SYNTHESIS, PHOTOCHEMISTRY AND DNA PHOTOCLEAVAGE OF COMPOUNDS CONTAINING TETRAZOLETHIONE SCAFFOLDS

by

ADITYA SWAROOP V GUNDUGOLA

B. Tech, University of Mumbai, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Chemistry
College of Arts and Sciences

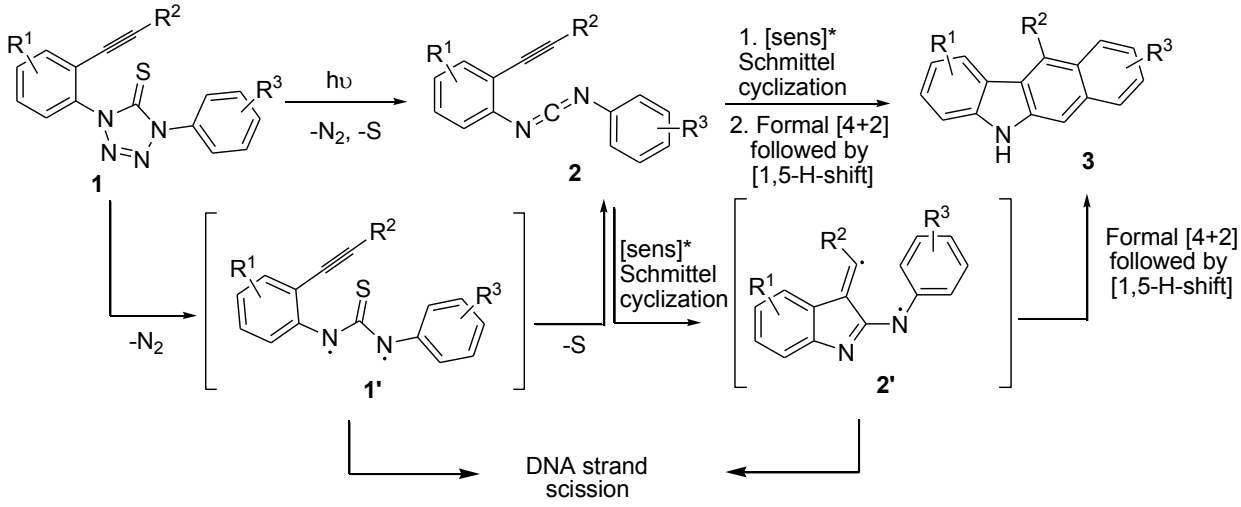
KANSAS STATE UNIVERSITY
Manhattan, Kansas

2011

Abstract

This dissertation focused on the synthesis of 1-(2-ethynylphenyl)-4-phenyl tetrazole-5-thione derivatives **1** and evaluating their potential as a new class of photoactivated DNA cleaving prodrugs. We hypothesized that light activation of **1** would cause the decomposition of the tetrazolethione ring system to enyne-carbodiimide **2** with simultaneous loss of dinitrogen and sulfur via 1,3-triplet biradicals **1′**, which would be spontaneously followed by Schmittel cyclization to indoloquinolines **3** via benzofulvene type biradicals **2′.** The biradicals **1′** and **2′** would have the potential to cause DNA cleavage by abstracting hydrogens from its sugar phosphate backbone, analogous to the mechanism of action of naturally occurring enediyne antitumor antibiotics. Note that our proposed prodrugs contained a 1,4-diaryl tetrazolethione functionality and a direct synthetic route for their construction was lacking in the literature despite their wide spread applications. Therefore, our initial efforts were directed towards developing a general strategy to obtain these ring systems. We employed a highly versatile and efficient copper mediated *N*-arylation to first obtain a series of 1,4-diaryl tetrazol-5-ones which were thionated with Lawesson's reagent to afford the corresponding 1,4-diaryl tetrazole-5-thiones in moderate yields. Specifically, the synthesis of **1** involved Sonogashira coupling of the obtained 1-(2-bromophenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione with the appropriate ethynyl compounds (Chapter 2). Since the tetrazole ring is an important structural component in many biologically and medicinally relevant compounds, we were interested in evaluating the anticancer activity of these compounds in the absence of photochemical activation. The moderate IC_{50} values against leukemia and breast cancer cell lines showed that the anticancer activity of these compounds prior to photoirradiation was minimal (Chapter 3). Independent studies have shown that the photodecomposition of tetrazolethiones gives carbodiimides *via* biradicals, and

photocyclization of enyne-carbodiimide forms indoloquinoline also *via* biradicals. However, it was not known whether these two photoreactions could happen sequentially in one pot with one light source from a substrate like **1,** generating biradicals **1′** and **2′** which could later be employed for DNA photocleavage as hypothesized. Therefere, we photolysed **1** in acetonitrile, and our results show clean formation of a mixture of enyne-carbodiimides and indoloquinolines *via* biradicals **1′** and **2′** (Chapter 4). Finally, we investigated DNA photocleavage by **1** at 350 nm and our results showed significant DNA cleavage in concentrations as low as 100 μM (Chapter 5).



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Approved by;

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Major Professor

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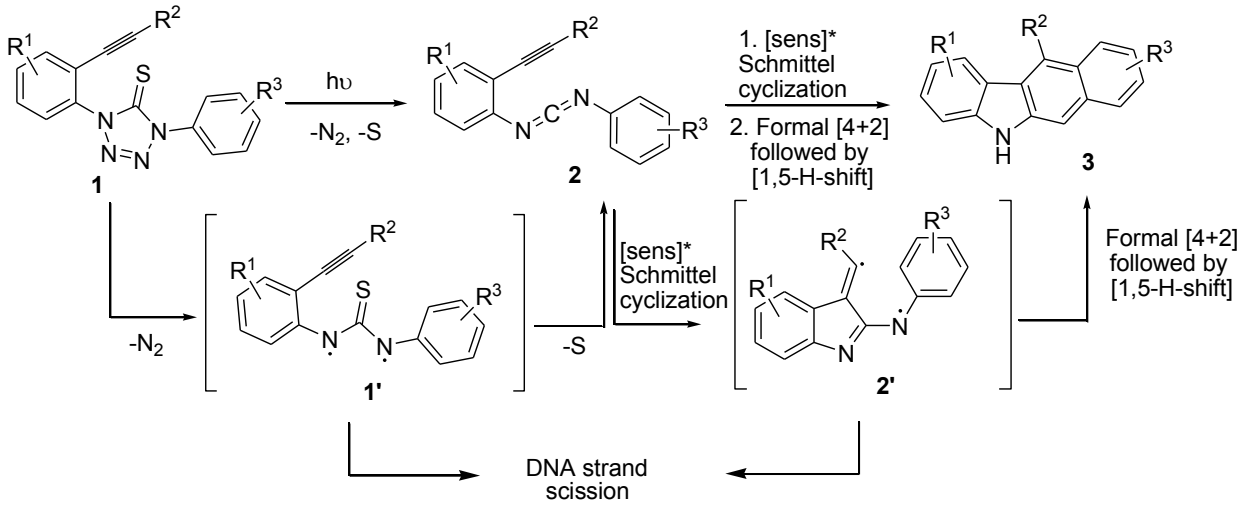

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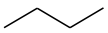
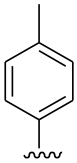
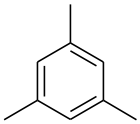
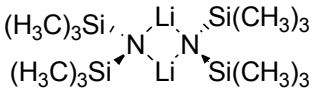
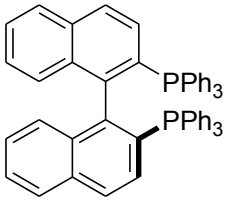
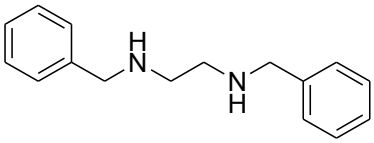
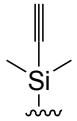
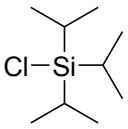
Abbreviations

Abbreviation	Full name	Structure
Ph	Phenyl	
Me	Methyl	**CH_3**
n-Bu	*n*-butyl	
p-Tol	*para*-Toluene	
Mes	Mesityl	
LiHDMS	Lithium bis(trimethylsilyl)amide	
BINAP	2,2'-bis(diphenylphosphino)-1,1'-binaphthyl	
-	N,N'-Dibenzylethylenediamine	
TMS	Trimethylsilyl	
TIPS-Cl	Triisopropylsilyl chloride	
1,4-CHD	1,4-cyclohexadiene	

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Acknowledgements

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Dedication

To all the greatest men to ever lived in this world and contributed to the society with their selfless love and service.

Chapter 1 - Enediynes and enyne-heteroallenes: Photoactivated DNA cleavage via biradicals

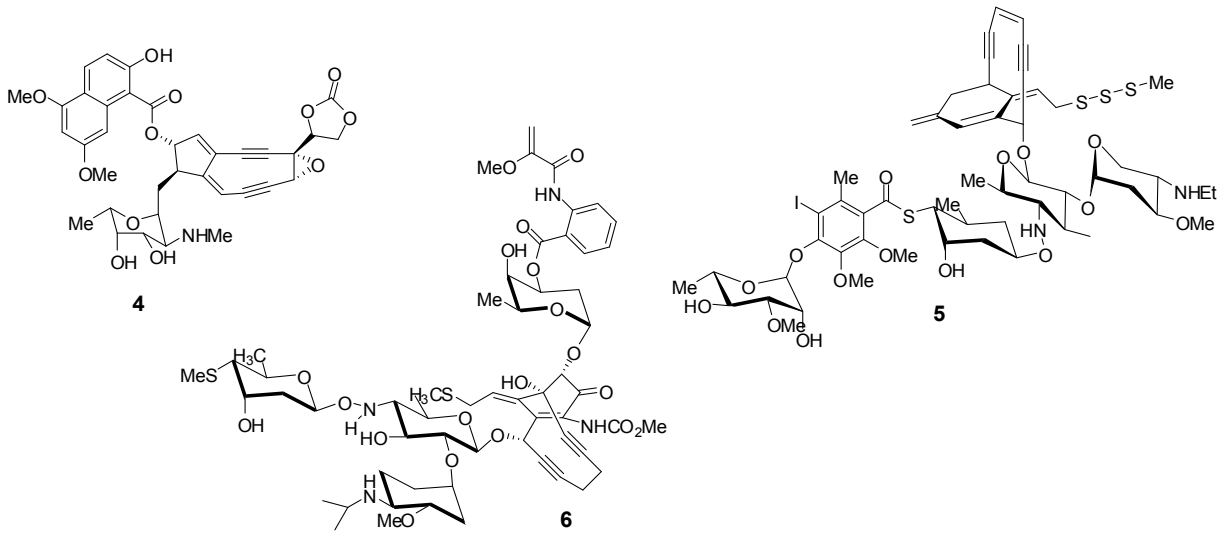
1.1. Introduction

Cancer which is characterized by an uncontrolled growth of cells in the body, is the second leading cause of death after cardiac disease in the United States.[1] Nearly half of the men and one-third of the woman develop cancer in their lifetime.[1] Numerous causes have been identified for the outgrowth of cancer such as exposure to carcinogenic chemicals, obesity, excessive drinking of alcohol, smoking, radiation etc.[2] Different modes of treatment have been developed depending on the type and the stage of cancer. For instance, surgery is employed for the treatment of localized tumors such as skin cancer, lung cancer etc.[1, 3] Other modes of treatment such as radiation and chemotherapy have been established for the damage of tumors where surgery cannot be performed.[3] The former employs high energy X-rays and gamma rays for selective demolition of cancer cells that damages their DNA,[3] and also, for the palliative treatment to reduce the tumors in the brain, esophagus etc.[3] While the latter involves the destruction of cancer cells using chemical drugs. These chemotherapeutic drugs based on their mechanism of action are classified into alkylating agents, antimetabolites, topoisomerase inhibitors[4] and DNA cleaving agents. Examples of alkylating drugs include carboplatin and cisplatin.[5] They impair the cell function by coordinating with amino, carboxyl and phosphate groups present on various biomolecules such as DNA, RNA, proteins etc.[6] Antimetabolite drugs like azathioprine and mercaptopurine act as disguised purines and pyrimidines and thus, interfere with DNA or RNA synthesis.[7] Topoisomerase inhibitors such as irinotecan,

amsacrine, etoposide[4] target DNA topoisomerases that play an important role in the replication of the DNA.[8] DNA cleaving agents induce antitumor activity by causing single or double stranded breaks in the DNA. The naturally occurring enediynes are the most potent antitumor antibiotics that belong to this class of chemotherapeutic drugs.

1.2. Enediyne antitumor antibiotics

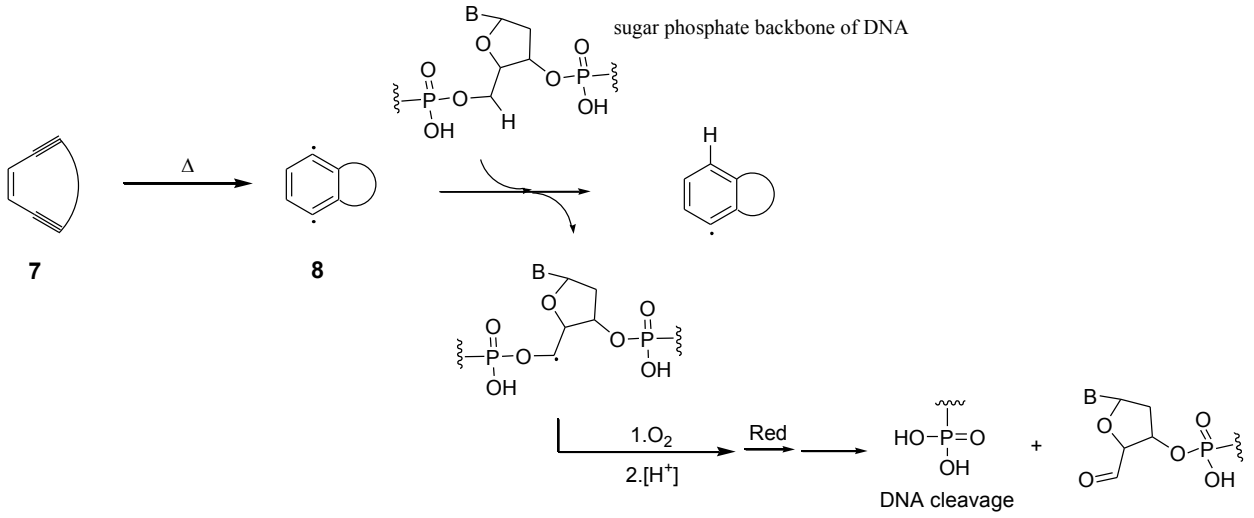
The natural enediyne antibiotics have unique molecular structure and striking biological activities.[9] As mentioned earlier, these are the powerful antitumor agents reported to date exhibiting cytotoxicity in a variety of cancer cells in the concentration range 1-100 pg/ml.[9] Neocarzinostatin is the first enediyne natural product isolated by Ishida in 1965.[9] Several other enediyne toxins like lidamycin, calcheamicin, esparamicin, dynemicin, C-1027, maduropeptin and kedaricidin etc. have been discovered in the subsequent years. The structures of neocarzinostatin **4**, lidamycin **5** and calcheamicin **6** are shown in Scheme 1.1.[9, 10]



Scheme 1.1. Natural enediyne antibiotics

1.2.1. Mechanism of action

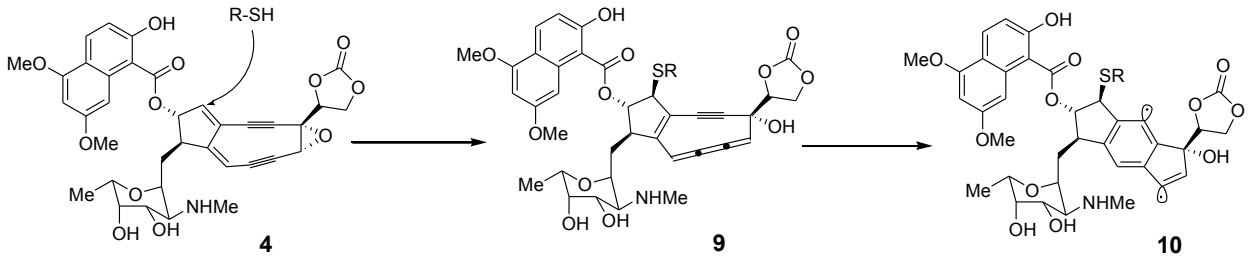
The enediyne antibiotics contain a (*Z*)-hexa-1,5-diyn-3-ene **7** unit usually embedded within a 9- or a 10- membered ring.[11] The excellent antitumor activity of these compounds is attributed to thermal Bergman cyclization (BC) of the enediyne moiety, leading to the formation of a highly reactive aromatic 1,4-diradical **8** (Scheme 1.2).[12, 13] The latter is known to abstract hydrogen atoms from the C5′ position of the sugar phosphate backbone of the DNA resulting in the formation of a radical, that further undergoes a series of oxidation and reduction steps that ultimately leads to oxidative cleavage of the DNA (Scheme 1.2).[14, 15]



Scheme 1.2. DNA cleavage mechanism of enediyne **7**

Note that neocarzinostatin **4** is a prototypical enediyne and has a slightly different mode of action from that of other enediynes antibiotics. It consists of a 1:1 mixture of an apoprotein and chromophoric molecule. The apoprotien mainly assists in stabilizing the chromophore and also carries it to the minor groove of the DNA where it is activated by nucleophilic attack from a

thiol to a highly reactive enyne-cumulene species **9**.[9] The cumulene **9** undergoes cyclization to the biradical **10**, which abstracts hydrogen atoms from the sugar phosphate backbone of the DNA resulting in the double-stranded cleavage (Scheme 1.3).[9, 11]

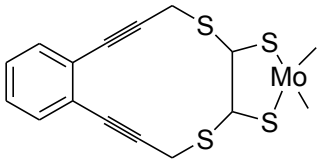


Scheme 1.3. Mechanism of biradical formation from neocarzinostatin **4**

1.2.2. Problems associated with natural enediyne antibiotics

Despite their remarkable antitumor activity, only two enediynes have been approved for clinical use. This includes neocarzinostatin which was approved by Japanese government in 1993 for the treatment of leukemia, gastric carcinoma and pancreatic adenocarcinoma,[9, 11] and Mylotarg©, a combination of calcheamicin **5** and CD33 antigen, which is approved for clinical use in the United States since 2000 for the treatment of acute myelogenous leukemia.[10] However, these natural enediyne antibiotics induce serious side effects such as anorexia, nausea, vomiting, loss of hair, mild gastrointestinal disorders, and oral mucositis, due to indiscriminate destruction of normal and tumor cells.[14, 16] As a result, some synthetic enediynes **11-13** have been developed (Scheme 1.4).[14, 17-20] Although synthetically designed enediyne systems have lower toxicity as compared to natural analogs, they have also been found to be less potent. For instance, synthetic

enediynes cause the DNA strand scission in microgram amounts while the natural enediynes accomplish this task in nano/pico gram amounts.[14, 17]



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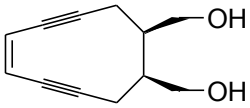

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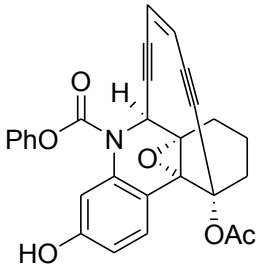

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Scheme 1.4. Designed enediynes[19, 20]

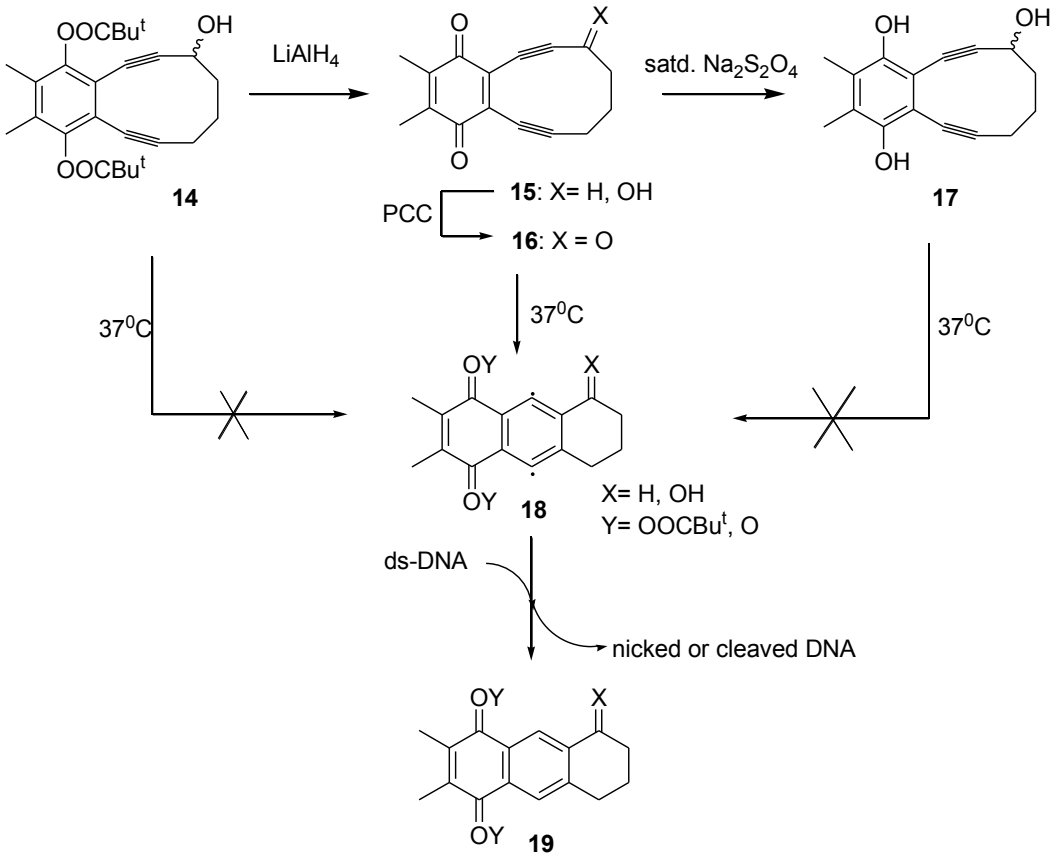
1.3. Synthetic enediyne prodrugs

In order to retain the potency of enediynes in cancer cells and eliminate the side effects, scientists explored methods to activate the enediynes from their prodrugs by an external trigger so as to allow spatial and temporal selectivity. Examples of some of these proposed enediyne prodrugs utilizing this strategy are discussed below.

1.3.1. Redox controlled Bergman cyclization

Nicolaou et al. proposed an enediyne system in which the rate of Bergman cyclization (BC) can be controlled by a hydroquinone ↔ quinone conversion by employing redox processes in the cell (Scheme 1.5). For example, hydroquinone **14** was synthesized and its reduction with $LiAlH_4$ produced the corresponding quinone **15**. Oxidation of the hydroxy group with PCC furnished the ketone **16**, while the reduction of **15** produced hydroquinone **17**. These researchers

independently tested the synthesized compounds towards their ability to undergo BC and reported that **14** and **17** were stable towards cycloaromatization while **15** and **16** underwent BC to **18** at physiological temperatures. This could be attributed to the presence of a stable aromatic ring in **14**, where the double bond is part of the aromatic sextet and hence, not available for BC. However, in hydroquinone **15**, the double bond becomes part of the enediyne system which is perfectly set for the BC.[9, 21] In accordance with this chemistry, Nicolaou and coworkers observed that **15** and **16** cause cleavage of circular supercoiled DNA while **14** and **17** showed no DNA damaging properties. Nicolaou proposed that the conversion of **14→15,** and **15→17** could be carried out by employing the redox chemistry in the cell and thus the formation of biradicals and subsequent DNA cleavage can be controlled.

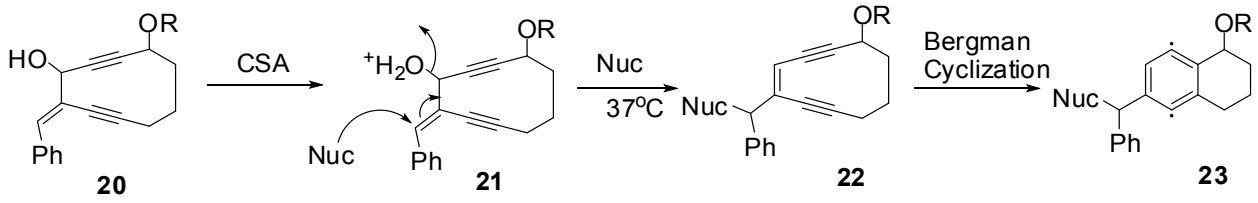


Scheme 1.5. Redox controlled Bergman cyclization of enediynes

Limitation: The redox potential for the quinone to hydroquinone conversion is approximately 0.715,[22] while the redox potential for a biological process such as NAD^+/NADH is much lower (0.32). Therefore, application of this method in vivo may not be plausible.

1.3.2. pH controlled Bergman cyclization

The cycloaromatization induced by exploiting the variations in pH between the normal and cancer cells have been explored that offer promise in enediyne mediated cancer therapy with reduced side effects.[14] For instance, the alcohol **20** upon protonation in the presence of an acid yields **21**. The latter was shown to undergo nucleophilic attack *via* S_N2' reaction to generate reactive enediyne **22** that cycloaromatized under ambient conditions to produce the biradical intermediate **23** (Scheme 1.6) and cleaves single strand DNA.[14, 23] The researchers hypothesized that the mild acidic environment in the tumor cell (6.8±0.07), as opposed to the normal cells (7.4)[24] could exclusively protonate the inactive enediyne **20** to generate the active enediyne **22**, which could be followed by an attack from a biological nucleophile to generate the active enediyne.

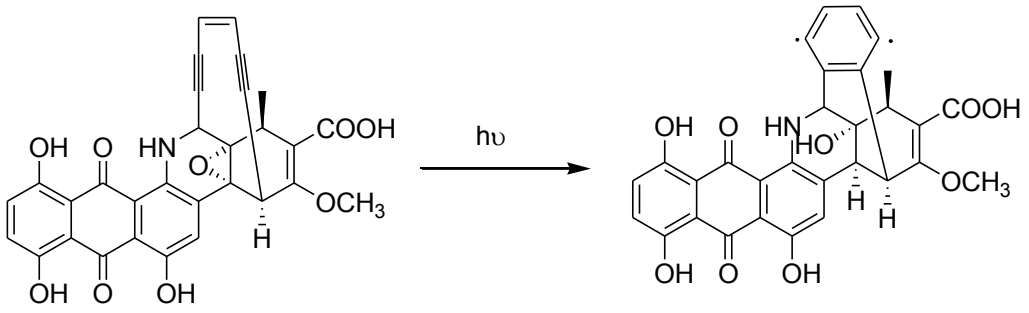


Scheme 1.6. Mild acid catalyzed action of enediynes[23]

Limitation: The camphor sulfonic acid (CSA) employed for the protonation of alcohol **20** **(**Scheme 1.6) is very acidic (pK_a 1.2), the acidity that cannot be attained inside the biological systems. At pH 6.8, the unprotected alcohol will predominate by 4900 times over its conjugated acid. The concentrations of enediyne required (millimolar) to induce DNA cleavage were too high.

1.3.3. Light controlled Bergman cyclization

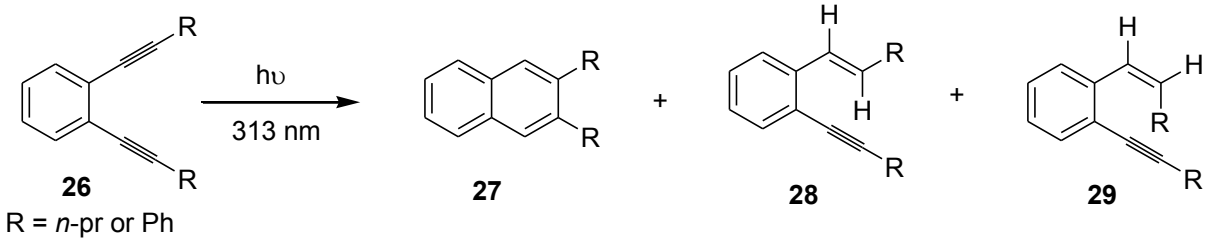
The natural antibiotic dynemicin A (**24**) exhibits *in vivo* potency against a variety of cancer cells which is further enhanced by irradiation that causes direct visible light induced cycloaromatization to **25** (Scheme 1.7).[25] However, the quantum and chemical yields of this photochemical process are very low thereby limiting its application.[26] Nevertheless, this novel mode of activation has encouraged researchers to design light triggered enediyne derivatives. Some of the approaches that are employed for the photochemical activation are mentioned below.



24 **25**

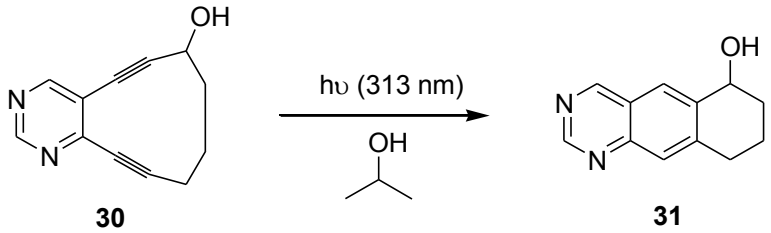
Scheme 1.7. Photochemical activation of natural enediyne Dynemycin A (**24**)[25]

Inspired by the mechanism of action of Dynemycin, Turro and his coworkers first demonstrated the possibility of a photochemical cycloaromatization of synthetic enediynes. They reported the formation of the naphthalene derivatives **27 – 29** by irradiation of *n*-propyl and phenyl substituted enediynes **26** (Scheme 1.8).[27] However, the photo BC is not as versatile as the thermal counterpart and also the quantum yields for the reaction are very low.



Scheme 1.8. Photochemical BC of enediynes[27]

Further investigations have revealed that the nature of substituent near the acetylene terminus greatly influence the rate of photochemical Bergman cyclization.[14, 28] For instance, Russell and coworkers reported facile photocyclization of pyrimidine based enediyne systems **30** in isopropanol to quinazolines **31** (Scheme 1.9).[14, 28] Replacing the alcohol group in **30** with a ketone failed to produce the photo BC product.[28] Irradiation of **30** with super coiled DNA produced nicked DNA at concentrations as low as 40 μM.



Scheme 1.9. Facile photochemical BC of pyrimidine based enediynes **30**

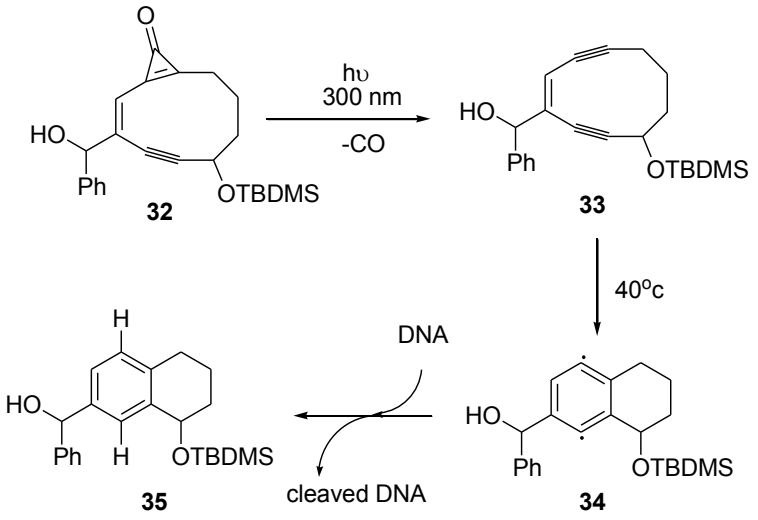
Limitation: One could conveniently think of applying this chemistry for DNA photocleavage in cells. But the quantum yields for the photochemical Bergman cyclization are low and the factors that affect the rate of cycloaromatization are not fully explored to date. Hence, the application of this method *in vivo* is an open question.

1.3.4. Light activated generation of reactive enediyne system

The researchers have also focused on an alternative strategy for selective DNA cleavage that involves photogeneration of the reactive enediyne system at the diseased site from its precursor followed by thermal Bergman cyclization under ambient conditions.[14, 26, 27, 29-32] Thus, these photoswitchable analogs of enediynes are inactive in dark but become active upon irradiation with light of appropriate wavelength. Some of the examples that employ this strategy are discussed below.

1.3.4.1. Photodecarbonylation of cyclopropenone

Popik et al. efficiently synthesized enediyne precursors **32**,[26] in which the acetylene moiety was masked with a cyclopropenone ring. Irradiation of **32** at 300 nm resulted in decarbonylation leading to the generation of an active enediyne **33** which underwent thermal Bergman cyclization at 40^{o}C forming the biradical intermediate **34** and thus, induced DNA strand cleavage resulting in the formation of **35** (Scheme 1.10).[26]

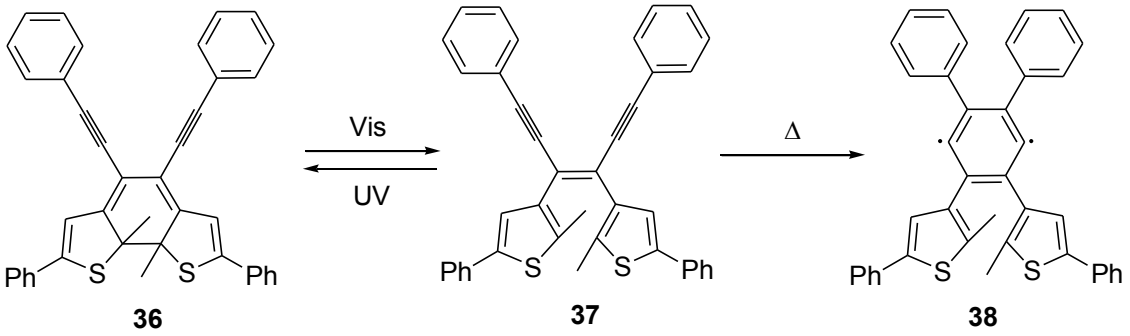


Scheme 1.10. Phototriggered activation of masked enediynes followed by DNA cleavage[26]

Limitation: The thermal cyclization of the reactive enediyne **33** takes place at temperatures >40°C that is not feasible under physiological conditions. Moreover, the synthesis of **32** is complicated.

1.3.4.2. Photocontrolled Bergman cyclization

Branda et al. utilized UV and visible light to generate an active enediyne from its precursor by efficiently making use of the reversible ring closing-opening mechanism of dithienylethane (DTE) backbone.[31] The enediyne precursor **36** was synthesized by placing the ethynyl structures on DTE. **36** was inactive towards BC in dark. Exposure to visible light resulted in the ring opening of DTE by electron transfer generating the active enediyne system **37**. The latter underwent *in situ* thermal cycloaromatization to produce the biradical **38**. Irradiation of **37** with UV light (365 nm) resulted in the ring closure of DTE backbone and thus, disrupting the active enediyne system and terminating the Bergman cyclization (Scheme 1.11).



Scheme 1.11. Reversible photocontrol of Bergman cyclization using DTE[31]

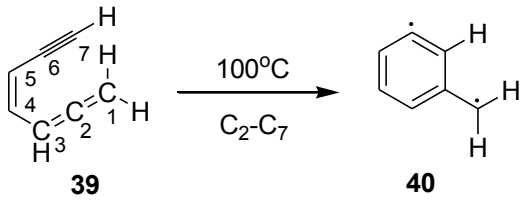
Limitation: The synthesis of the DTE substituted enediyne system is tedious (19 steps) and very low yielding. In addition, the DNA cleavage by these compounds has not been tested.

Numerous other enediyne systems that can be activated photochemically have been reported but these also suffer from limitations.[30, 32] This led researchers to investigate other compounds that generate biradicals upon photocyclization and thus, could mimic the mechanism of action of enediynes for DNA cleavage.

1.4. Enyne-allenes and their hetero analogs

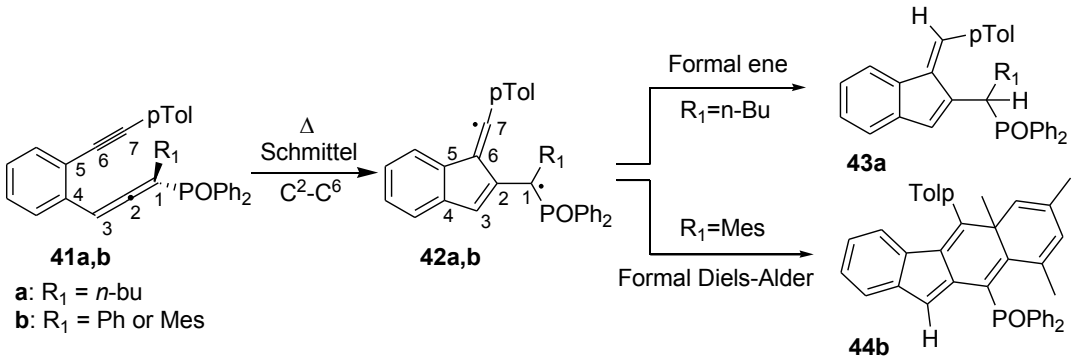
1.4.1. Thermal cyclization

Inspired by the mechanism of action of neocarzinostatin, Myers et. al synthesized a simple enyne-allene (Z)-1,2,4-heptatrien-6-yne **39**[33] and showed its thermal cyclization to σ,π-biradical **40** (Scheme 1.12). The biradicals produced by derivatives of **39** are less reactive towards hydrogen abstraction from the DNA than the one produced by the cycloaromatization of enediynes.[33, 34]



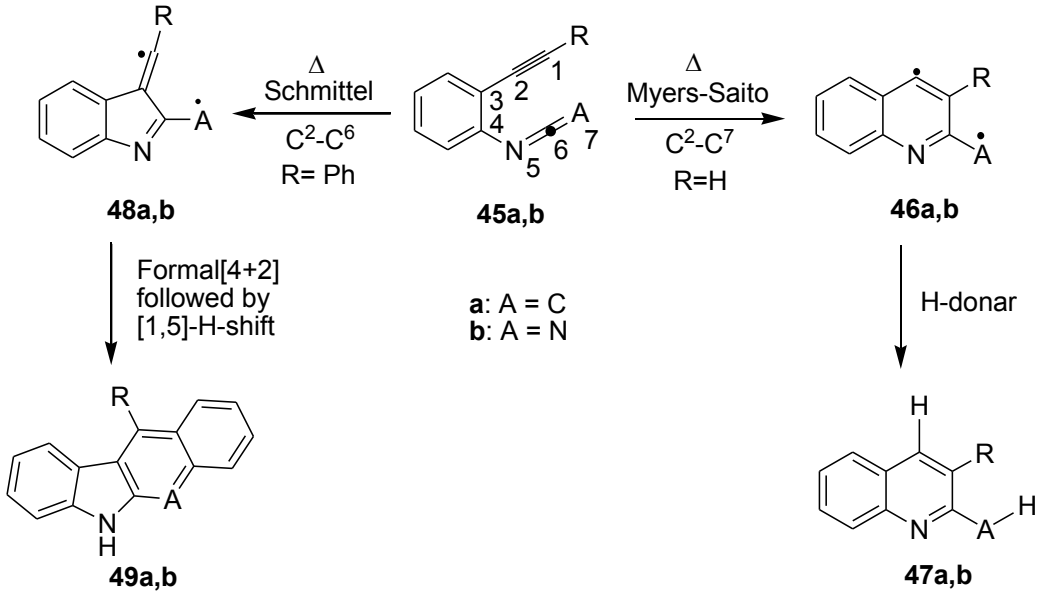
Scheme 1.12. Myers-Saito cyclization of simple enyne-allene[33]

Schmittel and coworkers later demonstrated that by an appropriate choice of the substituent at the alkyne terminus, one can allow the thermal biradical cyclizations of enyne-allenes **41a**,**b** to be steered away from the Myers-Saito (C_2-C_7) pathway towards a C_2-C_6 cyclization, leading to a benzofulvene type biradicals **42a**,**b** (Scheme 1.13).[35] The latter is facilitated by the presence of bulky substituents such as an aromatic ring, trimethyl silyl, *t*-butyl group etc. on the alkyne terminus. The biradical **42a** undergoes an intramolecular formal ene [2+2] reaction to form the indene derivative **43a**, while **42b** undergoes intramolecular formal Diels-Alder [4+2] reaction to form the benzo[*b*]fluorene product **44b** (Scheme 1.13). The former is favored by the presence of an alkyl group on the allene terminus (*e.g.* *n*-Bu) while the latter is favored by the presence of a bulky aromatic group (*e.g* Ph or Mes).[36] The discovery of these biradical cycloaromatizations has led researchers to exploit this chemistry for the synthesis of carbocyclic ring systems.[36, 37]



Scheme 1.13. Schmittel cyclization of enyne-allenes[36]

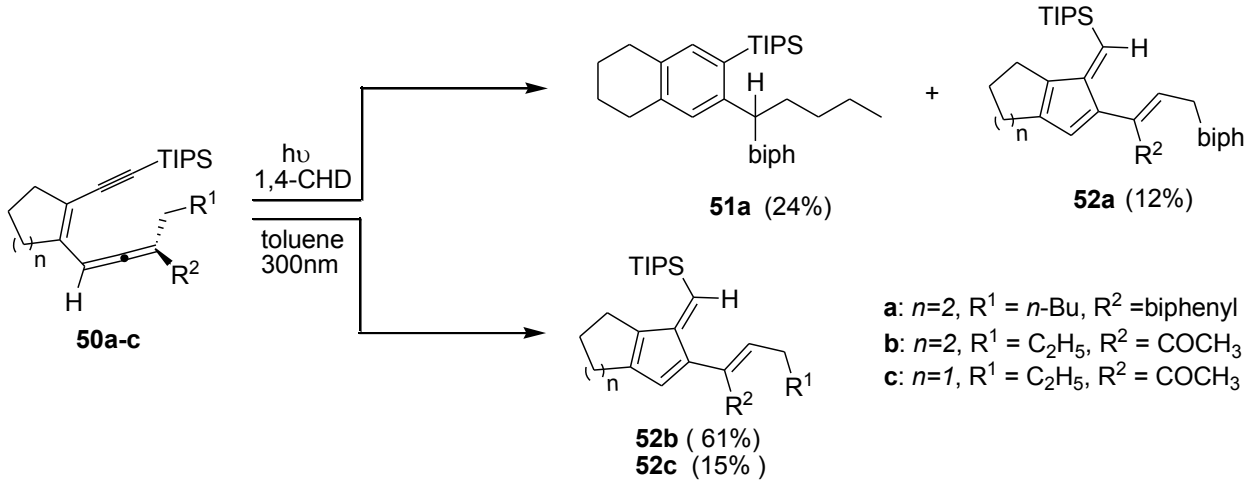
The organic chemists further speculated whether the replacement of the carbon atom in the enyne-allene system with heteroatoms could allow the synthesis of fused heterocyclic ring systems.[38] Therefore, the thermal cyclization of enyne-heteroallenes such as enyne-ketenimines **45a** and enyne-carbodiiimides **45b** was investigated and these were also shown to undergo C^2-C^7 and C^2-C^6 cyclizations depending on the substituent present at the acetylene terminus resulting in the formation of quinolines **47a**,**b**, or benzocarbazole derivatives **49a** and indoloquinolines **49b** *via* biradicals **46a**,**b** and **48a**,**b**, respectively (Scheme 1.14).[39] The formal [4+2] cyclization of enyne-carbodiimide followed by a 1,5-[H-shift] to form indoloquinoline **49b** (A = nitrogen) was efficiently utilized in the synthesis of plant alkaloid cryptotackieine.[40]



Scheme 1.14. Thermal cyclization of enyne-heteroallenes

1.4.2. Photochemical cyclization

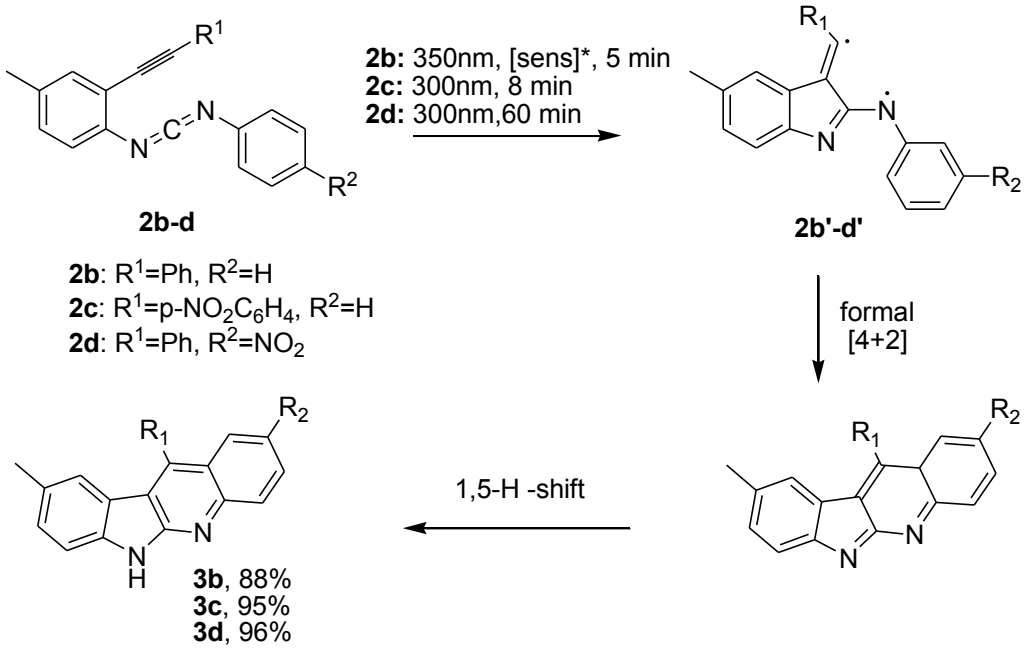
Later on, studies by Schmittel et al. revealed that these C_2-C_7 and C_2-C_6 cyclizations of enyne-allenes and their hetero derivatives can also be carried out photochemically analogous to photo Bergman cyclization of enediynes.[41, 42] The syntheses of various non-benzannulated enyne-allenes **50a – c** was carried out. Irradiation of the enyne-allene **50a** in toluene resulted in the mixture of Myers-Saito **51a** and the C_2-C_6 products **52a** while the irradiation of enyne-allenes **50b** and **50c** solely gave the Schmittel product **52b**,**c** (Scheme 1.15). It was observed that the energy of the substituent R_2 that acts as an internal triplet sensitizer determines the type of products formed during the reaction. However, the yields of these photochemical transformations were low.



Scheme 1.15. Photochemical cyclization of enyne-allenes **50a-c**

These researchers also explored the photochemistry of variously substituted enyne-carbodiimides **2b – d** (Scheme 1.16).[41] The photoirradiation of **2b** in *n*-hexane resulted in less than 5% conversion. However, the addition of a triplet sensitizer such as acetophenone resulted in complete conversion to the indoloquinoline cyclized product **3b** *via* biradical **2b′** followed by

a formal [4+2] cyclization and a subsequent 1,5-[H-shift] as shown. However, the presence of an electron-withdrawing group at the acetylene or carbodiimide terminus such as in **2c**,**d** respectively afforded the cyclized products **3c,d** under direct irradiation. The yields of these photochemical transformations were also close to 100% (Scheme 1.16). The reaction in the presence of a triplet quencher such as 1,4-dipheynl-1,3-butadiene inhibited the formation of the photoproduct **3** and hence, supported the photoreaction from a triplet manifold.



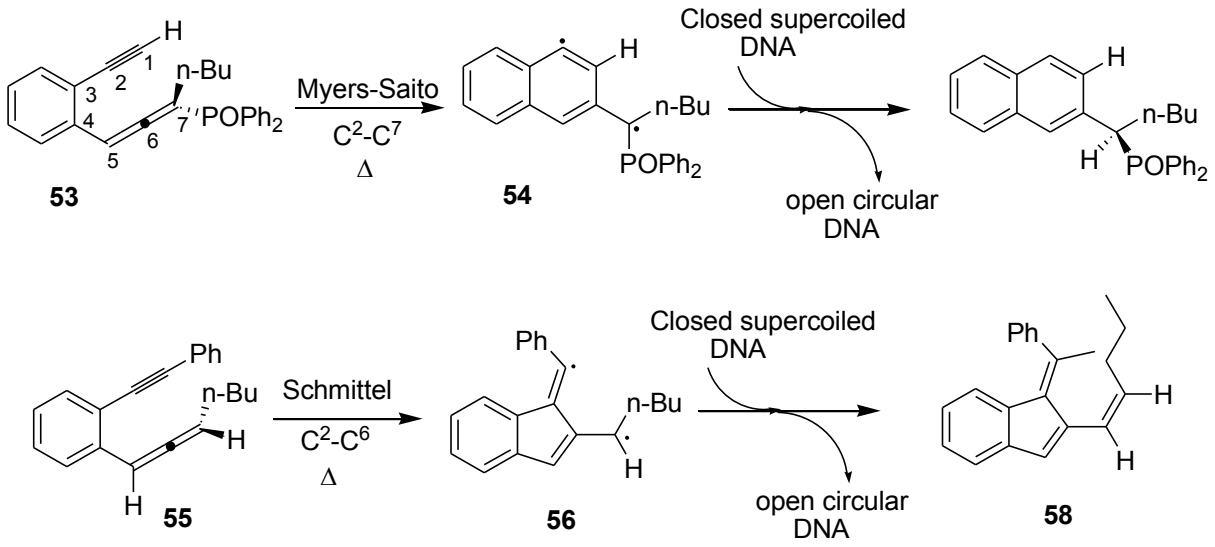
Scheme 1.16. Photochemical cyclization of enyne-carbodiimides[41]

The results obtained gave evidence that enyne-carbodiimides undergo more efficient photochemical C_2-C_6 cyclization than the corresponding enyne-allenes.[41] Independent theoretical investigations carried out by Engels et al.[43] showed that the geometry optimization of the triplet excited state of enyne-carbodiimide directly leads to the biradical intermediate of the C_2-C_6 cyclization through a barrierless transition state possessing a steadily descending energy profile. However, in the case of enyne-allenes and enyne-ketenimines, the molecule is predicted to relax

to a local minimum from which the barriers for both modes of cyclization are small.[43] The mechanistic studies are discussed more in detail in Chapter 4 (Section 4.2).

1.4.3. DNA cleaving ability of enyne-allenes

Shortly after the discovery of enyne-allenes, Nicolaou and Saito were able to demonstrate the DNA cleaving properties of phosphine oxide-substituted enyne allenes **53** via Myers-Saito biradical **54** pathway.[43] Later, Schmittel demonstrated that the enyne-allenes **55** that produce benzofulvene radicals **56** also effectively induce DNA strand cleavage (Scheme 1.17).[44]

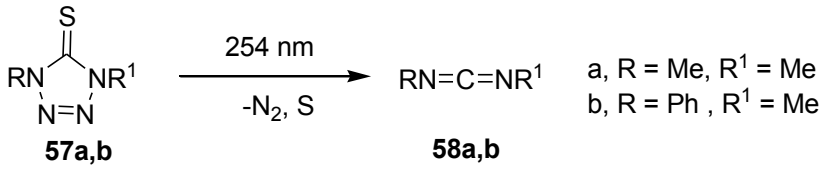


Scheme 1.17. DNA cleavage of enyne-allenes **53** and **55**

1.4.4. 1-(2-ethynylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione based photoactived DNA cleaving drugs

As discussed earlier (Section 1.4.1), the photochemical cyclization of enyne-carbodiimides is highly efficient producing the reactive biradicals that leads to formation of photoproducts in high yields. Therefore, we were interested in exploring whether these compounds could be employed as DNA photo cleaving agents. The intermediacy of a biradical **2′** in the photocyclization of enyne-carbodiimides **2** above (Scheme 1.16) and its high efficiency suggests the possibility to exploit this reaction for DNA photo cleavage and thus, mimicking the naturally occurring antitumor antibiotics enediynes in their mechanism of action. The enyne-carbodiimides offer advantage over enediynes in terms of structural simplicity and easier synthesis. However, these also present problems that must be circumvented for their application in biology: Firstly, carbodiimide is a highly reactive functionality and thus, expected to be extremely susceptible to nucleophilic attack *in vivo* where water is abundantly present. Secondly, in order to make the DNA photocleavage by enyne-carbodiimides selective with fewer side effects, one needs to think of ways to control the formation of biradicals in cells (especially, because of thermal cyclization of enyne-carbodiimides (Scheme 1.14) that can cause undesired side effects). One way to achieve this would be to mask the carbodiimide moiety with a photocleavable group. This masking group would not only protect the enyne-carbodiimide from biological nucleophiles on the way to its target, but it would also enable the generation of biradicals at a desired location and time with the help of light as an external trigger, which would ultimately lead to enhanced selectivity and reduced toxicity.

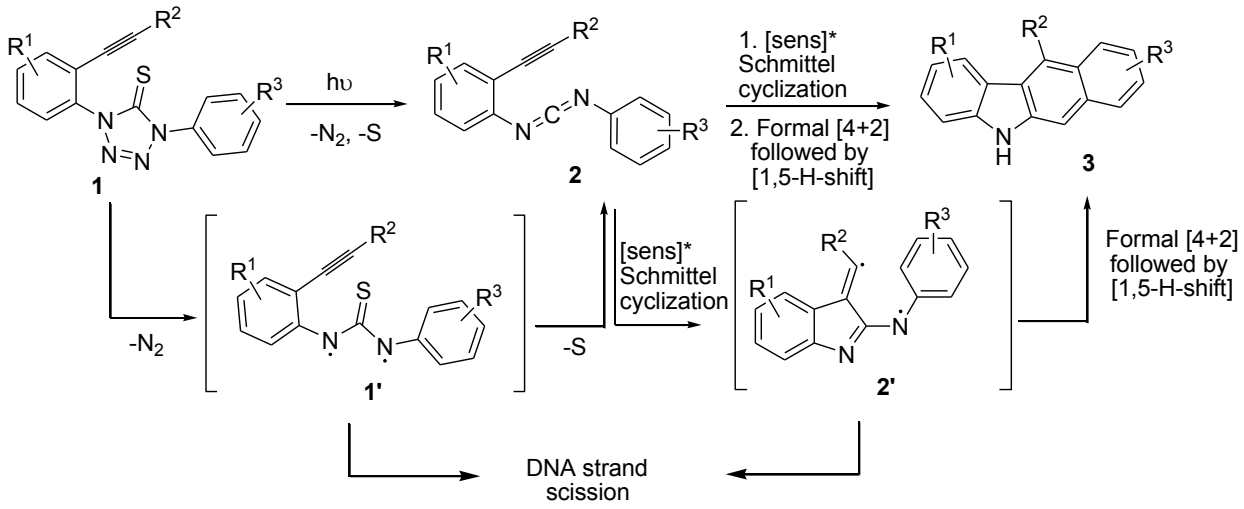
To our delight, Quast had reported the formation of carbodiimides **58** in the photodecomposition of tetrazole-5-thiones **57**.[46] Studies in our laboratory have revealed that this photochemistry involves the formation of biradicals (Scheme 1.18).



57a,b **58a,b**

Scheme 1.18. Photodecomposition of 1,4 substituted tetrazole-5-thiones

Therefore, we decided to use a tetrazolethione ring system to design masked enyne-carbodiimide prodrugs, such as the 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1**). We hypothesized that upon photoexcitation, **1** would generate the enyne-carbodiimide **2** via **1′**. The formation of **2** would be spontaneously followed by cyclization to indoloquinolines **3** via the biradical **2′**. Both **1′** and **2′** would have the ability to abstract the hydrogen atoms from the sugar phosphate backbone of the DNA leading to strand scission (Scheme 1.19). Therefore, the irradiation of **1** would bombard the cells with highly reactive and cytotoxic species, the biradicals **1′** and **2′** at a targeted location leading to cell death.



Scheme 1.19. Proposed DNA cleavage mechanism of 1-(2-ethynylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (**1**).

In order to accomplish our goal, following questions needed to be addressed:

- **How easy it is to synthesize the 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (1)?**

The proposed prodrug **1** contained a 1,4-diaryl tetrazolethione functionality and a direct synthetic route for the construction of these ring systems was lacking in the literature despite their wide spread applications. Therefore, our initial efforts were directed towards developing a general strategy to obtain 1,4-diaryl tetrazolethiones. We have efficiently utilized copper catalyzed *N*-arylation to synthesize 1,4-diaryl tetrazole-5-ones which underwent thionation with Lawesson's reagent to yield the 1,4-diaryl tetrazole-5-thiones. The synthesis of **1** involved Sonogashira coupling of the 1-(2-bromophenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione so obtained with the appropriate ethynyl source. These synthetic efforts are discussed in detail in Chapter 2.

- **Can 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thiones (1) induce antitumor activity in the absence of light?**

Since the tetrazole ring is an important framework in a number of medicinally relevant compounds,[47-51] we were interested in evaluating the antitumor activity of synthesized tetrazole-5-ones and tetrazole-5-thiones in the absence of photochemical activation. The moderate IC_{50} values obtained against fast and slow growing leukemia and breast cancer cell lines, respectively showed that the anticancer activity of these compounds before photoirradiation is minimal. The results obtained and the mechanism of their action is explained in Chapter 3.

- **Can prodrug 1 undergo two sequential photochemical activations generating biradicals 1′ and 2′ in one pot with the help of a single light source?**

Independent studies have shown that the photodecomposition of tetrazolethiones gives carbodiimides *via* biradicals (Scheme 1.18),[46, 52] and photocyclization of enyne-carbodiimide forms indoloquinoline also *via* biradicals (Scheme 1.16).[41] However, it was not known whether these two photoreactions could happen sequentially in one pot as is proposed in Scheme 1.18. Therefore, in Chapter 4, we studied the photochemical decomposition of 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione **1** at 300 nm which showed clean conversion to enyne-carbodiimide **2** and indoloquinoline **3 (**Chapter 4**)**.

- **Can 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thiones (1) induce photochemical DNA cleavage?**

Finally, we wanted to test if proposed prodrugs **1** have the ability to cause DNA strand scission upon light activation. Our experiments show that the irradiation of the samples at

>300nm containing 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1**) with circular supercoiled DNA in 20% acetonitrile: 80% water cause significant DNA damage at concentrations as low as 100 μM. The results are disclosed in Chapter 5.

1.5. References

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Chapter 2 - Synthesis of a series of 1,4-diaryl substituted tetrazol-5-ones and tetrazole-5-thiones

2.1. Introduction

Tetrazole and its derivatives have attracted interest in various fields due to their applications in major areas such as medicine,[1-5] agriculture,[6-8] and photoimaging technology[9] etc. The tetrazole ring systems are present in drugs patented for the treatment of central nervous system disorders,[1] HIV,[10] sexual dysfunction,[11] asthma,[2] obesity[3] and diabetes,[5] antihypertensive,[12] antiallergic,[13] antibiotics[14] and anticonvulsants.[15] The oxo and thio substituted tetrazole derivatives are excellent non-classical carboxylic acid bioisosteres in biological molecules.[16] Tetrazole scaffolds containing the 5-oxo substituent are used as potent and selective agonists for β3 human adrenergic receptors (HAR), which are useful in the treatment of obesity.[17-19] (Refer to section 3.2 in chapter 3 for a detailed discussion of the biological applications).

Tetrazole derivatives also find applications in agriculture as plant growth regulators, herbicides[6] and fungicides.[8] In addition, these are used as stabilizers in photography and photoimaging.[9]A tetrazole molecule loses dinitrogen upon decomposition and releases a high amount of energy because of their high enthalpy of formation and therefore, these scaffolds have been explored as explosives, propellents for missiles and gas generators in air bags.[20]

Supramolecular chemists have been interested in utilizing the coordination ability of the electron donating nitrogen atoms present on the tetrazole ring to build supramolecular

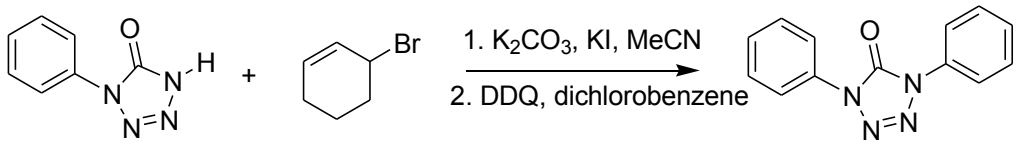
assemblies.[21] Studies have shown that tetrazole ligand can bind to seven different coordination modes with a variety of metal ions such as nickel, ruthenium, palladium etc, leading to the construction of novel metal-organic frameworks such as bis(carbodiimido)complexes,[22] which is a novel tetrazole based ligand for heck reaction.[22-24] Recently, novel benzoisothiazole-tetrazole derivatives have also been developed that can coordinate with manganese to form benzoisothiazole-tetrazole manganese complexes; these are under investigation for their potential catalytic applications.[21]

The electron-withdrawing properties of tetrazolyl halide derivatives have been efficiently utilized for heterogeneous catalytic transfer reductions of benzylic and allylic alcohols.[25-27] The tetrazole derivitizes these alcohols to form allyloxy[26] or benzyloxy tetrazole, that undergo chemical modification by hydrogenolysis,[27, 28] reduction[26, 29] etc to form alkanes or alkenes.

In addition, tetrazoles are interesting molecules because they exhibit tautomerism[30] and shows rich photochemistry.[31, 32] Photochemistry of these rings is discussed in detail inChapter 4. A review on the role of the photochemistry of tetrazoles in organic synthesis has recently been published.[33]

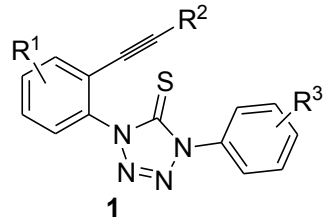
Because of the aforementioned applications, new and versatile methods that can provide quick access to a tetrazole scaffold are highly desirable. There are several reports in the literature that discuss the preparation of 1,4-phenyl alkyl substituted tetrazol-5-ones,[34-37] but the synthesis of the corresponding 1,4-diaryl derivatives has not been directly carried out . For instance, there is a method reported by Quast and Nahr in which the *N*-alkylation of 1-phenyl-1*H*-tetrazol-5-(4*H*)-one with 3-bromocyclohexene was carried out; this was followed by dehydrogenation of the resulting product with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone to yield 1,4-diaryl

tetrazol-5-one (Scheme 2.1).[38] However, there are no reports on the synthesis of 1,4-diaryl tetrazole-5-thiones.



Scheme 2.1. Synthesis of 1,4-diaryl tetrazol-5-ones-Literature methods[38]

. We are interested in the synthesis of 1,4-diaryl tetrazole-5-thiones because this unit forms an important structural framework of our proposed prodrug 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1)** as reported in Chapter 1 (Section 1.4.4.) and also shown below (Scheme 2.2)



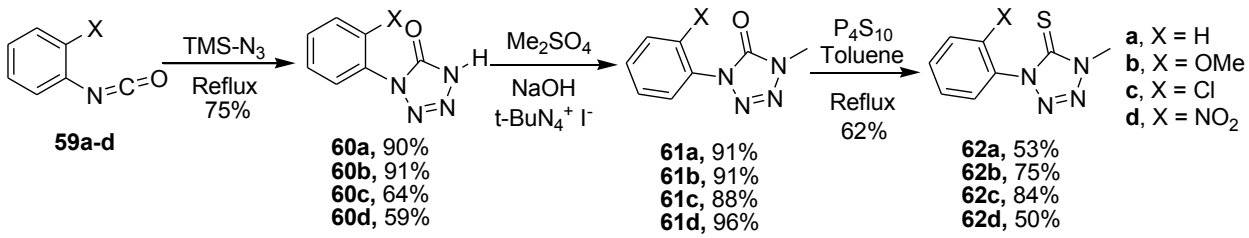
1

Scheme 2.2. Proposed prodrug 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1)**

2.2. Previous work in Rayat's lab [Chabbra, R.; MS Thesis]

Our lab has carried out synthesis of a series of 1-phenyl-4-methyl tetrazole-5-thiones **7** bearing different substituents,[39] by 1,3-dipolar cycloaddition of phenyl isocyanate **59a-d** with trimethyl silyl azide to form the tetrazole ring **60a-d**. *N*-Methylation reaction of **60a-d** with dimethyl sulfate in the presence of 20% NaOH and a phase transfer catalyst afforded 1-phenyl-4-

methyl tetrazole-5-one derivatives **61a-d**. Thionation of the latter in the presence of phosphoruspentasulfide furnished the 1-phenyl-4-methyl tetrazole-5-thiones **62a-d** in moderate to good yields (Scheme 2.3)



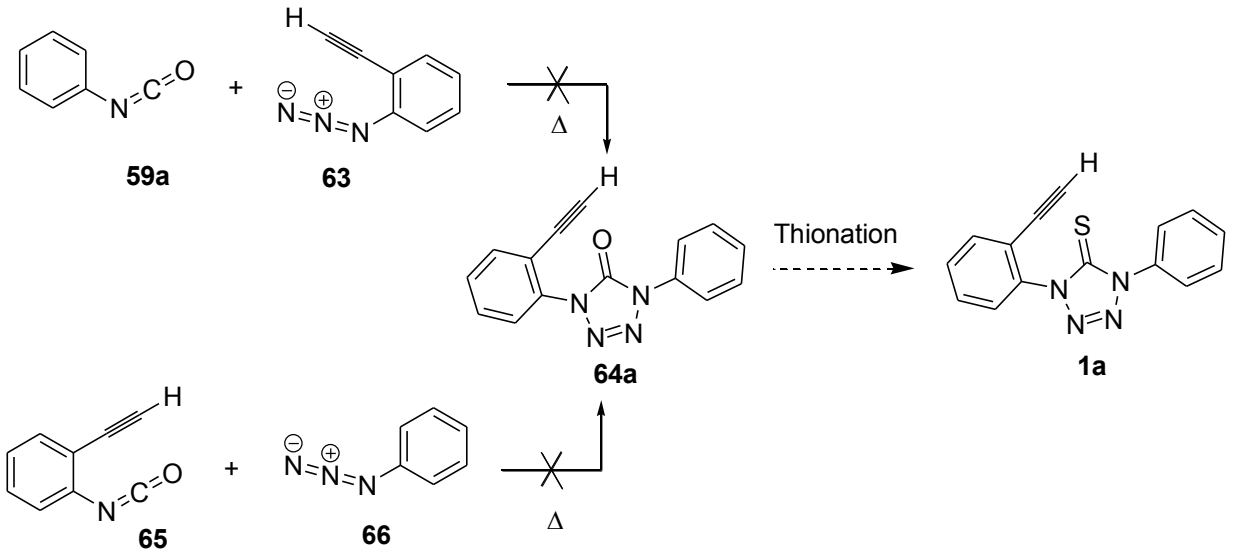
Scheme 2.3. Synthesis of 1-phenyl-4-methyl tetrazole-5-thiones **62a-d**

2.3. Results and discussion

2.3.1. Attempts toward the synthesis of 1-(2-ethynylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thiones 1

2.3.1.1. Direct 1,3-dipolar cycloaddition of the precursors

Since we successfully constructed 1-phenyl-1*H*-tetrazol-5(4*H*)-one **1a – d** by 1,3-dipolar cycloaddition of the phenyl isocyanate **59a-d** with trimethylsilyl azide (Scheme 2.3), we decided to attempt the synthesis of the compound 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazol-5(4*H*)-one (**64a)** by the 1,3-dipolar cycloaddition reaction of 1-azido-2-ethynylbenzene **(63)** and phenyl isocyanate (**59a**). **63** was synthesized by diazotization of 2-ethynylbenzenamine in the presence of sodium azide and the product obtained was used without purification (Scheme 2.4). We anticipated that the thionation of **64a** would yield the desired prodrug derivative **1a**. Unfortunately, no product was observed during the 1,3-dipolar cycloaddition reaction of **59a** and **63** and the starting materials remained unreacted after several hours of the reaction.



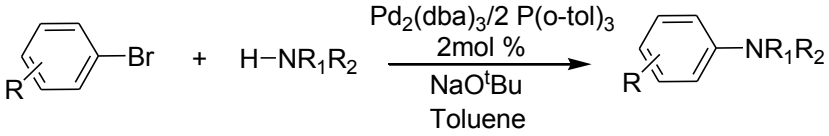
Scheme 2.4. Synthesis of **64a** by dipolar cycloaddition (Dashed arrows indicate planned synthesis that were not attempted)

We also attempted to obtain **64a** by the 1,3-dipolar cycloaddition of 1-ethynyl-2-isocyanatobenzene (**65**) with the commercially available phenyl azide (**66**). The isocyanate **65** was synthesized by the oxidation of the 2-ethynylbenzenamine using triphosgene. This reaction also failed to yield the diaryl tetrazole-5-one **64a** (Scheme 2.4). We believe that the presence of silicon atom in one of the reactant (such as in the reaction of **59** with $TMSN_3$) is essential for 1,3-dipolar cycloaddition reaction as it temporarily links the two substrates, and reduces the entropy of the reaction and also increases the selectivity towards cycloaddition.[40]

2.3.1.2. Palladium catalyzed N-arylation

The inability to obtain the 1,4-diaryl tetrazole-5-thione derivatives using 1,3-dipolar cycloaddition reaction prompted us to search for other alternative methods to construct this ring system. Since we have successfully synthesized 1-phenyl-4-methyl tetrazole-5-ones **60a – d** by *N*-methylation of **60** with dimethyl sulfate (Scheme 2.3), we considered performing *N*-arylation of **60** with various aryl sources to directly attach the phenyl ring to the tetrazole-5-ones. The thionation of the corresponding 1,4-diaryltetrazolethione would provide the desired compound **1**.

Transition metal catalyzed *N*-arylation has emerged as powerful strategy in the recent times due to utility in the synthesis of a wide range of complex heterocycles with application in a variety of fields.[41] The discovery of palladium based coupling of aryl halides with amines by Buckwald[42, 43] and Hartwig[44] was a major breakthrough in this field. The catalyst generated by mixing $Pd_2(dba)_3$ and two equivalents $(o\text{-tol})_3P$ has been shown to achieve the C-N bond formation with comparable efficiency. NaOt-Bu was used as base for this transformation (Scheme 2.5).[42]

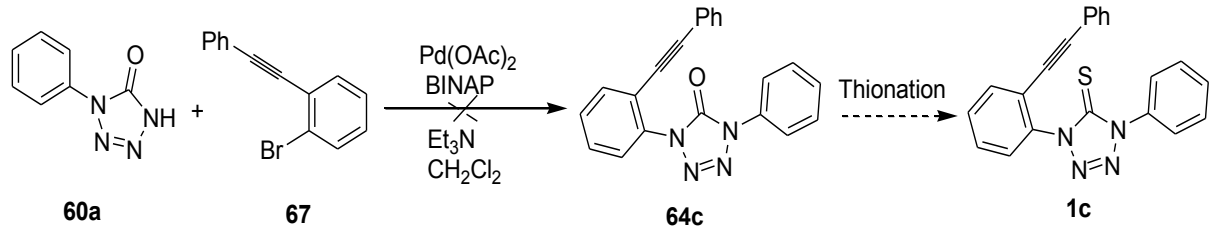


Scheme 2.5. C-N bond formation; Buchwald and Hartwig method[42]

A number of improvements have been provided over a period of time for this method.[41] For instance, Hartwig and Louie have reported that LiHDMS is a better base to induce such transformations.[45] Moreover, $Pd(OAc)_2$ is proven to be a better catalyst than $Pd(dba)_3$ for secondary amines.[41] The use of $Pd(OAc)_2$ was also advantageous when the reactions are carried

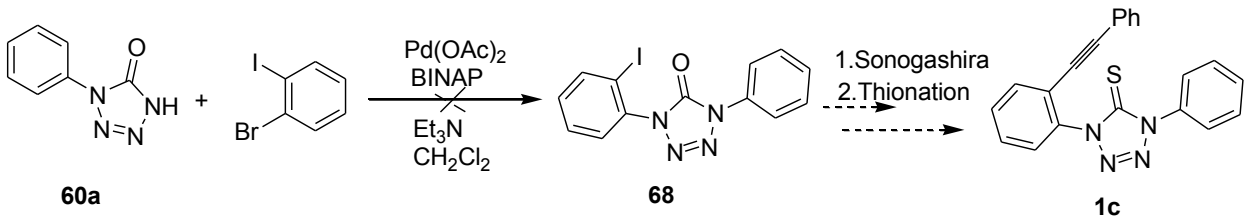
out in large scale because of the ease of handling.[43] BINAP was documented to be the best ligand.[41] This method was successfully applied for the coupling of a variety of substrates such as amines,[46] amides, indoles[47] etc with an aryl moeity. Pd catalysed *N*-arylation is used in the key step during the synthesis of many drugs, *e.g.*oxcarbazepine which is an anticonvulsant used for the treatment of epilepsy.[41]

Because of the enormous potential of the Pd catalyzed *N*-arylations in organic synthesis, we decided to employ this protocol to synthesize tetrazole-5-one **64c** through a coupling reaction between 1-phenyl-1*H*-tetrazol-5(4*H*)-one (**60a**) with the corresponding ethynyl substituted aryl halides **67** using BINAP as the ligand (Scheme 2.6). Thionation of **64c** would yield 1-phenyl-4-(2-(2-phenylethynyl)phenyl)-1*H*-tetrazole-5(4*H*)-thione (**1c**). Unfortunately, no product was observed during the reaction of **60a** with **67a**, and the starting materials remained unreacted after several hours of the reaction.



Scheme 2.6. Palladium catalyzed *N*-arylation reaction (Dashed arrows indicate planned synthesis that were not attempted)

We assumed that the presence of acetylene group *ortho* to the bromine in **67** may sterically hinder the attachment to the tetrazole ring **60a**. Alternatively, we tried to synthesize 1-(2-iodophenyl)-4-phenyl-1*H*-tetrazol-5(4*H*)-one (**68**) by *N*-arylation of **60a** with 1-bromo-2-iodobenzene but in vain (Scheme 2.7).



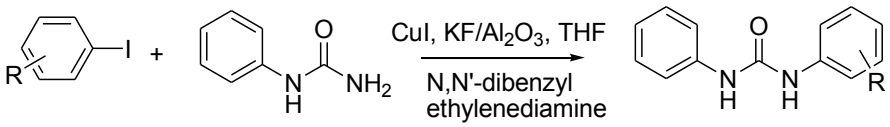
Scheme 2.7. Palladium catalyzed *N*-arylation reaction (Dashed arrows indicate planned synthesis that were not attempted)

2.3.2. Copper mediated N-arylation

Failure to obtain the desired 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1**) by the palladium catalyzed reaction diverted our attention to investigate other transition metals for *N*-arylation. In the recent years, copper mediated C (aryl)-N bond formation[48] is being successfully employed for the *N*-arylation of wide range of functional groups such as amines, amides,[47] indoles and sulfonamides.[49] The advantage of copper is that it is less toxic and cheaper than palladium. However, its application has been limited due to the requirement of harsh conditions and the reactions produce moderate yields.[41] To overcome these problems, highly efficient polydentate Schiff base and oxime type ligands have been developed that enables the reaction to be done under milder conditions (90-100^{o}C) and good yields.[50, 51]

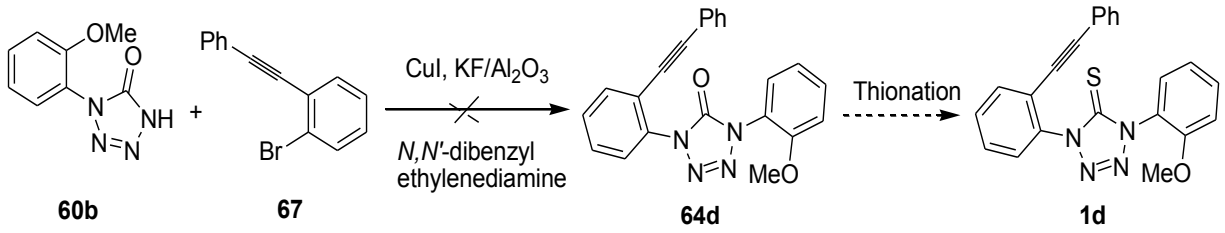
2.3.2.1. Cu mediated N-arylation with aryl halides

Copper iodide catalyzed C-N arylation has been successfully utilized to couple aryl halides with phenyl ureas for the synthesis of *N, N′*-diarylureas.[52] For instance, N-arylation reaction of iodobenzene and phenyl urea in the presence of KF/Al_2O_3 afforded N, N′-diaryl urea in 83% yield (Scheme 2.8). *N, N′*-dibenzylethylenediamine was employed as a ligand in this reaction.



Scheme 2.8. Copper catalyzed arylation of phenyl ureas[52]

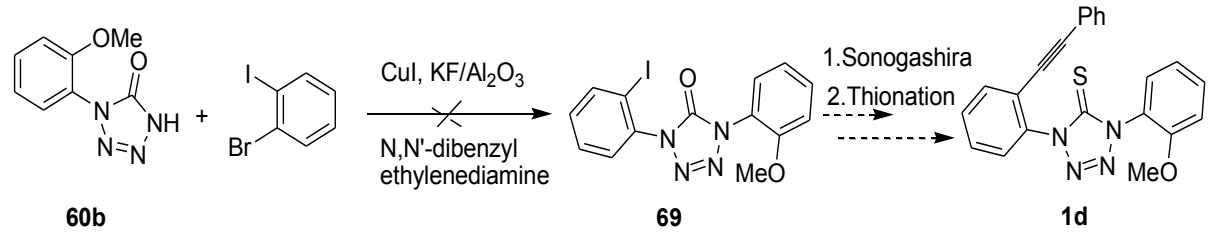
We tried to synthesize 1-(2-methoxyphenyl)-4-(2-(2-phenylethynyl)phenyl)-1*H*-tetrazol-5(4*H*)-one (64d) using the above method by reacting 1-bromo-2-(2-phenylethynyl)benzene (67) with 1-(2-methoxyphenyl)-1*H*-tetrazol-5(4*H*)-one (60b) in the presence of copper iodide, KF/Alumina and indicated ligand. We hoped that the thionation of 64d would yield a derivative of the desired prodrug, the 1-(2-methoxyphenyl)-4-(2-(2-phenylethynyl)phenyl)-1*H*-tetrazole-5(4*H*)-thione (1d). However, we were not successful and the starting materials remained unreacted after several hours (Scheme 2.9).



60b **67** **64d** **1d**

Scheme 2.9. Copper (I) Iodide catalyzed coupling with aryl halides (Dashed arrows indicate planned synthesis that were not attempted)

We hypothesized that the presence of a bulky ethynyl group *ortho* to the bromine in **67** may sterically hinder the coupling reaction. Therefore, we tried to couple tetrazolone **60b** with 1-bromo-2-iodobenzene. We anticipated that the Sonogashira coupling of 1-(2-iodophenyl)-4-(2-methoxyphenyl)-1*H*-tetrazol-5(4*H*)-one (**69**) with phenylacetylene followed by thionation would yield **1d**. However, the *N*-arylation was unsuccessful and the starting materials remained unreacted (Scheme 2.10).



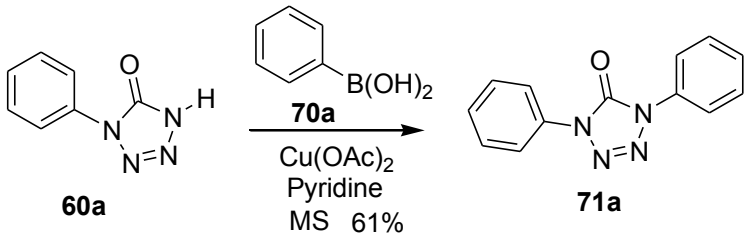
60b **69** **1d**

Scheme 2.10. Copper (I) Iodide catalyzed coupling with aryl halides (Dashed arrows indicate planned synthesis that were not attempted)

2.3.2.2. Copper catalyzed N-arylation with phenyl boronic acids

Chan and Lam[53] have introduced copper mediated *N*-arylation of pyridines, amines and sulfonamides using stoichiometric amounts of copper (II) acetate and aryl boronic acids. The advantages of boronic acids are that these do not require the use of ligands which were often air sensitive.[41] Moreover, the reactions usually proceed at room temperatures. However, the reactions with boronic acids are usually very slow and require several hours or days for completion.[41] This method has been successfully applied for the coupling of wide range of functional groups. These advantages drove us to investigate this reaction for the synthesis of desired 1-(2-ethynylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thiones **1**.

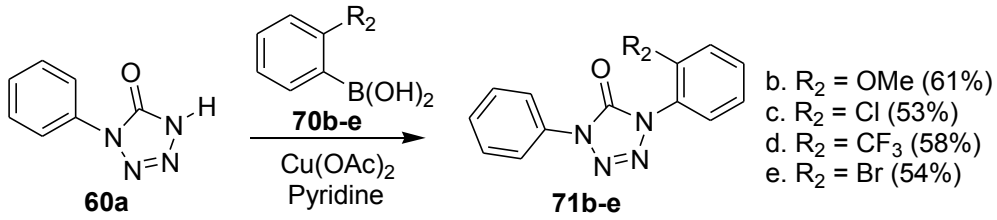
To our delight, the coupling of 1-phenyl-5*H*-tetrazol-5(4*H*)-one (**60a)** with phenyl boronic acid (**70a)** produced 1,4-diphenyl tetrazol-5-one (**71a)** in good yields. Pyridine was used as a base in the reaction and 3A^{o} molecular sieves were added to absorb the excess water generated during the course of the reaction (Scheme 2.11). [54]



Scheme 2.11. Synthesis of 1,4-diaryl tetrazol-5-one **71a**

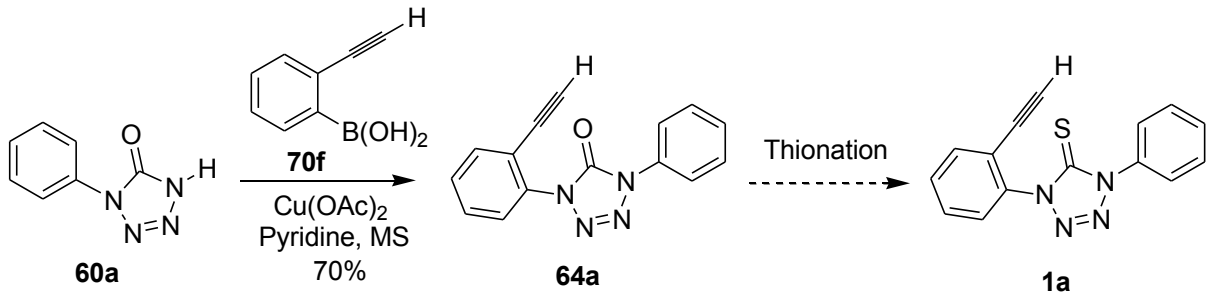
Encouraged by the result, we checked the versatility of this protocol for the *N*-arylation of the various other commercially available series of *ortho* substituted boronic acids **70b-e**. The

reaction was successful and the desired products **71b-e** were isolated in good yields (Scheme 2.12).



Scheme 2.12. Synthesis of 1,4-diaryl tetrazol-5-ones **71b-e**

After successfully *N*-arylating a series of substituted boronic acids, we tested this protocol for the synthesis of the tetrazolone precursor for the proposed compound **1a**. The 2-ethynylphenylboronic acid (**70f**) was prepared from phenyl acetylene by a reported method[55] and was used without further purification due to its comparatively lower yields. Copper catalyzed *N*-arylation of tetrazolone **60a** with boronic acid **70f** successfully yielded the coupled product **64a** (Scheme 2.13). Thionation of the latter to form **1a** is dicussed in Section 2.3.5.

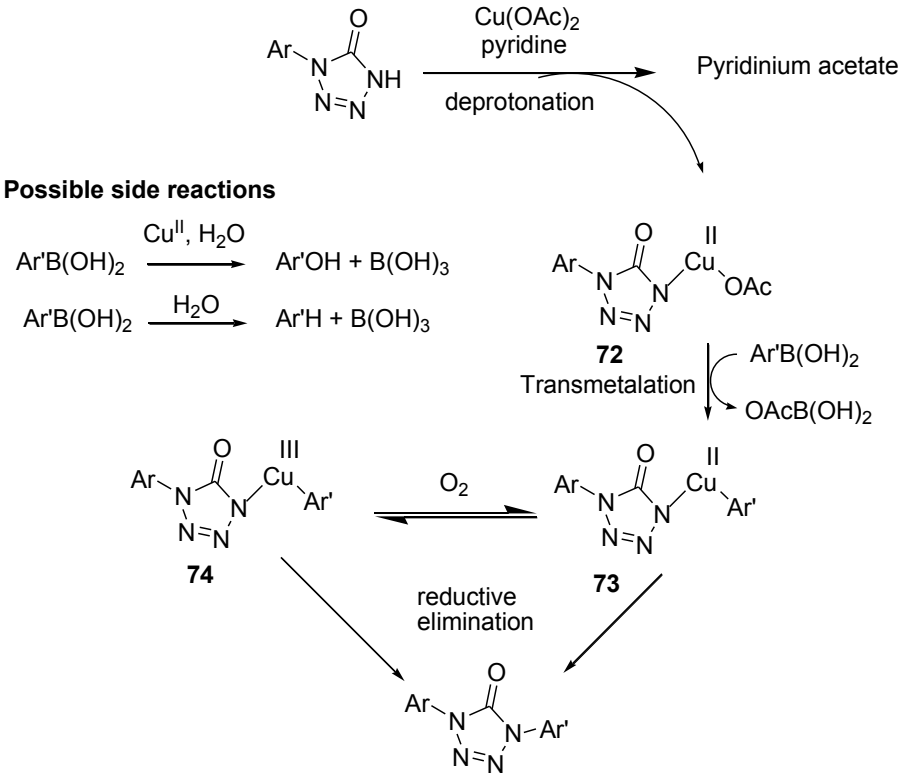


Scheme 2.13. Synthesis of 1-(2-ethynylphenyl)-4-phenyl-1H-tetrazol-5(4H)-one **64a**

(Dashed arrows indicate planned synthesis that were not attempted)[55]

2.3.2.3. Mechanism of Copper catalyzed N-arylation with aryl boronic acids

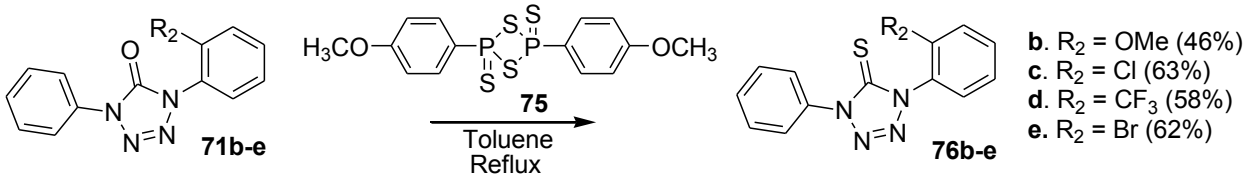
The plausible mechanism for the *N*-arylation reaction is shown[54] in Scheme 2.14. Pyridine assists in the formation of the copper complex **72**. The latter undergoes transmetallation with arylboronic acid to yield Cu (II) complex **73**. The reaction is carried out in the presence of air that oxidizes copper (II) complex **73** to Cu (III) complex **74** which undergoes reductive elimination faster. The excess water formed during the course of the reaction may result in possible side reactions with the arylboronic acids. For this reason, the arylboronic acid is added in excess to the reaction mixture. Molecular sieves are also added to the reaction mixture to absorb the water (Scheme 2.14).[54]



Scheme 2.14. Mechanism of *N*-arylation with phenyl boronsic acids[54]

2.3.3. Synthesis of 1,4-diaryl-1H-tetrazole-5(4H)-thiones 76b - e

Next, we wanted to determine if the thionation reaction could be efficiently carried out on 1,4-diaryl tetrazole-5-ones **71a-e** analogous to 1-methyl-4-phenyl tetrazole-5-ones **61** (Scheme 2.3). Numerous reagents are available in the literature for the thionation of a ketone to thioketone. Among them, phosphorus pentasulfide and Lawesson's reagent **75**[56] are the most commonly used due to their versatility and ability to tolerate different functional groups. We subjected **71a-e** to thionation with Lawesson's reagent. The tetrazol-5-ones **71b-e** successfully underwent thionation to yield the thionated products **76b-e** respectively (Scheme 2.15). However, thionation of **71a** did not yield any product whereas thionation of **64a** produced a complex mixture of products. Our efforts to synthesize the desired thionated products **76a** and **1a** were outlined below.

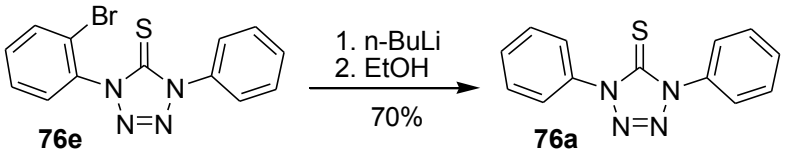


Scheme 2.15. Thionation of tetrazol-5-ones **71b-e** to tetrazole-5-thiones **76b-e**

2.3.3.1. Synthesis of 1,4-diphenyl-1H-tetrazole-5(4H)-thione (71a)

The thionation of **71a** to **76a** with Lawesson's reagent failed to yield the desired product. Several other thionation agents were tried to induce this transformation, such as P_4S_{10}/Al_2O_3,[57] $PSCl_3/H_2O/Et_3N$[58] at 63°C, and $PSCl_3/H_2O/Et_3N$[59] under microwave conditions, all of them in vain. The starting material remained unreacted after several hours of reaction. Since all our

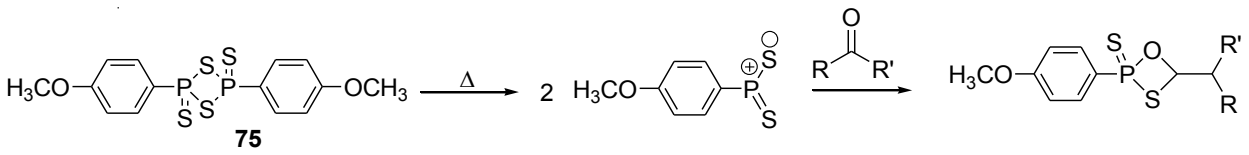
methods to directly thionate **71a** failed, we looked at alternate ways to obtain **76a**. The debromination of aromatic compounds in the presence of organolithium reagents is a well known reaction. To our delight, the debromination of 1-(2-bromophenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**76e)** with *n*-butyl lithum followed by protonation worked well to give **76a** in good yields (Scheme 2.16). The success of this reaction also confirms the stability of tetrazole-5-thione ring to withstand strong nucleophilic conditions.



Scheme 2.16. Synthesis of tetrazole-5-thione **76a** from **76e**

2.3.3.2. Why thionation reaction failed: Proposed hypothesis

The lack of reactivity of **71a** toward Lawesson's reagent may be attributed to the following reason: the mechanism of thionation is believed to involve a dissociative equilibrium involving the formation of a highly reactive dithiophosphine ylide that reacts with the carbonyl compounds via the formation of the Wittig-type intermediate (Scheme 2.17).[48, 56, 60]

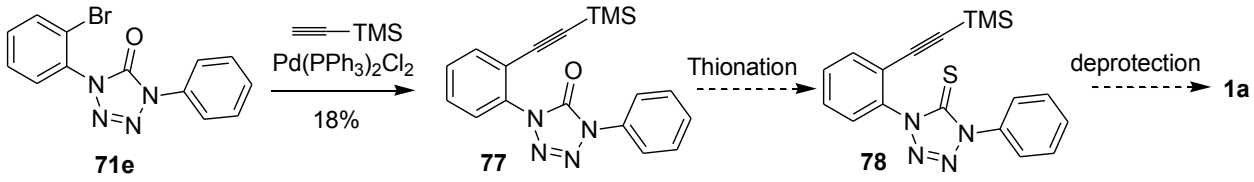


Scheme 2.17. Mechanism of Lawesson's reagent: Formation of Wittig reagent[56, 60]

We believe that the lone pairs present on the ortho substitutents in **71b-e** may provide the coordination site for electrophilic phosphorus of the ylide and thus, facilitate the attack at nearby carbonyl group.

2.3.5. Synthesis of the target compound 1-(2-ethynylphenyl)-4-phenyl tetrazole-5-thione (1a): Revised approach

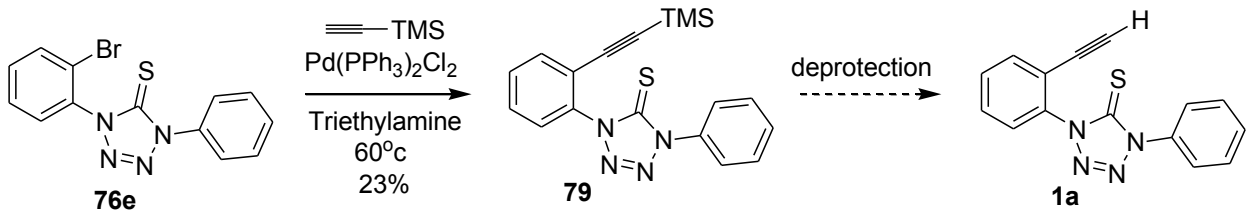
Since we were unsuccessful in obtaining the desired product **1a** by thionation of **64a**, we attempted to synthesize **1a** through the Sonogashira coupling of **71e** with ethynyltrimethylsilane[61-63] to form **77** followed by thionation and subsequent removal of TMS group. Unfortunately, the Sonogashira coupling reaction did not undergo completion in 48 h and resulted in an extremely low yield of **77** (Scheme 2.18).



Scheme 2.18. Synthesis of **1a**: Sonogashira coupling of **71e** (Dashed arrows indicate planned syntheses that were not attempted)

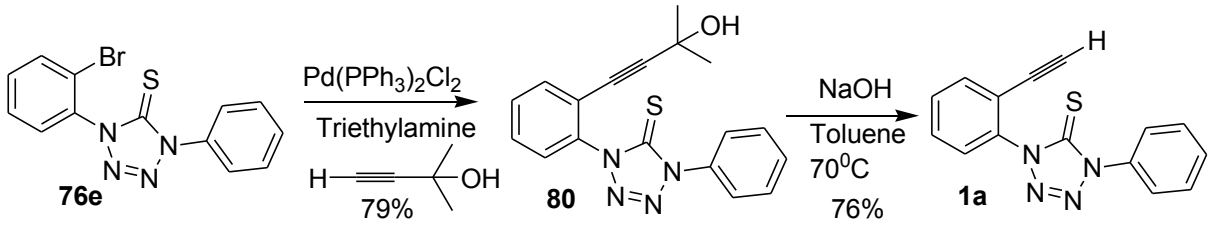
Lack of success in the above reaction prompted us to consider the Sonogashira coupling of **76e** with ethynyltrimethylsilane to obtain **79** followed by thionation to afford **1a**. However, the coupling reaction did not go to completion as more than 50% of **76e** remained unreacted after 60 h.

Moreover, the reaction produced two products that eluted closely with unreacted **76e** on silica gel column and thus, were inseparable and could not be identified (Scheme 2.19).



Scheme 2.19. Synthesis of **1a**: Sonogashira coupling of **76e** (Dashed arrows indicate planned synthesis that were not attempted)

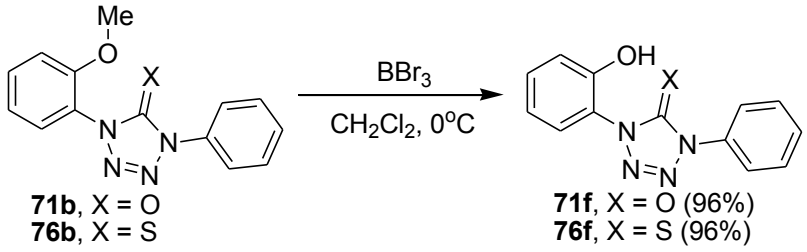
Since, one of the problems associated with Sonogashira coupling of ethynyltrimethylsilane with **76e** was the close elution of reactant and the products; we searched for other reagents to introduce the ethynyl group into organic structures via Sonogashira coupling. We came across report by Sabourin and co-workers in which 3-methyl-2-butynol was used as a useful and inexpensive alternative to ethynyltrimethylsilane in palladium catalyzed cross coupling reaction with aryl halides.[64, 65] We expected that the presence of an hydroxyl group in the ethynyl source would increase the polarity of the resulting Sonogashira product thereby enabling better separation. To our delight, the Sonogashira coupling of 1-(2-bromophenyl)-4-phenyl tetrazole-5-thione (**76e**) with 3-methyl-2-butynol yielded **80** which upon treatment with sodium hydroxide gave a derivative of the desired prodrug, the 1-(2-ethynylphenyl)-4-phenyl tetrazole-5-thione (**1a**) in good yields (Scheme 2.20).



Scheme 2.20. Synthesis of target compound **1a**: Sonogashira coupling of **76e**

2.3.6. Synthesis of –OH substituted derivatives 71f and 76f

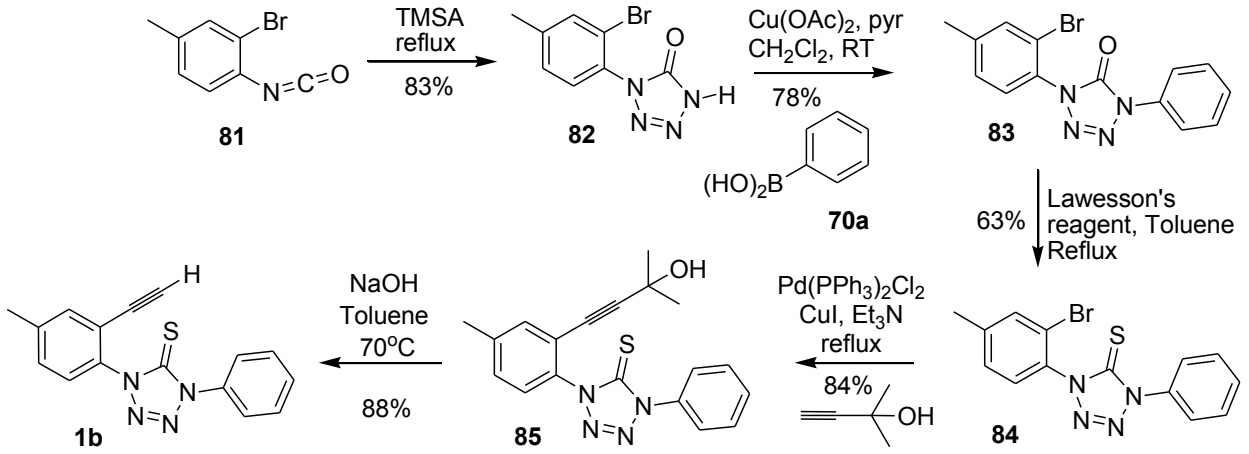
In our quest to explore the stability of the tetrazolone and tetrazolethione scaffolds 71a-e and 76a-e, we exposed these compounds to various reaction conditions such as organolithiation, Sonogashira coupling, strong basic conditions etc. We also wished to investigate the stability of the ring in strong Lewis acidic conditions. To explore this, we subjected the methoxy substituted derivatives **71b** and **76b** to treatment with boron tribromide to produce 1-(2-hydroxyphenyl)-4-phenyl tetrazol-5-one **71f** and its thio derivative **76f**, respectively[66] (Scheme 2.21). This reaction thus demonstrates the ability of tetrazol-5-one and tetrazole-5-thiones to withstand strong Lewis acid conditions.



Scheme 2.21. Acid catalyzed conversion of **71b** and **76b** to **71f** and **76f** respectively

2.3.7. Synthesis of 1-(2-ethynyl-4-methylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (1b)

After streamlining a general route for the synthesis of 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1a**), we used this protocol for the construction of another derivative of the proposed prodrug, the 1-(2-ethynyl-4-methylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1b**). 1,3-dipolar cycloaddition of 2-bromo-1-isocyanato-4-methylbenzene (**81**) with trimethylsilyl azide gave 1-(2-bromo-4-methylphenyl)-1*H*-tetrazol-5(4*H*)-one (**82**). Copper mediated *N*-arylation reaction of the latter with phenyl boronic acid **(70a)** resulted in the biphenyl tetrazol-5-one derivative **83**. Treatment of the latter with Lawesson's reagent yielded 1-(2-bromo-4-methylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione **(84)**. The thione was subjected to Sonogashira coupling with 3-methyl-2-butynol to yield **85** which upon treatment with sodium hydroxide in toluene gave the desired 1-(2-ethynyl-4-methylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione **(1b)** (Scheme 2.22). The synthesized compounds were characterized by IR and NMR spectroscopy and high resolution mass spectrometry.



Scheme 2.22. Synthesis of 1-(2-ethynyl-4-methylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione **(1b)**

2.4. Conclusion

A facile synthesis of the target molecules 1-(2-ethynylphenyl)-4-phenyl tetrazole-5-thione **(1a)** and 1-(2-ethynyl-4-methylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione **(1b)** were carried out in moderate to good yields. In addition, we devised general method for the synthesis of 5-oxo and 5-thio derivatives of 1,4-diaryl tetrazolones and their *thio* derivatives. The compound 1-(2-bromophenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione **(76e)** thus obtained is an excellent precursor to introduce other type of substituents on tetrazole-5-thione through metalation followed by electrophilic quenching, or *via* palladium catalyzed cross coupling reactions. The above reactions also demonstrate the stability of the tetrazole-5-thione ring to diverse reaction conditions.

2.5. Experimental

2.5.1. General

Thin layer chromatography was carried out on 250 μm silica gel plates and UV-light was used as a visualizing agent. Standard column chromatography was performed using 63–200 μm silica gel. Methylene chloride was dried by distillation over calcium hydride. Dry THF was obtained by distillation over sodium and benzophenone. ^{1}H and ^{13}C NMR spectra were recorded on 400 MHz and 200 MHz spectrometers as indicated. The carrier frequencies were 199.98 MHz (^{1}H) and 50.29 MHz (^{13}C) for the 200 MHz and 399.75 MHz (^{1}H) and 100.53 MHz (^{13}C) for 400 MHz spectrometers, respectively. Number of scans used was 64 for ^{1}H NMR spectra and for ^{13}C, it ranged from 3–5K depending on the sample concentration. Both the ^{1}H and ^{13}C

spectra were recorded with longer relaxation time (10 s). Chemical shifts and the coupling constants are reported in parts per million and Hertz, respectively. The infrared frequencies are reported in cm^{-1}. High resolution mass spectra were acquired on an MDS SCIEX/Applied Biosystems QStar Elite hybrid quadrupole/time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). The samples were prepared in acetonitrile containing 0.1% formic acid and were introduced by continuous infusion into the electrospray ionization (ESI) source at a rate of 30 µL/min. TOF scans were carried out in positive ionization mode. The ion spray voltage was set at 5 kV, the source temperature was 150ºC, the curtain gas was at 25 (arbitrary units), and the ion source gases were set at 20 and 30 (arbitrary units). The declustering potential was set to 80 V, the declustering potential 2 at 15 V, and the focusing potential at 300 V. The collision gas, nitrogen, was set at 3 (arbitrary units). Data were collected over the range of m/z 100-500, 300 cumulative scans over 5 minutes. Data were collected and smoothed using the Analyst QS 2.0 software. In most cases, both $[M+H]^+$ and $[M+Na]^+$ ions were detectable for each species.

2.5.2. Synthesis of 1-Phenyl-1H-tetrazol-5(4H)-one (60a)

A mixture of phenyl isocyanate (1.5 g, 12.6 mmol) and trimethylsilyl azide (TMSA) (2.2 g, 19.0 mmol) was heated to reflux at 100°C for 24 h. After the TLC indicated the completion of the reaction, excess TMSA was removed under reduced pressure. Pure product **60a** was obtained by flash chromatography on silica gel. Yield (1.6 g, 77%) as white solid. R_f (hexane:ethyl acetate 2:3) 0.22; FTIR (neat): 3170, 3077, 2848, 2745, 1704, 1597, 1492, 1462, 1382, 1346, 1293, 1238, 1167, 1148, 1081, 970, 909, 809, 751, 733, 698, 687, 655, 577, 515,

501; ^{1}H NMR (400 MHz, DMSO–d_6) δ 7.42 (t, J = 7.5 Hz, 1H), 7.56 (t, J = 7.6 Hz, 2H), 7.85 (d, J = 7.7, 2H). ^{13}C NMR (400 MHz, DMSO–d_6) δ 119.5, 127.5, 129.4, 134.1, 150.2.

2.5.3. General procedure for the synthesis of 1,4-diaryl tetrazol-5-one derivatives 71a–f

To a stirred suspension of dry copper acetate (0.9 mmol), 1-phenyl-1*H*-tetrazol-5(4*H*)-one **60a** (0.6 mmol), phenyl boronic acid **70** (1.2 mmol) and activated molecular sieves 3A^{o} (0.3 g) in dry methylene chloride (30 mL), was added pyridine (1.2 mmol). The reaction was vigorously stirred at room temperature in air. Upon completion of the reaction, the mixture was diluted with methylene chloride, filtered through celite and washed with aqueous EDTA solution (1.7 mmol). The colorless organic phase was dried over magnesium sulfate and was evaporated to dryness in vacuo. The residue was subjected to flash chromatography.

2.5.3.1. 1,4-diphenyl-1H-tetrazol-5(4H)-one (71a)

Compound **71a** was prepared from **60a** (0.1 g, 0.6 mmol) and phenylboronic acid **70a** (0.15 g, 1.2 mmol) using the general procedure. White solid (0.9 g, 61%), mp 117–118^{o}C. R_f (hexane:ethyl acetate 3:7) 0.79; FTIR (neat): 3068, 3035, 1723, 1592, 1497, 1483, 1464, 1380, 1359, 1292, 1187, 1153, 1113, 1096, 1064, 1025, 960, 906, 835, 749, 723, 685, 660, 623, 503; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39–7.43 (m, 1H), 7.51–7.55 (m, 2H), 7.98–8.01 (m, 2H); ^{13}C NMR (200 MHz, $CDCl_3$) δ 119.9, 128.2, 129.7, 134.7, 148.2; HRMS (ESI) for $C_{13}H_{10}N_4ONa$ $[M+Na]^+$: *m/z* calcd: 261.0747, measured 261.0753.

2.5.3.2. 1-(2-methoxyphenyl)-4-phenyl-1H-tetrazol-5(4H)-one (71b)

Compound **71b** was prepared from **60a** (0.1 g, 0.6 mmol) and 2-methoxyphenylboronic acid **70b** (0.18 g, 1.2 mmol) using the general procedure described above. White solid (0.08 g, 61%), mp 102–103^{o}C. R_f (hexane:ethyl acetate 3:2) 0.79; FTIR (neat): 3015, 2831, 1727, 1593, 1499, 1458, 1385, 1282, 1249, 1111, 1019, 962, 762, 752,744, 730, 723, 685, 663, 621, 501; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.88 (s, 3H). 7.14–7.18 (m, 2H), 7.49–7.61 (m, 5H), 8.08–8.10 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 56.3, 112.7, 119.5, 121.1, 127.8, 128.7, 129.6, 132.1, 135.0, 148.6, 155.2. HRMS (ESI) for $C_{14}H_{12}N_4O_2Na$ $[M+Na]^+$: *m/z* calcd: 291.0852, measured 291.0850.

2.5.3.3. 1-(2-chlorophenyl)-4-phenyl-1H-tetrazol-5(4H)-one (71c)

Compound **71c** was prepared from **60a** (0.1 g, 0.62mmol) and 2-chlorophenylboronic acid **70c** (0.19 g, 1.23mmol) using the general procedure. White solid (0.09 g, 53%), mp 107–108^{o}C. R_f (hexane:ethyl acetate 1:1) 0.44; FTIR (neat): 2962, 2361, 2325, 1730, 1581, 1487, 774, 752, 725, 698, 688, 506; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.58 (m, 8H), 7.62–7.64 (dd, *J* = 1.5, 8.1 Hz, 1H), 8.02–8.05 (m, 2H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 119.6, 128.1, 129.3, 129.7, 130.8, 131.1, 132.0, 132.3, 134.8, 148.0; HRMS (ESI) for $C_{13}H_9ClN_4ONa$ $[M+Na]^+$: *m/z* calcd: 295.0357, measured 295.0362.

2.5.3.4. 1-phenyl-4-(2-(trifluoromethyl)phenyl)-1H-tetrazol-5(4H)-one (71).

Compound **71d** was prepared from **60a** (0.39 g, 2.4 mmol) and 2-(trifluoromethyl)phenylboronic acid **70d** (0.91 g, 4.8 mmol) using the general procedure. Yellow oil (0.4 g, 57.5%). R_f (hexane:ethyl acetate 1:1) 0.66; FTIR (neat): 3081, 1734, 1589, 1501, 1459, 1314, 1178, 1132, 1096, 1065, 1036, 960, 911, 676, 642, 622, 503; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (t, J = 6.8 Hz, 1 H), 7.53-7.62 (m, 3H), 7.72 (dt, J = 7.5 Hz, 9 Hz, 2H), 7.90 (d, J = 5 Hz, 1H), 8.01 (d, J = 7.5 Hz, 2H) . ^{13}C NMR (400 MHz, acetone–d_6) δ 119.5, 122.0, 124.7, 128.0, 128.1, 129.7, 131.0, 131.9, 134.2, 135.0, 148.4. HRMS (ESI) for $C_{14}H_9F_3N_4ONa$ $[M+Na]^+$: *m/z* calcd: 329.0621, measured 329.0611.

2.5.3.5. 1-(2-bromophenyl)-4-phenyl-1H-tetrazol-5(4H)-one (71e)

Compound **71e** was prepared from **60a** (0.2 g, 1.2 mmol) and 2-bromophenylphenylboronic acid **70e** (0.5 g, 2.5 mmol) using the general procedure. White solid (0.3 g, 54%), mp 83–84 ^{o}C. R_f (hexane:ethyl acetate 1:1) 0.84; FTIR (neat): 1731, 1593, 1483, 1368, 1348, 1176, 1098, 1033, 958, 772, 748, 721, 688, 681, 663, 642, 529, 514, 507; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37–7.44 (m, 2H), 7.47–7.54 (m, 4H), 7.76 (dd, J = 1.2, 8.2 Hz, 1H), 8.01 (dd, J = 1.2, 7.8 Hz, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 119.8, 122.1, 128.4, 129.1, 129.9, 130.0, 132.5, 132.6, 134.5, 135.0, 148.2; HRMS (ESI) for $C_{13}H_9N_4BrO$ $[M+H]^+$: *m/z* calcd: 319.0013, measured 319.0019.

2.5.3.6. 1-(2-ethynylphenyl)-4-phenyl-1H-tetrazol-5(4H)-one (64a)

Compound **71f** was prepared from **60a** (0.28 g, 1.7 mmol) and 2-ethynylphenylboronic acid **70f** (0.5 g, 3.4 mmol) using the general procedure. Pale yellow solid (0.22 g, 49%), mp 122–123^{o}C. R_f (hexane:ethyl acetate 1:1) 0.66; FTIR (neat): 3048, 1729, 1593, 1483, 1450, 1380, 1152, 1065, 957, 903, 745, 685, 662, 624, 554, 526, 507; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.27 (s, 1H), 7.40 (t, J = 7.2 Hz, 1H), 7.50–7.57 (m, 5H), 7.72 (d, J = 7.2 Hz, 1H), 8.04 (d, J = 7.6 Hz, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 78.2, 83.6, 119.6, 120.6, 127.1, 128.0, 129.7, 130.1, 130.3, 134.5, 134.9, 148.0; HRMS (ESI) for $C_{15}H_{10}N_4O$ $[M+Na]^+$: *m/z* calcd: 285.0747, measured 285.0739.

2.5.4. General procedure for the synthesis of 1,4-diaryl tetrazol-5-thione derivatives 76b–e

Lawesson's reagent (LR) **75** (5.4 mmol) was added to a solution of **71** (1.8 mmol) in dry toluene and the reaction mixture was refluxed under nitrogen at 110^{o}C for 2 – 3 days. Once the TLC indicated the disappearance of the starting material, the reaction mixture was concentrated under reduced pressure. Purification by flash chromatography on silica gel afforded the desired product **76**.

2.5.4.1. 1-(2-methoxyphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (76b)

Compound **76b** was prepared from **71b** (0.1 g, 0.4 mmol) and LR **75** (0.45 g, 1.14mmol) using the general procedure. Pale yellow solid (0.05 g, 46%), mp 102–103^{o}C. R_f (hexane:ethyl

acetate 1:1) 0.74; FTIR (neat): 2974, 2823, 1787, 1597, 1499, 1381, 1282, 1111, 1012, 763, 752, 744, 730, 723, 685, 501; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.80 (s, 3H, CH_3), 7.14–7.18 (m, 2H), 7.49–7.61 (m, 5H), 8.08–8.10 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 56.2, 112.9, 121.0, 122.8, 123.9, 129.2, 129.3, 129.6, 132.7, 135.1, 155.1, 164.70; HRMS (ESI) for $C_{14}H_{12}N_4OSNa$ $[M+Na]^+$: *m/z* calcd: 307.0624, measured 307.0625.

2.5.4.2. 1-(2-chlorophenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (76c)

Compound **76c** was prepared from **71c** (0.1 g, 0.4 mmol) and LR **75** (0.45 g, 1.1 mmol) using the general procedure. White solid (0.07 g, 63%), mp 132–133 ^{o}C. R_f (hexane:ethyl acetate 4:1) 0.54; FTIR (neat): 2962, 1720, 1593, 1483, 1376, 1302, 1085, 1075, 1066, 1030, 976, 907, 687, 675, 577; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50–7.62 (m, 6H), 7.66–7.68 (m, 1H), 8.07–8.09 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 123.9, 128.1, 129.5, 129.9, 130.1, 131.1, 131.9, 132.6, 132.8, 135.0, 164.5; HRMS (ESI) for $C_{13}H_9ClN_4SNa$ $[M+Na]^+$: *m/z* calcd: 311.0129, measured 311.0130.

2.5.4.3. 1-phenyl-4-(2-(trifluoromethyl)phenyl)-1H-tetrazole-5(4H)-thione (76d)

Compound **76d** was prepared from **71d** (0.1 g, 0.3 mmol) and LR **75** (0.28 g, 0.7 mmol) using the general procedure. White solid (0.06 g, 58%), mp 98–99 ^{o}C. R_f (hexane:ethyl acetate 1:1) 0.63 ; FTIR (neat): 3048, 1941, 1605, 1491, 1458, 1376, 1314, 1302, 1269, 1167, 1128, 1114, 1063, 1030, 1014, 770, 759, 686, 641, 596, 579; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55–7.68 (m, 4H), 7.78–7.83 (m, 2H), 7.93 (d, J = 7.3 Hz, 1H), 8.06–8.07 (m, 2H); ^{13}C NMR (400 MHz,

$CDCl_3$) δ 121.3, 124.0, 128.2, 129.5, 129.9, 131.0, 131.6, 131.8, 133.5, 134.9, 164.2; HRMS (ESI) for $C_{14}H_9F_3N_4SNa$ $[M+H]^+$: *m/z* calcd: 323.0573, measured 323.0585.

2.5.4.4. 1-(2-bromophenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (76e)

Compound **76e** was prepared from **71e** (0.2 g, 0.6 mmol) and LR **75** (0.76 g, 1.88 mmol) using the general procedure. White solid (0.1 g, 52%), mp 123–124 ^oC. R_f (hexane:ethyl acetate 4:1) 0.4; FTIR (neat): 3036, 1593, 1483, 1375, 1317, 1301, 1072, 1063, 756, 736, 710, 644, 578; ^1H NMR (400 MHz, $CDCl_3$) δ 7.48–7.62 (m, 6H), 7.83 (m, 1H), 8.07 (dd, J = 1.2, 7.8 Hz, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 122.4, 124.0, 128.9, 129.5, 129.9, 130.3, 132.8, 133.6, 134.4, 135.0, 164.4; HRMS (ESI) for $C_{13}H_9N_4BrS$ $[M+H]^+$: *m/z* calcd: 332.9804, measured 332.9809.

2.5.5. Synthesis of 1, 4-diphenyl-1H-tetrazole-5(4H)-thione (76a)

76e (0.1 g, 0.3 mmol) was dissolved in dry THF (10mL) under argon atmosphere and the reaction mixture was cooled to -78^{o}C. This was followed by the dropwise addition of *n*-butyl lithium (1.6 M in hexanes, 0.6 mmol). The solution was slowly allowed to warm to room temperature and stirred under argon until the completion of the reaction as indicated by TLC. The reaction was quenched with ethanol (10 mL). The product was extracted using ethyl acetate and the solvent was evaporated in vacuo. Pure **76a** was obtained by flash chromatography on silica gel. White solid (0.05 g, 66%), mp 162–163^{o}C. R_f (hexane:ethyl acetate 4:1) 0.38; FTIR (neat): 3068, 3015, 1728, 1589, 1497, 1368, 1325, 1297, 1285, 1091, 1065, 752, 729, 685, 576; ^1H NMR (200 MHz, $CDCl_3$) δ 7.54–7.66 (m, 3H), 7.95–8.00 (m, 2H). ^{13}C NMR (200 MHz,

$CDCl_3$) δ 124.7, 129.5, 130.1, 139.6; HRMS (ESI) for $C_{13}H_{10}N_4ONa$ $[M+Na]^+$: *m/z* calcd: 255.0699, measured 255.0693.

2.5.6. Synthesis of 1-(2-hydroxyphenyl)-4-phenyl-1H-tetrazole-5(4H)-one (71f) and 1-(2-hydroxyphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (76f)

To a stirred solution of **71b** (0.2 g, 0.75 mmol) in dry CH_2Cl_2 (2 mL) at 0^oC was added BBr_3 (0.2 g, 0.8 mmol) dropwise under argon. The temperature was maintained at 0^oC for about 30 min after which the reaction mixture was slowly allowed to warm up room temperature and stirred overnight. BBr_3 was quenched by the addition of methanol. The reaction mixture was evaporated to dryness and purified by flash chromatography on silica gel. White solid (0.18 g, 96%), mp 130–131^oC. R_f (hexane:ethyl acetate 4:1) 0.12; FTIR (neat): 3256, 1700, 1580, 1496, 1377, 1302, 1191, 841, 750, 686, 665, 620, 532, 504; ^1H NMR (200 MHz, $CDCl_3$) δ 7.05–7.19 (m, 2H), 7.34–7.61 (m, 4H), 7.66–7.70 (dd, J = 0.5 Hz, 8 Hz , 1H), 7.96–8.00 (m, 2H); ^{13}C NMR (200 MHz, $CDCl_3$): δ 120.2, 121.1, 121.6, 123.5, 128.9, 129.8, 130.9, 134.1, 149.2; HRMS (ESI) for $C_{13}H_{10}N_4O_2$ $[M+H]^+$: *m/z* calcd: 255.0877, measured 255.0881.

Similarly, compound **76f** was prepared from compound **76b** (0.1 g, 0.35 mmol). White solid (0.1 g, 96%), mp 177–178 ^oC. R_f (hexane:ethyl acetate 4:1) 0.13; FTIR (neat): 3252, 2962, 2921, 2844, 2365, 2325, 1699, 1601, 1585, 1495, 1377, 1302, 1191, 1072, 753, 716, 688, 678, 666, 513; ^1H NMR (200 MHz, $CDCl_3$) δ 7.14–7.28 (m, 2H), 7.36(s, 1H), 7.49–7.68 (m, 5H), 7.93–7.98 (m, 2H); ^{13}C NMR (200 MHz, $CDCl_3$) δ 122.0, 122.4, 124.5, 126.7, 129.7, 130.5, 131.3, 132.5, 132.3, 150.4, 161.8; HRMS (ESI) for $C_{13}H_{10}N_4OS$ $[M+H]^+$: *m/z* calcd: 271.0648, measured 271.0649.

2.5.7. Preparation of 1-(2-(3-hydroxy-3-methylbut-1-ynyl)phenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (80)

To a stirred solution of **76e** (0.1 g, 0.3 mmol), $Pd(PPh_3)_2Cl_2$ (0.006 g, 0.009 mmol), CuI (0.006 g, 0.03 mmol) in distilled triethylamine (5 mL) under argon at 40°C, was added 3-methyl-2-butynol (0.05 g, 0.6 mmol) dropwise over a period of 10 min. The reaction mixture was heated at 85°C for about 2 days until the starting material was completely consumed. The solution was concentrated in vacuo and reaction mixture was purified by silica gel column chromatography. Pale yellow solid (0.06 g, 79%), mp 111–112°C. R_f (hexane:ethyl acetate 4:1) 0.28; FTIR (neat): 3264, 3232, 2096, 1728, 1589, 1493, 1379, 1366, 1298, 1266, 1064, 976, 759, 711, 685, 638, 577, 555, 532, 515; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 6H), 7.50–7.69 (m, 7H), 7.98–8.03 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) *δ* 65.6, 101.0, 122.4, 124.3, 128.2, 129.5, 129.6, 130.0, 131.1, 133.3, 135.0, 135.6, 164.4; HRMS (ESI) for $C_{18}H_{16}N_4OS$ $[M+Na]^+$: *m/z* calcd: 359.0937, measured 359.0939.

2.5.8. Preparation of 1-(2-ethynylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (1a)

To a solution of compound **80** (0.1 g, 0.3 mmol) in anhydrous toluene (10 mL) was added finely powdered NaOH (0.001 g, 0.035 mmol). The mixture was stirred at 80°C until the reaction was complete and then filtered. The solvent was removed in vacuo and product was purified by flash chromatography on silica gel. Pale yellow solid (0.06 g, 76%), mp 93–95°C. R_f (hexane:ethylacetate 3:7) 0.25; FTIR (neat): 3305, 2974, 1585, 1495, 1446, 1374, 1363, 1302, 1205, 1164, 1144, 1082, 954, 899, 805, 759, 724, 690, 651, 576, 560, 525; **;** ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 3.22 (s, 1H), 7.50–7.62 (m, 6H), 7.74–7.77 (m, 1H), 8.05-8.08 (m, 2H); ^{13}C

NMR (400 MHz, $CDCl_3$) δ 78.3, 84.0, 121.6, 124.0, 128.4, 129.4, 129.8, 130.0, 131.1, 134.4, 135.0, 135.7, 164.4; HRMS (ESI) for $C_{15}H_{10}N_4S$ [M+H]: *m/z* calcd: 271.0648,measured 271.0649.

2.5.9. Preparation of 1-(2-bromo-4-methylphenyl)-1H-tetrazol-5(4H)-one (83)

Compound **83** was prepared from **82** (0.23 g, 0.9 mmol) and phenylboronic acid **70a** (0.22 g, 1.8 mmol) using the general procedure for the synthesis of diaryl boronic acids. White solid (0.26 g, 85%), mp 88-89 ^oC. R_f (hexane:ethyl acetate 1:1) 0.84; FTIR (neat): 2960, 2850, 1715, 1647, 1600, 1581, 1500, 1459, 1391, 1360, 1260, 1188, 1064, 1034, 867, 749; ^1H NMR (400 MHz, $CDCl_3$) δ 2.45 (s, 3H), 7.32 (d, *J* = , 2H), 7.41–7.46 (m, 1H), 7.52-7.56 (m, 1H), 7.62 (s, 1H), 8.02-8.04 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 21.4, 115.4, 119.5, 121.5, 128.1, 129.7, 134.6, 134.8, 143.1, 148.1; HRMS (ESI) for $C_{14}H_{11}N_4BrO$ $[M+H]^+$: *m/z* calcd: 331.0189, measured 331.0194.

2.5.10. Preparation of 1-(2-bromo-4-methylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (84)

Compound **84** was prepared from **83** (0.2g, 0.3 mmol) and Lawesson's reagent (0.73g, 0.6 mmol) using general procedure used for the synthesis of 1,4-diaryl tetrazole-5-thiones **76b-e** (Chapter 1). White solid (0.14 g, 63%), mp 131-132 ^oC. R_f (hexane:ethyl acetate 1:1) 0.4; FTIR (neat): 2909, 2843, 1732, 1588, 1497, 1373, 1300, 1085, 1057, 827, 670; ^1H NMR (400 MHz, $CDCl_3$) δ 2.45 (s, 3H, CH_3), 7.31 (m, 1H), 7.39–7.43 (m, 2H), 7.52-7.56 (m, 2H), 7.62 (S, 1H),

8.02-8.04 (m , 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 21.4, 121.9, 123.9, 125.5, 129.5, 129.6, 129.9, 134.7, 135.0, 143.8, 164.5; HRMS (ESI) for $C_{14}H_{11}BrN_4SNa$ $[M+Na]^+$: *m/z* calcd: 346.9966, measured 346.9950.

2.5.11. Preparation of 1-(2-(3-hydroxy-3-methylbut-1-ynyl)-4-methylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (85)

To a stirred solution of **84** (0.1 g, 0.3 mmol), $Pd(PPh_3)_2Cl_2$ (0.006 g, 0.009 mmol), CuI (0.003 g, 0.01 mmol) in distilled triethylamine (5 mL) under argon at 40^{o}C, was added 3-methyl-2-butynol (0.05 g, 0.6 mmol) dropwise over a period of 10 min. The reaction mixture was heated at 85^{o}C for about 3 days until the starting material was completely consumed. The solution was concentrated in vacuo and reaction mixture was purified by silica gel column chromatography. Pale yellow solid (0.09 g, 85%), mp 119-120 ^{o}C. R_f (hexane:ethyl acetate 3:1) 0.3; FTIR (neat): 3304, 2906, 2850, 2366, 1904, 1511, 1495, 1366, 1320, 1303, 1265, 1109, 1071, 1043, 984, 753; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1,48 (s, 3H), 2.46 (s, 6H), 7.38 (s, *J* = , 2H), 7.56–7.63 (m, 3H), 7.65–7.68 (m, 1H), 7.84 (m , 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 21.6, 31.2, 65.6, 100.9, 124.3, 128.2, 129.5, 130.2, 130.1, 133.2, 135.6, 140.4, 164.4; HRMS (ESI) for $C_{19}H_{18}N_4OS$ $[M+Na]^+$: *m/z* calcd: 351.1279, measured 351.1274.

2.5.12. Preparation of 1-(2-ethynyl-4-methylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (1b)

To a solution of compound **85** (0.05 g, 0.1 mmol) in anhydrous toluene (5 mL) was added finely powdered NaOH (0.001 g, 0.035 mmol). The mixture was stirred at 80^{o}C until the reaction was

complete and then filtered. The solvent was removed in vacuo and product was purified by flash chromatography on silica gel. White solid(0.03 g, 76%), mp 124–125 ^{o}C. R_f (hexane:ethylacetate 3:1) 0.4; FTIR (neat): 3300, 2968, 2917, 2863, 2108, 1596, 1511, 1494, 1282, 1071, 982, 817, 753, 730; **;** ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.46 (s, 3H), 3.19 (s, 1H), 7.40 (m, 1H), 7.49-7.69 (m, 5H), 8.06-8.08 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 21.5, 78.4, 83.5, 121.2, 124.0, 125.3, 128.1, 129.5, 129.8, 130.9, 133.2, 134.9, 141.7, 164.4; HRMS (ESI) for $C_{16}H_{12}N_4S$ [M+H]: *m/z* calcd: 293.0861 ,measured 293.0855.

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Chapter 3 - Biological activity of 5-oxo and 5-thio tetrazole derivatives: Study of their antiproliferative activity

(Studies in this Chapter were carried out by Prof. Jean-Pierre H. Perchellet, Division of biology, Kansas State University)

3.1. Introduction

Among various heterocycles, tetrazoles and their derivatives have attracted attention of researchers because of their wide spread applications particularly in biology and medicine[1-5]. Tetrazoles possess abnormally high acidity and very weak basicity compared to other chemically and thermally stable azoles.[6-9] This property has been efficiently utilized in the design of an antihypertensive drug Losartan[10]. The negatively charged acidic tetrazole ring present in the drug molecule interacts with the positively charged site in the receptor. Recently, a novel tetrazole containing angiotension receptor agonist for the treatment of hypertension has also been developed which is found to be about 8 times more potent than Losartan.[11]

The tetrazole fragment, CN_4H, has similar acidity to that of carboxylic acid, CO_2H and is almost isosteric with it, but it is metabolically more stable at physiological pH[12]. The replacement of carboxylic group with the tetrazole ring increased the efficiency of many drugs[12]. For instance, Abell and coworkers[14] developed a novel HIV protease-I inhibitor by replacing the carboxylic group in the drug with the tetrazole ring and this is found to increase the inhibition of the enzyme . The metabolic stability of 5-oxo tetrazole rings is also utilized in the design of selective agonists for β3 human adrenogenic receptors (HAR) that are useful in the treatment of obesity. The tetrzolone ring has also replaced some other functionaliteies in drug molecules for increased efficacy. For instance, Substitution of the previously used imidazolone and

imidazolidine rings with the tetrazole scaffold is found to increase the oral bioavailability due to the better stability of the tetrazole ring[15-18]. The 5-*oxo* and 5-*thio* derivatives of tetrazole also form a structural framework of many drugs that are patented for the treatment of central nervous system disorders[1], antibacterial[19], sexual dysfunction[20], asthma[2], obesity[3], diabetes[5], antiallergic[19], anticonvulsants[21], anticancer drugs[22] and injuries caused by minor chemical warfare agents[23].

In addition, tetrazole molecules are interesting because exhibit rich photochemistry. The focus of this dissertation was to provide a new class of compounds based on 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thiones **1** and utlilize their photochemistry for the cleavage of the DNA in the cell. Therefore, in Chapter 2 we discussed the synthesis of 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione **(1a)** and 1-(2-ethynyl-4-methylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione **(1b).** During our efforts to optimize the synthesis of these compounds, we also obtained a series of other 5-oxo and 5-thio substituted 1,4-diarylated tetrazole derivatives (Section 2.4 in Chapter 2). In this Chapter, we report the antiproliferative activity of tetrazole-5-ones **71a-f** and tetrazole-5-thiones **76a-f**. The main goal of this study was to determine whether **1a** and **1b** has antiproliferative activity in the absence of light. We also evaluated the antiproliferative activity of a series of other synthesized tetrazolones **60a**, **64a**, **71a-f** and tetrazole5-thiones **76a-f** since such ring systems have not been previously assayed for this purpose.

The antiproliferative of the novel diaryl tetrazole-5-ones **71a-f** and tetrazole-5-thiones **76a-f** was evaluated in rapidly growing suspension cultures of L1210 leukemia cells and slow growing monolayer cultures of SK-BR-3 mammary tumor cells. Since the fraction of Ki-67-positive tumor cells was correlated with the clinical course of cancer, it was also of interest to

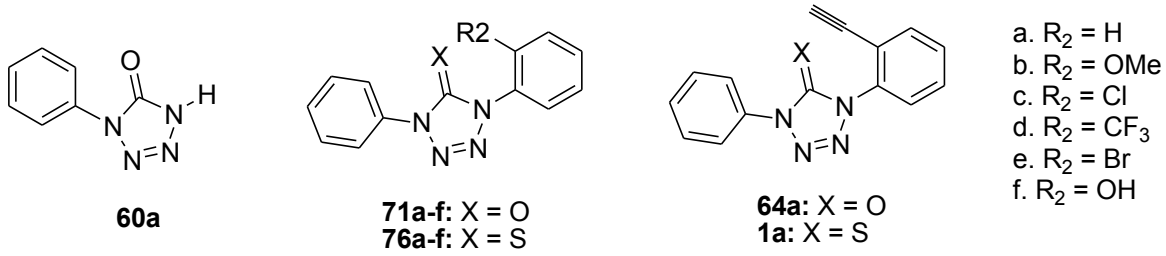
determine whether tetrazole derivatives would inhibit the expression of human Ki-67 nuclear protein, which is an excellent marker of tumor cell proliferation. The most active tetrazole derivative **76f** was evaluated for the inhibition of DNA, RNA and the protein synthesis in L1210 cell lines. The compound was assayed for its ability to induce DNA fragmentation and thereby induce apoptosis. Finally, the 5-*oxo* and 5-*thio* derivatives of tetrazole were also explored using a cell-free assay *in vitro* for their ability to directly inhibit the catalytic activity of human 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme of cholesterol biosynthesis targeted by statin drugs. Long term use of statin lowering drugs raise the risk of cancer since the lack of cholesterol may effect the cell functions.[24]

3.2. Results and discussion

3.2.1. Study of the antiproliferative activty

3.2.1.1. Inhibition of L1210 leukemia cancer cells

Compounds **60a**, **71a-f**, **76a-f** and **1a** (Scheme 3.1) , were evaluated for their antiprofilerative activity based on their ability to reduce the metabolic activity of fast growing L1210 leukemia cells after 2 and 4 days in culture



Scheme 3.1. Synthesized 5-oxo and 5-thio tetrazole derivatives

Based on the results (Table 3.1), 13 of the 15 tetrazol-5-ones and tetrazole-5-thiones tested showed *in vitro* tumor cell proliferation with IC_{50} values ranging 2.5-59-8 μM, where as two compounds were inactive.

Compound	IC_{50} values (μM)[a]	
	Day 2	Day 4
60a	NE[b]	NE[b]
71a	51.2 ± 2.0	36.4 ± 1.9
76a	NE[b]	NE[b]
71b	33.4 ± 2.3	29.1 ± 3.0
76b	59.8 ± 3.6	25.7 ± 3.5
71c	28.3 ± 4.0	24.9 ± 5.2
76c	29.1 ± 2.7	23.6 ± 4.4
71d	33.2 ± 2.4	29.6 ± 2.2
76d	33.6 ± 1.3	26.8 ± 4.8
71e	31.1 ± 1.2	26.4 ± 2.8
76e	30.7 ± 2.9	25.6 ± 5.4
71f	12.0 ± 2.4	7.1 ± 2.7
76f	3.8 ± 1.5	2.5 ± 2.4
64a	16.1 ± 2.6	12.9 ± 2.3
1a	48.0 ± 2.3	37.5 ± 5.4
Daunorubicin	22.9 ± 6.3 nM	10.3 ± 1.5 nM
Mitoxantrone	1.7 ± 0.2 nM	0.6 ± 0.1 nM

Table 3.1. Concentrations of novel synthetic compounds required to inhibit by 50% (IC_{50} values) the metabolic activity of L1210 tumor cells, using the MTS:PMS assay after 2 or 4 days of culture *in vitro*. IC_{50} values (μM, except otherwise specified for reference anticancer drugs used as positive controls) were calculated from linear regression of the slopes of the log-transformed concentration-survival curves. [a]Means ± SD (n = 3); [b]No effect on tumor cell growth even at the highest 156.25 μM concentration tested

Compounds **64a**, **71f** and **76f** were found to be most effective in inhabiting the mitochondrial ability of L1210 leukemia cell lines to metabolize the MTS:PMS reagent at days 2 and 4, whereas compounds **60a** and **76a** were inactive and do not significantly alter the control rate of L1210 tumor cell growth even at the highest concentrations tested. The target compound 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1a)** was found to have moderate

antiproliferative activity after days 2 and 4. This showed that the side effects associated with the compound before the photo irradiation were minimal. All the other compounds showed moderate antiproliferative properties. Full concentration-response curves indicated that the inhibitions of L1210 tumor cell growth by the most effective compounds **71f** and **76f** began around 1.6-4 μM and become maximal around 25-62.5 μM (Figure 3.1). Moreover, the magnitudes of the antiproliferative effects were generally more pronounced after 4 than 2 days in culture (Table 3.1 and Figure 3.1), suggesting that the effectiveness of these bioactive compounds against L1210 tumor cell growth is a combination of drug concentration and duration of action. Although less potent, the other compounds also induce concentration-dependent inhibitory effects that are able to block L1210 tumor cell proliferation by 90-100% at 62.5-156.25 μM.

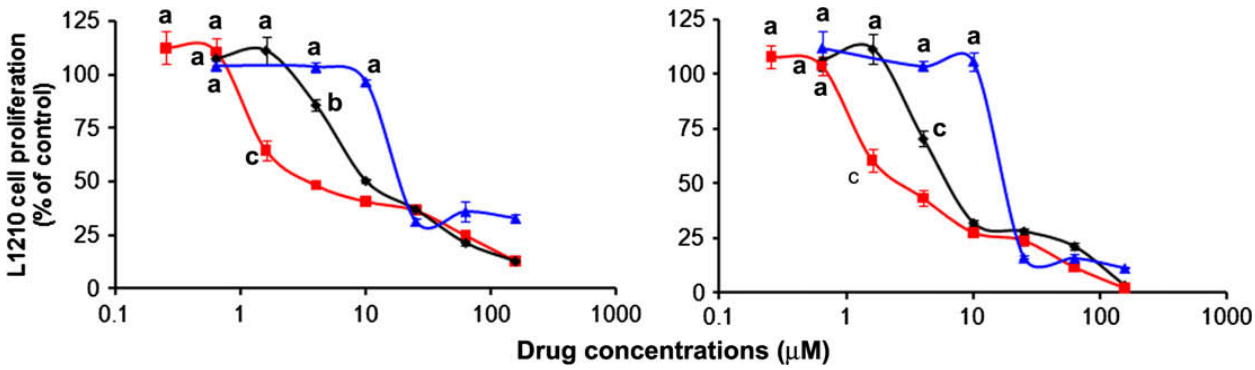


Figure 3.1. Comparison of the abilities of serial concentrations (plotted on a logarithmic scale) of **64a** (blue), **71f** (black) and **76f** (red) to inhibit the metabolic activity of L1210 tumor cells at days 2 (left) and 4 (right) *in vitro*. Cell proliferation results were expressed as % of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells after 2 ($A_{490\ nm}$ = 1.171 ± 0.044, 100 ± 3.8%, striped area in A) and 4 ($A_{490\ nm}$ = 1.188 ± 0.055, 100 ± 4.6%, striped area in B) days in culture. The blank values ($A_{490\ nm}$ = 0.447 at day 2 and 0.471 at day 4) for cell-free culture medium supplemented with MTS:PMS reagent were subtracted from the results. Bars: means ± SD (n = 3). [a]Not different from respective controls; [b]$P < 0.01$ and [c]$P < 0.005$, smaller than respective controls

The inactivity of **60a** suggests that the presence of a second aromatic ring may be critical to reveal the antitumor effect of the tetrazole derivatives.

In general, the related tetrazole-5-ones and tetrazole-5-thiones have similar antiproliferative activities but there were notable exceptions as **76a** was totally inactive while **71a** shows bioactivity, and also **1a** was much weaker than its tetrazole-5-one counterpart **64a**. The presence of a hydroxyl group **71f** and **76f** appeared to be an asset in enhancing the bioactivity of this framework. This may be attributed to the fact that an OH group was an excellent hydrogen bond donor which may result into a stronger bonding with its biological target, thereby increasing the antiproliferative activity of **71f** and **76f**. However, under similar experimental conditions, daunorubicin and mitoxantrone, the commonly available anticancer drugs inhibit L1210 tumor cell proliferation with IC_{50} values in the low nanomolar range[25] (Table 3.1).

3.2.1.2. Inhibition of human SK-BR-3 breast cancer cells

Compounds **64a**, **71f** and **76f** have been tested for their antiproliferative activity against slow growing SK-BR-3 breast cancer cell lines. They were observed to inhibit the proliferation of cells at days 2 and 4 (Figure 3.2), however higher micromolar concentrations of these compounds must be used to achieve inhibitory effects similar to those of L1210 cells. For instance, 156.25 μM **76f** inhibited SK-BR-3 cell proliferation by 60.6% at day 2 and 79.3% at day 4 (Fig 3.3) but these magnitudes of proliferation are achieved by 25 μM of **76f**. The tetrazole-5-thione was at least 6.25 times more effective against leukemic than breast cancer cell lines. The fact that the concentrations of **64a**, **71f** and **76f** required to inhibit the mitochondrial

ability of tumor cells to metabolize the MTS:PMS reagent at day 2 and 4 are somewhat higher in SK-BR-3 than L1210 cells suggests that the antiproliferative action of these drugs was generally greater against unsynchronized populations of rapidly-growing suspensions of leukemic cells that were frequently turning through the cell cycle than against unsynchronized populations of a relatively slow-growing adherent monolayers of solid tumor cells that have smaller growth fractions.

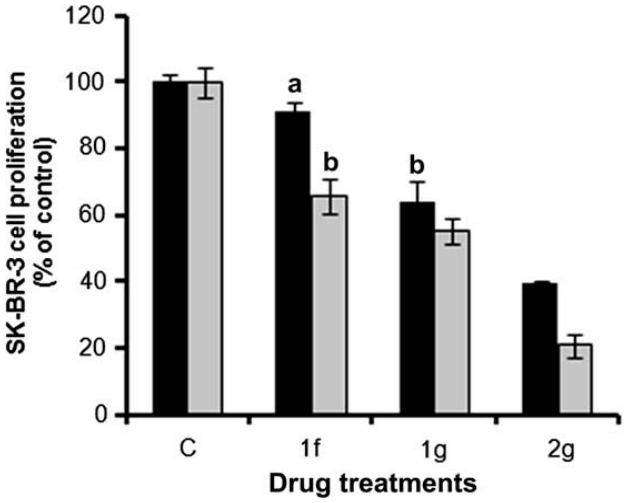


Figure 3.2. Comparison of the abilities of 156.25 μM concentrations of **64a**, **71f** and **76f** to inhibit the metabolic activity of SK-BR-3 tumor cells at day 2 (black columns) and 4 (grey columns) in vitro. SK-BR-3 cell proliferation results were expressed as % of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control (C) cells after 2 ($A_{490\ nm}$ = 1.396±0.034, 100±2.4%) and 4 ($A_{490\ nm}$ = 1.291±0.061, 100±4.7%) days in culture. The blank values ($A_{490\ nm}$ = 0.230 at day 2 and 0.249 at day 4) for cell-free culture medium supplemented with MTS:PMS reagent were substracted from the results. Bars: means±SD (n-3). $^{a}P < 0.0025$ and $^{c}P < 0.005$, smaller than respective controls.

3.2.2. Inhibition of human Ki-67 marker expression

The antitumor activity of **76f** was substantiated by finding that this compound inhibits the expression of human Ki-67 marker of cell proliferation in SK-BR-3 mammary tumor cells after 24h (Scheme 3.4). The Ki-67 nuclear protein, which was absent from resting cells (G_0), was

exclusively detected within the nuclei of cells progressing through all active phases of the cell cycle (G_1, S, G_2) and relocates to the surface of the chromosomes during mitosis. Since Ki-67 expression may be absolutely required to maintain cell proliferation, it was an excellent marker for determining the growth fraction of tumor cell populations and a fraction of Ki-67 protein detected by immunolabeling in untreated SK-BR-3 control tumor cells, the ability of 62.5-156.25 μM 2g to inhibit Ki-67 expression by 36.5-52.4% at 24h suggests that this novel antiproliferative compound maintains surviving tumor cells in the resting stage and prevents them from reentering the cell cycle to divide (Figure 3.3). After antiproliferative **76f** treatment, there were a few tumor cells and they failed to express Ki-67, indicating that the growth fraction of tumor cells progressing through the cell cycle has been reduced.

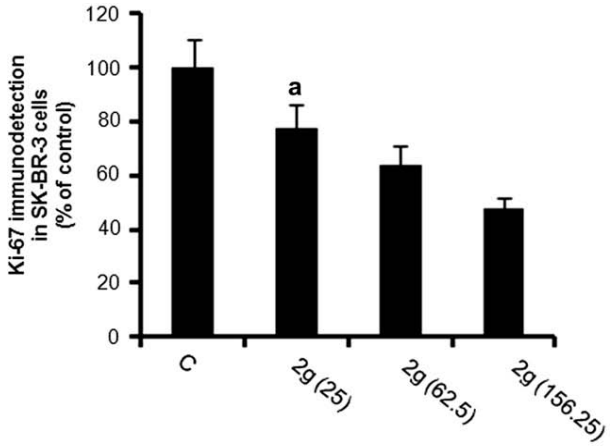


Figure 3.3. Whole cell immunodetection of Ki-67 protein level in vitro. Comparison of the abilities 25, 62.5 or 156.26 μM concentrations of **76f** to inhibit the Ki-67 marker of cell proliferation in SK-BR-3 cells at 24h. The results were expressed as % of the ratio of Ki-67 protein level (relative luminescence intensity at the anti-Ki-67 primary antibody-antigen immune complex bound to horseradish peroxidase-linked secondary antibody) : cell number ((relative luminescence intensity of the Hoechst reagent-DNA complex) in vehicle treated control SK-BR-3 tumor cells at 24h (C: 1.6248 ± 0.1771,100 ±10.9%). Bars: means ±SD (n=3). $^{a}P < 0.05$, smaller than control.

3.2.3. Inhibition of rate of DNA synthesis

A 90-minute treatment with **76f** was sufficient to inhibit the incorporation of [^{3}H]thymidine into DNA used to assess the rate of DNA synthesis over a 30-min period of pulse labeling in L1210 tumor cells *in vitro* (Figure 3.4). Although it may be somewhat misleading to compare the biological responses measured at different times, the concentration-dependent inhibition of DNA synthesis by **76f** suggests that the ability of this compound to prevent tumor cells from synthesizing DNA at 2h may play a vital role in its inhibition of Ki-67 expression at 24h and the antiproliferative activities at days 2 and 4 (Scheme 3.5).

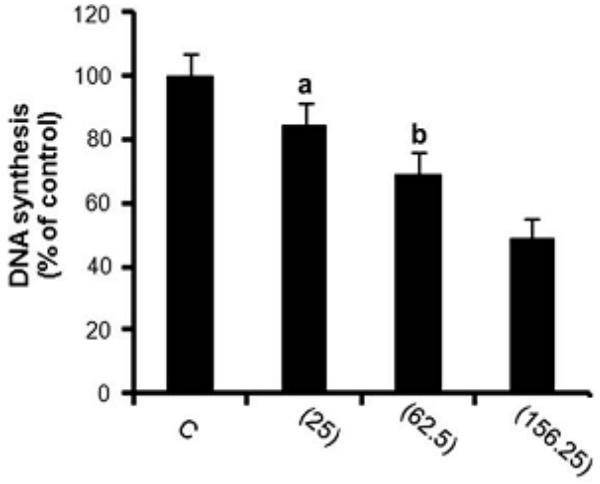


Figure 3.4. Comparison of the abilities 25, 62.5 or 156.26 μM concentrations of **76f** to inhibit the rate of incorporation of [^{3}H]thymidine into DNA measured in L1210 cells over 30 mon following a 90 min incubation at 37^{0}C *in vitro*. DNA synthesis ijn vehicle controlled (C) cells at 37^{0}C was 11,314 ±769 cpm (100 ± 6.9%). The blank value (1282 ± 112 cpm) for control cells incubated and pulse-labelled at 2^{0}C with 1 μCi of [^{3}H]thymidine has been subtracted from the results. Bars: means ±SD (n=3). $^aP < 0.05$ and $^bP < 0.005$, smaller than control.

Concentrations of **76f** somewhat higher than those sufficient to maximally inhibit the tumor cell proliferation must be used to partially inhibit Ki-67 expression and DNA synthesis.

Such apparent discrepancy may be simply due to different experimental conditions and cellular responses to various periods of drugs exposure: the rate of DNA synthesis over 30 min inhibited in cells treated for only 2h with **76f** and the level of Ki-67 protien was reduced in cells incubated for 24 h in the presence of antitumor compound, whereas the more spectacular inhibitions of L1210 and SK-BR-3 tumor cell proliferations were the result of 2- and 4-day long drug treatments.

3.2.4. Inhibition of the rate of RNA and protein synthesis

A 3-h treatment with **76f** also inhibited the rate of incorporation of [^{3}H]uredine into RNA and [^{3}H]leucine into protein, in a concentration-dependent manner and thereby assessing the rates of RNA and protein syntheses respectively determined over 1-h periods of pulse-labeling in L1210 tumor cells (Figure 3.5). Although it may be somewhat misleading to compare biological responses measured at very different times, the concentration-dependent inhibitions of RNA and protein syntheses by **76f** suggested that the ability of this compound to prevent tumor cells from synthesizing macromolecules at 2-3 h may play a role in its inhibition of Ki-67 expression at 24 h and antiproliferative activity at days 2 and 4.

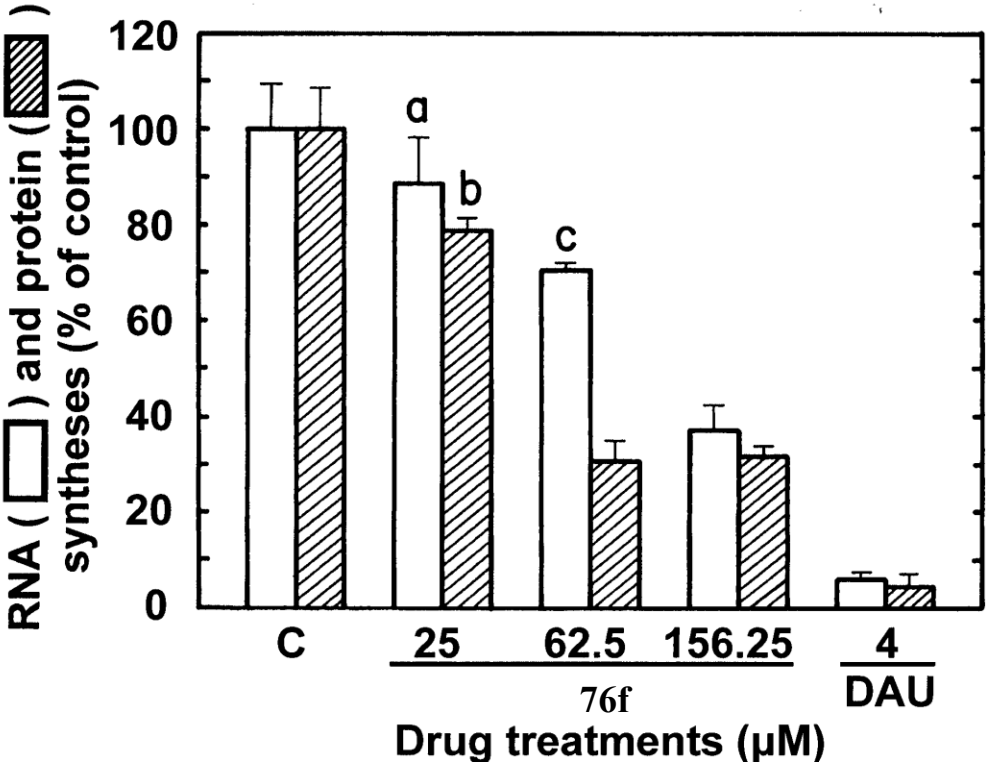


Figure 3.5. Comparison of the abilities of 25, 62.5 or 156.25 μM concentrations of **76f** to inhibit the rates of incorporation of [^{3}H]uridine into RNA (open columns) and [^{3}H]leucine into protein (striped columns) measured in L1210 cells over 1 h following a 3-h period of incubation at 37°C *in vitro*. The magnitudes of RNA and protein synthesis inhibition caused by 4 μM daunorubicin (DAU) were used as positive controls. In vehicle-treated control cells (C) at 37°C, RNA synthesis was 28,057 ± 2,609 cpm (100 ± 9.3%) and protein synthesis was 7,324 ± 637 cpm (100 ± 8.7%). The blank values for control cells incubated and pulse-labeled at 2°C with 2 μCi of [^{3}H]uridine (6,305 ± 719 cpm) or 2.5 μCi of [^{3}H]leucine (4,032 ± 375 cpm) have been subtracted form the results. Bars: means ± SD (n = 3). [a]Not different from control; [b]$P < 0.025$ and [c]$P < 0.01$, smaller than respective controls

3.2.5. DNA fragmentation study

The compound **76f** was tested for its ability to induce DNA cleavage. The levels of DNA fragmentation caused by 1.6 μM daunorubicin (DAU) and 0.256 μM mitoxantrone (Mitox) were used as positive controls. The ability of **76f** to induce DNA cleavage at 24 h, demonstrated using L1210 cells containing ^{3}H-thymidine-prelabeled DNA to detect low molecular weight DNA

fragments after intact chromatin precipitation, suggests that this antitumor drug may also trigger an apoptotic pathway of internucleosomal DNA fragmentation (Figure 3.6).

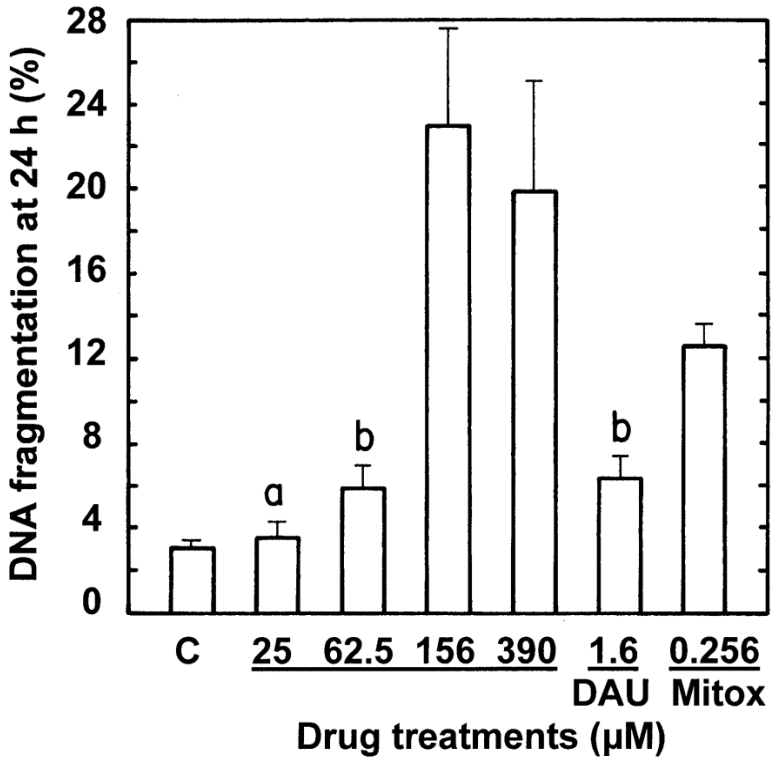


Figure 3.6. Comparison of the abilities of 25, 62.5, 156.25 or 390.625 μM concentrations of **76f** to induce DNA fragmentation at 24 h in L1210 cells containing ^{3}H-prelabeled DNA *in vitro*. The results were expressed as [cpm in supernatant/cpm in supernatant + pellet] x 100 at 24 h. For vehicle-treated control (C) tumor cells (3.13 ± 0.74% DNA fragmentation), the supernatant (DNA fragments) was 2,447 ± 583 cpm and the pellet (intact DNA) was 75,732 ± 17,753 cpm. Bars: means ± SD (n = 3). [a]Not different from control; [b]$P < 0.025$, greater than control.

3.2.6. Inhibition of human HMG-CoA reductase

The synthesized tetrazoles were screened at 10, 25 and 62.5 μM for their statin-like activity in a cell-free assay. All but one of these tetrazole derivatives failed to directly inhibit the specific catalytic activity of purified human HMG-CoA reductase. The lone exception was 1-(2-

ethynylphenyl)-4-phenyl-1*H*-tetrazol-5(4*H*)-one **64a**, which at 62.5 μM inhibited HMG-CoA reductase activity by 74.6%. At 156.25 μM, 8 other compounds had weak or moderate activity (inhibition by 50% or less) but **76c** and **76d** had interesting HMG-CoA reductase-inhibiting activity (inhibition by 66.2 and 71.2%, respectively). The results were summaraized in the table below

Compounds and concentrations tested	Specific activity of HMG-CoA reductase[a] (% of control)[b]		
	256 nM	62.5 μM	156.25 μM
76c		85.3 ± 9.3[c]	33.8 ± 2.6[d]
76d		93.3 ± 3.1[c]	28.8 ± 1.9[d]
71f		96.6 ± 5.9[c]	92.9 ± 5.4[c]
76f		101.7 ± 6.7[c]	86.0 ± 5.1[c]
64a		25.4 ± 9.3[d]	8.9 ± 1.8
Pravastatin	19.3 ± 1.1		
Fluvastatin	26.0 ± 2.5		
Simvastatin (Na salt)	15.7 ± 3.0		
Mevastatin (Na salt)	18.5 ± 1.6		
Lovastatin (Na salt)	34.5 ± 2.4[d]		

Table 3.2. Comparison of the abilities of known statins and novel synthetic tetrazole derivatives with antiproliferative activity to directly inhibit the catalytic activity of HMG-CoA reductase in a cell-free assay *in vitro*

The concentration dependent inhibition of HMG-CoA reductase by **64a** begins at 4 μM, is maximal at 156.25 μM (inhibition by 91.1%) and is characterized by an IC_{50} value of 34.1 μM, suggesting that this antiproliferative drug can also directly interact with the rate-limiting enzyme in cholesterol biosynthesis. But there was no apparent correlation between the ability of these

compounds to directly inhibit HMG-CoA reductase activity within 10 min in a cell-free assay and their effectiveness against tumor cell proliferation after 2-4 days in culture.

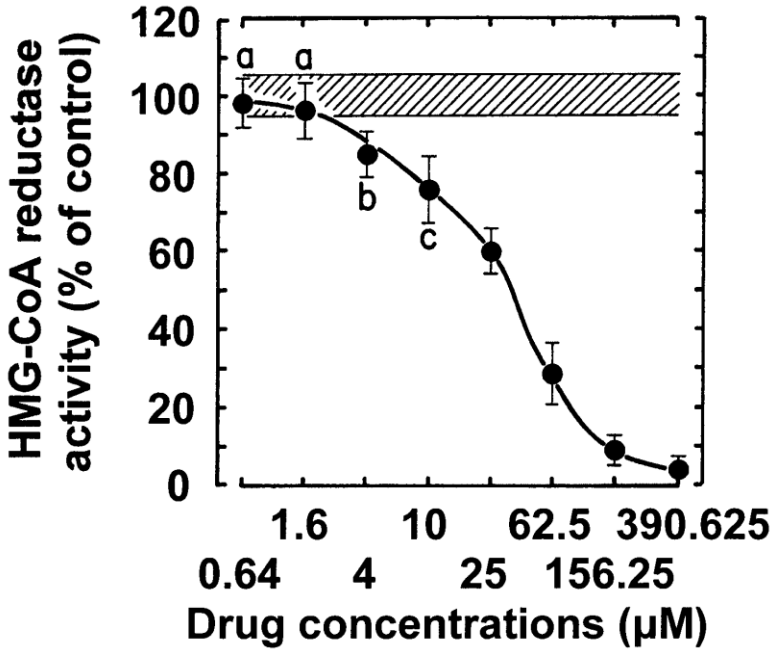


Figure 3.7. Ability of serial concentrations (plotted on a logarithmic scale) of **64a** (●) to directly inhibit the catalytic activity of human HMG-CoA reductase in a cell-free assay *in vitro*. The concentration of the HMG-CoA reductase stock solution was 0.52 mg protein/ml. Reactions were incubated at 37°C in quartz microcells and the rates of NADPH consumed were calculated from the decreases in spectrophotometric absorbance at 340 nm between 0 and 720 sec after addition of the enzyme. Results are expressed as % of the control specific activity of the enzyme in the absence of drug (0.346963 ± 0.019337 μmol of NADPH oxidized/min/mg protein, 100 ± 5.6%, striped area). Bars: means ± SD (n = 3). [a]Not different from control, [b]$P<0.05$ and [c]$P<0.025$, smaller than control.

3.3. Conclusion

The novel tetrazole derivatives were found to inhibit cell proliferation of fast growing L1210 leukemia cancer cell lines and slow growing SK-BR-3 breast cancer cell lines in

microgram concentrations *in vitro*. The most active tetrazole **76f** was observed to inhibit Ki-67 marker expression in SK-BR-3 breast cancer cell lines. The tetrazole **76f** was also found to inhibit the rate of DNA, RNA and protein syntheses in L1210 cells, thereby reducing the rate of cell proliferation. The latter was also investigated for its ability to cleave the DNA and it was found to induce DNA fragmentation in L1210 cells after treatment of 24h. Most of the synthesized tetrazoles were also found to inhibit the human HMG-CoA reductase, which was a rate limiting enzyme for the cholesterol biosynthesis pathway. The presence of OH substituent in **71f** and **76f** was found to be an asset for pronounced bioactivity. These compounds may have interesting bioactivity but more compounds based on 5-oxo and 5-thio 1,4-diaryl tetrazole scaffolds must be synthesized to elucidate structure–activity relationships, identify more potent antitumor lead compounds, and investigate their molecular targets and mechanism of action.

3.4. Experimental methods

The proliferation of control and drug-treated tumor cells was assessed from their mitochondrial ability to bioreduce the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (**MTS**) reagent in the presence of phenazine methosulfate (**PMS**) into a water-soluble formazan product that absorbs at 490 nm. Cell proliferation results were expressed as % of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells after 2 or 4 days in culture. The blank value for cell-free culture medium supplemented with MTS:PMS (2:0.1) reagent was subtracted from the results[26].

For immunodetection of Ki-67 protein level at 24 h, control and drug-treated SK-BR-3 cells were washed with PBS, fixed with MeOH, lysed in PBS containing 0.1% Triton X-100, blocked with SuperBlock and incubated with 1) rabbit anti-Ki-67 primary polyclonal antibody and 2) horseradish peroxidase-linked anti-rabbit IgG secondary monoclonal antibody and Hoechst 33342 reagent. After adding the SuperSignal West Pico chemiluminescence (**CL**) substrate, plates were scanned in the CL mode (425 nm) and finally read in the fluorescence mode (340 nm excitation/461 nm emission). The relative fluorescence intensities of the Hoechst reagent-DNA complexes were used to normalize the relative CL unit values of the primary antibody-antigen immune complexes bound to the horseradish peroxidase-linked secondary antibody in order to compare Ki-67 protein levels on an equal cell number basis. And the ratio of relative luminescence: fluorescence was expressed as % of that for vehicle-treated control cells.

To estimate the rate of DNA synthesis, L1210 cells were incubated for 90 min in the presence or absence (control) of drugs and then pulse-labeled for an additional 30 min with 1 μCi of ^{3}H-thymidine. For RNA and protein syntheses, cells were incubated for 3 h in the presence or absence of drugs and then pulse-labeled for an additional 1 h with 2 μCi of ^{3}H-uridine or 2.5 μCi of ^{3}H-leucine, respectively. The acid-insoluble macromolecules were extracted with 10% trichloroacetic acid (**TCA**), recovered over Whatman GF/A glass microfiber filters and washed trice with 5% TCA and twice with 100% EtOH. After drying the filters, the radioactivity bound to the acid-precipitable material was determined by liquid scintillation counting (**LSC**). Drug-induced DNA cleavage was determined by intact chromatin precipitation, using L1210 cells which were prelabeled with 1 μCi of ^{3}H-thymidine for 2 h at 37°C, washed with 3 x 1 ml of PBS, collected by centrifugation, resuspended in fresh medium at a density of 1.2 x 10^{6} cells/ml, and then incubated at 37°C for 24 h in the presence or absence of drugs. After centrifugation at 200 *g*

for 10 min to discard the drugs and wash the cells, the cell pellets were lysed for 20 min in 0.5 ml of ice-cold 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 0.2% Triton X-100, and centrifuged at 12,000 *g* for 20 min to collect the supernatants. After the intact pelleted chromatin had been solubilized for 2 h at 60°C in the presence of 0.6 ml of TS-1 tissue solubilizer (Research Products International), the radioactivity in the supernatants (detergent-soluble low molecular weight DNA fragments) and the pellets (intact chromatin DNA) was determined by LSC.

For the HMG-CoA reductase screening assay, the concentration of the purified human enzyme stock solution was 0.52 mg protein/ml. Reactions, containing 4 µl of NADPH (to obtain a final con-centration of 400 µM) and 12 µl of HMG-CoA substrate (to obtain a final concentration of 400 µM) in a final volume of 0.2 ml of assay buffer (Sigma kit), were initiated (time 0) by the addition of 2 µl of the catalytic domain of human recombinant HMG-CoA reductase and incubated in quartz microcells at 37°C in the presence or absence (control) of 1-µl aliquots of drugs dissolved in DMSO. The rates of NADPH consumed were monitored every 20 sec for up to 720 sec by scanning spectrophotometrically the decrease in absorbance at 340 nm. Results were expressed as % of the control specific activity of the enzyme (µmol of NADPH oxidized/min/mg protein) in the absence of drugs.

3.5. References

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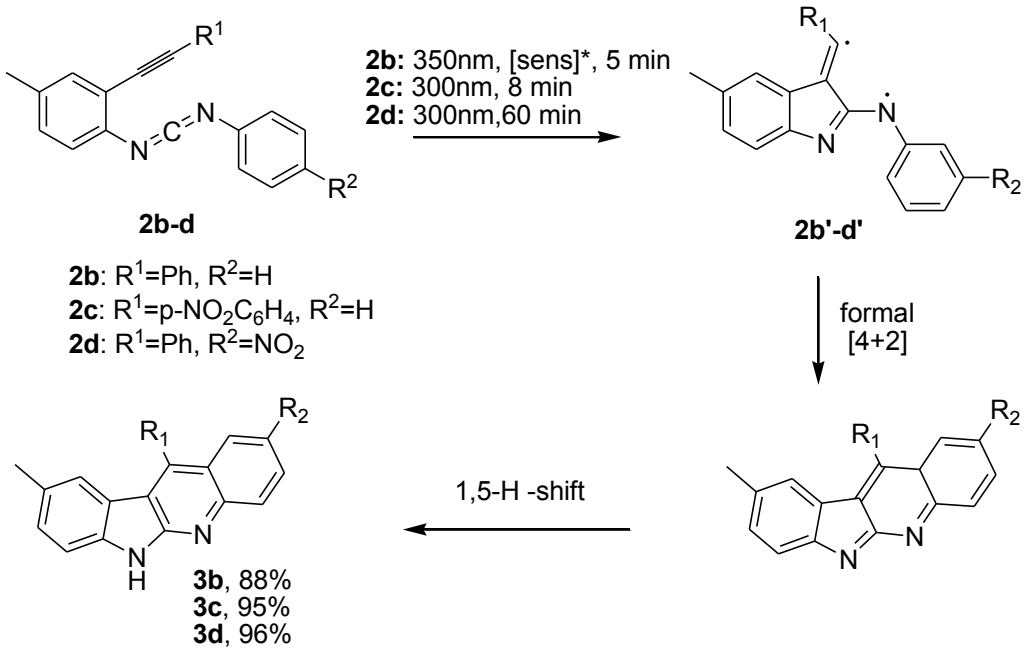
Chapter 4 - Photodecomposition of 1-ethynyl-4-phenyl tetrazole-5-thione: One step clean conversion to indoloquinoline *via* enyne-carbodiimide

4.1. Introduction

Under triplet sensitization, enyne-allenes and enyne-carbodiimides undergo photochemical cyclization *via* biradical intermediate(s) (Scheme 1.16.).[1] In case of enyne-allenes, a mixture of Myers-Saito (C_2-C_6) and Schmitel (C_2-C_7) cyclization products are produced and the yields are low (24% and 13% respectively),[2] while enyne-carbodiimides **2b** undergo complete conversion in the presence of aceptophenone and toluene to exclusively yield indoloquinoline **3b** *via* the Schmittel cyclization pathway in more than 90% yield. As mentioned in Chapter, the presence of an electron-withdrawing group at the acetylene or carbodiimide terminus such as in **2c**,**d** respectively afforded the cyclized products **3c,d** under direct irradiation. The yields of these photochemical transformations were also close to 100%. The presence of a triplet biradical intermediate during the photolysis of enyne-carbodiimides was further verified by performing the reaction in the presence of a triplet quencher *e.g.* 1,4-dipheynl-1,3-butadiene that inhibited the formation of the photoproduct.[1]



Scheme 4.1. Photochemical cyclization of enyne-carbodiimides **2b-d**

The reason for different product distribution and yields in case of enyne-allenes and enyne-carbodiimides was explained by computational studies (B3LYP/6311G+*) by Engels and coworkers. Their work showed that triplet excited state of enyne-allenes is a local minima on the potential energy surface possessing nearly similar activation barriers for C_2-C_7 and C_2-C_6 cyclization which results in a mixture of products arising from the two pathways. (Figure 4.1, right), while in the case of enyne-carbodiimides the geometrical optimization of the triplet excited state directly led to the C_2-C_6 biradical without a barrier (Figure 4.1, left),[3] which possessed a perfect nuclear arrangement for subsequent formal [4+2] cyclization reaction to form the final product. All the attempts to find a reaction pathway from the triplet excited state of enyne-carbodiimide to C_2-C_7 biradical were unsuccessful.[3]

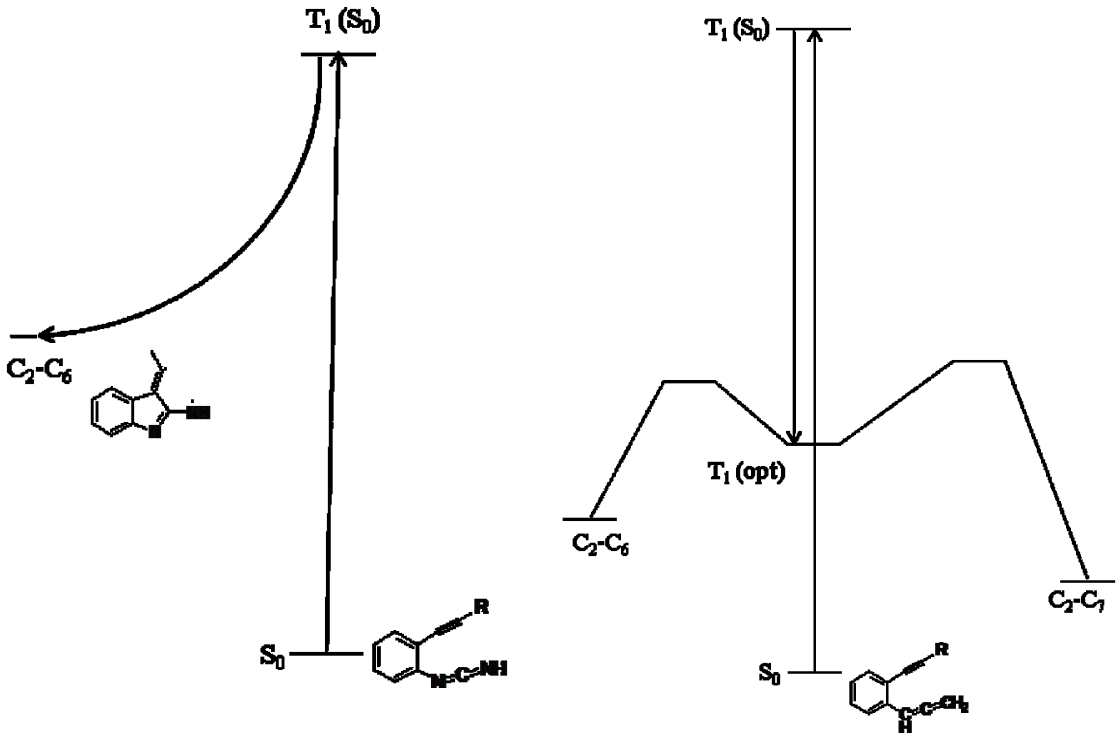
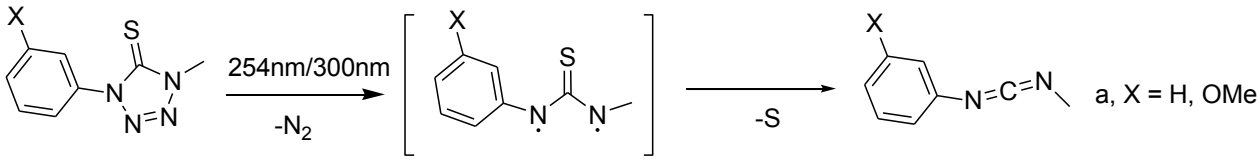


Figure 4.1. Theoretical investigation of photochemical cyclization of enyne-carbodiimides (left) and enyne-allenes (right)

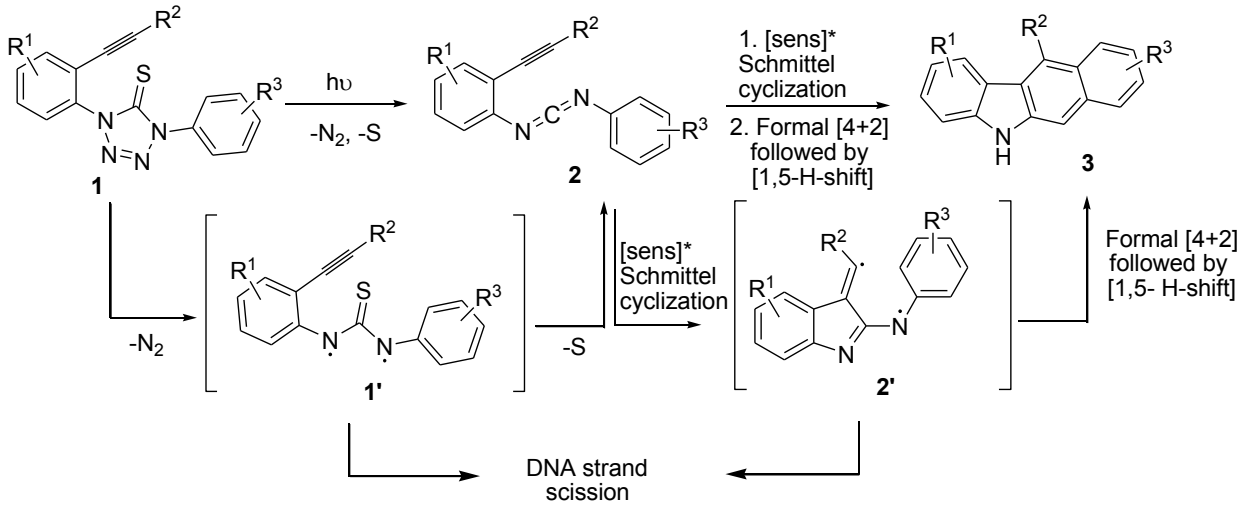
The enyne-allenes induce DNA strand scission in μg quantities.[4] We were interested in exploiting the biradical from the Schmittel cyclization of enyne-carbodiimides for DNA cleavage. However, the high reactivity of electrophilic carbon towards biological nucleophiles would prevent their application as DNA cleaving agents. Studies in our lab and also by others[5,6] revealed that photodecomposition of tetrazolethione yields carbodiimide as the major product. The mechanism of photodecomposition of 1-phenyl-4-methyl tetrazole-5-thiones **62a-d** (Scheme 2.3, Page 30) involves a 1,3-triplet biradical formed by the loss of dinitrogen, and subsequent loss of sulfur forms the carbodiimide (Scheme 4.2). [7],[8] The clean conversion of tetrazolethione to

the carbodiimide prompted us to use this ring system to mask the carbodiimide functionality so as to protect it from the biological nucleophiles.



Scheme 4.2. Photodecomposition of tetrazolethione: Formation of biradical intermediate

We hypothesize that the irradiation of 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione **(1a**,**b)** at appropriate wavelength would result in the formation of corresponding enyne-carbodiimide **2a,b** via the formation of biradical **1′a,b**. The enyne-carbodiimide in the presence of a triplet sensitizer would undergo *in situ* cyclization to form the highly reactive biradical intermediate **2′a,b** that would undergo intramolecular formal [4+2] reaction followed by [1,5]-H-shift to form the indoloquinolone product **3a,b** (Scheme 4.3). Both the biradical intermediates **1′** and **2′** would have the ability to abstract the hydrogen atoms from the sugar phosphate backbone of the DNA and thereby inducing cleavage.



Scheme 4.3. Photodecomposition of **1a,b** : Expected mechanism

Independent studies have shown that the photodecomposition of tetrazolethiones gives carbodiimides *via* biradicals (Scheme 1.18),[46, 52] and photocyclization of enyne-carbodiimide forms indoloquinoline also *via* biradicals (Scheme 1.16).[41] In this Chapter, we wanted to investigate whether 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione **1** at 300 nm could undergo these two photochemical activations sequentially in one pot with the help of a single light source resulting into a clean conversion to enyne-carbodiimide **2** and indoloquinoline **3** *via* biradicals **1′** and **2′**. Therefore, we studied the photochemistry of 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1a)** and its analog 1-(2-ethynyl-4-methylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione **(1b)** and the results are discussed.

4.2. Results and discussion

4.2.1. Photolysis of 1,4-diaryl tetrazole-5-thione

We have previously reported that photochemical decomposition of 1-phenyl-4-methyl tetrazole-5-thiones gave carbodiimide as the major product (Scheme 4.4). We were interested in determining whether the photodecomposition of 1,4-diaryl tetrazolethiones would also yield carbodiimide as the sole product. Therefore, we studied the photochemistry of 1,4-diaryl tetrazole-5-thione **76a**. The UV-Vis spectrum of the **76a** recorded in acetonitrile exhibited four bands (Figure 4.2).

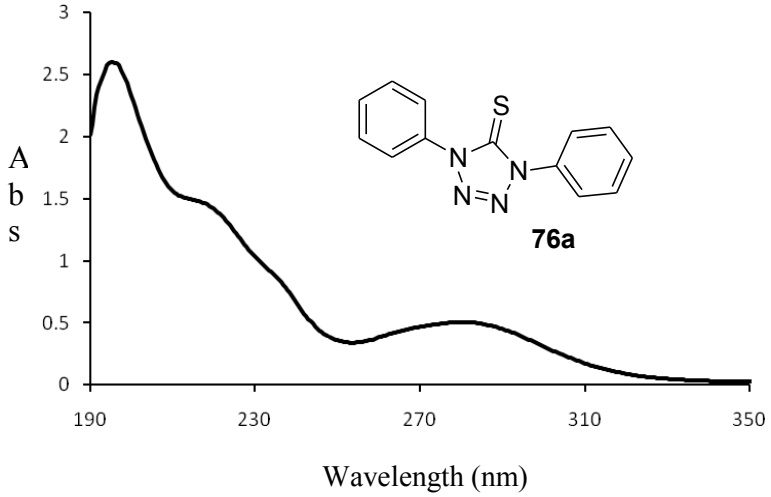
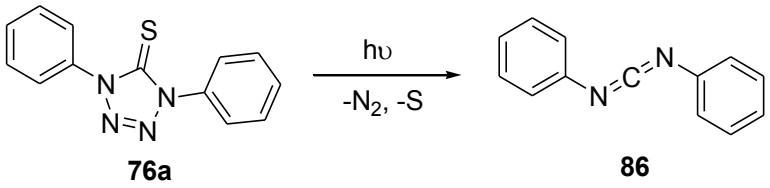


Figure 4.2. UV-Vis spectrum of 1,4-diphenyl tetrazole-5-thione **76a** in CH_3CN

1,4-Diaryl tetrazole-5-thione was dissolved in acetonitrile-d_3 and the photolysis was carried out in a quartz cuvette at 254 nm for 60 min. The comparison of the product peaks in the ^{1}H-NMR at 60 min to that of the pure carbodiimide[9] confirmed the formation of carbodiimide **86**. The photolysis was also carried out at 300 nm and found to be very slow because of the low

molar absorptivity value of the compound at this wavelength. Minor quantities of other products were also observed during the photolysis but they were not characterized (Scheme 4.4).



76a **86**

Scheme 4.4. Photolysis of 1,4-diphenyl-1*H*-tetrazole-5(4*H*)-thione (**76a)**

Once the formation of carbodiimide **86** was confirmed, we then proceeded to study the photochemistry of target compounds **1a-b** and the results of their photodecomposition studies are discussed in section 4.3.2.

4.2.2. Photolysis of 1-(2-ethynylphenyl)-4-phenyl tetrazole-5-thione (1a)

The UV-Vis spectra of the **1a** and **1b** were recorded in acetonitrile and are shown below (Figure 4.3).

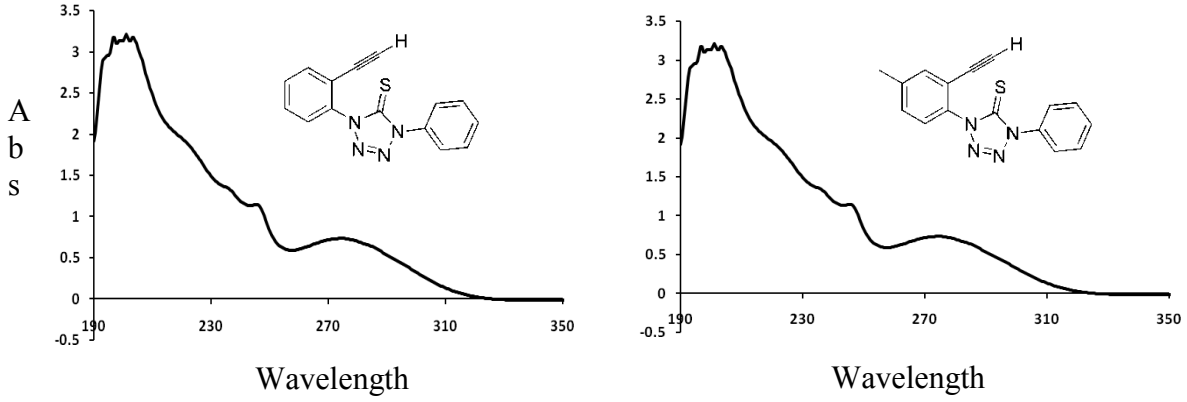
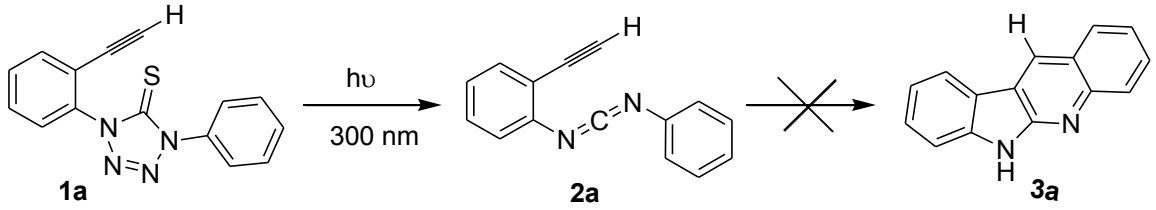


Figure 4.3. UV-Vis spectrum of **1a** and **1b** in CH_3CN

4.2.2.1. Direct Irradiation

An argon-saturated solution of the tetrazole-5-thione **1a** in acetonitrile-d_3 was taken in a quartz cuvette and the photolysis was carried out at 254 nm and 300nm. In both the cases, carbodiimide **2a** was found to be the only product as indicated by the NMR. The photolysis at 254 nm was observed to be faster compared to that of 300 nm as expected since the molar absorptivity of **1a** is higher at this wavelength (Scheme 4.5). In either case, no further cyclization of the enyne-carbodiimide **2a** to indoloquinolines **3a** was observed as expected since photochemical cyclization of the enyne-carbodiimide involves triplet sensitization.[1]

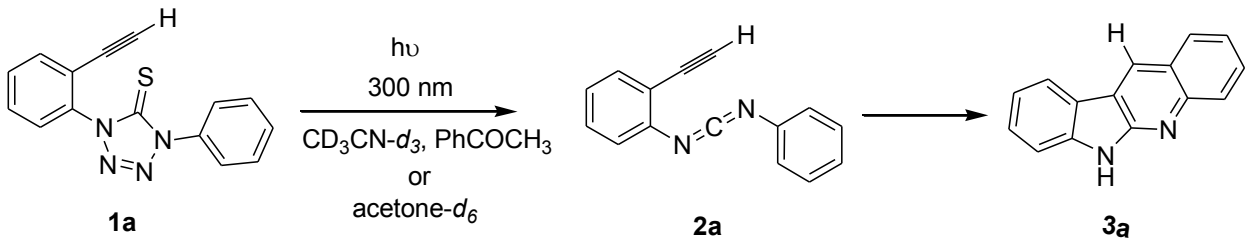


Scheme 4.5. Photoirradiation of **1a** in the absence of triplet sensitizer

4.2.2.2. Triplet sensitized irradiation

Next, photolysis was attempted by the addition of a triplet sensitizers of varying energy e.g. acetopheonone (E_T = 74 Kcal/mol) and acetone (E_T = 82 Kcal/mol). When acetophenone was used, **1a** and the sensitizer were taken in 1:1 molar ratio and the photolysis was performed in acetonitrile-d_3; while acetone-d_6 acted as a solvent as well as a triplet sensitizer. The ^{1}H NMR spectrum after 60 min of irradiation at 300 nm showed some unreacted **1a**, enyne-carbodiimide **2a** and the indoloquinoline **3a**, thus confirming that *in situ* Schmittel cyclization of the enyne-carbodiimides takes place in the presence of a triplet sensitizer followed by a formal [4 +2] and

subsequent [1,5]- H-shift (Scheme 4.6). The formation of the **2a** and **3a** was confirmed by comparison of their ^{1}H NMR spectrum with that of authentic samples in the literature. We also studied the effect of doubling the moles of triplet sensitizer (acetophenone) on the photolysis (2:1). However, the product yields were unaffected.



Scheme 4.6. Triplet sensitized photodecomposition of **1a**

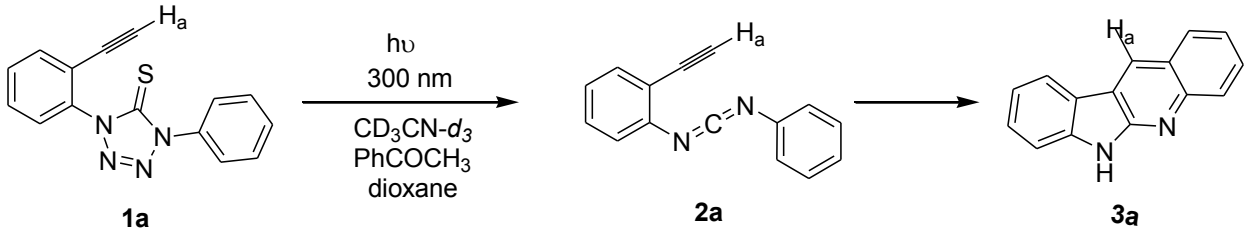
In order to determine the product yields, we attempted to purify the reaction using column chromatography by performing the reaction in large scale but the starting materials required for the synthesis of **1a** were very expensive, and in addition the synthesis was too long and low yielding. So these efforts were unsuccessful. Moreover, when the photoreaction was carried out at large scale, longer periods of irradiation were required that promoted the formation of secondary photochemical products. Therefore, the application of a different quantification method was essential in solving this complication and we considered using quantitative NMR spectroscopy for this purpose.

4.2.3. Application of Quantitative NMR spectroscopy to determine product ratios

The use of proton NMR spectroscopy for structure elucidation is widespread, but its potential as a quantitative technique is not widely utilized.[10, 11] The unique feature of NMR is

that the signal intensity is directly proportional to the number of nuclei producing the signal[10]. An internal[12, 13] or external standard[11] can be substituted for calibration curves that require analysis of a series of samples. Our laboratory had previously utilized ^{1}H NMR spectroscopy for the quantitative assessment of some other photochemical reactions[8], and with the results obtained we demonstrated the feasibility of accurate and precise measurements.

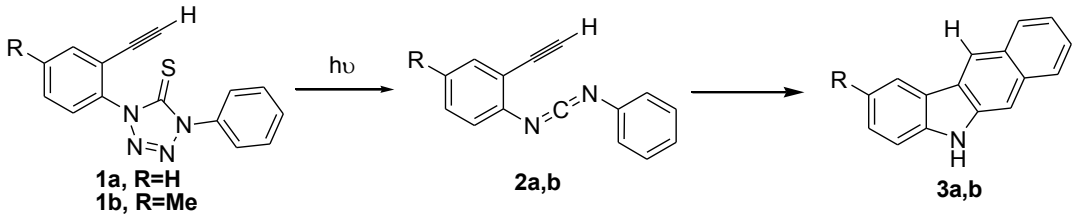
At first, we used 1,4-dioxane as an internal sensitizer for quantitative analysis since it has a single peak in the NMR and a reasonably higher boiling point. We decided to use protons labeled as H_a of 1-(2-ethynylphenyl)-4-phenyl tetrazole-5-thione **(1a)**, enyne-carbodiimide **2a** and the indoloquinoline **3a** to determine the quantitative yields (Scheme 4.8). However, the chemical shift value of dioxane (3.57 ppm) interfered with the acetylenic proton of the carbodiimide (3.60 ppm). So, quantitative measurement of the enyne-carbodiimide yield using dioxane was not reliable.



Scheme 4.7. Quantitative NMR analysis of **1a** using dioxane as internal standard

Therefore, we considered other internal standards, e.g. cyclohexane. The cyclohexane protons appear at 1.44 ppm. This signal is well separated from the protons (Ha) of **1a** and the photoproducts **2a** and **3a**. Samples **1a** and **1b** were irradiated at 300 nm for 60 min in the absence and presence of triplet sensitizer. The percent conversions of the **1a,b** to the corresponding

enyne-carbodiimides **2a,b** and the cyclized product indoloquinoline **3a**,**b** are summarized in Table 4.1.



Substrate	Wavelength (λ nm)	Sensitizer	1 unreacted (%)	2 (%)	3 (%)
	300	None	59	52	0
1a	300	Benzophenone	49	29	26
	300	Acetophenone	47	34	23
	300	None	46	49	0
1b	300	Benzophenone	43	29	18
	300	Acetophenone	42	43	6

Table 4.1. Photodecompositon of **1a** and **1b** with cyclohexane as internal sensitizer

The results obtained from the photodecomposition studies demonstrate that there was approximately 40-50% conversion of the **1** after 60 min of irradiation. Carbodiimide was the only product observed when no sensitizer was used during the reaction and the cyclized product indoloquinoline **3** was observed in the presence of triplet sensitizers, acetophenone and benzophenone.

4.2.4. Photodecomposition of 1-(2-ethynylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (1a) in water

Next, we wished to verify whether **1a** is stable and can undergo photochemical conversion to **2a** and **3a** in aqueous media. Therefore, we performed photolysis in a mixture of acetone and water. Dioxane, was used for quantitative NMR calculations in this study.

The acetone: water solutions were prepared in 4:1, 3:2 and 2:3 ratios respectively. Irradiation of these samples was carried out for 60 min at 300 nm and then analyzed using NMR spectroscopy. The NMR spectrum confirmed the formation of enyne-carbodiimide **2** and indoloquinoline **3** as major products. Quantitative NMR measurements were not possible because of the presence of large water peak in these samples. Moreover, some of the product precipitated due to its insolubility. A solution of **1a** was prepared in a acetone:water and photolysis was carried out at 300 nm for 60 min.

4.3. Conclusion

Photochemical decomposition of 1-(2-ethynylphenyl)-4-phenyl tetrazole-5-thione (**1a)** in acetonitrile at 300 nm yields carbodiimide **2a** as a major product. In the presence of a triplet sensitizer, the cyclization of the formed enyne-carbodiimide **2a** to indoloquinoline **3a** takes place. Quantitative NMR techniques were efficiently utilized to achieve the percentage yields of the products. 1-(2-Ethynyl-4-methylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione **(1b)** which is the methylated derivative of **1a** furnished the quantitative NMR results more reliable and reproducible. **1a** can also undergo activation to form biradicals in aqueous media.

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4.4. Experimental

4.4.1. General procedures

The quantitative analysis of the photoreaction mixtures were performed by NMR spectroscopy using either 1, 4-dioxane or cyclohexane as an internal reference standard. These experiments were carried out on a 500 MHz NMR spectrophotometer equipped with a 3 mm triplet resonance inverse detection pulse field gradient probe operating at 499.848 MHz for ^{1}H. The spectra were accumulation of 64 individual scans. The photoproducts were assigned by comparison of their chemical shift values of that of authentic samples.

4.4.1.1. Photodecomposition of 1,4-biphenyl tetrazole-5-thione 76a

Two separate argon purged solutions of **76a** (0.7 mL) in acetonitrile was irradiated in a quartz NMR tube for 60 min at 254 nm and 300 nm respectively, and an NMR spectrum obtained on a 400 MHz NMR spectrometer.

4.4.2. Photodecomposition of 1a and 1b

4.4.2.1. By direct irradiation

Two separate solutions of **1a** and **1b** (0.7 mL) in acetonitrile-d_3, were taken in quartz NMR tubes, purged in argon for 15 min, and irradiated with broad band 254 and 300 nm UV lamp for 60 min. Subsequently, a NMR spectrum was obtained.

4.4.2.2. Triplet sensitized irradiation

Two separate solutions of **1a** and **1b** (0.7 mL) in acetonitrile-d_3, containing acetophenone and benzophenone (0.5-1equiv) respectively, were taken in quartz NMR tubes, purged in argon for 15 min, and irradiated with broad band 300 nm UV lamp for 60 min. Subsequently, a NMR spectrum was obtained.

4.4.2.3. Photodecomposition of 1a in acetone-d_6

An argon purged solution of **1a** (4 mM) in acetone-d_6 was irradiated in a quart NMR tube, and irradiated with a broad band 300 nm UV lamp for 60 min. Subsequently, a NMR spectrum was obtained.

4.4.2.4. Photodecomposition of 1a in aqueous media

Four separate solutions of **1a** (0.005 M), containing acetophenone (0.5 equiv) were prepared in a mixture of acetone-d_6 and D_2O taken in different ratios of 1, 4:1, 3:2 and 2:3 (v/v) respectively. The samples were taken in quartz NMR tubes, purged in argon for 15 min, and irradiated with broad band 300 nm UV lamp for 60 min. Subsequently, a NMR spectrum was obtained.

4.5. References

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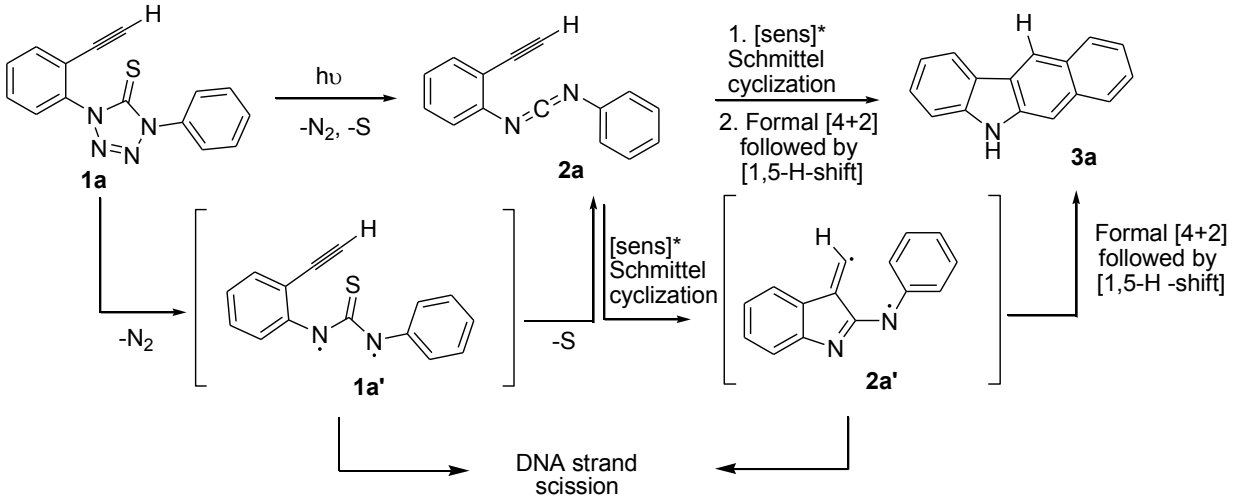
Chapter 5 - DNA photocleavage by of 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione

5.1. Introduction

DNA cleaving molecules have numerous important biochemical and biomedical applications that make the design of these compounds an interesting area of research.[1] For instance, Barton *et al.* studied the DNA conformation of simian virus T-antigen 40 using a designed metal complex tris (4,7-diphenyl-1,10-phenanthroline) rhodium (III) that specifically targets the double-stranded plasmids.[2] The knowledge about the DNA structure is important to demarcate the intron and exon boundary.[2] DNA cleaving agents are also efficiently being utilized to study the DNA-protein interactions.[3, 4] In addition, these agents have application as potential antitumor agents,[5] artificial nucleases or photonucleases,[1, 6] and in the design of gene-selective drugs.[4]

Among various DNA damaging agents, enediyne antibiotics have attracted attention because of their extremely potent antitumor activity at very low concentrations.[7] These compounds undergo thermal Bergman cyclization (BC) to generate cytotoxic biradicals that are known to abstract hydrogen atoms from the sugar phosphate DNA backbone leading to stand scission. However, the lack of tumor selectivity has limited their therapeutic applications. [8, 9] Inspired by the mechanism of action of enediynes, researchers have investigated the DNA cleaving ability of other compounds that generate biradicals *e.g.* enyne-allenes undergo Myers-Saito and Schmittel cyclization, thermally as well as photochemically, and have been shown to cleave DNA. (Scheme 1.17, page 17). [10, 11] Similar thermal and photochemical cyclizations of

their hetero analogs *e.g.* enyne-carbodiimides is also known. [12, 13] However, the DNA damaging ability from the corresponding biradicals of enyne-carbodiimides has not been explored. One obvious reason may be due to the high propensity of the electrophilic carbon in the carbodiimide functionality toward nucleophilic attack from the biomolecules that would destroy the enyne-carbodiimide system, and thus render these scaffolds incapable of forming biradicals *in vivo*. As a result, we designed masked the enyne-carbodiimides, 1-(2-ethynylphenyl)-4-phenyl tetrazole-5(4*H*)-thione **1a** in which the carbodiimide functionality was protected in a tetrazolethione ring system. We demonstrated in Chapter 4 that irradiation (300 nm) of the 1-(2-ethynylphenyl)-4-phenyl tetrazole-5(4*H*)-thiones **1a** generates enyne-carbodiimide **2a** *via* biradical **1a′**. The formation of **2a** is spontaneously followed by cyclization to indoloquinolines **3a** *via* the bsiradical **2a′** (Scheme 5.1).

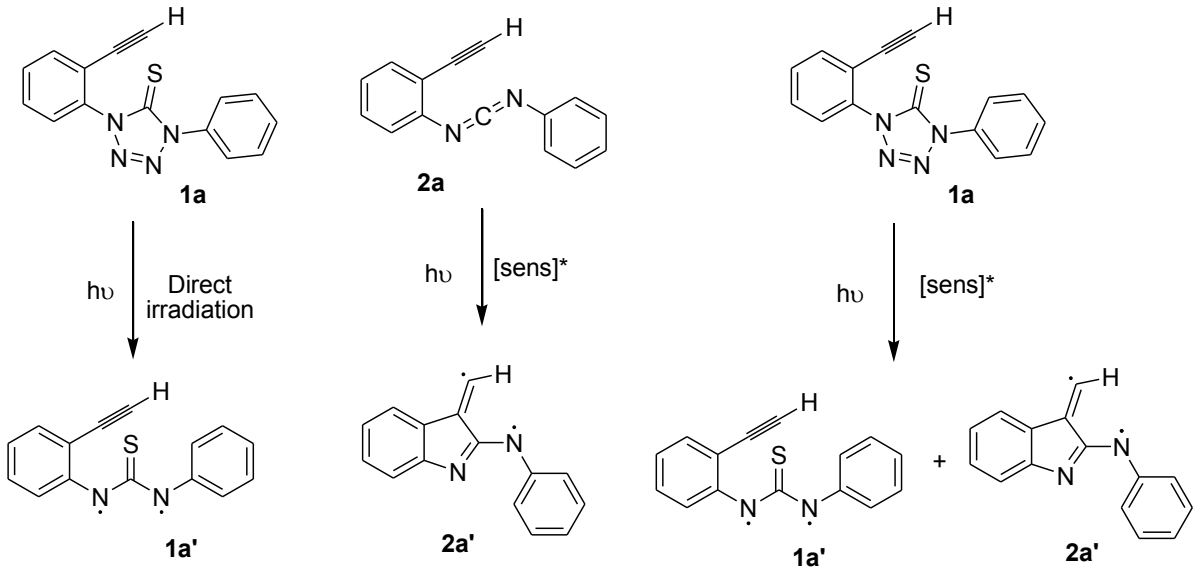


Scheme 5.1. Photodecomposition of 1-ethynyl-4-phenyl tetrazole-5(4*H*)-thione **1a**

In this chapter, our goal was to investigate the DNA cleaving ability of **1a** upon photoirradiation through the formation of biradicals **1a′** and **2a′**. The former is generated by the

photodecomposition of the tetrazolethione ring of **1a,** while the latter is formed by photochemical Schmittel cyclization of the intermediate enyne-carbodiimide **2a** as shown in Scheme 5.1. We were interested in independently evaluating the hydrogen-atom abstraction capability of the biradicals **1a′** and **2a′** as well as their combined effect on DNA photocleavage. In order to examine this, we planned three different experiments:

1. Direct irradiation of **1a** with supercoiled DNA was employed to independently study the DNA photocleavage by biradical **1a′** (since the second photochemical step (**2a→3a**) and formation of corresponding **2a′** is hindered as it requires triplet sensitization) (Scheme 5.2).
2. The enyne-carbodiimide **2a** was synthesized and photoirradiated with DNA in the presence of a triplet sensitizer to independently examine DNA photocleavage by biradical **2a′** (Scheme 5.2).
3. Sensitized irradiation of **1a** with DNA was used to probe the photocleavage by both the biradicals **1a′** and **2a′** (Scheme 5.2).

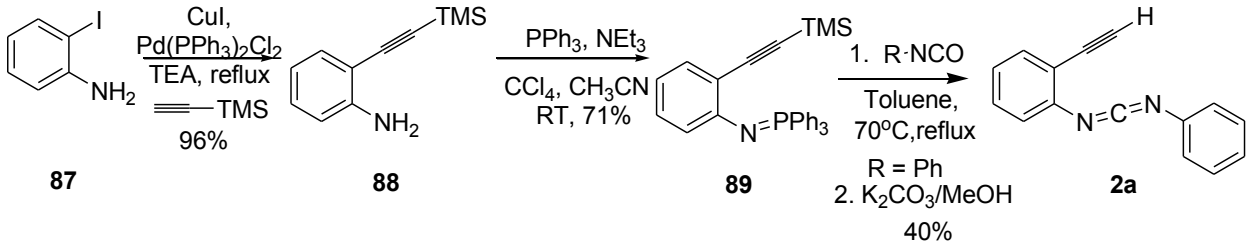


Scheme 5.2. Biradicals expected to be generated from **1a** under direct irradiation and from **2a** and **1a** under sensitized irradiation.

5.2. Results and discussion

5.2.1. Synthesis of N-((2-ethynylphenylimino)methylene)benzenamine (2a)

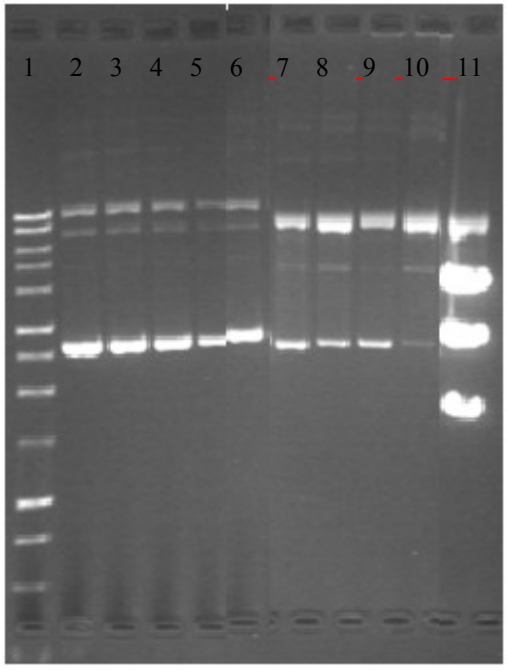
Sonogashira coupling of 2-iodobenzenamine **87** with trimethylsilylacetylene yielded 2-(2-(trimethylsilyl)ethynyl)benzenamine **(88)**.[14] Aza-wittig reaction[15] of **88** with triphenyl phosphine gave nitrogen ylide **89** in good yields. Treatment of **89** with phenyl isocyanate in refluxing toluene followed by the deprotection of the TMS group produced the expected product N-((2-ethynylphenylimino)methylene)benzenamine **(2a)** in good yields (Scheme 5.4).[15]

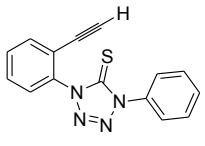


Scheme 5.3. Synthesis of enyne-carbodiimide **2a**

5.2.2. DNA photocleavage

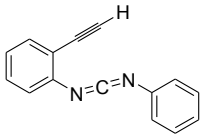
Six separate samples containing (1) super coiled DNA alone (2) super coiled DNA with acetophenone (3) supercoiled DNA with **1a** (4) supercoiled DNA with **1a** and acetophenone (5) supercoiled DNA with **2a** (6) supercoiled DNA with **2a** and acetophenone were prepared in 20% acetonitrile/water mixture. There samples were irradiated using a broad band 300 nm UV lamp for 60 min. The appropriate control experiments in dark were also carried out. The results of gel electrophoresis are shown below (Figure 5.1).





Thione

1a



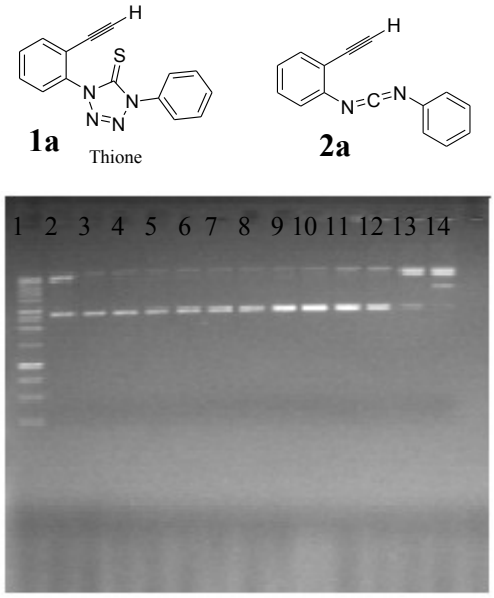
2

Lane 1: DNA Ladder;
Lane 2: DNA, 37°C, 1 h
Lane 3: DNA, **2a** (300 μM), 37°C, 1 h
Lane 4: DNA, **1a** (300 μM), 37°C, 1 h
Lane 5: DNA, **1a** (300 μM) acetophenone, 37°C 1 h
Lane 6: DNA, acetophenone, 37°C, 1 h
Lane 7: DNA alone, 300 nm, 1 h
Lane 8: DNA, **2a** (300 μM) 300 nm, 1 h
Lane 9: DNA, **1a** (300 μM) 300 nm, 1 h
Lane 10: DNA, **1a** (300 μM) acetophenone, 300 nm, 1 h
Lane 11: DNA, ECoR1, 37°C, 15 min

Figure 5.1. DNA cleavage of **1a** at 300 nm

Lane 1 is the DNA ladder. Lanes 2-6 are the unirradiated controls kept at 37°C. No DNA cleavage was observed in these samples. Irradiation of DNA alone at 300 nm produced some linear DNA (lane 7). When supercoiled DNA was irradiated with **2a** in the absence of a sensitizer (lane 8), amount of linear DNA seemed to increase slightly as compared to lane 8. This may be attributed to the low photochemical conversions of **2a** to indoloquinoline **3a** (<5%)[13] (lane 9). In lane 9, we wished to investigate the DNA cleavage by biradicals **1a′** and therefore, **1a** (300 μM) was irradiated with DNA in the absence of acetophenone. However, compared to the control (lane 8), no noticeable DNA damage could be detected in this lane. Almost complete photocleavage of the DNA was observed with **1a** in the presence of acetophenone which may be due to the formation of biradicals **1a′** and **2a′**, and subsequent H-atom abstraction for the DNA (lane 10). ECoR1 acted as a positive control for DNA cleavage

(lane 13). Since the irradiation of DNA alone at 300 nm produced damage (lane 7), we decided to change the irradiation wavelength to 350 nm for all the future experiments.



1a Thione

2a

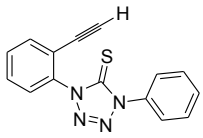
Lane 1: DNA Ladder
Lane 2: DNA, Fe-EDTA-ascorbate, 60 min
Lane 3: DNA alone, 37°C, 2 h
Lane 4: DNA, acetophenone, 37°C, 2 h
Lane 5: DNA, **2a** (300 μM), 37°C, 2 h
Lane 6: DNA, **2a** (300 μM) acetophenone, 37°C, 2 h
Lane 7: DNA, **1a** (300 μM) 37°C, 2 h
Lane 8: DNA, **1a** (300 μM) acetophenone, 37°C, 2 h
Lane 9: DNA alone, 350 nm, 2 h
Lane 10: DNA, acetophenone, 350 nm, 2 h
Lane 11: DNA, **2a** (300 μM) 350 nm, 2 h
Lane 12: DNA, **2a** (300 μM), acetophenone 350 nm, 2 h
Lane 13: DNA, **1a** (300 μM) 350 nm, 2 h
Lane 14: DNA, **1a** (300 μM) acetophenone, 350 nm, 2 h

Figure 5.2. DNA cleavage of **1a** at 350 nm

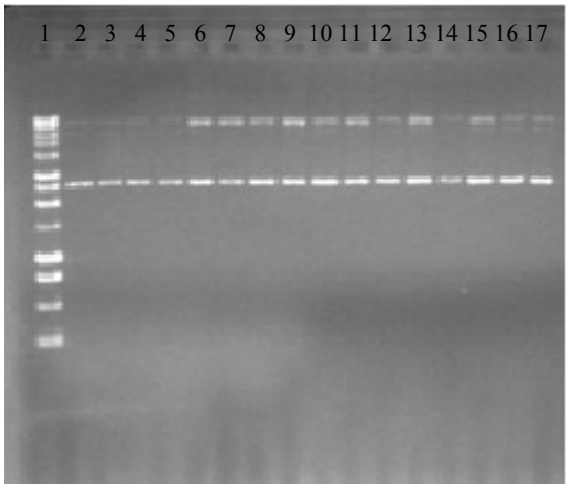
DNA ladder is seen in lane 1. Fe-EDTA-ascorbate complex was used as a positive control for DNA cleavage (lane 2). Lanes 3-8 are the unirradiated samples and no DNA damage was observed in these samples as expected. DNA remained in supercoiled form when irradiated alone at 350 nm in the absence of compounds (lane 9). Photolysis of DNA with **2a** (300 μM) in the absence of acetophenone induced some DNA damage which may be attributed to low photochemical conversions of **2a** as discussed above (lane 11). Irradiation of DNA with **2a** in the presence of acetophenone produced cleavage (lane 12), however, the extent of cleavage was not very different from that observed in lane 11. At this time, the reason for this discrepancy is not clear as we expected **2a** to efficiently generate Schmittel biradical **2a′** at 350 nm in the presence of acetophenone leading to enhanced DNA cleavage in lane 12.[13] Significant cleavage of

circular super-coiled DNA was observed when the samples containing DNA and **1a** (300 μM) were irradiated at 350 nm in the absence of acetophenone (lane 13). This may be attributed to DNA cleavage by biradicals **1a′**. Notably, irradiation of supercoiled DNA with **1a** and acetophenone produced nicked and linear DNA (lane 14). This demonstrates that both the biradicals **1a′** and **2a′** have ability to cause DNA damage.

Next, we assayed the DNA photo cleaving ability of **1a** in the concentration range 300 – 3 μM in the absence and presence of a triplet sensitizer to investigate the effect of biradicals **1a′** and **2a′,** respectively and the results of the gel electrophoresis are shown in Figure 5.3.



1a



Lane 1: DNA Ladder
Lane 2: DNA alone, 37°C, 2 h
Lane 3: DNA, acetophenone, 37°C, 2 h
Lane 4: DNA alone, 350 nm, 2 h
Lane 5: DNA, acetophenone, 350 nm, 2 h
Lane 6: DNA, **1a** (300 μM) 350 nm, 2 h
Lane 7: DNA, **1a** (300 μM) acetophenone, 350 nm, 2 h
Lane 8: DNA, **1a** (200 μM) 350 nm, 2 h
Lane 9: DNA, **1a** (200 μM) acetophenone, 350 nm, 2 h
Lane 10: DNA, **1a** (150 μM) 350 nm, 2 h
Lane 11: DNA, **1a** (150 μM) acetophenone, 350 nm, 2 h
Lane 12: DNA, **1a** (100 μM) 350 nm, 2 h
Lane 13: DNA, **1a** (100 μM) acetophenone, 350 nm, 2 h
Lane 14: DNA, **1a** (50 μM) 350 nm, 2 h
Lane 15: DNA, **1a** (50 μM) acetophenone, 350 nm, 2 h
Lane 16: DNA, **1a** (3 μM), 350 nm, 2 h
Lane 17: DNA, **1a** (3 μM), acetophenone, 350 nm, 2 h

Figure 5.3. DNA cleavage by **1a** at 350 nm: Concentration profile

Lane 1 is the DNA ladder. Lanes 2 and 3 are the unirradiated samples. Lanes 4 and 5 are the irradiated controls. Lanes 6 – 17 show results of irradiation of DNA with varying concentrations of **1a** (300, 200, 150, 100, 50 and 3μM) in the absence and presence of

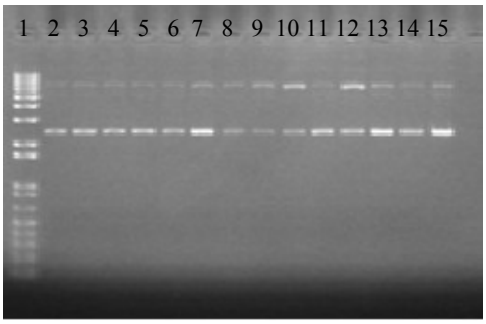
acetophenone. As expected, amount of DNA cleavage reduced as the concentration of **1a** was decreased both under direct and sensitized irradiation.

In order to mimic the physiological conditions, we also investigated DNA cleavage by **1a** in phosphate buffer at pH 7.4 under direct and sensitized irradiation. However, no significant DNA strand scission was observed. Therefore, we replaced the phosphate buffer with its non-nucleophilic version, the arsenate or cacodylate buffer (pH 7.4). To our dismay, no DNA cleavage was seen. The absence of DNA damage in buffers was frustrating and more experiments needed to be carried out to explain this behavior.

5.2.3. Mechanism of DNA cleavage

The mechanism of DNA cleavage by **1a** is expected to involve a biradical mechanism generating biradicals **1a′** and **2a′** that are expected to abstract hydrogen atoms from the sugar phosphate DNA backbone resulting into strand scission (Scheme 5.1). In order to verify this mechanism, we performed the irradiation experiments in the presence of a hydrogen atom donor such as 1,4-cyclohexadiene (1,4-CHD). If the DNA cleavage discussed above is indeed caused by the biradicals **1a′** and **2a′**, then the increasing concentration of 1,4-CHD should be able to reduce the extent of cleavage. Therefore, we carried out the experiments with varying concentrations of 1,4-CHD and gel is shown in Figure 5.4.

We believe that we had some error in loading the gel, especially, lanes 8 – 10 which were controls and therefore, no conclusive data could be obtained from this experiment. On a positive note, comparison of lanes 12 and 14 show that as the concentration of 1,4-CHD is increased, the extent of DNA cleavage seem to reduced.



Lane 1: DNA Ladder
Lane 2: DNA alone, 37^{O}C, 2 h
Lane 3: DNA, acetophenone, 37^{O}C, 2 h
Lane 4: DNA, 1,4-CHD 37^{o}C, 2 h
Lane 5: DNA, **1a** (300 μM), 1,4-CHD, 37^{O}C, 2 h
Lane 6: DNA, **1a** (300 μM), 1,4-CHD, incubated 37^{O}C, 2 h
Lane 7: DNA alone, 350 nm, 2 h
Lane 8: DNA, acetophenone, 350 nm, 2 h
Lane 9: DNA, **1a** (300 μM), 350 nm, 2 h
Lane 10: DNA, **1a** (300 μM), acetophenone, 350 nm, 2 h
Lane 11: DNA, 1,4-CHD, 350 nm, 2 h
Lane 12: DNA, **1a** (300 μM), 1,4-CHD (0.6 μM), 350 nm , 2 h
Lane 13: DNA, **1a** (300 μM), acetophenone, 1,4-CHD (0.6 μM), 350 nm, 2 h
Lane 14: DNA, **1a** (300 μM), 1,4-CHD (1 μM), irradiated, 350 nm , 2 h
Lane 15: DNA, **1a** (300 μM), Sensitizer, 1,4-CHD (1 μM), 350 nm, 2 h

Figure 5.4. Mechanism of photo DNA cleavage of **1a**

5.3. Conclusion

The compound 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (**1a)** caused DNA damage in the concentration as low as 100 μM. The experiments with 1,4-CHD showed that the mechanism of photocleavage of **1a** may involve the generation of cytotoxic biradicals. However, further investigations have to be carried out to completely understand the mechanism of photocleavage.

5.4. Experimental

5.4.1. DNA cleavage of 1-(2-ethynylphenyl)-4-phenyl-1H-tetrazole-5(4H)-thione (1a)

The DNA cleavage experiments were performed using KS^{+} blue script super coiled DNA in a volume of 25 μL. Acetophenone was used as the triplet sensitizer for the irradiations. Ultra pure water was used to make the solutions. The irradiations were carried out using Rayonet UV

reactor at 300 nm and 350 nm independently for 2 hours and the results of the experiment were analyzed using 1% agarose gel electrophoresis and detected with ethidium bromide. The electrophoresis gels were immediately visualized on a UV transilluminator and photographed using black and white instant film.[16]

5.4.1.1. DNA cleavage in water

Separate reaction samples containing the tetrazolethione **1a** (300 μM) and the enyne-carbodiimide **2a** (300 μM) with KS^+ blue script super coiled DNA (450 ng) in a volume of 25 μL containing 20% acetonitrile in ultra pure water (pH 7.6) at 25^{o}C. The irradiations were performed using the conditions described in section 5.5.3.

5.4.1.2. DNA cleavage in buffers

Separate reaction samples containing the tetrazolethione **1a** (300 μM) and the enyne-carbodiimide **2a** (300 μM) with KS^+ blue script super coiled DNA (450 ng) in a volume of 25 μL containing 20% acetonitrile in phosphate buffer (pH 7.4) at 25^{o}C. The same solutions were prepared in cacodylate buffer (pH 7.4). The irradiations were performed using the same conditions described above (section 5.5.3).

5.4.2. Concentration profile

The DNA was treated with **1a** in the concentrations of 300, 200, 150, 100, 50, 30, 10, and 3mM with photoirradiation at 25^{o}C for 2 h with a UV reactor (350 nm, 30 W) placed 10 cm from the mixture.

5.5. References

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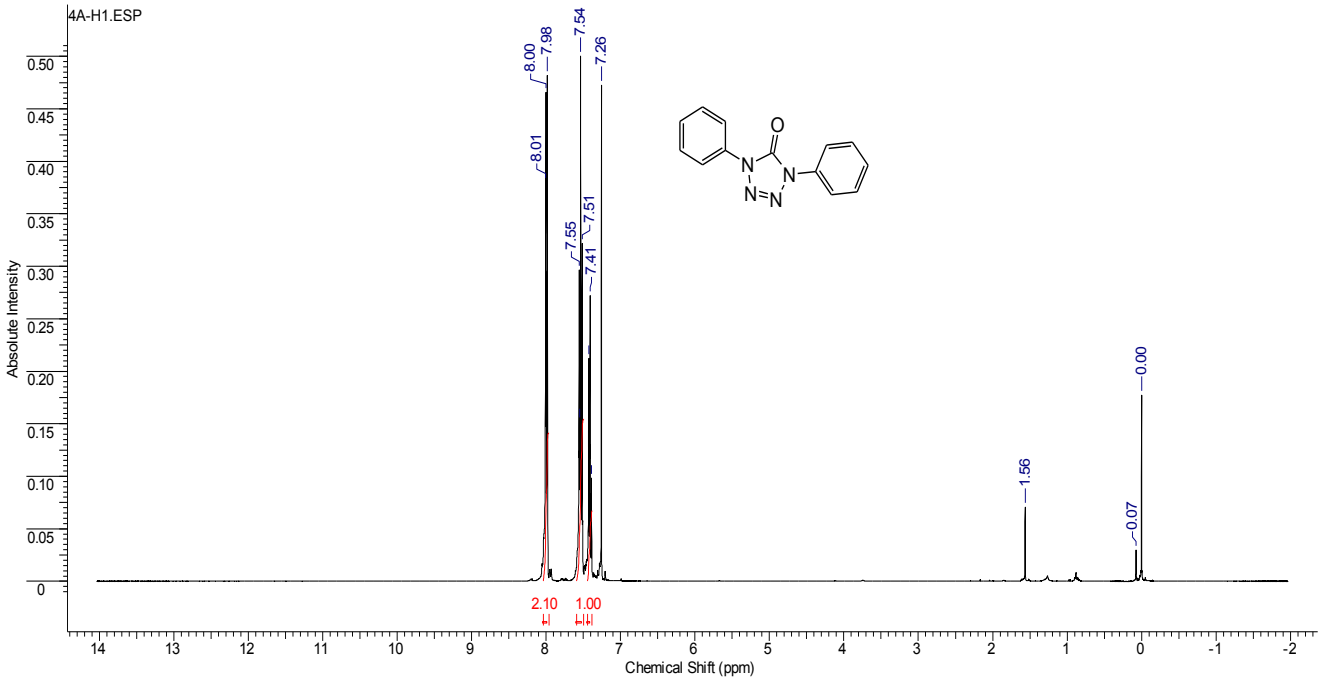
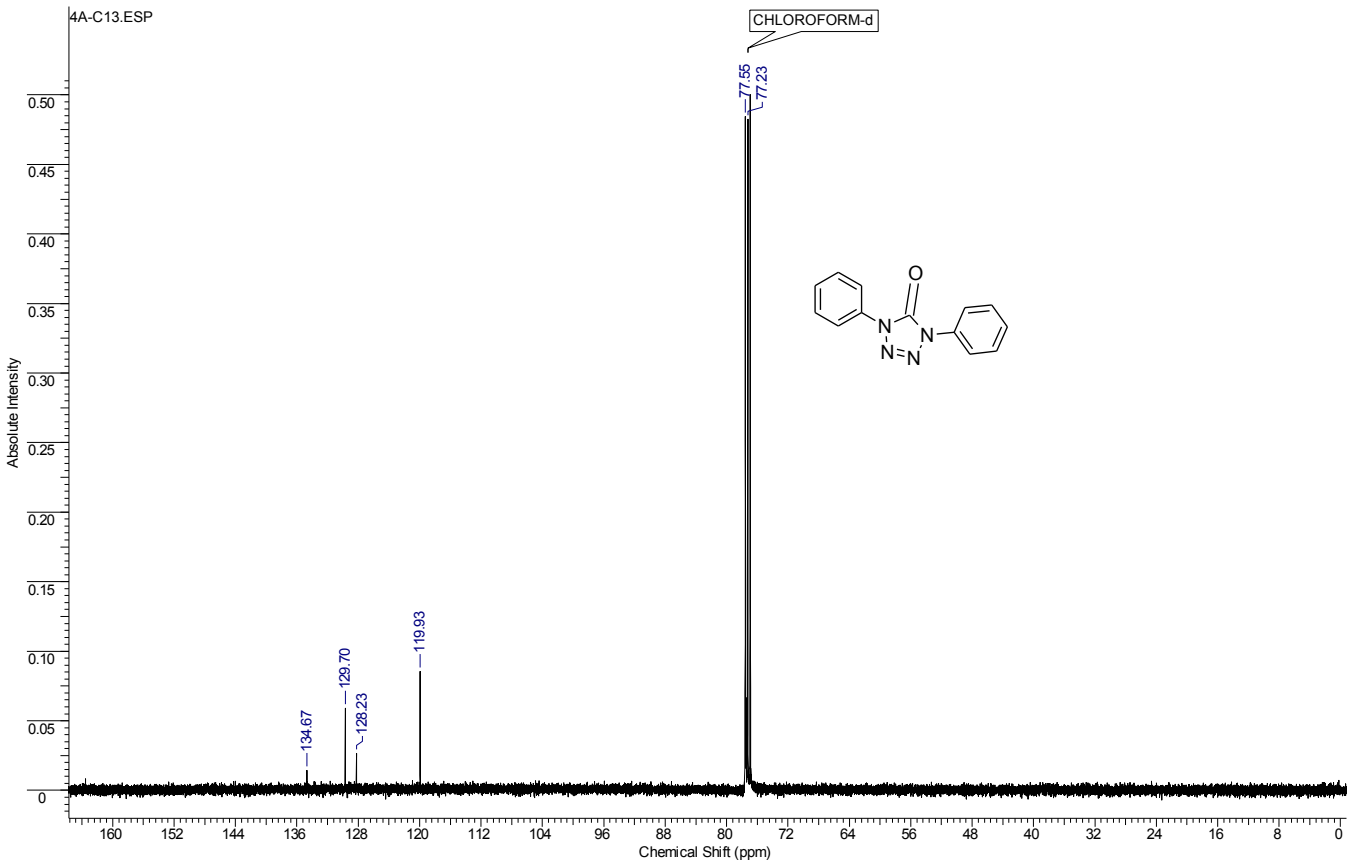
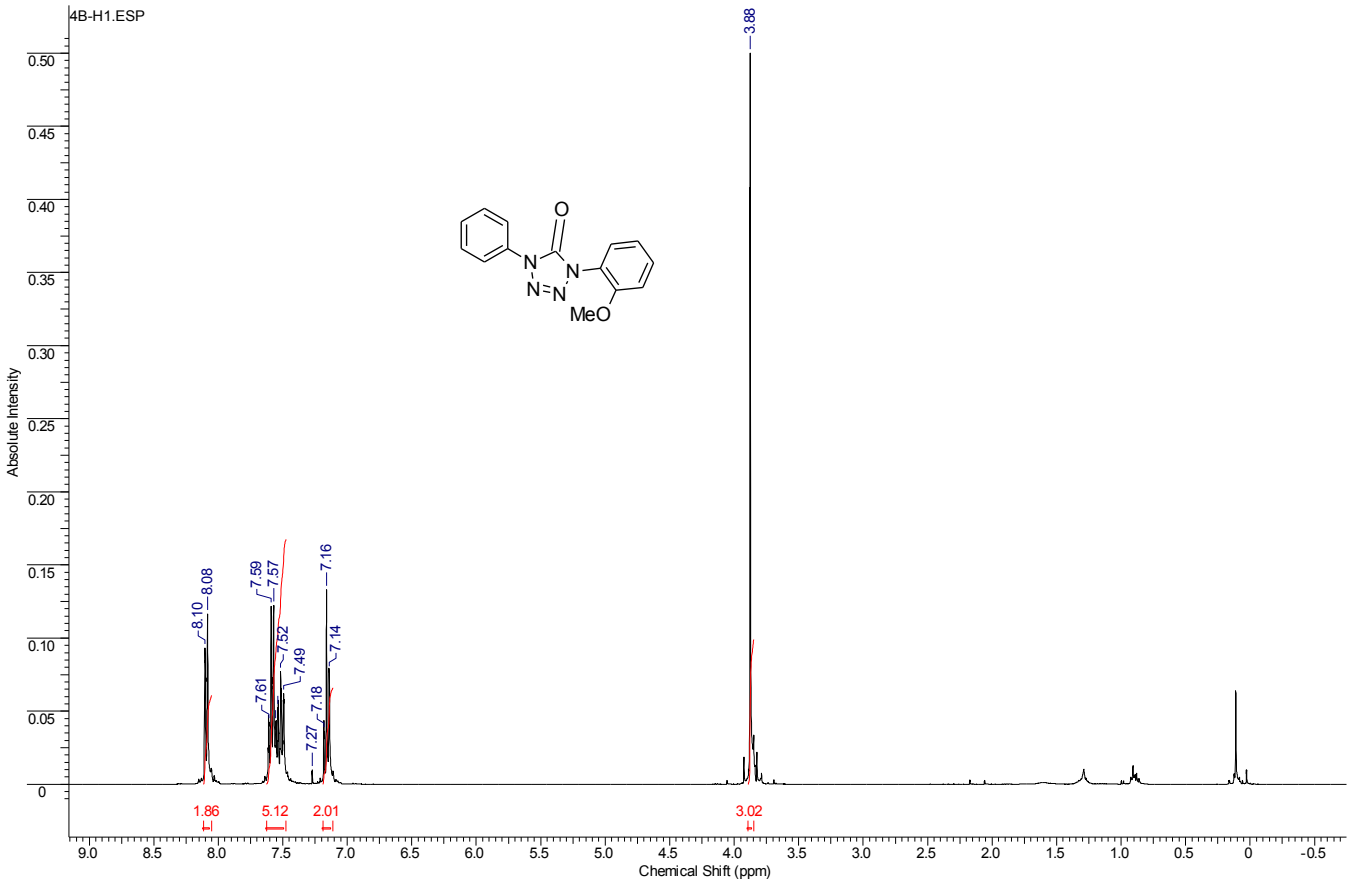
Appendix A – ^{1}H and ^{13}C spectra

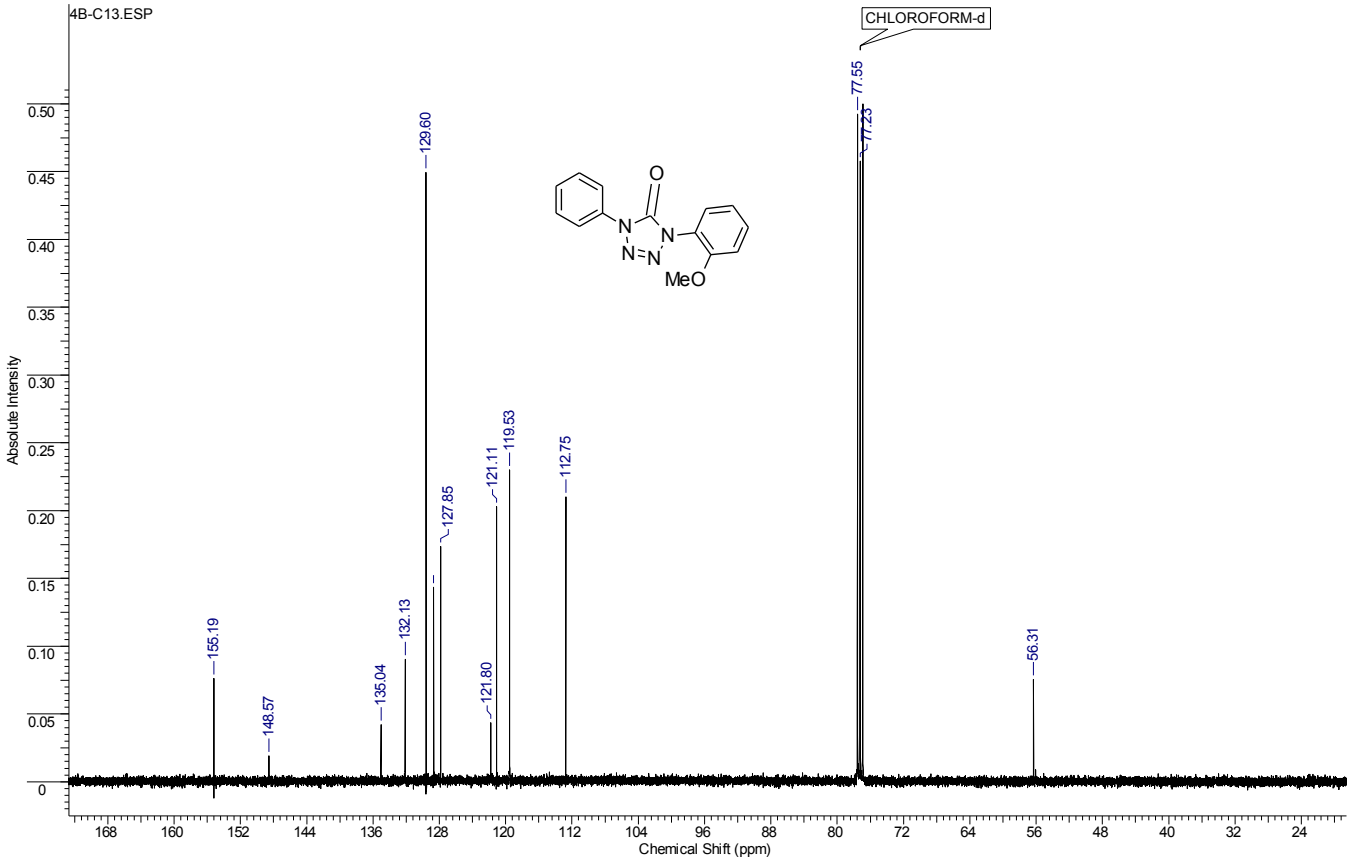
A 1. Chapter 2

Synthesis of 1,4-diaryl tetrazol-5-ones and 1,4-diaryl tetrazole-5-thiones




4A-C13.ESP
CHLOROFORM-d
77.55
77.23
134.67
129.70
128.23
119.93
Absolute Intensity
Chemical Shift (ppm)


4B-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
8.10
8.08
7.61
7.59
7.57
7.52
7.49
7.27
7.18
7.16
7.14
3.88
1.86
5.12
2.01
3.02
O
N
N
N
MeO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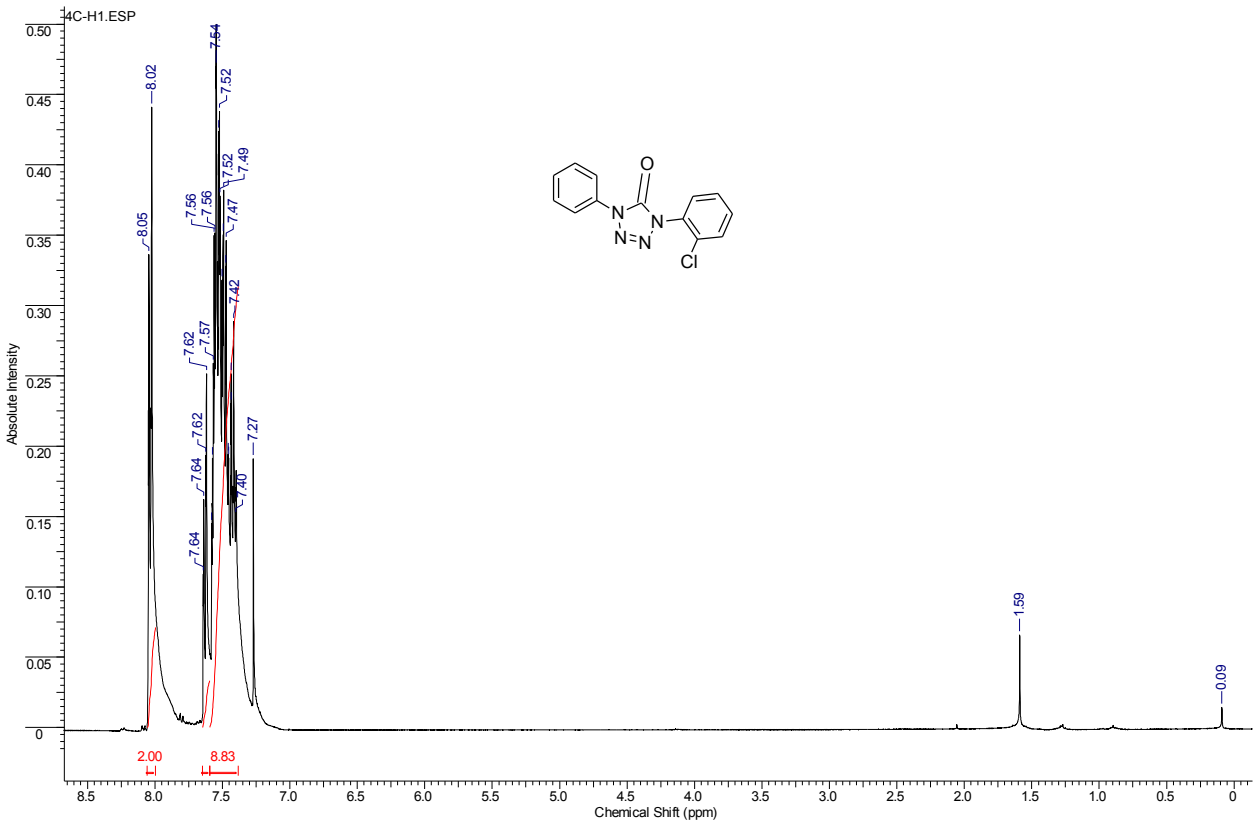
CHLOROFORM-d
77.55
77.23
129.60
155.19
148.57
135.04
132.13
127.85
121.80
121.11
119.53
112.75
56.31
O
N
N
N
N
MeO
Absolute Intensity
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
168
160
152
144
136
128
120
112
104
96
88
80
72
64
56
48
40
32
24
Chemical Shift (ppm)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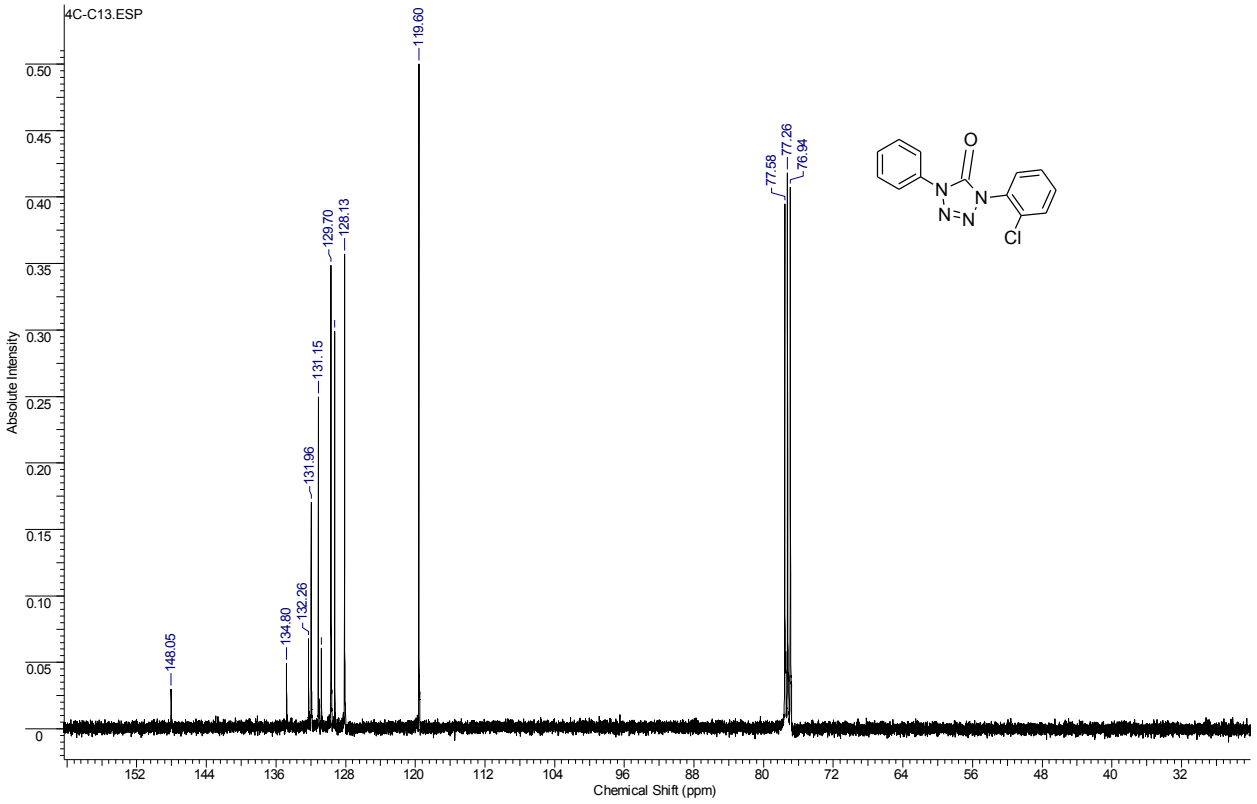
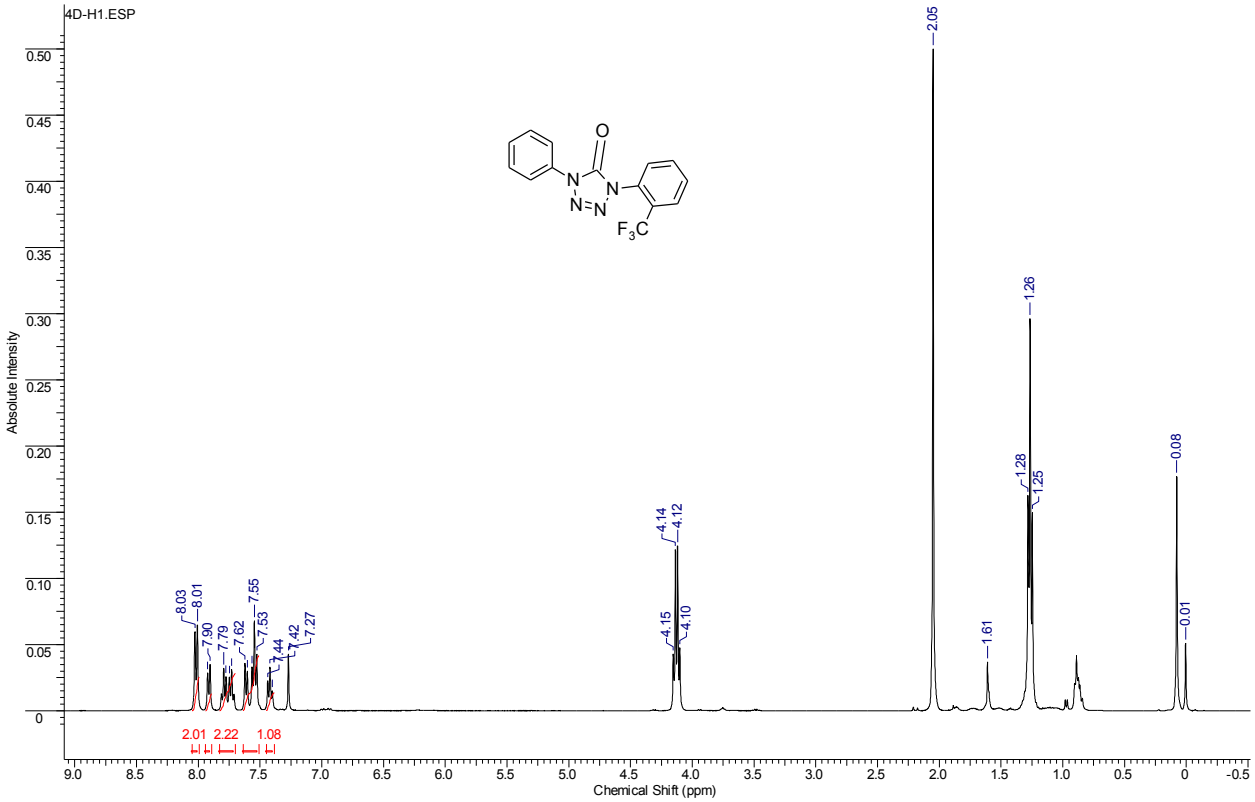
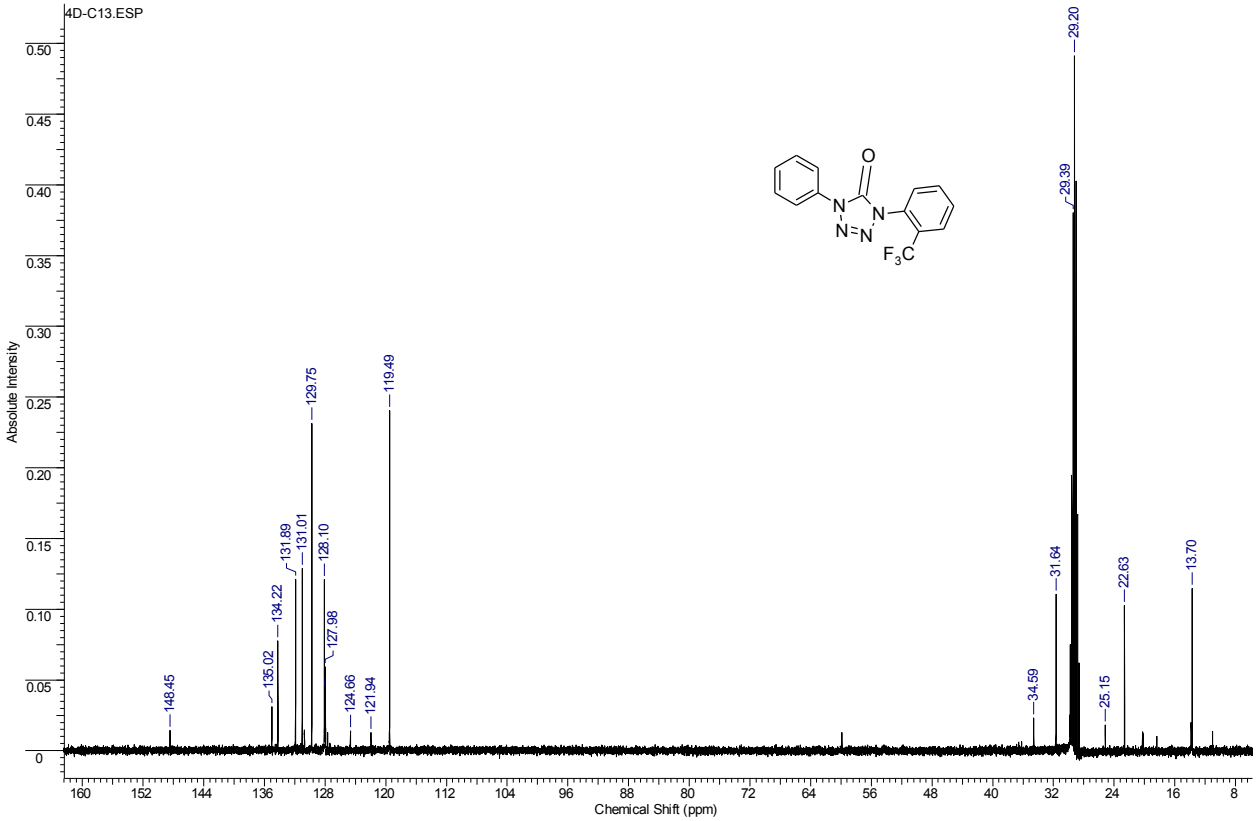
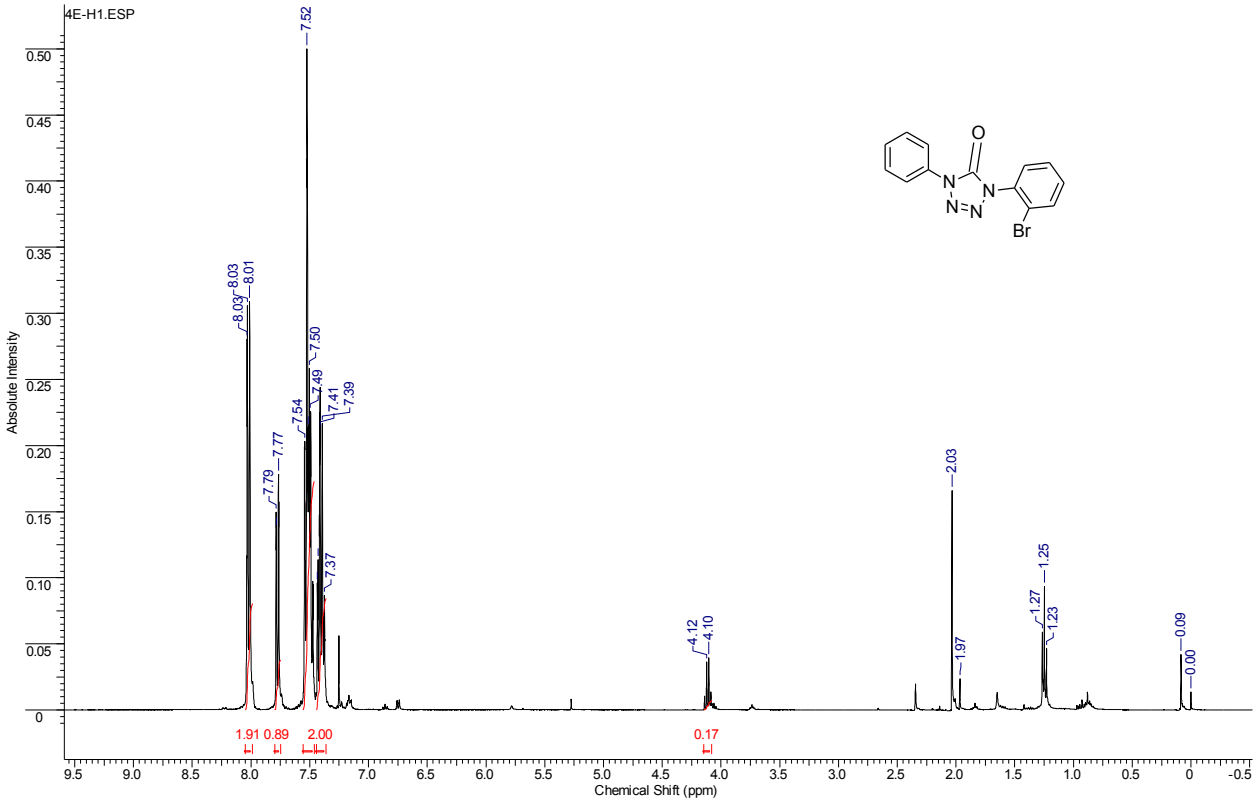
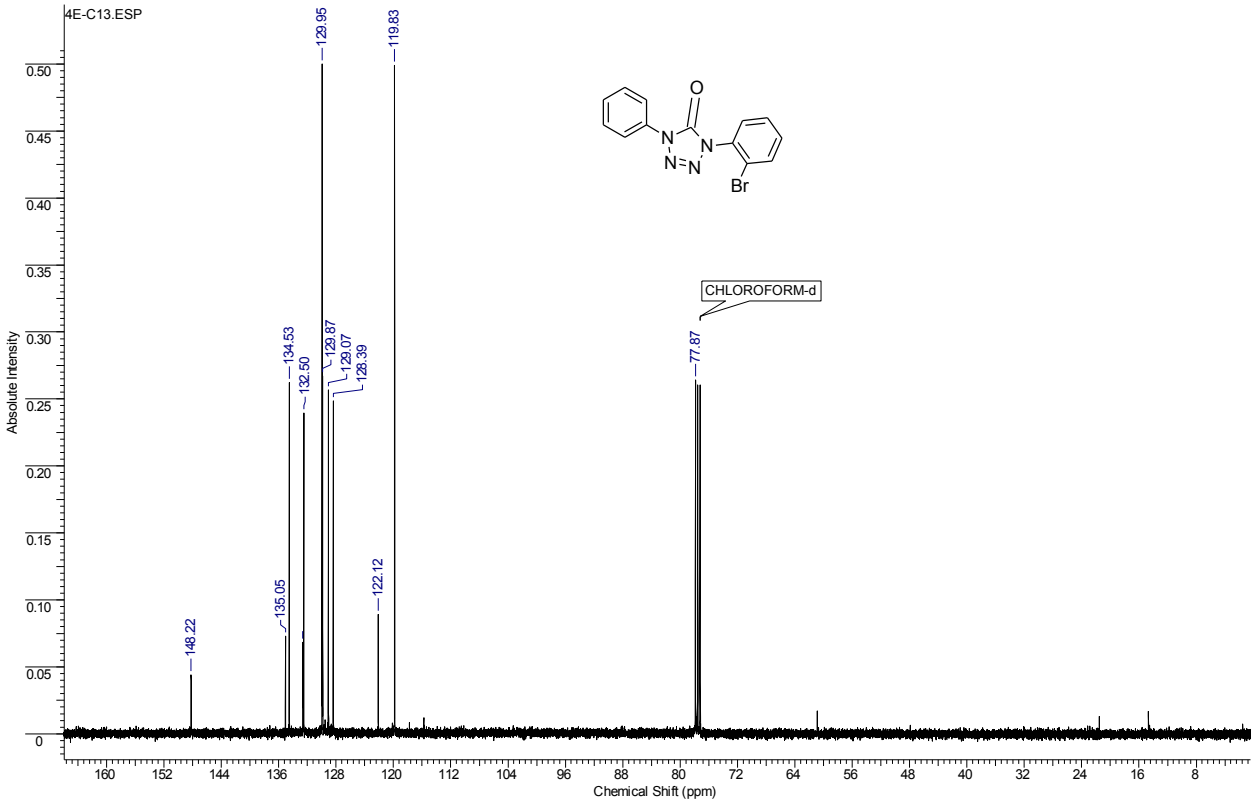
Absolute Intensity
Chemical Shift (ppm)
8.02
8.05
7.64
7.62
7.57
7.56
7.52
7.49
7.47
7.42
7.27
7.40
1.59
0.09
2.00
8.83
O
N
N
N
N
Cl

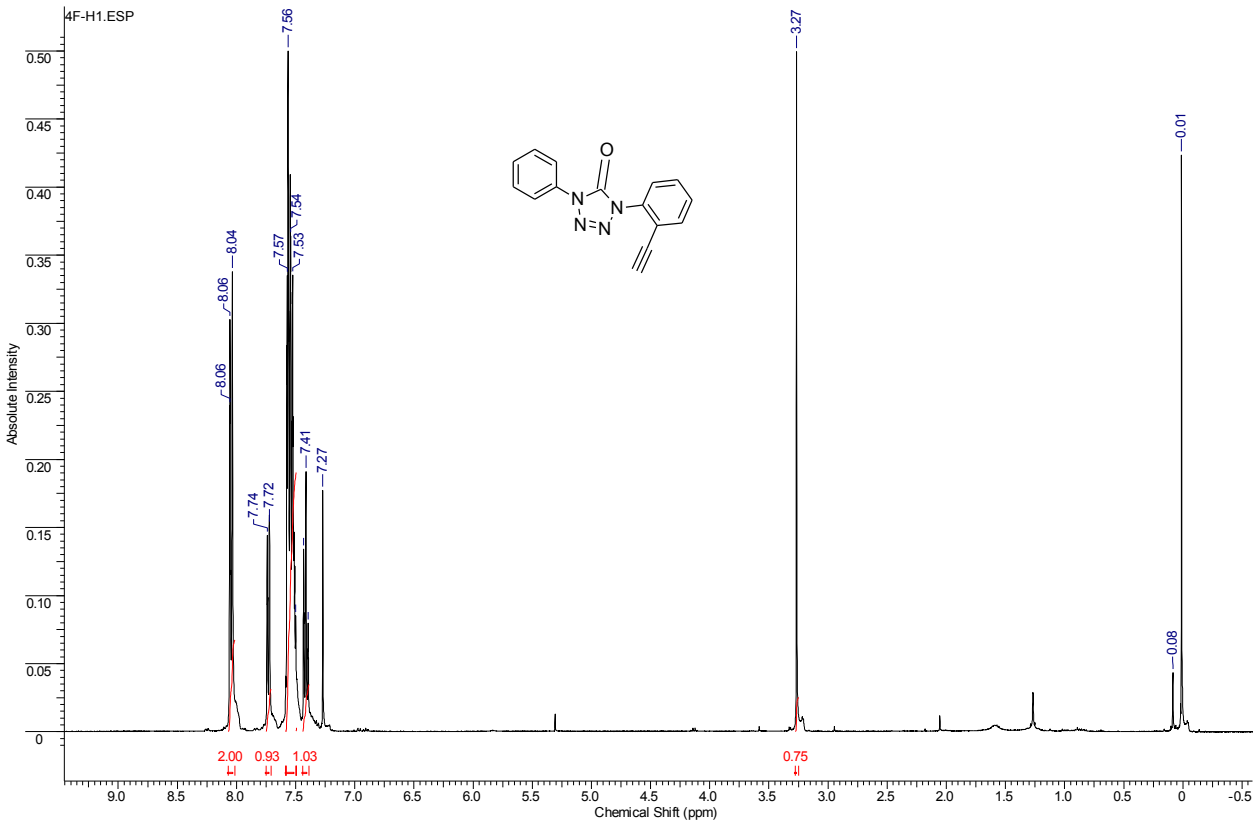

4C-C13.ESP
Absolute Intensity
Chemical Shift (ppm)
119.60
77.58
77.26
76.94
129.70
128.13
131.15
131.96
134.80
132.26
148.05
O
N
N
N
N
Cl

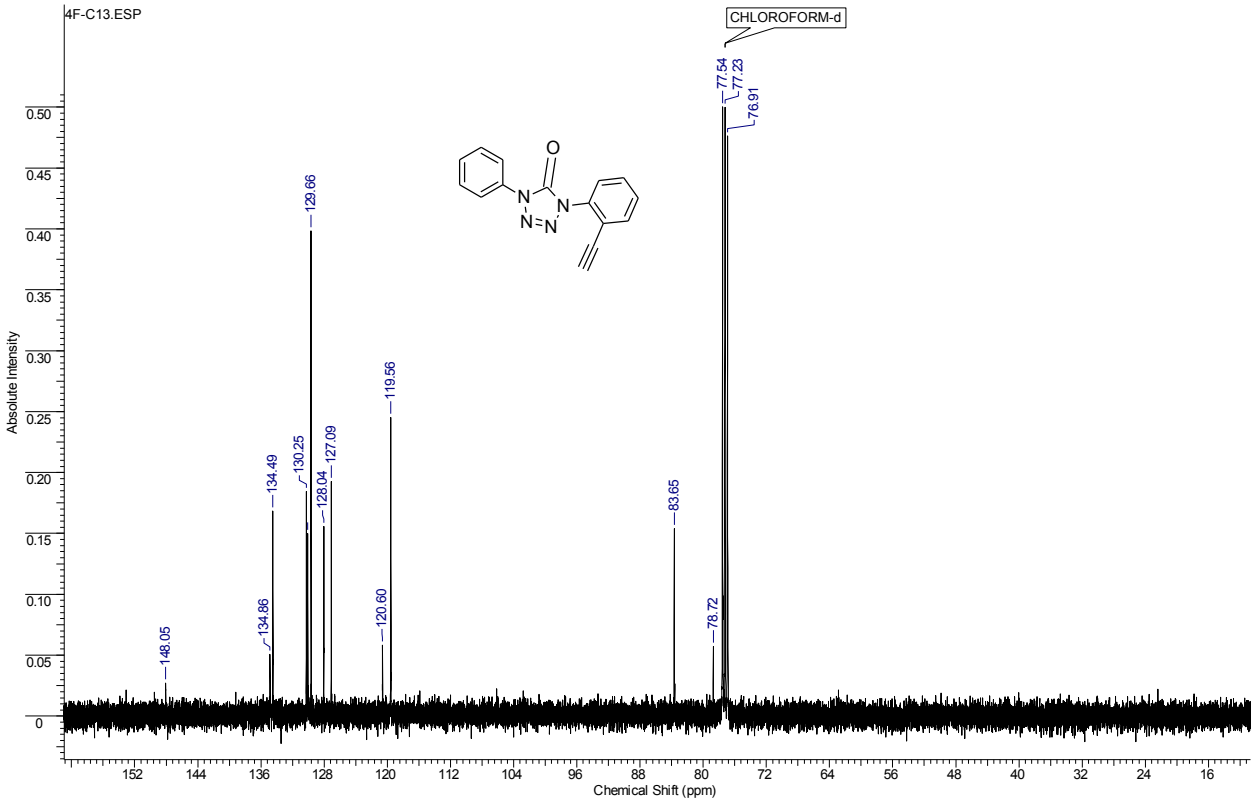

4D-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
8.03
8.01
7.90
7.79
7.62
7.55
7.53
7.44
7.42
7.27
4.14
4.12
4.15
4.10
2.05
1.61
1.26
1.28
1.25
0.08
0.01
2.01 2.22 1.08


4D-C13.ESP
Absolute Intensity
Chemical Shift (ppm)
148.45
135.02
134.22
131.89
131.01
129.75
128.10
127.98
124.66
121.94
119.49
34.59
31.64
29.39
29.20
25.15
22.63
13.70


4E-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
8.03
8.01
7.79
7.77
7.54
7.52
7.50
7.49
7.41
7.39
7.37
4.12
4.10
2.03
1.97
1.27
1.25
1.23
0.09
0.00
1.91
0.89
2.00
0.17
O
N
N
N
Br


4E-C13.ESP
Absolute Intensity
Chemical Shift (ppm)
129.95
119.83
134.53
132.50
129.87
129.07
128.39
77.87
CHLOROFORM-d
148.22
135.05
122.12
O
N
N
N
Br


4F-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
7.56
8.04
8.06
8.06
7.57
7.54
7.53
7.74
7.72
7.41
7.27
3.27
0.01
0.08
2.00
0.93
1.03
0.75


4F-C13.ESP
CHLOROFORM-d
Absolute Intensity
Chemical Shift (ppm)
129.66
119.56
134.49
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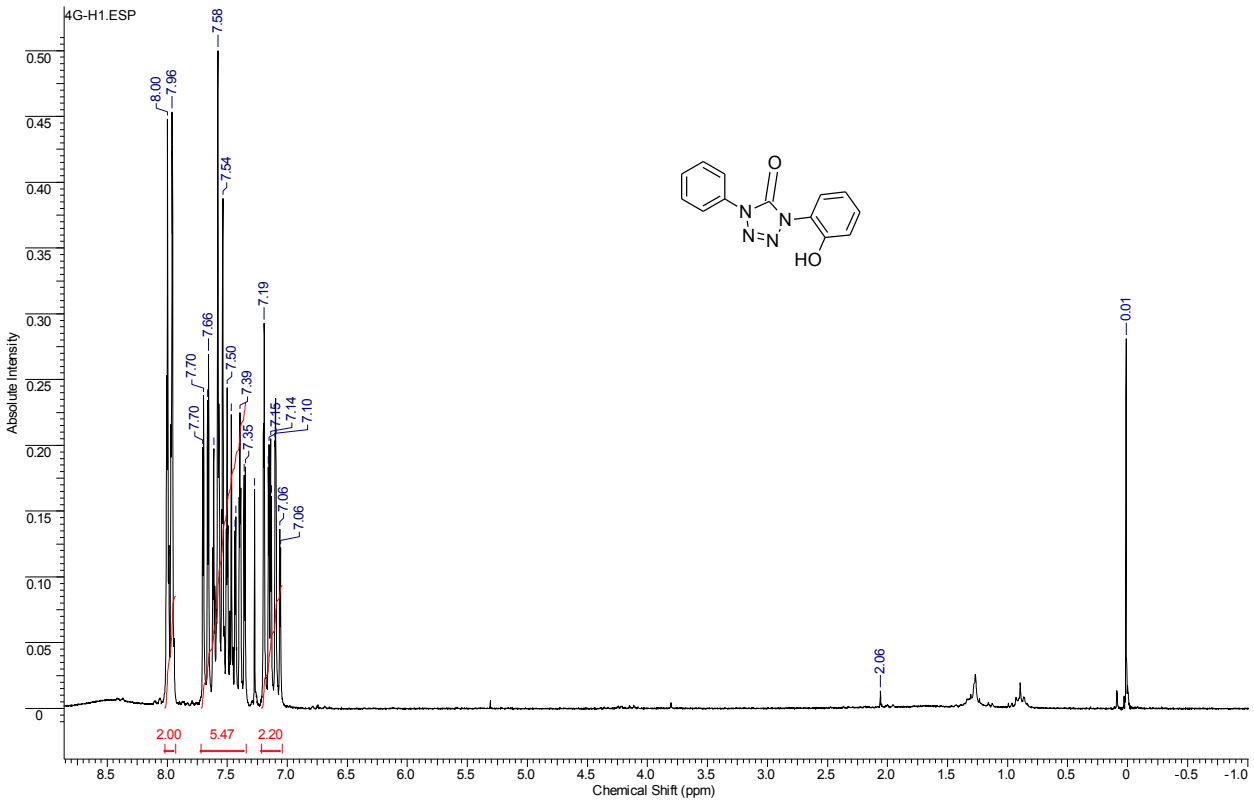
128.04
127.09
77.54
77.23
76.91
83.65
148.05
134.86
120.60
78.72


4G-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
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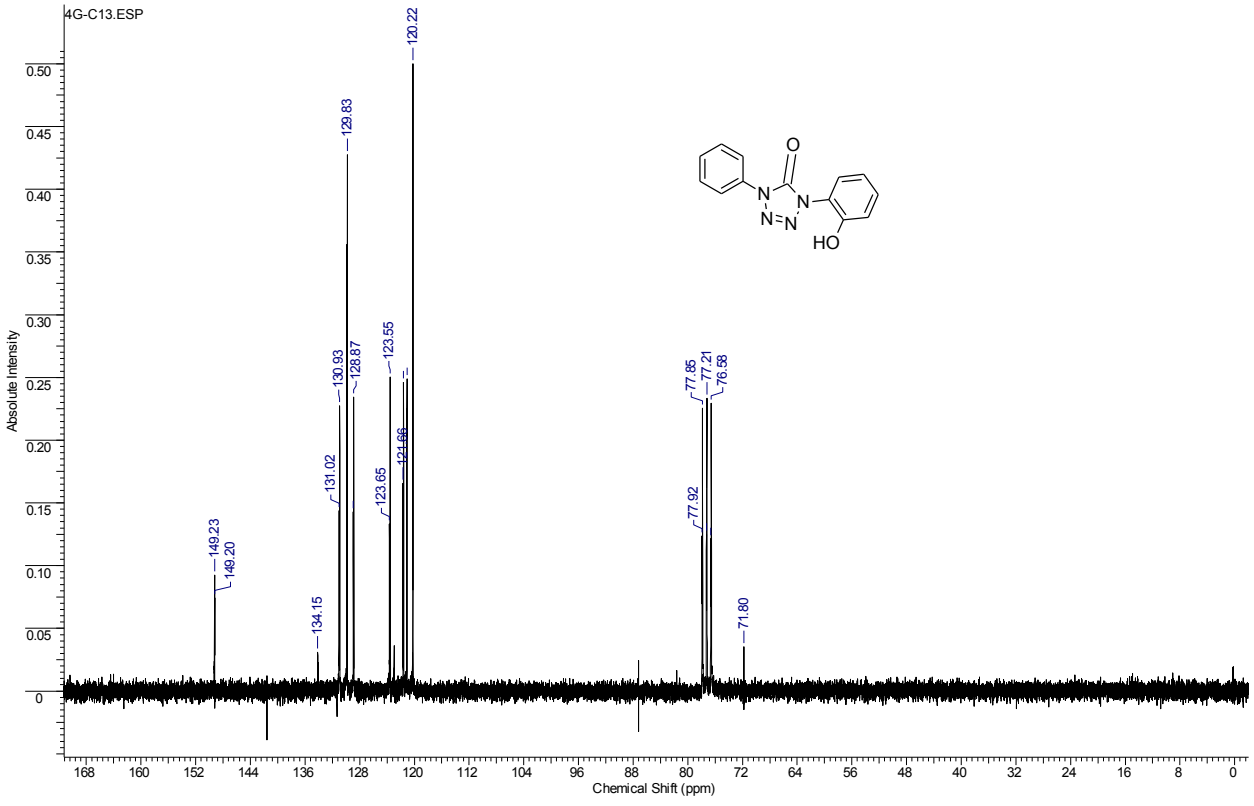
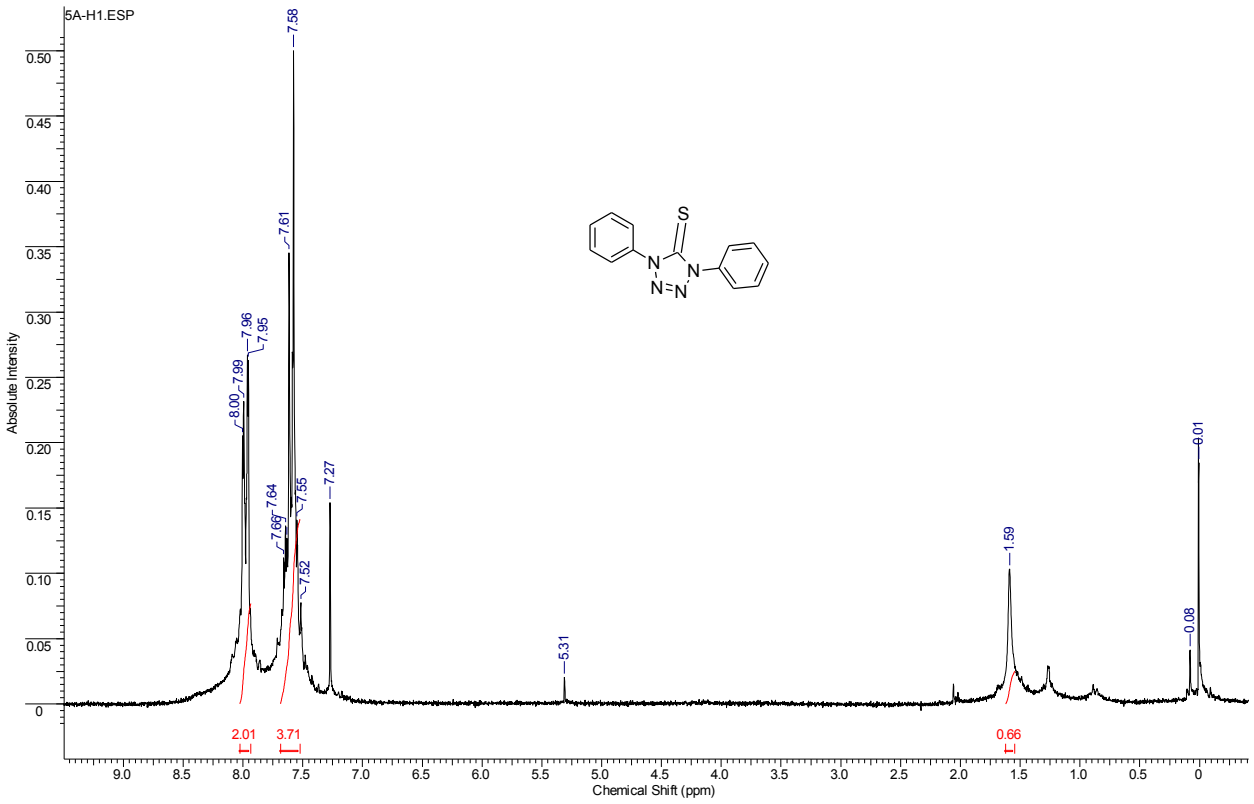
7.96
7.58
7.54
7.70
7.70
7.66
7.50
7.39
7.35
7.19
7.15
7.14
7.10
7.06
7.06
2.06
0.01
2.00
5.47
2.20
O
N
N
N
HO


4G-C13.ESP
Absolute Intensity
Chemical Shift (ppm)
149.23
149.20
134.15
131.02
130.93
129.83
128.87
123.65
123.55
121.66
120.22
77.85
77.21
76.88
77.92
71.80
O
N
N
N
HO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Absolute Intensity
Chemical Shift (ppm)
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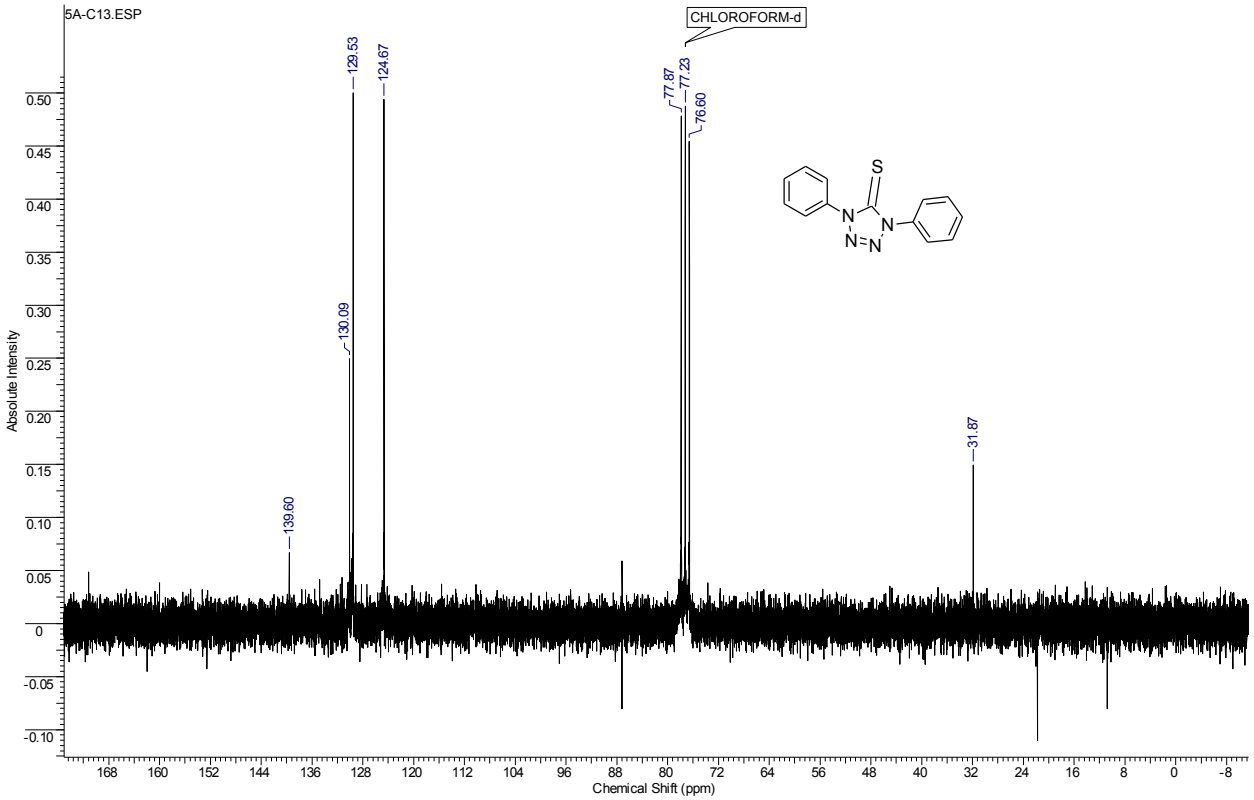
7.61
7.96
7.95
7.99
8.00
7.64
7.69
7.55
7.52
7.27
5.31
1.59
9.01
0.08
2.01
3.71
0.66
S
N
N
N=N


5A-C13.ESP
CHLOROFORM-d
Absolute Intensity
Chemical Shift (ppm)
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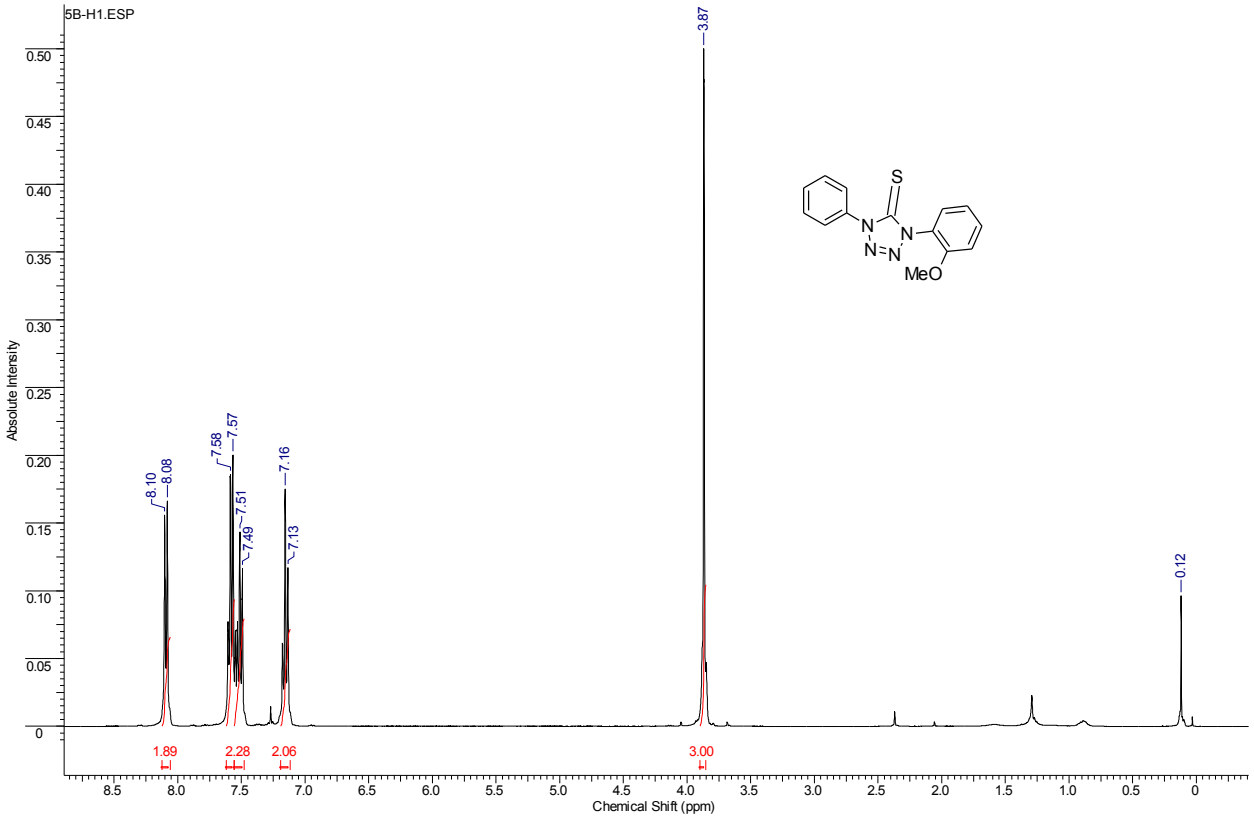
124.67
77.87
77.23
76.60
130.09
139.60
31.87
S
N
N
N=N


5B-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
8.10
8.08
7.58
7.57
7.51
7.49
7.16
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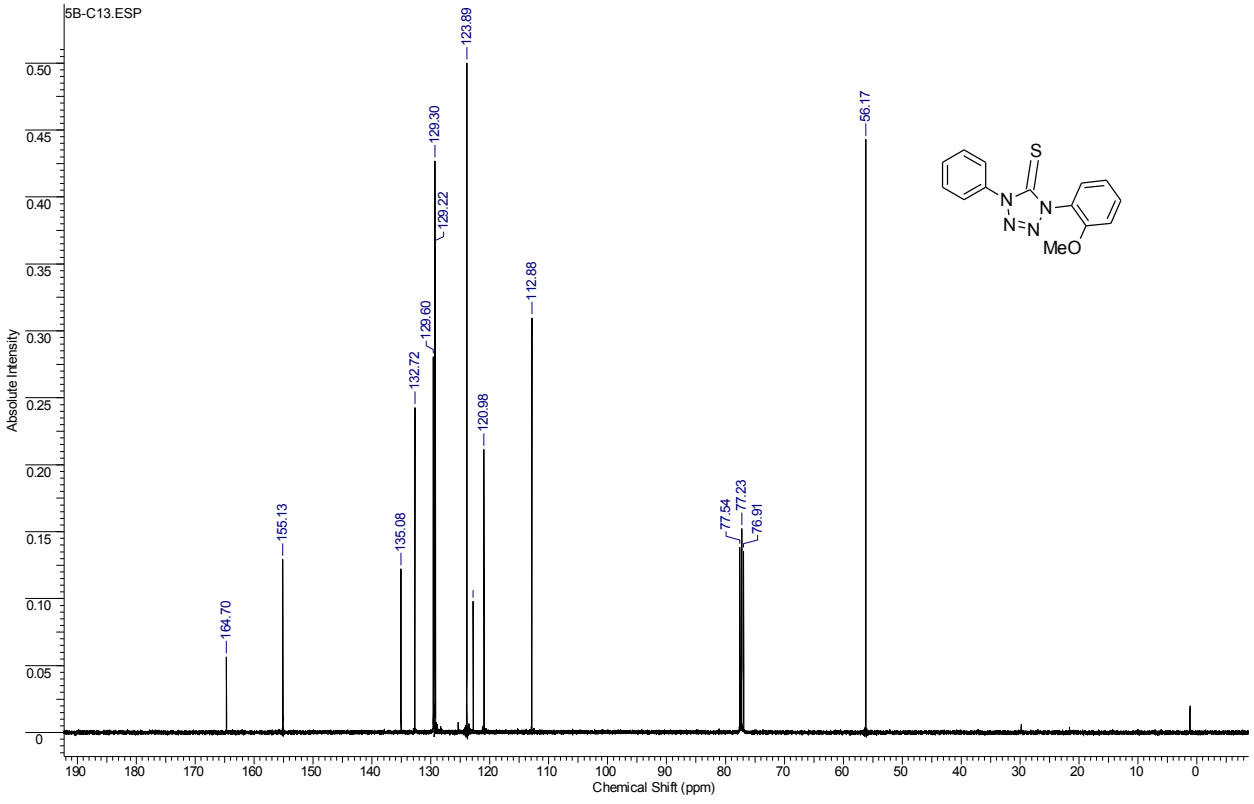
3.87
0.12
1.89
2.28
2.06
3.00
S
N
N
N
N
MeO


5B-C13.ESP
Absolute Intensity
Chemical Shift (ppm)
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135.08
132.72
129.60
129.30
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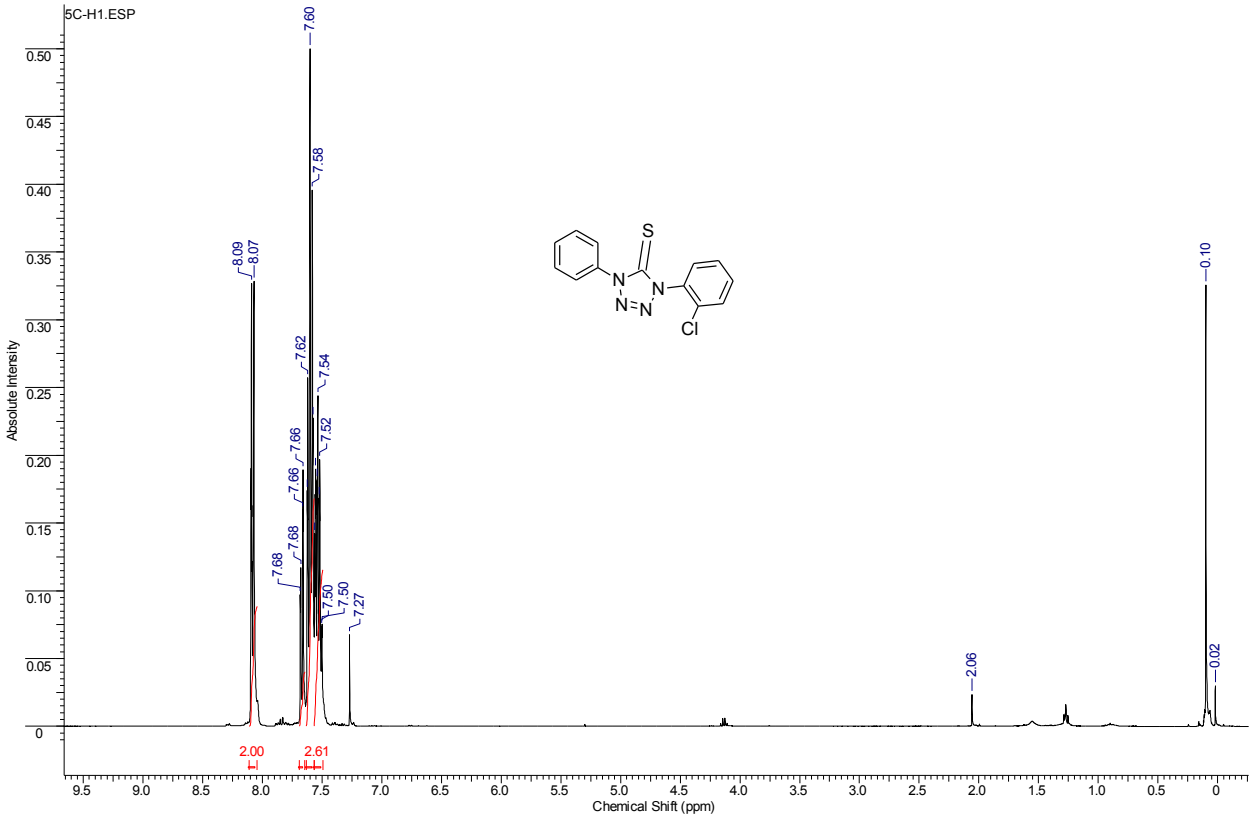
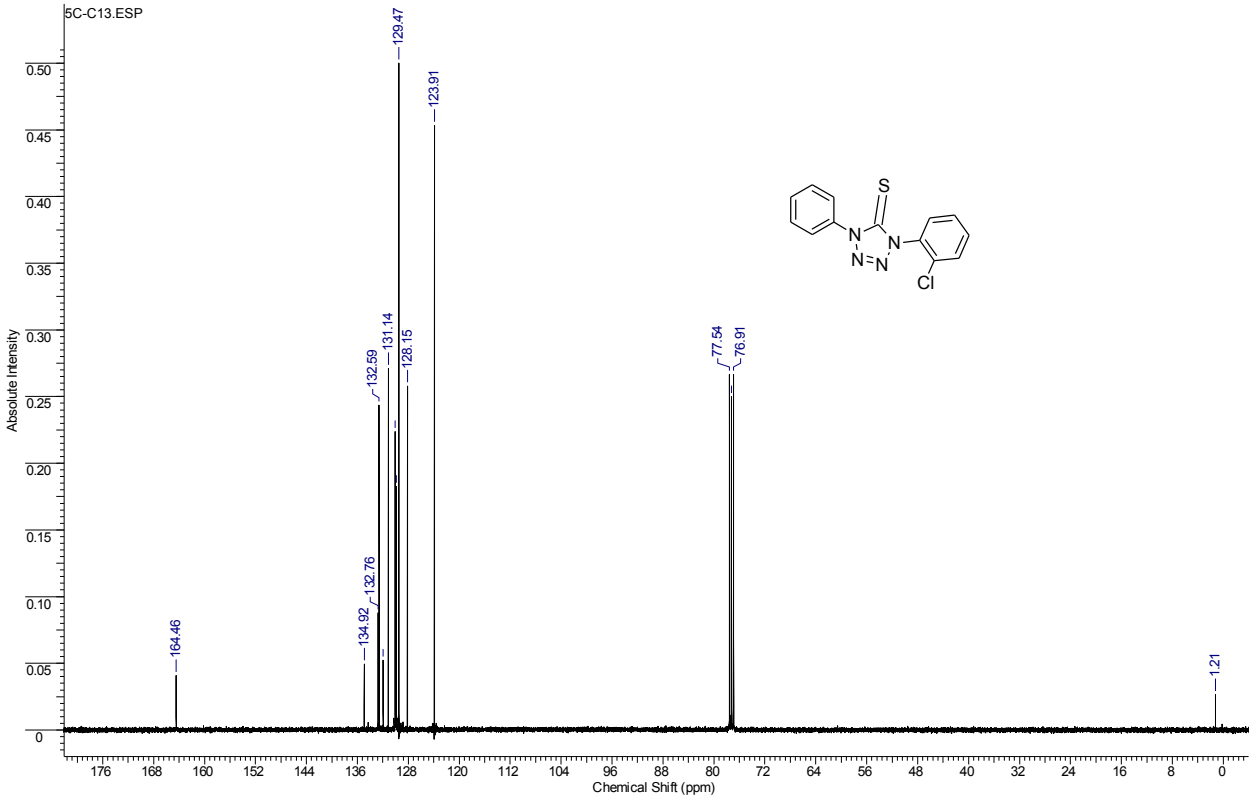
120.98
112.88
77.54
77.23
76.91
56.17
S
N
N
N
N
MeO


5C-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
7.60
7.58
8.09
8.07
7.62
7.54
7.66
7.52
7.66
7.68
7.68
7.50
7.50
7.27
2.06
0.10
0.02
2.00
2.61
S
N
N
N
N
Cl


5C-C13.ESP
Absolute Intensity
Chemical Shift (ppm)
129.47
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131.14
132.59
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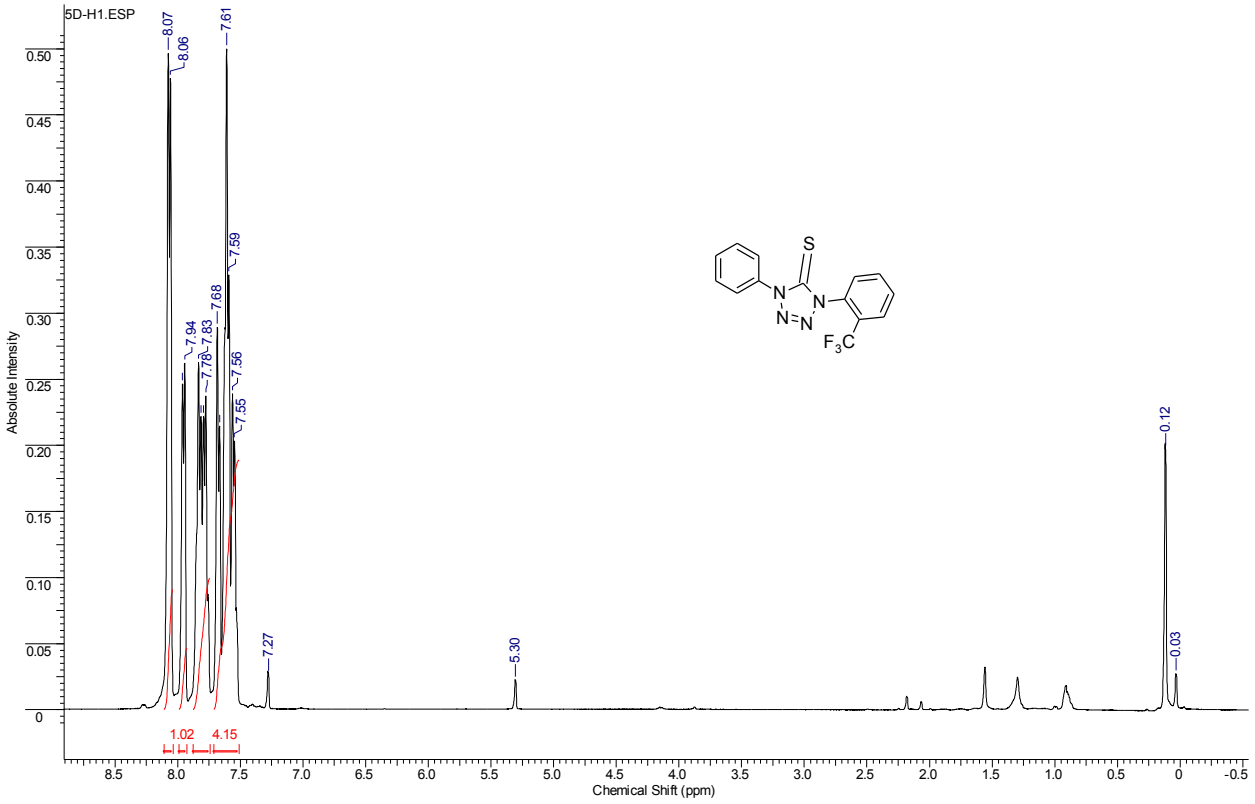
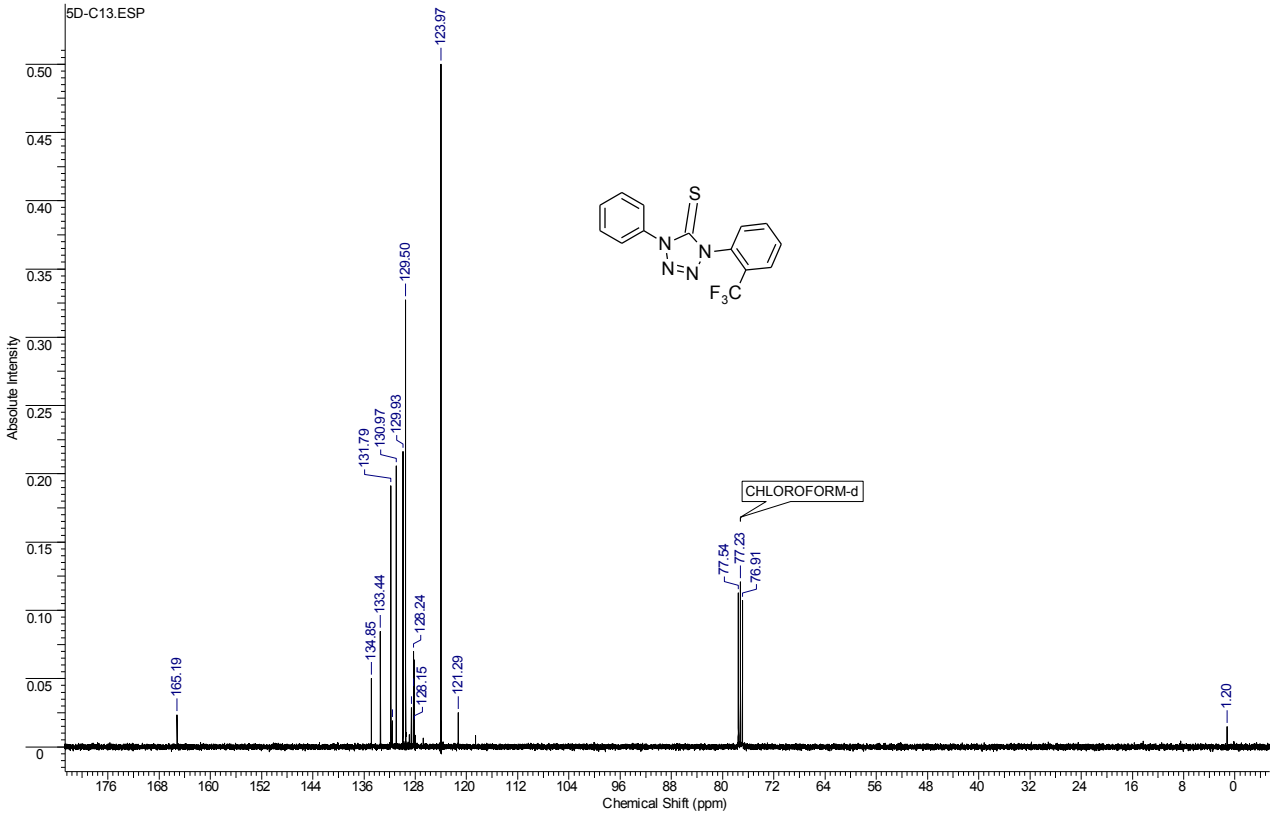
77.54
76.91
164.46
134.92
132.76
1.21
S
N
N
N
N
Cl


5D-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
8.07
8.06
7.94
7.83
7.78
7.68
7.61
7.59
7.56
7.55
7.27
5.30
0.12
0.03
1.02
4.15
S
N
N
N
N
F3C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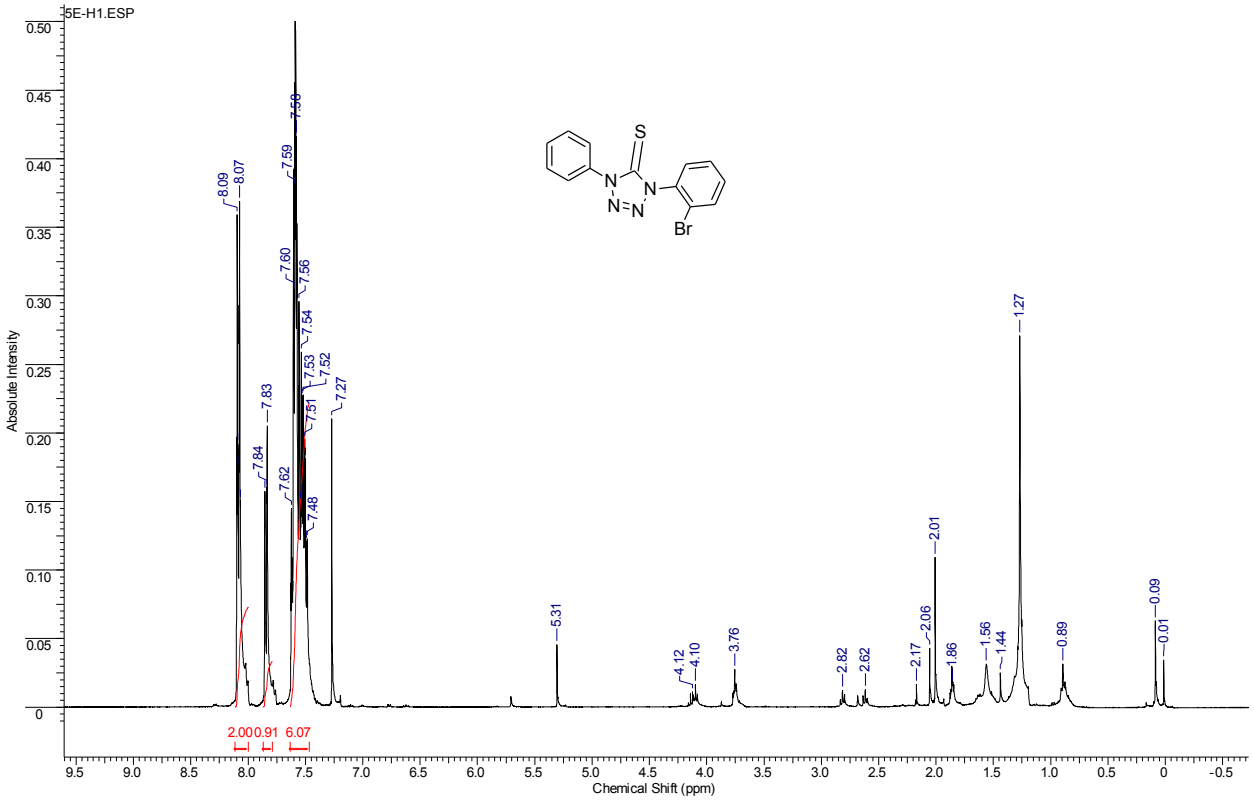
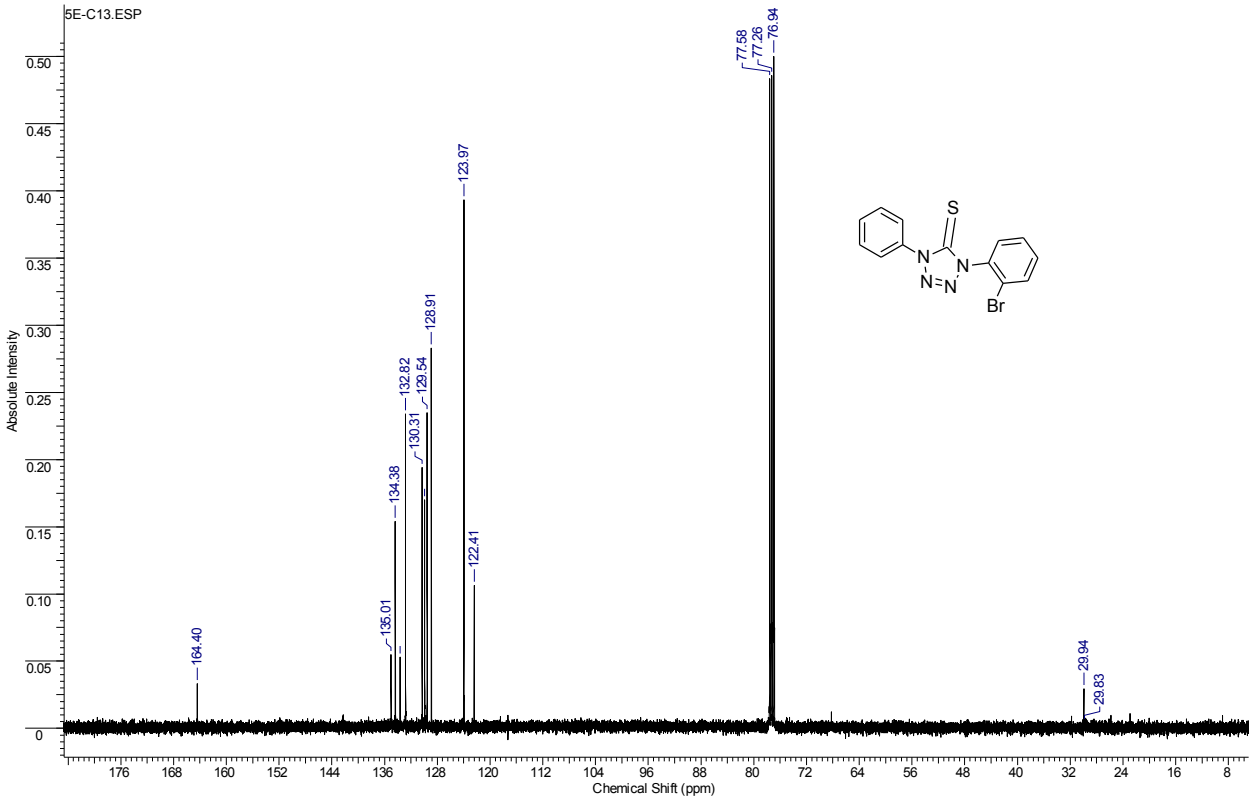
Absolute Intensity
Chemical Shift (ppm)
123.97
129.50
131.79
130.97
129.93
133.44
134.85
128.24
128.15
121.29
165.19
CHLOROFORM-d
77.54
77.23
76.91
1.20
S
N
N
N
N
F3C


5E-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
8.09
8.07
7.84
7.83
7.62
7.60
7.59
7.56
7.54
7.53
7.52
7.51
7.48
7.27
5.31
4.12
4.10
3.76
2.82
2.62
2.17
2.06
2.01
1.86
1.56
1.44
1.27
0.89
0.09
0.01
2.00
0.91
6.07
S
N
N
N
Br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Absolute Intensity
Chemical Shift (ppm)
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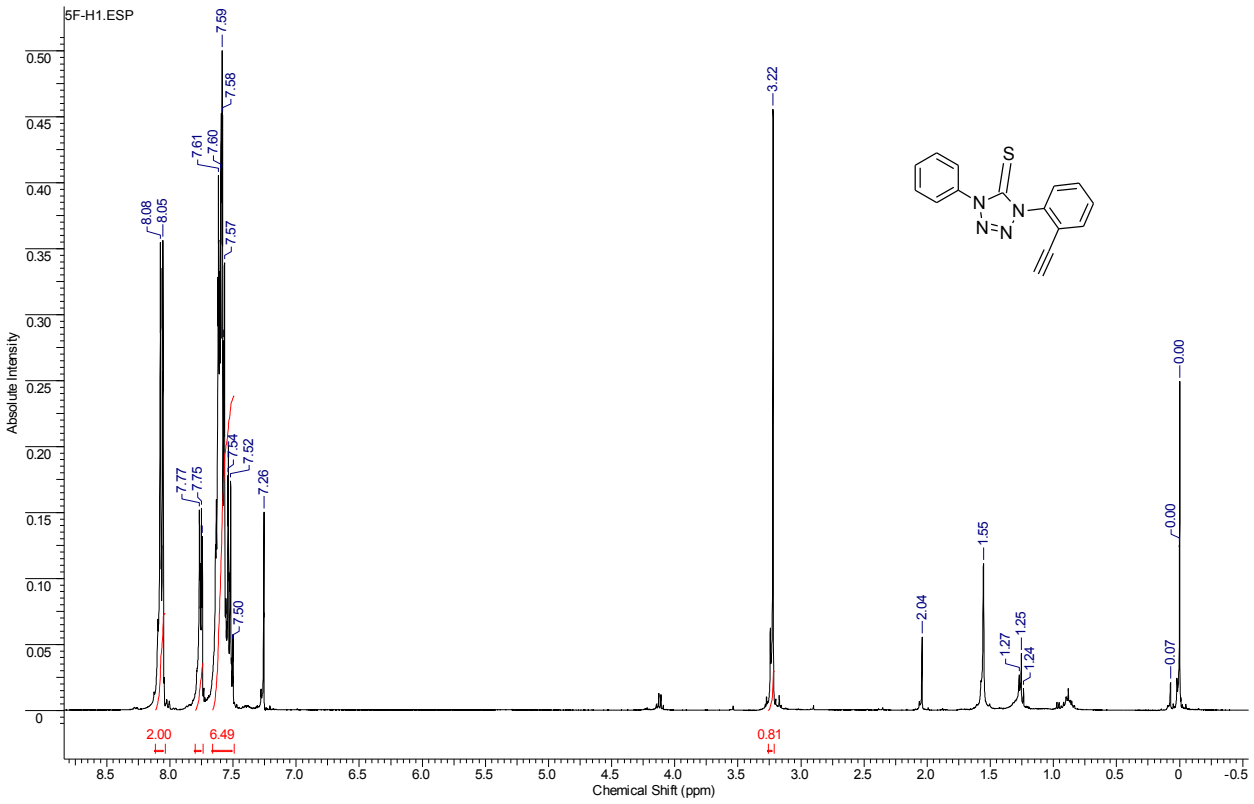
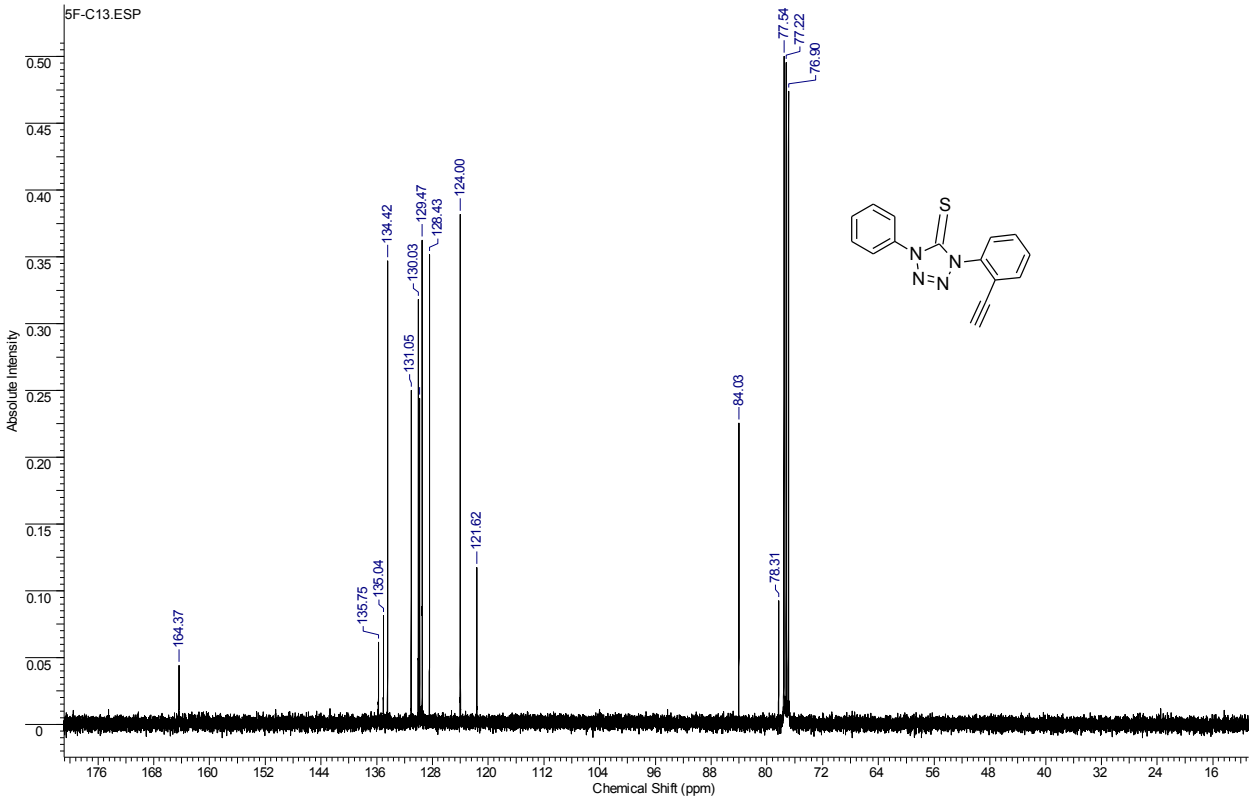
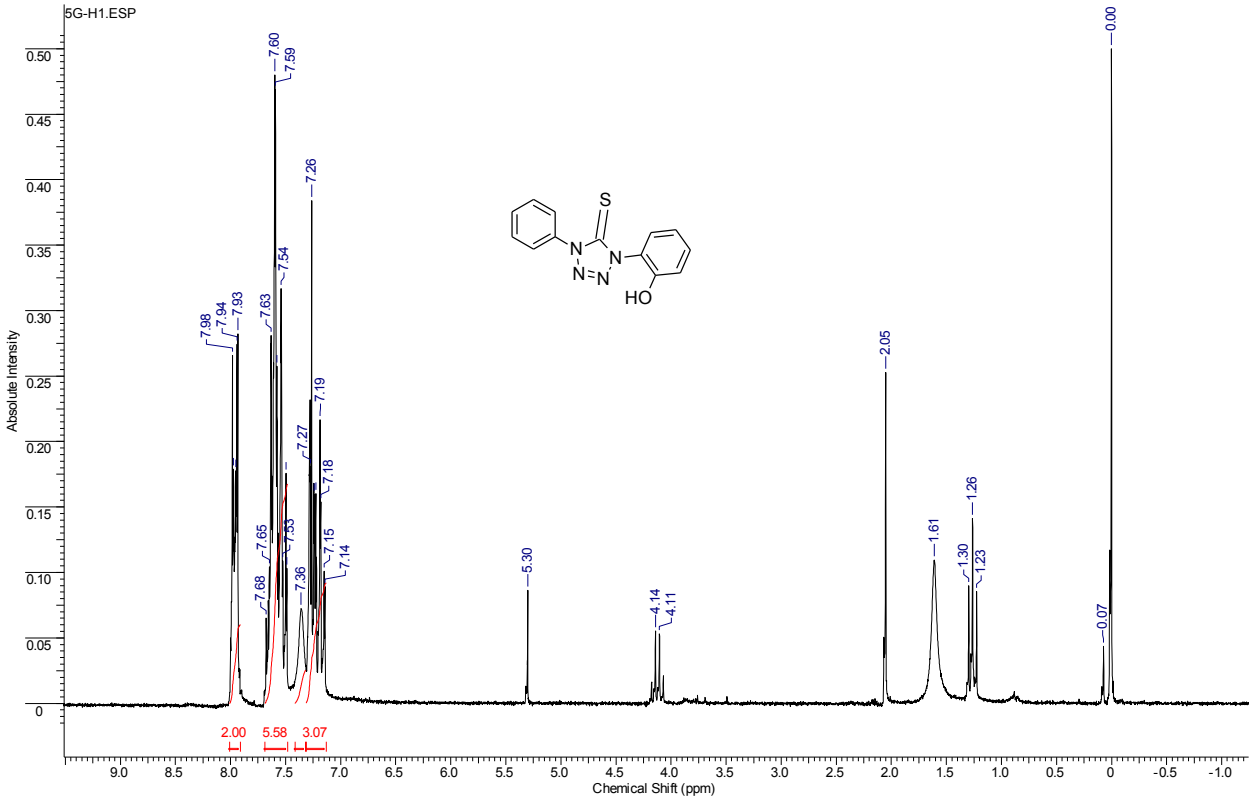
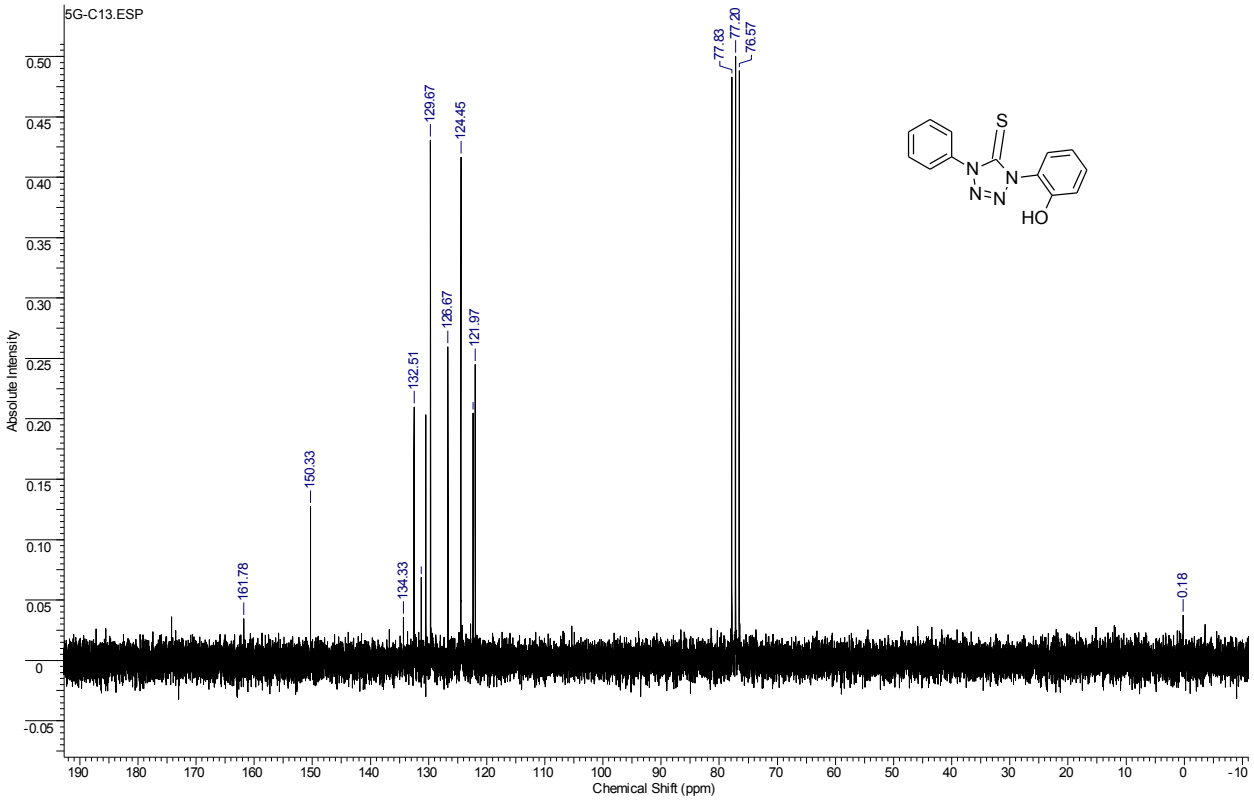
135.01
134.38
132.82
130.31
129.54
128.91
123.97
122.41
77.58
77.26
76.94
29.94
29.83
S
N
N
N
Br


5F-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
8.08
8.05
7.77
7.75
7.61
7.60
7.59
7.58
7.57
7.54
7.52
7.50
7.26
3.22
2.04
1.55
1.27
1.25
1.24
0.00
0.00
0.07
2.00
6.49
0.81
S
N
N
N
N

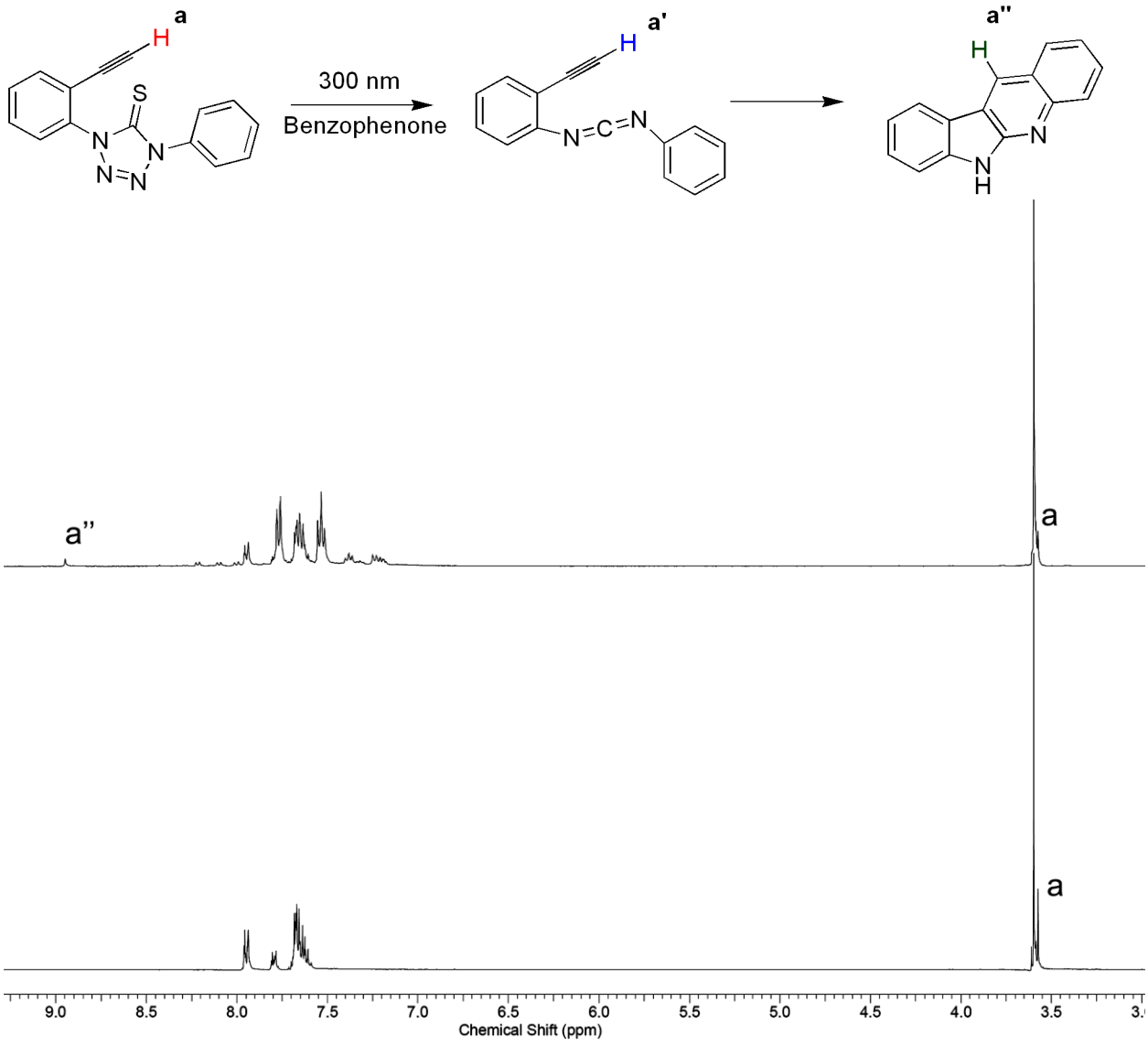

5F-C13.ESP
Absolute Intensity
Chemical Shift (ppm)
164.37
135.75
135.04
134.42
131.05
130.03
129.47
128.43
124.00
121.62
84.03
78.31
77.54
77.22
76.90
S
N
N
N
N

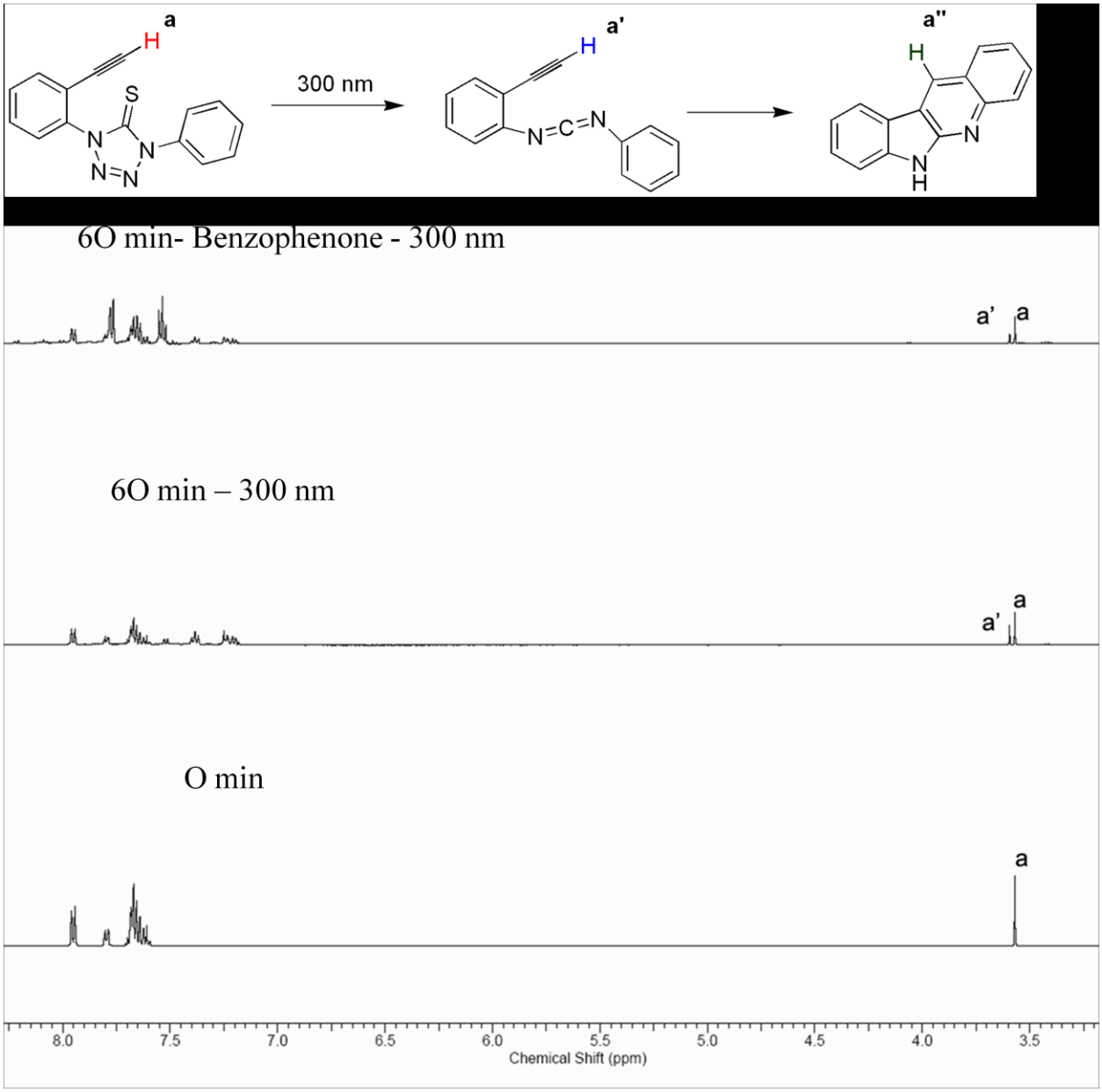

5G-H1.ESP
Absolute Intensity
Chemical Shift (ppm)
7.98
7.94
7.93
7.60
7.59
7.63
7.54
7.26
7.19
7.27
7.18
7.65
7.53
7.15
7.14
7.68
7.36
5.30
4.14
4.11
2.05
1.61
1.26
1.30
1.23
0.00
0.07
2.00
5.58
3.07
S
N
N
N
HO

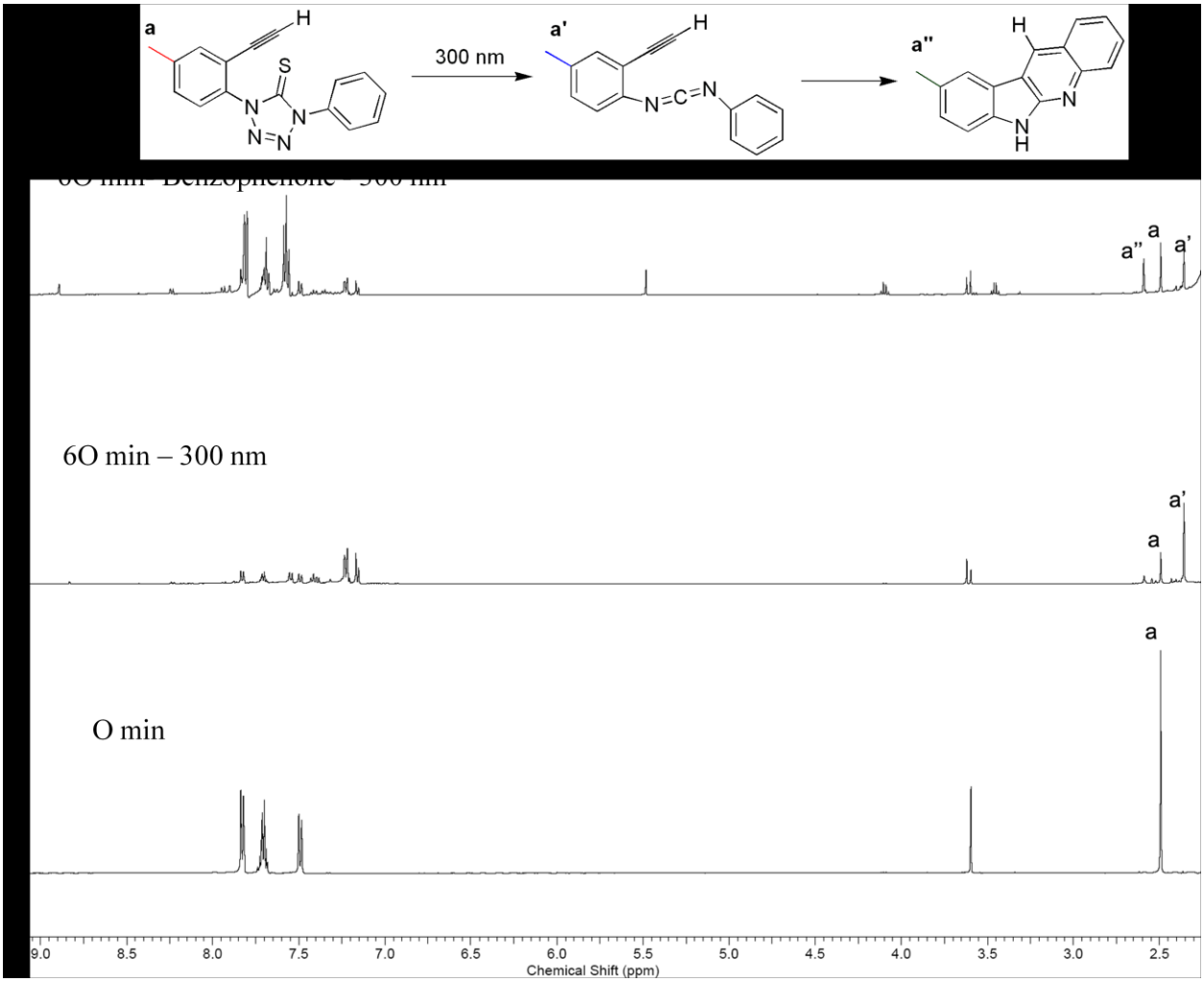

5G-C13.ESP
Absolute Intensity
Chemical Shift (ppm)
77.83
77.20
76.57
129.67
124.45
126.67
121.97
132.51
150.33
161.78
134.33
0.18
S
N
N
N
HO

A 2. Chapter 4

Photodecomposition of 1-(2-ethynylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (1a) and 1-(2-ethynyl-4-methylphenyl)-4-phenyl-1*H*-tetrazole-5(4*H*)-thione (1b)




a
H
S
N
N
N
N
300 nm
a'
H
N=C=N
a''
H
N
N
H
6O min- Benzophenone - 300 nm
a'
a
6O min – 300 nm
a'
a
O min
a
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
Chemical Shift (ppm)


a
H
S
N
N
N
N
300 nm
a'
H
N=C=N
a''
H
N
N
H
a''
a
a'
6O min – 300 nm
a'
a
a
O min
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
Chemical Shift (ppm)