 HZ, DMSO-d6, δ 8.31 (d, 2H), 8.19-8.15 (m, 4H), 7.74-7.61 (m, 3H)).[13]

2.2 Synthesis of Guanosine-Tetrazole Conjugate

In previous research by the Turecek group, Zima et al. has successfully attached 4,4′-azobis(4-cyanovaleric acid) to the ribose 5'-O position in guanosine by using an esterification reaction, while the 2'-O and 3'-O positions were protected with the isopropylidene group.[16] To synthesize the guanosine-tetrazole conjugate, a similar reaction was applied in which the tetrazole acid was attached to the ribose 5'-O position in guanosine, and this conjugate was used for a cross-linking study by MS.[15]



Scheme 4. The synthetic process for the 5'-(4-(2-phenyl-2H-tetrazol-5-yl)benzoyl) riboguanosine conjugate.[15]

2′,3′-O-Isopropylideneguanosine (II) was synthesized by dissolving 1g (3.53 mmol) guanosine and 1g (5.26 mmol) *p*-toluenesulfonic acid monohydrate in 40 ml acetone with 3.8 ml of 2,2-dimethoxypropane. The solution was stirred overnight at room temperature.[16] After filtration with a Büchner funnel, the solution was concentrated to a half of volume, and the product was precipitated by adding 1 mL of ammonium hydroxide.[16] The product was filtered out and dried in vacuum with an oil pump. The structure of the protected guanosine was confirmed by MS on a Bruker ESI-MS (*m/z* 324, dimer at *m/z* 647).[16]

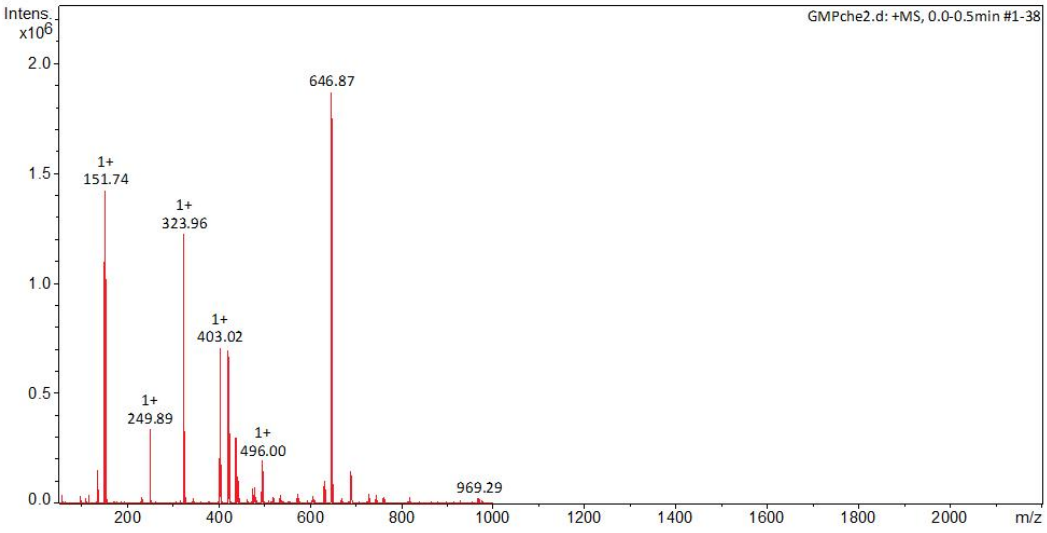


Figure 2. The ESI-MS spectrum for 2′,3′-O-isopropylideneguanosine (II), *m/z* 324, and the dimer of 2′,3′-O-Isopropylideneguanosine (II), *m/z* 647.[15]

The esterification reaction was carried out in a strictly dried round bottom flask by dissolving 2',3'-O-isopropylideneriboguanosine (II) (99 mg, 0.3 mmol) and diaryltetrazole acid (78mg, 0.293 mmol) into 40 mL of dichloromethane with catalysis by 4-dimethylaminopyridine (39 mg, 0.32 mmol) and N,N'-dicyclohexylcarbodiimide (63 mg, 0.3 mmol). The mixture was stirred overnight, and the intermediate product was crystallized by adding hexane which can also remove the byproduct, dicyclohexylurea.[15]

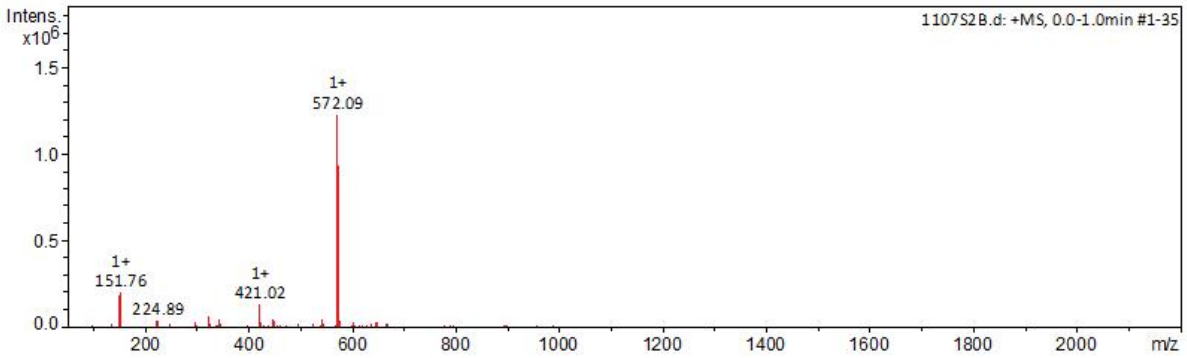


Figure 3. The ESI-MS spectrum for 2',3'-isopropylidene-5'-(4-(2-phenyl-2H-tetrazol-5-yl) benzoyl) riboguanosine, *m/z* 572.09.[15]

The final step of deprotection for the guanosine-tetrazole conjugate was performed by pipetting 3 mL of trifluoroacetic acid (TFA) solution (TFA:H_2O = 1:1) into the intermediate tetrazole-guanosine product and stirring for two hours. The TFA solvent was removed by a flow of compressed air for three hours.[15]

The synthesized compound was analyzed by MS on a Bruker LC-MS ion trap and Thermo Fisher Ascend Tribrid mass spectrometers.[15] The spectra confirmed that 5'-(4-(2-phenyl-2H-tetrazol-5-yl) benzoyl) riboguanosine was successfully synthesized, as indicated by the peak at *m/z* 532.10.[15] The compound was also analyzed with CID-MS^n and UVPD-MS^n methods from Thermo Fisher Ascend Tribrid mass spectrometers for testing the crosslinking of the compound.[15]

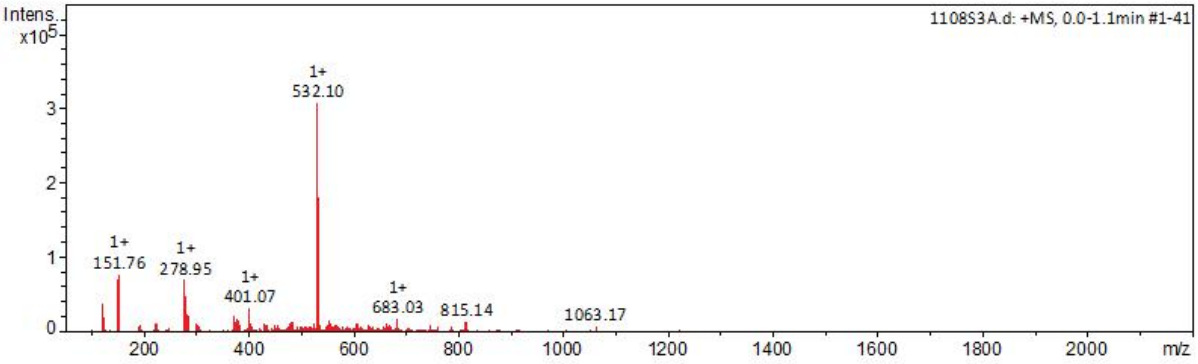


Figure 4. The ESI-MS spectrum for 5'-(4-(2-phenyl-2H-tetrazol-5-yl) benzoyl) riboguanosine, *m/z* 532.10.[15]

Chapter 3. Analysis of the Guanosine-Tetrazole Conjugate

After the synthesis of the guanosine conjugate, tandem mass spectrometry, MS^2 and MS^3, were also carried out with the compound. In these spectra, a loss of nitrogen ion at m/z 504.16 was discovered, which indicated self cross-linking by the tetrazole label that attached at the 5'-O position of the guanosine. In addition, a guanine fragment ion was detected at *m/z* 152.06 (Figure 5a).[15]

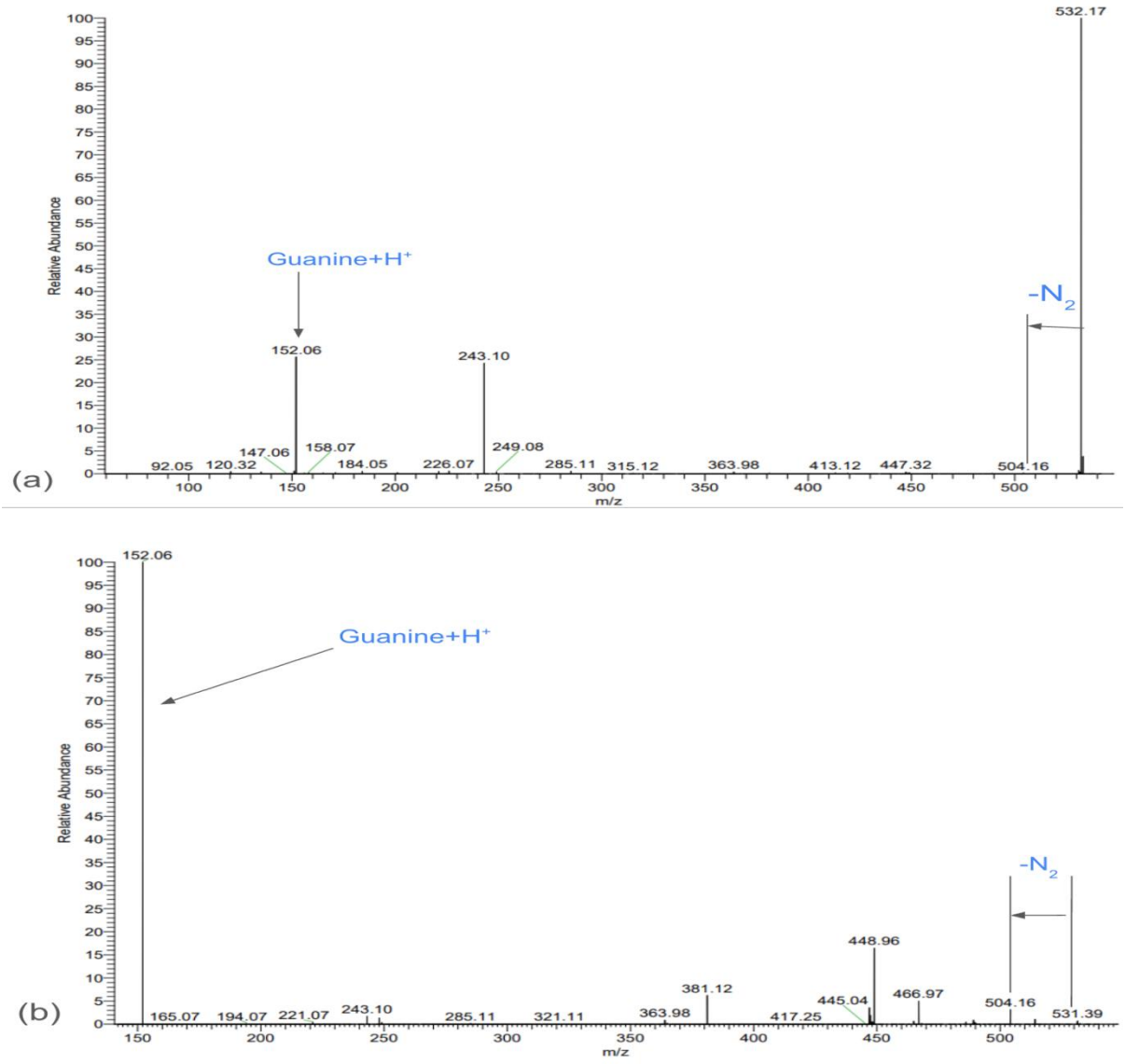


Figure 5. UVPD-MS^2 (Figure 5a) and CID-MS^2 (Figure 5b) of the tetrazole guanosine conjugate detected by the Orbitrap. The guanosine conjugate is at *m/z* 532.17 for [M+1] in UVPD-MS and at *m/z* 531.39 in CID-MS. The cross-linked peak is at *m/z* 504.16.[15]

The UVPD-CID-MS^3 of the m/z 504 ion further indicated that potential crosslinking occurred. This was evident from the fragment ions at *m/z* 315.12 and *m/z* 285.11 that contained guanine and the ribose ring with the loss of CH_2O and $C_3H_4O_2$. Moreover, the nitrile imine moiety was retained as a C_6H_5N group attached to the guanosine fragment. In addition, the MS^3 spectrum did not display a free guanine ion at m/z 152.06 which hinted that the guanine ring was involved in the crosslink with the nitrile imine.[15]

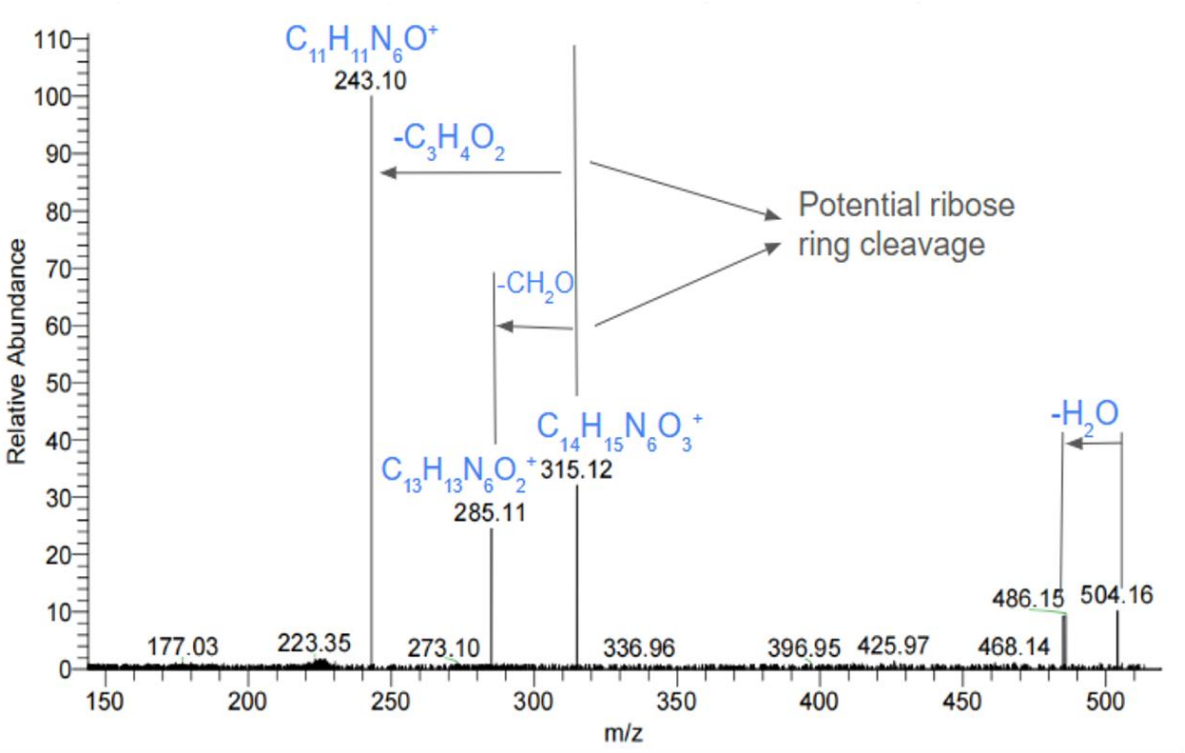
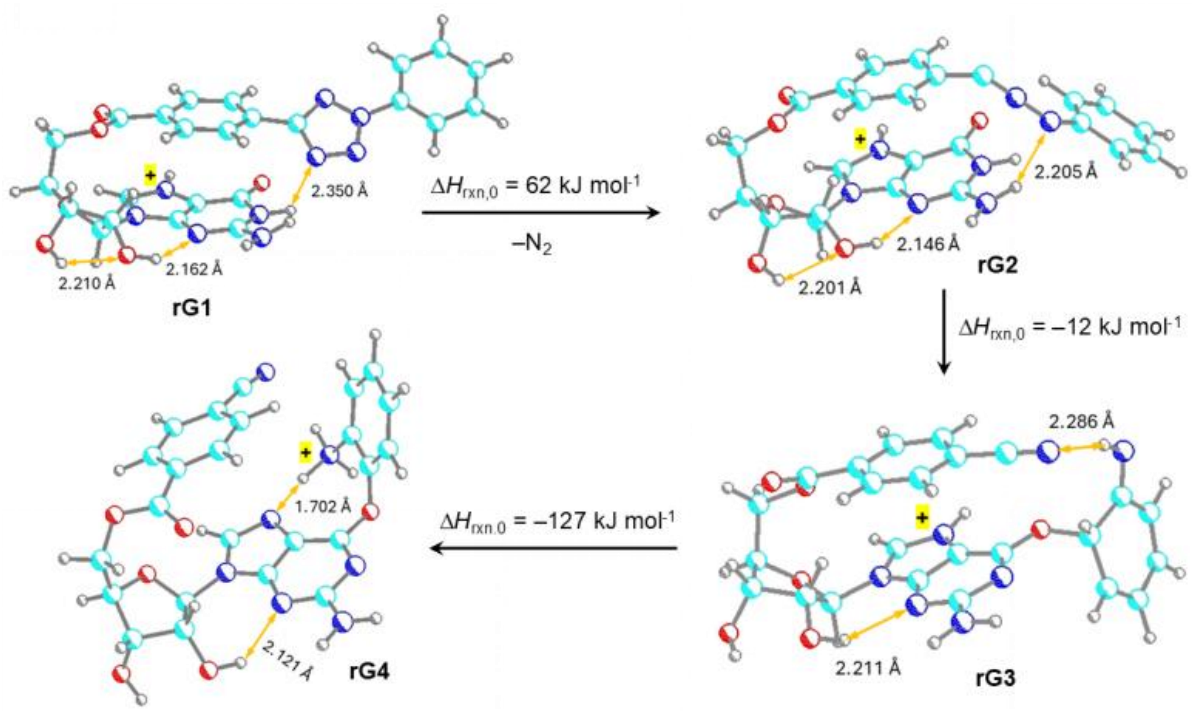


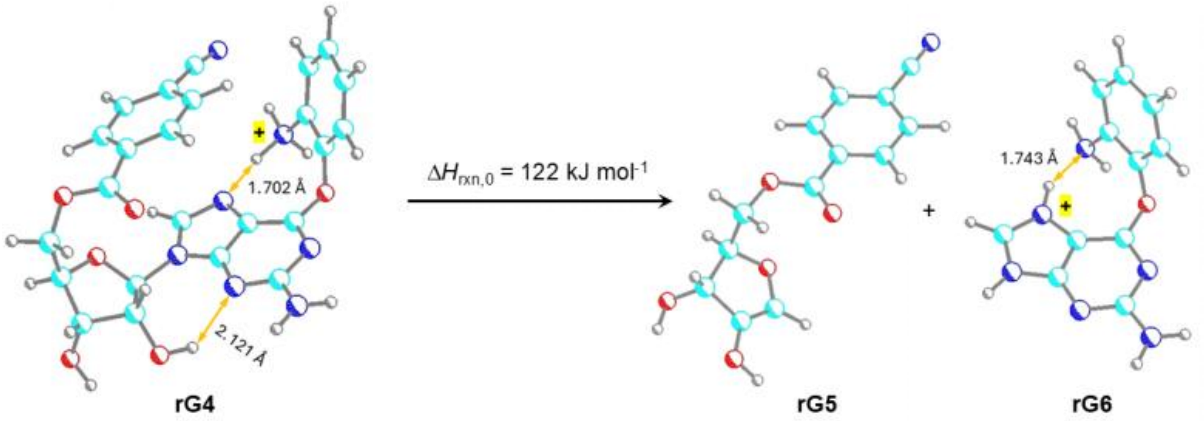
Figure 6. UVPD-CID-MS^3 of the tetrazole guanosine conjugate detected by the Orbitrap. The peaks at *m/z* 315.12 and *m/z* 258.11 indicate cleavage of the ribose ring.[15]

With the help of structure and energy calculations from Wan et al. (Scheme 5), the potential cross-linking reactions are expected to be exothermic and thus can proceed spontaneously. After nitrogen loss, proton transfer onto the nitrile imine facilitates cleavage of the N-N bond and cyclization to the benzene ring. This is presumably coupled with the formation of the stable nitrile group and attack by the guanine carbonyl at the phenylimine. The overall cyclization was calculated to be 139 kJ mol^{-1} exergonic, which provided the driving force for the reaction. The UVPD-CID-MS^3 spectrum also showed a facile glycosidic

bond cleavage, leading to the dominant *m/z* 243 fragment ion. This dissociation was calculated to require a modest activation energy, as shown in Scheme 6. [15]



Scheme 5. Cross-linking estimation for guanosine conjugate based on calculations.[15]



Scheme 6. Estimation for crosslinked guanosine conjugate dissociation based on calculations.[15]

Chapter 4. CONCLUSIONS

The results obtained in this research project contributed to our understanding of crosslinking mechanisms. In particular, the self photocrosslinking in 5'-(4-(2-phenyl-2H-tetrazol-5-yl) benzoyl) riboguanosine, which was associated with glycosidic cleavage in ribose brought more light on guanine reactivity with nitrile imines that takes place in gas-phase ions, as determined by tandem mass spectrometry. However, these experiments were conducted with guanosine only, and nitrile imine crosslinking still needs to be investigated with other types of nucleotides and short oligonucleotide sequences which will be the topic of future investigations.

Chapter 5. ACKNOWLEDGEMENTS

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