CHARLES UNIVERSITY IN PRAGUE

Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Chemistry and Drug Control

Synthesis of microRNA-21inhibitors

DIPLOMA THESIS

Supervisors: PharmDr. Jan Marek, Ph.D., Prof. Morten Grötli, Marta P. Carrasco, Ph.D.

2015 Jan Vosátka

Univerzita Karlova v Praze

Farmaceutická fakulta v Hradci Králové

Katedra farmaceutické chemie a kontroly léčiv

University of Gothenburg

Faculty of Science

Department of Chemistry and Molecular Biology

Synthesis of microRNA-21 inhibitors

DIPLOMOVÁ PRÁCE

ERASMUS Project

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Jan Vosátka

DECLARATION

Prohlašuji, že tato práce je mým autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla použita k získání jiného nebo stejného titulu.

Hereby I declare that this paper is my own work. All literature and sources of information I used are listed in the list of used literature and they are properly cited. This work has not been used to gain equal or different degree.

Hradec Králové 2015 Jan Vosátka

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My very frankly thanks belong to PharmDr. Jan Marek, Ph.D. for helping me with writing my diploma thesis.

This thesis is product of many hours spent in laboratory, extensive literature research and discussion of problems that occurred during my work.

ABSTRACT

Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra farmaceutické chemie a kontroly léčiv

Student: Jan Vosátka

Školitel: PharmDr. Jan Marek, Ph.D.
Školitel specialista: Prof. Morten Grötli, Marta P. Carrasco, Ph.D.

Název diplomové práce: Syntéza inhibitorů microRNA-21

V nedávné době byly popsány struktury, které negativně ovlivňují některé patologické procesy v organismu, jako například malé molekuly - microRNA. Pro různá onemocnění, typu rakovina nebo srdeční fibrosa, byly popsány specifické typy microRNA, které se účastní patologického růstu v těchto postižených tkáních. MiRNA, jakožto "žhavý" problém, vedly k vývoji inhibitorů těchto malých molekul v různých fázích jejich vývoje. Pro tuto práci byla použita miR-21, která se nachází v různých patologických procesech, hlavně v některých druzích rakoviny a srdeční fibróze. Testy ukázaly, že potlačení miR-21 pozitivně ovlivňuje tyto patologické procesy a výchozí inhibitor byl zvolen jako 4-(2-phenylhydrazinyl)-N-prop-2-ynyl-benzamide. Inhibitor obsahuje azobenzenové jádro, které sloučenině umožňuje po iradiaci přecházet mezi dvěma izomery. Na základě těchto vlastností ji lze považovat za "photoswitchable".

Hlavní cíl této práce byla syntéza nových potencionálních microRNA-21 inhibitorů, založených na známé struktuře, vedoucí ke zvýšení jejich stability a poměru konvrze mezi dvěma izomey po vystavení UV/VIS světlům. Různé skupiny v *ortho* nebo *para* pozicích umožňují změnu biologických a fyzikálních vlastnosti každé molekuly. V navrhovaných derivátech byly studovány rozdíly na těchto pozicích mezi elektron dotujícími, elektron odtahujícími a objemnými substituenty. Vnějšími podmínkami byly zvoleny světla UV/VIS v příslušných vlnových délkach, pro pozorování změn chování každé sloučeniny.

Výsledky ukázaly výrazné rozdíly vlastností azobenzenů na základě změn substituentů benzenového kruhu. Byly vyzkoušeny různé syntetické cesty k získání finálních sloučenin, které byly klasifikovány jako úspěšné/neúspěšné. Velký podíl v úspěšnosti syntézy hrály odlišné substituenty.

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ABSTRACT

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Student: Jan Vosátka

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Consultant: Prof. Morten Grötli, Marta P. Carrasco, Ph.D.

Title of diploma thesis: Synthesis of microRNA-21 inhibitors

Nowadays structures which negatively suppress some pathological processes in the organism have been revealed, for example small molecules - microRNAs. For various diseases, such as cancer or cardiac fibrosis, specific types of microRNAs, which participate in pathological growth of these affected tissues, have been discovered. MicroRNAs, as a hot issue, has resulted in focusing on the creation of inhibitors of these small structures in different stages of their genesis. For this work miR-21 occurring in some kinds of cancer and cardiovascular disease, mainly in cardiac fibrosis, has been used. The assays showed that the suppressing of miR-21 positively influences these pathological processes and 4-(2-phenylhydrazinyl)-*N*-prop-2-ynyl-benzamide was determined to be the appropriate inhibitor. This inhibitor contains azobenzene´s moiety which allows the compound to interconvert between two isomers upon irradiation. In this way, this compound can be considered a photoswitchable molecule.

The main goal of this work was to synthesize novel potential microRNA-21 inhibitors based on the structure of a known inhibitor in order to increase their stability and the conversion ratio between the two isomers after the exposure to the UV/Vis lights. The various groups in *ortho* and *para* positions allow to change the biological and photophysical properties of each compound. In the designed derivatives differences among EDG, EWG and bulky substituents in these positions were studied. External conditions in appropriate UV/Vis lights wavelengths were chosen for observing the changes in the behaviour of each compound.

The results show significant differences of properties of azobenzenes based on changes of the substituents at the benzene ring. Distinct synthetic-methods to obtain the final compounds have been tried and classified as successful/unsuccessful procedures. Varied substituents played a big portion of success in the synthetic pathways.

1. Table of contents

2. List of abbreviations

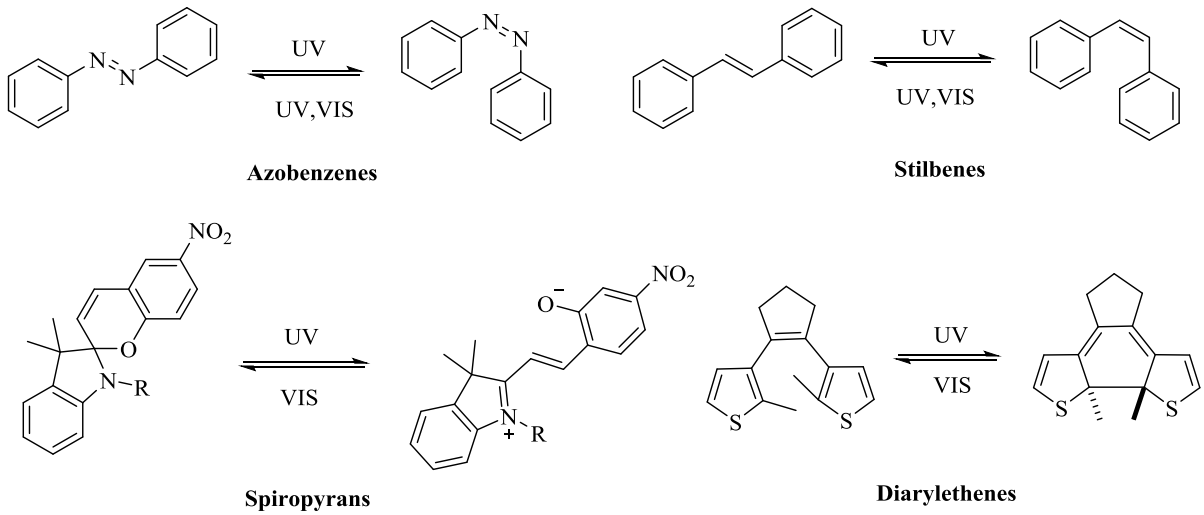
BMP	bone morphogenetic protein
DCM	dichloromethane
DIC	*N,N'*-Bis(isopropyl)carbodiimide
DMF	dimethylformamide
DMSO	dimethylsulfoxide
E	entgegen
EC_{50}	effective concentration
EDG	electron-donating groups
ERK	extracellular signal-regulated kinase
EWG	electron-withdrawing groups
GSH	glutathion
HEX	hexane
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
LC/MS	liquid chromatography – mass spectrometry
MARKs	microtubule-affinity-regulating kinase
miR-122,91,155	microRNA-122,91,155
miR-21	microRNA-21
miRNA	microRNA
MMP	matrix metalloproteinase
mRNA	messengerRNA
NMR	nuclear magnetic resonance
PDCD4	programmed cell death
pre-miRNA	precursor-microRNA
pri-miRNA	primary-microRNA
PSS	photostationary state
PTEN	phosphatase and tensin homolog
RECK	reversion-inducing cysteine
R_f	retention factor
RISC	RNA-induced silencing complex
RT	room temperature
***t*BuOI**	*tert*-butyl hypoiodite
THF	tetrahydrofuran
TIMP3	tissue inhibitor of metalloproteinases 3
TLC	thin layer chromatography
TPM1	tropomyosin α1
UV	ultraviolet light
Vis	visible light
Z	zusammen

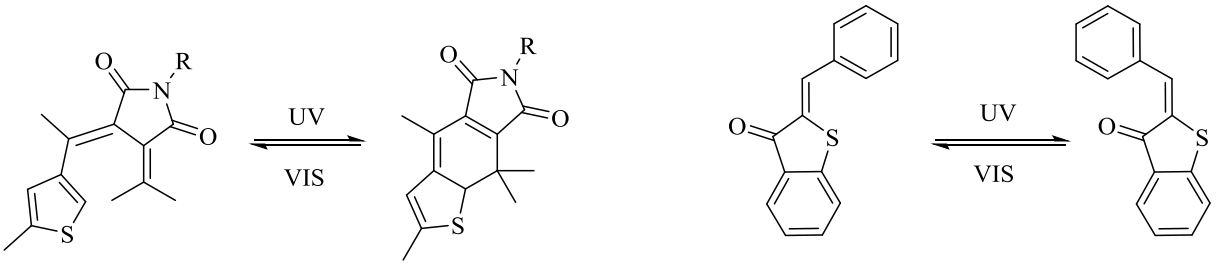
3. Introduction

Photoswitchable compounds are sensitive upon irradiation and occur in many fields - biology, materials, etc. Different features after the irradiation are an important aspect of this class of compounds [1].

Spatial and temporal controlling of compounds which are able to interact with biological material can bring lots of advantages, mainly for biological and other fields in the future. The possibility to control the function drugs allows to decrease their side effects or additional adverse effects for healthy tissues and directly aim at the target area. The method which includes light (UV,Vis), called photoswitching, can trigger appropriate compounds in the right place and switch them off afterwards. Disadvantages of this method comprise the following: it can cause some problems with using UV light since it can damage healthy cells and its penetration into the tissues is low[1, 2].

Nowadays some groups of compounds which are sensitive upon irradiation and can swap their conformation between two or more states are known. These changes are responsible for modifying abilities and properties of different conformations, for instance, biological activity, different absorption of light and fluorescence emission. The structure-changes of molecules caused by light can be divided into two groups where the first one is based on swapping between *trans* and *cis* isomers (azobenzenes, stilbenes, hemithioindigos) and the second one is characterized with *open* and *close* forms (spiropyrans, diarylethenes, thiophenefulgides) (Figure 1.) [1-3].





Thiophenefulgides **Hemithioindigos**

Figure 1. Compounds sensitive upon irradiation divided into various groups characterized with changes of their structures. Azobenezenes E/Z, Stilbenes E/Z, Spiropyrans Open/Close, Diarylethenes Open/Close, Thiophenefulgides Open/Close, Hemithioindigos E/Z.

3.1 General information about the photoswitchable compounds

In order be considered a molecular switch, the compound must include these requirements[4, 5].

- The isomerisation between two structures that contain molecular switch must be produced easily and selectively by irradiation with light in appropriate wavelength.
- The compound must be resistant to fatigue (a couple of cycles without degradation), to handle several structure changes without thermal degradation or photochemical side-products.
- The isomers should be easily detectable.
- High quantum yields should be achieved upon short irradiation periods.
- All properties have to be unchanged when the compound used as a photoswitch forms a part of a macromolecular structure.

4. Aim of the work

This work focuses on the synthesis and description of photo-physical properties of asymmetric-azobenzenes. The starting point was a known inhibitor of microRNA-21(miR-21) (Figure 9.), containing an azobenzene moiety. In this way, the main goal was to prepare derivatives of this compound in appropriate yield and to investigate the influence of substituents in *ortho* and *para* positions in their photochemical properties (Figure 2.). The distinct biological activity and photo-physical properties relate to two possible isomers E/Z.

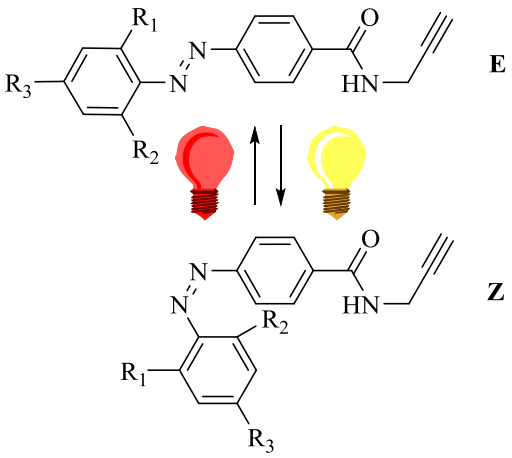


Figure 2. Two possible isomers of the miR-21 inhibitor upon irradiation of UV/Vis light. The red bulb characterizes UV and the yellow one Vis light.

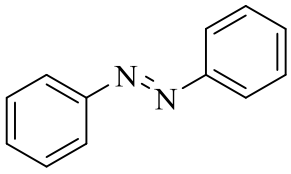
5.Theory

In the theoretical part areas for understanding my project including some theoretical parts and some general information will be described.

5.1 Azobenzenes

Azobenzenes, with the core of diazobenzene (Figure 3.), are the most studied class of photochromic molecules because of their *cis-trans* isomeration in the presence of appropriate radiation. Nowadays, aromatic azo compounds are used as a dyes, pigments[6], food additives, indicators[7], radical reaction initiators and therapeutic agents [8, 9]. They are also important units in the area of optical storage media[10], chemo sensors[11], photochemical switches[12] and electronic devices[13]. In biological systems azo compounds can be involved in the modification of the activity of enzymes and polypeptides due to the photoresponse[14].

Nowadays, 10 thousand of these molecules have been described and more than 2 thousand are used as dyes[15].



Diazobenzene

Figure 3. Diazobenzene. Basic structure of the inhibitor.

5.1.1 Azobenzenes as a molecular switch

The molecular switch is characterized by the molecular system that is able to perform mechanical movement upon exposure of some an external stimulus, such as light, resulting in conformational and environmental changes of the switch. The molecular switch can be also defined as a reversible transformation of chemical compounds caused by light between two states of a molecule with different absorption spectra[16].

The azobenzenes, as chromophores, contain an azo-bridge which is sensitive to various suggestions which modify the structure between two isomers, *cis* and *trans*, where *trans* is more stable than the other one because of planar conformation. The stability in different states is very a interesting tool to aim at. The stability of these isomers can be influenced either by the substituents on one of the rings or by the surrounding conditions[1, 2].

The possibility to change the structure between *trans, cis* and back is one of the most interesting things that azobenzenes offer. In most cases, to obtain *cis* isomer from the *trans* is reached upon irradiation by blue light with wavelengths between 320 - 380 nm which is not so harmful for biological materials in short intervals. This crossing can be reversible; to obtain *trans* isomer back from *cis* various approaches can be applied. Some of these reverse-pathways to obtain *trans* isomer can be achieved by light 400 - 450 nm or thermally - this way usually requires darkness[1].

This crossing opens new perspectives for their biological application. In previous studies this tool was used for changes of biological activity in peptides, proteins, nucleic acids, lipids and carbohydrates[17]. The decreasing or increasing of the activity of azobenzenes leads via reversible photoisomeration, “on and off ”, of the azobenzene moiety[15].

The isomeration can be reached by 4 possible ways - rotation, inversion, concerted inversion, inversion-assisted rotation. All of these transformations are characterized by mutual changing of angles of the phenyl rings or by crashing of the N=N bond[3, 18]. Two photoisomeration mechanisms have been described, one involving simple rotation around the N=N bond and another is characterized by an inversion, in-plane lateral shift. (Figure 4.)[19].

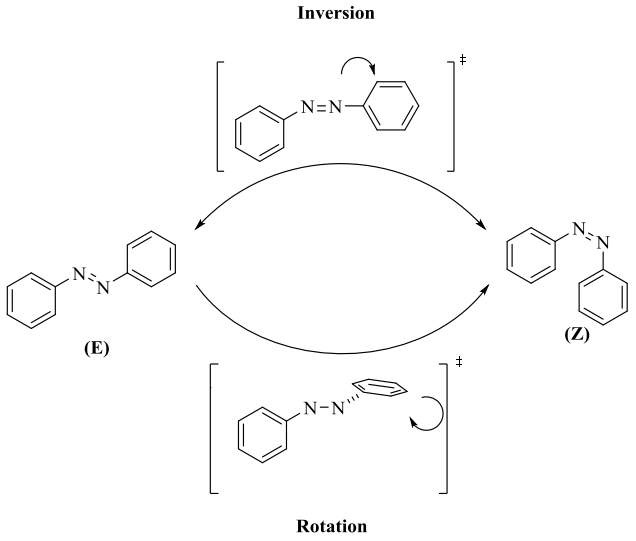


Figure 4. Two photoisomeration pathways. Mechanisms of rotation and inversion.

It is almost impossible to obtain pure isomers of unmodified azobenzenes. After irradiation they usually provide maximally 80% of *cis* isomer from the *trans*. Anyway, photo-isomerization of *cis* isomer is much better and the final yield of the *trans* form is around 95% whereas thermal reverse-isomerization might provides >99,99% of *trans* isomer[20]. The thermal relaxation of unsubstituted azobenezenes can take a couple of days (3-4) and mostly depends on surrounding conditions - polarity of solvents, low Ph, substituents on benzene rings [1].

The thermal relaxation means half-life of the *cis* isomer after the irradiation of *trans*-isomer by UV-light. It can be increased by substituents at the benzene rings in *ortho* positions. Substituents can influence the azo-bond either with steric hindrance by some bulky substituents, for instance methyl, isopropyl-groups, or by some electron-donating (EDG)/ withdrawing groups (EWG)[21]. These substituents probably create a hydrophobic pocket and are able to defend the azo-bond against the reduction. The half-life can also be reduced by the presence of glutathion (GSH) in life cells which is able to reduce the azo-bond. The effort to

avoid this effect is aimed at the preparation of azo-compounds with various substituents in different positions (mainly in *ortho,para* positions)[22].

5.1.1.1 Synthetic strategies

The synthesis of the azobenezenes is quite simple and some common strategies have been summarized in articles and reviews. By the reduction of the nitro group to the amino, or by the oxidation of the amino group to the nitro an aromatic azo compound can be obtained. Another possible method of diazotization/coupling reaction can be used. The first two synthetic pathways are useful mainly for the preparation of the symmetrical azo compounds. From the two different substrates a mixture of three azo products is produced: two symmetric ones and an asymmetric one. The third and fourth reactions are mostly used for the preparation of asymmetric compounds. In the fifth reaction electrophilic aromatic substitution occurs where the diazonium salt performs as an electrophilic reagent. Some EDG have to be located on another benzene ring due to the successful reaction. The substitution goes into *ortho* and *para* positions (Figure 5.) [15, 19].

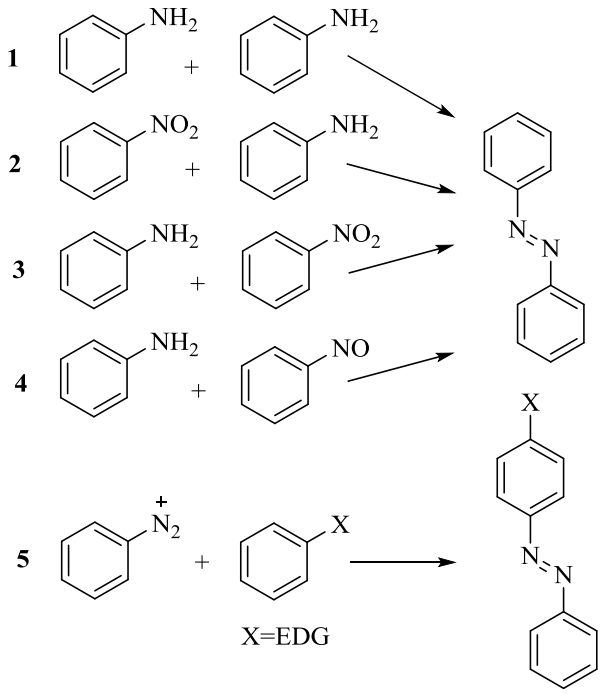


Figure 5. Pathways which lead to the preparation of symmetric and asymmetric azobenzenes.

5.1.2 MicroRNA

MicroRNA (miRNA) is a single-stranded non-coding RNA which consists of 21-23 nucleotides. About 1000 miRNAs occur in the human body and they participate in control of 30% of all genes. Each individual miRNA has lots of messengerRNA (mRNA) targets and effects on/off gene expression (Figure 6.). The occurrence of miRNA is increased under pathological conditions and diseases, which is why it is an attractive target for the drugs influence[23].

MiRNA has been discovered recently and provides a great opportunity for treating various illnesses, mostly cancer, cardiovascular disease, viral infection and inflammatory diseases[23, 24]. The miRNAs can participate in cancer development and progression. In solid tumours over-expression of miRNAs such as miR-21, microRNA-92 (miR-92), microRNA-155 (miR-155) etc. was indentified. The supposed tumour targets with over-expressed miRNAs were enriched with protein-coding tumour suppressors and oncogenes. It means that miRNAs are involved in cancer pathogenesis of solid tumours and support their function as a cancer genes[25].

Some important properties of miRNA have been revealed as possibility to influence gene expression. The function of miRNA leads via binding of mRNA´s 3´ untranslated region and is able to inactivate genes by suppression of specific mRNA, inhibition of translation or can trigger degradation of mRNA[26] .

The most of miRNAs are transcribed as primary miRNAs (pri-miRNAs), then are processed in the nucleus by the Drosha enzyme due to the creation of shorter, stem-loop-structure, double stranded RNAs called precursor miRNAs (pre-miRNAs). Pre-miRNAs go through the nucleus into the cytoplasm and are transformed by Dicer into mature miRNAs. Mature miRNAs are able to target single stranded complementary mRNAs as an effector complex, called the RNA-induced silencing complex (RISC.)[26] .

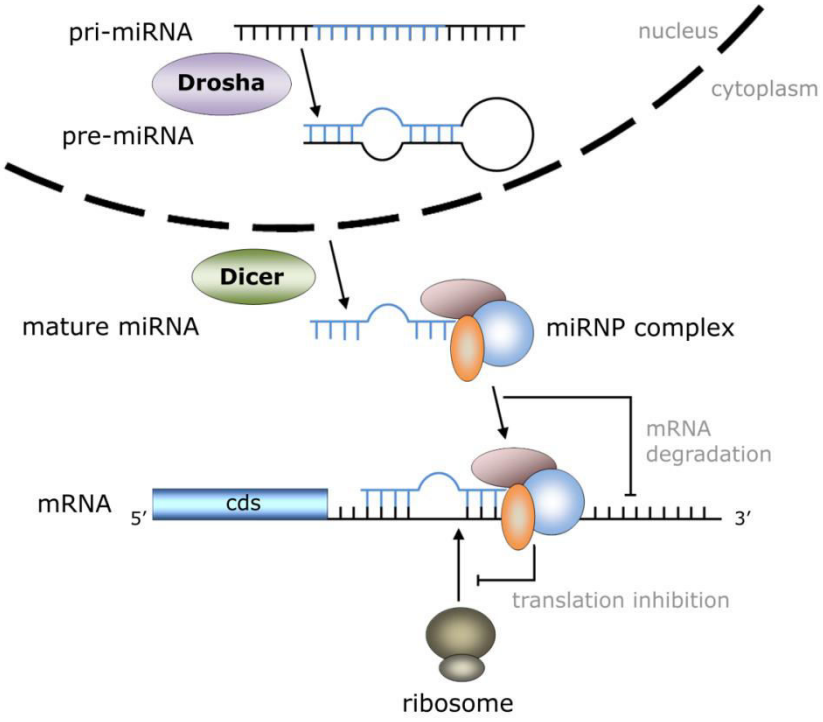


Figure 6. The pri-miRNA is transformed by Drosha into double stranded structure. Then it is transported by Dicer to cytoplasm as a mature miRNA. In the complex (RISC) it is able to target mRNA and induce mRNA degradation or inhibition of translation [27].

The possibility that miRNA can influence some pathways in gene expression has led to the development of some drugs which can interact with these small biological structures. Therapeutic targeting at miRNA can be divided into three groups of structures which have been already discovered - expression vectors[28], small molecule inhibitors[26] and antisense oligonucleotides[29].

The small molecule inhibitors are able to interact with miRNAs and suppress their function and expression (Figure 7.). Azobenzenes which suppress expression of miR-21 and some other compounds that inhibit microRNA-122 (miR-122) have the ability to inhibit the expression of miRNA. At least three ways of inhibition by these small compounds are known in miRNA assembly and function. First**,** small molecules can influence the procedure of transcription of the pri-miRNAs. The second way is via the inhibition of pri-miRNA processing by Dicer and the suppression of RISC activation. The last way is the inhibition by means of small molecules in order to prevent RISC from binding to mRNA. All of these mechanisms decrease the suppression of the targeted mRNA by miRNAs and miRISC. Their

therapeutic potential is rather limited because of their high EC_{50} that occurs in micromolar spread, and because of lack of information on targets[26, 29].

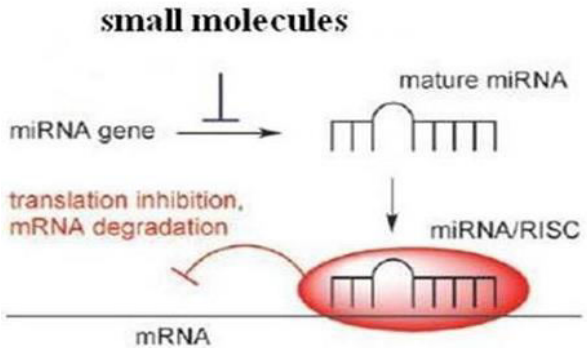


Figure 7. The scheme shows how can miRNA be regulated by small molecules. Without the molecules miRNA can bind the target (mRNA) and inhibit translation or mRNA degradation[26].

5.1.3 miR-21

In some kinds of cancer and cardiovascular diseases, for example in liver and breast cancer cells, the presence of miR-21was identified. Because of high amount of this small molecule inside the tumour cells some regulation mechanisms are suppressed, such as phosphatase and tensin homolog (PTEN)[30], tropomyosin 1 (TPM1)[31] and programmed cell death protein 4 (PDCD4) [32] (Figure 8.). It has led to a discussion about affection of miR-21. In the case of glioblastoma, liver and breast cancer cells it was observed that inhibition of mir-21 increased the degradation of cancer cells [30, 33]. Assays showed that inhibition of miR-21 can be promising in cardiac diseases as well. High amount was observed in cardiac fibroblasts as a starter of a cardiac fibrosis. With inhibition cardiac function could be improved and cardiac fibrosis reversed[34].

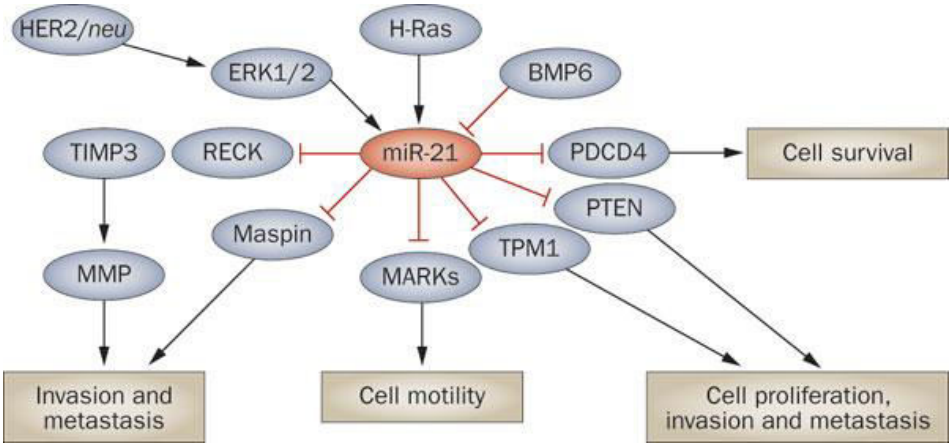


Figure 8. Mir-21 can influence many pathways in the cell with various outcomes. BMP – bone morphogenetic protein, ERK – extracellular signal – regulated kinase, MARKs – microtubule – affinity – regulating kinases, MMP – matrix metalloproteinase, PDCD4 – programmed cell death protein 4, PTEN – phosphatase and tensin homolog, RECK – reversion – inducing cysteine – rich protein with Kazal motifs, TIMP3 – tissue inhibitor of metalloproteinnases 3, TPM1 – tropomyosin α1 [35].

5.1.3.1 Mir-21 in cancer cells

MiR-21 plays a crucial role as an oncomir in tumour-genesis. The role of miR-21 as an antiapoptotic factor was studied in glioblastoma cells where some regulation mechanisms were suppressed. It was shown that the PTEN, tumour suppressor gene that decreases cell proliferation and cell survival, was down-regulated in pathological tissues by miR-21[30]. The down-regulated targets of miR-21 are also PDCD4 and TPM1 in cancer cells. PCDC4 is classified as a tumour suppressor gene that inhibits transformation, tumour promotion and progression[36, 37].The down-regulation of TPM1 leads to uncontrolled cell proliferation and invasion [38].

Presence of miR-21 has been confirmed in a number of distinct cellular processes and pathways including upstream effectors and downstream targets. MiR-21 influences many mechanisms which increase the cell invasion. The inhibitors of matrix metalloproteinases (MMPs) like a reversion – inducing cysteine – rich protein with Kazal motifs (RECK) and tissue inhibitor of metalloproteinnases 3 (TIMP3) are targeted, resulting in increased activity of MMPs [39, 40]. The tumour suppressor maspin is also inhibited, which contributes in cellular invasion and metastasis[41].

5.1.3.2 Mir-21 in cardiac fibrosis

In cardiac fibrosis upregulation of miR-21 in response to pathological stresses that promote cardiac fibrosis has been observed. The delivery of antagomirs aimed at miR-21 has

been reported to prevent fibrosis and cardiac hypertrophy. Antagomirs can also start the reparation of fibrosis and restore the function of injured heart tissue. Based on these beneficial effects of antagomirs in cardiac fibrosis some attempts to ihibit mitogen-activated protein kinase (MAPK), which is the driver of cardiac dysfunction[34] with positive outcomes, were performed [34]. In assays with mice, with deleted gene for miR-21, no diminution in fibrosis in response to multiple cardiac stress was observed. MiR-21 does not play the crucial role in this pathological process[42].

5.1.4 Potential inhibitor of mir-21 (small molecule)

An assay focused on small-molecule inhibitor with azobenzene moiety 4-(2-phenylhydrazinyl)-*N*-prop-2-ynyl-benzamide (**A**) (Figure 9.), which influences mir-21,was presented by group Gumireddy, K., et al.[26]. HeLa cells where miR-21was expressed in relatively high amounts were chosen for the assay. To control inhibition of miR-21, Luciferase-miR-21-linker, which was complementary to miR-21, was also expressed in the cells. After mutual binding of these sequences the signal of Luciferase was decreased. In cells fed by compound **A** the increase of Luciferase signal was observed more than fivefold as compared to cells fed by DMSO as a control measurement. In another test inhibitor **A** was applied at different microRNAs where no positive results were observed. It means that compound **A** is an inhibitor purely for the pathway of miR-21 genesis and does not function as a common inhibitor of microRNAs. The next test found out that compound **A** is an inhibitor that targets the transcription of the miR-21 gene into pri-miR-21 [26].

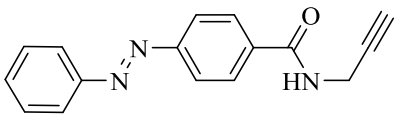


Figure 9. Structure of the inhibitor (**A**) miR-21 with core derived from azobenzene.

6. Chemistry

Lab-work with some comments and figures including reactions have been described in the chemistry part. It includes a general plan of synthesis and table with final yields of reactions.

6.1 General plan of synthesis

In my work three methods for obtaining final compounds were used (Figure 10.). Just one method worked properly and could be used for proposed compounds. Final reaction consists of three steps which are described in detail in the following sections hereof (Figure 11., 12., 13.). The unsuccessful pathways have been described as well.

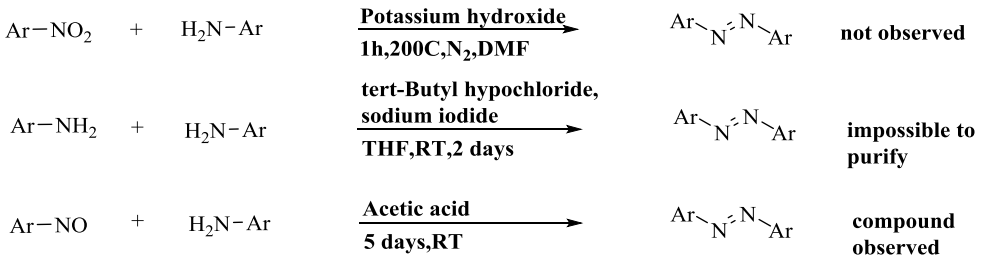


Figure 10. The methods used for preparation of final compounds: the pathways including nitro + amine or amine + amine coupling weren´t successful. The reaction with nitroso + amine was suitable, but not for all compounds which were designed.

6.1.1 General synthesis of the final derivatives JV_39, 41, 21, 34

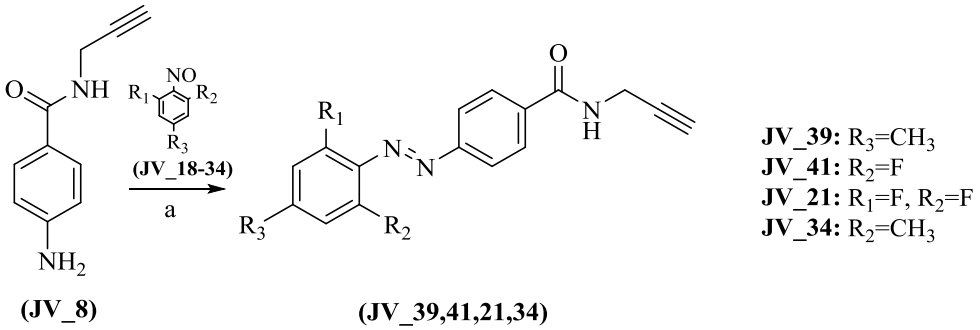


Figure 11. **Reagents and conditions:** (a) acetic acid, RT, 5 days, light protected by aluminium foil.

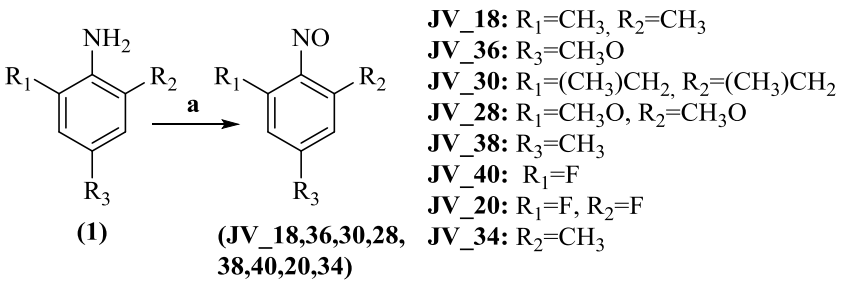


Figure 12. **Reagents and conditions:** (a) water, DCM, oxone, RT, 2 hours

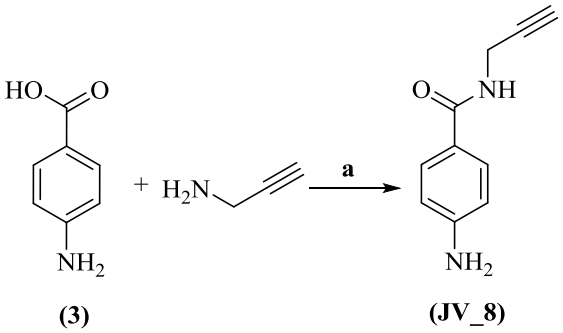


Figure 13. **Reagents and conditions:** (a) dry-DCM, DIC, HOBt,RT,12 hours

6.2. The synthesis of inhibitor

Three steps were used for the preparation of azobenzenes. These steps include: amide formation, oxidation, azo-bond formation. The mechanisms of each step are described in the parts bellow.

6.2.1 Amide formation

The first reaction included the preparation of amide from initial substances such as aminobenzoic acid and propargylamine with the goal of creating an amide bond (Figure 14.). The applied procedure was taken from supporting information in an article by Gumireddy, K., et al. [26].

The carboxylic group had to be activated by N,N'-Bis(isopropyl)carbodiimide (DIC) (**1**) where carbon was an electrophilic center and could be attacked by dissociated carboxylic acid. In the next step the complex is coupled with hydroxybenzotriazol (HOBt) (**2**) in order to avoid a side product of the reaction. Without HOBt the side product *N*-acyl-urea (**3**) rises because of an overlap, and the final yield is much lower. In the last step of the reaction the required amide was prepared by adding propargylamine (**4**), which is better nucleophile, and HOBt is a good leaving group [43].

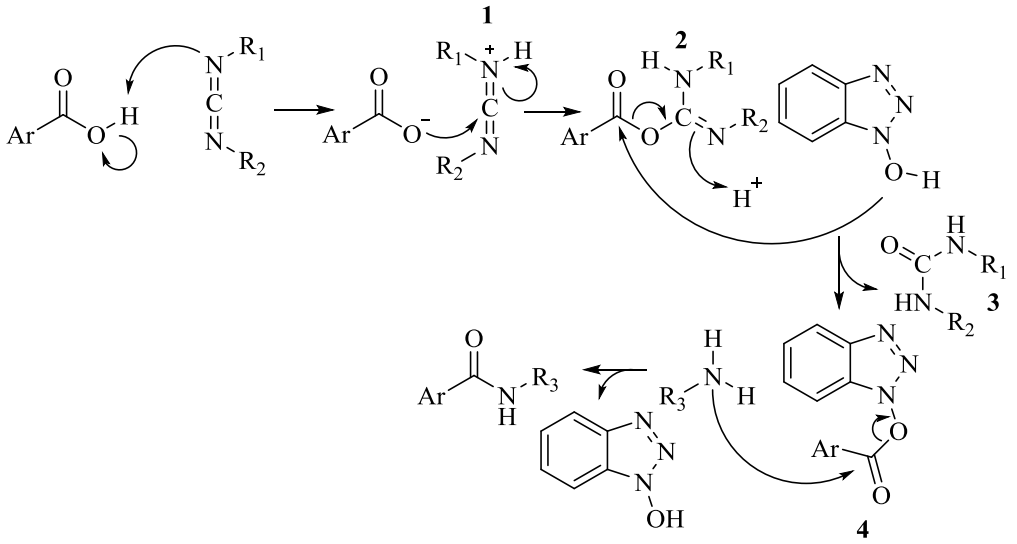


Figure 14. General synthesis of an amide group with the use of DIC and HOBt. During the reaction a small amount of urea derivative **(3)** arose which was observed in NMR spectrum.

The general procedure was followed by supporting information from an article by Gumireddy, K., et al .[26]. 4-Phenylazobenzoic acid was dissolved in dry-dichloromethane (dry-DCM) and then DIC and HOBt were added. After a while propargylamine was added which coupled with the carboxyl group to provide an amide group. The reaction was stirred for 12 hours at room temperature (RT) and was quenched with water and extracted with DCM. The organic layer was dried and purified.The procedure was the same as in the article, the only differences were in the scale of aminobenzoic acid and the final amount of the product. The yield reported in the article was 86% while the yield of 74% was found in my project.

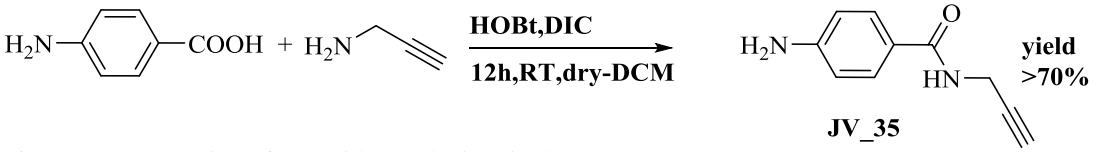


Figure 15. Preparation of an amide bond with yield more than 70%.

6.2.2 Oxidation

In the second reaction the initial substance was oxidized by potassium peroxymonosulfate (Oxone) (Figure 16). The main procedure with some modifications was used from article Efficient Preparation of Nitrosoarenes for the Synthesis of Azobenzenes [44].

In general oxidation is removing hydrogens and adding oxygens in the parallel with the reduction. In this reaction primari-amine was oxidated to nitroso group. Initial compounds were substituted anilines in *ortho* or *para* positions with bulky or EDG/EWG substituents. By oxidation of amine group, in the presence of oxone, nitroso-arenes were prepared, which were

used for the preparation of an azo-bond with derivatived amine in the next step (Figure 17.)[44].

The general procedure was about the preparation of the solution of the required aniline in DCM and oxone/water. This reaction was stirred (average 2 hours) at RT for various time periods depending on the substances until the colour of the solution changed. The next step was the separation of two different phases (organic/aqueous) from each other. The aqueous phase was extracted twice with DCM. The combined organic layers were washed with diluted HCl, saturated sodium hydrocarbonate solution, twice with water and once with brine. Then the solution was dried over the sodium sulfate, the DCM was removed at the rotary evaporator. Final yield of the nitroso-arenes was 30-70% (Figure 17.), depended on the used amino-derivatives.

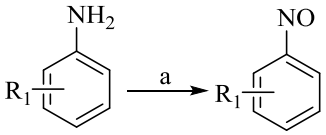


Figure 16. Oxidation of substituted anilines in the presence of oxone (**a**).

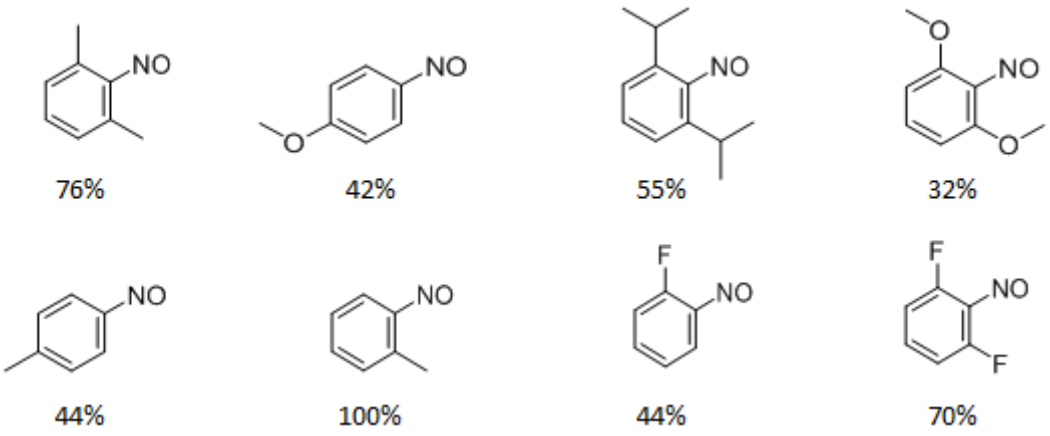


Figure 17. Nitroso-arenes with reported yields. The numbers are rounded on the integers.

6.2.3 Mills reaction

The last step is Mills reaction[45] where anilines and nitroso-arenes react with each other in the presence of acid media. The procedure was inspired by article Organic-chemical practical course, Summer semester 2007[46].

The Mills reaction consists of the nucleophilic attack of aniline on a nitroso group and of dehydration in acid media. In this case an acetic acid 37% was used which has hygroscopic

properties. After the dehydration of the intermediate azobenzenes were observed (Figure 18.) [45, 47]. This reaction was used for the coupling of nitroso + amine groups in the final step of this work to gain modified inhibitors of miR-21.

According to the general procedure the required nitrosobenzene was diluted in acetic acid, and 4-amino-*N*-(prop-2-yn-1-yl)benzamide was added. The solution was stirred for 5 days at RT in average, light protected by aluminum foil.

The reaction mixture was diluted with water and extracted three times with ethyl acetate (EtOAc). The combined organic layers were washed with water, half-concentrated HCl , again with water and dried over sodium sulfate. Then the solvent was evaporated. The reported yield was 14-22% (Table 1.).

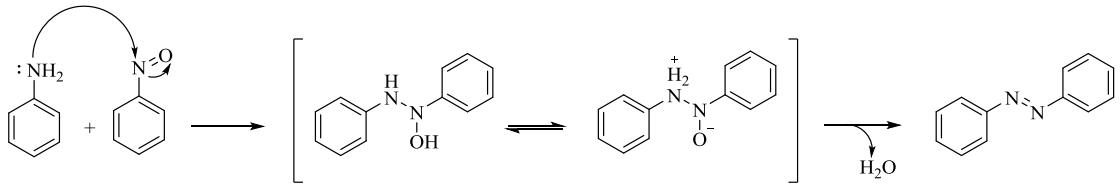
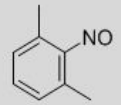
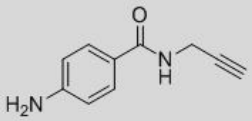
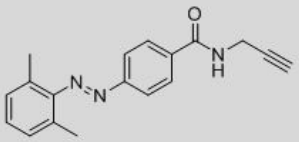
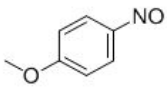
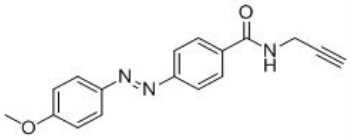
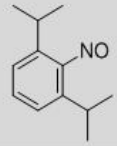
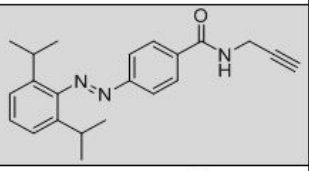
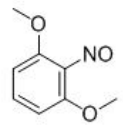
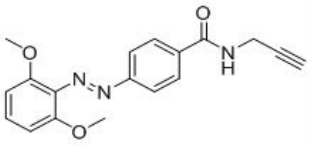
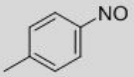
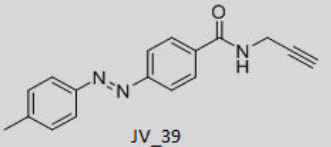
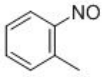
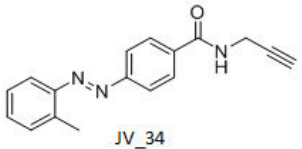
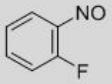
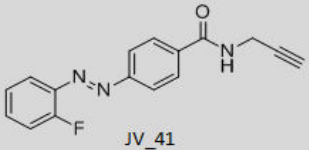
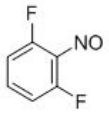
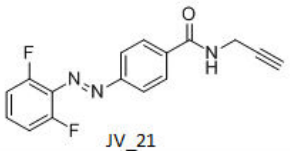


Figure 18. Mechanism of Mills reaction. Coupling of an amine with nitroso-arenes in presence of acetic acid.

Table 1. **Reaction conditions**: room temperature (RT) , nitroso-derivatives(1eq.), aniline (0.5 – 1 eq.), reaction time (5 days), solvent acetic acid 37%. In the table shows final products which were possible to be synthetized with reported yields. The numbers are rounded in the integers.

Used nitroso-arenes	Used aniline	Theoretical final product	Yield (%)
			0
			0
			0
			0
		 JV_39	20
		 JV_34	14
		 JV_41	20
		 JV_21	22

6.3 Preparation of the azo-bond/unsuccessful pathways

In the project two other pathways to reach an azo-bond were applied. The strategies were chosen according to articles dealing with the preparation of azobenzenes.

The first attempt was focused on coupling of nitro group with amine in presence of potassium hydroxide. The conditions were changing during the labwork to optimize the

reaction. Then coupling with two amines in presence of *tert*-butyl hypochlorite (tBuOI) with sodium iodide was tried.

6.3.1 Reduction of nitro group by potassium hydroxide

The first unsuccessful pathway to obtain an azo-bridge was reduction of nitro group by potassium hydroxide inspired by article One step synthesis of azo compounds from nitroaromatics and anilines[48].

In this reaction nitro group was reduced by potassium hydroxide to nitroso group. The possible mechanism shows nucleophilic attack of aniline at nitro group of the nitroso-arene; during the reaction an intermediate was formed. In the presence of KOH and in consequence of cleavage of the intermediate under heating a nitroso compound was produced. The coupling of nitroso compound and aniline was expected to provide an azo compound (Figure 19.).

The reaction was performed under N_2 atmosphere at 200 °C for 25 hours. Upon check of LC/MS no final product was observed; just starting materials were identified in the solution. This reaction was tested under various modified conditions (temperature, duration), but results kept being negative [48].

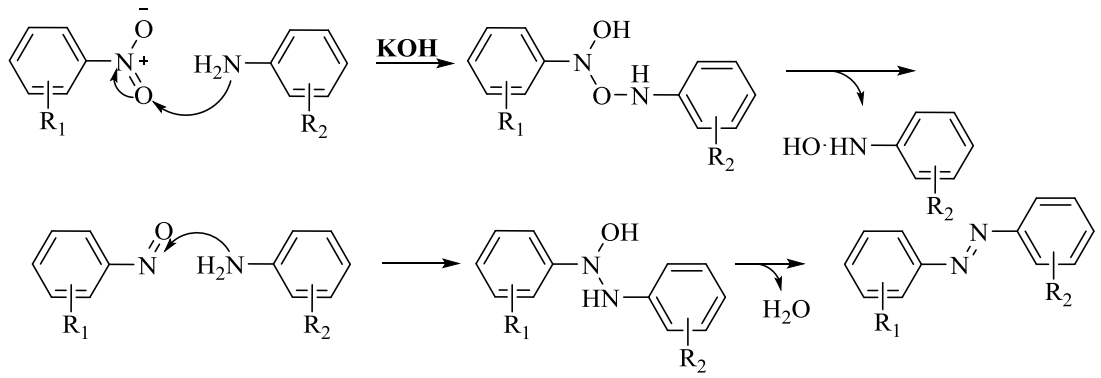


Figure 19. Reduction of nitro group by potassium hydroxide

6.3.2 Oxidative dimerization of aromatic amines using *t*BuOI

In the second reaction *t*BuOI was used for obtaining an azo bond. This procedure was based on article Oxidative Dimerization of Aromatic Amines using tBuOI: Entry to Unsymmetric Aromatic Azo Compounds[49] .

In this reaction quite acidic hydrogens atoms of aniline with EDG were attacked by iodine (*t*BuOI) to generate $ArNI_2$ through a hydrogen-iodine exchange process. Then by

means of elimination of 2HI from $ArNI_2$ and coupling with unreacted $ArNH_2$ the process was expected to produce an azo bond (Figure 20.).

THF was used as a solvent and the reaction was going at RT for 48 hours. Final products were checked by LC/MS with result of mixture of final compounds. Every possible combination between two different anilines could be observed. The reason for the side-products in the solution can be justified by the fact that the reaction is suitable for symmetric compounds. The product was impossible to purify because of very similar properties of each compound[49].

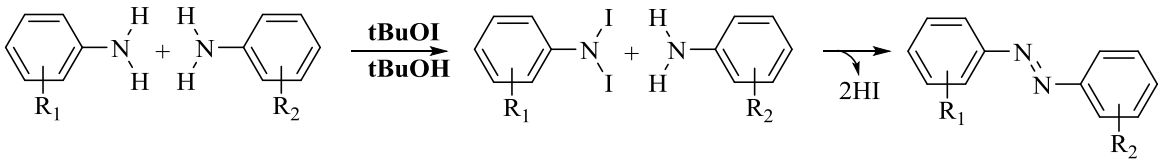


Figure 20. Oxidative dimerization of aromatic amines using *t*BuOI to gain symmetric azobenezenes

7. Methods

This section of methods includes the main methods used during the lab work. They are divided into sections of purification, analysis and spectroscopy. Some theoretical parts are included in each section.

7.1 Purification

The purification is used for removing some additional products of synthesis such as side products, starting material and other impurities added during the lab work. All purification methods are based on some kind of chromatography.

7.1.1 Chromatography

Lot of kinds of chromatography are known and they are characterized by stationary and mobile phases. Very common is the stationary phase of porous solids, SiO_2 or Al_2O_3, which bind mobile phase and crude product that is supposed to be purified. The mobile phase occurs in two variants (gas and liquid) which are able to carry the crude product through the solid. Each different molecule in product can bind the stationary phase with different properties (these properties are shown as an elution speed of the products through the stationary phase). This speed of moving ofthe products can be caused by different polarity of mobile phases or

by the mixture of mobile phases. At the end of the column(stationary phase) the mobile phase is collected in fractions and the product inside is divided into appropriate fractions[50].

7.1.2 Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a quick method for analysing how many components are in the mixture or to identify the substances in the solution. It is possible to compare some new retention factor (R_f) with already known R_f of these substances. The concept of the TLC is almost the same as the aforementioned type of chromatography, but in this case the stationary phase is spread out on thin glass or aluminum plates. The sample is spotted at the bottom of the plate and carried with mobile phase to the top of the plate by capillary action. When the eluent reaches the top of the plate then the plate is removed from the chamber with eluent and the spots are analyzed. The stationary phase is usually mixed with some fluorescent substances which help to see the spots of the substances upon the irradiation by UV-light. Solid absorbent is usually prepared from silica or alumina[51].

7.1.3 Crystallization

Crystallization is a method used to purify solid compounds. Each compound has a different solubility in the solvents (temperature, polarity) and according to these properties it is possible to remove undesirable impurities from the solids. Crystals form with the cooling of the solution or by adding more or less polar solvents as compared to the initial one. Impurities are excluded from the crystals and the pure solid can be removed by filtration[51].

7.2. Analysis

These methods are used for identification of products. One of the most important methods is nuclear magnetic resonance (NMR) where the structural information of the molecule is normally described. Liquid chromatography – mass spectrometry (LC/MS) is a method very commonly used for quick identification of required substances in a solution. TLC which was described in the previous part hereof was also used.

7.2.1 Nuclear Magnetic Resonance

Spin is a complicated property of an atomic nuclei with an odd mass. Spin is generated in the presence of magnetic field. In a uniform magnetic field different spin states occurring in different energy states are reached. The energy state of the nuclei is affected by the surroundings. As an example it is possible to mention hydrogen nucleus on an aromatic ring compared to hydrogen in methyl group - two different amounts of energy are needed to

change the energy states. So the main finding is that the nucleus absorbs exactly the amount of energy necessary to pass over one state to another.[52]

A studied sample inserted in a uniform magnetic field is scanned with radio waves at different frequencies which form a spectrum with peaks at wavelengths where energy was absorbed. The environment of the specific nuclei produces characteristic peaks which are necessary for analysis of the sample. The power of the magnetic field can be modified for specific types of nuclei, in general for ^{1}H and ^{13}C. The integrals of the peaks inform about the proportions of distinct nucleus environments in a sample and form ”splitting of the peaks” that is characterized by the peak into several closely placed peaks because of the interaction with adjacent nuclei[53].

7.2.2 Liquid Chromatography/Mass Spectroscopy

The connection of high performance liquid chromatography (HPLC) with Mass Spectrometry gives a very sensitive LC/MS instrument. Mass spectrometers (MS) work on the basis of converting the analytes to charged molecules (ions) and some additional fragments during the ionization process. In general a mass spectrometer scans the sample for different weights and provides a spectrum of different masses. The MS-system consists of a high vacuum pump, a sample inlet, an ion source, an analyzer system and a detector[54]. It is very necessary to deliver the ions into the vacuum where they are sent by pumps to avoid a collision with other molecules. The sample inlet is responsible for loading of analyzed molecules. The ion source transforms the molecules of the sample into the charged form and can be tuned up by electric field. There are several possibilities to create ions – impact ionization, chemical ionization, fast atom bombardment and electrospray ionization. These techniques are characterized by types and size of the fragments. Each technique is unique for some molecules because not all of them are suitable for the analyzed molecules, they can´t survive the process. The analyzer system distinguishes the ions so that only one weight could enter the detector at a time. Some types of analyzers are distinguished, such as the quadropole analyzer, the ion trap analyzer, and the magnetic sector analyzer. The MS system connected to HPLC divides various molecules in the sample and provides weight of the each molecule individually[50].

7.3 Spectroscopy

The spectroscopy was used in this case for analysis of E/Z isomers in the solution and for determination of the ratio between both states. During the measuring the duration between changes was observed. The results are attached in Appendix.

7.3.1 UV/Vis Spectroscopy

UV/Vis is the most common analyzing physical-chemistry method. It works in range 200 – 800 nm. The measuring is based on light transmission through the sample. During the absorption valence electrons which are part of bonding molecular orbitals are excited. Absorption of the photons leads to excitation of the electron to a higher energy state and as a result of that the molecule passes between the vibration – rotation states. That process creates the spectrum which can be observed[50].

8. Photo-physical testing

In this part the properties of molecules upon the irradiation and the structure changes in time are described.

For the photo-physical testing 2 different lights (UV/VIS) were used to modify the structures of inhibitors. After the irradiation, inhibitors have demonstrated changes of their structure between two isomers *cis-trans* (Figure 21.). The possibility to swap the structure is the main reason why azobenzenes were chosen for this work.

In general, the wavelengths needed for obtaining E/Z forms are around 350 – 450 mn as described in the theoretical part hereof. All prepared compounds changed their structures after the irradiation for some time until they achieved the photostationary state (PSS).

The used lights were within range 302 – 432 nm and the presence of compound in VIS - light was maximally 50 minutes, in UV it was much less because of the harmful impact for biological materials – 4.5 minutes. The tested substances were dissolved in the mixture of DMSO/water. Each isomer shows the absorption maximum in a different spectrum; it is described in graphs (Appendix) with maximums in UV and Vis spectrums which are decreased or increased depending on the presence of appropriate lights.

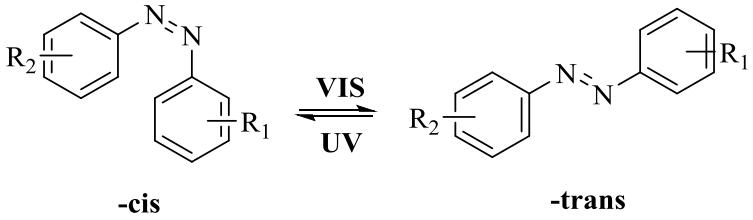
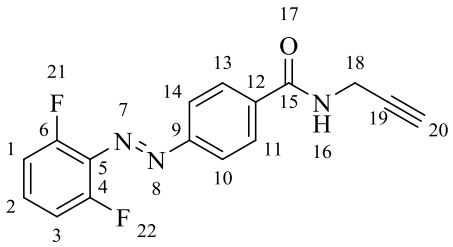


Figure 21. Changes between *cis* and *trans* isomers influenced by various wavelenghts of UV/VIS 302 – 432 nm.

9. Experimental

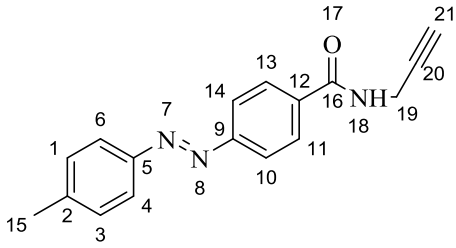
In the laboratory basic equipment for laboratory work was used – rotary evaporator for solvents, manual silica columns. For analysis of final substances a Varian 400Hz NMR Spectrometer was used. The LC/MS-system was also used for quick analysis of the solutions. For the samples of LC/MS solvent water:acetonitrile (1:1) was used, the applied ionization method was electro spray ionization and the analysis method was quadropole mass. For the spectroscopy UV and Vis lamps with various filters were used.

9.1 Synthesis of the potential inhibitors



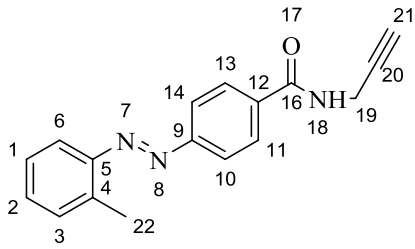
Chemical Formula: $C_{16}H_{11}F_2N_3O$
Molecular Weight: 299,28

JV_21-4-((2,6-difluorophenyl)diazenyl)-*N*-(prop-2-yn-1-yl)benzamide : 1,3-difluoro-2-nitrosobenzene (78 mg, 0.55 mmol) was dissolved in acetic acid (5.06 ml). 4-amino-N-(prop-2-yn-1-yl)benzamide was added (94.96 mg, 0.55 mmol). The mixture was stirred at room temperature for 5 days, light protected by aluminum foil. The reaction mixture was diluted with water (30 ml) and extracted three times with EtOAc (30 ml each). The organic layer was washed with water (30 ml), half-concentrated HCl (30 ml), again with water (30 ml) and dried over sodium sulfate. For purification column (8:2, penthene:EtOAc) was used, (35 mg, 21,5 %) **^{1}H NMR (400 MHz, Chloroform-*d*)** δ 8.00 - 7.93 (m, 4H - $4H_{10,11,13,14}$), 7.72 - 7.70 (m, H – H_2), 8.10 - 7.04 (m, 2H-$2H_{1,3}$), 6.36 (br, 1H - $1H_{16}$), 4.30 (dd, J = 7.6, 2.4 Hz, 2H – H_{18}), 2.32 (t, J = 5.2 Hz, 1H – H_{20}) **^{13}C NMR (101 MHz, cdcl3)** δ 166.18, 157.20, 154.91, 154.65, 136.20, 131.17, 128.04, 127.79, 123.12, 72.22, 29.90.



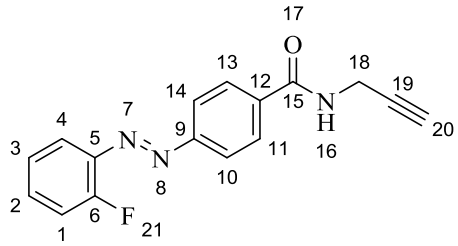
Chemical Formula: $C_{17}H_{15}N_3O$
Molecular Weight: 277,33

JV_34-*N*-(prop-2-yn-1-yl)-4-(o-tolyldiazenyl)benzamide : 1-methyl-2-nitrosobenzene (208.55 mg, 1.72 mmol) was dissolved in acetic acid (14 ml). 4-amino-N-(prop-2-yn-1-yl)benzamide was added (200 mg, 1.15 mmol). The mixture was stirred at room temperature for 5 days, light protected by aluminum foil. The reaction mixture was diluted with water (30 ml) and extracted three times with EtOAc (30 ml each). The organic layer was washed with water (30 ml), half-concentrated HCl (30 ml) and again with water (30 ml) and dried over sodium sulfate. For purification column (6:4, penthene:EtOAc) was used and it wasn´t still enough clean for NMR. The next step was crystalization with DCM + HEX (couple of drops from each) (42 mg, 13.2 %) **^{1}H NMR (400 MHz, Chloroform-*d*)** δ 7,97– 7.92 (m, 4H - $4H_{10,11,13,14}$), 7.65 (dd, J = 9,2, 1.2 Hz, 1H - $1H_{3,6}$), 7.42 – 7.28 (m, 2H-$2H_{1,2}$), 6.35 (br, 1H - $1H_{18}$), 4.30 (dd, J = 7.6, 2.4 Hz, 2H - $2H_{19}$), 2.74 (s, 3H - $3H_{22}$), 2.32 (t, J = 2.6 Hz, 1H - $1H_{21}$). **^{13}C NMR (101 MHz, cdcl$_3$)** δ 166.53, 154.93, 150.75, 138.99, 135.31, 131.81, 131.56, 128.16, 126.61, 123.22, 115.51, 72.28, 30.10, 30.01, 17.69.



Chemical Formula: $C_{17}H_{15}N_3O$
Molecular Weight: 277,33

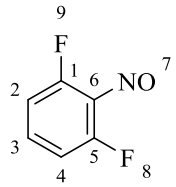
JV_39-*N*-(prop-2-yn-1-yl)-4-(p-tolyldiazenyl)benzamide : 1-methyl-4-nitrosobenzene (250 mg, 2.06 mmol) was dissolved in acetic acid (18.96 ml). 4-amino-N-(prop-2-yn-1-yl)benzamide was added (179.43 mg, 1.03 mmol). The mixture was stirred at room temperature for 5 days, light protected by aluminum foil. The reaction mixture was diluted with water (30 ml) and extracted three times with EtOAc (30 ml each). The organic layer was washed with water (30 ml), half-concentrated HCl (30 ml) and again with water (30 ml) and dried over sodium sulfate. For purification column (8:2, penthene:EtOAc) was used. (114 mg, 19.9 %) **^{1}H NMR (400 MHz, Chloroform-*d*)** δ 7.96 - 7.91 (m, 4H - $4H_{10,11,13,14}$), 7.87 - 7.84 (m, 2H - $2H_{4,6}$), 7.33 (d, J = 8.4 Hz, 2H - $2H_{1,3}$), 6.351 (br, 1H – H_{18}), 4.29 (dd, J = 7.6, 2.4 Hz, 2H – H_{19}), 2.45 (s, 3H – H_{15}), 2.31 (t, J = 5.2 Hz, 1H – H_{21}) **^{13}C NMR (101 MHz, cdcl$_3$)** δ 166.53, 154.70, 150.82, 142.57, 135.21, 129.99, 128.15, 123.29, 123.02, 72.27, 30.08, 21.74.



Chemical Formula: $C_{16}H_{12}FN_3O$
Molecular Weight: 281,29

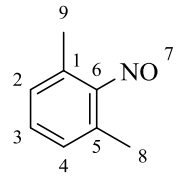
JV_41-4-((2-fluorophenyl)diazenyl)-*N*-(prop-2-yn-1-yl)benzamide : 1-fluoro-2-nitrosobenzene (200 mg, 1.60 mmol) was dissolved in acetic acid (14.75 ml). 4-amino-N-(prop-2-yn-1-yl)benzamide was added (139.25 mg, 0.80 mmol). The mixture was stirred at room temperature for 5 days, light protected by aluminum foil. The reaction mixture was diluted with water (30 ml) and extracted three times with EtOAc (30 ml each). The organic layer was washed with water (30 ml), half-concentrated HCl (30 ml) and again with water (30 ml) and dried over sodium sulfate. For purification column (8:2, penthene:EtOAc) was used. (89 mg, 19.8 %) **^{1}H NMR (400 MHz, Chloroform-*d*)** δ 8.02 - 7.98(m, 2H - $2H_{11,13}$), 7.96 - 7.93(m, 2H - $2H_{10,14}$), 7.729 - 7.696(m, H - $H_{2,4}$),7.096 - 7.040(m, 2H-$2H_{1,3}$), 6.358 (d, *J* = 11,2 Hz, $1H_{16}$), 4.299 (dd, *J* = 7.6, 1,6 Hz, 2H – H_{18}), 2.317(t, *J* = 5.2 Hz, 1H – H_{20}) **^{13}C NMR (101 MHz, cdcl$_3$)** δ 166.25, 161.72, 159.14, 154.52, 140.52, 135.76,123.05, 117.23, 105.30, 79.24, 72.17, 29.96.

9.2 Synthesis of nitoroso derivatives



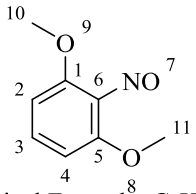
Chemical Formula: $C_6H_3F_2NO$
Molecular Weight: 143,09

JV_20-1,3-difluoro-2-nitrosobenzene : 2,6-difluoroaniline (100 mg, 0.77 mmol) dissolved in DCM (1.73 ml) and oxone (952 mg, 1.55 mmol) dissolved in water (8.93 ml) was mixed each other. The reaction mixture was stirred at room temperature for 1 hour. There was obvious change of colour when the reaction started to react. All starting materials were checking by TLC (pentane) and LC/MS during the reaction until disappeared. The phases were seperated and the aqueous phase extracted twice with DCM. The organic layer was washed with diluted hydrochloric acid (30 ml, 3 %), saturated sodium hydrocarbonate solution (30 ml), twice with water (30ml) and once with brine (30 ml). After drying over sodium sulfate, the DCM was evaporated at the rotary evaporater. (58 mg, 70.4 %)



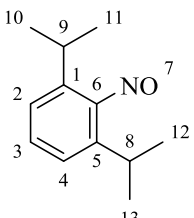
Chemical Formula: C_8H_9NO
Molecular Weight: 135,17

JV_18-1,3-dimethyl-2-nitrosobenzene : 2,6-dimethylaniline (100 mg,0.83 mmol) dissolved in DCM (2.5 ml) and oxone (1015 mg, 1.65 mmol) dissolved in water (7 ml) was mixed each other. The reaction mixture was stirred at room temperature for 1 hour. There was obvious change of colour when the reaction started to react. All starting materials were checking by TLC (DCM:Methanol 97:3) and LC/MS during the reaction until disappeared. The phases were seperated and the aqueous phase extracted twice with DCM. The organic layer was washed with diluted hydrochloric acid (30 ml, 3 %), saturated sodium hydrocarbonate solution (30 ml), twice with water (30ml) and once with brine (30 ml). After drying over sodium sulfate, the DCM was evaporated at the rotary evaporator. (85 mg, 76 %)



Chemical Formula: $C_8H_9NO_3$
Molecular Weight: 167,16

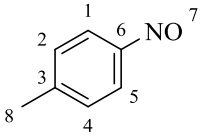
JV_28-1,3-dimethoxy-2-nitrosobenzene : 2,6-dimethoxyaniline (150 mg,0.98 mmol) dissolved in DCM (3 ml) and oxone (1204 mg, 1.96 mmol) dissolved in water (11.31 ml) was mixed each other. The reaction mixture was stirred at room temperature for 2 hour. There was obvious change of colour when the reaction started to react. All starting materials were checking by TLC (hexene:EtOAc 80:20) and LC/MS during the reaction until disappeared. The phases were seperated and the aqueous phase extracted twice with DCM. The organic layer was washed with diluted hydrochloric acid (30 ml, 3 %), saturated sodium hydrocarbonate solution (30 ml), twice with water (30ml) and once with brine (30 ml). After drying over sodium sulfate, the DCM was evaporated at the rotary evaporater. (53 mg, 32.4 %)



Chemical Formula: $C_{12}H_{17}NO$
Molecular Weight: 191,27

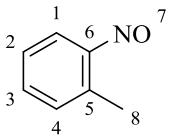
JV_30-1,3-diisopropyl-2-nitrosobenzene : 2,6-diisopropylaniline (300 mg,1.70 mmol) dissolved in DCM (5 ml) and oxone (2078 + 2600 mg – reaction was going slowly - , 3.38 mmol) dissolved in water (19 ml) was mixed each other. The reaction mixture was stirred at room temperature for 3 hours. There was obvious change of colour when the reaction started to react. All starting materials were checking by TLC (hexene:EtOAc 80:20) and LC/MS during the reaction until disappeared. The phases were seperated and the aqueous phase extracted twice with DCM.

The organic layer was washed with diluted hydrochloric acid (30 ml, 3 %), saturated sodium hydrocarbonate solution (30 ml), twice with water (30ml) and once with brine (30 ml). After drying over sodium sulfate, the DCM was evaporated at the rotary evaporater. (179 mg, 55.3 %)



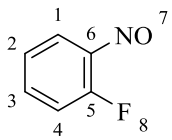
Chemical Formula: C_7H_7NO
Molecular Weight: 121,14

JV_34-1-methyl-2-nitrosobenzene : For this reaction, the pure starting material was used.



Chemical Formula: C_7H_7NO
Molecular Weight: 121,14

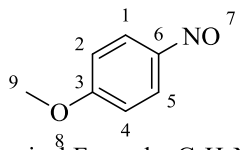
JV_38-1-methyl-4-nitrosobenzene : p-toluidine (500 mg,4.67 mmol) dissolved in DCM (25.7 ml) and oxone (5740 mg, 9.34 mmol) dissolved in water (56 ml) was mixed each other. The reaction mixture was stirred at room temperature for 2 hour. There was obvious change of colour when the reaction started to react. All starting materials were checking by TLC (hexene:EtOAc 80:20) and LC/MS during the reaction until disappeared. The phases were seperated and the aqueous phase extracted twice with DCM. The organic layer was washed with diluted hydrochloric acid (30 ml, 3 %), saturated sodium hydrocarbonate solution (30 ml), twice with water (30ml) and once with brine (30 ml). After drying over sodium sulfate, the DCM was evaporated at the rotary evaporater. (250 mg, 44.2 %)



Chemical Formula: C_6H_4FNO
Molecular Weight: 125,10

JV_40-1-fluoro-2-nitrosobenzene : 2-fluoroaniline (400 mg,3.60 mmol) dissolved in DCM (20 ml) and oxone (4426 mg, 7.20 mmol) dissolved in water (43 ml) was mixed each other. The reaction mixture was stirred at room temperature for 3 hours. There was obvious change of colour when the reaction started to react. All starting materials were checking by TLC (hexene:EtOAc 80:20) and LC/MS during the reaction until disappeared. The phases were seperated and the aqueous phase extracted twice with DCM. The organic layer was washed with diluted hydrochloric acid (30 ml, 3 %), saturated sodium hydrocarbonate solution (30 ml), twice with water (30ml) and once with

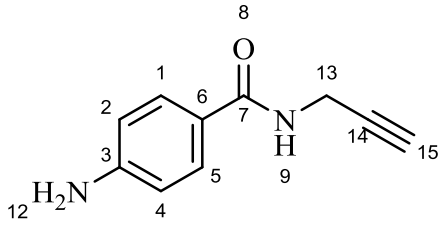
brine (30 ml). After drying over sodium sulfate, the DCM was evaporated at the rotary evaporater. (200 mg, 44.4 %)



Chemical Formula: $C_7H_7NO_2$
Molecular Weight: 137,14

JV_36-1-methoxy-4-nitrobenzene : p-anisidine (500 mg, 4.06 mmol) dissolved in DCM (12 ml) and oxone (4991 mg, 8.12 mmol) dissolved in water (46.8 ml) was mixed each other. The reaction mixture was stirred at room temperature for 2 hours. There was obvious change of colour when the reaction started to react. All starting materials were checking by TLC (hexene:EtOAc 80:20) and LC/MS during the reaction until disappeared. The phases were seperated and the aqueous phase extracted twice with DCM. The organic layer was washed with diluted hydrochloric acid (30 ml, 3 %), saturated sodium hydrocarbonate solution (30 ml), twice with water (30ml) and once with brine (30 ml). After drying over sodium sulfate, the DCM was evaporated at the rotary evaporater. (234 mg, 42 %)

9.3 Synthesis of 4-amino-*N*-(prop-2-yn-1-yl)benzamide



Chemical Formula: $C_{10}H_{10}N_2O$
Molecular Weight: 174,20

JV_8-4-amino-*N*-(prop-2-yn-1-yl)benzamide : 4-aminobezoic acid (500 mg, 3.45 mmol) was dissolved in DCM (12.5 ml), followed by the addition of diisopropylcarbodiimide (803.79 mg, 14.60 mmol) and HOBt (586.32 mg, 4.34 mmol). Propargylamine (803.79mg, 14.59 %) was added and the reaction was stirred at room temperature for 12 hours. The reaction was quenched with (10 ml) water and extracted two-times with DCM (10ml). The organic layer was dried with sodium sulfate. Solvent was evaporated. The product was purified by column chromatography (chloroform/methanol 97:3). (503 mg, 79.2 %) **^{1}H NMR (400 MHz, Chloroform-*d*)** δ 7.63 - 7.60 (m, 2H - $2H_{1,5}$), 6.68 - 6.64 (m, 2H – $H_{2,4}$), 6.12 (br, 1H - $1H_9$), 4.22 (ddd, J = 8.8, 2.8 Hz, 2H – H_{13}), 3.95 (s, 2H - $2H_{12}$), 2.26 (td, J = 5.2 Hz, 1H – H_{15})

10. Results and discussion

The effort to prepare designed derivatives during the labwork was only partly successful and it was not possible to achieve a relevant discussion about the groups in *ortho* and *para* positions regarding their influence on physical properties. On the other hand, the synthetic pathways for obtaining an azo bond were deeply tested and it is possible to say that substituents in *ortho* position feature a big portion of successful reaction.

During the synthetic route it was possible to prepare only **JV_21, JV_41, JV_39 and JV_34** compounds with reportable yields. The intermediates as appropriate nitroso-arenes were proved by analytical methods – LC/MS, TLC and no starting materials have been observed. It proves the proper selection of substituents.

In the synthetic part the Mills reaction was identified as a suitable reaction for preparation of an azo-bridge. The reaction was going well for half of the designed compounds. The problem was probably in very bulky substituents in *ortho* positions that caused steric hindrance and the coupling couldn´t be reached. The yields were in the range of 13.5 – 21.5%. These results show that even Mills reaction wasn´t the best method for obtaining an azo-bridge, but the yield was sufficient for another testing.

The other method comprising reduction of the nitro group by pottasium hydroxide was simply unsuitable for the preparation of the assymetric azobenezenes. No final product was observed there.

The oxidative dimerization was tested for the preparation of the azo-bridge, but this reaction is very suitable for symmetric azobenzenes. In the reaction a mixture of possible combinations between the amines with very similar properties was created, which was impossible to purify.

The nitroso-arenes were obtained by oxidation of the appropriate anilines and the yields were within range 32-76%. The losses of the compounds were probably caused by final washing and instability of the nitroso group.

The prepared compounds were tested for the stability upon irradiation and showed different properties in the ratio between *E/Z* forms. The compounds were irradiated for maximally 3000s by VIS and for 150s by UV light in order to avoid the harmful impact for biological materials. The most interesting compound was **JV_21** which had the best ratio between E/Z upon irradiation, more than 60/40. **JV_34** was even fully converted to *E* form during the reverse isomeration. The rest of the compounds showed insufficient properties upon irradiation.

The results from the physical measurements demonstrate that the EWG in *ortho* position induces conversion probably more than the EDG.

11. Conclusion

In conclusion new potential approaches of synthesis of a new ihibitors of miR-21 were investigated. In the theoretical part the latest information regarding the using of small compounds as inhibitors of microRNA and properties of azobenzenes were reviewed in detail. In the chemistry part successful/unsuccessful pathways used during the synthesis performed to provide products in practical and reproducible yields and purity were described. Another tested method proved that bulky substituents in *orto* positions do not enable the coupling to obtain an azo-bridge. The photo-physical properties of the prepared compounds were summarized and give an opportunity for further research in future project. Unfortunately none of the substances was with perfect results.

Manipulation with photoswitchable compounds in biological materials just with light seems very perspective for the future. Maybe it will take a long time until the right photochromic molecule is discovered for therapeutic purposes. But it is a big challenge with huge benefits for patients.

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13. Appendix – Graphs, results from irradiation

13.1 Compound JV_34

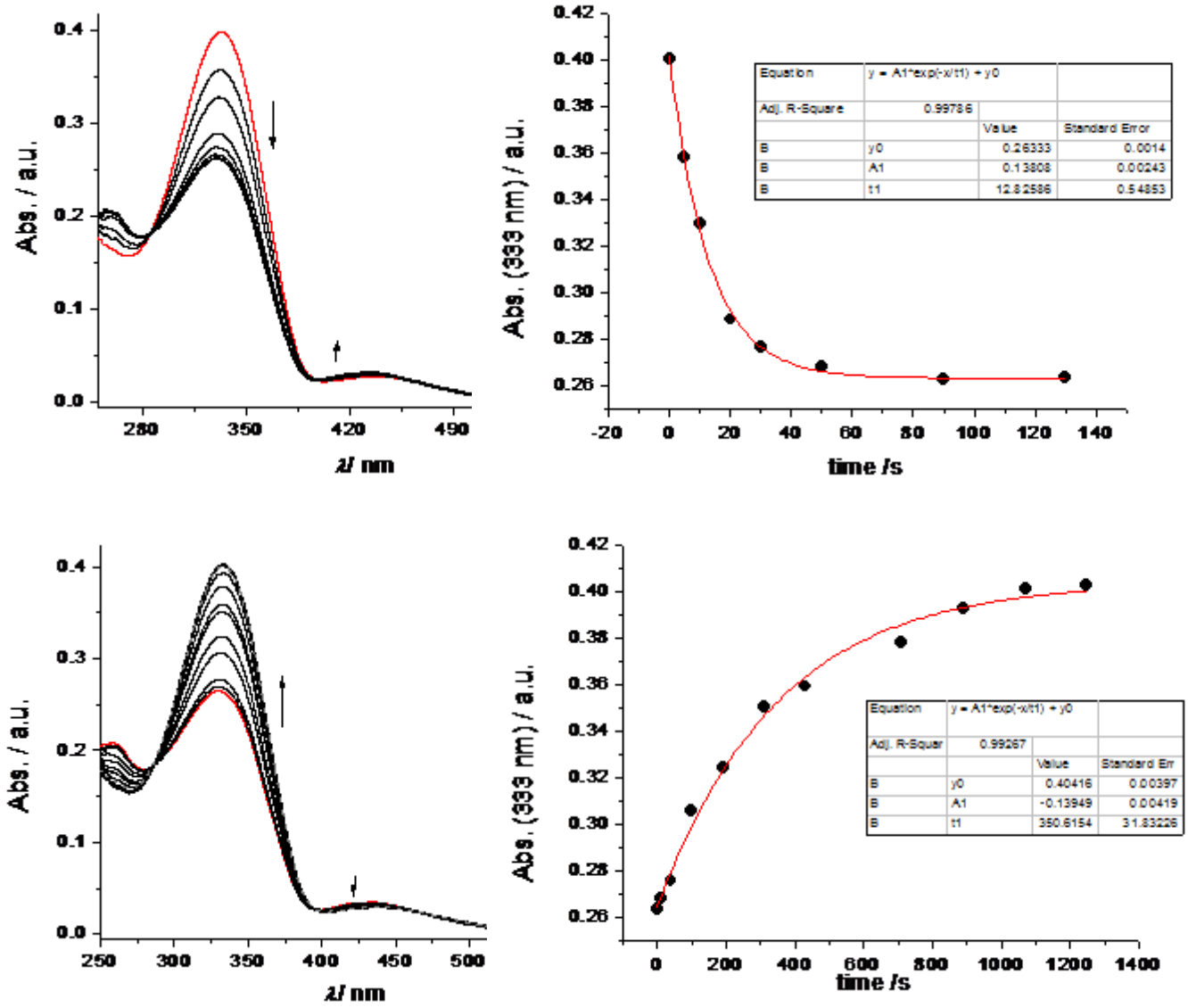

Figure 22. This figure characterizes JV_34 compound upon irradiation and its behaviour in time. The upper graphs demonstrate the time duration needed to convert E form as much as possible to Z in an appropriate wavelength. The red line shows the initial state of compound (100% E form) until it reaches the PSS. The light used for switching from E to Z had 302 nm wavelength. The compound was irradiated for 150s as long as the conversion was in progress. The mixture (E and Z forms) of compound contains probably 60:40 (E/Z) of each form after the irradiation. The lower graphs shows the behaviour of the mixture upon irradiation by Vis light (432nm) for 1300s. The red line demonstrates PSS after the irradiation by 302 nm. In this case the compound was completely converted back to Z form. Absorption maximum is 333 nm. The compound was dissolved in mixture of 20% DMSO/H_2O.

13.2 Compound JV_39

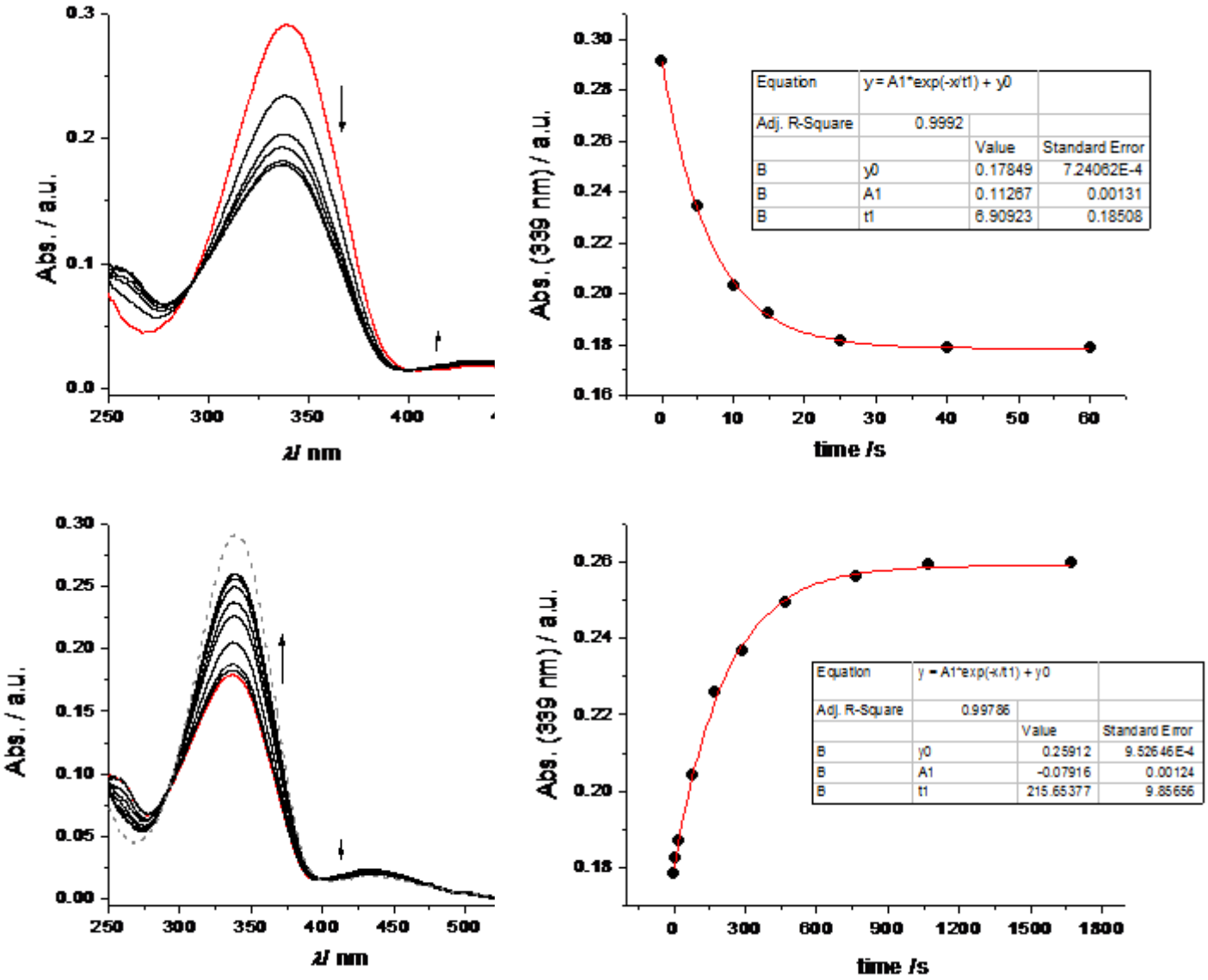

Figure 23. This figure characterizes compound JV_39 upon irradiation and its behaviour in time. The upper graphs demonstrate the time duration needed to convert E form as much as possible to Z in appropriate wavelength. The red line shows initial state of compound (100% E form) until it reaches the PSS. The light used for switching from E to Z had 302 nm wavelength. The compound was irradiating for 60s as long as the conversion was in progress. The mixture (E and Z forms) of compound contains probably 70:30 (E/Z) of each form after the irradiation. The lower graphs shows behaviour of the mixture upon irradiation by Vis light (432nm) for 1800s. The red line demonstrates PSS after the irradiating by 302 nm. In this case compound wasn´t completely converted back to Z form and dashed line shows the initial state. Absorption maximum is 339 nm. The compound was dissolved in mixture of 20% DMSO/H_2O.

13.3 Compound JV_41

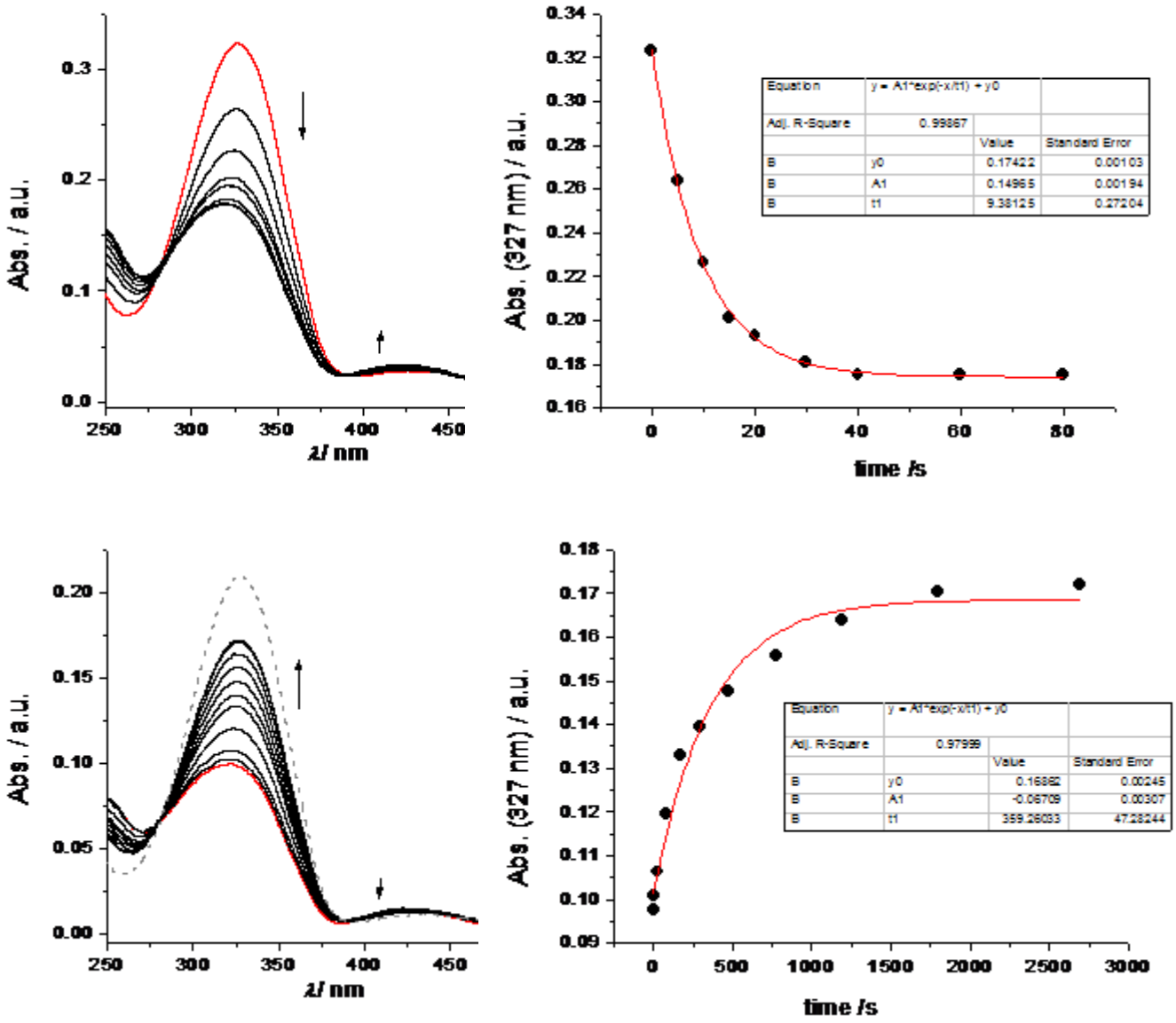

Figure 24. This figure characterizes compound JV_41 upon irradiation and its behaviour in time. The upper graphs demonstrate the time duration needed to convert E form as much as possible to Z in appropriate wavelength. The red line shows initial state of compound (100% E form) until it reaches the PSS. The light used for switching from E to Z had 302 nm wavelength. The compound was irradiating for 80s as long as the conversion was in progress. The mixture (E and Z forms) of compound contains probably 40:60 (E/Z) of each forms after the irradiation. The lower graphs shows behaviour of the mixture upon irradiation by Vis light (432nm) for 2750s. The red line demonstrates PSS after the irradiating by 302 nm. In this case compound wasn´t completely converted back to Z form and dashed line shows the initial state. Absorption maximum is 327 nm. The compound was dissolved in mixture of 1% DMSO/H_2O.

13.4 Compound JV_21

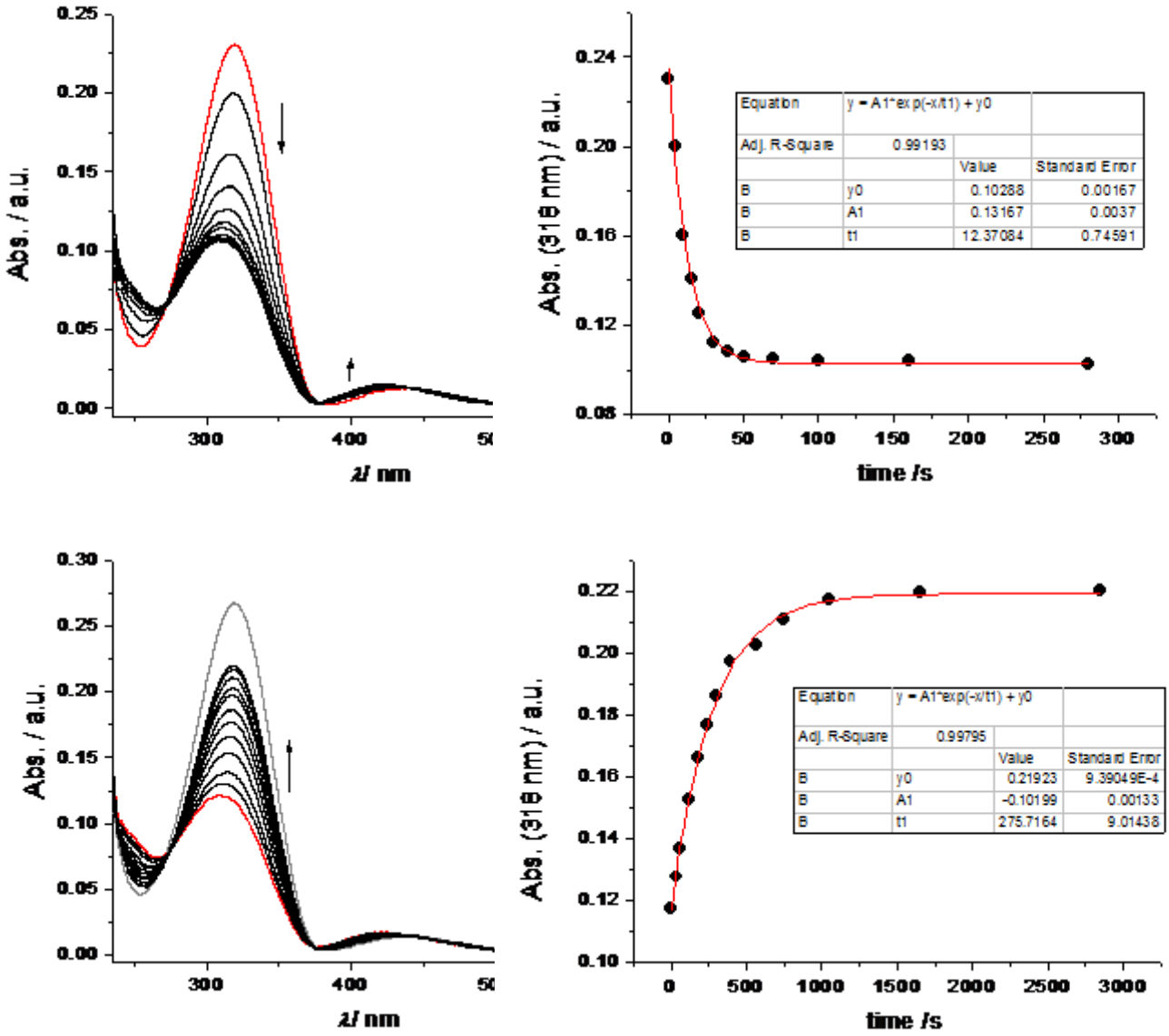

Figure 25. This figure characterizes compound JV_21 upon irradiation and its behaviour in time. The upper graphs demonstrate the time duration needed to convert E form as much as possible to Z in appropriate wavelength. The red line shows initial state of compound (100% E form) until it reaches the PSS. The light used for switching from E to Z had 302 nm wavelength. The compound was irradiating for 80s as long as the conversion was in progress. The mixture (E and Z forms) of compound contains probably 30:70 (E/Z) of each forms after the irradiation. The lower graphs shows behaviour of the mixture upon irradiation by Vis light (397nm) for 3000s. The red line demonstrates PSS after the irradiation by 302 nm. In this case compound wasn´t completely converted back to Z form and the gray line shows the initial state. Absorption maximum is 318 nm. The compound was dissolved in mixture of 1% DMSO/H_2O.