MARILIA VALLI

Produtos naturais do NuBBE, fonte de diversidade molecular para o planejamento racional de novos agentes antitumorais

Tese apresentada ao Instituto de Química, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutora em Química

Orientadora: Prof[a]. Dr[a]. Vanderlan da Silva Bolzani
Coorientador: Prof. Dr. Adriano Defini Andricopulo

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MARILIA VALLI

NuBBE natural products, source of molecular diversity for the design of new anticancer agents

Thesis submitted in part fulfillment of the requirement for the degree of Doctor of Philosophy

Supervisor Prof. Vanderlan da Silva Bolzani
Co-supervisor Prof. Adriano Defini Andricopulo

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CURRICULUM VITAE

Marilia Valli

São Paulo State University - UNESP

Institute of Chemistry

Nucleus of Bioassays, Biosynthesis, and Ecophysiology of Natural Products - NuBBE

Rua Professor Francisco Degni, 55 - CEP: 14800-900 - Araraquara/SP.

mariliava@iq.unesp.br

Education

- PhD in Chemistry - Institute of Chemistry – Sao Paulo State University (UNESP), Araraquara. March 2014.
- Master in Chemistry - Institute of Chemistry – Sao Paulo State University (UNESP), Araraquara. March 2010.
- Bachelor in Chemistry - Institute of Chemistry – Sao Paulo State University (UNESP), Araraquara. December 2007.

Graduate Courses (Ph.D.)

1. Stereochemistry and reactivity of organic compounds (12 credits), grade: A. Lecturer: Prof. Dulce Helena S. Silva
2. Medicinal chemistry applied to natural products (12 credits), grade: A. Lecturer: Prof. Vanderlan da Silva Bolzani
3. Mechanism of organic reactions (12 credits), grade: A. Lecturer: Prof. Maria Fátima das Graças Fernandes da Silva, course taken at the Federal University of São Carlos - UFSCAR.
4. Introduction to NMR Spectroscopy with demonstrations of data processing and analysis (2 credits), grade: A. Lecturer: Prof. Arthur S. Edison (University of Florida)

Graduate Courses (Master)

1. Advanced Organic Chemistry (12 credits), grade: A. Lecturer: Prof. Ian Castro-Gamboa and Prof. Wagner Vilegas.
2. Spectrometric methods (12 credits), grade: A. Lecturer: Prof. Wagner Vilegas.

3. Theory and method of separation, isolation and purification of organic compounds (12 credits), grade: A. Lecturer: Prof. Alberto José Cavalheiro.
4. Organic Synthesis (12 credits), grade: A. Lecturer: Prof. Ângela Regina Araújo
5. Medicinal Plants: an advanced view (3 credits), grade: A. Lecturer: Prof. Robert Verpoorte.

Teaching Training:

Organic Chemistry III for the undergraduate in Chemistry (IQ-UNESP)
Lecturer in Charge: Prof. Isabele R. Nascimento
Lecture attendance and teaching of 2 classes, from 1st August 2011 to 13th December 2011.

Undergraduate Supervision

Supervision of the experimental work developed by the undergraduate student Cíntia Hiromi Nakajima regarding literature review and the organization of the database of natural products ($NuBBE_{DB}$) from 1st October 2010 to 30th May 2011.

Presentation of Mandatory Seminar

Title: "Strategies for the design of drugs for the treatment of cancer"
This conference aimed to describe the basic chemical features of the topic and show some recent advances. This presentation was evaluated and approved by four Professors in the Chemistry area.
Date: 18th May 2012
Duration: 40 minutes

Research Internship

1. *Short training at Faculté des Sciences Pharmaceutiques et Biologiques*, U*niversité Paris V – Descartes*, under the supervision of Prof. Sylvie Michel and Prof. Xavier Cachet. From 4th December 2012 to 28th February 2013.
2. *Short training at Laboratório de Psicofarmacologia Experimental*, *Faculdade de Farmácia – Universidade Federal do Rio Grande do Su*l (UFRGS), under the supervision of Prof. Stela Maris Kuze Rates, January 2010.

3. *Short training at Laboratorio de Experimentación Animal* (LEA), *Facultad de Química – Universidad de la República* (UdelaR), 3rd October 2009 to 15th November 2009, under the supervision of Prof. Jenny Carolina Saldaña.

Published Papers

1. Valli, M.; Danuello, A.; Pivatto, M.; Saldaña, J.C.; Heinzen, H.; Domínguez, L.; Campos, V.P.; Marqui, S.; Young, M.C.M; Silva, D.H.S.; Bolzani, V.S. Anticholinesterasic, Nematostatic and Anthelmintic Activities of Pyridinic and Pyrazinic Compounds. *Current Medicinal Chemistry*, v. 18, n. 22, p.3423-3430, 2011.
2. Bolzani, V.S.; Valli, M.; Pivatto, M.; Viegas, Jr., C. Natural products from Brazilian biodiversity as a source of new models for medicinal chemistry. *Pure and Applied Chemistry*, v. 84, n. 9, p. 1837-1846, 2012.
3. Valli, M.; Pivatto, M.; Danuello, A.; Silva, D.H.S.; Castro-Gamboa, I.; Cavalheiro, A.J.; Araújo, A.R.; Furlan, M.; Lopes, M.N.; Bolzani, V.S. The Tropical Biodiversity: has it been a potential source of secondary metabolites useful for medicinal chemistry? *Química Nova*, v. 35, n. 11, p. 2278-2287, 2012.
4. Valli, M.; dos Santos, R.N.; Figueira, L.D.; Nakajima, C.H.; Andricopulo, A.D.; Bolzani, V.S. Development of a Natural Products Database from the Biodiversity of Brazil. *Journal of Natural Products*, v. *76,* n. 3, p. 439-444, 2013.

Conference Proceedings and Abstracts

1. Bolzani, V.S.; Cavalheiro, A.J.; Castro-Gamboa, I.; Valli, M.; Pinto, M.E.; Pivatto, M.; Andricopulo, A.D.; Pessoa, C.; Garcia, C.R. Tracing secondary metabolites on Brazilian biodiversity: how to do it usefully to find new biologically active compounds? Oral session Abstract of PSNA Meeting 2011. *Pharmaceutical Biology*, v.50, n.5, p.662-663, 2012.
2. Valli, M.; Santos, R.N.; Figueira, L.D.; Vieira, Jr., G.M.; Funari, C.S.; Regasini, L.O.; Lopes, M.N.; Cavalheiro, A.J.; Araújo, A.R.; Furlan, M; Silva, D.H.S.; Castro-Gamboa, I.; Andricopulo, A.D.; Bolzani V.S. Tubulin ligands identified on screening natural products from NuBBE database. *Planta Medica*, v. 78, p. 1101-1101, 2012.

Book Chapters

1. Flausino, Jr., O.; Valli, M; Bolzani, V.S. Biodiversidade brasileira: uma fonte potencial de agentes terapêuticos ainda inexplorada. Book Chapter '*Química de produtos naturais: novos fármacos e a moderna farmacognosia*' Editores: Rosendo A. Yunes e Valdir Cechinel Filho, 3rd ed., UNIVALI. Published on 13th June 2012.

Languages*:* English (Cambridge CAE certificate, C1*), Spanish (DELE certificate, B2*), German (Der *Online-Einstufungstest Deutsch als Fremdsprache* certificate (onDaF), B1*), French. *Levels from the "*Common European Framework of Reference for Languages*".

Oral Presentations

Development of a Natural products database from the Brazilian Biodiversity. *36ª Reunião Anual da Sociedade Brasileira de Química*. 2013. Águas de Lindóia, SP. 24th May 2013.

Awards & Honors:

Honor in the XIX Congress of Undergraduate Research of UNESP for the presentation of the poster: Tiopyridinic molecular hybrids as candidates for inhibitors of acetylcholinesterase. 2007.

Scientific Meetings Attended (2010-2013)

1. 36ª *Reunião Anual da Sociedade Brasileira de Química*. Águas de Lindóia, SP 2013.
2. São Paulo Advanced School on Bioorganic Chemistry. Araraquara, SP. 2013.
3. International Congress on Natural Products Research. New York, USA. 2012.
4. 3rd Brazilian Conference on Natural Products. Ouro Preto, MG. 2011.
5. Conferences for the International Year of Chemistry - Medicinal Chemistry: challenges and perspectives. FAPESP - São Paulo, SP. 2011.
6. ESPCA - São Paulo Advanced School on Chemistry. 2011. Unicamp - Campinas, SP. 2011.

7. Conferences for the International Year of Chemistry - *A contribuição de Marie Curie para a ciência e um olhar sobre o papel das mulheres cientistas*. FAPESP - São Paulo, SP. 2011.
8. Conferences for the International Year of Chemistry - *A Química inteligente a serviço da medicina*. FAPESP - São Paulo, SP. 2011.
9. 33ª *Reunião Anual da Sociedade Brasileira de Química*. Águas de Lindóia, SP 2010.
10. Biota FAPESP International Workshop on Metabolomics in the Context of Systems Biology. FAPESP - São Paulo, SP. 2010.

Scientific Events Organization

1. Organizing Committee of the São Paulo Advanced School on Bioorganic Chemistry. Araraquara, SP. 2013.
2. Organizing Committee of the 3rd Brazilian Conference on Natural Products – BCNP. Ouro Preto, MG. 2011.

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Araraquara, 28 de março de 2014.

BANCA EXAMINADORA

Profª. Drª. VANDERLAN DA SILVA BOLZANI (Orientadora)
Instituto de Química – UNESP, Araraquara.

Prof. Dr. IAN CASTRO-GAMBOA
Instituto de Química – UNESP, Araraquara.

Prof. Dr. FLAVIO DA SILVA EMERY
Faculdade de Ciências Farmacêuticas de Ribeirão Preto – USP, Ribeirão Preto.

Prof. Dr. NORBERTO PEPORINE LOPES
Faculdade de Ciências Farmacêuticas de Ribeirão Preto – USP, Ribeirão Preto.

Profª. Drª. SYLVIE MICHEL
Faculdade de Ciências Farmacêuticas e Biológica – Université Paris Descartes, Paris.

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RESUMO

Os produtos naturais são uma importante fonte de inspiração para o desenvolvimento de novos fármacos. O presente trabalho visou identificar produtos naturais bioativos que pudessem ser usados como modelo para o planejamento de novos compostos com propriedades antitumorais. A falta de dados organizados é ainda uma das dificuldades das áreas de produtos naturais e química medicinal. Portanto, a compilação de dados disponíveis sobre os metabólitos secundários sejam de espécies vegetais ou de outras fontes é de grande valor. Esse fato nos motivou a propor como primeiro objetivo deste projeto, a criação de uma base de dados contendo informações botânicas, químicas e biológicas dos metabólitos secundários obtidos e publicados pelo NuBBE durante 15 anos. A base de dados poderá ser útil não apenas para a pesquisa em química de produtos naturais atual do grupo, mas para todos interessados em estudos de planejamento de moléculas bioativas, metabolômica e *dereplication*, já que está disponível para acesso livre na internet. Um artigo científico descrevendo a criação da base de dados foi publicado na revista *Journal of Natural Products* em 2013. Os compostos da base de dados foram utilizados como fonte de moléculas para uma triagem virtual baseada na estrutura do receptor com a proteína tubulina para a identificação de moduladores dessa proteína. Baseado nos resultados de triagem virtual foi realizada a avaliação biológica *in vitro* das substâncias utilizando a proteína tubulina e ensaios de migração celular (*wound healing* e câmara de Boyden). Os ensaios biológicos indicaram uma série de guanidinas e a piplartina como principais compostos bioativos dentre os avaliados. A piplartina foi selecionada como modelo para o planejamento de novos compostos, pois apresentou relevante inibição de migração celular, além de estar descrito na literatura como citotóxico e seletivo, perfil interessante na busca de protótipos antitumorais. Uma série de 5 novos compostos análogos de piplartina foi planejada e sintetizada visando maior compreensão de mecanismos de ação e melhoria na atividade biológica. O análogo planejado por simplificação molecular se mostrou ativo no ensaio de migração celular com resultado comparável à piplartina. Os objetivos propostos neste projeto de pesquisa foram alcançados por meio de trabalho cooperativo e interdisciplinar nas áreas de química computacional, biológica, medicinal e de produtos naturais.

Palavras-chave: Base de dados. Produtos naturais. Atividade antitumoral.

RESUMO EXPANDIDO

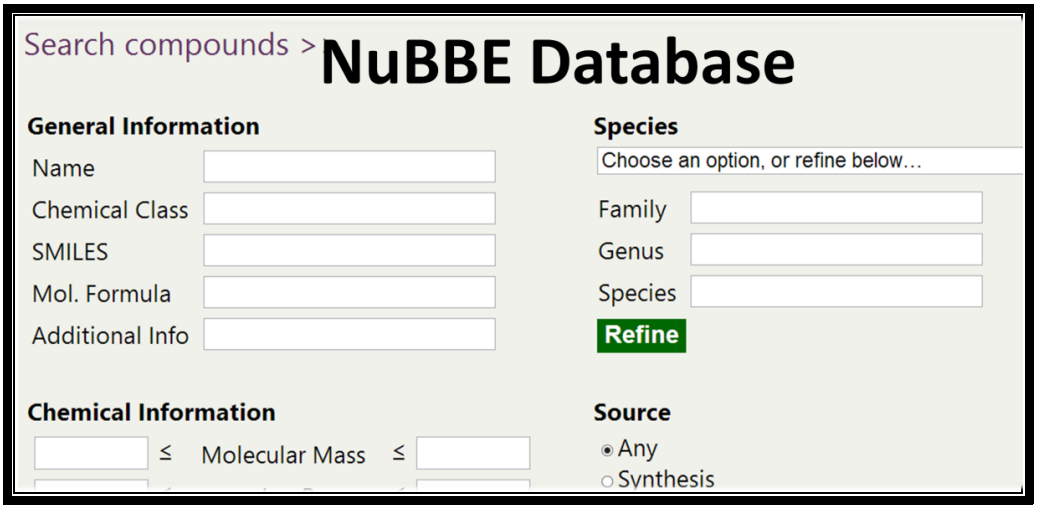
Produtos Naturais do NuBBE, fonte de diversidade molecular para o planejamento racional de novos agentes antitumorais

O Brasil possui uma das maiores biodiversidades do mundo, contabilizando cerca de 20% de todas as espécies vivas existentes, distribuídas por importantes biomas como o Cerrado e as florestas Amazônica e Atlântica [BOLZANI; CASTRO-GAMBOA; SILVA, 2010]. A biodiversidade provê diversidade química única que tem sido usada como modelo para o desenvolvimento de novos fármacos [KOEHN; CARTER, 2005; CHIN, 2006; NEWMAN; CRAGG, 2007; NEWMAN, 2008; KINGHORN *et al*., 2011; NEWMAN; CRAGG, 2012]. O presente trabalho visou identificar produtos naturais bioativos que pudessem ser usados como modelo para o planejamento de novos compostos com propriedades antitumorais.

Uma das dificuldades nas áreas de química medicinal e produtos naturais é a falta de bases de dados acessíveis de metabólitos secundários sejam de espécies vegetais ou de outras fontes. Existem diversos grupos no Brasil focados em explorar essa rica biodiversidade. Um deles é o Núcleo de Bioensaios, Biossíntese e Ecofisiologia de Produtos Naturais (NuBBE) que desenvolve pesquisa na área de química de produtos naturais, e tem se envolvido com aspectos inovadores como a metabolômica e a química medicinal. Um dos objetivos do grupo de pesquisa tem sido a busca por compostos biologicamente ativos de plantas e fungos da biodiversidade brasileira, resultando em mais de 600 metabólitos secundários e análogos sintéticos publicados em cerca de 170 publicações científicas. Devido à ausência de uma base de dados de metabólitos secundários da biodiversidade brasileira e o número significante de compostos obtidos pelo NuBBE, foi proposto o primeiro objetivo deste projeto, a criação de uma base de dados ($NuBBE_{DB}$) contendo informações botânicas, químicas e biológicas dos metabólitos secundários obtidos e publicados pelo NuBBE.

Algumas propriedades químicas foram disponibilizadas na base de dados, por serem importantes descritores para a química medicinal, tais como LogP, número de doadores e aceptores de ligação de hidrogênio, número de violações da regra de Lipinski, número de ligações rotacionáveis e volume molecular. Para todos os compostos da $NuBBE_{DB}$ também estão disponíveis as estruturas 3D em formato Mol2. Adicionalmente, dados quali- e quantitativos de algumas propriedades biológicas foram extraídos dos artigos científicos, quando disponíveis, e

disponibilizados na base de dados, bem como a origem do composto, a espécie de onde foi isolado. A $NuBBE_{DB}$ pode ser livremente acessada no portal do NuBBE, em http://nubbe.iq.unesp.br/nubbeDB.html [NÚCLEO..., 2014]. Para o fácil acesso das informações, foi criada uma ferramenta de busca onde é possível fazer uma pesquisa por propriedades, estrutura química ou uma combinação de critérios.

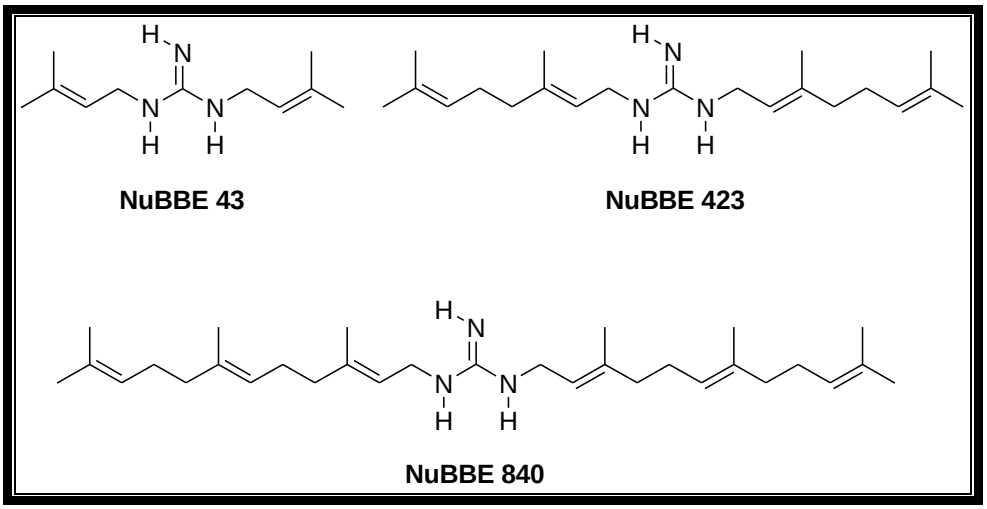


O objetivo é que a base de dados seja útil não apenas para a pesquisa atual do grupo, mas para toda a comunidade científica interessada em estudos de planejamento de moléculas bioativas, metabolômica e *dereplication*. Um artigo científico descrevendo a criação da base de dados foi publicado na revista *Journal of Natural Products* em 2013 [VALLI *et al*., 2013]. A divulgação deste trabalho tem sido intensa para que os objetivos da base de dados sejam alcançados.

A $NuBBE_{DB}$ foi utilizada como fonte de moléculas para uma triagem virtual baseada na estrutura do receptor com a proteína tubulina para a identificação de moduladores dessa proteína. Tubulina é a unidade formadora dos microtúbulos, com massa molecular aproximadamente 55 kDa [JORDAN; WILSON, 2004; LODISH *et al*., 2007]. Os microtúbulos são essenciais no processo de mitose celular, o que os faz um importante alvo macromolecular para fármacos antitumorais. A perturbação da dinâmica dos microtúbulos bloqueia a mitose e induz a morte celular, e pode ser dividida em dois tipos: (a) os fármacos que os desestabilizam e inibem a formação de microtúbulos e (b) os que os estabilizam e aumentam a polimerização de tubulina. O mecanismo do tipo (a) é descrito, por exemplo, para a colchicina, vincristina e vinblastina, e do tipo (b) é descrito para o

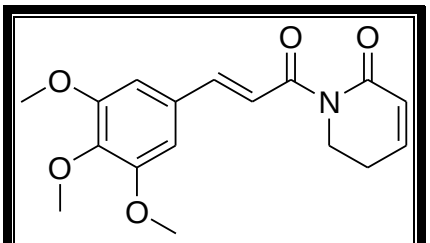
paclitaxel. Os compostos também variam quanto à forma de ligação e aos sítios de modulação. Tanto a desestabilização quanto a estabilização dos microtúbulos levam à morte celular. Sendo assim, o estudo de triagem virtual foi realizado em três sítios da tubulina, os sítios do paclitaxel, colchicina e vinblastina. Os resultados da triagem revelaram como promissoras uma série de casearinas, chalconas, flavonoides, guanidinas, entre outras classes de compostos. O estudo de *docking*, especialmente os valores de *score*, nos guiou para selecionar 35 substâncias para realizar a avaliação *in vitro* da polimerização de microtúbulos.

O objetivo da avaliação da polimerização de tubulina é identificar compostos que modulem a proteína tubulina como alvo antitumoral [CYTOSKELETON, 2013; BONNE *et al*., 1985; DYRAGER *et al*., 2011]. Os resultados dos ensaios indicaram que uma série de três análogos de guanidina (NuBBE 43, NuBBE 423 e NuBBE 840) se mostraram inibidores da polimerização de tubulina. Observa-se que a atividade decresce com a diminuição do número de unidades prenílicas nas duas cadeias laterais, sendo as EC_{50} de inibição de polimerização de tubulina medidas por fluorescência de NuBBE 43, NuBBE 423 e NuBBE 840 respectivamente 63 ± 1.4 µM, 27.5 ± 1.1 µM e 19.9 ± 1.1 µM (EC_{50} da colchicina = 2.77 µM).



Os outros compostos testados não mostraram efeito na polimerização de tubulina. A piplartina foi também avaliada nesse ensaio em diversas concentrações para explorar de forma extensiva se ela provocava alguma alteração na polimerização da tubulina, já que esse composto mostrou atividade interessante nos

ensaios celulares. Entretanto, os resultados indicaram que a piplartina (NuBBE 18) não inibiu nem potencializou a polimerização de tubulina

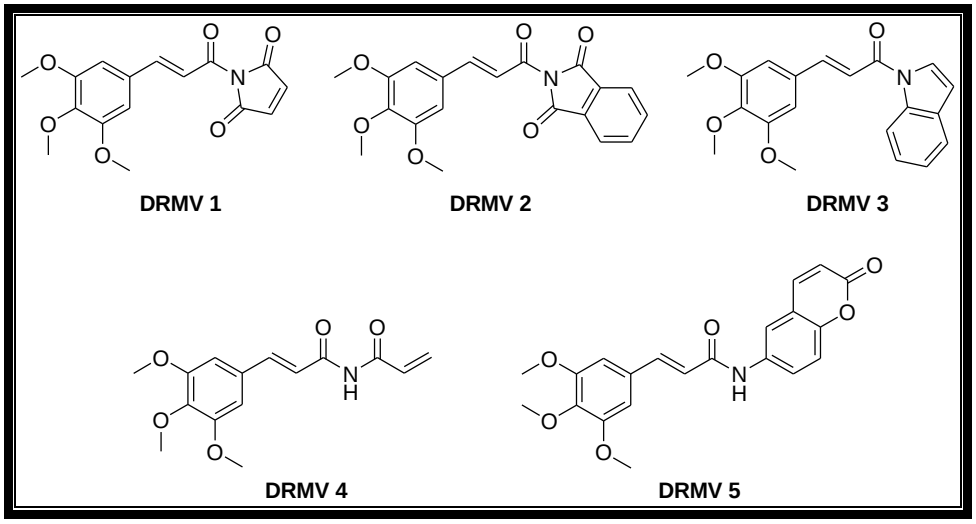


Piplartine (NuBBE 18)

Uma característica associada ao câncer é a metástase, processo biológico que permite às células cancerígenas migrarem para outras regiões do organismo sem diferenciação celular [STEEG, 2006; CHANG *et al*., 2011]. A metástase pode estar associada a maior agressividade de um câncer e, portanto a uma menor expectativa de vida do paciente [CHAMBERS; GROOM; MACDONALD, 2002]. Os Ensaios celulares *in vitro wound healing* [YUE *et al*., 2010] e de migração celular em câmara de Boyden (quantitativo) [ALBINI *et al*., 1987; SHAN *et al*., 2005] foram realizados a fim de avaliar os compostos quanto à capacidade de inibir a migração celular. A avaliação da citotoxicidade foi realizada visando testar a toxicidade dos compostos com as linhagens celulares cancerígenas MDA-MB-231 e MCF-7 [BARLTROP *et al*., 1991].

Os resultados obtidos no ensaio *wound healing* indicaram que as guanidinas NuBBE 43, NuBBE 423 e NuBBE 840 inibiram a migração celular, sendo que o composto NuBBE 840 apresentou-se como o mais potente entre os três. O grupo guanidínico mostrou-se importante para essa atividade, uma vez que a substituição isostérica do átomo de nitrogênio por um átomo de oxigênio ou de enxofre ocasionou a perda da atividade. A piplartina também apresentou atividade bastante relevante, inibindo a migração celular em 97%. A EC_{50} determinada pelo ensaio em câmara de Boyden para o composto NuBBE 840 foi de 2.98 ± 1 µM e para a piplartina 2.65 ± 1.1 µM (EC_{50} da colchicina = 0,5 µM). O ensaio citotóxico com as guanidinas NuBBE 423 e NuBBE 840 e a piplartina mostraram que esses compostos possuem menor toxicidade que o padrão doxorrubicina, como apresentado na Table 3, p. 55 desta tese.

Devido à relevante atividade apresentada, a piplartina foi selecionada como modelo para o planejamento de novos compostos, uma vez que apresentou relevante inibição de migração celular, além de estar descrito na literatura como citotóxico e seletivo, um perfil interessante na busca de protótipos antitumorais. Uma série de 5 novos compostos análogos de piplartina (DRMV 1-5) foi planejada e sintetizada visando maior compreensão de mecanismos de ação e melhoria na atividade biológica.



O análogo planejado por simplificação molecular (DRMV 4) se mostrou ativo na concentração de 10 µM inibindo 97% de migração celular no ensaio wound healing, com resultado comparável à piplartina. O composto DRMV 3 se mostrou medianamente ativo na concentração de 10 µM inibindo 50% da migração celular, sendo que os demais compostos sintetizados não apresentaram atividade. Uma avaliação da relação estrutura-atividade indica a importância do grupo aceptor de Michael presente na piplartina e em DRMV 4. Apesar deste grupo também estar presente no composto DRMV 1 ele não apresentou atividade na migração celular.

Os objetivos propostos neste projeto de pesquisa foram alcançados por meio de trabalho cooperativo e interdisciplinar nas áreas de química computacional, biológica, medicinal e de produtos naturais.

ABSTRACT

Natural products are an important source for the design of new drugs. This thesis aimed at the identification of bioactive natural products to be used as models for the design of compounds with antitumor properties. The lack of organized data is still one of the drawbacks in the natural products and medicinal chemistry area. Therefore, the compilation of accessible data of secondary metabolites from plant species or other sources is of great value, especially for the identification of molecular leads. This fact inspired us to propose as first objective of this thesis, the creation of the NuBBE database ($NuBBE_{DB}$) containing botanical, chemical, and biological information of the secondary metabolites obtained and published by NuBBE in 15 years. This database can be useful not only for the current research in natural products of the group, but for the scientific society interested in bioactive compounds, metabolomics, and dereplication. A scientific paper describing the creation of the database was published in the *Journal of Natural Products* in 2013. $NuBBE_{DB}$ compounds were used as molecular source for the virtual screening with the protein tubulin. Based on the results of the virtual screening the biological evaluation of selected compounds was performed with the protein tubulin, and cell migration assays (Wound Healing and Boyden Chamber). The results of the biological assays indicated a series of guanidines and piplartine as active compounds. Piplartine was selected to be a model for the design of new compounds because it inhibited cell migration and is described in the literature as cytotoxic and selective, an interesting profile for this project. A series of 5 analogue compounds were designed and synthesized aiming at a better understanding of structure activity relationship and improvement of the biological activity. The compound designed by molecular simplification showed activity in the cell migration assay comparable to piplartine. The objectives proposed in this research project were achieved by the interdisciplinary collaboration in the areas of computational, biological, medicinal, and natural products chemistry.

Keywords: Database. Natural products. Anticancer.

LIST OF ABBREVIATIONS

CAS number	Numerical identifiers assigned by the Chemical Abstracts Service
CDK	Chemistry Development Kit
cLogP	Calculated logarithmic of partition coefficient
DAPI	4',6-diamidino-2-phenylindole
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
EC_{50}	Compound concentration that caused a certain effect by 50%
EGTA	Ethylene glycol-bis(b-amino-ethyl ether) *N*,*N*,*N′*,*N′*-tetra-acetic acid
ESI-TOF	Electrospray ionization - Time of Flight
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HMBC	Heteronuclear Multiple Bond Correlation
GTP	guanosine triphosphate
HRMS	High Resolution Mass Spectrometry
LQMC	Laboratory of Computational and Medicinal Chemistry
MCF-7	human breast cancer cell line
MDA-MB-231	human breast cancer cell line
n-BuLi	*n*-Butyllithium
NMR	Nuclear Magnetic Resonance
nRotb	Number of rotatable bonds
NuBBE	Nucleus of Bioassays, Biosynthesis and Ecophysiology of Natural Products
$NuBBE_{DB}$	NuBBE database
PDB	Protein Data Bank
PIPES	Piperazine-*N*,*N′*-bis[2-ethanesulfonic acid] sequisodium salt
PK/DB	Database for Pharmacokinetic Properties
ROS	Reactive Oxygen Species

SAR	Structure-Activity Relationships
SBDD	Structure-Based Drug Design
SMILES	Simplified Molecular Input Line Entry System
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TPSA	Topological Polar Surface Area

SUMMARY

1. INTRODUCTION

1.1. Natural Products and the Brazilian Biodiversity

Brazil possesses an extremely rich biodiversity, accounting for approximately 20% of all known living species globally, which are found in several important biomes, such as the Amazonian and *Cerrado* regions [BOLZANI; CASTRO-GAMBOA; SILVA, 2010]. Most of the Brazilian plant species have not yet been studied [DE LUCA *et al*., 2012], which represents an economic potential to be explored in the identification of new bioactive agents [BOLZANI; CASTRO-GAMBOA; SILVA, 2010]. Tropical environments such as the Brazilian biomes offer particular potential for this task.

As reviewed extensively, biodiversity provides unique chemical scaffolds that have been used as templates for medicinal chemistry and drug discovery. [KOEHN; CARTER, 2005; CHIN, 2006; NEWMAN; CRAGG, 2007; NEWMAN, 2008; KINGHORN *et al*., 2011; NEWMAN; CRAGG, 2012]. Structural diversity is not the only reason that natural products are interesting for the development of new drugs. They often provide high selectivity and specific biological activity, based on distinct and in some cases new mechanisms of action. [NEWMAN; CRAGG; KINGSTON, 2008; LI; VEDERAS, 2009]. From a natural selection point of view, it is expected that compounds produced by an organism must be of value to the producer and therefore, natural products have naturally been developed in evolution for specific interactions with biomolecules. Previous to the establishment of modern synthetic medicinal chemistry, natural products were the primary source of medicinal compounds [MA; CHAN; LEUNG, 2011].

An analysis of the number of approved drugs shows that approximately 64% of all available drugs had a natural product involved in its development [NEWMAN; CRAGG, 2012]. This fraction includes the original natural products, derivatives and drugs which synthesis was inspired by a natural product. Furthermore, from 17 Plant Natural Products and Derivatives Approved by the U.S. Food and Drug Administration (FDA) from 2001 to 2010, two are new formulations of paclitaxel, revealing the great potential of natural products in the search for new classes of compounds with anticancer activity [KINGHORN *et al*., 2011].

The huge biodiversity present in Brazil is not consistent with the few examples of new drugs developed in this country. The complex system of drug discovery remains incipient in Brazil, and the transfer from basic research to preclinical and clinical research is quite new. Even with the latest advances in science and technology, few national pharmaceutical companies are involved in the research and development of new medicines from Brazilian natural resources. The topical anti-inflammatory *Acheflan*® is an interesting example of a medicine researched and developed completely in Brazil. It was introduced to the market by the pharmaceutical company *Aché Laboratórios* S.A. as an herbal medicine and contains a standardized mixture of the sesquiterpenes α-humulene and *trans*caryophyllen as active components. This mixture is present in the essential oil of *Cordia verbenacea* D.C. [PASSOS *et al*., 2007; FERNANDES *et al*., 2007]. This topical medicine is currently very successful in Brazil, and, following FDA approval, has been introduced in the USA, Canada, and Japan.

Another example of an herbal medicine recently launched on the market is *Fitoscar*®. It is a mixture of phenolic derivatives present in standardized dry extract of *Stryphnodendron adstringens* (Mart.) Coville, which was developed by *Apsen Farmacêutica*. This product is the first example of an herbal medicine developed from a plant from the Brazilian *Cerrado* biome [MINATEL *et al*, 2010], a genuine Brazilian ecosystem considered a hotspot of biodiversity [MYERS *et al*., 2000].

There are several research groups in Brazil that focus on exploring this rich biodiversity rationally. One of these is the Nucleus of Bioassays, Biosynthesis and Ecophysiology of Natural Products (NuBBE) research group, which has been involved in the latest advances in natural product chemistry, including metabolomics, medicinal chemistry, and the search for biologically active compounds from plants of the *Cerrado*, the Atlantic forest and plant endophytic fungi [BOLZANI; CASTRO-GAMBOA; SILVA, 2010; SILVA *et al*., 2006; JOLY *et al*., 2010].

Furthermore, in 1999, NuBBE was one of the first Brazilian natural products chemistry research groups involved in the creation of the Virtual Institute of Biodiversity, BIOTA-FAPESP, an ongoing successful program in the state of São Paulo, Brazil, nowadays a recognized Worldwide Biodiversity Program, a useful example of how conservation initiatives with a solid scientific basis can be achieved [JOLY *et al*., 2010]. The bioprospection project of Biota FAPESP afforded about 2000 extracts of over 800 species. The pharmacological and chemical diversity of

compounds isolated in NuBBE can be seen in the Table 1 and Figure 1. The chemical classes are diverse and the isolated compounds include terpenes, alkaloids, flavonoids, iridoids, lignans, phenylpropanoids, and chromenes. This diversity has great potential to be exploited by medicinal chemistry and drug discovery programs.

Table 1. Pharmacological properties of the natural products isolated in NuBBE for caseobliquin A (**1**, *Casearia* sp.), (–)-spectaline (**2**, *Senna* sp.), leptomerine (**3**, *Esenbeckia leiocarpa*), 6α-malonoyloxymanoyl oxide (**4**, *Stemodia foliosa*), indole alkaloids (3,4-dehydro-strictosidine, **5**, *Chimarrhis turbinata*), (+)-erythravine (**6**, *Erythrina mulungu*), piplartine (**7**, *Piper* sp.), piperamides (*Piper* sp.), pterogynidine (**8**, *Alchornea glandulosa*), guanidine alkaloids (*Pterogyne nitens*), chromenes ((2*S*)-2-methyl-8-(3''-methylbut-2''-enyl)-2-(4'-methylpent-3'-enyl)-2*H*-chromene-6-carboxylic acid, **9**, *Piper* sp.), tetrahydrofuran lignans (*Peperomia blanda*), iridoids (*Alibertia* sp.), *nor*-lignans (*Styrax ferrugineus*) and xanthones (*Arrabidaea samydoides*).

Pharmacological diversity of natural products from NuBBE			
Acetylcholinesterase inhibitors (–)-spectaline leptomerine	**Anti-trypanosomal** chromenes piplartine tetrahydrofuran lignans	**Antifungal** piperamides iridoids *nor*-lignans	**Antibacterial** *nor*-lignans 6α-malonyloxymanoyl oxide
Antioxidant indole alkaloids xanthones flavonoids	**Anxiolytic** (+)-erythravine	**Antiangiogenic** pterogynidine	**Cytotoxic** caseobliquin A guanidine alkaloids piplartine

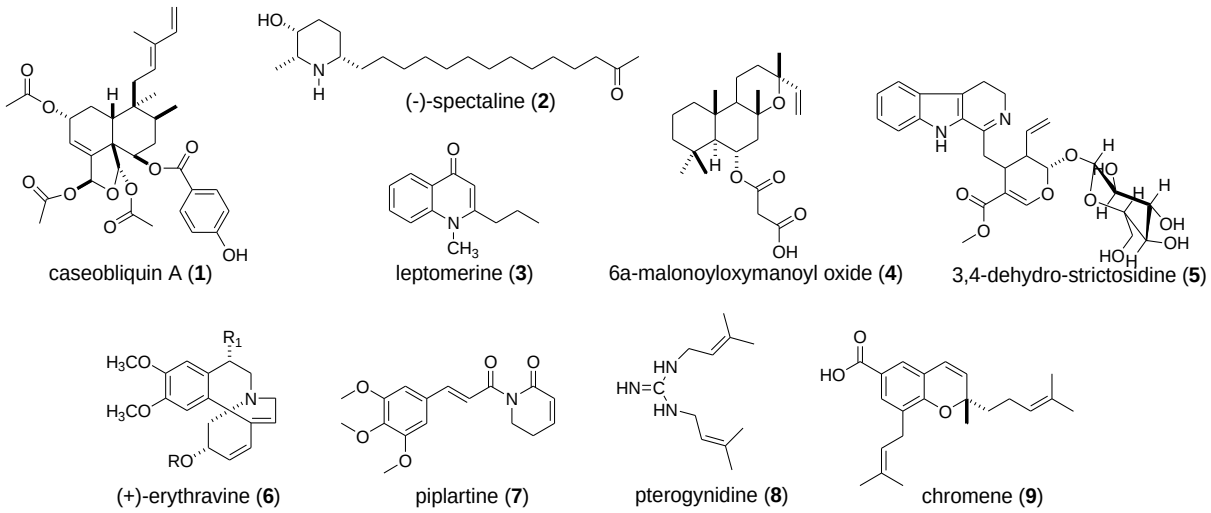


Figure 1. Some examples of the chemical diversity of the natural products isolated in NuBBE.

The classical phytochemical analysis consisted in randomly isolating compounds. Although this method has resulted in the isolation of many bioactive compounds during the years, it is time consuming and often results in a simplistic analysis of the system. New analytical, computational, and biotechnological techniques have been used with considerable success, especially for metabolomics and proteomics, considered modern approaches, involving the state of the art in the area of natural products [SHRIPSEMA, 2010; VERPOORTE; CHOI; KIM, 2010; CHIN *et al*., 2006; NEWMAN; CRAGG; KINGSTON, 2008].

The availability of natural compounds libraries is of significant importance for the integration of natural products and medicinal chemistry areas, especially for the rational design of compounds in drug discovery [DE LUCA *et al*., 2012].

1.2. Rational Design of Compounds

The definition of a modern medicinal chemistry strategy, with a strong multidisciplinary interaction is essential for the research success in this area. Chemo and bioinformatics tools are integrated with experimental techniques, being very useful in these studies aimed at the identification, selection and optimization of compounds with biological activity, and may evolve into the discovery of new drug candidates.

The design of new bioactive compounds includes the identification of the macromolecular target, selection of bioactive ligands (hits), optimization of lead compounds and the development of structure-activity relationships (SAR) [SALUM; ANDRICOPULO, 2009]. SAR studies have gained special attention in the optimization of molecular properties. Typically, the structure of lead compounds are modified by synthesis with the purpose of enhancing the pharmacodynamic (potency, affinity, selectivity) and pharmacokinetic properties (absorption, distribution, metabolism, excretion), and minimize or eliminate unwanted properties (toxicity) [ANDRICOPULO; MONTANARI, 2005]. A brief description of one of the drug discovery models is described in Figure 2.

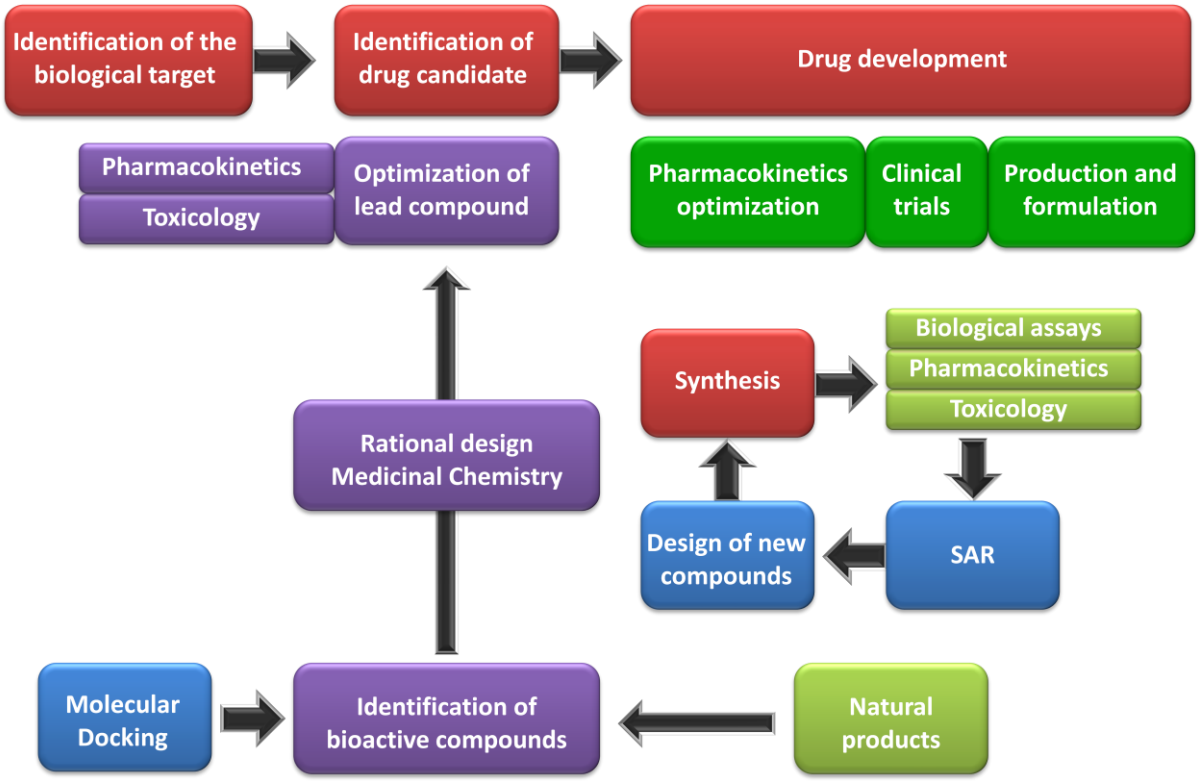


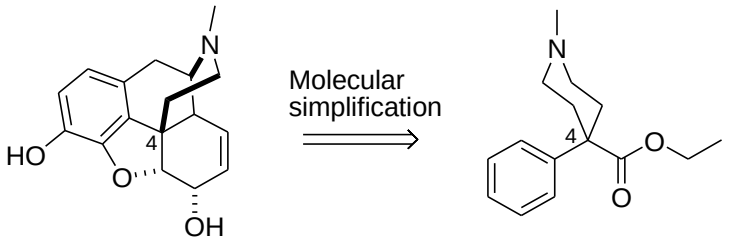
Figure 2. Natural products and medicinal chemistry as tools for drug discovery

For the design of modifications of the lead compound, techniques such as molecular simplification and hybridization have been extensively used. These techniques were used in this work and will be briefly described herein.

1.2.1. Molecular Simplification

Molecular simplification represents a drug design strategy to shorten synthetic routes while keeping or enhancing the biological activity of complex structures. Initially, this concept was applied empirically to natural products in order to obtain active derivatives with a simplified molecular structure. Furthermore, removal of non pharmacophoric subunits makes possible the reduction of unwanted chemical and pharmacological properties. Classical examples are the molecular simplification of the natural alkaloids morphine and quinine, which led to several drugs currently used in therapy. Morphine (**10**, Figure 3) is the model for the 4-phenylpiperidine system represented by pethidine (**11**, Figure 3) used in analgesia. Pethidine structure contains the piperidine ring in which the C-4 carbon is linked to the benzene ring,

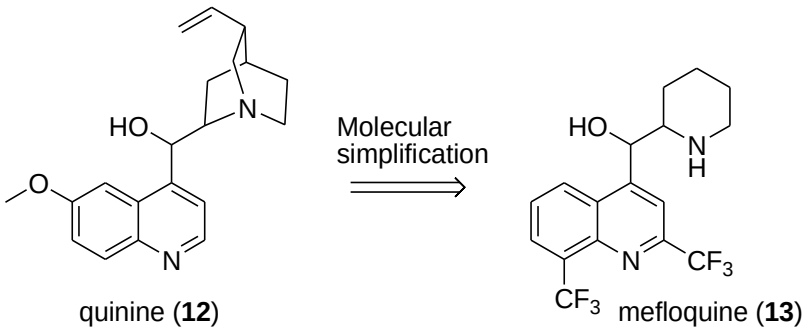
which altogether is the pharmacophoric group for the analgesic opioids (Figure 3) [BARREIRO; FRAGA, 2008].



morphine (**10**) pethidine (**11**)

Figure 3. Molecular simplification of morphine.

Quinine (**12**, Figure 4) is one of the main components of the bark of *Cinchona officinalis*. This alkaloid has recently been a model of molecular simplification for the design of new antimalarial derivatives, such as mefloquine (**13**, Figure 4). This molecular simplification of quinine mainly consisted in the change of the quinuclidine ring for a piperidine ring. The trifluoromethyl substituents were added for metabolic reasons. Mefloquine appeared as an innovation for old drugs such as chloroquine (Figure 4).



quinine (**12**) mefloquine (**13**)

Figure 4. Molecular simplification of quinine.

The molecular simplification approach is particularly interesting in the development of new anticancer drugs, since a large number of current anticancer chemotherapeutics is derived from natural products that have complex chemical structures [GARCÍA *et al*., 2010; BARREIRO; FRAGA, 2008].

1.2.2. Molecular Hybridization

Molecular hybridization is another strategy for the rational design of new compounds and consists in the combination of defined structural features of two or more bioactive compounds in a new designed compound. It is based in the recognition of pharmacophoric sub-unities in the known bioactive compounds, and through the appropriate fusion of these sub-unities, leads to the design of new hybrid architectures that maintain pre-selected characteristics of the original templates.

There are some examples of molecular hybridization for cytotoxic agents [DECKER, 2011; ROMAGNOLI *et al*., 2013; BARALDI *et al*., 2002], one of them is the synthesis of a series of hybrids of uramustine, a derivative of nitrogen mustard and uracil, (**14**, Figure 5) and the natural product distamycin A, originally isolated from *Streptomyces distallicus* (**15**, Figure 5) [BARALDI *et al*., 2002, DECKER, 2011]. The result of this hybridization was six new compounds with superior cytotoxic activity for leukemia cell line K562, when compared with the two initial model compounds.

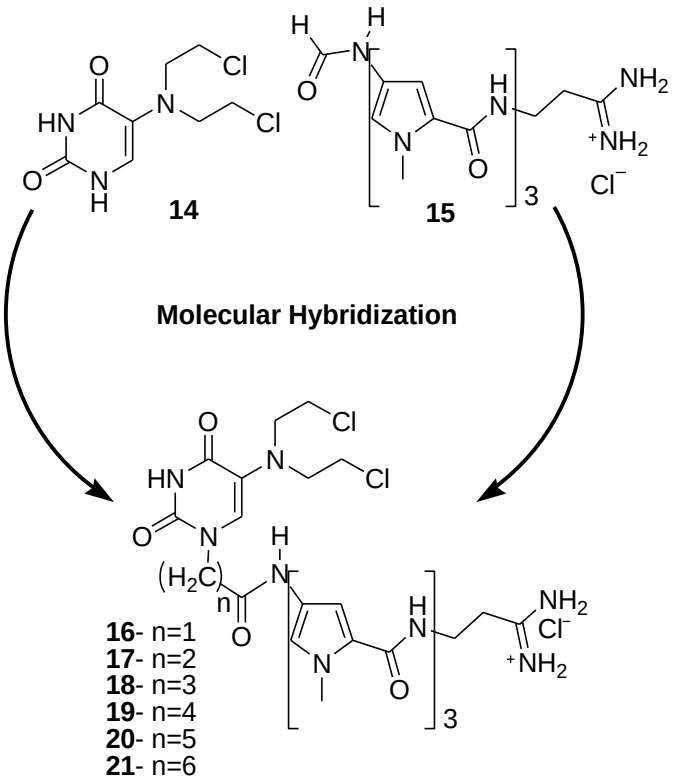


Figure 5. Molecular hybrids of distamycin A and uramustine

Compound **16** (EC_{50} = 4.06 µM), **17** (EC_{50} = 2.54 µM), and **18** (EC_{50} = 7.26 µM), which have a lower spacer showed moderate activity. The activity was enhanced with the increase of the spacer (**19**, EC_{50} = 0.11 µM; **20**, EC_{50} = 0.14 and **21**, EC_{50} = 0.07 µM). The cytotoxic activity of **21** is more than 1000 times higher than distamycin A (**15**, EC_{50} = 0.07 µM). This example shows the potential of the molecular hybridization strategy and highlights the importance in designing the appropriate spacer [VIEGAS JUNIOR *et al*., 2007; BARALDI *et al*., 2002].

1.3. Mechanism of Action and Molecular Targets of Anticancer Agents

The purpose of this thesis is to design new compounds with antitumor properties. Cancer is a group of diseases characterized by uncontrolled cell growth, invasion of surrounding tissues and spreading to more distant tissues of the body (metastasis) [PATRICK, 2009]. Only 5-10 % of cancer cases are attributed to genetic factors, while the remaining 90-95 % is attributed to external or environmental factors such as smoking, diet, radiation, and virus [ANAND *et al*., 2008]. These factors promote the mutation of the genetic material in specific genes resulting in cancer development [CROCE, 2008]. In recent decades, this disease has reached alarming proportions, becoming an evident public health problem worldwide. The World Health Organization (WHO) estimated that in 2030, we expect the incidence of 21.4 million cases of cancer and 13.2 million deaths because of this disease. In Brazil, estimates for the year 2014, and also valid for 2015, indicate the occurrence of approximately 576 thousand new cases of cancer [INSTITUTO NACIONAL DE CÂNCER, 2014].

The treatments available for this disease include surgery, radiotherapy, and chemotherapy. The design of new drugs for cancer treatment has been in focus for greater selectivity and is currently held in a rational and interdisciplinary way. Natural products have proved as a source of valuable chemical and biological diversity, especially for their intricate structural complexity and functional diversity. They are involved in the development of about 77% of drugs available for cancer treatment [NEWMAN; CRAGG 2012]. Strategies such as the search for compounds with mechanisms of action in specific biological targets have accelerated and directed the design of drugs, including antitumor, in which molecular targets are already known.

Tumors and cancer are the result of uncontrolled cell division. Normally, cell division is regulated by a family of extracellular growth factors, proteins that cause resting cells to divide and, in some cases, differentiate. Defects in the synthesis, regulation, or recognition of growth factors can lead to cancer [PATRICK, 2009].

In general, the pathogenesis of cancer is associated with mutation of genetic material in specific genes, the proto-oncogenes that encode proteins involved in processes of cell division and differentiation. After mutation, the proto-oncogenes become the oncogenes, which encode abnormal proteins, favoring uncontrolled growth of cells. Furthermore, cancer cells have defects in tumor suppressor genes, removing the natural barriers of cell division. These genes act as defense mechanisms against genetic faults, encode proteins that identify and correct flaws in the DNA, and induce apoptosis if the damage is irreversible. The gene *TP53* is a tumor suppressor gene, as it encodes for the protein p53, which has a role in conserving stability by preventing genome mutation. Mutations in the gene p53 are present in about 50% of all human cancers, stating the relevance of these genes in the pathogenesis of this disease. Generally, cancer is the result of an accumulation of mutations in oncogenes and tumor suppressor genes [PATRICK, 2009; NELSON; COX, 2004]. Through these mutations, cancer develops a set of functional abilities such as sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [HANAHAN; WEINBERG, 2011].

Several strategies have been developed to fight the abnormal cellular mechanisms acquired by cancer. Often these strategies include modulation of proteins involved in the cell cycle. Several proteins that are molecular targets for treating cancer are presented in Figure 6. Among theses, telomerase, topoisomerase, tubulin, are intensively studied. Several natural products of different classes act to modulate these targets as also shown in Figure 6 [NEWMAN, D.J.; CRAGG, G.M.; KINGSTON, 2008].

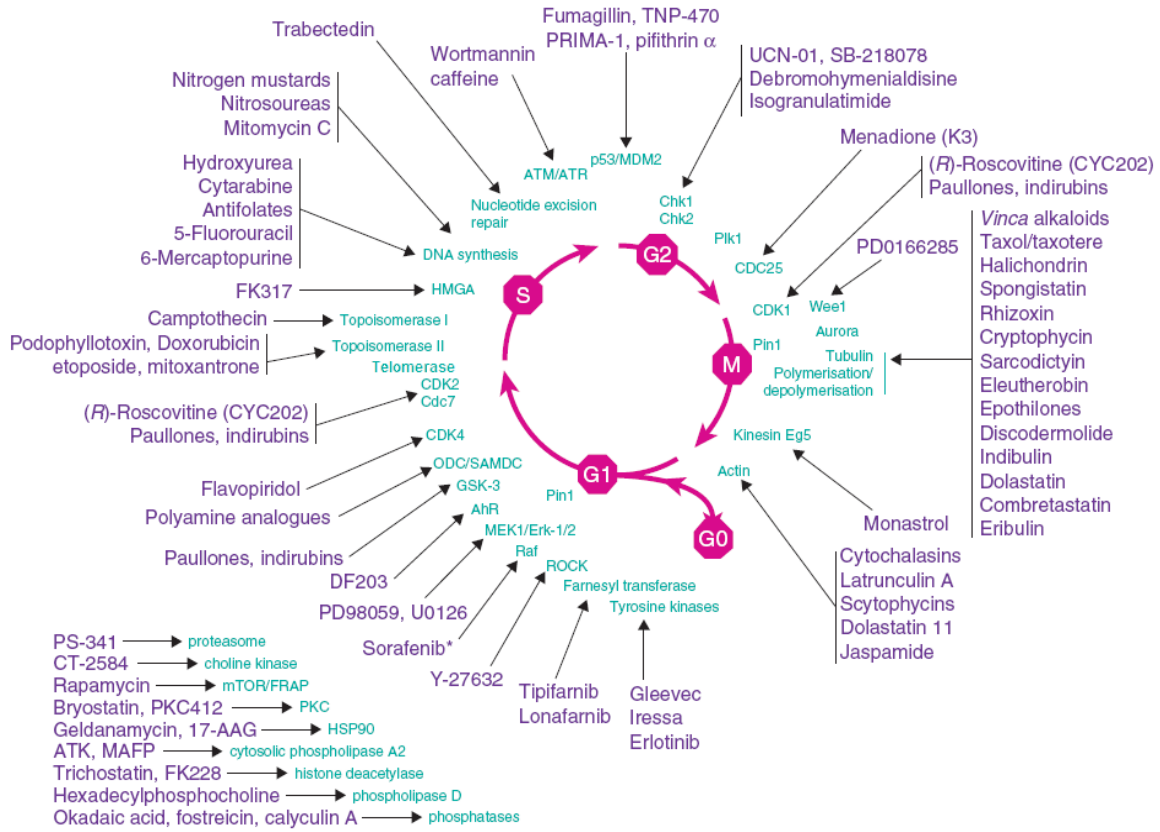


Figure 6. Natural products and the cell cycle. (NEWMAN, D.J.; CRAGG, G.M.; KINGSTON, 2008).

Microtubules are an important target for anticancer drugs, they are essential in cell mitosis, during which the duplicated chromosomes of a cell are separated into two identical sets before the cell dividing. Microtubules are essential in the development and maintenance of cell shape, in the transport of vesicles, mitochondria and other components throughout cells, in cell signaling, cell division and mitosis. They are composed of α-tubulin and β-tubulin heterodimers, and their polymerization dynamics are tightly regulated both spatially and temporally. Tubulin is the repeat unit of microtubules, with a molecular weight of approximately 55 kDa [JORDAN; WILSON, 2004; LODISH *et al*., 2007].

The interference in the microtubule dynamics blocks mitosis and induces cell death. Pharmacological agents that act in this way can be divided into two groups: (a) drugs that inhibit and destabilize microtubule formation; (b) drugs that stabilize and increase tubulin polymerization. The type (a) mechanism is described, for example, for colchicine (**22**), vincristine and vinblastine (**23**, **24**) and type (b) is

described for paclitaxel (**25**) (Figure 7). Both destabilization and stabilization of microtubules induce cell death [JORDAN; WILSON, 2004].

Taking into account the success of these classes of drugs, microtubules represent an important cancer target, and continue to be important chemotherapeutic agents [JORDAN; WILSON, 2004].

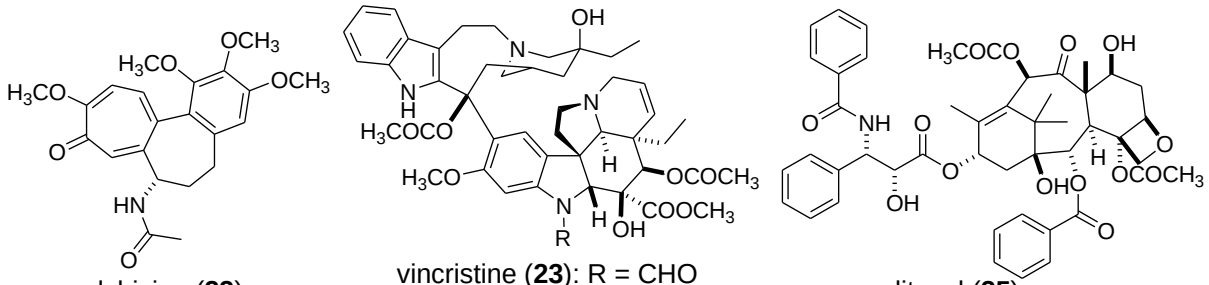


Figure 7. Anticancer compounds tubulin-targeting agents.

Another characteristic of cancer is metastasis, the biological process of cancer cells migrating to other body areas without cellular differentiation [STEEG, 2006; CHANG *et al*., 2011]. Metastasis is associated with more aggressive cancer and thus a lower life expectancy for the patient [LANG *et al*., 2006; CHAMBERS; GROOM; MACDONALD, 2002]. Many patients die not because of the primary tumor, but of metastasis. It is responsible for more than 90% of cancer-associated mortality [BRABLETZ, 2012; GUPTA, 2006]. The suppression of metastasis is an urgent need in cancer treatment; however, most existing drugs only inhibit cell proliferation [WEBER, 2013]. Traditionally, chemotherapy is based on therapeutic agents with cytotoxic property, which inhibit cell proliferation and cause cell death. Recently, the strategy of inhibiting cell migration has gained considerable interest [GAUL *et al*., 2004].

Some compounds that inhibit the process of metastasis are described in the literature [OGASAWARA; MATSUBARA; SUZUKI, 2001a; LANG *et al*., 2006]. Paclitaxel is originally found to be an antimitotic agent acting through stabilizing microtubules. Additionally, paclitaxel exhibited marked and selective inhibition of tumor cell migration [OGASAWARA; MATSUBARA; SUZUKI, 2001b]. Evodiamine is one of the main constituents of *Evodiae Fructus* that possess antitumor activity. This compound is also effective in inhibiting invasion and metastasis of several cancer

cell lines, being regarded as a promising agent in tumor metastasis [OGASAWARA; MATSUBARA; SUZUKI, 2001b; OGASAWARA *et al*., 2002]. Further examples are the natural product migrastatin, secreted by *Streptomyces* and their synthetic analogues. They are potent inhibitors of tumor cell migration, invasion and metastasis [DIAS *et al*., 2010; GAUL *et al*., 2004; NAKAE *et al*., 2000; NJARDSON *et al*., 2004].

1.4. Chemical Databases

A literature review of databases for medicinal and biological chemistry and natural products revealed some useful available databases. One of them is ZINC, a database of commercially available compounds for structure-based virtual screening. ZINC contains information of over 21 million compounds and is the most used database for computational chemistry and virtual screening [ZINC..., 2014; IRWIN; SHOICHET, 2005]. The Dictionary of Natural Products [DICTIONARY OF NATURAL PRODUCTS, 2014] and the Super Natural Database are databases of general data of natural compounds [Super Natural Database, 2014; DUNKEL *et al*., 2006]. There are also regionally specific databases such as the Traditional Chinese Medicine Database, containing three-dimensional structural information of traditional Chinese medicine constituents [TRADITIONAL CHINESE MEDICINE DATABASE, 2014; CHEN, 2011]; NeMedPlant [MEETEI *et al.*, 2012] containing information on medicinal plants from India; the Customary Medicinal knowledgebase (CMKB) [GAIKWAD *et al*., 2008] containing Australian native medicinal plants; specific database for biological activity such as NPACT [MANGAL *et al*., 2013] containing antitumor compounds; and specific databases for chemical classes such as the Alkamid database [BOONEN *et al.*, 2012]. A few more important databases are the noncommercial repository for ethnobotanical data available from the International Ethnobotany Database (ebDB) [INTERNATIONAL ETHNOBOTANY DATABASE, 2014]; NAPRALERT [LOUB *et al*., 1985; SHARMA; SARKAR, 2012] a paid database designed for the identification and analysis of experimental data related to natural products, the UCSD Marine Natural Products Database that collects all the publically available natural products data regarding their own and publically available collections [UCSD, 2014] and Universal Natural Products Database, a Peking University database with more than 200 thousand compounds [GU, *et al*., 2013].

Additionally, information on taxonomy, biosynthetic pathways and genomic are accessible through the National Center for Biotechnology Information (NCBI) [SAYERS *et al*., 2010], the Kyoto Encyclopedia of Genes and Genomes (KEGG) [KANEHISA; GOTO, 2000] and KNApSAcK [AFENDI *et al*., 2012].

The availability of natural products databases is of significant importance for *in vitro* and *in silico* screening studies in drug discovery [DE LUCA *et al*. 2012]. Danishefsky stated in 2002 that “a small collection of smart compounds may be more valuable than a much larger hodgepodge collection mindlessly assembled” [BORMAN, 2002]. This statement places natural products libraries in a remarkable position [HENKEL *et al*., 1999]. It is a common situation that a natural product is isolated, characterized and biologically evaluated, but there is no sequence of more advanced research for input in the process for product development. The difficulties in the complex pharmaceutical innovation process are frequently associated with the basic research carried out in academia for lack of an interaction between groups of different expertise.

The absence of a database of natural products from the Brazilian biodiversity, led us to develop the NuBBE database ($NuBBE_{DB}$) as one of the objectives of this thesis. This database can represent the starting point for cataloguing all chemical and pharmacological information of the Brazilian biodiversity.

2. OBJECTIVES

The main objective of this Ph.D. thesis is to identify natural products that may serve as models for the design of new compounds with antitumor properties. The specific goals are outlined below.

(i) Creation of a database of natural products containing the secondary metabolites published by NuBBE including botanical, chemical, and biological information;

(ii) Tubulin based virtual screening of NuBBE database, and biological activity evaluation of selected compound by *in vitro* tubulin polymerization assay and cell migration assays;

(iii) Design of new compounds based in a natural product model, synthesis and biological evaluation aiming at a better understanding of the structure-activity relationship and an enhanced biological activity.

3. RESULTS AND DISCUSSIONS

3.1. NuBBE Database Creation

The chemical information on Brazilian biodiversity collected over the years is fragmented and, thus, very difficult to access readily. Owing to the absence of any database of secondary metabolites from the Brazilian biodiversity, herein is described the design and development of a Web-based, freely available, and easy to access database of natural products and synthetic analogues from the Brazilian biodiversity called the NuBBE database ($NuBBE_{DB}$) [VALLI *et al*., 2013]. The $NuBBE_{DB}$ is the result of an effective collaborative project between the NuBBE group and the Laboratory of Computational and Medicinal Chemistry (LQMC, USP−São Carlos), which has experience in the development of innovative databases, such as PK/DB, a robust tool for pharmacokinetic studies and *in silico* ADME predictive models [MODA *et al*., 2008]. The scientific community may benefit from this specialized tool in studies of natural products and medicinal chemistry, including dereplication, metabolomics, virtual screening, and the design of new biologically active compounds.

The data published by the NuBBE research group, a total of 170 scientific papers containing information on pure compounds, were analyzed, and, to date, the $NuBBE_{DB}$ features a total of 640 compounds, which will be continually updated as new information becomes available. The database can be freely accessed via a Web interface at http://nubbe.iq.unesp.br/nubbeDB.html [NÚCLEO..., 2014].

The $NuBBE_{DB}$ includes a variety of information for each compound including chemical class and name; code, molecular formula, and mass; and source. Whenever available, biological, pharmacological, and toxicological information (qualitative and quantitative) are also included, with the corresponding references. Other molecular and physicochemical properties provided are lipophilicity (cLogP), Topological Polar Surface Area (TPSA), number of hydrogen-bond acceptors and donors, number of rotatable bonds (nRotb), the number of Lipinski's "rule of five" violations, and molecular volume. Chemical structures are represented by a SMILES string, and the 3D conformations are available in Mol2 file format, widely employed in molecular modeling studies [COLE; NISSINK; TAYLOR, 2005; JAIN, 2003; EWING *et al*., 2001; RAREY *et al*., 1996]. These properties were selected to be available in

the database due to the importance of these parameters for medicinal chemistry, and may be used to assist the understanding of the pharmacokinetic behavior of drug candidate compounds. For all compounds included in the database, a NuBBE code is assigned for identification.

To search compounds in the database, a Web-based search tool is available, incorporating a molecular drawing interface enabling the user to search for compounds by property, chemical structure, or a combination of criteria. The results are displayed in the same Web session, where it is possible to see the main properties, the chemical structure of the compound, a link to download the 3D structure in Mol2 file format, and a table of information from each compound in pdf format (or for all compounds in one click). By clicking the compound, all information is displayed. The Web system was designed to allow the scientific community to search, browse, and download molecules, providing a rapid response to specific queries.

In order to facilitate the analysis of the database, the compounds were grouped by acquisition source: 80% of the compounds were isolated from plants, 7% are semi synthetic, 6% were isolated from fungi/microorganisms, 5% are synthetic (inspired by a natural product), and 2% are products of biotransformation using a plant or fungi extract (Figure 8). The next step of this work, and one of the most striking achievements of the creation of this database, is to add as much as possible information on all secondary metabolites isolated from species of other representative Brazilian ecosystems, including compounds from marine organisms, which were not studied in the same depth as terrestrial organisms.

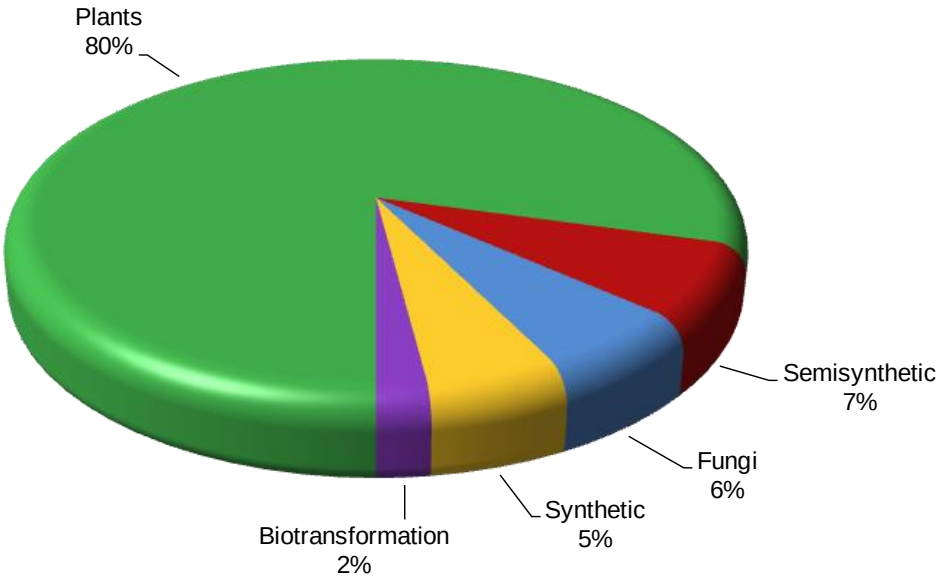


Figure 8. Source statistics of $NuBBE_{DB}$ compounds. It can be seen that plant compounds represents the majority source. The semisynthetic and synthetic sources represent 11% of the database and these compounds are inspired on natural products.

Molecular mass, cLogP, nRotb, TPSA and the number of hydrogen-bond donors and acceptors are useful descriptors for predicting “drug-likeness” of small molecules [LIPINSKI *et al*., 1997; VEBER *et al*., 2002; LIPINSKI, 2004; CLARK; PICKETT, 2000; LIPINSKI, 2000]. The average molecular mass of the set of compounds is 386.3, indicating the presence of large compounds, although, below the threshold proposed by Lipinski [LIPINSKI *et al*., 1997]. Altogether 485 compounds (75% of the database) violate fewer than two of Lipinski’s four parameters. If two or more parameters are out of range, a poor absorption or permeability is predicted [LIPINSKI *et al*., 1997]. As this is a natural product database, it is expected that it should be chemically rich and highly diverse in its contents. The database presents a suitable profile for the evaluation and identification of bioactive compounds for drug design studies [VALLI *et al*., 2013].

The importance of $NUBBE_{DB}$ is demonstrated by its inclusion in the ZINC database platform, created by Shoichet Laboratory in the Department of Pharmaceutical Chemistry, University of California, San Francisco (UCSF). ZINC is a

reference in the field of medicinal chemistry, and the cross-link ZINC/NuBBE$_{DB}$ is as international recognition of the data generated in this project. The cross-link provides additional information such as CAS number, and suppliers for the compounds which are commercially available. The NuBBE$_{DB}$ has also been considered by the Royal Society of Chemistry to be included in the ChemSpider database and is beginning to be currently used by the scientific community [VILLOUTREIX *et al*., 2013]. Also, its importance has being mentioned by different scientific magazines and websites [INTERNATIONAL COOPERATIVE..., 2014; ARANTES, 2014; MARQUES, 2012; MARQUES, 2014].

Particularly for this thesis, NuBBE$_{DB}$ was the source of compounds for virtual screening studies, in order to select compounds with higher biological potential for a selected target.

3.2. Virtual Screening (Docking)

Computational methods have become a crucial component for compound identification and optimization of many drug discovery programs [KITCHEN *et al*., 2004]. Virtual screening can be broadly defined as the use of computational analysis of a database of chemical structures to identify possible candidates for interaction with a specific biomolecular target. The main benefit of virtual screening is the remarkable reduction in time and resources required to screen an entire chemical library in a drug discovery project. By identifying non-binding compounds, the number of compounds to be tested *in vitro* is reduced. Additionally, due to the elimination of these inactive compounds, the hit rates in the assays are often much higher. Today, computational chemistry and chemoinformatics are widely used in early phase drug research, by identifying the most promising candidates for experimental investigations [MA; CHAN; LEUNG, 2011].

In this work, the objective of the virtual screening was to select potential microtubule modulators within NuBBE$_{DB}$ compounds to test them in the *in vitro* biological activity with tubulin. The method used to make the virtual screening was the molecular docking, one of the most popular for structure-based drug design (SBDD). This method allows the analysis of the interaction between compounds and a target protein and the prediction of the likely bioactive conformation [LEACH; GILLET, 2007]. There are many docking programs available, which are differentiated

by the search method and score function. Most of the approaches consider the protein as rigid and the ligands flexible [KITCHEN *et al*., 2004; TAYLOR; JEWSBURY; ESSEX, 2002].

The virtual screening was done with the compounds available in $NuBBE_{DB}$ and additional ones obtained by NuBBE but not yet published in the literature. Issues with supply can become a major bottleneck in natural products drug discovery, and therefore, the additional compounds were included in the virtual screening because they were available in sufficient quantities for further *in vitro* biological assay. They received an internal NuBBE code for control but, to date, they are not available in $NuBBE_{DB}$.

Both destabilization and stabilization of microtubules induce cell death [JORDAN; WILSON, 2004]. Therefore, three tubulin binding sites were separately considered for the docking studies, (paclitaxel, vincristine/vinblastine, and colchicine binding sites) as interaction with any of the binding sites would be interesting for this screening (Figure 9).

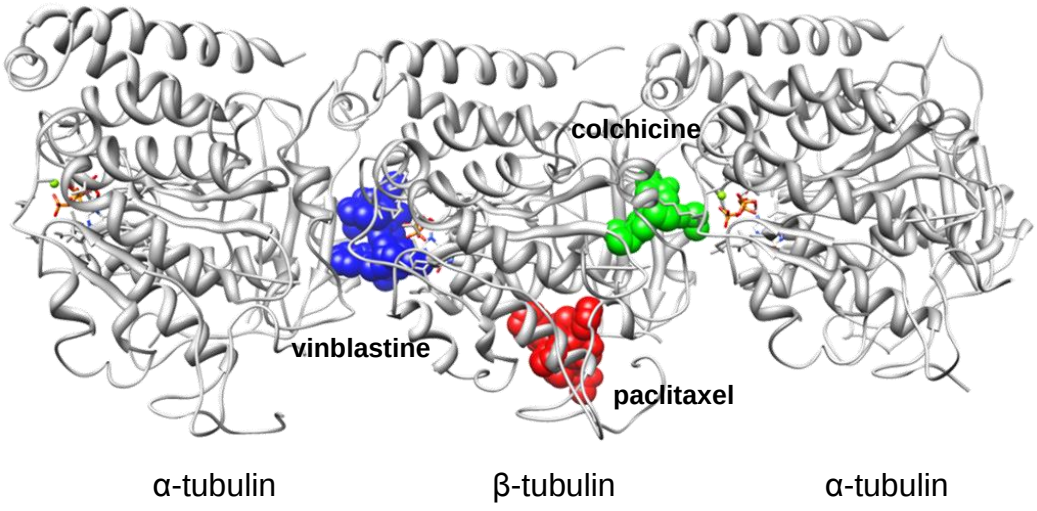


Figure 9. Graphical representation of vinblastine, paclitaxel and colchicine in their binding sites of tubulin.

The original results of the virtual screening are available at Appendix A. The docking hits were selected as the compounds with the best score values for any of the binding sites. Although the calculated score values are often not accurate, they are still broadly useful for selecting the most promising compounds for the *in vitro* evaluation [MA; CHAN; LEUNG, 2011]. The selected docking hits were evaluated in

the *in vitro* assays and some of the classes of compounds of these hits were casearins, chalcones, flavonoids, and guanidines.

3.3. Tubulin Polymerization Assay

In this work we evaluated the tubulin polymerization with two different assays. Light scattering is the classical method for the evaluation of microtubule polymerization. The intensity of the scattered light is proportional to the concentration of microtubule [SHELANSKI; GASKIN; CANTOR, 1973]. Polymerization can also be followed by fluorescence enhancement due to the incorporation of a fluorescent reporter, such as 4',6-diamidino-2-phenylindole (DAPI), into microtubules as polymerization occurs [BONNE *et al*., 1985; DYRAGER *et al*., 2011]. DAPI exhibits a maximum electronic transition in 342 nm and emits a maximum fluorescence at 470 nm. This fluorophore binds to tubulin dimers with a K_d of 43±5 μM and to polymerized tubulin with a K_d of 6±2 μM. The difference in affinity of DAPI for tubulin dimers and microtubules results in a difference in fluorescence intensity, which is quantified in the biological assay. Compounds that interact with tubulin can change the fluorescence intensity. The increase of polymerization caused by a compound like paclitaxel provides an increase in fluorescence. The opposite occurs with compounds which inhibit tubulin polymerization (Figure 10).

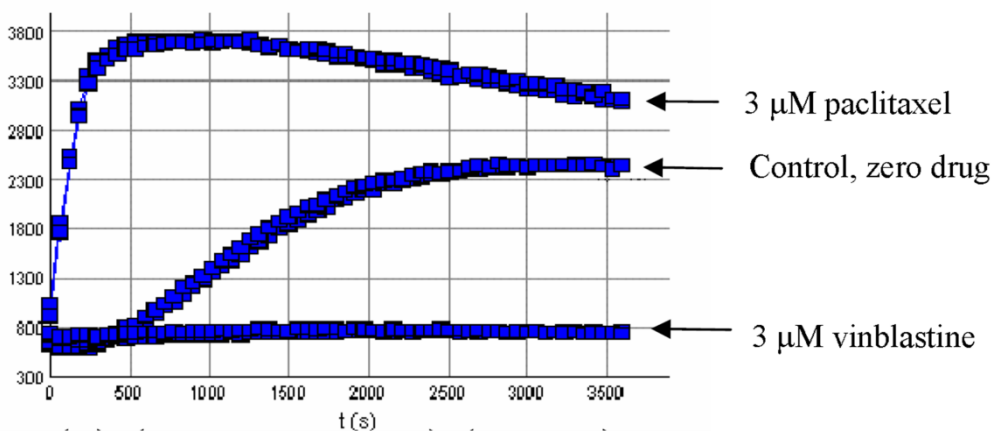


Figure 10. Standard polymerization observed in the fluorescence based tubulin polymerization assay. For control, there is a normal polymerization. The activity of paclitaxel cause an increase of polymerization and therefore an increase in the fluorescence measured. In contrast, the effect of vinblastine or colchicine decrease the polymerization of tubulin and the fluorescence measured [CYTOSKELETON, 2014].

DAPI interacts at a different binding site to that of paclitaxel, colchicine, or vinblastine and it does not interfere with the polymerization of microtubules. Even in substoichiometric amounts, this fluorophore can be used to assess the kinetics of microtubule formation. There is an interference that could possibly occur in this test, which is the interference of the reporter with the test compound.

The fluorescence assay, although more sensitive, is subject to interference of DAPI with the test compound. Therefore, tubulin polymerization was also measured by light scattering for further evaluation of the results with higher reliability. *In vitro* assays are important in the search for new bioactive compounds; however they should be constantly discussed and evaluated.

3.3.1. Fluorescence Based *in vitro* Tubulin Polymerization Assay

The purpose of this test is to identify natural products that modulate the activity of the protein tubulin. Based on the results of virtual screening and with a restriction of the compounds that were available in sufficient quantity (1 mg), 35 compounds (Appendix B) were selected for the *in vitro* fluorescence based evaluation of tubulin polymerization.

Initially, the assay was performed in single concentration (250 μM) and the compounds that showed some activity were further tested for the determination of EC_{50}. The single concentration results revealed that the casearins (NuBBE 57, 59+60, 61 and NuBBE 635-640) increased the polymerization of tubulin, being NuBBE 59+60 mixture and NuBBE 640 the most potent. Figure 11 presents the results obtained for the casearins, together with the positive (colchicine) and negative controls (DMSO). These results show an increase in tubulin polymerization assessed by the increase of fluorescence when compared to negative control. The observed effect is similar to that of paclitaxel, and it is noteworthy that casearins are also diterpenes such as paclitaxel. Amentoflavone (NuBBE 200) acted as inhibitor of tubulin polymerization. Figure 12 shows the results obtained in the polymerization assay for this compound.

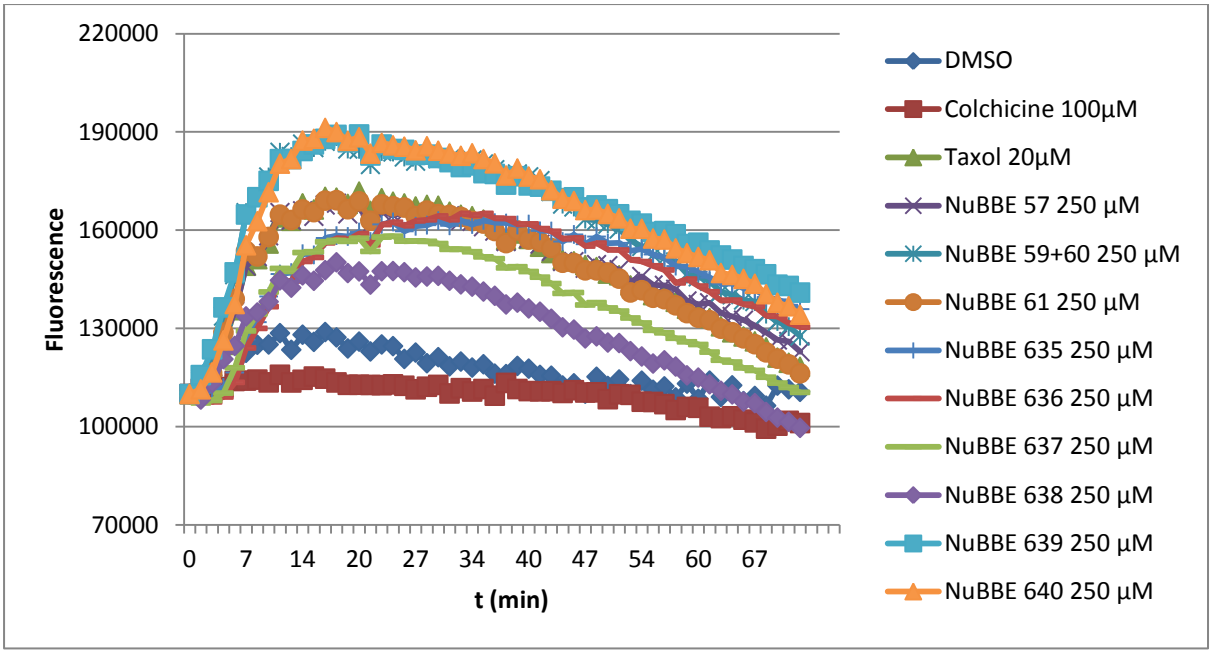


Figure 11. Results obtained for the *in vitro* tubulin polymerization assay of casearins (NuBBE 57, 59+60, 61 and 635-640). Mean values of triplicates were used.

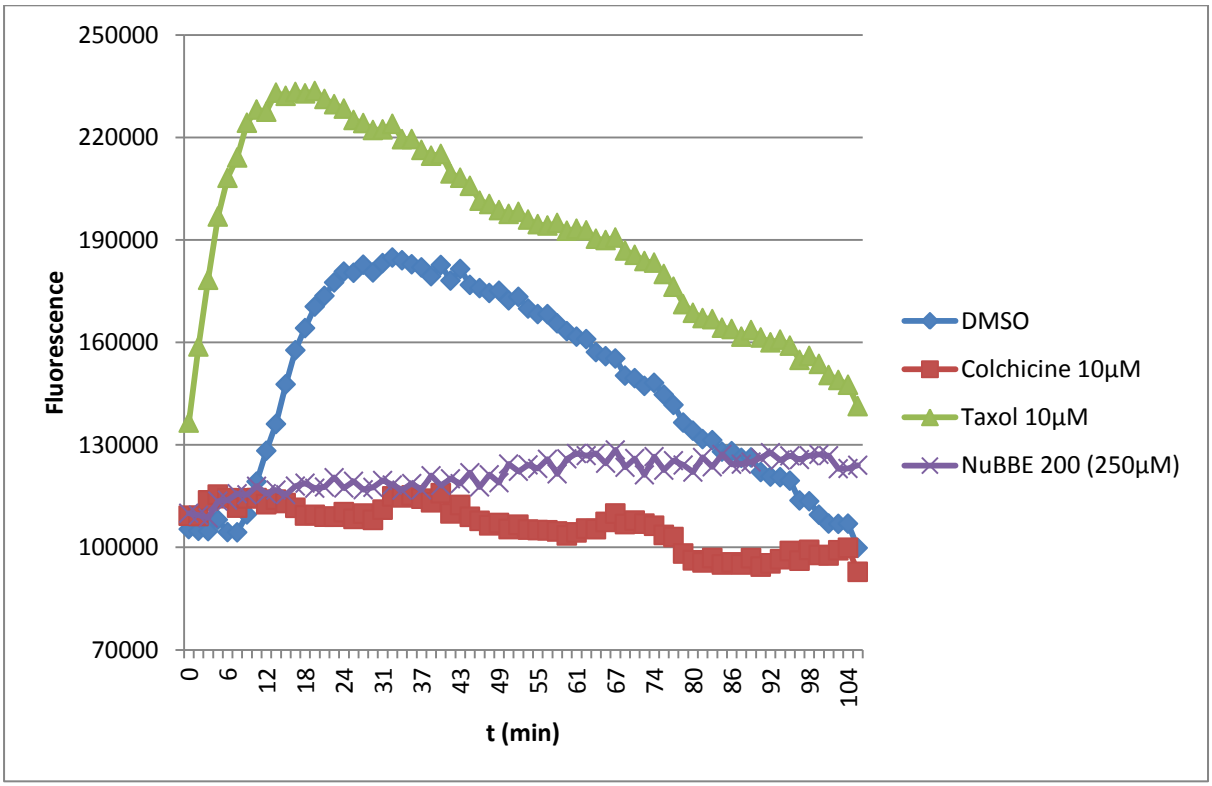


Figure 12. Results obtained for the *in vitro* tubulin polymerization assay of amentoflavone (NuBBE 200, 250 µM). Mean values of triplicates were used.

Additionally, a series of three guanidine analogues (NuBBE 43, NuBBE 423 and NuBBE 840) revealed inhibition of tubulin polymerization and are shown in Figure 13. It can be noted that the activity decreases with the decrease of the number of prenyl units in the two side chains.

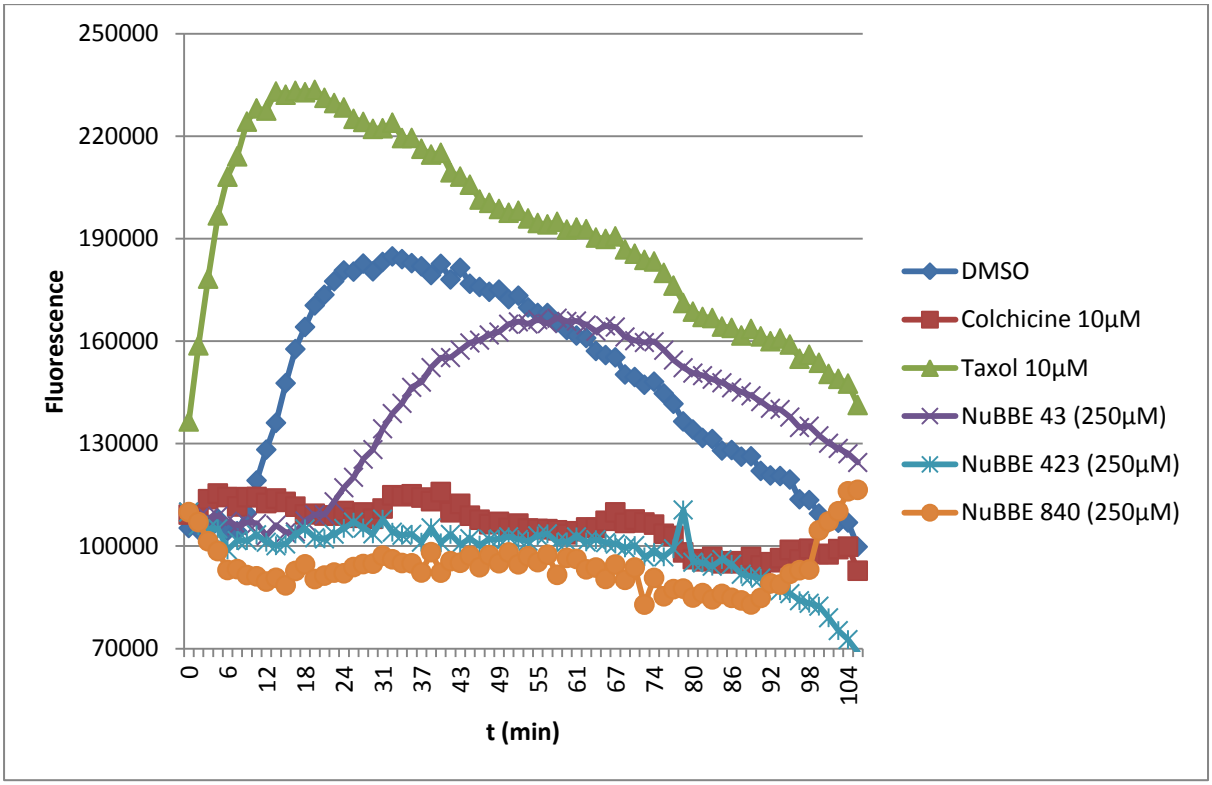


Figure 13. Results obtained for the *in vitro* tubulin polymerization assay of the series of guanidines NuBBE 43, NuBBE 423 and NuBBE 840 (250 µM). Mean values of triplicates were used.

The other compounds tested showed no effect on tubulin polymerization in this fluorescence based assay. The determination of the EC_{50} was performed for the active compounds and is presented as follows.

For the determination of EC_{50} of tubulin polymerization, the fluorescence based assay was used [CYTOSKELETON, 2013; BONNE *et al*., 1985; DYRAGER *et al*., 2011]. 6 different concentrations to generate EC_{50} curves were evaluated for the casearins NuBBE 57, NuBBE 59+60 and NuBBE 61, amentoflavone, the series of three guanidines and piplartine. Piplartine was also evaluated in this assay at various concentrations to explore extensively the effect of this compound in tubulin

polymerization, since it showed interesting activity in the cell assays that will be shown in the following sections.

The results obtained of the effect of the series of guanidines in several different concentrations in the polymerization of tubulin are shown in Figure 14-Figure 16.

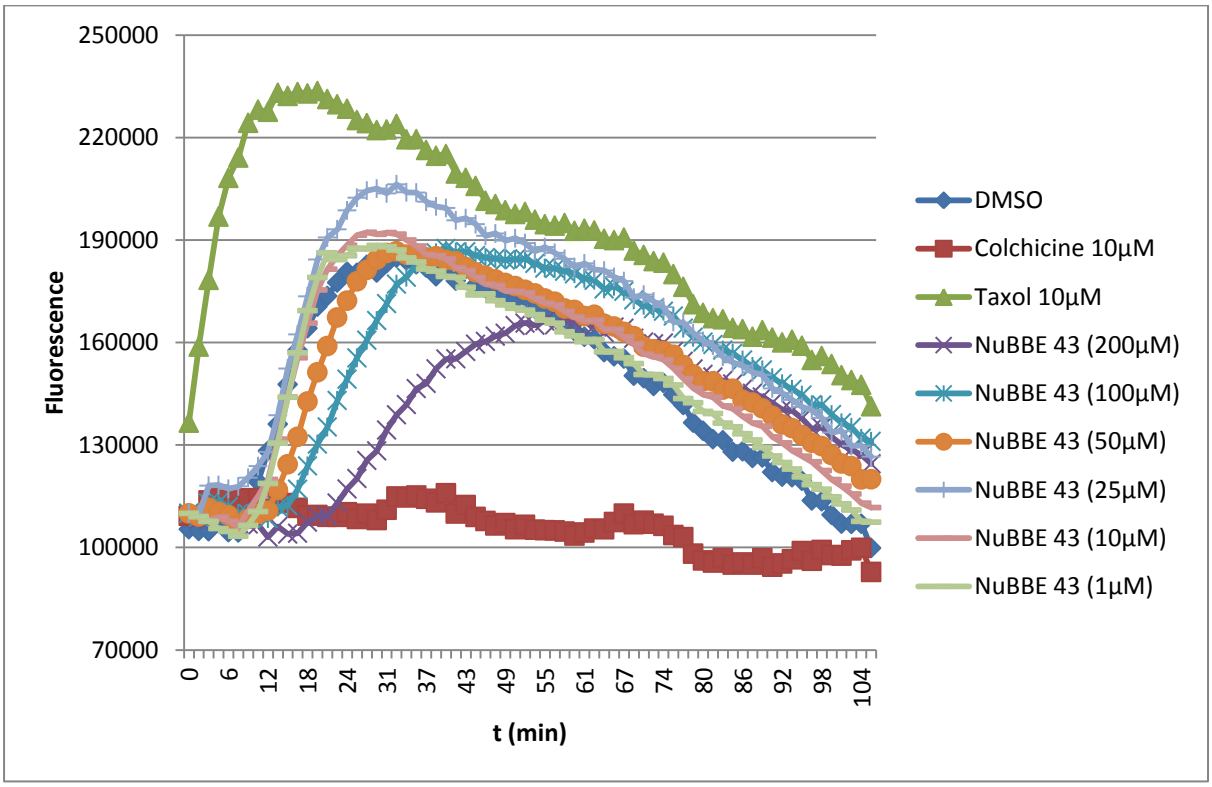


Figure 14. Results obtained for the EC_{50} determination of the guanidine NuBBE 43. Mean values of triplicates were used.

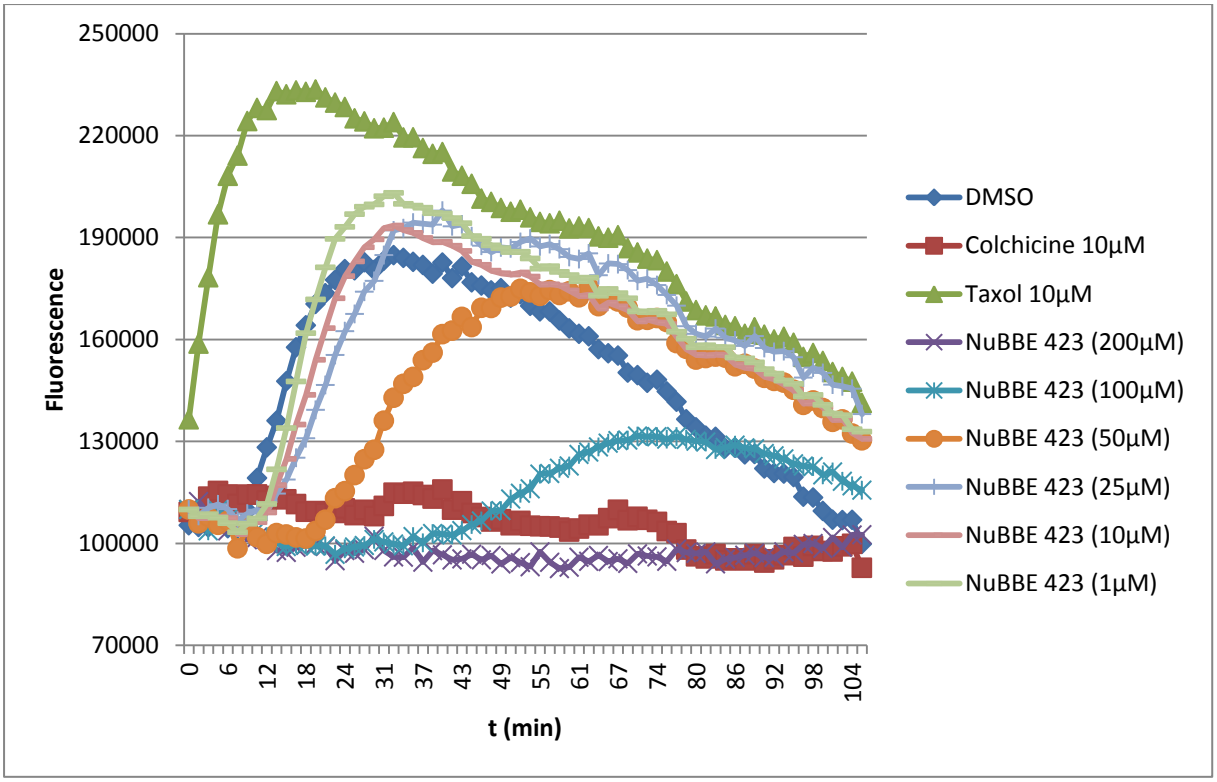


Figure 15. Results obtained for the EC_{50} determination of nitensidine A (NuBBE 423). Mean values of triplicates were used.

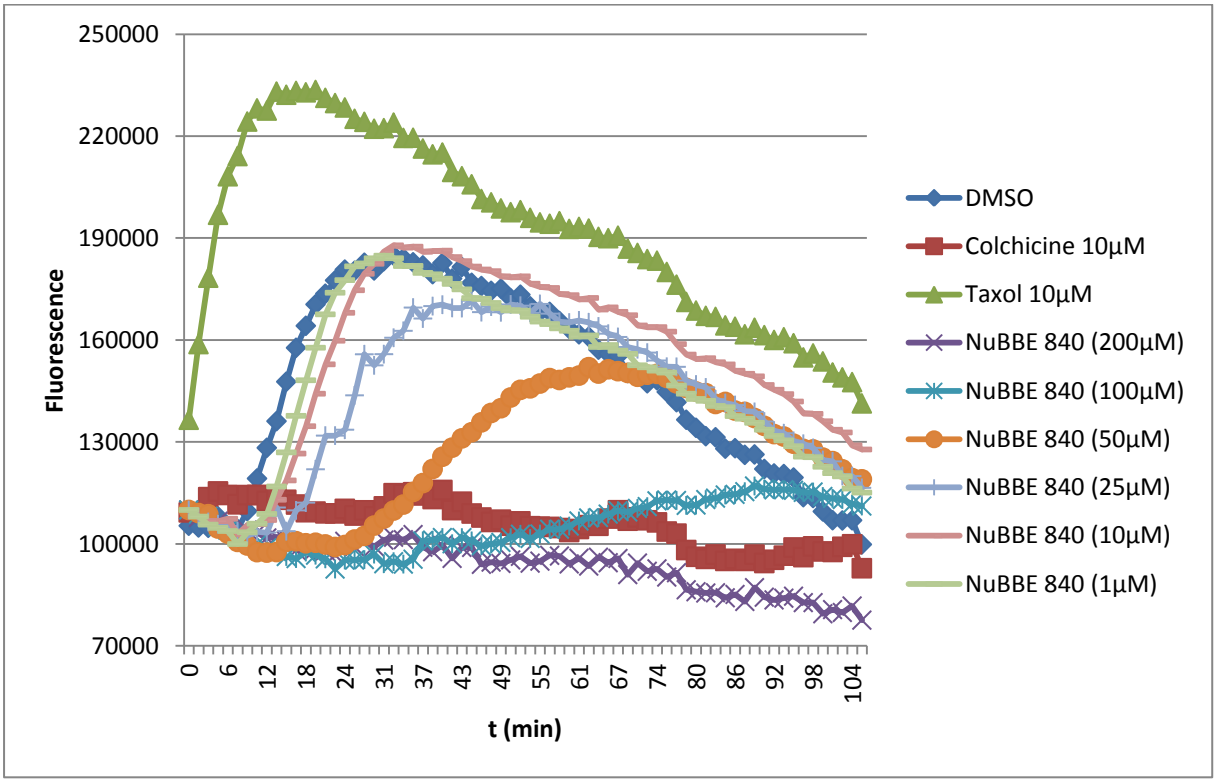
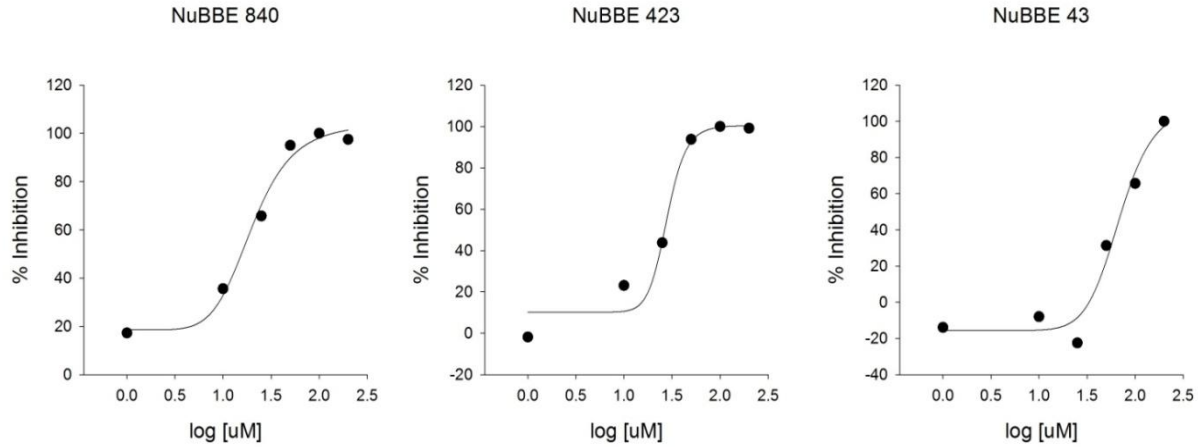


Figure 16. Results obtained for the EC_{50} determination of the guanidine NuBBE 840. Mean values of triplicates were used.

The EC_{50} was defined as the compound concentration that affected the extent of assembly by 50% after 20 min incubation, when the polymerization achieves steady state equilibrium phase (Figure 17) [RUAN *et al*. 2011].



EC_{50} = 19.9 ± 1.1 µM EC_{50} = 27.5 ± 1.1 µM EC_{50} = 67 ± 1.4 µM

Figure 17. Tubulin polymerization assay $EC_{50}$for the guanidines NuBBE 840, NuBBE 423 and NuBBE 43.

It can be noted that the activity increases with the number of prenyl units in the side chain. The EC_{50} for tubulin polymerization inhibition for NuBBE 43, NuBBE 423 and NuBBE 840 is 63 ± 1.4 µM, 27.5 ± 1.1 µM e 19.9 ± 1.1 µM, respectively (EC_{50} of colchicine = 2.77 µM).

The results indicated that piplartine (NuBBE 18) did not inhibit or increase the polymerization of tubulin (Figure 18).

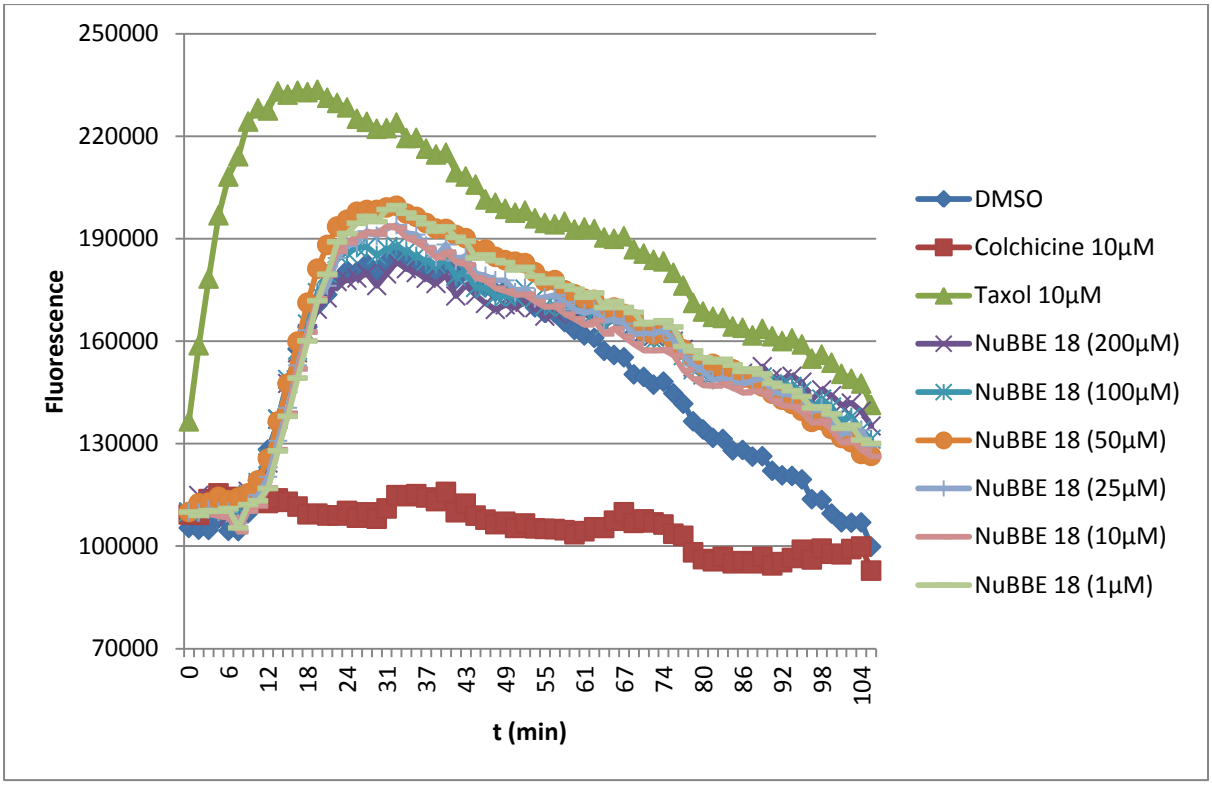


Figure 18. Results obtained for the determination of EC_{50} piplartine (NuBBE 18). Mean values of triplicates were used.

The assays for the determination of EC_{50} of the casearins, and amentoflavone did not presented coherence for the effect concentration x activity expected. The assays were repeated several times and no conclusive results were obtained. Thus, the light scattering based tubulin polymerization assay was performed to further study the data with greater reliability. These data will be discussed in the next section.

3.3.2. Light Scattering Based *in vitro* Tubulin Polymerization Assay

The evaluation of tubulin polymerization by light scattering was performed in order to compare and validate the activity measured by fluorescence. Thus, this assay was performed with representatives of the series of active compounds in the fluorescence, i.e. amentoflavone (NuBBE 200), the mixture of casearins NuBBE 69+60 and two guanidines (NuBBE 423 and NuBBE 840).

The results obtained in this assay for the mixture of casearins (NuBBE 59+60) and for amentoflavone (NuBBE 200) are equivalent to the negative control, indicating that these compounds do not have an effect in tubulin polymerization (Figure 19-Figure 20). Despite the satisfactory results obtained in the fluorescence assay, the

light scattering assay showed that these compounds are not active. In contrast, the results obtained for the guanidines revealed an inhibition of tubulin polymerization in both assays measured by fluorescence and light scattering, providing more evidence of biological activity (Figure 21).

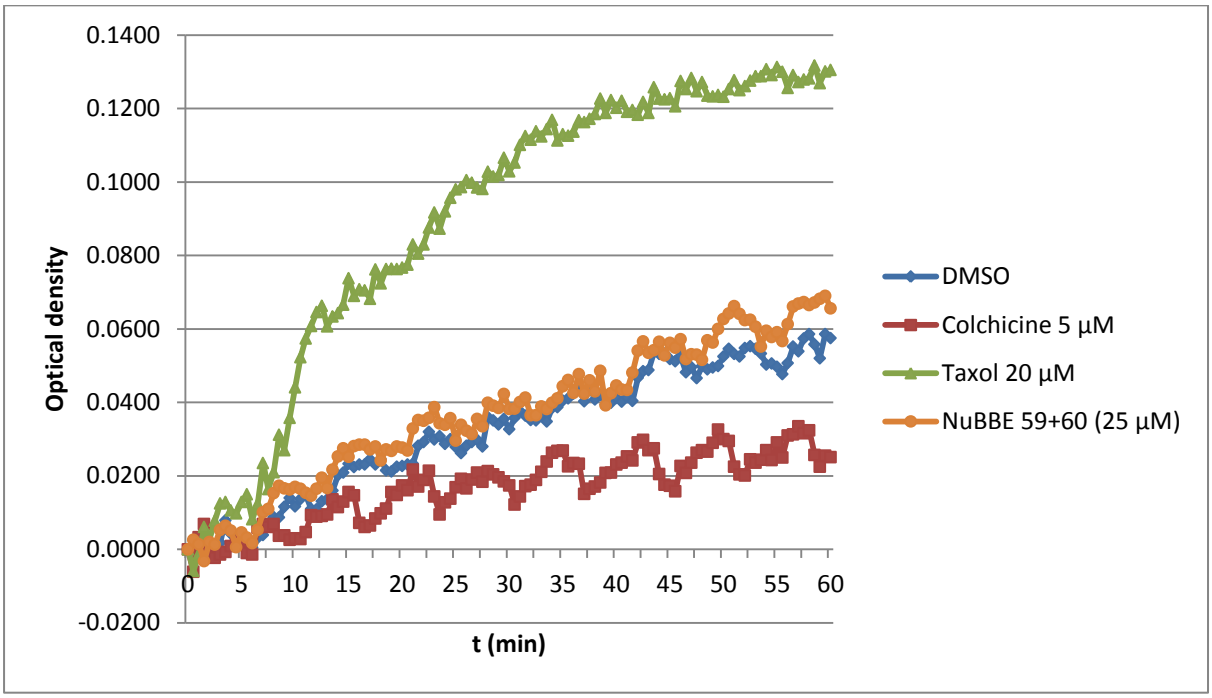


Figure 19. Results obtained for the evaluation of tubulin polymerization by light scattering with the mixture of casearins NuBBE 59-60. Mean values of triplicates were used.

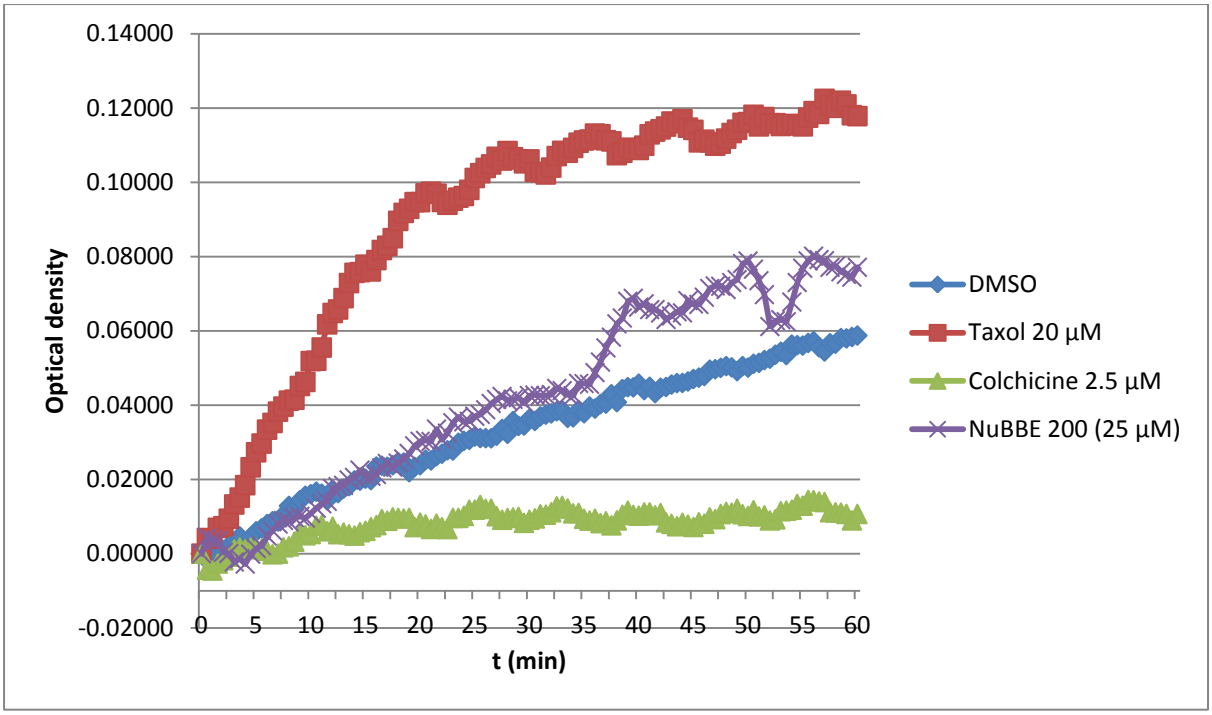


Figure 20. Results obtained for the evaluation of tubulin polymerization by light scattering with amentoflavone (NuBBE 200). Mean values of triplicates were used.

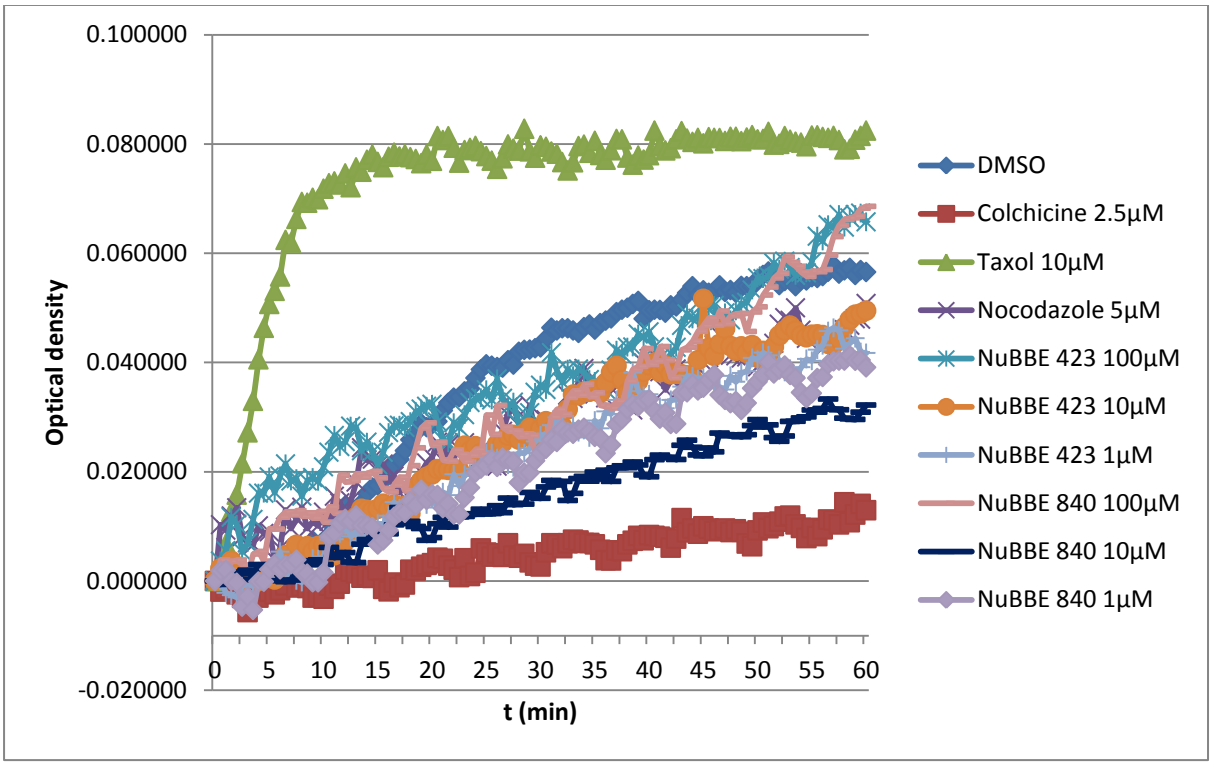


Figure 21. Results obtained for the evaluation of tubulin polymerization by light scattering with the guanidines NuBBE 423 e 840 in three different concentrations. Mean values of triplicates were used.

The tubulin polymerization assay measured by fluorescence can have interference because of the use of the reporter DAPI. Thus, comparing the same biological activity (tubulin polymerization) by two different techniques provides a more reliable result.

3.4. Cell Assays

The study of cell migration and its underlying mechanisms is of great significance in various fields of research, including cancer drugs development. The invasiveness of tumor cells represents one of the properties necessary for the development of metastasis. Several evaluation systems have been used to assess the capacity of cells to cross tissue barriers [ALBINI *et al*., 1987]. In this thesis, the *in vitro* cell assays Wound Healing [YUE *et al*., 2010] and Boyden chamber [SHAN *et al*., 2005] were conducted to evaluate compounds for their capacity to inhibit cell migration with the cancer cell line MDA-MB-231. The cytotoxicity assay was also performed for the active compounds in order to test the toxicity for the cancer cell lines MDA-MB-231 and MCF-7 [BARLTROP *et al*., 1991].

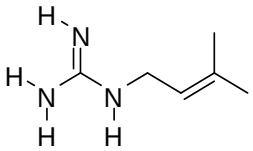
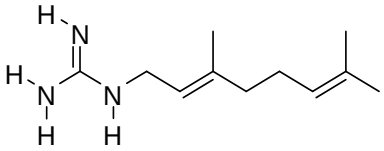
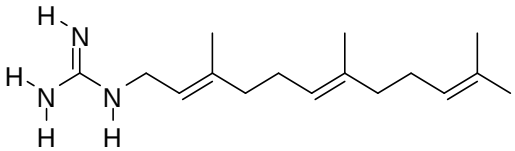
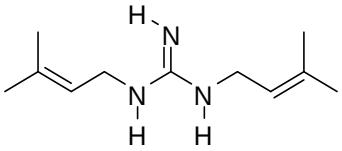
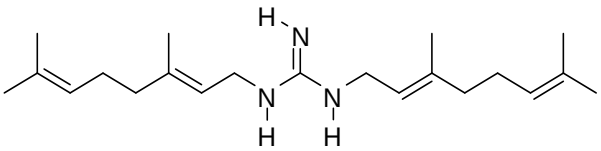
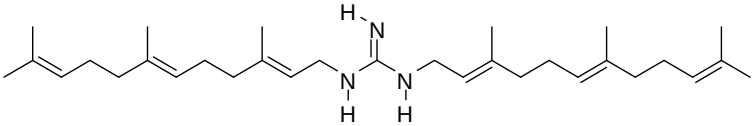
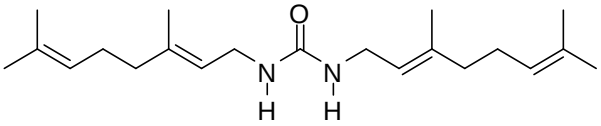
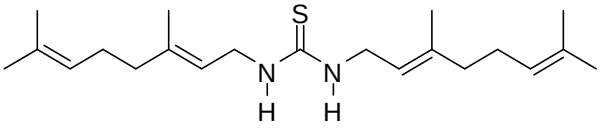
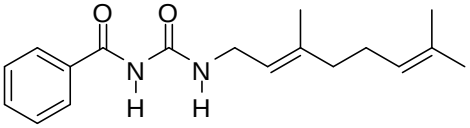
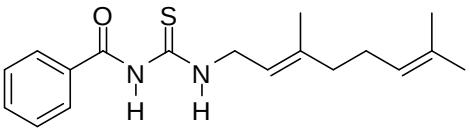
3.4.1. Wound Healing Cell Assay

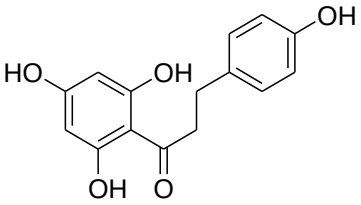
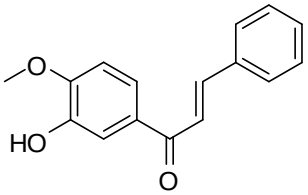
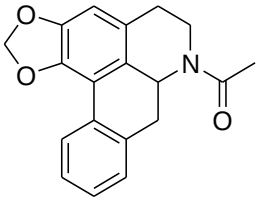
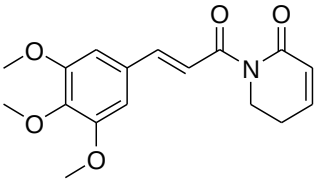
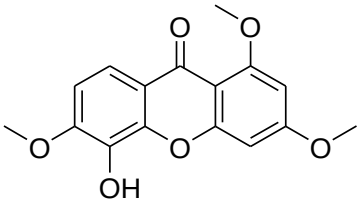
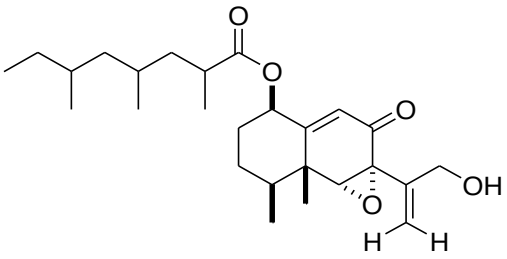
The wounding healing assay is an easy and economical *in vitro* method for assessing influence of compounds in cell migration. The wounds are usually created by the removal of a proportion of confluent cells grown on a multiwell plate using pipette tip. The closure of the uncovered area can then be observed and measured over time using microscopy or a computer imaging system [YUE *et al*., 2010].

A series of 10 guanidine synthetic derivatives inspired by natural products and 6 more compounds were selected to be evaluated for their influence in inhibiting cell migration in the wound healing cell assay, based on the results of the *in vitro* tubulin polymerization assay and on the literature review.

The results for a single concentration (10 μM) of the compounds are shown in Table 2. The percentage inhibition shown in the table refers to the capacity of the compound to inhibit cell migration compared to the negative control in which there is 0% of inhibition of migration.

Table 2. Results obtained for the wound healing assay for a single concentration of each compound.

Compound	Structure	% inhibition (10 µM)
NuBBE 044		0
NuBBE 040		0
NuBBE 839		0
NuBBE 043		58
NuBBE 423		78
NuBBE 840		73 (5 µM)
NuBBE 841		0
NuBBE 842		0
NuBBE 843		0
NuBBE 844		0

NuBBE 872		40
NuBBE 851		27
NuBBE 215		0
NuBBE 018		97
NuBBE 876		0
NuBBE 893		20
Colchicine	Positive control	72 (1 μM)
Evodiamine	Positive control	78

The results of % inhibition were obtained by software treatment of the photomicrographs. In the photomicrograph it can be noted that for the assay where no compounds were added, cells closed the created wound. Compounds with the capacity to inhibit cell migration, are identified by the remained unclosed wound.

The results obtained indicated that the guanidines NuBBE 43, NuBBE 423 and NuBBE 840 inhibit cell migration, and compound NuBBE 840 revealed to be the most potent among them. The guanidine moiety group showed to be important for the activity, since the isosteric replacement of nitrogen by oxygen or sulfur led to a loss of activity. Piplartine also presented quite relevant activity, inhibiting cell migration by 97%.

These guanidines (NuBBE 43, NuBBE 423 and NuBBE 840) and piplartine were also evaluated at different concentrations of 1, 5 and 10 μM, providing information on the concentration x activity relationship (Figure 22 and Figure 23). The compound NuBBE 840 induced cell death at a concentration of 10 μM, and it was not possible to make the evaluation of cell migration at this concentration.

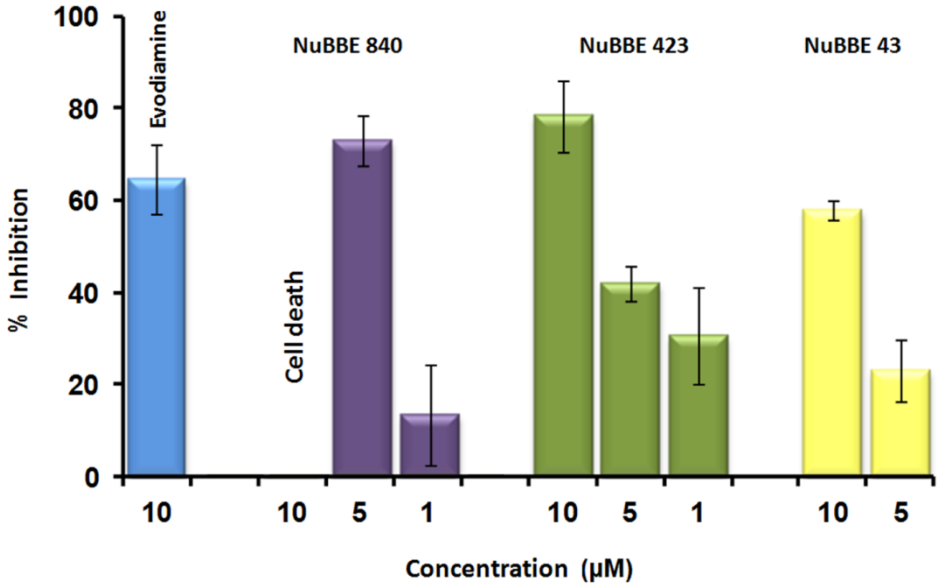


Figure 22. Results for the inhibition of cell migration in the wound healing assay with guanidine NuBBE 43, NuBBE 423 and NuBBE 840 in different concentration.

A brief review of the structure-activity relationship shows that the presence of prenyl side chains, as well as its size (number of carbon atoms) are important for the activity.

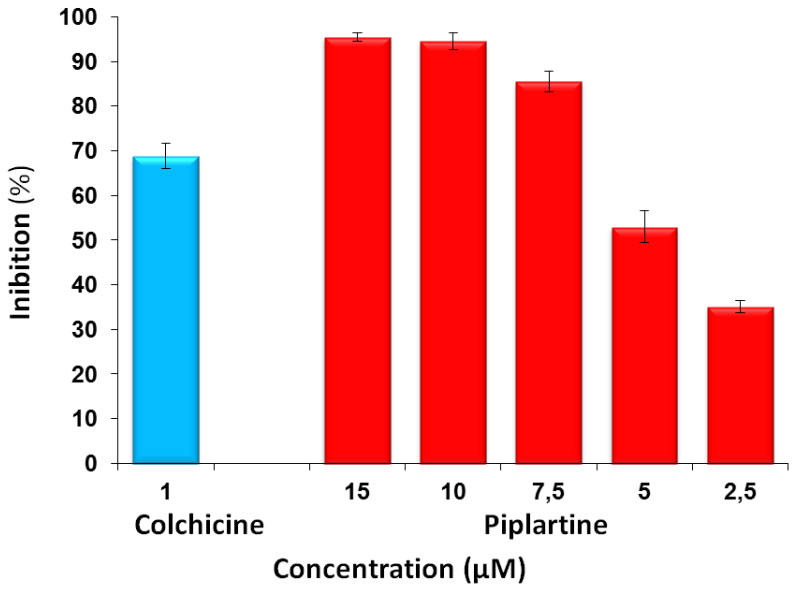


Figure 23. Results for the inhibition of cell migration in the wound healing assay with piplartine (NuBBE 18) in different concentration.

The wound healing assay presented great importance in the identification of new inhibitors of cell migration. Subsequent assays for quantification of the activity are presented in the next section.

3.4.2. Boyden Chamber Cell Assay

Tumor invasion of basement membranes is a crucial step in the complex multistage process which leads to the formation of a metastasis. Tumor cells cross basement membranes, invade the lymphatic or vascular system during dissemination, and then they penetrate into their target tissue. The Boyden chamber assay uses a two compartment system separated by a membrane to quantitatively evaluate the effect of compounds in migration of cancer cells [ALBINI *et al*., 1987; SHAN *et al*., 2005].

The most potent compounds in the wound healing assay, the guanidine derivative NuBBE 840 and piplartine (NuBBE 18) were evaluated in the Boyden chamber assay for determination of EC_{50}. The EC_{50} was defined as the compound concentration that arrested the extent of cell migration by 50%. The EC_{50} determined for compound NuBBE 840 is 2.98 ± 1 µM (Figure 24).

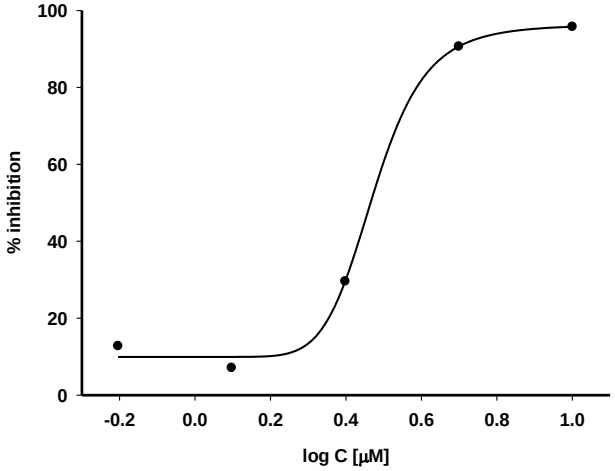


Figure 24. EC_{50} obtained for guanidine derivative NuBBE 840 in the Boyden chamber assay.

The guanidine alkaloids showed significant activity in the tubulin polymerization and cell assays. *In vitro* tubulin polymerization performed with NuBBE 43, NuBBE 423 and NuBBE 840 showed that tubulin is the possible target responsible for the activity of these compounds in tumor cells. The guanidine alkaloids are a class of compounds that have cytotoxic activity already described in the literature [BOLZANI; GUNATILAKA; KINGSTON, 1995; REGASINI et al, 2009.]. Its cytotoxic activity is caused by cell apoptosis and two other series of guanidines are described in the literature as inhibitors of tubulin polymerization [QIAN *et al*, 2010; ZHANG *et al*, 2009].

The EC_{50} for piplartine (NuBBE 18) is 2.65 ± 1.1 µM (Figure 25) (EC_{50} of colchicine = 0.5 µM).

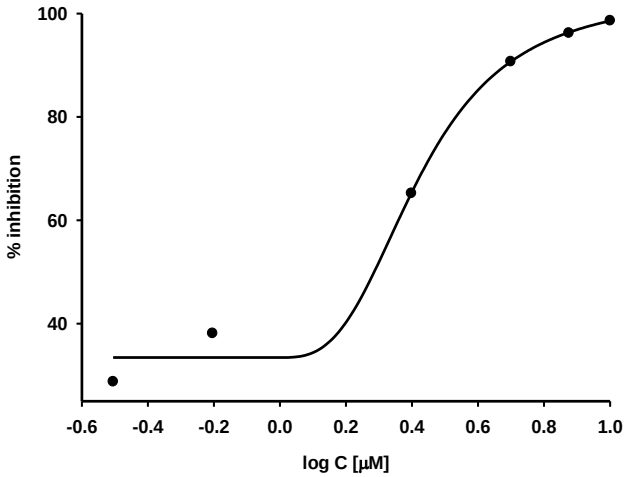


Figure 25. EC_{50} obtained for piplartine NuBBE 18 in the Boyden chamber assay.

The results obtained for the Boyden chamber assay were promising, indicating a series of guanidines and piplartine as inhibitors of cell migration.

3.4.3. Cytotoxicity Evaluation

The cytotoxicity assay was performed to evaluate the compounds that were active in the cell migration tests. Tetrazolium salts have been used to distinguish living cells from dead ones. They are reduced to formazans by the cytochrome systems of viable cells, and the color developed can be directly measured by absorbance and related to the viability of the cell culture [BARLTROP *et al*., 1991].

The tested compounds, guanidines NuBBE 423, NuBBE 840 and piplartine (NuBBE 18) presented toxicity to the cell lines used in the assay, and EC_{50} results are shown in Table 3.

Table 3. Results obtained for the cytotoxicity assay.

Compound	EC_{50} (μM) MDA-MB-231	EC_{50} (μM) MCF-7
NuBBE 840	9.9 ± 1.8	20.5 ± 9
NuBBE 423	9.6 ± 1.5	13.2 ± 1.2
Piplartine NuBBE 18	7 ± 1	Not tested
Doxorubicin	3 ± 1	Not tested

The results of the cell assays indicate that both piplartine and the guanidines have the capacity to inhibit cell migration with comparative potency to the positive control colchicine. The cytotoxic evaluation shows that these compounds are cytotoxic in greater concentration than for inhibiting cell migration. Additionally, combining the capacity of inhibiting cell migration and cytotoxicity is a suitable characteristic for anticancer compounds.

As a future perspective, it will be important to evaluate the effect of the compounds for normal cell lines, in order to determine if the compounds are

selective. The selectivity is a parameter that has gained increasing importance in the development of new drugs.

3.5. Design of New Compounds Using a Natural Product as Model

The main purpose of the biological assays performed in this work was the identification of bioactive compounds to be models for the design of new antitumor agents. Piplartine is presented as a useful scaffold for the structure-activity relationship studies, based on the results obtained in the cell assays. Although it presented no activity in the tubulin polymerization assay, indicating that is not the target for piplartine, this compound has a very interesting chemical structure that could be further explored by medicinal chemistry. Additionally, there are no studies in the literature of this compound regarding cell migration.

Piplartine is a natural amide isolated from several species of *Piper* (Piperaceae). It is described to be antifungal [NAVICKIENE *et al*., 2000], cytotoxic for several cancer cell lines [BEZERRA *et al*., 2006; BEZERRA *et al*., 2007], between other activities [BEZERRA *et al*., 2013]. It selectively induces cell death in cancer cells but do not reduce cell viability in normal cells [RAJ *et al*., 2011]. This natural compound also demonstrated to reduce angiogenesis and inhibited the formation of blood vessels in xenograft tumor mice [THE GENERAL HOSPITAL CORPORATION, 2009; RAJ *et al*., 2011]. Piplartine seemed to be more effective in tumor growth inhibition than paclitaxel and also showed excellent oral bioavailability [RAJ *et al*., 2011]. This compound caused an increase in reactive oxygen species (ROS) levels in cancer cells, but not in normal cells [RAJ *et al*., 2011]. This activity may be at least partly responsible for the selective activity of piplartine and also due to the inhibition of proteasome, leading to apoptosis [JARVIUS *et al*., 2013, GOLOVINE *et al*., 2013]. Recently a series of piplartine analogues were synthesized and it was proposed that the two reactive electrophilic sites are essential for the toxicity of the compounds. The double bond at C12-C13 is a key pharmacophore and at C7-C8 play significant role in cell toxicity (Figure 26) [ADAMS *et al*., 2012]. Even though pharmacokinetic data are important to better understand in vivo pharmacological and toxicological effects, little information about the absorption and metabolism of piplartine is available, [BEZERRA *et al*. 2013]. The bioavailability of piplartine following oral administration at 5 mg/kg and 10 mg/kg were 76.39% and

50.08%, respectively [RAJ *et al*., 2011]. In order to mimic cytochrome P450 enzymes metabolism, oxidation of piplartine using metalloporphyrins were performed. The oxidation of piplartine can take place both at the lactam ring and at the trimethoxycinnamic moiety of the molecule [SCHAAB *et al*., 2010].

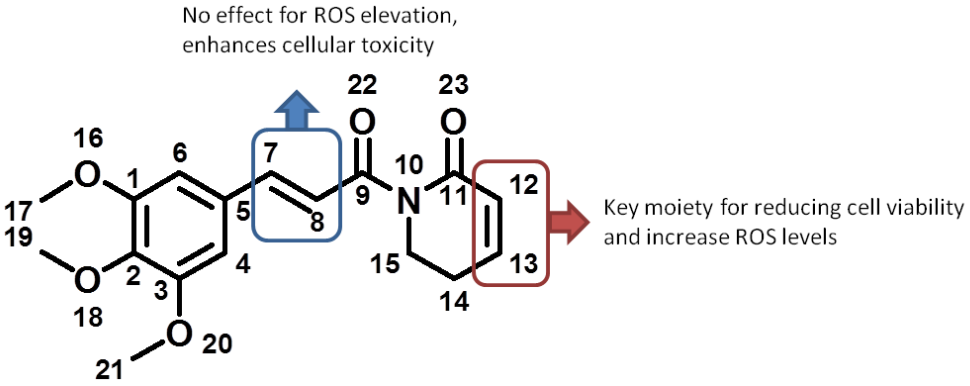
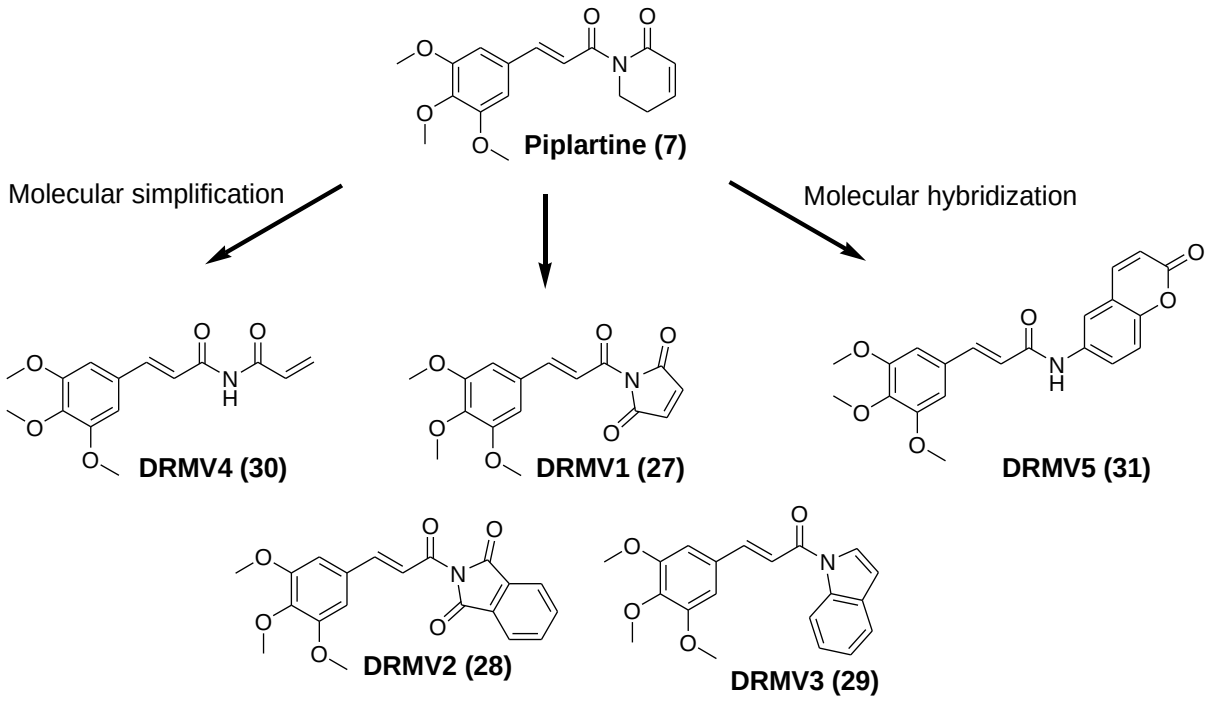


Figure 26. Piplartine structure and responsible moieties for biological activity.

To explore the structure of this lead compound further, 5 analogues were designed taking into account the literature information and using concepts of molecular simplification and hybridization. The design of new compounds was carried out in collaboration with Prof. Sylvie Michel and Prof. Xavier Cachet from the *Faculté de Pharmacie, Université Paris V - Descartes*.

Considering that the substitutions in the aromatic ring of the 3,4,5-trimethoxy cinnamic moiety does not have an effect in the antitumor activity, we did not design any modifications in this part of the molecule [ADAMS et al., 2012; RAJ et al., 2011]. Thus, we planned modifications in the δ-valerolactam ring. Three of the designed compounds have other rings in place of the δ-valerolactam (DRMV 1-3). We also designed one compound using molecular simplification, by keeping the Michael adduct present in piplartine, but removing the lactam ring (DRMV 4). Finally, we used molecular hybridization concept and designed a hybrid of piplartine and a chromene (DRMV 5). Chromenes are widely known for their cytotoxicity and induction of apoptosis. The designed compounds are presented in Scheme 1.

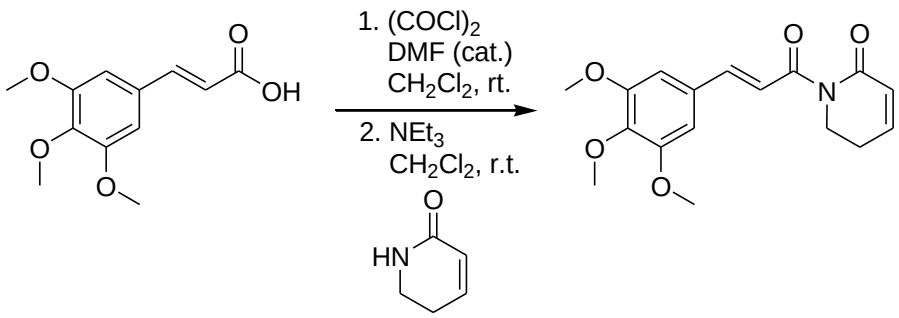


Scheme 1: Design of new compounds with piplartine as the natural product model

3.6. Synthesis of the Designed Compounds

In medicinal chemistry, the synthetic methodologies should be simple, fast and accomplished in a few steps. The synthetic route was planned and developed in collaboration with Prof. Sylvie Michel and Prof. Xavier Cachet from the *Faculté de Pharmacie, Université Paris V - Descartes*, and the accomplishment of the synthesis also had the collaboration of Ph.D. student Emilio Carlos de Lucca Júnior and Dr. Marco Aurélio Dessoy, under the supervision of Prof. Luiz Carlos Dias.

The synthesis of piplartine is described in the literature by different authors [CHATERJEE; DUTTA, 1967; BOLL; HANSEN; SIMONSEN, 1984; RAO *et al*., 2012; ADAMS *et al*., 2012; BOSKOVIC *et al*., 2013], and one of the latest and simple approaches is described by the coupling of the lactam and 3,4,5-trimethoxy cinnamoyl chloride (Scheme 2) [ADAMS *et al*., 2012].



Scheme 2: Synthetic route described for the synthesis of piplartine [ADAMS *et al*., 2012].

Accordingly, the designed compounds were synthesized by coupling commercially available reagent A and 3,4,5-trimethoxy cinnamoyl chloride [ADAMS *et al*., 2012]. The conditions to enhance nucleophilicity of reagent A and the solvent were dependent on the reactivity of this reagent. In Table 4 it is described the conditions used for each reaction. Detailed description of the synthetic methods together with their characterization is available in the *Experimental* section.

Table 4. Conditions used for the synthesis of the designed compounds. The less reactive, the Reagent A was, the stronger should be the base to increase nucleophilicity.

compound	solvent	base	reagent A	yield
DRMV 1	CH_2Cl_2	NEt_3	maleimide	45%
DRMV 2	CH_2Cl_2	NEt_3	phthalimide	40%
DRMV 3	THF	NaH	indole	41%
DRMV 4	THF	*n*-BuLi	acrylamide	30%
DRMV 5	CH_2Cl_2	NEt_3	6-amino-chromen-2-one	21%

The reaction was performed in two steps. First the 3,4,5-trimethoxy cinnamic acid was treated with oxalyl chloride for the formation of the acyl chloride. Then, reagent A was treated with a base to increase nucleophilicity, and it was coupled with the chloride derivative.

3.7. Biological Evaluation of the Synthesized Compounds

To determine the activity of the synthetic analogues of piplartine the wound healing and cytotoxic assays were performed. The results obtained are presented in Table 5-Table 6.

Table 5: Results obtained for the wound healing assay (MDA-MB-231) for the piplartine analogues (DRMV 1-5).

Compound	Concentration (μM)	Inhibition of cell migration (%)
colchicine	1	79
piplartine	10	97
DRMV 1	10	0
DRMV 2	10	0
DRMV 3	10	50
DRMV 4	10	97
DRMV 5	10	0

The analogue designed by molecular simplification (DRMV 4) was active with 97% of inhibition cell migration at 10 μM concentration. The compound DRMV 3 was moderate active with the inhibition of 50% of cell migration at 10 μM concentration. The other synthesized compounds were inactive in this assay. The most promising designed compound, DRMV 4, maintained the biological activity and possesses a simpler structure. The cytotoxic assay also demonstrated that the activity of this compound is similar to that of piplartine.

Table 6. Results obtained for the cytotoxicity assay for the biologically active piplartine analogue.

Compound	EC_{50} (μM) - MDA-MB-231
Doxorubicin	3 ± 1
Piplartine	7 ± 1
DRMV 4	9 ± 1

4. CONCLUSIONS AND PERSPECTIVES

One of the objectives of the present thesis was the creation of $NuBBE_{DB}$, an innovative database of all the compounds isolated along the 15 years of NuBBE research with species of the Brazilian biodiversity, especially from Cerrado and Atlantic Forest. The 640 compounds catalogued in this database present rich chemical diversity, comprising several classes of natural products, and a wide spectrum of biological and pharmacological activities. This information is now available to aid research on medicinal chemistry, chemical ecology and chemotaxonomy. The goal is that this database can be useful not only to the current research of NuBBE group, but also for others researchers interested in virtual screening, dereplication, metabolomics, and the design of new bioactive compounds. As a perspective, we aim to include all natural products published from the Brazilian biodiversity in $NuBBE_{DB}$. Additionally, the expansion of the database system is being accomplished in order to add analytical information, such as NMR and MS data of the compounds catalogued.

The $NuBBE_{DB}$ was the source of compounds used in this thesis for virtual screening aiming at the identification of hits to be tested for antitumor biological assays, and for the design of new synthetic analogues of the model selected in the virtual screening. From the compounds available in $NuBBE_{DB,}$ 35 were tested in the tubulin polymerization assay, and a series of three guanidines NuBBE 43, NuBBE 423 and NuBBE 840 presented a significant activity, with EC_{50} of 63 ± 1.4 µM, 27.5 ± 1.1 µM and 19.9 ± 1.1 µM respectively, using colchicine as positive control (EC_{50} = 2.77 µM).

In the preliminary wound healing assay, 16 compounds were tested and the series of guanidines (NuBBE 43, NuBBE 423 and NuBBE 840) and piplartine (NuBBE 18) were active. In the sequence, the Boyden chamber assay was performed to determine EC_{50} of NuBBE 840 and piplartine, which were 2.98 ± 1 and 2.65 ± 1.1 µM respectively, using colchicine as positive control (EC_{50} = 0.5 µM). These results revealed a good strategy to search for antitumor natural products from $NuBBE_{DB}$.

Piplartine was selected as the model for the design of new compounds, provided that there are no studies of this compound regarding cell migration assays. Thus a series of 5 compounds was planned and synthesized. The structure-activity

relationship studies revealed the importance of the Michael Acceptor group. The analogue designed by molecular simplification (DRMV 4) was the most active of the series, inhibiting 97% of cell migration at 10 µM in the wound healing assay. The perspective of this study is to determine EC_{50} using Boyden Chamber assay, evaluate the selectivity of these compounds, and to obtain a lead for inhibition of cell migration.

The objectives in this thesis were achieved through cooperative work with interdisciplinary concepts in the areas of computational, biological, medicinal and natural products chemistry.

5. EXPERIMENTAL

5.1. Design of NuBBE Database

5.1.1. Molecular Structure Preparation

The 3D chemical structure of each compound was generated using the standard tools available in the molecular modeling software package Sybyl 8.0 (Tripos, St. Louis, MO, USA), running on Red Hat Enterprise Linux workstations. The hybridization of every atom was verified using the Sybyl "Atom Types" option. All molecules were considered to have a neutral charge. The single 3D representation of each molecule from the database had its conformational energy minimized using the *Tripos* force field and Powell's method [SANTOS *et al*., 2011]. Partial atomic charges of the minimized structures were calculated using the Gasteiger−Hückel method, also available in the Sybyl 8.0 package. During these steps, the molecules were considered to be in an implicit aqueous environment (dielectric constant of 80.0). The 3D conformation of each molecule was used for the virtual screening and is available in the database in the Mol2 file format. NuBBE database was designed in collaboration with the Ph.D. student Ricardo Santos and supervision of Prof. Adriano Andricopulo.

5.1.2. Physicochemical Property Determination

The physicochemical properties of all molecules in $NuBBE_{DB}$ were predicted using the Web Property Calculation Service available as part of the Web-based Molinspiration software [MOLINSPIRATION..., 2013; ERTL; ROHDE; SELZER, 2000]. These properties include molecular mass, molecular volume, cLogP, TPSA, number of hydrogen-bond acceptors and donors, nRotb, and number of Lipinski's "rule of five" violations. The fragment-based approach used by Molinspiration has proven to be reliable and is employed in relevant scientific publications and chemical databases [IRWIN; SHOICHET, 2005; AKHOON *et al*., 2011; PIERONI *et al*., 2010; HSIN *et al*., 2011].

5.1.3. Database Interface

An integrated system was developed to allow easy and efficient retrieval of the compounds. The NuBBE Web system is installed on a Linux server with Apache Tomcat as the Web server and PostgreSQL as the relational database server, where

the set of data is stored. The Web interface was designed using standard Web technologies such as HTML, CSS, and JavaScript (AJAX), while the server itself is implemented using Java/Servlets with Hibernate, an object-relational mapping database framework. All of the software packages used are open source and recognized by the industry and the community as robust and reliable software. The Web interface was designed to work in all browsers equally, with special attention given to the ease of use. The molecular drawing interface WebME provided by Molinspiration [MOLINSPIRATION..., 2014], in association with the substructure search engine provided by CDK (Chemistry Development Kit) [STEINBECK *et al*., 2003] enables the user to search for compounds by chemical structure, offering a more user friendly search and retrieval system. The graphical interface was designed in collaboration with the computational expert M.Sc. Leandro D. Figueira.

5.2. Virtual Screening

Molecular modeling studies were performed as a virtual screening of NuBBE database for the *in vitro* assays. The protein structures of tubulin were obtained in the Protein Data Bank (PDB), available online with free access [PROTEIN DATA BANK, 2014; BERMAN *et al*., 2000]. In this project, 3 tubulin binding sites were separately considered for docking studies, named the paclitaxel, vincristine/vinblastine, and colchicine binding sites (Figure 9).

For docking in each binding site, a specific protein with the respective original ligand (paclitaxel, vinblastine, or colchicine) was used. The protein structures were selected in PDB with the best resolution and the original ligand present in the binding site, as detailed in Table 7.

Table 7. Protein structures obtained in PDB and used for the docking studies.

Binding site	**PDB ID**	**Resolution**
Paclitaxel	1JFF	3.5 Å
Colchicine	1SA0	3.58 Å
Vinblastine	1Z2B	4.1 Å

The proteins were prepared for docking in the Sybyl 8.0 program and hydrogen atoms were added using the tool Biopolymer available in this program. The docking program used for the docking studies was GOLD 5.0 (CCDC, Cambridge, UK). This program uses molecular mechanics expressions to generate the fitness score and a genetic algorithm to explore the full range of ligand conformational flexibility [JONES *et al*, 1997; VERDONK *et al*., 2003]. The molecular modeling studies were performed in collaboration with the Ph.D. student Ricardo Santos and supervision of Prof. Adriano Andricopulo.

5.3. Biological Assays

5.3.1. Tubulin Polymerization Assay (Fluorescence Based)

To analyze the polymerization of tubulin *in vitro*, the commercial kit BK011P of Cytoeskeleton was used (Cytoskeleton Inc., Denver, CO, EUA) [CYTOSKELETON, 2014]. The assay was performed as described herein, an adaptation of an assay originally described in literature [BONNE *et al*., 1985; DYRAGER *et al*., 2011]. The tubulin polymerization assays were performed in collaboration with the Ph.D. student Ricardo N. Santos and the supervision of Prof. Adriano Andricopulo in the Laboratory of Medicinal and Computational Chemistry of the Institute of Physics of São Carlos - USP.

Microtubule-associated protein rich tubulin protein (2 mg/mL, porcine, Cytoskeleton) in buffer containing 80 mM Piperazine-*N*,*N'*-bis[2-ethanesulfonic acid] sequisodium salt (PIPES) pH 6.9; 2.0 mM Magnesium chloride; 0.5 mM Ethylene glycol-bis(b-amino-ethyl ether) *N*,*N*,*N'*,*N'*-tetra-acetic acid (EGTA), 1.0 mM guanosine triphosphate (GTP) and 10% glycerol was placed in each well of the 96 wells plate OptiPlate F -1536 - Black (Perkin Elmer), 45 µL/well, and incubated with the solution of desired concentration of each compound, 5 µL/well. All assays were performed in triplicate, with 1% dimethyl sulfoxide (DMSO) as negative control and paclitaxel and colchicine as positive controls. The rate of polymerization was followed at 37 ^{o}C, using an excitation wavelength of 360 nm, and the fluorescence was collected at 440 nm with a Fluorimeter plate reader Victor3, software Wallac 1420 Multilabel Plate Counter (Perkin Elmer Inc; Waltham, MA, USA). 70 measurements were made for each well at intervals of about 1 min [CYTOSKELETON, 2014].

5.3.2. Tubulin Polymerization Assay (Light Scattering Based)

To analyze the *in vitro* tubulin polymerization by light scattering, commercial kit HTS02-B from Cytoskeleton was used (Cytoskeleton Inc., Denver, CO, USA) [CYTOSKELETON, 2014]. Microtubule-associated protein rich tubulin protein (3 mg/mL, porcine, Cytoskeleton) in buffer containing 80 mM PIPES, pH 6.9, 0.5 mM Magnesium chloride, 1 mM EGTA, 1 mM GTP, and 5% glycerol was placed in each well of the 96 wells clear plate 180 µL/well, and incubated with the solution of desired concentration of each compound, 20 µL/well. Assays were performed in triplicate, 1% DMSO was used as negative control and paclitaxel and colchicine as positive controls. Absorbance was read at 340 nm with a Spectramax-plus 384, software Softmax Pro, (Molecular Devices). The plate was kept at 37°C, 120 measurements were made in each well at intervals of about 30 seconds [CYTOSKELETON, 2014; XIE *et al*., 2011].

5.4. Cell Assays

5.4.1. Cell Cultures

MDA-MB-231 human breast cancer cells were cultured at 37°C in 5% CO_2 in Leibovitz medium and MCF-7 human breast cancer cells in DMEM (Dulbecco's modified Eagle's medium – Cultilab). The cells were stored in liquid nitrogen in culture medium 20% fetal bovine serum (FBS, sterile, Cultilab) containing 5% DMSO. The cell assays were performed in collaboration with the Ph.D. student Wanessa Altei and supervision of Prof. Adriano D. Andricopulo.

5.4.2. *Wound Healing Assay*

The wound healing assay was used to qualitatively assess the potential of a compound to inhibit cell migration [YUE *et al*., 2010]. Cells were cultured in 24-well plates (1 X 10^5 cells/well) containing 1 ml of culture medium and 10 % FBS. After the cultures were confluent, the culture medium was removed and, with the aid of a sterile pipette tip, a wound was made in the cell monolayer. The test compounds were dissolved in DMSO, diluted in culture medium and added to each the well (final

DMSO concentration = 0.1%). The system was photographed with an inverted microscope (4X magnified) Nikon Eclipse TS100. The images were taken at 0 h and after 22 h of incubation at 37°C in 5% CO_2. The percentage of inhibition shown by the test compound is determined by the variation of the wound area at times 0h and 22h. The extent of the areas is measured with the software NIS - Elements. The assays were performed in triplicate, using colchicine (or evodiamine) as positive control and DMSO as negative control [YUE *et al*., 2010].

5.4.3. Boyden Chamber Assay

The Boyden chamber assay was performed to quantitatively evaluate the effect of compounds on cell migration [ALBINI *et al*., 1987; SHAN *et al*., 2005]. This assay uses a hollow plastic chamber, sealed at one end with a porous membrane. This chamber/insert is suspended over a larger well which contain medium and a chemoattractant. Cells are placed inside the insert and allowed to migrate through the pores, to the other side of the membrane. Migratory cells are then stained and counted. The assays were performed in 24-well plates (BD BioCoatTM Migration/Invasion Chambers) with inserts with 6.5 mm diameter and 8 µm pore size. In the inner part of the insert (upper chamber) 4 X 10^4 cells/300 µL culture medium (without FBS) and test compound were added. In the lower compartment (well of the plate), 700 µL of culture medium supplemented with 10% FBS and the test compound were added. This system was incubated for 6 h at 37°C with 5% CO_2. After incubation, the medium in the insert was removed along with cells that have not migrated to the bottom of the membrane. The cells that migrated to the lower portion of the membrane were fixed and stained with aqueous toluidine blue 1% in borax for 5 min. The membranes were cut using a surgical lancet, placed on a slide and the migrated cells were counted in the optical microscope Nikon Eclipse E600 (NIS-Elements Image processing software). The assays were performed in duplicate, using colchicine as positive control and DMSO as negative control. The EC_{50} was determined by the curve of log concentration versus percent inhibition. The curves were plotted from the data obtained for six different concentrations of the compounds tested in duplicate. The curves were fitted in the software Sigmaplot 11.0, with the method of nonlinear regression - dynamic fitting. Figure 27 shows a schematic representation of the system employed.

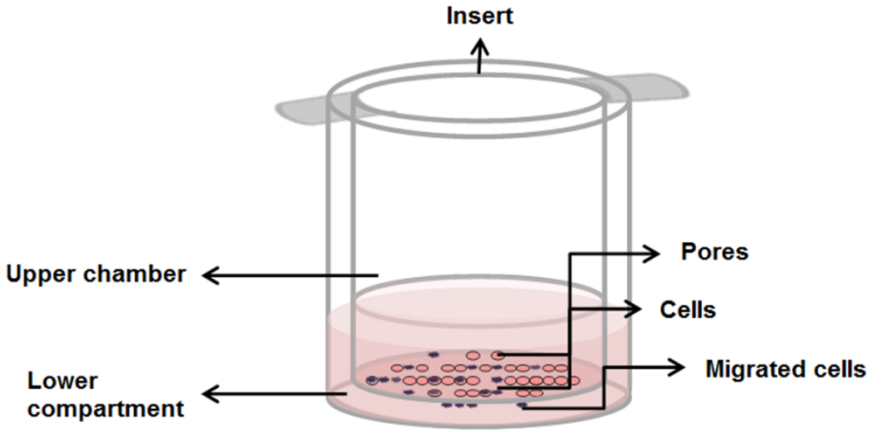


Figure 27. Schematic representation of the system used for the quantitative assessment of cell migration in Boyden chamber.

5.4.4. Cytotoxicity Assay

The cytotoxicity evaluation was performed to test the toxicity of compounds for the cancer cell lines MDA-MB- 231 and MCF-7. The assay was performed in 96-well plates where about 5 X 10^3 cells/well were added. After checking the cell adhesion (about 24 hours), the solution containing the test compound was added. The plate was incubated for 24 h and after this period, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) were added to each well. After 4h incubation, absorbance was read at 490 nm in a SpectraMax Plus384 - Absorbance Microplate Reader (Molecular Devices, Sunnyvale, USA). The plate was kept at 24°C, and assays were performed in triplicate, using doxorubicin as positive control and DMSO as negative control [BARLTROP *et al*., 1991].

5.5. Materials and Instruments

Reagents were purchased from Sigma-Aldrich, Acros Organics, or Fluka and were used without further treatment; solvents were purified according to standard methods [PERRIN; ARMAREGO, 1988]. Reaction progress was monitored by TLC on aluminum plates pre-coated with silica gel F254 layers (0.2 mm thickness; Whatman) and visualized under UV light (254 and 366 nm). Column chromatography

was carried out over silica gel (particle size 35 - 70 µm; Acros Organics). ^{1}H NMR spectra were recorded on Bruker AC 300 (300 MHz), Bruker Avance 400 (400 MHz) or Bruker Avance III 600 (600 MHz) spectrometers; spectra were referenced against the internal standard TMS (δ_{TMS} 0.00) or against the residual solvent resonances of $CDCl_3$ (δ 7.26 [^{1}H] and 77.0 [^{13}C] ppm, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet), coupling constant (Hz). ^{13}C NMR spectra were recorded on Bruker AC 300 (75 MHz) or Bruker Avance III 600 (150 MHz) spectrometers. ^{13}C NMR chemical shifts are reported in parts per million relative to solvent. All ^{13}C NMR spectra were determined with broadband decoupling. When necessary, the structures of the compounds were ensured and the signals unambiguously assigned by 2D NMR techniques: ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{13}C-^{1}H HMQC, and ^{13}C-^{1}H HMBC. Some ^{13}C values were attributed using evidence from HMBC and are marked with an asterisk in the *synthetic methods*. Original spectra are available at Appendix C. Electrospray ionization - time-of-flight (ESI-TOF) high resolution mass spectra were measured on MicrOTOF Q II spectrometer (Bruker Daltonics, Billerica, MA, USA) operating in the positive ion mode. ChemDraw Ultra 8.0 was used to calculate the values of the pseudo-molecular ions $[M+H]^{+}$ and $[M+Na]^{+}$.

5.6. Synthetic Methods

5.6.1. (*E*)-3-(3,4,5-trimethoxyphenyl)acryloyl chloride (26)

To a solution of (*E*)-3,4,5-trimethoxycinnamic acid (200mg, 0.84 mmol) in CH_2Cl_2 under nitrogen atmosphere was added oxalyl chloride (358 µl, 4.2 mmol) and catalytic amount of dimethylformamide (DMF) (catalytic). The reaction was stirred under room temperature for 2 hours. The residue, compound **26**, a yellow solid, was dried and used to the next step without any further purification [ADAMS *et al*., 2012].

5.6.2. 1-((*E*)-3-(3,4,5-trimethoxyphenyl)acryloyl)-1*H*-pyrrole-2,5-dione (27, DRMV 1)

CH_2Cl_2 (8 ml), triethylamine (352 µl, 2.52 mmol), and maleimide (96 mg, 1 mmol) were added to **26** (0.84 mmol). The reaction mixture was stirred at room temperature for overnight and then quenched by the addition of saturated aqueous ammonium chloride solution. The product was extracted with CH_2Cl_2 and dried over

$MgSO_4$ [ADAMS *et al*., 2012]. The product was purified by column chromatography eluted with ciclohexane:ethyl acetate (7:3) to afford DRMV 1 (**27**, 121 mg, 45% yield) as a pure reddish brown solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 3.95 (s, 6H), 3.94 (s, 3H), 6.88 (s, 2H), 6.92 (s, 2H), 7.23 (d, 1H, J = 15.5 Hz), 7.91 (d, 1H, J = 15.5 Hz); ^{13}C{^{1}H}-NMR (75 MHz, $CDCl_3$) δ ppm: 56.2, 61.0, 106.0, 117.9, 129.6, 135.4, 141*, 148.4, 153.5, 162.5*, 167.9; HRMS (ESI-TOF) *m/z*: 318.0977 $[M+H]^+$ (calcd for $C_{16}H_{16}NO_6$, 318.0972), 340.0796 $[M+Na]^+$ (calcd for $C_{16}H_{15}NNaO_6$, 340.0792).

5.6.3. 2-((*E*)-3-(3,4,5-trimethoxyphenyl)acryloyl)isoindoline-1,3-dione (28, DRMV 2)

CH_2Cl_2 (8 ml), triethylamine (352 µl, 2.52 mmol), and phthalimide (147 mg, 1 mmol) were added to **26** (0.84 mmol). The reaction mixture was stirred at room temperature for overnight and then quenched by the addition of saturated aqueous ammonium chloride solution. The product was extracted with CH_2Cl_2 and dried over $MgSO_4$ [ADAMS *et al*., 2012]. The product was purified by column chromatography eluted with ciclohexane:ethyl acetate (7:3) to afford DRMV 2 (**28**, 121 mg, 40% yield) as a pure light yellow solid. ^{1}H-NMR (600 MHz, $CDCl_3$) δ ppm: 3.90 (s, 3H), 3.92 (s, 6H), 6.86 (s, 2H), 7.28 (d, 1H, J = 15.5 Hz), 7.86 (dd, 2H, J = 3 and 5.5 Hz), 7.90 (d, 1H, J = 15.5 Hz), 8.00 (dd, 2H, J = 3 and 5.5 Hz); ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 56.2, 61.0, 106.0, 118.7, 124.4, 129.7, 131.4, 135.4, 141.0, 147.9, 153.5, 163.7, 165.7; HRMS (ESI-TOF) *m/z*: 368.1132 $[M+H]^+$ (calcd for $C_{20}H_{18}NO_6$, 368.1129), 390.0948 $[M+Na]^+$ (calcd for $C_{20}H_{17}NNaO_6$, 390.0948).

5.6.4. (*E*)-1-(1*H*-indol-1-yl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (29, DRMV 3)

CH_2Cl_2 (4 ml), sodium hydride (33 mg, 0.84 mmol), and indole (59 mg, 0.5 mmol) were added to **26** (0.42 mmol). The reaction mixture was stirred at room temperature for overnight and then quenched by the addition of saturated aqueous ammonium chloride solution. The product was extracted with CH_2Cl_2 and dried over $MgSO_4$ [ADAMS *et al*., 2012]. The product was purified by column chromatography eluted with ciclohexane:ethyl acetate (7:3) to afford DRMV 3 (**29**, 58 mg, 41% yield) as a pure reddish brown solid. ^{1}H-NMR (400 MHz, CDCl3) δ ppm: 3.96 (s, 3H), 3.99

(s, 6H), 6.74 (d, 1H, *J* = 3.7 Hz), 6.89 (s, 2H), 7.16 (d, 1H, *J* = 15.3 Hz), 7.32 (dd, 1H, *J* = 8.0 and 7.8 Hz), 7.41 (dd, 1H, *J* = 8.0 and 8.2 Hz), 7.63 (d, 1H, *J* = 7.8 Hz), 7.69 (d, 1H, *J* = 3.7 Hz), 7.94 (d, 1H, *J* = 15.3 Hz), 8.57 (d, 1H, *J* = 8.2 Hz); ^{13}C-NMR (75 MHz, CDCl3) *δ* ppm: 56.3, 61.0, 105.6, 109.2, 116.4, 116.9, 120.9, 123.8, 124.6, 125.1, 129.9*, 130.5*, 135.8*, 140.5*, 146.7, 153.5, 164.2*; HRMS (ESI-TOF) *m/z*: 338.1386 $[M+H]^+$ (calcd for $C_{20}H_{20}NO_4$, 338.1387), 360.1219 $[M+Na]^+$ (calcd for $C_{20}H_{19}NNaO_4$, 360.1206).

5.6.5. (*E*)-*N*-acryloyl-3-(3,4,5-trimethoxyphenyl)acrylamide (30, DRMV 4)

To a solution of acrylamide (710 mg, 10 mmol) in THF (50 ml) at −78^{o}C, *n*-BuLi (4.8 ml, 12 mmol; 2.5 M in hexane) was added dropwise. The reaction mixture was warmed to room temperature and the reaction was carried out for 7 hours. The reaction mixture was cooled to −78^{o}C and a solution of **26** (20 mmol) in THF was added dropwise. The reaction was warmed to room temperature, carried out for 15 hours and then quenched by the addition of saturated aqueous ammonium chloride solution. The reaction mixture was extracted with diethyl ether, washed with saturated solution of sodium chloride, and dried with magnesium sulfate filtered. The product was purified by recrystallization with ethyl ether (30 ml) and subsequently with ethanol (70 ml) to afford DRMV 4 (**30**, 920 mg, 30% yield) as a pure yellow solid [BOSKOVIC *et al*., 2013]. ^{1}H-NMR (600 MHz, CDCl3) *δ* ppm: 3.89 (s, 3H), 3.90 (s, 6H), 5.94 (dd, 1H, *J* = 1.2 Hz and 10.5 Hz), 6.56 (dd, 1H, *J* = 1.2 Hz and 16.6 Hz), 6.77 (dd, 1H, *J* = 10.5 and 16.6 Hz), 6.83 (s, 2H), 7.31 (d, 1H, *J* = 15.6 Hz), 7.79 (d, 1H, *J* = 15.6 Hz), 9.17 (s, 1H); ^{13}C-NMR (150 MHz, $CDCl_3$) *δ* ppm: 56.2, 61.0, 105.8, 118.6, 129.8, 130.3, 131.2, 140.6, 146.5, 153.4, 165.6, 166.9; HRMS (ESI-TOF) *m/z*: 292.1188 $[M+H]^+$ (calcd for $C_{15}H_{18}NO_5$, 292.1179), 314.1016 $[M+Na]^+$ (calcd for $C_{15}H_{17}NNaO_5$, 314.0999).

5.6.6. (2*E*)-3-(3,4,5-trimethoxyphenyl)-*N*-(2-oxo-2*H*-chromen-6-yl)acrylamide (31, DRMV 5)

CH_2Cl_2 (1 ml), triethylamine (352 µl, 2.52 mmol), and 6-amino-chromen-2-one (161 mg, 1 mmol) were added to **26** (0.84 mmol). The reaction mixture was stirred at room temperature for 2 hours and then quenched by the addition of saturated

aqueous ammonium chloride solution. The product was extracted with ethyl acetate and dried over $MgSO_4$ [ADAMS *et al*., 2012]. The product was purified by column chromatography eluted with and *n*-hexane:EtOAc gradient from 7:3 to 0:1 to afford DRMV 5 (**31**, 80 mg, 21% yield) as a pure light yellow solid [ADAMS *et al*., 2012]. ^{1}H-NMR (600 MHz, CDCl3) δ ppm: 3.89 (s, 3H), 3.91 (s, 6H), 6.47 (d, 1H, *J* = 9.5 Hz), 6.54 (d, 1H, *J* = 15.3 Hz), 6.78 (s, 2H), 7.30 (d, 1H, *J* = 8.9 Hz), 7.51 (dd, 1H, *J* = 2.5 and 8.9 Hz), 7.71 (d, 1H, *J* = 15.3 Hz), 7.73 (d, 1H, *J* = 9.5 Hz), 7.81 (s, 1H), 8.18 (bs, 1H; obs.: broad signal, constant couple not observed); ^{13}C-NMR (150 MHz, $CDCl_3$) δ ppm: 56.2, 61.0, 105.3, 117.2, 117.3, 118.5, 119.1, 119.6, 123.5, 129.9, 134.6, 140.1, 142.9, 143.5, 150.5, 153.5, 160.8, 164.1; HRMS (ESI-TOF) *m/z*: 382.1285 $[M+H]^+$ (calcd for $C_{21}H_{20}NO_6$, 382.1285), 404.1097 $[M+Na]^+$ (calcd for $C_{21}H_{19}NNaO_6$, 404.1105).

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APPENDICES

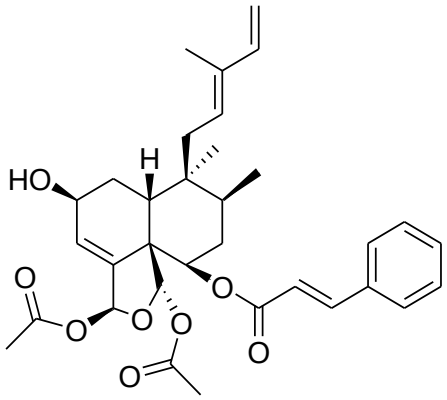
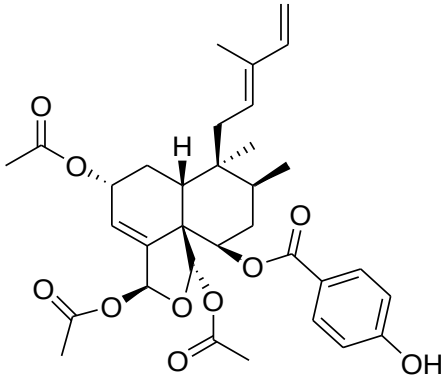
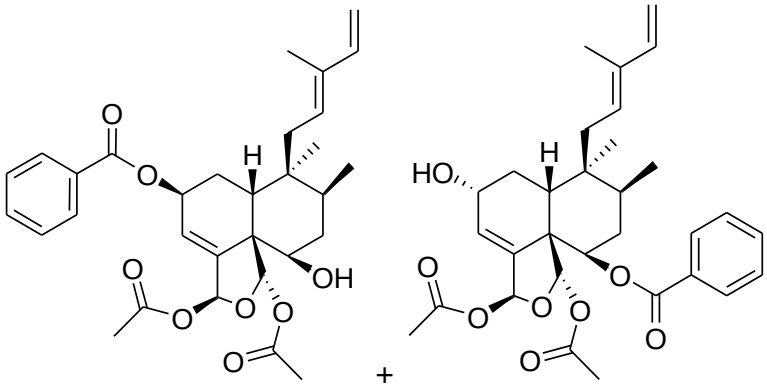
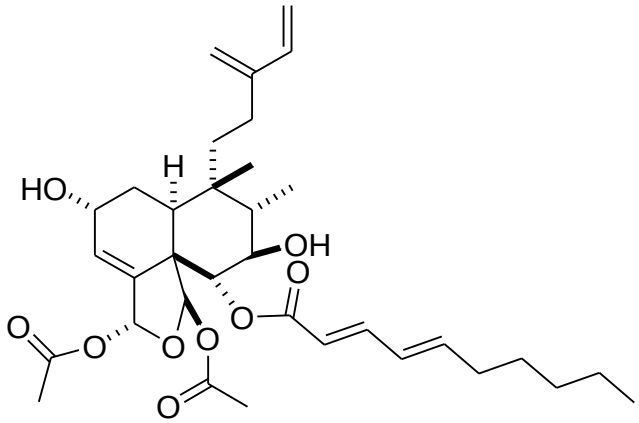
Appendix A. Results obtained for the virtual screening. The dark cells indicate the compounds that were selected for the *in vitro* assays.

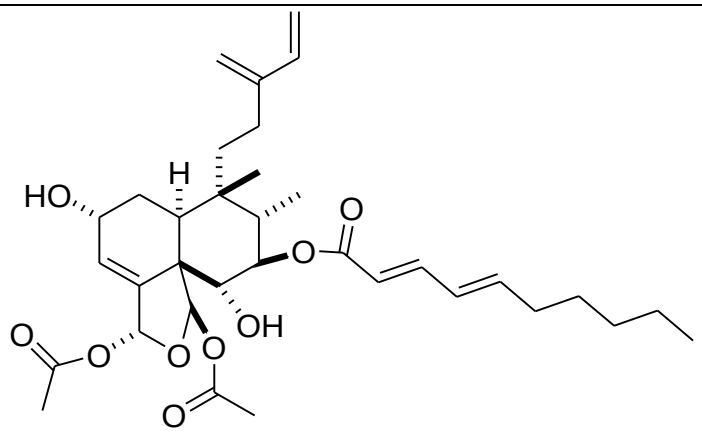
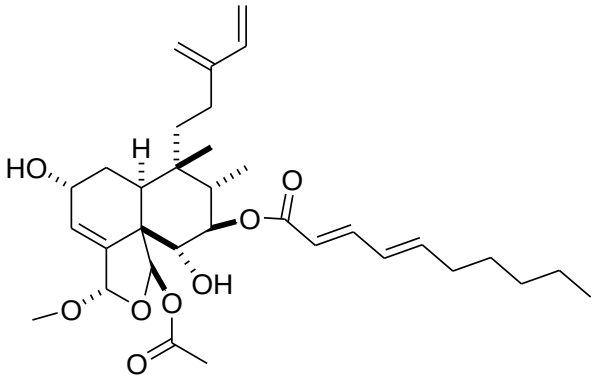
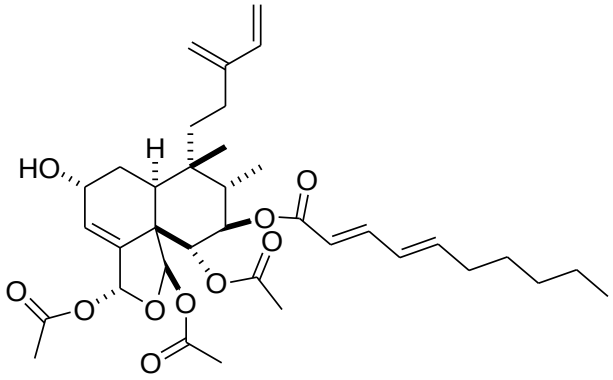
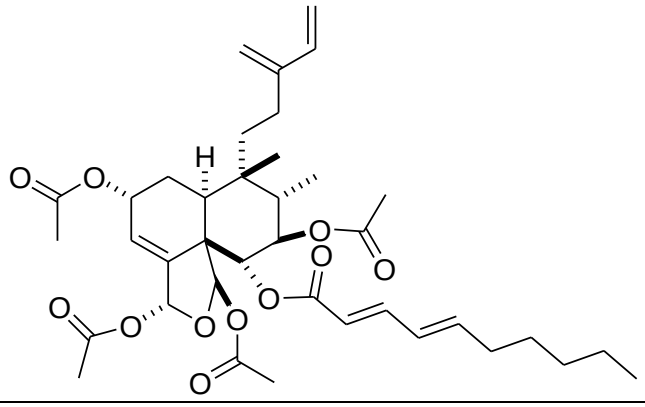
Taxol binding site		Colchicine binding site		Vinblastine binding site	
Fitness	**Ligand name**	**Fitness**	**Ligand name**	**Fitness**	**Ligand name**
71.54	'NuBBE640'	78.71	'NuBBE228'	91.49	'NuBBE640'
69.73	'NuBBE200'	72.85	NuBBE893'	80.67	'NuBBE639'
69.21	'NuBBE639'	71.16	NuBBE840'	78.82	'NuBBE638'
66.79	NuBBE853'	70.24	'NuBBE200'	77.02	'NuBBE636'
65.61	NuBBE840'	69.62	NuBBE892'	75.88	'NuBBE863'
63.99	'NuBBE639b'	69.02	'NuBBE226'	73.6	'NuBBE640b'
63.96	'NuBBE635b'	67.47	NuBBE863'	71.11	'NuBBE635'
62.6	'NuBBE636'	66.96	'NuBBE639'	70.33	'NuBBE200'
62.42	NuBBE842'	66.6	'NuBBE423'	70.03	'NuBBE637'
62.21	'NuBBE635'	66.47	'NuBBE635b'	69.73	'NuBBE636b'
61.95	'NuBBE228'	65.51	NuBBE859b'	68.67	'NuBBE865'
61.5	'NuBBE637'	65.23	'NuBBE806'	66.95	'NuBBE638b'
61.39	NuBBE870b'	65.06	NuBBE854'	66.51	'NuBBE635b'
61.36	'NuBBE423'	64.58	'NuBBE47'	65.07	'NuBBE637b'
60.33	NuBBE891'	64.55	'NuBBE61'	64.41	'NuBBE228'
60	NuBBE854'	64.53	NuBBE853'	64.36	'NuBBE59'
59.85	'NuBBE640b'	63.83	'NuBBE59'	63.93	'NuBBE891'
59.82	'NuBBE57'	63.72	NuBBE841'	63.36	'NuBBE57'
59.73	'NuBBE821'	63.58	'NuBBE820'	62.03	'NuBBE639b'
59.39	'NuBBE214'	62.97	'NuBBE639b'	61.71	'NuBBE864'
58.91	'NuBBE638b'	62.26	'NuBBE636'	61.39	'NuBBE842'
58.89	'NuBBE47'	62.11	'NuBBE78'	61.37	'NuBBE61'
58.83	NuBBE864'	61.89	'NuBBE637b'	60.85	'NuBBE47'
58.7	NuBBE863'	61.72	NuBBE865'	59.93	'NuBBE423'
58.66	'NuBBE48'	61.35	NuBBE870a'	59.22	'NuBBE857'
58.4	NuBBE870a'	61.19	'NuBBE819'	58.63	'NuBBE840'
57.85	'NuBBE536'	60.71	'NuBBE637'	58.04	'NuBBE48'
57.83	'NuBBE632'	59.79	NuBBE855'	58.03	'NuBBE60'
57.58	'NuBBE638'	59.54	NuBBE848'	57.48	'NuBBE821'
57.55	'NuBBE61'	59.51	NuBBE869'	56.77	'NuBBE841'
57.14	NuBBE859a'	58.98	NuBBE860b'	56.33	'NuBBE853'
57.06	NuBBE860a'	58.96	NuBBE870b'	56.2	'NuBBE11'
56.99	NuBBE892'	58.92	NuBBE856'	56.08	'NuBBE226'
56.43	'NuBBE636b'	58.91	'NuBBE632'	55.43	'NuBBE856'
55.73	'NuBBE538'	58.19	'NuBBE538'	55.16	'NuBBE806'
55.53	NuBBE865'	58.05	'NuBBE60'	54.3	'NuBBE893'
54.94	NuBBE862'	57.96	'NuBBE214'	54.21	'NuBBE860b'
53.98	'NuBBE637b'	57.25	'NuBBE821'	53.8	'NuBBE843'
53.8	'NuBBE60'	57.23	'NuBBE817'	53.62	'NuBBE869'

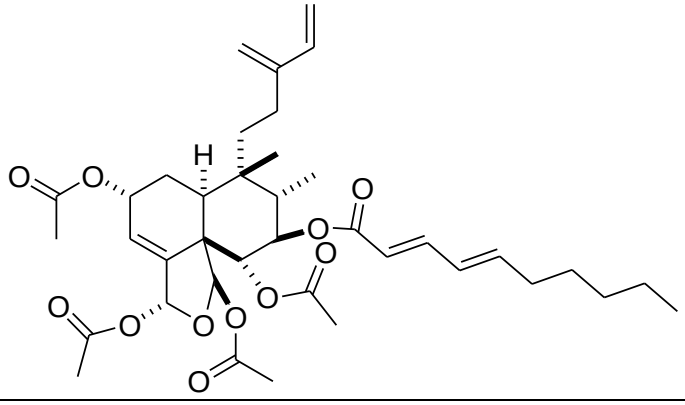
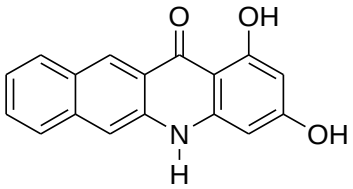
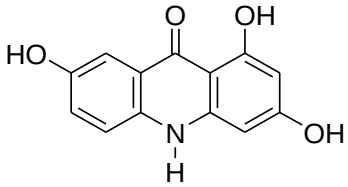
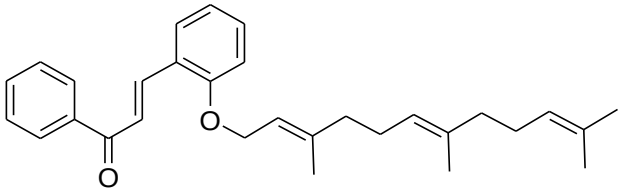
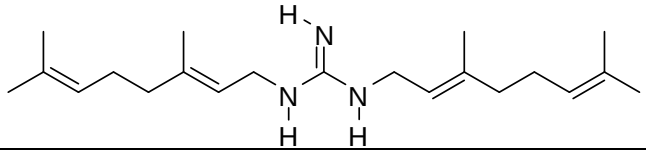
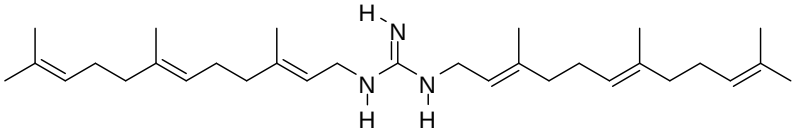
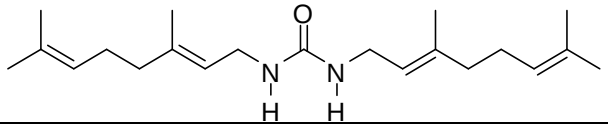
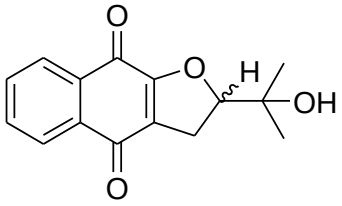
53.7	'NuBBE806'	56.96	'NuBBE536'	53.53	'NuBBE848'
53.51	'NuBBE52'	56.71	'NuBBE635'	53.21	'NuBBE214'
53.3	NuBBE869'	55.77	NuBBE891'	53.06	'NuBBE884'
53.09	'NuBBE226'	55.73	NuBBE859a'	52.62	'NuBBE854'
53.04	'NuBBE59'	55.37	'NuBBE258'	52.56	'NuBBE536'
52.92	NuBBE844'	55.32	NuBBE860a'	52.47	'NuBBE632'
52.76	NuBBE846'	55.03	NuBBE886'	52.06	'NuBBE880'
52.45	'NuBBE11'	54.78	'NuBBE11'	51.35	'NuBBE538'
52.44	'NuBBE819'	54.68	'NuBBE19'	51.35	'NuBBE882'
52.1	NuBBE859b'	54.68	NuBBE890'	51.31	'NuBBE52'
51.75	NuBBE860b'	54.56	'NuBBE636b'	51.21	'NuBBE892'
51.64	NuBBE893'	54.14	NuBBE843'	50.95	'NuBBE859b'
51.46	'NuBBE817'	53.98	NuBBE885'	50.88	'NuBBE819'
50.59	NuBBE839'	53.74	'NuBBE52'	50.71	'NuBBE805'
50.22	'NuBBE820'	53.64	NuBBE862'	50.45	'NuBBE256'
49.95	NuBBE843'	53.62	NuBBE844'	50.38	'NuBBE820'
49.77	'NuBBE78'	53.45	NuBBE880'	49.56	'NuBBE407'
49.59	NuBBE848'	53.31	'NuBBE805'	49.45	'NuBBE881'
49.53	NuBBE885'	53.25	'NuBBE638b'	49.39	NuBBE870b'
49.03	NuBBE841'	52.85	NuBBE845'	48.45	'NuBBE871'
48.94	'NuBBE805'	52.74	NuBBE842'	48.22	'NuBBE847'
48.09	'NuBBE407'	52.42	'NuBBE18'	48.16	'NuBBE862'
47.9	NuBBE884'	52.37	NuBBE847'	47.85	'NuBBE19'
47.22	NuBBE887'	52.3	'NuBBE256'	47.77	'NuBBE803'
47.17	'NuBBE258'	51.96	NuBBE857'	47.58	'NuBBE859a'
46.7	NuBBE877'	51.7	'NuBBE191'	47.41	'NuBBE18'
46.37	'NuBBE803'	51.58	NuBBE868'	47.25	'NuBBE78'
46.05	NuBBE880'	51.5	'NuBBE216'	47.13	'NuBBE860a'
46.04	'NuBBE19'	51.46	NuBBE881'	46.84	'NuBBE888'
45.9	'NuBBE128'	51.25	NuBBE839'	46.27	NuBBE870a'
45.78	'NuBBE21'	50.77	'NuBBE128'	46.16	'NuBBE844'
45.65	NuBBE868'	50.6	'NuBBE803'	46.06	'NuBBE889'
45.52	'NuBBE18'	50.55	'NuBBE71'	46.05	'NuBBE846'
45.22	NuBBE847'	50.21	'NuBBE215'	45.93	'NuBBE128'
44.92	NuBBE886'	50.07	NuBBE846'	45.93	'NuBBE868'
44.48	'NuBBE773'	49.87	'NuBBE21'	45.36	'NuBBE258'
44	NuBBE850'	49.59	NuBBE887'	45	'NuBBE885'
43.84	NuBBE867'	49.49	'NuBBE79'	44.58	'NuBBE79'
43.83	'NuBBE215'	49.48	NuBBE866'	44.46	'NuBBE886'
43.79	'NuBBE79'	48.96	NuBBE884'	44.41	'NuBBE855'
43.76	NuBBE856'	48.66	NuBBE861'	44.15	'NuBBE850'
43.69	NuBBE883'	48.63	'NuBBE48'	44.04	'NuBBE817'
43.3	NuBBE889'	48.62	NuBBE850'	43.57	'NuBBE887'
43.28	'NuBBE256'	48.59	NuBBE867'	43.5	'NuBBE851'
43.28	NuBBE852'	48.38	NuBBE858'	43.49	'NuBBE877'
43.25	NuBBE845'	48.36	NuBBE882'	43.32	'NuBBE839'

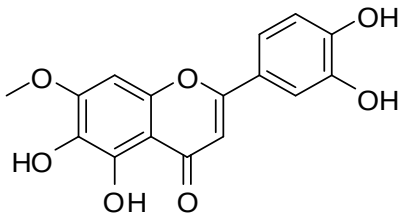
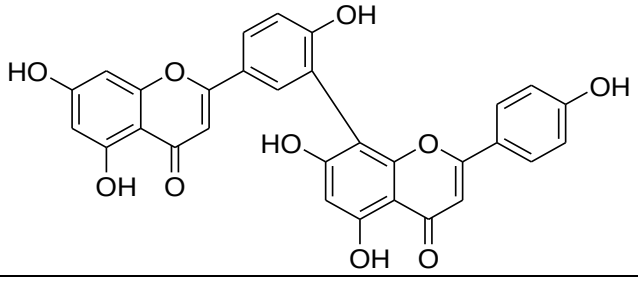
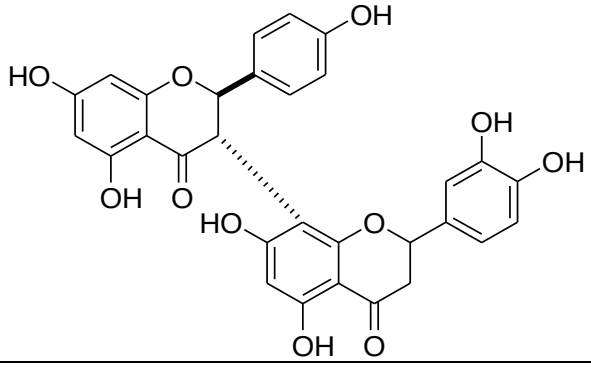
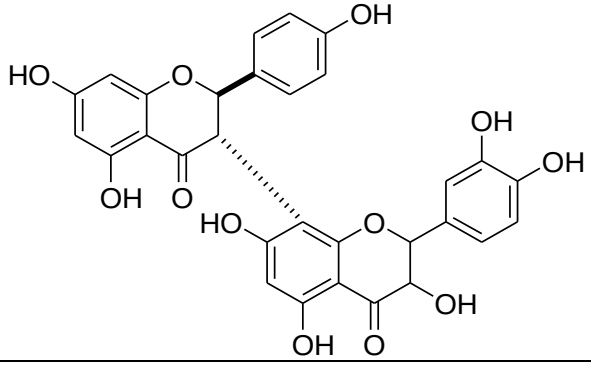
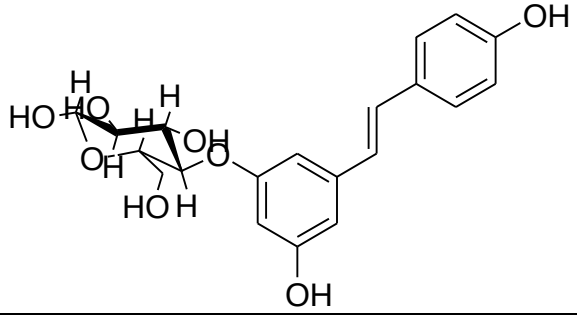
43.12	NuBBE855'	48.15	'NuBBE700'	43.15	'NuBBE24'
42.98	'NuBBE700'	47.46	NuBBE889'	42.94	'NuBBE43'
42.82	NuBBE876'	47.32	NuBBE875'	42.88	'NuBBE21'
42.74	'NuBBE71'	47.29	NuBBE888'	42.72	'NuBBE700'
42.72	NuBBE881'	47.01	'NuBBE693'	42.68	'NuBBE861'
42.64	'NuBBE216'	46.9	NuBBE852'	42.66	'NuBBE191'
42.33	NuBBE861'	46.63	'NuBBE24'	42.53	'NuBBE876'
42.26	NuBBE851'	46.53	'NuBBE726'	42.52	'NuBBE539'
42.15	NuBBE890'	46.08	'NuBBE638'	42.02	'NuBBE773'
42.06	NuBBE858'	45.45	NuBBE873a'	41.76	'NuBBE770'
42.04	'NuBBE24'	45.36	'NuBBE40'	41.31	'NuBBE873b'
41.91	NuBBE872'	44.7	NuBBE849'	41.3	'NuBBE40'
41.28	NuBBE882'	44.46	NuBBE851'	41.22	'NuBBE883'
40.67	'NuBBE191'	44.44	'NuBBE539'	40.77	'NuBBE71'
40.57	NuBBE857'	44.35	'NuBBE407'	40.66	'NuBBE867'
40.46	NuBBE871'	44.18	'NuBBE770'	40.6	'NuBBE215'
40.03	NuBBE849'	44.12	NuBBE876'	40.34	'NuBBE216'
39.8	'NuBBE40'	43.99	NuBBE883'	39.93	'NuBBE852'
39.57	'NuBBE770'	43.92	'NuBBE773'	39.81	'NuBBE726'
39.43	NuBBE875'	43.73	NuBBE871'	39.65	'NuBBE866'
39.29	NuBBE866'	43.69	NuBBE873b'	39.56	'NuBBE849'
38.51	'NuBBE538'	43.51	NuBBE878'	39.47	'NuBBE872'
38.47	'NuBBE726'	43.38	NuBBE877'	38.73	'NuBBE873a'
37.74	NuBBE888'	43.01	'NuBBE43'	38.55	'NuBBE890'
37.61	'NuBBE693'	42.78	NuBBE872'	38.45	'NuBBE858'
37.51	'NuBBE43'	42.02	NuBBE879'	38.37	'NuBBE845'
36.36	NuBBE873b'	40.15	'NuBBE57'	38.29	'NuBBE693'
36.24	NuBBE879'	39.27	NuBBE874'	38.27	'NuBBE875'
35.53	NuBBE873a'	32.65	'NuBBE640'	36.26	'NuBBE44'
35.46	NuBBE874'	32.33	'NuBBE44'	35.96	'NuBBE879'
34.82	NuBBE878'	24.68	'NuBBE640b'	33.26	'NuBBE878'
34.61	'NuBBE44'	19.13	NuBBE864'	32.7	'NuBBE874'

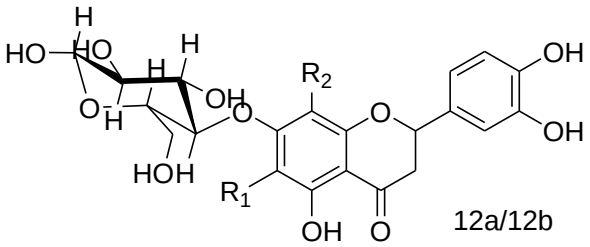
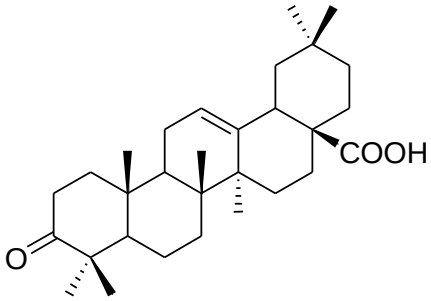
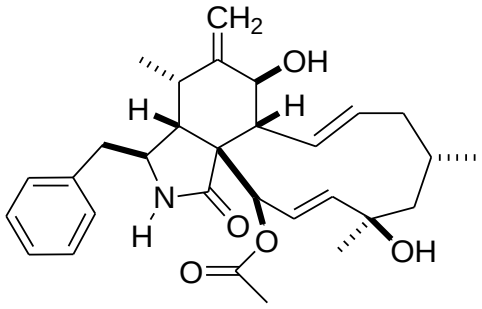
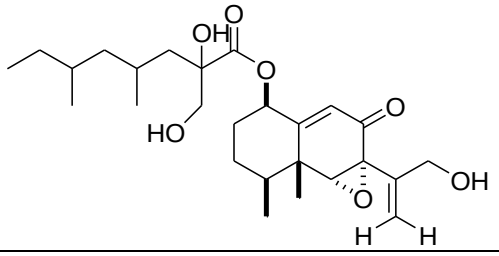
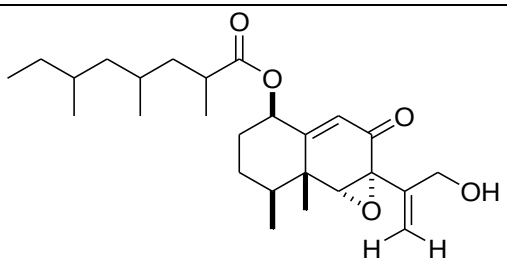
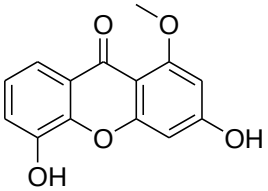
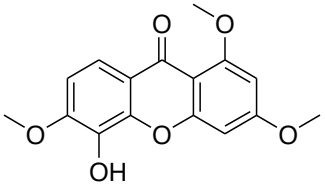
Appendix B. Docking hits tested in the *in vitro* fluorescence based evaluation of tubulin polymerization.

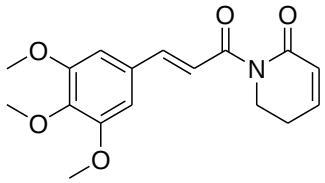
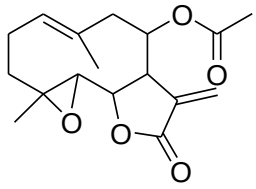
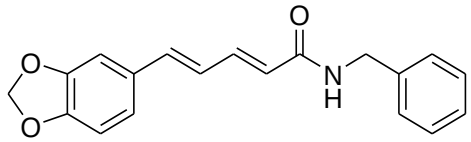
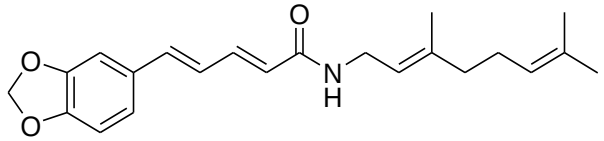
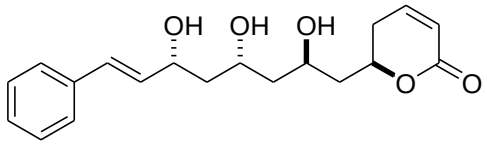
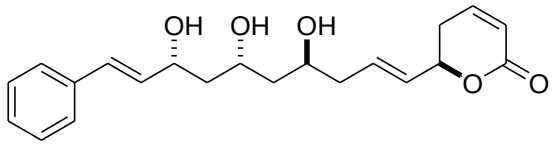
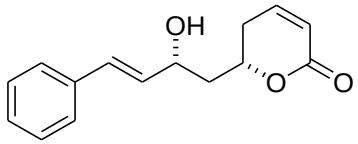
Compound	Concentration in the assay	Structure
NuBBE 61	250µM	
NuBBE 57	250µM	
Mistura NuBBE 59+60	250µM	
NuBBE 635	250µM	

NuBBE 636	250µM	
NuBBE 637	250µM	
NuBBE 638	250µM	
NuBBE 639	250µM	

NuBBE 640	250µM	
NuBBE 693	50µM	
NuBBE 852	250µM and 50µM	
NuBBE 853	250µM and 50µM	
NuBBE 423	250µM and 50µM	
NuBBE 840	250µM and 50µM	
NuBBE 841	250µM and 50µM	
NuBBE 873	250µM and 50µM	

NuBBE 128	250µM and 50µM	
NuBBE 200	250µM and 50µM	
NuBBE 856	250µM and 50µM	
NuBBE 857	50µM	
NuBBE 862	250µM and 50µM	

NuBBE 859	250µM and 50µM	 R1 R2 12a/12b OH H 13a/13b H OH
NuBBE 861	50µM	
NuBBE 855	250µM and 50µM	
NuBBE 0892	50µM	
NuBBE 0893	50µM	
NuBBE 875	50µM	
NuBBE 876	50µM	

NuBBE 0018	EC_{50}	
NuBBE 0878	50µM	
NuBBE 0885	50µM	
NuBBE 0891	50µM	
NuBBE 536	50µM	
NuBBE 538	50µM	
NuBBE 539	50µM	

Appendix C. NMR and HRMS Spectra

Figure A1. ^{1}H NMR (400 MHz, $CDCl_3$) of DRMV1.

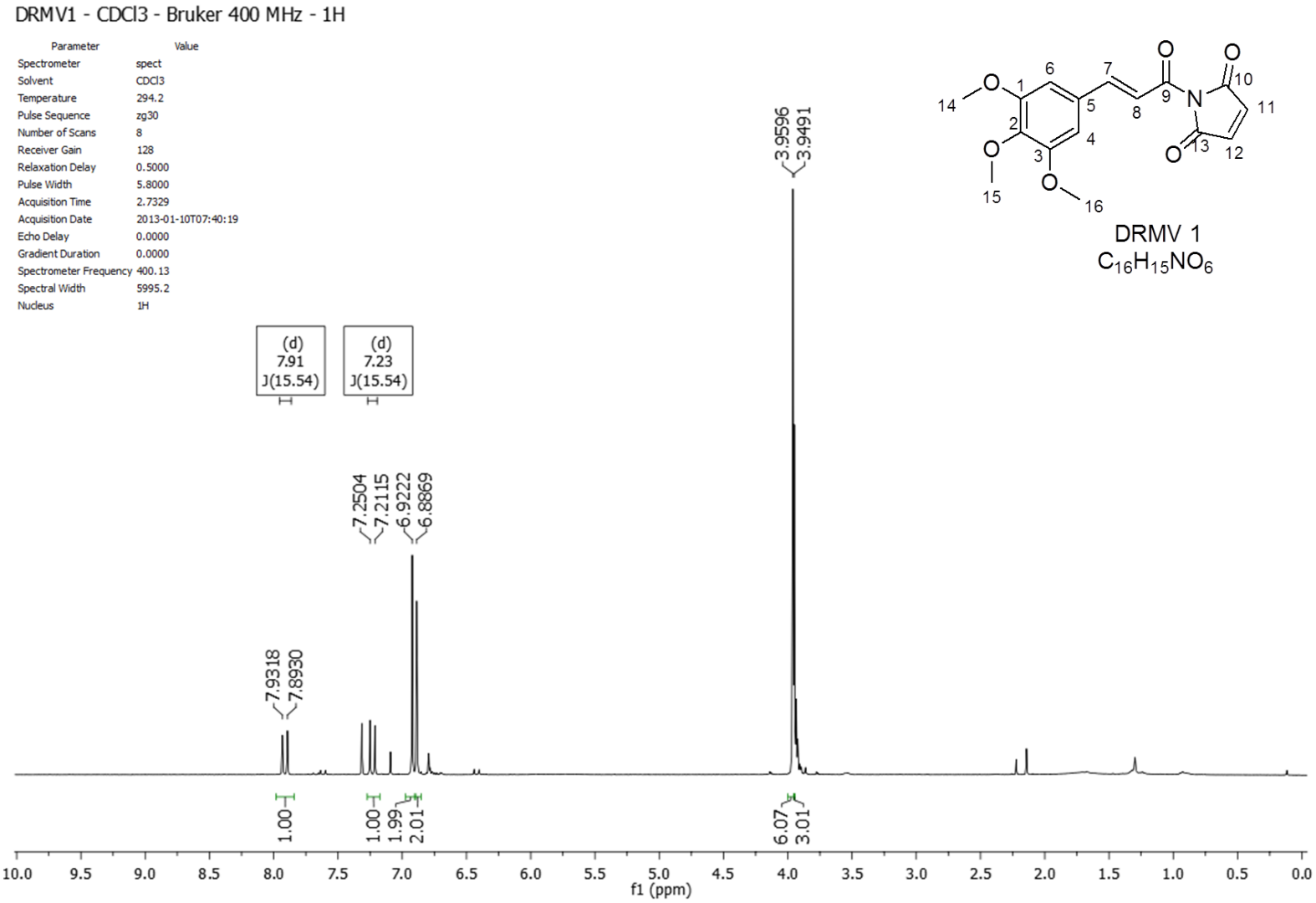


Figure A2. ^{13}C NMR (75 MHz, $CDCl_3$) of DRMV1.

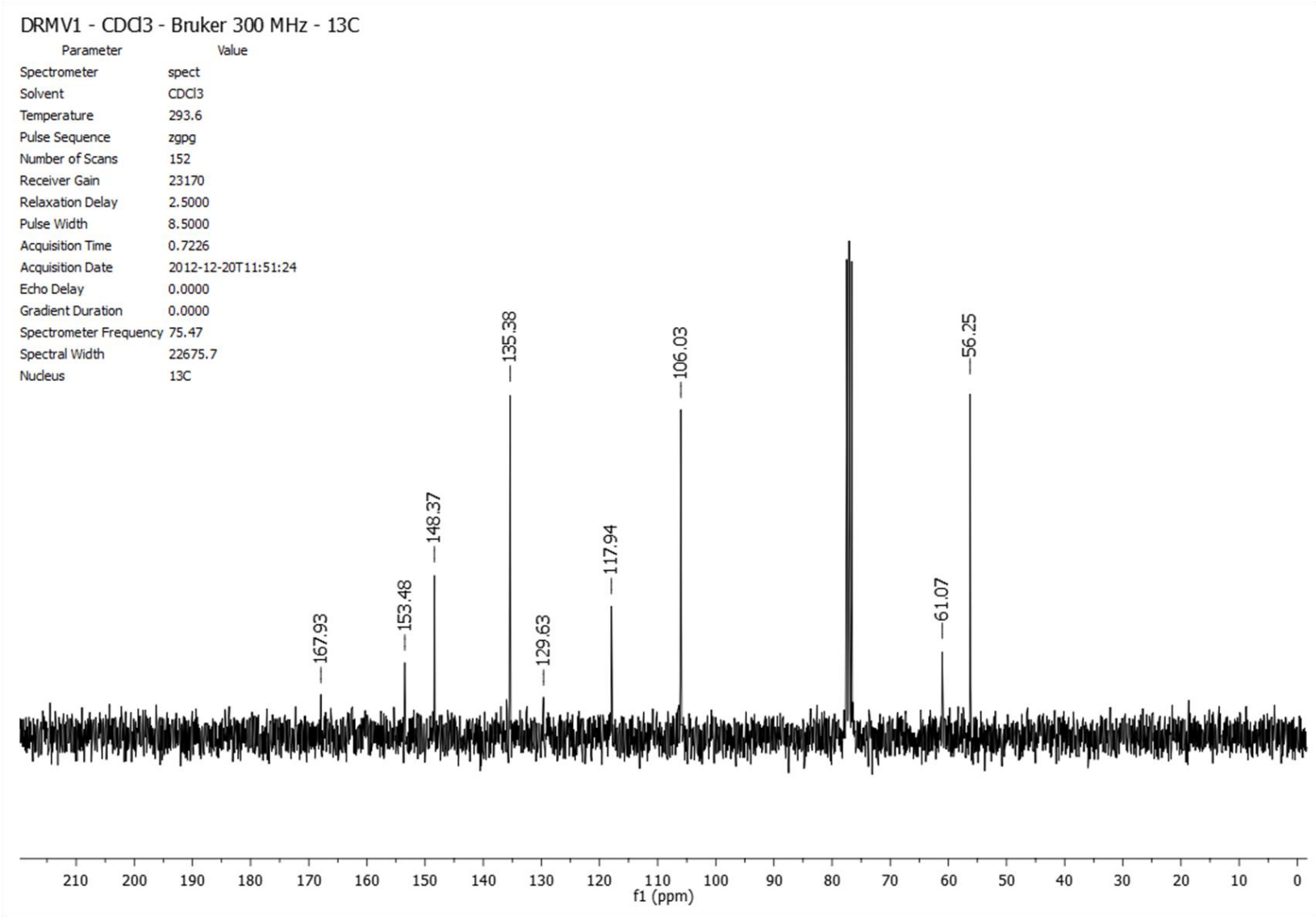


Figure A3. HMBC (400 MHz, $CDCl_3$) of DRMV1.

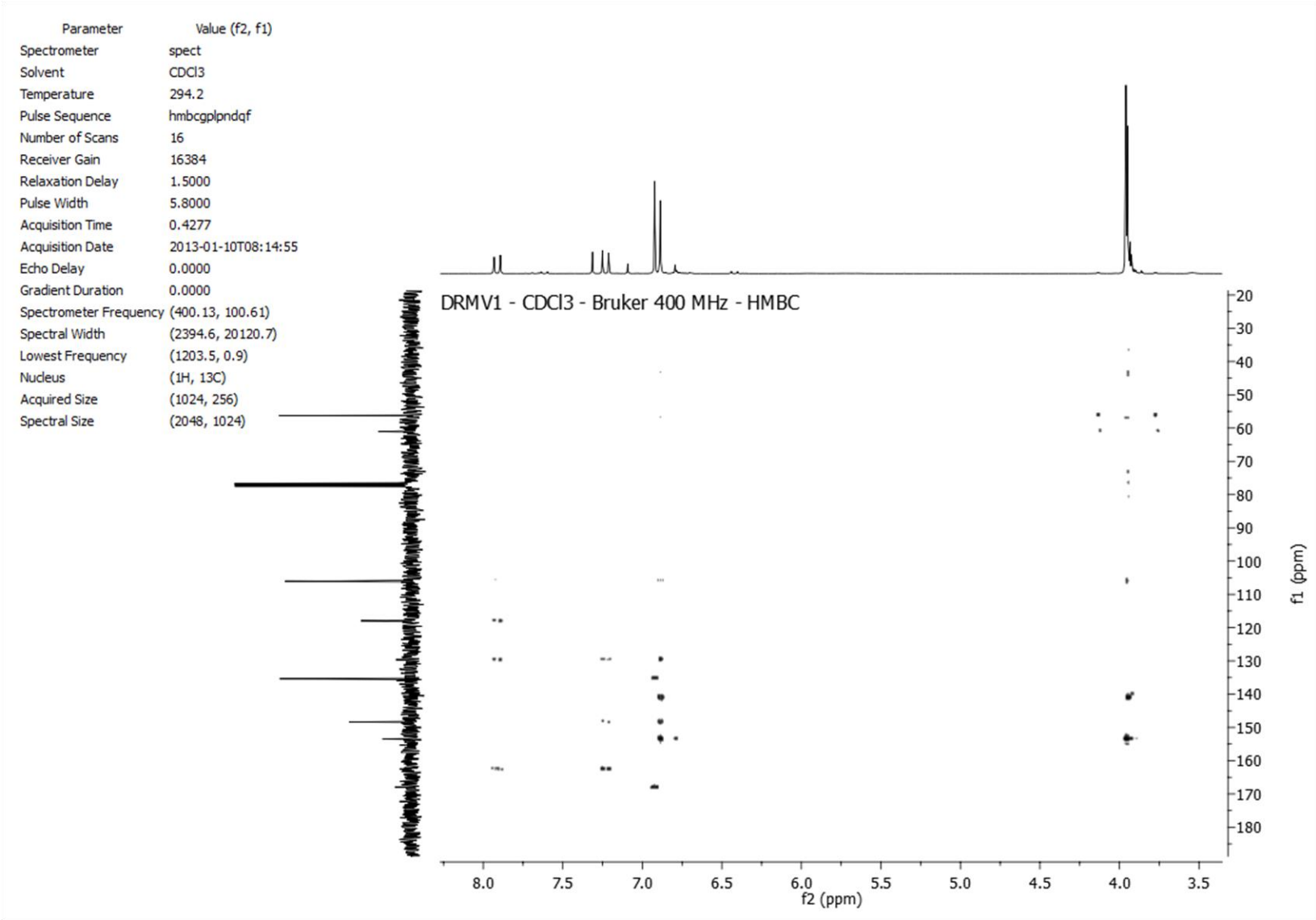


Figure A4. ^{1}H NMR (600 MHz, $CDCl_3$) of DRMV2

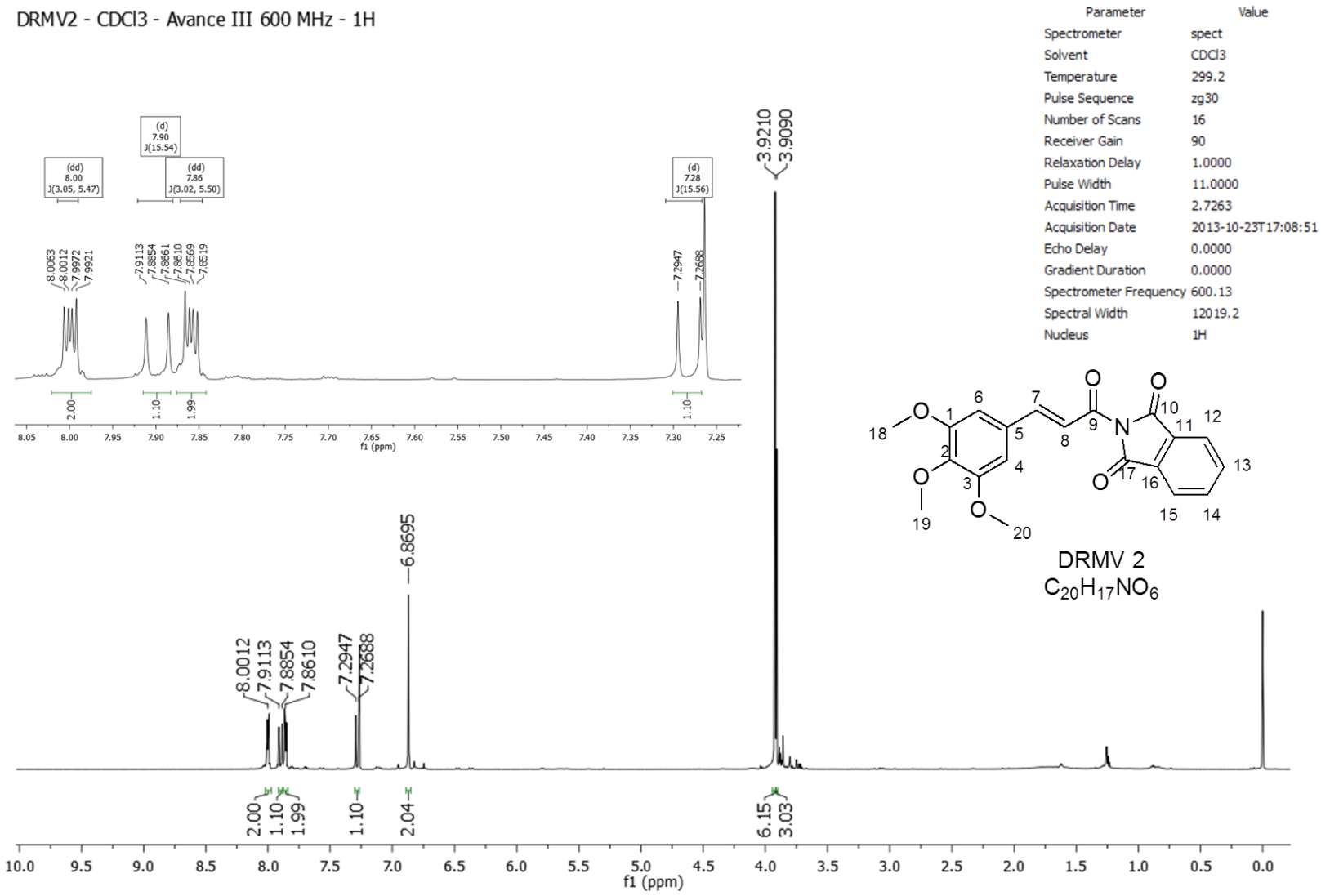


Figure A5. ^{13}C NMR (150 MHz, $CDCl_3$) of DRMV2

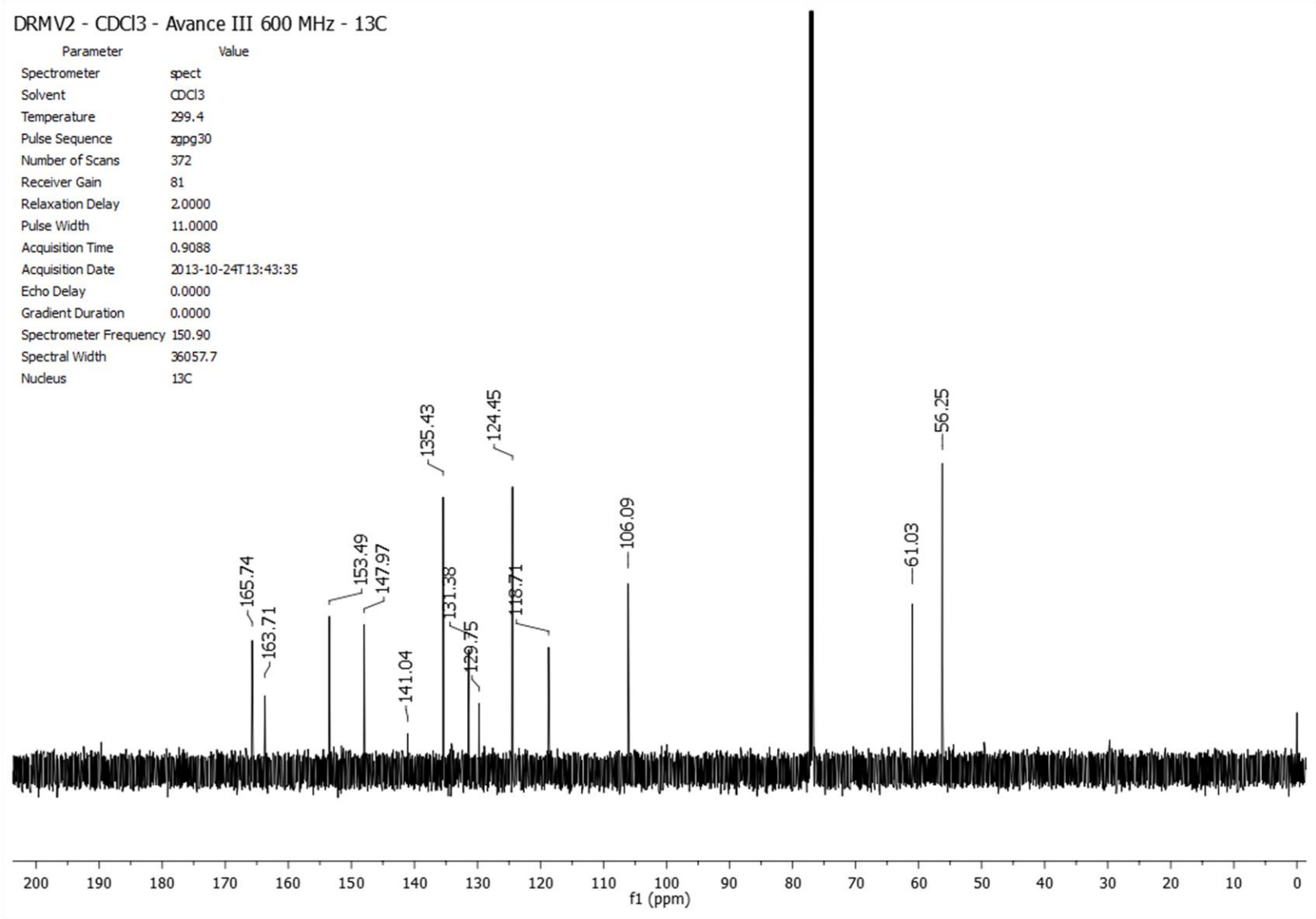


Figure A6. HMBC (600 MHz, $CDCl_3$) of DRMV2

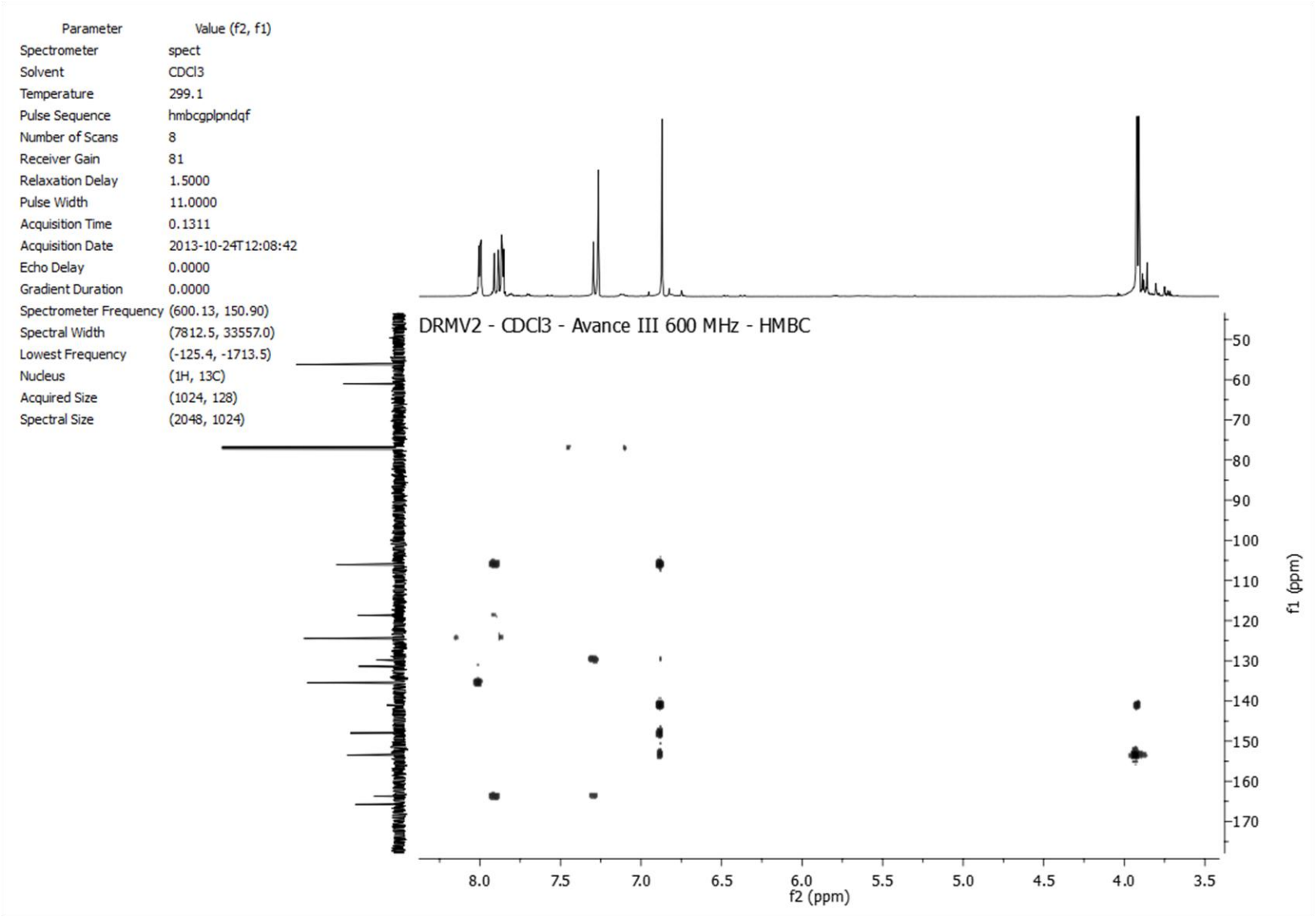


Figure A7. ^{1}H NMR (400 MHz, $CDCl_3$) of DRMV3

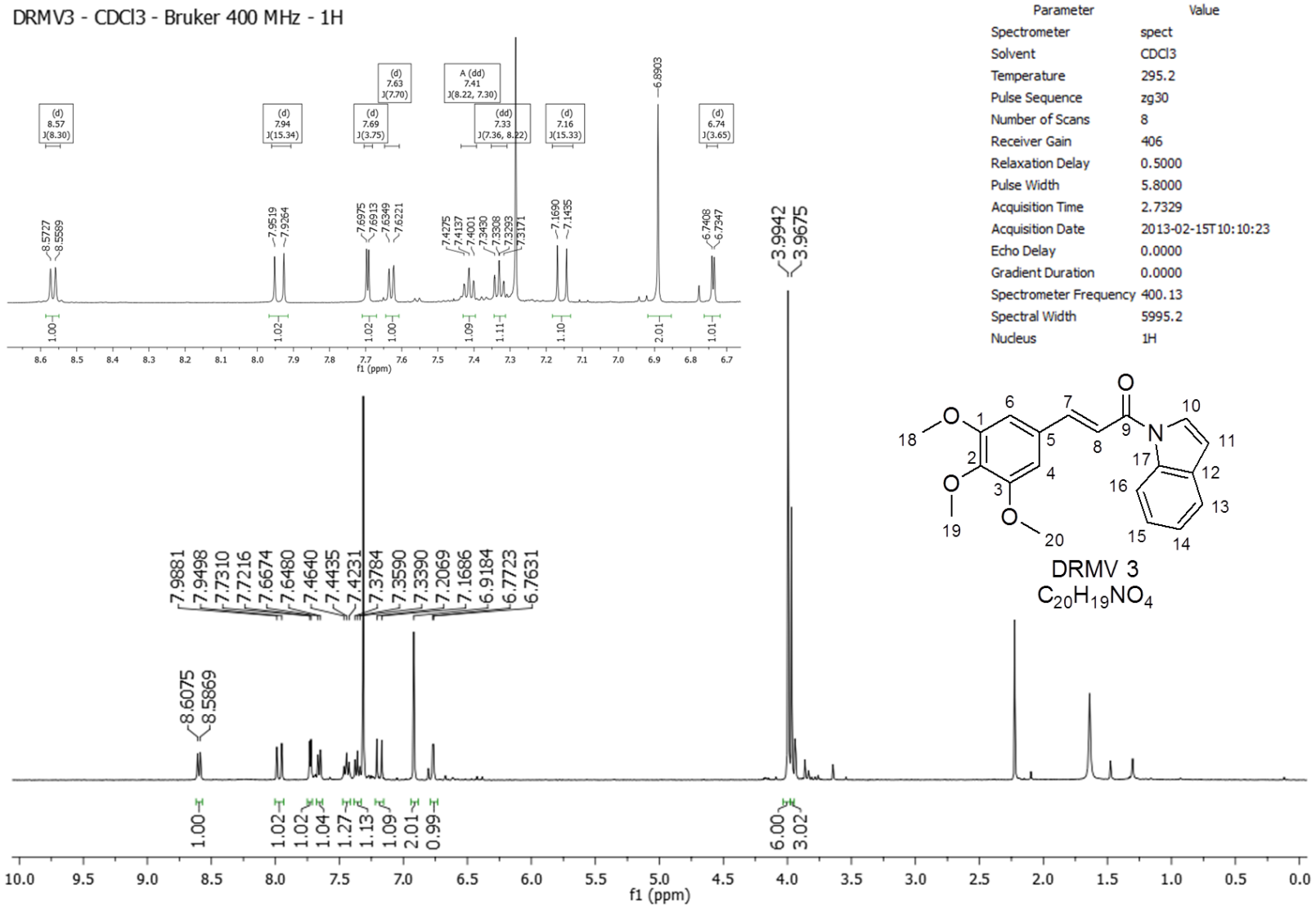


Figure A8. ^{13}C NMR (75 MHz, $CDCl_3$) of DRMV3

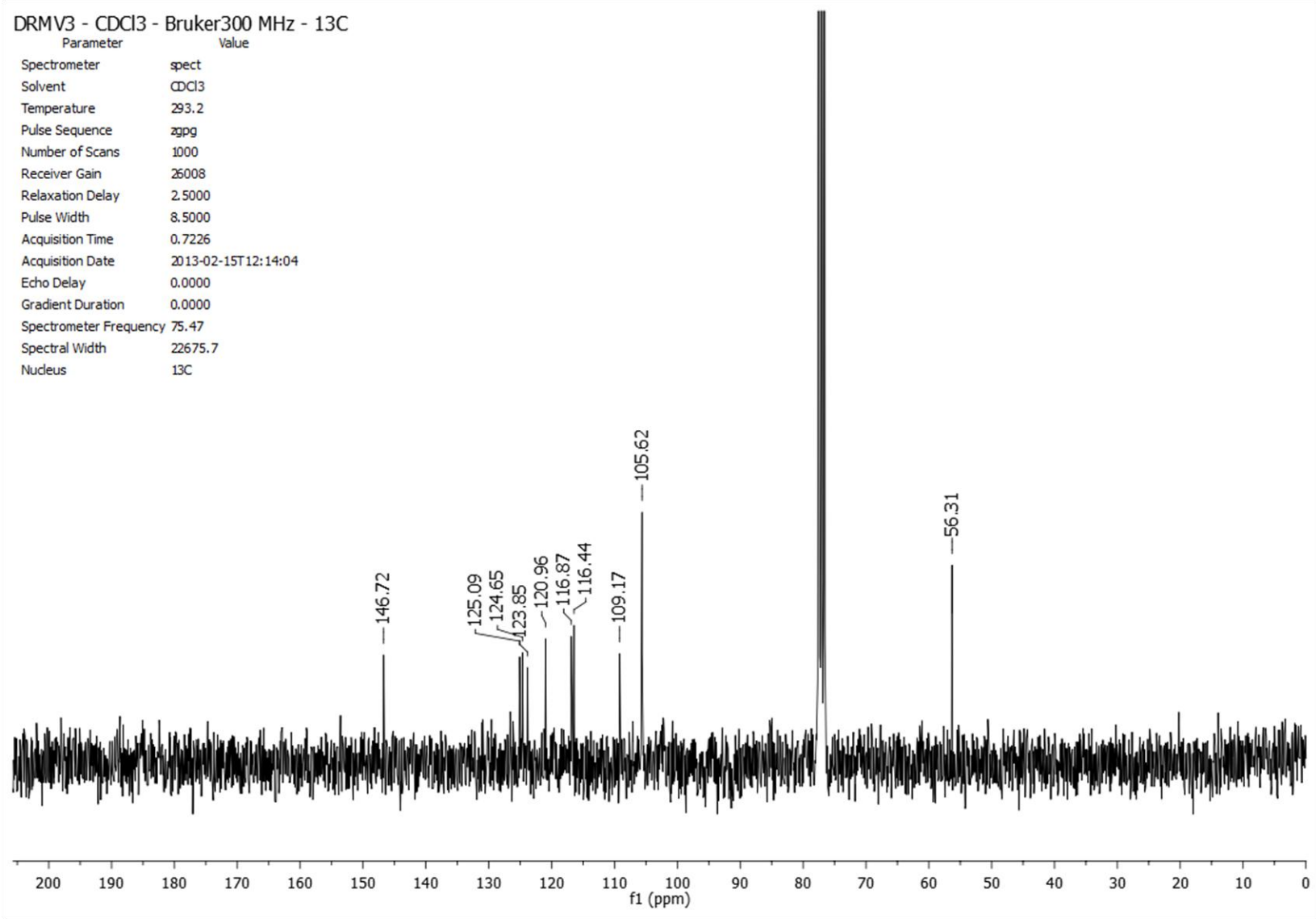


Figure A9. HMBC (400 MHz, $CDCl_3$) of DRMV3

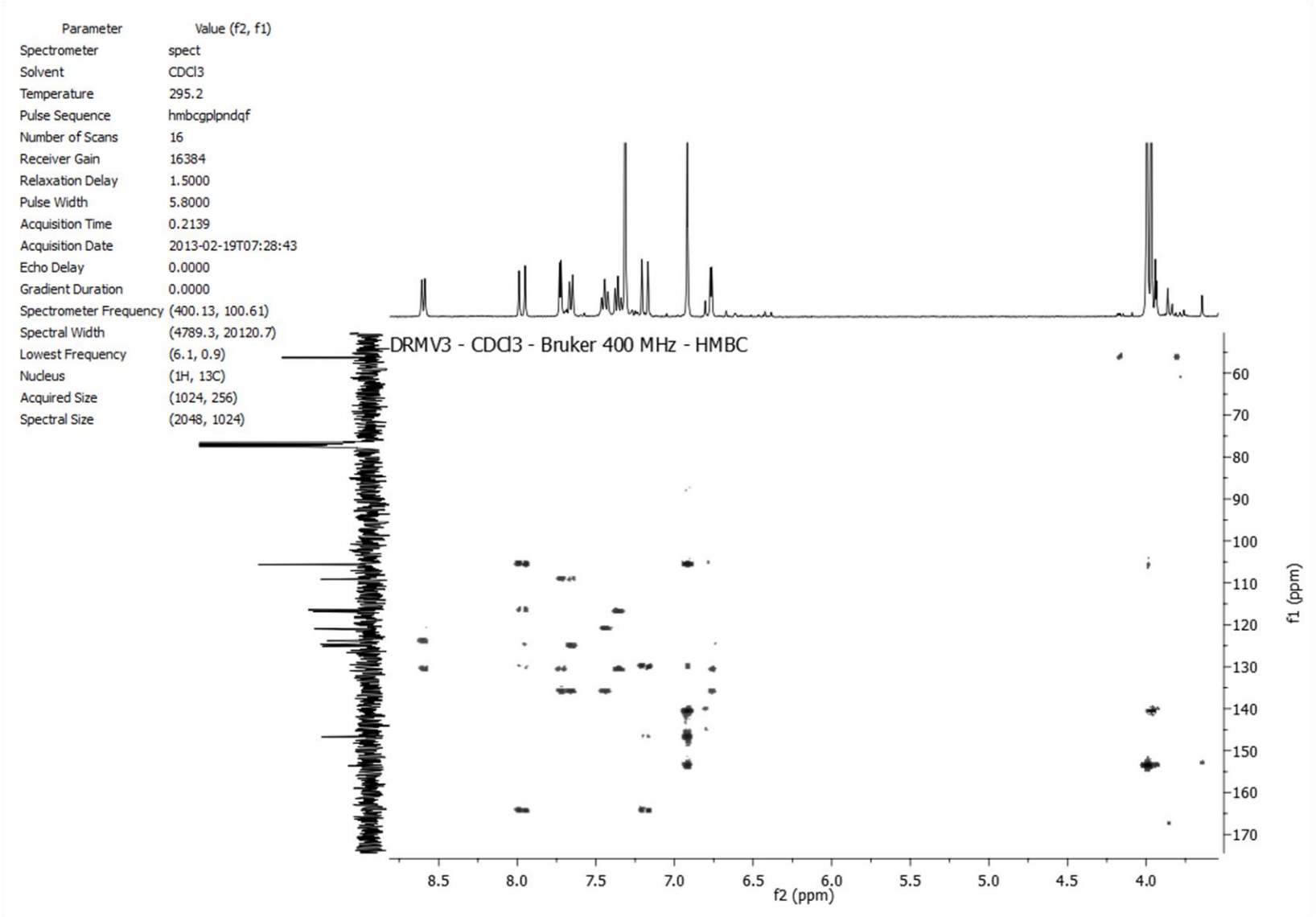


Figure A10. ^{1}H NMR (500 MHz, $CDCl_3$) of DRMV4

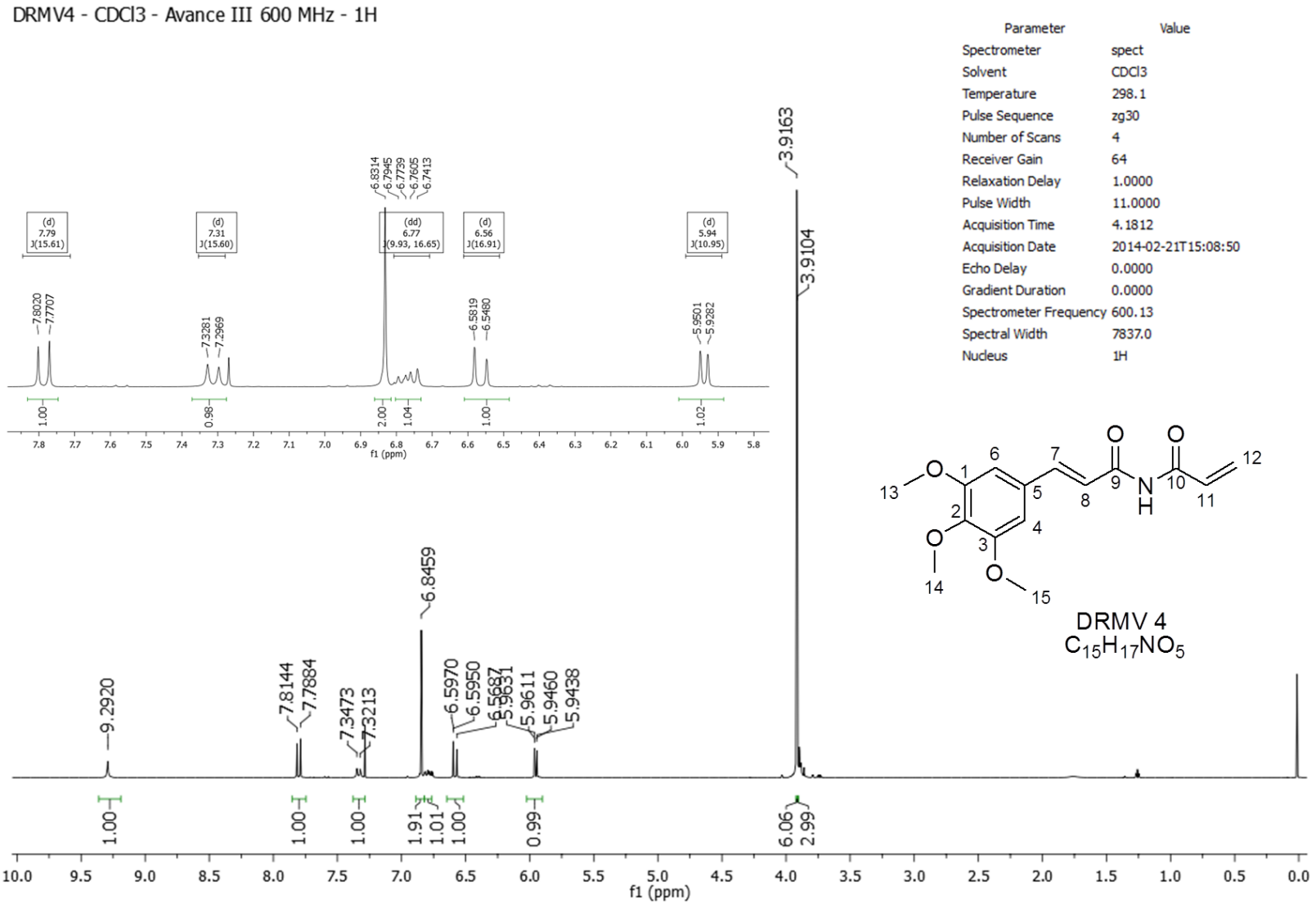


Figure A11. ^{13}C NMR (125 MHz, $CDCl_3$) of DRMV4

DRMV4 - CDCl3 - Avance 600 MHz - 13C

Parameter	Value
Spectrometer	spect
Solvent	CDCl3
Temperature	298.3
Pulse Sequence	zgpg30
Number of Scans	144
Receiver Gain	2050
Relaxation Delay	2.0000
Pulse Width	11.0000
Acquisition Time	0.9088
Acquisition Date	2014-02-21T15:11:28
Echo Delay	0.0000
Gradient Duration	0.0000
Spectrometer Frequency	150.90
Spectral Width	36057.7
Nucleus	13C

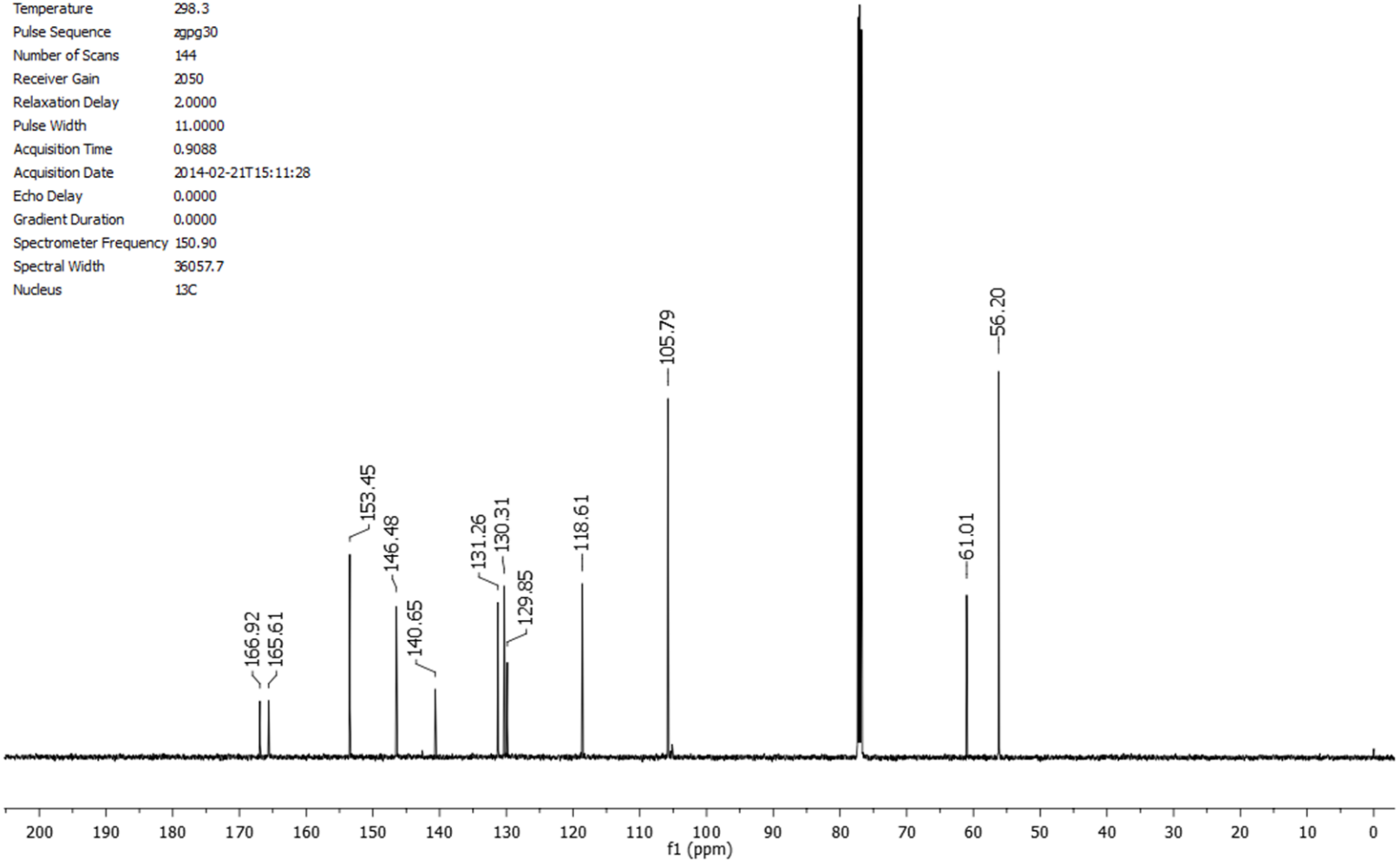


Figure A12. HMBC (500 MHz, $CDCl_3$) of DRMV4

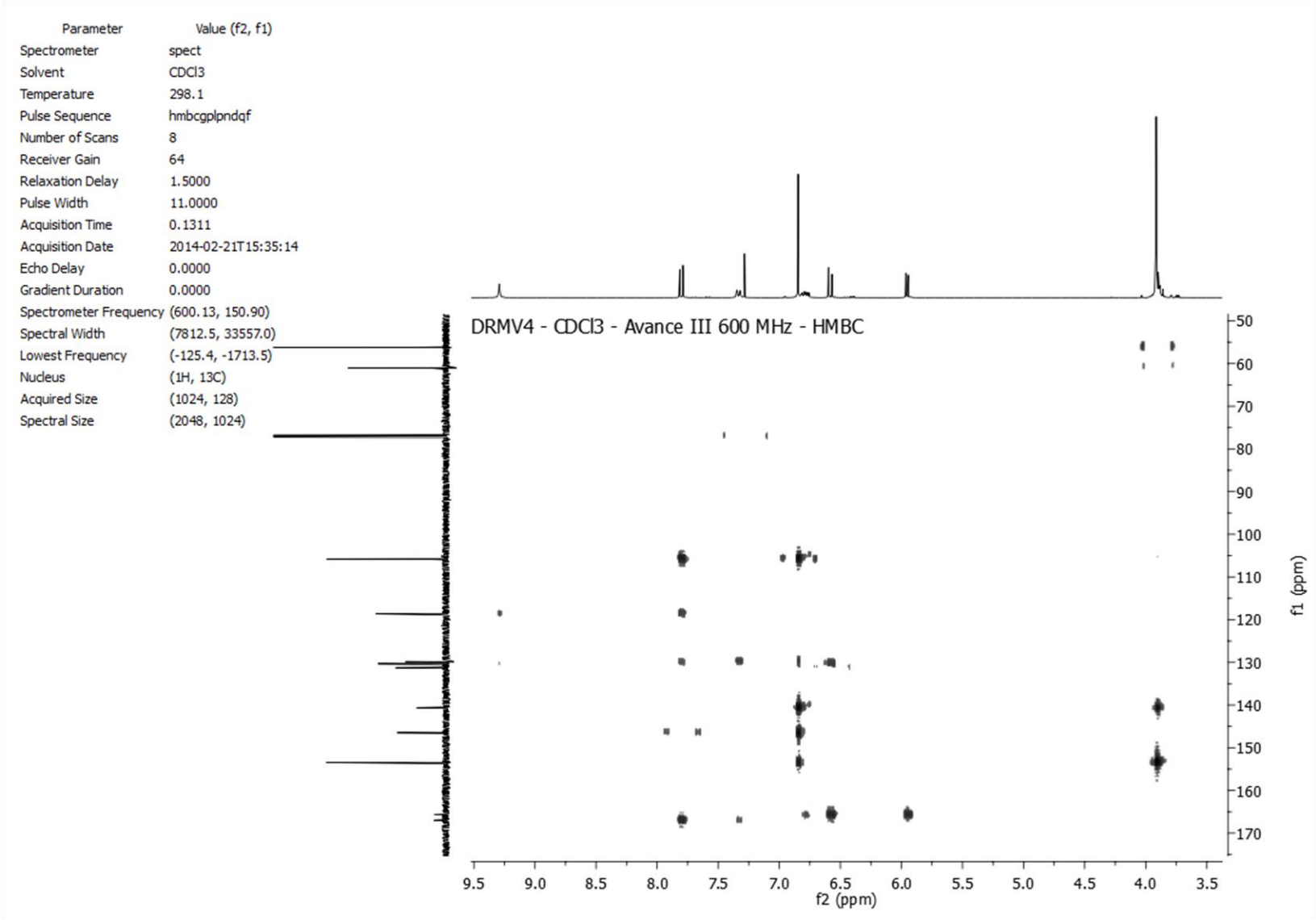


Figure A13. ^{1}H NMR (600 MHz, $CDCl_3$) of DRMV5

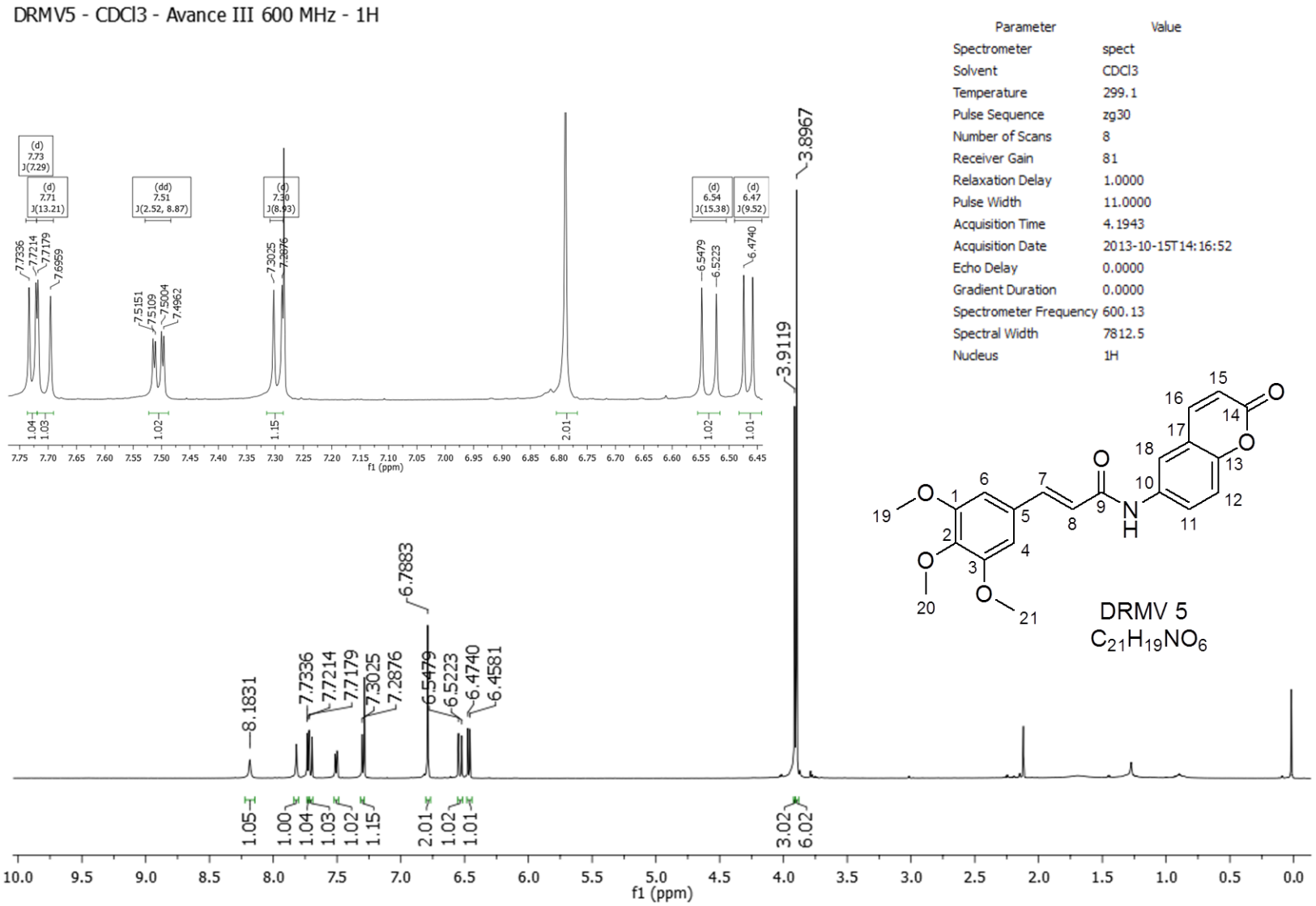


Figure A14. ^{13}C NMR (150 MHz, $CDCl_3$) of DRMV5

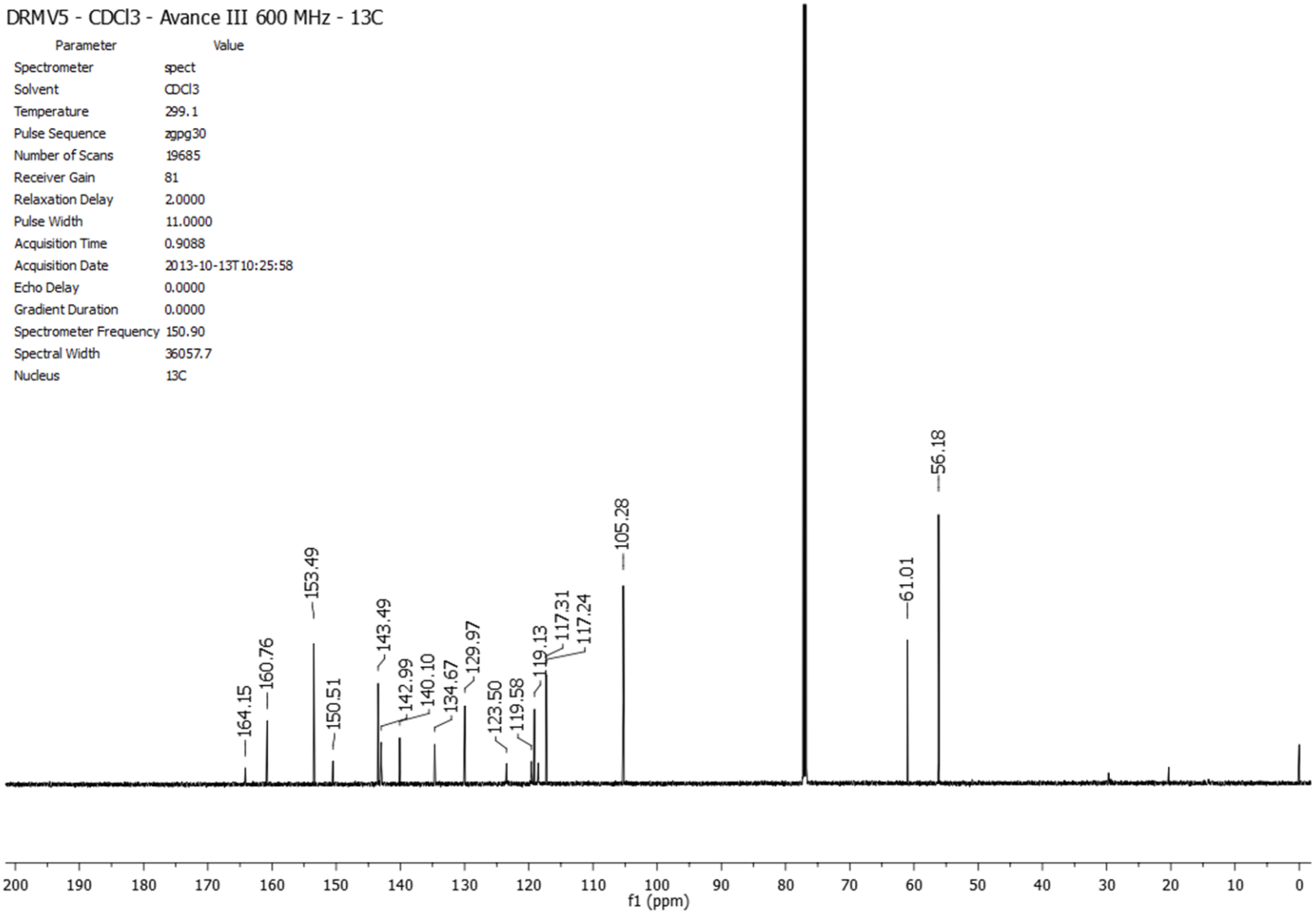


Figure A15. HMBC (600 MHz, $CDCl_3$) of DRMV5