UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

FACULTAD DE CIENCIAS QUIMICAS

“Synthesis and characterization of luminescent compounds of Sn IV with potential application as cellular marker *in vitro*”

By:

I.Q Eduardo Gabriel Pérez Pérez

AS PARTIAL REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN MATERIALS CHEMISTRY

APRIL 2016

“SYNTHESIS AND CHARACTERIZATION OF LUMINESCENT COMPOUNDS OF SN IV WITH POTENTIAL APPLICATION AS CELLULAR MARKER *IN VITRO*”

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RESUMEN

I.Q Eduardo Gabriel Pérez Pérez Abril 2016

Universidad Autónoma de Nuevo León

Facultad de Ciencias Químicas

Título del estudio: "SÍNTESIS Y CARACTERIZACIÓN DE COMPUESTOS LUMINISCENTES DE Sn IV CON POTENCIAL APLICACIÓN COMO MARCADOR CELULAR IN VITRO".

Número de páginas: 56

Candidato para el grado de Maestría en Ciencias con Orientación en Química de los Materiales.

Área de estudio: Química de los Materiales.

Propósito y Método de estudio: Los biomarcadores o marcadores biológicos, son aquellos que indican un estado biológico sea bioquímicos, fisiológicos o morfológicos. Es decir, son unos indicadores que pueden medirse objetivamente, el cual nos indica si un proceso biológico es normal o patológico (indicativo de una enfermedad). Recientemente se ha reportado que los compuestos organometálicos de estaño IV han servido para teñir células cancerígenas además de que se puede modular la citotoxicidad de estos modificando estratégicamente los diferentes sustituyentes a los que se encuentran enlazado. Con base en lo anterior, se realizó la síntesis de cuatro compuestos de estaño derivados de benzohilidrazinas, se determinó su capacidad citotóxica y su capacidad para teñir células cancerígenas *in vitro*.

Conclusiones y contribuciones: Fueron reportadas cuatro nuevas estructuras de compuestos derivados de benzoilhidrazinas con estaño IV, las cuales fueron completamente caracterizados por técnicas espectroscópicas y espectrométricas. Fue determinada también su capacidad citotóxica a diferentes parámetros de concentración y su capacidad de tinción de células de igual forma a diferentes parámetros de concentración *in vitro*. Los resultados fueron alentadores en comparación con los reportes ya existentes, además, se realizaron otros acoplamientos con otros sustituyentes de boro, lo cual dio resultados excelentes y prometedores que dieron pauta a la elaboración de 1 artículo científico y la posibilidad de generar una patente, con esto se cumplió con la hipótesis planteada rebasando las expectativas.

FIRMA DEL ASESOR:

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Dr. Víctor M. Jiménez Pérez

SUMMARY

I.Q Eduardo Gabriel Pérez Pérez April 2016

Universidad Autónoma de Nuevo León

Facultad de Ciencias Químicas

Study Title: "SYNTHESIS AND CHARACTERIZATION OF LUMINESCENT COMPOUNDS SN IV WITH POTENTIAL APPLICATION AS CELLULAR MARKER IN VITRO".

Number of pages: 56

Candidate for the degree of Master of Sciences with Orientation in Materials Chemistry.

Study area: Materials Chemistry.

Purpose and study method: Biomarkers or biological markers indicate a biological state like biochemical, physiological or morphological. These are indicators that can be measured objectively, which would indicate that a biological process is normal or pathological (indicative of a disease). Recently it has been reported that organo-tin compounds have been used to stain cancer cells and also modulate the cytotoxicity modifying strategically the substituents that is bonded to tin. Based on the above, we are interested in the synthesis of four compounds of tin derivatives benzohidrazines, determine their cytotoxic capacity and ability to stain cancer cells in vitro.

Conclusions and contribution: There were reported four new structures derivatives of derivatives benzohidrazines with tin IV, which were fully characterized by spectroscopic and spectrometric techniques. It was also determined their cytotoxic capacity to different concentration parameters and cell staining to different concentration parameters *in vitro*. The results were promising compared to existing reports, also other coupling with other substituents of boron were made, which gave excellent and promising results that gave guidelines for the development of one scientific articles and the possibility of generate a patent, this was fulfilled with the hypothesis surpassing expectations.

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Dr. Víctor M. Jiménez Pérez

This thesis was realized at the Laboratory of Materials III, into the postgraduate area of Facultad de Ciencias Químicas from Universidad Autónoma de Nuevo León under the management of Ph.D. Victor Manuel Jiménez Pérez.

This project was carried out whit the financial support from CONACYT (558050), and it was complemented with two short stays with the collaboration of Ph.D. Arturo Chávez and Ph.D. Rosario Nuñez Aguilera.

DIVULGATION PROJECT AND STAYS PERFORMED

Two shorts stays were performed

April 23 – August 24 2015 in Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional in Monterrey (Cinvestav), under de management of Dr. Arturo Chávez Reyes.

August 31 2015 – January 31 2016 in Instituto de Ciencia de Materiales de la Autónoma de Barcelona, Consejo de Investigaciones Científicas, Barcelona España, under the management of Dra. Rosario Núñez Aguilera.

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To the teachers who taught me during this master's stage, without their teachings i would not had succeeded.

To PhD. Idalia Gómez, for always being aware of the progress and stages involved in achieving this.

To my brothers for supporting me in all my decisions.

To my parents for always being with me.

DEDICATORY

I thank God for having accompanied me and guided me throughout my career, for being strength in moments of weakness and for always giving me a life full of learning, experiences and above all happiness.

I thank my parents Guadalupe and Gabriel for supporting me at all times, for the values they have instilled in me, and for giving me the opportunity to have an excellent education throughout my life. But above all for being an excellent example of life to follow.

To my brothers Alejandra and Gabriel, for being an important part of my life and representing the family unit.

To my friends Mauricio, Denain, Antulio, Misael and Ramiro for believing in me and having made this stage of mastery a journey of ups and downs that I will never forget.

To my grannies Emma and Irene, who, although they no longer meet us physically, are always present in my heart, for feeling proud and having believed in me until the last moment. Grannies, I've finished my master's degree!

To my now wife Anay for all the support she gave me, for putting up with me and for always looking for a way to have a good time with me. For enduring my hysteria. For being an excellent friend and without hesitation, magnificent wife and for the best gift in the entire world, my beautiful son, Leo, I hope someday you will read this, and feel proud and motivated by your father.

Eduardo

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ATR attenuated total reflection µg microgram

Å Armstrongs

MP melting point

CO_2 carbon dioxide µm micrometer

^{13}C carbon NMR

ºC Celsius degrees

δ chemical shift

COSY correlation spectroscopy

NMR Nuclear magnetic resonance

J coupling constant

OLED organic light-emitting diode

DNA deoxyribonucleic acid

PBS phosphate-buffered saline

DMEM Dulbecco's Modified Eagle's medium

1H proton NMR

HETCOR 2-D heterocorrelation

FBS fetal bovine serum

RNA ribonucleic acid

IC50 inhibitory concentration of 50% of cell growth

TOF time of flight

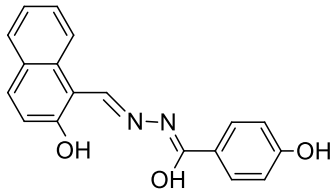
IR infrared spectroscopy

119 Sn tin NMR

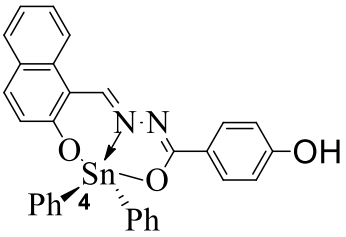
MHz Megahertz

B16F10 melanoma murine cells

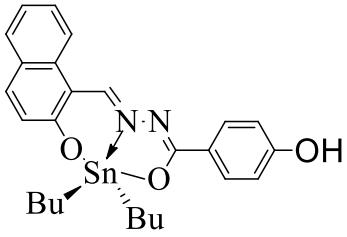
LIST OF SYNTHESIZED COMPOUNDS.



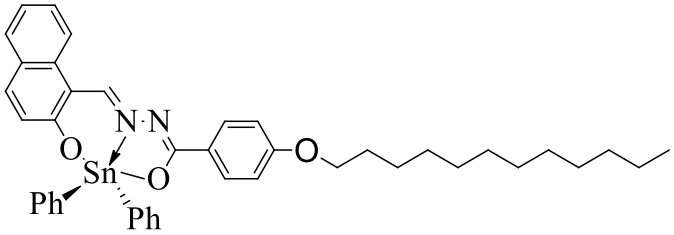
1. (1*Z*,*N*'*E*)-4-hydroxy-*N*'-((2-hydroxynaphthalen-1 yl)methylene)benzohydrazonic acid



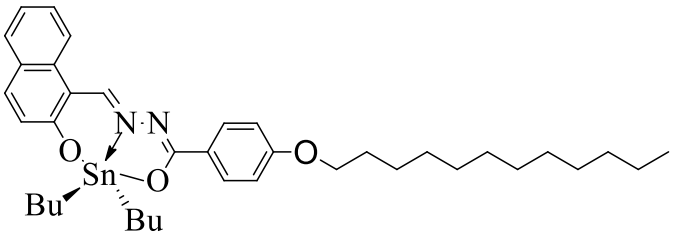
2. 4-((1*E*,3*Z*)-6,6-diphenylnaphtho[1,2-*h*][1,3,5,6,2] dioxadiazastannonin-4-yl)phenol



3. 4-((1*E*,3*Z*)-6,6-dibutylnaphtho[1,2-*h*][1,3,5,6,2]dioxadiazastannonin-4-yl)phenol



4. (1*E*,3*Z*)-4-(4-(dodecyloxy)phenyl)-6,6-diphenylnaphtho[1,2-*h*][1,3,5,6,2]dioxadiazastannonine



5. (1*E*,3*Z*)-6,6-dibutyl-4-(4-(dodecyloxy)phenyl)naphtho[1,2-*h*][1,3,5,6,2]dioxadiazastannonine

CONTENTS

1. INTRODUCTION

A luminescent material is defined as a substance that emits photons due to the return of electrons from the excited state to the ground state. There are materials that transform this energy in visible light and others such as X-rays or gamma rays. Understand and eventually manipulate this phenomenon is very important for exploitation and application [1]. Have been reported that these materials are applied to manufacture of LED [2] and bioimaging [3].

The luminescence is phenomenon of emission of electromagnetic radiation from an excited state. Depending on the energy that originates is possible to speak of various kinds of luminescence: photoluminescence, fluorescence, phosphorescence, thermoluminescence, chemiluminescence, triboluminescence, electroluminescence and radioluminescence [4]. Many organic or inorganic materials exhibit luminescence, that means emit visible light or not visible during and after exposure to the excitation of an energy source [5].

Currently there are luminescent organometallic compounds based in indium (In) [4], gold (Au) [6], platinum (Pt) [7] copper (Cu) [7], boron (B) [8] and tin (Sn) [9] which allow to develop a variety of complex and in turn be able to use this property for various applications.

The versatility of the light emitting organometallic compounds has been a goal for various applications, these include the manufacture OLEDs from organometallic compounds of tin [2] indium [4] and boron [8], to mention a few, fluorescent probes for detection of metal ions and for use as a biomarker [10]. In the latter include that there currently report of tin compounds applied for this purpose.

The advantages of working with synthesis of organic tin compounds are: synthesis mechanisms are relatively simple, does not require complex instrumentation, are friendly to the environment, their toxicity is relatively low and can be manipulated in a practical way, in addition to compounds are more accessible economically compared to other organometallic complexes such as gold, iridium and rhodium which require more

complex instrumentation and procedures as well as having a considerable degree of toxicity [7].

Bioimaging techniques are based primarily on the ability of fluorescence that some molecules have present naturally. In the case of the human body, some cells and molecules have fluorescent [11] properties; as mitochondria, hair, elastin arteries or most of the liver cells [12]. However, to watch those that are not fluorescent, and to study living organisms, scientists use different techniques, as stick other animal proteins, especially of jellyfish and coral. In the case of the investigation of dead cells, another way to obtain fluorescence is to attach antibodies to which previously have been treated with a certain color or chemical complexes, mostly organic compounds or fluorescent organometallic known as biomarkers [13].

The biomarkers are substances that indicate biological states as biochemical, physiological or morphological. There are different types of biomarkers according to the characteristics of its own composition, these serve to mark different cell structures, from the cell nucleus (an example of this marker is the DAPI that stained strongly DNA) to their different structures (ribosomes, mitochondria, cytoplasm, etc.) [14].

The incorporation of this type of fluorescent compounds opens the way to the use of different techniques for bioimaging, which could allow, for example, the study of infectious agents such as viruses and bacteria within the human body and in real time as well as most effective drug design [15].

The use of these techniques means a great scientific and clinical advancement, being that their applications are attributed to the diagnostic of cancer. An example more studied, because today there are advanced studies referring to this, is the analysis of cultured HeLa cells *in-vitro* for which have been developed various fluorescent dyes (Hoechst and DAPI) that when placed in the cells, are capable of fluoresce and contribute to better illumination of biological tissues that characterize this cell line [16].

The cytotoxicity of these markers plays an important role in the application as it is vital that these compounds are the least toxic possible and allow enough lifetimes to develop a good cells analysis. It has been reported that the cytotoxicity of organotin compounds correlates with the number and length of alkyl groups attached to tin, whereas counter anions not affect [17]. Thus, organotin compounds with short carbon chains are more toxic [18].

Recently in our research group we have developed new luminescent tin compounds which allowed observe a promising application to cell bioimages *in-vitro*. In the report is performed the synthesis and characterization of 6 complexes pentacordinated tin derivatives of salicidilenbenzoylhydrazine where it was observed how a homogeneous staining is achieved in the cytoplasms and vesicles of murine melanoma cells B16F10 and in turn have an acceptable cytotoxicity [19].

Because of this the research group are interested in strategically develop of 4 new tin compounds Schiff bases which enhance their luminescent properties for application in bioimages due to chromophore 2-hydroxy-naphthaldehyde and the new electrodonor group OH of the hydroxybenzoylhydrazine. Also propose a new synthesis with these new complexes modifying the molecule by adding an alkyl chain of 12 carbons attacking the OH group located in the para position of benzene ring from the hydroxybenzoylhydrazine, since it has been reported that the decreased toxicity relates with the number and length of alkyl groups attached to tin [17, 18], this in order to evaluate their cytotoxicity and see the effect in the cells *in-vitro*.

2. BACKGROUND

2.1 Photoluminescence

Many inorganic and organic materials exhibit luminescence, it means that emit light during and after being exposed to excitation of an energy source. For photoluminescent materials the excitation source is the visible light (usually UV). The basic principle of photoluminescence is simple: electrons orbiting atoms or molecules absorb energy due to collision with protons during exciting. Then the excess energy is emitted as photons for some time [20].

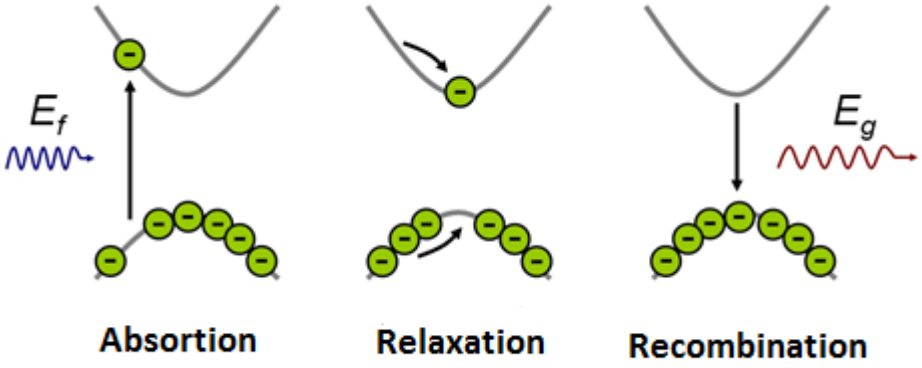


Figure 1“Principle of photoluminescence”.

There are two types of photoluminescence: fluorescence and phosphorescence. The difference between them is the time of light emission. Fluorescent materials emit light from seconds to nanoseconds when excited electrons to a higher energy state. It means that they don’t need continually lighting source to increase energy and emit light. Therefore, fluorescent materials, need a continuous excitation source. In contrast, phosphorescent materials, once excited by an energy source, emit light for a prolonged time ranging from minutes to hours [21].

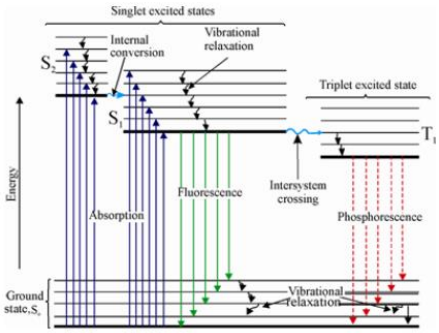


Figure 2"Activation and deactivation process"

2.2 Confocal microscope.

In the last decade have been developed procedures to significantly increase the resolution of the optical microscope; however this has already developed decades ago. Minsky [22], in 1961, proposed a new type of microscope to observe living specimens with high contrast and improved resolution. This new type of microscope is based on eliminating the veil that is produced for regions outside the plane of focus in a normal image optical. For this, have chosen to pass the light incident on the sample by a small hole and focus the image plane with a high numerical aperture objective. Thus, the light that is reflected by the point in the focal plane of the target, returns and is refocused and transmitted in turn through a small hole without any loss. Thus, an image with high contrast and definition of a focal point [23] plane is obtained.

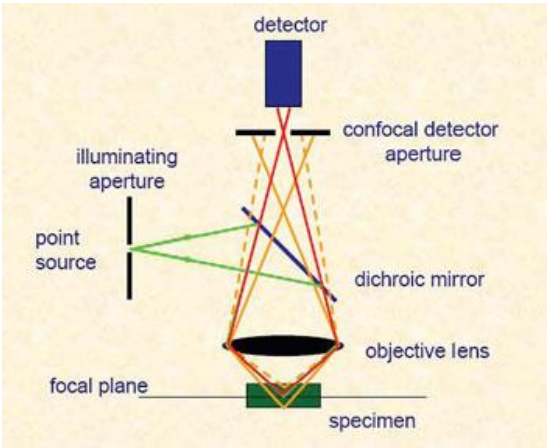


Figure 3 "Confocal microscope Scheme"

In Figure 3 is shown a particular type of confocal microscope, in which the light source used is a laser beam. The light beam is stopped by a slot (P1) and impacts in a dichroic mirror (which totally reflects light at an angle of about 45 °), later is focused on the sample using the microscope objective. The light emitted by the sample is collected by the same objective and passing through the dichroic mirror is focused on a detecting groove (P2). The light that penetrates less or greater depth in the sample (planes out of focus), incise in front of or behind of the sensing slot (light beams shown in dotted lines in figure) [23].

Today the laser scanning confocal microscopy has been improved to obtain higher resolution allowing to obtain high-quality resolutions.

2.3 Biomarkers and bioimaging.

The bioimages are regularly obtained by microscopic to observe cellular structures, molecular structures and moreover behaviors and mechanisms of them from non-invasive techniques, these can be taken *in-vivo* or *in-vitro* [24].

A biomarker, or biological marker, is a substance that helps to measure biological process, a disease or response to specific treatment. Use of biomarkers in risk assessment against certain diseases has increased significantly in the last decade. Mainly particular interest in assessing of progressive disease where symptoms occur after a long period of exposure [25].

2.4 Classification of Biomarker.

Biomarkers are differentiated into three types [25]:

- Biomarker of exposure, which evaluates in an organism the presence of an exogenous substance, a metabolite or one product of the interaction between xenobiotic agent (synthetic natural compounds in the environment that the body metabolizes and accumulates) and a molecule.

- Biomarker of effect, which evaluates the biochemical, physiological or behavioral alteration produced in the body that can be associated with a disease.
- Biomarkers of susceptibility, is an indicator of inherited or acquired capacity of an organism to respond to exposure to xenobiotic substance.

2.5 Medical importance of organometallic materials such as biomarkers and bioimages.

In 2013 Ji Young Choi, Gun-Hee Kim and colleagues developed fluorescent radiometric probes called chemo-radiation monitors for the detection of Au^{+3} ions in in-vitro cell synthesized from a naphtalamide with propargylamine, which resulted in a sensor of Au^{+3} ions in real time which was analyzed by confocal microscopy determining bioimages in HeLa cells and mouse embryonic fibroblasts. Bioimages shows that the compound penetrate efficiently the cells showing green fluorescence as pattern without Au^{+3} ions, in the case of treated Au^{+3} ions a blue fluorescence is observed due to react with the cells, attributing the change staining for interaction with CTAC (Cetyl Trimethyl Ammonium chloride) surfactants, to the lipids of the cells exhibiting a faster response of adipocytes and growth structures Au^{+3} ions in aqueous media. This opens the way for the development of tools related with chemical and biological studies and new systems to detect other ions fixed in cellular lipids and their characteristics of reaction mechanisms of chemical processes that entails [6].

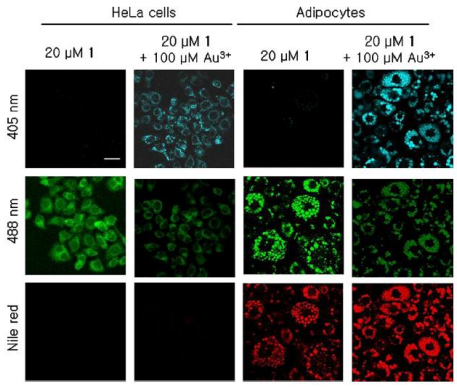


Figure 4 “Bioimages of HeLa cells and adipocytes treated with gold ions and radiometric probe”

In the same year Diego Montagnier *et al*, develop a series of probes capable of detecting cis-platinum which is a drug used in chemotherapy for the treatment of various cancers understanding of a better way how these drugs are processed after enter the cell, developing a non-fluorescent probe linking the spirolactam of Rhodamine "B" to a part of TCDD that it works like platinum detector (Rho-TCDD). The compound will join the platinum species through the link ditiocarbonile; therefore *trans* ligands are transformed to react with the part of the spirolactam and so the fluorescence is selectively activated.

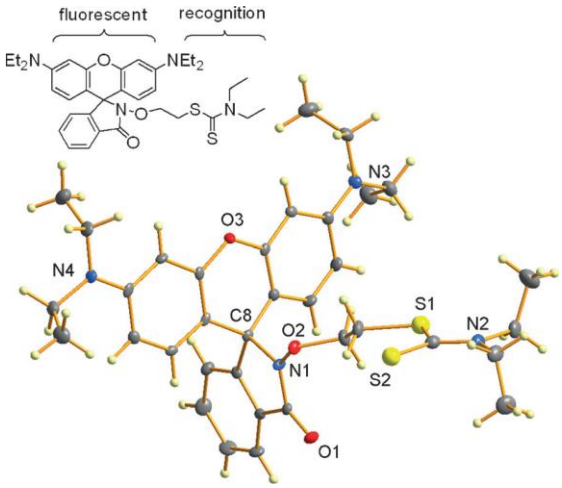


Figure 5“Molecular representation of the Rho-TCDD probe”

Using confocal microscopy they determine the presence of Pt in HeLa cells treated *in-vitro* with cisplatinum comparing with biomarker Hoechst at different concentrations. The compounds named as **3** and **6** were selected for their high selectivity and reaction activity on the Pt (IV). Compound **6** is hydrophobic while compound **3** is highly hydrophilic. Both compounds are stable in culture medium for 72h however compound **3** resisted reduction due its high reduction potential. Using a confocal microscope a dispersed staining observed in the cytoplasm of the HeLa cells also corroborated the interaction of the probes with Pt ions (IV) consistent with characteristic fluorescence color. This proved to be a very versatile tool for locating clinical anticancer platinum drugs [27].

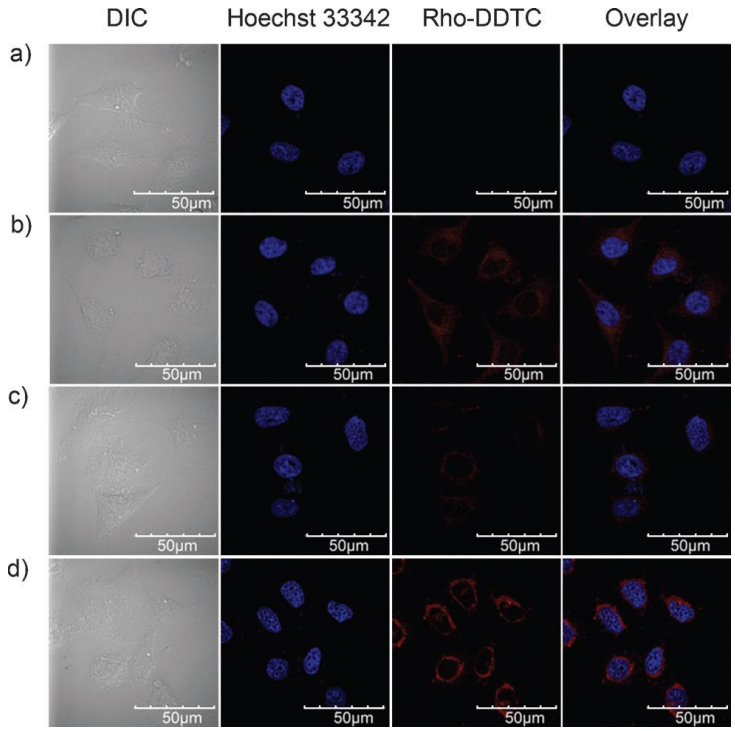


Figure 6 "Fluorescence images of HeLa cells a) Untreated (control), and exposed to b) cisplatin, c) 3 and d) 6 for 3h and further incubated with Rho-TCDD for 1h at 37 ° C"

At present there is only one report of complex tin (IV) used for staining cells and bioimaging.

2.6 Commercial Biomarkers, Hoechst and DAPI.

Hoechst is a fluorescent dye of DNA used in fluorescence microscopy and cell sorting by flow cytometry (FACS) [28]. For its staining ability of DNA, it is also used to visualize cores and mitochondria. Two related compounds are usually used Hoechst 33258 [29] and Hoechst 33342 [30]. Both dyes are excited by ultraviolet light with a wavelength near to 350 nm and emit blue fluorescence with an emission maximum of about 461nm. Hoechst can be used in fixed or living cells, and are often used to replace another marker for nucleic acid stain: DAPI [30]. DAPI differ from Hoechst in the additional ethyl group (Hoechst 33342), which gives a more lipophilic behavior, thus is more easily cross the cell membrane [31].

DAPI (4', 6-diamino-2-phenylindole) is a fluorescent marker that binds strongly to regions enriched in adenine and thymine in DNA sequences. It is widely used in fluorescence microscopy. DAPI can pass through the cell membrane, due of that, is used to stain live cells and fixed cells, although it passes through the cell membrane, it doesn't works very well in living cells thus the efficacy of the fluorescent signal is less [30, 31].

Cytotoxicity of these two compounds is really low because in the different applications that have been used have been reported low levels of mortality, toxicity and low presence of mutations, and also has good photostability, so they are the most commercial and used for clinical purposes, for example for morphologic cells analysis and watch different histological structures [28, 29, 31].

2.7 Activity of organotin compounds IV in cancer cells.

The first study, in 1929, about the cytotoxic activity of organotin compounds derived from Sn (IV) were contradictory. Later, in 1972, showed that the triphenyl tin acetate delay tumor growth in mice. Since then, a large number of organic tin derivatives have been prepared, tried and tested *in-vivo* and *in-vitro* for different panels of human cancer cell lines [32].

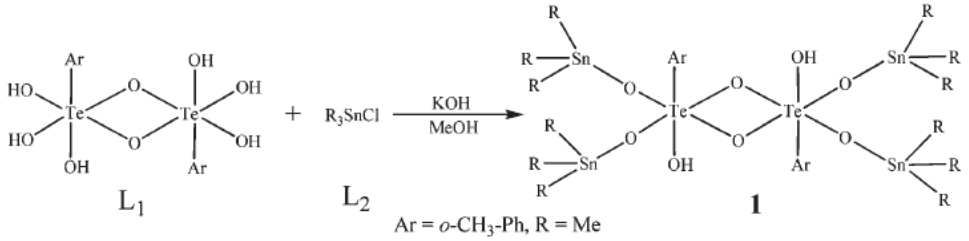
In 2009 Lorenzo Pellerito and László Nagy, make a deep analysis which demonstrates that tin compounds have excellent antitumor activity *in vitro*. There are two compounds which have exceptional antitumor activity, $Ph_3Sn\ (IV)^+$ benzoates exhibit a greater antitumor activity *in-vitro* than cisplatin and comparable to mitomycin C, and Ph_3Sn (IV) esters, they have greater activity *in vitro* against four human tumor cell lines [33]. Also they discovered that a number of different structures of carboxylates $Bu_2Sn\ (IV)^{2+}$ and other derivatives exhibit high antitumor activity *in vitro* and in addition some have low mammalian toxicity and higher activity *in vivo* than Cisplatin compounds [34] .

2.8 Cytotoxicity of tin compounds.

Cellular cytotoxicity is defined as a change in basic cellular functions leading to damage. Different authors have developed batteries *in vitro* to predict the toxic effects of drugs and chemicals compounds, using as experimental models primary cultures and isolated organs with established cell lines [35].

Organic tin compounds are characterized by having a tetravalent structure with at least one C-Sn bond and are classified as mono, di, tri and tetra alkyl tin depending on the number of alkyls. Its toxicity is related to the number and length of alkyl groups attached to tin. Trialkyl tin compounds with short carbon chains are the most toxic, however toxicity decreases by substituting methyl derivative to n-hexyl or n-octyl [36].

In 2013 Chun Li Ma et al, synthesized a new triorganotin ester prepared by reaction of acid o-tolilteluronic and Me_3SnCl in the presence of potassium hydroxide for 12 h to obtain a 72% yield.



Scheme 1. "Synthesis of complex $(Me_3Sn)_4[o\text{-}Me\text{-}PhTe(\mu\text{-}O)(OH)O_2)]_2$ (2)"

To evaluate the cytotoxicity of the complex performed several experiments in A549 cells (alveolar epithelium cells). These cells were treated for 24 h with various concentrations of complex **1**. Cytotoxicity was evaluated by cell viability using MTT assay during exposure to the complex along the exponential growth phase. Viability tests showed that dose complex decreases viability of A549 cells with an IC50 value of 2.4 μM and this response occurred in a time-dependent course, but the ligands caused a slight attenuation viability as shown in figure 7.

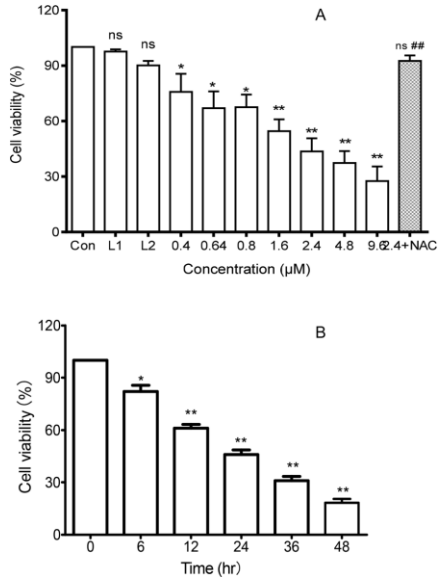


Figure 7. ”Graph shows how complex reduces the viability of A549 cells. (A) Effect of complex concentration expressed as a percentage of MTT values. (B) Effect of the complex with time on the viability of A549 cells with 2.4 μM treatment for 48 h"

The data show that the complex can induce death of A549 cells or stop the proliferation and suggested that the complex may be a promising potential therapeutic agent.

Induction of apoptosis in malignant cells is an important mechanism of action of anticancer drugs. Therefore were analyzed morphological changes that occurred in the cells by fluorescence microscopy. Changes of A549 cells treated with complex **1** also were stained with DAPI. Intact cells showed a homogeneous morphology with cores stained uniformly by DAPI. In the groups treated with different concentrations from 1 to 24 h cells showed a change in morphology with the characteristics of apoptosis, including cell shrinkage which is directly correlated with the concentration of complex[37].

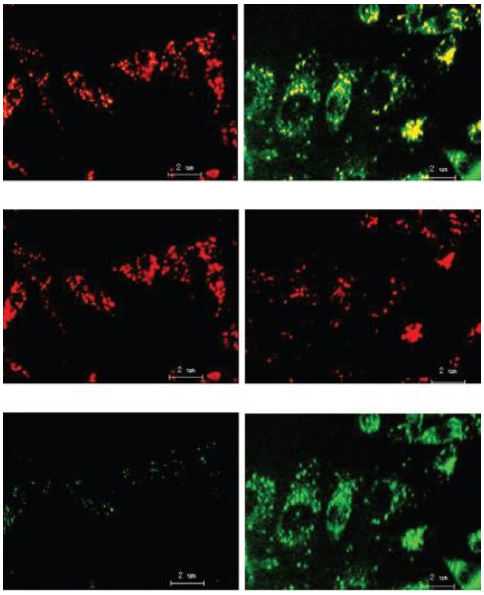
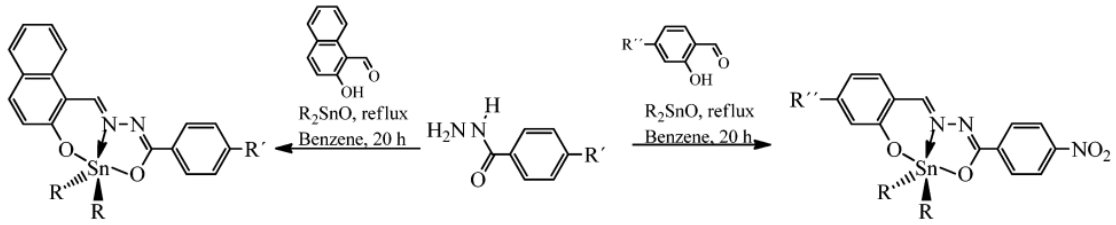


Figure 8. “Images representative fluorescence dual channel green (bottom row) and red (middle row), and the combination (top row) of dye JC-1 in A549 cells (left panel) and A549 cells exposed to 2.4 µM of complex (panel right)"

With this they deduce that the synthesized complex esters showed that tin could be a clinically relevant and potent suppression of growth of A549 cells agent.

2.9 Organotin compounds and their potential application to cellular bioimages.

Recently in our research group it has developed the first study of organotin compounds derivatives of Schiff bases for use as cellular markers. They were synthesized and characterized eight novel compounds derivatives of Schiff base. These are fluorescent in solution at room temperature where the emission peaks are shifted to the blue with respect to the ligand, an also have higher quantum yield. Besides the potential of these compounds was studied as a cytoplasm marker of B16F10 murine melanoma cells and found that the compound **5** is a good candidate for this purpose and also was able to stain cells at low concentrations not showing significant cytotoxicity, on the other hand compound **3** is proposed to be used as a dye of contrast cytoplasmic on fixed cells [43].



1: R = *n*Bu, R´= NO_2
2: R = Ph, R´= NO_2
3: R = *n*Bu, R´= H
4: R = Ph, R´= H

5: R = *n*Bu, R´´ = Et_2N
6: R = Ph, R´´ = Et_2N
7: R = *n*Bu, R´´ = MeO
8: R = Ph, R´´ = MeO

Scheme 2. Synthesis of organometallic tin compounds 1-8.

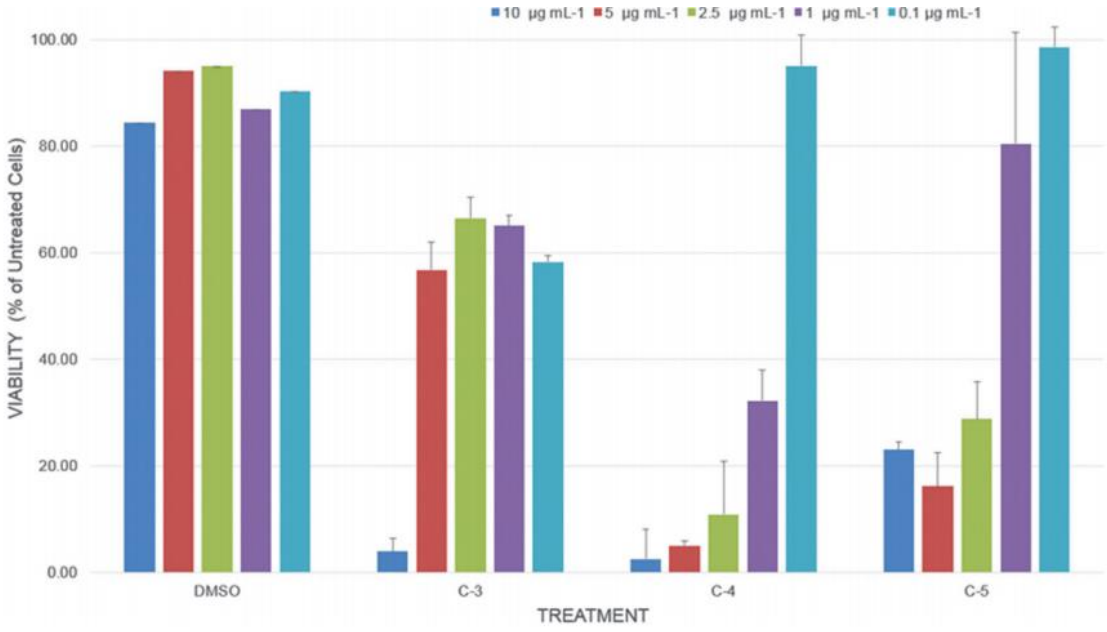


Figure 9. "Cytotoxicity effects of organotin IV compounds 3, 4 y 5"

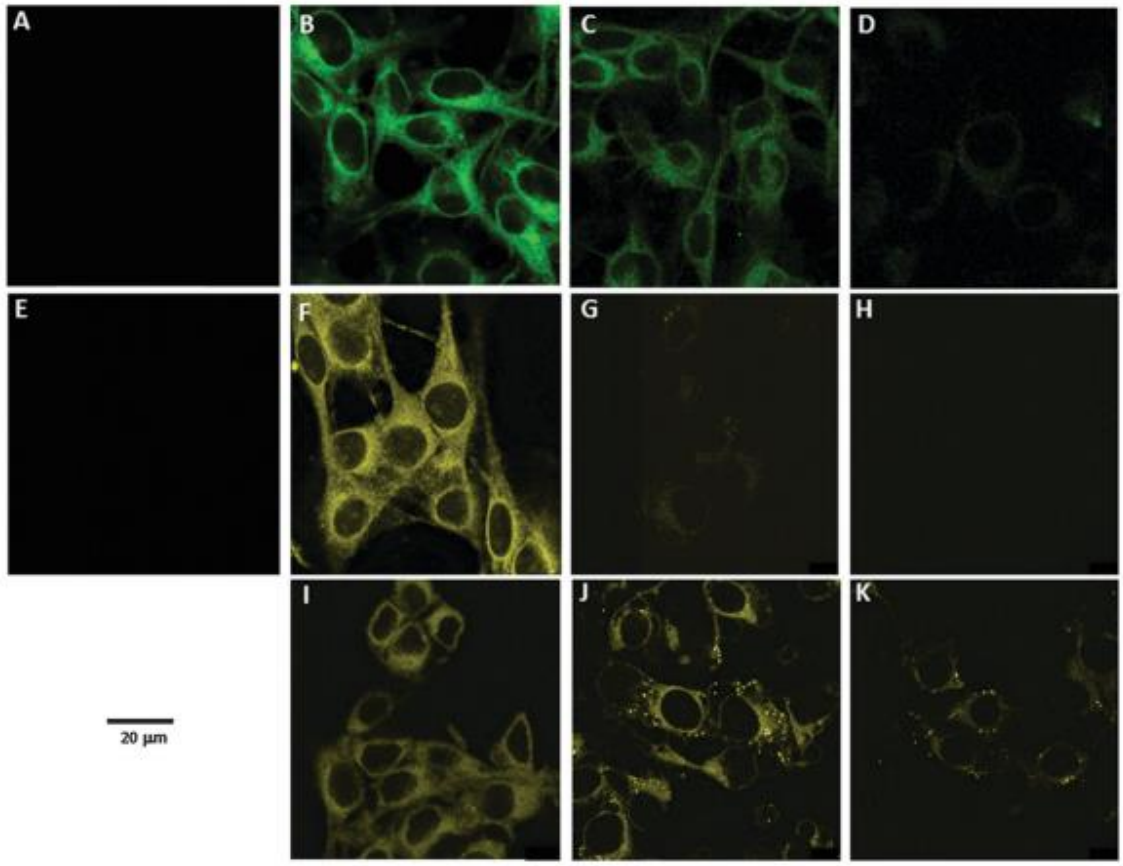


Figure 10. “Staining of cells with organotin IV compounds 3, 4 and 5”

Based on this, we propose a new synthesis of 4 new tin IV compounds derivative from Schiff bases using for the synthesis of ligand hydroxy-naphthaldehyde and hydroxy-benzoylhydrazine. We take advantage of the part of naphthyl as fluorophore to ensure that the complex present luminescence and moreover the OH^- electron donor group benzoylhydrazine serve to increase the electronic dislocation in the complex causing luminescence increases and, on the other hand, we can make an attack with a strong base to form an alkoxide and subsequently perform a nucleophilic attack by adding alkyl chains, which will allow us to modulate the cytotoxicity of our molecules.

3. HYPOTHESIS

The novel compounds of tin IV developed from benzoylhydrazines have luminescent properties and due to their structure allow to have low cytotoxicity adding long-chain alkyl groups, making them potential *in vitro* biomarkers.

4. GENERAL OBJECTIVE

Synthesize two new complexes of tin IV, add alkyl groups attacking the radical (OH), characterize by spectrometric and spectrophotometric techniques, measure their cytotoxicity and apply them in *in-vitro* cells to determine their efficiency as cellular marker.

5. SPECIFIC OBJECTIVES

- Synthesize 8 tin compounds and purify them.
- Characterize the compounds using analytical techniques: UV-VIS, NMR, IR, MS.
- Determine the cytotoxicity of four compounds.
- Apply the compounds to cells *in vitro* to determine their efficiency as a cell biomarker.

6. MATERIALS AND METHODS

6.1 Materials.

All starting materials were purchased from Sigma-Aldrich Chemical Company. Solvents were used without further purification.

Melting points were determined on an Electrothermal Mel-Temp apparatus, and are not corrected. Infrared spectra were recorded using a Burker Tensor 27- FT-IR spectrophotometer equipped with a Pike Miracle TM ATR accessory with a single reflection ZnSe ATR cristal.

Multinuclear magnetic resonance experiments as ^{1}H, ^{13}C, and ^{119}Sn- NMR spectra were recorded on a Bruker advance DPX 400. Chemical shifts (ppm) are relative to (CH3)4Si for ^{1}H and ^{13}C, and ^{119}Sn-NMR spectra were referenced externally to $(CH_3)_4Sn$. Mass spectra was recorded on an AB Sciex API 2000 TM LC/MS/MS System.

Elemental analyses were carried out on a Thermo Finngan Flash EA 1112 elemental microanalyzer. High resolution mass spectra were acquired by source. UV-Vis absorption spectra were measured on a Shimadzu 2401 PC spectrophotometer. The emission spectra have been recorded with a Fluorolog 3 spectrofluorometer, by exciting 10 nm below the longer wavelength absorption band.

6.2 Bioassays.

To perform the in vitro studies we used cell line B16F10 murine melanoma. The culture medium used was DMEM and DMEM/F12, both supplemented with 5% fetal bovine serum and 1x antibiotic-antimycotic, all purchased from GIBCO Invitrogen Life Technologies. Other reactants used were 0.25% TrypsinEDTA (1X), antibiotic-antimycotic (100x), purchased from GIBCO Invitrogen Life Technologies. Alamar blue used for the metabolic viability assay from Biosource Invitrogen Life Technologies.

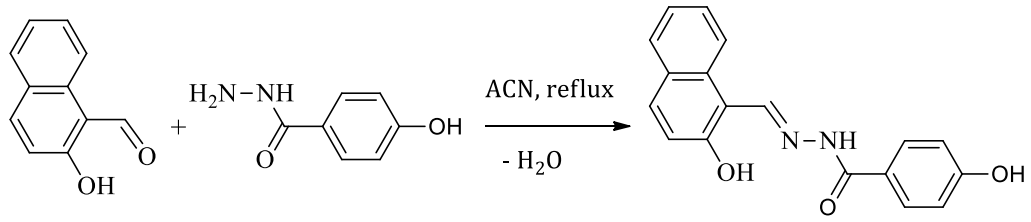
Equipment:

- Incubator for cell culture model 3554 Thermo Electron Corporation.
- Bell laminar flow biosafety Nuaire A-425-400 model.
- ELISA plate reader Labsystems Multiskan model ELx800.
- Confocal microscope TCS SP5 model Leica.

6.3 Methods.

*6.3.1 Synthesis of ligand (compound **1**).*

The ligand was synthesized by a condensation reaction in a reflux system with Dean-Stark trap mixing 0.5gr (0.29mmol) of 2-hydroxy-naphthaldehyde with 0.044gr (0.29mmol) of 4-hydroxybenzyl hydrazine, using as solvent acetonitrile with vigorous stirring and constant reflux for 48 h; obtaining a Schiff base (Scheme 3).

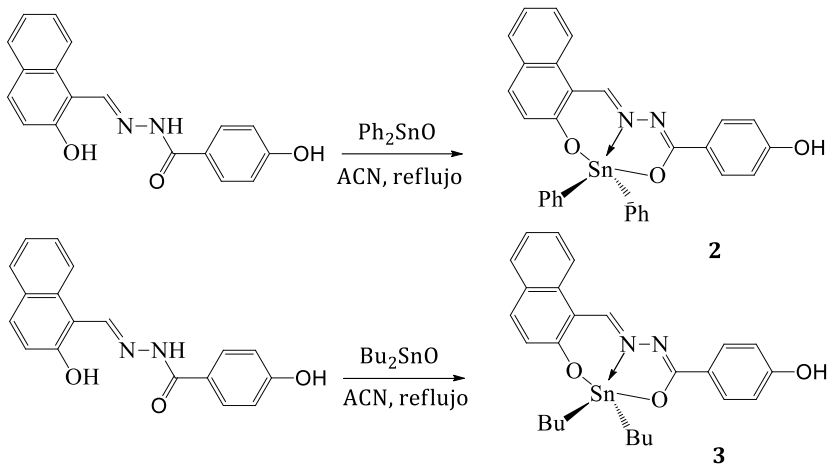


1

Scheme 3. "Synthesis of ligand"

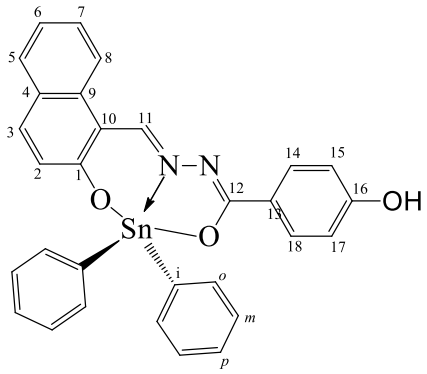
*6.3.2 Synthesis of **2** and **3**.*

Subsequently compounds **2** and **3** were similarly synthesized by a condensation reaction in a reflux system with Dean-Stark trap in equimolar mixture of the respective oxides of tin IV using as solvent acetonitrile and with vigorous stirring is left to constant reflux for 48 h (Scheme 4).



Scheme 4. "Synthesis of 2 and 3"

*6.3.3 4-((1E,3Z)-6,6-diphenylnaphtho[1,2-h][1,3,5,6,2] dioxadiazastannonin-4-yl) phenol (**Compound 2**).*

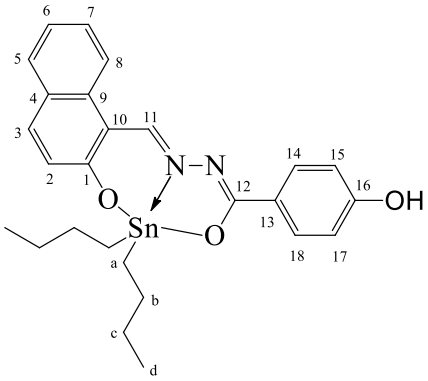


2

A solution of **1** (0.5 g, 1.63 mmol) with diphenyltin (IV) oxide (0.47 g, 1.63 mmol) in acetonitrile (50 ml), after were heated for 48 h in a Dean-Stark tramp for the azeotropic removal of water. The solution was cooled to room temperature and filtered. Each one of the solutions was precipitated (CH_2Cl_2:Hexane, 1:4, *v*/*v*) obtaining a yellow powder (Yield: 85 %, MP: 170-175 °C. ^{1}H NMR (400.13 MHz, $(CD_3)_2CO$, 298 K) δ: 6.9 (d, 2H, ^{3}J=8.8 Hz, H-17, H15), 7.33 (m, 2H, ^{3}J=8 Hz, H-2, H-7), 7.47 (m, 6H, ^{3}J=6.8 Hz, H-*m*, H-*p*), 7.57 (t, 1H, ^{3}J=15.6 Hz, H-6), 7.83 (d, 1H, ^{3}J=7.6 Hz, H-5), 7.92 (m, 4H, ^{3}J= 6.3 Hz, H-*o*), 8.19 (d, 2H, ^{3}J=8.8 Hz, H-14, H-18), 8.27 (d, 1H, ^{3}J=8.4 Hz, H-3), 9.80 (s, 1H, $^{3}J(^{1}H–^{119}Sn)$ = 55.2 Hz, H-11). ^{13}C NMR (100.61 MHz, $(CD_3)_2CO$, 298 K) δ: 107.52 (C-10), 114.45 (C-15, C-17), 119.70 (C-3), 123.38 (C-7), 124.16 (C-13), 125.44 (C-8), 127.60 (C-4), 128.25 (C-6), 128.99 (C-*m*), 129.13 (C-5), 129.28 (C-*p*), 130.60 (C-14, C-18), 133.76 (C-9), 135.94 (C-*o*), 136.90 (C-2), 139.03 (C-*i*), 156.64 (C-16), 161.79 (C-11), 167.69 (C-12), 168.98 (C-1). ^{119}Sn NMR (128 MHz, $CDCl_3$,298 K) δ: -329.50. FTIR ν máx

cm^{-1}: 3090, 1620 (C=N), 755. Emission $\lambda_{máx}$ (nm) = 537 (CD_3CN, $1X10^{-3}$ M). HRMS calculated: 578.065; found: 579.072, error: 0.2097 ppm.

*6.3.4 4-((1E,3Z)-6,6-dibutylnaphtho[1,2-h][1,3,5,6,2]dioxadiazastannonin-4-yl)phenol (**Compound 3**).*

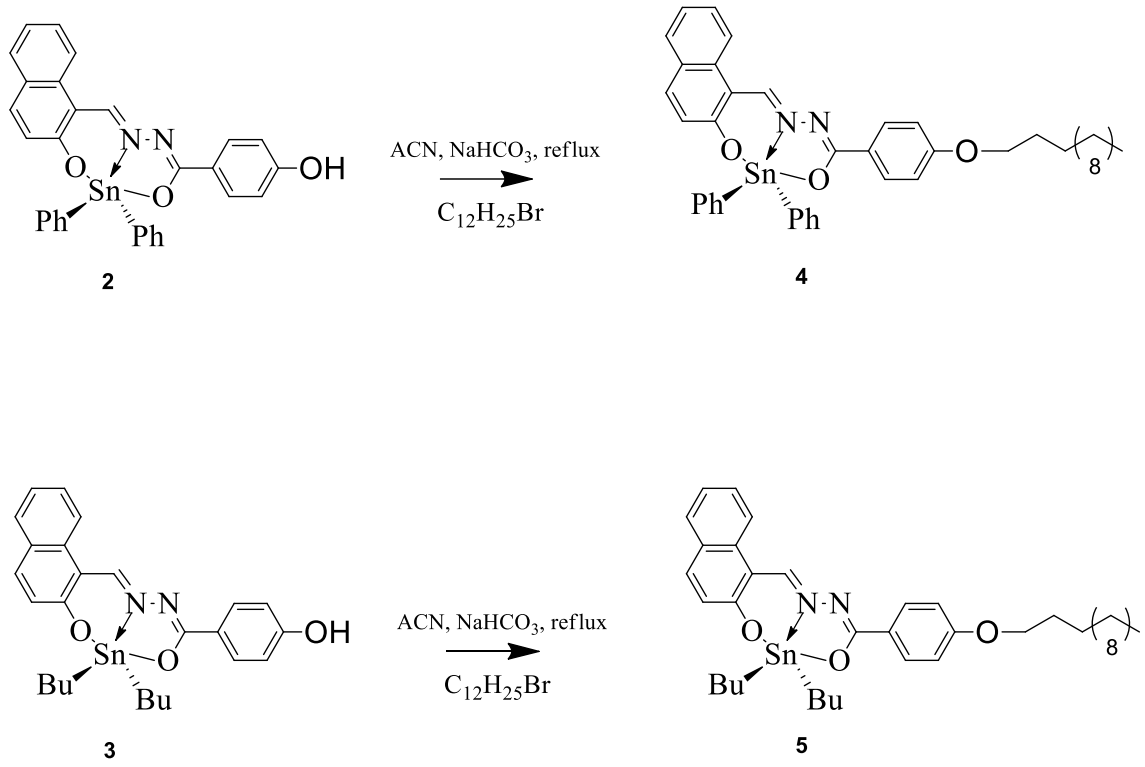


3

A solution of **1** (0.5 g, 1.63 mmol) and dibutyltin (IV) oxide (0.40 g, 1.63 mmol) in acetonitrile (50 ml) were heated for 48 h in a Dean-Stark tramp for the azeotropic removal of water. The solution was cooled to room temperature and filtered. Each one of the solutions was precipitated (CH_2Cl_2:Hexane, 1:4, *v*/*v*) obtaining an orange powder (Yield: 82 %, MP: 185 °C). ^1H NMR (400.13 MHz, $(CD_3)_2CO$, 298K) δ: 0.848 (t, 6H, ^3J=14.84 Hz, CH_3-d), 1.381 (m, 4H, ^3J=32.8 Hz, CH_2-c), 1.55 (t, 4H, 3^J=4.4 Hz, CH_2-a), 1.69 (m, 4H, ^3J=31.2 Hz, CH_2-b), 6.92 (d, 6H, ^3J=8.8 Hz, H-17, H15), 6.97 (d, 1H, ^3J=8.8 Hz, H-2), 7.33 (t, 1H, ^3J=14.8 Hz, H-7), 7.56 (t, 1H, ^3J=15.2 Hz, H-6), 7.78 (d, 1H, ^3J=8 Hz, H-5), 7.86 (d, 1H, ^3J=9.2 Hz, H-8), 8.01 (d, 2H, ^3J=8.8Hz, H-14, H-18), 8.23 (d, 1H, ^3J=8.8 Hz, H-3), 8.81 (s, 1H, ^3J= 3524 Hz, H-OH), 9.72 (s, 1H, $^3J(^1H–^{119}Sn)$ = 48.6 Hz, H-11). ^{13}C NMR (100.61 MHz, $(CD_3)_2CO$, 298 K) δ: 12.958 (C-d), 21.39 (C-b), 26.09 (C-a), 26.77 (C-c), 107.01 (C-10),114.86 (C15, C-17), 119.27 (C-3), 122.83 (C-13), 124.00 (C-7), 125.13 (C-2), 127.37 (C-4), 128.06 (C-6), 129.16 (C-5), 129.36 (C-14, C-18), 133.93 (C-9), 136.05 (C-8), 155.78 (C-11), 159.84 (C-12), 168.16 (C-16), 169.05 (C-1).^{119}Sn NMR (128 MHz, $CDCl_3$, 298 K) δ: -190.31. FTIR ν máx cm^{-1}: 3450 (C-OH), 2982, 1525 (C=N), 755. Emission $\lambda_{máx}$ (nm) = 537 (CD_3CN, $1X10^{-3}$ M). HRMS calculated: 538.128; found: 539.136, error: 0.1864 ppm.

*6.3.5 Synthesis of **4** and **5**.*

After obtaining the coordinated tin IV compounds we proceed to the synthesis of compounds **4** and **5** in equimolar mixture in refluxing acetonitrile for 48 hours by performing a nucleophilic attack using $NaHCO_3$ causing deprotonation of group OH- generating an alkoxide to be attacked by a nucleophile alkylated and attaching an alkyl chain of 12 carbons. (Scheme 5).

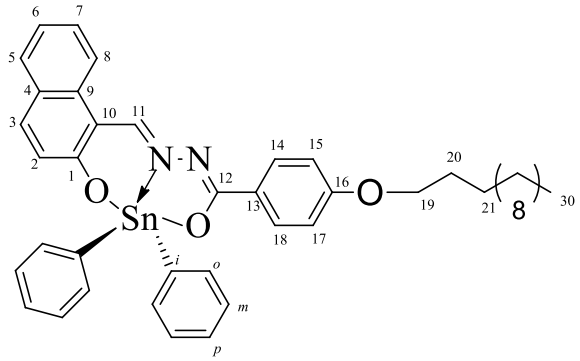


2 4

3 5

Scheme 5. "Synthesis of 4 and 5"

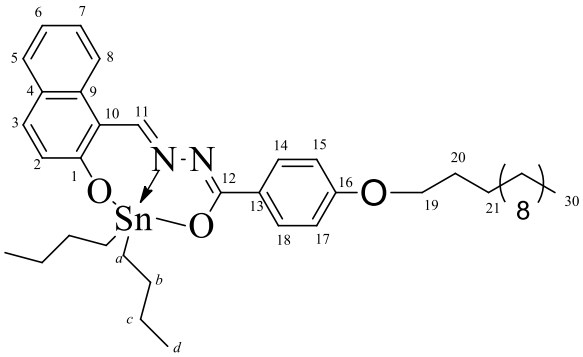
*6.3.6 (1E,3Z)-4-(4-(dodecyloxy)phenyl)-6,6-diphenylnaphtho[1,2h] [1,3,5,6,2] dioxadiazastannonine (**Compound 4**).*



Compound **2** (0.1 g, 0.17 mmol) are mixed in a flask with 1-bromododecane (0.043 g, 0.17 mmol) and 10 equivalents of $NaHCO_3$ (0.14 g, 1.7 mmol) in acetonitrile (50 ml), then heated to reflux for 48 h. The solution is cooled to room temperature and then filtered to remove excess salt. Subsequently it puts in vacuum to remove the solvent to give oil. ^{1}H NMR (400.13 MHz, $(CD_3)_2CO$, 298K) δ: 0.806 (t, 1H, J=14.00 Hz, H-30), 1.185 (m, H-22, H-23, H-24, H-25, H-26, H-27, H-28, H-29), 1.30 (m, 2H, H-21), 1.73 (m, 2H, J=28.40 Hz, H-20), 3.10 (t, 2H, J=14.00 Hz, H-19), 6.91 (d, 2H, J=8.80 Hz, H-15, H-17), 7.18 (d, 1H, J=8.80 Hz, H-2), 7.23 (t, 1H, J=19.80 Hz, H-6), 7.32 (m, 4H, H-*m*), 7.33 (m, 1H, H-*p*), 7.42 (t, 1H, J=16.40 Hz, H-7), 7.63 (d, 1H, J=16.40 Hz, H-5), 7.76 (d, 1H, J=9.20 Hz, H-3), 7.80 (m, 4H, J=83.62 Hz, H-*o*), 7.98 (d, 1H, J=8.80 Hz, H-8), 8.13 (d, 2H, J=8.80 Hz, H-14, H-18), 9.62 (s, 1H, $^{3}J(^{1}H–^{119}Sn)$ = 55.61 Hz, H-11). ^{13}C NMR (100.61 MHz, $(CD_3)_2CO$, 298 K) δ: 6.20 (C-19), 13.09 (C-30), 21.66 (C-28), 27.53 (C-29), 28.32 (C-27), 28.40 (C-26), 28.53 (C-25), 28.60 (C-24, C-23), 29.49 (C-21), 30.89 (C-22), 32.56 (C-20), 106.45 (C-10), 113.23 (C-15, C-17), 118.23 (C-3), 122.19 (C-7), 123.36 (C-13), 124.41 (C-8), 126.39 (C-4), 127.03 (C-6), 127.81 (C-*m*), 128.12 (C-5), 128.33 (C-*p*), 129.40 (C-14, C-18), 132.67 (C-9), 135.10 (C-o, J=54.12 Hz), 135.63 (C-2), 138.06 (C-*i*), 155.19 (C-11), 160.70 (C-16), 167.08 (C-12), 168.12 (C-1).

*6.3.7 (1E,3Z)-6,6-dibutyl-4-(4-(dodecyloxy)phenyl)naphtho[1,2 h][1,3,5,6,2] dioxadiazastannonine (**Compound 5**).*

Compound **2** (0.04 g, 0.074 mmol) are mixed in a flask with 1-bromododecane (0.018 g, 0.074 mmol) and 10 equivalents of $NaHCO_3$ (0.62 g, 0.74 mmol) in acetonitrile (50 ml), then heated to reflux for 48 h. The solution is cooled to room temperature and then



filtered to remove excess salt. Subsequently it puts in vacuum to remove the solvent to give oil. ^{1}H NMR (400.13 MHz, $(CD_3)_2CO$, 298K) δ: 0.88 (m, 9H, H-d, H-30), 1.26 (m, 21H, H-22, H-23, H-24, H-25, H-26, H-27, H-28, H-29, H-c), 1.53 (t, 4H, J= 16.45 Hz, H-a), 1.67 (m, 4H, J=26.00 Hz, H-b), 3.18 (t, 2H, J=14.00 Hz, H-19), 6.92 (d, 2H, J=5.60 Hz, H-15, H-17), 6.95 (d, 1H, J=5.50 Hz, H-2), 7.29 (t, 1H, J=14.80 Hz, H-7), 7.57 (t, 1H, J=16.00 Hz, H-6), 7.68 (d, 1H, J=6.80 Hz, H-5), 7.74 (d, 1H, J=9.20 Hz, H-8), 8.04 (d, 2H, J=8.80 Hz, H-14, H-18), 8.07 (d, 1H, J=8.80 Hz, H-3),9.67 (s, 1H, $^{3}J(^{1}H–^{119}Sn)$ = 38.01 Hz, H-11). ^{13}C NMR (100.61 MHz, $(CD_3)_2CO$, 298 K) δ: 7.29 (C-19), 10.97 (C-d), 13.58 (C-b), 14.12 (C-a, C-c, C-28), 22.69 (C-28), 28.56 (C-29), 29.35 (C-27), 29.43 (C-26), 29.56 (C-25), 29.63 (C-23, C-24), 30.52 (C-21), 31.92 (C-22), 33.59 (C-20), 107.14 (C-10), 114.12 (C-6), 119.16 (C-3), 122.86 (C-13), 124.36 (C-7),125.73 (C-2), 127.15 (C-4), 127.88 (C-6), 128.80 (C-15, C-17), 128.18 (C-5), 130.87 (C-14, C-18), 132.46 (C-8), 133.80 (C-9), 156.02 (C-11), 161.47 (C-12), 168.43 (C-16), 168.98 (C-1).

6.4 Microwave.

Microwave energy has provided an efficient method for the synthesis of various materials. Significantly improves the uniformity of product properties processed and facilitate obtaining microstructures and properties that sometimes are impossible to reach by other ways. This also allows significant reductions in manufacturing costs resulting from significant energy savings and shorter processing times.

Therefore the synthesis of compounds **1** (ligand), **2** and **3** under these conditions was also developed (compounds **4** and **5** not were success to do), using the following procedure:

For **1** was mixed in a G10 vial 0.04 gr of hydroxynaphtaldehyde and 0.05 gr hidroxybenzoilhidrazine in 5 ml of acetonitrile at 180 °C for 30 min, then filtered and precipitated with a hexane solution and dichloromethane 1: 1. The precipitate is filtered with conventional filter paper and dried in high vacuum to give a yellow powder with a yield of 90 %.

Synthesis of **2** was mixed in a G10 vial 0.04 gr of hydroxynaphthaldehyde, 0.05 g of 0.08 g hydroxybenzoylhydrazine and diphenyl tin oxide in 5 ml of acetonitrile at 170 °C for 25 min, then filtered and precipitated with a solution of hexane and dichloromethane 1: 1. The precipitate is filtered with conventional filter paper and dried in high vacuum to give a canary yellow powder with a yield of 93%.

Synthesis of **3** was mixed in a G10 vial 0.04 g of hydroxynaphthaldehyde, and 0.072 g hydroxybenzoylhydrazine 0.05 g of dibutyl tin oxide in 5 ml of acetonitrile at 170 °C for 30 min, then filtered and precipitated with a solution of hexane and dichloromethane 1:1. The precipitate is filtered with conventional filter paper and dried in high vacuum to give an orange powder with a yield of 88 %.

Yields with this methodology have resulted higher than traditional method, so that was carried out an analysis of advantages and disadvantages, methodology microwave is best, however, in this case was chosen to synthesize by the traditional method, due we required a large amount of our tin compounds for coupling with alkyl chains.

COMPOUND	M.W	CHARACTERISTICS	M.P	WORK TEMPERATURE	YIELD
L (1)	306.315 g/mol	Light yellow powder	390°C	170°C	90%
C-PH (2)	577.217 g/mol	Yellow powder	400°C	170°C	93%
C-BU (3)	537.23 g/mol	Orange powder	185°C	185°C	87.93%

Table 1. Conditions for microwave synthesis compounds **1**, **2** and **3**

6.5 Bioassays.

6.5.1 Cell culture.

B16F10 cell line was cultured in 25 cm^2 flasks, DMEM / F12 was used as culture medium supplemented with 5 % fetal bovine serum (FBS). Cell passage was performed twice a week and the culture was maintained at 37 °C in an atmosphere of 5 % CO_2 and 95 % humidity. For the manipulation of cells and culture medium the trypsin was heated at 37 ºC. When the culture plates reach a confluence of 80 % or more, is necessary to pass the cells to have enough cells to work. For the first pass of cells, the culture medium was removed from the bottle and subsequently 1 ml trypsin was added, ensuring that the trypsin completely covers the floor of the plate, after 5 minutes, when the cells are detached, the trypsin was inactivated with 3 ml of culture medium and carefully shaken with a pipette several times to make sure there are no clumps of cells in the culture. After 8 ml of medium was added in an empty flask properly labeled and subsequently added 3-5 drops of cell culture (depending on the junction) and stirred gently before narrow plate, and allowed to incubate at 37 ºC. The remaining cell culture is counting, for later use in the bioassays.

6.5.2 Cytotoxicity.

The method of Alamar Blue is a technique that is designed to quantitatively measure the proliferation of human and animal cells, bacteria and fungi. The bioassay can be used to establish the relative cytotoxicity of various chemical agents. This technique uses the advantage of the reducing environment within healthy cells to reduce resarzurine (blue reagent) to resorufin (red reactive) (Figure 11). The color change is directly proportional to the number of living cells. Can thus relate the change in the color of the cell culture with the viability and cytotoxicity.

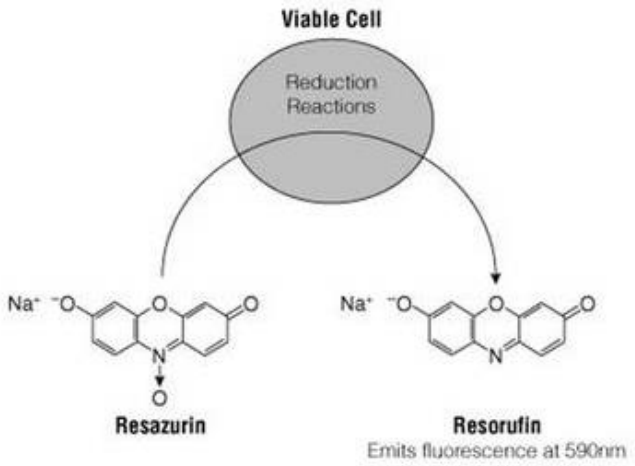


Figure 11"Conversion of the non-fluorescent dye resazurin to the fluorescent dye resorufin"

B16F10 murine melanoma cells were seeded in 96 wells plates at a density of $1x10^4$ per well in 100 ml of media and incubated for 24 hours at 37 °C. Media was exchanged and compounds were added at concentrations ranging from 0.01 µg/ml to 10 µg/ml. Cells treated with dimethyl sulfoxide (DMSO, Sigma-Aldrich Co, St. Louis, MO) were used as controls. Twenty four hours later 10 µl of alamarBlue (Biosource Invitrogen Life Technologies, Carlsbad, CA) were added to each well. Viability of the cultures was measured as reagent colour change by spectrophotometry on an ELISA Microplate Reader (Biotek Multiskan ELX800, BioTek Instruments, Inc, Winooski, VT) at dual wavelength of 570 and 600 nm. The data obtained were then analyzed by computer.

6.5.3 Cell imaging.

B16F10 murine melanoma cells were seeded in 6 wells plates on sterile coverslips at a density of $2x10^5$ cells/well in 2 ml of DMEM/F12 media and maintained at 37 °C in a controlled humid atmosphere of 5 % CO_2 and 95 % air. After that, 24 h later media was exchanged and cells were exposed to the compounds at concentration of 10 µg/ml, for two hours. Cells treated with DMSO were used as controls. Supernatants were removed and coverslips were washed once with 2 ml of PBS, mounted on microscope slips using glycerol 75% and imaged using confocal laser microscopy (Leica TCS SP5 Confocal System). Samples were excited at 405 and 458 nm and the fluorescence emission was measured at 478-612 nm.

Each experiment was performed in duplicate and repeated 3 times and stains at concentrations of 10 µg/ml and 5 µg/ml. Each compound was excited to a specific wavelength and reading the emission wavelengths according to the absorption and emission values of each compound (Table 2).

Compound	***Excitation***	***Emission***
L-1	*436 nm*	*524 nm*
C-Ph (2)	*459 nm*	*524 nm*
C-Bu (3)	*462 nm*	*525 nm*
Ph-CH (4)	*461 nm*	*523 nm*
Bu-CH (5)	*461 nm*	*524 nm*

Table 2. "Parameters used for bioimaging for compounds **1**, **2, 3, 4** and **5**"

6.6 Disposal of hazardous waste.

The organic waste generated in the reactions like benzene, as well as for the work up: hexane, acetone, ethyl acetate were collected in the container C, which is assigned for halogen free-solvents. Waste of solvents like chloroform and dichloromethane were collected in container D, which is assigned for halogen containing solvents. Biological waste was disposed in the appropriate containers.

6.7 Results and discussion.

6.7.1 Synthesis.

We carry out the synthesis of Schiff bases 1 - 3 by condensation reaction of the napthaldehyde with the corresponding hydroxybenzohydrazide in benzene for 48 h. The Schiff bases were isolated with good yields (75-85 %). Also we made the coupling of compounds **2** and **3** with 1-bromododecane for the addition of alkyl chains. We obtained compounds **4** and **5** with good yields (68-78 %). All compounds were soluble in common organic solvents such as dichloromethane, chloroform and acetone (Table 3) and this is a good parameter to know for further analysis.

Solvent	L-1	C-Ph (2)	C-Bu (3)	Ph-CH (4)	Bu-CH (5)
Acetone	O	O	O	O	O
Methanol	X	O	O	ЭЌ	O
Chloroform	ЭЌ	O	O	O	O
Benzene	ЭЌ	O	O	O	O
Dichloromethane	ЭЌ	O	O	O	O
Toluene	X	O	O	O	O
Acetonitrile	O	O	O	O	O

Table 3. “Table of solubility **1**, **2, 3, 4** and **5,** where **O** is soluble, **ЭЌ** partially soluble and **X** insoluble”

6.7.2 Chemical structure elucidation.

6.7.3 UV Analysis.

The UV-Vis spectra of the compounds and its ligand were obtained in chloroform (Figure 12).

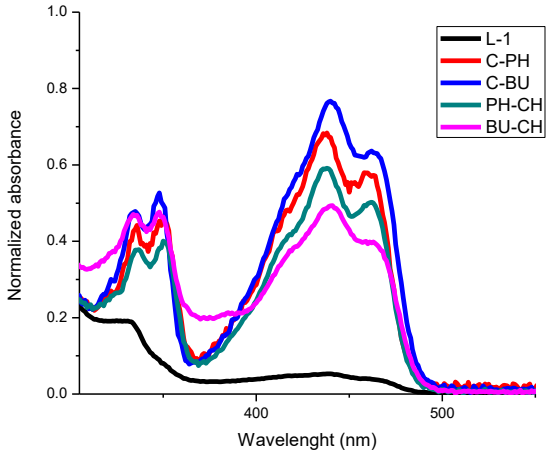


Figure 12"Absorption spectrum of compounds 1-5 in chloroform"

We can observe that the ligand present a maximum absorption peak at 436 nm, but when the ligand is coordinated with its respective tin oxide the bands are shifted to red window (459-561 nm) due to the larger electronic delocalization. The same phenomenon happens for the alkylated compounds and all the coordinated compounds shows relatively stronger absorbance than the ligand. As we can see, there is a uniform shift of the maximum absorption peaks for compounds **2**, **3**, **4** and **5** due to the presence of the naphthyl group in the molecules that is acting like fluorophore. The emission spectrum, showed wavelengths for all compounds including ligand between 520-525 nm (Figure 13), being all compounds red-shifted, attributed to the presence of naphthalene group. Also we can see that the intensity of compounds **4** and **5** decrease respect to their analogous compound, this little decrease is attributed to the alkyl chains attached to the molecule; however this decrease doesn't affect considerably their quantum yield. The data are represented in Table 4.

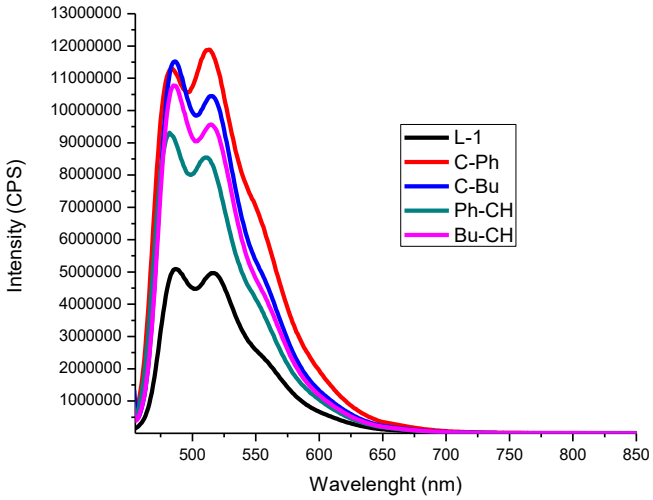


Figure 13"Emission spectra of compounds 1-5 in chloroform"

Compound	Máx. Absortion	Emission	Φ
L-1 (1)	**436 nm**	**524 nm**	**-**
C-Ph (2)	**459 nm**	**525 nm**	**33.7**
C-Bu (3)	**462 nm**	**525 nm**	**33.0**
Ph-CH (4)	**461 nm**	**523 nm**	**24.2**
Bu-CH (5)	**461 nm**	**524 nm**	**25.7**

Table 4. "Absortion and emission data for compounds **1**, **2, 3, 4**, **5**, and their respective quantum yield"

6.7.4 Analysis of NMR data.

^{1}H NMR spectra confirmed the formation of the Schiff bases 1-5 (see appendix), with signals for H-11 (all compounds) in the range of 8.67 to 9.62 ppm, typical of an imine proton, which are according to reported by Ariadna Garza et. al [44]. Also we can see in all de proton NMR spectra satellite signals for this protons ($^{n}J^{1}$H-^{119}Sn: 48-56 Hz) due coupling with ^{119}Sn and its isotopic abundance, which are according to reported by

Smith for organotin compounds [45]. For compounds **2** and **4** the coupling of the *ortho* protons due to the proximity of tin is appreciated. (Table 5).

In the ^{13}C NMR spectra of tin compounds, the principal signals correspond to C-11 (C=N) between 155.11 to 161.79 ppm, C-1 (C-O) with shifts between 168.12 to 169.05 ppm and C-12 (N=C-O) that appear between 159.84 to 167.69 ppm. The existence of the N→Sn coordination bond was evidenced by ^{119}Sn NMR spectra. Can be observed values for the chemical shifts that confirm the compounds are pentacoordinated. Five- and six-coordinate organotin compounds show ^{119}Sn signals, which occur at much lower frequencies than those of the four-coordinate derivatives. Has been reported that signals between -204 to -395 ppm are indicative of pentacoordinated tin atoms, which is consistent with the chemical shifts obtained. (Table 5).

	^{1}H	^{13}C				
Compound	**H-11**	**C-*ortho***	**C-11 (C=N)**	**C-1 (C-O)**	**C-12 (N=C-O)**	**^{119}Sn**
2	9.8	135.94	161.79	168.98	167.69	-329.5
3	9.72	-	155.78	169.05	159.84	-190.31
4	9.62	135.1	155.19	168.12	167.08	-325.15
5	9.67	-	156.02	168.98	161.47	-188.72

Table 5. "Principals signals 1H (nJ1H-119Sn), 13C [nJ13C-119Sn], and 119Sn (ppm)"

6.7.5 Analysis of IR data.

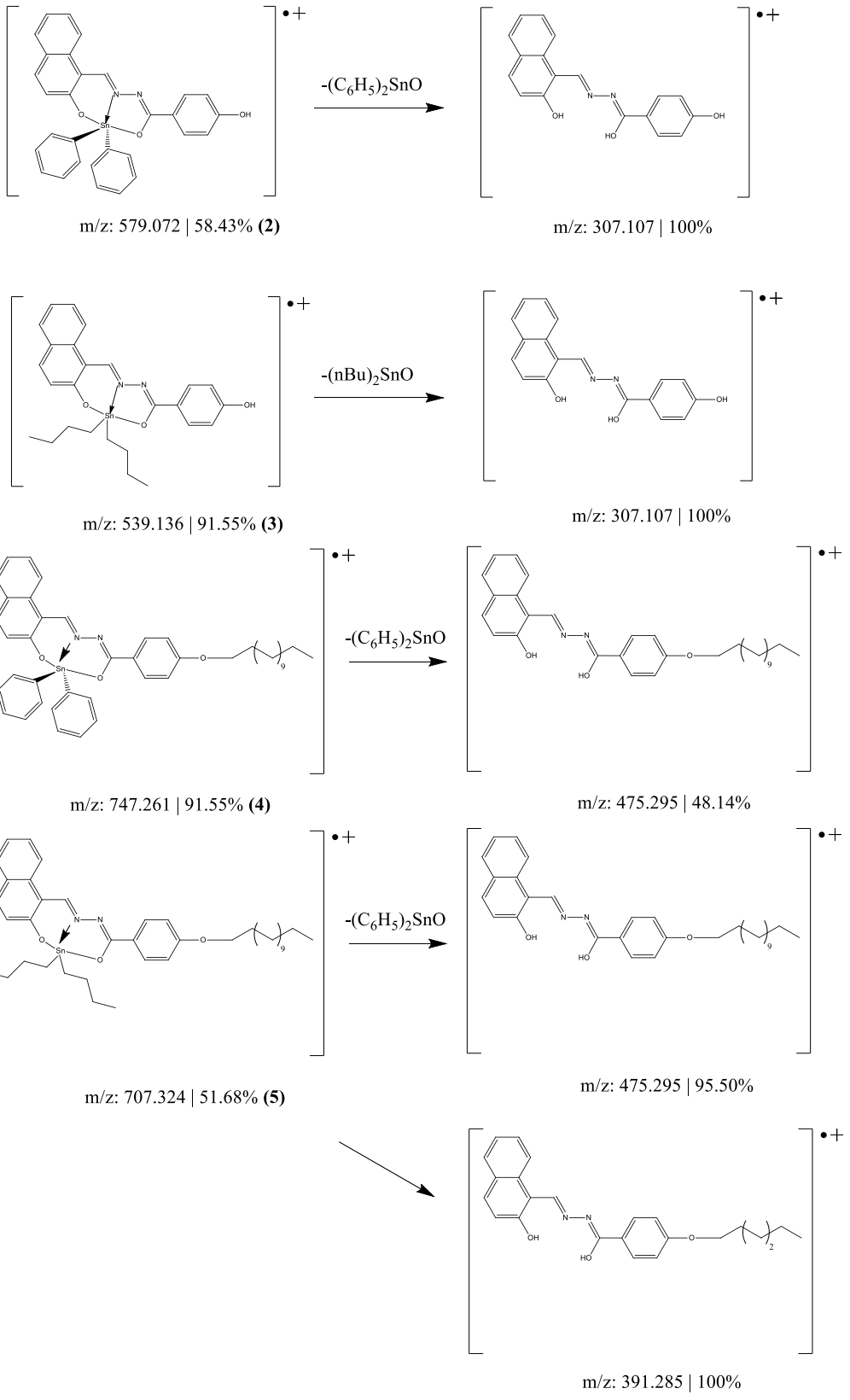
The IR spectral analysis showed that the C=N stretching vibration bands for compounds **2** and **3** (1605-1525 cm-1) were shifted to lower wavenumbers in comparison with the ligand (1620 cm-1), demonstrating a decrease in strength as the new coordination bond with tin is formed. Main bands are shown in Table 6. (It was not possible to develop IR for compounds **4** and **5** because they were oils).

	Compound	
	1	2
C-H arom	3090 cm^{-1}	3090 cm^{-1}
C-H sp3 stre.	2990 cm^{-1}	2990 cm^{-1}
C=N	1605 cm^{-1}	1525 cm^{-1}
C-H sp3 bend.	1400 cm^{-1}	1400 cm^{-1}
C-H arom. IP	1200 cm^{-1}	1200 cm^{-1}
C-H arom. OP	770 cm-1	790 cm-1

Table 6. "Main IR bands of **1** and **2**"

6.7.6 Mass spectra. Molecular ion and fragmentation.

The mass spectra of the four molecules, was obtained by TOF method. The tin atom presents several isotopes, because of this many molecular ions are observed in the mass spectra, thus the most intense peaks correspond to a mixture of most abundant isotopes. All the compounds shows in the spectra one peak that correspond to the molecular ion and for compounds **2**, **3**, **4** and **5** showed a base peak after loss of the di-n-butyltin and di- phenyltin and correspond to the ligand for **2** and **3** and ligand plus alkyl chain for **4** and **5**. (Scheme 6). The presence of the base peak of each compound is consistent with the theoretical molecular mass that confirm de formation of the tin compounds.



Scheme 6“Proposed fragmentation of tin compounds”

The recorded high-resolution mass spectra of 2–5 and molecular ion peaks are in agreement with the proposed structures. The MS peaks present the correct isotopic distribution, as expected for the tin isotope distribution. (Figure 17).

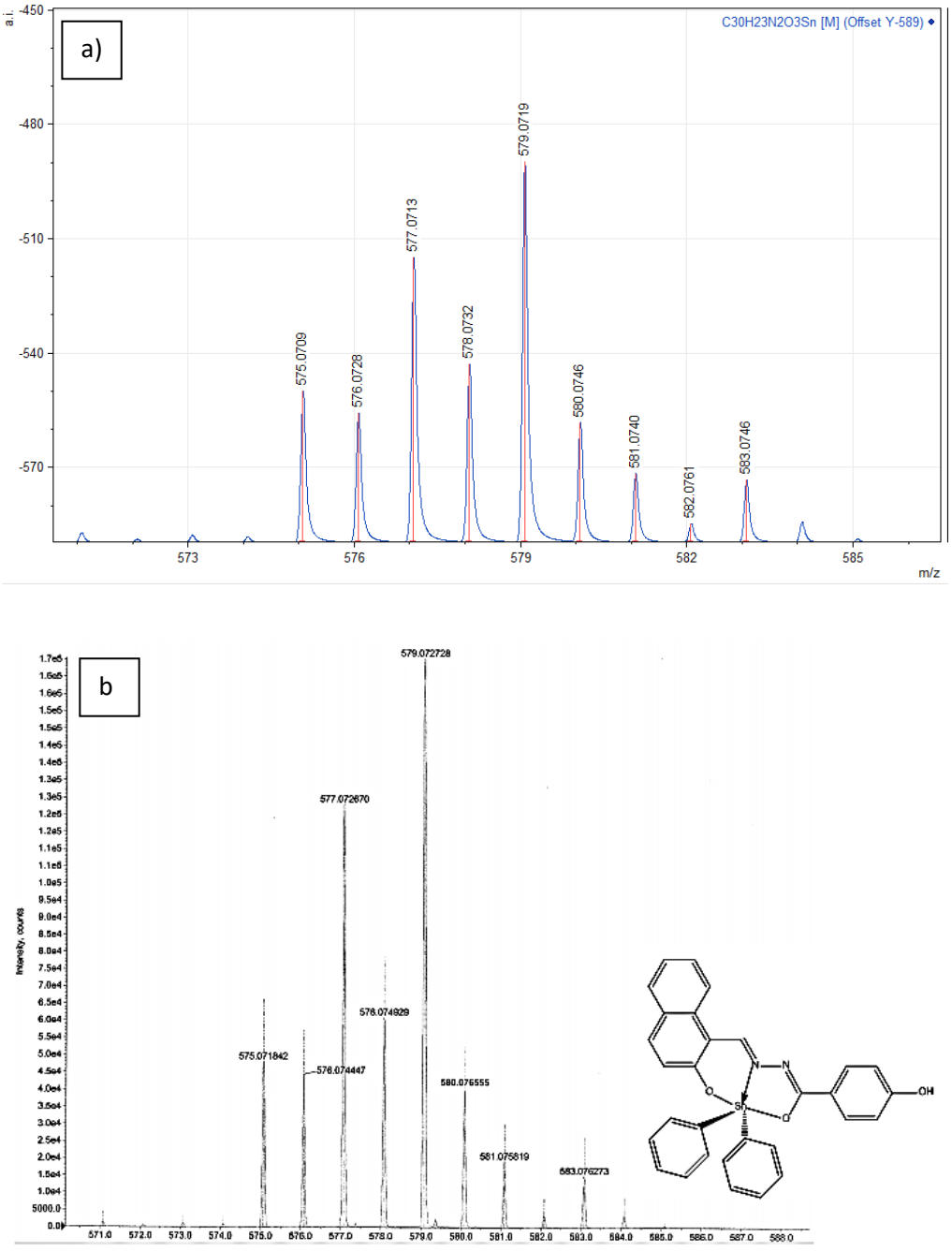


Figure 14“Observed mass spectrum (b) and simulated spectrum (a) of compound 2”

6.7.7 Cytotoxicity assays.

Organotin compounds were used on tissue culture in order to assess their cytotoxic effects. B16F10 murine melanoma cells were treated with six different concentrations of the compounds (20, 10, 5, 2.5, 1 and 0.1 µg/ml) for 24 h and then viability was determined by Alamar Blue. As DMSO was the solvent used for the compounds, its toxicity was analyzed as well (Figure 18). The viability of the cells was greatly affected by the higher concentrations tested, being compounds **2** and **3** the more toxic (showing less than 8% of viability in concentration of 20 µg/ml), and also compound **3** presents considerable toxicity, while compound **4** and **5** present less toxicity at the same concentration. However, when lower concentrations of compounds were used, a dose-response effect tendency was observed for **2**, **4** and **5** compounds.

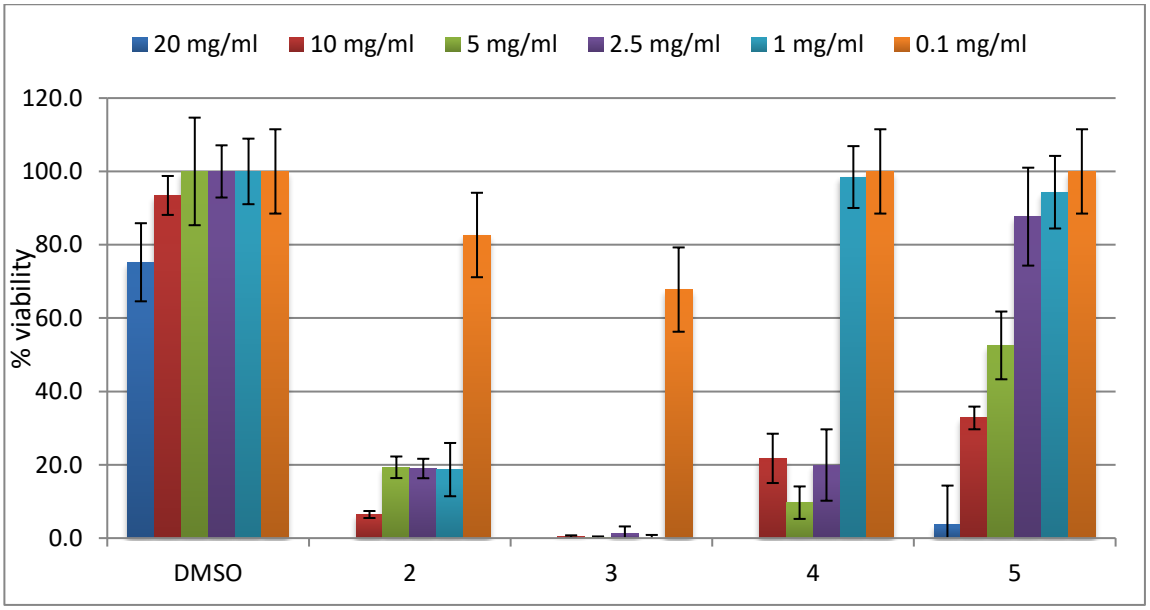


Figure 15. "Cytotoxic effect of organotin compounds. Melanoma cells B16F10 treated with 20 µg/ml (blue bars), 10 µg/ml (red bars), 5 µg/ml (green bars), 2.5 µg/ml (purple bars), 1 µg/ml (light blue) and 0.1 µg/ml (orange bars) of each compound for 24 h"

Clearly we can observe that the addition of the alkyl chains in the molecular structure decreases cytotoxicity corroborating the hypothesis and therefore increase the viability considerably. As we can see in the concentration of 10 µg/ml of **3** and its analog **5** the

viability increases from 2 % to 32.8 %. That means not direct addition of alkyl chains to tin helps to reduce the cytotoxicity too. Comparing with the previous research in the group.

6.7.8 Bioimages.

To evaluate the behavior of the luminescent organotin compounds in cells, confocal fluorescence microscopy measurement was carried out. Melanoma cells B16F10 were seeded at 2 x 10^5 cells/2 ml media per well in 6 wells plates on sterile coverslips, 24 h later media was removed and fresh media and the compounds at 10 and 5 µg/ml were added. Two hours later media and compounds were removed and cells were washed twice with PBS. Coverslips were recovered and mounted on microscope slides using 75 % glycerol. Cells were analyzed by confocal microscopy in a Leica SP5 system using a 458 nm laser to excite the luminescence while emission was registered between 478 and 612 nm. These experiments were performed three times in duplicate.

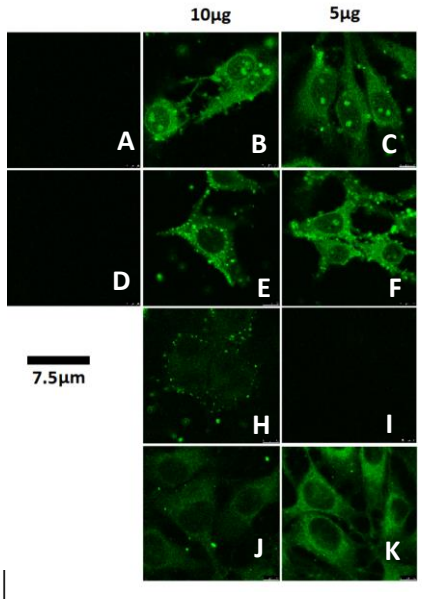


Figure 16. “Staining of cells with organotin compounds. Confocal microscopy of melanoma cells B16F10 treated with 10 and 5 µg/ml of each compound for 2 h. A, Untreated cells; D, DMSO control; B-C, Compound 2; E-F, Compound 3; G-H, Compound 4; I-J, Compound 5. (Scale bar shown represents 7.5 µm).

Cells exposed to compound **2** and **3** showed a strong green cytoplasmic staining with focalized vesicle-like structures showing stronger staining (Figure 19 B-C, E-F) while compounds **4** and **5** produced weak cytoplasmic staining (Figure 19 G-H, I-J). It is important to notice that compound **2** and **3** is able to stain nucleoli showing a characteristic pattern of two points in the nucleus (Figure 20). We attribute this entrance to the electrodonor group OH^- of hydroxybenzohidrazine because in the case of compounds **4** and **5** it doesn't happen. It could be due alkyl chains attached to the molecule; these chains don't allow the total entrance of the compounds which is reflected in the intensity of bioimages. Is worth mentioning that compound **4** and **5** presented photobleaching, but it was most notorious in bioimaging of compound **4** due it doesn't allow us to make good bioimaging because it presented beaching until 10 seconds after being subjected to radiation. On the other hand, compound **5** presented bleaching until 2 minutes approximately allowing us to make a good bioimaging.

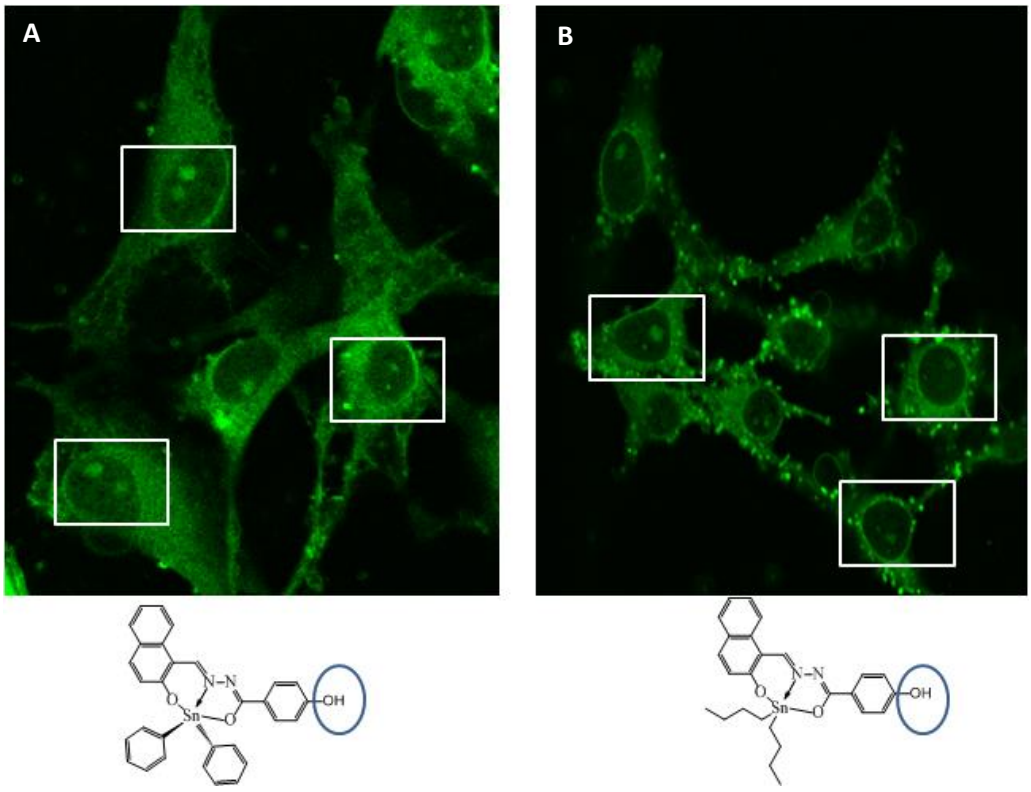


Figure 17. "Bioimages of compounds 2 and 3 showing nuclei staining"

The cytoplasmic staining in the cells with numerous vesicle-like structures showing a stronger staining, suggests that the possible internalization mechanism of the organotin

compounds is endocytosis. We hypothesize that after endosome processing the compound and/or its metabolites are released to the cytosol. Once in the cytoplasm compounds **2** and **3** are able to get into the nucleus showing a characteristic pattern of staining due to the electrodonor group OH^-. Vesicle formation may be influenced by the solubility of the molecule, if not completely solubilized microprecipitates are formed which lead to the internalization of the compound by endosomes, but this no total solubility increase in the compounds **4** and **5** due to the alkyl chain attached, however this compounds show a best staining of cytoplasm without formation of too much vesicles.

Another possible mechanism is that the compounds are being recognized by cell surface receptors inducing in this way endocytosis. Important to mention, high intensity luminescence from the vesicles might be explained on the basis of the acidic internal pH.

7. CONCLUSIONS.

In summary, we have synthesized four new organotin compounds derivative from Schiff bases in good yields.

Cytotoxic assays with melanoma murine cells B16F10, shows that compound **2** and **3**, have most toxic activity, even with the lowest concentration. Compounds **4** and **5** have a good cytotoxic capacity at 10 µg/ml, but a lower concentration is better than its own analogous.

The cytotoxicity comparison between the compounds **2** and **3** versus **4** and **5**, suggest that the alkyl chain of twelve carbons have good influence on the cytotoxic capacity.

The cell images of **2** and **3** show very strong cytoplasmic staining in the cells with numerous vesicle-like structures which suggests that the possible internalization mechanism of the organotin compounds is endocytosis and it's promoted for the OH^- but also this compound stain nucleolus which suggest that could be potential biomarker of nucleolus in B16F10 cells.

The cell images of **4** and **5** show uniform cytoplasmic staining in the cells with which suggests a good candidate for cytoplasmic biomarker for B16F10 cells due of their low cytotoxicity.

The results obtained in this work contribute to the tin cytotoxic capacity knowledge, and know some more about the behavior in cells.

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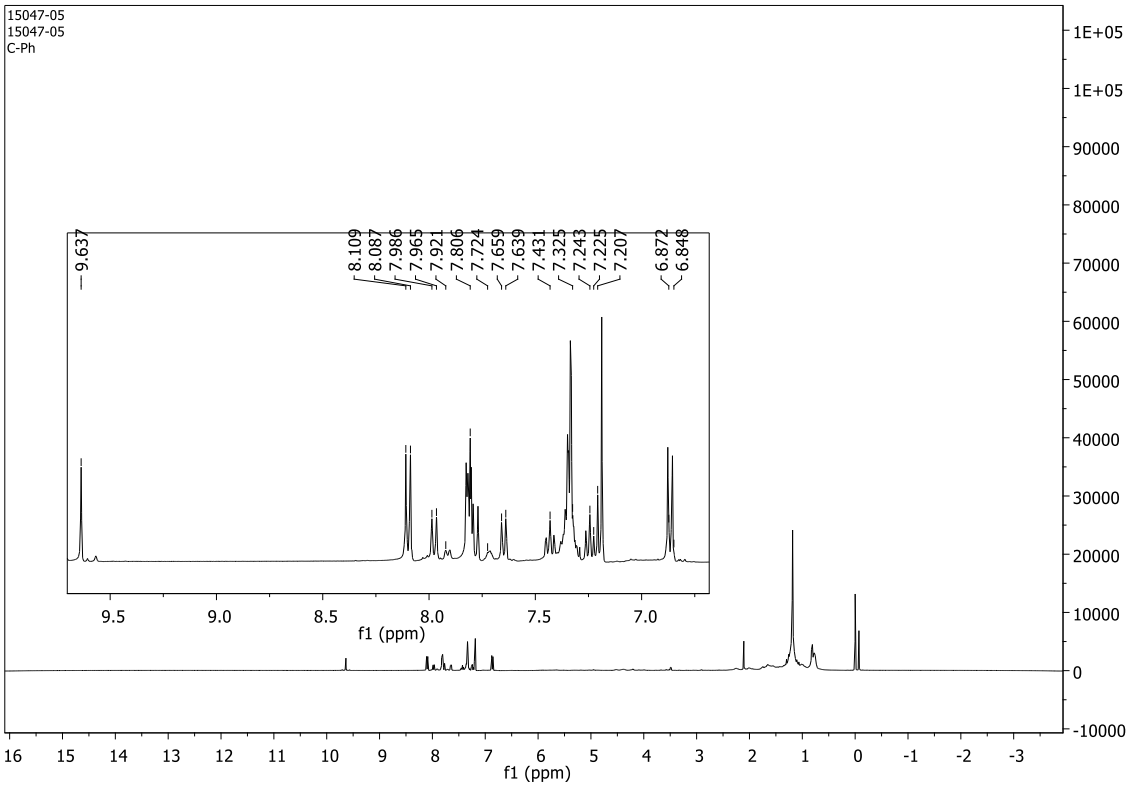
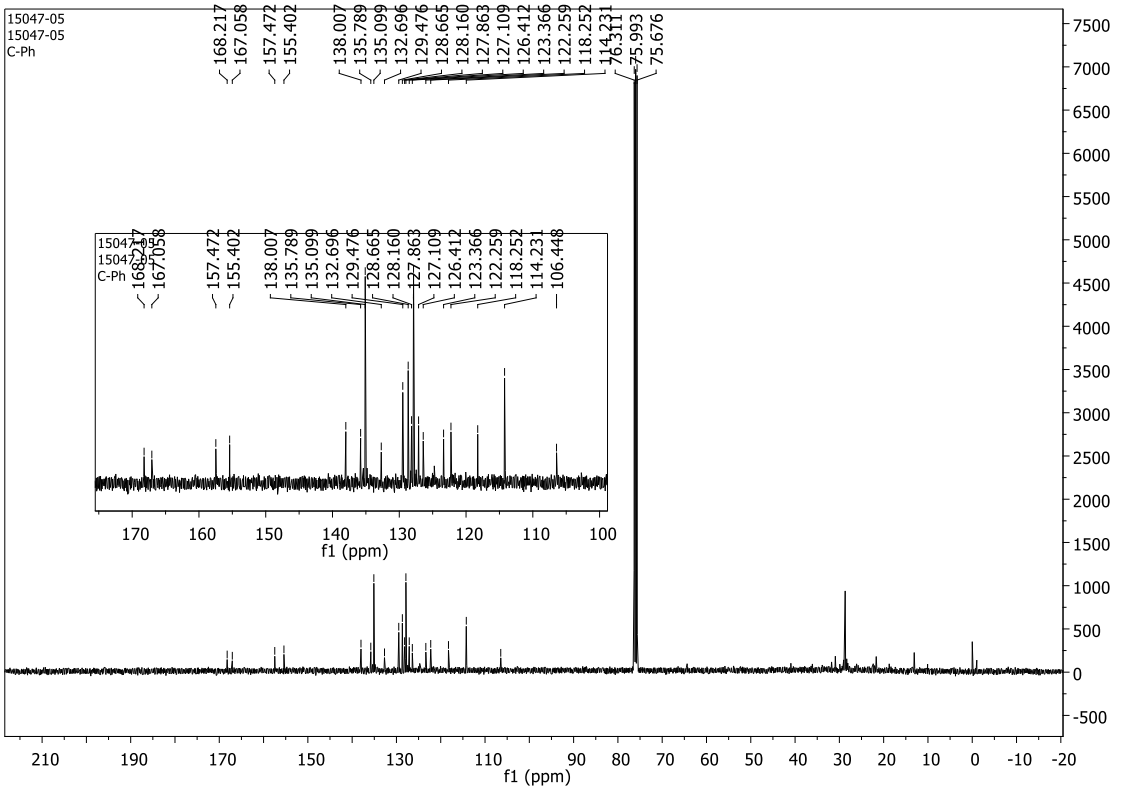
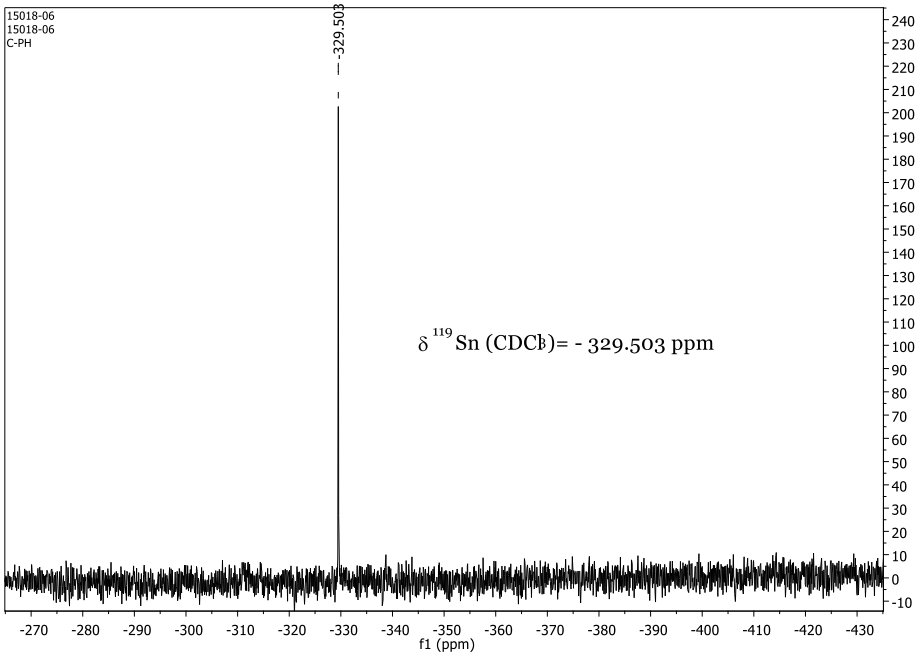
9. APPENDIX



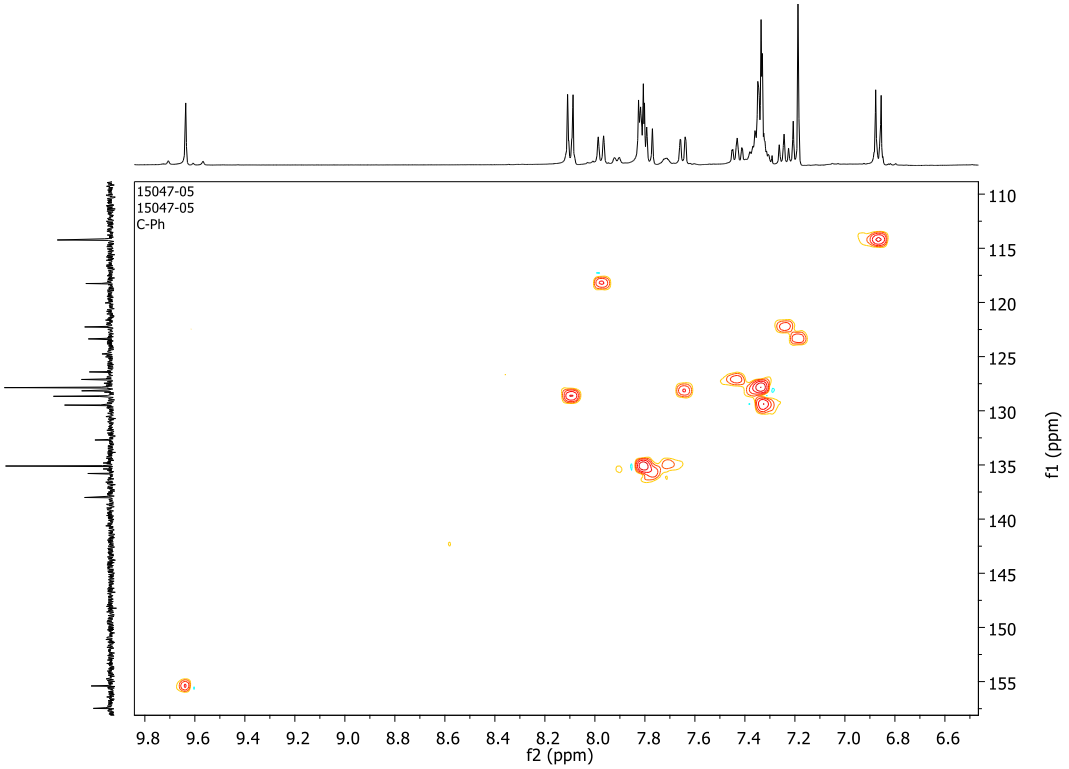
^{1}H -NMR (400 MHz, $CDCl_3$) spectrum of compound 2.



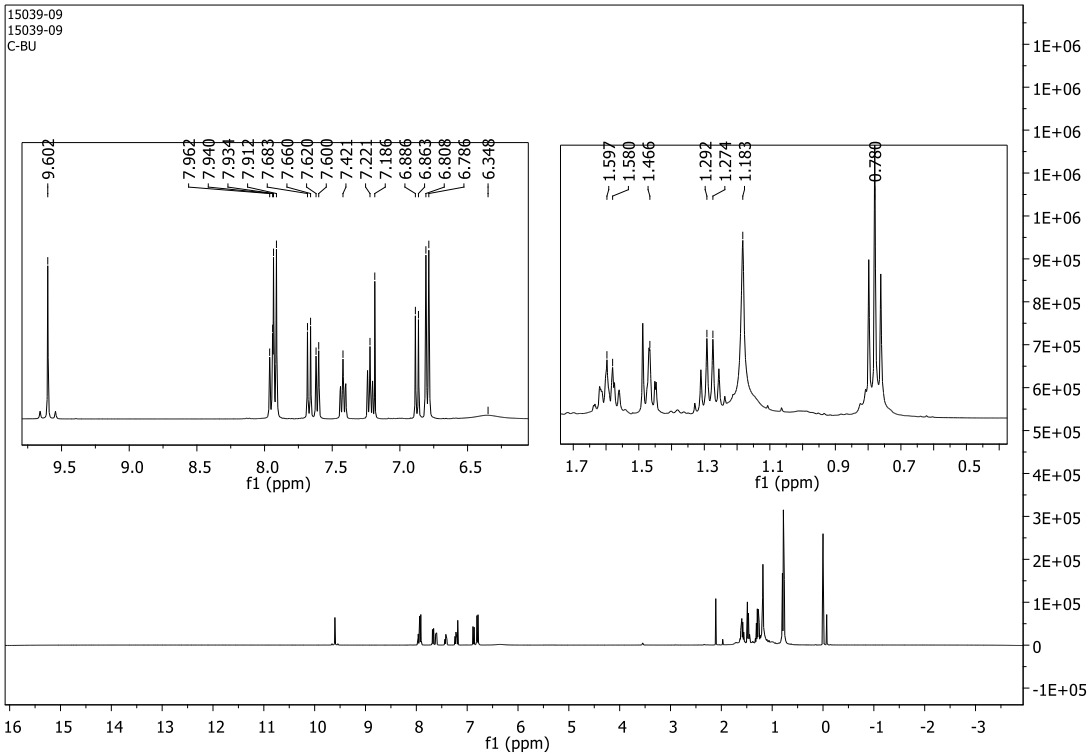
^{13}C-NMR (100 MHz, $CDCl_3$) spectrum of compound 2.



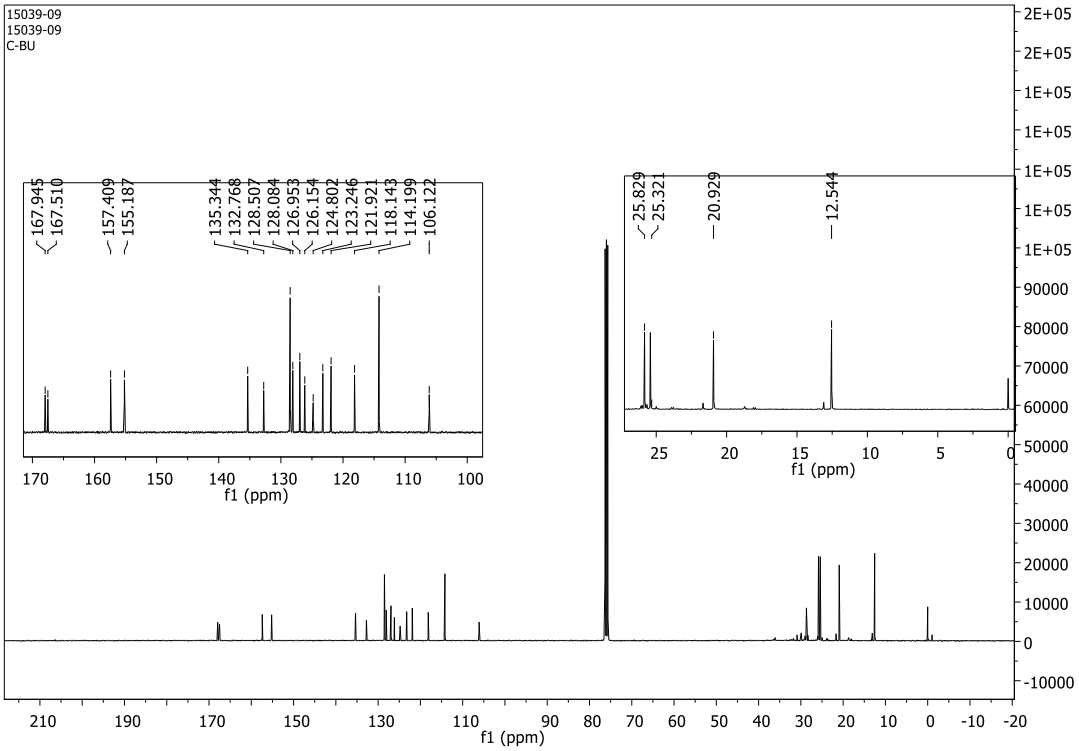
^{119}Sn-NMR (149.14 MHz, $CDCl_3$) spectrum of compound 2.



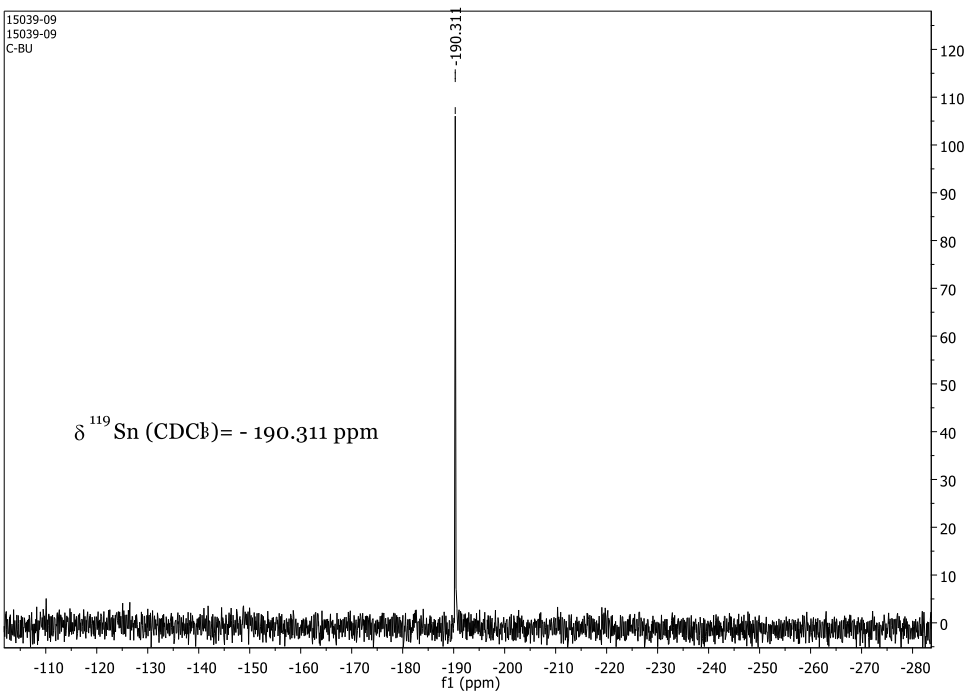
^{1}H/^{13}C HETCOR spectrum of compound 2 (aromatic region).



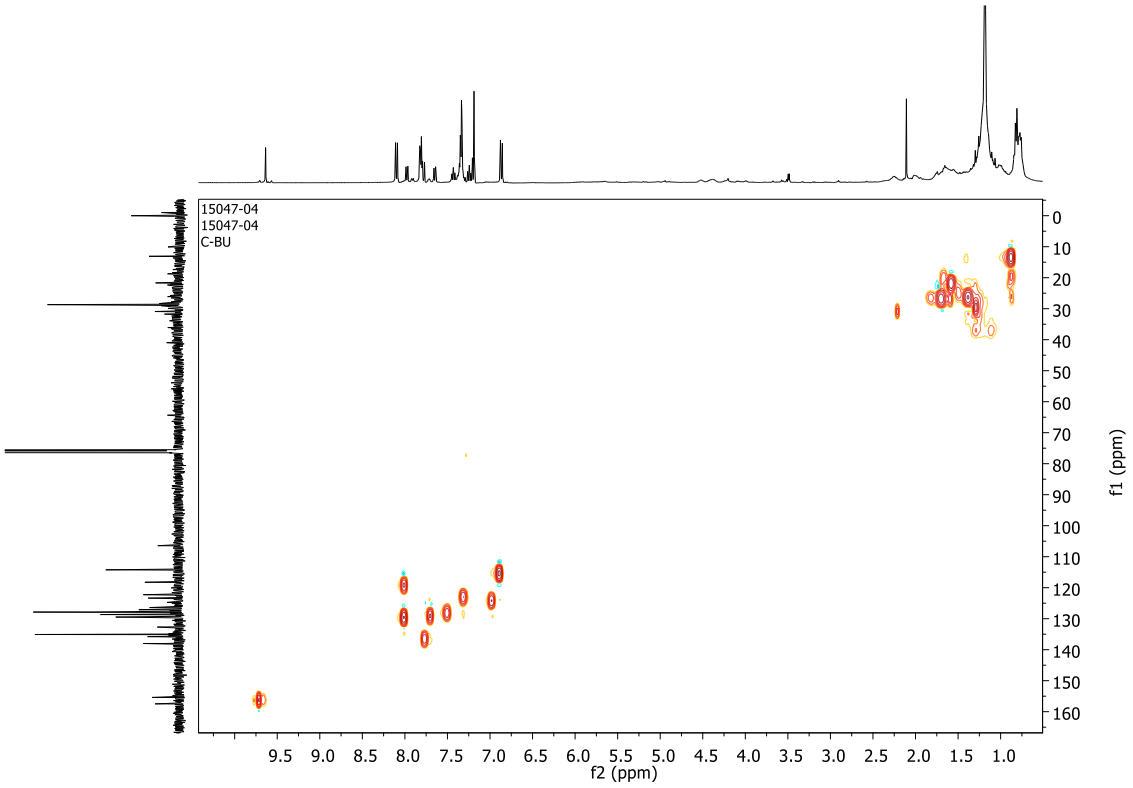
^{1}H -NMR (400 MHz, $CDCl_3$) spectrum of compound 3.



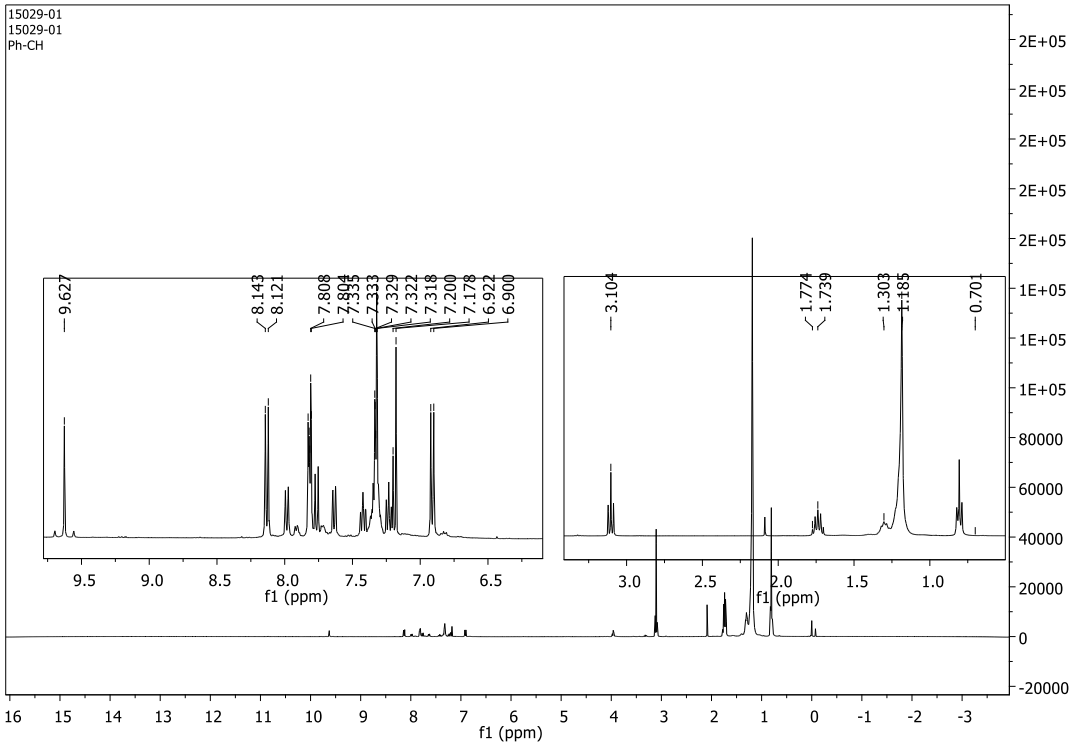
^{13}C-NMR (100 MHz, $CDCl_3$) spectrum of compound 3.



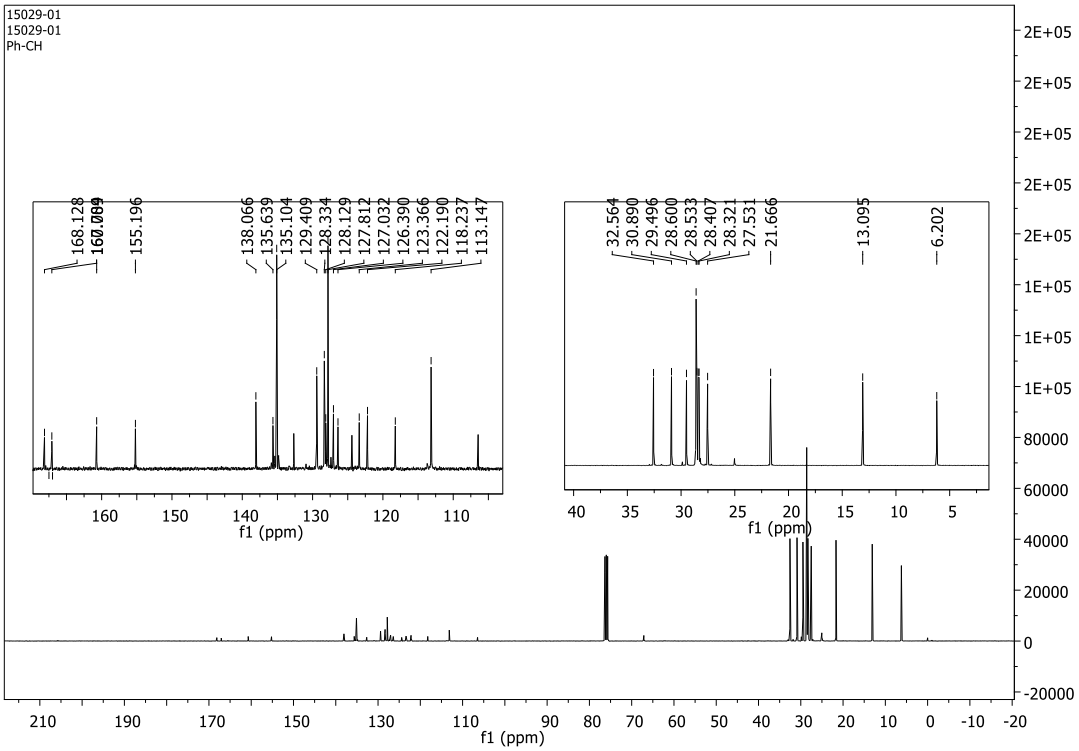
^{119}Sn-NMR (149.14 MHz, $CDCl_3$) spectrum of compound 3.



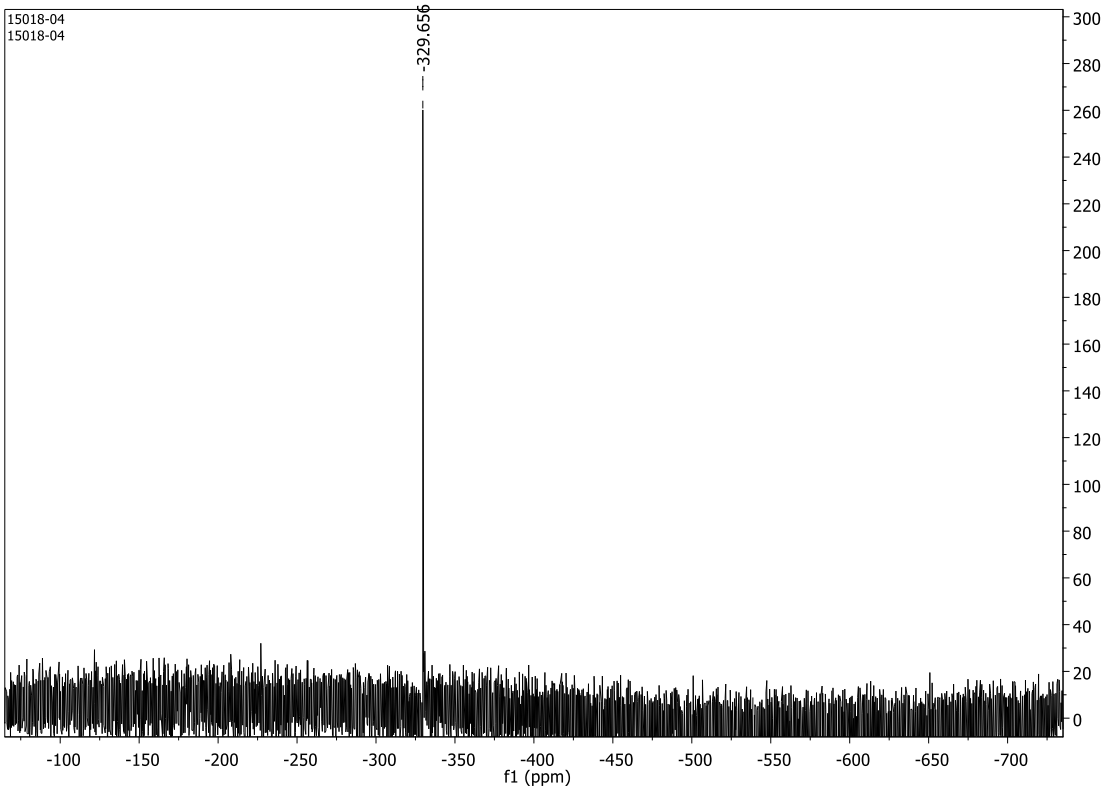
^{1}H/^{13}C HETCOR spectrum of compound 3.



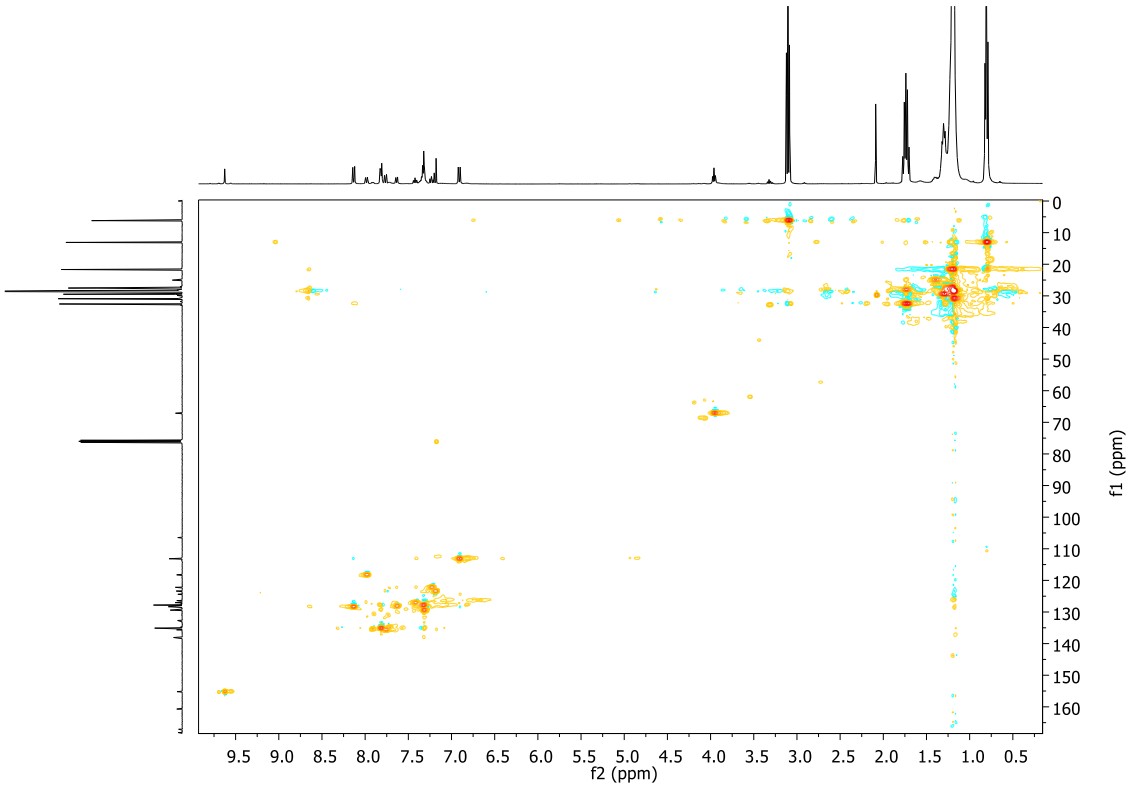
^{1}H -NMR (400 MHz, CDCl3) spectrum of compound 4.



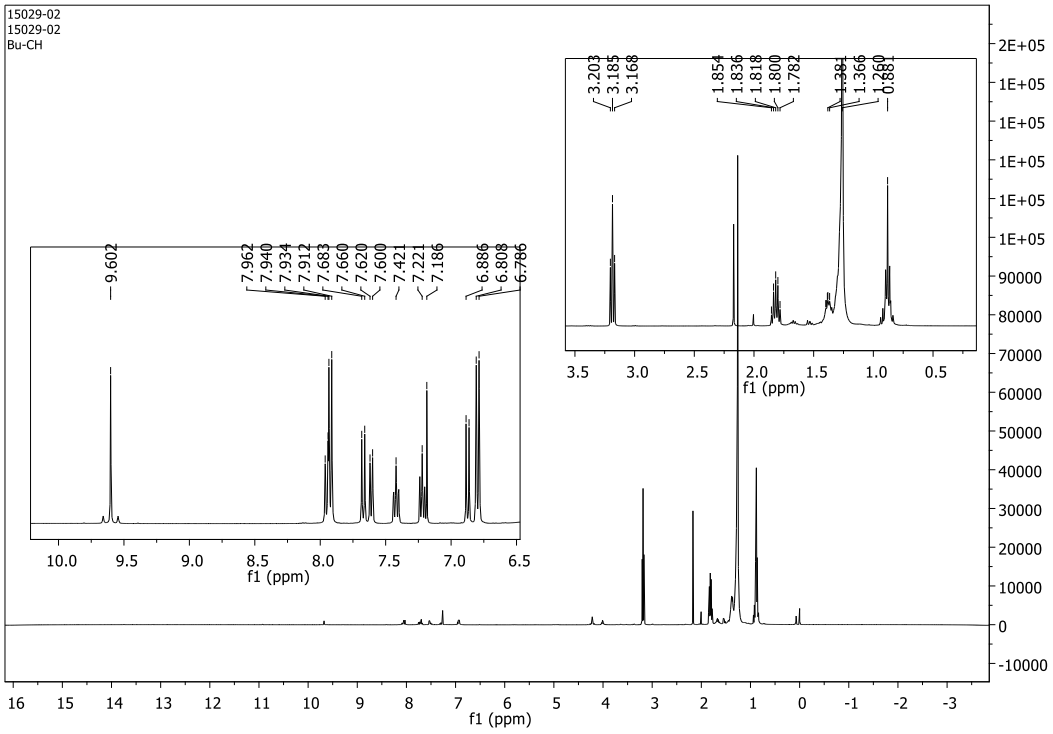
^{13}C-NMR (100 MHz, CDCl3) spectrum of compound 4.



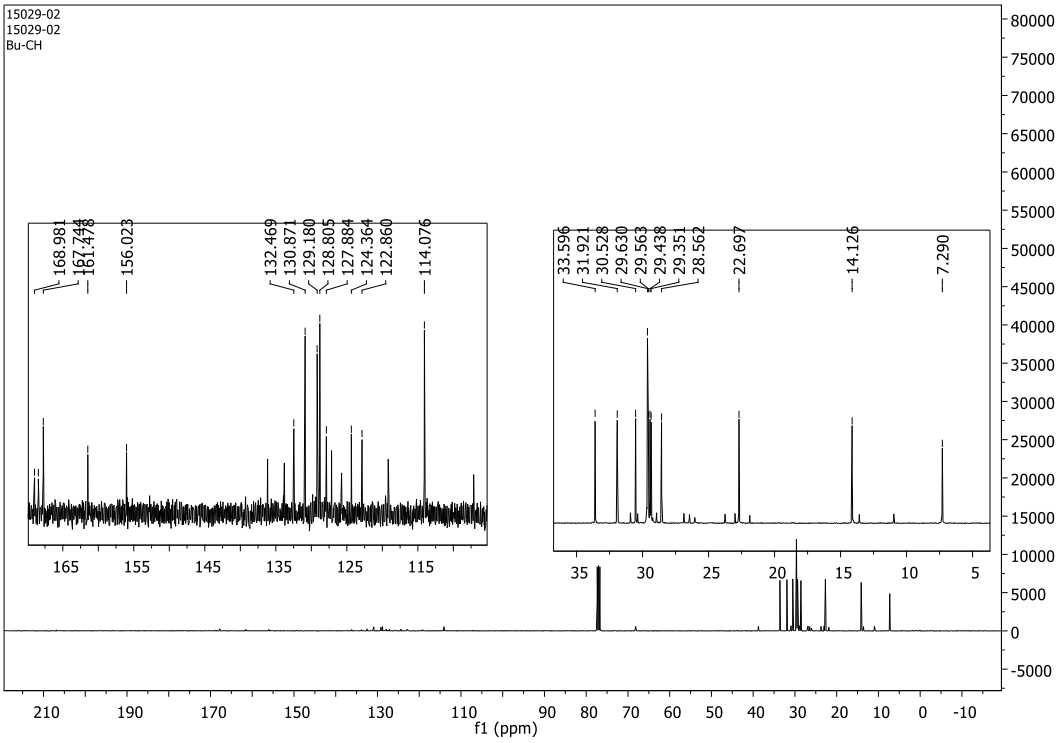
^{119}Sn-NMR (149.14 MHz, $CDCl_3$) spectrum of compound 4.



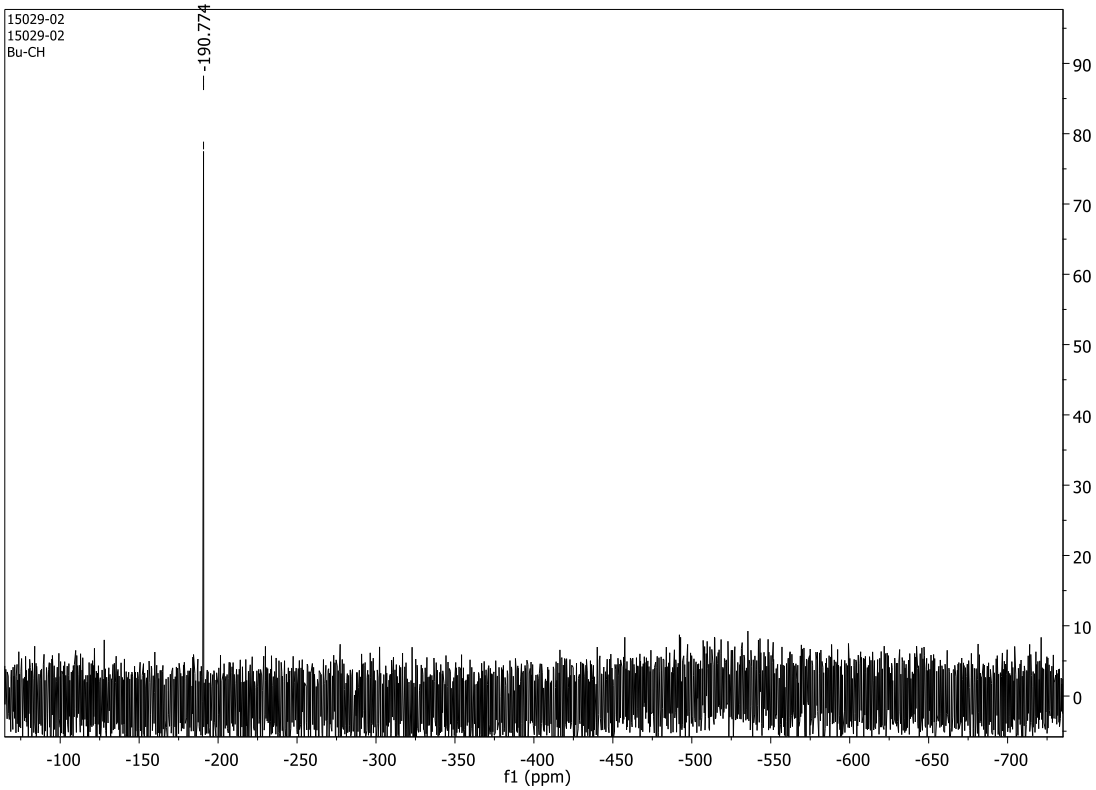
^{1}H/^{13}C HETCOR spectrum of compound 4.



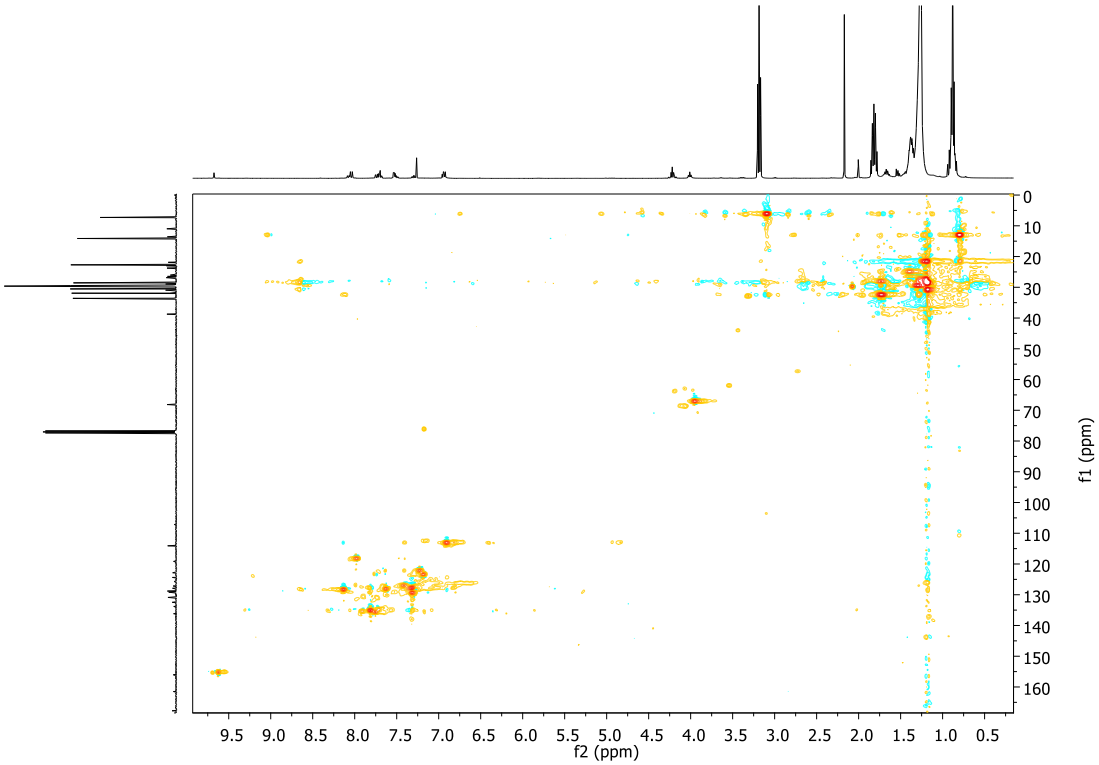
^{1}H -NMR (400 MHz, $CDCl_3$) spectrum of compound 5.



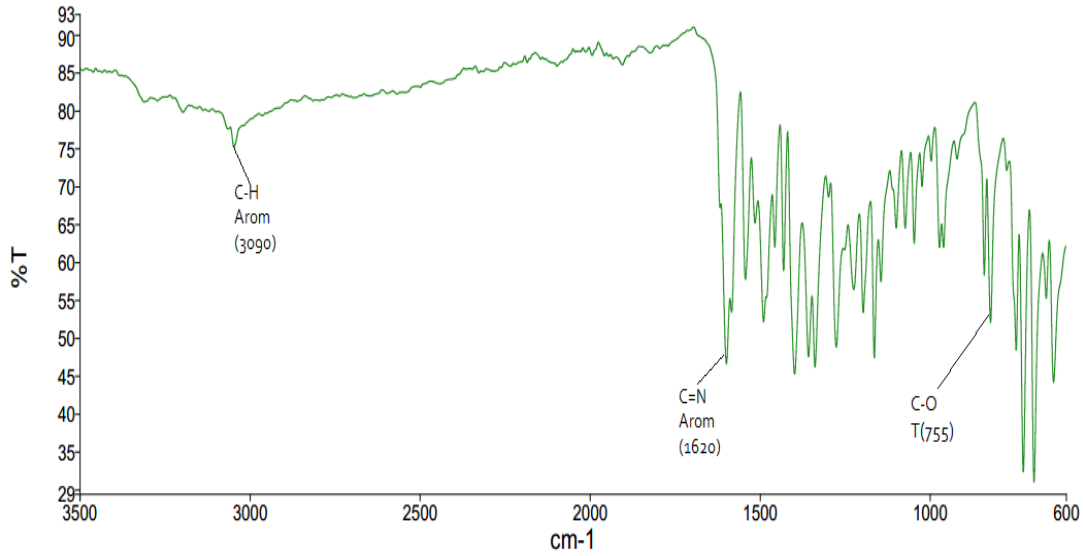
^{13}C-NMR (100 MHz, $CDCl_3$) spectrum of compound 5.



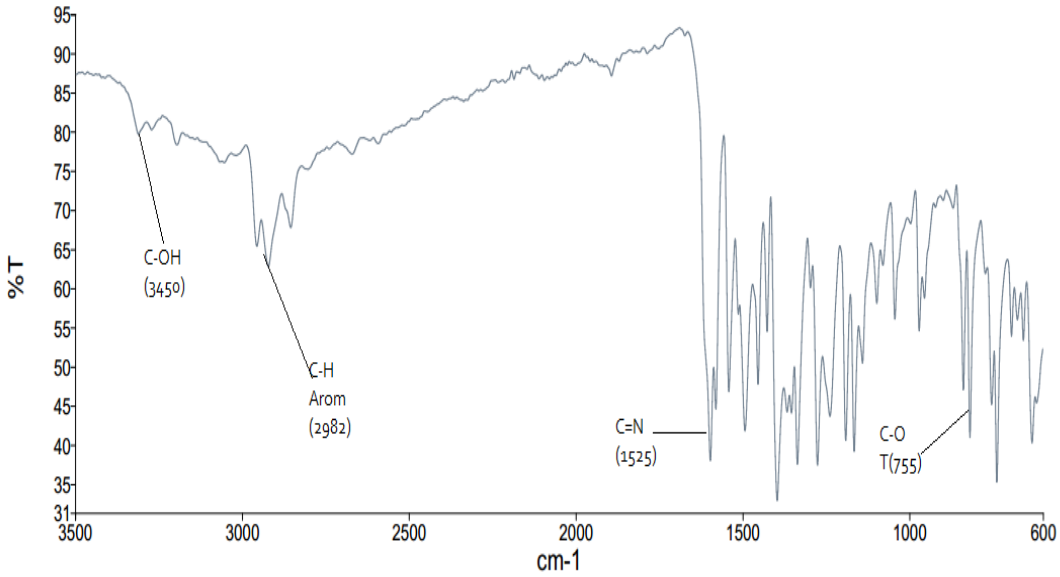
^{119}Sn-NMR (149.14 MHz, $CDCl_3$) spectrum of compound 5.



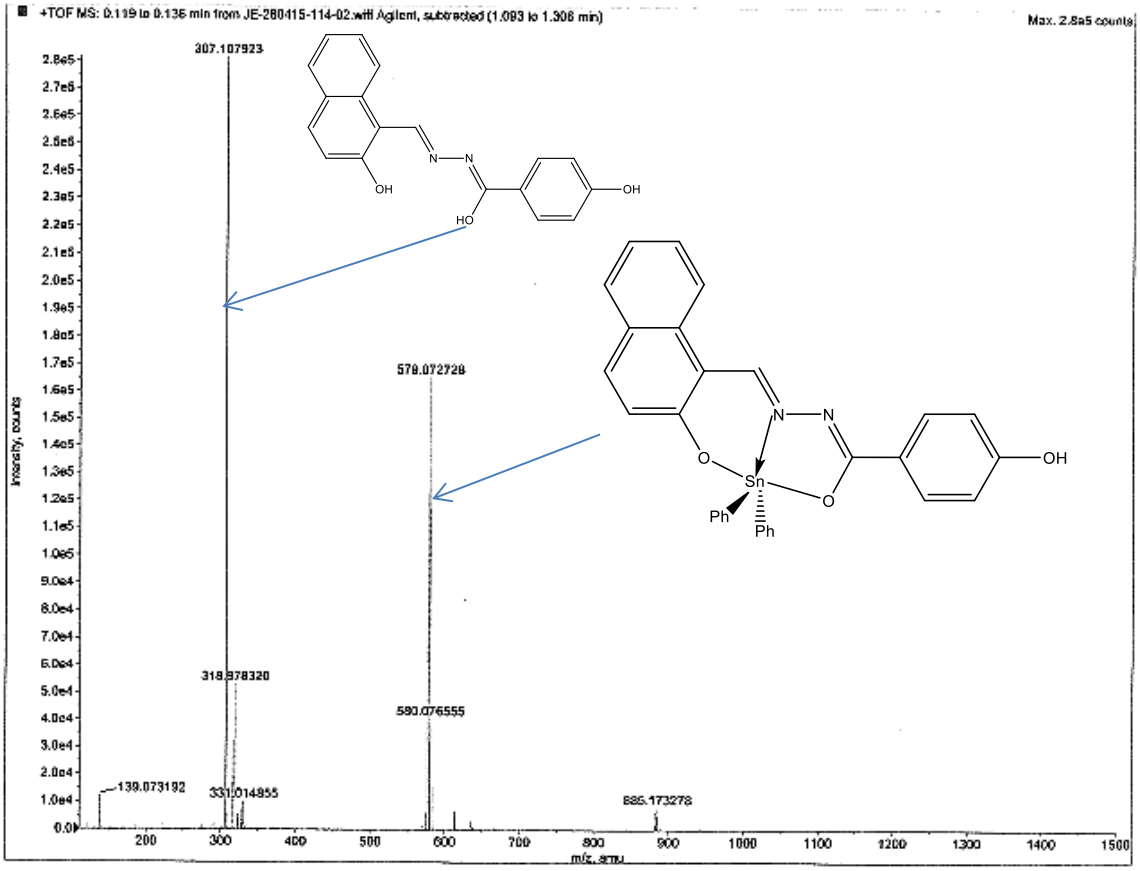
^{1}H/^{13}C HETCOR spectrum of compound 5.



IR spectrum of compound 2.

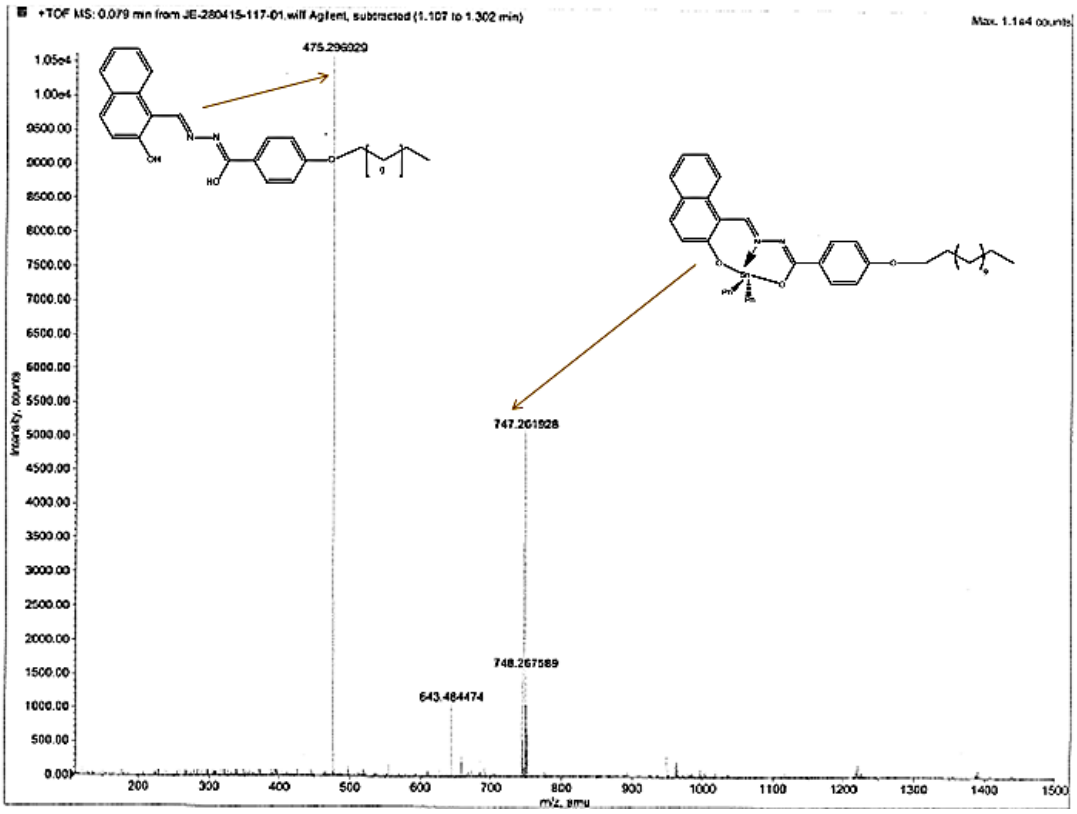


IR spectrum of compound 3.

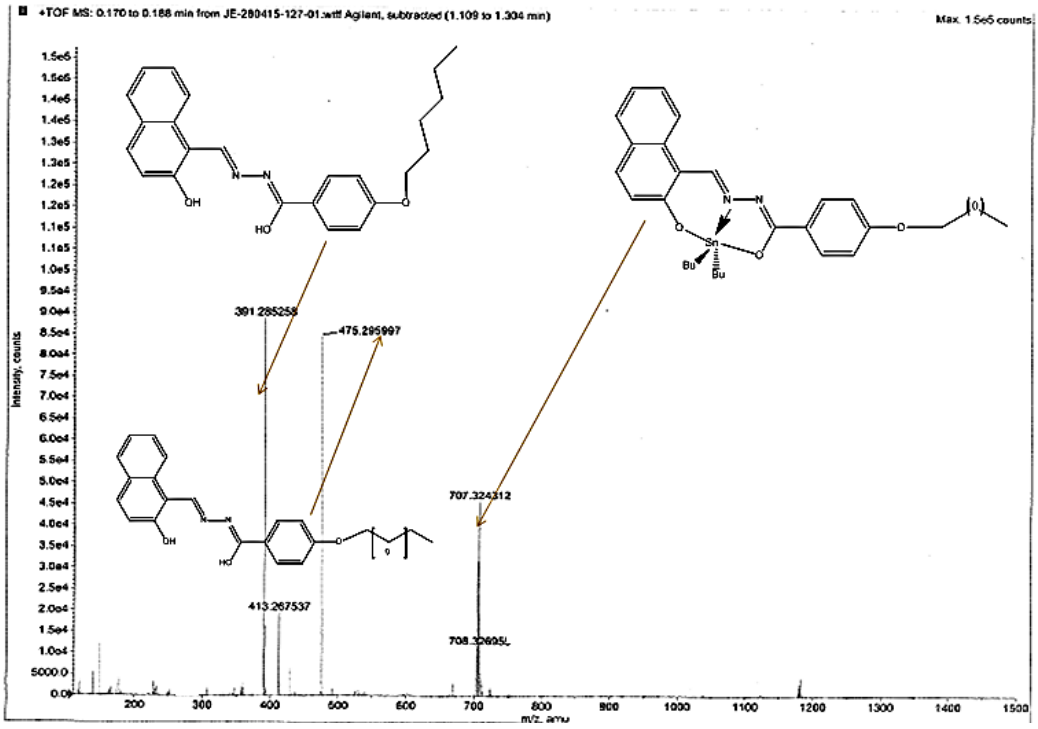


Mass spectrum of compound 2.

Mass spectrum of compound 3.



Mass spectrum of compound 4.



Mass spectrum of compound 5.

AUTOBIOGRAPHIC SUMMARY

I.Q EDUARDO GABRIEL PEREZ PEREZ

Candidate for the degree of:

Master of Science with Orientation in Chemical Materials

Thesis:

SYNTHESIS AND CHARACTERIZATION OF LUMINESCENT COMPOUNDS OF Sn IV WITH POTENTIAL APPLICATION AS CELLULAR MARKER IN VITRO

Field of study: Industrial Chemistry

Biography: Born in Puebla, Puebla, México, on September 17th of 1985.

Education: Graduated from the Universidad Politecnica de Tlaxcala, degree obtained: Chemical Engineer in 2013.

Professional experience: Supervisor in Porcelanite/Lamosa 2013. Logistics specialist in RESIRENE 2009. Professional practices in Universidad Politecnica de Tlaxcala 2010.