Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Pharmaceutical Chemistry and Drug Control

Diploma Thesis

Synthesis of Model Water-Soluble Azaphthalocyanine Fluorescence Quencher

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Hradec Králové, 2010 **Ljiljana Subara**

This is to declare that this diploma thesis is my own work and I worked on it on my own. All literature sources are properly cited in reference list.

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Abstract

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Azaphthalocyanine (AzaPc) or tetrapyrazinoporphyrazine as they are often termed in literature, and its substituted derivatives have been investigated extensively for applications as photodynamic therapeutics, dyes, catalysts, liquid crystals, non-linear optical materials and as a red fluorophore.

Quenching in general is a process that decreases the fluorescence of another compound. Azaphthalocyanine quenchers are synthetic dyes that decrease fluorescence by absorbing energy over a wide range of wavelengths to dissipate their absorbed energy as non fluorescence. However, in order to do so they must remain in close contact with the fluorescent compound.

In this work, we describe the approaches used for the synthesis of azaphthalocyanines (AzaPc) and ways to increasing quenching properties while attaining water solubility. We synthesized a compound which had water solubility as a hydrochloride and no aggregation, however it lacked sufficient quenching properties, and remained difficult to purify on TLC. For improvement of quenching an alternative precursor substituted with a tertiary amine instead of the methyl substituent was used, but purification could only be solved using a shielding group such as phthalimide; in which basicity would be removed and purification would no longer be an issue. The use of a shielding group is still under investigation and remains to be seen whether in vitro results will have positive results.

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Acknowledgments

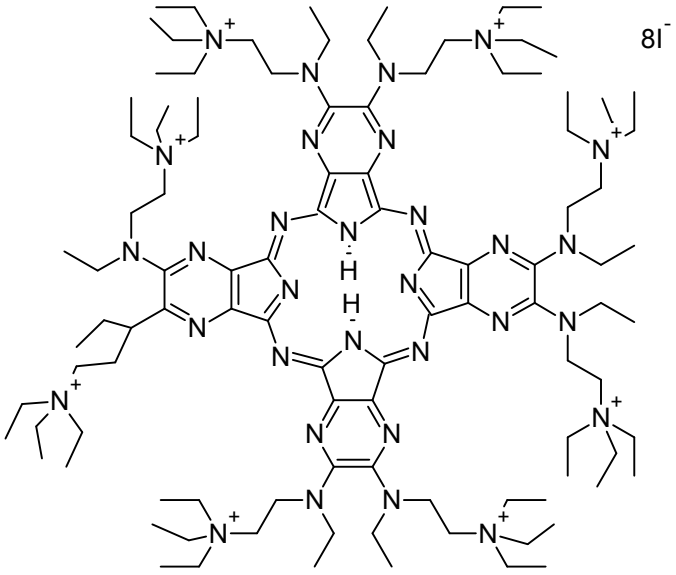
I would like to thank my supervisor Dr. Petr Zimčík Ph.D. for his guidance, help, and patience in the completion of this diploma thesis.

1. Abbreviations

ANTS	8-aminonaphthalene-1,3,6-trisulfonate
AzaPc	Azaphthalocyanine
BuOH	Butanol
DMF	Dimethylformamide
DPX	*p*-xylene-*bis*-pyridinium bromide
FAM	fluorescein
FRET	(Förster or Fluorescent) Resonance Energy Transfer
IR	Infra Red
MV^{2+}	Methylviologen
NMR	Nuclear Magnetic Resonance
PET	Photoinduced Electron Transfer
RET	Resonance Energy Transfer
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography

2. Aim of the research

In the past, our lab has carried out extensive research on the tetrapyrazinoporphyrazine molecule, also known as azaphthalocyanine (AzaPc), in the application of quenching of fluorescence[1]. Up until now synthesis of AzaPcs for this application have only resulted in water-insoluble molecules, however interest remained high due to their many favorable attributes. They display not only stable physical properties but chemical as well, and they show a strong absorption in different regions of the electromagnetic spectrum[2], which has been of interest in dark quencher studies. In this work, I focused on the approaches used for the synthesis of AzaPc and ways to increasing water solubility of the final compounds. Below I present the desired molecule that should retain quenching properties due to eight amino groups attached directly to macrocycle and eight aliphatic tertiary amines that may be quaternized or converted to hydrochloride leading thus to water-soluble derivatives.



Proposed model of water soluble AzaPc

3. Theoretical part

3.1. Foresight into photochemistry

Luminescence is the emission of light from any substance, and occurs from electronically excited states[3]. Luminescence can be divided into two categories, fluorescence and phosphorescence, depending on the nature of the excited state.

3.1.1 Phosphorescence

Phosphorescence is emission of light from triplet-excited states, in which the electron in the excited orbital has the same spin orientation as the ground-state electron[3]. Transitions to the ground state are forbidden and the emission rates are slow (100-1000 s^{-1}), so phosphorescence lifetimes are typically milliseconds to seconds[3]. Phosphorescence is usually not seen in fluid solutions at room temperature.

3.1.2 Fluorescence

Fluorescence is emission of light from singlet-excited states, in which the electron in the excited orbital is paired (of opposite spin) to the second electron in the ground-state orbital[3]. Return to the ground state is spin-allowed and occurs rapidly by emission of a photon. These emission rates of fluorescence are typically 10^8 s^{-1}, so that a typical fluorescence lifetime is near 10 ns. Fluorescence spectral data are generally presented as emission spectra. Emission spectra vary widely and are dependent upon the chemical structure of the fluorophore and the solvent in which it is dissolved.

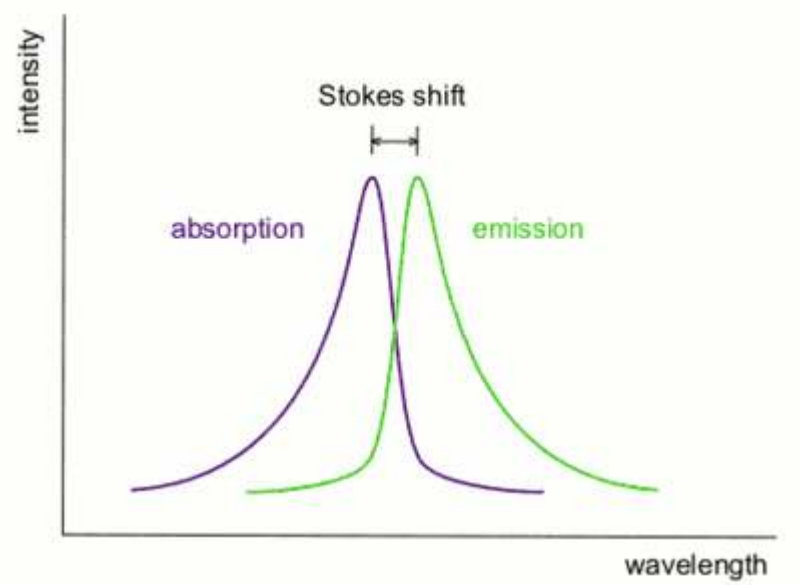


Figure 1. Fluorescence spectra with absorption, emission and stokes shift[4].

Examination of Figure 1 reveals that the energy of the emission is typically less than that of absorption. The Irish physicist, Sir. G. G. Stokes noticed that fluorescence most often occurs at lower energies or longer wavelengths, and this difference between positions of the band maxima of absorption and emission spectra of the same electronic transition became known as Stokes shift[3].

Sir G. G. Stokes noticed energy losses universally for fluorescent molecules in solution. He noted that one cause of the Stokes shift is due to the rapid decay to the lowest vibrational level of S_1, while fluorophores generally decay to higher vibrational levels of S_0 (Figure 2), resulting in further loss of excitation energy by thermalization of the excess vibrational energy[3]. Other causes of Stokes shift are due to solvent effects, complex formation and energy transfer[3].

3.1.3 Basic photophysics module and Jablonski Diagrams

Absorption of a photon raises a molecule to its lowest singlet excited state, for which three internal decay pathways exist[5]. They include *fluorescence* in which the molecule returns to the ground state with the emission of radiation; *internal conversion*, in which the energy of

the molecule is converted into vibrational energy; and *intersystem crossing*, in which the singlet state is converted to the triplet state[6]. These processes that occur between the absorption and emission of light can be illustrated by the Jablonski diagram (Figure 2). These diagrams are named after the Polish professor Alexander Jablonski who is still to this day regarded as the father of fluorescence spectroscopy because of his many accomplishments in this evolving field[3].

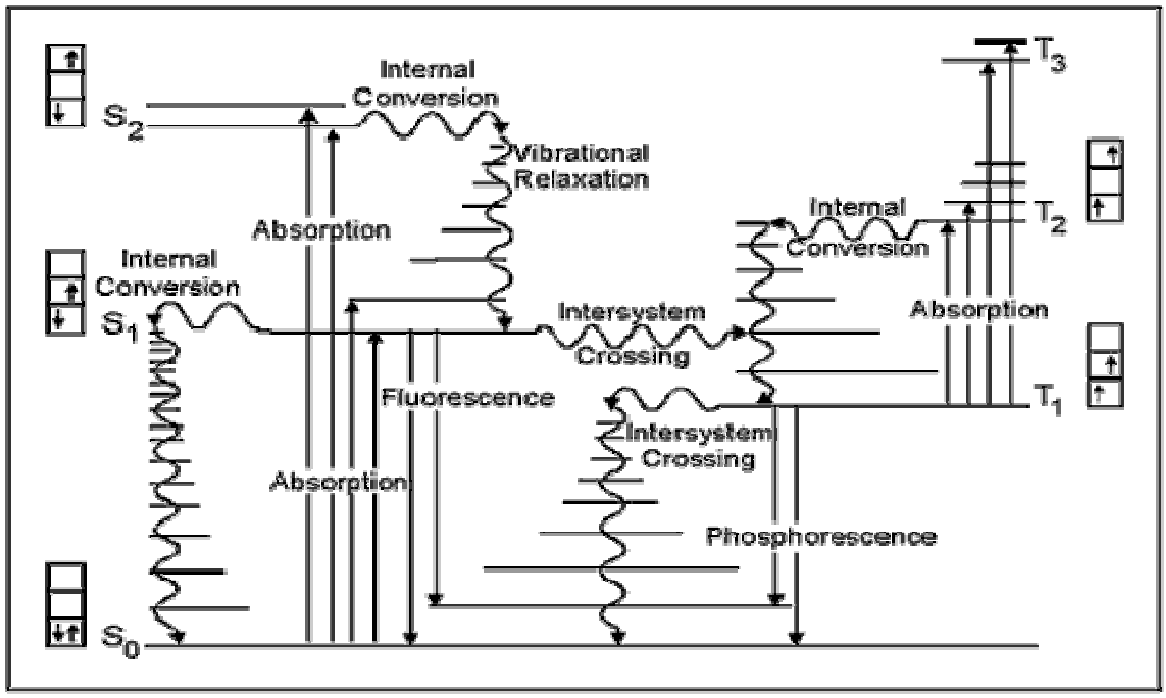


Figure 2: A Jablonski diagram illustrating the Basic Photophysics Module for photophysical radiative and non-radiative processes displayed by organic molecules in solution. The symbols S_0, S_1, T_2, etc., refer to the ground electronic state (S_0), first excited singlet state (S_1), second excited triplet state (T_2), and so on. The horizontal lines represent the vibrational levels of each electronic state. Straight arrows indicate radiative transitions, and curly arrows indicate non-radiative transitions. The boxes detail the electronic spins in each orbital, with electrons shown as up and down arrows, to distinguish their spin[6].

It should be noted that all transitions from one electronic state to another originate from the lowest vibrational level of the initial electronic state. For example, fluorescence occurs only from S_1, because the higher singlet states (S_2, etc.) decay so rapidly by internal conversion that fluorescence from these states cannot compete[3].

3.2 Fluorescence Quenching

Fluorescence quenching in general refers to any process that decreases the fluorescence intensity of another compound[3]. The energy emitted from a fluorophore is characteristically in the form of light. However, energy emission from some fluorophores can be in the form of heat dissipation. Molecules that dissipate absorbed energy as heat are the molecules of interest known as quenchers. A wide range of molecular interactions can lead to quenching. These include excited-state reactions, energy transfer, molecular arrangements, ground-state complex formations and collisional quenching. In this work we shall focus our attention on the three quenching processes usually encountered, namely resonance energy transfer (RET) static quenching (ground-complex formation) and collisional quenching.

Both collisional and static quenching requires molecular contact between the fluorophore and quencher[3]. In either case a close distance is required for quenching, in which the extent of quenching is dependent of molecular factors that affect the rate and probability of contact. These factors include steric shielding and charge-charge interaction[3]. RET is considered a form of dynamic quenching because its energy transfer occurs while the donor is in the excited state[7] and it occurs through space over long distances.

3.2.1 Collisional Quenching

Collisional quenching, a part of dynamic quenching, occurs when the excited-state fluorophore is deactivated by contact with a quencher[8]. The molecules are not chemically altered in the process. For collisional quenching, the decrease in intensity is described by the ratio of the fluorescence in the absence of quenching to the presence of the quencher by applying the Stern-Volmer equation[3]:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities observed in the absence and presence, respectively, of quencher, [Q] is the quencher concentration and K_{SV} is the Stern-Volmer quenching constant.

The accessibility of fluorophores to quenchers can be used to determine the location of fluorescent probes on macromolecules[3], and a wide variety of molecules can act as collisional quenchers, and the mechanism varies with the fluorophore-quencher pair. There are at least three mechanisms for collisional quenching. They include; intersystem crossing, electron exchange and photoinduced electron transfer (PET). Common substances used for quenching are halides: bromide and iodide (I^-), oxygen and acrylamide[3]. In the case of oxygen, which is paramagnetic, it is thought that it may cause the fluorophore to undergo intersystem crossing to the triplet state. In fluid solutions this long-lived triplet states decay before phosphorescence can occur. Halides such as iodine and bromine are also thought to cause intersystem crossing to the excited triplet state, promoted by spin-orbit coupling of the excited state fluorophore and the halogen. Other quenchers, such as Cu^{2+}, Pb^{2+}, Cd^{2+} and Mn^{2+} are thought to cause the donation of an electron from the fluorophore in the excited state[3].

As mentioned above, the quenching efficiency of collisional quenching depends on the quencher concentration. In the simplest case, then, a plot of F_0/F versus [Q] should yield a straight line with a slope equal to K_{SV}. Such a plot, known as a Stern-Volmer plot, is shown below (Figure 3) for the case of fluorescein quenched by iodide ion (I^-).

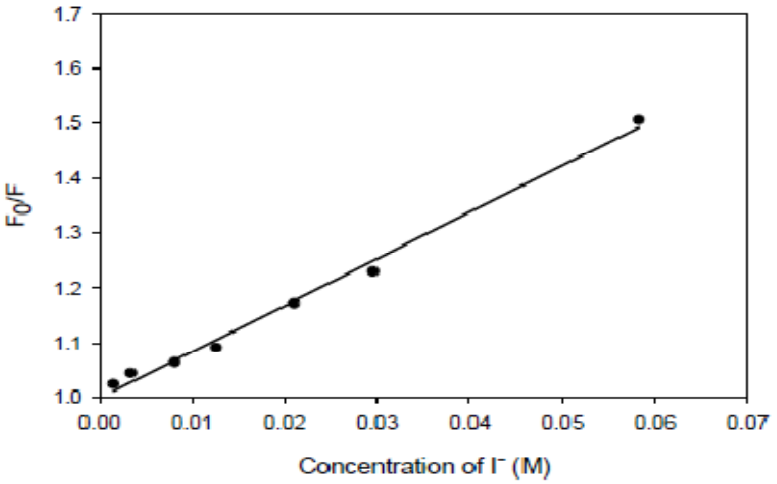


Figure 3: A Stern-Volmer plot of collisional quenching[3]

To get a sense for how efficient collisional quenching can be, it is informative to consider the root mean square distance over which a quencher can diffuse during the lifetime of the excited state (τ). This distance (x) is given by the Einstein equation[3]:

$$\Delta x^2 = 2D\tau$$

where D is the diffusion coefficient of the quencher. Consider an oxygen molecule at 25 °C. The diffusion coefficient in water is 2.5×10^{-5} cm^2 s^{-1}. In a typical tryptophan lifetime (4 ns) the oxygen molecule can travel 44 Å. If the lifetime is longer, then the average distances are longer.

3.2.2 Static Quenching

Static quenching occurs through formation of a ground state complex[7]. During this process the fluorophore forms a stable complex with another molecule that does not rely on diffusion in the excited state but rather an interaction by proton-coupled electron transfer through the formation of hydrogen bonds[3] (figure 4). If this *ground-state* is non-fluorescent then we can assume that the fluorophore has been statically quenched. In such a case, the dependence of the fluorescence as a function of the quencher concentration follows the relation:

$$F_0/F = 1 + K_a[Q]$$

where K_a is the association constant of the complex. Such cases of quenching via complex formation were first described by Gregorio Weber[3].

During static quenching the lifetime of the sample will not be reduced since those fluorophores which are not complexed and hence are able to emit after excitation will have normal excited state properties[3]. The fluorescence of the sample is reduced since the quencher is essentially reducing the number of fluorophores which can emit. Unlike collisional quenching which only affects the excited states of fluorophores, and thus no changes in absorption spectra are visible, ground-state complex formation frequently results in

perturbation of the absorption spectrum of the fluorophore[3]. This is because ground-state complex formation results in creation of a reporter-quencher dimer. The intramolecular dimer effectively decreases the concentration of the fluorescent reporter by creating a new, nonfluorescent reporter-quencher dimer with a unique absorption spectrum[7].

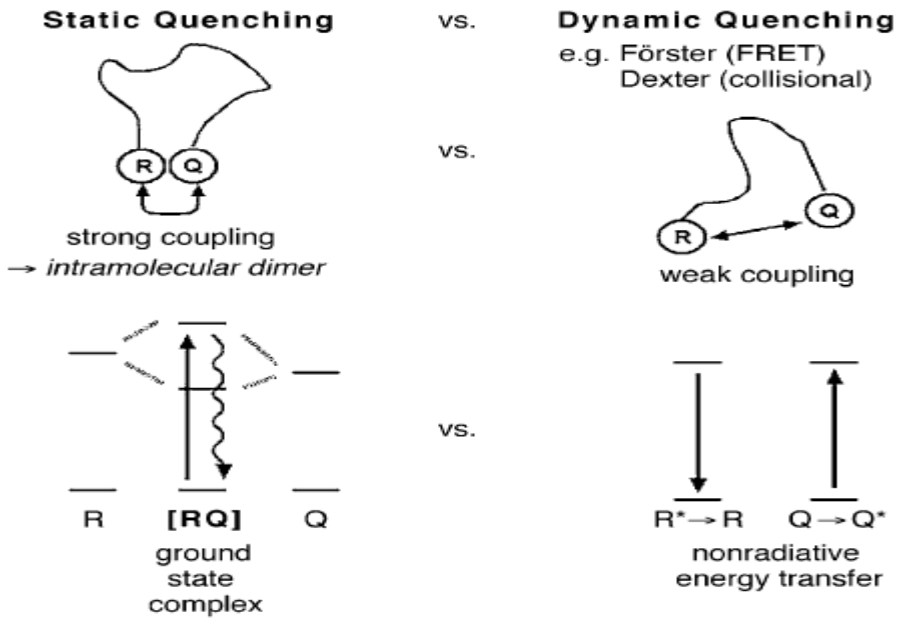


Figure 4: Comparison of static and dynamic quenching mechanisms[7].

3.2.3 RET

Resonance energy transfer (RET) is a type of dynamic quenching. In this non-radiative process, a photon from an energetically excited fluorophore, the "donor", raises the energy state of an electron in another molecule, the "acceptor", to higher vibrational levels of the excited singlet state[9]. As a result, the energy level of the donor fluorophore returns to the ground state, without emitting fluorescence. This mechanism is dependent on the dipole orientations of the molecules and by the distance between the donor and the acceptor molecule. The typical effective distances between the donor and acceptor molecules are in the 10 to 100 Å range[3]. In contrast to the short range collisional quenching, RET occurs via a long range electrostatic dipole-dipole interaction between the donor fluorophore and the acceptor

fluorophore[10]. Because RET has no intermediate photon, does not involve emission of light by the donor, and donor-acceptor are couples by dipole-dipole interactions; for these reasons the term RET should be preferred over the term fluorescence resonance energy transfer (FRET) which is commonly used in literature[3].

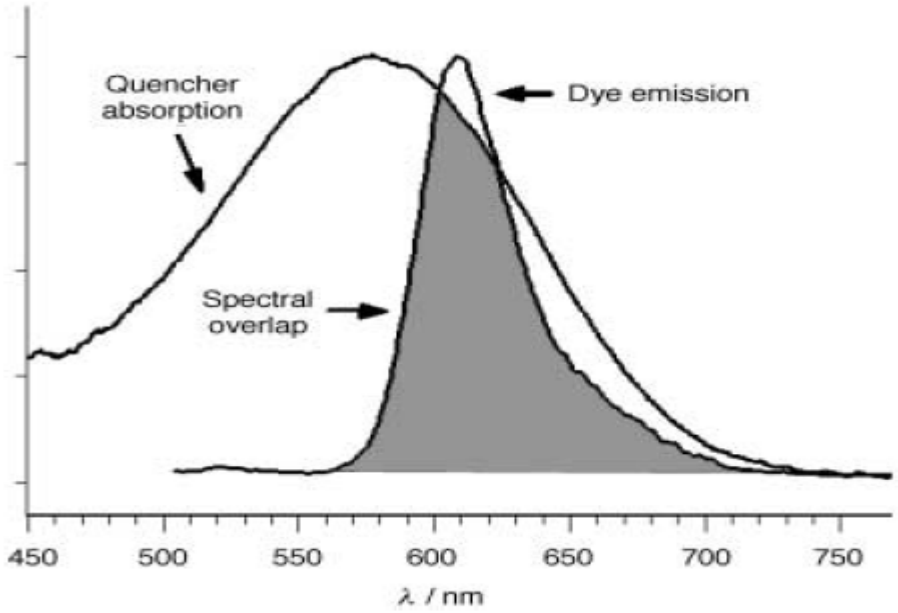


Figure 5: Emission and quencher absorption with a large spectral overlap[11].

An essential requirement of RET is overlap between the donor emission spectrum and the acceptor absorption spectrum[7]. If there is not sufficient overlap, the donor will not couple to the acceptor and RET cannot occur (Figure 5). If the absorbance spectrum of a molecule does not overlap the emission spectrum of the donor, the emitted energy will not be able to excite the potential acceptor[12]. If the absorbance spectrum of the acceptor does overlap the emission spectrum of the donor, energy from the donor will excite the acceptor molecule provided that they are within proximity.

The mechanism for excitation energy transfer between a compatible donor-acceptor fluorophore pair is known as the Förster mechanism[3]. Förster mechanism is proportional to the interaction of two transition dipoles; it is therefore a dipole-dipole coupling. Because this

mechanism does not require physical overlap of the wavefunctions, energy transfer by this mechanism can occur over much larger distances[13].

Additional physical characteristics that govern RET include: the quantum yield of the donor (the fraction of absorbed photons it reemits as fluorescent photons), the extinction coefficient of the acceptor (how efficiently it absorbs photons at its excitation wavelength), and the relative spatial orientation of the donor and acceptor probes[14]. These parameters, influencing strongly the Förster distance, determine how efficiently energy is transferred from the donor to the acceptor[14].

The relationship between the transfer efficiency and the distance (R) between the two probes illustrated in figure 6 is given by the equation:

$$E = \frac{R_0^6}{R_0^6 + R^6}$$

Where R_0 is the Förster distance, which is the distance at which 50% of the excitation energy is transferred from the donor to the acceptor[3]. This equation shows that the transfer efficiency is strongly dependent on distance. From figure 6, we can see that as distance increases there is a rapid decrease of efficiency, and the opposite is true when the distance decreases below R_0

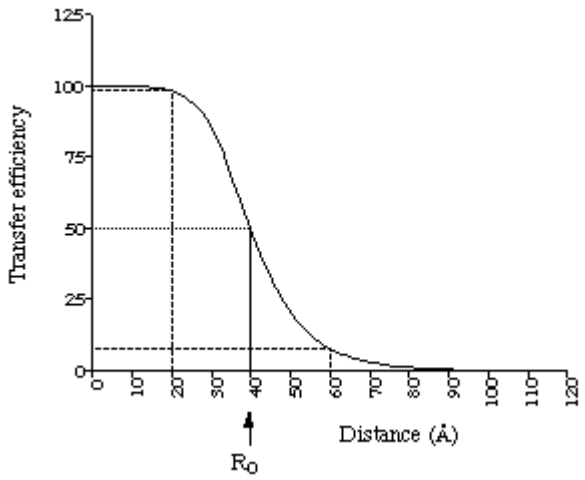


Figure 6: The solid curve represents the relationship between the efficiency of the RET and the distance separating the donor and the acceptor. The importance of this relationship is that there is a limited range of donor-acceptor distances which can be probed by any particular donor-acceptor pair [15]

3.3 Applications of quenching

The requirement of molecular contact for quenching results in the numerous applications for quenching. For example, quenching measurements can reveal the accessibility of fluorophores to quenchers[3]. In the case of proteins, the presence or absence of quenching can reveal whether the fluorophore is located on the interior of the macromolecule or externally. Thus quenching studies can be used to reveal the localization of fluorophores in proteins, membranes, and their permeability to quenchers.

RET offers many opportunities and advantages to fluorescence quenching. Energy transfer occurs when donor and acceptor are within the Förster distance and changes in energy transfer occur due to changes in analyte proximity or changes in absorption spectra of analyte[3]. For collisional quenching to occur the probe must be sensitive to the analyte however, if RET is used the donor and acceptor can be on specific molecules. Therefore, a specific advantage of RET is that it simplifies the design of the fluorophore.

3.3.1 Application of quenching in DNA

Molecular Beacons are probes which contain a stem-loop structure, a fluorophore and a quencher at their 5' and 3' ends of DNA, respectively. The 'stem' sequence keeps the fluorophore and the quencher together, but only in the absence of a sequence complementary to the 'loop' sequence[16]. As long as the fluorophore and the quencher are in close vicinity, any photons emitted by the fluorophore are absorbed by the quencher. This phenomenon is called static quenching. In the presence of a complementary sequence, the probe unfolds and hybridizes to the target, the fluorophore is displaced from the quencher, that can no longer absorb the photons emitted by the fluorophore, and the probe starts to fluoresce.

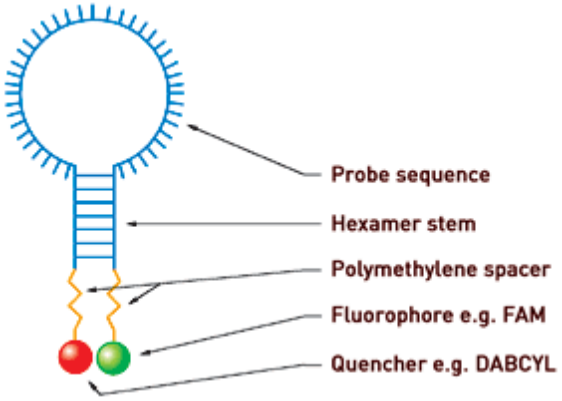


Figure 7: Static quenching within a molecular beacon probe using a fluorophore (fluorescein, FAM) and a compatible quencher (DABCYL)[16].

RET is being extensively used in DNA and RNA analysis as well. However, in contrast to DNA, RNA can adopt a variety of three-dimensional conformations. Ribozymes are molecules that are catalytically active, and RET is has found use in studies of ribozyme folding[3]. Figure 8 illustrates how the substrate (S) is cleaved by the ribozyme (Rz). The next step that follows is binding of loops A and B via a motion called docking. This motion brings the donor (fluorescein) and acceptor (hexachlorofluorescein) closer together so that RET can result. The docking motion leading to proximity is illustrated in the lower panel.

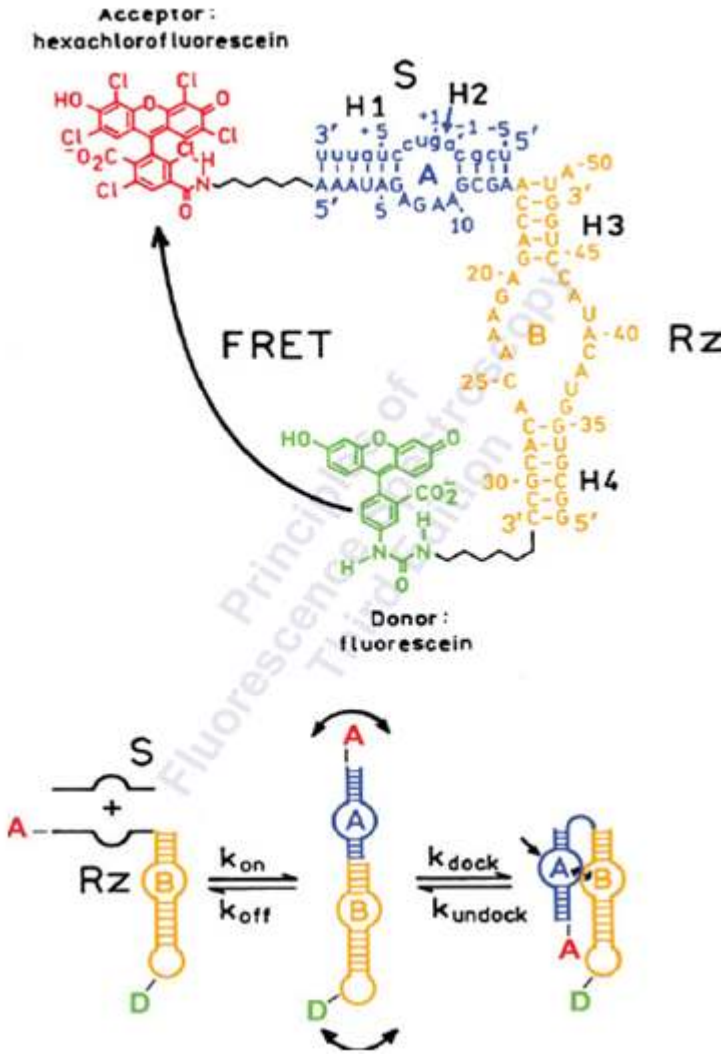


Figure 8: Structure of hairpin ribozyme labeled with a donor and acceptor. The lower panel shows the docking conformational change that occurs upon binding of the substrate [3].

3.3.2 Application of quenching to membranes and proteins

The following example (figure 9) is an illustration of collisional quenching. This assay is based on collisional quenching of ANTS (8-aminonaphthalene-1,3,6-trisulfonate) fluorescence by quencher DPX (*p*-xylene-*bis*-pyridinium bromide**)**. In this example, separate vesicles are loaded with ANTS or DPX, respectively. When content mixing happens, ANTS and DPX collide and fluorescence of ANTS monitored at 530 nm (with excitation at 360 nm), is quenched[17].

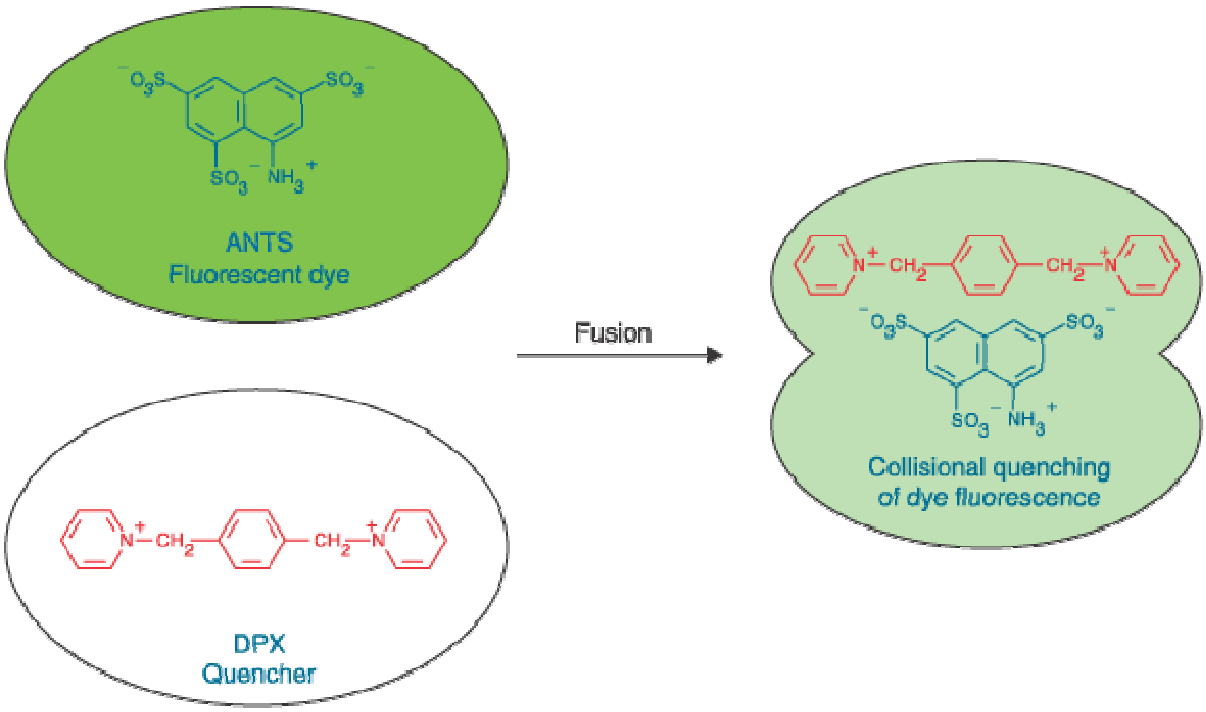


Figure 9: The ANTS/DPX fluorescence quenching assay used to detect membrane fusion. This assay is based on collisional quenching of the polyanionic fluorophore ANTS by the cationic quencher DPX[17].

This method of monitoring membrane fusion was originally developed by Smolarsky and co-workers to follow complement-mediated immune lysis, ANTS/DPX quenching include studies of the role of amino lipids and phospholipids asymmetry in membrane fusion glycoprotein-mediated viral fusion with membranes; and the interactions of vesicle membranes with annexins, HIV fusion peptide, and apolipoprotein[17].

In addition to collisional and static quenching, RET is employed to study the conformational changes in proteins (Figure 10). In this application RET is employed to study the conformational changes that occur in Type II myosin during the ATP-driven movement of this molecular motor[18]. Small organic fluorophores (acceptors and donors) were attached to different parts of the myosin protein. The changes in distance between these regions of the protein during the myosin power stroke can then be readily detected as changes in the efficiency of energy transfer between the fluorophores[18].

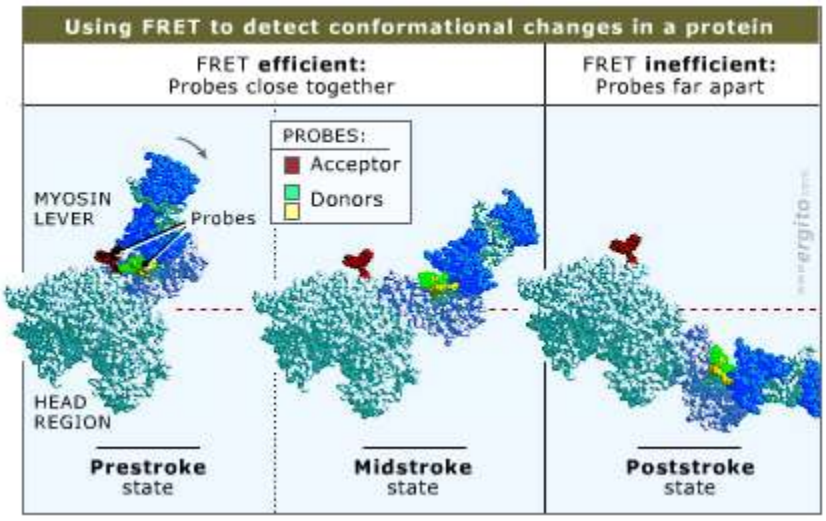


Figure 10: FRET/RET detection of conformational changes that occur in Type II myosin during ATP-driven movement[14].

3.3.3 Application of quenchers for sensing and imaging

Collisional quenching results in decrease of intensity and lifetime of fluorescence[3]. In order to use the collisional quenching as the sensing mechanism requires the fluorescent probe be sensitive to quenching by the desired analyte. Because $[Ru(Ph_2phen)_3]^{2+}$ is a long lived fluorophore, it has a high selectivity to oxygen, and thus it is one of the most commonly used fluorophores in oxygen sensors[3]. Currently sensors include remote fiber optic-based luminescence systems for measuring O_2, pH, partial pressure of CO_2, temperature, and for immunoassays.

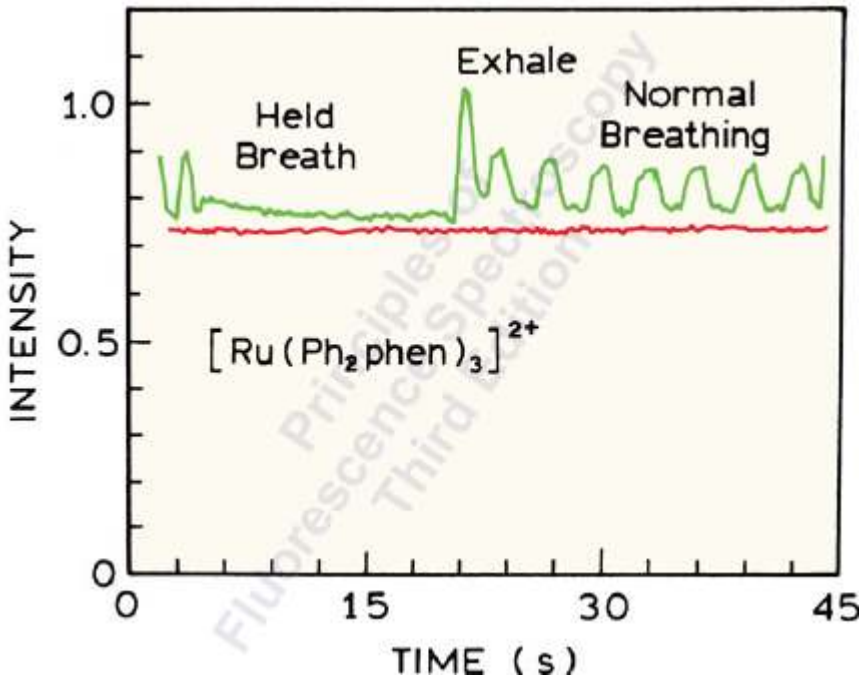


Figure 11: Luminescence intensity of an oxygen sensor with $[Ru(Ph_2phen)_3]^{2+}$ as the probe when exposed to breathing[3].

From Figure 11, we can see the intensity of $[Ru(Ph_2phen)_3]^{2+}$ in silicone while exposed to exhaled air. The intensity increases with each exhale because of the lower O_2 and higher CO_2 content of exhaled air. The higher intensity of the first exhale after the breath was held is due to the lower oxygen content in the air that was retained longer in the lungs[3].

The use of RET in sensing can also be applied to protein-protein interactions (Figure 12). In this example we can see how RET can be used to study the mechanism of assisted protein folding by the molecular chaperone GroEL[19]. The donor fluorophore was AEDANS and it was attached to GroEL, the acceptor fluorophore was 5IAF, and it was attached to the cochaperonin GroES.

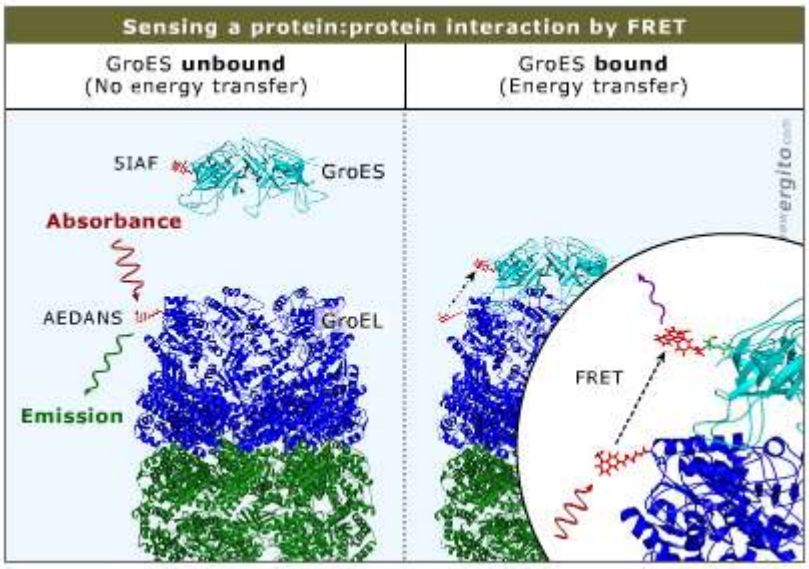


Figure 12: Attachment of donor fluorophore (AEDANS) to GroEL, and acceptor fluorophore (5IAF) to GroES to study the mechanism of assisted protein folding[14].

The inset in Figure 12 shows a magnified view of how the donor and acceptor fluorophores are positioned adjacent to each other when the GroEL-GroES complex forms. Upon this complex formation, resonance energy transfer occurs.

3.4 Examples of quencher and fluorophore pairs

When choosing a fluorescence quencher and fluorophore pair for design of fluorescent hybridization probes that utilize RET, it is important that the fluorophore-quencher pair has sufficient spectral overlap. Fluorophores with an emission maximum between 500 and 550 nm, such as FAM, TET and HEX, are best quenched by quenchers with absorption maxima between 450 and 550 nm, such as dabcyl and BHQ-1[9] as illustrated in figures 13 and 14. Those fluorophores with an emission maximum above 550 nm, such as rhodamines (including TMR, ROX and Texas red) and Cy dyes (including Cy3 and Cy5) are best quenched by quenchers with absorption maxima above 550 nm (including BHQ-2)[9].

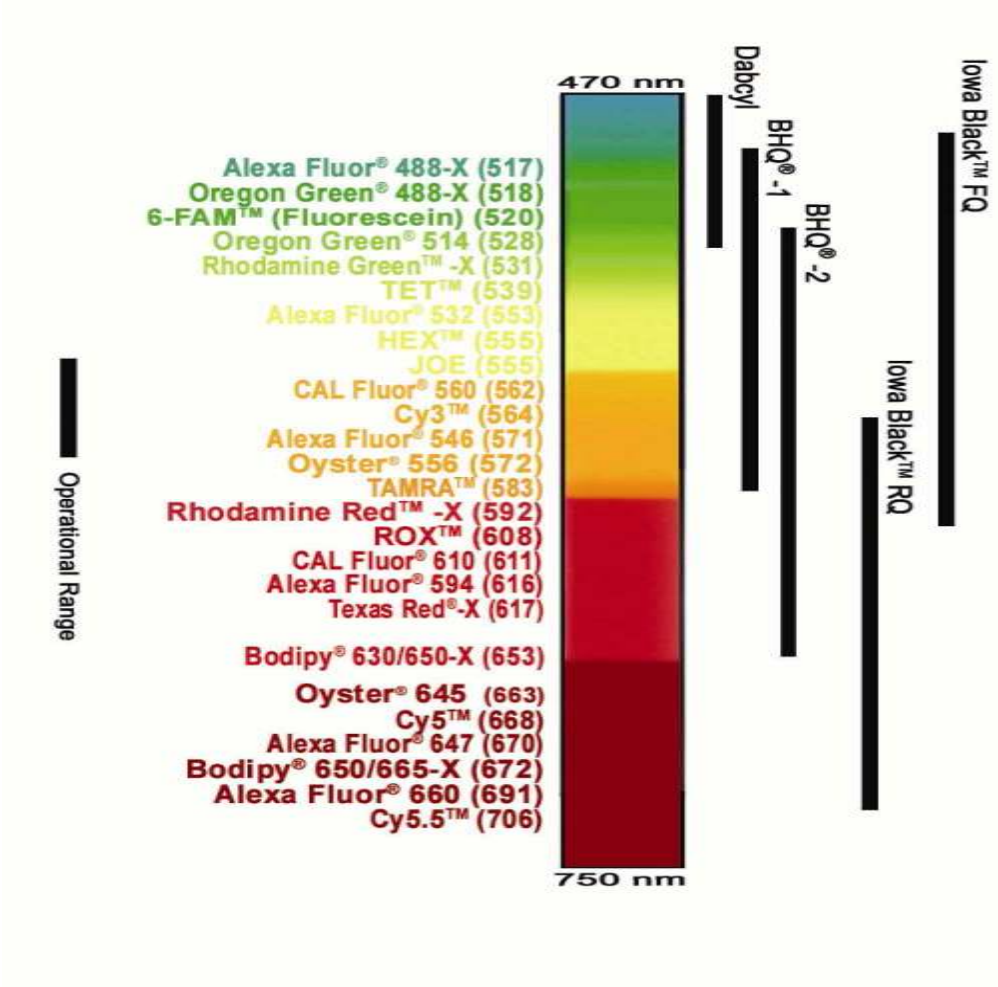


Figure 13: fluorescence quenchers and their fluorophore pairs. The number of wavelengths over which one of these quenchers will absorb fluorescence energy provides flexibility in choosing fluorophores for multiplex assays[12].

For the design of fluorescent hybridization probes that utilize contact quenching, any non-fluorescent quencher can serve as a good acceptor of energy from the fluorophore. However, research performed by Marras et al. suggests that Cy3 and Cy5 are best quenched by the BHQ-1 and BHQ-2 quenchers[9]. Figure 14, illustrated a list of fluorescence quenchers and spectrally compatible fluorophores.

Fluorophore	E_{max}	Dabcyl	BHQ-1	BHQ-2	BHQ-3	Cy5Q	Cy7Q	Iowa Black FQ	Iowa Black RQ	IRDye QC-1	QSY35	QSY7	QSY21	QXL520	QXL570	QXL610	QXL680
Coumarin	472	■									■						
Alexa Fluor® 488-X	517	■	■					■			■			■			
Oregon Green 448-X	518	■	■					■			■			■			
Fluorescein	520	■	■					■		■	■	■		■			
Rhodamine Green-X	531		■					■		■		■					
Alexa Fluor 532	553		■			■		■	■	■		■					
Cy3	564		■	■		■		■	■	■		■			■		
BODIPY® 558/568	568		■	■		■		■	■	■		■	■		■		
Alexa Fluor 546	571		■	■		■		■	■	■		■	■		■		
TAMRA	583		■	■				■	■	■		■	■		■		
ROX	608			■				■	■	■		■	■		■	■	
Alexa Fluor 594	616			■				■	■	■			■			■	
Texas Red®-X	617			■	■			■	■	■			■			■	
BODIPY 630/650-X	653			■	■		■		■	■			■				
Cy5	668			■	■		■		■	■			■				■
Alexa Fluor 647	670			■	■		■		■	■			■				■
IRDye 680	702				■					■							■
IRDye 700DX	700				■					■							
Cy5.5	706				■					■							
Alexa Fluor 750	775									■							
IRDye 800CW	789									■							
IRDye 800	809									■							

Figure 14: List of quenchers and spectrally compatible fluorophores [20].

4. Methodology

4.1. Nucleophilic substitution

The synthesis of AzaPc quenchers is the result of nucleophilic reactions. In organic and inorganic chemistry, nucleophilic substitution is a fundamental class of substitution reaction in which an "electron rich" nucleophile selectively bonds with or attacks the positive or partially positive charge of an atom attached to a group or atom called the leaving group; the positive or partially positive atom is referred to as an electrophile[21].

The most general form for the reaction may be given as

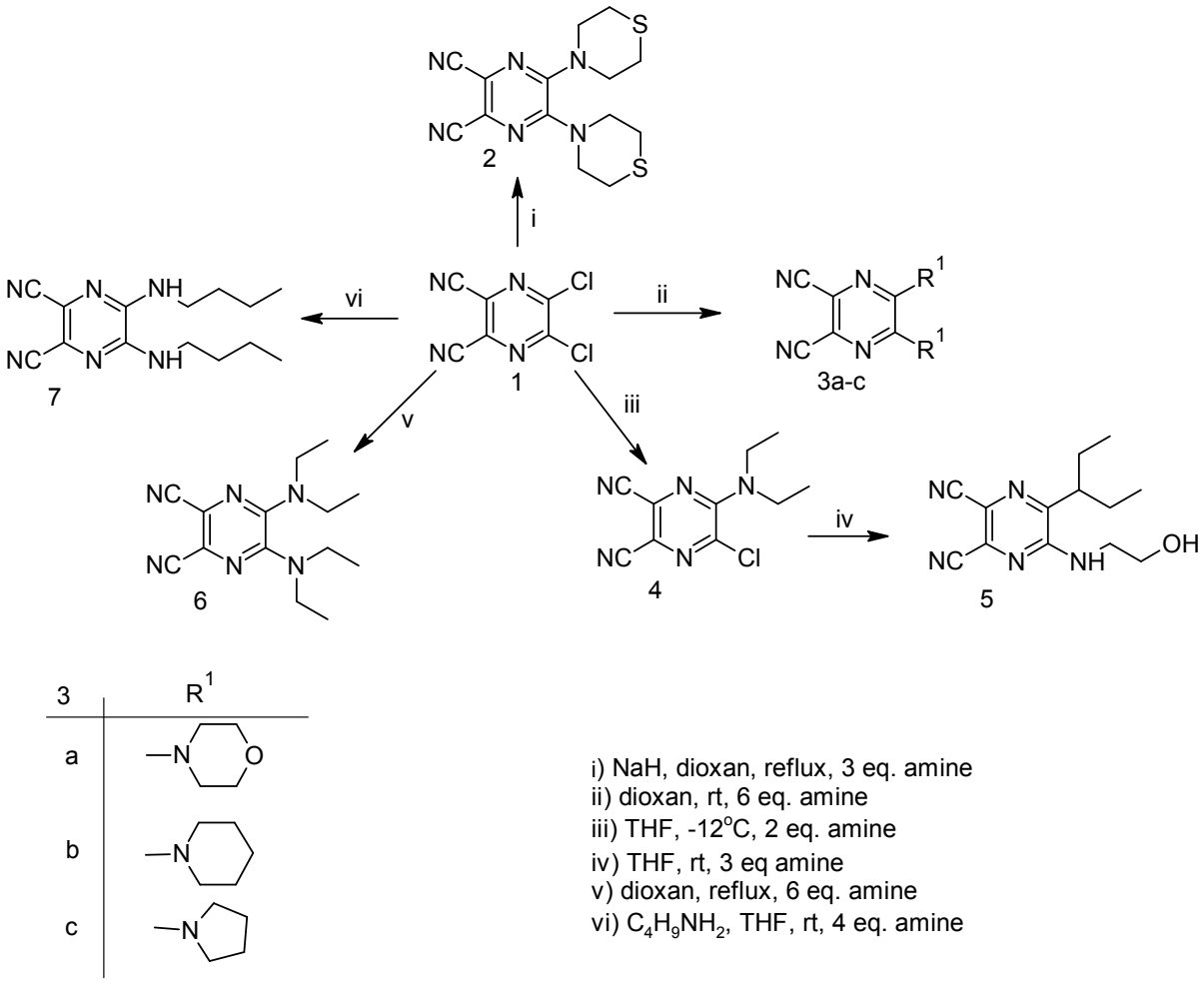
Nuc**:** + R-LG → R-Nuc + LG**:**

The electron pair (**:**) from the nucleophile (Nuc) attacks the substrate (R-LG) forming a new bond, while the leaving group (LG) departs with an electron pair. The main product is R-Nuc. The nucleophile may be electrically neutral or negatively charged, whereas the substrate is typically neutral or positively charged[21].

4.2. Nucleophilic substitution in 5,6-dichloropyrazine-2,3-dicarbonitrile using amines

The synthesis of precursors for heteroatom substituted AzaPc is easiest to start from 5,6-dichloropyrazine-2,3-dicarbonitrile[22] (**1**) since it is a suitable starting material for synthesis of (di)alkylamino substituted pyrazine-2,3-dicarbonitriles. It easily undergoes nucleophilic substitution with primary or secondary amines under mild conditions to obtain desired mono- or disubstituted compounds in reasonable yields, by simply changing the reaction conditions such as stoichiometry and temperature[22] as illustrated in Scheme 1.

Mørkved et al. reacted **1** with thiomorpholine in dioxan in the presence of NaH (Scheme 1) to obtain the disubstituted product in very good 75% yield[23]. In another reaction, Reid and Tsiotis reacted **1** with morpholine in dioxan at room temperature to obtain the disubstituted product **3a** with 69% yield[24]. In the same experiment Reid and Tsiotis reacted **1** with piperidine under the same conditions to obtain a disubstituted product **3b** in 67% yield[24]. A similar experiment by the same researchers involved the reaction of **1** with pyrrolidine in dioxan to again attain a disubstituted product **3c** in 79% yield[24]. Reaction of **1** with only 2 eq of amines at low temperatures may lead to only monosubstituted derivatives, but an increase in the amount amines at a higher temperature leads to disubstituted derivatives. The monosubstituted product **4** was obtained when **1** was reacted with diethylamine in tetrahydrofuran at -12°C with an excellent yield of 93%[22]. The same researchers Kopecky et al. again reacted **4** with 2-aminoethanol in tetrahydrofuran (at rt) to obtain the disubstituted derivative **5**. In another reaction Kopecky et al. found when they increased the ratio of (di)alkylamine they obtained a disubstituted product **6**. They reacted **1** with diethylamine in dioxan to obtain a product with a yield of 64%[22]. Similar work performed by Zimcik et al. produced the disubstituted amine **7**. In which the researchers reacted **1** with butylamine in tetrahydrofuran to obtain the product with 87% yield[25].



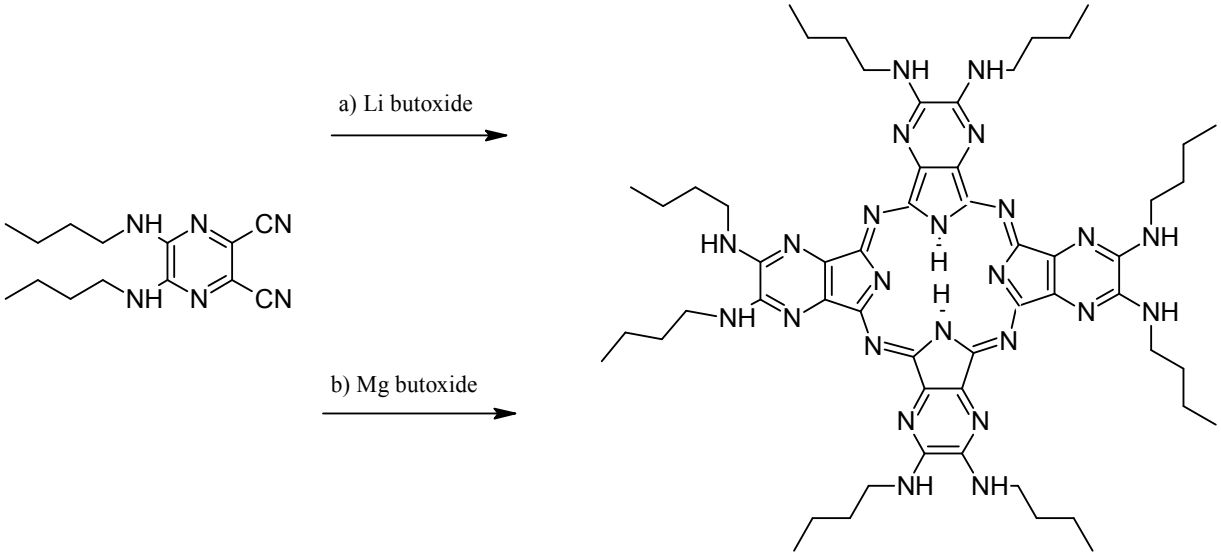
Scheme 1: synthesis of mono- and di-substituted (di)alkylamino-pyrazine-2,3-dicarbonitriles.

4.3 Synthesis of AzaPc macrocycle

There exist various approaches for cyclotetramerization of dinitriles to synthesize an AzaPc macrocycle. In most cases cyclotetramerization of the precursors using lithium or magnesium alkoxide in refluxing alcohol (i.e. the Linstead reaction) was found to proceed smoothly to give the metal-free macrocycle. Besides the Linstead reaction, there are lots of other procedures introduced for cyclization of starting derivatives of pyrazine-2,3-dicarbonitrile, e.g. the use of 1,8-diazabicyclo[5,4,0]-7-undecene, urea and appropriate metal salt in formamide or quinoline and also conversion of dicarbonitrile to highly reactive isoindolinediimines[25]. Zinc AzaPc can also be readily prepared from metal-free AzaPc by reaction with zinc acetate in dimethylformamide. Metal-free AzaPc can be obtained from

either dilithium or magnesium AzaPc using weak or strong acid, respectively. The most widespread procedure of obtaining dilithium or magnesium AzaPc's is the use of alcoholates[25] as mentioned above.

One approach is illustrated in Scheme 2. The metal-free AzaPc was obtained from either dilithium or magnesium AzaPc using a weak or strong acid respectively[25].



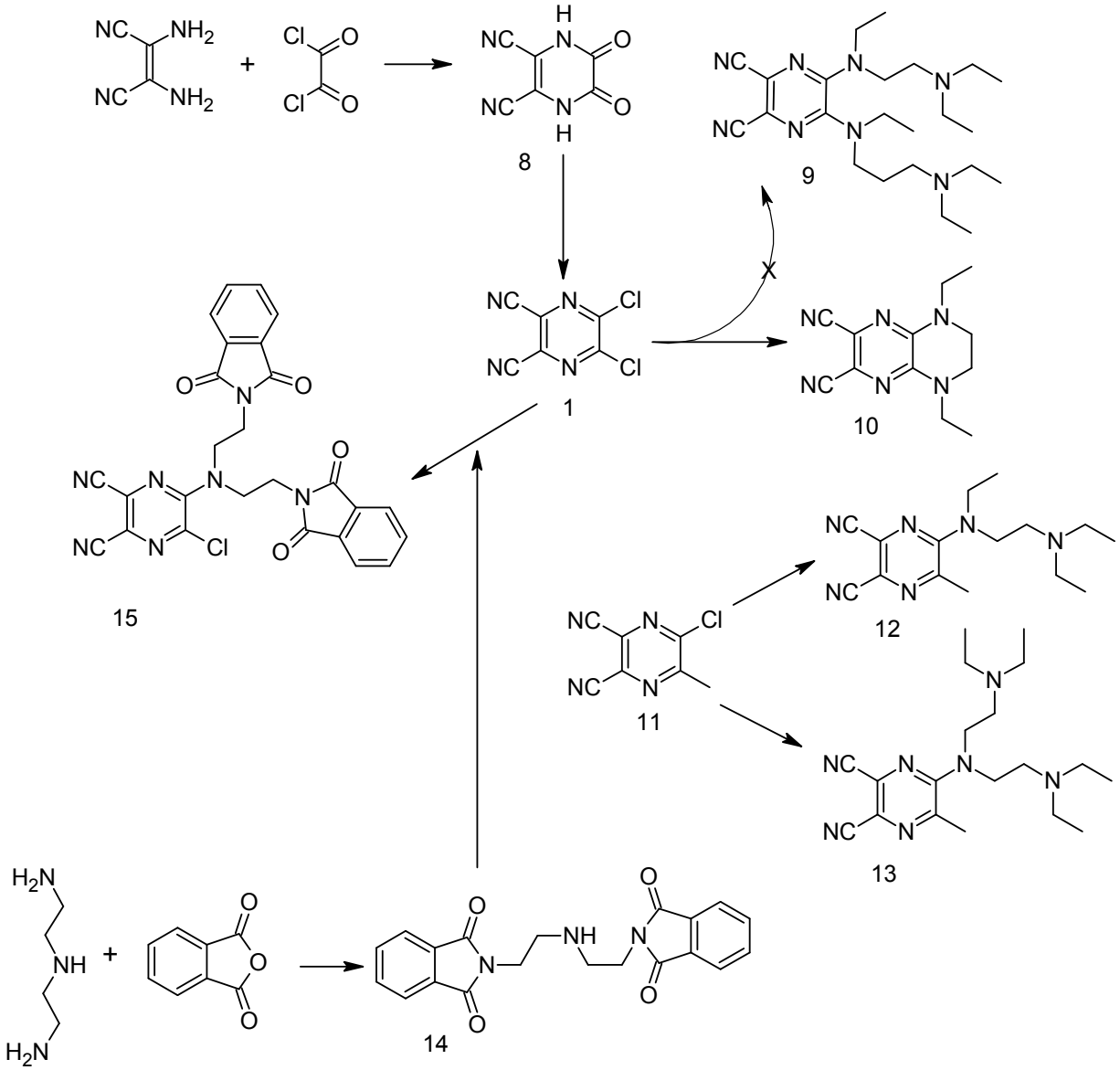
Scheme 2: Synthesis of magnesium AzaPc and lithium AzaPc using alcoholates.

Although the same principle is applied for the synthesis of this metal-free AzaPc there were big differences in reaction time for synthesis of metalated species. The reaction using Li butoxide, was much faster compared to reactions with Mg butoxide which was slower, and the maximum yields were reached after 3 and 24 hours, respectively[25].

5. Experimental

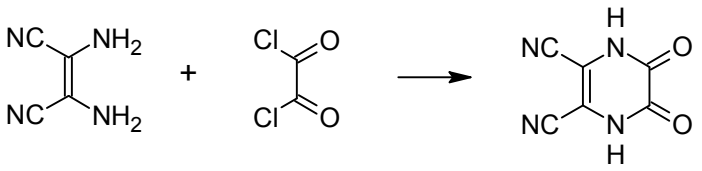
Butanol and dioxan were stored over magnesium and sodium, respectively, and distilled prior to use. All other organic solvents used were of analytical grade and used as received. TLC was performed on Merck aluminium sheets with silica gel 60 F254. Merck Kieselgel 60 (0.040-0.063 mm) was used for column chromatography. Melting points were measured on Electrothermal IA9200 Series Digital Melting point Apparatus (Electrothermal Engineeering Ltd., Southend-on-Sea, Essex, Great Britain). ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury – Vx BB 300 (299.95 MHz – ^{1}H and 75.43 MHz – ^{13}C). Chemical shifts reported are given relative to $Si(CH_3)_4$. The elemental analysis was carried out on Automatic Microanalyser EA1110CE (Fisons Instruments S.p.A., Milan, Italy). Diaminomaleonitrile, *N,N,N'*-triethylethylene-1,2-diamine and *N,N,N',N'*-tetraethyldiethelynetriamine were supplied from established suppliers (Aldrich, Merck, Acros, TCI Europe). Microwave used was CEM Discovery Explorer 24.

5.1 General scheme of performed reactions



Scheme 3: Preparation of precursors

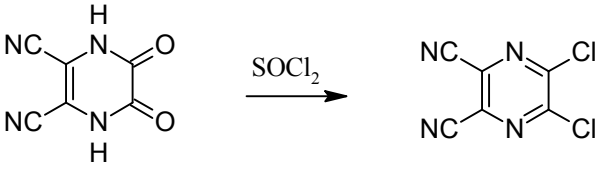
5.1.1 Preparation of 5,6-dioxo-1,4,5,6-tetrahydropyrazin-2,3-dicarbonitrile (8)



A suspension of anhydr. dioxane (200 ml) with diaminomaleonitrile (15 g, 89 mmol) was added dropwise into a solution of oxalyl chloride (20 ml, 233 mmol) in anhydr.dioxane (250 ml) within 3 hours. Reaction temperature was slowly increased to 50°C during that time. The mixture was heated for next 3 h at a temperature of 50 °C. The dioxane was partially removed under reduced pressure. The precipitated product was filtered and washed with hexane.. Product was recrystallized from water (approx. 200 ml) and left to air dry for 2 days. Brownish crystals were collected and yielded 21.9 g (95%).

The reaction was performed according to previously published method[26]. This reaction is frequently performed in the lab with similar results.

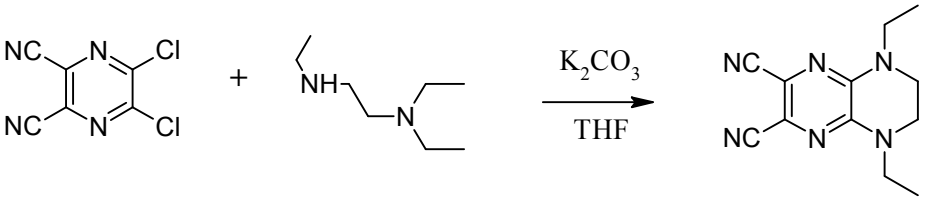
5.1.2 Synthesis of 5,6-dichloropyrazine-2,3-dicarbonitrile (1)



A mixture of 2,3-dioxo-1,2,3,4-tetrahydropyrazine-5,6-dicarbonitrile (22 g, 173 mmol), thionyl chloride (48.5 ml, 667 mmol) and anhydr. dioxane (600 ml) was stirred at 60 °C for 1.5 h. After that time, DMF (20 ml) was added, and the mixture was stirred and heated with a condenser for a further 1.5 h at 60 °C. The solvent was removed under reduced pressure, and the residue was extracted with hot toluene 3 times (200 ml). After removal of toluene by evaporation, a brownish solid was obtained. The product was purified using column chromatography on silica with toluene-chloroform 1:1 as an eluent. Off-white solid was obtained, 7.9 g (30%).

The reaction was performed according to previously published method[26]. This reaction is frequently performed in the lab with similar results.

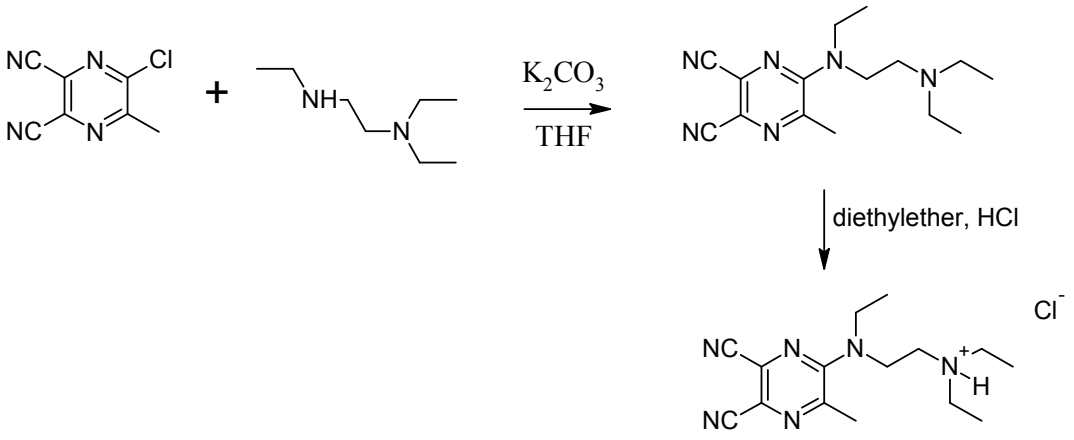
5.1.3 Reaction of 5,6-dichloropyrazine-2,3-dicarbonitrile with *N*,*N*,*N*'-triethylethylenediamine



Compound **1** (100 mg, 0.5 mmol) was dissolved in THF (5 ml) and *N*,*N*,*N*'-triethylethylene-1,2-diamine (0.55 ml, 3 mmol) was added in microwave test tube. Upon addition of potassium carbonate (69.1 mg, 1 mol) the colorless solution turned a pale yellow color. The test tube was placed into microwave and heated with the following settings: 150 °C for 10 min, 17 bar and power 200 W. After the reaction was finished, the solvent was evaporated; remains were dissolved in chloroform and water and washed with NaOH. The organic layer was dried (Na_2SO_4), examined by TLC (eluent dichloromethane or acetone/triethylamine 30:1) and evaporated. The product was purified on silica using dichloromethane as eluent to obtain off white solid (82 mg, 66 %). M.p. 192.8-194.2 °C All analyses did not confirm the intended product (compound **9)** but 5,8-diethyl-5,6,7,8-tetrahydropyrazino[2,3-*b*]pyrazine-2,3-dicarbonitrile **10** was formed instead. ^{1}H NMR ($CDCl_3$) δ (ppm) 3.65 (q, 4H, J=7 Hz, NC*H*$_2$CH$_3$), 3.57 (s, 4H, CH_2), 1.19 (t, 6H, J=7Hz, CH_3), ^{13}C NMR ($CDCl_3$) δ (ppm) 142.5, 119.3, 115.8, 43.7, 43.5, 11.2. Elemental analysis calc. for $C_{12}H_{14}N_6$: C 59.49, H 5.82, N 34.69, found: C 59.38, H 5.97, N 35.27.

The reaction was performed also at room temperature or with different ratio of **1** and diamine (1:1) but the results were similar.

5.1.4 Synthesis of 5-((2-(diethylamino)ethyl)(ethyl)amino)-6-methylpyrazine-2,3-dicarbonitrile (12)



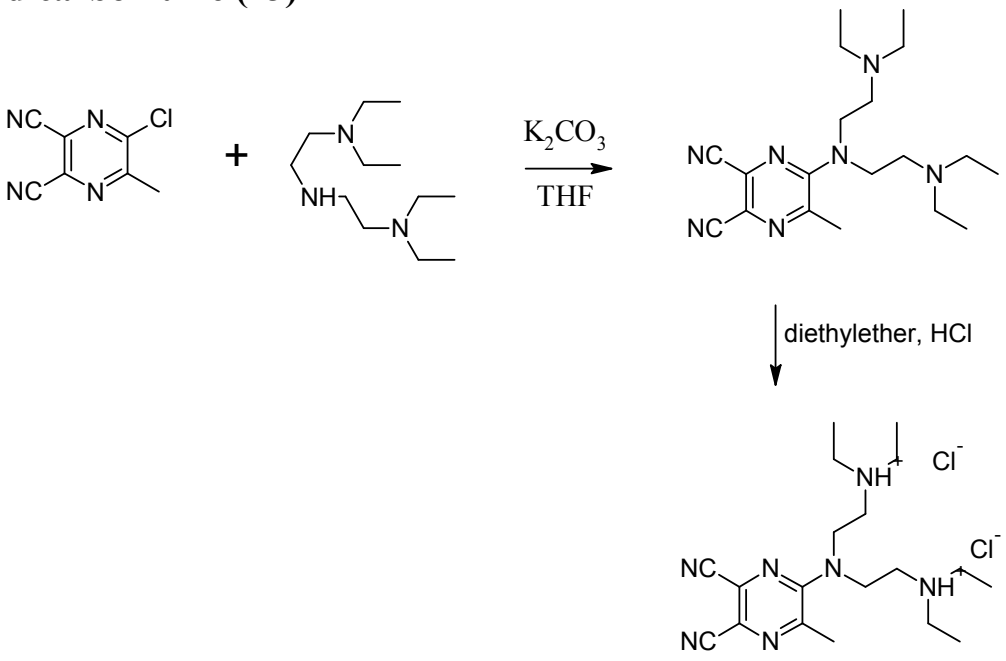
6-methyl-5-chloropyrazine-2,3-dicarbonitrile (359.2 mg, 2 mmol) was dissolved in THF (6 ml) and anhydrous potassium carbonate (347.2 mg, 2.5 mmol) was added. The colorless solution turned a dark pink. After stirring at room temperature, the *N,N,N*'-triethylethylenediamine (360 mg, 2.5 mmol) was added and initial TLC plates with dichloromethane or acetone/triethylamine 30:1 were run. The mixture was heated at reflux for 4 hr after which the TLC examination showed that the all starting material had reacted. Solvents were evaporated and the resulting mixture was dissolved in ethylacetate and water and acidified with HCl. Then the mixture was placed in separatory funnel and washed three times with ethylacetate collecting water phase. NaOH was then added to the collected water phase to the basic reaction and the mixture was washed three times with ethylacetate collecting organic phase. The organic phase was dried (Na_2SO_4) and evaporated. The product was purified on silica with acetone as eluent to receive 190 mg (26.9%) of yellow oil.

Due to stability reasons, the compound was converted to its hydrochloride. The oily product was dissolved in diethylether and the solution was bubbled with HCl (g) and white-yellow sticky solid appeared. Diethylether was decanted and the product was dissolved in small amount of methanol and precipitated with diethylether. The product was stored over diethylether before further reactions or analysis.

^{1}H NMR (CD_3OD) δ (ppm) 3.99-3.90 (m, 2H, CH_2), 3.72 (q, 2H, J=7Hz, CH_2), 3.43-3.31 (m, 6H, CH_2), 2.70 (s, 3H, CH_3), 1.40 (t, 6H, J=7Hz, CH_3), 1.34 (t, 3H, J=7Hz, CH_3).

^{13}C NMR (CD_3OD) δ (ppm) 156.0, 148.8, 129.6, 121.3, 115.6, 114.9, 49.1, 47.3, 45.1, 25.4, 14.5, 9.3. One signal overlapped with the signal of solvent. The NMR was measured for hydrochloride.

5.1.5 Synthesis of 5-(bis(2-(diethylamino)ethyl)amino)-6-methylpyrazine-2,3-dicarbonitrile (13)



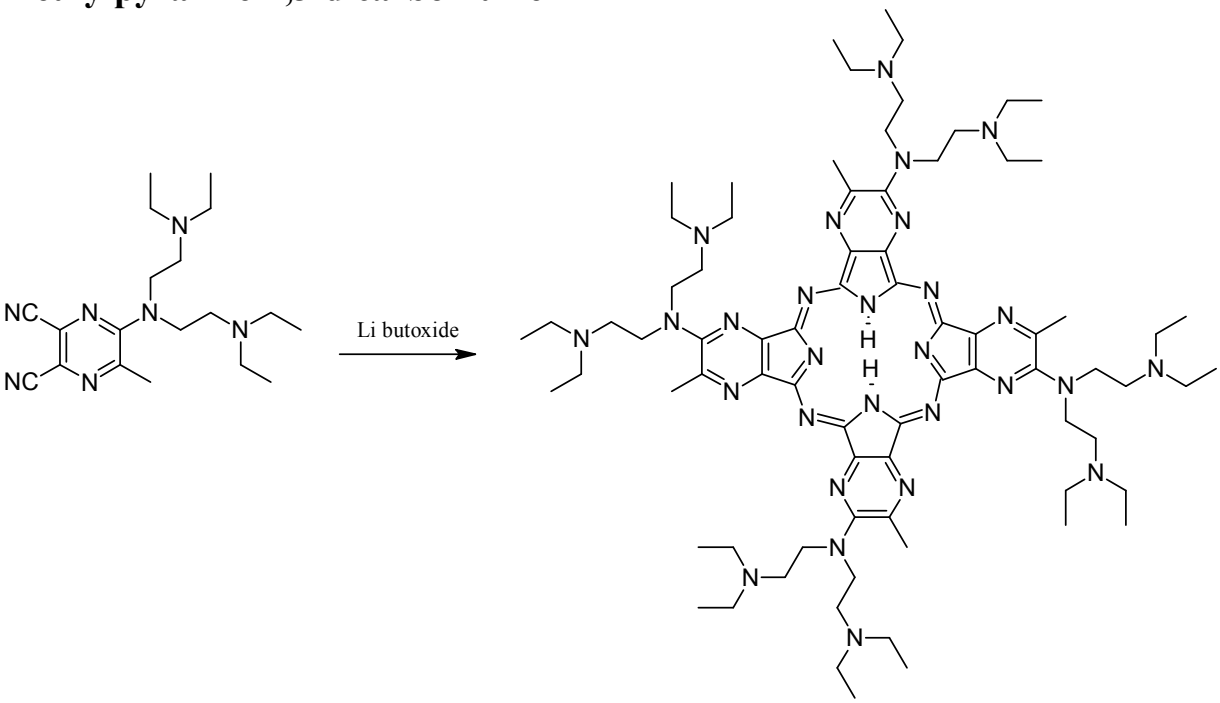
6-methyl-5-chloropyrazine-2,3-dicarbonitrile (359.2 mg, 2 mmol) was dissolved in THF (6 ml) and anhydrous potassium carbonate (347.2 mg, 2.5 mmol) was added. The colourless solution turned a pink. After stirring at room temperature, the *N,N,N'*,*N'*-tetraethyldiethelynetriamine (360 mg, 2.5 mmol) was added and initial TLC plates with dichloromethane or acetone/triethylamine 30:1 were run. The mixture was heated at reflux for 4 hr after which the TLC examination showed that the all starting material had reacted. Solvents were evaporated and the resulting mixture was dissolved in ethylacetate and water and acidified with HCl. Then the mixture was placed in separatory funnel and washed three times with ethylacetate collecting water phase. NaOH was then added to the collected water phase to the basic reaction and the mixture was washed three times with ethylacetate collecting organic phase. The organic phase was dried (Na_2SO_4) and evaporated. The product was purified on silica with acetone/methanol 5:1 as eluent to receive 200 mg (28.3%) of dark yellow oil.

Due to stability reasons, the compound was converted to its hydrochloride. The oily product was dissolved in diethylether and the solution was bubbled with HCl (g) and white-yellow sticky solid appeared. Diethylether was decanted and the product was dissolved in small

amount of methanol and precipitated with diethylether. The product was stored over diethylether before further reactions or analysis.

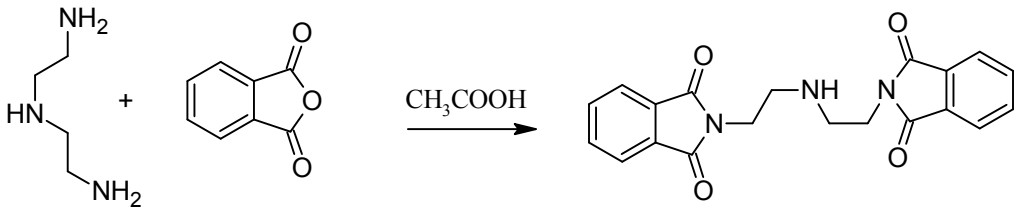
^{1}H NMR (CD_3OD) δ (ppm) 4.11-4.02 (m, 4H, CH_2), 3.55-3.46 (m, 4H, CH_2), 3.42-3,27 (m, 8H, CH_2), 2.77 (s, 3H, CH_3), 1.40 (t, 12H, J=7 Hz, CH_3).
^{13}C NMR (CD_3OD) δ (ppm) 156.2, 149.9, 129.5, 123.3, 115.3, 114.8, 49.8, 48.9, 46.3, 25.2, 9.2. The NMR was measured for hydrochloride.

5.1.6. Cyclotetramerization of 5-(bis(2-(diethylamino)ethyl)amino)-6-methylpyrazine-2,3-dicarbonitrile



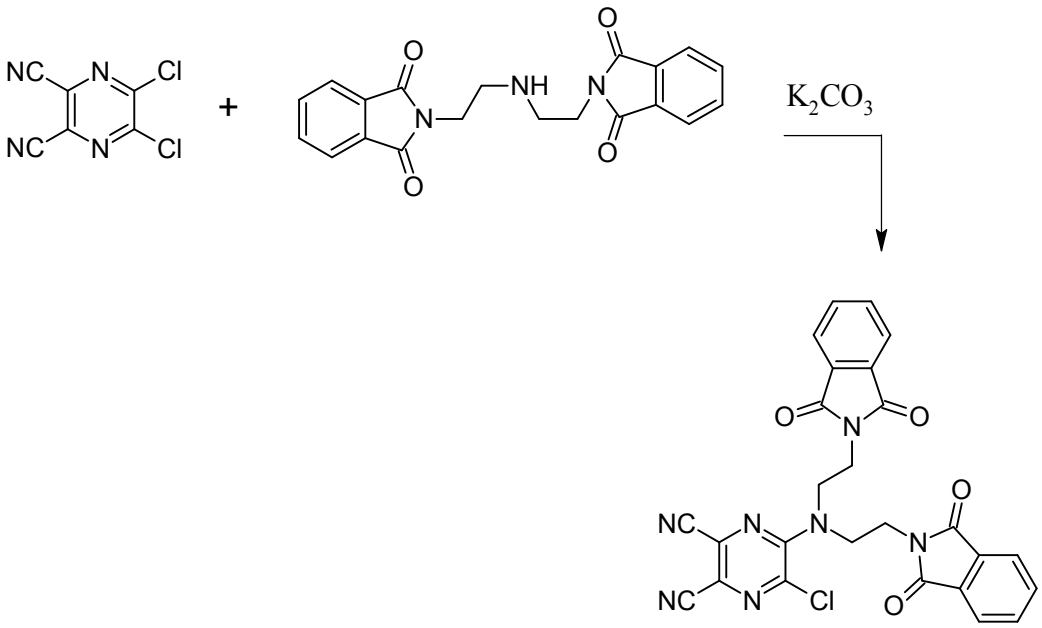
The hydrochloride of **7** (193 mg, 0.45 mmol) was converted to free base before the reaction. It was dissolved in water and made basic by few drops of 10% solution NaOH and extracted three times with ethylacetate. The organic layers were combined, dried (Na_2SO_4) and evaporated to dryness. Anhydrous butanol (10 ml) was added and the reaction mixture was heated to reflux. After refluxing, metal lithium (22 mg, 3.14 mmol) was added through the condenser and the reaction ran for 2 hours at reflux. After that time, butanol was evaporated and the remains were dissolved in 1% HCl. Then the mixture was placed in separatory funnel and washed three times with ethylacetate collecting water phase. NaOH was then added to the collected water phase to the basic reaction and the mixture was washed three times with ethylacetate collecting organic phase. The organic phase was dried (Na_2SO_4) and evaporated. The green product was dissolved in chloroform and TLC plates were examined. The product stuck to the start in methanol, pyridine/THF 1:1 or methanol/pyridine 1:3 as eluents. Strong tailing was observed in pyridine/THF/triethylamine 10:10:1. The use of alumina as sorbent did not help in separation as well.

5.1.7 Synthesis of 1,5-diphthalimido-3-azapentane (14)



A mixture of 1,5-diamino-3-azapentane (5.12 g, 0.05 mol) and phthalic anhydride (16.3g, 0.11 mol) in glacial acetic acid (80 ml) was refluxed for 4 hours. The solvent was removed under reduced pressure and then hot ethanol (160 ml) was added with continuous stirring until yellow product was formed. To resulting sticky product an excess of ethanol was added and placed under ultrasound for 10 minutes. After a further heating in ethanol for 45 minutes and cooling, the product was filtrated under suction and fine yellow crystals were collected. Yield 3.78 g (17.7 %), mp 178-179 °C. (lit[27]. 81-183 °C).

5.1.8 Reaction of 5, 6-dichloropyrazine-2,3-dicarbonitrile with 1,5-diphthalimido-3-azapentane



The 1,5-diphthalimido-3-azapetane (400 mg, 1.1 mmol) and potassium carbonate (345 mg, 2.2 mmol) were added to a solution of 5,6-dichloropyrazine-2,3-dicarbonitrile (100 mg, 0.5 mmol) in THF (6 ml). The mixture was stirred under reflux for 1 h and a TLC plate was run in chloroform. Yellow product (R_f = 0.44) was detected together with some starting material (R_f = 0.93). The reaction was heated for further couple of hours but no change on TLC was observed. That is why another 1,5-diphthalimido-3-azapetane (1.8 g, 5 mmol) was added and reaction heated further. No new products were observed, only the starting material disappeared. The reaction was performed also without the potassium carbonate with the same result. However, due to time constraint, the reaction was not further investigated.

6. Discussion

In Figure 15, we see the range absorption for AzaPc and emission spectra of two commonly used fluorophores – fluorescein and Cy5. It is evident that AzaPc quenchers are capable of covering the range of both fluorescein and Cy5 together. That is why, they seem to have a great potential in RET-based probes. However, as mentioned in the beginning of the thesis, the AzaPc prepared till this time suffered from water-insolubility.

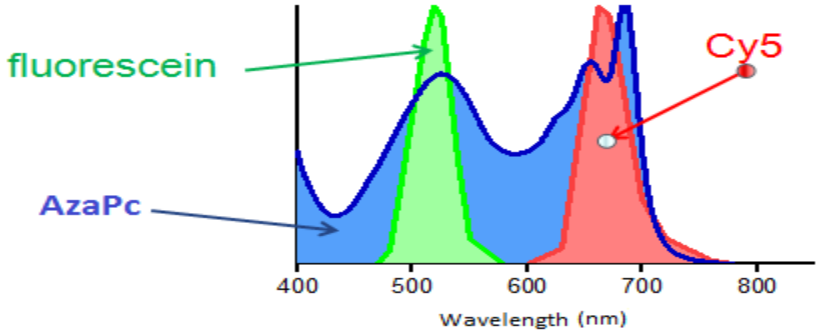
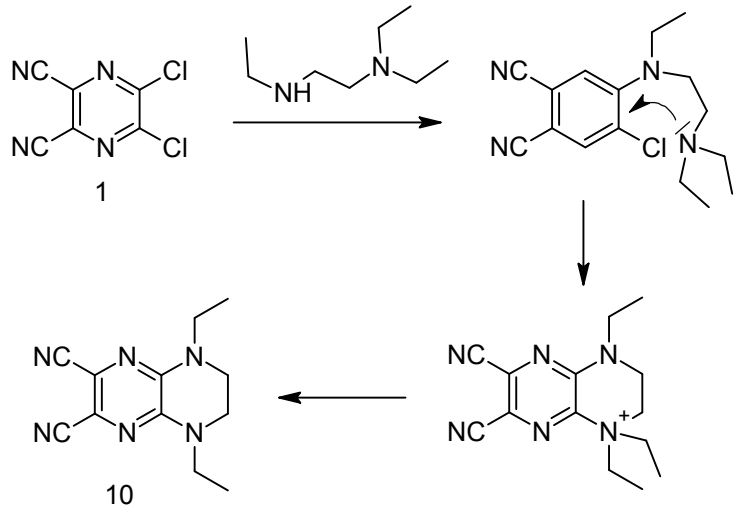


Figure 15: Emission spectra for two commonly used fluorophores (Cy5 and fluorescein) and the absorption spectrum of AzaPc quencher.

The goal of this research was to synthesize an AzaPc quencher with water solubility and strong quenching ability as suggested in the aim of the work. We started off by synthesizing 5,6-dioxo-1,2,3,4-tetrahydropyrazin-2,3-dicarbonitrile **8** from the reaction of oxalylchloride with diaminomaleonitrile in dioxane, which we then reacted with thionyl chloride to synthesize 5,6-dichloropyrazine-2,3-dicarbonitrile **1**. Via nucleophilic substitution of the chlorine groups, the preparation of precursor **9** should be possible. However, the precursor **9** was obtained in only trace amounts following a reaction of **1** with *N,N,N*-triethylethane-1,2-diamine and potassium carbonate. The TLC examination showed that the main compound that was obtained in yield of 66% was 5,8-diethyl-5,6,7,8-tetrahydropyrazino[2,3-*b*]pyrazine-2,3-dicarbonitrile **10**. This cyclization was believed to result from a nucleophilic attack of the free electron pair of tertiary amine on the carbon attached to the chlorine group as illustrated in Scheme 4. Upon performing an NMR, and elemental analysis the structure of 5,8-diethyl-5,6,7,8-tetrahydropyrazino[2,3-*b*]pyrazine-2,3-

dicarbonitrile was confirmed. Several attempts were performed to change the reaction conditions and eliminate this side reaction, however unsuccessfully. That is why we failed in synthesis of compound **3**.



Scheme 4: Proposed cyclization of precursor 5,8-diethyl-5,6,7,8-tetrahydropyrazino[2,3-*b*]pyrazine-2,3-dicarbonitrile.

Due to development of the predominantly cyclized product **10**, we attempted to avoid this reaction by introducing 2-chloro-3-methyl-5,6-dicyanopyrazine **11,** as the starting material as illustrated in scheme 3. We reacted **11** with *N,N,N*'-triethylethylene-1,2-diamine to give rise to compound **12**. However, this precursor carries only one aliphatic tertiary amino group that may not be sufficient for good solubility of the final AzaPc that would carry in this case only four substituents increasing water solubility. For this reason we synthesized compound **13** using similar procedures to those used for compound **12**.

Subsequently, compound **13** was introduced to cyclotetramerization using lithium butoxide as the initiator. Unfortunately, the compound could not be properly purified due to very strong silica or (alumina) binding as a consequence of strong basicity. Some preliminary properties were therefore investigated in the unpurified form. The compound had good water solubility as a hydrochloride and no aggregation was observed after investigation of absorption spectrum (Figure 16). However it lacked sufficient quenching properties because

its solution showed some evidence of fluorescence, although weak. For this reason we propose that having eight amino substituents attached directly to the macrocycle or their different arrangement will be necessary in order to synthesize a fluorescence quencher with water solubility.

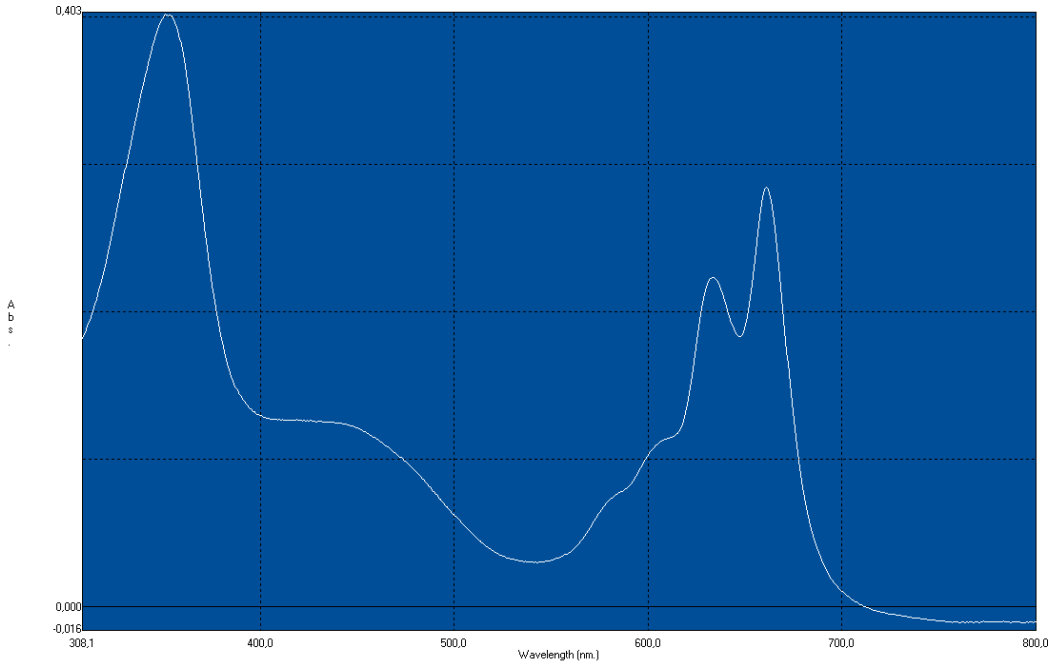


Figure 16: Absorption spectrum of metal-free AzaPc (synthesized from compound **13**) in water acidified with few drops of HCl. The sample was taken directly from the reaction mixture.

For improvement of purification of the final AzaPc, an alternative precursor substituted with a primary amino groups protected with phthalimide was considered. The basicity would be removed and purification of the final AzaPc would no longer be an issue. The first results from the reaction showed that only monosubstituted derivative, 5-chloro-6-{bis [2-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)ethyl]amino}pyrazine-2,3-dicarbonitrile will be most likely accessible due to increased bulkiness of the protected group. The use of the protecting group is still under investigation and remains to be seen whether it will lead to some positive results.

7. Conclusion

In this work we have explored a reaction sequence from diaminomaleonitrile to some *N*-substituted pyrazinedicarbonitriles in attempts of synthesizing an azaphthalocyanine quencher with improved water solubility while maintaining a strong quenching efficiency. While efforts were made to attain these two properties by introducing various diamine and triamine substituents, we were still unsuccessful of attaining both properties simultaneously. Initially, we had also difficulties in purification as a result of strong basicity of final AzaPc leading to strong silica binding. Another unforeseen problem was observed fluorescence due to only four amines being attached directly to the macrocycle. In spite of these difficulties encountered in synthesizing a model AzaPc quencher, the possibility still remains that it could become a powerful tool for future research in medicine and study of endogenous life processes.

8. References

[1] Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové and Generi Biotech, s.r.o. (2009) Nefluoreskující deriváty ftalocyaninů a azaftalocyaninů jako zhášeče fluorescence, CZ Patent 300906.

[2] MUSIL, Z., ZIMCIK, P., MILETIN, M., KOPECKY, K., PETRIK, P., LENCO, J. Influence of electron-withdrawing and electron-donating substituents on photophysical properties of azaphthalocyanines. *J. Photochem. Photobiol. A- Chem.*, **2006**, vol. 186, no. 2-3, pp. 316-322.

[3] LAKOWICZ, J. R. *Principles of Fluorescence Spectroscopy*. Springer: New York, **2006**.

[4] NATION MASTER. Stoke's shift [online]. **2003**, [viewed. 2010-07-03]. Available from: <http://www.nationmaster.com/encyclopedia/Stokes-shift>

[5] MINAGAWA, Jun. Fluorescence quenching analysis [online]. **2010**, [viewed. 2010-12-05]. Available from: < http://www.chlamy.org/companion/Fluorescence_Quenching.pdf>

[6]SMITH, K. C. Basic Photochemistry [online]. Last revised 10.03.2009, [viewed. 2010-07-03]. Available from: < http://www.photobiology.info/Photochem.html>.

[7]JOHANSSON, M. K., COOK, R. M., Intramolecular Dimers: A New Design Strategy for Fluorescence-Quenched Probes. *Chem. Eur. J*. **2003**, vol. 9, pp. 3466-3471.

[8]MARRAS, A. E. S. Interactive Fluorophore and Quencher Pairs for Labeling Fluorescent Nucleic Acids Hybridization Probes. *Mol. Biotechnol*. **2008**, vol. 38, pp. 247-255.

[9]MARRAS, A.E. SALVATORE. Selection of Fluorophore and Quencher Pairs for Fluorescent Nucleic Acid Hybridization Probes, In: Methods in Molecular Biology: Fluorescent Energy Transfer Nucleic Acid Probes: Designs and Protocols. Edited by: V.V. Didenko. Humana Press Inc.: Totowa, NJ, **2006**.

[10]KAMINSKI, C.F., Fluorescence Imaging of Reactive Processes. *Phys. Chem. Chem. Phys*., **2005**, vol. 219, no. 6, pp. 747-774.

[11] ChemPep Inc. [online] **2005**, [viewed. 2010-06-04]. Available from: < http://www.chempep.com/ChemPep-Generic-Term_Fluorescent_Dyes_clip_image002.jpg>

[12]BEHLKE M.A, HUANG L., BOGH L., ROSE S., DEVOR E. J. Fluorescence and Fluorescence Applications for *Integrated DNA Technologies* [online]. 2005, [viewed. 2010-05-02]. Available from: <http://www.idtdna.com/support/technical/TechnicalBulletinPDF/Fluorescence_and_Fluorescence_Applications.pdf>

[13] ANSLYN, V. ERIC, DOUGHERTY, A. DENNIS. Modern Physical Organic Chemistry. University Science Books: New York, **2006**.

[14] RYE, Hays. Fluorescence Resonance Energy Transfer (FRET) [online]. 2007, last revised 09.12.2004 [viewed. 2010-05-10]. Available from: < http://bioscience.jbpub.com/cells/Tech4.aspx>

[15] MOENS, Pierre. Fluorescence resonance energy transfer spectroscopy [online]. **2009**, [viewed. 2010-15-05]. Available from: <http://www.anatomy.usyd.edu.au/mru/fret/abot.html#fig2>.

[16]Eurogentec [online]. 1985, [viewed. 2010-05-05]. Available from: <http://www.eurogentec.com/applications/molecular-beacons.html>.

[17] Invitrogen [online]. 2010, [viewed. 2010-05-05]. Available from: < http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Technical-Notes-and-Product-Highlights/Assays-of-Volume-Change-Membrane-Fusion-and-Membrane-Permeability.html>.

[18] SHIH, W. M., GRYCZYNSKI, Z., LAKOWICZ, J. R., AND SPUDICH, J. A. A FRET-based sensor reveals large ATP hydrolysis-induced conformational changes and three distinct states of the molecular motor myosin. *Cell,* **2000**, Vol. 102, pp. 683-694.

[19] RYE, H. S., ROSEMAN, A. M., CHEN, S., FURTAK, K., FENTON, W. A., SAIBIL, H. R., HORWICH, A. L. GroEL-GroES cycling: ATP and nonnative polypeptide direct alternation of folding-active rings. *Cell*, **1999**, vol. 97, pp. 325-338.

[20] OSTERMAN L. H. The next step in near infrared fluorescence: IRDye QC-1 Dark Quencher [online]. 2009, [viewed. 2010-19-04]. Available from: <http://biosupport.licor.com/docs/QC-1DarkQuencher_v5.pdf>

[21] MARCH, J. *Advanced Organic Chemistry*. Wiley: New York, **1992**.

[22]KOPECKY, K., ZIMCIK, P., NOVAKOVA, V., MILETIN, M., MUSIL, Z., STRIBNA, J. The Synthesis and Characterization of metal-free, unsymmetrical azaphthalocyanines with hydroxyl groups and their complex formation with pyridine. *Dyes Pigment.*, **2008**, vol. 78, pp. 231-238.

[23] MØRKVED, E.H., KJØSEN, H., OSSLETTEN, H., AND ERCHAK, N. Synthesis of Octa(dialkylamino)azaphthalocyanines. *J. Porphyrins Phthalocyanines,* **1999**, vol. 3, pp. 417-423.

[24]REID, W., TSIOTIS, G. Neue kondensierte Stickstoff-reiche Heterocyclen aus 5,6-Dichlorpyrazin-2,3-dicarbonitril. *Liebigs Ann. Chem*., **1988**, pp. 1197-1199.

[25]ZIMCIK, P., MILETIN M., KOSTKA M., SCHWARZ J., MUSIL Z., KOPECKY K. Synthesis and comparison of photodynamic activity of alkylheteroatom substituted azaphthalocyanines. *J. Photochem. Photobio.l A–Chem.,* **2004**, vol. 163, pp. 21–28.

[26] MØRKVED, E.H., HOLMAAS, L. T., KJØSEN, H., AND HVISTENDAHL, G. Preparation of Magnesium Azaphthalocyanines by Cyclotetramerisation of S-Substituted 4,5-Disulfanylpyrazine-2,3-dicarbonitriles. *Acta Chem. Scand.,* **1996**, vol. 50, pp. 1153–1156.

[27] MIRANDA, C., ESCARTI, F., LAMARQUE, L., YUNTA, M.J.R, NAVARRO, P., GARCIA-ESPANA, E., JIMENO, M.L. New 1H-Pyrazole-Containing Polyamine Receptors Able to Complex L-Glutamate in Water at Physiological pH Values. *J. Am. Chem. Soc*., **2004**, vol. 126, no. 3, pp. 823-833.