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Synthesis of photofunctional molecules. Photoelectron transfer and bond cleavage study.

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June 2019

UNIVERSITAT DE BARCELONA

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REPORT

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1. SUMMARY

Photochemical reactions show a great interest in bioengineering applications as they can be selectively activated by light. In this research project photochemical release of different reactive molecules such as carboxylic acids is achieved by bond photocleavage and photoelectron transfer using riboflavin as the photosensitizer.

The study of three different photoreleasable protecting groups was achieved by the irradiation of a N-alkyl-picolinium salt, ethylenediamine tetraacetic acid disodium salt and 1,2-bis-(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid dipotassium salt. The experiments were followed by ^{1}H NMR spectroscopy and UV-Visible measurements leading to a similar rate of schism for the NAP salt and EDTA disodium salt in the reported experiences.

This project attempts to find the best conditions of concentration substrate molecule and riboflavin to obtain the highest rate of bond cleavage. The final goal of this study is to introduce the substrate molecule and the photosensitizer in a vesicle and making it burst by light irradiation due to the increase of osmotic pressure inside the vesicle caused by the molecule bond cleavage.

Keywords: Photoelectron transfer, Photoreleasable protecting groups, Photobleaching, Bond cleavage, Riboflavin.

2. RESUM

Les reacciones fotoquímiques presenten un gran interès en aplicacions de bioenginyeria ja que poden ser selectivament activades amb la llum. En aquest projecte d'investigació l'alliberament fotoquímic de diferents molècules reactives, com àcids carboxílics, s'assoleix per trencament d'enllaços i transferència fotoelectrònica utilitzant la riboflavina com a fotosensibilitzador.

L'estudi de tres grups protectors fotoalliberats es va assolir mitjançant la irradiació d'una sal de N-alquil-picolini, la sal disòdica de l'àcid etilendiamintetraacètic i la sal dipotàssica de l'àcid 1,2-bis-(o-aminofenoxi)età-N,N,N',N'-tetraacètic. Els experiments es van seguir per espectroscòpia de RMN de ^{1}H i es fan mesures de UV-Visible donant lloc a un índex de trencament similar per la sal de NAP i la sal disòdica d'EDTA.

Aquest projecte es proposa trobar les millors condicions de concentració per a la molècula substrat i per a la riboflavina per tal d'obtenir el rendiment més elevat de trencament d'enllaços. L'objectiu final d'aquest estudi és la introducció de la molècula substrat i el fotosensibilitzador a una vesícula i fer-la explotar gràcies a la irradiació que genera un increment a la pressió osmòtica dins la vesícula degut al trencament d'enllaços.

Paraules clau: Transferència electrònica fotoinduïda, grups protectors fotoalliberats, Fotoblanquejament, Trencament d'enllaç, Riboflavina.

3. INTRODUCTION

3.1. BACKGROUND

Photochemistry is the branch of chemistry that studies the interaction between the molecules and light. When a molecule absorbs a photon, its electronic structure changes to a higher energetic state with increased reactivity. The light absorption is schematically described in Jablonski diagram (Figure 1) where the different ways for molecule de-excitation are shown.

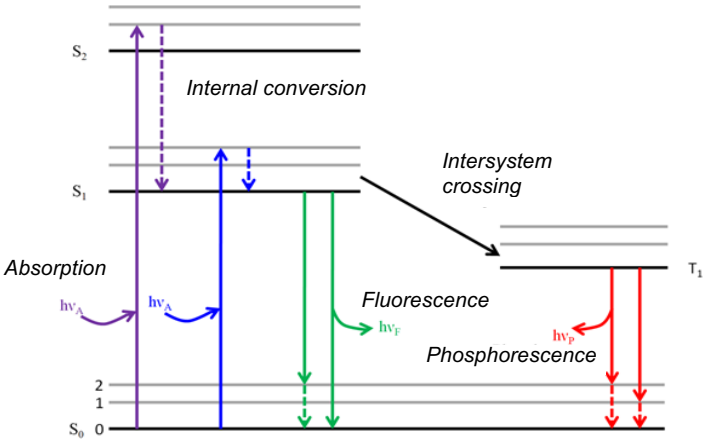


Figure 1: Jablonski diagram
(Addapted from: 07/04/19 https://nptel.ac.in/courses/102103044/module2/lec6/1.html)

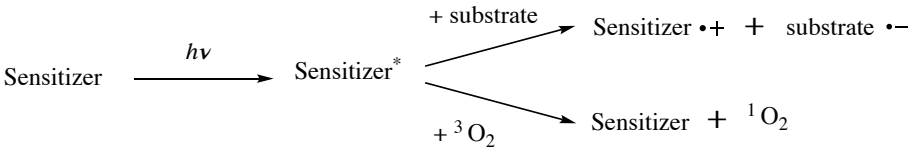
When a molecule absorbs light, one of the electrons on its highest occupied molecular orbital (HOMO) is excited to its lowest unoccupied molecular orbital (LUMO). Then, the electron can return to its ground state, losing the absorbed energy either by radiative or by non-radiative pathways. The first case is called fluorescence and the emitted light has a less energetic wavelength than the absorbed light. The ratio between the number of photons emitted and absorbed is known as quantum yield. This parameter is very useful in photochemistry to compare

the efficiency of different fluorophores, the higher quantum yield, the brighter the fluorescence emission.

Other ways to relax are the internal conversion (the energy is lost in form of heat), the intersystem crossing (a process in which the electron decays to a less energetic state and with longer lifetime) and the quenching (in which the energy is donated to another molecule that is called the quencher). It is possible to observe that the quantum yield diminishes in presence of a substrate whose orbitals have a similar energy to the excited molecule (called *quenchers*).

The interest in photochemistry has lately increased as it offers a way to trigger or activate chemical reactions with temporal and spatial control by the use of light. The use of "caged" compounds for biological processes study is gaining importance as they achieve the same role than protecting groups in organic chemistry but with the main benefit that their deprotection is undergone via irradiation of light. In biology, chelate molecules are usually used as inhibitors as they have photofunctional groups, which can retain or release metals such as Calcium or Magnesium when they are irradiated.

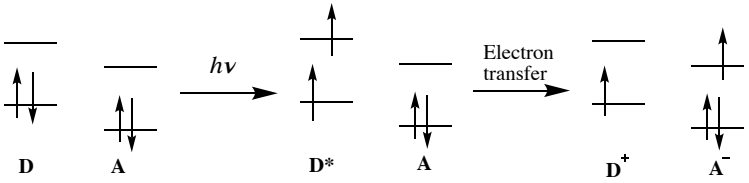
Photosensitization is a reaction to light in which a molecule absorbs light, is chemically altered and alters another molecule in the system. The light absorbing molecule is called photosensitizer and it can either return to its original state when the photoreaction is finished or stay permanently in a state where it is unable to fluoresce. The latter case is called photobleaching and occurs when there are irreversible modifications in covalent bonding. All photosensitizers photobleach but each one does at a certain number of absorption-emission cycles. Photobleaching forms a molecule, which is uncapable to absorb light, and competes with the photosensitization reaction.[1]



Scheme 1: Sensitizer activation and reactivity.

Once the photosensitizer is excited, in the photosensitization reaction there is a competition between a substrate molecule (which usually is the ultimate target of the reaction) and other reactive molecules in the medium such as triplet oxygen (Scheme 1). Usually, to assure a better efficiency of the reaction, a sacrificial reactant is added to reduce the number of secondary

reactions. When the sensitizer reacts with the substrate molecule, there is an electron transfer process between the excited electron of the photosensitizer and the ground state of the acceptor molecule, leading to a oxidized donor and an reduced acceptor.



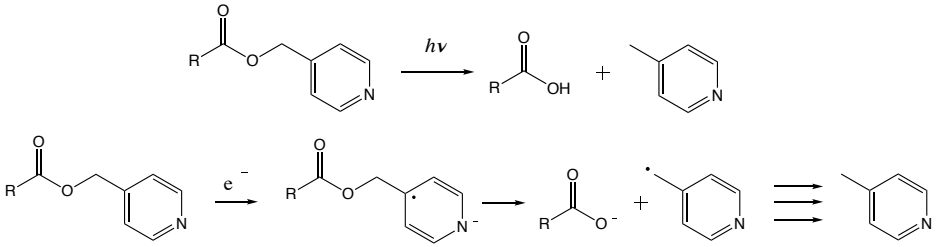
Scheme 2: Photoelectron transfer (PET) process between a donor (D) and an acceptor

Photoreleasable protecting groups (PPGs) are photosensitive molecules, which are linked to a substrate molecule to mask their reactivity[2,3,4,5].The PGGs can be cleaved to release the substrate molecules by irradiating the samples with light. To return to their activated state, PPGs must absorb light. There are two ways of photolysis: through direct light absorption or by photoelectron transfer (PET). PET is an excited state electron transfer between a donor and an acceptor. Usually, the donor is a photosensitizer, which is excited by light absorption, and the acceptor is the substrate molecule linked to the PPGs.

As reported in previous studies[6,7], the use of PET processes is more effective and allows the use of a greater range of wavelengths to release the protecting group due to the fact that the photosensitizer can be either the substrate or another photosensitive molecule. During the last years, different PPGs have been studied in order to improve the solubility in aqueous medium, the absorption in low energy wavelengths such as UV-Visible light and the generation of less harmful by-products.

On the other hand, one of the main interesting characteristics of PET processes is their capability of undergoing bond fragmentation. When photosensitization takes place with a substrate molecule bonded to a PPG, it is possible to break strong bonds, such as C-O and release high energy radicals that lead to reactive species such as carboxylic acids. For instance, 4-pyridylmethyl group has been used to protect carboxyl substrates as it is easily removed by irradiation or PET process as shown in Scheme 3. Taking into consideration previous studies[8], “the bond cleavage may occur via the anion radical of the ester” and this mechanism permits a better selectivity in the bond schism (the direct light excitation usually leads to unwanted

derivatives due to supplementary absorptions). The neutral pyridyl molecule is formed due to several steps not well known and not described in the literature.



Scheme 3: Bond cleavage mechanism for 4-pyridylmethyl group.

In this TFG a study of bond scission rate using different substrate molecules (bond cleavable molecules) and photosensitizers has been performed. The concentration of both substances has been improved in order to achieve a better yield of bond scission with the main goal of assuring a notable pressure increment.

3.2. *OPTONUTRICS* PROJECT

The experimental work performed belongs to a collaborative multidisciplinary project called *Optonutrics* carried out by the *Institut de Sciences Moléculaires* (ISM), *Laboratoire de Chimie de Polymères Organiques* (LCPO) and *Laboratoire NutriNeuro* of Bordeaux University. The aim of this project is to insert a medicine in a localized area of the brain by using a polymer vesicle that explodes releasing it. The bursting of the vesicle is achieved by the inset of a molecule that can undergo a PET process when irradiated, making the osmotic pressure inside the vesicle increase, as reported in previous studies[9] (Figure 2).

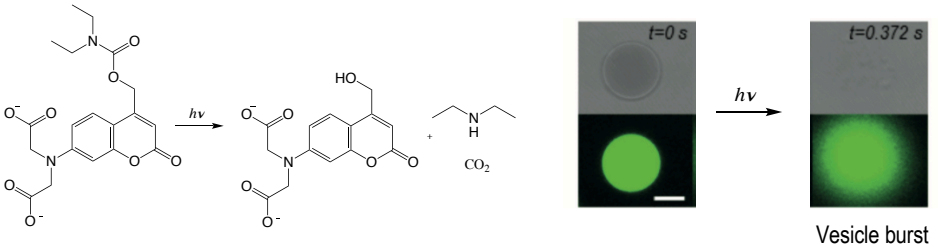
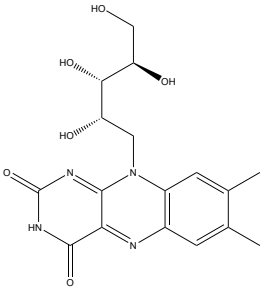


Figure 2: Process for vesicle rupture via substrate molecule bond scission.
Addapted from 16/05/19 A. Peyret, et al. *Angew. Chem.* ***2017***. Ed. 56 (6) 1566–1570.

This TFG reports the study performed in Dr McClenaghan research group (*ISM*) where different substrates at different concentrations were tested in order to achieve a better breaking rate. One of the several things that were taken into account is that the photosensitizer, the substrate, the radicals that are formed and the other products that will be needed (such as oxidant agents, buffer solutions, etc) must be biocompatible, stable and soluble in aqueous solution at pH 7. Moreover, the photosensitizers have their maximum absorption band in the blue region (around 450 nm) in order to avoid the excitation of other organic molecules that are present in the irradiated part of the body (that absorb in the UV region).

3.3. PHOTOSENSITIZER CHOICE

Previous knowledge in the research group, suggest that Riboflavin is the most suitable photosensitizer for the project needs: is biocompatible, does not interact with the polymerosome membrane of the vesicle and absorbs in the UV-Vis region.



Scheme 4: Riboflavin structure.

Riboflavin (RF) which is also known as Vitamin B2 is an organic yellowish substance present in dairy products such as meat, milk and fruits, which is sensitive to light and high temperatures[10,11,12,13]. Previous studies have reported its degradation after light exposure. Riboflavin stability mainly depend on light intensity and wavelength, exposure time, presence of oxygen and pH.

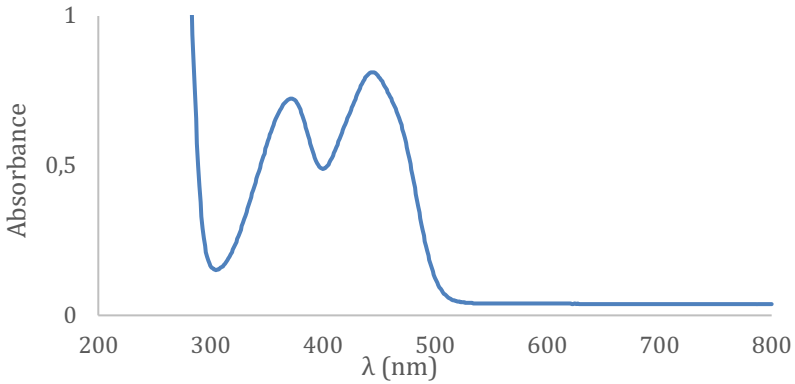
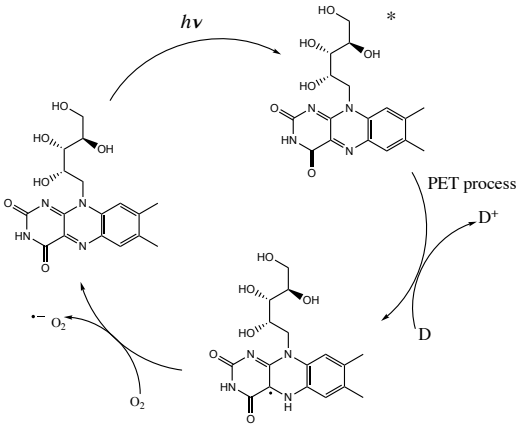


Figure 3: Riboflavin absorption spectrum at 70 μM concentration in deuterium oxide.

RF absorbs in the UV- Visible region with maximum of absorption at 223, 267, 373 and 444 nm (Figure 3).

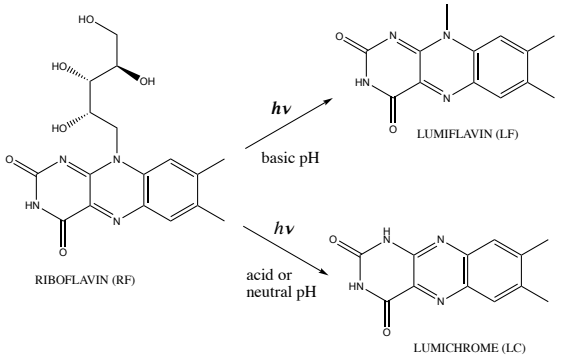
When a molecule is irradiated several mechanisms can take place for its relaxation, as stated above. Nevertheless, as the molecule to be excited is a photosensitizer, the relaxation mechanism by a substrate quenching is one of the main goals to be studied in this project.

When the RF is excited, a PET process takes place reducing the RF and oxidizing the substrate. Then, riboflavin is re-oxidized to the ground state molecule by the reaction with an oxidant[14] (Scheme 5).



Scheme 5: Riboflavin excitation-de-excitation cycle by photoelectron transfer process.

Although the re-oxidation process is highly efficient and RF is capable of bearing the absorption-emission cycle more than once, after a particular number of absorption cycles, RF undergoes photobleaching. The formation of photoproducts unable to fluoresce stops the PET process and the substrate fragmentation. The two photoproducts shown in Scheme 6 can be formed when RF photobleaches and they are lumiflavin (LF) and lumichrome (LC) which are selectively formed depending on the medium pH[15].



Scheme 6: Degradation of riboflavin by light absorption.

Even though the study of the formation of these photoproducts is not crucial for this report, the changes in the absorption bands of the UV-Vis spectra support the hypothesis of the photoproduct that is generated for RF degradation.

For the experimental work that has been performed herein, the quenching mechanism is studied depending on the concentration of substrate that is present in the reaction mixture.

4. OBJECTIVES

The first objective of this work is to find the substrate molecule which participating in riboflavin PET process gives the highest bond fragmentation rate: the highest percentage of broken molecule in the less time.

Secondly, it is important to optimize the concentrations of photosensitizer and substrate molecule in order to achieve the fastest rate of conversion and not saturating the vesicle. These concentrations must be regulated because these products are going to be introduced in a vesicle, which, afterwards will be, ideally, injected in a human body.

Additionally, for a real application of this project, the most critical condition that is taken into account in the experimental work executed is that every molecule that is used or formed in the reaction mixture is needed to be harmless and in the lowest concentrations as possible for the process to occur.

5. EXPERIMENTAL SECTION

5.1. MATERIALS AND PHYSICAL MEASUREMENTS

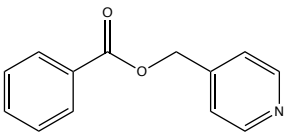
All the reactions were set-up under N_2 atmosphere. Reactants and reagents used were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), Fischer Scientific UK and Acros Organics. BAPTA tetraethyl ester was previously synthetized by other researchers in the group, following the literature procedure[16]. Solvents were dried using solvent Purification System (dry DCM) or used directly from VWR Chemicals. Deuterated solvents used for ^{1}H NMR spectroscopy were purchased from Sigma-Aldrich and Eurisotop.

Excitation lamps used were a Luzchem LED Illuminator in the blue wavelength (4550 W/m^2-450 nm) mode and a UV mercury lamp (295×76 mm-15W-365 nm). Absorption spectra were recorded on a Varian Cary 100 Bio UV Spectrophotometer at room temperature. NMR spectra were obtained on a Bruker 300 MHz spectrometer at room temperature. Column chromatography was performed using automated Flash chromatography system (Intershim Puriflash 430 with 50 μm Silica) and Silica 40-63 μm for manual chromatographic columns.

5.2. SYNTHESES

5.2.1. Synthesis of pyridin-4-ylmethyl benzoate

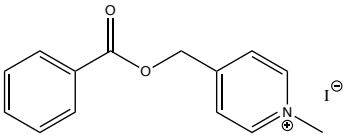
Following a literature procedure[8], pyridine 4-methanol (2.022 g, 18.54 mmol) was dissolved in benzene (25 ml) and triethylamine (4.7 ml, 3.36 g, 33.27 mmol) in a round bottom flask under N_2 atmosphere. A solution of benzoyl chloride (4.098 g, 29.27 mmol) in benzene (8.5 ml) was added dropwise. During the addition, the solution changed from white to dark orange color. The reaction mixture was stirred for 24 hours at room temperature. The product was washed with water (3×10 ml). Combined organic layers were dried over anhydrous $MgSO_4$, filtered and the solvent was removed *in vacuo*. Crude reaction mixture was purified by flash column chromatography (SiO_2, 6:4 EtOAc: cyclohexane) to provide pyridine-4-ylmethyl benzoate (1.598 g, 40% yield) as a brown oil.



^{1}H NMR (DMSO-d_6, 300 MHz, ppm): δ 8.59 (2H,dd, J = 3.0, 6.0 Hz), 8.05 (2H, m), 7.70 (1H, tt, J = 3.0, 7.4 Hz), 7.56 (2H, m, J = 1.3, 7.7 Hz), 7.46 (2H, d, J = 6.0 Hz), 5.41 (2H, s).

5.2.2 Synthesis of N-methyl picolinium benzoate iodide

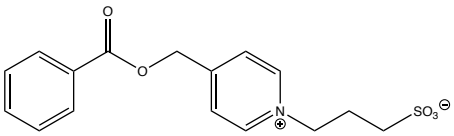
According to literature procedure[8], pyridine-4-ylmethyl benzoate (0.643 g, 2.78 mmol) was dissolved in dried AcCN (10 ml) under N_2 atmosphere. Methyl iodide (0.26 ml, 4.23 mmol) was added and the solution was refluxed at 75°C for 24 h. The solvent was removed *in vacuo* and a brown solid was obtained. The solid was redissolved in EtOAc and filtered to provide N-methyl picolinium benzoate iodide (0.698 g, 65% yield).



^{1}H NMR (DMSO-d_6, 300 MHz, ppm): δ 8.96 (2H, d, J = 6.6 Hz), 8.19 (2H, d, J = 6.6 Hz), 8.11 (2H, m), 7.75 (1H, tt, J = 1.4, 7.4 Hz), 7.60 (2H, t, J = 7.5 Hz), 5.69 (2H, s), 4.35 (2H, s). ^{13}C NMR (DMSO-d_6, 75.5 MHz, ppm): δ 165.2 (Ar-CO-O), 155.3, 145.5, 134.0, 129.6, 129.0 (aromatic carbons), 63.9 (O-CH_2-Ar), 47.6 (N+-CH_3).

5.2.3 Synthesis of 4-[(benzoyloxy)methyl]-1-sulfopropyl- pyridinium

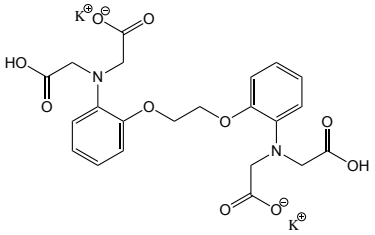
According to a literature procedure[17], pyridine-4-ylmethyl benzoate (0.802 g, 3.76 mmol) was dissolved in THF (10 ml) under N_2 atmosphere and 1,3-propanesultone (0.551 g, 4.52 mmol) was added. The solution was stirred and refluxed at 60°C for 90 minutes and then, cooled at room temperature. The solid was filtered and washed with DCM to provide 4-[(benzoyloxy)methyl]-1-sulfopropyl- pyridinium (1.202 g, 95% yield).



^{1}H NMR (DMSO-d_6, 300 MHz, ppm): δ 9.07 (2H, d, J = 6.6 Hz), 8.21 (2H, d, J = 6.6 Hz), 8.12 (2H, d, J = 7.1 Hz), 7.73 (1H, t, J = 7.5 Hz), 7.59 (2H, t, J = 7.5 Hz), 5.68 (2H, s), 4.74 (2H, t, J = 6.0 Hz), 2.40 (2H, t, J = 6.0 Hz), 2.23 (2H, m). ^{1}H NMR (D_2O, 300 MHz, ppm): δ 8.90 (2H, d, J = 9.0 Hz), 8.17 (4H, m), 7.75 (1H, tt, J = 3.0, 11.3 Hz), 7.61 (2H, tt, J = 2.6, 7.5 Hz), 5.73 (2H, s), 3.76 (2H, d, J = 6.0 Hz), 3.01 (2H, t, J = 7.2 Hz), 2.49 (2H, q, J = 7.5 Hz). ^{13}C NMR (DMSO-d_6, 75.5 MHz, ppm): δ 165.2 (Ar-CO-O), 155.8, 144.9, 134.0,129.6, 128.9 (aromatic carbons), 69.9 (O-CH_2-Ar), 63.9 (N+-CH_2), 47.0 (CH_2-SO_3), 27.3.

5.2.4 Hydrolisis of 1,2 -bis (o-aminophenoxy) ethane-N, N, N',N'- tetraethyl ester (BAPTA tetraethyl ester)

According to a literature procedure[16], BAPTA tetraethyl ester (20 mg, 0.034 mmol) was dissolved in a mixture MeOH/THF 1:1 (2 ml). $LiOH \cdot H_2O$ (28.5 mg, 0.68 mmol) and a drop of water were added to the solution and the resulting mixture was stirred at room temperature for 24h. The solvent was removed *in vacuo*. The white solid was dispersed in EtOAc and centrifuged. The liquid was pipetted off and the solid was redissolved in water. The solution was set to neutral pH by the addition of HCl and KOH to provide 1,2-bis (o-aminophenoxy)ethane- N,N,N',N'- tetraacetic acid dipotassium salt (18.3 mg, 97% yield) as a white solid.

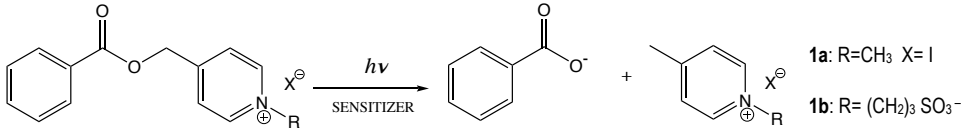


^1H NMR (D_2O, 300 MHz, ppm): δ 6.90-7.18 (8H, m), 4.43 (4H, s), 3.79 (8H, s).

6. SYNTHESIS AND IRRADIATION EXPERIENCES

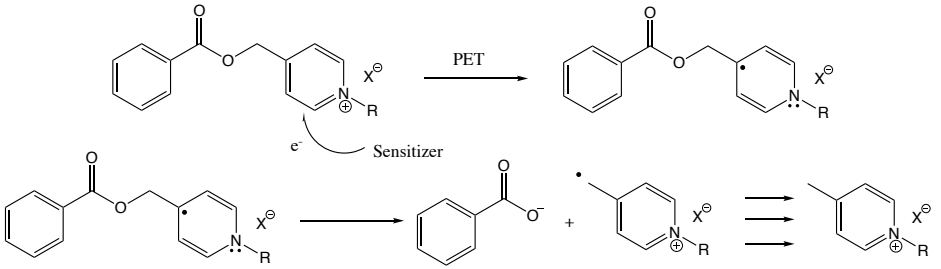
6.1. PYRIDINIUM SALTS

The first family of molecules to be synthetized was the pyridin-4-ylmethyl benzoate derivative. In this case, the photoreleasable protecting group is N-alkyl-picolinium (known as NAP) which masks benzoic acid reactivity by an ester bond[8]. In order to assure a better solubility in water of the substrate and the products obtained after the photocleavage, the synthetized derivatives are electrically charged.



Scheme 7: Bond cleavage for N-alkyl-4-methylpyridinium salt.

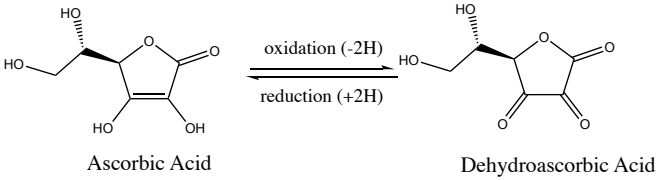
When irradiating the molecule in the presence of a photosensitizer, the PET process takes place and the molecule gains an electron. This new configuration leads to a bond cleavage which forms an ester anion an a picolinium radical. The final neutral species are formed by the gain of two protons. This mechanism remains unknown as it is not described in the literature.



Scheme 8: Mechanism for pyridinium salt photoelectron transfer process and bond cleavage.

Several irradiation experiences were carried out for **1b** pyridinium salt. Its bond fragmentation was studied in terms of its concentration in presence of riboflavin as the photosensitizer and ascorbic acid as a reducing agent to avoid RF loss. Different previous studies have reported the efficiency of ascorbic acid as an active oxygen quencher: it is known that when ascorbic acid is added to a RF solution, the photosensitizer content after a certain time of irradiation is much higher than without the reductant.

Ascorbic acid, also known as Vitamin C, is one of the strongest antioxidants as it is able to reduce oxygen dissolved in water. In the experimental work carried out, it is used as a sacrificial reductant in order to avoid RF oxidation[18]. If excited RF reacts with oxygen, the photobleaching reaction takes place and the fluorescence of the molecule is lost. In order to avoid riboflavin degradation, ascorbic acid is added to the solution. Ascorbic acid rapidly reacts with oxygen giving dehydroascorbic acid (Scheme 9) and the oxidation of the photosensitizer is prevented[19].



Scheme 9: Ascorbic acid oxidation reaction.

All the solutions were prepared in deuterium oxide as the solvent because the course of the reaction was monitored by ^{1}H NMR spectroscopy. Before starting the irradiation, a ^{1}H NMR in DMSO-*d*$_6$ was performed in order to assign the protons in the 4-[(benzoyloxy)methyl]-1-sulfopropyl-pyridinium molecule (Figure 4).

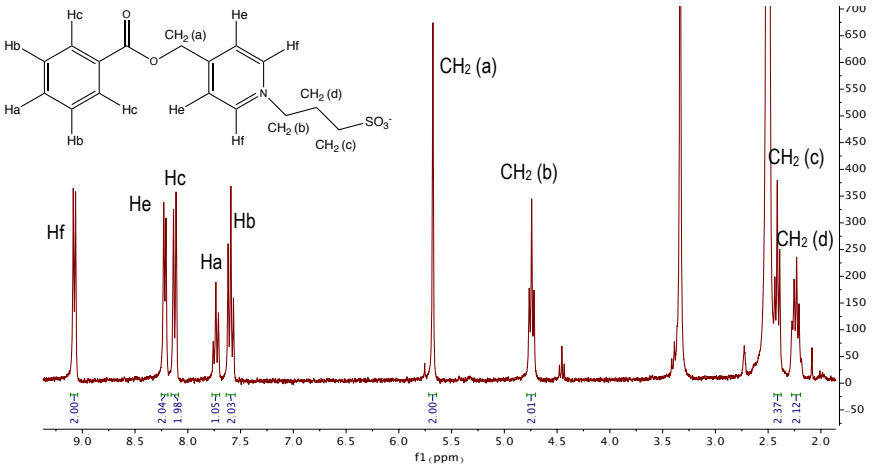


Figure 4: ^{1}H NMR spectrum of 4-[(benzoyloxy)methyl]-1-sulfopropyl- pyridinium in dimethyl sulfoxide (DMSO-*d*$_6$).

There are five different peaks from 9.1 to 7.5 ppm assigned to the aromatic protons. The two protons of the CH_2 appear at 4.7 and 2.4 ppm and the protons of the sulphonyl chain, at 2.2 ppm.

The course of the reaction was followed by ^{1}H NMR spectroscopy and it was observed that new peaks appeared after irradiation as shown Figure 5. Thanks to a ^{1}H-^{1}H COSY NMR all protons were assigned: the red circled peaks correspond to ascorbic acid protons[20] (which change after the irradiation due to its oxidation) and the black circled peaks correspond to the new molecules that have been formed (Scheme 7-**1b**). The violet circled signals are the triplet of triplets of the aromatic molecules (**1b** and benzoic acid) which overlap due to their similar chemical shift. It was observed that the black circled peaks relative integration increased with the irradiation time because the bond fragmentation process was taking place. The peaks corresponding to riboflavin could not be observed due to its low concentration (around 100 times lower than the quencher and ascorbic acid).

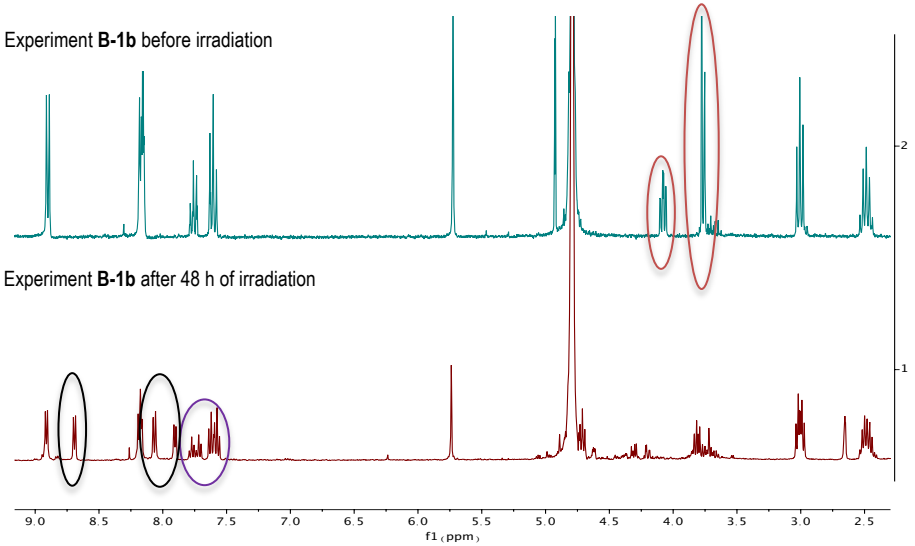


Figure 5: ^{1}H NMR for **B-1b** experiment in D_2O before and after 48 h of 450 nm irradiation.

The concentration of 4-[(benzoyloxy)methyl]-1-sulfopropyl- pyridinium which underwent photoelectron process and bond cleavage was calculated by the relative intensities of the two doublets at 8.7 and 9 ppm. The samples (Table 1) were prepared at different concentration of **1b** pyridinium, taking into account the salt solubility in water (20 mM) and previous experiments of

the research group[21], of RF and ascorbic acid in order to understand the behavior of the mechanism in relation with the ratio of concentrations. The concentration of photosensitizer in all the samples was much lower than for the quencher as the RF is restored after PET process when it reacts with oxygen, as seen in Scheme 5.

Table 1: Experiments performed and irradiation conditions at 450 nm for 4-[(benzoyloxy)methyl]-1-sulfopropyl- pyridinium in presence of riboflavin and ascorbic acid in D_2O medium.

	A-1b	**B-1b**	**C-1b**	**D-1b**	**E-1b**	**F-1b**	**G-1b***
[1b] [mM]	2	5	5	5	5	5	5
[RF] [μM]	70	70	35	70	70	70	70
[Ascorbic acid] [mM]	2	5	5	25	0	10	2.5

(a) The irradiations were carried out in NMR tubes at 5 cm from the light beam.

(b) The lamp used for the irradiations was the Luzchem LED Illuminator (450 nm).

(c) Sample solution pH was neutral.

*The irradiation was performed in absence of stopper in the NMR tube.

Table 2: Percentage of 4-[(benzoyloxy)methyl]-1-sulfopropyl- pyridinium which has undergone photoelectron process and bond cleavage.

	[broken 4-[(benzoyloxy)methyl]-1-sulfopropyl- pyridinium] [%]						
	A-1b	**B-1b**	**C-1b**	**D-1b**	**E-1b**	**F-1b**	**G-1b***
1h	0	0	0	0	0	0	0
6h	23	20	20	0	0	2	0
24h	33	40	50	17	0	2	17
48h	-	45	56	-	-	-	-

In Table 2, the percentage of **1b** pyridinium salt that has been photocleaved is calculated for the different irradiation times. It is important to consider when comparing the experiences that all the samples have an initial **1b** concentration of 5 mM except for **A-1b**, whose

concentration was 2 mM. In Figure 6, the variation of concentration for **1b** pyridinium salt in solution is plotted versus the irradiation time.

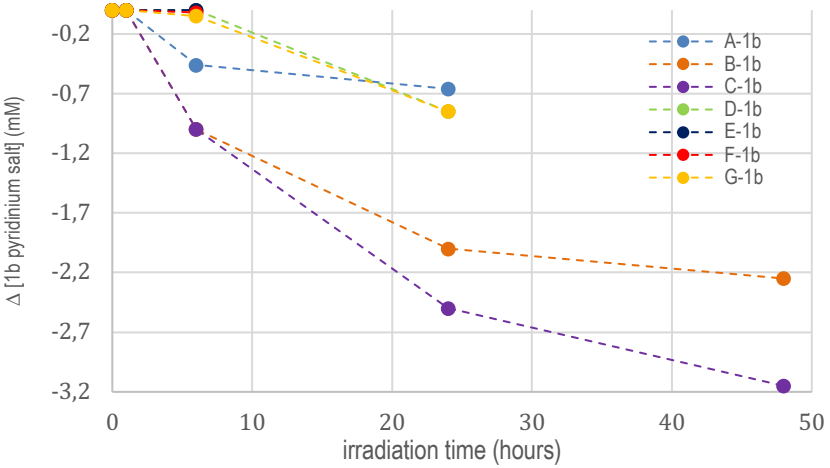


Figure 6: Variation of concentration of **1b** pyridinium at different irradiation times.

There are several results from Figure 6 that must be analyzed: on the one hand **E-1b** and **F-1b** experiences do not undergo bond cleavage after 6 hours of irradiation. This fact may be caused by a shortage and excess of reducing agent. In **E-1b**, as there is no sacrificial reductant, there is oxygen in the solution and RF can be oxidized to photoproducts decreasing the rate of photoelectron transfer process. In **F-1b,** the excess of ascorbic acid may avoid the oxidation of RF after the PET process and RF is not restored to its ground state, as stated above. The hypothesis of lack of oxygen in the solution as the cause for a slower reaction process is the reason why in experiment **G-1b** the stopper was removed during the irradiation. In addition, it is possible to observe that the experiences **D-1b** and **G-1b** present the same behavior and even the reason of this similarity remains unknown, the same exposition made for **E-1b** and **F-1b** could be used for these experiments low bond cleavage rate. The ideal equivalent ratio between ascorbic acid and pyridinium salt is observed to be the unity.

On the other hand, in samples **A-1b**, **B-1b** and **C-1b**, the rate of reaction diminishes with time and that may be due to a decrease of RF concentration by photobleaching. If there are

less photosensitizer molecules able to absorb light, less substrate molecules undergo bond cleavage.

The experience whose bond cleavage was quicker is **C-1b**, in which the concentration ratio between **1b** and riboflavin is the highest. This result may seem to go against the system expected behavior because if there are more sensitizer molecules for the same number of substrate molecules, the bond cleavage rate should be higher. Nevertheless, as RF de-excitation is an equilibrium between different relaxation ways, a higher concentration of quencher makes the PET process more efficient. Moreover, the samples with more RF concentration have a minor reaction rate because the restorage of the RF that has undergone PET process decreases if there are still RF molecules not being excited. The different compounds are in equilibrium.

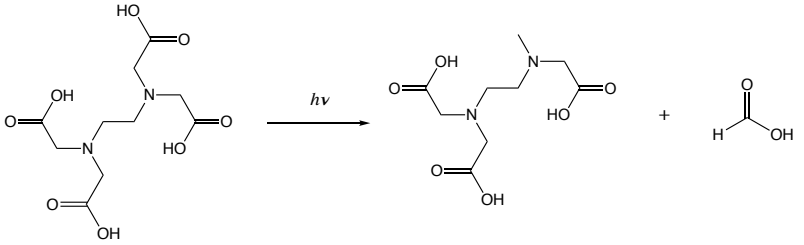
In addition, the same trend is observed between experiences **A-1b** and **B-1b** in which the sample with a higher concentration of pyridinium (**B-1b**) presents a higher bond cleavage rate. In conclusion, the photoelectron process may be thought in terms of the probability for a sensitizer molecule to find a substrate molecule: the higher number of substrate molecules the sensitizer is able to encounter, the higher probability for the PET to take place.

Even though the irradiation of the **1b** pyridinium salt presents bond cleavage and its reaction rate was improved by studying the behavior of the different **1b**/RF concentration ratios, the bond cleavage rate was not very high for any of the experiences. Taking into consideration the final aim of this study, it is necessary a system whose bond scission is more efficient: with a high concentration of broken substrate molecule in less irradiation time (around minutes).

6.2. EDTA DISODIUM SALT

The second molecule whose bond cleavage was tested was ethylenediaminetetraacetic acid (known as EDTA). EDTA is one of the most common complexing agents of metals and it is versatile used to isolate di and trivalent ions (such as Calcium and Magnesium) bonding via four carboxylate and two amine groups. In biology, EDTA is commonly used as inhibitor because most nucleases require Magnesium to function and EDTA is able to control its concentration.

According to literature, EDTA shows a significant bond cleavage rate when riboflavin is used as the photosensitizer[14]. In this molecule, the photoreleasable protecting group is 2,2'- (2-((carboxymethyl)(methyl)amino) ethylazanediyl)-diacetic acid which masks formic acid reactivity.



Scheme 10: Ethylenediaminetetraacetic acid structure (EDTA) and bond photocleavage.

As the tetra-protonated acid presents a low solubility in water, the EDTA disodium salt was used in these irradiation experiences in order to increase its solubility (60 mM). In addition, the pH was set to 7.4 by adding a phosphate-buffered saline (PBS) solution. Following the literature procedure, the samples were prepared using different concentrations of EDTA disodium salt and riboflavin (Table 3) in D_2O. No ascorbic acid or other sacrificial reductant was added.

Table 3: Experiments performed and irradiation conditions for ethylenediaminetetraacetic acid disodium salt in presence of riboflavin in D_2O medium.

	EDTA-A	**EDTA-B**	**EDTA-C**	**EDTA-D**
[EDTA-2Na$^+$] [mM]	12,5	12,5	5	60
[RF] [μM]	70	600	70	12000

(a) The irradiations were carried out in NMR tubes at 5 cm from the light beam.

(b) The irradiation lamp was the UV mercury lamp (295×76 mm-15W) with 365 nm mode.

(c) Sample solution pH was 7 using pbs buffer.

The irradiations were monitored by ^{1}H NMR and UV-Visible before and after the irradiation in order to comprehend when all the riboflavin in the solution had been degraded. A benzene-d_6 capillary was introduced in the NMR tube to be used as a reference and facilitate the integration of the peaks. Benzene was chosen as the reference as it shows only one signal in ^{1}H NMR at 7.17 ppm and it does not overlap with the signals of the EDTA neither before nor after the bond cleavage.

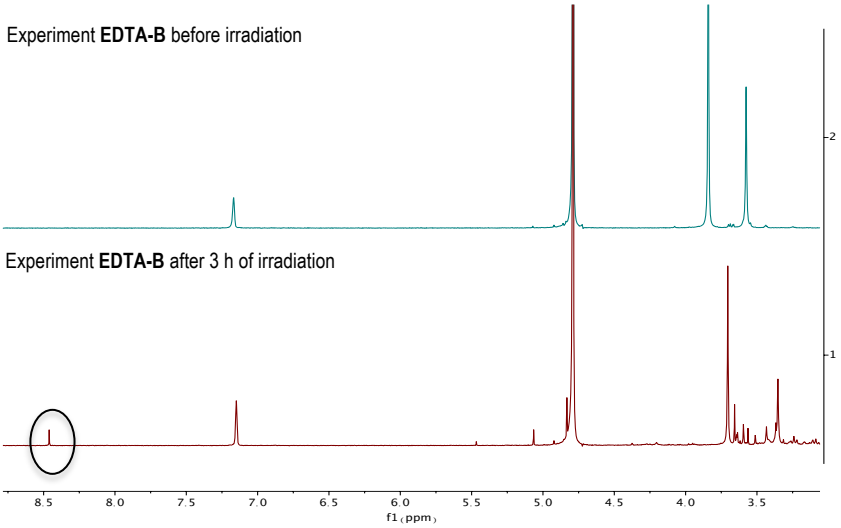


Figure 7: ^{1}H NMR for EDTA-B experiment in D_2O before and after 3 h of 365 nm irradiation.

In Figure 7 it is possible to observe the benzene reference peak at 7.17 ppm. The EDTA protons appear at 3.84 and 3.58 ppm with 2:1 relative intensities. After irradiation, the peaks shift to lower chemical shifts, new signals appear around 3.5 ppm and a singlet at 8.46 ppm due to formic acid proton.

Each time the sample was irradiated, the NMR tube solution presented a colour change from intense yellow to colourless. Then the tube was shaken and the colour was restored due to RF oxidation. Some UV-Vis measurements were performed at different irradiation times (Figure 8) in order to understand the decrease of RF concentration. It is possible to notice that the intensity of riboflavin characteristic absorption bands at 373 and 444 nm diminish with time because a part of RF molecules have undergone photobleaching.

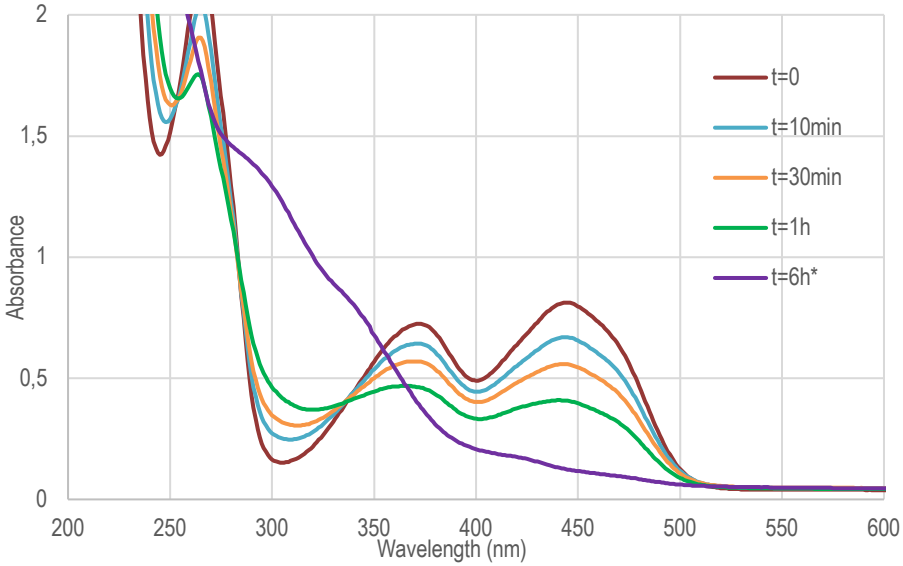


Figure 8: UV-Visible measurements for EDTA-A experience at different irradiation times.

*Measurement before and after 1 h of oxygen bubbling.

After 6 hours of irradiation, the NMR tube did not present the yellowish colour of the initial solution. UV-Visible absorption was measured (violet line) and the spectrum did not present riboflavin bands. The solution was bubbled with oxygen for one hour in order to restore RF and the measurement was repeated. However, the obtained spectrum did not change. This fact indicates that all the RF in solution has photobleached forming a photoproduct incapable to fluoresce. There is an intensity increase from 300 nm to lower wavelengths. According to literature data, the absorption band for lumichrome appears around 350 nm and for lumiflavin at 450 nm. This evidence and the neutral pH in the solution confirms the formation of lumichrome.

The obtained NMR data for the EDTA irradiation experiences was used to calculate the relative intensity of the formic acid proton and its concentration. In Table 4 the calculated concentration of formic acid is summarized for the EDTA - $2Na^+$ experiences at the different irradiation times. The formic acid concentrations were obtained using the relative intensities of the EDTA: benzene in the initial spectrum and formic acid: benzene in the following spectra.

Table 4: Calculated concentrations of formed formic acid for the EDTA disodium salt experiences at the different irradiation times.

	[Formic acid] [mM]			
	EDTA-A	**EDTA-B**	**EDTA-C**	**EDTA-D**
10 min	0	0,102	0	0,129
30 min	0	0,111	0	0,551
60 min	0	0,154	0,025	0,763
100 min	-	-	-	1,19
120 min	0,046	0,174	0,037	-
160 min	-	-	-	2,289
240 min	0,479	0,260	0,068	2,289
300 min	0,839	-	-	-
360 min	-	0,370	0,074	-
420 min	0,899	-	-	-

In Figure 9, the variation of EDTA disodium salt concentration versus irradiation time for the four performed experiences is shown. It is clearly observed that the **EDTA-D** experience (in which the concentration of EDTA disodium salt and riboflavin were up to their solubility value) leads to the fastest bond cleavage. This fact suggests the opposite to the results stated above for **1b** pyridinium salt. Nevertheless, the concentrations for RF and substrate in this experience are respectively 170 and 5 times more than for the others. Taking into consideration that experience **EDTA-D** is saturated of RF and quencher, it is reasonable for this sample to have the highest bond scission rate.

As expected, when the concentration values are comparable to the analyzed for pyridinium salt, the same tendency is observed. Comparing **EDTA-A**, **EDTA-B** and **EDTA-C**: **EDTA-A** has the greatest bond cleavage rate because the number of substrate molecules for one of sensitizer's is higher and a greater substrate/sensitizer concentration ratio leads to a higher number of photoelectron transfer and bond scission processes.

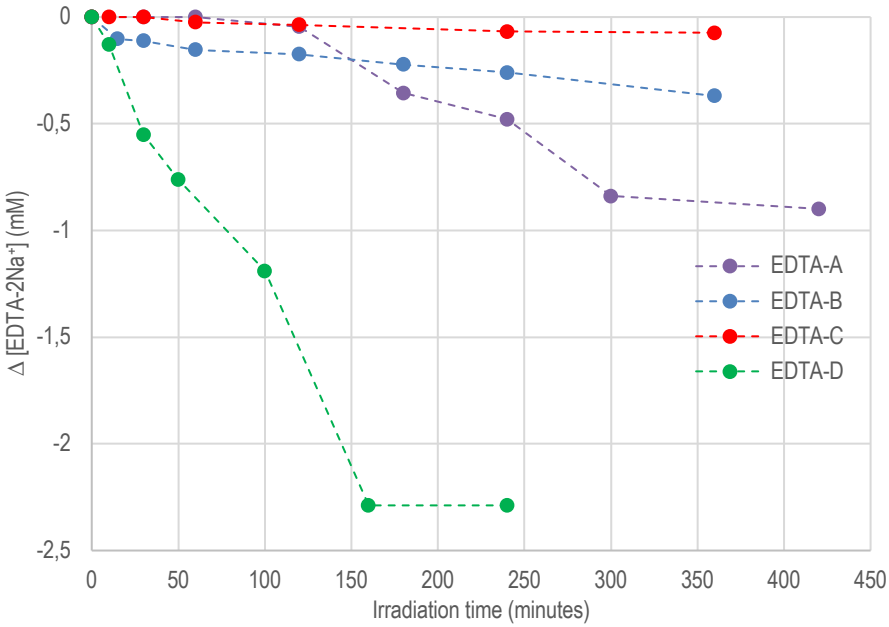
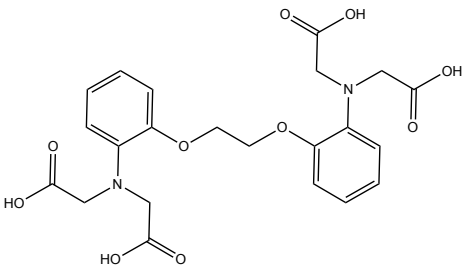


Figure 9 : Variation of concentration of EDTA-2Na$^+$ experiences at different irradiation times.

While comparing the experiences with the highest rate for the salts tested: **C-1b** presents a higher bond cleavage rate than **EDTA-A** because in 6 hours 1mM of **1b** underwent bond scission, while less than 0.9 mM of EDTA-2Na$^+$ did. Nevertheless, taking into account that the irradiation lamp used for **1b** was 7 times more powerful than for EDTA experiences and that the concentration of EDTA was around 4 times higher than for **1b**, it is not possible to assure which of the substrates has the highest bond cleavage rate. These results are currently under investigation to be improved by adding an oxidant, which rapidly restores riboflavin activated state for it to react with other substrates molecules in solution or by the use of other photosensitizer.

6.3. BAPTA DIPOTASIUM SALT

The third molecule to be irradiated was 1,2-bis(o-aminophenoxy)ethane-N,N,N', N'-tetraacetic acid (known as BAPTA). This molecule has a great similarity with EDTA because it is also a very good mono and divalent metal chelator thanks to its four carboxylic acid groups that lead to a flexible molecule.



Scheme 11: 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid structure.

BAPTA importance is mainly due to its biological activity in the control of Calcium concentration. As a large number of intracellular processes depend on free Ca^{2+} concentration, its control is very important. BAPTA is able to release or retain calcium cations and this can be controlled by light irradiation as this molecule is photosensitive[22].

Even if there are no studies that report BAPTA bond scission, its resemblance to EDTA and the group previous experience[23] make this molecule a good candidate to be irradiated. It was hypothesized that the proximity between the amine CH_2COOH arms could reduce the probability to obtain more than one formic acid for one molecule of EDTA. In BAPTA structure the aromatic rings maintain the amines distant and we expected the formation of a larger number of formic acid molecules.

Table 5: Experiments and irradiation conditions at 365 nm for 1,2- bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid dipotassium salt in presence of riboflavin in D_2O medium.

	BAPTA-A	**BAPTA-B**
[BAPTA-2K$^+$] [mM]	5	12,5
[RF] [μM]	70	70

(a) The irradiations were carried out in NMR tubes at 5 cm from the light beam.

(b) The irradiation lamp was the UV mercury lamp (295×76 mm-15W).

(c) Sample solution pH was 7 using pbs buffer.

The BAPTA tetraethyl ester was hydrolyzed in order to obtain the dipotassium salt, whose solubility in water is higher (15 mM). The irradiation was performed following the same procedure

reported for EDTA (See Table 5) but using acetonitrile reference instead of benzene in order to avoid the aromatic signals to overlap.

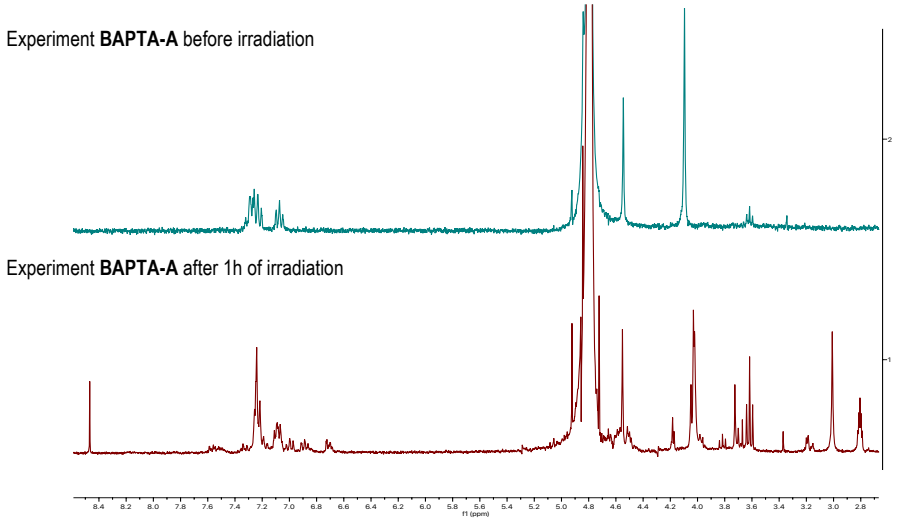
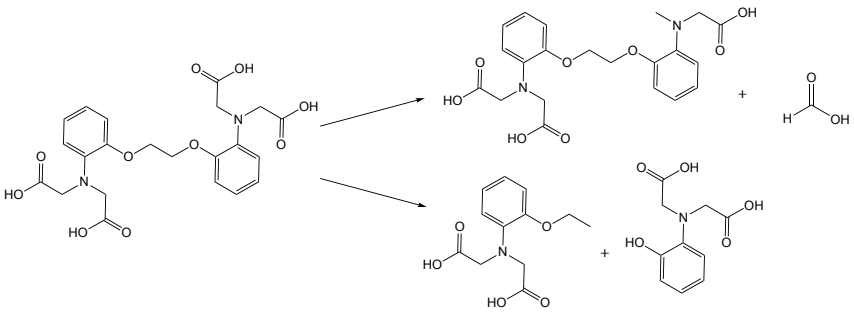


Figure 10: ^{1}H NMR for **BAPTA-A** experience in D_2O before and after 1 h of 365 nm irradiation.

The ^{1}H NMR spectrum for BAPTA dipotassium salt (Figure 10) presents two multiplets around 7.1 and 7.25 ppm due to the aromatic protons, and two singlets at 4.55 and 4.10 ppm with 1:2 relative intensities due to CH2 chain protons. After the irradiation, a considerable number of new signals are observed caused by BAPTA bond cleavage. The most characteristic ones are the singlet at 8.47 ppm (which corresponds to formic acid chemical shift) and the singlet at 3 ppm.



Scheme 12: Possible decomposition products for 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid in the dark in aqueous solution.

Even if the same behavior observed in EDTA was expected, according to a reported study about the decomposition of a BAPTA derivative[24], the molecule could also break by the ether bond. The large number of new signals is a proof that more than one fragmentation has taken place in BAPTA molecule. In addition, in both experiences, the irradiated molecule rapidly underwent bond cleavage and after 1 hour of irradiation. The intensity of the new peaks is kept constant for the following hours and that the bond scission has cease. It was also observed that the initial colour of the solution (yellow) changed to a darker brown as the irradiation time increased.

The experience **BAPTA-A** was going to be studied for the first hour of irradiation but shorter interval of time. Nevertheless, the molecule underwent decomposition in the prepared aqueous solution without the presence of a photosensitizer. To identify if the cause of the decomposition was direct light absorption or other factors such as temperature or the fact that the molecule was in solution, a dark NMR control was performed using an amberized NMR tube (*NORELL 508 UP*). The sample in colourless NMR tube presented decomposition after 3 days, while the sample at dark presented decomposition after 7 days. In both cases, the molecule undergoes schism at room temperature and aqueous solution.

The fact that BAPTA bond cleavage can not be externally controlled in terms of light irradiation time, quencher and photosensitizer concentration, because it naturally decomposes in aqueous solution at room temperature, makes the molecule unsuitable for *Optonutrics* project aim.

7. CONCLUSIONS

The present study has proved that photosensitizers are able to produce bond cleavage in target molecules by a photoelectron transfer process when irradiated. In addition, reactive species such as carboxylic acids can be obtained by this schism when they are bonded to photoreleasable protecting groups.

The rate of bond cleavage depends on the concentration of photosensitizer and substrate because the de-excitation can happen through different pathways. In the reported experiments, the samples with the highest concentration ratio substrate/photosensitizer presented the highest bond cleavage rate because each excited molecule could encounter more molecules to which donate an electron. In addition, the samples with a higher concentration of photosensitizer show a lower bond scission rate due to the equilibrium between the different compounds.

It has been also shown that the addition of a sacrificial reductant can accelerate the reaction. Nevertheless, its concentration must be controlled because an excess reduces the reaction rate. This excess could avoid the restorage of the photosensitizer ground state molecule leading to the end of the reaction. In contrast, a shortage of sacrificial reductant allows the oxygen molecules in solvent to react with the excited photosensitizer and diminish the rate of scission.

The bond cleavage reaction finishes when all the riboflavin in the medium has photobleached, even though in most of the cases, the rate of reaction decreases with time. The photobleaching of riboflavin has been trailed by UV-Visible measurements in which the characteristic absorption bands of riboflavin decreased with the irradiation time. Besides, the yellow colour of the solution was recovered when oxygen reacted with riboflavin by shaking the sample.

In overall, the **1b** pyridinium salt and EDTA disodium salt presented a similar bond cleavage rate using riboflavin as photosensitizer in aqueous solution.

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9. ACRONYMS

PPG: Photoreleasable protecting group

PET: Photoelectron transfer

RF: Riboflavin

LF: Lumiflavin

LC: Lumichrome

DCM: Dichloromethane

AcCN: Acetonitrile

MeOH: Methanol

THF: Tetrahydrofurane

EtOAc: Ethyl Acetate

DMSO: Dimethyl sulfoxyde

Et_2O: Diethyl ether

$CDCl_3$: Deutered chloroform

D_2O: Deuterium oxide

NAP: N-alkyl-picolinium

EDTA: ethylenediaminetetraacetic acid

BAPTA: 1,2- bis (o-aminophenoxy)ethane- N,N,N',N'- tetraacetic acid